Short Circuit Co–Evolution by the Perfect Parasites: Antifreeze Glycoproteins in Antarctic Fish Leeches (Hirudinea, Piscicolidae)

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Zoology

at Massey University in Auckland

New Zealand

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2013

For my wife Kirsten and my sons Luca and Theo,

and the children of this world who kindly lent it to me.

So eine Arbeit wird eigentlich nie fertig,

man muß sie für fertig erklären,

wenn man nach Zeit und Umständen

das Mögliche getan hat.

A work as this is never really finished,

one must declare it finished

when one has within limits of time and circumstances,

done what is possible.

Johann Wolfgang von Goethe

German poet and philosopher (1749 – 1832), *Italienische Reise*, 16th March 1787

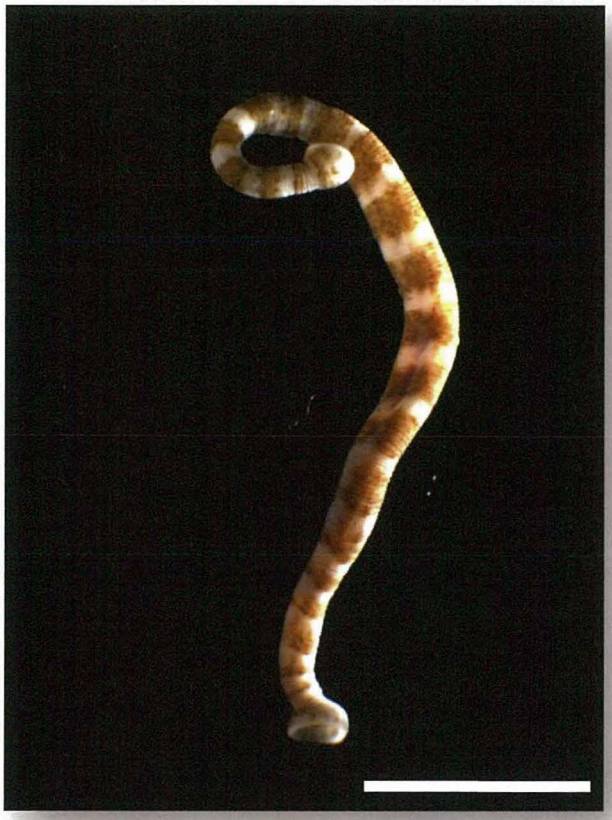

Image of *Cryobdella antarctica* (Epshtein, 1970), scale bar 5mm.

Image of *Cryobdella levigata* (Harding, 1922), scale bar 5mm.

Abstract

Antifreeze glycoproteins (AFGPs) play an important role in biochemical adaptation to supercooled waters and hence in the survival of notothenioid fish in Antarctica. These fishes have a well developed parasitic epifauna, which in turn is also exposed to freezing conditions. In order to retain their association with Antarctic fishes as the environment progressively cooled during the Miocene, leeches as fish–associated ectoparasites had either (i) to evolve a short circuit mechanism to acquire the necessary life–saving chemical compounds from their host, (ii) to adapt their own genome to confer protection from freezing, or (iii) to develop a combined tactic unique to their parasitic life strategy according to requirements during ontogenesis.

I have found that Antarctic leeches (Hirudinea, Piscicolidae), that feed on a variety of notothenioid fish species, contain antifreeze compounds at the cellular level. I present evidence that strongly indicates an absorption pathway of AFGPs in the parasitic organisms from the fish blood as source. The physiological processes of AFGPs uptake from the intestine and circulatory distribution by haemolymph would be analogous to those enabling the fish hosts to distribute these peptides by blood within their bodies, as fish absorb AFGPs through the gut after production in the pancreas.

The analysis of protein chemical structures in leech material revealed characteristics typical of fish AFGPs. Further, there are high capacities for freezing point suppression *in vivo*, thus biological activity of antifreeze proteins in the leech parasites *Cryobdella antarctica* and *Cryobdella levigata*. A combination of this thermal hysteresis (TH) with a specific bi–pyramidal ice crystal growth has been observed, which is typical for fish AFGPs. This confirms the presence not only of functional antifreeze macromolecules but also of true AFGPs in these parasite species.

Finally, to trace the potential origin of these proteins to leech genomic information, mRNA molecules were successfully detected in *C. levigata*, as the intermediate step necessary for any *de novo* AFGP biosynthesis. These results suggest the possibility of a

horizontal gene transfer (HGT) event in this host–parasite system and if proven would mark a further record of such a gene transfer for antifreeze molecules in Antarctica but for the first time outside the surface sea ice zone.

I conclude that Antarctic fish leeches have developed a novel means of an evolutionary shortcut by co–opting mechanisms for survival in supercooled waters from their hosts in the form of biochemical exploitation and possibly in addition by HGT. To the best of my knowledge, the use of functional AFGPs after digestive absorption would represent the first example in the animal kingdom of an instantly effective adaptive advantage provided by another species under natural conditions in a quasi short circuit co–evolution.

I also present results from a first survey on the leech fauna in the Ross Sea across nine species of Antarctic fishes and report one new host record for *C. antarctica* and three new leech–host associations for *C. levigata*.

Finally, one new species belonging to the Piscicolidae is described, *Megapodibdella kirsteni,* gen. et sp. nov., from the Antarctic eelpout *Lycodichthys dearborni*.

Preface

This thesis is written in a traditional format with a general introduction to the topic, followed by four chapters presenting data and a final conclusion with a summary of my contributions to the field of polar science and perspectives for future research. The introductory chapter is written to provide a general understanding of the Antarctic habitat and life at sub–zero temperatures and the context for this research. Each chapter has an abstract to summarise the aim, method and findings. The introductions include explanations of and commentaries on the research questions addressed with the discrete methodologies. There are detailed discussions with specific future perspectives as well as concluding summaries at the end of each data chapter.

I have structured my thesis in a succession of methodologies providing increased depths of information, detail and complexity. The reader will see how the different chapters build upon each other to investigate different aspects leading to the final conclusions.

The idea to this PhD work was my own and I was fortunate in obtaining a scholarship for an extended six week period of field and laboratory work in Antarctica. I organised, executed and planned the fieldwork followed by experiments at laboratory facilities at Scott Base in Antarctica as well as Massey University and Auckland University in New Zealand. Apart from the actual mass analysis, I conducted all experiments and data analysis myself with technical assistance and guidance of supervisors.

Acknowledgements

I am greatly indebted to Prof. Dianne Brunton for being my primary supervisor in this demanding task of completing my PhD. It is rare to find a personal advocate in the academic field combined with her interpersonal skills and supportive personality. I have been fortunate to have her as my kind and considerate mentor.

I greatly appreciated and am very grateful to my co–supervisor Assoc. Prof. Clive Evans for taking me in to work on the project of fish leech parasites associated with his Antarctic fish pets and for his knowledge in the laboratory and the field while *"on the ice"* at McMurdo Sound. I shall never forget our ice fishing efforts and the catch of Toby, the Antarctic toothfish.

I would like to extend my gratitude to my co–supervisor Dr. Rosemary Barraclough for her constructive topic discussions, detailed commentaries to improve funding applications and early manuscripts of this thesis as well as her moral support. I thank Dist. Prof. Paul Rainey as co–supervisor for his kind support in facilitating laboratory work and the very comprehensive and challenging discussions we had on various aspects of evolution.

I am grateful to Dr. Karen Stockin for her support throughout the PhD on academic as well as other topics. I wish to thank Prof. David Lambert and Dr. Leon Huynen for their support and expertise in the laboratory work, and Mark Delaney for his logistical support. I appreciated the time Dr. Justin Sullivan, Dr. Austen Stanley and Dr. Xue–Xian Zhang have spent with me and for the inspiring discussions with them on genomics and RNA research. I thank Prof. David Raubenheimer for his critical reviews of grant applications as well as detailed topic discussions.

I also extend my thanks to the Massey librarians, Anne Hall and Jane Leighton, for their endless efforts to search university libraries around the globe in order to obtain copies of classic literature from the most exotic locations.

The role of the Utevsky brothers, Assoc. Prof. Andrei Utevsky and Dr. Sergei Utevsky, with their infectious enthusiasm for polar fish leeches should not be left unmentioned. Their scientific work inspired me to aim for a PhD in polar biology and for that I am especially grateful. I very much appreciated the time I could spend with them discussing my project and in particular for the latest literature on Piscicolidae in English, rather than Russian, to facilitate my work.

I am grateful to Prof. Eugene Burreson for our discussions at the initial stage of my PhD and particularly for his knowledgeable input on leech taxonomy and biology. His expertise acquired through his numerous discoveries of new leech species around the world was very much appreciated. I want to express my profound gratitude to Dr. Victoria Metcalf for our discussions on fish parasites and her generous donation of Antarctic leech material at the start of this PhD project. I am grateful to Dr. Craig Marshall and Prof. David Wharton for the many interesting ideas and comments I received during and after my visit to Dunedin in 2008 as part of the Antarctica New Zealand annual conference. I very much appreciated the intriguing and very interesting discussions with Prof. Craig Cary and Dr. Charles Lee on numerous occasions at conferences as well as at Scott Base after my science lecture on leeches. I would also like to express my appreciation for all those anonymous but supportive discussion partners I had the opportunity to meet during conferences and seminars and during my Antarctic expedition to Scott Base and McMurdo Station. The time spent discussing my research was gratefully appreciated and helped to clarify many aspects. I wish to thank Prof. Arthur DeVries for sharing with me his 50 years of expertise as an Antarctic researcher and field scientist. Thanks are also due to Dr. Andrew Bowie and Dr. Edward Butler for their kind supply of CTD data which enabled me to include a graph illustrating seawater temperature ranges.

I wish to thank Natasha Lucas and Martin Middleditch for their technical support with the mass spectrometry work and in particular M. Middleditch for sharing his profound expertise with me. I thank the team of field event K160, Dr. Nimrod Kiss and Lauren Fields for invaluable help on the ice as well as the Scott Base team of the field season 2010/11. The support and help provided by the staff of Antarctica New Zealand and in particular by Shulamit Gordon and Dr. Ed Butler was greatly appreciated. The invaluable cooperation with the United States Antarctic Programme and United States Air Force in the provision of the

logistical infrastructure for Antarctic field work is gratefully acknowledged. I thank Dr. Anna Gsell for her support during my PhD and for her scientific intellect in many discussions. I would like to extend my gratitude to my father–in–law Robert Luther for his detailed commentaries. Thanks are also in order to every past and present student at the Ecology and Conservation Group at Massey University who provided help and support during my time there. I very much enjoyed being part of this dynamic group.

I am very grateful for the financial support for my research on the biology and ecology of fish leeches provided by Kelly Tarlton, Antarctica New Zealand and Massey University. I am deeply indebted to Kelly Tarlton and Antarctica New Zealand for their financial support for Antarctic field logistics which were key to the realisation of my PhD. The stipends from Antarctica New Zealand as well as the universities of Otago, Waikato and Canterbury were greatly appreciated as they permitted me to present my research at their conference events.

I am immeasurably grateful to my family. First of all to my mother Eva–Maria and my father Prof. Dietmar Kolb for the nurturing home in which I grew up. Their patience and support for my efforts to become a biologist and polar scientist were paramount over the years during which one cannot count the number of academic discussions I had with my father. I would have never seen it through to the PhD level without their inspiration and everlasting encouragement. And secondly to my late grandfather Dr. Joseph Kolb for being a source of my natural curiosity and interest to understand the science behind our world. His ever ready phrase to spur academic ambitions in his grandson to study and follow the family tradition of an occupation in the field of natural sciences *"zu höchsten akademischen Ehren"* (continually strive to achieve the highest academic honours) will always echo in my mind. To you lieber Opa, I have reached this goal against all odds.

Finally, the patience of my wife Kirsten and her undying support for my polar passion allowed me the freedom to work day and night on my research and were paramount to this PhD. I am forever grateful for her understanding and independence which allowed me to go on a prolonged expedition to Antarctica when our first born was just nine weeks old. I conclude with thanks to my two sons Luca and Theo for letting me spend long days at work instead of playing with them and still recognising and loving their Papa after I submitted my thesis.

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Abbreviations

A = Ala	alanine
ACC	Antarctic circumpolar current
ACCF	Antarctic circumpolar current front
AFGP	antifreeze glycoprotein or glycopeptide
AFP = THP	antifreeze protein = thermal hysteresis protein
AFPP	antifreeze potentiating protein
AMCA	aminomethylcoumarin
amu	atomic mass unit
API	atmospheric pressure ionisation
AZ	Antarctic zone
BM	body mass
BSA	bovine serum albumin
CA	*Cryobdella antarctica* (Epshtein, 1970)
cDNA	complementary deoxyribonucleic acid
CID/CAD	collision–induced/activated dissociation
CL	*Cryobdella levigata* (Harding, 1922)
CLSM	confocal laser scanning microscopy
CPS	counts per second
CTMax	critical thermal maximum
DAPI	4',6–diamidino–2–phenylindole
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimetry
EDTA	2,2',2'',2'''–(ethane–1,2–diyldinitrilo)tetraacetic acid
EI	electron ionisation
ESI	electrospray ionisation
eV	electronvolt

EW	Evans Wall (sampling location)
FITC	fluorescein isothiocyanate
FP	freezing point
Gal	β–D–galactose
GalNAc	*N*–acetyl–D–galactosamine = *N*–acetylgalactosamine
GC	gas chromatography
gDNA	genomic DNA
gen. nov.	genus novum / new genus
GFP	green fluorescent protein
GlcNAc	*N*–acetyl–D–glucosamine = N–acetylglucosamine
GP	gas phase
GPMAW	General Protein/Mass Analysis for Windows software
Hex	hexose
HexNAc	α–*N*–acetyl–D–hexosamine = *N*–acetylhexosamine = aminohexose
HexNAc–Hex	β–D–galactosyl–(1→3)–α–*N*–acetyl–D–galactosamine = hexose–hexosamine
HGT = LGT	horizontal (or lateral) gene transfer
HPLC	high pressure/performance liquid chromatography
IAS	ice–active substance
IBP	ice–binding protein
IC	intestinal chamber
ICT	International Critical Tables of Numerical Data, Physics, Chemistry and Technology
IHC	immunohistochemistry
IMS	imaging mass spectrometry
INI	Inaccessible Island (sampling location)
JGI	Joint Genome Institute
K	Kelvin

LC	liquid chromatography
LC–MS	liquid chromatography–mass spectrometry
LC–MS/MS	liquid chromatography–tandem mass spectrometry
M	mouth pore
M^+	molecular ion
MALDI	matrix–assisted (soft) laser desorption ionisation
mOsmol	milli Osmole
MP	melting point
M_r	relative molecular mass or molecular weight
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mya	million years ago
m/z	mass–to–charge ratio
NCBI	National Centre for Biotechnology Information database
NCBInr	National Centre for Biotechnology Information protein database
NMR	nuclear magnetic resonance
NO	nanolitre osmometry
nt	nucleotide
P = Pro	proline
PBD	antibody blocking solution
PCR	polymerase chain reaction
PepDraw	peptide drawing software
PF	polar front
PFZ	polar front zone
PTM	posttranslational modification
OCT	optimal cutting temperature polymer
RL	rat liver
RPC	reversed phase chromatography

RP–HPLC	reversed phase high pressure/performance liquid chromatography
RT	reverse transcription
SAF	subantarctic front
SAZ	subantarctic zone
SB	southern boundary
SE	standard error
SL	standard body length
SPE	solid phase extraction
sp. nov.	species nova / new species
ssDNA	single stranded DNA
STCZ	subtropical convergence zone
STF	subtropical front
Swiss–Prot	Swiss protein sequence database
T = Thr	threonine
TB	*Trematomus bernacchii* (Boulenger, 1902)
TBE	TRIS–borate–EDTA buffer
TH	thermal hysteresis
THA	thermal hysteresis activity
THP = AFP	thermal hysteresis protein = antifreeze protein
TOF	time–of–flight
TrEMBL	Translated European Molecular Biology Laboratory Nucleotide Sequence Data Library
TRIS	2–amino–2–hydroxymethyl–propane–1,3–diol
u	unified atomic mass unit
UniProt	Universal Protein Resource database
UniProtKB	Universal Protein Resource Knowledgebase
WGA	whole genome analysis
WoRMS	World Register of Marine Species database
WQB	Winter Quarters Bay (sampling location)

CHAPTER ONE

Introduction

1.1 Abstract

The rationale for this thesis was to explore how ectoparasites have evolved mechanisms to survive in freezing conditions. The study organisms of this PhD are marine fish leeches, the Piscicolidae, previously thought to be rare ectoparasites on Antarctic Notothenioidei. The ecophysiology and genetic composition of fish leeches is studied to explain how these survive in the sub–zero waters of the Southern Ocean.

In this first chapter I provide an overview of the Southern Ocean and the particular oceanographic conditions resulting in the sub–zero marine habitat surrounding the Antarctic continent, with implications for the benthos. The endemic fish species are introduced as well as the model organisms of my PhD, the Antarctic Piscicolidae or fish leeches. The physiological adaptations of life to freezing conditions in the form of antifreeze molecules and how these function are presented in general and for the Antarctic fishes in particular. Finally, the concept of horizontal gene transfer (HGT) as an important development in evolutionary research is introduced and explained with examples of recent literature including Antarctic species, followed by the chapter outline and aims of this thesis.

1.2 Antarctic conditions

Ice can function as a refuge as well as a feeding habitat for notothenioid fishes of the McMurdo Sound. For instance, the borch or bald notothen *Pagothenia borchgrevinki* (Boulenger, 1902; first described as *Trematomus borchgrevinki*) is cryopelagic (DeWitt, Heemstra & Gon, 1990) and specialised to live and forage in the platelet ice layer

underneath the sea ice where it feeds on invertebrates as well as Antarctic silverfish *Pleuragramma antarctica* (Boulenger, 1902) (Janssen, Montgomery & Tien, 1992). Borch also use ice as hiding spots from predation, such as from the Antarctic toothfish *Dissostichus mawsoni* (Norman, 1937) (Fuiman, Davis & Williams, 2002), the Weddell seal *Leptonychotes weddellii* (Lesson, 1826) and the Emperor penguin *Aptenodytes forsteri* (Gray, 1844) (DeVries & Steffensen, 2005).

In the Arctic, bottom–dwelling fish can supercool and live in sub–zero waters (Scholander, Flagg, Hock & Irving, 1953). However, the presence of ice is problematic in these environments, as demonstrated when fish taken from shallow water froze to death on contact with ice crystals in freezing experiments using tanks (Scholander, van Dam, Kanwisher, Hammel & Gordon, 1957). Nevertheless, species such as the Arctic mummichog killifish *Fundulus heteroclitus* (Linnæus, 1766) can survive the winter in the ice–free deep waters by being permanently supercooled (Umminger, 1967) based on changes in the chemical composition of the fish at these sub–zero temperatures (Umminger, 1975).

In the harsh Antarctic winter the temperature of the Southern Ocean falls to the freezing point (FP) of seawater, depending on salinity, about –1.93°C (Littlepage, 1965), and free–floating ice crystals are common in Antarctic shallow waters. Ice in the form of crystals at the sea ice surface or ice attached to the substrate, referred to as anchor ice, has also been observed to depths of 33 m from October to December, during the warming austral spring period in SCUBA–diving studies (Dayton, Robilliard & DeVries, 1969). Moreover, results from an annual oceanographic survey in McMurdo Sound demonstrated that the physical conditions for floating crystals exist to a depth of 75 m (Littlepage, 1965). Supercooled waters between –2°C to –2.1°C have been recorded along the seafloor of more than 1,000 m depth within 200 km of the Filchner Ice Shelf and ice crystals collected as deep as 250 m (Dieckmann, Rohardt, Hellmer & Kipfstuhl, 1986). The depth in which floating crystals as well as anchor ice occur are likely to reach lower than the 33 m previously documented (Mager, Smith, Kempema, Thomson & Leonard, 2013) and the McMurdo Ice Shelf could potentially produce stable average conditions for sea ice to exist down to 40 m depth with some cold pockets reaching as low as 70 m (Sarah M. Mager, pers. comm.). Since fishes and their parasitic leeches are caught throughout these depths, this strongly suggests they are capable of tolerating contact with ice as seeding nucleators.

1.3 Antarctic fish taxonomy

The isolation of the Antarctic waters is believed to have been caused by the progressive cooling after the Eocene epoch 34 million years ago (mya) and then the formation of the eastward flowing Antarctic Circumpolar Current (ACC) during the late or upper Oligocene 28–23 mya (Møller, Nielsen & Anderson, 2005). This resulted in the geographical isolation of the enclosed fish species in a cold environment of 5°C or less from the middle Miocene at 16–11 mya (Kennett, 1977). The evolution of preadapted AFGP bearing fish from one benthic common ancestor into the diverse modern notothenioid species occurred predominantly during the late Miocene 11.6–5.3 mya (Near, et al., 2012).

The dominant fish species of the Southern Ocean, and in the region of the marine field activities for this work, namely the Ross Sea, belong to the suborder Notothenioidei or notothenioids of the family Nototheniidae or notothens as part of the order Perciformes (Møller, et al., 2005). Fish of the family Nototheniidae, dominate the high–latitude Antarctic waters in terms of diversity, with 32 valid species in the Southern Ocean, as well as in terms of abundance (Gon & Heemstra, 1990). On the circum–continental Antarctic shelf the benthic or epibenthic species of the genus *Trematomus* (Boulenger, 1902) are the dominating fishes (DeWitt, et al., 1990) and represent the most diverse fish group of the Ross Sea benthos (La Mesa, Eastman & Vacchi, 2004).

Early descriptions by C. T. Regan of the Antarctic ichthyofauna included 25 species by June 1914 (Regan, 1914a, 1914b). This work was mainly concerned with taxonomy in order to obtain a polar species inventory. The two benthic fish species the emerald rockcod *Trematomus bernacchii* (Boulenger, 1902) and the striped notothen *Trematomus hansoni* (Boulenger, 1902) were described in further detail (Fig. 1.1), which are the two primary host species for the marine invertebrates in this work.

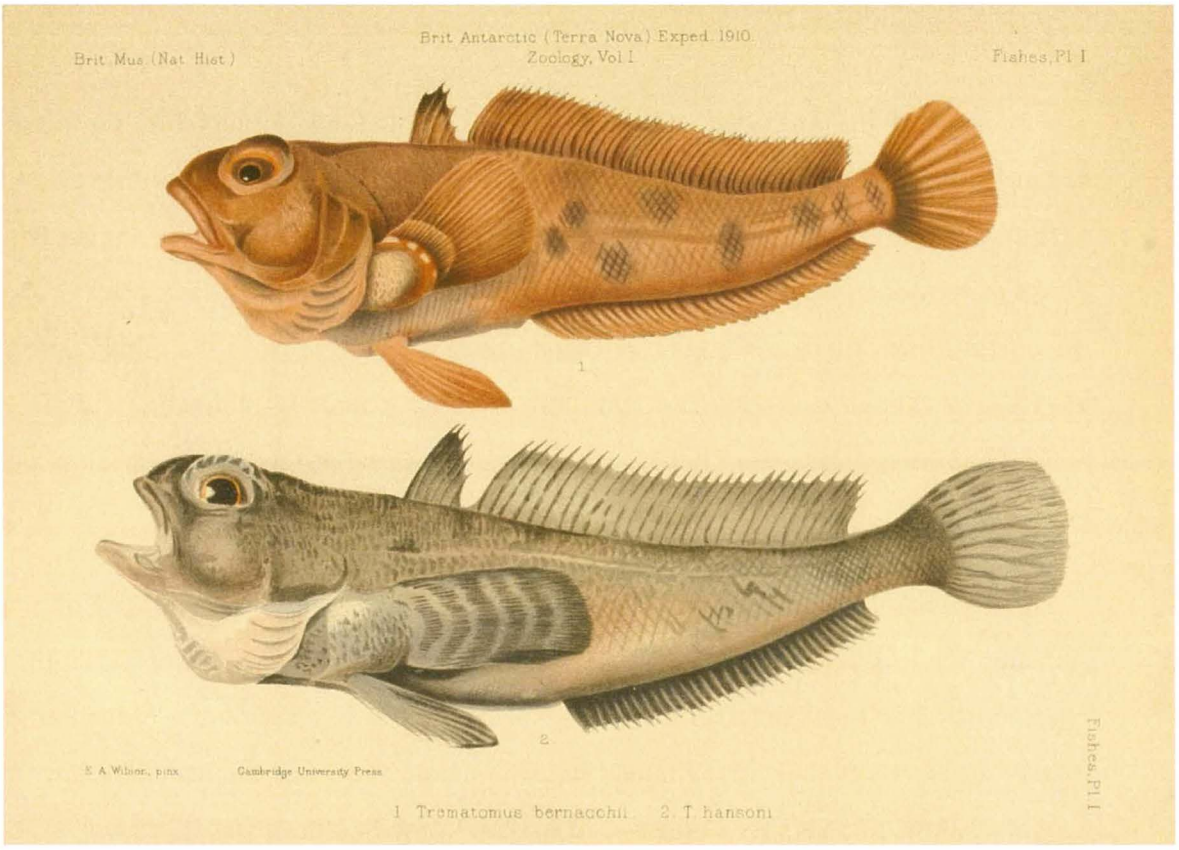


Figure 1.1 Antarctic fish host species of the genus *Trematomus* (Boulenger, 1902).

Trematomus bernacchii (Boulenger, 1902) and *Trematomus hansoni* (Boulenger, 1902) historical drawings as water–colour sketches illustrating their natural colourations (Regan, 1914a, p. 68, plate 1 by E. A. Wilson).

It is noteworthy that, parallel to the fish discovery in 1911, the first record of Antarctic leeches was made which were later described by W. A. Harding (1922) as possibly derived from either of these two *Trematomus* species. Regan included some comments on the distribution of the fish species and their general biology, and further stated that the taxonomy of the recorded fish fauna supports the early Tertiary connection of Antarctica with Australia and South America. Regan also speculated on the evolutionary origins of the coastal fish fauna as stemming from the isolation of the Antarctic continent during the Tertiary (Regan, 1914a, p. 40), from 65 to 2.6 mya.

1.4 Antarctic fish leeches

The leeches of the Southern Ocean surrounding the Antarctic continent belong to the family of fish leeches the Piscicolidae (Johnston, 1865) [synonym Ichthyobdellidae (Leuckart, 1863) (Sawyer, 1986b, p. 660)], and all three subfamilies Platybdellinae (Epshtein, 1970), Pontobdellinae (Llewellyn, 1966) and Piscicolinae (Johnston, 1865) are found in Antarctica (Utevsky, 2005, 2007). They are part of the order Rhynchobdellida (Blanchard 1894) of the subclass Euhirudinea (Lukin, 1956), the true leeches, and class Hirudinea (Lamarck, 1818) (Sawyer, 1986b, pp. 646-706; Utevsky, 2007). The Hirudinea and Oligochaeta (Huxley, 1875) form the subphylum Clitellata and together with the subphylum Polychaeta comprise the phylum Annelida, the segmented worms.

The two leech species on which this PhD is based are from the genus *Cryobdella* (Harding, 1922) that was discovered in material from the British *"Terra Nova"* expedition collected on 10th and 16th May 1911 (Harding, 1922) by Robert Falcon Scott's party and sampling efforts at various localities between Cape Royds and Hut Point in McMurdo Sound off the Ross Island (Harmer & Lillie, 1914) as within the vicinity of their erected hut at Cape Evans as their chosen winter quarters for 1911 (Leiper & Atkinson, 1915). A total of seven specimens were taken from gills of fishes belonging to the genus *Trematomus* and subsequently described by W. A. Harding as a new and first genus of Piscicolidae from the Antarctic: *Cryobdella levigata* (Harding, 1922) as from the Greek *κρύος* (*cryos*) for *icy cold* and *βδέλλα* (*bdella*) for *leech*. Harding only had information about the fish genus from which the leeches were sampled because the initial description of fish caught from the area being solely *T. bernacchii* was later revised to specimens of *T. bernacchii* as well as *T. hansoni* (Leiper & Atkinson, 1915). Thus, Harding pointed out that either species must serve as fish host as both are common in Antarctic waters, a suggestion that after my field observations I can now confirm to be correct for this sampling area.

The second leech species *Cryobdella antarctica* (Epshtein, 1970) (Fig. 1.2) was first discovered much later and at the opposite side of Antarctica (67° 40' S, 45° 48' E), 100 m off the coast at a glacier tongue near the Russian Antarctic station Молодёжная or *Molodyozhnaya*.

Figure 1.2 Images of one *Trematomus bernacchii* with multiple *Cryobdella antarctica*.

Field observation *in situ* of attached and dislodged parasitic *C. antarctica* on a large and gravid *T. bernacchii* (left). Detailed image of *C. antarctica* located on the dorsal side of the same host fish (right). The fish was caught by rod and line off Inaccessible Island in approximately 20 m depths.

The presence of the ectoparasitic Piscicolidae on Antarctic fish species is generally overlooked in the literature, with a recent inventory list of the Antarctic marine benthic community excluding them completely (Clarke & Johnston, 2003). Nonetheless, new species of fish leeches are still being discovered in Antarctic waters (Meyer & Burreson, 1990; Epshtein & Utevsky, 1994; Utevsky, 1994a, 1994b, 1997) and redescribed (Meyer & Burreson, 1990; Utevsky, 1995). The largest fish leech ever caught had a body length of 340 mm and 13 mm width and was found in 1990 in the collections of the 1962–68 cruises of the *USNS Eltanin* and described as *Megaliobdella szidati* (Meyer et Burreson, 1990). It was collected in Antarctic waters of 549–556 m depth in November 1968 with a 5 or 10 foot Blake trawl at 77° 42.0′ S, 167° 44.0′ W. The host and internal anatomy of this species is still undescribed. However, the phylogeny of piscicolids in the Antarctic (Moore, 1938; Epshtein, 1968; Epshtein, 1970; Meyer & Burreson, 1990) and in general (Llewellyn, 1966) have been and remain a focus of research (Epshtein, Utevsky & Utevsky, 1994; Utevsky & Trontelj, 2004; Williams & Burreson, 2005) and debate (Williams & Burreson, 2006; Utevsky, Utevsky, Schiaparelli & Trontelj, 2007). Recently, A. Y. Utevsky (2005, 2007) compiled and revised the present knowledge of 21 species in 13 genera. This advanced the initial descriptions by Harding (1922) and Moore (1938) and revisions by Autrum (1939). However, it is clear that this group is still understudied in the Southern Ocean (Meyer & Burreson, 1990; Bielecki,

Rokicka, Ropelewska & Dziekońska-Rynko, 2008) as well as on a global scale. In an effort to provide up to date estimates of all species in the world's marine environment W. Appeltans and M. J. Costello involved 121 taxonomic specialists as part of the World Register of Marine Species (WoRMS) database (2012). I contributed to the total number standing now at 0.7–1 million species with a revision of the marine Piscicolidae. This group is identified as one of the taxonomic units in the world's oceans with the highest potential for the discovery of new species. This has further been confirmed by my discovery of a new benthic fish leech in McMurdo Sound on the Antarctic eelpout *Lycodichthys dearborni* (DeWitt, 1962) (pers. discovery, description in prep.). This host species is endemic to the Ross Sea of the Southern Ocean as a member of the family Zoarcidae or eelpouts of the Perciformes (Anderson, 1990; Kelley, Aagaard, MacCoss & Swanson, 2010). With 20,000 new marine species discovered and described during the last decade alone (Pimm, 2012) it is not surprising to find new species in remote and inaccessible locations such as the Ross Sea, particularly when species may have a small geographical range. However, this information on the oceans' taxonomic inventory by Appeltans and colleagues does not deal with the biology or the degree of endemism of the Antarctic representatives or their ecophysiological adaptations to freezing habitats. This PhD aims to further our knowledge on these aspects.

A biochemical necessity for Piscicolidae is related to their life strategy. The blood sucking or haematophagous or sanguivorous Rhynchobdellida maintain an indigenous bacterial flora (Mootz, 1936) as endosymbionts (Dinand & Bottenberg, 1935; Hornbostel, 1942; Büsing, 1951; Büsing, Döll & Freytag, 1953; Jennings & van der Lande, 1967; Graf, 1999; Kikuchi & Fukatsu, 2002). These are essential for the digestion of the blood meal and lysis of erythrocytes and hydrolysis of proteins (Büsing, 1951; Büsing, et al., 1953) as demonstrated by a dramatically reduced efficiency of protein digestion if the bacteria are removed or killed (Graf, 2002). This symbiotic relationship has practical implications in the medical application of *Hirudo medicinalis* (Linnæus, 1758), the European medicinal leech, as the bacterial flora consists of human pathogens and can cause infections during leech therapy, as demonstrated by two German doctors who injected themselves with pure leech bacterial cultures (Dinand & Bottenberg, 1935). Leeches play an important role in plastic and reconstructive surgery with proven benefits for recovery (Mutimer, Banis & Upton, 1987; Whitaker, et al., 2012; Senchenkov & Jacobson, 2013) despite early concerns regarding infections (Whitlock, O'Hare, Sanders & Morrow, 1983; Dickson, Boothman & Hare, 1984;

Nonomura, et al., 1996). And, yet, despite medical records for leech therapy stretching four millennia from ancient Egypt, Greece, Rome to China and the Middle Ages (Adams, 1988; Whitaker, Rao, Izadi & Butler, 2004; Papavramidou & Christopoulou-Aletra, 2009; Singh, 2010), the biology of most leech species and especially those from the marine habitat remains generally unknown.

1.5 Life at sub–zero

The first written record of fishes tolerating freezing dates back to the Roman 'Pliny the Elder' (Gaius Plinius Secundus, 23 – 79 AD), who described how fish from the Black Sea revived after being frozen (Knauthe, 1891). R. Boyle (1627 – 1691) described how poikilotherm vertebrates such as frogs and fishes came back to life after being frozen stiff inside an ice block (1683) while J. Franklin (1786 – 1847) noted how Arctic frogs revived from the frozen grounds in spring (Franklin, 1824a, pp. 271-272) and freshwater carp survived 36 h of freezing (Franklin, 1824b, pp. 16-17). However, W. Kochs (1890) and G. F. Rahm (1924) independently concluded that Teleosts cannot survive being frozen in ice. Although some freeze tolerance was shown for frogs (Müller-Erzbach, 1891; Kodis, 1898; Cameron & Brownlee, 1913) and conclusively for the European wall lizard *Lacerta muralis* (Laurenti, 1768) [accepted today as *Podarcis muralis* (Laurenti, 1768) (Arribas, et al., 2013)] (Weigmann, 1929).

In pure water, spontaneous ice nucleation occurs at temperatures between –36°C and –37.8°C (Stan, et al., 2009). Although undisturbed water might be kept supercooled at –8°C to –10°C for more than 10 months without freezing solid (Dorsey, 1948) this physical property is irrelevant to living organisms that cannot escape contact with ice. An ice crystal can act as a nucleus for a crystallisation event when it touches a supercooled liquid. Such liquids are defined as being below their standard FPs and can include the body fluids within an organism. Supercooled body fluids will abruptly crystallise when in contact with ice, putting the life of an organism at risk.

The freezing temperature of the blood and body fluids in most euryhaline teleost fishes, those adapted to different water salinities, lies between –0.5°C and –0.8°C (Black, 1951). The

natural presence of ice in the marine habitats of Antarctica made the reliance on supercooling alone not a stable evolutionary strategy for the survival of fish species (DeVries, 1982). Consequently, to live in the hostile conditions these marine organisms have had to develop a survival strategy to overcome the temperature gap between the FP of their body and that of the surrounding seawater. One option would be to alter their behaviour in order to avoid icy waters by vertical migration into warmer water masses. However, fish ectoparasites such as leeches are not independent in regards to their mobility, and cannot simply shift into more favourable water temperatures unless they detach from their host, thus risking the loss of their food source. Additionally, this would not have been a viable option in Antarctica where, apart from a few localised warm water currents, the bottom water temperatures for most of the Southern Ocean are below 0°C down to a depth of 1,000 m (Deacon, 1982) and can remain below zero for most or even the entire year in McMurdo Sound (Hunt, Hoefling & Cheng, 2003; Cheng & Detrich III, 2007). The cold water front extends a considerable distance along the seabed of the continental shelf (Fig. 1.3; with permission by A. R. Bowie), of less than –1.3°C down to 150 m depth for latitudes greater than 65° even during the late austral summer period (March–April 2008). Additionally, the shallow water surface layers can be freezing polar waters in the summer (Bowie, Griffiths, Dehairs & Trull, 2011).

The fish leech life cycle includes an obligate benthic stage, hence they are unable to disperse from the potentially lethal temperature zone of Antarctic sub–zero waters. It should be noted that Antarctic leeches are obligate fish parasites, and it is unlikely that they evolved this dependency after their isolation in the Southern Ocean, thus making the separation from the fish host an unstable evolutionary strategy. Consequently, these parasites had to become truly freeze–resistant.

If migration out of the ice zone is not an option for leech parasites to exist successfully in the Antarctic habitat, then what mechanisms could lead to their tolerance to freezing conditions? Osmolytes are potentially protective agents that are essential and ubiquitous parts of cell life. However, any adaptive advantage gained by increasing their concentrations within the cells for freezing tolerance by their colligative effects would have an immediate consequence on the natural function of the cells. Any substance that has a strong impact on vital mechanisms in the cell can, at some concentrations, result in toxicity to the cell.

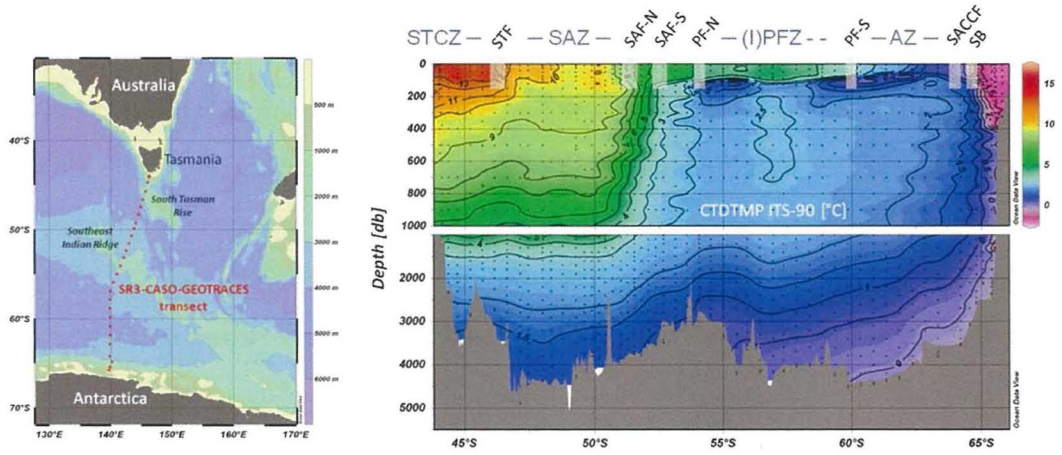


Figure 1.3 Oceanographic images to water temperatures in the Southern Ocean.

Ocean water temperatures from surface to bottom layers obtained during an oceanographic north–south transect survey from Tasmania to the sea ice barrier of Antarctica during the late austral summer of 2008 (STCZ – Subtropical Convergence Zone, STF – Subtropical Front, SAZ – Subantarctic Zone, SAF–N – station north of Subantarctic Front, SAF–S – station south of SAF, PF–N – station north of Polar Front, PFZ – Polar Front Zone, PF–S – station south of Polar Front, AZ – Antarctic Zone, SACCF – south of Antarctic Circumpolar Current Front, SB – Southern Boundary with permission by Andrew R. Bowie & Edward C.V. Butler, Antarctic Climate and Ecosystems Cooperative Research Centre, University of Tasmania, Australia).

Thus, there are various tolerable concentration levels for different solutes. Salt ions in particular have a strong cellular impact when in higher quantities. Furthermore, energetic costs for a hyperosmotic marine organism could represent a limiting factor due to the osmotic pressure and the necessity for permanent and increased active extraction of water from the cell.

Teleosts are typically hyposmotic to their environment with up to 370 mOsmol and only the small class Myxini or hagfish contains isosmotic fish species (Bone & Moore, 2008, pp. 161-187). The notothenioids of the Southern Ocean increased their blood concentrations to around 550 mOsmol with an equilibrium FP of –1.0°C based on colligative effects and their survival depends on antifreeze glycoproteins (AFGPs) to bridge 0.9 K, a small yet lethal gap between the colligative FP of their blood and the ambient water of below –1.9°C (DeVries, 1983). The haemocoelomic fluid of the medicinal leech *H. medicinalis* is hyposmotic to seawater with an osmolarity of 200 mOsmol (Nicholls & Kuffler, 1964; Zerbst-Boroffka, 1970)

and elevated FP of circa –0.4°C or 1/5 of the surrounding salt water in McMurdo Sound with an osmotic concentration of around 1030 mOsmol (Cheng & Detrich III, 2007). Thus, based on colligative effects alone, the Antarctic piscicolids could face a larger thermal gap than their hosts or other teleosts. As a result the leech fauna required an adaptive response to survive in the ice–laden waters and this PhD addresses the reasons for the presence of AFPs in the parasites.

1.6 Thermal hysteresis (TH) and antifreeze proteins (AFPs)

An alternative strategy to migration into warmer habitats as well as freeze tolerance is freeze avoidance by means of a biochemical adaptation to cope with the hostile environment in the form of antifreeze proteins (AFPs) or thermal hysteresis proteins (THPs). These proteins and smaller peptides have the capacity to lower the temperature of the FP of an aqueous solution away from the melting point (MP) and as such push the point at which the animal freezes further into the negative temperature scale. The resulting temperature gap or difference in the two points is referred to as thermal hysteresis (TH) or thermal hysteresis activity (THA) as it is caused by antifreeze activity and is measured in Kelvin (K).

The TH gap observed in work with a coleopteran insect, the mealworm *Tenebrio molitor* (Linnæus, 1758) (Ramsay, 1964), was caused by AFPs (Tomchaney, Morris, Kang & Duman, 1982) from a gene family with a repetitive structure of 30–50 units per gene in analogy to genetics with repetitive motifs encoding AFPs in Arctic and Antarctic fishes (Liou, Thibault, Walker, Davies & Graham, 1999). Beetles like *Dendroides canadensis* (Latreille, 1810) also have a gene family (Wu, Duman, Cheng & Castellino, 1991) and excrete AFPs for their antifreeze properties into the urine (Nickell, Sass, Verleye, Blumenthal & Duman, 2013). AFPs were even identified in the desert beetle *Anatolica polita* (Frivaldsky, 1889) of central Asia exhibiting THA (Mao, Liu, Li, Ma & Zhang, 2011). This beetle was found to have a 30 ku protein with an ice–binding surface (Mao, Liu, Ma, Pang & Zhang, 2011) which is now known to have a seasonal gene expression induced by cold (Ma, Wang, Mao & Wang, 2012). Such cold stress stimulation was also found in *T. molitor* whose larvae increased their concentration of THPs 20–fold after a 4°C cold exposure of four weeks (Graham, Walker & Davies, 2000).

Insect AFPs can be 30 times (Tyshenko, Doucet, Davies & Walker, 1997) and up to 100 times more active than those found in fish (Graham, Liou, Walker & Davies, 1997). The most potent AFP studied so far was recently discovered in the longhorn beetle *Rhagium inquisitor* (Linnæus, 1758) surviving –25°C (Hakim, et al., 2013). AFPs have been additionally discovered in spiders (Duman, 1979) and plants (Atici & Nalbantoglu, 2003) such as the perennial ryegrass *Lolium perenne* (Linnæus, 1758) (Kumble, et al., 2008; Lauersen, Brown, Middleton, Davies & Walker, 2011). And, in Antarctic springtail *Gressittacantha terranova* (Wise, 1967), a terrestrial arthropod, a family of AFPs also seems to have evolved for THA (Hawes, Marshall & Wharton, 2011). These different proteins are of highly diverse structures (Cheng, 1998) yet they all function as ice–binding proteins (IBPs) and freezing point depressants.

The most prominent and evolutionary relevant example is found, however, in the Antarctic fishes where this class of proteins represents the key biochemical adaptation to life in sub–zero waters. Arthur L. DeVries discovered the class of antifreeze glycoproteins (AFGPs) which contribute to the survival of notothenioid fishes by depressing the FP of their body fluids (DeVries, Komatsu & Feeney, 1970; DeVries, 1971). Early work established the presence of eight differently sized AFGPs 1–8 in fish blood (DeVries, et al., 1970). Their antifreeze effect is possible through binding of the AFGPs onto the ice crystals and inhibiting their growth inside the animal (DeVries, 1988).

As the result of convergent evolution, Arctic fishes on the opposite side of the planet were also found to have AFPs (Chen, DeVries & Cheng, 1997a). Yet for Arctic fish such as winter flounder *Pseudopleuronectes americanus* (Walbaum, 1792) seasonality in FP levels (Pearcy, 1961) occurs as the result of varying AFP concentrations (Duman & DeVries, 1974), with the highest concentrations found in winter (25 mg/ml) and lowest in summer (1 mg/ml) (Petzel, Reisman & DeVries, 1980). This occurs despite the fish burying itself in warmer sediments with an average of 0.2°C above seawater temperatures during the winter period of December to May (Fletcher, 1977).

AFPs in general are a unique class of proteins and can function even outside the organisms in which they naturally occur. For instance, AFPs whose TH effect was described in *T. molitor* by Ramsey (1964) could function outside the beetle's body in a foreign environment such as in the bacterium *Escherichia coli* (Migula, 1895) (Liou, et al., 2000). A

similar study with IBP genes from plants using *L. perenne* as model organism showed ice interactions of such proteins when expressed in *E. coli* but without a clear TH gap, though low and insufficient concentrations might have caused this lack of antifreeze activity in the bacterium (Kumble; et al., 2008). And lately IBPs from fungi of the genus *Leucosporidium* (Fell, Statzell, Hunter et Phaff, 1970) were expressed in yeast *Pichia pastoris* ((Guilliermond) Phaff, 1956) with a TH gap of 0.42 K, reached concentrations in culture media of over 270 mg/l and remained there active and stable for six days of fermentation (Lee, et al., 2013).

An earlier cross–species experiment with AFPs in fish of the northern hemisphere also demonstrated their functionality in a teleost normally devoid of such proteins (Fletcher, Kao & Fourney, 1986). Furthermore, when in solution, ice–active substances (IASs) of one Antarctic diatom *Navicula* sp. (Bory de Saint–Vincent, 1822) could enhance the survival rate of a temperate marine diatom *Nitzschia frustulum* ((Kützing) Grunow, 1880) and another Antarctic diatom *Fragilariopsis* sp. (Hustedt, 1913) that lack IASs under natural conditions (Raymond & Knight, 2003).

TH based on active AFPs has been found in Antarctic life forms other than notothenioid fishes, such as in the nudibranch *Tergipes antarcticus* (Pelseneer, 1903) (Kiko, Kramer, Spindler & Wägele, 2008), the copepod *Stephos longipes* (Giesbrecht, 1902) (Kiko, 2010) and the nemertean worm *Antarctonemertes valida* (Bürger, 1893) (Waller, Worland, Convey & Barnes, 2006). Furthermore, even unicellular organisms have IBPs, such as Arctic yeast *Leucosporidium* sp. present in a frozen 1 m core from a freshwater pond soil in Spitsbergen (Lee, et al., 2010).

Other organisms that express IBPs include fungi such as *Flammulina populicola* (Redhead et Petersen, 1999) (Raymond & Janech, 2009), snow moulds (Hoshino, Xiao & Tkachenko, 2009), the diatoms *Navicula glaciei* (Van Heurck, 1909) (Raymond & Janech, 2003) and sea ice diatom *Fragilariopsis kerguelensis* ((O'Meara) Hustedt, 1952) (Janech, Krell, Mock, Kang & Raymond, 2006), as well as one mobile alga *Chlamydomonas* sp. (Ehrenberg, 1833) (Raymond, Janech & Fritsen, 2009). IBPs and THA have even been found in Antarctic bacteria, in a strain of *Flavobacterium xanthum* (McCammon et Bowman, 2000) (Kawahara, et al., 2007), *Marinomonas primoryensis* (Romanenko et al, 2003) (Gilbert, Davies & Laybourn-Parry, 2005) and an ancient bacterium of Flavobacteriaceae from an ice core more than 400,000 years old (Raymond, Christner & Schuster, 2008).

AFPs are beyond doubt a fascinating example of protein evolution as they have evolved either independently from taxonomic relationships (Cheng & DeVries, 1989; Cheng, 1998) or present a unique case of gene exchange (Raymond & Kim, 2012) within the biochemical evolution of multiple species. These organisms have in common a group of molecules that serve the same protective function.

1.7 How AFPs work

DeVries discovered AFGPs in Antarctic fishes and suggested the means by which AFGPs work is by acting as ice growth inhibitors (1971). It was later proposed that the methyl groups of each amino acid residue could serve as hydrophobic areas and the hydroxyl groups of the sugars as hydrophilic areas in specific intervals along the entire length of the molecule. This could then form a structure with membrane characteristics that separates water molecules in the liquid phase from the solid surface of the ice crystal and thus interfere with ice crystal development (Vandenheede, Ahmed & Feeney, 1972). The adsorption–inhibition model was then suggested after the FP depression was discovered to be caused by a non–colligative mechanism with AFGPs not altering the ice crystal structure but instead adhering to the crystal's surface and thereby inhibiting its growth (Raymond & DeVries, 1977).

It has been demonstrated that the variety of proteins and glycopeptides providing different polar fishes with protection from ice formations have an astonishing similarity in their physical mechanisms (Raymond, Wilson & DeVries, 1989). Attachment of AFGPs occurs on the prism face of the hexagonal ice crystal (Fig. 1.4 A & B), restricting its extension yet inhibiting only in limited form the attachment of water molecules to the larger basal planes (Raymond, et al., 1989). This leads to a pyramidal extension on both sides of the ice crystal in the presence of AFPs and AFGPs when the temperature falls below the FP. During the initial phase of ice crystal propagation one can observe this very characteristic ice crystal behaviour as a bi–pyramidal growth with a hexagonal basis (Fig. 1.4 C) before a change to an extension in the form of a rectangular crystal occurs (Fig. 1.4 D; for video demonstration see digital file Appendix A.3).

The binding of fish AFGPs to the ice structure was found to be the result of a different number of hydrogen bonds available to form between the hydroxyl groups of disaccharides from the peptides and the ice surface, leading to a nearly complete halt of any crystal growth due to a nearly irreversible attachment on a chemical basis (Knight, Driggers & DeVries, 1993). Thus confirming earlier suggestions that the TH difference between smaller and larger AFGPs is caused by molecule length alone and not conformations, and that hydroxyl groups are presented on one side of the peptides at regular intervals (Bush, Feeney, Osuga, Ralapati & Yeh, 1981). The extreme effectiveness of Antarctic fish AFGPs 1–5 was demonstrated by THA and the influence on ice crystal shape and growth at concentrations from above 1% (w/w) (Knight, DeVries & Oolman, 1984). Further, Knight and colleagues found these proteins to actively inhibit ice recrystallisation even at very low concentration levels of circa 10^{-9} g/g or 10^{-12} M.

The interactions of AFPs from the spruce budworm *Choristoneura fumiferana* (Clemens, 1865) have been visualised by labelling AFPs with green fluorescent protein (GFP) (Pertaya, et al., 2007). The authors illustrated with the resulting AFP–GFP conjugates how higher protein concentrations are present at all six corners of a small hexagonal ice crystal. Furthermore, during crystal growth the already attached AFPs were overgrown by ice and additional AFPs adhered to the prism face. It is noteworthy that some AFPs attach also to the basal planes (Pertaya, Marshall, Celik, Davies & Braslavsky, 2008) and warrant a classification of some insect AFPs as hyperactive with prism as well as basal plane affinity or moderate AFPs, like in fish, binding to the six prism planes only (Bar-Dolev, Celik, Wettlaufer, Davies & Braslavsky, 2012).

The solid bonding of AFPs onto ice appears to be a universal principle of antifreeze molecules. In one of the latest experiments on THA, the AFPs of *T. molitor* were also discovered to bind nearly irreversibly to the ice crystal surface (Celik, et al., 2013).

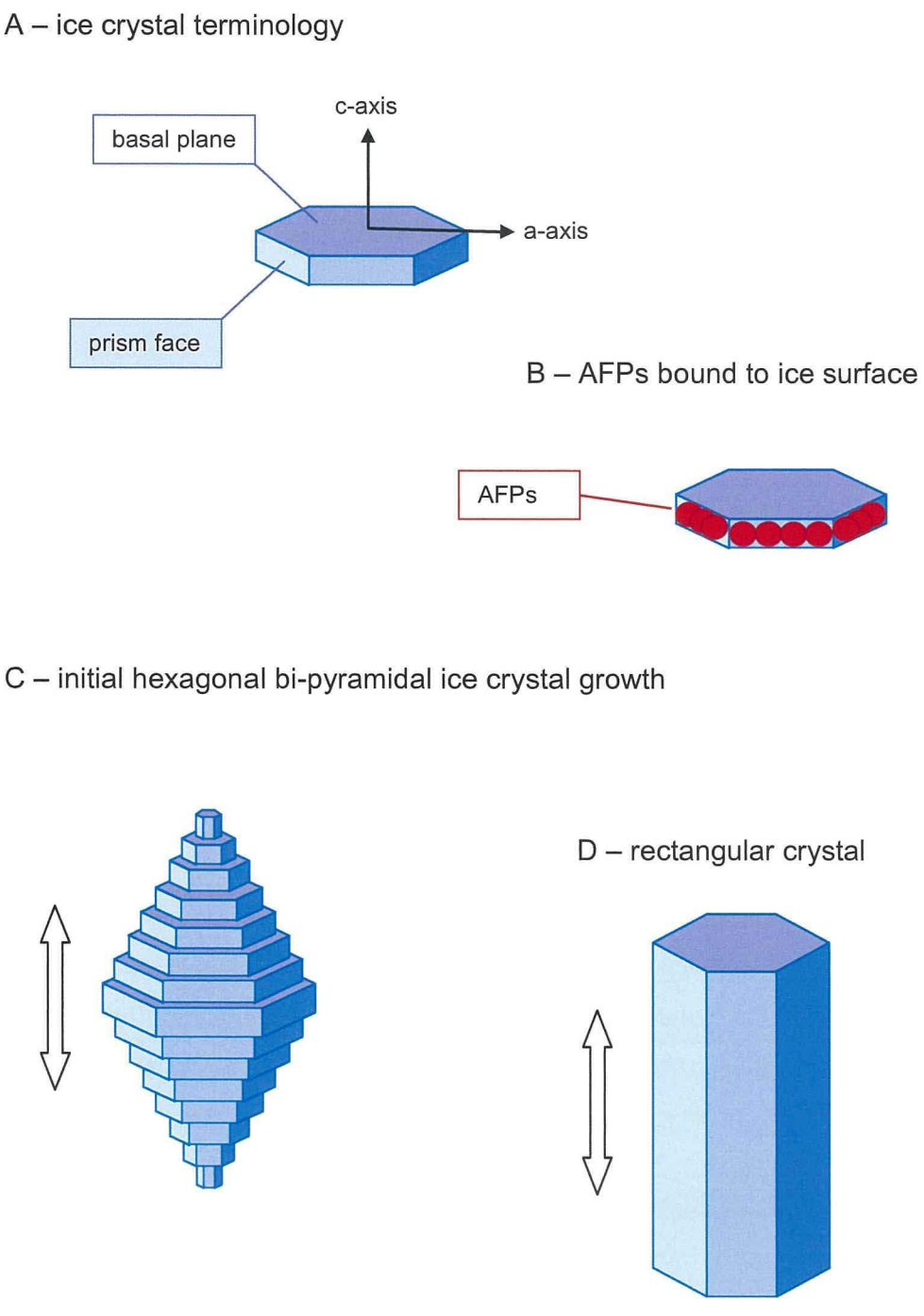


Figure 1.4 Schematic representation of ice crystal growth inhibiting AFGPs.

Illustration of fish AFPs' interaction with a seed ice crystal resulting in the characteristic initial hexagonal and bi–directional crystal growth. (A) Single crystal terminology. (B) AFPs (in red) attached to the prism face of the crystal but not the basal planes. (C) Ice propagation as initial growth of hexagonal bi–pyramidal crystal. (D) Ice crystal growth in rectangular form due to AFPs' interferences as can be observed during nanolitre osmometry (see Chapter 5).

A visual experiment involving fluorescently labelled AFPs from a strain of the bacterium *M. primoryensis* demonstrated that these hyperactive AFPs with a high antifreeze capacity (Gilbert, et al., 2005) cover the surface of an ice crystal with a green overcoat and thus attach to the ice plane but are released and dispersed into the solution when the crystal melts (Celik, et al., 2010). It was further demonstrated that mixtures of fish AFP types I, II and III resulted in slight changes of the ice crystal shapes, yet the THA of the different mixtures remained unaffected, thus the molecules act each on their own and the different types do not need to interact with each other in order to bind to the ice planes (Chao, DeLuca & Davies, 1995).

Since the 1970s it has been established that AFGPs work effectively as ice growth inhibitors, yet four decades on there is still debate as to the exact nature of the mechanisms by which the proteins bind to the specific planes of the ice crystals to inhibit ice propagation. This is because the scale of investigations into the protein–ice interactions are at a molecular level and thus the primary reason for the lack of experimental evidence into the exact nature of the inhibition (Nada & Furukawa, 2012). Nonetheless, an increasing number of experiments on AFP analogues now aim to further our knowledge and to produce effective cryoprotectants for practical applications (Balcerzak, Febbraro & Ben, 2013).

1.8 Unique evolution of AFGPs in Antarctic notothenioid fishes

H. F. Osborn (1857 – 1935) termed the concept *"adaptive radiation"* (1902) and elaborated on the concept for mammalian evolution (1910, pp. 22-32) while giving credit to Charles R. Darwin (1809 – 1882) and even Jean–Baptiste de Lamarck (1744 – 1829) for the discovery. The evolution of AFGPs in ancestral notothenioid fishes enabled this taxon to undergo an extensive adaptive radiation into the vacant niches of the supercooled waters of the Southern Ocean, as the majority of the original inhabitants emigrated to avoid the cold or perished through freezing (Chen, DeVries & Cheng, 1997b; Near, et al., 2012). This was a rare evolutionary event, which only occurred once in Antarctica and the notothenioid phylogeny, and the ectoparasites carried by these fishes would also have experienced strong selective pressure to develop strategies to cope with sub–zero temperatures. The convergent evolution of AFGPs in polar fishes of the north and south was based on a

common teleost genome with pre–cursor proteins at hand composed of AAT repeats but originating from dissimilar genes in the fishes from different regions (Chen, et al., 1997a, 1997b). Although it could not be further clarified within the scope of this PhD, it seems unlikely that Antarctic leech parasites as annelids would have evolved independently a set of invertebrate AFGPs of extremely similar if not identical structure to their local fish hosts or even identical or extremely similar gene sequences to the fish encoding such AFGPs. Only a future genomic study into the Antarctic piscicolid fauna can provide us with a conclusive answer, yet it is highly questionable whether any underlying genes even exist in leeches to allow for a convergent evolution to form analogous protein structures based on similar genetic motifs.

1.9 Horizontal gene transfer (HGT)

An alternative, although controversial case for the presence of AFGPs in parasites of notothenioids may be by a horizontal or lateral gene transfer (HGT or LGT). This mechanism has been suggested as a key element in prokaryotic evolution (Hotopp, et al., 2007) and recently a megaplasmid of 165 kb in *Arthrobacter nicotinovorans* (Kodama, Yamamoto, Amano et Amachi, 1992) was shown to serve as vehicle for HGT amongst bacteria (Mihasan & Brandsch, 2013). HGT provides an additional evolutionary pathway to vertical transmission from parent to offspring as the result of natural selection after Darwin (1859). There are increasing numbers of studies in recent years that reveal possible cases of gene transfers in eukaryotic systems, a process that until recently was regarded as a near impossible or extremely rare evolutionary event in higher taxonomic groups (Boto, 2010). In vertebrates, for example, HGT has been used to explain a recorded transfer of genes from Squamata to Ruminantia some 40–50 mya (Kordis & Gubensek, 1998), as well as a gene transfer for endogenous AFPs between different fish species (Graham, Lougheed, Ewart & Davies, 2008). Despite the unknown nature of transfer mechanisms via parasites, viruses or transposons, it has been shown that under natural selection any advantageous trait, such as an antibiotic resistance for bacteria (Kurland, 2005) or an antifreeze for a fish (Graham, et al., 2008; Graham, Li, Davidson & Davies, 2012; Sorhannus, 2012), has the potential to establish itself in a new species genome. Viruses (Matveev & Okada, 2009) and transposons (Pace et al.,

2008) have been identified as promoting gene transfer between species, which can lead to incorporation of host genes in a parasitic genome, as has been demonstrated in retroposons in Salmonoidei, which presumably was a viral mediation system to transfer genes to its trematode parasite, the blood fluke *Schistosoma japonicum* (Katsurada, 1904) (Matveev & Okada, 2009).

A gene transfer with metabolic significance has been demonstrated in the host–parasite system of the microscopic alga *Emiliania huxleyi* ((Lohmann) Hay et Mohler, 1967) and its DNA virus EhV–86, involving the transfer of seven genes for the pathway and complete *de novo* biosynthesis of sphingolipids (Monier, et al., 2009). Based on the law of parsimony the authors suggested the acquisition of the entire metabolic pathway by the virus from its eukaryotic host in a series of HGT events and a gradual transfer of all genes involved. These lipids and their derivatives play fundamental roles in eukaryotes and some prokaryotes for cell responses and signal pathways (Hannun, Luberto & Argraves, 2001) and the biosynthesis of ceramide, a sphingolipid known to play a central role in eukaryotic stress responses and inducing apoptosis or programmed cell death (Guenther, et al., 2008). One of the latest examples of HGT was demonstrated by sequencing a 160 kb genome fragment, which showed that a lateral transfer for the AFP type 2 gene between three fish species with distant phylogenetic relationships had occurred (Graham, et al., 2012). The authors interpreted the highly conserved exon and intron sequences as evidence of a natural HGT event in vertebrates.

The combination of AFGPs available in the food source and the potential for HGT events presents the leech parasites simultaneously with two evolutionarily unique opportunities for ecophysiological and genomic adaptations to their ice–laden environment. As the Antarctic fishes have evolved at least eight types of AFGPs, distinct only in molecular length or mass, and in addition an antifreeze potentiating protein (AFPP) (Jin & DeVries, 1997) enhancing the THA further, any parasite feeding on such a host could theoretically gain these life–saving components *ad libitum*. The digestive absorption and distribution of AFGPs within the body tissues would provide protection against any intra– and extracellular freezing events. The parasites could therefore take advantage of the abundant supply of AFGPs in their normal diet in order to survive in the hostile conditions in which their notothenioid fish hosts feed and breed. If the leech had no free living life stage and assuming it has access to AFGPs

sourced from the fish host blood, then the adult parasite were under less selective pressure to evolve antifreeze genes independently in a convergent development. However, the juvenile stage of Antarctic piscicolids is benthic, thus these animals must have protective mechanisms in order to withstand the freezing water conditions.

There is an increasing number of studies providing examples of HGT in the sea ice zone as a habitat of frequent genetic exchange of genes relating to functions of TH or ice–binding to inhibit ice crystallisation (Janech, et al., 2006; Kiko, 2010). Consequently, there could well be an evolutionary advantage for the leech to carry antifreeze genes in its genome to provide freeze protection at the juvenile stages prior to the initial blood meal on its definitive fish host as the main source of AFGPs for the adult.

1.10 Outline and aims of thesis

This research aims to establish whether hirudinean fish parasites utilise specific macromolecules for antifreeze protection in the sub–zero living conditions of Antarctica, where their notothenioid hosts underwent an adaptive radiation based on AFGPs. The principal aim of my PhD is to understand the mechanisms leading to the co–existence of a marine group of parasites with their fish host species after the major environmental change of sub–zero water temperatures occurred in the Southern Ocean. This includes exploring the structure and role of AFPs in piscicolids and the potential pathways to how they arose. The host–parasite system also represents a unique opportunity to study processes of co–evolution as well as potentially a HGT in eukaryotic evolution. A further aspect of this thesis was to potentially answer the intriguing question in terms of Darwinian evolution as to whether environmental constraints have led to a gene transfer outside the classic theorem of vertical gene flow in this Antarctic sea ice biota.

I introduce in **Chapter Two** the sampling locations for my field work and provide information to extend our limited knowledge on the biology of Antarctic Piscicolidae. I present a new marine leech species, *Megapodibdella kirsteni,* gen. et sp. nov., as well as new host records and comments on the biogeography and abundance of *Cryobdella antarctica* and *Cryobdella levigata* along with field notes on sampling technique,

experiments to parasite attachment and their heat tolerance. In **Chapter Three** I investigate the presence and distribution of AFGPs within Antarctic leeches by use of immunohistochemistry (IHC) and a two step immunological process for the visualisation and localisation of AFGPs *in situ* on tissue slides of *C. levigata*. **Chapter Four** focuses on the characterisation of these peptides by mass spectrometry (MS) and analyses their chemical structure in piscicolid samples of *C. antarctica* and *C. levigata*. The antifreeze activity of AFGPs embedded in parasite tissues is tested in **Chapter Five** with nanolitre osmometry (NO) to illustrate the relevance of the peptides *in vivo* for these two marine invertebrates living in ice–laden waters. **Chapter Six** uses messenger RNA (mRNA) technology to characterise the underlying gene motif of fish AFGPs in the genomes of *C. antarctica* and *C. levigata*. The concluding **Chapter Seven** provides a synthesis of findings and future research outlook.

CHAPTER TWO

A new marine leech and Antarctic piscicolid biology

2.1 Specimen collection in McMurdo Sound, Ross Sea

A variety of fish, predominantly belonging to the genus *Trematomus*, with their associated leech parasites, were caught using a simple rod and line with various baits of either fish meat or coloured rubber pieces in 5–35 m depths. The different localities in McMurdo Sound of the Ross Sea along the shoreline of Ross Island (Fig. 2.1) were from north to south:

Evans Wall (EW)

A sampling site at a glacier wall close to Cape Evans,

77°38'28.89"S, 166°31'15.38"E.

Inaccessible Island (INI)

The sampling location was next to the island which is also close to Cape Evans,

77°39'17.13"S, 166°22'23.10"E.

Haskell's Hole

A location situated on the sea ice in McMurdo Sound to sample deep water fish,

77°46'52.1"S, 166°18'42.6"E.

Winter Quarters Bay (WQB)

This is the harbour site off the U.S. Antarctic station in McMurdo Sound,

77°50'47.07"S, 166°39'0.87"E.

Scott Base

A site close to the shoreline at the station,

77°51'1.57"S, 166°46'3.63"E.

These ice fishing locations were within a day's return excursion from Scott Base. Fish and leeches were transferred into chilly bins containing cool and aerated water for transport to the holding aquaria at Scott Base where they were kept until needed at a temperature of no more than –1.0°C under a constant flow of fresh seawater.

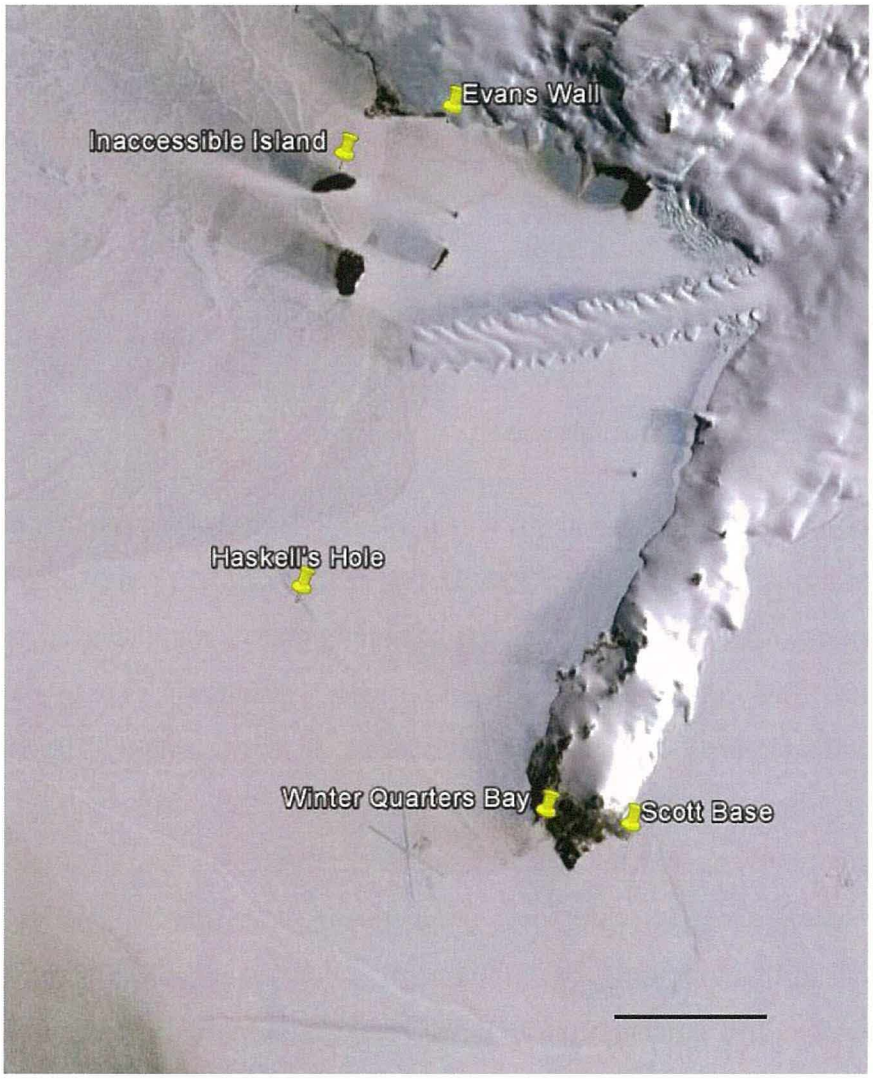


Figure 2.1 Aerial map of sampling locations in McMurdo Sound, Antarctica.

Aerial view of the field sites in McMurdo Sound, Antarctica, from Scott Base to Inaccessible Island at an eye altitude of 46 km above mean sea level (scale bar 5 km; copyright U.S. Geological Survey, Google Earth v.7.1.1.1888, 2013 Google Inc.).

2.2 A new marine fish leech *Megapodibdella kirsteni,* gen. et sp. nov., from the Ross Sea, Antarctica (Hirudinea, Piscicolidae)

2.2.1. Abstract

A new leech species is described, which was collected in McMurdo Sound, Ross Sea, off an Antarctic eelpout *Lycodichthys dearborni* (DeWitt, 1962) during the austral spring of 2010. It is the first record of a piscicolid on the genus *Lycodichthys*. The external morphology was examined and found to differ from any presently described piscicolid of the Southern Ocean. This leech is small and has the largest presently recorded ratio of a posterior sucker diameter to body width and length (body length to sucker ratio 1:0.6). The extreme size of the sucker in relation to body size is presumably related to the substantial external mucus layer of the fish host species. This leech belongs to the new genus and species *Megapodibdella kirsteni* based on its unique morphological characteristics.

2.2.2. Material and Methods

The collection of leech specimens was performed by targeting benthic fish species using a bottom trap at Haskell's Hole, 77°46'52.1"S, 166°18'42.6"E, in McMurdo Sound, Antarctica. Fishes were caught at a depth of approximately 450 m and inspected for the presence of leeches. The discovered specimens were attached to an Antarctic eelpout *Lycodichthys dearborni* (DeWitt, 1962) collected on 4th November 2010. This fish host is only known from McMurdo Sound of the Ross Sea and belongs to the genus *Lycodichthys* (Pappenheim, 1911) of the family Zoarcidae or eelpouts of the Perciformes which contains only *L. antarcticus* (Pappenheim, 1911) and *L. dearborni* as two species endemic to the Southern Ocean (Anderson, 1990; Kelley, et al., 2010). Two leeches were preserved in 70% ethanol after an initial observation in the field. A total of five individuals were observed in the field on a single fish host. At time of capture, three were at the base of the right and two at the base of the left pectoral fin. The latter two either actively or passively moved to the region of the dorsal fin during handling and transportation to Scott Base.

2.2.3. Description

Megapodibdella gen. nov.

Type species *Megapodibdella kirsteni* sp. nov.

Diagnosis:

External characters:

Small leech of circa 4 mm length and 0.6–1 mm body width. Cylindrical, short body with smooth surface. Body widening with narrowest point 0.6 mm in diameter below anterior sucker of trachelosome to 1 mm urosome at mid body length, not narrowing towards posterior end. Maximum/minimum body width to length ratio 1:4/1:6.6.

Somites lack segmental external features of tubercles, papillae, ocelli, marginal flanges or obvious pulsatile vesicles. Gills, branchia or tentacles absent.

Body pale translucent, without darkened or brown pigmentation or any pattern present in live animals before fixation. Indistinct division into trachelosome and urosome. Urosome without definite shoulders, annulation indeterminate. Not obvious clitellum with male and female gonopores. Anterior and posterior sucker thin and circular.

Small anterior or oral sucker of 1 mm diameter demarcated or separated from trachelosome or rest of body, typical for Piscicolidae (Llewellyn, 1966), excentrically attached to trachelosome, smooth edge and surface with diameter equal to urosome. Mouth pore located centrally in ventral side of anterior sucker. On dorsal side no eyes, eye–like spots, ocelli or pigment patches. Tubercles, papillae or tentacles absent.

Very large, powerful posterior sucker centrally attached to urosome and directed towards the posterior. Diameter of 2.5 mm greater than body width at 2.5–4.2 times, body length to sucker ratio 1:0.6. Edge not rough but eccentrically undulated with smooth surface. Papillae or ocelli absent.

Internal characters:

Restricted to seven blood filled intestinal chambers (ICs) observed in posterior third of the body (Fig. 2.2 A). Not obvious internal pulsatile vesicles in one or more pairs per somite. No crop chambers with lateral processes visible in anterior half.

This description is based on one leech functioning as holotype, as host fish–handling permit considerations made it impossible to secure all the five individuals discovered in the field and to preserve more than one paratype. Consequently, no further information on the internal anatomy, such as the digestive, reproductive and coelomic systems, can be provided. A yellow–green discoloured content of the intestine was regurgitated upon transfer in ethanol.

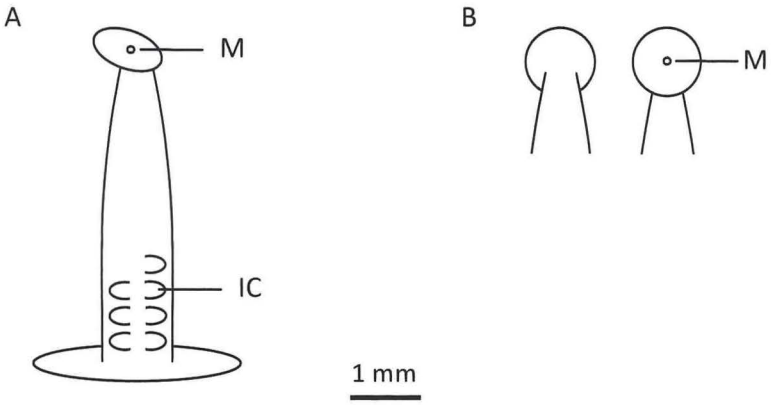


Figure 2.2 *Megapodibdella kirsteni*, gen. et sp. nov., schematic drawings of characters.

(A) External overview of new leech with seven intestinal chambers (ICs) and mouth pore (M). (B) Anterior sucker in dorsal (left) and ventral (right).

2.2.4. Taxonomic summary

***Type host*:** Antarctic eelpout *Lycodichthys dearborni* (DeWitt, 1962), other hosts unknown. First record of a leech parasite from *L. dearborni* and generally a first record for any fish species belonging to the Antarctic Zoarcidae.

***Type location and collection date*:** Collected in McMurdo Sound at 77°46'52.1"S, 166°18'42.6"E and circa 450 m depth on 4th November 2010 by Jürgen Bertram Kolb.

***Site of infection*:** Attached to the body at the base of both pectoral fins towards the posterior with the fins covering the parasites. In resting position the fish lies with the fin against its body surface.

***Holotype & paratype*:** Both in 95% ethanol will be made available and deposited at Te Papa, Wellington, New Zealand.

***Etymology*:** Genus for its main feature named after the Greek μέγαϲ or *megas* for *big* and πόδι or *podi* for *foot* with βδέλλα or *bdella* for *leech*. Species named to honour my wife Kirsten.

2.2.5. Remarks

A new genus was attributed to this leech based on the unique external morphology, namely the very large posterior sucker used to attach to the host, the total body length which is small or very small for Antarctic leeches and the unique ratios of the sucker to body dimensions.

M. kirsteni is the first leech recorded on *L. dearborni* in the Antarctic marine habitat. Furthermore, no previous report exists (Utevsky, 2007) to any piscicolid found on any fish species in the Antarctic belonging to the family Zoarcidae. Although the sex of the host specimen for *M. kirsteni* could not be determined, it was a mature eelpout which are known to be slow growing with a life expectancy of generally less than 8 years and never more than 9–12 years (Anderson, 1990, p. 256). In Antarctica a number of zoarcid species have only records from specific localities and exhibit limited or no geographic distribution around the continent with *L. dearborni* being endemic with a restricted geographic distribution to the

McMurdo Sound (Anderson, 1990). The holotype of *L. dearborni* was caught in McMurdo Sound circa 3 km south off the coastline from Observation Hill next to McMurdo Station at 77°53'S, 166°44'E in a wire trap at the bottom in 585 m depth (DeWitt, 1962).

The body ratios of *M. kirsteni* are unique among records for any genus belonging to the family Piscicolidae. *M. kirsteni* is thereby ranked as a new genus and placed provisionally in the subfamily Platybdellinae after Sawyer (1986b, pp. 591-630, 660-669) and the taxonomic identification key constructed for Antarctic fish leeches by Utevsky (2007).

The placement of the new species within the subfamily Platybdellinae is based on the three main external characteristics for this group after Utevsky (2007) as a body that is smooth or has only small tubercles and papillae, the indistinctly divided annuli and finally the absence of pulsatile vesicles. However, in many aspects, the external features of this new leech do not match with typical traits of the known marine genera of this subfamily after Sawyer (1986b, pp. 660-669) and Utevsky (2007). According to Utevsky's key the new leech shares most similarities with species of the genus *Cryobdella* (Harding, 1922). However, *M. kirsteni* deviates from the four known leech species that constitute the exclusively Antarctic genus *Cryobdella* in that these have a well developed anterior sucker with a serrated edge of circa 160 fine teeth, some pigmentation and a posterior sucker facing towards the ventral side that is smaller in terms of their body ratios.

Another genus present at this site is *Glyptonotobdella* (Sawyer et White, 1969), a monotypic genus with the type species *Glyptonotobdella antarctica* (Sawyer et White, 1969). It is found in McMurdo Sound and was originally thought to be a permanently associated and feeding parasite of the giant isopod *Glyptonotus antarcticus* (Eights, 1852). One specimen of this crustacean with presumably leech egg cocoons on it was trapped accidentally in the water filter system of Scott Base in 2010 (pers. obs.). Therefore, this host seemed present in McMurdo Sound at the time of the discovery of *M. kirsteni*. The isopod's leech has also been recorded on two members of the crocodile icefish genus *Chionodraco* (Lönnberg, 1906) and *Chaenocephalus aceratus* (Lönnberg, 1906). However, the type species of *G. antarctica* is large with up to 26 mm body length, with 10–12 pairs of lateral tubercles on the urosome, 5 red–brown pigment bands on the trachelosome and 14 such bands on the urosome, has eye–like spots on its anterior sucker and a small, cup–

shaped posterior sucker with striped pigmentation. Therefore, *M. kirsteni* does differ significantly in its external appearance from *G. antarctica*.

M. kirsteni is also unlike leeches of the genus *Notostomum* (Levinsen, 1882) which are found in the Arctic, are large and often more than 100 mm in body length, have a subcylindrical body shape and a mouth pore at the anterior margin of the oral sucker. The genus *Platybdella* (Malm, 1863) is characterised by a relatively large, discoidal posterior sucker but according to Diesing (1850, pp. 439-443 as Ichthiobdella (Blainville)) and Malm (1863) of smaller proportions than in *M. kirsteni*. The type species of the genus is *Platybdella anarrhichae* (Diesing, 1859), found in the Arctic and North Atlantic as longer (22 mm) and wider (2.75 mm) specimens than the new leech, found confined to the gill cavity of the host and according to Malm with a different shaped and sized posterior sucker (1863, Plate 4, Fig. 14 A & B). The length of 25 mm and width of 2 mm was later confirmed by Leigh–Sharpe (1916), but the posterior sucker described to be folded ventrally and to reach a diameter only twice that of the 2 mm wide anterior sucker. *Platybdella fabricii* (Malm, 1863) has a relatively wide urosome and a posterior sucker of a much smaller diameter than the widest body width. The species described by Selensky (1914) as *Crangonobdella murmanica*, as synonym to *P. fabricii* (Sawyer, 1986b), is 13–20 mm long, has a division between the urosome and trachelosome, pigmentation and pattern on its finely annulated body, three pairs of eyes on the dorsal side of the anterior sucker and most importantly a posterior sucker of approximately the diameter of the maximum body width. Finally, *Platybdella olriki* (Malm, 1863) has a posterior sucker of a slightly smaller diameter than its posterior body. And the closely related *Platybdella pinnara* (de Silva et Burdon–Jones, 1961) in the Celtic Sea has a laterally folded posterior sucker to enclose fin–rays. Additionally, the genus *Platybdella* comprises species exclusively described from the Arctic and adjacent seas without Antarctic representatives. Sawyer (1986b, p. 611) stated that potentially geological changes of the world's oceans resulted in the biogeographic separation of the Arctic genus *Platybdella* from its nearest related marine genera in the Antarctic as *Cryobdella–Glyptonotobdella* as an unsolved issue in polar biology and in need of further research.

It should be noted that the traditional use of annulations for taxonomic identification and phylogenetic classification has previously been found the least reliable character in leeches

(Siddall, 2002). Consequently, the observed lack of annuli and external subdivision of somites in *M. kirsteni* might not provide further information for its description and taxonomic classification.

The lack of the single morphological character of one pair of externally visible pulsatile vesicles per somite excludes *M. kirsteni* from the subfamily Piscicolinae. However, in this subfamily several marine genera without eyes were discovered in the southern parts of the Pacific Ocean in waters of New Zealand, Tasmania and Australia (Sawyer, 1986b, pp. 671-677).

The new Antarctic species has further neither a body with large tubercles nor distinctly divided annuli or visible pulsatile vesicles under the epidermis as the three main characteristics required for its classification as a Pontobdellinae after Utevsky (2007). Additionally, Llewellyn (1966) and Sawyer (1986b) regarded the absence of distinct external tubercles on each or at least the major annulus of each somite as a key external characteristic for this subfamily. However, Sawyer (1986b, pp. 669-671) stated that the presence of two pairs of pulsatile vesicles per somite was an important internal character for Pontobdellinae. Such presence or absence could not be ascertained for the new species and might result in a revision of the proposed taxonomic relationship after an internal examination on additional material in the future.

As eelpouts have a considerable quantity of external mucus, and amongst the fish species encountered in the field, they possess the least feasible handling properties. Thus, it seems likely that a leech would require a specially adapted organ to maintain a solid attachment. Although Antarctic leeches generally have a large posterior sucker for their life style as long–term ectoparasites (Utevsky, 2007), this new leech has the anatomical trait in an apparently extreme form. The benthic eelpouts display little spontaneous activity and if are only slow moving (Pörtner, Lucassen & Storch, 2005, p. 100). I predict, therefore, that *M. kirsteni* is a host–specific piscicolid to this one fish species *L. dearborni* endemic to the sampling area of McMurdo Sound.

The base of the pectoral fin as attachment position has previously been recorded from other marine fish leeches (Knight-Jones, 1940). The location of *M. kirsteni* attachment was close to a biomechanical joint where thinner skin facilitates blood sucking. More

importantly, this position on the host offers better protection from abrasion impacts which place strenuous demands on the leech grip on a mucilaginous fish. A brush against hard substrate might generate a force too strong for the suction power of the small leech and lead to its dislodgement. The reattachment experiments performed with both *C. antarctica* and *C. levigata* (see 2.7.3) are suggestive that a loss of host contact results in the termination of the leech–host association. The basal positions behind the pectoral fins represent the two microhabitats offering best protection on the fish.

M. kirsteni fulfils the criteria for a new species and genus within the subfamily Platybdellinae. However, I am unable to conclusively determine where *Megapodibdella* should be placed within the present classification of Piscicolidae. The temporary identification as a genus belonging to Platybdellinae remains subject to verification and possibly revision in the future as further information of the internal anatomy can only be gained after additional specimens are collected for examination.

2.3 New fish host records and comments on parasite abundance

Piscicolids were observed attached to their natural fish host species in the course of my field research campaign. A number of new records of fish species serving as hosts were made.

2.3.1 New host record for *Cryobdella antarctica* (Epshtein, 1970)

I report one new fish host record for *C. antarctica* which, according to the latest literature, has not been found on *Trematomus newnesi* (Boulenger, 1902) before. The new host *T. newnesi* was caught at Evans Wall (EW) on 16.11.2010 with a body mass (BM) of 158 g and fish body standard length (SL), defined as from snout to base of caudal fin, of 19.7 cm. Previous host information to species level in this fish genus was restricted to the collection of a single specimen from the body of one *Trematomus bernacchii* (Meyer & Burreson, 1990; Utevsky, 2007). The latter host can be confirmed with many sightings of 1–49 individuals on *T. bernacchii* from EW (individual fish specimens ranging from BM of 87 g and SL of 15.8 cm to fish with BM=134 g and SL=18.7 cm) and Inaccessible Island (INI) (one fish of BM 177 g with SL 19.2 cm and second of 439 g at 22 cm). Yet circa 23 km to the south not a single *C. antarctica* was found on any *T. bernacchii* caught in Winter Quarters Bay (WQB).

2.3.2 New host records for *Cryobdella levigata* (Harding, 1922)

C. levigata was initially suggested by Harding (1922) to have either *T. bernacchii* or *T. hansoni* as the most likely fish host species. Records have since been made on *T. hansoni* (Moore, 1938), *T. bernacchii* (Epshtein, 1970) and *Trematomus centronotus* (Regan, 1914) [accepted today as *Trematomus pennellii* (Regan, 1914) (Bailly, 2013)] (Meyer & Burreson, 1990; Utevsky, 2007). I can confirm this with actual sightings of this leech species on both *T. bernacchii* and *T. hansoni* during the sampling period at the McMurdo field sites of EW (fish specimens ranging from BM=99 g and SL=17.2 cm to BM=108 g and SL=17.3 cm), INI (fish of BM=61 g with SL=14.7cm) and WQB (fish of BM=141.2 g and SL=18.7 cm).

Sightings of *C. levigata* on *T. pennellii* were on one small fish of 34 g BM and 12.2 cm SL caught at WQB in a depth of circa 35 m on 22.11.2010 and two other fish of 32 g and 33 g BM with 11.8 cm and 12.1 cm SL, respectively, caught on 2.12.2010 from around 10 m depth

also in WQB. The deeper site, at the level of Hut Point, has an underwater topography with a steep wall as a rocky fall which separates the shallower bay from the McMurdo Sound area (Adam Marsh, pers. comm.).

Additionally, I can add three more new host records for *C. levigata* in McMurdo Sound. First, the cryopelagic borch *Pagothenia borchgrevinki* to which a single specimen of *C. levigata* was attached protruding from the operculum of the left gill cavity, caught under the ice in circa 5 m depth at EW on 16.11.2010, as well as attached to a borch which was no longer alive upon inspection of the fish at Scott Base caught at INI on 5.11.2010. The second new host species is *T. newnesi* of which a small fish of 37 g and 12.7 cm caught at INI on 5.11.2010 was infected by one *C. levigata*. Finally as the third new host record a similarly small *Trematomus nicolai* (Boulenger, 1902) with one *C. levigata* was caught on 25.11.2010 at Scott Base some 20 m off the coastline in circa 20–25 m depth.

2.3.3 Fish leech abundance in McMurdo Sound, Ross Sea

I present the first survey on the leech parasite fauna of demersal fish in McMurdo Sound. The highest intensity in terms of the number of individual leeches attached to one host recorded for *C. antarctica* was 49 specimens on a large (BM=439 g, SL=22 cm), gravid female *T. bernacchii* caught at INI on 7.11.2010. For *C. levigata* the highest was 32 specimens of various sizes attached in both gill cavities of a large *T. hansoni* from WQB. Therefore, the range of 1–36 individuals of *C. antarctica* and 1–4 for *C. levigata* found previously attached to a single host (Utevsky, 2007) can be considerably extended to 1–49 and 1–32 respectively.

All data gathered to obtain detailed information with respect to the absence or presence and abundance of piscicolids on different notothens in the McMurdo Sound is presented in table 2.1. This complete record of all fish species in the catchment area of the 2010 campaign provides sampling numbers for the different fishes at five sites ordered from north to south, although not all fish were accounted for due to logistical or time constrains and other considerations. The fishes caught were *Gymnodraco acuticeps* (Boulenger, 1902) (GA), *L. dearborni* (LD) and the notothens *Dissostichus mawsoni* (DM), *T. bernacchii* (TB), *T. hansoni* (TH), *Trematomus loennbergii* (Regan, 1913) (TL), *T. newnesi* (TNE), *T. nicolai* (TNI), *T. pennellii* (TP) and *P. borchgrevinki* (PB).

Table 2.1 Abundance data of piscicolid parasites on notothens in McMurdo Sound.

Prevalence with standard error (SE) and median intensity including range of *Cryobdella antarctica* (CA), *C. levigata* (CL) and *Megapodibdella kirsteni* (MK) on *Dissostichus mawsoni* (DM), *Gymnodraco acuticeps* (GA), *Lycodichthys dearborni* (LD), *Trematomus bernacchii* (TB), *T. hansoni* (TH), *T. loennbergii* (TL), *T. newnesi* (TNE), *T. nicolai* (TNI), *T. pennellii* (TP) and *Pagothenia borchgrevinki* (PB). Sampling sites for fishes ordered from north to south.

	Host			Parasite			
Collection site	Species	Total n	Infested n	Species	Total n	Prevalence ± SE [%]	Intensity (Range)
Evans Wall	TB	26	4	CA	5	15.38 ± 7.08	1 (1–2)
			2	CL	2	7.69 ± 5.23	1 (1)
	TNE	3	1	CA	1	33.33 (–)	1 (–)
			0	CL	0	0	0
	PB	19	0	CA	0	0	0
			1	CL	1	5.26 ± 5.12	1 (–)
Inaccessible Island	TB	27	2	CA	50	7.41 ± 3.77	(–) (1–49)
			3	CL	6	11.11 ± 6.27	2.0 (1–3)
	TH	19	0	–	0	0	0
	TNE	7	0	CA	0	0	0
			1	CL	1	14.29 ± 13.23	1 (–)
	TNI	15	0	0	0	0	0
	TP	1	0	0	0	0	0
	PB	4	0	CA	0	0	0
			1	CL	1	25.00 (–)	1 (–)
Haskell's Hole	DM	3	0	–	0	0	0
	LD	31	0	CA	0	0	0
			0	CL	0	0	0
			1	MK	5	3.23 ± 3.18)	5 (–)
Winter Quarters Bay	GA	23	0	–	0	0	0
	TB	10	0	CA	0	0	0
			1	CL	2	10.00 ± 9.49	2 (–)
	TH	102	0	CA	0	0	0
			31	CL	73	30.39 ± 4.55	1.5 (1–32)
	TL	6	0	–	0	0	0
	TNE	34	0	–	0	0	0
	TNI	17	0	–	0	0	0
	TP	9	0	CA	0	0	0
			3	CL	3	33.33 ± 15.71	1 (1)
	PB	16	0	–	0	0	0
Scott Base	TNI	1	0	CA	0	0	0
			1	CL	1	(–)	1 (–)

The prevalence including standard error (SE) and median intensity with range are listed for the encountered piscicolids *Cryobdella antarctica* (CA) and *Cryobdella levigata* (CL) for all sites and fish species, while *Megapodibdella kirsteni* (MK) was a sole record from Haskell's Hole and found attached to a single eelpout. The prevalence is given as the proportion of fish infested by leeches in percent. As the true population proportion is not known, the SE is provided, although only for samples with n≥5. Additionally, the median intensity with range is shown as the median number of conspecific parasites found living on a fish host. This provides an estimation of infection levels for the affected fish species. A mean intensity cannot be used due to the small sample sizes available for this study. Furthermore, parasites in general do not follow a normal distribution but show a tendency towards aggregations on some host individuals (Poulin, 1993; Rózsa, Reiczigel & Majoros, 2000). The few catches of higher numbers of piscicolids on individual fish demonstrate that these Antarctic ectoparasites are no exception to the rule.

In total, the following numbers of leeches were found attached to the different fish hosts (see Table 2.1):

On 63 *T. bernacchii* were 65 leeches (7 at EW, 56 at INI, 2 at WQB) and 73 *C. levigata* were found on 121 *T. hansoni* caught at INI and WQB. While no leech was discovered on any *T. nicolai* from INI or WQB there was one *C. levigata* on a single fish caught at Scott Base. For *T. pennellii* a total of three *C. levigata* on three fish at WQB were caught.

The notothens *T. newnesi* and *P. borchgrevinki* as new host records had two leeches each with a single *C. antarctica* on one *T. newnesi* at EW and one *C. levigata* on another at INI, while in *P. borchgrevinki* it was equally a *C. levigata* at EW and INI.

The 23 specimens of the naked dragonfish or ploughfish *G. acuticeps* collected for inspection during the one field season were exclusively caught from the seafloor at WQB. Although this sampling site is now known to harbour vast numbers of *C. levigata* on other fishes of the genus *Trematomus*, not a single piscicolid was attached to any dragonfish found at McMurdo Sound in the Antarctic spring of 2010.

In addition, the pelagic toothfish *D. mawsoni* was free of any piscicolid, while the benthic eelpout *L. dearborni* had neither *C. antarctica* nor *C. levigata* but only the newly discovered leech species *M. kirsteni.*

The prevalence values can vary across collection sites for the different notothens with the exception of *T. bernacchii* that was infected at all three sites in 2010 with two specimens at EW and one at INI inhabited by both *C. antarctica* and *C. levigata.*

T. hansoni without leech parasites at INI (n=19) differs from the group of *T. hansoni* at WQB (n=102) with a prevalence of 30.4% for *C. levigata*. This remarkable observation requires further investigation as both parasite species are present at INI and variations in life strategy or other biochemical characteristics might equally explain the situation.

T. newnesi had one *C. antarctica* attached to one host caught at EW in a sample size of three fish, while at INI one out of seven specimens of this host species had a single *C. levigata*. Thus, both parasite species attach to *T. newnesi* at sites merely separated by circa 4 km and situated less than 23 km due north of WQB. Remarkably, not a single *T. newnesi* was found infested in the much larger sample size from WQB (n=34) despite the presence of at least *C. levigata* as demonstrated by the infestation of one *T. bernacchii* with two leeches, three *T. pennellii* with one leech each and 31 individually infested *T. hansoni.* The supposedly krill eating and active semi–pelagic *T. newnesi* (Gon & Heemstra, 1990, p. 41) might be a slightly more active species or swimmer above ground than other members of its genus. As a consequence, *T. newnesi* might represent a more challenging target host for the ectoparasites. Although without conclusive evidence, the *Trematomus* species of McMurdo Sound might vary in either their attractiveness or ease of successful attachment for the piscicolids.

Equally discriminating behaviour of a potential host might explain the observation of complete piscicolid absence on *T. nicolai* at INI (n=15) and WQB (n=17) with merely one *C. levigata* discovered on a single fish caught at Scott Base.

It should also be noted that, despite the largest sample size of over 218 fish at WQB, not one *C. antarctica* was caught but exclusively *C. levigata*. Yet both piscicolid species were encountered at close proximity in McMurdo Sound at EW and INI. This puzzling result cannot be explained simply in biogeographical terms as the distance is too short for a segregation of parasite species to have occurred during the evolution of the Southern Ocean biota. The underwater topography and other aspects such as glacial freshwater run–off and resulting water currents and temperatures do not themselves provide a sufficient explanation of the

absence of *C. antarctica* from WQB. Instead I hypothesise that a potential interspecific competition avoidance has led to the larger *C. levigata* dominating this one part of the McMurdo Sound habitat.

A further intriguing aspect of piscicolid biology can be found in the distribution of individual parasites amongst their potential fish host specimens. On the premise that the likelihood of encountering a parasite on a host was independent of the presence of other leeches, or of any other factors, then the likelihood would be the proportion as one parasite on the total number of fish inspected. However, as independent findings the 49 *C. antarctica* attached to a single *T. bernacchii* at INI or 32 *C. levigata* on one *T. hansoni* from WQB are statistically highly improbable with a merely theoretical chance to be seen. Consequently, the observed leech clusters on some hosts demonstrate that the parasites' dispersion is not independent but based upon yet unknown factors. The aggregations can only be explained from a biological point of view and after further research into intraspecific as well as host–parasite interactions. These might include the need to find copulation partners despite the strong dependency on a constant supply of nutrients as well as antifreezes. As a result, in evolutionary terms, a strong preference might have developed to mate while in contact with the fish host.

A total of 151 leeches from five different sites were collected during one field research campaign and individual fish were seen to be infested with multiple leech species. The parasite abundances encountered completely contradict the general view amongst Antarctic scientists involved in marine research that leeches might generally be present but merely detected sporadically and in very low numbers. Furthermore, I provide irrefutable evidence that McMurdo Sound is a suitable area for field–based ecological research on the Antarctic piscicolid fauna.

2.4 Comments on host specificity of Antarctic piscicolids

During the field season, more than 32 individual *T. nicolai* were sampled at INI and WQB. However, none was found with a leech attached to it, although all these *T. nicolai* were of similar BM and SL to those individual fish of other species having parasites. Furthermore, a single *T. nicolai* specimen was found as a new host record with one *C. levigata* at Scott Base. Yet in behavioural tank experiments, no leech of either species attached to *T. nicolai* as new host. The same absence of any piscicolids when caught was observed for *G. acuticeps* (n=23) as well as *T. loennbergii* (n=6). In further artificial tank experiments no parasite of either species attached to either of these fishes.

Finally, close inspection of three *D. mawsoni* revealed no leech. This large and engibenthic species endemic to Antarctic seas (DeWitt, et al., 1990) was not exposed to leech attachment experiments as the fish held in one large aquarium at Scott Base were hardly moving and thus represented inaccessible targets for a small leech sitting with its posterior sucker attached on the hard substrate of the tank walls.

These observations derived from sampling as well as experiments in the field and could be simply due to limited information, as is typical for remote localities and in particular the polar regions. The complete lack of records for ectoparasites on some Antarctic fish species in the current literature (Utevsky, 2007) is further substantiated by my field observations. Future investigations geared towards sampling a higher number of fishes might result in these species being evaluated as potentially prohibitive to Antarctic fish parasitic leeches. Yet as demonstrated by my new fish host records, an increase in the number of fishes being inspected as well as an increase in sampling localities might likewise change the current leech–free status of some hosts. Nonetheless, apart from an obligate benthic coupling which would allow contact and any host–parasite interactions, there is another aspect one should consider for the encountered notothenioids in McMurdo Sound. These fish species lack any marked behavioural differences with generally low activity levels and speed of movements (Gon & Heemstra, 1990; Pörtner, et al., 2005). During my field work some host species had never had an ectoparasite attached although all fishes were caught with the same sampling equipment and technique at identical times and locations. This could potentially indicate a repelling chemical adaptation by some fishes to the presence of the leeches in their benthic

habitat instead of, or in addition to, any subtle behavioural differences. In a series of experimental trials to assess reattachment success and host avoidance, one might in the future include a detailed chemical analysis by mass spectrometry of the external mucus covering the different fish host species. This might determine whether the observed absence of piscicolids is due to slight variations in the life cycles or other attributes of particular notothens. In fact, it may be possible to determine whether it is caused by one or numerous biochemical adaptations of all or some parasite–free host species as a defence and typical outcome from an arms race in a host–parasite co–evolution (Dawkins & Krebs, 1979).

The two Antarctic leech species were never observed attached to a fin but always with the posterior sucker firmly attached to the body surface. There seems no variability in the site of infection as observed in other fish leeches (Nagasawa, Kikuchi, Arakane & Yusa, 2009).

In conclusion, for the general biology of piscicolids and their relevance in the Antarctic ecosystem with fish interactions, I can provide further evidence that at least the two species belonging to the genus *Cryobdella* have no host specificity in Antarctica, but can be found attached to different species of the genus *Trematomus*. I interpret this observation as a biological adaptation to the unique life at sub–zero temperatures when the slow metabolic rates of physiological processes lead to reduced fish mobility. This sessile and stationary behaviour of the fish resting on the seabed in the habitat they share with their predators, the piscicolids, resulted in a unique opportunity for the parasites in the Antarctic benthos. The fishes are active swimmers and generally do not move much and thus are easy prey for the leeches. Consequently, an evolutionary adaptation of a leech species to the life cycle of one specific fish host species was probably not subject to strong selective pressure, as failing to develop such a strategy would not have resulted in the extinction of that particular species by selection of phenotypes. This marine habitat is thus unlike any other. The reduced evolutionary pressure to focus on one host species amongst the benthic or demersal notothenioids is in my opinion a primary cause for the well developed piscicolid fauna encountered in the Antarctic. The field observations made during this study added host information for two out of 21 leech species known at present and provide the first information for a new taxon. The gathering of more information in the future might shed similar light on other piscicolids associated with benthic fishes. We are thus presented with an unrivalled field site of stable and available leech populations for fish parasite research.

2.5 Comments on biogeography

This study provides the first record of *C. antarctica* in the Ross Sea, a shoreline distance of circa 4,000 km to its nearest recorded finding to the east in the Davis Sea. There have been only two previous records for the genus *Cryobdella* across the entire Pacific sector of Antarctica between 60°W and 95°E, namely that of *C. antarctica* at 66°16'60.00"S and 110°32'0.00"E Newcomb Bay (Yang, 1987) and *C. levigata* close to Scott's winter quarters at Cape Evans in the Ross Sea (Harding, 1922; Utevsky, 2007). My work now confirms that the Ross Sea is populated by both *C. antarctica* and *C. levigata*, thus extending the geographic distribution of *C. antarctica*.

The density of leeches encountered during field work establishes their presence as two stable populations in the benthos of McMurdo Sound. Previous studies have suggested that benthic organisms of the coastal waters in Antarctica often exist in isolated populations (Hedgpeth, 1969). However, these populations, seemingly isolated over extensive distances, might in reality be part of an ocean–wide panmictic population. Deacon (1982) suggested that more research results in the revision of isolation patterns were needed. Indeed, the dearth of sampling across the Antarctic means that we still lack complete distribution information. Further investigations are, therefore, needed to clarify fundamental aspects of polar research such as whether species are isolated to specific regions or have a wider or continental distribution.

2.6 Interspecific competition avoidance on fishes as microhabitats

Specific observations were made on the ecology and behaviour of piscicolids. Leeches were more likely to be found attached to *T. hansoni* than to *T. bernacchii* as their host. Whether this is due to fish size or species abundance in the benthic community has yet to be determined. In principle, there seems no reason to assume a difference in the capability of either species to attach onto a host at any point. However, the distribution of the ectoparasitic leeches on their respective fish hosts was consistent. During my field work *C. levigata* was found, apart from the single instance mentioned above on a dead

P. borchgrevinki, exclusively in the gill cavities of their hosts. In contrast, *C. antarctica* was dispersed over the fish body.

A host can be regarded as a microhabitat for parasites (Aznar, Balbuena, Bush & Raga, 1997; Montero, Kostadinova & Raga, 2009). The total of all individuals on a host belonging to one species is referred to as an infrapopulation. Some fish caught were infested with both leech species so that two infrapopulations of sympatric species shared one habitat. I hypothesise that the difference in attachment locations represents an ecological niche segregation for these two congeneric ectoparasites. As a result, the parasites avoid interspecific interferences while they exploit a fish as a spatially very limited habitat for their energy and reproduction needs.

2.7 General notes from field work

Three additional observations drawn from the field work of this PhD:

2.7.1 How to catch fish leeches in Antarctica

The fauna of marine leeches is especially difficult to quantify due to their elusive habits as ectoparasites attached to their host. The method of choice to sample for piscicolid leeches is in *ad hoc* findings of fishermen or in the by–catch aboard fishing expeditions. This method of finding specimens is inefficient at best as leeches are often dislodged during the fishing process.

Nonetheless, there are some marine taxa for which it is far easier to undertake work in Antarctica than anywhere else on earth. This is mainly due to the extreme cold water conditions in which organisms evolved physiological and behavioural adaptations for survival. The demersal fish fauna consists mostly of lethargic moving species (Gon & Heemstra, 1990; Pörtner, et al., 2005). This is an important behavioural trait for their ectoparasites such as fish leeches, as they can more easily attach themselves effectively to their new host.

During the Antarctic field season of 2010 working in coastal waters of McMurdo Sound, piscicolid leeches were readily collected by targeting their respective demersal fish species hosts. The sampling technique involved the use of a simple fishing rod with a standard hook and line through ice holes at different localities. This simple yet efficient method yielded a good collection of fish leech parasites attached to fish. However, sampling efficiency and biological data acquisition for fish leeches could be further improved through manual collection by SCUBA–diving which has, however, one main disadvantage, namely that it is a major undertaking for an Antarctic field event bearing serious risks as ice diving operation.

2.7.2 CTMax – Critical thermal maximum experiments

I tested the heat tolerance of both leech species, *C. antarctica* and *C. levigata*, in a simple critical temperature or CTMax experiment, with specimens acclimatised to their natural spring water temperatures of around –1.9°C. The heat tolerance levels of leeches were well above 6°C, and they survived exposure to 24°C, well above the lethal temperature recorded for any Antarctic fish tested thus far (Beers & Sidell, 2011 & A. L. DeVries, pers.comm.). Although this level of water temperature might never be reached in their natural habitat, it demonstrates that the eurythermy of the piscicolids has endured alongside their stenothermal fish hosts. Antarctic notothenioids might have some thermal plasticity but they have a reduced heat tolerance compared to temperate fishes as a result of a genetic adaptation to the cold (Bilyk & DeVries, 2012). This is not the case for their ectoparasites.

2.7.3 Reattachment experiments

A series of simple behavioural experiments with leech parasites and their host species was undertaken at Scott Base. The detached parasites did not readily reattach to their original fish hosts, neither to the individual fish they derived from, nor did they choose a larger fish of the same species or any sized specimens of a different species. Instead, the adult leeches reproduced and perished after they attached egg cocoons to a hard substrate such as the tank walls or the plastic tubes in which they were held. The larger individuals of *C. antarctica* and *C. levigata* deposited a greater number of cocoons than their smaller congeners.

CHAPTER THREE

Visualisation and localisation of AFGPs in Antarctic piscicolid parasites by immunohistochemistry

3.1 Abstract

In this chapter I targeted the fish–derived AFGPs in cells and tissues of the piscicolid ectoparasites using the structural characteristics of peptides as they are presented in the fish hosts. Any protein has one specific amino acid sequence or primary structure which defines its three–dimensional properties and functionality, in this case antifreeze activity. Immunohistochemistry (IHC) was used as targeting method for the visual localisation by fluorescent labelling of AFGPs inside the leech parasite *Cryobdella levigata*. Cryomicrotomy results indicated punctiform AFGPs localisations in tissue sections of *C. levigata*. Signals were observed in the tissues of the digestive system indicating endocytosis of AFGPs in the form of protein containing vesicles at a cellular level. Assuming that the AFGPs detected in the parasite tissues originated from the host fish, rather than being produced by the leech, the data suggests that the absorption mechanisms of *C. levigata* to obtain substances for its energetic and nutritional requirements have additionally led to the assimilation of AFGPs from the same fish blood meal. Fish leeches found in the freezing waters of Antarctica thereby appear capable of gaining antifreeze agents directly from their hosts' blood.

3.2 Introduction

3.2.1 The detectability of proteins by IHC

IHC has a long experimental tradition of being exploited for the discovery of foreign substances in biological samples. The detection methods for antigens were initially analytic (Breinl & Haurowitz, 1932; Heidelberger, Kendall & Soo Hoo, 1933; Marrack, 1934), then visual (Coons, Creech & Jones, 1941), and finally advanced to localising specific target proteins in cells and thus within the appropriate tissue context (Coons, Creech, Jones & Berliner, 1942).

Over 70 years ago, Coons and colleagues (1942) used IHC for the first time to detect and identify a bacteria causing pneumonia, possibly *Streptococcus pneumonia* (Klein, 1884), in tissue sections of infected liver cells of mice. In their pioneering work photographs were taken using a purpose–built fluorescence microscope with special components by Leitz and Zeiss. An ever expanding repertoire of fluorescent dyes and microscopes, combined with various protocols for enhanced immunoreactivities, has established their classic technique as a standard method in laboratories for clinical diagnostics (Brinkhuis, et al., 1995; Kalebi & Dada, 2007; Fetsch & Abati, 2010) as well as in life science research (Kurzik-Dumke, Kaymer, Gundacker, Debes & Labitzke, 1997; Ohashi, Nakakita, Sumiyoshi & Takegawa, 2010; Ramsay, 2013).

The use of IHC for protein detection by means of visualisation of the targeted molecules with help of one or more fluorescently labelled antibodies has a good track record in vertebrates (Fabbrizio, Léger, Anoal, Léger & Mornet, 1993; Meiser & Schulz, 2003; Holzinger, et al., 2013), and has also been successfully applied in invertebrate species, as outlined below. The application of specific antibodies to target a group of similar or identical proteins across species barriers has also been successfully demonstrated, for example with the protein dystrophin. This large protein of 427 ku is part of the cytoskeleton in vertebrate muscle cells (Koenig, Monaco & Kunkel, 1988) and functions as a connection between the extracellular matrix, the sarcolemma and the actin filaments of the cytoskeleton (Winder, 1997), but dystrophin can also be found in neurons (Hoffman, Hudecki, Rosenberg, Pollina & Kunkel, 1988).

The dystrophin found in the elasmobranch fish *Torpedo californica* (Ayres, 1855) as peripheral membrane protein in cells of the electric organ, a modified skeletal muscle, is an evolutionary conserved protein (Chang, Bock & Bonilla, 1989). The authors used a immunodetection with polyclonal antibodies and fluorescent dyes (rhodamine and a streptavidin–fluorescein isothiocyanate (FITC) conjugate solution) on frozen sections obtained with a cryomicrotome of the electric organ and concluded that a homology must exist between the fish proteins and those labelled with the same 1:1,000 dilution of antiserum in samples of human, mouse and rat muscle tissues.

The gene encoding dystrophin in the sea urchin *Strongylocentrotus purpuratus* (Stimpson, 1857) was discovered to be a genuine homologue to this protein in vertebrates (Wang, Pansky, Venuti, Yaffe & Nudel, 1998) and an evolutionary link between invertebrate and vertebrate genes for dystrophins suggested (Roberts & Bobrow, 1998). Similarly the gene of the fruit fly *Drosophila melanogaster* (Meigen, 1830) was found to be a homologue to the human gene encoding the protein (Neuman, Kaban, Volk, Yaffe & Nudel, 2001). Neuman and colleagues stated that an ancestral gene must have existed before the phylogenetic separation of the Deuterostomia (Grobben, 1908) and Protostomia (Grobben, 1908) some 600 million years ago.

IHC was then used to visualise dystrophin at the cell membranes in caudal muscles of the marbled electric ray *Torpedo marmorata* (Risso, 1810), a benthic elasmobranch (Royuela, Hugon, Rivier, Fehrentz, et al., 2001). In this study mono– and polyclonal antibodies were used with cyanine or fluorescein dyes to establish the presence and absence of targeted proteins using light as well as confocal laser microscopy to detect, or not, fluorescent signals in different muscle cells.

Finally an example for a successful use of the immunodetection approach in combination with cryostat sectioning can be given for work on the piscicolid leech *Pontobdella muricata* (Linnæus, 1758) (Royuela, et al., 1999). Here the authors used light and electron microscopy for analysis and detection of a compacted form of an invertebrate dystrophin protein as the first such record at protein level in an invertebrate species. The finding of this protein in *P. muricata* can be explained further by the evolutionary path of this important molecule which could have similar properties in species phylogenetically far removed as it has a

similar mass and occupies the same localities in the leech muscle cells as dystrophin does in muscles of the skeleton in vertebrates (Royuela, Hugon, Rivier, Paniagua & Mornet, 2001).

The appropriate detection of proteins with a common genetic ancestry, although not necessarily identical structures, by immunological techniques is established. The cross–reactivity of antibodies targeting molecules in taxa of very distant phylogenetic relationships is possible and can produce patterns that are convincing to the expectant observer. Consequently, the application of an immunofluorescence protocol for targeting AFGPs authenticated on Antarctic teleost fishes could equally function in their piscicolid ectoparasites.

3.2.2 How does IHC work?

The IHC method applied in this thesis uses an indirect approach (Fig. 3.1). First, the primary and polyclonal antibodies, rabbit anti–AFGPs, target antifreeze molecules as antigens within the cells and tissues of specimen samples cut in 5 µm thin sections and placed on glass microscopy slides. These antibodies derive from fish blood and target the AFGPs as antigens known from fish. The protein domains involved in the antifreeze properties of the AFGPs in fish are inevitably a stable genetic component, as selection in Antarctica eliminates any deviation with a loss of functionality as it causes immediate fatality. Thus, the essential building blocks as AAT repeats in the peptide are identical between the different types 1 to 8 in the Antarctic notothenioids.

Next, a second antibody, goat anti–rabbit, is added which specifically binds to the first antibody and is chemically labelled with a fluorophore (or fluorochrome) stain tag, thus forming a complex with an AFGP. The fluorescent label can be excited with UV light of a defined wavelength (488 nm) to emit a light in the visible spectrum, in this case cyan–green (519 nm). This allows an observer to qualitatively detect the signal using a compound microscope set up for fluorescence imaging analysis. This two–step technique for the detection of light signals allows the direct visualisation of the distribution of immunological complexes attached to AFGPs *in situ*. Consequently, this enables the observer to localise the specific structures within cells and their proper tissue context, and thus physiological processes.

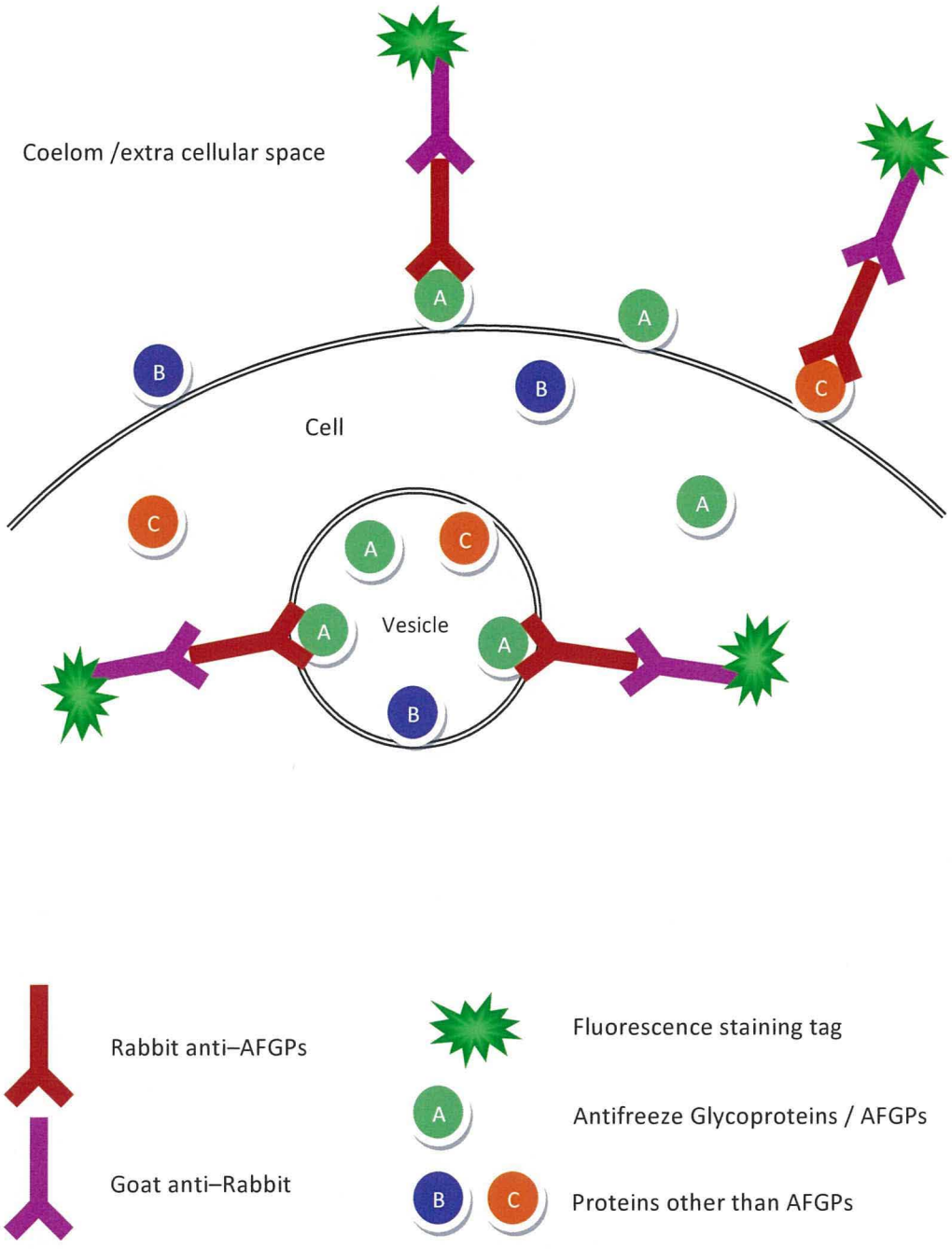


Figure 3.1 Illustration of the applied immunohistochemistry (IHC) in a hypothetical cell.

A two–step sandwich technology is used, involving primary (anti–AFGPs) and secondary (fluorochrome labelled) antibodies, for a qualitative analytic visualisation and localisation of the AFGP target proteins.

3.2.3 Aim of chapter

This chapter investigated the potential presence and distribution of fish AFGPs in tissues of their respective piscicolid ectoparasites using an immunodetection methodology. The approach to target specific fish peptides in these invertebrates was made possible using a previously established and authenticated system of polyclonal antibodies binding to and appropriately identifying AFGPs in notothenioids.

Fish AFGPs should be identifiable inside leech tissues under the premise that the molecules regain their functional domains upon rehydration after fixation and preservation in leech section cuts in a way that is analogous to what is known for fish tissue samples. Consequently, the structural characteristics, quintessential to the function of AFGPs, would allow the application of an IHC protocol with an immunofluorescent labelling to successfully target and then visualise these molecules within the parasites.

3.3 Material & Methods

3.3.1 Piscicolid specimens of *Cryobdella levigata* for IHC

The collection of Antarctic fish and their respective leech parasites was undertaken by ice hole fishing and use of hook and line at different localities in McMurdo Sound, Ross Sea (see Chapter 2). The material was immediately fixed in 70% ethanol after manually collecting *C. levigata* specimens from their respective fish hosts *Trematomus hansoni* while alive at the seawater holding facility of Scott Base. Specimens were used for paraffin embedded sectioning as well as cryomicrotomy. The molecular constitution of AFGPs has been studied in the Antarctic notothenioid fish *T. hansoni* (DeVries & Wohlschlag, 1969; DeVries, 1971), thus representing an ideal antifreeze source for this study.

3.3.2 Sectioning of paraffin embedded samples with a rotary microtome

In a first approach using classic histology methods eight specimens of *C. levigata* (n=8) were transferred into paraffin by an automatic tissue processor (Tissue–Tek VIP 2000, Miles Scientific, Minneapolis, Minnesota, USA). This included a pressurised heating cycle at 65°C

for 60 min to penetrate and fully saturate the leech specimen samples with paraffin. Afterwards the specimens were individually placed into plastic holding cassettes for mounting using a paraffin embedding machine (Leica EG 1150H, Leica Biosystems, Wetzlar, Germany). Each block was trimmed carefully to ensure minimal folding and 5 µm thin sections were cut off by use of a rotary microtome (Microm HM 330, Microm, Heidelberg, Germany) at room temperature. The individual sections were carefully placed on a warm distilled water bath (set to 45°C) in order to stretch them out on the water surface and where necessary any folds of the cuts were removed by using a fine brush. The tissue sections were arranged in consecutive order and picked up from the water surface with object slides. Each slide was coated with a thin film of albumin and placed into a drying oven at 60°C for 10 min to improve adhesion of the sections to the glass during the following water based staining protocol. A standard xylol (100% twice) and ethanol series (100% to 70%) was used to remove paraffin from the sections and to rehydrate the samples.

3.3.3 Sectioning using a cryomicrotome

Cryotomy was used for cutting four ethanol fixed and frozen specimens of *C. levigata* (n=4). The animals were transferred onto a small mounting piece to hold the specimens during sectioning. A highly viscous and water soluble optimal cutting temperature (OCT) polymer (Frozen Section Compound/FSC 22, Leica Biosystems) was used as the embedding compound to improve cutting by making the tissue more rigid. Each specimen was frozen at –20°C to solidify the substance and to mount as well as enclose the animal entirely for appropriate support during cryosectioning. The mounted specimen block was tentatively trimmed by hand to minimise curling of the 5 µm thin sections at –20°C in a cryomicrotome (Leica CM 1850, Leica Biosystems). The samples had an increased hardness at this low temperature compared to ambient room temperature, which facilitated and enhanced the cutting results. Each section was carefully stretched with fine brushes while in the cooling chamber of the cryostat. Sections were transferred in succession onto microscope object slides coated with albumin, an adhesive to avoid detachment of tissues during subsequent steps, and immediately processed for immunological staining.

3.3.4 IHC protocol

A modified IHC protocol established and based on notothenioid work by Clive W. Evans (pers. comm. & Evans, et al., 2012) was used for paraffin embedded and cryomicrotome sections. This included the quintessential polyclonal primary as well as secondary fluorophore labelled antibodies being tested to positively identify AFGP epitopes in Antarctic fishes. Variations in the incubation times and antibody concentrations were trialled to enhance antibody retrieval in piscicolid sections and to reduce unspecific background staining. The secondary fluorescence dye used was Alexa Fluor 488 Dye (formerly Invitrogen, now Thermo Fisher Scientific, Waltham, Massachusetts, USA), which emits a cyan–green colour at a wavelength of 519 nm upon excitement by UV light at 495 nm during microscopic analysis of the whole–mount tissue sections.

3.3.5 Immunological staining

Each section was pretreated for 30 min with an antibody blocking solution (PBD) which consisted of Sorensen's phosphate buffer at pH 7.4 (35.61 g Na_2HPO_4 [$2H_2O$] and 27.6 g NaH_2PO_4 [H_2O], each dissolved in 1 l distilled water, then 40.5 ml and 9.5 ml, respectively, taken and combined and diluted to 100 ml with distilled water), 1% bovine serum albumin or BSA (Sigma–Aldrich, St. Louis, Missouri, USA) and 1% dimethyl sulfoxide or DMSO (Sigma–Aldrich). The samples were incubated by merely covering the tissue under a film of primary polyclonal rabbit anti–AFGP antibody (diluted 1:1,000 in PBD) for 2 h in a moist dark chamber and then washed four times with PBD. The secondary fluorophore labelled antibody (goat anti–rabbit Alexa Fluor 488 Dye, diluted 1:1,000 in PBD) was applied for 1 h in the moist dark chamber. It was removed by one wash with PBD.

In an attempt to improve imaging contrast for better results with a reduced unspecific background staining, the concentrations (up to 1:10,000) and incubation times (from 30 min to 3 h) were varied for the blocking agent bovine serum albumin as well as the primary and secondary antibodies. A 4',6–diamidino–2–phenylindole (DAPI) fluorescent stain diluted to 0.2 μg/ml or 300 nM in PBD was used to visualise DNA inside the cell nucleus, applied for 5 min and washed off four times with PBD solution. The sections serving as negative controls

were treated in the same manner as described above but without the application of the primary antibody solution.

All stained samples were then sealed under glass coverslips with a small amount of anti–fading mounting media (FluoroGuard, Bio–Rad Laboratories, Hercules, California, USA) to prevent photobleaching of the fluorophore label as well as solubilisation of the AFGP–antibody products by enzymatic activities.

Finally, the edges were sealed with household nail polish to prevent evaporation and air penetration during the immediate subsequent but lengthy visual inspection of the immunofluorescence under a light microscope. The observations were made at varying magnifications from x100 to x1,000 using an epifluorescence microscope (Zeiss AxioSkop HBO 50, Zeiss, Jena, Germany), which provides fluorescent illumination with appropriate filters from above the samples for visualisation and photographic documentation (Zeiss AxioCam, Zeiss).

3.4 Results

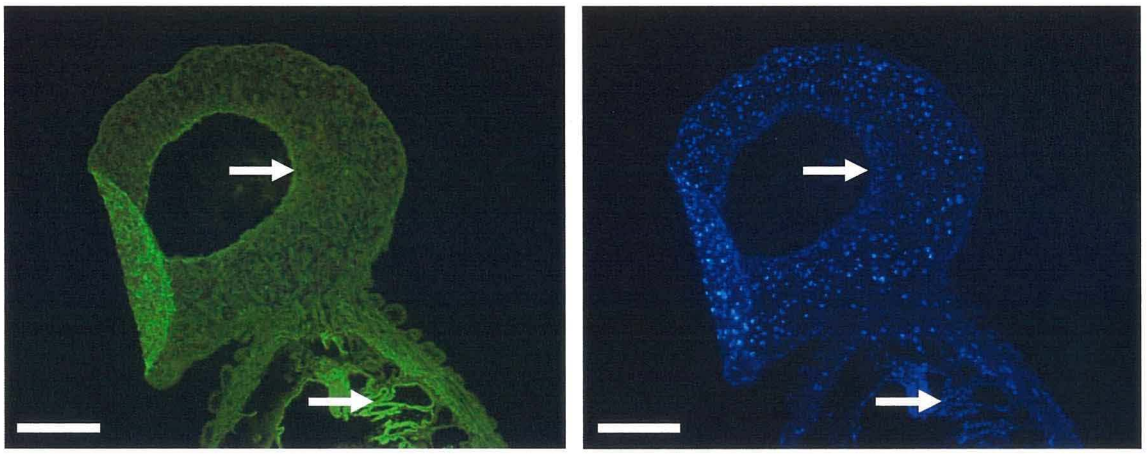
In total twelve individual *C. levigata* were analysed. The immunological method used successfully indicated punctiform AFGPs in cryomicrotomy tissue sections of the piscicolid. However, the staining success varied between cross sections as well as the two methods to obtain tissue slides. The different trials to enhance signal–to–noise ratios by altering the concentrations and incubation times of blocking buffer, primary as well as secondary antibodies did not result in visible improvements for either method of sectioning.

The specimens of *Cryobdella antarctica* collected during the 2010 field season proved extremely difficult to handle in the preparative steps for IHC and resulted only in unsatisfactory microtome sections. The preserved specimens were too brittle, thus despite best efforts, no images and results can be presented.

3.4.1 Paraffin sections

The paraffin embedded samples of *C. levigata* show a generally pronounced cyan–green fluorescence signal, while the DAPI stain allocates cell nuclei of intact cells within the sections (Figs. 3.2–3.7). The positive fluorescence for both dye labels can be seen against an otherwise clear and black background. In these anterior sections a difference in the intensity of signals could be identified for different tissues.

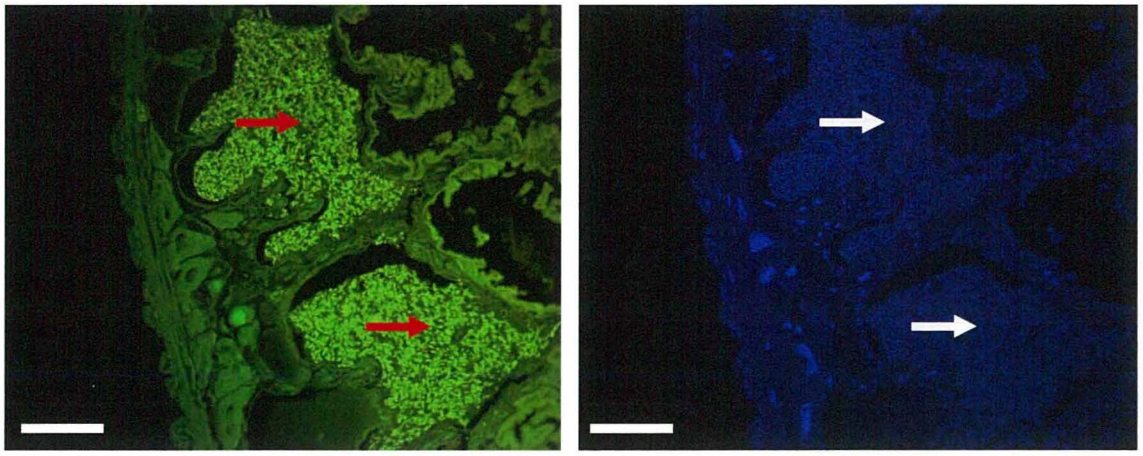
A remarkably brighter illumination can be observed from some cells possibly lining the lumen of the oesophagus and the cells lining the circular ring of the anterior sucker in which the mouth pore for blood sucking is located (Fig. 3.2 A). There are strong IHC based fluorescence signals in parts of the digestive tract (Fig. 3.3–3.6 A) where DAPI staining with a high level of fluorescence in every slide reveals vast numbers of cell nuclei in dense and compact form indicating notothenioid blood cells in a clotted state (Fig. 3.3–3.6 B). The muscular tissues under the epidermis show a diminished IHC based fluorescence intensity compared to tissues in direct contact on either sides with large cells in connective tissues containing bright and punctiform fluorescence signals (Fig. 3.7 A).



A B

Figure 3.2 Fluorescence images of anterior sucker and oesophagus region in *C. levigata*.

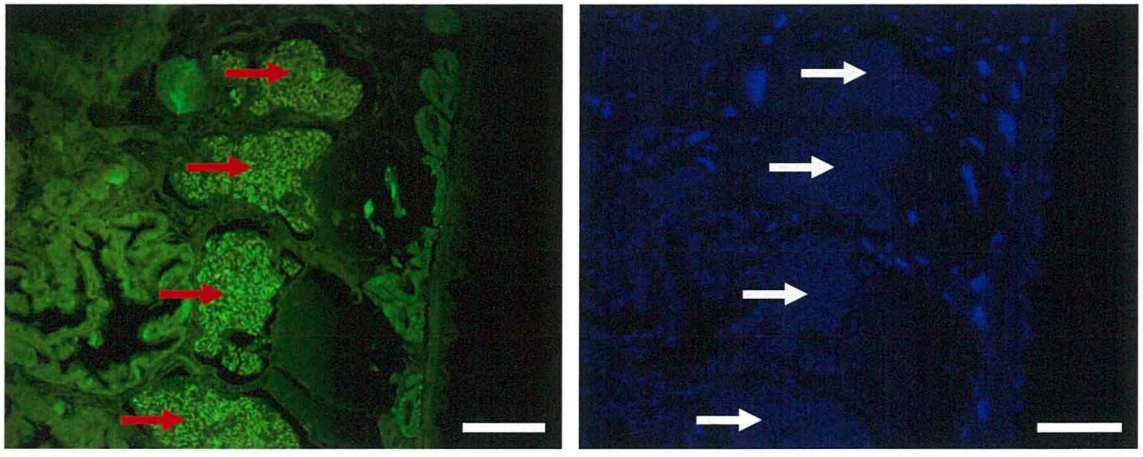
(A) Fluorescence from IHC staining illustrating variations in signal intensities with lining cells of anterior sucker and pharynx–oesophagus region (arrows) of brighter colouration compared to surrounding tissues indicating AFGP concentration gradients.
(B) DAPI stain showing cell nuclei at appropriate areas (arrows).
(A) & (B) include a tissue folding (on left side of sucker) to visually demonstrate an artefactual increase of signal intensities (scale bars 200 µm).



A B

Figure 3.3 Images of crop region and epidermis in *C. levigata*.

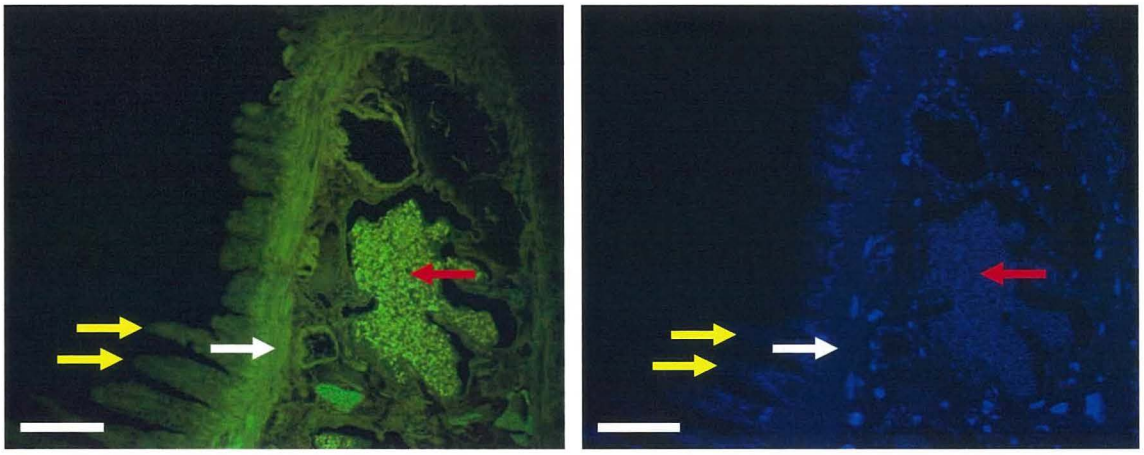
(A) Fluorescence signals obtained by IHC illustrating a differentiation in the cyan–green fluorescence intensities between tissues and compartments inside the parasite including an area of possibly clotted notothenioid blood with positive signals as indicated by red arrows.
(B) DAPI stain showing cell nuclei (indicated by arrows) in compartments potentially containing swallowed fish blood.
(A) & (B) show tissues in close proximity to anterior sucker (scale bars 100 µm).



A B

Figure 3.4 Images of intestinal tract and tissues with epidermis in *C. levigata*.

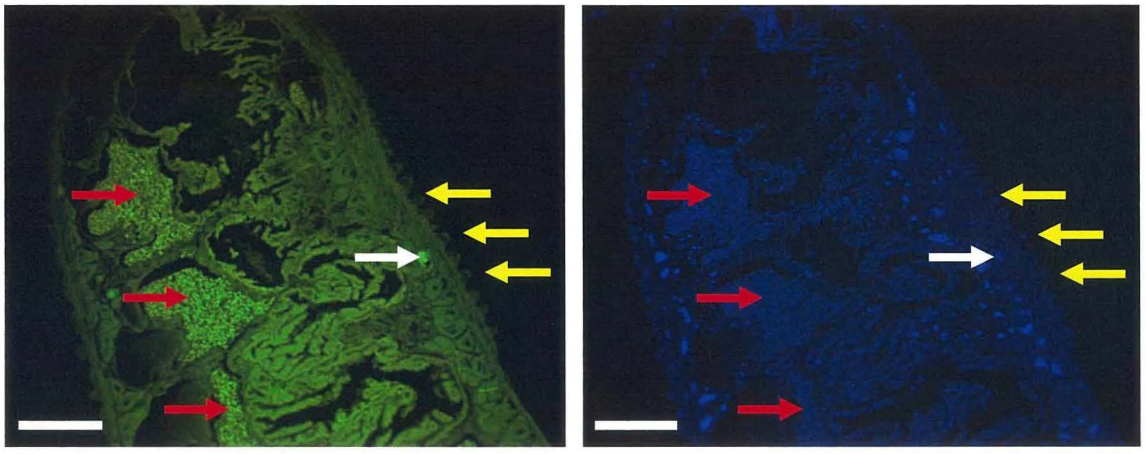
Fluorescence deriving from IHC (A) and DAPI (B) illustrating different signal intensities of piscicolid tissues close to anterior sucker. Several compartments, potentially containing fish blood, illustrate positive signals as indicated by red arrows for IHC (A) and white arrows for nuclei (B) (scale bars 100 µm).



A B

Figure 3.5 Epidermal annulations, muscular layers and tissues of *C. levigata*.

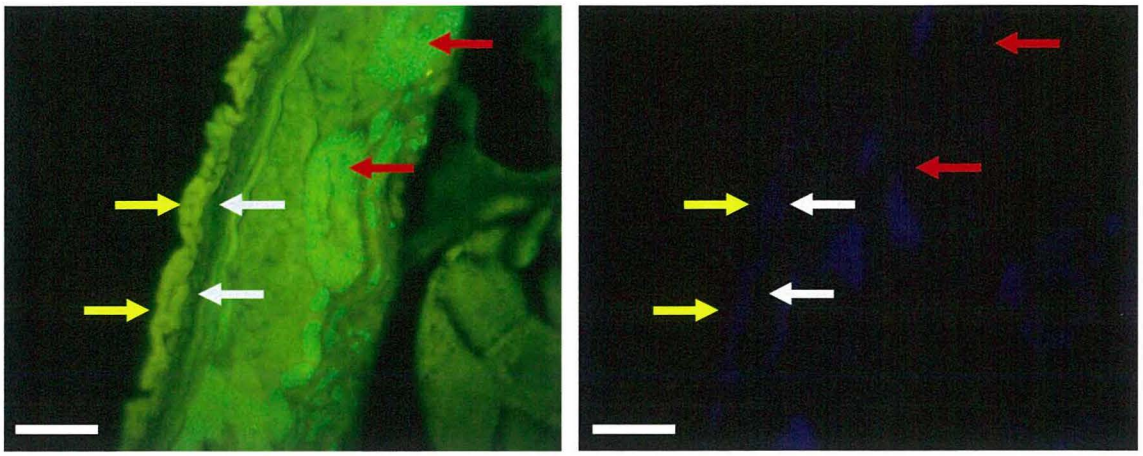
Fluorescence deriving from IHC (A) and DAPI (B) illustrating different signal intensities of piscicolid tissues including epidermal annulation (yellow arrows to left) and epidermis (white arrow in middle) as well as potentially swallowed notothenioid blood (red arrow to right) in close proximity to the anterior sucker (scale bars 200 µm).



A B

Figure 3.6 Fluorescence images of body cross–section of *C. levigata*.

Fluorescence deriving from IHC (A) and DAPI (B) illustrating various fluorescence green signal intensities of possibly notothenioid blood (red arrows to left), an artefact (white arrow in middle) and piscicolid tissues including annulation (yellow arrows to right) in images of a body width cut showing epidermis, connective tissues, intestinal tract and presumably reproductive organ tissues close to the anterior sucker (scale bars 200 µm).



A B

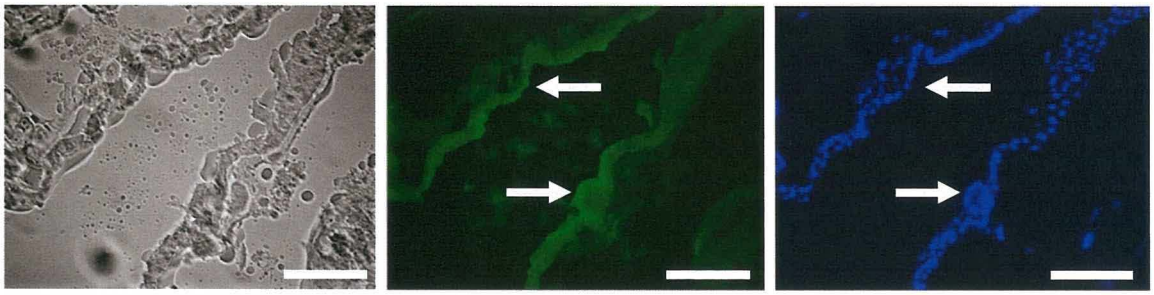
Figure 3.7 Images of epidermis, muscular layers and connective tissues in *C. levigata*.

Fluorescence deriving from IHC (A) and DAPI (B) illustrating marked differences in the cyan–green signal intensities of epidermis showing annulation (yellow arrows to left), the muscular layers beneath (white arrows in middle) and connective tissues including large cells containing bright and punctiform fluorescence signals (red arrows to right) in detailed images close to anterior sucker (scale bars 50 µm).

3.4.2 Cryotomy sections

The results obtained from sectioning tissues by cryomicrotomy show three images of the same frame at two positions within the digestive system of *C. levigata* (Figs. 3.8 & 3.9). The light microscopy photographs provide an overview of the tissue context, while the DAPI stain allocates the cell nuclei of intact cells with good contrast against a dark background.

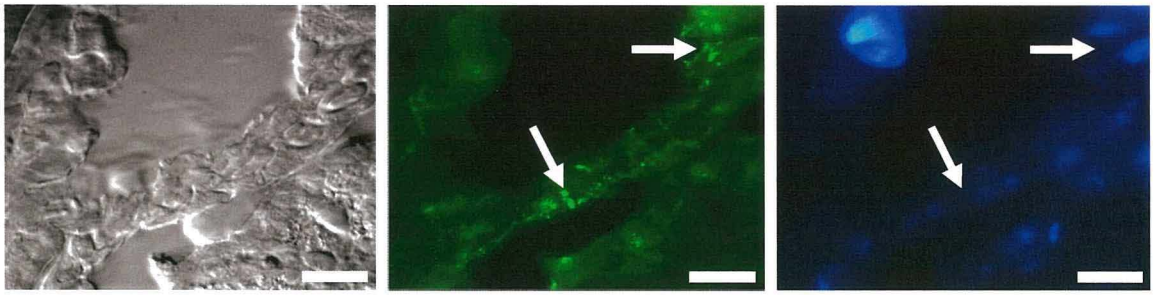
The tissues obtained by cryotomy are of slightly weaker fluorescence intensity than the previous paraffin embedded samples. A gradient in the immunological staining can be identified at the anterior part within the oesophagus area (Fig. 3.8). The lumen is lined by tissue cells which shine marginally brighter at the side of the lumen compared to their distal side with further cells in the surrounding tissue of a lesser intensity than those lining the lumen. The second series of images shows signals in the posterior region of the intestine with lateral chambers where punctiform fluorescence spots in respective cells can be allocated (Fig. 3.9).



A B C

Figure 3.8 Images of oesophagus region with lumen anteriorly in *C. levigata*.

Images of lining tissue cells along lumen of the oesophagus region in anterior body part of one *C. levigata* specimen (scale bars 20 µm).
(A) Light microscopic overview of lining tissue cells.
(B) Decreasing cyan–green fluorescence intensity (arrows) across each cell line indicates a potentially underlying gradient in AFGP concentrations.
(C) DAPI stain visualising cell nuclei to demonstrate intact cell lines to either side of the lumen.

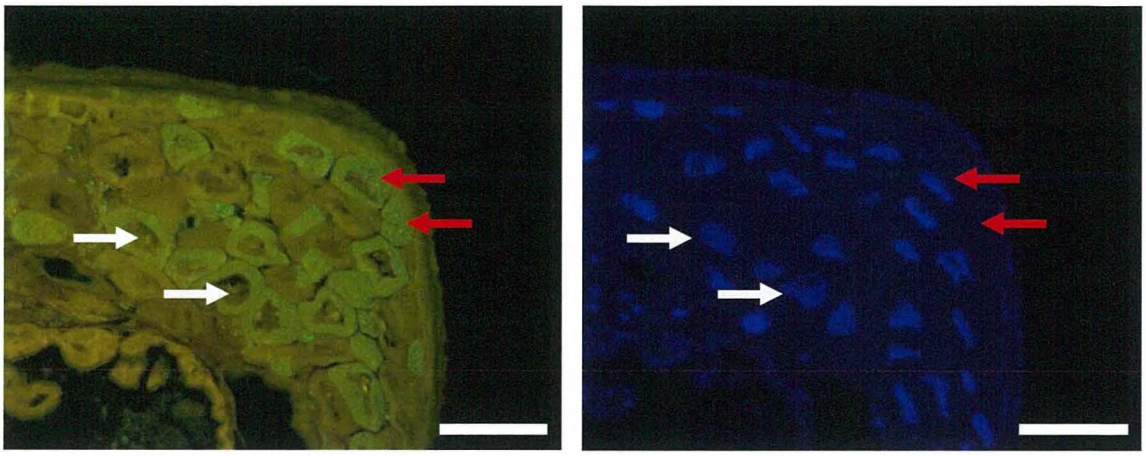


A B C

Figure 3.9 Images of tissue in digestive system posteriorly in *C. levigata*.

Images of tissue cells in digestive tract in posterior body part of one *C. levigata* specimen (scale bars 10 µm).
(A) Light microscopic overview of tissue with cells.
(B) Punctiform fluorescence signals (arrows) inside single cells indicate AFGP accumulations. A slight decrease in fluorescence intensity can be seen from cell membrane to plasma indicating a potential for a gradient in AFGP concentrations.
(C) DAPI stain visualising cell nuclei to demonstrate intact cells.

The staining controls were not observed to be of a clear dark but with some degree of background colouration as was seen for example in a paraffin section of the epidermis with connective tissues (Fig. 3.10).



A B

Figure 3.10 Images of IHC and DAPI control slides for *C. levigata*.

Section cut close to anterior sucker with epidermis and connective tissues illustrating the level of autofluorescence in *C. levigata* for IHC (A) and DAPI (B) staining of an area containing large cells (some cells indicated by arrows) typical for specific piscicolids. Some large cells contain bright and punctiform fluorescence signals (red arrows) (scale bars 200 µm).

3.5 Discussion

Using IHC it was possible to demonstrate the presence of AFGPs in tissue sections of the piscicolid parasite *C. levigata*. The primary and polyclonal antibodies targeting specific structures of the antifreeze molecules as epitopes, which are known to exist in notothenioid fish, can also detect these structures with sufficient staining capability in a cross–species application in the invertebrate piscicolids.

The results demonstrate that this methodology has a successful cross–species application, effectively making further testing and use of different fluorescent dyes as well as the development of new protocols redundant. Consequently, the visualisation of distribution

and therefore localisation of AFGPs within piscicolid tissue samples are possible using this classic immunological staining technique.

The paraffin embedded tissues illustrated the presence of antifreeze proteins at the appropriate localities within the parasite's digestive system. The ingestion of proteins can be in the form of physiological processing of nutrients from the crop and intestine by active but undifferentiated endocytosis. As AFGPs are macromolecules of varying molecular sizes, these Antarctic invertebrates have almost certainly developed a number of selective absorption mechanisms to ensure an optimal energy yield from the fish blood meal which is its food source.

The images obtained from the anterior body tissues of *C. levigata* are anatomically the furthest distance away from the fish blood as AFGP source stored towards the posterior part of the intestine. The results are suggestive of the ability of a fish parasite to absorb AFGPs across the epithelial barrier of the digestive lumen as per an analogous physiological process demonstrated previously for polar fishes (Evans, et al., 2012). IHC staining with the identical set of primary and secondary antibodies to the present study demonstrated that epithelial cells in the rectum of the Antarctic *T. hansoni* as well as the Arctic polar cod *Boreogadus saida* (Lepechin, 1774) contain AFGPs and thus function as the location of peptide uptake (Evans, et al., 2012). AFGPs from northern Arctic cod and southern Antarctic notothenioid fish species are nearly indistinguishable in their primary structure, yet derive from an independent convergent evolution (Chen, et al., 1997a). I hypothesise therefore that in a functional analogy to polar fishes their respective leech parasites can also utilise epithelial absorption mechanisms for an uptake of functional AFGPs from their digestive system.

The detection of AFGP epitopes demonstrates further a successful transformation of proteins from hydrated states in the living parasites to fixative and reverse as required in the methodology. The quintessential prerequisite for any immunological result is the successful attachment of the antibodies to specific antigen conformities defined by the protein structures. The molecular integrity is paramount to the antifreeze properties and in particular the hydroxyl groups of the sugar residues are essential for antifreeze functionality (DeVries, et al., 1970) which can be lost entirely by enzymatic degradation (Komatsu, DeVries & Feeney, 1970) or chemical (Komatsu, et al., 1970; DeVries, 1971) alterations of the

glycoproteins. For the present study this translates into a successful restoration of a protein into its original native state upon rehydration, effectively allowing the protein to obtain its functional characteristics prior to fixation and preservation. Therefore, the ethanol treatment, which effectively removed water from the animal tissues, and equally important the sequential steps of the applied methodology, in case of paraffin including heating, have no significant effects on the immunological reactivity of AFGPs in piscicolid tissues.

This was to be expected as heat and ethanol have been reported to have no negative or irreversible effects on glycoproteins (DeVries, et al., 1970). Yet the antifreeze polypeptide type 3 from the ocean pout *Zoarces americanus* (Bloch et Schneider, 1801) has been reported to lose its antifreeze properties upon denaturation at 60°C for only 30 min (Gaede-Koehler, Kreider, Canfield, Kleemeier & Grunwald, 2012). Thus, the validity of the protocol in use had to be established to work on AFGPs in leech material.

In conclusion, the IHC results show that AFGPs are detectable and present in the tissues and cells of the digestive tract and indicate their active digestive absorption by the piscicolids followed by a distribution within the body.

3.5.1 Comments on fluorescence in paraffin sections

The photos of samples embedded using the automated paraffin process showed a much higher and all over fluorescence (Figs. 3.2–3.7) compared with those using the cryomicrotome technique (Figs. 3.8 & 3.9). There are several potential explanations for this. It might be caused by thermal dispersal of AFGPs in the tissue during the paraffin embedding steps. Alternatively it might indicate a variation in staining successes amongst cross sections even though a standardised methodology was followed. Another reason could be that the tissues prepared using the paraffin method have a higher affinity to the antibodies than do those from the cryosectioning using the OCT polymer medium which has been reported to inhibit protein detection (Schwartz, Reyzer & Caprioli, 2003; Seeley & Caprioli, 2008). This slight masking effect caused by the embedding medium might explain the differentiated visualisation of AFGPs seen in cryostat but not in paraffin tissues and constitutes a beneficial experimental condition in this IHC application.

If considered without the cryostat results, one might be tempted to explain the differences in the quality of the immunologically stained slides in paraffin as mere artefacts. However, aside from the inevitable unspecific binding typical for any IHC work, more crucial aspects must be taken into consideration as main contributors to the high background noise observed in this work, rendering the unquantifiable aspect of unspecific staining insignificant for the interpretation of results.

First, the leech specimens used for IHC staining originated from different fish hosts and were of varying nutritional and physiological and possibly even ontogenetic states. As a result it can be expected that parasites collected in the field have variations in the overall antifreeze quantities and possibly even in the different AFGP types and their respective concentrations. Due to the nature of this work, involving live animals and their sampling and preservation in the field, it was impractical to standardise the physiological state in which leeches were fixed for subsequent work. This natural limitation for biological field samples could only be eliminated in the future by use of a breeding facility for Antarctic piscicolids or more advanced methodologies which allow concentration measurements, such as imaging mass spectrometry (IMS), as will be discussed in the following chapter. The mixture of animal conditions could lead to variable success in the IHC procedures, although the level of variation in the results observed was unexpected.

Second, varying antibody concentrations and incubation times had no notable effect in terms of a reduction of background noise, especially in the paraffin embedded samples. This might be due to the high fluorescent intensity of the Alexa dye used, combined with the efficient characteristics of the secondary antibody to attach and eventually form stable AFGP–conjugates even at very low concentrations of primary and secondary antibodies. Additionally, one can consider a scenario in which the parasites contain substantial quantities of AFGPs within their digestive tract and tissues. Thus, a high concentration of target proteins would inevitably result in strong and generally more pronounced staining representations. This would be the case even when applying diluted antibody solutions onto the tissue sections.

Third, the PBD medium, used to block epitopes for a reduction or elimination of unspecific binding of primary antibodies to similar antigens in the leech samples, was a mixture of chemicals known to enhance IHC results (Cattoretti, et al., 1993). The bovine

serum albumin as protein blocker with an applied concentration of 1% was at least double that of other successful applications (Cattoretti, et al., 1993).

Fourth, a widespread distribution of AFGPs confers a biological advantage in terms of physiological protection of cells and tissues against ice crystal growth. In evolutionary terms, the control over ice crystal formation is paramount for individual survival. The results are consistent with this in that an omnipresence of proteins functioning as antigens would be the key factor causing a high intensity of fluorescence observed as a general green colouration of all cells and tissues. Varying the blocking agent's concentration to reduce unspecific staining might have no visible effect in samples with generally high AFGP quantities. Furthermore, unlike in the cryostat sections it was not possible in the paraffin slides to identify similarly small and single point signals as being AFGP aggregates or clustered densities. This further supports the view that AFGPs are widely dispersed in the piscicolids rather than being restricted to cells and tissues of the digestive tract.

And finally, a study comparing results for the same antigen in 172 different laboratories concluded that the antigen retrieval or staining techniques in use must be the main factors for the varying results (Mengel, et al., 2002). This clearly illustrates the variability of IHC outcomes. Therefore, considerable variation in staining results can be expected when working with a novel array of organisms of non–standardised physiological condition and exhibiting other differences unknown at time of collection.

In summary, any experimental alterations would not have a significant effect on reducing background fluorescence due to limitations of chemicals and protocol as well as unspecific cross–reactions with other invertebrate macromolecules and salt ions, which cannot be quantified with the available samples or the current IHC methodology. Additionally, the underlying concentrations of dispersed AFGPs inside the leech tissues might noticeably contribute towards the varied outcome of staining results. Indeed the unknown levels of AFGP concentrations within and between different leech individuals might explain the observed variability.

3.5.2 Signal amplification by antigen retrieval with heat treatment

The fluorescence signals observed in the cryosections were of weaker visual intensities than those from the paraffin embedding method which seems to improve the poor antigen retrieval or undetectable antifreeze proteins in the fluorescence imaging. The results indicate a high concentration of AFGPs in the sections in the form of a generally high background staining alongside a differentiated and significantly stronger illumination of some tissue areas (Figs. 3.2–3.7). This was seen in paraffin embedded sections and suggests a substantial unmasking for antigen retrieval as the result of the 60 min heat treatment at 65°C in the automated embedding machine and under pressure leading to even penetration of the tissue samples with paraffin. The unmasking of an antigen by heat (Cattoretti, et al., 1993; Cuevas, et al., 1994; Kahveci, Minbay, Noyan & Cavusoglu, 2003; Wisztorski, et al., 2013) and with the aid of a pressure cooker for improvement of results has been well demonstrated in tissue samples (Norton, Jordan & Yeomans, 1994; Hayat, 2002, pp. 117-154). The underlying mechanisms of such unmasking which presents previously hidden epitopes for a subsequent detection by antibodies is not entirely understood but has been attributed to the removal of calcium ions (Hayat, 2002, pp. 120-123) or a denaturation of proteins (Cattoretti, et al., 1993; Hayat, 2002, pp. 125-142). The overall high staining intensity provides substantial support to the hypothesis that the pointed fluorescence signals observed in cryotomy sections are not artefacts but true AFGP signals.

3.5.3 Limitations of the study

The method of immunological staining used here has limitations regarding its specificity. The protein mixture of any organic tissue is highly complex and inevitably leads to *ad hoc* binding of antibodies to macromolecules other than the targeted AFGPs. This produces artefacts and a certain level of background interference, as exemplified in figure 3.1 with protein C. Therefore in this study, as in any similar investigation, such erroneous signals have to be taken into consideration when interpreting the results. It is possible to conclude that AFGPs are present in the leech tissue despite false positives additional to the actual pool of fluorescent peaks correctly indicating AFGPs.

The negative controls were not of a clear black without green contrast in the tissues, as they should be in theory, but showed weak signal strengths. This might be autofluorescence caused by various cell structures and at least partially due to a less specific immunological activity of the antibodies used for this cross–species application and as such represent one major limitation of the methodology. The specificity of the secondary antibody in the leech system remains to be tested with higher resolution methods as the immunological interactions in the invertebrate might bind the secondary antibody to more than merely the target protein structures, as discussed above.

The exact reasons for the failed sectioning of *C. antarctica* specimens for use in IHC remain unclear but presumably are linked to the species' size and anatomy. The blood filled lumen equates to a considerably higher proportion of body volume in these very slim animals when compared to *C. levigata*. The structural support by connective and other tissues might be insufficient in *C. antarctica* to facilitate proper microtome cutting.

Finally, it must be noted that with the available technique a higher number of IHC slides, even including *C. antarctica*, would not be beneficial to this part of the PhD as it would not improve the interpretation accuracy and thus cannot provide further insight.

3.5.4 Alexa fluorochrome potential for background fluorescence

The fluorescence dyes of the Alexa family are insensitive in the pH 4–10 range, show higher intensities and are more resistant to bleaching and fading compared with other conventional dyes such as aminomethylcoumarin (AMCA), Lucifer Yellow, fluorescein or rhodamine (Panchuk-Voloshina, et al., 1999). The study illustrated quantitatively that Alexa dyes 350 and 430 can generate background fluorescence to the extent that a decrease in intensity by photobleaching cannot be observed (Panchuk-Voloshina, et al., 1999). Further, Alexa dyes are ionic fluorochromes and have a negative net charge, with Alexa 488 being the strongest with three negative charges. This can cause electrostatic interactions with any positively charged structures within the cell or tissue of the invertebrate, as is the case for fluorescein isothiocyanate (or fluorescein) (Mahmudi-Azer, Lacy, Bablitz & Moqbel, 1998), causing challenges in the interpretation of results due to the additional staining by the fluorochrome. Thus, the dye might add considerably to the noise of unspecific and

undesirable background fluorescence in the leech invertebrate. However, it is impossible to prevent any interactions of the negatively charged ionic fluorochrome or to quantify its influence on the observed staining intensity. Only the use of a different fluorochrome might allow one to state what impact the particular detection molecule had in this study. However, alternative dyes were not available to evaluate the paraffin embedded tissues further, and their use would have exceeded the financial scope of this PhD. Furthermore, their use was rendered redundant after the results from mass spectrometry, and in particular nanolitre osmometry, as will be demonstrated and discussed later in this thesis.

3.5.5 AFGP concentration gradient

The anatomy of a leech allows us to distinguish areas of the digestive tract within a microtome section. In the anterior body, just posterior to the sucker with its mouth pore, lies the oesophagus. This strong muscular tube produces the suction required for the physical extraction of blood from the fish host. Upon close inspection, this body part shows as strong a fluorescence as the area of the beginning of the digestive tract. At a low magnification of x100, numerous differentiated areas of high and low fluorescence intensity can be distinguished (Figs. 3.2–3.7). These variations coincide with the pathway of fish blood and thus AFGPs. These imaging results could represent concentration gradients within the leech tissues. The signal is weaker outside the crop and shows a decrease away from this AFGP source.

Future work could investigate the AFGP absorption mechanism as well as tissue allocations. This could be done by tracing of fluorescently labelled nanoparticles coated with AFGPs to mimic antifreeze encompassing entities. This would require injection of particles into living parasite specimens to facilitate active *in vivo* interactions and could be undertaken analogous to previously successful work on the Antarctic fish *Pagothenia borchgrevinki* (Evans, et al., 2011). Visually similar to the staining results from the present study, the method of nanoparticles would visualise pointed and distinguishable fluorescence signals within tissue sections of leeches. And this time with the knowledge that signals can only derive from marked particles as no antibody for immunological labelling was used. This would prove and verify beyond doubt the proposed absorption mechanisms for AFGPs into the piscicolid parasites and provide visual proof to antifreeze dispersion patterns.

In order to study AFGP concentration levels in different body compartments such as epithelia or muscle tissues or the endolymph the use of substances with radioactive labels such as C^{14} might prove suitable as a proxy, like previously demonstrated to trace AFGPs in the fish host *Trematomus bernacchii* (Ahlgren, Cheng, Schrag & DeVries, 1988). This approach could provide answers to concentration levels as well as dispersion pathways.

3.5.6 Confocal laser scanning microscopy (CLSM)

A commonly used technology to overcome the resolution limitations of the epifluorescence method is the conventional compound microscope set up for confocal laser scanning microscopy (CLSM). In this imaging technique, a laser beam is focused on a specific point in the sample and allows a scan of the three–dimensional space of a specimen for a target fluorochrome, giving superior depth selectivity and resolution. However, as powerful as this method might be, the limitation of it in this case lies in the many reasons which could lead to the overall positive and strong fluorescence of tissue sections. Just like LM the CLSM cannot differentiate between unspecific conjugates in the tissues and actual AFGP attachment sites.

3.5.7 MALDI–IMS for analysis and high resolution *in situ*

A higher resolution method alone, such as CLSM, is not, however, sufficient to conclusively show whether these pointed fluorescence signals in the cryotome sections are caused by AFGPs or represent methodological artefacts using fluorescence markers. One requires an analytic tool in combination with a much improved imaging precision to overcome the current limitations. The technology ideally suited for this purpose would be the use of an imaging mass spectrometer (IMS) in combination with matrix–assisted laser desorption/ionization (MALDI–IMS) or three–dimensional electron microscopy (3DEM). These superior high resolution imaging tools are becoming key methodologies in cell biology to determine structural properties at the cellular level and can detect target proteins within cell tissues with unprecedented accuracy.

3.6 Conclusions

The technique of immunological staining as a classic method in histology allows us to conclude that AFGPs are in fact present in piscicolid specimens, either by means of digestive endocytosis or more selective absorption mechanisms. The cyan–green staining visible in every slide emitting off cell walls and structures is not merely unspecific staining, as to a certain degree is inevitable for IHC, but represents true AFGP–fluorochrome conjugates.

I conclude that by the natural processes of digestion AFGPs are absorbed alongside other nutrients by the parasite from the blood meal taken off the fish host. I postulate a similar nutritional absorption mechanism of AFGPs in leeches as a physiological analogy to the distribution pathway of antifreezes in fish. The resolution power of light microscopy does not allow us to distinguish further details in the images, taken at maximum magnification. Thus, the interpretation of results as fluorescence signals indicating AFGP accumulations cannot be conclusive without a more powerful analytic tool. The logical next step is the verification of the presence of AFGPs in leech tissue material by mass analysis. This technique allows the identification of AFGPs as molecular species rather than by their spatial properties. This can overcome the limitations of specificity in this immunofluorescence work and is the focus of the next chapter.

CHAPTER FOUR

Detection and identification of AFGPs in Antarctic fish leeches by mass spectrometry

4.1 Abstract

The amino acid sequences of leech antifreezes were determined by mass spectrometry (MS) and peptide sequencing. The comparison of results with fish antifreeze glycoprotein (AFGP) types 1 to 8 provided evidence that types 7 and 8 fish AFGPs are present in whole specimen homogenate of *Cryobdella antarctica* as well as in individual body compartment samples of *Cryobdella levigata* from digestive content, skin and the posterior sucker muscle. This detection by characterisation of AFGPs in two piscicolid species demonstrates active peptide absorption unaffected by either species digestion even with help of an endosymbiotic bacterial flora. These undigested peptides consequently offer an *in vivo* protection against freezing events in the sub–zero temperatures of the Southern Ocean benthic habitat with a potential co–evolution alongside the notothenioid fish hosts.

4.2 Introduction

One hundred years ago Sir Joseph John Thomson published in *Science* his finding that the element neon exists in two forms with different atomic masses (Thomson, 1913), marking the first proof of the existence of isotopes for non–radioactive stable elements and at the same time the first application of mass spectrometry (MS). The importance of this tool for science was immediately recognised and numerous improvements in the instrumentation and its analytic power occurred over the following century, with computational advances being at the centre of recent developments. Today, MS is a well proven and established

laboratory technique, not just for the detection and identification of substances in biological samples but also for the description of novel protein characteristics in proteomics. A summary of previous MS applications is provided here to put the methodology used in the current research on proteins and antifreezes into context. This is by no means a comprehensive review as this lies outside the scope of this thesis.

Early investigators aiming to identify proteins and peptides suggested experimental methods, such as pyrolysis, that allowed descriptive work on molecules by means of their respective amino acids at the level of microgram quantities (Simon & Giacobbo, 1965) and to use pyrolysis to perform sequencing analysis of proteins such as bovine insulin (Merritt & Robertso, 1967). This was soon followed by the suggestion of utilising peptide fragments to obtain biomolecule sequences when identifications were possible even of mass equivalent amino residues such as leucine and isoleucine (Levsen, Wipf & McLafferty, 1974).

The challenging task of the interpretation of mass spectra resulting from MS is greatly facilitated when the atomic composition of the target substance is known. The *relative molecular mass* (M_r) or *molecular weight* can then be calculated based on the sum of the *relative atomic mass* or *atomic weight* of all elements present in the structural formula of the molecule. Whereas the *relative atomic mass* represents the average weight of all naturally occurring isotopes of any element and thus there is no atom with that actual mass in nature.

This elementary physics was the basis for the identification of peptides and proteins and their characterisation by MS in combination with their degradation in an Edman reaction (Edman, 1950) that resulted in two high impact publications in *Nature*. The sequence of enkephalin as a very short pentapeptide isolated from the brain of pigs was resolved in its fifth position by using both techniques (Hughes, et al., 1975). The direct combination of the Edman's reaction followed by a mass spectrometric analysis of the resulting fragments allowed not only to identify but to fully characterise other peptides, such as the adipokinetic hormone of the migratory locust *Locusta migratoria* (Linnæus, 1758) and the desert locust *Schistocerca gregaria* (Forsskål, 1775) (Stone, Mordue, Batley & Morris, 1976). It is noteworthy that 3,000 corpora cardiaca resulted in the 750 µg of extracted peptide mass necessary for the full description of the hormone.

The chemical structures of Antarctic fish AFGPs were studied using *nuclear magnetic resonance* (*NMR*) with a considerable sample size of 3 mg total AFGPs isolated from *Pagothenia borchgrevinki* (Shier, Lin & DeVries, 1972; in original as former *Trematomus borchgrevinki*). In the same year, the sequences of AFGP 7 and 8 were determined based on the Edman degradation (1950) followed by NMR spectra and amino acid analysis as well as polyamide chromatography with 150 nmol or 177 µg starting material for each protein digest (Lin, Duman & DeVries, 1972).

These results were soon succeeded by a comprehensive study for this fish species comparing AFGP 8 sequences obtained by an Edman degradation of the peptide in an elastase hydrolysis followed by an automated sequencing parallel to MS analysis of product fragments (Morris, et al., 1978). Fourney et al. used gradient HPLC to fraction the antifreeze proteins (AFPs) from blood sample of winter flounder *Pseudopleuronectes americanus* to then use an automated amino acid sequencer, a Beckman 121, for the description of the peptides (Fourney, et al., 1984). The general quest to obtain primary sequences of oligopeptides and proteins utilised the characteristic array of fragmentation products in MS combined with specific fragmentation techniques (Hunt, Buko, Ballard, Shabanowitz & Giordani, 1981).

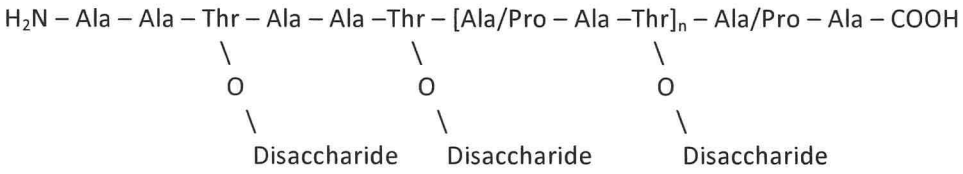
Finally, MS can also serve in the reverse approach. The exact knowledge of peptide sequences enables the search for specific protein sequences in different biological tissues. This has been demonstrated for the AFPs of the beetle *Dendroides canadensis* where the presence or absence of types 1, 2 and 4 in different body compartments was established (Duman, Verleye & Li, 2002). One can also search for, detect and identify AFGPs accurately in different body locations in Antarctic fishes (Evans, et al., 2011; Evans, et al., 2012).

Consequently, if there are any fish AFGPs, or closely related proteins, present in tissues of the Antarctic leech parasites, then these could be detected using MS as the analytical method. Proper molecular identification of a potential AFP must include a chemical analysis to obtain information on the primary structure or amino acid sequence. MS is thereby utilised here as another classic technique for the tracing of the target proteins in leech tissue samples.

4.2.1 Background on chemical characteristics of AFGPs

AFGPs have some distinguishing properties that facilitate their analytical detectability in a mass spectrometer. These proteins consist of alanine (Ala, A), threonine (Thr, T), β–D–galactose (galactose or Gal) and α–*N*–acetyl–D–galactosamine (GalNAc), arranged in sequence of one repeat unit as the glycotripeptide Ala–Ala–Thr with the two Gal sugars as one disaccharide attached to threonine, as depicted in figure 4.1 (DeVries, et al., 1970; Komatsu, et al., 1970; DeVries, 1971; DeVries, Vandenheede & Feeney, 1971). The peptide backbone is common to all AFGPs (DeVries, et al., 1971; Shier, Lin & DeVries, 1975; Ahlgren & DeVries, 1984) but AFGP types 6, 7 and 8 can contain proline (Pro, P) in some positions instead of alanine (DeVries, et al., 1970; Lin, et al., 1972; Morris, et al., 1978). This is relevant when mass spectra are interpreted as it leads to a varying M_r for the proteins of these AFGP classes.

A



B

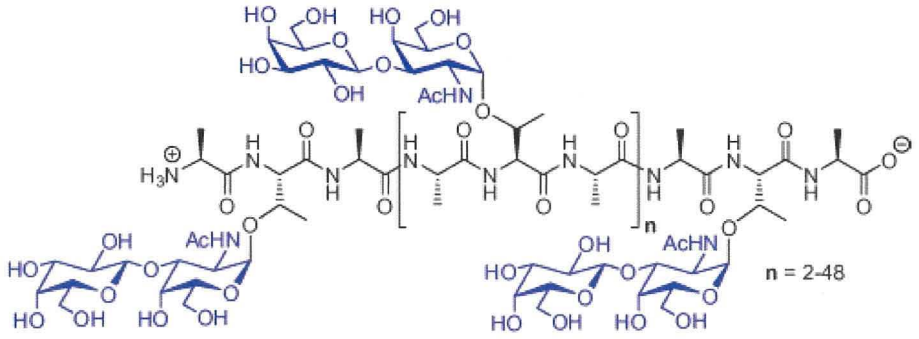


Figure 4.1 Amino acid structure and chemical formula of notothenioid AFGPs.

(A) Illustration of the repetitive tripeptide AAT sequence of notothenioid AFGPs with the possible substitutions of alanine with proline after the 6th position in AFGP type 7 (n=4) and type 8 (n=2) (Figure A modified from Lin, et al., 1972).
(B) Chemical formula of native AFGPs with the repetitive unit in paraphrases including the number of repeats (n) ranging from 2–48 resulting in a total of 4–50 repeats of the A/P–A–T subunit (Figure B from Corcilius, et al., 2013).

It was demonstrated in *P. borchgrevinki* that AFGP types 7 and 8 represent a group of AFGPs with a varying number of proline instead of alanine residues, thus with a ratio of A:P in the amino acid chain from position 7 onwards (Lin, et al., 1972). It was then discovered (Morris, et al., 1978) that type 8 represents a protein group in which proline substitutes alanine at positions 7, 10 and 13, resulting in a mixture with mainly three amino acid sequences in proportions of circa 7:2:1 as follows:

1) Ala-Ala-Thr-Ala-Ala-Thr-**Pro**-Ala-Thr-Ala-Ala-Thr-**Pro**-Ala
2) Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-Thr-**Pro**-Ala-Thr-**Pro**-Ala
3) Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-Thr-**Pro**-Ala

The authors interpreted their data further in that AFGP 8 only exists in very small quantities as a pure alanine species without any proline residues or as a molecule containing all three proline substitutions:

4) Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala
5) Ala-Ala-Thr-Ala-Ala-Thr-**Pro**-Ala-Thr-**Pro**-Ala-Thr-**Pro**-Ala

The antifreeze properties of the glycoproteins derive from their sugar residues or moieties, as any chemical alterations in these lead to the reduction or entire loss of any antifreeze activity (Komatsu, et al., 1970; DeVries, 1971; Shier, et al., 1972; Vandenheede, et al., 1972; Ahmed, Osuga & Feeney, 1973).

The molecular analysis by MS must therefore detect the entire antifreeze molecule or parts thereof as the monosaccharide entities and the amino acids are essential building blocks of the protein primary structure.

The basic unit of a sugar or saccharide is a single molecule or monosaccharide, such as the β–D–galactose in AFGPs. This sugar is a hexose (Hex) as it consists of six carbon atoms, and it has the chemical formula $C_6H_{12}O_6$. A hexose has different classifications according to its functional group. The latter can either be an aldehyde at position C–1 in the case of aldohexoses or a ketone at C–2 in the case of ketohexoses. β–D–galactose is one

stereoisomer out of the eight naturally occurring conformations of an aldohexose. α–*N*–acetyl–D–galactosamine is a derivative of galactose, and is termed an amino sugar as it contains an amine group ($–NH_2$) in place of one hydroxyl group (–OH). In the case of AFGPs, one galactose molecule is attached by an α–glycosidic 1–3 linkage with *N*–acetylgalactosamine to form the disaccharide β–D–galactosyl–(1→3)–α–*N*–acetyl–D–galactosamine or β–D–galactopyranosyl–(1→3)–2–acetamido–2–deoxy–α–D–galacto–pyranose (Gal–α–(1→3)GalNAc or GalNAc–Gal or HexNAc–Hex) (DeVries, et al., 1970; Komatsu, et al., 1970; DeVries, et al., 1971; Shier, et al., 1972; Vandenheede, et al., 1972; Shier, et al., 1975). The two monosaccharides form a linear heteropolymer, termed a glycan. In an enzyme–mediated process known as glycosylation, this glycan is attached to a molecule such as a peptide. In AFGPs the glycan GalNAc–Gal is covalently O–linked to the hydroxyl group of the threonine of the structural peptide backbone (Komatsu, et al., 1970; DeVries, et al. 1971). As a result, the disaccharide becomes the carbohydrate moiety of a conjugate with a protein, and is now referred to as a glycoprotein. Hence the terminology of freezing point depressing entities in Antarctic fishes as antifreeze glycoproteins or AFGPs.

4.2.2 Introduction to MS for interpretation of a peptide mass spectrum

Knowledge of the exact molecular structure allows the analysis and detection of AFGPs in a sample from fish parasites. The fundamentals necessary to understand and interpret the results are outlined in full elsewhere (see Appendix A.2). In principle, molecule ions pass through the instrument and separate based on their masses and the time of travel is determined for each in a *time–of–flight* (TOF) mass spectrometer. The amino acid sequence of a protein is determined with aid of *tandem mass spectrometry* (MS/MS) in which two mass analysers are separated by a collision cell to selectively fragment molecular ions for analysis (Fig. 4.2).

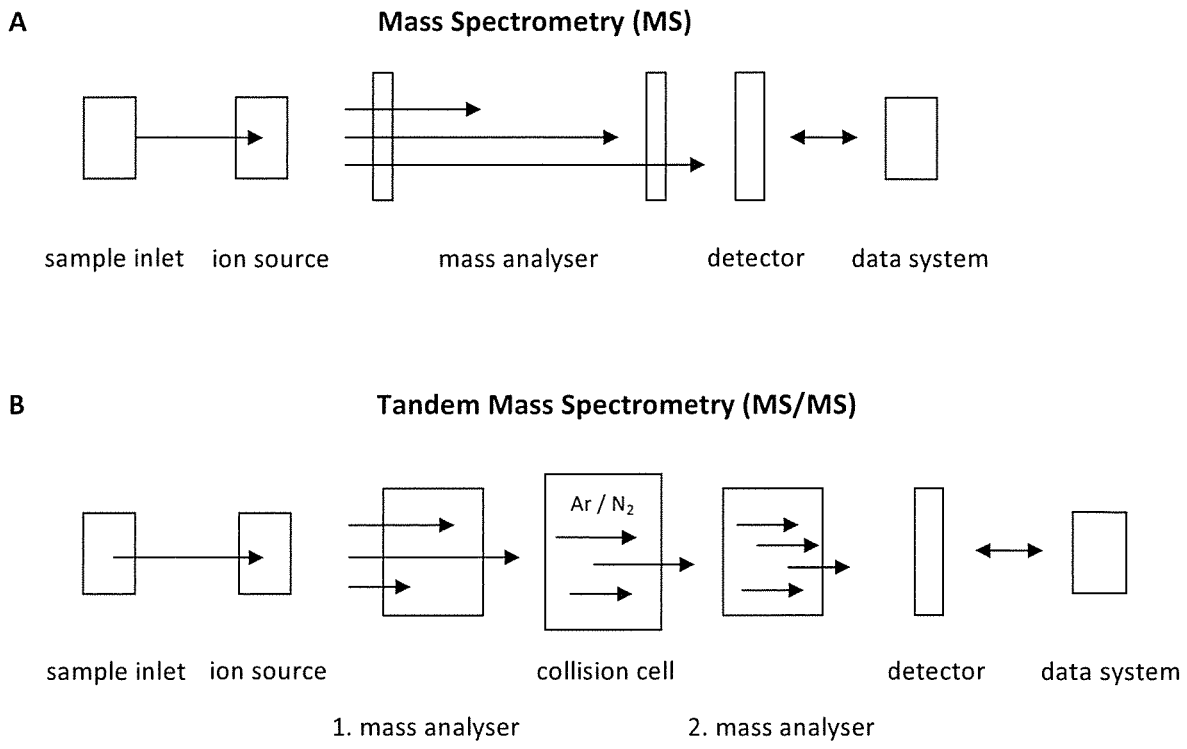


Figure 4.2 Schematic illustration of *time–of–flight* (TOF) mass spectrometry.

Principles for (A) mass spectrometry (MS) and (B) tandem mass spectrometry (MS/MS). Both systems are *time–of–flight* (TOF) instrumentations in which the time for the flight of the ions from source to detection are recorded to determine their *mass–to–charge* (*m/z*) ratios. A single–stage MS consists of an ion source where the molecular ionisation of the inserted sample occurs, a mass analyser and a detector with data system and analogue–to–digital conversion of signals. This setup is extended in tandem MS by a gas–phase collision cell (argon or nitrogen) and a further mass analyser in the flight path. In practice, this allows for the selection of a specific ion species by its *m/z* value from the first analyser to enter the collision cell for further fragmentation before a second mass analysis determines the atomic masses for this one specific ion (Figure adapted from Yates, 2000; Gross, 2011, p. 8).

The protein backbone breaks specifically at the amide bonds and the process results in fragmented product ions termed *b*–type ions from the N–terminus and *y*–type from the C–terminus (Fig. 4.3). Specific previous knowledge as well as databank use allows for the description of the underlying protein sequence based on the variety of different molecule fragmentations in the MS/MS experiments.

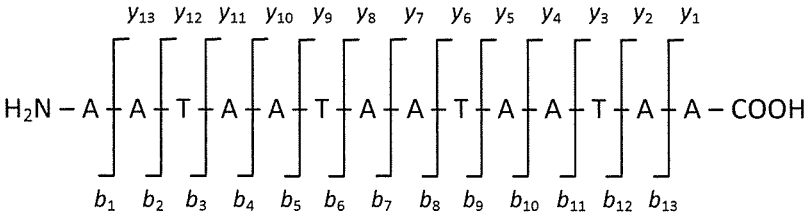


Figure 4.3 Naming scheme for product ions of a protein fragmentation.

Typical fragmentation patterns occurring with tandem MS leading to the production of *b*– and *y*–type fragmentation ions.

4.2.3 Aim of chapter

The aim of this chapter was to use MS for the analysis of the chemical properties of AFGPs previously visualised by immunological fluorescence staining. In order to conclude whether AFGPs are present in piscicolids, this analytic method must first detect the molecular mass of one AFGP type or more. Subsequently, the existence of AFGP derived ion fragmentations must be demonstrated. These are the distinguishing tripeptide AAT subunits as the repetitive building blocks for the primary structure and the sole disaccharide residues HexNAc–Hex. Furthermore, the results must include a variety of ions whose formations are possible when the basic components of AFGPs are combined and chemically altered during the MS experimentation.

4.3 Material & Methods

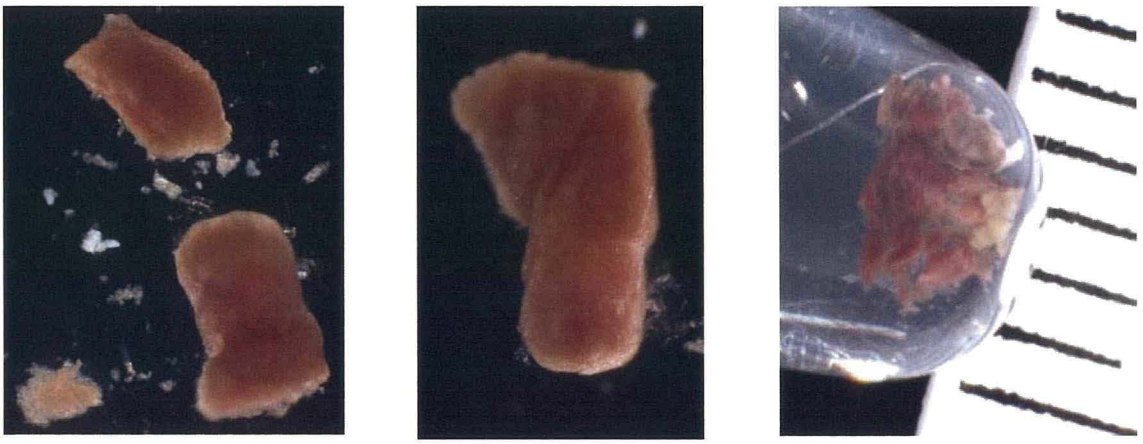
The antifreeze protein analyses were undertaken on specimens of *C. antarctica* and *C. levigata* collected off *Trematomus bernacchii* or *T. hansoni* and ethanol fixed in the field at McMurdo Sound, Antarctica. The material was analysed in singular repeats derived from the homogenisation of an entire specimen of *C. antarctica,* whereas selective body compartments were investigated for *C. levigata*. For the latter these were the contents of the digestive system, the epidermis including some of the attached subdermal epi– and endothelial as well as muscular tissues, and the posterior sucker, which is mainly muscular with some dermal tissue included. In total four different MS experiments were performed using fresh material from specimens in their natural state of the two piscicolid species available to this PhD.

The protocol followed established MS work on notothenioids by Clive W. Evans and Martin Middleditch (pers. comm. & Evans, et al., 2011; Evans, et al., 2012) with specific modifications and adjustments for work on piscicolid samples as a series of difficulties had to be overcome and taken into consideration during the preparation procedures especially for the body parts and tissue material of *C. levigata*.

4.3.1 Leech material preparation

An entire specimen of *C. antarctica* was used for homogenisation and MS analysis (n=1).

A portion of the content from the digestive tract was extracted from one larger *C. levigata* specimen (n=1) by opening the body cavity lengthwise from anterior to posterior using a fine disposable stainless steel syringe needle as a microscalpel. The outer tissues were removed by fine cuts using the needle as a microscalpel and Dumont forceps. The hardened gut content could then be removed with minimal contamination from the associated tissue (Fig. 4.4). The recovered granules were ground in a small volume of 0.1% (v/v) formic acid (Scharlau, Barcelona, Spain) in HPLC–grade Milli–Q water.



A B C

Figure 4.4 Images of gut content after dissection of *C. levigata.*

(A) & (B) Individual pieces of gut content extracted as very small particles of hardened material from one large specimen of *C. levigata* (magnification x40).
(C) Collected material in Eppendorf tube prior to homogenisation (ruler in mm scale; magnification x10).

The sample of epidermis from one large *C. levigata* (n=1), with subcutaneous tissues including muscles, was obtained by separating and carefully folding the outer skin from the internal tissues with fine cuts of a syringe needle used as a microscalpel, dissecting needles and Dumont forceps.

Finally, the posterior sucker as muscle covered with dermal tissues was used as a sample void of any fish contaminations other than by metabolic processes. The material was obtained from four individual *C. levigata* and then pooled for analysis (n=1). The anus was located, and material from below this point was carefully separated so as to exclude artefactual fish AFGPs deriving from erroneous preparative cuts and also any natural contents of the digestive tract.

Each sample was transferred into a small 1.5 ml Eppendorf tube for manual homogenisation as pretreatment to analysis. It was found that a small volume of liquid as well as a micropestle were necessary to mechanically crush the hard gut contents and leathery tissues (Fig. 4.5). This simple technique of a manual micropestle proved highly effective for a complete tissue destruction resulting in even homogenisation of the different samples. A minimal volume of 10–50 µl of 0.1% formic acid was added so as to achieve

homogenisation of the tissue samples using the lowest dilution factor possible. The samples were stored frozen at –30°C until processed.

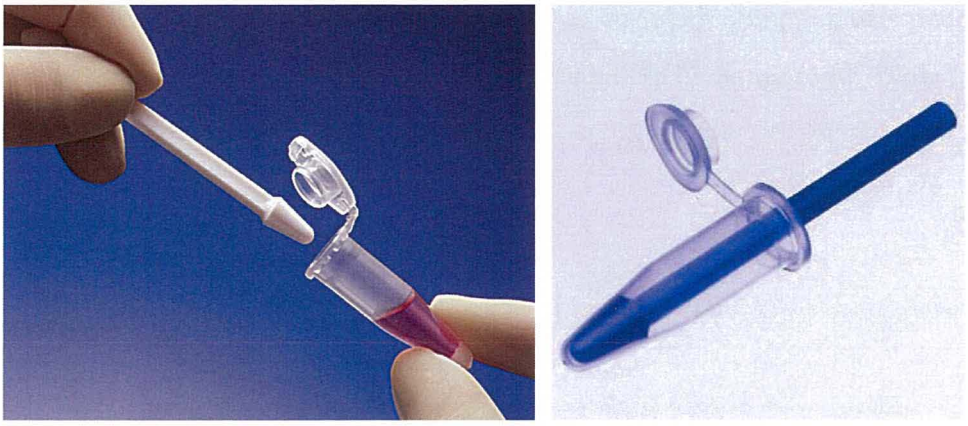


Figure 4.5 Illustration of micropestle use for tissue homogenisation.

Demonstration of use of micropestle and Eppendorf tube for manual homogenisation of tissue samples (Copyright by Eppendorf, Hamburg, Germany; image retrieved from Eppendorf's homepage).

4.3.2 Solid phase extraction (SPE)

Prior to HPLC a solid phase extraction (SPE) was performed for an initial cleaning of the homogenised samples. This was accomplished with a small Oasis HLB 10 mg SPE extraction cartridge (Waters, Milford, Massachusetts, USA), which was preconditioned and activated with 1 ml HPLC–grade 100% methanol (Merck, Haar, Germany) and equilibrated with 0.1% formic acid before each sample was loaded. For all samples of the homogenised piscicolid material, with volumes ranging from 20–50 µl, the insoluble material was centrifuged (16,000 g; 3 min) to form a pellet. The supernatant was transferred into 0.5 ml 0.1% formic acid and applied onto the SPE cartridge at a flow–rate of circa 1 ml/min, applied manually with a syringe as positive pressure. One wash with 0.5 ml 5% (v/v) methanol was followed by the elution of the target proteins in a final volume of 0.3 ml 10% (v/v) HPLC–grade acetonitrile (Scharlau) to obtain a first protein fraction. A second application of 0.3 ml 20% (v/v) acetonitrile was carried out to test and ensure that a full recovery of all protein material from the SPE column was achieved for every leech sample. This analyte was

concentrated by acetonitrile evaporation in a vacuum centrifuge (Thermo Savant SPD121P, Thermo Savant, Holbrook, New York, USA) until only circa 5 µl remained before it was reconstituted by addition of 0.1% formic acid to a volume of 20 µl. This final volume was used for subsequent MS analysis in the case of the posterior sucker material of *C. levigata*. Other samples had a dilution factor of 2x for the skin and 3x for the gut content samples of *C. levigata*, which were obtained by the addition of 10 or 20 µl 0.1% formic acid to a volume of 10 µl taken from the above 20 µl final volume. The whole leech homogenate of *C. antarctica* was 10x diluted.

4.3.3 Mass spectrometry analysis

4.3.3.1 Reversed phase high pressure liquid chromatography (RP–HPLC)

The RP–HPLC was run as a gradient elution with a flow–rate of 6 µl per min. The final SPE volume of 20 µl was injected into a trap column PepMap C18 (300 µm inner diameter x 5 mm; LC Packings, Amsterdam, Netherlands) for a sample enrichment, using a mobile phase consisting of 2% acetonitrile in 0.1% formic acid.

For each analysis, after 3 min the protein sample in the trap was switched in line, flushed by the mobile phase and thus transferred into the analytical column, a Zorbax 300SB–C18 (300 µm inner diameter x 100 mm; Agilent, Santa Clara, California, USA), with the mobile phase consisting of solvent A as Milli–Q water with a final concentration of 0.1% formic acid and solvent B as 99.9% acetonitrile with 0.1% formic acid. Prior to this, the analytic column was run with 2% solvent B for 3 min before the trap column was switched in line for sample injection.

A linear gradient for the protein elution was applied in the form of an increase in solvent B from 2% to 35% over the next 20 min at a flow–rate of 6 µl per min. This marked the end of the sample run and a wash step followed in which solvent B was rapidly increased within 3 min to 95%. The column was cleaned at this level for several minutes before it was reset and re–equilibrated for subsequent runs with 2% solvent B for 5 min.

For maximum data acquisition the column effluent for each sample was continuously monitored by transfer into the ion source of the tandem mass spectrometer for the entire

duration of each sample run of around 35 min. This made the classic spectrometric analysis obsolete in which the elutions are monitored at 230 and 280 nm UV absorbance to analyse the purity of the glycoprotein fractions. An absorbance at 230 nm but not at 280 nm occurs only in the absence of contaminating proteins with aromatic residues (DeVries, 1986).

4.3.3.2 Tandem mass spectrometry (MS/MS)

The QSTAR XL hybrid system (Applied Biosystems, South San Francisco, California, USA), in which the HPLC is coupled via the *electrospray ionisation* (ESI) source to the mass spectrometer, was used for the MS/MS analysis. The transfer of samples from HPLC into the first mass spectrometer, at the low flow–rate of 6 µl per min, was accomplished by ESI as *atmospheric pressure ionisation* (API) at room temperature. A spray voltage of 5,000 V was applied with a continuous coaxial nitrogen flux of circa 5 l/min to desolvate the ions from any mobile phase chemicals that may cause adverse effects as impurities in MS.

In the tandem mass analysis the selection process occurred during full survey scan mode in which selected ion species at *mass–to–charge ratio m/z* 300–1,600 were continuously analysed as they emerged. Ion species of unassigned or single charge state in survey scans were filtered out by being rejected from entering the collision cell prior to MS/MS. This ensured that only the most abundant multiply charged molecular ions passed into the collision cell with *collision–induced dissociation* (CID) settings of 20–40 eV, followed by subsequent mass spectrometric measurements of all product ions. The empirically predetermined software settings of the manufacturer were used according to ion charge and *m/z* values for peptides. The raw data sets obtained were deconvoluted using the Analyst QS software package (Applied Biosystems) to translate TOF information into molecular masses. The correct identification of peptide and carbohydrate entities was performed by manual interpretation of the raw data based on the reasonable expectation that spectra are based on AFGPs and their characteristic fragmentations.

The MS/MS data acquisition was set to start at *m/z* 100 and lower *m/z* ranges (< 100) were not acquired as these provide merely single amino acid ions with no sequence information and thus hold little value as they do not provide unambiguous information in MS. Standard scans of protein samples start at *m/z* 300, as peaks below this value represent normally single and not multi–charged ion species as artefacts and background noise. These

can derive from impurities such as residual air (range *m/z* 18–44), silicon grease or from HPLC column bleed of the silicon dioxide (silica) matrix both in the form of silicon Si_X isotopic patterns at *m/z* 73–429 or hydrocarbons from grease at *m/z* 27–500 as well as common plasticisers in laboratory equipment and vials such as phthalates, especially di–2–ethylhexyl phthalate or DOP (e.g. 149, 167, 279 for DOP), amongst many other possible impurities (Gross, 2011, pp. 299, 729).

A computer simulation was used to provide an image of the peptide structure derived from the AFGP type 8 amino acid sequence AAT AAT PAT AAT PA with *monoisotopic accurate formula mass* of 1,184.6017 u including one water (Fig. 4.6; produced with software PepDraw, v. 1, image retrieved from http://www.tulane.edu/~biochem/WW/PepDraw/, 2011 copyright by Tulane University, Louisiana, USA, with courtesy of Universal Protein Resource Knowledgebase (UniProtKB) and Swiss–Prot science collaborations).

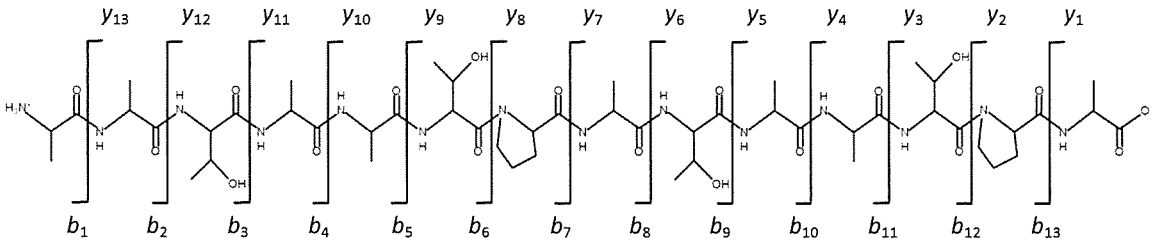


Figure 4.6 Chemical formula of AFGP type 8 with naming of fragmentation ions.

Peptide structure illustration derived from 14 amino acid sequence AAT AAT PAT AAT PA with monoisotopic accurate formula mass 1,184.6017 u (obtained using PepDraw).

Additionally, General Protein/Mass Analysis for Windows software (GPMAW; v. 4, Lighthouse Data, Odense, Denmark) was used for protein and peptide analysis in MS to calculate a full list of theoretical fragmentation ions deriving from AFGP type 8 as per the above amino acid sequence variant of AAT AAT PAT AAT PA.

4.4 Results

The two leech parasites *C. antarctica* and *C. levigata* show monoisotopic molecular ion peaks $[M+H]^+$ in MS for oligopeptides of equivalent M_r to AFGP types 7 and 8 described for their notothenioid fish host species.

The identification by *m/z* values (Duman, et al., 2002) or deconvoluted M_r (Li, Andorfer & Duman, 1998) are both equally accurate and accepted methods used to interpret MS results on antifreeze proteins in the beetle *D. canadensis* as well as in the bald notothen or borch *P. borchgrevinki* (Morris, et al., 1978; Evans, et al., 2012) and naked dragonfish *Gymnodraco acuticeps* (Evans, et al., 2011).

The ESI mass spectra of fish and leech samples obtained from survey scans, as well as tandem MS, show striking similarities in regards to the distribution pattern of molecular ion peaks and to their relative abundances.

The nominal *m/z* values detected in the two parasite species and the sampled body compartments are identical to results found in their hosts. Significantly, their accurate values are in agreement to 1 d.p. In conclusion, the match of leech with fish data at this level of accurate mass determinations supports the interpretation that the ion peaks represent the same M_r. This, in combination with the sequencing information provided below, indicate that these ion peaks detected are identical molecular species and true AFGP signals detected in each parasite sample.

The mass spectra are of high quality and only show minimal background noise with low signal intensities. These artefacts are caused by common MS impurities at low *m/z* values and can derive amongst others from residual air, hydrocarbons and silicon from grease or LC column bleeding (Gross, 2011, p. 729).

The results include a formula of AFGP 8 set to the most common sequence variant for this peptide group known from notothenioids as 14 amino acid residues AAT AAT PAT AAT PA (Morris, et al., 1978) (Fig. 4.6).

A comprehensive calculation and list of the theoretical LC–MS/MS fragmentations of the AFGP 8 peptide as AAT AAT PAT AAT PA sequence molecule includes all *b*– and *y*–type ions

(Table 4.1 & 4.2; using GPMAW), with the undetectable first fragments b_1 and y_1 added for completeness.

The LC–MS/MS results are shown for the posterior sucker material of *C. levigata* as measured accurate masses of internal *b*– and *y*–type fragmentation ions of an AFGP 8 as sequence variant AAT AAT PAT AAT PA (Table 4.3). The results are within the expected error of measurement of MS at $\Delta m \leq 0.2$ u (Table 4.4).

The fixation of material in the field and its preservation with 70% ethanol had no negative effect on the detectability of AFGPs in MS, nor did the further treatment of samples with chemicals used during the preparations and MS protocol show adverse effects on the primary structures of peptides extracted from piscicolid samples. This was as expected and in agreement with previous findings on AFGPs treatment with, for example, ethanol, acetone (DeVries, et al., 1970) and acetonitrile or formic acid (Evans, et al., 2011).

Table 4.1 Theoretical LC–MS/MS fragmentations of AFGP 8.

Peptide sequence set to 14 amino acids AAT AAT PAT AAT PA (as in Fig. 4.6) results in the respective amino acid residues [u] with position number in bond breakages for *b*–type and for *y*–type fragment ions. The first fragments b_1 and y_1 are added for completeness.

b fragment	Position	Residue	Position	*y* fragment
(72.045)	1	Ala	14	–
143.082	2	Ala	13	1114.574
244.130	3	Thr	12	1043.537
315.167	4	Ala	11	942.490
386.204	5	Ala	10	871.453
487.252	6	Thr	9	800.415
584.304	7	Pro	8	699.368
655.342	8	Ala	7	602.315
756.389	9	Thr	6	531.278
827.426	10	Ala	5	430.230
898.463	11	Ala	4	359.193
999.511	12	Thr	3	288.156
1096.564	13	Pro	2	187.108
1167.601	14	Ala	1	(90.056)

Table 4.2 Assorted mass list of theoretical LC–MS/MS fragmentation ions of AFGP 8.

The potential MS/MS fragment ions derived from the peptide AAT AAT PAT AAT PA (as in Fig. 4.6) including the undetectable first fragments b_1 and y_1 for completeness (calculated using GPMAW).

Sorted mass	Fragment type	Position
(72.045)	*b*	1
(90.056)	*y*	14
143.082	*b*	2
187.108	*y*	13
244.130	*b*	3
288.156	*y*	12
315.167	*b*	4
359.193	*y*	11
386.204	*b*	5
430.230	*y*	10
487.252	*b*	6
531.278	*y*	9
584.304	*b*	7
602.315	*y*	8
655.342	*b*	8
699.368	*y*	7
756.389	*b*	9
800.415	*y*	6
827.426	*b*	10
871.453	*y*	5
898.463	*b*	11
942.490	*y*	4
999.511	*b*	12
1043.537	*y*	3
1096.564	*b*	13
1114.574	*y*	2
1167.601	*b*	14

Table 4.3 LC–MS/MS results from *Cryobdella levigata* posterior sucker material.

List of fragment ions derived from AFGP 8 peptide AAT AAT PAT AAT PA obtained for $[M+H]^+$ at *m/z* 1,185.612. Different sequences for internal fragments from the 14 amino acid residue backbone can be detected in the ESI mass spectra (Fig.4.14). The measured accurate mass 1,167.464 u for AFGP 8 deviates by Δm 0.1224 u after deprotonation from the formula mass 1,166.5864 u and is within the marginal MS measuring tolerance. Note not all variations for *y*–type fragments are detected and y_8^* shows as y_8 at *m/z* 699.368 with loss of one water molecule. The first fragments b_1 and y_1 cannot be detected in MS without ambiguities.

b fragment	Position	Residue	Position	*y* fragment
–	1	Pro / Ala	14	–
169.062	2	Ala / Pro	13	1114.574
270.172	3	Thr	12	–
341.225	4	Ala	11	–
412.157	5	Ala	10	–
513.197	6	Thr	9	800.377
584.250	7	Ala	8	681.329
655.320	8	Ala	7	602.315
756.308	9	Thr	6	531.218
827.340	10	Ala	5	430.230
898.322	11	Ala	4	359.179
999.370	12	Thr	3	270.110
1167.464	13+14	Pro + Ala	1+2	–

Table 4.4 Deviation for *C. levigata* posterior sucker results from expected values.

List of *b*–type fragment ions from the monoisotopic molecular ion peak $[M+H]^+$ at *m/z* 882.7 as AFGP 8. The measured accurate mass of each fragment (Fig. 4.14) is compared to its theoretical accurate formula mass in order to obtain the mass error Δm in unified atomic mass [u] with ≤ 0.2 u as expected MS tolerance. The first fragment b_1 lies below the cut off at 100 u.

Product ion	Measured mass [u] for $[M+H]^+$ ion	Theoretical mass [u] for $[M+H]^+$ ion	Δm [u]
$b_{14}^{\bullet}$	1167.464	1167.46	0.005
b_{12}	999.384	999.370	0.014
b_{11}	898.377	898.322	0.055
b_{10}	827.345	827.340	0.005
b_9	756.368	756.3079	0.060
b_8	655.287	655.3203	–0.033
b_7	584.234	584.250	–0.016
b_6	513.205	513.197	0.008
b_5	412.262	412.157	0.105
b_4	341.209	341.225	–0.016
b_3	270.110	270.172	–0.062
b_2	169.065	169.062	0.003
b_1	–	98.025	–

4.4.1 Identification of notothenioid AFGPs in *Cryobdella spp.* by their M_r

All MS data were obtained from SPE elutions using 10% acetonitrile (analogous to fish samples) as the subsequent second application of 20% acetonitrile did not provide peaks, and thus yielded no material from the SPE columns.

The monoisotopic molecular ion peaks $[M+H]^+$ at *m/z* 882.724 and 1,085.482 were very similar in the mass spectra of each piscicolid sample and represent the nominal molecular masses of 2,646 u and 3,254 u. These protein fractions elute between 12:30 and 15 min running time from the HPLC column in all piscicolid samples as can be seen in different survey scans of *C. antarctica* (Fig. 4.7 A) and *C. levigata* (Figs. 4.8–4.10).

The data is in agreement with 2,600 u as type 8 (DeVries, et al., 1970) as well as 2,600 u and 3,500 u for types 8 and 7, respectively, (Ahlgren & DeVries, 1984) determined in both studies by ion exchange chromatography. Ahlgren and colleagues (1984) illustrated in their gel electrophoresis some slight molecular size differences for both types of AFGP in nine fish species caught from the Southern Ocean: *Chaenocephalus aceratus, Dissostichus mawsoni, G. acuticeps, Gobionotothen gibberifrons* (Lönnberg, 1905) [former *Notothenia gibberifrons* (Lönnberg, 1905)], *Lepidonotothen larseni* (Lönnberg, 1905) [former *Notothenia larseni* (Lönnberg, 1905)], *Notothenia coriiceps* (Richardson, 1844), *Notothenia rossii* (Richardson, 1844), *T. hansoni* and *T. loennbergii*. The M_r calculated from the structures determined by Lin et al. (1972) were given as 2,600 u for AFGP 8 and 3,800 u for type 7.

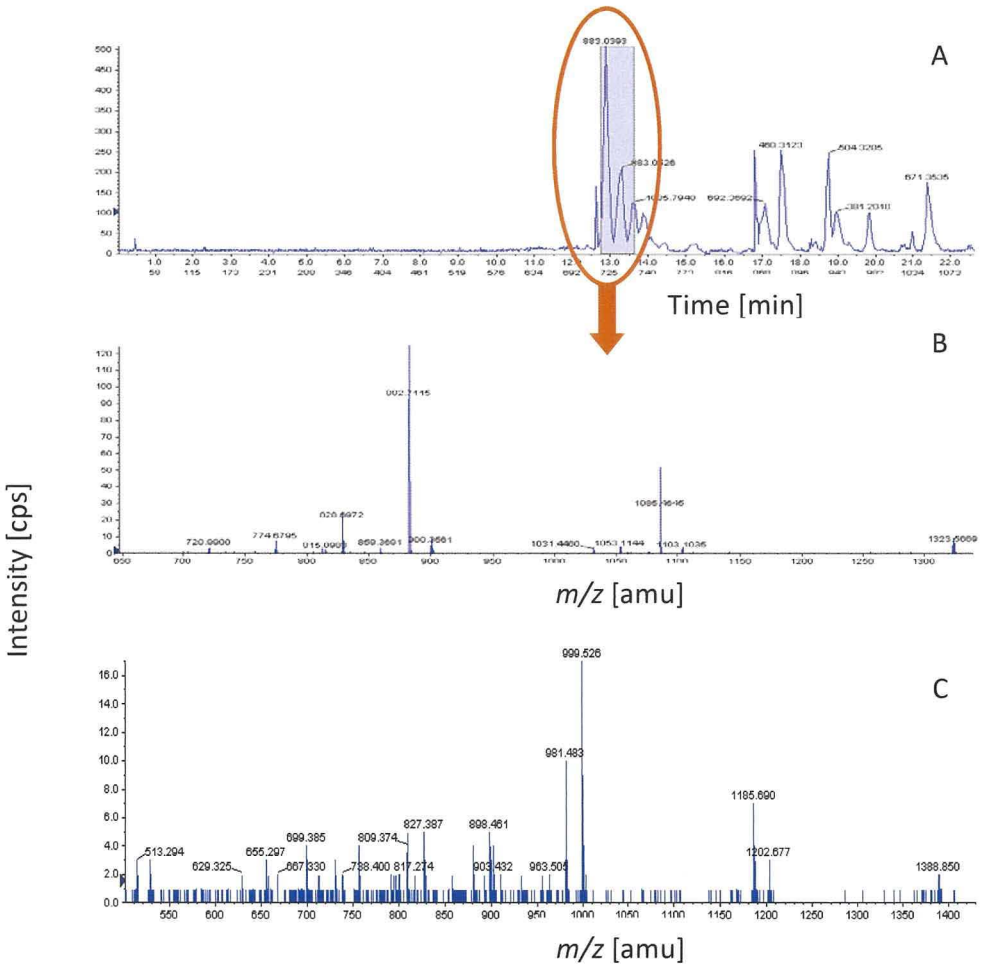
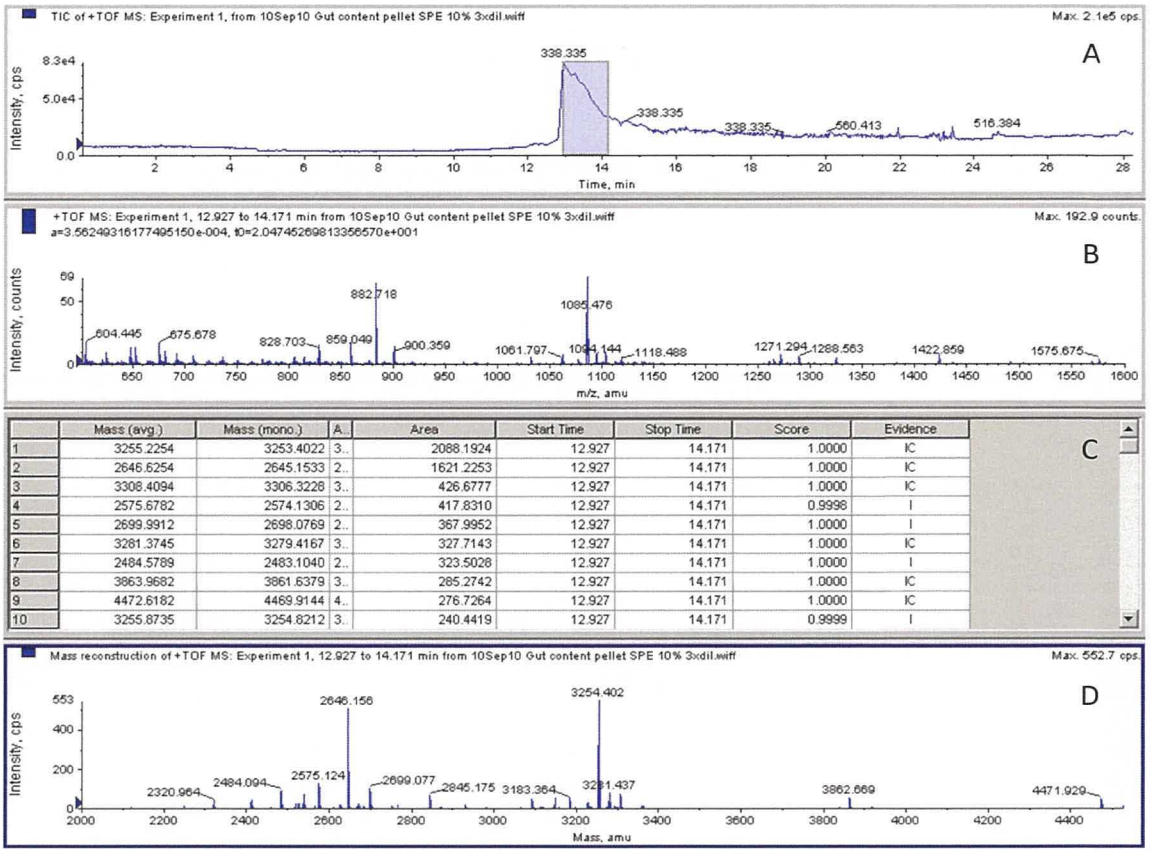


Figure 4.7 Mass spectra of *C. antarctica* whole specimen homogenate.

(A) ESI–MS survey scan with elution times.
(B) ESI–MS spectrum of the two smallest AFGPs, type 8 and 7, as peaks at *m/z* 882.7 and 1,085.5 respectively at elution time frame 12.757 to 13.672 min.
(C) ESI–MS spectrum of M^+ ions at *m/z* 999.5 and 1185.7 as AFGP 8 and 7 at 13.057 min elution time.

The accurate formula mass of the AFGP 8 peptide backbone as the most common AAT AAT PAT AAT PA species is 1,184.6017 u, which lacks the sugar residues. The addition of one galactose residue of 162.0528 u and an *N*–acetylgalactosamine residue of 203.0794 u or 365.1322 u per disaccharide residue results in 1,460.5288 u as the sum of the four attached sugars, and finally in a calculated accurate formula mass of 2,645.1305 u for the AFGP 8 molecule. The M_r for AFGP 8 was detected *in P. borchgrevinki* at 2,645.3 u (Evans, et al., 2012).



	Mass (avg.)	Mass (mono.)	A..	Area	Start Time	Stop Time	Score	Evidence
1	3255.2254	3253.4022	3..	2088.1924	12.927	14.171	1.0000	IC
2	2646.6254	2645.1533	2..	1621.2253	12.927	14.171	1.0000	IC
3	3308.4094	3306.3228	3..	426.6777	12.927	14.171	1.0000	IC
4	2575.6782	2574.1306	2..	417.8310	12.927	14.171	0.9998	I
5	2699.9912	2698.0769	2..	367.9952	12.927	14.171	1.0000	I
6	3281.3745	3279.4167	3..	327.7143	12.927	14.171	1.0000	IC
7	2484.5789	2483.1040	2..	323.5028	12.927	14.171	1.0000	I
8	3863.9682	3861.6379	3..	285.2742	12.927	14.171	1.0000	IC
9	4472.6182	4469.9144	4..	276.7264	12.927	14.171	1.0000	IC
10	3255.8735	3254.8212	3..	240.4419	12.927	14.171	0.9999	I

Figure 4.8 Mass spectra of *C. levigata* intestine sample.

Composite summary illustration of ESI–MS data from digestive content analysis during elution time of 12.927 to 14.171 min.
(A) ESI–MS survey scan with elution times.
(B) ESI–MS spectrum with M^+ ions of AFGP 8 and 7 at *m/z* 882.7 and 1085.5 respectively.
(C) Deconvoluted M^+ as average and monoisotopic molecular masses listed according to their peak area sizes with the two largest being AFGP 7 (3253.4 u) and 8 (2645.2 u).
(D) Mass reconstruction of ion peaks shown in (B) with AFGP 8 of 2646.2 u and 7 of 3254.4 u.

As ESI produces protonated monoisotopic molecular ions with a measured accurate mass in samples of *C. levigata*, the $[M+H]^+$ mass is determined to 2,646.156 u for the intestine (Fig. 4.8 D), 2,646.167 u for the skin (Fig. 4.9 D) and 2,645.135 u for the sucker (Fig. 4.10 C). Their deprotonated and nominal values are perfect matches with the fish data, and furthermore can be seen as within tolerance levels of MS for the accurate values.

AFGP 7 as AAT AAT PAT AAT AAT AAT PA has an accurate formula mass of 1,670.8447 u (with one water and 1,652.8294 u for the actual peptide backbone). The addition of 2,190.7932 u from the six attached disaccharide residues results in 3,861.6379 u as accurate formula mass of AFGP 7 or 3,862 u nominal mass.

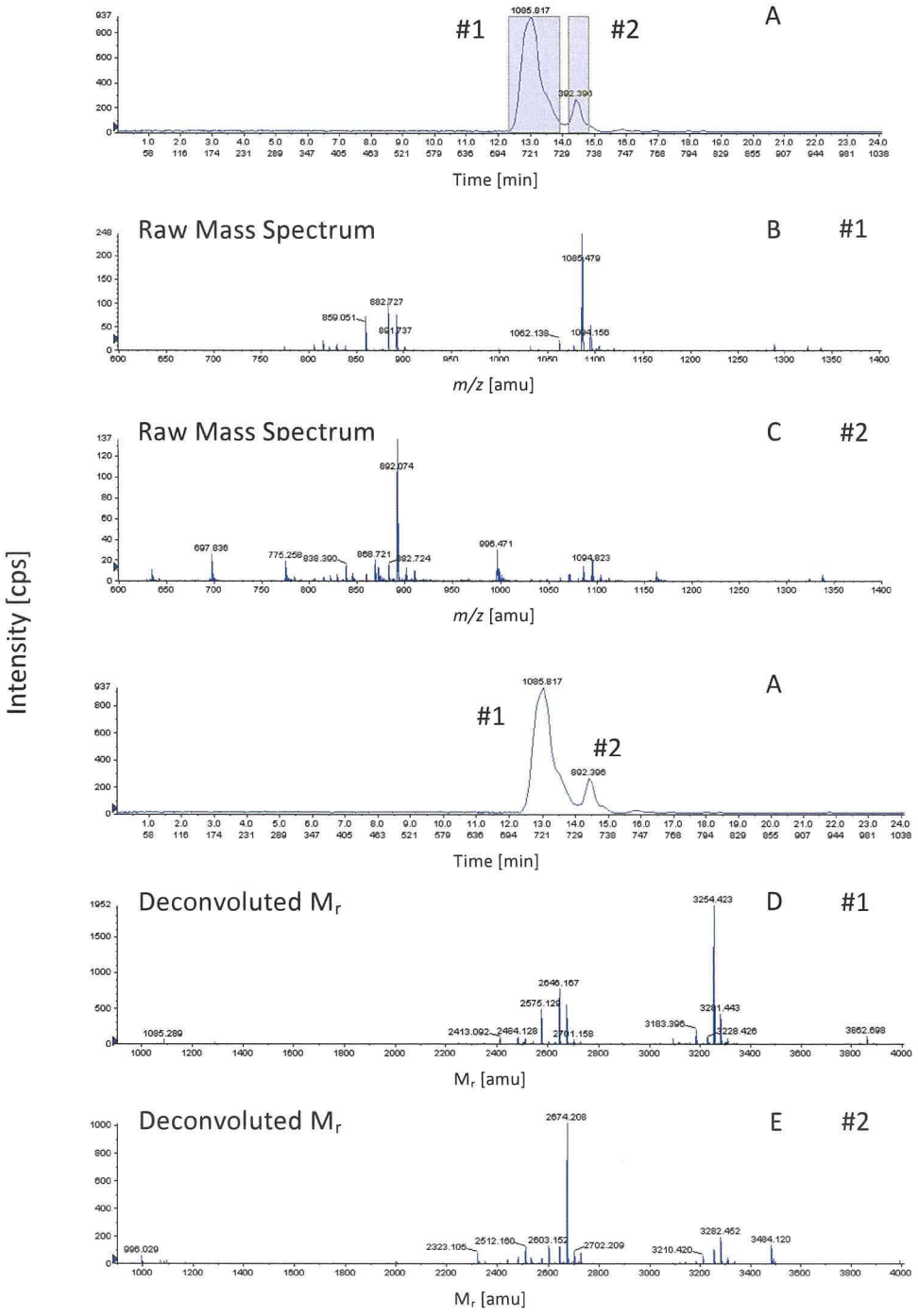


Figure 4.9 Mass spectra of *C. levigata* epidermal sample.

Results of peptide samples at two different elution times (#1 & 2) in the survey scan (A) followed by LC–MS results for these as presented in raw data *m/z* (B & C) with their respective deconvoluted mass reconstructions (D & E) to illustrate the two main peaks which reveal the presence of AFGP 8 (2,646 u) and AFGP 7 (3,254 u) with the proline variants eluting later in both types of AFGP.

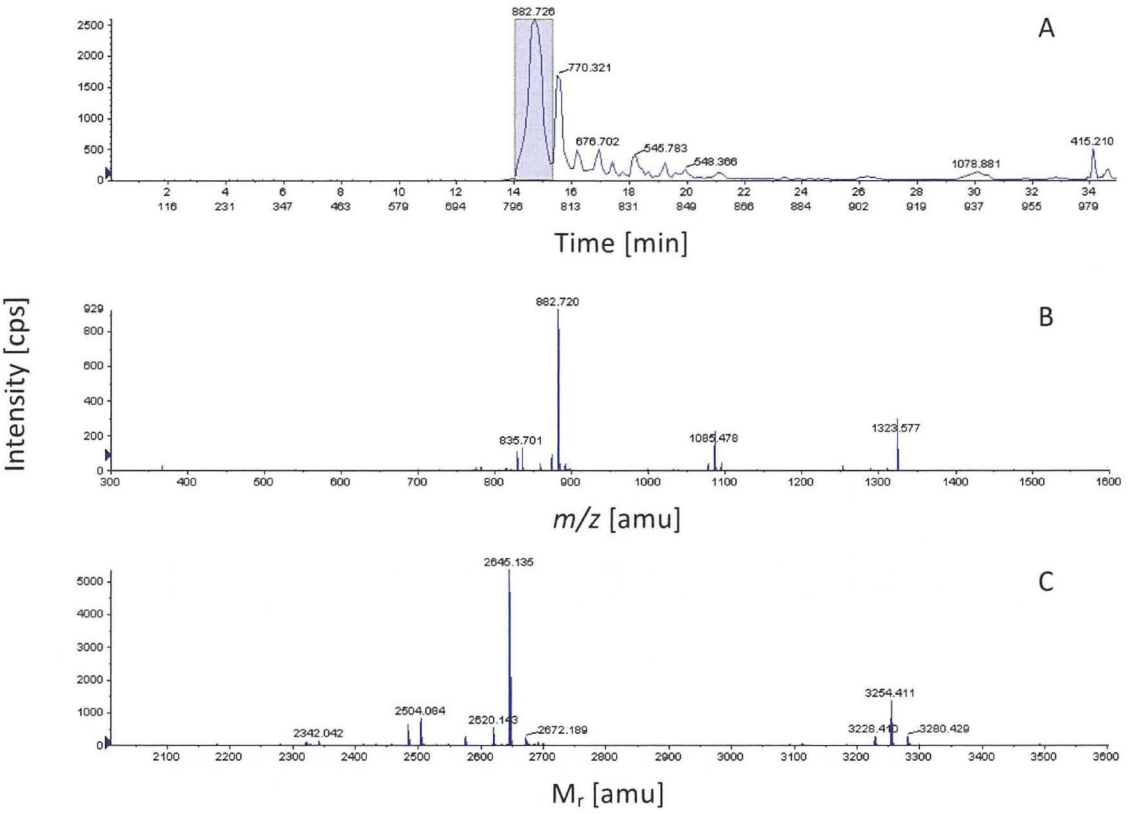


Figure 4.10 Mass spectra of *C. levigata* posterior sucker sample.

(A) ESI–MS survey scan with elution times.
(B) ESI–MS spectrum with M^+ ions of AFGP 8 and 7 at *m/z* 882.7 and 1085.5 respectively during elution time 14.031 to 15.388 min.
(C) Mass reconstruction of ion peaks shown in (B) with AFGP 8 of 2645.1 u and 7 of 3254.4 u.

The measured mass of $[M+H]^+$ species can be detected at low abundance in *C. antarctica* and in higher intensity in samples of *C. levigata*, for the intestine with 3,862.669 u (Figs. 4.8 D & 4.11) and for the skin at 3,862.698 u (Fig. 4.9 D) or 3,862.706 u (Fig. 4.12 A). These agree with the accurate formula mass to the maximal extent as being within the measuring tolerance of MS.

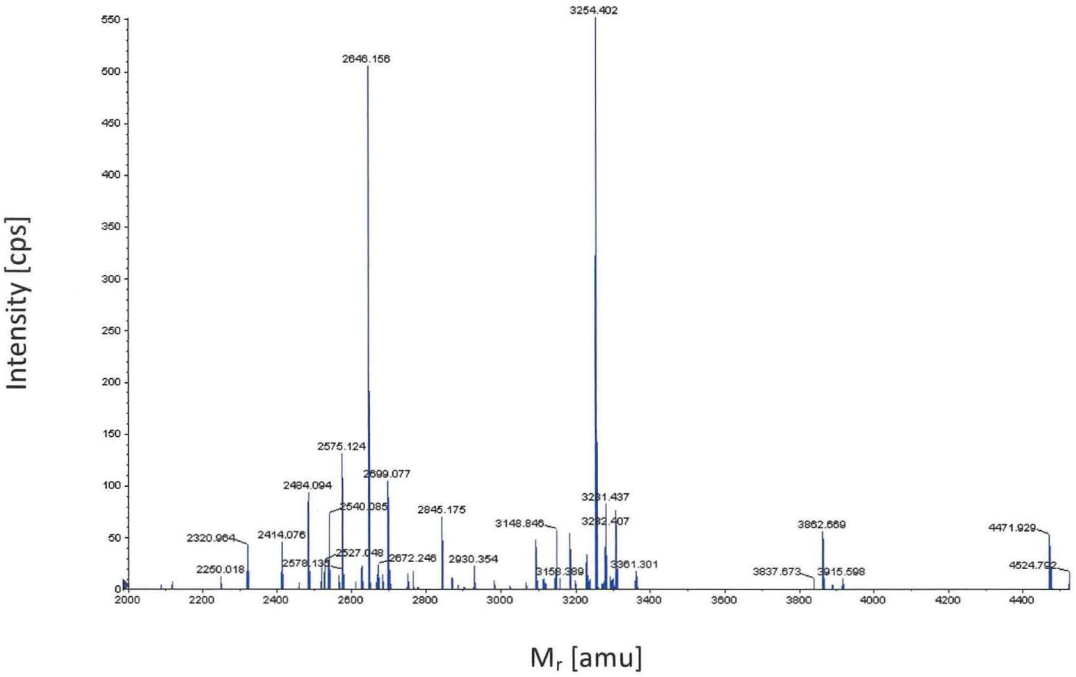


Figure 4.11 Deconvoluted mass spectra of *C. levigata* digestive tract sample.

The mass reconstruction illustrates two main peaks, which reveal the presence of AFGP 8 (2,646 u) and AFGP 7 (3,254 u).

However, the dominant peak with the highest relative abundance for AFGP 7 can be found in all ESI spectra as a fragment ion with the loss of one AAT subunit including sugar residues. The disaccharide residue HexNAc–Hex as $C_{14}H_{23}O_{10}N$ of 365.3347 u plus 243.1219 u from two A with 142.0742 u and T 101.0477 u leads to a total mass of 608.4566 u before protonation by ESI–MS/MS, a value confirmed from literature (Knight, et al., 1993). Thus, a nominal mass loss of 609 u results in a nominal type 7 ion of 3253 u as an intermediate state between 7 and 8. The accurate formula mass of AAT is 261.1320 u (including water) and the disaccharide residue 365.1322 u (residues of Hex 162.0528 u + HexNAc 203.0794 u), which adds to 626.2642 u. After the loss of water 18.0153 this results in 608.2489 u accurate formula mass for the subunit without the proton in ESI–MS.

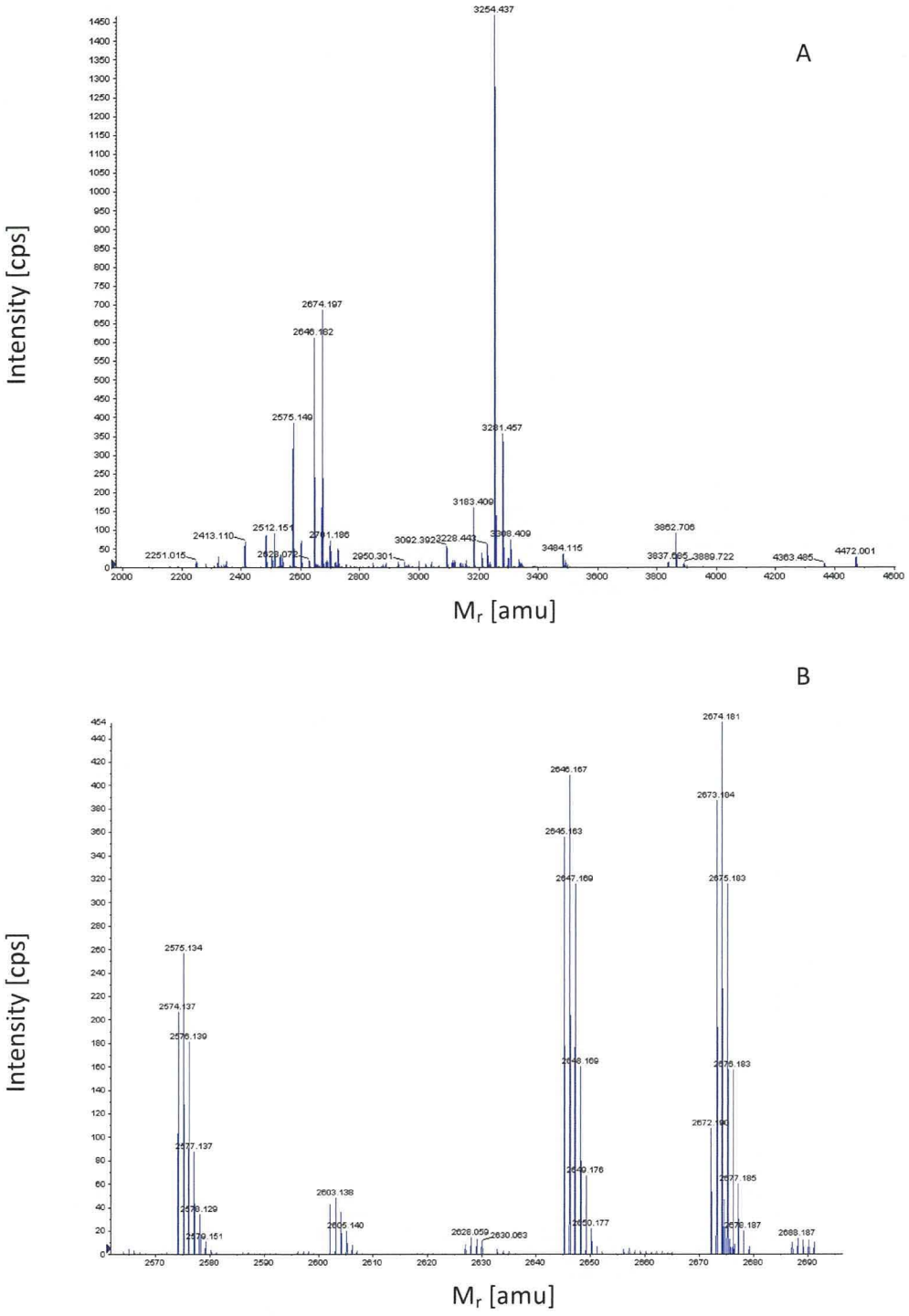


Figure 4.12 A–B Details of mass spectra for *C. levigata* epidermal sample.

Deconvoluted mass reconstructions from ESI mass spectra at elution time 12.24 to 15.99 min. (A) Peaks of AFGP 8 (2,646 u) and AFGP 7 (3,254 u) (A) with A to P shifts and mass increases for AFGP 8 and AFGP 7 and (B) showing isotopic patterns of AFGP 8 ion fragments.

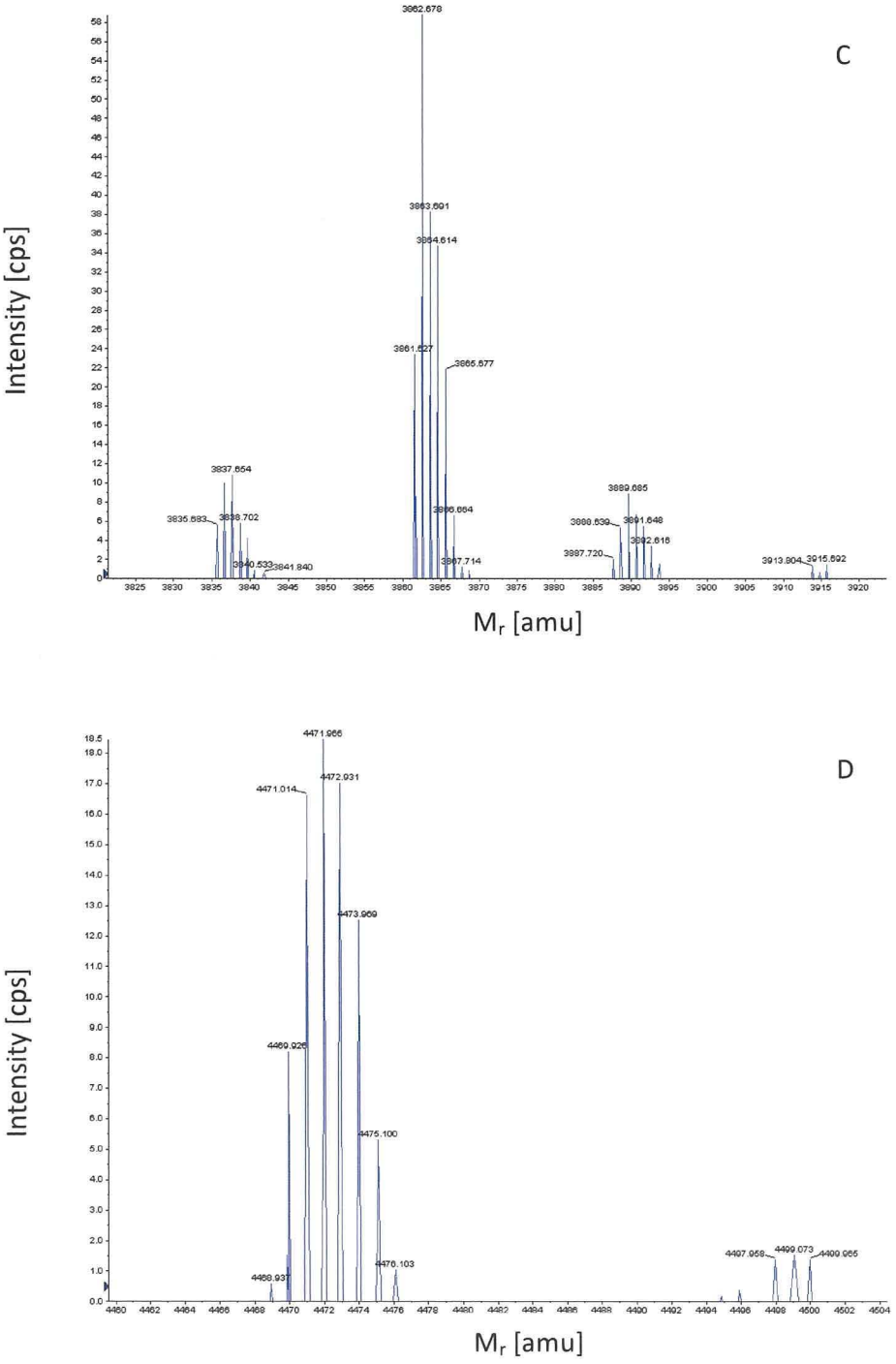


Figure 4.12 C–D Isotopic peaks for *C. levigata* epidermal sample.

Deconvoluted mass reconstructions from ESI mass spectra at elution time of 12.24 to 15.99 min. (C) Isotopic pattern of four sequence variations for AFGP 7 with proline substitutions for alanine as 26 u mass difference between peaks and (D) as higher resolution for one species including a further sugar residue with two peaks of 26 u difference.

The protonated monoisotopic molecular ions in ESI–MS/MS spectra measured accurate masses in *C. levigata* of 3,254.402 u for the intestine (Figs. 4.8 D & 4.11), 3,254.423 u for the skin (Fig. 4.9 D) or 3,254.437 u (Fig. 4.12 A) and 3,254.411 u for the sucker (Fig. 4.10 C). These results also represent matches with the theoretical values and can be seen as being within tolerance levels of MS for their respective accurate mass values.

Although it must be noted that their respective molecular ion peaks are not necessarily the base peaks in the mass spectra obtained, the fish AFGP type 8 equivalent at 2,646 u and type 7 at 3,254 u can be identified in all leech material. These results are identical to values provided for AFGPs 8 and 7 as 2,646 u and 3,254 u, respectively, obtained at an elution time of 12:30 to 15 min in one of the latest publications of LC–MS/MS results for these AFGPs in the external mucus of *G. acuticeps* (Evans, et al., 2011). AFGP samples from blood sera of this fish species were run as a control, under the same experimental conditions and identical instrumentation as leeches in this PhD work, and have an elution of these peptides at around 11 min (Fig. 4.13 A) producing ESI mass spectra of high resolution (Fig. 4.13 B–E).

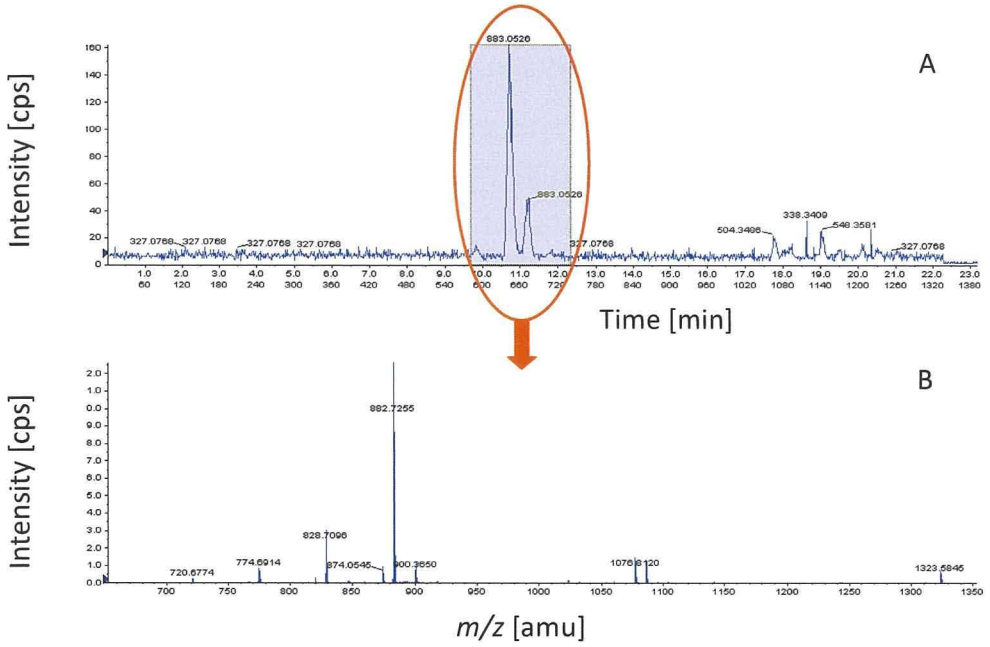


Figure 4.13 A–B AFGPs extract from blood serum of *Gymnodraco acuticeps*.

(A) ESI–MS survey scan with elution times.
(B) ESI–MS spectrum of the two smallest AFGPs, type 8 and 7, as peaks at *m/z* 882.7 and 1,076.8 respectively at elution time frame 9.701 to 12.367 min.
AFGPs extract from Antarctic fish *Gymnodraco acuticeps*, LC–MS output with $[M+H]^+$ at nominal *m/z* 883 and 1,076 representing AFGP type 8 and 7.

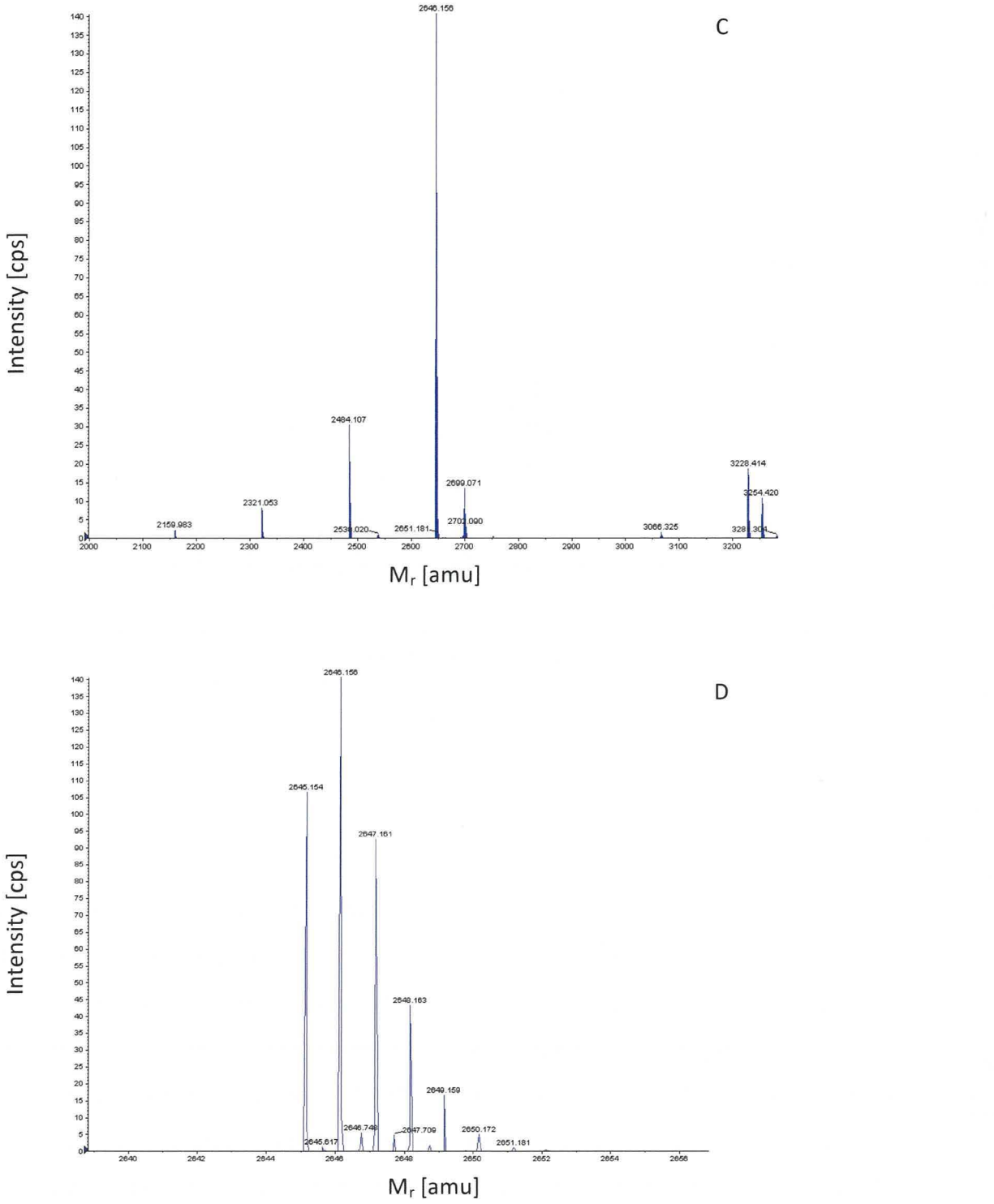


Figure 4.13 C–D AFGPs extract from blood serum of *G. acuticeps*.

AFGPs extract sample from Antarctic fish *G. acuticeps*. Deconvoluted LC–MS output with M_r peaks of sequence variants for AFGP 8 (C) and isotopic patterns for AFGP 8 with M_r nominal 2,646 u (D).

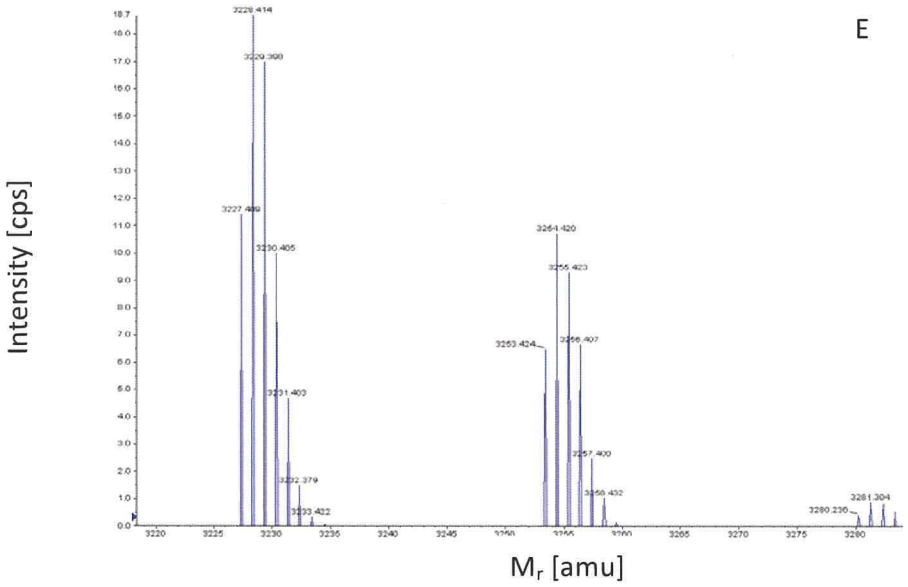


Figure 4.13 E Isotopic pattern for AFGP 7 from blood serum of *G. acuticeps*.

AFGPs extract sample from Antarctic fish *G. acuticeps*. Deconvoluted LC–MS output with M_r nominal 3,254 u representing AFGP type 7 ion fragments.

4.4.2 Nitrogen rule

The nitrogen rule as a coincidental principle observed in MS is applicable in the mass spectrum for the molecular ion peak as well as fragment ions. It states that a compound with an even number of nitrogen atoms has an even number of nominal molecular mass and vice versa, i.e. an odd number of nitrogens leads to an odd–numbered nominal *m/z* (Ekman, Silberring, Westman-Brinkmalm & Kraj, 2009, pp. 164-166; Gross, 2011, pp. 265-266). This rule is based on the number of covalent bonds between atoms in naturally occurring organic molecules composed by the elements hydrogen, carbon, nitrogen and oxygen, as well as silicon, phosphorus and sulphur, and the halogens.

Applying the nitrogen rule to AFGP 8 with A, P and T each containing one nitrogen in their amino acid residues of the tripeptide–disaccharide subunit lack the additional fourth N atom from the attached HexNAc sugar in the native state of the protein. Thus, the monoisotopic

molecular ions have an even number of 14 nitrogen atoms in the chemical formula $(AAT)_4$ AA.

The detected sequence of AAT AAT PAT AAT PA as $[M+H]^+$ species of AFGP 8 in posterior sucker material of *C. levigata* has an even number of 14 N atoms with a measured nominal *m/z* value 1,167 (Tables 3 & 4). As the protonation caused by ESI adds 1 u, this has to be subtracted to reduce it to the monoisotopic molecular ion M^+, and thus the AFGP 8 results agree with the nitrogen rule.

The samples of *C. antarctica* (Fig. 4.7) as well as the digestive content (Figs. 4.8 & 4.11), skin (Fig. 4.9) and sucker muscle (Fig. 4.10) of *C. levigata* present odd nominal *m/z* 883 and 1,085 traced as $[M+H]^+$ species in ESI spectra obtained by LC–MS/MS and appropriately identified as AFGP 8 and 7 respectively. These peptides are hence all in agreement with the nitrogen rule of MS and do not contain any atomic abnormalities in their chemical structures. This result demonstrates that the analysed peptides were in their natural states and unaltered conditions to their naturally occurring biological compositions.

4.4.3 Isotopic pattern resolution

The relevance of N, C, O and H isotopes can be seen in the isotopic patterns for AFGP 7 and 8 in leech skin (Fig. 4.12 B–D & 4.15). The findings are comparable to the results obtained in parallel runs for fish AFGP 7 and 8 (Fig. 4.13 D & E). This level of peak resolution further demonstrates the general high quality of ESI mass spectra and data obtained in this study.

In the skin sample the peak at *m/z* 882.727 represents an antifreeze molecule as a pure monoisotopic species, while other peaks in the pattern contain one (883.062), two (883.396), three (883.729) or four (884.064) heavier isotope elements. At weak signal levels some AFGPs ions could contain five isotopes (884.396) and the pattern also includes, at a very diminished intensity level, molecular species with six or seven carbon isotopes (Fig. 4.15 A). In addition to the isotopic patterns of type 8 at 2,646 u, a variant at 2,674 u is resolved as well as a third AFGP species at 2,702 u with prolines instead of alanines (Fig. 4.15 B).

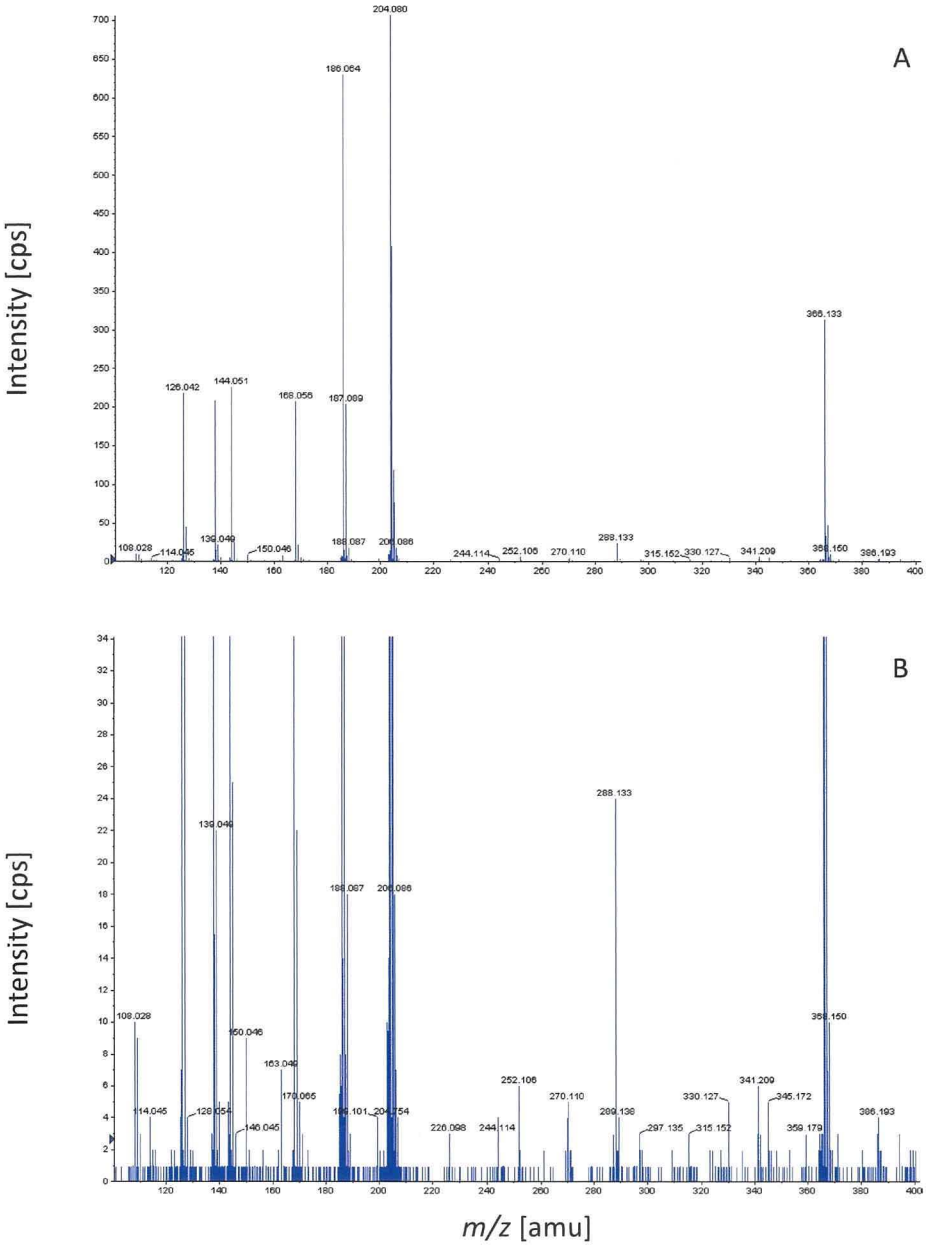


Figure 4.14 A–B ESI–MS/MS spectra of *C. levigata* posterior sucker sample.

ESI–MS/MS spectra with high resolution for full peptide sequencing by manual interpretation of pooled material from four posterior suckers off individual *C. levigata*. (A) & (B) with *m/z* 100–400 range but different intensity scaling to detect lower peaks as separate from baseline.

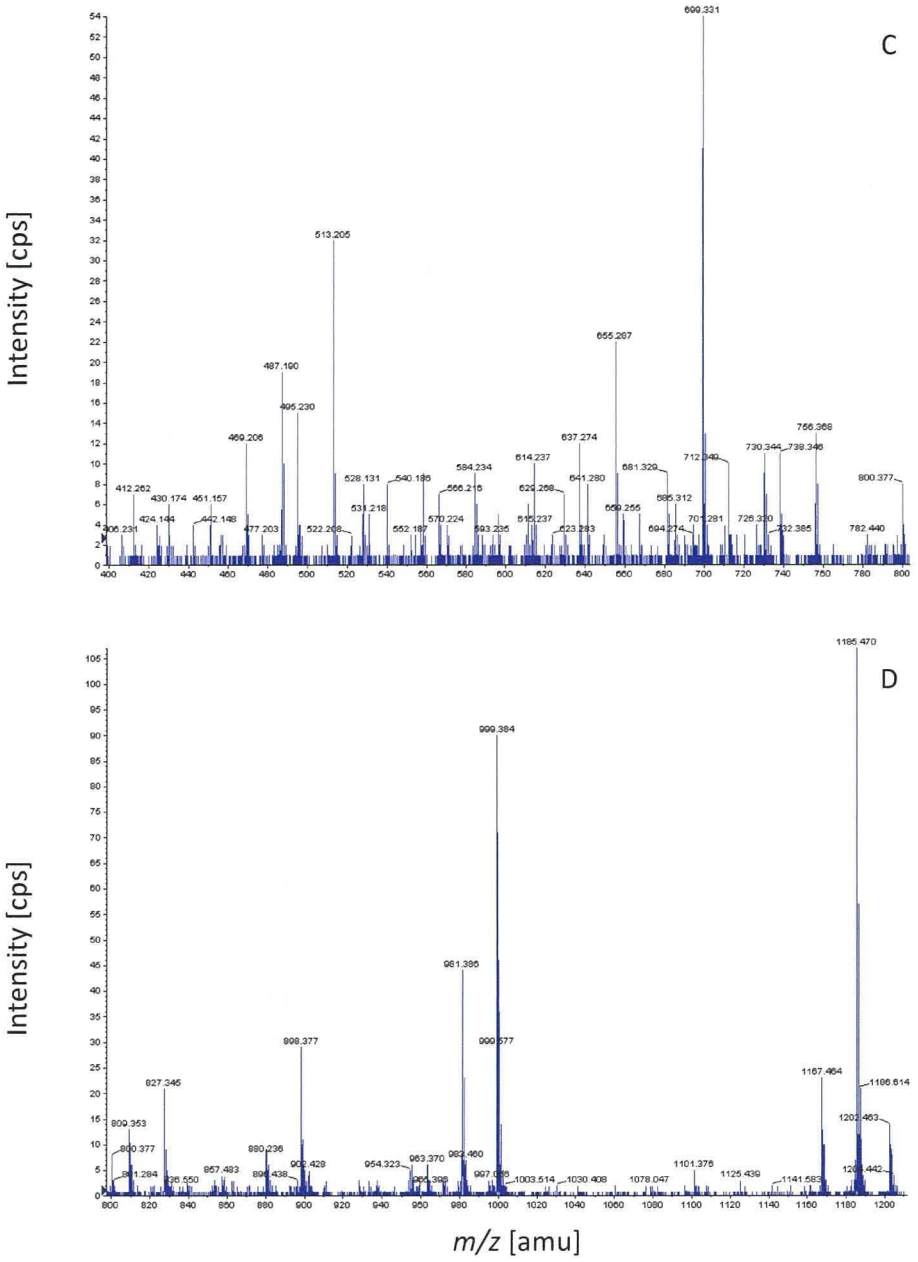


Figure 4.14 C–D ESI–MS/MS spectra of *C. levigata* posterior sucker sample.

ESI–MS/MS spectra with high resolution for full peptide sequencing by manual interpretation, (C) with *m/z* 400–800 range and (D) with *m/z* 800–1200 range.

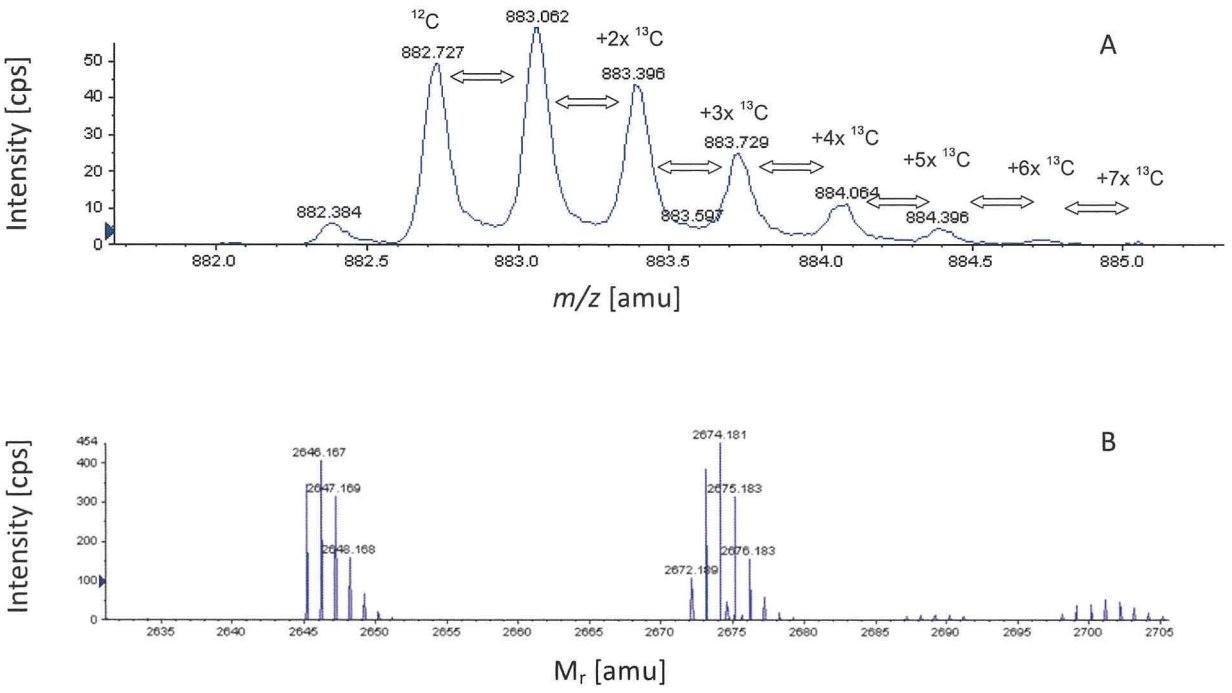


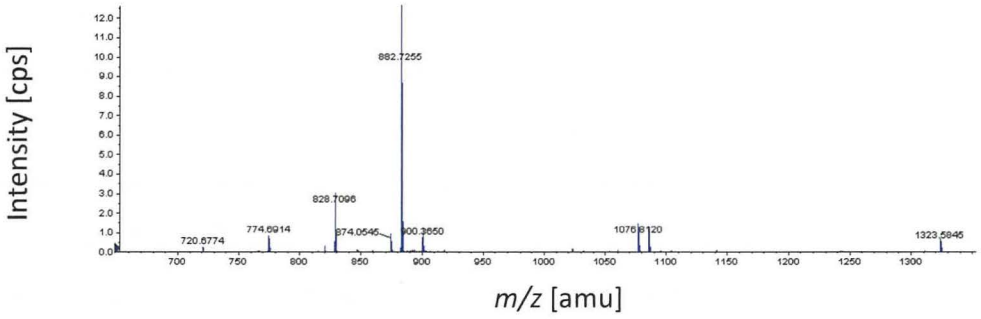
Figure 4.15 Isotopic pattern in AFGP 8 peak of *C. levigata* epidermal sample.

(A) Isotopic pattern of AFGP 8 ion at *m/z* 882.727 illustrating the effect on the molecular weight and deviation from the monoisotopic molecular mass with increasing numbers of isotopes.
(B) The isotopic pattern of type 8 ion at *m/z* 882.727 shown in (A) deconvoluted to M_r. Two variants of type 8 of 2674 u and 2702 u mass (*m/z* not shown in A) can be detected with one and two proline respectively substituting alanine.

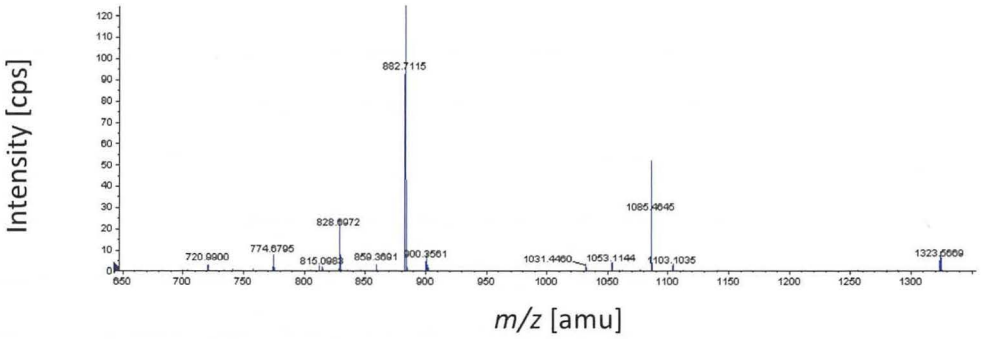
4.4.4 Relative abundance of AFGPs

The ESI mass spectra of leech and fish blood samples show a striking similarity in the distribution of ion peaks at appropriate retention times and *m/z* values (Fig. 4.16). However, the relative intensities of the highest peak at *m/z* 883 to the second highest at *m/z* 1,085 for leeches, representing type 8 and 7 respectively, are in different relative proportions than in fish. The sample of fish blood serum shows one main peak for type 8, yet the *m/z* measurements indicating the presence of type 7 are subdued and considerably below the

Gymnodraco acuticeps



Cryobdella antarctica



Cryobdella levigata

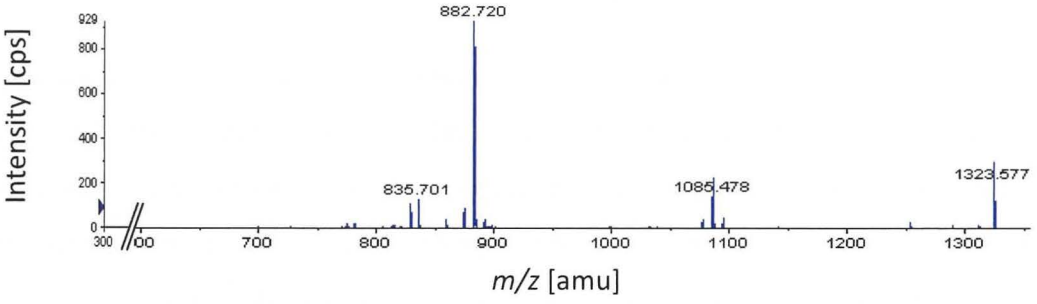


Figure 4.16 Interspecies comparison of AFGP mass spectra.

ESI–MS spectra of *G. acuticeps* (blood serum) AFGP type 7 and 8 in comparison to piscicolid parasite species *C. antarctica* (one whole specimen homogenate) and *C. levigata* (four pooled posterior suckers).

intensity counts of type 8 fragmentations. In contrast, type 7 can be detected in considerably higher proportions relative to AFGP 8 in both leech species.

There are differences in the body compartments of *C. levigata*, with the two peptides in near equilibrium in the intestine (Fig. 4.11), which is in sharp contrast to the fish blood in which type 7 is nearly absent (Fig. 4.13 B & C). The epidermal sample shows twice the relative abundance of type 7 than type 8 (Fig. 4.12 A) and is the reverse pattern to the whole specimen sample of *C. antarctica,* which has double the intensity count of type 8 compared to type 7 (Fig. 4.7). The situation changes dramatically in *C. levigata* from intestine and skin to the posterior sucker, which exhibits an approximately fourfold higher quantity of type 8 (Fig. 4.10 B & C) and is identical to the ratio detectable in the dragonfish *G. acuticeps* (Evans, et al., 2011).

The relative abundances of AFGP 8 sequence variants with A (2,646 u) compared to P substitutions (2,674 u) show a similar contribution of these sequence variations to the overall type 8 content in dermal material of the leech *C. levigata* (Fig. 4.12 A & B). This is not the case for type 7 variants, as one containing an additional proline (3,863 u) dominates over a species of 3,837 u with one A instead or when one (3,889 u) or two (3,915 u) prolines are added (Fig. 4.12 C). This result demonstrates the detectability of the heterogeneous isoforms for these AFGPs in piscicolids.

4.4.5 *C. antarctica* specimen homogenate

In the leech homogenate of *C. antarctica* the chromatographic separation produced base peaks at different retention times for an initial mass analysis in survey scan mode (Fig. 4.7 A) as well as the fish sample containing AFGPs of type 1 to 8 with approximately 200 ng of material applied (Fig. 4.13 A). For both samples the relative intensities are highest for the base peaks at nominal *m/z* 883 but with a 2 min shift in the retention time from around 11 min in the fish to around 13 min in the leech sample.

For the MS/MS measurements these base peaks at nominal *m/z* 883, as indicated by the blue background underlay, with accurate *m/z* 883.0393 in leech and 883.0526 in fish, were subjected to fragmentation in the collision cell and subsequent mass analysis. The towering

base peaks for both are identical and represent the molecular ion $[M+H]^+$ of type 8 glycoprotein at *m/z* 882.7115 in leech (Fig. 4.7 B) and *m/z* 882.7255 in fish (Fig. 4.13 B).

4.4.6 Material from *C. levigata*

4.4.6.1 Intestinal content

Material was analysed at an elution time of circa 12:30 to above 14 min. A composite overview of the results is illustrated in figure 4.8. The deconvoluted data for mass reconstruction is presented enlarged so as to visualise the characteristic pattern with more detailed information (Fig. 4.11).

The two main peaks at 2,646.156 u and 3,254.402 u are AFGP type 8 and 7 respectively. The remaining peaks can be explained as variations of these masses as fragment ions and artefacts specific to MS. For example, the peak at nominal *m/z* 4,472 is 609 atomic units heavier than the native AFGP 7 of 3,863 u. This correlates to one molecular ion mass of the peptide subunit Ala–Ala–Thr–O–Disaccharide as addition to the AFGP 7.

The mass spectra from MS/MS allow for appropriate classification of alanine and threonine from the tripeptide backbone of fish AFGPs. The disaccharide consisting of two hexose molecules can be detected as *N*–acetylhexosamine (HexNAc) alone or in combination with a further hexose molecule (HexNAc–Hex).

4.4.6.2 Epidermis and subcutaneous tissues with muscles

Two elution fractions were obtained, a first at circa 13 min and a second at 14:30 min (Fig. 4.9). The first shows MS raw data at *m/z* 882.727 and 1,085.479, which deconvoluted translates into a measured molecular mass of 2,646.167 u as AFGP 8 and 3,254.423 u as AFGP 7. In the second elute these values change to *m/z* 892.074 and 1,094.823, representing molecular weights of 2,674.208 u and 3,282.452 u. The data agree with the literature and AFGP results from the external mucus of *G. acuticeps* (Evans, et al., 2011). The triple–charged species at *m/z* 882.7246 and 1,085.482 in *G. acuticeps* (Fig. 4.13 A) can be seen in both elutes for *C. levigata*.

4.4.6.3 Posterior sucker

The elution at near to 15 min provided data at *m/z* 882.720 and 1,085.478 with accurate molecular weights of 2,645.135 u and 3,254.411 u for a pooled sample of four individual posterior suckers (Fig. 4.10). The mass spectrum resulting from an initial single preparation showed weak signals at low intensities at the appropriate *m/z* values, but had to be repeated with a higher quantity of material which verified the previous results. The complete ESI–MS/MS spectrum for the pooled sample of posterior suckers was used for peptide sequencing (Fig. 4.14).

The larger ion fragments demonstrate the possible isotopic composition of peptide molecules, and at higher *m/z* the composition of carbon isotopes in the spectra become more evident with the pure monoisotopic ^{12}C ion species increasingly less abundant.

4.4.7 Sequence variations in heterogeneous AFGP type 7 and 8

The ESI mass spectra illustrate the differences of the primary structures within each fraction of glycoproteins in the form of a M_r discrepancy matching the substitution of Pro for Ala. The nominal mass difference of the residues from one A with 71 u to one P with 97 u is 26 u and can be observed in the mass spectra of AFGP 7 and 8 as sequence variants.

The accurate formula mass calculated from the molecular structure is 2,645.1305 u for AFGP 8 as AAT AAT PAT AAT PA sequence variant and 3,861.6379 u for AFGP 7 with two additional AAT subunits to the smaller type 8 peptide. The variations in the sequences of the two AFPs can be detected in every ESI mass spectrum (Figs. 4.8–4.12).

The substitution of one proline for alanine as a change from one AAT to a PAT subunit marks a mass increase of 28 u. In AFGP 8 this can be detected as a 2,646 u molecular ion species with AAT to a 2,674 u ion with PAT instead. In the ESI spectra obtained for leeches the latter variant can be of higher abundance and represent the base peak (Fig. 4.9 E).

The loss of one alanine residue of 71 u in the AAT variant of 2,646 u leads to the next highest peak in the spectra at *m/z* 2,575 (Figs. 4.8 D, 4.9 D, 4.11, 4.12 B). The addition of a full subunit adds 609 u and brings the mass to the peak at *m/z* 3254 (Figs. 4.8–4.12), which is the ion of highest abundance in all ESI spectra except for one (Fig. 4.10 C).

For example, the skin of *C. levigata* was analysed for the two eluates separated at different retention times off the HPLC column as detected during the survey scan (Fig. 4.9 A), as can be seen in *C. antarctica* (Fig. 4.7 A) and fish blood (Fig. 4.13 A). The LC–MS results for these two peptide samples from the first (#1) and second (#2) elution times are presented with their raw data *m/z* (Fig. 4.9 B & C) and respective deconvoluted M_r (Fig. 4.9 D & E). The two main peaks can be identified as AFGP 8 as 2,646 u and type 7 as 3,254 u as alanine variants and the dominant peaks in the first elute. These are followed by their proline variations dominating the second elute with peaks at 2,674 u as type 8 and 3,282 u as type 7. The respective proportions of A to P sequence variants reverses from the first to the second eluate. This illustrates a very successful HPLC application as for each AFP the respective variant peaks are nearly absent in one of the two elutions.

The deconvoluted data for the skin sample as measured accurate mass for AFGP 7 is 3,862.698 u (Fig. 4.9 D) or 3,862.706 u (Fig. 4.12 A) and a mere 0.0601 or 0.0681 u mass deviation (after deprotonation or isotopic considerations) from the theoretically expected formula mass of 3,861.6379 u. For AFGP 8 the protonated ions have the measured accurate mass of 2,646.167 u (Fig. 4.9 D) to 2,646.182 u (Fig. 4.12 A) with Δm of 0.0365 u to 0.0515 u from the accurate formula mass of 2,645.1305 u. These results represent excellent measurements as a deviation of 0.05 u or below is regarded as impressively accurate in MS results (Morris, Paxton, Panico, McDowell & Dell, 1997). A constant percentage tolerance equates to a slightly higher absolute error rate of $\leq$ 0.2 u as expected MS tolerance in peptides of up to 5,000 u and in particular at M_r above such as proteins (M. Middleditch, pers. comm.).

4.4.8 Peptide sequencing by fragmentation analysis

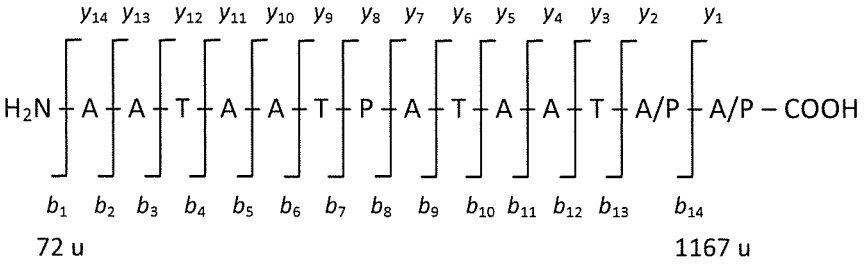
The data from the posterior sucker muscle of *C. levigata* was used for the complete protein sequencing (Fig. 4.14). This material was chosen for in–depth analysis of ion fragments as it represents the purest leech tissue sample obtainable for a fish parasite. The manually accomplished *de novo* peptide sequencing was undertaken on the molecular ion at *m/z* 882.7 in the ESI survey scan.

In nominal measures starting from the monoisotopic molecular ion peak $[M+H]^+$ of AFGP 8 at 1,185 u from MS/MS the sequential subtraction of appropriate mass equivalents of A, P or T and other expected specific reactions in MS (Table 4.5) allows the reconstruction of the full length 14 residues comprising the polypeptide with the primary sequence AAT AAT PAT AAT PA/AP (Fig. 4.6; fragment summaries in Tables 4.1–4.4).

The calculations leave one uncertainty at the end positions 13 and 14 as P–A or A–P:

PA
AAT AAT PAT AAT
AP

The primary structure is of accurate length for AFGP type 8 described from notothenioid fishes (Fig. 4.6) with one proline substitution for an alanine at position 13 or 14 as characteristic for type 8.



The calculated monoisotopic molecular weight of AFGP 8 is 1,184.6017 u as formula mass by the sum of all monoisotopic masses of elements in the amino acid residues and with the addition of 18.0153 u as one water equivalent mass. The accurate measured molecular mass is without water 1,166.464 u (Fig. 4.14) for the AFGP 8 with an error Δm of 0.1224 u from its theoretical formula mass 1,166.5864 u, which lies within MS tolerance levels.

The b_1 as the first as well as the last y_1 fragment ions cannot be assigned any sequential information as these exist as solitary compounds without any associated other molecules.

Table 4.5 Amino acids and monosaccharides present in notothenioid AFGPs.

Formula, accurate mass and chemical structures for each of the three amino acids with their residue information as necessary for interpretation of ESI mass spectra.

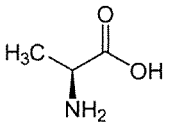
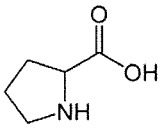
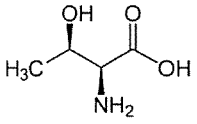
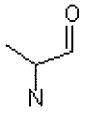
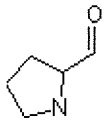
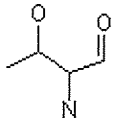
	Alanine	**Proline**	**Threonine**
Formula	$C_3H_7NO_2$	$C_5H_9NO_2$	$C_4H_9NO_3$
Accurate mass [u]	89.0477	115.0633	119.0582
Structure			
	Alanine residue	**Proline residue**	**Threonine residue**
Residue formula	C_3H_5NO	C_5H_7NO	$C_4H_7NO_2$
Residue accurate mass [u]	71.0371	97.0528	101.0477
Residue structure			
Immonium ion [u]	44	70	74

Table 4.5 Amino acids and monosaccharides present in notothenioid AFGPs.

Formula, accurate mass and chemical structures for each of the three amino acids with their residue information as necessary for interpretation of ESI mass spectra.

	Galactose	***N*–acetylgalactosamine**
Formula	$C_6O_6H_{12}$	$C_8O_6NH_{15}$
Accurate mass [u]	180.0634	221.0899
	Galactose residue	***N*–acetylgalactosamine residue**
Residue formula	$C_6O_5H_{10}$	$C_8O_5NH_{13}$
Residue accurate mass [u]	162.0528	203.0794

The measured values of *b*–type fragment ions (Fig. 4.14) of the monoisotopic molecular ion $[M+H]^+$ at *m/z* 882.7 as AFGP 8 deviate from the theoretical accurate formula mass by less than the error rate of MS of Δm 0.2 u (Table 4.4). The isotopic variations as demonstrated for AFGPs in this work could also cause these slight deviations from theoretical values.

The skin sample of *C. levigata* (Fig. 4.12 D) shows a peak at *m/z* 4,472 and as another proline species of it at 4,500 u.

The respective masses for the molecular building block subunits and their components of alanine, proline, threonine, galactose and N–acetylgalactosamine can be detected in the

different ESI spectra of parasitic materials (Figs. 4.7–4.12, 4.14 & 4.16) at specific elution times and with characteristic distribution patterns established from fish blood samples (Fig. 4.13).

The detection of appropriate molecular fragments provides further evidence that piscicolid samples contain peptides of identical composition to AFGPs described for Antarctic fishes.

The b_2 fragment with a sequence of AA and theoretical protonated mass 143.0742 u in the ESI spectrum or as PA dimer with 169.0899 u can be detected. Yet these peaks are within the *m/z* range containing the sugar ions with identical M_r values (Fig. 4.14 A & B). An alternative explanation for the mass equivalent of AA would be a HexNAc of measured 144.051 u. The peak with a mass equivalent of PA could also represent a HexNAc ion of measured 204.080 u after the loss of two water molecules to a measured 168.056 u as base peak followed by two characteristic isotopic peaks for this ion at 169.0562 u and 170.065 u.

4.4.9 Internal fragmentation analysis

The peaks at lower *m/z* represent galactose (Gal) as detected mass identity at nominal *m/z* 163 and *N*–acetyl–D–galactosamine (GalNAc) as *N*–acetyl–hexosamine (HexNAc) at *m/z* 204 as a monosaccharide or in combination with a further hexose molecule of 162 u as disaccharide HexNAc–Hex at *m/z* 366 (Fig. 4.14 A & B). The peaks at *m/z* 186, 168 and 150 are HexNAc ions after the loss of one, two or three water molecules with further variants at *m/z* 126, 138 and 144.

The fragmentation variations of AAT AAT PAT AAT PA species with a proline not before position 7 and 10 allow us to explain fragment ions in the ESI mass spectra as internal sequence fragments in the sample of *C. levigata* posterior sucker (Fig. 4.14). The values match fragmentation ion masses known from notothenioid fish AFGP standards (Table 4.1).

The sequential fragmentation patterns from either the 5'–end or the 3'–end reveal the characteristic subunits in the molecular ion peaks of the sucker sample (Fig. 4.14; Table 4.3) when the theoretical fragmentations are considered (Tables 4.1 & 4.2). The measured masses of the product ions next to the calculated masses for the respective units in theory

are provided (Table 4.4). The calculated Δm shows the error or value by which the measured accurate mass of the various product ions deviate from their expected theoretical masses. The accuracy is impressive, with < 0.06 u for all but one measurement, the fragment b_5 with Δm = 0.105 u above expected value. These calculated values represent instrument errors of ± 0.2 u and are as close to the theoretical values of molecular measurements as the MS technology allows. Consequently, the MS results are considered high quality data sets.

The immediate ion peak of smaller mass next to the monoisotopic molecular ion $[M+H]^+$ of measured 1,167.464 u is caused by the loss of one water unit. This is a mere Δm 0.1224 u difference from the theoretical monoisotopic formula value 1,167.5864 u of the pure protonated backbone ion of type 8 as AAT AAT PAT AAT PA peptide and thus lies well within the marginal MS measuring tolerance. The following three peaks can be explained as mass losses of one immonium ion of A as nominal 44 u to a 1,141 u ion or two such ion losses and a $[M+3H]^+$ ion at *m/z* 1,125. Alternatively, the latter could be caused by the loss of 43 u as carbenium ions and one more protonation to a $[M+2H]^+$ ion species of identical mass. The slightly higher ion peak amongst these low intensity peaks at *m/z* 1,101 represents the loss of measured 84.094 u from the molecular peak at 1,185.470 u. This could be caused by dissociation of one cyclohexane C_6H_{12} element of exact mass 84.0939 u.

Starting at the nominal mass of the monoisotopic molecular ion peak $[M+H]^+$ of AFGP 8 at 1,185 u the subtraction of 186 u as equivalent loss of a P–A or A–P dimer and one water molecule, the next peak is reached at 999 u as the b_{12} fragment (Fig. 4.14; Tables 4.1 & 4.3). From there a loss of one T with 101 u leads to b_{11} with a mass of 898 u, the loss of one A to b_{10} with 827 u, another A to b_9 and 756 u, one T to b_8 at 655 u, and the loss of A to b_7 with 584 u. Then a further loss of one P can theoretically lead to b_6 at 487 u (Table 4.1) or one A to 513 u as detected (Table 4.3). Subsequent reductions could theoretically either be by A to b_5 (386), A to b_4 (315) and T to b_3 (244), to conclude with AA to b_2 (143) (Table 4.1) or result in measured alternative fragments of subsequent reductions by A to b_5 (412), A to b_4 (341) and T to b_3 (270), to conclude with PA to b_2 (169) (Table 4.3).

The first peak in the ESI mass spectrum of the sucker sample as b_2 at nominal 168 u is a combined P–A or A–P dimer, which with the addition of 101 u as one T forms PAT or APT with 269 u after protonation as b_3 at 269 u. The following additions of A to b_4 PATA at 341 u,

A to b_5 PATAA at 412 u, T to b_6 PATAAT at 513 u, A to b_6 PATAAT at 513 u, with a measured accurate mass of 513.205 u.

Further fragments at the lower *m/z* range could be two alanine after double protonation as $[M+2H]^+$ weighing nominal 144 u as possible peak b_2. The addition of T with 101 u and loss of one proton leads to the $[M+H]^+$ species of AAT at 244 u as b_3 and a further A to b_4 at 325 u. The sequence fragments y_8 as PAT AAT PA or PAT AAT AP with measured accurate mass of 699.331 u can also be detected and explained as internal fragmentations of the known AFGP 8 or even 7 sequences. The addition of a cyclohexane C_6H_{12} of 84.0939 u to the theoretical $[HexNAc+H]^+$ of 204.08 u leads to 288.1733 u and a signal can be found at 288.133 u in the mass spectrum. This peak can also be caused by a loss of a benzene C_6H_6 of exact mass 78.0469 u from the measured 366.133 u HexNAc–Hex ion, which results in a mass deviation error of 0.0469 u and the following two smaller peaks of 270.110 u and 252.106 u could be explained as water losses. Alternatively, these peaks could represent a PAT fragment ion with a formula mass of 288.1476 u after protonation and then one and two water losses to 270.1371 u and 252.1266 u respectively. Other fragments at 1,185.470 u and 1,141.583 u represent the loss of 44 u as either the mass equivalent of one immonium ion of A or a $CONH_2$.

There are peaks representing larger molecules than the monoisotopic ion $[M+H]^+$ of AFGP 8 (Fig. 4.14 D). These are at *m/z* 1,388.641 the peptide ion $[M+H]^+$ with a nominal mass increase of 203 u or a HexNAc residue still attached to one threonine. The two side peaks at *m/z* 1,370.563 and 1,390.875 can be explained as a loss of water and addition of two protons respectively forming a $[M+3H]^+$ peptide species. At *m/z* 1,551.659 with a difference of 163 u is the peptide ionised as a $[M+2H]^+$ with HexNAc and one additional hexose residue remaining. Finally, the last ion at *m/z* 1,591.538 is the molecular ion $[M+H]^+$ with an increased weight of 406 u or two HexNAc residues attached.

4.5 Discussion

The detected peaks of appropriate masses can be identified by tandem mass spectrometry and peptide sequencing to be of the characteristic amino acid sequence and the typical composition of fish AFGPs. Consequently, the detected peptides are either exclusively of fish origin, represent structural analogues produced in *de novo* biosynthesis by the leech itself, or are a mixture of fish type 7 and 8 AFGPs with similar leech peptides. These AFGPs, or similar and closely related leech derived proteins, are detected as the major ion peak species in MS/MS for both piscicolids. This was as expected as these two AFGPs are the most abundant types in fish blood for most notothenioids, in which the smallest type 8 and second smallest type 7 can comprise up to two thirds of the total AFGP content (DeVries & Cheng, 2005, p. 165).

As per the immunohistochemistry (IHC) applied in Chapter 3, these results from LC–MS/MS represent a new application of a protocol in a cross–species study. The conditions in LC prior to MS measurements were paramount for a successful separation, and a dysfunctional chromatographic separation of structural isoforms could theoretically inhibit the work entirely if they proved too complex to resolve. The gradient elution conditions achieved results with the provided silica base for bonding interactions of the peptides with a high resolution at the given time interval suitable to detect and identify AFGPs in leech specimens.

The results demonstrate the capacity of the LC–MS/MS methodology as well as the protocol followed here as a suitable analytic approach, as known from fish (Evans, et al., 2012), to accurately detect AFGPs and derivatives in the biologically complex leech samples. The high quality of ESI mass spectra are further underlined by the resolution of isotopic patterns for AFGP 7 and 8 ions in leech *C. levigata* (Figs. 4.12 B–D & 4.15) and fish *G. acuticeps* (Fig. 4.13 D & E).

The discovery of AFGPs in the homogenate of *C. antarctica* demonstrates the detectability of peptides in the complex mixture of invertebrate cellular components and fish blood. Furthermore, it allows us to conclude that AFGPs must either be of sole leech

origin, are not digested by the invertebrate at all, or are digested at a marginal rate so as to retain peptide concentrations in the parasite at levels able to be detected in MS.

In *C. levigata*, the content of the digestive tract tested positive for fish AFGPs. This is suggestive of a low digestive activity, if any, and theoretically presents the leech with the option of using the blood meal as an internal reservoir for these peptides. These undigested molecules are then available for distribution within the parasite. Consequently, under the assumption that AFGPs are not of leech origin alone, two central questions can be asked. Firstly, can the parasite absorb undigested AFGPs from the fish blood meal? Secondly, can the leech actively distribute these substances amongst its tissues?

The presence of intact AFGP type 7 and 8 in all samples of *C. levigata* demonstrates the general ability of the invertebrate for a passive or active absorption of these peptides followed by their dispersion within the body tissues. Therefore, the analyses of piscicolid proteins were performed with increasingly informative steps. The occurrence of peptides in samples of skin and posterior sucker material cannot be explained other than by active metabolic transport mechanisms. These organs are not attached or associated with the digestive tract and represent the most distal body compartments away from the digestive content as the fish AFGP reservoir. The posterior sucker represents the purest sample of leech material one can obtain for an ectoparasite feeding on its host blood. The analysis of the muscular and epidermal tissues guarantees the absence of any fish contamination deriving from blood cells and plasma as could initially be expected in the *C. antarctica* homogenate. The results permit the conclusion that AFGPs are present in every tissue with their proper primary structures despite any metabolic processes or digestive activities.

The ion in the intestine sample of *C. levigata* with nominal 4,472 u differs by 609 u from the true AFGP 7 observed at nominal 3,863 u (Fig. 4.8 D). This difference equates to the addition of an entire AAT subunit and can either be a MS artefact or a true sequence fragment. The latter could derive from one larger AFGP, such as a type 6, which are also known to contain proline instead of alanine. The 4,500 u molecule could be a second such sequence species of 609 u heavier than AFGP 7 containing a further proline. Although HPLC can separate the peptides effectively, this fragment might elute at this appropriate time due

to digestive processes inside the leech gut and as such represent a fragment of AFGP type 1–5 or 6.

The main limitations to this PhD work of sample size and sufficiently available material of high quality tissues could eventually be overcome. In terms of MS, however, this extremely resource intensive methodology forced a very selective utilisation of MS runs for data acquisition and analysis. Ideally, a larger number of specimens for *C. antarctica* (n=3) and both leeches in general (n>5) could be run for a statistical approach for each piscicolid species and their separate tissues including possibly blood sera samples of different fish species as controls and for additional information. Yet with the present results from merely four experiments one can conclude irrefutably the presence in both piscicolid species of glycoproteins with identical AAT repeat structures as known from notothenioid AFGPs.

4.5.1 Interpretation of ambiguous peaks

The interpretation limits of the ESI mass spectra obtained in this study should be noted. There are ion peaks with M_r values in the ESI spectra which have more than one possible composition of atoms resulting in the measured mass. These ambiguous interpretations to the molecular composition of some peaks are exemplary detailed for the posterior sucker sample from *C. levigata* in the following (Fig. 4.14). Alternative interpretations allow us to conclude that ion peaks at lower *m/z* values could consist, at least in part, of the smaller AFGP fragments in small quantities next to the sugar permutations.

The peak with measured accurate mass of 204.080 u could represent one *N*–acetylhexosamine residue of 203.0794 u with the addition of a proton H^+ as $[M+H]^+$ ion and a very small Δm of 0.0006 u. The subsequent loss of one water as 18.0105 u could lead to theoretical 186.0689 u as the neighbouring peak with a measured accurate mass of 186.064 u and a deviation of Δm 0.0049 u. The second loss of water leads to a theoretical 168.0584 u for the $[M+H]^+$ fragment ion of *N*–acetylhexosamine. The closest peak with measured accurate mass of 168.056 u can thus be this with Δm 0.0024 u. However, the dimer P–A equates to 168.0899 u or a mass increase of Δm 0.0339 u. This peak could therefore be composed out of the fragmented *N*–acetylhexosamine ion or the first two

amino acid residues proline and alanine attached to one another as a monoisotopic ion peak.

The peak b_3 with a measured accurate mass of 244.114 u can either be a protonated AAT species with a theoretical monoisotopic mass of 243.1320 u plus 1 u proton or represent a methylated *N*–acetylhexosamine with a theoretical monoisotopic mass of 245.1263 u.

It becomes evident that despite the best efforts it is not possible in MS to unambiguously correlate every single peak in ESI mass spectra to one specific molecular ion and thus underlying substance. This is due to fragments being altered by the manifold addition or loss of different atom groups. Furthermore, the naturally occurring isotopes become statistically important with increasing number of atoms in the structural formulas as shown in the detected range of molecular weights. Therefore, some signals were not consistent with one sole interpretation or could not be decoded.

Fortunately, the limitation to the methodology does not impinge on the appropriate interpretation of the detected fragmentations representing various peptides with the characteristic sequence of AFGPs. It must be noted that it was not essential to determine all ion fragmentations in order to support the conclusion of AFGP presence as was the aim of this chapter.

4.5.2 Circulatory system for AFGP distribution to distal organs

Terrestrial and marine leeches have a circulatory system that allows them to distribute nutrients and remove cellular end products in their body compartments (Sawyer, 1986a, pp. 67-99). Consequently, Antarctic piscicolids could distribute AFGPs absorbed at the digestive wall within the interstitial space and into every organ tissue and cell. This theoretical ability for AFGP distribution has been shown with the detection of AFGPs in skin and posterior sucker material.

4.5.3 Relative AFGP abundances indicate metabolic influences?

The observation of a relative higher abundance of AFGP type 7 to 8 in the two leech species when compared to pure fish data could indicate a selective metabolic activity by both

parasite species. This observation indicates that the antifreeze composition in the leech parasites are not merely the exact reproductions of the protein composition typical for fish blood. It seems that each parasite has either diminished AFGP 8 by digestion and use as a nutritional source or elevated the concentration for the second smallest AFGP in its body fluids by unknown but active mechanisms.

Fish blood can be assumed to be the main source of the AFPs peaks detected in the ESI spectrum of the digestive tract. The measured differences in the relative abundances of the gut content might be caused by a higher absorption rate of the smaller peptide AFGP 8 by the surrounding epithelial cells and therefore a faster decrease in its related concentration within the digestive lumen when compared to fish blood conditions. The differences could thus be caused by physiological processes that result in actual variations of relative concentrations of the two AFPs in the different body tissues of the leech parasite. The ratio of type 7 to 8 in the posterior sucker mirrors the situation measured in fish mucus covering the epidermis in dragonfish (Evans, et al., 2011). Therefore, this might represent a favourable composition for antifreeze protection adapted by the leech, analogous to the situation in the fish, as the adequate response to the naturally ice–laden habitat.

It remains puzzling then that the ratio is more than inverted in the skin of the parasites, with twice the quantity of type 7 than type 8. A plausible explanation for this might lie in the different amount of epidermal and muscular tissues in the skin and sucker samples. This could be explained by the epidermal tissue containing a higher proportion of type 7 and muscle a higher proportion of type 8. Whether this represents an undiscovered biochemical adaptation or is merely caused by physiological transportation processes remains to be investigated. Yet it seems unlikely that the larger AFGP is embedded into the skin in higher quantities when the smaller type 8 appears to be absorbed more readily from the digestive tract.

Furthermore, it is unclear why the larger AFGPs have not been detected. The MS spectra of blood sera from notothenioid fishes show no large proteins as one might expect (pers. obs. & C. W. Evans, pers. comm.). This might be caused by the extraction method or insufficient pressure by the manual application of the syringe. The larger AFGPs might adhere to the silica matrix better than the smaller type 7 and 8 and might be lost due to methodological issues. However, this remains to be clarified.

4.5.4 Peptide sequencing by fragmentation analysis

The fragmentations detected in the posterior sucker sample of *C. levigata* could either represent mere internal sequence products, as expected, or entail true and novel sequences of leech origin (Fig. 4.14; Table 4.3). The seemingly hypothetical argument of a leech derived *de novo* biosynthesis of AFGPs gains support by the mRNA discovery in piscicolid material as presented in the last data chapter of this thesis.

There are sequence fragments as PAT, PATA, PATAA, PATAAT and PATAATPA that can all be explained as dissociated ions from fish AFGP 7 and 8 sequences (Fig. 4.6) or from AFGP 6. As such these confirm the heterogeneity of type 7 and 8 AFGPs and that these groups of glycoproteins with varying peptide sequences are positively detected by MS in leech samples. Consequently, this reveals that HPLC can separate sequence variations of the alanine AFGP 8 with M_r 2646 u and AFGP 7 of 3254 u from those containing one or more substitutions with proline. This must be based on adsorption differences in the isomers interacting with the silica matrix in the pressure column and leads to proline richer AFGPs to elute later. As both types 7 and 8 can separate in this manner, the essential prerequisite conditions for a potential sequence analysis are provided at least in principle, and thus remain quantity dependent only.

The fragmentation of the peptides in the collision cell of tandem MS is independent of the N–terminus and can be one main advantage of the top–down approach in proteomics over the bottom–up incorporating the Edman degradation. The dissociation of ions at any position in the peptide backbone produces fragmentations independent from the end terminus of the protein to obtain information on the entire sequence. Consequently, the fragment ions in the piscicolid sample not matching the building concept of fish AFGPs represent either internal fragmentations or potentially true novel sequences of unknown origin.

The Antarctic borch *P. borchgrevinki* shows no P in any sequence variant before position 7, with further substitutions only at position 10 and 13, while the Atlantic cod *Gadus morhua* (Linnæus, 1758) and Arctic cod *Boreogadus saida* show this in position 4 already (Hew, Slaughter, Fletcher & Joshi, 1981). Thus, the fragments from the ESI spectra could also be

caused by a truly novel leech derived sequence analogous to polar fish of the northern hemisphere or be a yet undiscovered AFGP sequence for Antarctic notothenioids.

However, the interpretation of fragments is based on an expected composition resulting in the specific ions of mass equivalents. While a proline can substitute alanine at position 7, 10 and 13 in AFGP 8 there is no variation described in fish where P is incorporated at either end of the protein (Lin, et al., 1972). The detected fragments with sequence PATPA or PATAATPA could thus either conform with the fish sequences ending in PA as known sequence fragment mass equivalent (Lin, et al., 1972; Morris, et al., 1978) or have a dimer AP as a true leech specific peptide sequence. Regrettably, appropriate sequence information was not obtained that would allow a conclusive answer as to whether a sequence starting with PA/AP or ending in AP exists in *C. antarctica* or *C. levigata*.

The sequencing work of AFGP 7 and 8 by the Edman degradation started at the N–terminal end of the peptides and established unambiguously that there are no peptide species starting or ending with proline (Lin, et al., 1972). This advantage of the Edman over the MS techniques in association with the heterogeneity of these AFGP groups allowed the interpretation of the data from the intact protein in the bottom–up but not the presented top–down approach. To overcome this limitation would require further sequencing work, given the minute quantities of AFGPs available for Antarctic leeches.

Additionally, there is the complication that the adult leech might contain a potential mixture of leech as well as fish AFGPs from which any potentially novel sequence could emerge. In order to test leech derived material only, distal organs such as the skin and posterior sucker would need to be analysed further for the individual AFGP sequence variants. This reduces the available leech material currently used as wet weight below approximately 20 µg per adult *C. levigata* further into the ng range for the body parts. From this starting quantity each HPLC fraction containing the heterogeneous group 7 and 8 AFGPs would need to obtain sufficient quantities for every single sequence variant to be separated prior to sequencing steps. The aforementioned previous sequence study on AFGP 8 as AAT AAT PAT AAT PA variant with a formula mass of 1,184.6017 u or g/mol used a starting material of 0.15 µmol for the Edman reaction, which equates to 177.7 µg (1,184.6017 g/mol x 0.15 µmol) of peptide sample for each digest (Lin, et al., 1972). Even though the current technological limitation of MS reaches to very low detection and working quantities at below

the picomol and into the femtomol and potentially attomol range (Morris, et al., 1997) and modern Edman processors work with 10–100 picomol of material (Walk, et al., 1999), it is still highly questionable whether the material at hand could yield reproducible results even at the scope of an independent PhD project. Therefore the study of potentially new AFGP sequences in Antarctic leeches from *de novo* biosynthesis had to be deferred and is reserved for future investigators.

4.5.5 Verification of possible AFGP detections in IHC

The immunological staining technique in Chapter 3 was used to produce data to visualise immunoreactive species within piscicolid tissues and cells in a cross–species application of chemicals. However, those fluorescent signals could either have indicated the conjugates of the fluorochrome with antifreeze molecules or represent artefacts originating from alternative attachments to different cellular structures and biomolecules. Therefore, the MS analysis data provides conclusive verification that AFGPs were indeed present throughout the leech structures and were thereby available to form protein–dye conjugates observable as fluorescent signals. Although the exact figure of additional unspecific fluorophore attachments cannot be quantified further, these MS data provide strong support for the IHC examination.

It has previously been speculated that a possible transition of the small glycoproteins 7 and 8 through the cell membrane might serve as antifreeze protection for the intracellular space (Lin, et al., 1972; Theede, Schneppenheim & Béress, 1976). In particular, the detected AFGP 7 and 8 in ESI spectra of skin and sucker samples confirm the previous staining results as factual presence and visualisation for these peptides in body tissues. Although the results from IHC can no longer be regarded as pure artefacts, it remains unclear as to what level unspecific staining occurs. Nonetheless, the evidence provided here in favour of a transition hypothesis holds the potential of being biologically relevant for piscicolid survival. Furthermore, Lin and colleagues (1972) speculated on whether in fact these smallest AFGPs might serve other cellular and physiological functions in addition to that of antifreeze protection in fish. This potential could also be relevant for leeches.

4.5.6 AFGP heterogeneity as structural isomers and isobars and MS limitations

The initial separation of the protein mixture into individual fractions of AFGP types is based on the chemical structure of the peptides. As mentioned in the introduction to LC, it is a powerful methodology with very high resolution at the molecular level. However, this is based on the presence or absence of atom groups and differs from the situation in the heterogeneous group of AFGP 7 and 8. Although not discussed in the early literature (Lin, et al., 1972; Morris, et al., 1978), these AFGPs are a group of constitutional or structural isomers that exist with identical molecular formula but different structures. As such, these do not differ in their M_r as the underlying peptide sequences vary not in the composition of the particular amino acid residues but merely their succession. Although, as discussed above, HPLC can separate isomeric molecules of type 7 and 8 according to proline numbers, this process depends on dissimilar properties caused by differences in functional groups and has limitations. As a result, the fragmentations detected by ESI–MS/MS still represent some isomers and possibly isobars with inextricable sequence information. This methodological limitation of indistinguishable sequence variants has to be borne in mind when considering the aforesaid conclusions. However, these results with sequence variations are according to literature where, for example, in blood serum of *P. borchgrevinki* the isomeric forms of AFGP 8 with different A and P variants were also shown as separate peaks in LC–MS/MS (Evans, et al., 2012).

4.5.7 Exclusion of potential fish contaminations

It has been demonstrated for the dragonfish that AFGPs are present in the mucus covering the epidermis (Evans, et al., 2011). The authors commented that this might function as a first barrier for antifreeze protection, but that no physiological mechanism is known that could deposit these peptides. An adult leech specimen sampled off its notothenioid hosts *T. bernacchii* or *T. hansoni* might have been in contact with AFGPs at the fish skin surface. This potential source for fish derived AFGP contaminants in the leech samples was eliminated by fixation in ethanol: Upon insertion of the leech specimens in ethanol a very clear disassociation of the external mucus could be observed as a diffuse layer separating from the leech exterior. Thus, the ethanol treatment effectively removed any leech mucus

that had been in contact with the fish exterior and fish AFGPs. As a consequence, the source of AFGPs at the detection level in MS/MS must lie internally, and does not derive from an artefact contamination caused by external AFGPs on the fish. The initial assumption that fish contaminations were successfully removed is verified by the detection of AFGP specific mRNA as will be discussed later in this thesis.

4.5.8 Outlook

4.5.8.1 True protein sequencing with genome information

The MS/MS results show a clear fragmentation pattern matching the characteristic amino acid sequence of AFGPs 7 and 8 described from Antarctic fishes. As these types represent groups of glycoproteins of varied sequences the exact primary structure of the AFGPs cannot conclusively be provided by MS/MS. In theory, MS allows the analysis of different fractions for their individual protein sequences. However, this requires a pure protein sample of one type 7 or 8 of sufficient quantity from the target species. Herein alone lies an issue for work on Antarctic leeches. But furthermore, for a more accurate protein identification than already achieved, these protein mixtures would need to be further separated into their individual sequence species. At the end of this hypothetical work one might accomplish a list of AAT and PAT class AFGPs belonging to either type 7 or 8. Yet their origin from either leech or fish would still remain unsolved. Thus, even the information of a full primary sequence cannot provide an additional benefit to this work. Future investigations into this field might find a novel sequence, but this would require verification to its origin, which is currently a practical impossibility. This might change in the near future, with the genomic efforts currently put into the Antarctic fish biota by the DeVries group (A. L. DeVries, pers. comm.). Once the genome of one fish host and thus the entirety of gene sequences becomes available the further conclusions on the AFGP sequences could follow to take this and other antifreeze research to an entirely new level.

4.5.8.2 AFGP concentration gradients in body fluids and tissues

A further future research aspect could be an investigation into the concentration levels of AFGPs in the different parasite organ tissues. To study this in greater detail one would require an internal AFGP standard. One could use artificially synthesised antifreeze

molecules with a defined structure and concentration in an analogous procedure as used previously for THA studies (Peltier, et al., 2010). This would allow the comparison of concentration levels between the standard and the sample in the same MS run as a comparison of their respective calculated peak area sizes. This might prove difficult, albeit possible to achieve. I therefore promote the use of imaging mass spectrometry (IMS) as far superior methodology and emerging technology.

4.5.8.3 Imaging mass spectrometry (IMS)

The initial test for fish AFGPs in leech specimens involved immunological staining with its associated limitations and required further investigations by MS. The method of three–dimensional computational electron microscopy could not be realised during this PhD work, as mentioned in the IHC chapter, due to the time required to establish an appropriate protocol for AFGP localisation as well as logistical and financial constraints. However, the emerging research field of quantitative proteomics using IMS in conjunction with MALDI and MS/MS analysis for larger biomolecules promises to surpass 3D microscopy as it allows to identify biomolecules by determination of their monomer sequence instead of indirect fluorescence signals. This can include *in situ* protein hydrolysis followed by MALDI–MS/MS analysis without any impact on the tissue integrity at the specific sample area (Groseclose, Andersson, Hardesty & Caprioli, 2007).

MALDI–IMS allows the detection of biopolymers such as proteins and peptides and also of DNA, mRNA and other large or small organic molecules, like lipids and sugars, *in situ* on a tissue section by mass spectrometric analysis. This technology will thus allow the advance of the results of this study from IHC and MS work to the next analytical level. The localisation of specific AFGP molecules within cells and thus the tissue context reaches a resolution previously unachievable. The specimens can be scanned for specific mRNA presence or absence in different tissue cells. Thus, future work could focus on the question as to whether other peaks in leech ESI–MS are leech derived, and as such contain slight variations in the fish AFGP compositions, or are merely due to the invertebrate tissue compositions. MALDI–MS enables future studies to localise AFGPs inside the parasites with micrometre accuracy, which will allow the visualisation of the indigestion and postulated incorporation of fish antifreeze proteins in leech tissues. Furthermore, IMS can easily distinguish between molecular species, an aspect hardly achievable with other classic techniques of proteomics

such as autoradiography (Seeley & Caprioli, 2008). And as demonstrated on specimens of the medicinal leech *Hirudo medicinalis* the IMS can even generate cellular maps of proteins and identify these without previous knowledge of the target molecules (Bruand, et al., 2011).

Furthermore, after IMS has scanned for AFGPs and their distribution within the leech body, the concentration levels and proportions of the different types can be established. With this one can potentially also determine the presence and concentration of any other molecule serving as possible antifreeze protectant. IMS can provide crucial insight to resolve the question of cooperative influences on the thermal hysteresis of cell components such as sugars, alcohols and salt ions.

Additionally, one could scan for the different types of AFGPs in leech material. A core question to ask would be whether and to what extent the parasite tissue concentrations differ from the pure, native fish blood. This could give an indication to the main source of antifreeze protection in leech tissues. Are the same types relevant as found in fish tissues and what are their respective concentrations? Does the parasite really saturate its tissues with AFGPs to or beyond the concentration levels found in fish as indicated by the present study?

Furthermore, the leech might accumulate AFGPs inside the digestive tract or fish meal volume by active dehydration and only embed what is necessary. Therefore, does the parasite have an internal concentration gradient for AFGPs, from the leech digestive system filled with fish blood to the peripheral organ tissues?

Finally, IMS can open new research avenues and sources of study material. Recent work found that by virtue of modern IMS a museum sample of human spleen taken in 1899, preserved and stored since in formalin, was successfully analysed *in situ* and peptides identified (Seeley & Caprioli, 2008). Such results offer the possibility of future studies to work with Antarctic museum specimens reaching as far back as the first samples available, dated from the heroic age of Antarctic exploration by Roald Amundsen, Robert Falcon Scott, Ernest Shackleton, Douglas Mawson and others. Effectively this removes the main entry barrier to many research topics on organisms of remote regions, such as Antarctic leeches.

Yet despite exciting prospects for future work, one of the core questions I asked cannot conclusively be answered even with advanced MALDI–IMS results: Are the detected AFGPs present only by virtue of absorption or *de novo* protein expression within leech tissues, or is it a combination of both? The cells carrying the antifreeze agents might have absorbed or have in fact synthesised the AFGPs. This limitation of IMS means that no biologically relevant information can be added to what has been established already in this work. With the considerations that before 2012 MALDI–IMS instruments were only available overseas and no appropriate field sampling including flash freezing was undertaken before 2010, the logistical challenges and significant costs were prohibitive and were the main reasons why no further attempts in the field of protein localisation in leech tissues were undertaken as part of my PhD project.

4.6 Conclusions

Here I have demonstrated using MS that type 7 and 8 AFGPs can be detected in tissue samples of the two parasitic fish leeches *C. antarctica* and *C. levigata*. The components of the peptide backbone as alanine and threonine together with *N*–acetylhexosamine are characteristic for notothenioid AFGPs and were positively identified in all leech material. The presence of peaks in ESI mass spectra representing appropriate atomic masses of the shorter variations of fragment sequences are highly indicative for intact proteins. The detection of undigested AFGPs points towards fully functional molecules in both piscicolids *in situ*. The parasites are thus presented with the possibility of utilising the fish AFGPs for their own antifreeze protection. To test this hypothesis, a thermal effect in the form of an active freezing point depression must be observed and the genetic origin of these proteins further explored.

CHAPTER FIVE

Determination of thermal hysteresis activity and presence of AFGPs in two Antarctic piscicolid parasites by nanolitre osmometry

5.1 Abstract

The potential for piscicolid invertebrates to obtain antifreeze protection by means of digestive absorption of antifreeze glycoproteins and glycopeptides (AFGPs) from their notothenioid fish hosts was investigated by measuring the thermal hysteresis (TH) by nanolitre osmometry (NO) while observing the behaviour of single ice crystals in solution. The combined determination of TH activity (THA) with the characteristic bi–directional ice crystal growth was demonstrated in *Cryobdella antarctica* and *Cryobdella levigata*. It was concluded that these parasitic leeches have active *in vivo* antifreeze protection and bioactive molecules capable of providing chemical protection in a manner analogous to that observed in polar fishes.

5.2 Introduction

5.2.1 Animals and their freezing capabilities

The curiosity of man to investigate how animals and plants survive freezing winter temperatures produced vivid descriptions as early as three centuries ago. A compelling account written by Henry Power (1623 – 1668) entitled *"Observation XXX. Of the little white Eels or Snigs, in Vinegar or Aleger"* describes in great detail and with astonishment the survival of the vinegar eel (Power, 1664, p. 35):

Eightly, Now though heat hath that killing property, yet it seems that cold hath not: for I have taken a jar–glass full of the said Vineger, and by applying Snow and Salt to it, I have artificially frozen all the said Liquor into a mass of Ice, (wherein all these Animals it seemed lay incrystalled) though I could discover none of them in it (though I have taken the Icy–mass out on purpose to look at it) so that now I gave them gone for ever: yet when I came again (about two or three hours after) to uncongeal the Liquor, by keeping the glass in my warm hand, when the Vineger was again returned to its former liquidity, all my little Animals made their re–appearance, and danced and frisked about as lively as ever. Nay I have exposed a jar–glass full of this Vineger all night to a keen Frost, and in the morning have thaw'd the Ice again, and these little Vermin have appeared again and endured again that strong and long Conglaciation without any manifest injury done to them; which is both a pretty and strange Experiment.

This study on presumably the small invertebrate nematode or roundworm *Turbatrix aceti* (Müller, 1783) is to the best of my knowledge the first recorded experiment with the observation that an animal can tolerate freezing while submerged in water. Power previously described how heat kills these animals. It is also of note that Power followed modern scientific protocol in that he repeated the experiment for verification of his observation with a refreeze step. Such repeats of freezing and thawing cycles play a pivotal role in the determinations of freezing tolerances as well as antifreeze activities in aqueous solutions in modern research and literature.

Power further commented in the introductory preface dated 1st August 1661 (seventh page into the book) on the importance of an emerging optical revolution: *"Such, I am sure, our Modern Engine (the Microscope) wil ocularlyevince and unlearn them their opinions again: for herein you may see what a subtil divider of matter Nature is* [...]*"*. Although this statement was made 350 years ago, it has by no means lost its validity. A light microscope was one of the essential instruments necessary for the completion of this thesis on the freezing abilities in another invertebrate worm. Power was the first to publish his studies involving microscopy in English (Hughes, 2010), and a year before Hooke (1635 – 1703) presented his renowned *Micrographia* (Robinson, 1945). The latter refers to Power as the *"ingenious Doctor"*, while presenting his own observations on the same nematodes, which are neither leeches nor

insects as he suggested (Hooke, 1665, p. 284): *"I found that they could wriggle and winde their body, as much almost as a Snake, which made me doubt, whether they were a kind of Eal or Leech."*

Hooke introduced the freezing point (FP) of water as being point zero in the Celsius temperature scale (Andrade, 1953). In this system, the definition of the freezing or melting point (MP) of water is set to a fixed point at zero degree Celsius, as in the first volume of the International Critical Tables of Numerical Data, Physics, Chemistry and Technology, in short International Critical Tables (ICT) (Mueller, Adams, Fairchild & Wensel, 1926, pp. 52-53). It is defined as the temperature in degree Celsius where the crystalline and liquid phases are in equilibrium with dry air under one atmosphere of pressure (West, Dorsey, Bichowsky & Klemenc, 1928, p. 6) and is used as such in antifreeze literature.

Another observation by Hooke (1665, pp. 88-93 & scheme VIII) was the hexagonal characteristic of ice crystals as stars with six pointed branches (1665, p. 89): *"Some of the stems of the six branches proceeded straight, and of a thickness that gradually grew sharper towards the end"*. Although he does not comment on the biological relevance of this, the ice structures he described for the first time at the microscopic level are essentially the reason for the evolution of antifreeze proteins: They are to prevent the detrimental damage resulting from crystals propagating inside cells and tissues.

Finally, Hooke provided on page 112 under his *"Observation XVIII. Of the Schematisme or Texture of Cork, and of the Cells and Pores of some other such frothy Bodies"* the description of a cork slide, which he accurately reproduced in figure 1 B on scheme XI: *"I Took a good clear piece of Cork, and with a Pen–knife sharpen'd as keen as a Razor, I cut a piece of it off, and thereby left the surface of it exceeding smooth, then examining it very diligently with a Microscope* [...] " (1665, p. 112). He goes on to describe that when this slide was found too thick for any observation in his microscope he cut "[...] *an exceeding thin piece of it* [...] " (1665, p. 113), thus enabling him to finally conclude albeit stated in brackets that these pores or cells, "[...] *(were indeed the first microscopical pores I ever saw, and perhaps, that were ever seen, for I had not met with any Writer or Person, that had made any mention of them before this)* [...] " (1665, p. 113). Consequently, Hooke not only discovered the basic biological unit of tissues and coined the term *"cell"*, but also invented the microtome cut to facilitate microscopic work on biological samples,

something Hooke is rarely credited for today and which also was an important technique used in this PhD research.

Another early and revealing description of work focusing on freezing conditions comes from the French scientist René Antoine Ferchault de Réaumur (1683 – 1757). Seemingly, he was the first to systematically study the freeze tolerance of animals, yet there are very few citations to his credit in modern antifreeze research. Réaumur posed questions and used techniques that are the foundation of present investigations that still seek to provide answers to some of his questions. Réaumur published six volumes between 1734 and 1742 under the title *"Mémoires pour servir à l'histoire des insects"* or *"Memoirs relating to the history of insects"* (Torlais, 1958). The following transcripts from French to English are translated by myself. The second volume of 1736 contains detailed descriptions of the survival of insects at sub–zero temperatures (Réaumur, 1736, pp. 141-154).

Réaumur demonstrated that a common caterpillar of the Bordeaux region, the *"chenilles du pin"* or pine processionary moth *Thaumetopoea pityocampa* (Denis et Schiffermüller, 1775), can survive freezing treatments reaching below the frost record of –14°C in 1709 which represented the coldest winter temperature for decades (1736, pp. 141-142). He described experiments in which he froze specimens by aid of a salt–ice mixture to as low as –19°C. After 30 min the apparently dead caterpillars were placed on a block of ice to warm up and he observed movements indicating their live status within less than 15 min. Réaumur postulated, in the context of his time, the particularly bold conclusion (1736, pp. 142-143):

> *Ce seroit un étonnant prodige, si un insecte dont le sang, dont toutes les liqueurs auroient été gelées, revenoit à la vie, ce seroit là une vraye resurrection; car lorsque toute circulation, tout mouvement des liqueurs est arrêté, l'animal est un animal mort; du moins n'avons nous pas d'autre idée de l'état de mort.*
>
> *[This would be an astonishing miracle, if an insect whose blood, of which all the liquids would have been frozen, comes back to life, this then would be a true resurrection; because when all circulation, all movement of fluids is stopped, the animal is a dead animal; at least we have no other idea what constitutes the state of death.]*

Réaumur also noted that caterpillars whose liquids were truly frozen do not return to life as they are *"trop bien mortes"* or *"altogether too dead"* (1736, p. 143). Furthermore, he realised that physiological conditions and body size can be important factors in survival as they influence the temperature at which body fluids freeze (1736, pp. 144-145). He then determined the freezing points of blood samples from a pigeon and a lamb as –7°C to –8°C and –5°C respectively (1736, p. 145). His conclusion was that larger animals such as birds, quadrupeds and even man *"ont dans leurs corps une chaleur"* or *"have an inner heat inside their bodies"* (1736, p. 146), which insects lack and therefore larger animals do not need a blood FP as low as do insects. Although one cannot grant him the credit of foreseeing the results of antifreeze work carried out since the 19th century, including the present study, his observations, questions and experiments can without doubt be regarded as the beginning of present day cryobiology.

Réaumur later mentions earthworms and leeches as if he understood their annelid relationship, although he regarded these as insects (Réaumur, 1738, p. xxviii preface). He also states that the production of further memoirs is required. One might thus consider any research on cold survival strategies, especially on living organisms, as a continuation and sequel from this very early articulated request.

In 1872 Dönhoff studied, among many other animals, some *"Egel"* or *"leeches"*, presumably *Hirudo medicinalis*, and found that a short exposure of just minutes at –6.25°C killed all specimens. Mild freezing conditions of –1.5°C for a time of 3 h or even less also resulted in no survivors (Dönhoff, 1872). Two decades later, Kochs published a detailed description of how *H. medicinalis* does not survive freezing treatment in water supercooled to –3°C due to the crystallisation of tissue water (1892). After extensive research in modern and classic pre World War One literature published either in modern or old German, French or English, but excluding manuscripts in Japanese and Russian, Dönhoff's and Kochs' cold experimentations in this one Hirudinea seem to be the sole records to date for any species belonging to this family of annelid worms. Although there is no accurate information on the exact experimental conditions or the leech species tested, the group of leeches in Antarctic waters is confronted with an environment of below –1.9°C for many months of the year (Littlepage, 1965). This duration of freezing temperatures present extreme conditions for leeches as those hirudinean specimens tested by Dönhoff and Kochs could not tolerate

similar temperatures for even a few hours. The latest three–volume reference work for Hirudinea by R. T. Sawyer has a citation list spanning over 266 pages with more than 4,200 references (Sawyer, 1986b, 1986a, 1986c), yet Dönhoff and Kochs are not included and neither are comments on physiological adaptations of leeches to survive freezing water temperatures.

5.2.2 How does ice kill? The lethal effect of ice crystals

In order to fully appreciate the importance of antifreeze proteins (AFPs) for fishes in the marine environment of Antarctica a short overview of the history and the mechanisms involved is given here. The crucial observation that ice formation generally starts in the extracellular space and not inside the cells was to the best of my knowledge first made by Müller–Thurgau (1880). Although his conclusion that intracellular freezing is lethal derived from work centred around how plants survive the deep temperatures of European winters, it became evident that this principle equally applies in the animal kingdom.

The question on how ice formation exerts its detrimental impact on cells and tissues led to the hypothesis that this is particularly lethal when intracellular freezing occurs as this process causes minute crystals to interfere with or destruct the protoplasm of a plant cell (Maximov, 1914). This was supported by the idea of *"Eisnadeln"* or *"ice needles"* that destroy tissues in animals such as nematodes (Rahm, 1924, p. 110). Maximov (1914) further postulated that a low temperature kills the plant by changing the chemical consistency of the plasma as result of ice propagation and dehydration of the cells.

In a freezing study on plants investigating the prothallia of *Polypodium aureum* (Linnæus, 1758), it was concluded that intracellular freezing is never survived due to structural cell damage caused by ice crystals from within the cell resulting in permeable plasma membranes (Stuckey & Curtis, 1938).

One of the early publications into the lethal effect of intracellular freezing studied in detail isolated fibres of the sartorius muscle of a frog and epidermal cells of a red onion (Chambers & Hale, 1932). The authors used ice crystals to initiate internal freezing in the frog's muscle cells cooled to –1.3°C to –1.8°C. They described for the fibre a fast (3–8 s for 3 mm length) external surface freezing followed by slower ice propagation inside (8–15 s).

The authors concluded that internal freezing might be inhibited in either animals or plants by the cell or plasma membrane acting as a barrier. This function eventually breaks down at a critical temperature, at which point external ice expands into the cells. The lethal effect of intracellular freezing was attributed to the freezing of the sarcoplasm in frog muscle or in the plant due to ice forming between the plasma membrane and cell wall followed by plasmolysis after thawing. The authors stated that cold temperatures alone are not lethal to frog cells, which without the external initiation could be supercooled to –15°C without ice forming within them.

Asahina established the importance of extracellular ice formation to protect an animal from damaging intracellular ice crystal growth after such a mechanism was indicated from work on the oriental moth *Cnidocampa flavescens* (Walker, 1855) [accepted today as *Monema flavescens* (Walker, 1855) (Pan, Zhu & Wu, 2013)] (Asahina, Aoki & Shinozaki, 1954). In a series of freezing experiments published in *Nature*, Asahina demonstrated how extracellular ice formation leading to dehydration of cells protects an animal from intracellular ice propagation (Asahina & Aoki, 1958). He froze winter pupae of the swallow tail *Papilio machaon hippocrates* (Felder et Felder, 1864) at –30°C for one hour before storing them in liquid oxygen for two days and found that 50% resumed their natural development after reheating (Asahina, 1959b). Asahina concluded, in consideration of other very similar pretreatment experiments with the nematode plant parasite *Aphelenchoides ritzemabosi* (Schwartz, 1911) (Asahina, 1959a) and the caterpillar of *Cnidocampa flavescens* (Walker, 1855) (Asahina, 1959b), that there was a generally good survival rate after an initial freezing step. He argued (1959b) that the initial treatment leads to an extracellular freezing of more than 90% of the water and as a consequence hardly any water remains to form ice crystals intracellularly. He extended the experiments and found that most pre–frozen caterpillars survived after more than two months in liquid oxygen. Asahina thus established with his pretreatments, although performed under highly unnatural conditions, how animals could survive extremely low temperatures by using dehydration to avoid intracellular freezing and cellular death.

The interest on how intracellular freezing can be prevented led to work on egg cells of a sea urchin as marine invertebrate by Asahina (1961) who tested eggs of *Strongylocentrotus nudus* (Agassiz, 1863) [accepted today as *Mesocentrotus nudus* (Agassiz,

1863) (Tatarenko & Poltaraus, 1993; Drozdov, Vinnikova, Zezina & Tyurin, 2012)]. He observed that these cells did not freeze in temperatures above –15°C when submerged under silicone oil but could freeze intracellularly at –8°C in seawater. His explanation was that contact with ice crystals from outside the egg cells initiated the observed freezing process of the cells. In his experiments with fertilised and unfertilised eggs he saw a clotted cytoplasm as the result of intracellular ice formation in dead egg cells. Freezing experiments with a slow (1 K per min) or fast (9 K per min) cooling rate to –20°C resulted in a high mortality for unfertilised eggs at a fast but not a slow freezing speed. However, the freezing rate had no effect on the survival of the fertilised eggs. Asahina attributed the sufficient loss of water in fertilised eggs at either speed and the unfertilised eggs at the slow cooling rate to the chemical potential that exists between the external ice on the cell surfaces and the supercooled water inside the cells. This allows the cells to dehydrate sufficiently during the initial moments of freezing to prevent intracellular formation of ice crystals afterwards. He concluded that the ability of fertilised eggs to transfer water in or out of the cell more readily than unfertilised cells was the reason for their better survival. Thus, Asahina demonstrated that a marine invertebrate at its earliest life stage could supercool and potentially survive below the ambient sub–zero temperature of Antarctic waters and continue normal ontogenesis when returned to higher temperature seawater (up to +20°C).

Although Asahina found external ice crystals as freezing initiators could cause cell death, it remains unclear from his descriptions whether cells were readily contacted by ice crystals in his experimental tubes. Did ice crystals inserted during the experiment when the temperature reached –2°C to –3°C really function as ice seeds? Could they reach eggs on the bottom of the flasks without a mixing device? The experimental duration of between 2 and 30 min of ice exposure before reheating could in this context have a further influence on the survival rate. One important equivalent study to Åkerman's (1927) freezing resistance in crop, but in the animal kingdom, investigated the ability of killifish *Fundulus heteroclitus* and other fishes to tolerate and survive partial freezing (Borodin, 1934). After 151 experiments for up to 16.5 h, the author concluded that though some killifish specimens survived freezing treatments outside of water, they died under the same conditions when frozen within water. Borodin further established that partial freezing in fish leads to death. In the supplement he presents experimental data on the cold hardy Alaska blackfish *Dallia pectoralis* (Bean, 1880), which he received specimens of just before submission of his manuscript. While some of the

previously proven lethal conditions were tolerated, freezing in water was not and was lethal to the blackfish. This early study effectively demystified tales that the Alaska blackfish can survive the harsh polar winter being frozen in ice, something still widely believed today (pers. obs., Anchorage 2007).

According to Asahina (1961) intracellular freezing always leads to death for the eggs of the marine sea urchin *M. nudus*. He concluded the lethal effect is caused by cytolysis or coagulation of the cytoplasm and moreover that the fatal result is independent from the total quantity of intracellular ice. This supports Salt's site–of–freezing hypothesis (1961) in which an event leading to intracellular freezing is lethal, while the survival of extracellular ice formation depends on the extend of any injuries.

Furthermore, the mortality graph provided by Asahina (1961) illustrates the steady decline of survivors in the short interval of 30 min in which ice crystals were present at supercooled water temperatures. Thus, in evolutionary time scales, the Antarctic species are confronted with a potentially fatal situation as free floating ice crystals are common in this habitat (Littlepage, 1965; Dayton, et al., 1969).

The apparent lethalness of intracellular freezing for animals, as result of direct and indirect contact with external ice appeared universal until exceptions were discovered in Antarctica. *Panagrolaimus davidi* (Timm, 1971), a terrestrial nematode (< 1 mm body length) present in soil of the McMurdo Sound region, survives daily freeze–thaw cycles (Timm, 1971). Microscopic observations of a freezing procedure demonstrated their capacity to tolerate intracellular ice formation and growth (Wharton & Ferns, 1995). Although most animals and plants cannot survive intracellular freezing, this exception illustrates the potential in animals to adapt to life at sub–zero conditions in Antarctica including intracellular freezing.

5.2.3 Freezing waters and survival of teleosts

Freezing work in 1953 by P. Scholander investigated the partial freezing aspect for *D. pectoralis* and found it always to be lethal. A frozen tail did lead to death within days, even if the freezing was not to the extent that the body part separated, an observation contradicting what Borodin (1934) described for the survival of some test specimens of

F. heteroclitus and the brown bullhead *Ameiurus nebulosus* (Lesueur, 1819). The freezing of the head resulted in cataracts, and though the five specimens swam after the treatment in the tank, they died within three days (Scholander, et al., 1953). A frozen heart *in situ* could not return to its function and thus the authors concluded that a partially or entirely frozen fish cannot return to normal life. The experiments by Borodin (1934) and Scholander et al. (1953) both confirmed previous findings of the lethality of ice for fish in that they cannot survive being frozen stiff while enclosed by ice (Kochs, 1890; Rahm, 1924).

The puzzling question on how Arctic fishes can tolerate sub–zero waters during the winter months resulted in the formulation of the supercooled state hypothesis. This suggests that fish can cool down to the temperature of their surrounding water and have their blood cooled by 0.75–0.85 K below its FP (Scholander, et al., 1957). This requires the absence of external and internal ice, as seeding crystals killed supercooled fish of the genus *Fundulus* (Lacepède, 1803) (no species names provided) and Scholander et al. (1957) concluded that bottom dwelling fish species or those in the deep waters of the sampling site, the Hebron Fjord in northern Labrador, can exist during the Arctic winter in a supercooled state as *"they live so deep that ice can never reach them"* (1957, p. 16). However, in the presence of ice in an experimental tank the supercooled fish species taken from the shallow water mostly froze to death. This is intriguing considering that Fletcher (1977) described after a four year project on winter flounder *Pseudopleuronectes americanus* that ice crystals were frequently observed by SCUBA divers down to 6 m depth in coastal waters off Newfoundland. Additionally, this scenario of free floating ice crystals is the natural condition during the Antarctic spring (October–December) for the upper 33 m of the water column as observed previously by SCUBA–diving activities and ice formation up to this depth (Dayton, et al., 1969). And ice crystals could exist to a depth of 75 m as indicated from results of a one year oceanographic survey in McMurdo Sound (Littlepage, 1965) at sites where fish and leeches alike were caught before as well as for this PhD. Furthermore, the notothenioid species serving as hosts are not active swimmers but tend to rest on the bottom of holding tanks in captivity (pers. obs. & Eastman, 1993, p. 214) and do not behave differently in their natural habitat. *Trematomus bernacchii, Pagothenia borchgrevinki, T. centronotus, T. hansoni* and *T. nicolai* were all recorded resting on formations of anchor ice (Dayton, et al., 1969; DeVries & Wohlschlag, 1969). Supercooling alone is therefore not a viable survival strategy for fish in

the presence of ice crystals in general (Umminger, 1967) or the Southern Ocean in particular (DeVries, 1982).

The survival of animals in *"überschmolzenem Wasser"* or *"supercooled water"* was considered possible as long as no internal ice propagation occurred (Kochs, 1892, p. 339). The notothenioids were found to survive and thrive in such an environment as the result of a biochemical adaptation in the form of antifreeze glycoproteins or glycopeptides (AFGPs), which prevent the growth of ice crystals inside the fish by lowering the freezing point of the body fluids (DeVries, 1971). As a direct consequence of the fish behaviour in shallow water habitats, it is impossible for their ectoparasitic leeches to evade direct contact with ice. Such danger could only be avoided when attached to fish living below the sea ice zone at depths, depending on locality, greater than 70–1,000 m (S. M. Mager, pers. comm. & Dieckmann, et al., 1986; Mager, et al., 2013). This specific situation has been shown to be lethal for the related *H. medicinalis* which could not survive supercooling to –3°C even in artificially purified water to remove any ice nucleating particles (Kochs, 1892). Kochs observed no crystallisation events in distilled and filtered water cooled to –15°C. An observation in agreement with a measured FP of distilled water in the range of –13.6°C to –16.1°C provided in Dorsey's comprehensive study on the freezing properties of water (1948). But according to Kochs even a motionless, sedated leech could not be cooled to –5°C before it froze. Kochs correctly identified nucleators, either seed crystals, gas bubbles or even the body surface of the animal itself, to result in ice crystals propagating within tissues and to cause the death of animals rather than other factors related to low temperatures. As a consequence for the Antarctic environment the related piscicolids had to obtain protective mechanisms as the situation would otherwise be critical for the survival of these species in the presence of ice crystals in their natural habitat.

5.2.4 Marine invertebrates under freezing conditions

The generally accepted condition of marine invertebrates to be isosmotic, that is of same solute or osmotic pressure as the surrounding seawater, leads to the assumption that these animals have no greater tendency for freezing than seawater (Schmidt-Nielsen, 1997, pp. 229; 305-309). However, this could still lead to selective pressure in the intertidal zones of

the polar seas and in particular the Southern Ocean where conditions lead to anchor ice formation (Denny, et al., 2011) and inevitably affect benthic invertebrates.

Stephenson (1922) recorded that two oligochaetes, the limnic *Enchytraeus crymodes* (Stephenson, 1922) [accepted today as *Marionina crymodes* (Stephenson, 1922) (Erséus & Healy, 2001)] and littoral *Lumbricillus aegialites* (Stephenson, 1922) [accepted today as *Lumbricillus pagenstecheri* (Ratzel, 1869) (Erséus & Healy, 2001)], survived solid freezing in the Arctic archipelago of Svalbard. However, there appears to have been no further research on the mechanisms of their freezing abilities and these species are known and classified as terrestrial rather than marine worms of the northern hemisphere (Coulson & Refseth, 2004). It is thus questionable whether this is in fact an example of marine invertebrates tolerating freezing.

The Arctic macrofauna of the littoral or tidal zone lack sessile species with a few mobile animals present during warmer summer months leaving this ice zone during winter (Madsen, 1940; Gerlach, 1965). Gerlach (1965) commented on up to 50 nematode species found in the supra– and midlittoral zones of the Spitsbergen coastline. Whereas the sublittoral, as the constantly submerged zone of coastal waters below the tide level, has a rich fauna with for example large specimens of *Mytilus edulis* (Linnæus, 1758), which presumably can survive freezing conditions of the Greenland winter in littoral rock pools but at the cost of high mortality (Madsen, 1940). Kanwisher (1955) found that under natural conditions shore animals can supercool to a maximum of –7°C and that up to 75 ± 5% of body water can be frozen. His study included *M. edulis* as a cosmopolitan species and demonstrated the cold hardiness of intertidal species in the Arctic. Kanwisher included a short note on freezing experiments of sublittoral animals. Although he did not provide details to species names or quantitative information, he stated that annelids like any of the other tested arthropods, molluscs and echinoderms could not tolerate large formations of internal ice. Kanwisher concluded that these marine invertebrates cannot stand freezing despite living for the entire year at –1.7°C at the bottom of the Hebron Fjord, Labrador (Kanwisher, 1955).

Cold hardiness in marine invertebrates was tested for various marine bivalve species of the intertidal zone collected from the Atlantic Arctic Ocean as Arctic–boreal to subtropical and tropical waters of Florida and the Caribbean (American Atlantic coastline, northern

Norway, North Sea, Baltic Sea, Mediterranean Sea and Black Sea) including *M. edulis* (Theede, 1972). The author found that within 210 min half of the population was killed at freezing conditions of –10°C with *M. edulis* being able to resist up to 330 min. Although these molluscs were capable of surviving freezing temperatures it was only for short periods and came at the cost of greatly diminished population sizes which demonstrates the relevance of additional mechanisms necessary to survive in Antarctic waters.

The protection mechanisms against freezing were closer investigated for the Antarctic limpet *Patinigera polaris* (Hombron et Jacquinot, 1841) [accepted today as *Nacella polaris* (Hombron et Jacquinot, 1841) (Engl, 2012, p. 68)], which secretes a mucus when trapped in ice of the tidal zone (Hargens & Shabica, 1973). This mucus seemed to have a TH function analogous to that of AFGPs known from polar fishes. Noteworthy is that mucus as a freeze protectant was commented on much earlier from observations made in the shallow parts of European freshwater ponds where an often found remains as *"Schleimmasse"* or *"mucilaginous mass"* was suggested to serve as the protective mechanism of an overwintering frog (Müller-Erzbach, 1891).

The possibility of the existence of AFPs outside of fishes was studied in the mussel *M. edulis* of the North Sea, in which a TH gap was detected that seemed to stem from biomolecules of similar mass as AFGPs (Theede, et al., 1976).

Further studies have investigated the relevance of ice for benthic invertebrates (Gutt, 2001), yet it was not until recently that other cases of TH were discovered in marine invertebrates in Antarctica.

First in the nemertean *Antarctonemertes validum* (Bürger, 1893) [accepted today as *Antarctonemertes valida* (Bürger, 1893) (Clarke & Johnston, 2003)] collected during summer in the inter– and subtidal zones off Rothera Station with a TH gap of 1.4 K in the haemolymph due to AFPs (Waller, et al., 2006). Other examples of TH based on proteins in animals of higher taxonomic rank include the nudibranch *Tergipes antarcticus* (Kiko, et al., 2008) and the copepod *Stephos longipes* (Kiko, 2010). Both invertebrates live in association with the surface sea ice and though *S. longipes* is isosmotic to the surrounding seawater its haemolymph demonstrates THA (Kiko, 2010).

Proteins interfering with ice formation at a cellular level can also be found in unicellular species living in the Antarctic. For instance a cross–species sharing of *ice–active substances* (IASs) within unicellular organisms was observed in experiments using several Diatom species collected during summer from the sea ice and platelet ice in McMurdo Sound (Raymond & Knight, 2003). The authors showed that after one freezing cycle, the survival of *Fragilariopsis* sp. as well as a temperate marine diatom *Nitzschia frustulum* was significantly higher when IASs from a third species of *Navicula* were present. This experimental work together with recrystallisation inhibition of IASs found in species of the genus *Berkeleya* (Greville, 1827) and genus *Navicula* demonstrated that these are ice–binding proteins (IBPs).

The sea ice associated diatom *Navicula glaciei*, sampled from McMurdo Sound, was found to carry a circa 25 ku IBP (Janech, et al., 2006). In this case sea ice was melted and the supernatant consisting mainly of *N. glaciei* observed under a microscope found to have a clear interference with ice formation in form of ice–pitting. In addition, several isoforms of one IBP have been described in the mobile Antarctic alga *Chlamydomonas* sp. (Raymond, et al., 2009).

Finally, it shall be noted that even Antarctic bacteria possess proteins for intracellular protection. An active AFP providing a TH gap and demonstrating interference with ice crystals followed by the explosive bi–directional growth was detected in a cell extract of a *Flavobacterium xanthum* strain living in the mud of Antarctic pools (Kawahara, et al., 2007). The purified AFP at a concentration of 0.86 µg/ml has a THA of 1.19 K and with a molecular mass of 59,000 u is one of the largest AFPs discovered to date. And the evolution of IBPs might have a long tradition in Antarctica. A strain of *Marinomonas primoryensis*, a bacteria discovered and described in the coastal ice of the Sea of Japan (Romanenko, et al., 2003), was found in brackish water lakes separated from the Southern Ocean for around 10,000 years at the Vestfold Hills, Eastern Antarctica (Gilbert, et al., 2005). This AFP is dependent on Ca^{2+} ions for its full functionality, can produce a TH of up to 2 K and marked the first time a hyperactive AFP could be identified in a bacterium (Gilbert, et al., 2005). Further, at an age of over 400,000 years or in 3,519 m depth (Petit, et al., 1999) in the ice core at Lake Vostok of the glacial ice sheet of Eastern Antarctica, an ancient bacterial strain of Flavobacteriaceae was found which inhibits ice crystallisation (Raymond, et al., 2008).

The biological relevance of IBPs to deter freezing finds evidence for example in the planktonic foraminifera collected in the upper 300 m during the cruises of the research vessel *RV Eltanin* as part of the U.S. Antarctic Research Program between 1960 and 1965. While *Globigerinita glutinata* (Egger, 1893) has a global distribution and can be found in subantarctic waters and the seabed sediment of the Scotia Sea north of the ACC, it cannot be found in Antarctic surface waters of 0–10 m with measured temperatures at below –1°C (Bé, 1969). *Globigerina pachyderma* (Ehrenberg, 1861) [accepted today as *Neogloboquadrina pachyderma* (Ehrenberg, 1861) (Costello, Emblow & White, 2001, p. 73)] has been suggested to exist as sinistral or left–coiling form adapted to life in the Antarctic waters south of the ACC at –1°C to +8°C, as their highest abundances were found there in waters of less than 2°C, and as dextral or right–coiling form found in water of 9°C to 15°C (Bé, 1969). The author concluded that the sinistral *N. pachyderma* is the most cold–tolerant amongst the planktonic Foraminifera as found abundantly in subantarctic and Antarctic but also subarctic and Arctic waters. Regarding the recent results for sea ice associated organisms in the Antarctic it seems the distribution of *N. pachyderma* is correlated to some form of freeze tolerance in the sinistral but not the dextral form.

Although Antarctic sea ice diatoms, algae and bacteria are unicellular species they clearly demonstrate a biochemical adaptation to their sub–zero habitats. This contradicts previously proposed survival mechanisms in which it was suggested that single cell organisms are pushed along the ice front and remain in the liquid and unfrozen portion (Salt, 1961). The protection resulting from IBPs likely reduces the damage caused to cell membranes by growing ice crystals (e.g. Knight & Duman, 1986; Knight, Wen & Laursen, 1995; Thomashow, 1998; Costanzo & Lee, 2013), a phenomenon first articulated in the 19th century (e.g. Müller-Thurgau, 1880; Schander & Schaffnit, 1918) and postulated by Power and Réaumur.

The leeches of the Southern Ocean are in a unique position in regards to biologically active antifreezes. Their notothenioid fish host species distribute AFGPs within their bodies through the circulation of blood (Evans, et al., 2012) readily sampled by antifreeze researchers to obtain these peptides. This source of AFGPs will likewise be available to the leech parasites to exploit for their own freeze protection.

5.2.5 Aim of chapter

The results presented in the previous chapters illustrate the potential absorption of AFGPs, and established the presence of undigested peptides in piscicolid tissues. The aim of this chapter was to examine a potential protein functionality of AFGPs as antifreeze substances inside the parasites.

Nanolitre osmometry (NO) was used to measure a potential thermal hysteresis activity (THA), as the difference in the freezing and melting points, while concurrent observations of ice crystal behaviour are made by light microscopy *in vitro*. A characteristic bi–directional propagation of ice crystals together with a THA documents the presence of functional AFGPs and would establish their active antifreeze functionality *in vivo*.

5.3 Material & Methods

5.3.1 How does TH determination by NO work?

In water nucleation events at which ice crystals can form and grow eventuate at the FP of 0°C which is identical to the melting point (MP) at which these crystals disappear. This occurs without a measurable difference at zero milliOsmoles (mOsmol) and without a TH in the absence of AFGP or any other substance reducing the FP. The hexagonal ice crystals grow in all six directions in a snowflake pattern.

The assessment of antifreeze properties of a solution is performed by the determination of its freezing and melting points. If a gap or difference in temperature exists between these two events the solution exhibits TH. This difference expressed in Kelvin (ΔK) widens the further the FP is suppressed which is based on a non–colligative effect of AFGPs (Raymond & DeVries, 1977) and is referred to as antifreeze activity (DeVries, 1971) or THA.

A nanolitre osmometer is the control unit of a sample holder which can be cooled and heated. A small droplet of the solution for TH determination is frozen, reheated and frozen again while observing a minute ice crystal of 3–5 μm in diameter (A. L. DeVries, pers. comm. & e.g. Kiko, 2010). The temperature at which this crystal melts is defined as the MP of the

liquid, while the FP is reached when the crystal starts to grow in a monocrystalline process. For fish caught in McMurdo Sound a variability in size of larger seed crystals resulted in highly unreliable TH data, while small 5 µm crystals had a lower FP and were well reproducible (Jin & DeVries, 1997).

In addition to determining the temperature for ice crystal formation, in work with fish AFGPs it is important to observe the ice crystal behaviour during the cooling process. The antifreeze in fish force a particular pattern upon the ice crystal formation when the FP is reached. The presence of active AFGPs bound onto the ice planes inhibits the addition of water molecules to the ice nucleus and forces a specific elongation in two opposite directions, leading to the characteristic bi–pyramidal ice crystal growth in the presence of fish AFGPs (for video demonstration see digital file Appendix A.3).

The temperature difference between the freezing and melting points as the TH in a particular sample solution was determined by NO using a Clifton Technical Physics instrument (Hartford, New York, USA). The osmolality of the solution was measured in mOsmol of solute per kilogram of solvent. Standard NaCl solutions (290 & 1,000 mOsmol) and pure Milli–Q water were used for calibrating the set–up to 0°C following the manufacturer's instructions and the ICT (West, Dorsey, Bichowsky & Klemenc, 1926; West, et al., 1928). The raw data are converted into MP, FP and then TH in Kelvin by multiplication of the experimental values with the factor of –0.001858°C mOsmol/kg (Pauling, 1953, pp. 345-347) and the ICT as established (DeVries, et al., 1970; Komatsu, et al., 1970; DeVries, 1986; Ahlgren, et al., 1988). A value of for example a 100 mOsmol solution represents a temperature of –0.186°C. This set–up is in line with that used in numerous previous studies (DeVries, et al., 1970; Shier, et al., 1972; Duman & DeVries, 1975; Chakrabartty, Yang & Hew, 1989; Chakrabartty & Hew, 1991; Celik, et al., 2010; Ahn, et al., 2012; Gaede-Koehler, et al., 2012). The temperature range in which this Clifton instrument can operate is limited to 4,000 mOsmol or circa –7.5°C.

The definition of ice being present in an aqueous phase, such as the sample droplets observed in this study, is a darkening of the sample under the light microscope as the direct result of changes in the refraction in accordance with observations made and described by early investigators (Chambers & Hale, 1932). It is referred to as "*flashing*" when numerous small ice crystals suddenly form and after very fast propagation through the entire liquid

volume of a sample or a cell result in one solid piece of ice (Luyet & Gibbs, 1937; Asahina, 1953, 1956). The temperature at which an ice crystal starts to grow and changes its shape from round to hexagonal and bi–pyramidal was defined as the FP. At this moment a sudden and explosive growth of the single crystals can be observed (Celik, et al., 2010). The MP was determined as the temperature at which the last remaining ice crystal abruptly disappears (DeVries, 1971; Lin, et al., 1972).

The Piscicolidae available for this study were each of small mass and volume and the animals were weighed (± 0.1 mg) due to difficulties of accurate volumetric measurement. To estimate the volumetric dilution factors (v/v) the assumption was made that the mass calculated into volumetric units is 1 mg equal to 1 µl.

In a simple freezing experiment specimens of both leech species were frozen and reheated. This was achieved by placing specimens in Petri dishes at –18°C until either the animal was frozen in parts or as a whole with subsequent thawing at room temperature.

5.3.2 Sampling procedure for TH determination

The material for measurements of THA by NO and observation of ice crystal growth by light microscopy was performed at Scott Base, Antarctica. The piscicolid specimens were taken off the same fish host species *Trematomus hansoni* to reduce any potential variations in AFGP concentrations between different host species. The fresh samples were processed immediately to avoid possible errors caused by evaporation (Kiko, 2010).

Leech specimens were washed to remove external fish contaminants by placing them inside an Eppendorf tube of Milli–Q water and shaken. They were then put on paper towels to dry and any remaining leech mucus wiped off (Bielecki, et al., 2008) as this could also contain fish mucus with AFGPs (Evans, et al., 2011). Dried leeches were placed on a Petri dish under a dissecting microscope (Leica 2000 Zoom Stereo Microscope, Leica, Wetzlar, Germany) for sampling of fluids into a glass capillary (Figs. 5.1–5.6).

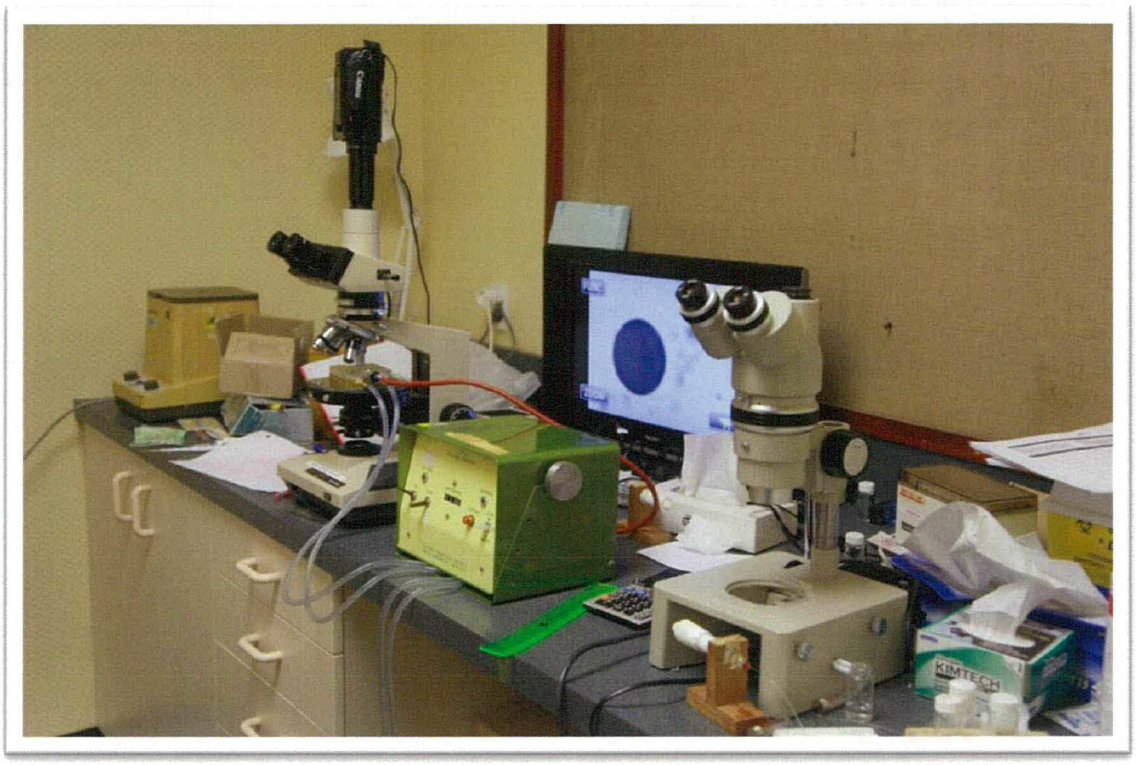


Figure 5.1 Image of the workbench setup for antifreeze activity determination.

The Clifton nanolitre osmometer (green instrument in the centre) is controlling the thermal block under the microscope (Olympus to the left) with a mounted video camera (Canon) for live observation on the display (Sony) behind as well as documentation. The cooling water supply through transparent hoses can be seen while the thermostat was below the bench (not pictured). The dissection microscope (Nikon) with one of the two mounted syringes for loading oil and then the samples into the wells can be seen to the right. At the far end of the table is the centrifuge (Eppendorf).

Samples of intestinal blood, body fluids and the anterior body part were taken from the larger leech species, *C. levigata*. Each of the four suckers used was dissected out and homogenised using a micropestle in an Eppendorf tube with the addition of Milli–Q water and liquid nitrogen.

Micropipettes (glass capillary tubes heated over a Bunsen burner and pulled manually into a fine point) were used to penetrate the leech integument and to extract minute volumes of body fluids. These micropipettes were placed on the end of a fine silicone hose attached to a 2 ml Gilmont micrometre syringe (Thermo Fisher Scientific, Waltham, Massachusetts, USA) filled with light grade mineral oil (Cargille type A immersion oil, Cargille Laboratories, Cedar Grove, New Jersey, USA). The collection of sample volumes was

performed under a dissecting microscope (magnification x10–12; Leica Z30–L, Leica Microsystems, Wetzlar, Germany) in order to see when the needle punctured the skin and which body cavity was sampled (Fig. 5.2).

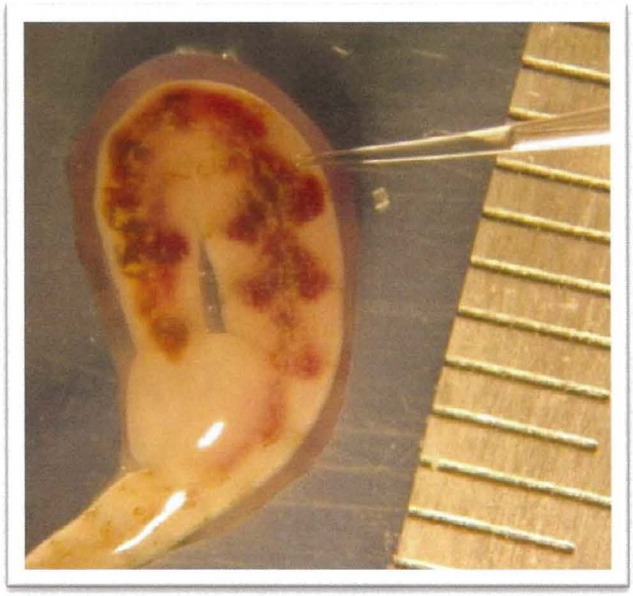


Figure 5.2 Illustration of sampling from *C. levigata* with a glass capillary.

The digestive tract can be readily identified as filled with red fish blood (ruler in mm scale).

Next, suction was applied using the syringe, which allowed the sampling of minute volumes of fluids into the capillary tip. The glass needle was removed from the hose and closed at the opposite end from the sample with a Plasticine–like sealant mass to allow for centrifugation. The capillary was then centrifuged (Eppendorf 5414 Microcentrifuge, Hamburg, Germany) at 15,000 rpm to spin down and separate the blood serum from the plasma or tissue fragments from the fluid sample, which were visible as small pellets (Fig. 5.3).

The micropipettes were used to prepare the well chambers of a small metal plate with oil and also to load the sample volumes for the freezing experiments into the individual well chambers under a dissecting microscope (Nikon SMZ–10 Binocular, Tokyo, Japan). This was achieved using a glass pipette and micrometre syringe as above containing first a heavy grade mineral oil (Cargille type B immersion oil) to fill the well with liquid (Fig. 5.4).

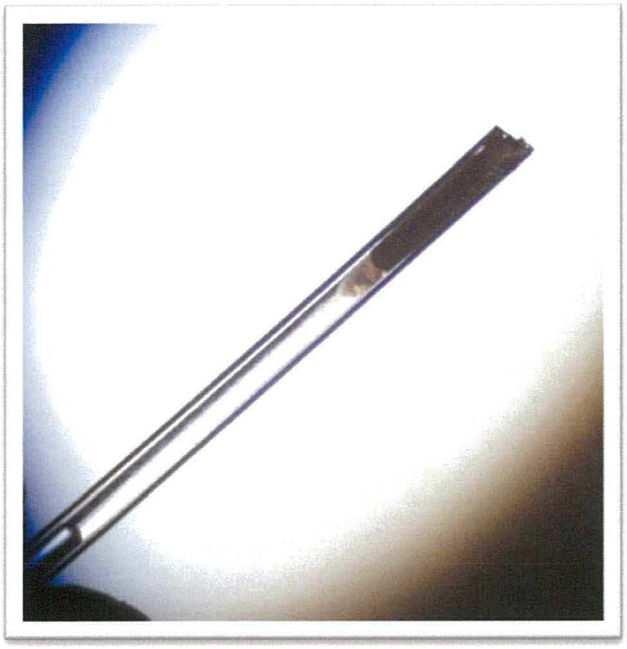


Figure 5.3 Image of sample volume in sealed glass capillary.

The aqueous sample inside the capillary can be seen with a meniscus (lower left corner) and with a small tissue pellet towards the sealed end (top right).

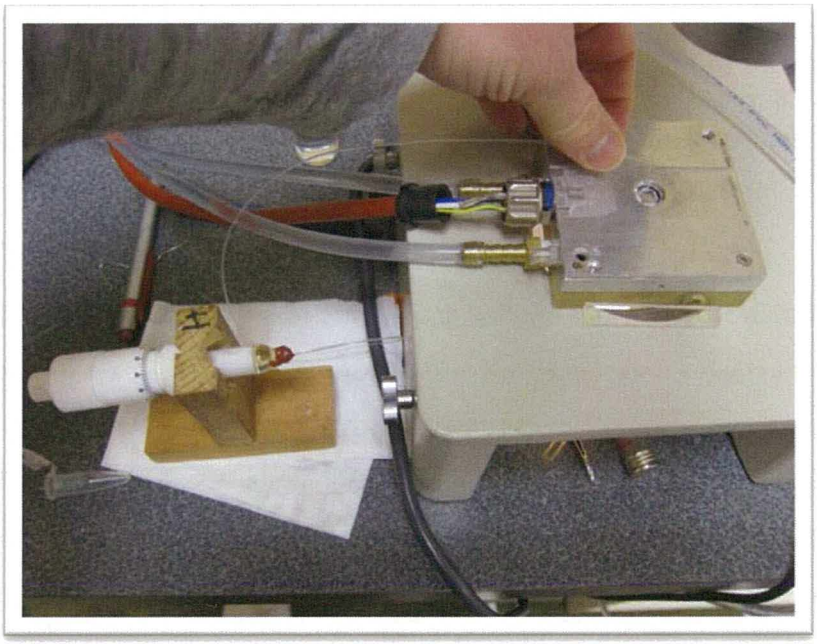


Figure 5.4 Preparation of the well chambers under the dissecting microscope.

The heavy grade mineral oil (indicated as "H" on the wooden frame for the syringe) was put into the wells. The next step was to load one droplet of the aqueous sample in the central position of each well.

The capillary with the spun sample was broken open with forceps and another micropipette was used as a loading tip using a syringe with light grade mineral oil to place one droplet of the aqueous sample centrally into each of the wells prepared with heavy grade mineral oil (Fig. 5.5). The inserted sample volume was kept to the technically feasible minimum.

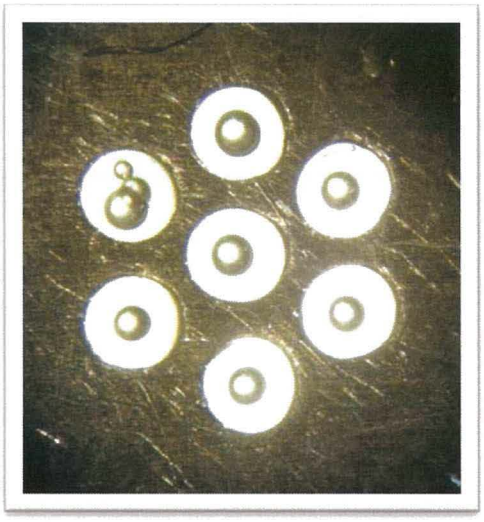


Figure 5.5 Illustration of loaded wells in the cooling–heating element of the NO.

One droplet of an aqueous sample for TH determination was inserted in each well filled with heavy grade mineral oil as can be seen in the central positions for six of the seven wells. The numbering of wells was marked clockwise: 2, 4, 6, 8, 10 and 12 at the top with one further well in the centre.

A small amount of thermal paste was applied around the four sides of the metal plate to optimise thermal conductivity of the inlay piece with the cooling and heating block. The plate was cleaned between runs of different samples with a degreasing solvent, then rinsed in water and dried before it was placed in the cooling–heating block as the cold stage controlled by the Clifton. The osmometer for manual temperature control was placed next to the microscope in order to simultaneously adjust the cold stage while observing the ice crystal behaviour (Fig. 5.6).

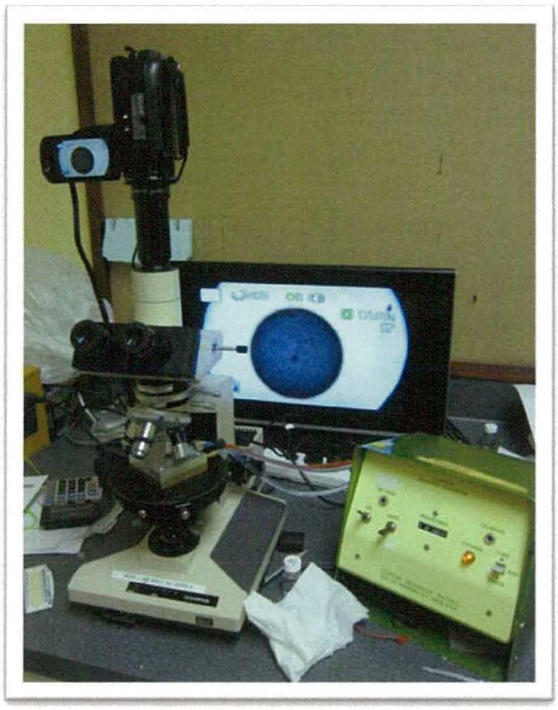


Figure 5.6 Observation of freezing experiment.

The one droplet of the aqueous sample inserted into the heavy oil filled well can be seen on the display of the video camera and the monitor in the background.

5.3.3 Preparative procedure for *C. antarctica*

Larger specimens of *C. antarctica* were collected off *T. hansoni* sitting externally on the body of the host. The THA test was performed on an entire specimen homogenate of one individual (n=1) and one pooled sample of posterior suckers off four *C. antarctica* (n=1). One specimen was selected to be starved for one month before TH determination. *C. antarctica* had a visibly smaller blood to body ratio than *C. levigata* while feeding on the fish host and it was assumed the blood meal content in the digestive tract would reduce fairly quickly with starvation.

The specimen that was immediately processed was washed in Milli–Q water and dried to remove any fish mucus with fish AFGPs. It required the addition of 15 µl Milli–Q water for homogenisation. As a conservative estimate, this resulted in a volumetric dilution of no less than fourfold. After centrifugation for 25 min, a small volume of a brownish coloured phase of 3.5 mm in the capillary above the solid pellet could be estimated to approximately 0.6 µl

(i.e. 5 µl glass capillary with a 32 mm total length, 3.5/6.4=0.55 µl). This volume was taken into the loading pipette and used to insert the small aqueous droplets inside the heavy grade oil of several wells for THA determination.

Four individuals were washed in Milli–Q and dried before their posterior suckers were cut off and weighed on a precision balance (Mettler Toledo AE 160, Columbus, Ohio, USA). The suckers were homogenised in 5 µl Milli–Q and centrifuged for 5 min. A clear supernatant of 10.8 mm in a 5 µl glass capillary could be used as a 1.7 µl sample volume (5 µl capillary of 32 mm, 10.8/6.4=1.69). Likewise, the starved leech was washed, dried, weighed and homogenised in 20 µl Milli–Q water and after 13 min centrifugation the capillary showed a 24.5 mm column or circa 3.8 µl of sample volume (5 µl capillary of 32 mm, 24.5/6.4=3.83).

5.3.4 Preparative procedure for *C. levigata*

Larger and blood filled specimens of *C. levigata* were collected from the gill cavities of *T. hansoni*. A fine glass pipette and suction were used to extract small volumes of the digestive contents from three specimens (n=3). After a short spin of 5 s, the supernatant was transferred undiluted into a loading pipette for THA determination, analogous to fish protocols.

To obtain body fluids one specimen (n=1) was washed in Milli–Q water, dried and cut approximately at the anterior third above the first pair of testisacs, with care being taken to exclude fish blood, which was visible below the cut. The anterior part of the specimen was then pressed to squeeze out a liquid and tissue mixture, which was collected in a 10 µl glass capillary filled to 1 mm or 0.24 µl (i.e. 10 µl glass capillary with a 41 mm total length, 1/4.1=0.24). The addition of 2 µl was necessary to obtain a usable and clear supernatant after 20 min of centrifugation.

In order to obtain a larger sample of leech tissue one specimen was washed and dried (n=1). It was then slightly flattened and fish blood from the oesophagus was removed into the posterior part of the digestive tract by starting to push from the anterior sucker at the top towards the posterior end with the rounded back of a forceps. A cut approximately at the anterior third above the first pair of testisacs was made, with care being taken to exclude fish blood, which was now stored more than 5 mm below the cut. The sample was weighed

and after 9 µl Milli–Q was added for homogenisation, a sample volume of 2 µl was obtained after 7 min centrifugation.

Four specimens were washed and dried before their posterior suckers were cut off and weighed (n=1). They were homogenised in 25 µl Milli–Q and centrifuged for 4 min. A clear supernatant (Fig. 5.3) of 4.2 mm in the glass capillary could be used as a 1 µl sample volume (10 µl capillary of 41 mm, 4.2/4.1=1.02).

5.3.5 Measuring procedure and ice crystal examination

The sample volume in the cold stage was rapidly frozen solid at a temperature below –20°C. The freezing process could be readily observed as a sudden change in transparency of the sample droplet from translucent to opaque inside the oil filled chamber. The temperature was slowly raised until the ice crystals started to melt. From then very slow adjustments and often repeated cooling and heating were necessary to obtain a single ice crystal located in the centre. The temperature was raised until a stable and very small hexagonal structure or nearly round crystal of below 5 µm in diameter was achieved (Figs. 5.7 A & 5.8 A). The moment at which this minute ice crystal disappeared was considered to be the MP of the sample.

A series of pilot runs were made to establish the mOsmol values of the MP and FP for each sample prior to final data recording. Time intervals of between 5 and 10 min were used to allow for the equilibrium to establish before the temperature was changed. The melting and FPs were determined by many repeats in which very slow and gradual temperature changes provided a precise value. The observations were documented by digital photography and video recording, using a video camera (Canon VIXIA HF S21, Tokyo, Japan) mounted onto the microscope (Olympus, BH–2, Olympus Optical, Tokyo, Japan), while the investigator observed and controlled the experiment on a 32" TV display (Sony Bravia, Tokyo, Japan).

5.4 Results

The results provided in tables 5.1–5.7 are listed in order of the data acquisition. There were at least 30 min and often some hours between the first and the last THA determination.

5.4.1 TH measurements for *C. antarctica*

The specimen in its natural and fed condition had a TH of 0.3 ± 0.04 K (mean ± SE) in the diluted sample of the homogenate with a MP of –0.61°C ± 0.02° (mean ± SE) (Table 5.1). The undiluted natural TH value is estimated, under the assumption of linear correlations, to reach 1.2 K. The total mass of material from four suckers was 1.1 ± 0.1 mg with a TH of 0.30 ± 0.03 K (mean ± SE) in the diluted sample (Table 5.2), representing possibly an estimated value of 1.66 K. The diluted sample had a MP of –1.7°C ± 0.07° (mean ± SE).

The specimen kept in isolation was visibly starved and its mass of 12.2 ± 0.1 mg results in a volumetric dilution factor for the THA value of no less than 2.6. As these invertebrates are heavier than water, a volume of less than 12.2 µl was assumed in order to provide a conservative estimate for the dilution factor. For the diluted volume of the starved leech homogenate the TH was 0.24 ± 0.02 K (mean ± SE) and the MP –0.66°C ± 0.03° (mean ± SE) (Table 5.3). Hence the TH in the living animal could be more than 0.63 K.

Table 5.1 Thermal hysteresis (TH) measurements from homogenate of *C. antarctica*.

Thermal hysteresis (TH) data for the diluted sample of a single specimen homogenate in natural condition from *C. antarctica* (n=1).

MP [mOsmol]	MP [–°C]	FP [mOsmol]	FP [–°C]	TH [mOsmol]	TH [K]
280	0.52	418	0.78	138	0.26
275	0.51	340	0.63	65	0.12
275	0.51	330	0.61	55	0.10
275	0.51	400	0.74	125	0.23
270	0.50	340	0.63	70	0.13
330	0.61	465	0.86	135	0.25
330	0.61	460	0.85	130	0.24
315	0.59	488	0.91	173	0.32
312	0.58	560	1.04	248	0.46
295	0.55	420	0.78	125	0.23
295	0.55	455	0.85	160	0.30
300	0.56	385	0.72	85	0.16
300	0.56	360	0.67	60	0.11
300	0.56	430	0.80	130	0.24
301	0.56	433	0.80	132	0.25
430	0.80	600	1.11	170	0.32
420	0.78	640	1.19	220	0.41
420	0.78	820	1.52	400	0.74
420	0.78	725	1.35	305	0.57
420	0.78	680	1.26	260	0.48
mean ± SE	0.61 ± 0.02		0.91 ± 0.06		0.30 ± 0.04

Table 5.2 TH measurements from pooled posterior sucker samples of *C. antarctica*.

TH data for diluted sample from four posterior suckers cut off four *C. antarctica* (n=1).

MP [mOsmol]	MP [–°C]	FP [mOsmol]	FP [–°C]	TH [mOsmol]	TH [K]
890	1.65	1000	1.86	110	0.20
890	1.65	1075	2.00	185	0.34
885	1.64	1080	2.01	195	0.36
1010	1.88	1080	2.01	70	0.13
1017	1.89	1085	2.02	68	0.13
1020	1.90	1090	2.03	70	0.13
1025	1.90	1095	2.03	70	0.13
1030	1.91	1100	2.04	70	0.13
1035	1.92	1105	2.05	70	0.13
1037	1.93	1110	2.06	73	0.14
1042	1.94	1115	2.07	73	0.14
1047	1.95	1120	2.08	73	0.14
1052	1.95	1130	2.10	78	0.14
1060	1.97	1135	2.11	75	0.14
1070	1.99	1140	2.12	70	0.13
880	1.64	1070	1.99	190	0.35
860	1.60	1030	1.91	170	0.32
865	1.61	1115	2.07	250	0.46
1060	1.97	1170	2.17	110	0.20
1060	1.97	1170	2.17	110	0.20
1055	1.96	1475	2.74	420	0.78
1054	1.96	1474	2.74	420	0.78
1050	1.95	1250	2.32	200	0.37
890	1.65	970	1.80	80	0.15
910	1.69	1070	1.99	160	0.30
910	1.69	1100	2.04	190	0.35
910	1.69	1105	2.05	195	0.36
910	1.69	1100	2.04	190	0.35
910	1.69	990	1.84	80	0.15
910	1.69	1090	2.03	180	0.33
910	1.69	1090	2.03	180	0.33
910	1.69	1230	2.29	320	0.59
910	1.69	1330	2.47	420	0.78
910	1.69	1335	2.48	425	0.79
910	1.69	1140	2.12	230	0.43
910	1.69	1085	2.02	175	0.33
910	1.69	1175	2.18	265	0.49
mean ± SE	1.70 ± 0.07		2.00 ± 0.08		0.30 ± 0.03

Table 5.3 TH measurements from homogenate of starved *C. antarctica*.

TH data for diluted sample of specimen homogenate from one starved *C. antarctica* (n=1).

MP [mOsmol]	MP [–°C]	FP [mOsmol]	FP [–°C]	TH [mOsmol]	TH [K]
300	0.56	400	0.74	100	0.19
300	0.56	385	0.72	85	0.16
300	0.56	470	0.87	170	0.32
298	0.55	400	0.74	102	0.19
300	0.56	420	0.78	120	0.22
300	0.56	380	0.71	80	0.15
300	0.56	380	0.71	80	0.15
300	0.56	440	0.82	140	0.26
300	0.56	442	0.82	142	0.26
430	0.80	560	1.04	130	0.24
430	0.80	515	0.96	85	0.16
430	0.80	555	1.03	125	0.23
430	0.80	560	1.04	130	0.24
430	0.80	550	1.02	120	0.22
430	0.80	610	1.13	180	0.33
430	0.80	725	1.35	295	0.55
mean ± SE	0.66 ± 0.03		0.90 ± 0.05		0.24 ± 0.02

5.4.2 TH measurements for *C. levigata*

The undiluted samples of the digestive system from three *C. levigata* (n=3) exhibited a TH gap of 1223–1750 mOsmol or 2.27 to 3.25 K (2.84 ± 0.09 K = mean ± SE) (Table 5.4). The MP was determined as –2.46°C ± 0.04° (mean ± SE). The sample of body fluids and a TH value of 0.07 ± 0.01 K (mean ± SE) diluted (Table 5.5). This could potentially represent a TH of 0.65 K or more in the fluids. For the diluted body liquids the MP was –0.32°C ± 0.01° (mean ± SE). The anterior third of the body weighing 4.6 ± 0.1 mg (with the remaining body of weighing 16.6 ± 0.1 mg). The diluted sample had a TH of 0.37 ± 0.04 K (mean ± SE) (Table 5.6), resulting in an estimated TH in the living animal of 1.09 K. For the diluted anterior body

sample the MP was determined as –1.18°C ± 0.01° (mean ± SE). The four posterior suckers, with a combined total mass of 8.5 ± 0.1 mg, when diluted had a TH value of 0.62 ± 0.05 K (mean ± SE) (Table 5.7). At a volumetric dilution of approximately 3.9 this could represent a TH *in vivo* of 2.44 K. The MP in the diluted sample was –5.82°C ± 0.33° (mean ± SE).

Table 5.4 TH measurements of samples from digestive system of *C. levigata*.

TH data for undiluted sample of digestive system content from three *C. levigata* (n=3).

MP [mOsmol]	MP [–°C]	FP [mOsmol]	FP [–°C]	TH [mOsmol]	TH [K]
1320	2.45	2640	4.91	1320	2.45
1260	2.34	2640	4.91	1380	2.56
1237	2.30	2460	4.57	1223	2.27
1270	2.36	2665	4.95	1395	2.59
1480	2.75	2930	5.44	1450	2.69
1360	2.53	2960	5.50	1600	2.97
1340	2.49	3090	5.74	1750	3.25
1300	2.42	3010	5.59	1710	3.18
1280	2.38	3010	5.59	1730	3.21
1310	2.43	3010	5.59	1700	3.16
1360	2.53	2920	5.43	1560	2.90
1400	2.60	2900	5.39	1500	2.79
mean ± SE	2.46 ± 0.04		5.30 ± 0.11		2.84 ± 0.09

Table 5.5 TH measurements from body fluids of *C. levigata*.

TH data for diluted sample of body fluids from one *C. levigata* (n=1).

MP [mOsmol]	MP [–°C]	FP [mOsmol]	FP [–°C]	TH [mOsmol]	TH [K]
160	0.30	210	0.39	50	0.09
165	0.31	200	0.37	35	0.07
135	0.25	160	0.30	25	0.05
140	0.26	170	0.32	30	0.06
135	0.25	170	0.32	35	0.07
140	0.26	160	0.30	20	0.04
140	0.26	180	0.33	40	0.07
150	0.28	180	0.33	30	0.06
146	0.27	170	0.32	24	0.04
150	0.28	200	0.37	50	0.09
150	0.28	170	0.32	20	0.04
150	0.28	195	0.36	45	0.08
150	0.28	180	0.33	30	0.06
150	0.28	170	0.32	20	0.04
245	0.46	295	0.55	50	0.09
150	0.28	180	0.33	30	0.06
150	0.28	180	0.33	30	0.06
245	0.46	270	0.50	25	0.05
245	0.46	285	0.53	40	0.07
245	0.46	290	0.54	45	0.08
220	0.41	250	0.46	30	0.06
210	0.39	252	0.47	42	0.08
210	0.39	260	0.48	50	0.09
210	0.39	270	0.50	60	0.11
210	0.39	240	0.45	30	0.06
210	0.39	290	0.54	80	0.15
120	0.22	140	0.26	20	0.04
140	0.26	180	0.33	40	0.07
135	0.25	175	0.33	40	0.07
mean ± SE	0.32 ± 0.01		0.39 ± 0.02		0.07 ± 0.01

Table 5.6 TH measurements from anterior third body material of *C. levigata*.

TH data for diluted sample of anterior third body part from one *C. levigata* (n=1).

MP [mOsmol]	MP [–°C]	FP [mOsmol]	FP [–°C]	TH [mOsmol]	TH [K]
630	1.17	780	1.45	150	0.28
630	1.17	880	1.64	250	0.46
660	1.23	780	1.45	120	0.22
650	1.21	970	1.80	320	0.59
660	1.23	780	1.45	120	0.22
670	1.24	970	1.80	300	0.56
660	1.23	960	1.78	300	0.56
620	1.15	730	1.36	110	0.20
620	1.15	820	1.52	200	0.37
620	1.15	810	1.50	190	0.35
620	1.15	810	1.50	190	0.35
620	1.15	730	1.36	110	0.20
620	1.15	820	1.52	200	0.37
620	1.15	810	1.50	190	0.35
mean ± SE	1.18 ± 0.01		1.55 ± 0.04		0.37 ± 0.04

Table 5.7 TH measurements from posterior sucker material of *C. levigata*.

TH data for pooled and diluted sample from four posterior suckers off four *C. levigata* (n=1).

MP [mOsmol]	MP [–°C]	FP [mOsmol]	FP [–°C]	TH [mOsmol]	TH [K]
2360	4.38	2560	4.76	200	0.37
2370	4.40	2680	4.98	310	0.58
2360	4.38	2560	4.76	200	0.37
2350	4.37	2800	5.20	450	0.84
2330	4.33	2580	4.79	250	0.46
3570	6.63	4050	7.52	480	0.89
3570	6.63	4030	7.49	460	0.85
3630	6.74	3850	7.15	220	0.41
3630	6.74	4010	7.45	380	0.71
3640	6.76	4010	7.45	370	0.69
3640	6.76	3990	7.41	350	0.65
3635	6.75	3985	7.40	350	0.65
3635	6.75	3985	7.40	350	0.65
mean ± SE	5.82 ± 0.33		6.44 ± 0.36		0.62 ± 0.05

5.4.3 TH in notothenioid hosts

At the same time that leech specimens were collected and TH measurements made, the identical set–up and protocol was used for blood serum samples of the notothenioid fish *T. hansoni* and *T. nicolai*. A. L. DeVries and L. Fields (pers. comm.) recorded TH values of around 1.4 K for *T. hansoni* and of around 2.26 K for *T. nicolai*, with little variance between the specimens.

5.4.4 Bi–pyramidal ice crystal behaviour in *Cryobdella* spp. samples

The samples from *C. antarctica* and *C. levigata* showed THA in all measurements *in vitro*. The undiluted samples of digestive fluid from *C. levigata* represent the direct measurement of an *in vivo* THA of this body compartment in leeches. For all samples, the typical explosive bi–pyramidal ice crystal growth in the presence of active AFGPs was observed at the freezing temperatures (Figs. 5.7 B & 5.8 B; for video demonstration see digital file Appendix A.3). One additional shape of ice crystals as ice formation was observed in a sample from the anterior body part of *C. levigata*.

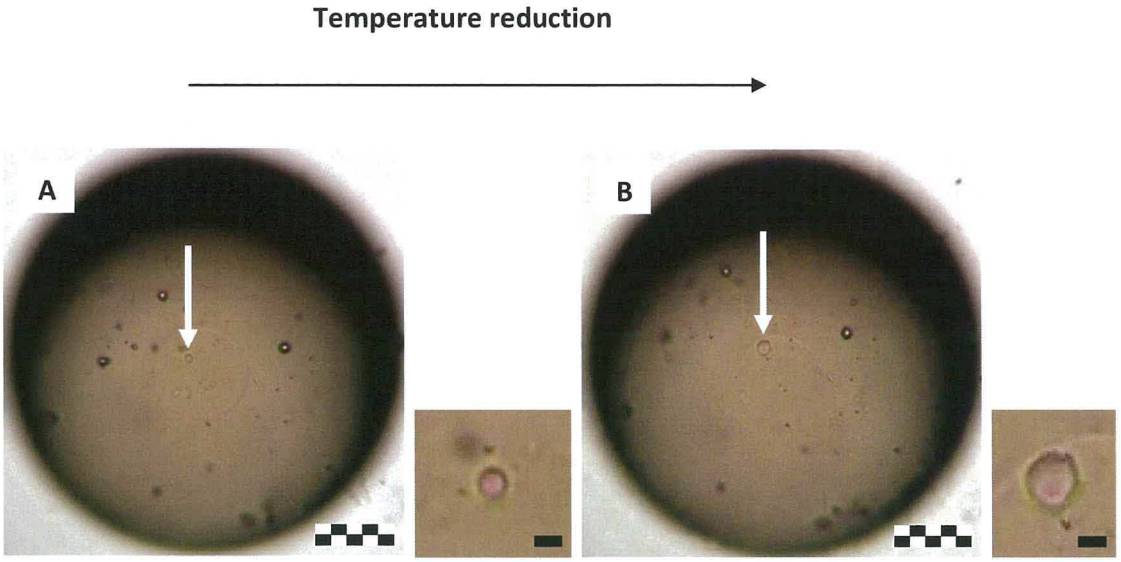


Figure 5.7 Images of the aqueous droplet inside the heavy grade oil with seed crystal.

(A) One single ice crystal located centrally (indicated by arrow) was produced before the temperature decrease in the freezing step (scale bar 50 µm with 10 µm divisions); with cropped image of the crystal as shown in smaller image to the right, scale bar 5 µm).
(B) The identical crystal at the start of propagation marking the freezing point and experimental end of this sample run (scale bar 50 µm with 10 µm divisions; with cropped image of the crystal as shown in smaller image to the right, scale bar 5 µm).

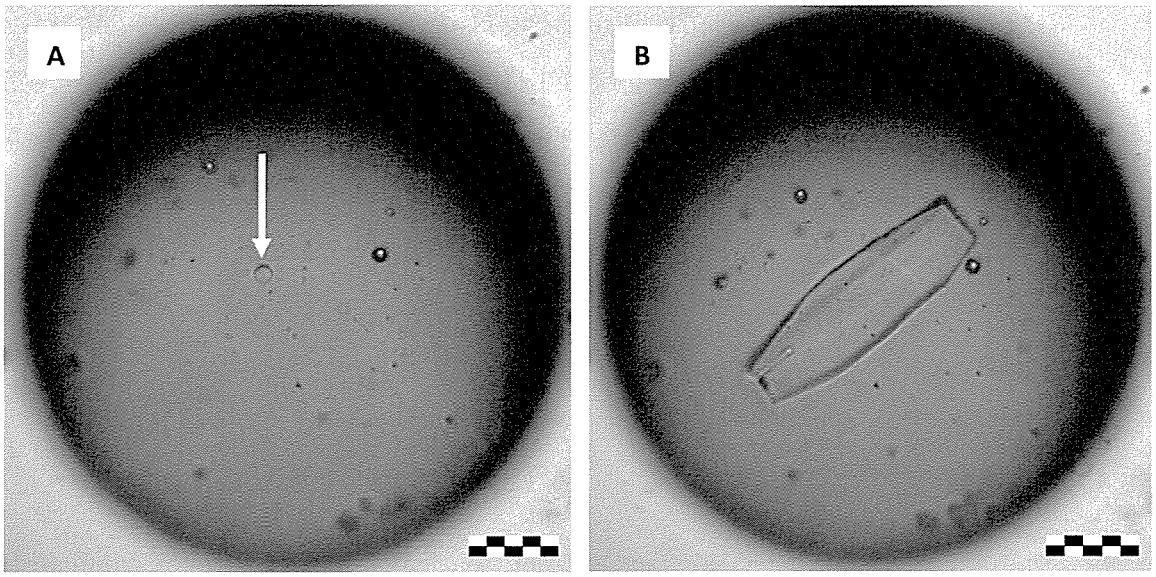


Figure 5.8 Images of ice crsytal growth behaviour in solution containing AFGPs.

(A) One ice crystal of minimal diameter size (3–5 µm) is centrally produced (indicated by arrow).
(B) The temperature is lowered until growth can be observed as characteristic bi–pyramidal elongation and recorded for determination of thermal hysteresis or antifreeze activity for the sample solution (scale bar 25 µm with 5 µm divisions; for video demonstration see digital file Appendix A.3).

5.5 Discussion

The presence of THA in every sample, including all dilutions, in combination with the characteristic bi–pyramidal ice crystal growth allows us to conclude that the two piscicolid species *C. antarctica* and *C. levigata* have an active antifreeze protection *in vivo*. These marine invertebrates cannot tolerate freezing and therefore require protection against cellular freezing events. This is the sixth report of THA in a marine invertebrate due to AFPs, after *M. edulis* in the North Sea (Theede, et al., 1976) and the four species *Homaxinella balfourensis* (Ridley et Dendy, 1886) (Wilkins, et al., 2002), *A. valida* (Waller, et al., 2006), *T. antarcticus* (Kiko, et al., 2008) and *S. longipes* (Kiko, 2010) in the Southern Ocean. The Piscicolidae are the first recorded Antarctic marine invertebrates possessing AFPs and demonstrating THA outside the intertidal as well as the surface sea ice associated biota.

These fish leeches are capable of surviving *in situ* in the Antarctic ice–laden water their entire annual life cycles and have no need to migrate to warmer water or change host to escape death by freezing. As in other leeches, the crop is filled and used for storage of blood, but as the species are obligate parasites it can be assumed that the crop contains the current fish host blood. However, even a mixture of AFGPs deriving from different host individuals of the same or different fish species would not have any effect on the conclusions of this ecophysiological study. It should be noted that artificially detached leeches did not readily reattach to either the same host or similar sized and aged fish of the same or other species (pers. obs.).

The AFGPs in the digestive tract are functional and the TH exceeds the activity level observed in fish at the same time during the Antarctic spring. *T. hansoni* previously caught in the same area of McMurdo Sound and at shallow water depths of 20 m had a FP for blood serum of –2.01°C ± 0.019° (mean ± SE) with 44% of the FP depression calculated to be due to NaCl (DeVries & Wohlschlag, 1969). Thus, the TH of 1.4 K measured for *T. hansoni* during the field season of 2010 is in agreement with the literature.

The demonstration of active peptides in the digestive system of the parasites and their use in the tissue samples represent a successful cross–species application under natural conditions. This is analogous to the experiments performed on northern teleosts with AFPs and thus THA transfers (Fletcher, et al., 1986), on marine diatom species (Raymond & Knight, 2003) or the insect AFP successfully transferred and active in *Escherichia coli* (Liou, et al., 2000). Consequently, the results from Antarctic leeches are further evidence for the universal characteristics of AFPs as functional ice inhibitors across phylogenetic divisions. This is the first record of notothenioid AFGPs to function as antifreeze protectant outside the taxonomic clade of teleost fishes.

The primary aim of this study was to establish the presence of FP depressing proteins in their active form in two marine invertebrates. This was achieved using values derived from fresh material in a sealed experimental environment so as to reduce artefacts caused by evaporation. However, the samples had varying calculated dilution factors and consequently the estimated maximal THA *in vivo* must be regarded as indicative only. It would require different techniques for sampling and analysis to investigate this further, these were either emerging technologies or not available at the time this work was undertaken.

The TH values demonstrate the ability of Antarctic leeches to survive in sub–zero waters by means of FP depression. As such, they are able to withstand freezing conditions that would be lethal to other Hirudinea as was demonstrated first 140 years ago by Dönhoff (1872) and Kochs (1892). The specimens selected for TH experiments of both species were at the upper end of the size range for individuals observed feeding on fishes. This was in order to facilitate the sampling and to maximise volumes, and at the same time to target adults. Potential variations within juveniles for both species were excluded from this study. Additionally, if these Antarctic fish leeches have a multi–year ontogenesis (Utevsky, 2007), the adult specimens might have not only had more time to assimilate AFGPs but individual differences might be minimised to below the level of biological relevance.

The discovery of TH as the result of bioactive AFGPs further establishes that the digestive processes inside the invertebrate leeches, either by endogenous exopeptidases or the endosymbiotic bacteria, as indigenous flora of the leech intestine (Hornbostel, 1942; Büsing, 1951; Büsing, et al., 1953), have little or no effect on AFGPs and detectable THA. The enzymatic reactions that occur in the fish prior to the proposed absorption of AFGPs from the rectum in Antarctic as well as Arctic fishes (Evans, et al., 2012) do not interfere with the core function of AFGPs in the host species. The nutritional processing by the leech or symbiotic activities do not seem to be more effective or result in any meaningful alterations of the peptides that could cause a loss of function prior to absorption. Although this conclusion derives from only two species tested, it seems plausible that functional AFGPs are available to all Antarctic hirudineans parasitic on fishes with AFGPs.

5.5.1 Supercooling and superheating effects

It has been demonstrated that an ice crystal is stabilised by AFP attachment. AFPs attached ice crystals do not grow at the equilibrium FP but at lower temperatures (supercooling). Furthermore, they do not melt at the equilibrium MP but at a higher temperature, resulting in superheating of the crystal in the presence of AFPs (Celik, et al., 2010). This phenomenon would provide inaccurate MP and FP values, and thus higher artefactual TH measurements, as the appropriate temperature readings at either of these two points would be affected. The use of very slow cooling or heating rates of 0.01 K per 5 min avoid artefacts caused by supercooling on an observed small ice crystal (Schrag & DeVries, 1983). In the current study

the THA was determined by many repeats in which a very slow and gradual temperature increase and decrease eventually provided an accurate value for the MP and FP. I followed working protocols established by A. L. DeVries and C. W. Evans (pers. comm.) aiming at very slow temperature alterations in either direction to heat or cool the sample droplet. These changes were chosen at the rates previously described and derived from using a water bath at a rate of 0.01 K per 5 min (DeVries, 1971) to cooling rates slower than 0.05 K per min (Jin & DeVries, 2006). A number of runs were also performed using faster temperature changes, with around 40–60 mOsmol change per min equivalent to 0.1 K per min as used before for TH determination with a Clifton instrument (Hansen & Baust, 1988). It can be concluded that AFGPs in leech material behave in similar manner as in fish and other animals tested for THA due to AFPs.

5.5.2 Comparison to TH of other Antarctic marine invertebrates

The TH of another Antarctic marine invertebrate, *T. antarcticus,* has been reported (Kiko, et al., 2008). In three egg clutches (n=3) of this mollusc the authors measured a TH of 0.16 K to 0.19 K, with melting points of –2.4°C to –4.6°C. The adults had a TH of 0.29 K in a diluted sample (circa 4x, Rainer Kiko, pers. comm.) and TH 1.49 K (undiluted sample) and their MPs were observed at –0.59 K (diluted sample) and at –2.35°C (undiluted sample). The data in my study from piscicolid tissues are comparable with the values measured in adult specimens of *T. antarcticus*. Considering a similar dilution for sucker material of around fivefold in *C. antarctica* and fourfold in *C. levigata* to the approximated fourfold in the mollusc, the similarities are striking. I believe this might prove in a future study with undiluted samples to be not merely coincidental but based on a relevant physiological adaptation.

The samples used to test for TH in the posterior sucker of pure leech tissue were pooled from four individuals in both leech species. Although the TH values for the diluted samples were of nearly a twofold difference, this discrepancy was markedly reduced when the dilution factors were taken into consideration. This could be indicative of a linear relationship between AFGP quantity and THA in leeches, as it has been found in fish when the overall AFGP concentrations were low and the TH plateau was not reached yet (DeVries, et al., 1970) as well as in the Antarctic springtail *Gressittacantha terranova* where the THA was also found to be linear to the protein concentration (Hawes, et al., 2011). At AFGP

concentrations of less than 4 mg/ml in *P. borchgrevinki* blood serum, the freezing temperature is decreased in a near linear relationship before changing into a curve and then plateau at higher concentrations (DeVries, 1971). The estimated TH of 1.66 to 2.44 K for Antarctic marine leeches might therefore represent values within close proximity to those that occur in nature. The provision of dilution factors in this PhD is the first attempt to estimate the *in vivo* THA for an Antarctic invertebrate.

It was not possible to present THA as a function of antifreeze concentration against temperature as has been given in the literature (Haymet, Ward & Harding, 1999) because the concentration levels could not be determined for the minute volumes obtained from leech material. However, the leech values are similar to those first measured in *M. edulis*, with a demonstrated TH of 0.38 K, being the average of 10 specimens, and with a MP of –1.75°C ± 0.02° and a FP of –2.13°C ± 0.04° (mean ± SE) (Theede, et al., 1976). My findings indicate that AFPs are biologically relevant at these levels.

The TH of diluted samples of sucker muscle of pure leech tissue had a mean MP of –5.82°C in *C. levigata*. This value far exceeds the MP measured in adult specimens of *T. antarcticus*. Although this could be an experimental error, it is more likely to indicate a considerable quantity of AFGPs in the tissues of *C. levigata* as a biological adaptation. This species lives attached to the fish under the operculum in the gill cavity and is well protected from physical abrasion in this microhabitat, which could cause injury or loss of contact to its host. Yet as crystals are present in Antarctic seawater at the depth of these benthic fishes, and as the gills experience constant water flow, the danger of interactions with ice crystals as ice nucleators is increased.

A comparison with the TH of 1.4 K discovered in the haemolymph of *A. valida* (Waller, et al., 2006) puts the results further into perspective. The experimental data for *A. valida* were not obtained by NO as for *T. antarcticus* but by a procedure known as differential scanning calorimetry (DSC) (Hansen & Baust, 1988). Nevertheless, the TH gap stems from undiluted samples for this worm, which lives in the intertidal ice zone, and yet is still below the values observed in diluted leech samples.

The calculations to potential THA in different body compartments of the leeches are speculative for the tissue samples with an error of measurement which could not be further

quantified. The body fluid sample represents the closest estimate to the *in vivo* conditions. This can be assumed based on the sample consisting of liquid volume with a minor quantity of tissue fragments. These hypothetical calculations aimed to provide an estimate of the maximum values one could expect to find with accurate measurements of the TH gap.

5.5.3 Relevance of blood supply on TH in Antarctic Piscicolidae

An extensive analysis of the metabolism of the medicinal leech *H. medicinalis* established that the water content in this species ranged between 77.5% and 78.9% of wet body mass under normal or fed conditions and up to 84% in extensively (five months) starved individuals (Pütter, 1907, p. 226). Faced with no further information in this respect, it was assumed that the water content in marine leeches is at a similar level as for their freshwater relative and within a range of 16–22% of dry body mass substance. The digestive processes in *H. medicinalis* lead to a rapid decrease in total body mass after a blood meal (Pütter, 1907). The leech *H. medicinalis* can engorge blood to more than tenfold of its starved body mass but reduces this mass rapidly within the first days and to half the mass by day 24 (Pütter, 1907). It can then exist for more than 1.5 years before the next feed must occur in order to survive (Pütter, 1907, 1908). The situation is different in Antarctic Piscicolidae of the genus *Cryobdella* as these are examples of permanent and obligate fish parasites (Utevsky, 2007) and have a visibly reduced blood storage capacity under natural conditions (Fig. 5.2; pers. obs.). The adult specimens of both species found in Antarctica were located on their fish hosts and able to feed *ad libitum*. These were not engorged manifold like *H. medicinalis* and the proportion of the host blood to leech body mass is much lower than in the well–studied haematophagous leech. Consequently, as was observed, a detached fish leech can be starved within a much shorter period of time than recorded for its European relative (Pütter, 1907, 1908). No further comments can be made on the metabolic activities and rate of nutrient adsorption in the Antarctic fish leeches at present. Nevertheless, a starved *C. antarctica* void of blood, either entirely or to an artificially low extent, still exhibits a TH gap with characteristic ice crystal shape and behaviour even at diluted levels. Furthermore, it can be concluded that AFGPs embedded in leech tissues are capable of inducing a similar TH value at diluted levels. The starved whole specimen had a similar TH gap as did an entire individual in its naturally fed condition, with both measurements being made on diluted samples of unknown but presumably different concentrations. The TH value

of the diluted anterior body part of *C. levigata* is also similar. The relevance of the fish blood as AFGP supply might therefore be diminished at this stage in the leech life cycle. However, this could also indicate a potential native source for AFGPs within the leech species themselves, an aspect that is focused on in the final data chapter.

5.5.4 General comments on methodology

The AFGPs stayed biologically active for hours during the NO experiments at room temperature. These peptides in the leech material have to be regarded as fairly heat tolerant and as such behave as do fish AFGPs, which have a good heat tolerance under experimental conditions (DeVries, et al., 1970, A. L. DeVries & C. W. Evans pers. comm., pers. obs.).

The method used for sample collection and purification of fish AFGPs was established as a standard procedure in the early work by DeVries and colleagues (DeVries, et al., 1970; DeVries, 1971; DeVries, et al., 1971). It follows blood sampling and analytic protocols as performed on other large fish like the living fossil *Latimeria chalumnae* (Smith, 1939) (Pickford & Grant, 1967) and the method is still applied today (Lin, et al., 1972; Shier, et al., 1972; Vandenheede, et al., 1972; Osuga & Feeney, 1978; Fletcher, et al., 1986; Knight, et al., 1993). It involves using a hypodermic syringe to take blood from the caudal vein or the bulbus arteriosus, the heart, of larger animals or for smaller individuals using a scalpel to cut open the caudal artery. The blood sample is left to clot for 4 h at 0°C before centrifugation at 2,000 x g for 10 min after which the serum is collected and stored at –20°C. Because of the obvious size differences to the model organisms, this procedure could not be followed for this work on fish parasites. However, the fish blood in the digestive tract of the leeches is not in its native state but is partly clotted and digested. As the fish leeches are permanently attached to their hosts in supercooled water, the first step of the fish protocol is technically not possible. It is not necessary either as the clotting processes in the blood inside the leech prior to the NO measurements can be assumed to exceed 4 h. This mimics the established protocols as closely as is feasible. The absorbed AFGPs inside the parasite are separated from fish blood cells but are immersed in leech tissues, thus making a centrifugation step necessary. In the applied protocol, the centrifugation step after sampling separated any substances deriving from clotted fish blood as well as any leech cellular material from the aqueous phase. The clarity of this aliquot varied amongst specimens, but it was either clear

or slightly coloured brown to orange. It is not possible to quantify the effect of remaining proteins on the antifreeze activity of AFGPs in the current samples. However, the FP depressing characteristics are evidently not negatively affected or even neutralised by such impurities. It can be noted though that any remaining impurities in the NO samples after centrifugation might at worst slightly diminish the antifreeze capacities. On the other hand, the effects might be neutral or additive.

It must be noted, that the Clifton was a shared instrument in high demand. Despite my best efforts in the field, any further data acquisition with larger sample sizes for piscicolids remains a task reserved for an Antarctic endeavour of the future with greater financial scope.

5.5.5 Removal of external fish contaminants

It was paramount to eliminate any contamination by fish AFGPs on the outside of the parasite samples used for homogenisation. In order to remove any AFGPs from the fish mucus (Evans, et al., 2011), which could interfere with the TH measurements, every leech specimen was carefully washed. Leeches too have a mucus layer, which can be observed to separate as a very fine and thin layer from an animal during ethanol fixation. In this PhD the external layer was cleaned off with Milli–Q water followed by its mechanical removal with paper tissues for all samples except the intestinal content. The mucus was removed in this manner in order to avoid artefactual influences of ethanol that could have led to the dehydration of tissues. A future study could aim to sample the external mucus layers off a higher number of leeches for a mass analysis and a test for presence or absence of AFGPs.

5.5.6 Detecting TH and mass dependent AFGP distribution

The two AFGPs 7 and 8 can be present in high concentrations in blood of Antarctic fishes, for example *P. borchgrevinki* (DeVries, et al., 1970). The MS results show these types of AFGPs to be present in all samples of piscicolid parasites. The effectiveness of the smaller AFGP types 7 and 8 compared to larger types 1–5 has been tested in blood samples of *P. borchgrevinki* (Lin, et al., 1972). It was found that while a Fiske osmometer could detect a THA for the larger peptides type 1–5 this was not the case for the two smallest antifreezes.

However, when the highly sensitive apparatus described by Ramsay and Brown (Ramsay & Brown, 1955) was used this changed, and solutions containing AFGP 7 and 8 demonstrated a FP suppression. The authors concluded that the interpretation of data for biological samples in solutions can be problematic and that the ice crystal growth is an important element in the analysis. Therefore, the observation of the ice crystal behaviour was an essential criterion in the present study for antifreeze activity caused by AFGPs. Although I cannot comment on the concentration levels of the different AFGP types in leech material, this information has no relevance for the detected THA as determined by osmolality measurements of the samples.

A study on the dispersion of the different types of AFGP in *T. bernacchii* found that these are distributed within a short time period of 12 h to the extracellular space of body tissues, this, however, depends on their molecular weight (Ahlgren, et al., 1988). The types 1–5 peptides with high molecular weights are not found in the intracellular space but are only distributed extracellularly. The smallest type 8 is evenly distributed in the extracellular space of the vascular and interstitial spaces (Ahlgren, et al., 1988).

If the transitions into leech cellular compartments are size–dependent, then types 7 and 8 as the smallest AFGPs might represent the highest proportion of all AFPs and at high concentrations, which would agree with previous findings in fish (DeVries, et al., 1970). AFGPs type 7 and 8 adhere to the ice surface nearly irreversibly to inhibit the ice propagation (Knight, et al., 1993). These types 7 and 8 were detected by MS to be present in leech samples. Therefore, even under the assumption that only these smaller type AFGPs of the fish reach the leech tissues, the TH effect observed can be explained. It remains to be demonstrated what part these types 7 and 8 play in the overall FP suppression activity as measured and observed in this chapter. The more effective larger types 1–5 (Osuga & Feeney, 1978) might also be present in the leeches as well as any AFPPs. If the physiological situation is identical to that described for fish (Ahlgren, et al., 1988), then larger peptides would need to contribute towards the final result independently from their intracellular absence. A future investigation could focus on the composition of the antifreeze mixture in piscicolid parasites to differentiate the effects of different type AFPs on the TH.

5.5.7 Differences in AFGP freezing suppression

Types 7 and 8 AFGPs are a group of glycoproteins that depress the FP of a solution, but not as effectively as do the larger types 1–5 (Lin, et al., 1972). A mixture of types 7 and 8 AFGPs cannot interfere with a growing ice crystal to force its bi–directional expansion along the length (c–axis) at a concentration level of 10 mg/ml (Raymond & DeVries, 1977). The authors found for blood samples of *P. borchgrevinki* that types 1–5 reached a saturation point at circa 20 mg/ml, from which a further increase in the AFGP concentration had no beneficial effect in terms of increasing THA. The two smaller AFGPs 7 and 8 had a much lower TH at comparable concentrations but their antifreeze activity continuously increased without reaching saturation before the end of the trial at 32 mg/ml (Raymond & DeVries, 1977).

A detailed analysis of FP suppression of the different type AFGPs from *Dissostichus mawsoni* found that the larger types have a greater TH effect than the smaller ones (Schrag, O'Grady & DeVries, 1982). The authors modified a type 5 peptide by Edman degradation in 14 consecutive turns to reduce its molecular weight M_r from 10,500 u to 7,900 u. The FP depression at identical µM concentrations increased by as much as 50% for the larger molecule. Schrag and colleagues (1982) concluded that THA is a function of the molecular mass for all AFGPs and that the substitutions of proline for alanine or alanine for threonine cause no measurable alterations.

The FP depression in the leech samples was, however, stronger than in serum from fish blood, the tissue with presumably the second highest AFGP concentrations after the exocrine pancreas within the fish. The previous MS results prove the presence of types 7 and 8, thus they can contribute to TH although they might only be active at high concentrations.

A possible explanation for the high THA could lie in the effects of salt ions, despite their potential Hofmeister effects on proteins, and other substances in the leech as well as higher AFGP 7 and 8 concentrations when compared to fish. Although it is likely that the TH effect of AFGPs reach saturation stages just like in fish serum, the effects of other substances to enhance the gap further, like in insects, remains to be further investigated and proven.

The TH in leeches could be the result of a combination of fish AFGPs and additive effects of ions. Because of the parasite's food source and physiology both of these components

could have been enhanced to their saturation limits. Some accumulative processes within the leech could further concentrate the proteins. These metabolic circumstances could lead to quantities of AFGPs inside the parasites that are above concentration levels found in fish for a sufficient bioactivity and antifreeze protection.

Contrary to their hosts as the source of AFGPs, the parasites that assimilate these molecules have no energetic cost associated with the biosynthesis of the proteins. Thus, a leech would be able to elevate their quantities above the biological cost–benefit–equilibrium that must exist in the fishes. This would allow for and explain a higher TH gap in the parasites when compared to their hosts. Therefore, under these special circumstances, types 7 and 8 antifreezes could reach concentration levels not found in the blood serum of any notothenioid host fish in McMurdo Sound where total AFGP concentrations range from 5.01 mg/ml to 35.10 mg/ml (Jin & DeVries, 2006). Thus, AFGP 7 and 8 might contribute significantly more to the THA observed in leeches than what is normal in the fish hosts.

5.5.8 Dehydration as possible cause of high TH

The TH in the leeches' digestive system was of greater magnitude than of their respective fish host species *T. hansoni* at the time of sampling. I speculate that the physiological processes during digestive activities and in particular dehydration are the main causes of this effect. Detailed experiments on blood stored by *H. medicinalis* indicated a rapid water loss of 2% per day (Pütter, 1907, p. 231). Although it remains unknown what the exact extraction rate is in Antarctic piscicolids, it is unlikely that no such water removal occurs. Consequently, this leads to a higher concentration of AFGPs as well as potentially other substances with the capacity of lowering the FP, such as salt ions (DeVries & Wohlschlag, 1969; Kilmartin, Reid & Samson, 2000), other proteins (Rogers, Roos & Goff, 2006), lipids (Lee, Kwon & Ramamoorthy, 2008) as well as sugars and carbohydrates (Smith & Bradley, 1983). Potentially, polyols such as glycerol and sorbitol, as chemicals known from insects (Salt, 1964), could advance antifreeze effects in leeches as well. Although such additional effects could exist, this would not alter the results of the present study as antifreeze capacities discovered in leech tissues are related to AFGPs.

Another unique aspect must be considered for the leech system in connection with active dehydration and enrichment of solutes. As previously demonstrated, the THA for insect AFPs can be increased by substances in high concentration levels not necessarily found under natural conditions (Li, et al., 1998). The inorganic ions, and in particular Na^+ and Cl^- with a virtually unlimited supply in the marine millieu, can serve as enhancing mediators to further suppress the FP. As indicated by the very high MP values in diluted sucker samples, the leech natural metabolism is likely to concentrate such ions to physiologically tolerated limits and as such might well be beneficial to AFGP activity in the parasite.

It could not be established as to what extent potential dehydration could function as a beneficial adaptation to increase salt concentrations in the leech as part of the antifreeze protection. It remains to be established as to whether leeches can use higher salt concentrations for an increased THA and which particular salt ions could be beneficial in this respect.

5.5.9 Error of measurement by ICT

As stated in the introduction, the definition of the MP is the temperature in degrees Celsius where the crystalline and liquid phases are in equilibrium with dry air under the pressure of one atmosphere (West, et al., 1928, p. 6) and is set for water to the fixed point of 0.000°C (Mueller, et al., 1926, p. 52). The ICT states that a measuring accuracy of MP or FP determinations for a one–component system in water has an accuracy of ± 0.05 K (West, et al., 1928, p. 6), which is dependent on the atmospheric pressure at the time of measurement (West, et al., 1928, p. 9). However, the influence of atmospheric pressure has not been considered by any of the authors in antifreeze research. Consequently, the accuracies claimed by some authors of up to 0.01 K are greater than the measuring error obtainable in a solution under standard conditions with a single component. A biological sample is a complex mixture of salt ions, AFGPs, sugars, lipids and other carbohydrates and is a multi–component system, which without doubt is more complex and cannot have a higher accuracy and reproducibility in measurements than the one–component NaCl standard.

The determination of THA can further vary due to AFGP species and salt interactions as well as supercooling and superheating and temperature change rates. As a result, the small

variations in osmolality data obtained and TH gaps observed can be due to non–standardised conditions during measurements as well as to other components such as biological functions that influence the MP and FP determinations of a solution. As a logical consequence, the TH values provided should be interpretated with an error of ± 0.05 K or 27 mOsmol. This should also be extended to historic data involving calibrations using NaCl solutions as standards for freezing measurements, such as fish AFGP experiments.

Important for the present work is to realise that the error rate provided by the ICT reference of ± 0.05 K is of the same magnitude as the standard error (SE) calculated for all but one of the leech samples. The highly diluted sample of body fluids from *C. levigata* showed a mean TH of 0.07 K with a SE of 0.01 K. Although considering the dilution factor, with very small deviations in the actual measurements, this could potentially be a TH of 0.65 K or 352mOsmol. Whether this value is similar to the FP of body fluids in hyposmotic teleosts of between –0.5°C to –0.8°C (Black, 1951) by coincidence or based on a biological function remains to be investigated.

Irrespective of the uncertainty to the exact value or a potentially larger error rate than ± 0.05 K, the conclusions drawn from the present data remain unaffected. If one were to argue, for example, that the highly diluted sample of body fluids allows only a TH gap determination slightly above the error rate and thus does not allow to state whether or not there are AFGPs in this fluid, then how else can one explain the presence of AFGPs in all other tested samples? The only explanation for THA in both leech species without the distribution by the invertebrate's haemocoelomic fluids would be a *de novo* synthesis of the peptides in every tissue. Consequently, such circumstances would not invalidate and not even diminish but instead substantially support the hypothesis of a biological relevance of AFGPs for these marine invertebrates in Antarctica.

5.5.10 Supercooling and superheating of sample volume

I observed slight variations in the temperatures of MP and FP for one particular ice crystal in individual freeze–reheat experiments of the same sample volume using the identical ice seed repeatedly. A crystal did not always disappear or propagate at the exact same mOsmol mark as it did in previous runs. Such differences between measurements have also been

previously described (Sayre, 1964; DeVries, 1971) and represent a temperature range in which the MPs or FPs are recorded. In Antarctic fishes the lowest to the highest FP values can be 0.29–0.68 K apart (DeVries, 1971). Although most, but not all, diluted leech samples fall within that range for the FP, this finding might indicate that AFGPs in leech solutions behave as it has been described for fish blood samples. However, the accuracy of the experiments with diluted volumes did not allow a further quantification beyond the observation of MP and FP differences and a comparison with pure fish serum.

It has been recorded that high concentrations of AFGPs can stabilise the ice crystal structure and thus lead to a higher temperature in the solution before the crystal melts and also to a lower FP before an explosive growth occurs (Celik, et al., 2010). This would then provide altered MP and FP values, independently of measuring accuracy. These effects, together with the previously discussed supercooling effects of a sample (DeVries, 1982), were reduced aiming for highly reproducible measurements by the use of a small sample volume in form of one very small ice crystal for experimentation centrally located inside the oil of the well and small temperature changes during TH determinations. In addition, a set of faster temperature changes was used to verify the MP and FP values for each sample run. The variations within the mOsmol values for the TH gap in the samples are indicative of supercooling and superheating effects in some of the experiments.

5.5.11 Interchanging ice formations

Previous work demonstrated that the form of the ice crystals can be slightly altered from the bi–directional shape when a mixture of three different types of AFPs are isolated from different fishes (Chao, et al., 1995). The authors stated that the THA of the aqueous solution remains unaffected by different mixtures of foreign AFPs. They concluded that these molecules act independently and the different types do not need to interact with each other in order to bind to the ice plane. Yet no two interchanging hybrid shapes for ice crystals were described from one sample.

A similar observation of hybrid shapes deviating from the forms observed otherwise in leech and fish samples was made in the homogenised anterior body sample of *C. levigata*. The bi–directional growth of one ice crystal interchanged occasionally with alternative forms

apparently without having an effect on the THA. Consequently, the finding of two different crystallisation events in the same liquid and with the identical crystal used as the nucleation point at the start of each measurement has to be noted as a novum not previously described. No explanation can be provided for this observation but interactions of AFPs with ions and further invertebrate proteins, including other glycoproteins, could potentially have concentration–dependent effects on ice crystals' behaviour in piscicolid tissues as well as body fluids.

5.5.12 Limitation of study because pure endolymph could not be extracted

A study into the distribution of AFGPs within the fish leech host *T. bernacchii* using a radioactive substance as proxy commented that the endolymph was a fluid compartment with a volume too small for sampling (Ahlgren, et al., 1988). This experimental limitation is certainly true for leeches as they are far smaller animals. The leeches of the Antarctic waters belong to the order Rhynchobdellida (Sawyer, 1986b, pp. 646-706; Utevsky, 2005, 2007) which have a reduced coelom (Sawyer, 1986a, pp. 69-71; Utevsky & Trontelj, 2004). As the Antarctic representatives are all part of the family Piscicolidae, their coelom is specialised to an evolutionary end point found for the entire group of Annelida. These animals have lymphatic hearts pushing the coelomic fluid through the lateral, ventral and dorsal lacunae as part of the haemocoelom at a temperature dependent pulsation rate of vesicles between 30 to 64 times per min (Sawyer, 1986b, pp. 597-602). The characteristics of the reduced coelom are used for the taxonomic classification in the subfamily Platybdellinae of the Piscicolidae and identification of *Cryobdella* (together with marine *Glyptonotobdella*, *Notostomum*, *Platybdella* against other genera of Platybdellinae) with the lack of a continuous lateral lacuna and marginal lacuna or vesicles (Sawyer, 1986b, p. 661).

The lacunae are not as readily visible in the living animals as they might be on a stained tissue slide. The issue of not being able to identify an entry point for their accurate sampling could not be overcome. The elasticity of the skin was of no additional help. Thus, the sampling of individual fluid cavities of these leeches was a technical challenge that could not be overcome.

5.6 Outlook

5.6.1 Polar comparison – THA under Arctic conditions without ice crystals?

The lower environmental temperatures in McMurdo Sound have led to the evolution of AFGPs that can lower the blood FP and fish freezing temperature further than AFPs evolved in fish of warmer water temperatures in the subarctic where fish and their blood serum FPs are higher (DeVries, 1980). This reported difference in freezing adaptation between the polar fishes must reflect its biological relevance. Therefore, the environmental conditions could have had the same effect on their respective ectoparasitic leech fauna.

Future investigations into the northern species of the Piscicolidae family might find a smaller TH gap in the Arctic species as compared to the Antarctic species. Such work could also establish whether Arctic marine invertebrates evolved as blood–sucking parasites in the absence of ice in water but at sub–zero temperatures in a supercooled state, as has been reported for their potential fish hosts (Scholander, et al., 1957). If these leeches were shown not to utilise the northern fish AFPs and yet survive supercooled this would be an evolutionary stable strategy. As a result, one could further hypothesise that these animals, isosmotic or not, might survive supercooled and require no AFPs or even the genes for their biosynthesis in the Arctic.

5.6.2 Osmotic conditions and regulations

Are Antarctic fish leeches able to reduce their risk of freezing by ways of active osmotic regulations of their body fluids? How fast do adult leeches change their osmotic pressure after the blood meal? These and related questions would require a substantial number of individuals for statistically sound statements to the potential osmoconformity of these marine invertebrates to the seawater as proposed by the generally produced isosmotic argument in the literature.

The simplest test would require a series of different salinities with a stepwise increase or decrease of seawater concentrations followed by the osmolality determination in mOsmol of the body fluids in animals by NO in analogy to previous work which required 108 specimens

per species (Shumway, 1977) or 69 to 72 individuals per species (Santos, Castellano & Freire, 2013). An interesting comparison to the Arctic fish leeches could provide further information to the osmotic reality of these blood parasites in the polar oceans.

5.6.3 Ecological questions and epigenetics on bacterial endosymbionts

How fast do Antarctic leeches digest the blood meal? Do they suck constantly, on demand or store blood intermittently? Do they use only one host, as may be assumed as they were observed not to reattach after manual and artificial separation from the host? Do they have enough energy for reproduction after their first blood intake upon attaching to a host?

It has been speculated (Dinand & Bottenberg, 1935) and known for more than 70 years that the digestion in species belonging to the true leeches or Euhirudinea is with the aid of a symbiotic bacterial flora (Hornbostel, 1942; Büsing, 1951; Büsing, et al., 1953).

An epigenetic study using the modern tools of whole genome analysis (WGA) could investigate the digestive symbiotic community inside Antarctic marine leeches. This might also shed light on other aspects of this group, such as developmental time, which is currently described as being between 30 and 300 days (Utevsky, 2007) and of more than 40 days for both *C. antarctica* and *C. levigata* (pers. obs.).

A further intriguing aspect would be to learn whether a different indigenous bacterial flora has evolved in the Antarctic environment. Any discovery could be compared to known bacteria *Aeromonas hydrophila* (Stanier, 1943) and *Aeromonas veronii biovar sobria* (Hickman–Brenner et al, 1987) (Indergand & Graf, 2000) from freshwater and other bacteria newly discovered living in marine leeches (Jennings & van der Lande, 1967; Goffredi, Morella & Pulcrano, 2012). Previous bacteriological studies on deep–sea and shallow–water piscicolids found the gammaproteobacteria genus *Psychromonas* (Mountfort et al, 1998) to dominate the flora, and was different in five leech species as well as from symbionts found in association with freshwater leeches (Goffredi, et al., 2012).

The discovery of new bacterial species in Antarctic leeches is likely considering that bacterial flora in other piscicolids show striking dissimilarities (Goffredi, et al., 2012) and discoveries of new endosymbionts in blood–sucking leeches continue with species of the

genus *Rikenella* (Collins et al, 1985) (Worthen, Gode & Graf, 2006) and *Psychromonas* spp. (Goffredi, et al., 2012).

5.6.4 AFGP effectiveness and model to estimate theoretical TH

The development of models to calculate the FP suppression of a solution (e.g. Ge & Wang, 2009) could provide data from thermodynamic theory against which the experimental data could be compared. This would allow us to assess the accuracy of TH data in leeches in general for a comparison with the levels of their fish hosts. It would also enable us to determine the relevance of dilution factors for AFGPs in their biological context.

5.7 Conclusions

I established by the use of NO and ice crystal behaviour that the effect of TH exists in tissue samples of the two Antarctic parasitic leeches *C. antarctica* and *C. levigata*. Thus, the functional application of fish AFGPs by the leech parasites is possible *in vivo*. Both species contained antifreeze compounds suppressing the FPs, with *C. levigata* to a lower temperature than observed in its respective fish host species at the time of sampling. The ice crystal growth observed during the TH experiments is analogous to the bi–pyramidal formation described as being characteristic of AFGPs in fish blood samples. Consequently, the parasitic leeches obtained not only types 7 and 8 amongst possibly other AFGPs in their primary structure as demonstrated by MS, but furthermore, these remain in their physically active forms as ice–binding proteins.

It has been demonstrated that a parasitic group of leeches exploits the physiological adaptation evolved in a phylogenetically distant vertebrate clade, the notothenioid fishes. The ability of these marine invertebrates to obtain and utilise an array of life–saving compounds by feeding on fish blood has importance in the ice–laden waters of Antarctica.

CHAPTER SIX

Genetic basis of AFGPs in Antarctic piscicolids?

6.1 Abstract

Antifreeze glycoproteins (AFGPs) were found to have biologically relevant and active freezing point depressing properties in *Cryobdella antarctica* and *Cryobdella levigata*. The genetic origin of the peptides was traced by mRNA analysis. A potential horizontal gene transfer (HGT) of the characteristic AFGP gene motif from host to parasite was investigated. The underlying nine amino acid sequence described from fish as the repetitive AAT AAT AAT was used to search for genes established in either leech genome by HGT events. The results of mRNA work are suggestive for an HGT event in *C. levigata* but not in *C. antarctica* and might indicate either an evolutionary process in transition, or possibly a difference in life cycle strategies with different requirements for AFGP biosynthesis.

6.2 Introduction

6.2.1 From Darwin to Mayr in evolutionary research

Charles Robert Darwin (1809 – 1882) formulated one of the most influential ideas in life sciences in his theory of evolution by means of natural selection (1859). He explained the diversity of nature as being caused by selection leading to the formation of new species or by speciation from one common ancestor. A declared joint discoverer to Darwin was the naturalist Alfred Russel Wallace (1823 – 1913), who promoted the same view of the gradual change of species (1869a, 1869b). However, the underlying mechanisms required to understand how evolution proceeds were debated until other key figures such as Ronald Aylmer Fisher (1890 – 1962) and Theodosius Grygorovych Dobzhansky (1900 – 1975)

[original name Feodosy Grigorevich Dobrzhansky] published their ideas on the principles of population genetics in 1930 and 1937 respectively. They incorporated the work of Gregor Johann Mendel (1822 – 1884) and his *"Vererbungslehre"* or the Mendelian laws of inheritance (1866), which although published in 1866 were unknown to Darwin. This work on the genetic aspects of evolution led Julian Sorell Huxley (1887 – 1975) to develop what he termed the *modern evolutionary synthesis* (1942), which today is referred to as *modern synthesis*. This idea was promoted by Ernst Walter Mayr (1904 – 2005) who described a species as a breeding entity (1942). In his view, a species represents a group not only with similar morphology but of a genetic type different from and excluding reproduction with other groups. For species with two sexes he explained the speciation of organisms as a process where a sub–population evolves in isolation from the remainder of the population into a new species. Mayr took Dobzhansky's idea of isolation as a requirement for species development further in that the means of separation of groups could be choice of mating partners or food selection as well as particular geographic locations. Mayr referred to geographic isolation as an essential aspect of the evolutionary synthesis and speciation in his second classic work *"Animal species and evolution"* (1963, p. 477): *"In not a single case is the sympatric model superior to an explanation [...] through geographic speciation."*

Sympatric speciation is defined as the evolutionary process in which new species within the same geographic location arise from one common ancestor. The evolutionary rate of genetic changes increases in small populations in extreme isolation, such as on islands.

6.2.2 Evolution of Antarctic notothenioids and AFGPs

The formation of the ACC in combination with extremely cold seawater temperatures resulted in a geographic isolation as defined by Mayr for species in the Southern Ocean from the rest of the world. This isolation can explain the adaptive radiation of one ancestral benthic form into the many extant Antarctic notothenioids, with the dominant genus *Trematomus* in the Ross Sea. These fish had a preadaptation to tolerate the new cold–water environment by means of AFGPs dating back 42–22 mya (Near, et al., 2012). The authors concluded that the diversification into the numerous notothenioid fish species occurred mainly in the late Miocene at 11.6–5.3 mya.

Notothenioid evolution in the Southern Ocean led to a family of AFGPs with a repetitive tripeptide of AAT with one disaccharide attached per repeat (DeVries & Cheng, 2005) at an estimated time of 5–14 mya (Chen, et al., 1997b). The sudden evolution of five notothenioid families, estimated at around 5–15 mya (Bargelloni, et al., 1994), coincides with the appearance of AFGPs and thus strongly indicates an ecological relevance of this biochemical adaptation.

The AFGPs present today are thought to have evolved from a short AAT AAT AAT coding element, which together with a trypsinogen–serine protease ancestor gene evolved into the tandem repeats of the AFGP gene family (Chen, et al., 1997b). Therefore, notothenioids have the identical repetitive motif AAT AAT AAT as is common in the amino acid sequence of all their AFGPs. There can be four repeats in the shortest AFGP 8 of 2,600 u and up to 56 repeats in the largest type 1 of 33,700 u (Cheng, 1996). These AFGPs are therefore unique in their basic unit, and also represent a large group of genes that share one common and identical sequence motif. As a result the underlying genetic code for the nine–letter word can be found in numerous locations in the fish genomes.

6.2.3 Evolution and HGT in the Antarctic

The Antarctic sea ice has been the focus of recent investigations into the mechanisms of organisms facing death by ice formation within their cells and tissues. An ice–binding protein (IBP) has been discovered in the diatom *Navicula glaciei* sampled in the sea ice at Cape Evans, McMurdo Sound (Janech, et al., 2006). This comprehensive study found that this protein of circa 25 ku interacted with ice and that the underlying genetic sequences were similar to those discovered in the snow mould *Typhula ishikariensis* (Imai, 1930) (Hoshino, Kiriaki & Nakajima, 2003). The sea ice diatom *Fragilariopsis cylindrus* ((Grunow) Krieger, 1954) has a genetic sequence encoding an IBP very closely resembling that of *N. glaciei*. However, such proteins are absent in the mesophilic Bacillariophyceae diatoms *Thalassiosira pseudonana* (Hasle et Heimdal, 1970) (Armbrust et al. 2004) and *Phaeodactylum tricornutum* (Bohlin, 1897) (Scala et al. 2002), leading Janech and colleagues (2006) to conclude that diatoms with IBPs are limited to the polar regions of sea ice, as had been postulated previously (Raymond et al. 1994).

Under cold conditions, unicellular plants produce IBPs that have a repeat motif previously found in antifreeze proteins (AFPs) of insects and higher plants (Raymond, et al., 2009). An HGT mechanism is suggested for Antarctic diatoms whereby bacteria living in association with algae in the sea ice transfer genes from other diatom species that contain IBP genes (Janech, et al., 2006).

In the deep glacial ice core just above Lake Vostok an ancient bacterium has been found that encodes a large IBP of 54 ku, which is similar to those in other sea–ice associated diatoms, bacteria and snow mould (Raymond, et al., 2008). Rainer Kiko (2010) discovered a copepod of the Antarctic sea ice that exhibited thermal hysteresis (TH) and speculated, due to the high similarity of its AFPs to the group known from snow mould, bacteria and diatoms, that the copepod protein could well have established in its genome as the result of HGT. Finally, the research group of Peter L. Davies found evidence of gene transfer between vertebrate species in the form of AFPs in fishes (Graham, et al., 2008; Graham, et al., 2012).

There are numerous examples of HGT events in the marine environment and in particular in the Antarctic involving AFPs. The necessity of freezing protection in an ice–laden environment seems vital. In the light of HGT transfer of AFP genetic material being observed in fishes, it seems likely that there would be similar events in the Antarctic. Therefore, this final chapter investigated the possibility of HGT between fish hosts and their leech parasites.

6.2.4 Aim of chapter

The final part of this PhD was to investigate the possibility of an HGT event for a gene encoding AFGPs in the piscicolid parasites of Southern Ocean notothenioid fishes. This chapter aimed to demonstrate the ability of these fish leeches to synthesise *de novo* the life–saving molecules that evolved in their fish hosts.

Molecular techniques targeting messenger ribonucleic acid (mRNA) as the genetic transcript and template for AFGP production in cells were used to indicate an active transcription in the adult leeches *in vivo* at the time of their collection in Antarctica. A comparison of their characteristic mRNA results with the genomic target sequence from the host fish *Trematomus bernacchii* was used to examine whether the fish leeches have the underlying genetic code for AFGPs.

6.3 Material & Methods

6.3.1 Collection of material

The fish hosts on which the parasites live attached were caught by ice fishing in McMurdo Sound. Individual specimens of *C. antarctica* and *C. levigata* were then manually collected off their fish hosts and starved in isolation micro–aquaria for up to four weeks until visibly void of fish blood so as to avoid contamination by host blood cells. This is analogous to the technique used in previous molecular work (Siddall & Burreson, 1998). Leech specimens were cleaned with Milli–Q water and dried to remove the external mucus (Bielecki, et al., 2008) before being transferred into 1.5 ml thermostable cryovials and flash frozen by immersion into liquid nitrogen for fixation and transport. Specimens were then stored at –80°C until processed for mRNA extraction.

The homogenised tissue material used to obtain the cDNA libraries from the total RNA extraction was restricted due to freezer failure to one set of pooled samples (n=1) for each species consisting of material from three entire specimens for *C. antarctica* and four posterior suckers cut off four individual *C. levigata*. A sample of rat liver cells served as quality control for mRNA extraction and at the same time as the negative control in the steps of reverse transcription (RT) and polymerase chain reaction (PCR).

The fish DNA was extracted from a small sand corn sized piece (circa 1/8 mm^3) of muscle tissue of *T. bernacchii* (cell digestion by proteinase K at 56°C overnight) and used directly for the final PCR step to provide a positive control to illustrate how the primers in use are working on the original gene templates and what typical molecular fragments were to be expected.

6.3.2 Total RNA extraction and purification

The extraction of total RNA from the tissue samples was performed using TRIzol (formerly Invitrogen, now Life Technologies, Carlsbad, California, USA) in combination with RNeasy Mini Kit (Qiagen, Hilden, Germany). RNase–free water was used containing diethyl pyrocarbonate (DEPC) as nonspecific RNase inhibitor. The centrifugation steps required

≥ 8,000 g or ≥ 10,000 rpm and were generally set to 12,000 rpm. The working spaces were sprayed with RNaseZAP (Ambion, Austin, Texas, USA), which is a surface decontaminant that destroys RNases on contact.

The samples were transferred into 1.5 ml Eppendorf tubes and 750 µl of cold (4°C) TRIzol was added. Samples were homogenised using a handheld rotor–stator homogeniser (TissueRuptor, Qiagen) and left to incubate at room temperature for 5 min before 150 µl chloroform [$CHCl_3$] was added. After 15 s vortex samples were incubated a further 3 min at RT. A spin for 5 min at 13,200 rpm followed and the aqueous phase was then transferred into a fresh tube that had been previously weighed in order to calculate for the addition of an equal volume of 70% ethanol made fresh from a 96% alcohol stock solution with DEPC water. The volume was loaded onto a Qiagen RNeasy Mini column and centrifuged for 15 s at RT. After adding 700 µl of buffer *RW1* into the column it was again spun for 15 s at RT and the collection tube discarded. 10 µl of RNase–free DNase stock was added to 70 µl buffer *RDD* and loaded into the column to be incubated at RT for 15 min. A further 700 µl buffer *RW1* was added and spun for 15 s, followed by 500 µl buffer *RPE* and 15 s centrifugation, and finally 500 µl buffer *RPE* and a 3 min spin. The elution was achieved by adding 15 µl DEPC water and centrifuging for 1 min.

The purity and quantity of total RNA material extracted was measured by spectrophotometry and absorbance of a 1 µl sample volume at 230, 260 and 280 nm to calculate the ratios of 260/280 nm and 260/230 nm using a NanoDrop ND–1000 (NanoDrop, Wilmington, Delaware, USA).

The quality or integrity of RNA samples was analysed by electrophoresis on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). A loading sample of 1 µl was heated for 2 min at 70°C followed by 1 min vortex at 2,400 rpm before transfer onto the chip for analysis.

6.3.3 Reverse transcription (RT) for complementary DNA (cDNA) library

The reverse transcription (RT) of mRNA into a complementary DNA (cDNA) library by use of a reverse transcriptase was performed with an iScript cDNA synthesis kit (Bio–Rad Laboratories, Hercules, California, USA) according to the manufacturer's specifications.

A mixture of oligo(dT) and random hexamer primers was used to obtain products of less than 1,000 nucleotide (nt) single stranded DNA (ssDNA) as a complete set of copies of all types of RNA present in the cells at time of collection.

A volume of 20 µl per cDNA reaction was used, containing 4 µl iScript reaction mix and 1 µl iScript reverse transcriptase, with the remaining 15 µl consisting of nuclease–free water and RNA template in calculated proportions so as to have approximately 750 ng of RNA material in each reaction. After careful mixing, the reactions were incubated for 5 min at 25°C, 90 min at 42°C, heated to 85°C for 5 min to inactivate the reverse transcriptase, and stored at –20°C until further processed.

6.3.4 Polymerase chain reaction (PCR) targeting AFGP motif in cDNA library

Following standard procedures and the manufacturer's (Bio–Rad Laboratories) recommendations the cDNA reactions were diluted 1:10 prior to use in the PCR so as to obtain a 10 µM final concentration. An iTaq DNA polymerase kit (Bio–Rad Laboratories) was used for PCR, set to international laboratory standards but with 10 µM final primer concentration, in a 25 µl PCR reaction volume consisting of 15.25 µl PCR grade nuclease–free water, 2.5 µl 10x iTaq buffer, 4 µl magnesium chloride solution ($MgCl_2$), 0.5 µl dNTP mix, 0.15 µl iTaq DNA polymerase, 0.8 µl of forward primer *afpFrpt* 5'–GCTGCAACAGCTGCAACAGCTGCAA–3' and 0.8 µl of reverse primer *afpRrpt* 5'–CTGTTGCAGCAAAAATCAATGCAGGT–3' plus 1 µl DNA sample. The PCR programme was run on a GeneAmp 2400 (Applied Biosystems, Foster City, California, USA) with a 10 min activation at 95°C to hot–start the DNA polymerase and then a total of 40 cycles in a touchdown style PCR as follows. The initial 10 cycles were run for 30 s at 95°C followed by 30 s annealing and then 30 s at 72°C for elongation, while the annealing temperature was decreased by 1 K per cycle, i.e. from 62°C to 52°C. The remaining 30 cycles were each for 30 s at 95°C, 52°C and 72°C. The final elongation of PCR fragments was allowed to continue for 10 min at 72°C.

6.3.5 Agarose gel electrophoresis of PCR fragments

A 2% agarose gel in 1x TBE (Tris–borate–EDTA) buffer (a 1:10 dilution with Milli–Q water from 10x TBE stock solution of 1 M TRIS base [tris(hydroxymethyl)aminomethane or 2–amino–2–hydroxymethyl–propane–1,3–diol], 0.9 M boric acid [or trihydroxidoboron] and 0.01 M EDTA [ethylenediaminetetraacetic acid or 2,2',2'',2'''–(ethane–1,2–diyldinitrilo)tetraacetic acid] in Milli–Q water) was produced by pouring the heated gel directly into the flat running bed and letting it settle before it was placed in the running chamber and covered by 3–5 mm of 1x TBE buffer. 2 µl of sample with 1 µl bromophenol blue dye was loaded into the wells of the gel and 90 V applied until the dye marker had migrated three–quarters of the way to the opposite end of the gel. At this time the run was terminated and the gel stained with ethidium bromide. A digital image was then taken under UV light.

6.4 Results

The homogenised leech samples showed in the NanoDrop ratios of 260/280 nm at ≈ 2.0 which are accepted as "pure" RNA and also had 260/230 nm ratios above the previous levels and in the required range of 1.8–2.2. This demonstrated a successful yield of total RNA from all samples and served as a methodological control of material preservation as well as protocol. The Bioanalyzer further demonstrated a high quality of RNA as sharp peaks above the noise threshold with a low baseline in the electropherogram for samples of *C. antarctica* and *C. levigata* (Figs. 6.1 & 6.2) as well as the extract from rat liver cells (Fig. 6.3).

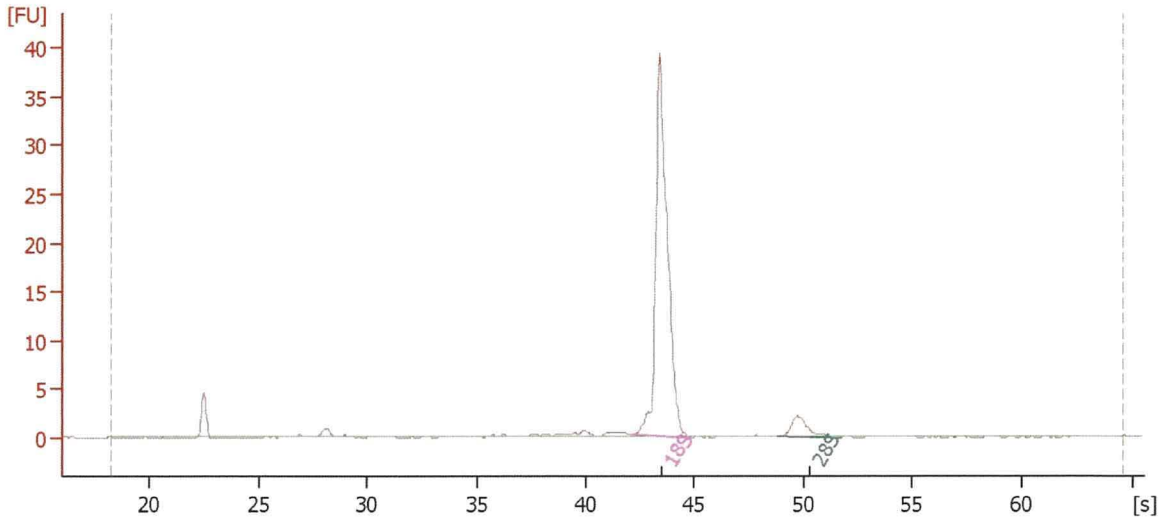


Figure 6.1 Electropherogram from specimens homogenate of *C. antarctica*.

Graphical output from Bioanalyzer run of total RNA from pooled whole animal homogenates of three *C. antarctica* specimens for quality and quantity assessments with peaks of 18S and 28S RNA as well as a 5.8S peak (unlabelled; detected at around 23 s).

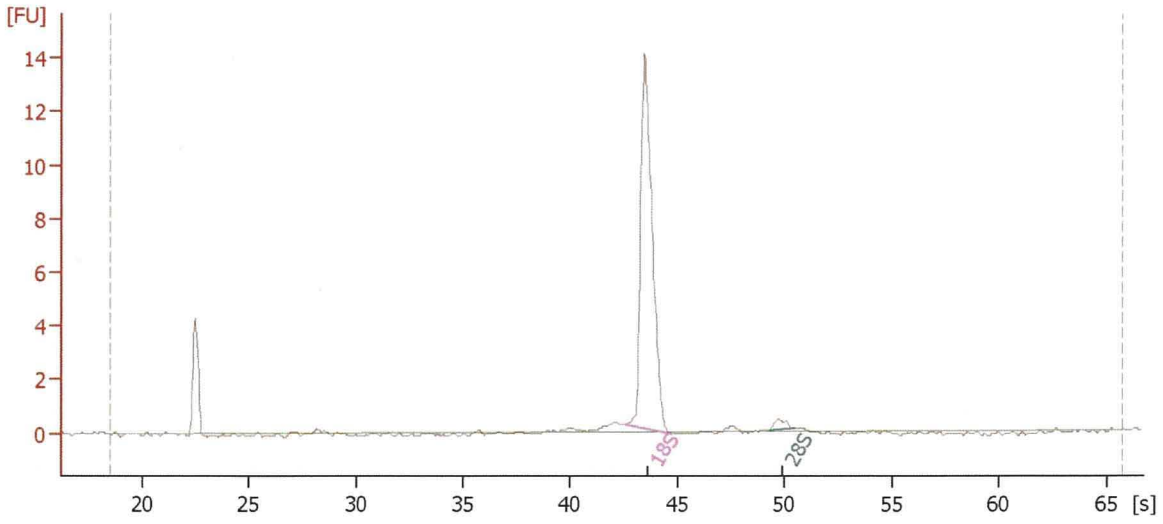


Figure 6.2 Electropherogram from posterior sucker material of *C. levigata*.

Graphical output from Bioanalyzer run of total RNA from pooled sucker material cut off four *C. levigata* for quality and quantity assessments with peaks of 18S and 28S RNA as well as a 5.8S peak (unlabelled; detected at around 23 s).

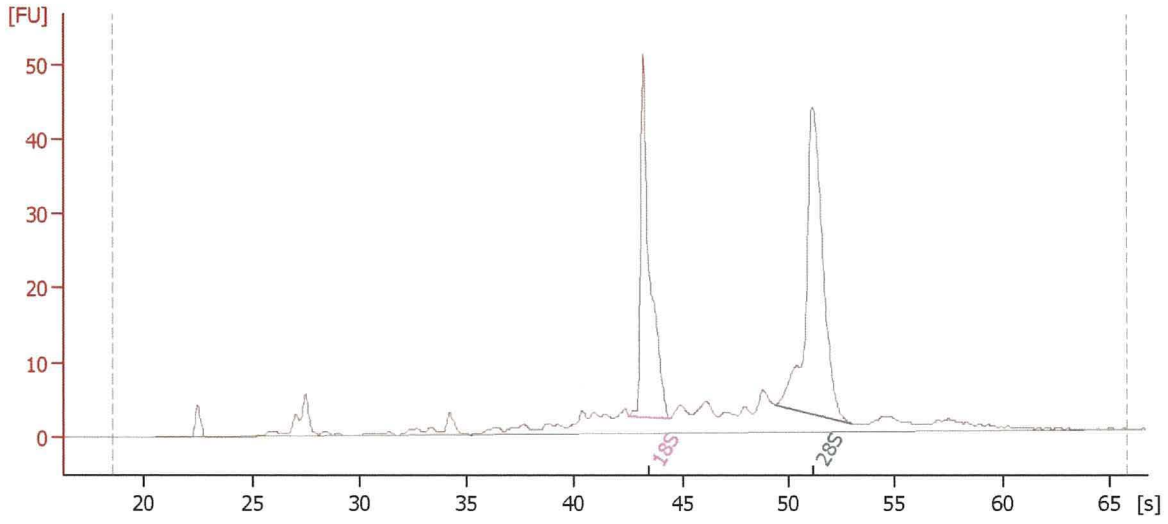


Figure 6.3 Electropherogram from rat liver cells' extract.

Graphical output from Bioanalyzer run of total RNA from rat liver cells for quality and quantity assessments with 5.8S (detected at around 23 s as first of three unlabelled signals), 18S and 28S RNA peaks.

If the time for heating and denaturing prior to the Bioanalyzer run is not long enough, then in insect samples there are still parts of the functional linkage in the 28S rRNA visible (Winnebeck, Millar & Warman, 2010). This was found to be the case for the leech samples as at the corresponding times to the 28S peak in the mammalian rat sample (Fig. 6.3) there are small residual 28S signals detectable in the electropherogram plots for both leech samples (Figs. 6.1 & 6.2).

All peaks are according to expectation, with the peak at 18S being dominant. The peak at 28S is still visible, but this is most likely due to the 70°C denaturation time being too short, as most likely its subunits would co–migrate as similarly sized fragments within the 18S rRNA peak. The 5.8S released from the hydrogen bonding to the 28S unit is visible. The very low baseline noises and lack of clear spikes or bulges indicate a high quality of the total RNA for all three extractions, despite an overall lower yield for the two leeches compared to the rat extract. The 18S peaks clearly dominate in the heated samples as the 28S peaks overlap into this elution time, thereby adding in mass and thus area size of the graphs.

The annealing of primers results in non–uniform fragment lengths for forward and reverse transcriptions of cDNA encoding AFGPs for this particular PCR application, as visualised on a 2% agarose gel (Fig. 6.4). There are two dilutions for each animal species, for *T. bernacchii* (TB) the left lane is 1:10 and the right lane is 1:100 diluted, while for *C. levigata* (CL) and *C. antarctica* (CA) the left lanes are each undiluted (1:1) and the right lanes are 1:10 diluted samples.

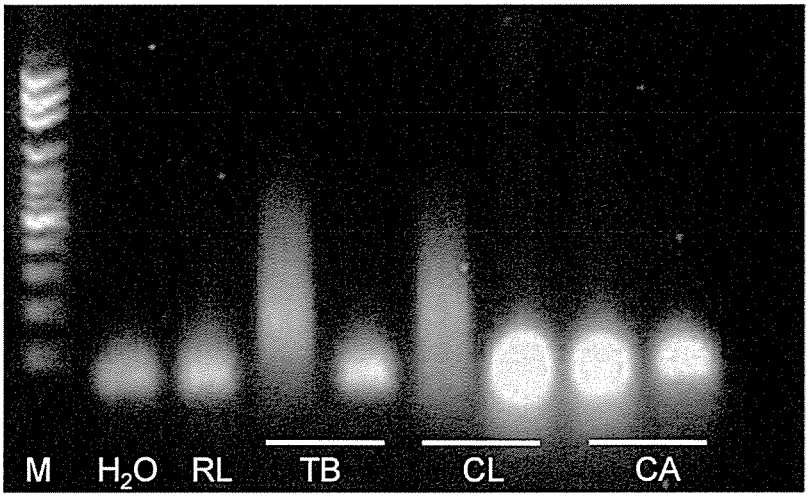


Figure 6.4 PCR fragments obtained from cDNA library targeting gene motif of AFGPs.

Electrophoresis gel of PCR fragments obtained from cDNA library targeting repetitive gene motif AAT AAT AAT common in fish AFGPs. Molecular–weight marker (M) as DNA ladder with defined fragment sizes (ascending 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,500, 2,000 base pairs); Milli–Q water (H_2O) and rat liver cell extract (RL) as negative controls; muscle cell extract of fish host *Trematomus bernacchii* (TB) as positive control; pooled homogenate of four posterior suckers off four *Cryobdella levigata* (CL), and pooled homogenate of three whole specimens of *Cryobdella antarctica* (CA). Dilution of samples for PCR: TB left lane 1:10, right lane 1:100; CL & CA left lane 1:1 undiluted, right lane 1:10).

The primers bound to DNA templates with the targeted part of the AFGP gene sequence in the genome of the piscicolid *C. levigata* (CL) as well as the fish host *T. bernacchii* (TB) but not in *C. antarctica* (CA). The reaction did not produce fragments visible on agarose gels at diluted levels for either fish or leech samples.

The results demonstrate a clear positive signal in *C. levigata* as equal presentation of a PCR mixture with fragments of various lengths as they originate from the genomic sequences in *T. bernacchii*. The results are conclusive and in the expected pattern technically achievable by mRNA work on a repetitive gene motif.

The negative controls, as Milli–Q water and total RNA extract from rat liver cells, produce visible artefacts as a result of the PCR. There are strong signals as the resulting sequence product of two primers binding to each other to form a dimer. This leads to a pool of fragments with slightly different lengths at approximately 30–50 bp. In fish and *C. levigata* the fragments have a minimal length of more than the artefactual fragments of primer dimerisation. The peak intensity caused by the higher number of fragments of this length lies at around 200 bp, but fragments have a lower limit of circa 50 bp and an upper limit of circa 800 bp. This characteristic fragment mixture is not visible in *C. antarctica* although a stronger primer band is visualised in the undiluted sample.

6.5 Discussion

In this chapter I used mRNA technology to obtain results for the expression of a gene motif in *C. levigata* that is otherwise found as a characteristic pattern for AFGPs in the genome of the notothenioid fish host. The mRNA levels detected in leech material were of quantities to produce positive results. This allows to speculate that a gene that is similar to those that have evolved in the fish host could be transcribed inside the parasite. At the time of sampling, which is in the Antarctic spring, such an active transcription of at least one underlying gene demonstrates its biological relevance for the piscicolid *C. levigata* living in the sub–zero waters.

The posterior sucker material of *C. levigata* was washed so as to remove the leech external mucus that had been in contact with the fish exterior. The use of a commercial silica matrix in the form of Qiagen's RNeasy kit provided high quality RNA with minimal DNA co–purification. Additionally, RNase–free DNase was applied to the columns as DNase treatment of the samples in order to avoid any contamination with fish DNA. The homogenate of three entire specimens of *C. antarctica* yielded no fragments that were similar to those found in

T. bernacchii and *C. levigata*. If the fragments in *C. levigata* were an artefact of fish–derived mRNA products even inside pure leech tissue, then an identical result would be present in starved *C. antarctica* which, unlike pure sucker muscle, could still contain some remains of fish blood. This was not found to be the case. Consequently, the PCR results and the signal in *C. levigata* cannot be interpreted as fish contamination or artefacts in the piscicolids.

C. levigata might encounter ice frequently as a result of the artificially increased water circulation through the gill cavity where this leech can be found (pers. obs.) The evolutionary pressure on this species might therefore have led to a successful integration of an AFGP gene after it entered the leech genome by HGT. The functional and phenotypical advantage of an ice–binding peptide as a new trait could then lead to the distribution of the gene within this species. If a juvenile leech attached to a host and fed on the fish blood without having a source of AFGP this might result in a mortality rate too high to be tolerated for the survival of the species.

The specimen homogenate of *C. antarctica* demonstrated no cDNA copy of the relevant target sequence within its genome. A comparison to *C. levigata* as its closest relative within the same genus leads to the conclusion that there are no other gene motifs of similar sequence that could cause similar PCR results but that originate from genes not encoding AFGPs. This is further supported by research on the glossiphoniid leech *Helobdella robusta* (Shankland, Bissen et Weisblat, 1992), which has been sequenced by the Joint Genome Institute (JGI), Walnut Creek, California, USA [http://www.jgi.doe.gov]. The whole genome data is freely available online [http://genome.jgi.doe.gov/Helro1/Helro1.home.html]. Although this leech has a total genome size of around 300 Mb and represents a small genome, it has been classified as a completed project and allows the comparison of the Antarctic results at least at a level of higher phylogenetic relationships. A FASTA blast yields no matches for a sequence of AAT AAT AAT or AAT AAT even in the data sets of "unplaced reads".

It could be argued that the apparently negative result for *C. antarctica* was due to circumstances other than the absence of the specific gene sequence, for instance the lack of sufficient material. However, the results of NanoDrop and Bioanalyzer measurements demonstrate not only the successful extraction of RNA molecules but also their purity and high quality in samples of both leech species. The limitation of minute mRNA quantities

presumably in the femto– or picogram range could, however, not be overcome in this study. First of all, material was not readily available for large–scale transcriptome work where an expressed sequence tag collection could have been used to obtain a full AAT protein sequence, including information on its relative abundance. Such work normally requires grams of tissue material for an RNA extraction from the model organism (Peter L. Davies, pers. comm.) in order to have sufficient RNA in milligram range. Secondly, the deep freezer holding the leech samples in the transitional facility failed shortly after the one successful mRNA extraction was performed. This restricted the piscicolid material to the single set of data presented in this thesis. In a future investigation a larger number of specimens ideally not pooled and treated separately could shed more light on the interspecific and potentially intraspecific differences of the AFGP underlying RNA production in Piscicolidae.

However, the individuals of *C. antarctica* available for this study might have sufficient levels of fish–derived AFGPs at the time of sampling due to an accumulative absorption of these peptides through their blood sucking life style. Whether this is due to seasonal changes, as has been demonstrated in fish (Petzel, et al., 1980; Liebscher, et al., 2006) as well as in insects (Ma, et al., 2012), in addition to biochemical reception of internal AFGP concentration levels in the adult leech, is mere speculation at this stage.

From a purely energetic perspective, *C. antarctica* might be preserving the energy involved in the biosynthesis of peptides that are currently not required. A halt in the translational processes would only occur once the genetic transcription had been deactivated and the gene silenced, resulting in the absence of mRNAs coding for AFGPs. At present, without the availability of juvenile specimens collected prior to bloodsucking, it cannot be answered conclusively whether *C. antarctica* has one or more silenced AFGPs genes or whether it lacks them entirely.

One could also argue that the observed stronger primer band in the undiluted sample compared to the diluted sample could be caused by protein biosynthesis. As with any other cellular or biological process there is no absolute deactivation of gene transcription and a very low number of mRNAs will always be present. This can be seen in Arctic fish such as *Pseudopleuronectes americanus*. While AFP concentrations were variable with 25 mg/ml during winter and a low in summer at 1 mg/ml (Petzel, et al., 1980) there was still an active AFP gene transcription leading to a baseline production of proteins. The absence of any

mRNA even at low levels which even by magnification of PCR steps still produce no visible result in *C. antarctica* is interpreted as the absence of any underlying gene AAT AAT AAT motifs in this species. But even a suppressed or minimal baseline transcription of AFGPs in the adult leech might cause only a slight elevation of PCR products within a pool of primer artefacts that are of insufficient numbers and quantity to be detected. The only means of confirming a biosynthesis activity in *C. antarctica* conclusively would be to use whole genome analysis (WGA).

Despite the aforementioned difficulties in interpreting these results, my conclusion that the fish parasite *C. levigata* has the AFGP gene motif, thus far unique to its notothenioid host, still holds. The Antarctic piscicolids might have as individual species or as an entire group, as do Antarctic fish, a genetic adaptation to survive in sub–zero waters.

A cDNA library effectively represents the gene activity at a specific point in time of the organism of interest. An analogy would be a snap–shot photo of a cell's activities at the time of death of the specimen, with details of every active gene. It can be regarded as the active part contributing to live functions at that particular point in time, and thus is a selection of active genes from the entire genome. It is a limited and simplified approach used to obtain information about the whole genome by targeting specifically relevant gene products in the form of the mRNA proteins present at the time of collection by drop–freezing of the leeches in this study. This technique allows us to allocate those genes actively involved in live processes in leeches rather than looking at the genetic level and genomic entirety. In the context of this study, the presence of the targeted gene product in the form of mRNAs inside the leech homogenates provides more information than merely a positive signal for the underlying gene from the leech genome could do. This is because the existence of short–lived mRNAs means that the leech actively produces its own antifreeze proteins based on the same genetic template and code that evolved in its hosts. The use of a cDNA library is thus a very suitable approach to show whether a gene has a functional role to play in metabolism or is only a dormant, non–expressed gene ballast of the species evolution that has remained in the genome. The results provide further support to the nature of AFGPs and their functional domains in an entirely different taxon to its fish group origin.

6.5.1 RT–PCR with varying fragments and primer dimers

The results using a set of primers designed for the repetitive gene motif encoding AAT AAT AAT as the part known to be present in fish AFGP genes shows a positive for the host *T. bernacchii* as well as for its parasite *C. levigata*. The various fragment lengths produced by PCR are in fact true results and are to be expected due to the experimental design. Any successful PCR depends on the use of a suitable primer pair targeting specific genetic sequences in or close to a gene of interest. Any primer pair thus produces fragments of definite nucleotide length, visible and distinguishable on an agarose gel under UV light. For this study, however, the only known sequence information is that of the common AFGP gene motif from the notothenioid host fishes, the short nine–letter AAT AAT AAT repeat of the peptide. The design of a primer for a repetitive sequence is particularly challenging as the initial step for each cycle in a PCR is the alignment of the primer to the target sequence. Ideally, for a gene with repeated motifs the forward and the reverse primer bind, but at various positions within the gene. As the target motif is repetitive within the host fishes AFGP genes, as far as is currently known, the primers can bind at many sites, producing a whole class of different fragments, all of which are true copies from the one gene but of different sequence and thus nucleotide lengths. Therefore, a positive result indicating the presence of a gene containing the repeat motif for AAT AAT AAT must show bands with a visible smear or tail in the lanes of an analysing gel. The fact that it does in the fish demonstrates functional primers and that an appropriate protocol was followed.

However, there is a bias towards shorter fragmentations. The experiment is prone to produce an overwhelming quantity of very short artefacts by primers binding to each other, so–called primer dimerisation. As one has a pair of primers constructed to aim for the same sequences within the gene, which is obviously essential to obtain fragments from a PCR, there is such artefact formation and it cannot be overcome by dilution or any other adjustment. This results in strong signals for the negative controls, Milli–Q water and rat liver, as well as the diluted samples, and is caused by this experimental limitation.

6.5.2 Methodology and limitations

The main complication of this study is the presence of two sets of genomic DNA (gDNA) within one organism, namely fish gDNA from the fish blood cells within the leech digestive tract and leech gDNA from the parasite itself. The aim was to exclude all fish–derived genomic information so as to find the respective AFGP motif that was solely of leech origin. While the method of choice would have been an all encompassing WGA of the parasite species, this approach was too time and resource demanding to be a viable option. A *de novo* data alignment to obtain even limited information in the form of a partial cover of the whole genome sequence would far exceed the scope of a PhD project, unless it was incorporated into a larger collaborative genome research effort and based upon substantial knowledge of previous genome, proteome or transcriptome research. None of these fields had been sufficiently tackled at time of this work, though each of these must be regarded as fast emerging fields in modern zoology. Therefore, an alternative method had to be used despite the associated limitations and potential inadequacies in regards to providing a conclusive result.

In order to eliminate contamination of the sample with fish gDNA, the methodology applied targeted the gene products only detectable in the presence of active gene transcription. RT–PCR targets the molecular product of genes that are in active mode of transcription, producing mRNA for the transfer of genomic information from the cell nucleus in the cell plasma and to the ribosomal apparatus for translation into proteins. Such mRNA molecules are only present within cells actively producing the specific protein they encode for. Furthermore, mRNAs are very short lived within a cell and are quickly degraded. This information about general eukaryotic cell metabolism is essential as it allows us to distinguish in this study between the presumably omnipresent fish DNA in an adult leech and the cell metabolic activity in the parasite itself. Consequently, mRNAs were the target in my work as being the intermediate step necessary between the gene at DNA and genomic level inside the cell nucleus and the respective proteins as gene end products inside the leech tissues. The mRNA results provide valuable insights into the potential of a HGT event even with the current limitations of sample size, number of repeats and the technologies used. Yet a future investigation could invest the necessary time and funding required by new and

more powerful technologies to obtain comprehensive information on a possible HGT by transcriptome and proteome analysis, both at qualitative and quantitative levels.

It must further be noted that the conclusions based on single PCR bands for each species are of course not according to best practice in the fields of genomics, proteomics or transcriptomics. A WGA would have been desirable, however, the applied RT–PCR, though not ideal, presented the least cost and resource intense technique manageable within the constraints of this PhD project. Although further confirmation is required, the current results, even if marginal, present at least some indication for a genetic basis of AFGPs in a piscicolid due to HGT from the host genome.

6.5.3 Flanking region information missing

It is not possible to discover the origin, i.e. fish or leech, of the mRNA detected in the parasite without further knowledge of the flanking sequences in the leech genome, which is required for the design of further primer pairs as well as for sequence comparisons. Thus far there can only be speculation for a fish–derived AFGP gene, either as a whole or in parts or even repeats, might have been inserted into the leech genome. Further primers cannot be designed unless information of the flanking regions on either side of any potential gene in the leech genome is known so as to utilise further forward and reverse primers. There was no attempt made for further experimentations with a large set of degenerative primers after the successful pair was found to produce the presented results. This because it is important to note that even if by chance, using an array of random PCR primers only one such gene sequence for a flanking region to an AFGP gene was actually obtained from a leech sample, the result would still not be conclusive. Even with the knowledge of a complete flanking gene sequence reaching into the AFGP gene motif at the genetic level in the parasite, the fragment's origin as to fish or leech remains to be deciphered. At present, this last requirement would prove an impossible task as any meaningful analysis would rely on a database of genetic references for sequence comparisons in order to trace the origin to either Antarctic leech or its fish host. Such work would require at least in parts a genome database of all the species involved, which is currently unavailable and thus has to be reserved for future investigations.

6.5.4 Cloning not the solution

The question is then how to tackle the inadequacies of the methodology as well as a lack of more detailed knowledge. The gene library obtained by RT–PCR consists of a mixture of all actively transcribed genes in the collective of the parasite's cells. Thus, it is impossible to differentiate whether one or more AFGP genes lie at the core of the sequences encoded in the mRNAs and in which tissue cells these are expressed. A further complication arises from the nature of the genes themselves, as the targeted motif is repeated within each fish AFGP gene, leading to a mixture of fragments with different lengths within each single PCR reaction. This effectively prevents a direct sequencing as a simple tool to obtain genomic information. The fragments obtained would need to be separated, which could be achieved by cloning, and subsequently be sequenced and studied.

One might thus assume that cloning could solve the issues at hand. However, cloning alone cannot provide a conclusive answer to the origin of the sequence discovered in leech samples simply because we lack a complete knowledge of all gene sequences for the various AFGP types in fish. These are yet to be described. Knowledge of the potential flanking regions is also lacking, as discussed above. Without this necessary additional information one cannot at present comment further as to the origin of the sequences found from work at the mRNA level.

Furthermore, even if one assumed a hypothetical scenario in which all relevant fish gene sequences were known, the genetic characteristics of the regions flanking the targeted genes within the leech genome would require clarification. This information on the flanking data is quintessential in order to be able to answer the question as to the origin of any sequences discovered in leeches. The genomic sequences on either side of the prospective AFGP gene motif in leech species would need to be identified as either fish or leech derived. Only with such knowledge could it be proven that the experimental results obtained from the present work are in fact not from the leech genome.

Until work incorporating genomic sequence data can be carried out, the mRNA method applied here is the best technique applicable as it allows us to gain information on gene activities in leeches and is superior to cloning of merely partial genomic sequences, which is available at present. Cloning cannot provide information on the biological relevance of a

sequence and whether it is actually transcribed and utilised *in vivo*. Furthermore, cloning does not present additional information as to the origin of the sequenced fragments as long as the flanking areas are missing for a detailed analysis.

In conclusion, cloning results would not provide any additional information to answer whether a gene with a similar AFGP motif to that of notothenioids has evolved in a piscicolid or was merely transferred in an HGT event. As work at genomic level is outstanding any data obtained provides no further answer to whether genes are in fact present in the leech genome as well as actively expressed. However, the proteomic work carried out here, at the level of mRNA molecules, allows to state that an active gene transcription occurs in at least one leech species while actively feeding on a host. Furthermore, protein biosynthesis is a metabolic process requiring energy, thus if exercised at the cellular level it must be of biological relevance for the parasite. If that was not the case, and the fish–derived AFGPs were absorbed by assimilation alone, the leech species would waste valuable resources in maintaining a functional gene with protein assembly machinery. In terms of evolution this would not be a stable strategy. As a direct consequence, the active gene transcription with a *de novo* biosynthesis would need to represent an advantage.

The only appropriate genomic tool to use to overcome the limitations of mRNA as well as any potential cloning efforts would be a WGA. Yet despite these desirable perspectives for further data by cloning and WGA, the conclusions of the results of my work are nonetheless of importance to Antarctic evolution of AFPs.

6.5.5 Darwin, HGT and modern synthesis

One might take the position that events of HGT are contradictory to Darwin and evolutionary theory. Instead, I suggest understanding such events as established evolutionary mechanisms that are only now being discovered as scientists start to understand mechanisms at this level that were previously hidden but are detectable today with the tools of molecular biology. Consequently, HGT can work additionally to the vertical transmission of genes after Darwin and as such represents a mechanism novel to science.

I believe Darwin's hypothesis is not challenged *per se* by the discovery of gene transfers between species as a source of new phenotypic characters or traits. Speciation was not

understood to be based on genes until Mendel's work was recognised and built on around 1900. HGT occurs and as such species evolve by means of another instrument than their natural genetic pool would otherwise allow.

The principle of mutations as the basis of a variability of traits, as Dobzhansky understood it, is not the only basis to secure the survival of a species by evolution in nature. In my view, the concept of HGT expands the modern synthesis in that genes are still the basic currency of evolution, but the mechanisms involved in the tree of life are more complex than biologists had previously assumed.

One could argue that Darwin, and in particular Mendel, were wrong in their beliefs of a vertical transfer of traits from adult animals to their offspring. The dogma of parental inheritance was questioned for prokaryotes in regards to their HGT of antibiotic resistances (Levy & Marshall, 2004). However, as shown above, HGT is becoming only recently established in more and more eukaryotic examples. This is without doubt due to scientists starting to investigate the phenomenon closely, but also is essentially dependent on modern molecular tools, which allow scientists to now study species not limited to a single or maybe a few genes but in their genetic entirety and complexity. The statistics behind discovering a lateral event without molecular tools were never in favour of Darwin, Mendel, Fisher or any of these pioneers of evolution and genetics.

The transfer of AFPs between fish species (Graham, et al., 2008; Graham, et al., 2012; Sorhannus, 2012) or the hot spot for their exchange in the Antarctic sea ice (Janech, et al., 2006; Kiko, 2010) can therefore be considered as extensions, 70 years in the making, to Huxley's modern synthesis. Genes are still the key to evolution, here of AFPs in polar organisms, yet their distribution is not necessarily limited to one species as a population of individuals. There are genes that can successfully cross Mayr's boundaries of isolation to enter new groups. If such genetic information contains the code for a beneficial character, such as a protein with ice–binding capability, then it can establish itself in the new pool of animals and henceforth be part of their evolutionary make–up. If these genes convey a function then they fend off the extinction of the organisms they inhabit. Thus, DNA sequences encoding AFPs might be considered mutualistic as a symbiotic relationship exists between a species or genome and such genes.

The two leech species investigated here were observed on *T. bernacchii* as well as *Trematomus hansoni*, *Trematomus nicolai* and *Pagothenia borchgrevinki* (pers. obs.) in agreement with the latest available literature on Antarctic Piscicolidae (Utevsky, 2007). The parasites are thus exposed to gene sets in different benthic fish species.

The data presented here on the Piscicolidae fits into the theoretical framework equally well, as do other discoveries related to fish and organisms associated with the Antarctic sea ice. None of these data contradict Darwin's view of natural selection as the force behind evolution or the modern view of genetics. However, the traditional concept of an exclusive intraspecific gene flow has to be accepted to be the main but not sole mechanism of evolution. As a consequence, the *species problem,* as 19th century scholars debated it, as well as the modern definition of a species have just been reignited. Mayr's important contribution to the species concept (1963), the biospecies, defined as a genetic unity with reproductive barriers towards other such groups, is still valid. Equally, the ideas of Darwin and even 18th century Carl Linnaeus or Linnæus in Latin, with the morphological approach to species from the perspective of a taxonomist, have not been rebutted but merely extended by the additional information. Species change constantly, which makes their definition complicated and as a concept artificially invented by man it is like much else in science, being merely the best feasible approach to describe nature and by definition the best approximation given at one time to the reality of a system.

6.5.6 Posttranslational modifications (PTMs) for adaptive advantage by HGT

In the context of HGT one might doubt the effectiveness of any AFGP gene transfer due to the posttranslational modifications (PTMs) required to achieve antifreeze functionality. The question is whether any precursor proteins would be adequately processed in the Antarctic parasites.

The physical property of any AFP is based upon its interaction with the ice surface. In case of notothenioid AFGPs, the GalNAc–Gal disaccharide moieties are O–linked to the threonine residues of the peptide backbone and are key to the THA (see Chapter 4). In the context of AFGPs, the importance of this gene family cannot exert any influence on the evolution of a species without a selective advantage manifested as a biochemical adaptation to the

freezing environment. At first glance, the necessary PTMs by glycosyltransferases for the enzymatic glycosylation of AFGPs might be considered a particularity reserved to fishes as higher taxonomic ranking vertebrates, thus questioning the existence of any appropriate mechanisms in piscicolids. However, this is by no means certain as the glycosylation of proteins is the most diverse as well as an abundant PTM (Schjoldager & Clausen, 2012) and common mechanism not only in higher vertebrates but also invertebrates (Lai, Nice & Schilling, 2013), despite the controlling factors reaching beyond the genome level (Uy & Wold, 1977). Glycosyltransferases, with a striking homology to proteins of the glycogen phosphorylase superfamily (Wrabl & Grishin, 2001), play a pivotal role in regulatory pathways and signal transduction in nearly all eukaryotes from filamentous fungi and plants to animals including animal parasites and eukaryotic viruses (Van den Steen, Rudd, Dwek & Opdenakker, 1998; Wells, Vosseller & Hart, 2001; Howerton, Morgan, Fischer & Bale, 2013; Li, Liu, Xu, Du & Xu, 2014).

The O–linked glycosylation of a protein is long known to be a step–wise process in which GalNAc can be added to serine or threonine residues (Strous, 1979). The processes involved during O–GalNAc glycosylation which attaches O–glycans onto proteins are complex as not only the sites but also the sugar substrates have to be selected (Gill, Clausen & Bard, 2011). The various isoforms of different glycosyltransferases families target specific substrates to be glycosylated, and thus several glycosyltransferases are required with their enzymatic specificity to modify residues accurately and synthesise one final glycoprotein (Ten Hagen, Fritz & Tabak, 2003). The protein O–glycosylation with GalNAc differs from most other glycosylations in that the first step requires a large number of isoenzymes deriving from an evolutionary old gene family found throughout the animal kingdom but not in prokaryotes, plants and yeast (Kawaguchi, et al., 1986; Bennett, et al., 2012). Notably, the view that glycosyltransferases have limited influence on basic plant physiology has changed (Vogt & Jones, 2000; Wang, 2009) as plants possess a multigene family of highly divergent and polyphyletic glycosyltransferases, including O–glycosyl transfers (Gutmann & Nidetzky, 2013) with a recent identification of more than 704 glycosyltransferases in the Korean ginseng *Panax ginseng* (C. A. Meyer) (Khorolragchaa, et al., 2014). These enzymes add sugar moieties to various substrates in plants such as hormones and secondary metabolites (Gachon, Langlois-Meurinne & Saindrenan, 2005; Bowles, Lim, Poppenberger & Vaistij, 2006; Modolo,

et al., 2009) or xenobiotics such as herbicides or pesticides (Jones & Vogt, 2001; Barvkar, Pardeshi, Kale, Kadoo & Gupta, 2012).

The cellular machinery for GalNAc–type O–glycosylation is restricted to the eumetazoans (Hashimoto, et al., 2009) with galactose representing an epimer and diastereomer of glucose. Although it is a very abundant and complex form of PTM, the particular mechanisms involved in this protein glycosylation are thus far the least understood with regard to attachment sites on the proteins. In principle, the O–linked N–acetylglucosamine transferase facilitates the O–glycosidic linkage between the intra– and extracellular protein's serine or threonine residue and the N–acetylglucosamine (GlcNAc) (Comer & Hart, 2000).

The primary sequences of the glycosyltransferases are very diverse (Campbell, Davies, Bulone & Henrissat, 1997) with most glycosyltransferases belonging to two (Franco & Rigden, 2003) or three monophyletic (Liu & Mushegian, 2003) structural superfamilies, indicating that only a few events in the tree of life resulted in successful solutions to the omnipresent task of glycosylation as PTM (Hu & Walker, 2002). The conserved three–dimensional structure has few (Breton, Snajdrova, Jeanneau, Koca & Imberty, 2006) or only two fold domains surrounding the active site of substrate access, recognition and catalysis (Hu & Walker, 2002; Albesa-Jové, Giganti, Jackson, Alzari & Guerin, 2014; Johal, et al., 2014). The latest literature stipulates further the pivotal role pH can have on glycosyltransferases in determining their open or close states to the catalytic site (Johal, et al., 2014).

There is evidence for the synthesis and thus native importance of glycoproteins in a vast variety of invertebrate organisms and I shall list but a few excerpts from the extensive literature on glycoproteins in the animal kingdom of lower taxonomic ranking than vertebrates.

The first ever record in any invertebrate of a glycoprotein with an O–linked N–acetylglucosamine (GlcNAc) was made in the blood fluke *Schistosoma mansoni* (Sambon, 1907), which coincidentally is yet another parasite but found in humans causing hepatosplenic disease (Nyame, Cummings & Damian, 1987). This endoparasite, as a member of the class Trematoda under the phylum Platyhelminthes, is taxonomically distant from higher vertebrate animals. Nyame et al. (1987) could, however, demonstrate a glycoprotein biosynthesis in *S. mansoni* to produce O–linked oligosaccharides containing the sugar

GlcNAc, including an unusual attachment at the terminal end of the protein. Such terminal O–linked GlcNAc was previously first described in mice as monosaccharide attached to proteins on the cell surface of lymphocytes (Torres & Hart, 1984). Presumably, the sugar moieties in glycoproteins of some human blood flukes of the genus *Schistosoma* (Weinland, 1858) have an antigenic function (Norden & Strand, 1984).

A detailed analysis of 36 eukaryotic genomes established no less than 53 families of glycosyltransferases with extremely diverse sequences, but each with at least one conserved region. No single motif in the amino acid primary sequences was, however, found to be conserved across the different families (Hashimoto, et al., 2009). The authors found these families to divide into the two groups of universal and diverged glycosyltransferases. From an evolutionary perspective, the former enzymes produce conserved glycans, while the latter result in diverged glycans that have different types of substrate specificities and are specific to kingdoms or clades (Hashimoto, et al., 2009).

In the marine context, a study of three invertebrates as part of their metamorphosis from free–living pelagic to benthic sessile stages demonstrated that the level of protein glycosylation as PTM decreased in the striped barnacle *Amphibalanus amphitrite* (Darwin, 1854) and the spionid polychaete *Pseudopolydora vexillosa* (Radashevsky & Hsieh, 2000), while it increased in the brown bryozoan *Bugula neritina* (Linnæus, 1758) (Chandramouli, Zhang, Wong & Qian, 2012). Furthermore, the number of proteins known to need PTMs is constantly rising as a result of methodological advances as well as improvements in protein identification software, in particular for organisms which had previously not been sequenced, for instance in polychaetes (Chandramouli, et al., 2013). The phylum Annelida or segmented worms contains the class of Polychaeta as the sister group to the class of Clitellata in which the subclass Hirudinea contains the Antarctic fish leeches. This illustrates not only the utilisation of protein glycosylation *per se* but also the vital importance of the underlying PTM mechanisms in marine taxa of the kingdom Animalia belonging to three very distant phylogenic clades, namely the phyla Annelida (polychaete), Arthropoda (barnacle) and Bryozoa.

Finally, the capacity of Piscicolidae for glycosylation as functional PTMs at cellular level has been shown by the presence of invertebrate dystrophin–like products in the obliquely striated muscle of *Pontobdella muricata*, the leech parasite of the electric fish

Torpedo marmorata (Royuela, et al., 1999). The annelid muscle contains at least β–dystroglycan, α–, β–, γ– and δ–sarcoglycan as integral dystrophin–associated glycoproteins very similar to those found on vertebrate striated muscles (Royuela, Hugon, Rivier, Paniagua, et al., 2001) and nerve cells (Hoffman, et al., 1988). The results highlight striking similarities in glycoproteins found in marine piscicolids and vertebrates, although the underlying PTMs remain to be described.

One large precursor protein might contain multiple copies of a biologically active peptide, each flanked by recognition sites for enzymatic cleavage as is the case for enkephalin peptides in proenkephalin (Gubler, Seeburg, Hoffman, Gage & Udenfriend, 1982; Noda, et al., 1982), the peptides in the pro–opiomelanocortin precursor (Nakanishi, et al., 1979) or the single AFGP copies within the fish precursor proteins (Hsiao, Cheng, Fernandes, Detrich III & DeVries, 1990; Wang, DeVries & Cheng, 1995). The GalNAc O–glycosylation of proteins can be tissue– and even site–specific and not all biologically active peptides are cleaved, resulting in different end products from identical precursor proteins (White, Gall & McKelvy, 1986; Birch, Estivariz, Bennett & Loh, 1991). Although a similar situation might occur in leech tissues, any functional AFGPs derived from a fish DNA template must contain sugar moieties. There is no reason to doubt the presence of cellular machineries for glycosylation of proteins in the parasite, however, there might be differences in the piscicolid isoenzymes for GalNAc O–glycosylation processes. This might lead to varying degrees of AFGP glycosylation success in leech protein templates compared to those of the fish hosts. Thus, one might consider the PTMs as an aspect of biochemistry which is an insuperable obstacle for the piscicolid metabolism that ultimately leads to non–functional AFGPs. However, as in the case of PTMs in general, the modification mechanisms usually lead to only small or moderate structural changes in proteins that are sufficient to modulate their functions (Xin & Radivojac, 2012). In this recent work Xin and Radivojac calculated that, although PTMs predominantly induce conformational shifts, they result in a surprisingly low percentage of large changes in their substrates since only 7% of glycosylated proteins exhibit a significant structural change of more than 2 ångström. It remains to be demonstrated whether piscicolids have in fact homologous cell structures and thus the same mechanisms necessary for the appropriate biosynthesis of glycoproteins with antifreeze activity. Given the genetically based differences in glycosyltransferases of leech to fish, the PTMs of any AFGP precursor protein inside leech cells and tissues might result in biosynthetic errors in form of missing sugars on the final

AFGP products. Nevertheless, any partial GalNAc O–glycosylation might still provide the fish parasites with proteins that exhibit some degree of THA and consequently entail an adaptive advantage.

In conclusion, at present it is only possible to speculate about the extent to which the glycosylation mechanisms as PTMs in marine Piscicolidae might serve as preadaptive biological attributes for *in vivo* biosynthesis of AFGPs after a potential HGT provided the genetic template. However, the vital importance of glycosylic PTMs is apparent for marine taxa in general and for the mobility of piscicolids in particular. Although there might be a strong probability of a selective advantage deriving from HGT, the plasticity of the leech cellular machinery, including pH condition, remains to be confirmed. Only future research in genomics and proteomics can facilitate O–glycoproteome analysis for a much improved understanding of the complexity of glycosyltransferases and functions as well as the necessary pathways of biosynthesis of the enzymes and their products (Wang, 2013), including extensive crosstalk between PTMs (Venne, Kollipara & Zahedi, 2014). Computational biology aims to understand and utilise the underlying mechanisms in order to describe the formation of glycans *in silico* and ultimately predict the proteome of any given organism *in vivo*. The recent and fast advances in the field are encouraging to comprehend protein evolution and PTM frameworks across taxa applicable to AFGPs in Antarctica. Furthermore, other claims might be substantiated for potentially similar adaptive advantages by AFPs provided by HGT events between phylogenetic distant animal groups.

6.5.7 Future research directions

In studying an organism that potentially contains genes of two different genomic origins, the classic molecular tools reach their limits. Although it could be shown that at least one gene is actively transcribed and produces mRNA encoding AFGPs in the fish leeches, it remains to be seen what the nature of that gene is. Until recently, the technology of WGA was financially prohibitive for a PhD project and also the technical and practical aspects of working with extremely limited material did not make it possible to obtain more data. However, technological advances in the past three years now allow the study of the genomes of *C. antarctica* and *C. levigata* in greater detail than ever before by using WGA. Material that could be used to undertake further studies using WGA is available frozen at

–80°C und also preserved in ethanol. The issue of only very limited quantities of juvenile leeches being available has also been resolved by research companies that now have specific protocols available to sample and boost gDNA prior to WGA. Consequently, the questions regarding the regions outside a leech gene for AFGPs, the exon and intron frequency, their length and the amount of non–coding DNA between them, amongst other information, could allow us to decipher within a short period of time what the main characteristics of the leech gene are. This could then be compared to the fish gene, with its typical repetitiveness, to see whether and where the gene might have transposons to either side of it in fish. This could provide more information on the currently unanswered question as to the mechanisms in eukaryotes leading to HGT.

6.6 Conclusions

The active transcription of a gene for the translation into functional AFGPs outside the Antarctic fish hosts is a novum. At least one HGT event must have occurred, which established the fish AFGP motif in the piscicolid parasite *C. levigata*. This gene is transcribed even though the end product is available in the fish blood. This indicates an evolutionary pressure for a *de novo* synthesis in the leech although this might be muted and the relevance amplified by the mRNA methodology used. A quantification of AFGPs inside the parasite based on its own biosynthesis in comparison to fish–derived peptides could establish the relevance of the observed active AFGP gene transcription.

In this study I found the first evidence for an HGT event outside the Antarctic surface sea ice zone. The unique AFGP motif found in notothenioid fishes as a biochemical adaptation to their sub–zero environment facilitated their radiation and represents a key evolutionary event in this animal group. Their parasitic fauna not only uses the fish peptides for their own protection but have also obtained the underlying genetic code in at least one species, *C. levigata.* A WGA could provide further information to identify and characterise the gene motif in *C. levigata*. Furthermore, WGA could establish the presence or absence of AFGP–characteristic motifs in *C. antarctica*.

CHAPTER SEVEN

Conclusions and future research directions

He would need practice if he was going to see the things above.

First he would most easily see shadows

and then the images of men and other things reflected in water,

and then those things themselves,

and the things in the heavens and the heavens themselves.

(Plato 428 – 348 BC in *The Republic*)

The main motivation behind this thesis was a desire to broaden our knowledge of Antarctic parasitology and the evolutionary processes that have shaped this unique biota under the extreme environmental conditions of the Southern Ocean. The Antarctic sea ice, including the shallow water zone containing ice crystals, is unique in presenting science with an evolutionary laboratory nearly undisturbed by human impact, challenging the most fundamental aspects of biology and enabling us to extend our understanding of life's mechanisms.

This thesis includes studies on the structure and function as well as the origin and evolution of antifreeze proteins in fish leech parasites. No other investigation has focused on the leech fauna of McMurdo Sound since the first true Antarctic leech species was discovered there during the Terra Nova expedition in May 1911. With the use of increasingly

more powerful technologies, the four consecutive results chapters present evidence for an active antifreeze protection in Antarctic Piscicolidae. The planning, design and development of methods came to fruition with the different steps demonstrating antifreeze glycoprotein (AFGP) absorption, integration as functional peptides in different tissues, and active thermal hysteresis (TH) *in vivo* of body fluids and tissues alike. The potential for a *de novo* biosynthesis of AFGPs for freeze protection at the juvenile stage is suggested by the presence of the underlying characteristic genetic sequence for AFGPs in *Cryobdella levigata*. This strongly suggests a further example of a horizontal gene transfer (HGT) event in the Antarctic in the presence of sea ice. I also report for the first time the use of AFGPs with their antifreeze properties outside of their natural milieu of the notothenioid fish, in other organisms of the Ross Sea, namely the parasitic leeches.

The life strategy of fish leeches provides the potential for opportunistic utilisation of a virtually unlimited supply of AFGPs at little energetic cost. This is because species such as those within the genus *Cryobdella* feed constantly from their hosts and lack the storage capacities for blood seen in other leeches. This anatomical characteristic is proof of their life strategy as an obligate parasite (Utevsky, 2007).

The potential HGT of the peptide–encoding gene sequence in parallel to the assimilation of the gene end products from its original source, the fish, can be seen as an extraordinary case in evolution. The initial juvenile stages of Piscicolidae would have been without protection against contact with ice crystals prior to their first blood meal. Thus, endogenous AFGPs originating from one or several genes incorporated in the piscicolid genome might allow the juvenile animal to bridge the time of peptide deficiency until AFGPs from the fish blood could be assimilated in sufficient quantity. In addition, for *C. levigata* an ectoparasite inhabiting the gill cavity of the host, the high flow rate of water and thus ice crystals over the gill epithelia likely provides a high risk.

The results from mass spectrometry (MS) and nanolitre osmometry (NO) demonstrated that AFGPs are incorporated in their functional forms in both *Cryobdella antarctica* and *C. levigata* adults. These could have stemmed exclusively from the fish by *ad libitum* availability and absorption, or the AFGPs found could be leech–derived, by peptide synthesis in the specific tissues. The exact mechanisms producing these detected AFGPs, assimilation or biosynthesis or both, remain to be seen. Current technology and time constraints in the

field, especially for rearing experiments, did not enable us to further our knowledge on these aspects. However, the application of imaging mass spectrometry (IMS) and whole genome analysis (WGA) could be used in the future to provide more data. The scientific progress made in recent years in methodology combined with the interest of the polar research community will facilitate fast progress in the metagenomics for the Antarctic biota and in the not too distant future will allow us to find the answers to the questions arising from this work.

7.1 Comments on THA

The results obtained by NO indicate a substantial AFGP concentration in fish leech parasites, expressed in a greater TH in the leeches compared to in blood samples of their fish hosts taken at the time of sampling. As a physiological necessity for optimal dispersion, the blood is the organ with the highest concentration of AFGPs inside a notothenioid. Consequently, the parasite can and apparently does concentrate this further by means of dehydration to the extent that a homogenate of an entire specimen reaches a deeper freezing point than native fish blood.

The AFGPs in the digestive tract are functional and the TH exceeds the activity level observed in fish at the same time during the Antarctic spring. Any artefacts due to methodology or sampling would have had little effect on the osmolality determined for the different samples even at diluted levels.

Schrag and DeVries (1983) found that using small cooling and heating rates (0.01 K in 5 min) during the observation of a single ice crystal prevents supercooling and thus allows for an accurate TH determination. This was found to be the case not only for the large types 1–5 AFGPs but also for the small AFGP 7 and 8. The MS results demonstrated the presence of type 7 and 8 AFGPs. Consequently, the observed TH and ice crystal behaviour in all leech samples derives from true AFGP activity. Although this THA could be based only on AFGP 7 and 8, I hypothesise that other larger peptides and probably AFPPs could also contribute towards the final TH manifestation.

The circulatory system of the piscicolid invertebrates allows the distribution of AFGPs into the posterior sucker as has been demonstrated by MS. The important extension is this work with the discovery of TH activity in pure samples of leech tissues for both Antarctic species. Consequently, piscicolid parasites have the ability to tolerate intra– and extracellular ice crystals *in vivo*, as their growth would be inhibited by an active antifreeze protection.

7.2 Antarctic marine organisms and antifreeze properties

The many studies into freeze tolerance of different marine invertebrates provide evidence that these can freeze under natural conditions independently of isosmotic conditions of the surrounding seawaters. Once an animal is frozen for either a short or an extended period of time, as is possible during the polar winter, its survival is threatened considerably, as is indicated by mortality rates reported in the literature (e.g. Asahina, 1961; Theede, 1972). Consequently, under the premise of natural selection marine invertebrates and unicellular organisms alike require protective mechanisms against freezing in their Antarctic environment. The presumption of isosmotic body condition seems insufficient as a strategy to enable them to avoid freezing in the sea ice or the upper supercooled water column, where ice crystals forming and floating freely present a constant danger of contact with ice as a seeding and inoculating element. This has been supported by the subsequent discoveries of peptides and proteins in diatoms and sea ice animals, which actively interfere with ice crystals.

The results of this study of the Antarctic fish leeches provide further evidence in support of the argument that marine organisms do indeed require specific freeze protecting agents in the presence of free ice in their natural habitat. Although further systematic experiments are required, the outcomes of this research indicate that the leeches are not of isosmotic conditions and consequently have physiological constraints that require solutions.

The AFGPs stay biologically active for hours at room temperature and have to be regarded as fairly heat tolerant and as such behave as fish AFGPs, which have a good heat tolerance under experimental conditions (pers. obs., A. L. DeVries & C. W. Evans pers. comm. & DeVries, et al., 1970). Consequently, it can be assumed that the previous heat treatment

during the IHC protocol for embedding leech specimens into paraffin for sectioning had little negative effect on the results. To the contrary, this heat treatment seemed to be an effective method for the unmasking of the AFGPs in ethanol fixed tissues, leading to the overall high fluorescence observed. Any negative effect of degrading AFGPs due to the higher temperature can thereby be assumed to be negligible for the discussion of the antifreeze capacities of fish leeches in general.

In the future, IMS could be used to investigate total AFGP concentrations from individual body parts, such as the intestine, as well as the different dermal tissues and the posterior sucker muscle or even the haemocoelomic lacunae. It might be that the interior concentration of AFGPs inside the digestive tract is considerably higher than in the source, the fish blood. Furthermore, a decreasing concentration gradient from the digestive content to the perimeter of the leech body in a juvenile would support the assimilation theory. The mass analysis of a juvenile prior to a fish blood meal could also establish the active transcription of a gene.

It is possible that THA in leeches could be related to their nutritional status. In one of the latest studies on intracellular freezing tolerance of the Antarctic nematode *Panagrolaimus davidi* it was demonstrated that their survival rate correlates with the nutritional status of individuals, where well fed specimens had a better chance of survival (Raymond & Wharton, 2013). The starving experiment prior to TH measurements of one leech in this work could be expanded to see what effect blood deprivation has on THA in adults.

7.3 Protein evolution, Darwin and HGT

A transplant experiment has proven that antifreeze proteins are the protecting agent against ice in northern fish of Newfoundland (Fletcher, et al., 1986). The AFPs from the blood plasma of an AFP–producing species, the winter flounder *Pseudopleuronectes americanus*, were injected into another species that naturally lacks AFPs, the rainbow trout *Salmo gairdneri* (Richardson, 1836) [accepted today as *Oncorhynchus mykiss* (Walbaum, 1792) (Costello, et al., 2001, p. 362)]. This cross–species study found that AFPs are in fact responsible for the

antifreeze properties of the serum, as the trout now showed THA proportional to the administered flounder's proteins. The authors concluded that TH functions independently from any specific receptors in the fish epithelia in order to position the proteins and to prevent ice propagation. Fletcher et al. (1986) commented that although the results were to be expected that no previous study demonstrated AFPs from one species to work with the same properties in another species of fish. In my study I argue that a potential exchange of active AFPs has occurred under natural conditions in the Antarctic.

I provide further evidence to support the claim that AFPs are universal in their molecular functionality and that their ice–inhibiting properties are species independent. The results of my cross–species application confirm previous findings in Antarctic diatoms (Raymond & Knight, 2003) for ice–binding properties in different species. I also confirm the hypothesis that no specific cellular receptors are necessary to produce a TH effect by AFPs (Fletcher, et al., 1986). These results suggest that it is likely that there are no specific absorption mechanisms required at the tissue or cellular level for AFGPs to work effectively.

Marcel Florkin proposed that the biological dimension of protein research should be regarded as a part of the phylogeny and taxonomy of life (1949; 1966). The study of biochemical properties and the evolution of molecules (Florkin & Swain, 1974) is challenged by HGT as an established mechanism of gene and thus protein exchange (e.g. Kiko, 2010; Mihasan & Brandsch, 2013), even between remotely related species (e.g. Matveev & Okada, 2009). Under Florkin's hypothesis, the results from Antarctic leeches are perplexing. We are presented with an example of how molecular evolution might provide conflicting evidence to the evolutionary theory proposed by Charles Darwin 150 years ago (1859). The increasing evidence that the phenomenon of HGT plays a pivotal role for AFPs and species evolution in Antarctica seems to make the biochemistry of proteins the black box of Darwin's evolution. In the context of HGT, the classic view of a tree of life as a hierarchical and phylogenetic dependency has to be reconsidered for the protein evolution in the Southern Ocean.

The *Red Queen hypothesis* (van Valen, 1973) states that in order to survive the evolutionary arms race between predator and prey every species must constantly develop and not stand still. The principle of co–evolution between and even within species was discussed by Dawkins and Krebs (1979), who saw a host–parasite interaction as an interspecific and asymmetric arms race, that is one side attacking while the other is

defending. In the Antarctic system the fish host evolved an adaptation to a potentially lethal environment for its parasites, and the Piscicolidae outflanked their hosts' move into new environments that could have caused the complete loss of the entire ectoparasitic fauna as co–existing taxa. The adaptive radiation from the ancestral fish since the Miocene to populate the vacant niches of the freezing Southern Ocean of today would inevitably have put the survival of the parasites at risk. However, the biochemical adaptation enabling the fish host to invade new habitats was immediately available to the leech by default through its life strategy and could be used for the same freeze protection. I refer to this unique situation as a short–circuit co–evolution in which a potentially ultimate form of an arms race between host and parasite could not build up any selective force and thus evaporated. Furthermore, this applies not only to the one parasite species as typical in host–parasite interactions but presumably to the entire group of Antarctic fish leeches.

Similarly, any blood parasites of fish in the Antarctic, such as Trematoda, live suspended in an aqueous solution with high antifreeze concentrations. As a consequence of my observations made in Piscicolidae and the experiments on IBPs in diatoms (Raymond & Knight, 2003) I hypothesise that such endoparasitic species utilise AFGPs in order to prevent crystallisation events like other unicellular and small organisms do by IBPs in this marine habitat. Likewise, Nematoda found in Antarctic fishes (Rocka, 2002; Oguz, Heckmann, Cheng, El-Naggar & Tepe, 2012) living as endoparasites in the intestine and thus a locality of high AFGP concentrations within their fish hosts (Evans, et al., 2012), could use the peptides in the adult stage as well as incorporate peptides into eggs and larvae for protection during their free living exposure to ice in their benthic habitat. A future investigation might consider this ecophysiological situation between endoparasites and their fish hosts in the Antarctic.

7.4 Climate change, stenothermy and Antarctic parasites

The extent of a rising temperature in the Southern Ocean caused by the predicted global climate change is still debated, but adaptive mechanisms will without doubt be crucial to the survival of stenothermal or cold–adapted species (Clarke, Johnston, Murphy & Rogers, 2007). There has been research on the temperature adaptation and heat tolerance of assumed stenothermal Antarctic fishes (Somero & DeVries, 1967) since before the discovery

of their cold adaptation by AFGPs (DeVries, 1971). Three fishes of McMurdo Sound, *Trematomus bernacchii, T. hansoni* and *T. borchgrevinki* [accepted today as *Pagothenia borchgrevinki*], were tested and found to reach their lethal temperature limits at 6°C. Recent work recorded for the same fishes and location a critical thermal maximum (CTMax) of 11.95°C to 13.62°C (Bilyk & DeVries, 2011) and even found that notothenioid fishes increased their CTMax after an initial heat treatment (Bilyk, Evans & DeVries, 2012). However, the influence of heat as a stress factor for these cold–adapted fishes remains (A. L. DeVries, pers. comm.).

To the best of my knowledge, no–one has yet considered the additional stressors in the form of leech parasites and fish endoparasites to the overall fitness of their hosts in Antarctica. Piscicolid parasites are likely to be costly for the energy household of their hosts. For example, the continuously feeding leech *Malmiana nuda* (Richardson, 1970) [accepted today as *Janusion nuda* (Richardson, 1970) (WoRMS, 2013)] costs its fish host the shorthorn sculpin *Myoxocephalus scorpius* (Linnæus, 1758) up to 1.6 kj per week (Mace & Davis, 1972).

Additionally, leeches are known as functional vectors for endoparasites (Burreson, 2006). For instance, Trematoda use freshwater leeches as intermediate hosts (Sawyer, 1986b, pp. 559-560) and *Trypanosoma* (Gruby, 1843) species are known to use freshwater and marine leeches in temperate Europe (Sawyer, 1986b, pp. 623-630) and marine Piscicolidae in the Arctic (Sloan, Bower & Robinson, 1984; Karlsbakk, Haugen & Nylund, 2005). The combined impacts of the leeches and any endoparasites that they may be transmitting, on the metabolism and health of the Antarctic fish hosts is currently unknown and is a question for future research.

Our knowledge of the life cycle of Antarctic leeches is limited, but it can be assumed to occur at a slower developmental pace in the low temperature regimes of the Southern Ocean compared to Piscicolidae in temperate marine habitats. Wägele (1988) undertook a rare laboratory study to learn more about the life cycle of an Antarctic marine invertebrate in which he held and reared in captivity the isopod and fish parasite *Gnathia calva* (Vanhöffen, 1914) [today accepted as *Caecognathia calva* (Vanhöffen, 1914) (Schotte, Kensley & Shilling, 1995 onwards)]. He kept eggs in artificial seawater at –1°C and found that the larvae hatched after an incubation time of 12 months. Wägele estimated the embryonic development of *C. calva* to last 1 year, with 3–4 years of larval stages before maturity is

reached at 4–5 years. He compared this with the complete embryonic development of the temperate isopod *Paragnathia formica* (Hesse, 1864) of 1–2 months and a life cycle of 1 year from egg to egg. The lack of experimental data for Antarctic invertebrates allows us only to comment on a rough figure for their developmental time, as provided by Utevsky of between 30 and 300 days (2007). Although Piscicolidae are annelids and not crustaceans involving several larval stages, I believe the low–temperature environment must inevitably lead to an extended embryogenesis in Antarctic leeches due to slower metabolic processes. The Arctic piscicolid *Oceanobdella sexoculata* (Malm, 1863) was previously described to have a bi–annual life cycle (Khan & Meyer, 1978) and under similar conditions in the Antarctic it is likely to have a similar time frame. Embryogenesis is likely to be considerably greater than the one month necessary in most of the temperate relatives (Sawyer, 1986a, pp. 32-54). An incubation period of over 40 days, tested under laboratory conditions at seawater temperature before termination of the experiments, was found to be insufficient for juveniles of either *C. antarctica* or *C. levigata* to hatch from their cocoons (pers. obs.).

It would be interesting to obtain quantitative data on the distribution, abundance and developmental time for leech species at present seawater temperatures as a proxy for future predictions. Furthermore, their relevance in the Antarctic benthos should be established in the form of infestation rates of fish. As personal observations of new host records indicate, it would be interesting to determine host–specificity for the full suite of Antarctic piscicolids or whether they can tolerate a wide range of fish species as their hosts.

7.5 McMurdo Sound: A new site for reliable piscicolid sampling

There are several important results from this work to be highlighted as of relevance for future Antarctic ecosystem investigations:

1. The McMurdo Sound benthos is home to a stable population of *C. levigata* with findings 100 years apart and also harbours *C. antarctica*.
2. Both leech species are present in numbers far above the occasional sighting previously reported and not a rarity to catch alive on their respective fish hosts.
3. There are still more fish host species for the known leeches to be identified.

4. The potential for new piscicolid species to be discovered in Antarctica has not ceased.

Whilst there is a heightened awareness of climate change and the predicted decline in worldwide biodiversity, there is a fundamental lack of data with which to decipher the impacts on the marine environment. Biodiversity is poorly documented for the world marine habitats (Costello & Wilson, 2011; Appeltans, et al., 2012) let alone the Southern Ocean (Griffiths, 2010; De Broyer, 2011). Warnings of the severity of already occurring alterations at the ecosystem level and to the distribution of polar species have been presented (Arctic Climate Impact Assessment, 2005; Intergovernmental Panel on Climate Change, 2007). Yet theoreticians have to make assumptions at levels as basic as the total species number for polar marine environments. This inevitably limits what conclusions can be drawn for species interactions to reliably formulate future modulations and judge the scope of changes for polar habitats. I utilised a simple method as a tool to gather primary data on ectoparasitic fish leeches (Hirudinea, Piscicolidae) from coastal waters of Antarctica.

Distribution data available for analysis in the case of the Southern Ocean has been patchy, due to logistical and financial constraints, with vast areas under–sampled, thus there is considerable scope for further species discoveries (De Broyer, 2011; Schiaparelli & Hopcroft, 2011; Appeltans, et al., 2012).

I suggest a simple sampling technique for piscicolids that directly targets the fish hosts by use of a standard recreational fishing rod with hook and line lowered through manually drilled ice holes. This unsophisticated method has not appropriately been credited before. Yet it has proven to be extremely effective, yielding large numbers of fishes for collection of ectoparasites, while being logistically and financially viable, even under the most challenging field conditions encountered in Antarctica.

7.6 Limitations of study

The main limitations for this work must be considered in the context of the numerous logistical problems at the time of collection leading to the low number of parasite species available, insufficient time in the field, as well as financial constraints to undertake a high

number of repeats for statistical analysis for some parts of this thesis (MS) and possible artefacts caused by preservation techniques (IHC).

The complexity of this thesis required a variety of methods from classic histology to modern molecular tools. It is in the nature of scientific research that one can never find definitive proof but only numerous indications to support a particular hypothesis. This thesis is an example of a field–based investigation, with all the associated limitations on sampling locations, specimen numbers and quantity of material available for analysis, although for the most part these issues were successfully overcome. Although not paramount to the core questions and thus the main conclusions of my thesis based on qualitative results, a larger number of repeats would have been desirable to additionally obtain statistically significant support.

The images obtained by IHC were not as straightforward to interpret as hoped. There could be many factors influencing this, such as limitations to the cross–species application of antibodies, the varying antigen concentration levels in different specimens, piscicolid proteins resembling antifreeze conformities, an excessively high concentration of salt ions interfering with the antibody system or simply an ineffective blocking of unspecific secondary antibody binding to cell structures under the experimental conditions in piscicolids as different organisms from the tested and established fish model.

The requirement for an internal standard with known concentration to measure proteins quantitatively in MS applications with one sample being run at a time prohibited further data acquisition in terms of AFGP quantities in leech samples as part of this PhD. However, IMS does not require an internal standard in order to estimate the concentrations of macromolecules of interest in tissues, even at cellular resolution. This new technology will enable future investigators to measure *in situ* the precise quantity of antifreeze agents present in real life under natural conditions.

Due to the nature of this work involving live animals and their sampling and preservation under natural conditions in the field, it was challenging to standardise the physiological conditions in which leeches were used in subsequent work. This natural limitation of any biological field sample could only be eliminated in the future by use of a breeding facility for

Antarctic piscicolids or the use of more advanced methodologies that allow concentration measurements such as IMS.

The fact that leeches have biologically active AFGPs undeniably leads to cryoprotection. Whether these invertebrate organisms are hyposmotic, isosmotic or even hyperosmotic and have additionally other compounds to reduce their freezing point could not be answered in the context of this thesis. It is difficult within the time frame of a PhD to undertake the necessary tests for AFGP presence and freeze tolerance in virgin leeches before their first blood meal. It remains for a long–term experiment to be set up under natural conditions, either in Antarctica or elsewhere, to demonstrate the extent of antifreeze resilience of juvenile leeches before and after obtaining fish blood. Furthermore, only such a setup could produce data to determine whether young leeches are in need of AFGPs.

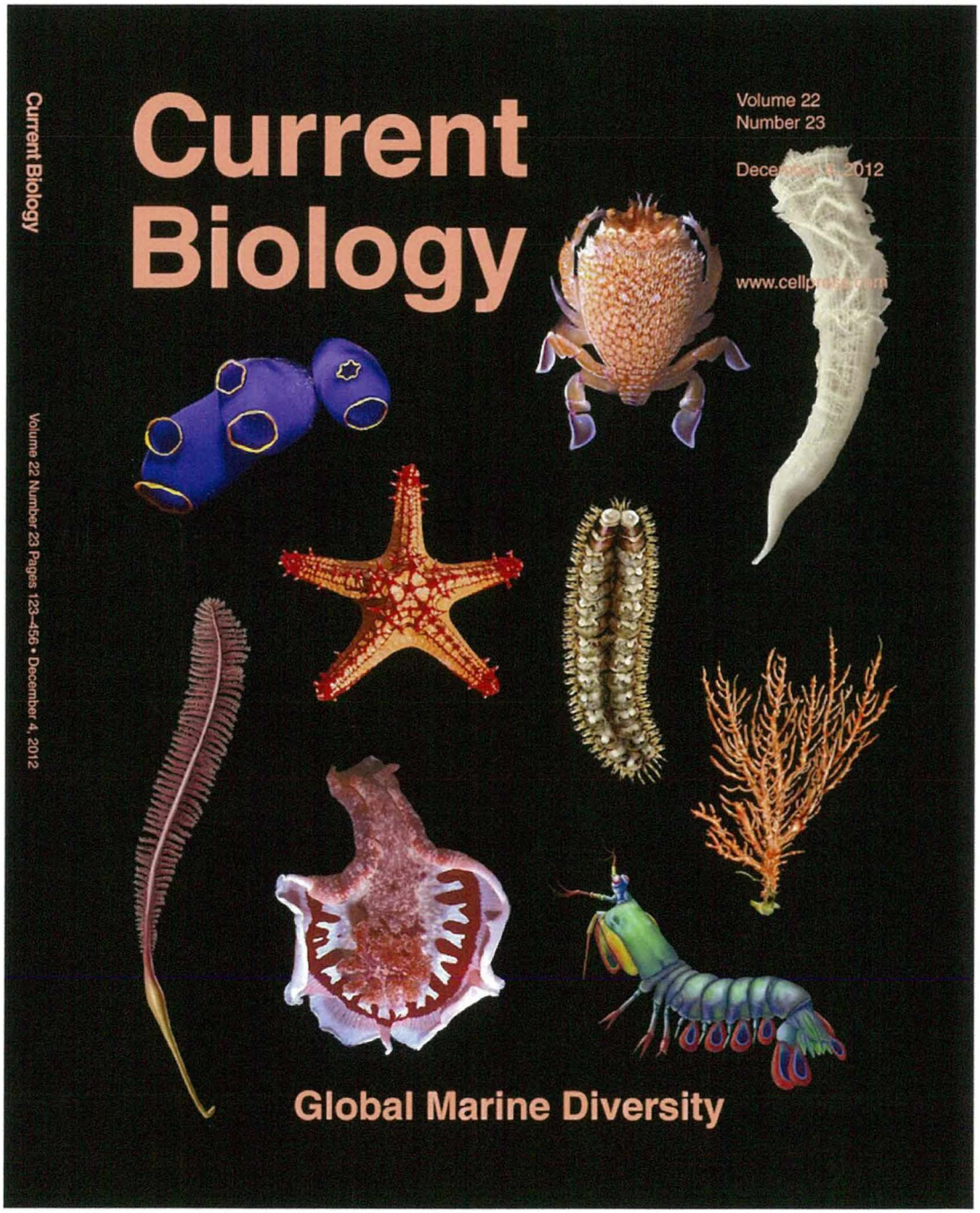
7.7 Concluding remarks

My ambition to discover previously unknown aspects of the biology of life in the most remote and captivating extremes of our globe, the polar regions, has materialised in the form of this PhD thesis. It marks the preliminary end of my quest into Arctic and Antarctic biology and my expeditionary endeavours from the Arctic at Spitsbergen and the Arctic Ocean as well as Lake Baikal to the Antarctic waters of McMurdo Sound. I hope to inspire others to venture into the challenging journey of polar research and possibly the intriguing Piscicolidae of the Ross Sea in one of the most remote and therefore still most pristine parts of our planet. The ultimate goal would be to enable our children to see the complexity of life and our global ecosystem from afar in the clarity and context as Plato described so vividly more than two millennia ago.

Finally, I urge the science community to act on the dire need for baseline information on host–parasite interactions in Antarctica. Such an undertaking is essential today in the context of climate change to elevate the predictive power for models of future species interactions and implications for the fragile and endangered polar biodiversity.

APPENDIX

A.1 Appeltans, W., Ahyong, S. T., & 119 WoRMS Taxonomic Editors (2012). The magnitude of global marine species diversity. *Current Biology, 22*(23), 2189–2202.



Please cite this article in press as: Appeltans et al., The Magnitude of Global Marine Species Diversity, Current Biology (2012), http://dx.doi.org/10.1016/j.cub.2012.09.036

 http://dx.doi.org/10.1016/j.cub.2012.09.036

Article

The Magnitude of Global Marine Species Diversity

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CURBIO 9885

Please cite this article in press as: Appeltans et al., The Magnitude of Global Marine Species Diversity, Current Biology (2012), http://dx.doi.org/10.1016/j.cub.2012.09.036

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CURBIO 9885

Please cite this article in press as: Appeltans et al., The Magnitude of Global Marine Species Diversity, Current Biology (2012), http://dx.doi.org/10.1016/j.cub.2012.09.036

Summary

Background: The question of how many marine species exist is important because it provides a metric for how much we do and do not know about life in the oceans. We have compiled the first register of the marine species of the world and used this baseline to estimate how many more species, partitioned among all major eukaryotic groups, may be discovered.

Results: There are ~226,000 eukaryotic marine species described. More species were described in the past decade (~20,000) than in any previous one. The number of authors describing new species has been increasing at a faster rate than the number of new species described in the past six decades. We report that there are ~170,000 synonyms, that 58,000–72,000 species are collected but not yet described, and that 482,000–741,000 more species have yet to be sampled. Molecular methods may add tens of thousands of cryptic species. Thus, there may be 0.7–1.0 million marine species. Past rates of description of new species indicate there may be 0.5 ± 0.2 million marine species. On average 37% (median 31%) of species in over 100 recent field studies around the world might be new to science.

Conclusions: Currently, between one-third and two-thirds of marine species may be undescribed, and previous estimates of there being well over one million marine species appear highly unlikely. More species than ever before are being described annually by an increasing number of authors. If the current trend continues, most species will be discovered this century.

Introduction

The most widely used metric of biodiversity is species richness, and much has been written about how many species may exist on land and in the sea [1–3]. Recent estimates of the number of extant described marine species vary from 150,000 to 274,000, and of those that may exist from 300,000 to over 10 million [4–14] (Table 1). Most of these estimates were made without the benefit of a global inventory of known marine species. The former estimates were based on experts' polls. The latter were based on extrapolation from past rates of description of species and higher taxa, proportions of undescribed species in samples, proportions that well-known taxa may represent of regional biota, and numbers of species in samples (Table 1). Here, we report on the near completion of such an inventory. The World Register of Marine Species (WoRMS) is an open-access online database created by an editorial board of 270 taxonomists from 146 institutions in 32 countries [15]. The first goal of WoRMS has been the compilation of a list of all taxonomically accepted marine species, commonly used synonyms, and key literature sources. Beyond complete taxonomic coverage, the longer-term aim is to provide or link to data on species distributions, biology, ecology, images, and guides to their identification. An important side benefit is that it facilitates communication within and beyond the taxonomic community, which can lead to increased rates of discovery of species and synonyms and a reduced rate of creation of new synonyms (and homonyms).

This collaborative database enabled the following set of marine biodiversity metrics to be compiled for the first time: (1) the number of nominal species, i.e., all species named, including those now recognized as synonyms due to multiple descriptions of the same species, and (2) the number of taxonomically accepted species, i.e., recognized species, excluding names that have been relegated to synonymy. In addition, we estimated the number of species that (3) have been collected but not yet described, (4) are undiscovered (unsampled), and (5) are molecular cryptics, i.e., only distinguishable by molecular analysis. Finally, we applied a statistical model that predicted how many more species might be discovered based on historical rates of species description and compared it with values from the above estimates. We omitted Bacteria and Archaea from our analysis because the species concept used for eukaryotes cannot be applied to these two taxa.

Our estimates of valid and nominal species are based on the WoRMS database as of February 17, 2012 and the literature on taxa for which WoRMS was not yet complete. The figures regarding species collected but not yet described, undiscovered, and cryptic are based on our own experience and that

Table 1. An Overview of the Estimated Numbers of Marine Species Described and Those that May Exist, as Published in the Literature

	Method	Reference (Year)
Number of Species Described		
150,000	expert opinion	van der Land [4] (1994)
160,000	expert opinion	Gordon [5] (2001)
204,000	expert opinion	Gibbons et al. [6] (1999)
222,000–230,000	inventory of 214,000 and expert opinion	present study
230,000	expert opinion	Bouchet [7] (2006)
250,000	literature and expert opinion	Winston [8] (1992)
274,000	expert opinion	Reaka-Kudla [9] (1996)
Number of Existing Species		
300,000	predicted based on description rate using WoRMS 2009	Costello et al. [10] (2012)
<500,000	proportion new species in samples	May [11] (1992)
320,000–760,000	predicted based on description rate using WoRMS 2012	stats model, present study
704,000–972,000	expert opinion	experts, present study
>1,000,000	expert opinion of proportions of undescribed species in regions of the world	Winston [8] (1992)
1,500,000	extrapolation from proportion of Brachyura in Europe	Bouchet [7] (2006)
2,200,000	extrapolation from rate of discovery of higher taxa	Mora et al. [12] (2011)
5,000,000	extrapolation from benthos samples off Australia	Poore and Wilson [13] (1993)
>10,000,000	extrapolation from deep-sea benthos samples	Grassle and Maciolek [14] (1992)

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of other experts, considering information on numbers of undescribed species that we observed in samples and our knowledge of particular habitats and geographic areas that remain little explored. The rationales for these estimates are provided in Table S2 available online. We each limited our estimates to groups for which we have close working knowledge. To indicate areas of uncertainty, we applied minimum and maximum estimates. The expert-opinion approach to estimating the magnitude of unknown biodiversity has been endorsed, for example, by Gaston [16] and used by many others (e.g., [7, 8]; Table 1). It complements macroecological approaches involving extrapolation from surrogate taxa, habitats, and/or geographic areas (reviewed in [2]). Our collective estimates are less likely to be biased than previous estimates made by fewer experts because we are most familiar with our particular taxa [17]. The 270 editors in WoRMS are among the world's top taxonomists. They represent ~5% of the active marine taxonomists today (based on ~4,900 marine taxonomists publishing during the last decade) and are involved in nearly one-third of new marine species descriptions in the past decade [15]. However, estimates based on expert opinion are subject to bias based on scientists' individual experiences, accuracy of their recollections and beliefs (e.g., how endemic a taxon is), and concerns about the consequences of their estimates on perceptions of the importance of their taxon [18]. For example, expert estimates tend to be optimistic [18], and they may feel it prudent to overestimate rather than underestimate the number of species in a taxon. Estimates can be substantially improved by combining empirical data with expert judgment [19]. Thus, we complemented the expert-opinion approach by fitting a statistical model with confidence limits to the species description rate for accepted species in WoRMS as of February 17, 2012 [20] (Supplemental Experimental Procedures). This model accounts for variation between years and identifies taxa whose rate of discovery is too variable for such extrapolation.

Results

Accepted Species

We recognized that 222,000–230,000 accepted eukaryotic marine species have been described. Of these, ~7,600 species belong to Plantae, ~19,500 to Chromista, ~550 to Protozoa, ~1,050 to Fungi, and nearly 200,000 to Animalia. We were unable to give a more precise number for Animalia due to the uncertainty in the total number of gastropod species (Table 2; see also Table S2).

Unaccepted Synonyms

Of ~400,000 species names established, ~170,000 (~40%) were currently not accepted, i.e., were synonyms (Table 2). This means that on average, for every five species described as new to science, at least two had already been described. The level of synonymy was greatest among the most-studied organisms, such as cetaceans, where 1,271 names existed for only 87 valid species. Taxa of which over 70% of names were considered synonyms were Cetacea, Reptilia, Sirenia, Sipuncula, Siphonophora, Zoantharia, and Bacillariophyceae. Taxa with over 50% synonymy rates included Pisces, Mollusca, Myriapoda, Scleractinia, Asteroidea, Pennatulacea, Chaetognatha, and Larvacea. Of the 170,000 synonyms we were aware of, 57,000 were entered into WoRMS. These entries indicated that the proportion of recognized synonyms has been steadily decreasing since the early 20th century (Figure 1). Of species described in the first decade of the 20th century, 25% were now synonyms, from the 1950s 15%, and the 1980s 5%. Adjusting for the fact that about 33% of synonyms were in WoRMS, and if this synonym trend was only due to the time it takes to discover synonyms, then a further 42,000 species remain to be synonymized since 1900.

Estimated Total Global Species Richness

Based on Past Rates of Species Descriptions

The marine species description rate has increased since the 1750s, with a very high discovery rate around 1900 (Figure 2). It declined during the two world wars and has recovered from 1950 to present. The curve dipped in the 1990s but has sharply increased again since 2000, with more than 20,000 marine species (9% of those currently known) described in the last decade. The number of marine species described per year reached all-time highs in the past decade, with over 2,000 species described in each of four different years (Figure 2).

The statistical model predicted a total of 540,000 marine species, with a 95% probability interval of 320,000 to 760,000. When limited to the different taxonomic groups, the estimates were comparable to or less than the experts' estimates (Table 2). For several taxonomic groups (especially where the majority of species remain to be described), the rate of discovery was still rising and the model could not make a meaningful estimate of total species numbers. This was the case for Acanthocephala, Polychaeta, Hirudinea, Oligochaeta, Cumacea, Isopoda, Tanaidacea, Copepoda, Ostracoda, Bryozoa, Cephalorhyncha, Chaetognatha, Hexacorallia, Octocorallia, Hydrozoa, Gastrotricha, Gnathostomulida, Bivalvia, Gastropoda, Cestoda, Digenea, and Porifera (Table 2).

Even in taxa of large body size or high economic value, new species continued to be discovered and described. Between 1999 and 2008, 780 new crabs, 29 lobsters, and 286 shrimps (of a total of 1,401 decapods), 1,565 marine fish, 4 sea snakes, and 3 new species and 7 subspecies of cetaceans [15] were described.

Our data also showed that the number of authors describing new species each year has been increasing, to 4,900 authors in the past decade (Figure 3). Moreover, the number of authors has been increasing faster than the number of new species. The number of valid species described per author decreased from between three to six species per year before 1900 to less than two species per author per year since the 1990s (Figure 3).

Based on Expert Opinion

Our collective estimates suggested that global marine species richness was between 704,000 and 972,000, so that only one-third to one-fourth of marine species have been described. However, this proportion varied greatly between taxa (Table 2). Of this number, 58,000–72,000 species, or 25%–30% of the known marine diversity, were already represented in specimen collections waiting to be described (Table 2). The estimated number of undiscovered molecular cryptic species was ~9,000–35,000 (Table 2) for 49 taxa that have a total of ~80,000 accepted described species—i.e., 11%–43% of their known species. Cryptic species were predicted not to occur in 9 taxa, and for 32 of the 98 remaining taxa, the experts did not have a basis on which to make an estimate. The proportion of cryptic species was highest in taxa with few externally visible diagnostic characters, such as Radiozoa, Placozoa, Hydrozoa, Zoantharia, Mesozoa,

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Table 2. Estimates of Known and Unknown Marine Species Diversity

			Total Known	Described (Accepted)	% Syn	Undescribed (Collected)	Undiscovered (Morpho)	Undiscovered (Molecular Cryptic)	Total Unknown (Experts)	Total Unknown (Model)	Total Estimated	% Known	New spp. (1999-2008)
Plantae			7,593							2,500-3,600	22,798-22,803	33	632
	Chlorophyta			1,300	19	?	1,200	-	1,200	-		52	
	Rhodophyta			6,150	49	?	14,000	-	14,000	-		31	
	Mangroves			75	29	?	0-5	-	0-5	-		94-100	
	Seagrasses			68	6	0	5	-	5	-		93	
Chromista			19,484							3,500-4,200	77,930-93,923	21-25	790
	Bigyra			76	?	?	75	-	75	-		50	
	Cercozoa			173	?	?	160	-	160	-		52	
	Ciliophora			2,615	39	?	1,058-4,648	3,173-14,526	4,231-19,174	-		12-38	
	Cryptophyta			88	?	?	150	-	150	-		38	
	Foraminifera			6,000	40	1,000	500	-	1,500	-		80	
	Haptophyta			241	?	?	100-150	-	100-150	-		62-71	
	Heliozoa			10	?	?	20	-	20	-		33	
	Myzozoa			2,688	?	?	575	-	575	-		82	
	Ochrophyta												
		Phaeophyceae		1,800	49	50	150-200	-	200-250	-		88-90	
		Bacillariophyceae		5,000	75	?	50,000	-	50,000	-		9	
		Chrysophyceae		51	-	?	1,000	-	1,000	-		5	
		Other Ochrophyta		283	?	?	100	-	100	-		62	
	Oomycota			43	?		225	-	225	-		16	
	Radiozoa			400	30	0	40	50-1,000	90-1,040	-		28-82	
Protozoa			542							150-400	2,207	25	23
	Amoebozoa			117	?	?	450	-	450	-		21	
	Apusozoa			3	?	?	15	-	15	-		17	
	Choanozoa			150	?	?	750	-	750	-		17	
	Euglenozoa			243	?	?	370	-	370	-		40	
	Excavata			29	?	?	80	-	80	-		27	
Fungi			1,035	1,035	10	200	14,800	-	15,000	1,100-1,500	16,035	6	125
Animalia													
	Acanthocephala		450	450	25	20	150	50-150	220-320	**	670-770	58-67	30
	Annelida		13,721								26,011-37,096	37-53	841
		Polychaeta		12,632	35	3,160	3,160	NB	6,320	**		67	
		Hirudinea		179	26	15-35	50-100	5-20	70-155	**		54-72	
		Oligochaeta		910	30	300	5,000-15,000	600-1,600	5,900-16,900	**		5-13	
	Arthropoda												
		Chelicerata	2,685							2,700-3,000	5,385-7,066	38-50	340
		Merostomata		4	-	1	0	NB	1	-		80	
		Pycnogonida		1,307	3	150-500	979-1,650	50-100	1,179-2,250	-		37-53	
		Acarina		1,218	-	100	1,220-1,830	150-200	1,470-2,130	-		36-45	
		Araneae		125	-	?	?	-	-	-		-	
		Pseudoscorpionda		31	-	?	?	-	-	-		-	
		Crustacea											
		Decapoda	12,029							4,500-5,100	21,073-24,204	50-57	1,611
		Dendrobranchiata		551	31	50	100	NB	150	-		79	
		Achelata		142	38	10	30-70	5-10	45-90	-		61-76	
		Chirostyloidea		206	2	250	580	10-55	840-885	-		19-20	

(Continued on next page)

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Table 2. *Continued*

		Total Known	Described (Accepted)	% Syn	Undescribed (Collected)	Undiscovered (Morpho)	Undiscovered (Molecular Cryptic)	Total Unknown (Experts)	Total Unknown (Model)	Total Estimated	% Known	New spp. (1999-2008)
	Galatheoidea		715	8	300	830	19-97	1,149-1,227	-		37-38	
	Hippoidea		81	19	3	10	NB	13	-		86	
	Lithodoidea		129	29	10	40	-	50	-		72	
	Lomisoidea		1	0	0	0	-	0	-		100	
	Paguroidea		1,106	17	150-200	400	NB	550-600	-		65-67	
	Enoplometopoidea		12	20	0	2-7	1-3	3-10	-		55-80	
	Glypheoidea		2	0	0	1-2	-	1-2	-		50-67	
	Nephropoidea		54	24	1	10-28	2-5	13-34	-		61-81	
	Brachyura		5,688	30	300	3,550-6,400	0	3,850-6,700	-		46-60	
	Procaridoidea		6	0	0	2	NB	2	-		75	
	Caridea		2,572	25	400	1,500	NB	1,900	-		58	
	Polychelida		38	27	0	7-15	1-3	8-18	-		69-83	
	Stenopodidea		68	16	10	50	NB	60	-		53	
	Gebiidea		203	10	50	100	-	150	-		58	
	Axiidea		455	10	50	200	-	250	-		65	
	Peracarida	17,115							**	132,297-228,231	7-13	2,276
	Amphipoda		6,947	-	?	20,000	-	20,000	4,600-4,300		26	
	Bochusacea		5	0	0	10	NB	10	-		33	
	Cumacea		1,444	2	45	6000	-	6,045	**		19	
	Isopoda		6,345	2	3,400	80,000-120,000	0	83,400-123,400	**		5-9	
	Lophogastrida		56	24	10	120	1-5	131-135	-		29-30	
	Mictacea		1	0	0	0	0	0	-		100	
	Mysida		1,180	32	80-100	2,000-4,000	10-20	2,090-4,120	340-450		22-36	
	Tanaidacea		1,130	6	900	22,600-56,500	NB	23,500-57,400	**		2-5	
	Thermosbaenacea		7	0	1	5	-	6	-		54	
	Other Crustacea	21,086								55,604-107,594	20-38	
	Branchiopoda		90	3	0	0	0	0	-		100	
	Cephalocarida		12	0	0	10	NB	10	-		55	
	Amphionidacea		1	-	0	0	0	0	-		100	
	Euphausiacea		86	42	0	0	0	0	-		100	
	Stomatopoda		468	19	52	200	-	252	-		65	
	Leptostraca		49	2	50-100	200-600	-	250-700	-		7-16	
	Branchiura		44	12	2-3	50-80	NB	52-83	-		35-46	
	Copepoda		10,000	17	1,500-2,000	28,500-48,000	125	30,125-50,125	**		17-25	
	Mystacocarida		13	0	1	10	NB	11	-		54	
	Pentastomida		10	-	?	?	-	-	-		-	
	Tantulocarida		36	0	60	1,000	NB	1,060	-		3	
	Thecostraca		1,400	7	?	100-200	NB	100-200	-		88-93	
	Ostracoda		8,653	7	1,000-2,000	1,825-32,000	NB	2,825-34,000	**		21-77	
	Remipeda		24	4	8	20-50	5-8	33-67	-		26-42	
	Hexapoda (Insecta and Collembola)	2,037	2,037	15	30-60	30-100	NB	60-160	110-250	2,097-2,197	93-97	30
	Myriapoda	61	61	58	?	190	-	190	-	251	24	2
Brachiopoda		388	388	-	0	?		-	65-175	388	?	21
Bryozoa		5,930	5,930	9	?	2,450-4,250	350-950	2,800-5,200	**	8,700-11,100	53-68	599
Cephalorhyncha		284							**	2,667-3,772	8-11	47
	Kinorhyncha		228	0	250-350	1,000-2,000		1,250-2,350			9-15	
	Loricifera		32	0	123	1,000	-	1,123	-		3	

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Table 2. Continued

		Total Known	Described (Accepted)	% Syn	Undescribed (Collected)	Undiscovered (Morpho)	Undiscovered (Molecular Cryptic)	Total Unknown (Experts)	Total Unknown (Model)	Total Estimated	% Known	New spp. (1998-2008)
	Nematomorpha		5	0	?	10-15	NB	10-15	-		25-33	
	Priapulida		19	-	?	?	-	-	-		-	
Chaetognatha		129	129	54	6-9	44	0-268	50-309	**	179-438	29-72	11
Chordata												
	Cephalochordata	33	33	-	?	?	-	-	-	33		
	Tunicata	3,020							2,700-4,300	4,600-5,100	59-66	391
	Ascidiacea		2,874	43	500	500-1,000	500	1,500-2,000	-		59-66	
	Larvacea		67	53	4	83	NB	87	-		50	
	Thaliacea		79	0	5	8	-	13	-		86	
	Pisces (incl. Agnatha)	16,733	16,733	49	500	4,200-4,300	200-300	4,900-5,100	6,700-10,700	21,833-21,833	77	1,577
	Mammalia	135							0-11	137-143	94-99	3
	Carnivora		44	14	0	0	-	0	-		100	
	Sirenia		4	89	0	0	0	0	-		100	
	Cetacea		87	93	0	1-5	1-3	2-8	-		92-98	
	Reptilia	110	110	82	?	20-30	-	20-30	-	130-140	79-85	4
	Aves	641	641	-	30-50	30-50	0	60-100	0-9	701-741	87-91	1
Cnidaria												
	Hexacorallia	3,152							**	3,976-5,105	62-79	286
	Actiniaria		1,093	25	?	?	NB		-		-	
	Antipatharia		250	11	50-75	50-100	NB	100-175	-		59-71	
	Ceriantharia		141	12	4-6	15-25	-	19-31	-		82-88	
	Corallimorpharia		47	15	?	?	NB	0	-		-	
	Zoantharia		101	78	30	150-330	60-760	270-1,170	-		8-27	
	Scleractinia		1,520	61	93	342	0-142	435-577	-		72-78	
	Octocorallia	3,171							**	4,671	65	290
	Alcyonacea, Helioporacea		2,951	18	100	1,500	NB	1,600	-		65	
	Pennatulacea		220	51	20	80	NB	100	-		69	
	Cubozoa	37	37	20	10-20	20-50	-	30-70	-	67-107	35-55	
	Hydrozoa (excl. Siphonophorae)	3,426	3,426	27	50-100	500-1,500	1,000-2,500	1,550-4,100	**	4,976-7,526	46-69	394
	Siphonophorae	176	176	74	50-60	50-60	0	100-120	-	276-296	59-64	
	Scyphozoa	201	201	1	35-80	77	22-25	137-182	-	338-383	52-59	
	Staurozoa	48	48	24	10-12	30-50	0-3	40-65	-	88-113	42-55	
Ctenophora		190	190	24	25-50	100-250	0-10	125-310	7-57	315-500	38-60	3
Cyclophora		2	2	0	3	10-125	-	13-128	-	15-130	2-13	1
Echinodermata		7,291							230-300	9,617-13,261	55-76	297
	Asteroidea		1,922	65	125-200	200-500	-	325-700	-		79-86	
	Echinoidea		999	37	20-50	45-150	306-1,080	371-1,280	-		44-73	
	Ophiuroidea		2,054	34	260-300	200-400	100-150	560-850	-		71-79	
	Crinoidea		623	32	20-30	50-100	-	70-130	-		83-90	
	Holothuroidea		1,693	20	200-400	800-2,600	-	1,000-3,000	-		36-63	
Echiura		175	175	14	5-10	30-40	-	35-50	12-44	210-225	78-83	5
Entoprocta		193	193	13	30	1,000	NB	1,030	16-57	1223	16	16
Gastrotricha		434	434	16	310	1,000-1,500	500-1,000	1,810-2,810	**	2,244-3,244	13-19	86
Gnathostomulida		98	98	10	15-20	200	NB	215-220	**	313-318	31	9
Hemichordata		118	118	7	10	?	-	10	0-2	128	?	4

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Table 2. *Continued*

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Mesozoa (Orthonectida, Dicyemida)		134	134	1	40-50	500-1,000	100-500	640-1,550	84-305	774-1,684	8-17	34
Mollusca		43,689-51,689							**	135,887-164,107	26-36	4,022
	Bivalvia		9,000	55	2000	3,000	-	5,000	**		64	
	Caudofoveata		133	8	?	500	-	500	-		21	
	Cephalopoda		781	-	150	500	-	650	-		54	
	Gastropoda		32,000-40,000	69-75	35,000-45,000	50,000-60,000	-	85,000-105,000	**		23-32	
	Monoplacophora		30	-	3	50	-	53	-		36	
	Polyplacophora		930	52	50	50-100	-	100-150	-		86-90	
	Scaphopoda		572	33	55	500	NB	555	-		51	
	Solenogastres		283	21	20-30	320-480	-	340-510	-		34-44	
Myzozoa		700	700	7	100-250	6,300-8,400	71-468	6,471-9,118	600-1,200	7,171-9,818	7-10	99
Nematoda		11,400								81,400	19	296
	Nematoda, free-living		6,900	9	?	50,000	NB	50,000	-		12	
	Nematoda, parasitic		4,500	-	?	?	-	-	-		-	
Nemertea		1,285	1,235	20	200-400	500-1,000	-	700-1,400	170-320	1,985-2,685	48-65	85
Phoronida		18	18	56	0	0	-	0	-	18	100	0
Placozoa		1	1	0	18	0	10-100	28-118	-	29-119	1-3	0
Platyhelminthes		11,690							3,000-3,900	35,296-73,441	16-33	1,142
	Cestoda		1,300	31	300	2,000	-	2,300	**		38	
	Monogenea		1,626	-	200-300	10,000-15,000	500-5,000	10,700-20,300	2,300-2,700		7-13	
	Aspidogastrea		18	25	0	6	-	6			75	
	Digenea		6,000	20	600	4,000-8,500	400-900	5,000-10,000	**		38-55	
	Catenulida		12	0	5	20	-	25	-		32	
	Rhabditophora		2,641	9	500-700	5,000-28,000	75-420	5,575-29,120	820-1,130		8-32	
Porifera		8,553	8,553	22	2,300-3,000	15,000	NB	17,300-18,000	**	25,853-26,553	32-33	621
Rotifera		114	114	-	20	?	300-2,500	320-2,520	20-140	434-2,634	4-26	17
Sipuncula		150	150	90	3-5	10-25	30-200	43-230	2-20	193-380	39-78	0
Tardigrada		183	183		?	1,120	-	1,120	40-250	1,303	14	16
Xenacoelomorpha		401							250-360	4,501	9	74
	Acoela		391	35	100	4,000	NB	4,100	-		9	
	Nemertodermatida		8	20	?	?	NB	0	-		-	
	Xenoturbellida		2	0	0	?	NB		-			
Total		222,201-230,201			58,279-72,326	415,205-533,872	8,792-35,753	482,776-741,951		704,977-972,152		

The following data are listed: number of currently described and taxonomically accepted species, percent of all nominal species names considered subjective synonyms (% Syn), undescribed species in specimen collections, unsampled and undiscovered morphospecies, undiscovered molecular cryptic species (only distinguishable by molecular methods), total species unknown (undescribed + undiscovered based on expert opinions), total species unknown based on the statistical model, total estimated number of species (expert-based), estimated percent of all existing species that are currently described (% known), and number of new species published in the last decade (1999–2008, data from WoRMS). Names of taxonomic groups for which data are broken down further by subgroups are listed in bold. The following symbols are used: ?, not estimated; -, no data; NB, no basis for judgment; **, rate of discovery still rising, so no meaningful estimate of total species numbers can be made using the statistical model.

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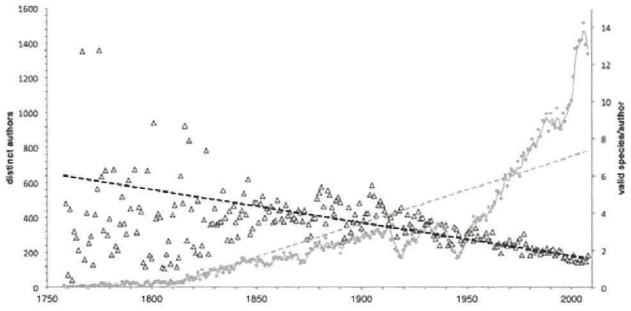

Figure 3. Number of Distinct Author Names per Year and Number of Valid Species per Author

The number of distinct author names per year (gray circles; solid gray line: two-year moving average; dashed gray line: linear with $r^2 = 0.721$) and the number of valid species per author (black triangles; dashed black line: linear with $r^2 = 0.056$).

than the median for New Zealand and the Southern Ocean (25% each). Averages for studies from Europe, deep sea, and tropics were close to the overall average (37%, 39%, and 33% respectively). These proportions can question estimates of total species richness. For example, the estimate of free-living nematode diversity reported here as 50,000 species suggests that 86% of the existing species remain to be discovered. Yet, field surveys have found only 6% to 56% undescribed species.

Discussion

Rate of Discovery

The description rate of marine species has been increasing steadily since 1955. Costello et al. [10] found a similar trend for marine and terrestrial (including freshwater) species, but the relative rate of description of marine species was higher than for terrestrial species. Evidently, the past decade has been the most productive period for marine species discovery. This may be due to more taxonomic effort, new technologies, exploration of new habitats and localities, use of molecular methods, or a combination of these factors.

Our analysis of temporal trends indicated a decreasing rate of species description (from six to two species per author per year) and an increased number of authors engaged in species descriptions. This increase in the number of taxonomists is likely to contribute to the continued high rates of species description. Other studies have similarly reported an increasing number of authors describing fossil North American mammals [21], marine fish [22], terrestrial vertebrates and plants in Brazil [23], flowering plants of the world [24, 25], cone snails, spiders, amphibians, birds, and mammals [25], as well as marine and nonmarine species globally [10].

The increasing number of authors publishing in taxonomy reflects the increasing number of scientists worldwide [26]. This has particularly been the case in Australasia and South America since the 1980s [27, 28]. The number of taxonomic publications has increased more than 8-fold from 1969 to 1996 [29]. Haas and Häuser [30] estimated there to be 5,000 professional and 35,000 amateur taxonomists worldwide. Our data suggest that this may be an underestimate. We found that 4,900 authors described marine species in the past decade alone, which accounted for about 12% of all species described. Although some of the marine taxonomists may also describe nonmarine species, this suggests that there are over 40,000 scientists involved in the taxonomic description of species. This number may be higher if the authors who could be considered taxonomists but have not recently described species are included, for example those who study taxa in well-studied geographic regions.

The change in the number of authors of species descriptions, a minimum indicator of authors involved in taxonomy, does not necessarily indicate increased taxonomic effort, because the individuals' effort may be declining. However, we found in WoRMS [15] that the proportion of authors who described only one species has been similar (42%–44%) over the past century. A previous study using WoRMS similarly found no trend in the proportion of the most prolific authors during that period [10].

The advent of scuba diving [31], deep-water tangle nets [32], submersibles, remotely operated vehicles (ROVs), and other technologies [22] has allowed sampling of previously unexplored habitats such as cold seeps, mud volcanoes, submarine canyons, and anchialine lakes and caves [33, 34] and of very fragile organisms previously unavailable to scientists [35]. For example, since 2002, the number of species of remipedes (crustaceans that live exclusively in coastal anchialine caves) has more than doubled from 11 to 24. The use of submersibles and deep diving resulted in the discovery of 30 new fish species around even such a highly studied area as the Galápagos Islands [22]. Thus, the greater number of taxonomists, the sampling of more remote geographic areas, and the use of a greater variety of sampling methods must all be contributing to the high rate of species description.

Molecular Methods and Cryptic Species

Estimating the diversity of cryptic species, i.e., species that remain unrecognized because of limitations of current morphotaxonomic methods, is a challenge because molecular surveys that most readily reveal them have been applied to only a fraction of marine diversity. For example, only 6,199 species (3% of all described) have been genetically "barcoded" by MarBOL (http://www.marinebarcoding.org, as of April 24, 2012). Furthermore, in all taxa except Placozoa (with only one species at present), these discoveries of "cryptic" species only apply to some of the presently known species, sometimes only within genera. For example, up to 18 cryptic species have been reported for parasite genera, but most (78%) only had one or two cryptic species [36]. It also needs to be considered that reports of cryptic species may be subject to sampling bias because these methods tend to be applied to taxa where positive findings are expected, and negative results may not be reported [36].

For two-thirds (in terms of described richness) of marine biota, experts were hesitant to provide, or indicated there was no good basis for, any estimate for the diversity of cryptic species, reflecting our poor understanding of this issue. For the remaining one-third, estimates ranged widely, reflecting the limited sampling and differences in the incidence of cryptic

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species among taxa. In some genera, molecular characters are more useful than morphological characters for distinguishing species (e.g., *Leptochonchus* gastropods [37]). In others, morphology is adequate to distinguish species, although molecular data can aid their classification. Thus in Pisces, a morphologically complex and visually communicating group of animals, the likely incidence of cryptic diversity is low, estimated here as ~1% of total diversity [38]. Most crustaceans have sufficient morphological characters to discriminate species, and so cryptic speciation may also be low (<5%) overall. Conversely, in Sipuncula, which have limited morphological complexity, cryptic species are estimated to represent between 10% and 55% of total diversity [39]. In some coral genera, molecular markers could better indicate the occurrence of cryptic species than reveal synonyms, because a lack of variation in one character does not necessarily suggest they are the same species [40]. Our knowledge is noticeably incomplete in the unicellular eukaryotes, where environmental sequencing is indicating that some of these groups may be more diverse than currently recognized based on conventional morphological taxonomy [41]. However, how this genetic diversity translates into species diversity is unknown.

Despite the uncertainty in the estimates of cryptic species, they help to illustrate the degree to which molecular methods may increase our knowledge of marine biodiversity, both in distinguishing and classifying species. Considering our numbers of cryptic species, molecular methods may add tens of thousands, rather than hundreds of thousands, of species to the currently accepted ~226,000 species. In a few cases, molecular methods have actually worked in reverse by assigning species to synonymy, though this is unlikely to have any more than a minor influence on total species numbers. Certainly, it is not valid to multiply up from examples of cryptic diversity discovered by molecular methods for a small group of species or genera to a phylum.

Synonyms

Our data showed that the proportion of described species that were later recognized to be synonyms of others was decreasing over time. This could be the result of fewer synonyms being created and/or could reflect the time it takes to discover synonyms. Taxa that had been studied more intensively tended to have more synonyms (e.g., fish, mollusks) but were also more likely to have had their taxonomy revised and thus more likely to have had such synonyms discovered. Even the same taxonomist can describe a species several times: for example, 9 of the sperm whale's 19 synonyms were by three authors, each naming the species three times [42]. With better access to publications and type specimens, improved communication among taxonomists, and the greater availability of systematic revisions, the introduction rate of synonyms should continue to decline.

Furthermore, molecular analyses complement morphological approaches and, where the latter are equivocal, have supported the raising of subspecies to species status [22]. For example, the killer whale and the common bottlenose dolphin have each been split into two or more species [43, 44]. WoRMS currently contains ~7,600 recognized infraspecific taxa (i.e., 3%). Molecular methods will also resurrect some names from synonymy. Assuming that pre-1900 names assigned to synonymy are mostly true synonyms, about 21,000 names of species described since 1900 were synonymized and another 42,000 may yet be synonymized due to the time delay in recognizing synonyms. It is highly unlikely that all 63,000 would be resurrected from synonymy by molecular methods. If all recognized subspecies and, say, 25% of synonyms were reestablished as accepted species, then the number of known species could increase by about 23,000.

The occurrence of as yet unrecognized synonyms is one of the most significant problems in estimating the true number of described species. Taxonomic revision may find more synonyms, but in some cases, often assisted by use of molecular methods, previously "sunken" species names may be found to be real. Although the significance of synonymy in biasing estimates of taxon and global species richness merits more in-depth study, action to reduce the reoccurrence of synonyms can be undertaken. This must include taxonomic revisions, rapid publication, open access to descriptions, online species identification guides, knowledge of where type specimens and genetic profiles are located, accessibility of taxonomic expertise, and continued revision of species inventories at global to local levels. An analysis of whether there is a trend of less time to discover synonyms could usefully clarify whether the creation of synonyms has been decreasing.

Global Species Richness

Both the sum of our individual estimates and the statistical analysis predicted that there were fewer than one million eukaryotic marine species on Earth. It was reassuring that the methods overlap, in contrast to most previous estimates, which have exceeded one million (Table 1). The estimates based on expert opinion were closest to ours, in the 1.0–1.5 million range. Winston [8] also considered the proportion of undescribed species in different geographic regions in her estimate of "over one million." This avoided extrapolation from one geographic area to the world, as was the case with the 5–10 million estimates. Local (α) diversity tends to overestimate regional (γ) diversity when few samples are available and thus spatial turnover (β diversity) is underestimated [45]. The relative species richness of higher taxa varies across geographic regions [46], although whether this is true or reflects variation in sampling and taxonomic effort is unclear. Further research is required before it can be assumed that the proportion that a higher taxon contributes to species richness in one region is the same as in other regions. Using the relationship of species richness in higher taxa to predict global species richness may compound several biases, including the changing proportions of species across higher taxa as classifications change, and dominance of richness by a few taxa. However, experts are not impartial [18]. They are subject to influence by such biases as the estimates of their peers and authority figures, widely reported hyperestimates, their personal experience and recollections, and not wishing to downplay the importance of their specialty. We have partly addressed this by independently eliciting experts by e-mail before exposing all experts to their peers' estimates. Experts were then asked to document their reasoning and review their numbers. This documentation was then compiled and circulated to experts, and they were asked to reconsider their estimates once again. Experts were not aware of the statistical model's predictions until a late stage in this process and thus did not consider them. By providing the rationale for our individual estimates (Table S2), we encourage them to be challenged as new data become available, as is the recommended best practice [19]. A future improvement on our approach may be to include direct discussion of all available

data and opinions between experts at a workshop or video conference [17].

Recent estimates of the richness of insects and terrestrial species have also been more modest, on the order of six million, compared to the 30–100 million species proposed by some authors (reviewed in [1, 10]). The same model we used here predicted that only 0.3 million marine species may exist on Earth using an earlier version of WoRMS [10]. This model is sensitive to the period of highest species description. Because the data now show that the highest marine species description rates occurred in the past decade, the present study predicted 0.5 million species. Both estimates will be inflated by undiscovered synonyms. Future modeling may be improved by distinguishing the taxa and geographic regions that are well known and by quantifying the effects of taxonomic effort.

Some of our higher estimates of undiscovered species may be questioned. Findings of high local species diversity do not necessarily imply high global species diversity [45]. Species with life stages that are easily dispersed (e.g., due to small body size, as in microbes, Fungi, and meiofauna) and can survive conditions suboptimal for growth tend to be cosmopolitan and thus have low spatial turnover (β diversity) in species (discussed in [10, 45]). This may be the case for the high predictions of undiscovered species for Fungi and Nematoda [47]. Indeed, one analysis suggested that there were 10,000–20,000 free-living marine nematodes [48] rather than the 50,000 listed in this paper. The present estimate of undiscovered Fungi was back calculated from an estimate of 1.5 million species on Earth, suggesting that only 7% of species are described. This seems unlikely by comparison with other taxa, and if there were so many undescribed species, one might expect the current rate of description to be relatively higher than it is for other taxa, because species would be easier to discover. However, comparable easily dispersed life stages are not common in macroinvertebrate taxa such as Crustacea (especially Isopoda, Tanaidacea, Amphipoda, Cumacea, and Leptostraca) and Mollusca, where thousands of undiscovered species are predicted as well. Moreover, more cosmopolitan species also tend to be discovered first, and the remaining species of such taxa are likely to be geographically rare (i.e., endemic to small areas). Thus, a particular problem in estimating global species richness is the lack of understanding of geographic patterns. It is well known that most species are geographically rare, but whether all taxa show similar β diversity is not clear. For example, are there equal proportions of parasitic and nonparasitic copepods that are cosmopolitan, and does the spatial occurrence of parasitic and symbiotic species scale similarly with their hosts? If taxa do scale similarly, then this will aid prediction of both global species richness and sensitivity to extinction [45]. However, the present evidence suggests that taxa have contrasting geographies, with pelagic megafauna (mammals, birds, reptiles) and meiofauna being more cosmopolitan than benthic macroinvertebrates (reviewed in [10]). Consequently, taxonomic research into this spectrum of rare and endemic species is critical for scientific discovery and to inform the selection of conservation priorities.

Field studies found that most samples have less than 37% undescribed species (median 31%), suggesting that our estimate of two-thirds to three-quarters of species being undiscovered may be too high rather than too low. However, field studies document common species better than rare species, whereas undescribed species are proportionally better represented among rare species. Because of this, field studies undersample undescribed species, except when they are exhaustive at the species level, a level of sampling that has yet to be attained in species-rich localities (see e.g. [49]). Alternatively, these averages may be overestimates because (1) authors do not report when all species in samples have been described or (2) upon closer analysis, some may prove not to be new to science (but are perhaps new to the observer). Europe has probably the best-studied sea area in the world, but one-third of its biodiversity may yet be undescribed [2]. Consequently, the proportion of undiscovered species is likely between one-third and two-thirds of all described marine species. However, this is a global figure, and some taxa provide exciting opportunities for discovering many new species, notably Mollusca, Rhabditophora, Oligochaeta, Tanaidacea, and Isopoda.

If we further consider that the number of authors describing species has been increasing at a higher rate than the number of new species described, then it seems that it has become harder to find new species [10]. If the description curves for taxa have not reached an asymptote because of the increasing taxonomic effort, then the model will overpredict marine species richness as well as bias our personal estimates. Consideration of the increasing effort suggests that we should be conservative in our estimates of the number of undiscovered species.

Rates of marine species description have never been higher and are driven by the increasing number of taxonomists and their ability to sample geographic areas and habitats previously undersampled. If the rate of 2,000 new species per year can be maintained by continued taxonomic effort and focus on the least-known places, habitats, and taxa, then another 100,000 species will be described in the next 50 years, and the number of described species will be within the 95% confidence limits of our statistical predictions.

As more species are described, the skills to diagnose them will be increasingly in demand. This applies to both the large, easily identified species that may be important for food, conservation, and ecosystem functioning and the less conspicuous taxa with small body size, because they will include parasites and pathogens of other species, may become pests, and may have as yet unrealized roles in ecosystem function.

The open-access online World Register of Marine Species has set the stage for our estimates of marine diversity. Collaborative international initiatives such as WoRMS help increase our knowledge, promote standardization in taxonomy, and bring the community together in a more coordinated and, because of the shared responsibility of maintaining the database, more sustainable way. We call on other taxonomic communities to similarly collaborate to publish online databases of their species as a synthesis of current knowledge and vehicle for improved scientific collaboration. The present study provides a baseline of the diversity of marine species and higher taxa, which the taxonomic editors of WoRMS should revisit in 5 to 10 years' time in the light of future discoveries.

Supplemental Information

Supplemental Information includes two tables and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.09.036.

Please cite this article in press as: Appeltans et al., The Magnitude of Global Marine Species Diversity, Current Biology (2012), http://dx.doi.org/10.1016/j.cub.2012.09.036

Acknowledgments

WoRMS has benefited from funding as part of several EU projects: Network of Excellence in Marine Biodiversity and Ecosystem Functioning (MarBEF), Pan-European Species directories Infrastructure (PESI), Distributed Dynamic Diversity Databases for Life (4D4Life), the Global Biodiversity Information Facility (GBIF), and the Census of Marine Life (CoML). It originated in the European Register of Marine Species (ERMS) that was funded by the EU Marine Science and Technology (MAST) research program. We thank Catherine McFadden (Harvey Mudd College, Claremont, CA) and Mark Brugman (University of Melbourne, Australia) for helpful discussion and the referees for helpful criticism. We wish to acknowledge the time contributed by many more scientists and their institutions in creating WoRMS than are currently listed in the author list. This paper is dedicated to Jacob van der Land (1935–2011), who began the compilation of a digital inventory of all marine species in the mid-1990s, which contributed to WoRMS.

Received: April 26, 2012
Revised: August 14, 2012
Accepted: September 18, 2012
Published: November 15, 2012

References

1. Hamilton, A.J., Basset, Y., Benke, K.K., Grimbacher, P.S., Miller, S.E., Novotný, V., Samuelson, G.A., Stork, N.E., Weiblen, G.D., and Yen, J.D. (2010). Quantifying uncertainty in estimation of tropical arthropod species richness. Am. Nat. *176*, 90–95.
2. Costello, M.J., and Wilson, S.P. (2011). Predicting the number of known and unknown species in European seas using rates of description. Glob. Ecol. Biogeogr. *20*, 319–330.
3. May, R.M. (2002). The future of biological diversity in a crowded world. Curr. Sci. *82*, 1325–1331.
4. van der Land, J. (1994). UNESCO-IOC Register of Marine Organisms: A common base for biodiversity inventories: Families and bibliography of keyworks, version 1.0 (DOS-formatted 3.5" floppy disk). http://www.marinespecies.org/urmo/.
5. Gordon, D.P. (2001). Marine Biodiversity (Wellington, New Zealand: The Royal Society of New Zealand).
6. Gibbons, M.J., Abiahy, B.B., Angel, M., Assuncao, C.M.L., Bartsch, I., Best, P., Biseswar, R., Bouillon, J., Bradford-Grieve, J.M., Branch, W., et al. (1999). The taxonomic richness of South Africa's marine fauna: crisis at hand. S. Afr. J. Sci. *95*, 8–12.
7. Bouchet, P. (2006). The magnitude of marine biodiversity. In The Exploration of Marine Biodiversity: Scientific and Technological Challenges, C.M. Duarte, ed. (Madrid: Fundación BBVA), pp. 31–62.
8. Winston, J.E. (1992). Systematics and marine conservation. In Systematics, Ecology, and the Biodiversity Crisis, N. Eldredge, ed. (New York: Columbia University Press).
9. Reaka-Kudla, M. (1996). The global biodiversity of coral reefs: a comparison with rain forests. In Biodiversity II: Understanding and Protecting our Biological Resources, M.L. Reaka-Kudla, D.E. Wilson, and E.O. Wilson, eds. (Washington, DC: Joseph Henry Press), pp. 83–108.
10. Costello, M.J., Wilson, S., and Houlding, B. (2012). Predicting total global species richness using rates of species description and estimates of taxonomic effort. Syst. Biol. *61*, 871–883.
11. May, R.M. (1992). Bottoms up for the oceans. Nature *357*, 278–279.
12. Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G.B., and Worm, B. (2011). How many species are there on Earth and in the ocean? PLoS Biol. *9*, e1001127.
13. Poore, G.C.B., and Wilson, G.D.F. (1993). Marine species richness. Nature *361*, 597–598.
14. Grassle, J.F., and Maciolek, N.J. (1992). Deep-sea species richness: regional and local diversity estimates from quantitative bottom samples. Am. Nat. *139*, 313–341.
15. Appeltans, W., Bouchet, P., Boxshall, G.A., Fauchald, K., Gordon, D.P., Hoeksema, B.W., Poore, G.C.B., van Soest, R.W.M., Stöhr, S., Walter, T.C., and Costello, M.J., eds. (2011). World Register of Marine Species (WoRMS). http://www.marinespecies.org.
16. Gaston, K.J. (1991). The magnitude of global insect species richness. Conserv. Biol. *5*, 283–296.
17. McBride, M.F., Garnett, S.T., Szabo, J.K., Burbidge, A.H., Butchart, S.H.M., Christidis, L., Dutson, G., Ford, H.A., Loyn, R.H., Watson, D.M., and Burgman, M.A. (2012). Structured elicitation of expert judgments for threatened species assessment: a case study on a continental scale using email. Methods Ecol. Evol. *3*, 906–920.
18. Burgman, M.A. (2004). Expert frailties in conservation risk assessment and listing decisions. In Threatened Species Legislation: Is It Just an Act?, P. Hutchings, D. Lunney, and C. Dickman, eds. (Mosman, Australia: Royal Zoological Society of New South Wales), pp. 20–29.
19. Martin, T.G., Burgman, M.A., Fidler, F., Kuhnert, P.M., Low-Choy, S., McBride, M., and Mengersen, K. (2012). Eliciting expert knowledge in conservation science. Conserv. Biol. *26*, 29–38.
20. Wilson, S.P., and Costello, M.J. (2005). Predicting future discoveries of European marine species by using a non-homogeneous renewal process. Appl. Stat. *54*, 897–918.
21. Alroy, J. (2002). How many named species are valid? Proc. Natl. Acad. Sci. USA *99*, 3706–3711.
22. Eschmeyer, W.N., Fricke, R., Fong, J.D., and Polack, D. (2010). Marine fish biodiversity: A history of knowledge and discovery (Pisces). Zootaxa *2525*, 19–50.
23. Pimm, S.L., Jenkins, C.N., Joppa, L.N., Roberts, D.L., and Russell, G.J. (2010). How many endangered species remain to be discovered in Brazil? Natureza & Conservação *8*, 71–77.
24. Joppa, L.N., Roberts, D.L., and Pimm, S.L. (2011). How many species of flowering plants are there? Proc. Biol. Sci. *278*, 554–559.
25. Joppa, L.N., Roberts, D.L., and Pimm, S.L. (2011). The population ecology and social behaviour of taxonomists. Trends Ecol. Evol. *26*, 551–553.
26. Ware, M., and Mabe, M. (2009). The STM Report: An Overview of Scientific and Scholarly Journal Publishing (Oxford: International Association of Scientific, Technical and Medical Publishers).
27. Zhang, Z.-Q. (2010). Reviving descriptive taxonomy after 250 years: Promising signs from a mega-journal in taxonomy. In Systema Naturae 250: The Linnaean Ark, A. Polaszek, ed. (Boca Raton, FL: CRC Press), pp. 95–107.
28. Gaston, K.J., and May, R.J. (1992). Taxonomy of taxonomists. Nature *356*, 281–282.
29. Winston, J.E., and Metzger, K.L. (1998). Trends in taxonomy revealed by the published literature. Bioscience *48*, 125–128.
30. Haas, F., and Häuser, C.L. (2005). Taxonomists: An endangered species? In Success Stories in Implementation of the Programmes of Work on Dry and Sub-Humid Lands and the Global Taxonomy Initiative: Abstracts of Poster Presentations at the 11th Meeting of the Subsidiary Body on Scientific, Technical and Technological Advice of the Convention on Biological Diversity, CBD Technical Series 21 (Montreal: Secretariat of the Convention on Biological Diversity), pp. 87–89.
31. Pyle, R.L. (2000). Assessing undiscovered fish biodiversity on deep coral reefs using advanced self-contained diving technology. Mar. Technol. Soc. J. *34*, 82–91.
32. Ng, P.K.L., Mendoza, J.C.E., and Manuel-Santos, M. (2009). Tangle net fishing, an indigenous method used in Balicasag Island, central Philippines. Raffles Bull. Zool. *20*(*Suppl.*), 39–46.
33. Becking, L.E., Renema, W., Santodomingo, N.K., Hoeksema, B.W., Tuti, Y., and de Voogd, N.J. (2011). Recently discovered landlocked basins in Indonesia reveal high habitat diversity in anchialine systems. Hydrobiologia *677*, 89–105.
34. Dennis, C., and Aldhous, P. (2004). Biodiversity: a tragedy with many players. Nature *430*, 396–398.
35. Haddock, S.H.D. (2004). A golden age of gelata: past and future research on planktonic cnidarians and ctenophores. Hydrobiologia *530/531*, 549–556.
36. Poulin, R. (2011). Uneven distribution of cryptic diversity among higher taxa of parasitic worms. Biol. Lett. *7*, 241–244.
37. Gittenberger, A., and Gittenberger, E. (2011). Cryptic, adaptive radiation of endoparasitic snails: sibling species of *Leptoconchus* (Gastropoda: Coralliomorphidae). Org. Divers. Evol. *11*, 21–41.
38. Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., and Hebert, P.D.N. (2005). DNA barcoding Australia's fish species. Philos. Trans. R. Soc. Lond. B Biol. Sci. *360*, 1847–1857.
39. Kawauchi, G.Y., and Giribet, G. (2010). Are there true cosmopolitan sipunculan worms? A genetic variation study within *Phascolosoma perlucens* (Sipuncula, Phascolosomatidae). Mar. Biol. *157*, 1417–1431.
40. McFadden, C.S., Alderslade, P., van Ofwegen, L.P., Johnsen, H., and Rusmevichientong, A. (2006). Phylogenetic relationships within the

Please cite this article in press as: Appeltans et al., The Magnitude of Global Marine Species Diversity, Current Biology (2012), http://dx.doi.org/10.1016/j.cub.2012.09.036

tropical soft coral genera *Sarcophyton* and *Lobophyton* (Anthozoa, Octocorallia). Invertebr. Biol. *125*, 288–305.

41. Liu, H., Probert, I., Uitz, J., Claustre, H., Aris-Brosou, S., Frada, M., Not, F., and de Vargas, C. (2009). Extreme diversity in noncalcifying haptophytes explains a major pigment paradox in open oceans. Proc. Natl. Acad. Sci. USA *106*, 12803–12808.
42. Perrin, W.F. (2011). The World Cetacea Database. http://www.marinespecies.org/cetacea.
43. Morin, P.A., Archer, F.I., Foote, A.D., Vilstrup, J., Allen, E.E., Wade, P., Durban, J., Parsons, K., Pitman, R., Li, L., et al. (2010). Complete mitochondrial genome phylogeographic analysis of killer whales (*Orcinus orca*) indicates multiple species. Genome Res. *20*, 908–916.
44. Möller, L.M., Bilgmann, K., Charlton-Robb, K., and Beheregaray, L. (2008). Multi-gene evidence for a new bottlenose dolphin species in southern Australia. Mol. Phylogenet. Evol. *49*, 674–681.
45. Stork, N.E. (1997). Measuring global biodiversity and its decline. In Biodiversity II: Understanding and Protecting our Biological Resources, M.L. Reaka-Kudla, D.E. Wilson, and E.O. Wilson, eds. (Washington, DC: Joseph Henry Press), pp. 41–68.
46. Costello, M.J., Coll, M., Danovaro, R., Halpin, P., Ojaveer, H., and Miloslavich, P. (2010). A census of marine biodiversity knowledge, resources, and future challenges. PLoS ONE *5*, e12110.
47. Vanreusel, A., Fonseca, G., Danovaro, R., da Silva, M.C., Esteves, A.M., Ferrero, T., Gad, G., Galtsova, V., Gambi, C., da Fonse Genevois, V., et al. (2010). The contribution of deep-sea macrohabitat heterogeneity to global nematode diversity. Mar. Ecol. *31*, 6–20.
48. Mokievsky, V., and Azovsky, A. (2002). Re-evaluation of species diversity patterns of free-living marine nematodes. Mar. Ecol. Prog. Ser. *238*, 101–108.
49. Bouchet, P., Lozouet, P., Maestrati, P., and Heros, V. (2002). Assessing the magnitude of species richness in tropical marine environments: exceptionally high numbers of molluscs at a New Caledonia site. Biol. J. Linn. Soc. Lond. *75*, 421–436.

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A.2 Theoretical background of MS important for AFGP analysis

A.2.1 Fundamental principles of MS

In MS a particular molecule is analysed by its M_r after ionisation, which means that the mass of ions deriving from the target molecule is measured. The molecular mass is given as the *atomic mass unit* [amu] in *dalton* [Da] or *unified atomic mass unit* [u].

A mass spectrum is a plot of signal *intensity* versus the dimensionless *mass–to–charge ratio m/z*. Each signal is called a *peak* and derives from a single ion. The abundance of an ion correlates to its measurement as relative intensity or *counts per second* (cps), with the most intense peak termed the *base peak*. The *molecular ion peak* M^+ represents the detection of the intact molecule of interest as a positively charged even–electron ion. This is most often, but not always, the peak at the highest *m/z* value in a series of signals and in most cases is equivalent to the base peak. Peaks close to the molecular ion are caused by fragmentation, dissociation or reaction of the M^+ ion with other molecules and represent fragment ions normally of lower *m/z*. For a single charged ion with *z* equals 1, the *m/z* scale of a mass spectrum is in fact, usually but not always, the atomic mass scale.

The deconvolution of MS raw data as *m/z* values can be done manually but is more often done by algorithms embedded in computer software to translate the signals of ion species detected in the instrument into the desired results of M_r as the sum of atomic masses for one particular peak.

It is important to realise that the data output of MS does not determine any unique composition of elements allowing an interpretation of the molecular constitutions and conformations. Rather, the result for a specific ion as its total sum of atomic units has to be related to preassigned masses of molecular units and their possible fragmentations. Consequently, the two monosaccharide units β–D–galactose and α–*N*–acetyl–D–galactosamine as well as their combination to β–D–galactosyl–(1→3)–α–*N*–acetyl–D–galactosamine can only be accounted for by their molecular masses as a hexose (Hex), aminohexose (HexNAc) or a hexose–hexosamine (HexNAc–Hex).

For the appropriate interpretation of MS data one therefore relies on previous experimentations as a prerequisite and quintessential basis for the correlation of ion peaks in mass spectra to their correct molecular representatives in the injected sample.

A.2.2 How does the MS analysis work?

The raw data obtained from MS was interpreted based on the following principles (following e.g. Ekman, et al., 2009; Gross, 2011; Ham, 2012). The tracing of a specific chemical or key component constitutes a challenge in experimentation and interpretation, especially when a biological system is under investigation. A living entity, such as a cell, and more so an entire organism, consists of a cocktail of organic molecules as well as inorganic ions. Such a complex mixture requires the segregation, ideally into individual samples each containing only a single substance, prior to mass spectrometric measurements.

Protein separation and purification is achieved by adsorption chromatography. A silica matrix as the stationary phase interacts with proteins and peptides of the sample in transfer as solutes in the mobile phase, which in the case of AFGPs are mainly hydrophobic and hydrogen–bonding interactions. The molecules undergo adsorption to the matrix and elute again at a later point into the mobile phase in a desorption process. This reverse step depends on pH and solvent strength in the mobile phase and can be fine–tuned to optimally suit the experiment, hence, the terminology *reversed phase chromatography* (RPC). The RPC method used for the separation process of AFGPs is based on a linear gradient elution. In this the composition of the mobile phase is not of a constant but of a changing ratio of two solutions, termed A and B. While A is the running buffer allowing adsorption to the matrix, B is the elution buffer of which the strength is increased in a linear gradient to allow for a gradual desorption of the proteins off the silica matrix and therefore separation with elution of proteins at different retention times. Such separation by retention is based on differences in the *hydrophobic foot* of a peptide or protein. It is defined by the primary amino acid sequence as well as conformation differences in proteins resulting from their secondary and tertiary structures.

The chemical properties of the silica matrix inside the column define its interactions with the proteins, thus the column characteristics are essential to the separation of the sample

mixture by retention processes. Effectively, the column stretches the sample into a chain of chemical partitions, with the smallest masses transitioning fastest. This process can be achieved using *chromatography*, either *gas chromatography* (GC), *liquid chromatography* (LC), or when high pressure pumps are used for work with low volatile samples such as AFGPs, *high pressure liquid chromatography* (HPLC).

The use of gas and liquid chromatography for high resolution sample separations with columns for the solid phase of 4–11 feet length (James & Martin, 1952) has not advanced in the way the authors predicted it would: *"Indeed it would seem feasible to use columns of the order of 100 metres long to carry out a particularly difficult separation."* Instead, reversed phase chromatography has allowed the separation of fatty acids on columns filled only with kieselguhr and of merely 11.7 cm length with 1–8 cm inner diameter (Wittenberg, 1957). This simple modification separated the equivalent of a C_2H_4 group in a mixture of hexanoic $C_6H_{12}O_2$, octanoic or caprylic $C_8H_{16}O_2$ and decanoic or capric acids $C_{10}H_{20}O_2$.

The ability of HPLC as a separation technique for isomers as molecules with similar structures was established early. For example a study investigated the separation of carbohydrates and flavonoids as typical organic compounds found in nature (Ward & Pelter, 1974). Here HPLC separated 2',7–dimethoxyflavone from its isomeric 2',7–dimethoxyisoflavone and 4',7–dimethoxyisoflavone, yet with different resolutions depending on the mobile phase. An improved clarity in peak separation and a shorter run time was achieved with isopropyl ether instead of hexane–tetrahydrofuran as the mobile phase, while the silica matrix remained unchanged and thus had no effect on separation results. This gives an insight into the potential complications using HPLC. Ward and Pelter (1974) also demonstrated that a mixture of three dipeptide derivatives, of which two were isomers with only a reverse order in amino acids, were separated and they fractioned carbohydrate isomers from methyl–D–glucopyranoside–2,3,6–tribenzoate based on the difference in one single asymmetric centre. Another example for the capacity of HPLC was the separation of molecules with very similar structures such as positional and configurational isomers in divinylbenzene (Lesec & Quivoron, 1979).

HPLC was recognised as prime method particularly in combination with MS for samples causing difficulties in GC (Arpino, Dawkins & McLafferty, 1974). In this paper McLafferty and his colleagues presented the first instrumentation for LC–MS analysis of eluates in a

continuous monitoring experiment. A mixture of three steroids (androstanolone, 5–α–3–androstanone and estrone methyl ether) were separated with the new apparatus running a linear gradient reversed phase HPLC (RP–HPLC) coupled with MS. The immense resolution power of this chromatography was demonstrated 30 years ago in the separation of rabbit from human insulin molecules (Rivier & McClintock, 1983). In this methodological study one small structural difference between the rabbit and human insulin molecules, in the form of a change at position 30 in the B chain from serine in rabbit to threonine in human, was sufficient for their separation by RP–HPLC. This demonstrates the effect a difference of a single methylene group ($=CH_2$) can have.

This technique was applied to Arctic fishes research a year later, where it also proved superior to other methods in separating AFPs from one another as well as from other blood proteins in a single run with the same resolution (Hew, Joshi & Wang, 1984). However, the authors commented on this independently or at least without referencing Rivier and McClintock. Hew and colleagues found that from a total of seven fractions in *Pseudopleuronectes americanus* the two main ones contain 37 amino acids yet differ by only one, which is sufficient for HPLC segregation. The difference is a replacement of glutamic acid in a smaller antifreeze molecule to aspartic acid in a larger AFP and equates again to the difference of one methylene group ($=CH_2$). This not only confirms the previous study and sensitivity of the method but also illustrates that RP–HPLC is the technique of choice for work on antifreezes in fishes with Fourney et al. (1984) confirming the method further. The procedure partitions AFPs as assorted and purified substances that can then be injected into the analytic chamber of the mass spectrometer for atomic mass measurements.

The first step in MS is the transfer of a molecule in solution into the gas phase (GP) as an ion, as only charged molecule species can be analysed in MS. This so–called *electron ionisation* (EI) is classically achieved by ionisation of a neutral molecule already evaporated in the GP with a beam of high energy electrons directed onto the neutral. This energy impact converts an entire molecule or its fragmented parts into ions by removing one or more electrons before the ions can disperse into the analytic chamber for mass analysis. However, for work on macromolecules with a mass of several kilodaltons, and biological samples that require the combination of LC and MS, there are two main methods to achieve ionisation of

such large neutrals: *Electrospray ionisation* (ESI) and *matrix–assisted (soft) laser desorption ionisation* (MALDI).

The ESI technique generates positively charged ions by single or multiple protonations, which are referred to as singly and multiply charged ions of the molecule and its fragments. ESI allows for the transfer of molecules into ions and at the same time from solution into GP, and as a soft ionisation technique works for large and non–volatile molecules such as antifreeze peptides. Consequently, ESI was used in this mass analytical work as being a simple and established technique for LC–MS/MS.

In naturally occurring stable peptides there is one hydrogen (H) at the N–terminus and a hydroxyl group (OH) at the C–terminal end associated with the amino acid backbone. In ESI spectra these can remain attached as the mass equivalent of one water molecule with the accurate formula mass of 18.0153 u. This must be considered in the interpretation of ESI–MS data. It translates for AFGP 8 with a sequence of AAT AAT PAT AAT PA and the complete loss of all sugar residues in the accurate formula mass of 1,184.6017 u with one water and 1,166.5864 u for the actual peptide backbone. Both ions carry at least one additional proton in ESI spectra, leading to an expectation of detecting two peaks of the molecular peptide ion at an accurate molecular mass of 1,185.6017 u and 1,167.5864 u.

The elongation by two AAT units of 243.1215 u each in AFGP 7 with a sequence of AAT AAT PAT AAT AAT AAT PA results in an accurate formula mass of 1,670.8447 u with one water and 1,652.8294 u for the actual peptide backbone. Both ions carry at least one additional proton in ESI spectra, leading to the expectation of detecting two peaks of the molecular peptide ion at 1,671.8447 u and 1,653.8294 u.

The positively charged ions now travel from the ion source to the detector through a field–free flight path of high quality vacuum and known length. The time it takes an ion to drift through this path is measured as *time–of–flight* (TOF) and depends solely on its mass, with lighter ions travelling faster and thus hitting the detector before heavier ones. This separates ions based on their mass and the ion's TOF directly correlates to *m/z* values. It is the simple principle behind molecule analysis in TOF–MS instruments.

In analytical applications, MS is often used in an experimental setup as *tandem mass spectrometry* (MS/MS). Here two mass analysers are arranged in succession, with a collision

cell, filled with the inert gas argon (Ar), helium (He) or as in this work nitrogen (N_2), between them (Fig. 4.2). The first mass spectrometer serves as a separator after which one *precursor ion* at a time is selected by its specific *m/z* ratio to pass through into the collision cell. Here it is subjected to a *collision–induced/activated dissociation* (CID/CAD) in which the precursor ion can further dissociate and fragment but also rearrange, react or isomerise before entering the second mass analyser as a series of newly formed *product ions*. The primary fragments continue to collide and dissociate into secondary fragments, leading to a high degree of fragmentations and thus more complex mass spectra with more information on the composition of the individual precursor ions and initially injected molecules. This collision cell thus allows for a further selection process within the instrumentation as well as the production of further ions to reconstruct the amino acid sequence of a protein.

Peptides are normally broken up using a low energy CID of less than 100 electronvolt (eV) and in this work between 20 eV to 40 eV was used. Each collision adds a small quantity of energy to the internal energy of the ion and this allows enough time for an internal distribution of the added energy amongst all atomic bonds before the next impact occurs. This causes the peptide backbone to dissociate at the amide bonds and the process results in sequence ions with the N–terminus as *b*–type and C–terminus as *y*–type product ions (Fig. 4.3), with the number of apostrophes indicating the often associated single or double protonation for *y*–fragments as y' and y'' respectively. It is important to note that the necessary proximity of atoms in the peptide backbone limits the fragmentation pathway to the production of *b*– and *y*–fragment ions by CID. In practice, this results in the absence of the b_1 and y_1 fragment ion peaks in the spectra.

The *m/z* selection step effectively filters out any other molecular fragments after the initial mass spectrometer. At the same time, it is the ultimate filtering process providing for a high purification step as well as enabling the mass analysis of one single molecular ion species at a time to produce highly reproducible results. The new array of product ions originating from one precursor ion entering the second mass analyser are detected by their *m/z* values and provides substantially more information on specific ions of interest.

This information together with the *monoisotopic molecular mass* of the entire peptide, as the sum of atoms containing only the lightest isotopes for each element N, C, O and H, facilitates the search for a suitable match in established databases for proteins and their

primary sequences. The *monoisotopic mass*, the exact mass of the most abundant isotope of an element, is used to calculate the sum of all elements known to compose the structural formula in order to obtain the *monoisotopic molecular mass*. This procedure ensures an accurate interpretation of MS data, as the underlying isotopic masses are the most likely representatives to contribute to the overall composition of a biological molecule. It should be noted that with increased molecular size the isotopic impact on the mass becomes relevant and can be detected in mass spectra as slight peak separations, termed *isotopic pattern,* for any given molecular fragment.

The two main reference sources are the National Centre for Biotechnology Information (NCBI) with its protein database NCBInr comprising most public databases in one, and the European Bioinformatics Institute with the Universal Protein Resource (UniProt) containing both the Swiss protein sequence database (Swiss–Prot) and the Translated European Molecular Biology Laboratory Nucleotide Sequence Data Library (TrEMBL) with extensive descriptions of protein functions, domain structures and posttranslational modifications (PTMs). Scientific authorities such as A.L. DeVries submit their protein data for antifreeze associated research to these databanks (Hsiao, et al., 1990).

The specific name for the entire application is reversed phase high pressure liquid chromatography–tandem mass spectrometry, RP–HPLC–MS/MS, which in literature is commonly abbreviated to LC–MS/MS.

The two step analysis of a tandem mass spectrometer allows for a *de novo* protein sequencing of a protein within the complex mixture of a biological tissue sample (Hunt, et al., 1981). It facilitates the measurement of an entire protein by MS with the intact amino acid sequence and without any proteolytic modifications, and is referred to as *"top–down" proteomics*. This differs from the earlier *"bottom–up" proteomics* reported in high impact publications by world leading authorities in MS such as Howard R. Morris and colleagues. In bottom–up proteomics, a target protein is initially digested by the Edman degradation (Edman, 1950), which removes one amino acid residue at a time starting at the N–terminal end, followed by analysis of the fragment mixtures using MS (Hughes, et al., 1975).

The first use of ESI–MS/MS with CID to sequence an intact protein was achieved on bovine ribonuclease A more than two decades ago (Loo, Edmonds & Smith, 1990). In this

work the authors stress the use of CID in tandem MS as being essential for the necessary fragmentation process required to sequence larger biomolecules with M_r greater than 4,000 u. The classic EI provides little structural information for such molecules as the collision energy is reduced by increased potential ion sizes.

However, the main associated problem for *de novo* protein sequencing lies at the very core of MS, where substances are identified by their respective mass determinations. The issues of isomers and isobars can hinder the interpretation of a correct molecule composition such as for the heterogeneous group of AFGP 7 and 8. In general, an isomer is a molecule of identical chemical formula but different structural arrangement. An isobar is a molecule with the identical or very similar molecular mass but of a different chemical formula and structure. The amino acid leucine has the isomer isoleucine, which has identical atomic mass 113.0841 u. Although they both can be incorporated in the protein, they cannot be differentiated by their respective masses. This is referred to as an isobaric situation and can occur with any of the remaining 18 amino acids, as numerous reactions within the instrument can result in residues of identical molecular masses. Consequently, it is only possible to obtain an unambiguous peptide sequence if by chance every single peptide bond is broken, with a representative species detected in the product ion spectrum after the collision cell in tandem MS. The accuracy of the instrument used for mass determination at high resolution, that is low signal–to–noise ratios, becomes of utmost importance and is achieved in modern MS.

A.3 Video file of ice crystal behaviour in *Cryobdella* spp. samples (on CD)

BIBLIOGRAPHY

Adams, S. L. (1988). The medicinal leech: A page from the annelids of internal medicine. *Annals of Internal Medicine, 109*(5), 399-405.

Ahlgren, J. A., Cheng, C.-H. C., Schrag, J. D., DeVries, A. L. (1988). Freezing avoidance and the distribution of antifreeze glycopeptides in body fluids and tissues of Antarctic fish. *Journal of Experimental Biology, 137*, 549-563.

Ahlgren, J. A., DeVries, A. L. (1984). Comparison of antifreeze glycopeptides from several Antarctic fishes. *Polar Biology, 3*(2), 93-97.

Ahmed, A. I., Osuga, D. T., Feeney, R. E. (1973). Antifreeze glycoprotein from an Antarctic fish - effects of chemical modifications of carbohydrate residues on antifreeze and antilectin activities. *Journal of Biological Chemistry, 248*(24), 8524-8527.

Ahn, M., Murugan, R. N., Kim, E., Lee, J. H., Cheong, C., Kang, S. W., et al. (2012). Studies on the effect of number of sugar moiety in the antifreeze activity of homodimeric AFGPs. *Bulletin of the Korean Chemical Society, 33*(7), 2411-2414.

Åkerman, E. Å. (1927). *Studien über den Kältetod und die Kälteresistenz der Pflanzen nebst Untersuchungen über die Winterfestigkeit des Weizens [Studies on the death by freezing and the cold resistance of plants together with investigations on the winter hardiness of wheat]* (J. B. Kolb, Trans.). Lund: Berlingska Boktryckeriet.

Albesa-Jové, D., Giganti, D., Jackson, M., Alzari, P. M., Guerin, M. E. (2014). Structure-function relationships of membrane-associated GT-B glycosyltransferases. *Glycobiology, 24*(2), 108-124.

Anderson, M. E. (1990). Zoarcidae - eelpouts. In O. Gon & P. C. Heemstra (Eds.), *Fishes of the Southern Ocean* (pp. 256-276). Grahamstown: J.L.B. Smith Institute of Ichthyology.

Andrade, E. N. d. C. (1953). Robert Hooke, 1635-1703. *Nature, 171*(4348), 365-367.

Appeltans, W., Ahyong, S. T., & 119 WoRMS Taxonomic Editors (2012). The magnitude of global marine species diversity. *Current Biology, 22*(23), 2189-2202.

Arctic Climate Impact Assessment (2005). *Arctic climate impact assessment - scientific report*. New York: Cambridge University Press.

Arpino, P. J., Dawkins, B. G., McLafferty, F. W. (1974). A liquid chromatography/mass spectrometry system providing continuous monitoring with nanogram sensitivity. *Journal of Chromatographic Science, 12*(10), 574-578.

Arribas, O., Ilgaz, C., Kumlutas, Y., Durmus, S. H., Avci, A., Uzum, N. (2013). External morphology and osteology of *Darevskia rudis* (Bedriaga, 1886), with a taxonomic revision of the Pontic and Small-Caucasus populations (Squamata: Lacertidae). *Zootaxa, 3626*(4), 401-428.

Asahina, É. (1953). Analysis of the freezing process of living organisms. X. Freezing process of egg cell of sea-urchin. *Low Temperature Science, Series B, 10*, 81-92.

Asahina, É. (1956). The freezing process of plant cell. *Contributions from the Institute of Low Temperature Science, 10*, 83-126.

Asahina, É. (1959a). Frost-resistance in a nematode, *Aphelenchoides ritzemabosi*. *Low Temperature Science, Series B, 17*, 51-62.

Asahina, É. (1959b). Prefreezing as a method enabling animals to survive freezing at an extremely low temperature. *Nature, 184*(4691), 1003-1004.

Asahina, É. (1961). Intracellular freezing and frost resistance in egg-cells of the sea urchin. *Nature, 191*(479), 1263-1265.

Asahina, É., Aoki, K. (1958). Survival of intact insects immersed in liquid oxygen without any antifreeze agent. *Nature, 182*(4631), 327-328.

Asahina, É., Aoki, K., Shinozaki, J. (1954). The freezing process of frost-hardy caterpillars. *Bulletin of Entomological Research, 45*(2), 329-339.

Atici, O., Nalbantoglu, B. (2003). Antifreeze proteins in higher plants. *Phytochemistry, 64*(7), 1187-1196.

Autrum, H. (1939). Hirudineen. Geographische Verbreitung [Hirudineans. Geographical distribution] (J. B. Kolb, Trans.). In H. G. Bronn (Ed.), *Dr. H.G. Bronn's Klassen und Ordnungen des Tierreichs, wissenschaftlich dargestellt in Wort und Bild* (Vol. 4, Abteilung III, Buch 4, Teil 2, pp. 497-520). Leipzig, Germany: C. F. Winter'sche Verlagshandlung.

Aznar, F. J., Balbuena, J. A., Bush, A. O., Raga, J. A. (1997). Ontogenetic habitat selection by *Hadwenius pontoporiae* (Digenea: Campulidae) in the intestine of Franciscanas (Cetacea). *Journal of Parasitology, 83*(1), 13-18.

Bailly, N. (2013). *Trematomus pennellii* (Regan, 1914). In R. Froese & D. Pauly (Eds.), *FishBase*: Accessed through: World Register of Marine Species at http://www.marinespecies.org/aphia.php?p=taxdetails&id=234709.

Balcerzak, A. K., Febbraro, M., Ben, R. N. (2013). The importance of hydrophobic moieties in ice recrystallization inhibitors. *Journal of the Royal Society of Chemistry Advances, 3*(10), 3232-3236.

Bar-Dolev, M., Celik, Y., Wettlaufer, J. S., Davies, P. L., Braslavsky, I. (2012). New insights into ice growth and melting modifications by antifreeze proteins. *Journal of the Royal Society Interface, 9*(77), 3249-3259.

Bargelloni, L., Ritchie, P. A., Patarnello, T., Battaglia, B., Lambert, D. M., Meyer, A. (1994). Molecular evolution at subzero temperatures - mitochondrial and nuclear phylogenies of fishes from Antarctica (Suborder Pototheniоidei), and the evolution of antifreeze glycopeptides. *Molecular Biology and Evolution, 11*(6), 854-863.

Barvkar, V. T., Pardeshi, V. C., Kale, S. M., Kadoo, N. Y., Gupta, V. S. (2012). Phylogenomic analysis of UDP glycosyltransferase 1 multigene family in *Linum usitatissimum* identified genes with varied expression patterns. *BioMed Central Genomics, 13*(175), 1-13.

Bé, A. W. H. (1969). Planktonic Foraminifera. In A. W. H. Bé (Ed.), *Distribution of Selected Groups of Marine Invertebrates in Waters South of 35°S Latitude* (Vol. 11, pp. 9-12). New York: Academic Press; Antarctic Map Folio Series, American Geographical Society.

Beers, J. M., Sidell, B. D. (2011). Thermal tolerance of Antarctic notothenioid fishes correlates with level of circulating hemoglobin. *Physiological and Biochemical Zoology, 84*(4), 353-362.

Bennett, E. P., Mandel, U., Clausen, H., Gerken, T. A., Fritz, T. A., Tabak, L. A. (2012). Control of mucin-type O-glycosylation: A classification of the polypeptide GalNAc-transferase gene family. *Glycobiology, 22*(6), 736-756.

Bielecki, A., Rokicka, M., Ropelewska, E., Dziekońska-Rynko, J. (2008). Leeches (Hirudinida: Piscicolidae) - parasites of Antarctic fish from Channichthyidae family. *Wiadomości Parazytologiczne, 54*(4), 345-348.

Bilyk, K. T., DeVries, A. L. (2011). Heat tolerance and its plasticity in Antarctic fishes. *Comparative Biochemistry and Physiology A - Molecular and Integrative Physiology, 158*(4), 382-390.

Bilyk, K. T., DeVries, A. L. (2012). Heat tolerance of the secondarily temperate Antarctic notothenioid, *Notothenia angustata*. *Antarctic Science, 24*(2), 165-172.

Bilyk, K. T., Evans, C. W., DeVries, A. L. (2012). Heat hardening in Antarctic notothenioid fishes. *Polar Biology, 35*(9), 1447-1451.

Birch, N. P., Estivariz, F. E., Bennett, H. P. J., Loh, Y. P. (1991). Differential glycosylation of N-POMC$^{1-77}$ regulates the production of γ_3-MSH by purified pro-opiomelanocortin converting enzyme. A possible mechanism for tissue-specific processing. *Federation of European Biochemical Societies Letters, 290*(1-2), 191-194.

Black, V. S. (1951). II. Osmotic regulations in teleost fishes. In W. S. Hoar, V. S. Black & E. C. Black (Eds.), *Some aspects of the physiology of fish.* (Vol. 71, Publication Ontario Fishery Research Laboratory, No. 59, pp. 53-89). Toronto: University of Toronto Press.

Bone, Q., Moore, R. H. (2008). *Biology of fishes* (3 ed.). New York: Taylor and Francis.

Borodin, N. A. (1934). The anabiosis or phenomenon of resuscitation of fishes after being frozen. *Zoologische Jahrbücher, Abteilung für allgemeine Zoologie und Physiologie der Tiere, 53*, 313-342.

Boto, L. (2010). Horizontal gene transfer in evolution: Facts and challenges. *Proceedings of the Royal Society B - Biological Sciences, 277*(1683), 819-827.

Bowie, A. R., Griffiths, F. B., Dehairs, F., Trull, T. W. (2011). Oceanography of the subantarctic and polar frontal zones south of Australia during summer: Setting for the SAZ-sense study. *Deep-Sea Research Part II - Topical Studies in Oceanography, 58*(21-22), 2059-2070.

Bowles, D., Lim, E.-K., Poppenberger, B., Vaistij, F. E. (2006). Glycosyltransferases of lipophilic small molecules. *Annual Review of Plant Biology, 57*, 567-597.

Boyle, R. (1683). *New experiments and observations touching cold, or, an experimental history of cold* (2 ed.). London: Richard Davis.

Breinl, F., Haurowitz, F. (1932). Aenderungen der Spezifität von Immunseren nach chemischer Vorbehandlung [Changes in the specificity of immune sera after chemical pretreatment] (J. B. Kolb, Trans.). *Zeitschrift für Immunitätsforschung und experimentelle Therapie, 77*, 176-186

Breton, C., Snajdrova, L., Jeanneau, C., Koca, J., Imberty, A. (2006). Structures and mechanisms of glycosyltransferases. *Glycobiology, 16*, 29R-37R, 2005.

Brinkhuis, M., Wijnaendts, L. C. D., van der Linden, J. C., van Unnik, A. J. M., Voûte, P. A., Baak, J. P. A., et al. (1995). Peripheral primitive neuroectodermal tumour and extra-osseous Ewing's sarcoma; a histological, immunohistochemical and DNA flow cytometric study. *Virchows Archiv, 425*(6), 611-616.

Bruand, J., Sistla, S., Meriaux, C., Dorrestein, P. C., Gaasterland, T., Ghassemian, M., et al. (2011). Automated querying and identification of novel peptides using MALDI mass spectrometric imaging. *Journal of Proteome Research, 10*(4), 1915-1928.

Burreson, E. M. (2006). Phylum Annelida: Hirudinea as vectors and diseases agents. In P. T. K. Woo (Ed.), *Fish Diseases and Disorders - Protozoan and Metazoan Infections* (2 ed., Vol. 1, pp. 566-591). Wallingford, UK: CAB International.

Bush, C. A., Feeney, R. E., Osuga, D. T., Ralapati, S., Yeh, Y. (1981). Antifreeze glycoprotein. Conformational model based on vacuum ultraviolet circular dichroism data. *International Journal of Peptide and Protein Research, 17*(1), 125-129.

Büsing, K.-H. (1951). *Pseudomonas hirudinis*, ein bakterieller Darmsymbiont des Blutegels (*Hirudo officinalis*) [*Pseudomonas hirudinis*, a bacterial symbiont in the gut of the

leech (*Hirudo officinalis*)] (J. B. Kolb, Trans.). *Zentralblatt für Bakteriologie und Parasitenkunde, 157*(7), 478-484.

Büsing, K.-H., Döll, W., Freytag, K. (1953). Die Bakterienflora der medizinischen Blutegel [The bacterial flora of medicinal leeches] (J. B. Kolb, Trans.). *Archiv für Mikrobiologie, 19*(1), 52-86.

Cameron, A. T., Brownlee, T. I. (1913). The effect of low temperature on the frog. *Proceedings and Transactions of the Royal Society of Canada - Series 3, 7*(4), 107-124.

Campbell, J. A., Davies, G. J., Bulone, V., Henrissat, B. (1997). A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochemical Journal, 326*(3), 929-941.

Cattoretti, G., Pileri, S., Parravicini, C., Becker, M. H. G., Poggi, S., Bifulco, C., et al. (1993). Antigen unmasking on formalin-fixed, paraffin-embedded tissue-sections. *Journal of Pathology, 171*(2), 83-98.

Celik, Y., Drori, R., Pertaya-Braun, N., Altan, A., Barton, T., Bar-Dolev, M., et al. (2013). Microfluidic experiments reveal that antifreeze proteins bound to ice crystals suffice to prevent their growth. *Proceedings of the National Academy of Sciences of the United States of America, 110*(4), 1309-1314.

Celik, Y., Graham, L. A., Mok, Y. F., Bar, M., Davies, P. L., Braslavsky, I. (2010). Superheating of ice crystals in antifreeze protein solutions. *Proceedings of the National Academy of Sciences of the United States of America, 107*(12), 5423-5428.

Chakrabartty, A., Hew, C. L. (1991). The effect of enhanced α-helicity on the activity of a winter flounder antifreeze polypeptide. *European Journal of Biochemistry, 202*(3), 1057-1063.

Chakrabartty, A., Yang, D. S. C., Hew, C. L. (1989). Structure-function relationship in a winter flounder antifreeze polypeptide. II. Alteration of the component growth rates of ice by synthetic antifreeze polypeptides. *Journal of Biological Chemistry, 264*(19), 11313-11316.

Chambers, R., Hale, H. P. (1932). The formation of ice in protoplasm. *Proceedings of the Royal Society of London. Series B - Containing Papers of a Biological Character, 110*(767), 336-352.

Chandramouli, K. H., Sun, J., Mok, F. S., Liu, L., Qiu, J. W., Ravasi, T., et al. (2013). Transcriptome and quantitative proteome analysis reveals molecular processes associated with larval metamorphosis in the polychaete *Pseudopolydora vexillosa*. *Journal of Proteome Research, 12*(3), 1344-1358.

Chandramouli, K. H., Zhang, Y., Wong, Y. H., Qian, P.-Y. (2012). Comparative glycoproteome analysis: Dynamics of protein glycosylation during metamorphic transition from pelagic to benthic life stages in three invertebrates. *Journal of Proteome Research, 11*(2), 1330-1340.

Chang, H. W., Bock, E., Bonilla, E. (1989). Dystrophin in electric organ of *Torpedo californica* homologous to that in human muscle. *Journal of Biological Chemistry, 264*(35), 20831-20834.

Chao, H., DeLuca, C. I., Davies, P. L. (1995). Mixing antifreeze protein types changes ice crystal morphology without affecting antifreeze activity. *Febs Letters, 357*(2), 183-186.

Chen, L. B., DeVries, A. L., Cheng, C.-H. C. (1997a). Convergent evolution of antifreeze glycoproteins in Antarctic notothenioid fish and Arctic cod. *Proceedings of the National Academy of Sciences of the United States of America, 94*(8), 3817-3822.

Chen, L. B., DeVries, A. L., Cheng, C.-H. C. (1997b). Evolution of antifreeze glycoprotein gene from a trypsinogen gene in Antarctic notothenioid fish. *Proceedings of the National Academy of Sciences of the United States of America, 94*(8), 3811-3816.

Cheng, C.-H. C. (1996). Genomic basis for antifreeze glycopeptide heterogeneity and abundance in Antarctic notothenioid fishes. In S. J. Ennion & G. Goldspink (Eds.), *Gene Expression and Manipulation in Aquatic Organisms* (Vol. 58, pp. 1-20). Cambridge: Cambridge University Press.

Cheng, C.-H. C. (1998). Evolution of the diverse antifreeze proteins. *Current Opinion in Genetics and Development, 8*(6), 715-720.

Cheng, C.-H. C., Detrich III, H. W. (2007). Molecular ecophysiology of Antarctic notothenioid fishes. *Philosophical Transactions of the Royal Society of London Series B - Biological Sciences, 362*(1488), 2215-2232.

Cheng, C.-H. C., DeVries, A. L. (1989). Structures of antifreeze peptides from the antarctic eel pout, *Austrolycicthys brachycephalus*. *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology, 997*(1-2), 55-64.

Clarke, A., Johnston, N. M. (2003). Antarctic marine benthic diversity. In R. N. Gibson & R. J. A. Atkinson (Eds.), *Oceanography and Marine Biology: An Annual Review* (Vol. 41, pp. 47-114). London: Taylor and Francis.

Clarke, A., Johnston, N. M., Murphy, E. J., Rogers, A. D. (2007). Introduction. Antarctic ecology from genes to ecosystems: The impact of climate change and the importance of scale. *Philosophical Transactions of the Royal Society of London Series B - Biological Sciences, 362*(1477), 5-9.

Comer, F. I., Hart, G. W. (2000). O-glycosylation of nuclear and cytosolic proteins: Dynamic interplay between O-GlcNAc and O-phosphate. *Journal of Biological Chemistry, 275*(38), 29179-29182.

Coons, A. H., Creech, H. J., Jones, R. N. (1941). Immunological properties of an antibody containing a fluorescent group. *Proceedings of the Society for Experimental Biology and Medicine, 47*(2), 200-202.

Coons, A. H., Creech, H. J., Jones, R. N., Berliner, E. (1942). The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *Journal of Immunology, 45*(3), 159-170.

Corcilius, L., Santhakumar, G., Stone, R. S., Capicciotti, C. J., Joseph, S., Matthews, J. M., et al. (2013). Synthesis of peptides and glycopeptides with polyproline II helical topology as potential antifreeze molecules. *Bioorganic and Medicinal Chemistry, 21*(12), 3569-3581.

Costanzo, J. P., Lee, R. E. (2013). Avoidance and tolerance of freezing in ectothermic vertebrates. *Journal of Experimental Biology, 216*(11), 1961-1967.

Costello, M. J., Emblow, C. S., White, R. (Eds.). (2001). *European register of marine species. A check-list of the marine species in Europe and a bibliography of guides to their identification. Collection patrimoines naturels* (Vol. 50). Paris: Muséum national d'histoire naturelle.

Costello, M. J., Wilson, S. P. (2011). Predicting the number of known and unknown species in European seas using rates of description. *Global Ecology and Biogeography, 20*(2), 319-330.

Coulson, S. J., Refseth, D. (2004). The terrestrial and freshwater invertebrate fauna of Svalbard (and Jan Mayen). In P. Prestrud, H. Strøm & H. Goldman (Eds.), *A Catalogue of the Terrestrial and Marine Animals of Svalbard* (Vol. Skrifter 201, pp. 57-122). Tromsø, Norway: Norwegian Polar Institute.

Cuevas, E. C., Bateman, A. C., Wilkins, B. S., Johnson, P. A., Williams, J. H., Lee, A. H. S., et al. (1994). Microwave antigen retrieval in immunocytochemistry: A study of 80 antibodies. *Journal of Clinical Pathology, 47*(5), 448-452.

Darwin, C. (1859). *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life* (1 ed.). London: John Murray.

Dawkins, R., Krebs, J. R. (1979). Arms races between and within species. *Proceedings of the Royal Society of London B - Biological Sciences, 205*(1161), 489-511.

Dayton, P. K., Robilliard, G. A., DeVries, A. L. (1969). Anchor ice formation in McMurdo Sound, Antarctica, and its biological effects. *Science, 163*(3864), 273-274.

De Broyer, C., Danis, B., & 64 SCAR-MarBIN Taxonomic Editors (2011). How many species in the Southern Ocean? Towards a dynamic inventory of the Antarctic marine species. *Deep-Sea Research Part II - Topical Studies in Oceanography, 58*(1-2), 5-17.

Deacon, G. E. R. (1982). Physical and biological zonation in the Southern Ocean. *Deep-Sea Research Part A - Oceanographic Research Papers, 29*(1), 1-15.

Denny, M., Dorgan, K. M., Evangelista, D., Hettinger, A., Leichter, J., Ruder, W. C., et al. (2011). Anchor ice and benthic disturbance in shallow Antarctic waters: Interspecific variation in initiation and propagation of ice crystals. *Biological Bulletin, 221*(2), 155-163.

DeVries, A. L. (1971). Glycoproteins as biological antifreeze agents in Antarctic fishes. *Science, 172*(3988), 1152-1155.

DeVries, A. L. (1980). Biological antifreezes and survival in freezing environments. In R. Gilles (Ed.), *Animals and Environmental Fitness* (Vol. 1, pp. 583-607). Oxford: Pergamon Press.

DeVries, A. L. (1982). Biological antifreeze agents in coldwater fishes. *Comparative Biochemistry and Physiology Part A - Physiology, 73*(4), 627-640.

DeVries, A. L. (1983). Antifreeze peptides and glycopeptides in cold-water fishes. *Annual Review of Physiology, 45*, 245-260.

DeVries, A. L. (1986). Antifreeze glycopeptides and peptides: Interactions with ice and water. *Methods in Enzymology, 127*, 293-303.

DeVries, A. L. (1988). The role of antifreeze glycopeptides and peptides in the freezing avoidance of antarctic fishes. *Comparative Biochemistry and Physiology B - Biochemistry and Molecular Biology, 90*(3), 611-621.

DeVries, A. L., Cheng, C.-H. C. (2005). Antifreeze proteins and organismal freezing avoidance in polar fishes. In A. P. Farrell & J. F. Steffensen (Eds.), *The Physiology of Polar Fishes* (Vol. 22, pp. 155-201). San Diego: Elsevier Academic Press.

DeVries, A. L., Komatsu, S. K., Feeney, R. E. (1970). Chemical and physical properties of freezing point-depressing glycoproteins from Antarctic fishes. *Journal of Biological Chemistry, 245*(11), 2901-2908.

DeVries, A. L., Steffensen, J. F. (2005). The Arctic and Antarctic polar marine environments. In A. P. Farrell & J. F. Steffensen (Eds.), *The Physiology of Polar Fishes* (Vol. 22, pp. 1-24). San Diego: Elsevier Academic Press.

DeVries, A. L., Vandenheede, J., Feeney, R. E. (1971). Primary structure of freezing point-depressing glycoproteins. *Journal of Biological Chemistry, 246*(2), 305-308.

DeVries, A. L., Wohlschlag, D. E. (1969). Freezing resistance in some Antarctic fishes. *Science, 163*(3871), 1073-1075.

DeWitt, H. H. (1962). A new genus and species of zoarcid fish from McMurdo Sound, Antarctica. *Copeia, 1962*(4), 819-826.

DeWitt, H. H., Heemstra, P. C., Gon, O. (1990). Nototheniidae - notothens. In O. Gon & P. C. Heemstra (Eds.), *Fishes of the Southern Ocean* (pp. 279-331). Grahamstown: J.L.B. Smith Institute of Ichthyology.

Dickson, W. A., Boothman, P., Hare, K. (1984). An unusual source of hospital wound infection. *British Medical Journal, 289*(6460), 1727-1728.

Dieckmann, G., Rohardt, G., Hellmer, H., Kipfstuhl, J. (1986). The occurrence of ice platelets at 250 m depth near the Filchner ice shelf and its significance for sea ice biology. *Deep Sea Research Part A - Oceanographic Research Papers, 33*(2), 141-148.

Diesing, K. M. (1850). *Systema helminthum* (Vol. 1). Vienna: Braumüller.

Dinand, F., Bottenberg, H. (1935). Experimentelle Studien zur Blutegelbehandlung [Experimental studies on leech therapy] (J. B. Kolb, Trans.). *Die medizinische Welt, 32*, 1147-1149.

Dobzhansky, T. G. (1937). *Genetics and the origin of species* (1 ed.). New York: Columbia University Press.

Dönhoff, H. (1872). Beiträge zur Physiologie I. Über das Verhalten kaltblütiger Tiere gegen Frosttemperatur [Contributions to physiology I. On the behaviour of cold-blooded animals at freezing temperature] (J. B. Kolb, Trans.). *Archiv für Anatomie und Physiologie*, 724-727.

Dorsey, N. E. (1948). The freezing of supercooled water. *Transactions of the American Philosophical Society, New Series, 38*(3), 247-328.

Drozdov, A. L., Vinnikova, V. V., Zezina, O. N., Tyurin, S. A. (2012). Morphology of gametes of mollusks, echinoderms, and brachiopods in systematics and phylogeny. *Paleontological Journal, 46*(8), 936-944.

Duman, J. G. (1979). Subzero temperature tolerance in spiders: The role of thermal-hysteresis-factors. *Journal of Comparative Physiology B - Biochemical Systemic and Environmental Physiology, 131*(4), 347-352.

Duman, J. G., DeVries, A. L. (1974). Freezing resistance in winter flounder *Pseudopleuronectes americanus*. *Nature, 247*(5438), 237-238.

Duman, J. G., DeVries, A. L. (1975). The role of macromolecular antifreezes in cold water fishes. *Comparative Biochemistry and Physiology, 52A*, 193-199.

Duman, J. G., Verleye, D., Li, N. (2002). Site-specific forms of antifreeze protein in the beetle *Dendroides canadensis*. *Journal of Comparative Physiology B - Biochemical Systemic and Environmental Physiology, 172*(6), 547-552.

Eastman, J. T. (1993). *Antarctic fish biology: Evolution in a unique environment*. San Diego: Academic Press.

Edman, P. (1950). Method for determination of the amino acid sequence in peptides. *Acta Chemica Scandinavica, 4*(2), 283-293.

Ekman, R., Silberring, J., Westman-Brinkmalm, A., Kraj, A. (Eds.). (2009). *Mass spectrometry: Instrumentation, interpretation, and applications* (Vol. 21, Issue 1). Hoboken: John Wiley and Sons.

Engl, W. (2012). *Shells of Antarctica*. Hackenheim: ConchBooks.

Epshtein, V. M. (1968). Zoological analysis of the fish leeches in the Antarctic and a revision of the genus *Trachelobdella* (Diesing, 1850) *Fifth All-Union Conference of the Diseases and Parasites of Fishes and Marine Invertebrates. Leningrad* (pp. 137-138). Leningrad: Nauka Dumka. (English translation, National Research Council of Canadian Libraries, Ottawa, Ontario, 1974).

Epshtein, V. M. (1970). Fish leeches (Hirudinea: Piscicolidae) in the Antarctic seas from the collection of the Zoological Institute of the Academy of Sciences of the USSR. In V. A.

Volyanitsky (Ed.), *Problems of Marine Parasitology. Materials of the First All-Union Symposium on Parasites and Diseases of Marine Animals, Sevastopol, 1970* (pp. 146-149). Kiev: Nauka Dumka. (English translation: National Research Council of Canadian Libraries, Ottawa, Ontario, 1974).

Epshtein, V. M., Utevsky, A. Y. (1994). A new marine leech *Cryobdella ljadovi* sp.n. from Antarctic seas (Hirudinea: Piscicolidae). *Zoosystematica Rossica, 3*(1), 23-25.

Epshtein, V. M., Utevsky, A. Y., Utevsky, S. Y. (1994). The system of fish leeches (Hirudinea: Piscicolidae). *Genus, 5*(4), 401-409.

Erséus, C., Healy, B. M. (2001). Oligochaeta. In M. J. Costello, C. S. Emblow & R. White (Eds.), *European register of marine species. A check-list of the marine species in Europe and a bibliography of guides to their identification. Collection patrimoines naturels* (Vol. 50, pp. 231-234). Paris: Muséum national d'histoire naturelle.

Evans, C. W., Gubala, V., Nooney, R., Williams, D. E., Brimble, M. A., DeVries, A. L. (2011). How do Antarctic notothenioid fishes cope with internal ice? A novel function for antifreeze glycoproteins. *Antarctic Science, 23*(1), 57-64.

Evans, C. W., Hellman, L., Middleditch, M., Wojnar, J. M., Brimble, M. A., DeVries, A. L. (2012). Synthesis and recycling of antifreeze glycoproteins in polar fishes. *Antarctic Science, 24*(3), 259-268.

Fabbrizio, E., Léger, J., Anoal, M., Léger, J. J., Mornet, D. (1993). Monoclonal antibodies targeted against the C-terminal domain of dystrophin or utrophin. *Febs Letters, 322*(1), 10-14.

Fetsch, P. A., Abati, A. (2010). The clinical immunohistochemistry laboratory: Regulations and troubleshooting guidelines. *Methods in Molecular Biology, 588*, 399-412.

Fisher, R. A. (1930). *The genetical theory of natural selection* (1 ed.). Oxford: Clarendon Press.

Fletcher, G. L. (1977). Circannual cycles of blood plasma freezing point and Na^+ and Cl^- concentrations in Newfoundland winter flounder (*Pseudopleuronectes americanus*): Correlation with water temperature and photoperiod. *Canadian Journal of Zoology, 55*(5), 789-795.

Fletcher, G. L., Kao, M. H., Fourney, R. M. (1986). Antifreeze peptides confer freezing resistance to fish. *Canadian Journal of Zoology, 64*(9), 1897-1901.

Florkin, M. (1949). *Biochemical evolution* (S. Morgulis, Trans. 1st English ed.). New York: Academic Press.

Florkin, M. (1966). *A molecular approach to phylogeny*. Amsterdam: Elsevier Scientific.

Florkin, M., Swain, T. (1974). Comparative biochemistry, molecular evolution. In M. Florkin & E. H. Stotz (Eds.), *Comprehensive Biochemistry* (Vol. 29, Part A). Amsterdam: Elsevier Scientific.

Fourney, R. M., Hew, C. L., Joshi, S. B., Fletcher, G. L. (1984). Comparison of antifreeze polypeptides from Newfoundland, Nova Scotia, New Brunswick and Long Island winter flounder. *Comparative Biochemistry and Physiology Part B - Comparative Biochemistry, 78*(4), 791-796.

Franco, O. L., Rigden, D. J. (2003). Fold recognition analysis of glycosyltransferase families: Further members of structural superfamilies. *Glycobiology, 13*(10), 707-712.

Franklin, J. (1824a). *Narrative of a journey to the shores of the polar sea, in the years 1819-20-21-22* (3 ed. Vol. 1). London: John Murray.

Franklin, J. (1824b). *Narrative of a journey to the shores of the polar sea, in the years 1819-20-21-22* (3 ed. Vol. 2). London: John Murray.

Fuiman, L. A., Davis, R. W., Williams, T. M. (2002). Behavior of midwater fishes under the Antarctic ice: Observations by a predator. *Marine Biology, 140*(4), 815-822.

Gachon, C. M. M., Langlois-Meurinne, M., Saindrenan, P. (2005). Plant secondary metabolism glycosyltransferases: The emerging functional analysis. *Trends in Plant Science, 10*(11), 542-549.

Gaede-Koehler, A., Kreider, A., Canfield, P., Kleemeier, M., Grunwald, I. (2012). Direct measurement of the thermal hysteresis of antifreeze proteins (AFPs) using sonocrystallization. *Analytical Chemistry, 84*(23), 10229-10235.

Ge, X. L., Wang, X. D. (2009). Calculations of freezing point depression, boiling point elevation, vapor pressure and enthalpies of vaporization of electrolyte solutions by a modified three-characteristic parameter correlation model. *Journal of Solution Chemistry, 38*(9), 1097-1117.

Gerlach, S. A. (1965). Über die Fauna in der Gezeitenzone von Spitzbergen [On the fauna in the intertidal zone of Spitsbergen] (J. B. Kolb, Trans.). *Botanica Gothoburgensia - Proceedings of the 5th Marine Biological Symposium, Göteborg, 3*, 81-92.

Gilbert, J. A., Davies, P. L., Laybourn-Parry, J. (2005). A hyperactive, Ca^{2+}-dependent antifreeze protein in an Antarctic bacterium. *Fems Microbiology Letters, 245*(1), 67-72.

Gill, D. J., Clausen, H., Bard, F. (2011). Location, location, location: New insights into O-GalNAc protein glycosylation. *Trends in Cell Biology, 21*(3), 149-158.

Goffredi, S. K., Morella, N. M., Pulcrano, M. E. (2012). Affiliations between bacteria and marine fish leeches (Piscicolidae), with emphasis on a deep-sea species from Monterey Canyon, CA. *Environmental Microbiology, 14*(9), 2429-2444.

Gon, O., Heemstra, P. C. (Eds.). (1990). *Fishes of the Southern Ocean* (1 ed.). Grahamstown: J.L.B. Smith Institute of Ichthyology.

Graf, J. (1999). Symbiosis of *Aeromonas veronii* biovar sobria and *Hirudo medicinalis*, the medicinal leech: A novel model for digestive tract associations. *Infection and Immunity, 67*(1), 1-7.

Graf, J. (2002). The effect of symbionts on the physiology of *Hirudo medicinalis*, the medicinal leech. *Invertebrate Reproduction and Development, 41*(1-3), 269-275.

Graham, L. A., Li, J. Y., Davidson, W. S., Davies, P. L. (2012). Smelt was the likely beneficiary of an antifreeze gene laterally transferred between fishes. *BMC Evolutionary Biology, 12*(190), doi:10.1186/1471-2148-1112-1190.

Graham, L. A., Liou, Y. C., Walker, V. K., Davies, P. L. (1997). Hyperactive antifreeze protein from beetles. *Nature, 388*(6644), 727-728.

Graham, L. A., Lougheed, S. C., Ewart, K. V., Davies, P. L. (2008). Lateral transfer of a Lectin-like antifreeze protein gene in fishes. *Plos One, 3*(7), e2616. doi:2610.1371/journal.pone.0002616.

Graham, L. A., Walker, V. K., Davies, P. L. (2000). Developmental and environmental regulation of antifreeze proteins in the mealworm beetle *Tenebrio molitor*. *European Journal of Biochemistry, 267*(21), 6452-6458.

Griffiths, H. J. (2010). Antarctic marine biodiversity - what do we know about the distribution of life in the Southern Ocean? *Plos One, 5*(8), 11.

Groseclose, M. R., Andersson, M., Hardesty, W. M., Caprioli, R. M. (2007). Identification of proteins directly from tissue: In situ tryptic digestions coupled with imaging mass spectrometry. *Journal of Mass Spectrometry, 42*(2), 254-262.

Gross, J. H. (2011). *Mass spectrometry: A textbook* (2 ed.). Berlin: Springer.

Gubler, U., Seeburg, P., Hoffman, B. J., Gage, L. P., Udenfriend, S. (1982). Molecular cloning establishes proenkephalin as precursor of enkephalin-containing peptides. *Nature, 295*(5846), 206-208.

Guenther, G. G., Peralta, E. R., Rosales, K. R., Wong, S. Y., Siskind, L. J., Edinger, A. L. (2008). Ceramide starves cells to death by downregulating nutrient transporter proteins. *Proceedings of the National Academy of Sciences of the United States of America, 105*(45), 17402-17407.

Gutmann, A., Nidetzky, B. (2013). Enzymatic C-glycosylation: Insights from the study of a complementary pair of plant O- and C-glucosyltransferases. *Pure and Applied Chemistry, 85*(9), 1865-1877.

Gutt, J. (2001). On the direct impact of ice on marine benthic communities, a review. *Polar Biology, 24*(8), 553-564.

Hakim, A., Nguyen, J. B., Basu, K., Zhu, D. F., Thakral, D., Davies, P. L., et al. (2013). Crystal structure of an insect antifreeze protein and its implications for ice binding. *Journal of Biological Chemistry, 288*(17), 12295-12304.

Ham, B. M. (2012). *Proteomics of biological systems: Protein phosphorylation using mass spectrometry techniques.* Hoboken: John Wiley and Sons.

Hannun, Y. A., Luberto, C., Argraves, K. M. (2001). Enzymes of sphingolipid metabolism: From modular to integrative signaling. *Biochemistry, 40*(16), 4893-4903.

Hansen, T. N., Baust, J. G. (1988). Differential scanning calorimetric analysis of antifreeze protein activity in the common mealworm, *Tenebrio molitor. Biochimica et Biophysica Acta, 957*(2), 217-221.

Harding, W. A. (1922). Hirudinea. In Trustees of the British Museum (Ed.), *British Museum Natural History. British Antarctic ("Terra Nova") Expedition, 1910. Natural History Reports. Zoology. Collecting Stations, Mollusca, Brachiopoda, and Worms* (1 ed., Vol. 2, No. 11, pp. 257-261 plus one plate). London: British Museum.

Hargens, A. R., Shabica, S. V. (1973). Protection against lethal freezing temperatures by mucus in an Antarctic limpet. *Cryobiology, 10*(4), 331-337.

Harmer, S. F., Lillie, D. G. (1914). List of collecting stations. In Trustees of the British Museum (Ed.), *British Museum Natural History. British Antarctic ("Terra Nova") Expedition, 1910. Natural History Reports. Zoology. Collecting Stations, Mollusca, Brachiopoda, and Worms* (1 ed., Vol. 2, No. 1, pp. 1-12 plus four maps). London: British Museum.

Hashimoto, K., Tokimatsu, T., Kawano, S., Yoshizawa, A. C., Okuda, S., Goto, S., et al. (2009). Comprehensive analysis of glycosyltransferases in eukaryotic genomes for structural and functional characterization of glycans. *Carbohydrate Research, 344*(7), 881-887.

Hawes, T. C., Marshall, C. J., Wharton, D. A. (2011). Antifreeze proteins in the Antarctic springtail, *Gressittacantha terranova. Journal of Comparative Physiology B, 181*(6), 713-719.

Hayat, M. A. (2002). *Microscopy, immunohistochemistry, and antigen retrieval methods for light and electron microscopy* (1 ed. Vol. 19). New York: Springer.

Haymet, A. D. J., Ward, L. G., Harding, M. M. (1999). Winter flounder "antifreeze" proteins: Synthesis and ice growth inhibition of analogues that probe the relative importance of hydrophobic and hydrogen-bonding interactions. *Journal of the American Chemical Society, 121*(5), 941-948.

Hedgpeth, J. W. (1969). Introduction to Antarctic zoogeography. In A. W. H. Bé (Ed.), *Distribution of Selected Groups of Marine Invertebrates in Waters South of 35°S Latitude* (Vol. 11, pp. 1-9). New York: Academic Press; Antarctic Map Folio Series, American Geographical Society.

Heidelberger, M., Kendall, F. E., Soo Hoo, C. M. (1933). Quantitative studies on the precipitin reaction: Antibody production in rabbits injected with an azo protein. *Journal of Experimental Medicine, 58*(2), 137-152.

Hew, C. L., Joshi, S., Wang, N. C. (1984). Analysis of fish antifreeze polypeptides by reversed-phase high-performance liquid chromatography. *Journal of Chromatography, 296*, 213-219.

Hew, C. L., Slaughter, D., Fletcher, G. L., Joshi, S. B. (1981). Antifreeze glycoproteins in the plasma of Newfoundland Atlantic cod (*Gadus morhua*). *Canadian Journal of Zoology, 59*(11), 2186-2192.

Hoffman, E. P., Hudecki, M. S., Rosenberg, P. A., Pollina, C. M., Kunkel, L. M. (1988). Cell and fiber-type distribution of dystrophin. *Neuron, 1*(5), 411-420.

Holzinger, D., Flechtenmacher, C., Henfling, N., Kaden, I., Grabe, N., Lahrmann, B., et al. (2013). Identification of oropharyngeal squamous cell carcinomas with active HPV16 involvement by immunohistochemical analysis of the retinoblastoma protein pathway. *International Journal of Cancer, 133*(6), 1389-1399.

Hooke, R. (1665). *Micrographia: Or some physiological descriptions of minute bodies made by magnifying glasses with observations and inquiries thereupon* (1 ed.). London: John Martyn and James Allestry, Printers for the Royal Society of London, at the Bell in St. Pauls Churchyard.

Hornbostel, H. (1942). Über die bakteriologischen Eigenschaften des Darmsymbionten beim medizinischen Blutegel (*Hirudo officinalis*) nebst Bemerkungen zur Symbiosefrage [On the bacteriological properties of gut symbionts in the medicinal leech (*Hirudo officinalis*), together with comments on the question of symbiosis] (J. B. Kolb, Trans.). *Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten, 148*, 36-47.

Hoshino, T., Kiriaki, M., Nakajima, T. (2003). Novel thermal hysteresis proteins from low temperature basidiomycete, *Coprinus psychromorbidus*. *CryoLetters, 24*(3), 135-142.

Hoshino, T., Xiao, N., Tkachenko, O. B. (2009). Cold adaptation in the phytopathogenic fungi causing snow molds. *Mycoscience, 50*(1), 26-38.

Hotopp, J. C. D., Clark, M. E., Oliveira, D., Foster, J. M., Fischer, P., Torres, M. C., et al. (2007). Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. *Science, 317*(5845), 1753-1756.

Howerton, C. L., Morgan, C. P., Fischer, D. B., Bale, T. L. (2013). O-GlcNAc transferase (OGT) as a placental biomarker of maternal stress and reprogramming of CNS gene transcription in development. *Proceedings of the National Academy of Sciences of the United States of America, 110*(13), 5169-5174.

Hsiao, K.-C., Cheng, C.-H. C., Fernandes, I. E., Detrich III, H. W., DeVries, A. L. (1990). An antifreeze glycopeptide gene from the Antarctic cod *Notothenia coriiceps neglecta* encodes a polyprotein of high peptide copy number. *Proceedings of the National Academy of Sciences of the United States of America, 87*(23), 9265-9269.

Hu, Y., Walker, S. (2002). Remarkable structural similarities between diverse glycosyltransferases. *Chemistry and Biology, 9*(12), 1287-1296.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., Morris, H. R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature, 258*(5536), 577-579.

Hughes, J. T. (2010). *Henry Power of Halifax: A seventeenth century physician and scientist* (1 ed.). Oxford: Rimes House.

Hunt, B. M., Hoefling, K., Cheng, C.-H. C. (2003). Annual warming episodes in seawater temperatures in McMurdo Sound in relationship to endogenous ice in notothenioid fish. *Antarctic Science, 15*(3), 333-338.

Hunt, D. F., Buko, A. M., Ballard, J. M., Shabanowitz, J., Giordani, A. B. (1981). Sequence analysis of polypeptides by collision activated dissociation on a triple quadrupole mass spectrometer. *Biomedical Mass Spectrometry, 8*(9), 397-408.

Huxley, J. S. (1942). *Evolution: The modern synthesis* (1 ed.). London: Allen and Unwin.

Indergand, S., Graf, J. (2000). Ingested blood contributes to the specificity of the symbiosis of *Aeromonas veronii* biovar sobria and *Hirudo medicinalis*, the medicinal leech. *Applied and Environmental Microbiology, 66*(11), 4735-4741.

Intergovernmental Panel on Climate Change (2007). Summary for policymakers. In S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor & H. L. Miller (Eds.), *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge: Cambridge University Press.

James, A. T., Martin, A. J. P. (1952). Gas-liquid partition chromatography. A technique for the analysis of volatile materials. *Analyst, 77*(921), 915-932.

Janech, M. G., Krell, A., Mock, T., Kang, J.-S., Raymond, J. A. (2006). Ice-binding proteins from sea ice diatoms (Bacillariophyceae). *Journal of Phycology, 42*(2), 410-416.

Janssen, J., Montgomery, J. C., Tien, R. (1992). Social rotational feeding in *Pagothenia borchgrevinki* (Pisces: Nototheniidae). *Copeia, 1992*(2), 559-562.

Jennings, J. B., van der Lande, V. M. (1967). Histochemical and bacteriological studies on digestion in nine species of leeches (Annelida: Hirudinea). *The Biological Bulletin, 133*(1), 166-183.

Jin, Y., DeVries, A. L. (1997). Purification and identification of a novel antifreeze-related protein-antifreeze potentiator protein. *American Zoologist, 37*(5), 138A.

Jin, Y., DeVries, A. L. (2006). Antifreeze glycoprotein levels in Antarctic notothenioid fishes inhabiting different thermal environments and the effect of warm acclimation. *Comparative Biochemistry and Physiology B - Biochemistry and Molecular Biology, 144*(3), 290-300.

Johal, A. R., Blackler, R. J., Alfaro, J. A., Schuman, B., Borisova, S., Evans, S. V. (2014). pH-induced conformational changes in human ABO(H) blood group glycosyltransferases confirm the importance of electrostatic interactions in the formation of the semi-closed state. *Glycobiology, 24*(3), 237-246.

Jones, P., Vogt, T. (2001). Glycosyltransferases in secondary plant metabolism: Tranquilizers and stimulant controllers. *Planta, 213*(2), 164-174.

Kahveci, Z., Minbay, F. Z., Noyan, S., Cavusoglu, I. (2003). A comparison of microwave heating and proteolytic pretreatment antigen retrieval techniques in formation fixed, paraffin embedded tissues. *Biotechnic & Histochemistry, 78*(2), 119-128.

Kalebi, A. Y., Dada, M. A. (2007). Application of immunohistochemistry in clinical practice: A review. *East African Medical Journal, 84*(8), 389-397.

Kanwisher, J. W. (1955). Freezing in intertidal animals. *Biological Bulletin, 109*(1), 56-63.

Karlsbakk, E., Haugen, E., Nylund, A. (2005). Morphology and aspects of growth of a trypanosome transmitted by the marine leech *Johanssonia arctica* (Piscicolidae) from Northern Norway. *Folia Parasitologica, 52*(3), 209-215.

Kawaguchi, N., Ohmori, T., Takeshita, Y., Kawanishi, G., Katayama, S., Yamada, H. (1986). Occurrence of Galβ(1→3)GalNAc-Ser/Thr in the linkage region of polygalactosamine

containing fungal glycoprotein from *Cordyceps ophioglossoides*. *Biochemical and Biophysical Research Communications, 140*(1), 350-356.

Kawahara, H., Iwanaka, Y., Higa, S., Muryoi, N., Sato, M., Honda, M., et al. (2007). A novel, intracellular antifreeze protein in an Antarctic bacterium, *Flavobacterium xanthum*. *CryoLetters, 28*(1), 39-49.

Kelley, J. L., Aagaard, J. E., MacCoss, M. J., Swanson, W. J. (2010). Functional diversification and evolution of antifreeze proteins in the Antarctic fish *Lycodichthys dearborni*. *Journal of Molecular Evolution, 71*(2), 111-118.

Kennett, J. P. (1977). Cenozoic evolution of Antarctic glaciation, the circum-Antarctic Ocean, and their impact on global paleoceanography. *Journal of Geophysical Research - Oceans and Atmospheres, 82*(27), 3843-3860.

Khan, R. A., Meyer, M. C. (1978). Evidence of a bi-annual life cycle in the marine leech *Oceanobdella sexoculata* (Hirudinea: Piscicolidae). *The Journal of Parasitology, 64*(4), 766-768.

Khorolragchaa, A., Kim, Y.-J., Rahimi, S., Sukweenadhi, J., Jang, M.-G., Yang, D.-C. (2014). Grouping and characterization of putative glycosyltransferase genes from *Panax ginseng* Meyer. *Gene, 536*(1), 186-192.

Kiko, R. (2010). Acquisition of freeze protection in a sea-ice crustacean through horizontal gene transfer? *Polar Biology, 33*(4), 543-556.

Kiko, R., Kramer, M., Spindler, M., Wägele, H. (2008). *Tergipes antarcticus* (Gastropoda, Nudibranchia): Distribution, life cycle, morphology, anatomy and adaptation of the first mollusc known to live in Antarctic sea ice. *Polar Biology, 31*(11), 1383-1395.

Kikuchi, Y., Fukatsu, T. (2002). Endosymbiotic bacteria in the esophageal organ of glossiphoniid leeches. *Applied and Environmental Microbiology, 68*(9), 4637-4641.

Kilmartin, P. A., Reid, D. S., Samson, I. (2000). The measurement of the glass transition temperature of sucrose and maltose solutions with added NaCl. *Journal of the Science of Food and Agriculture, 80*(15), 2196-2202.

Knauthe, K. (1891). Meine Erfahrungen über das Verhalten von Amphibien und Fischen gegenüber der Kälte [My experiences on the behaviour of amphibians and fish to the cold] (J. B. Kolb, Trans.). *Zoologischer Anzeiger, 14*(359), 104-106.

Knight-Jones, E. W. (1940). The occurrence of a marine leech *Abranchus blennii* n. sp. resembling *A. sexoculata* (Malm) in North Wales. *Journal of the Marine Biological Association of the United Kingdom, 24*(2), 533-541.

Knight, C. A., DeVries, A. L., Oolman, L. D. (1984). Fish antifreeze protein and the freezing and recrystallization of ice. *Nature, 308*(5956), 295-296.

Knight, C. A., Driggers, E., DeVries, A. L. (1993). Adsorption to ice of fish antifreeze glycopeptides 7 and 8. *Biophysical Journal, 64*(1), 252-259.

Knight, C. A., Duman, J. G. (1986). Inhibition of recrystallization of ice by insect thermal hysteresis proteins: A possible cryoprotective role. *Cryobiology, 23*(3), 256-262.

Knight, C. A., Wen, D. Y., Laursen, R. A. (1995). Nonequilibrium antifreeze peptides and the recrystallization of ice. *Cryobiology, 32*(1), 23-34.

Kochs, W. (1890). Kann die Kontinuität der Lebensvorgänge zeitweilig völlig unterbrochen werden? [Can the continuity of life's processes temporarily be stopped completely?] (J. B. Kolb, Trans.). *Biologisches Centralblatt, 10*(22), 673-686.

Kochs, W. (1892). Über die Vorgänge beim Einfrieren und Austrocknen von Tieren und Pflanzensamen [About the processes involved in freezing and drying of animals and plant seeds] (J. B. Kolb, Trans.). *Biologisches Centralblatt, 2*, 330-339.

Kodis, T. (1898). Die Unterkühlung der thierischen und pflanzlichen Gewebe [The supercooling of animal and plant tissues] (J. B. Kolb, Trans.). *Centralblatt für Physiologie, 12*(18), 593-595.

Koenig, M., Monaco, A. P., Kunkel, L. M. (1988). The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell, 53*(2), 219-228.

Komatsu, S. K., DeVries, A. L., Feeney, R. E. (1970). Studies of the structure of freezing point-depressing glycoproteins from an Antarctic fish. *Journal of Biological Chemistry, 245*(11), 2909-2913.

Kordis, D., Gubensek, F. (1998). Unusual horizontal transfer of a long interspersed nuclear element between distant vertebrate classes. *Proceedings of the National Academy of Sciences of the United States of America, 95*(18), 10704-10709.

Kumble, K. D., Demmer, J., Fish, S., Hall, C., Corrales, S., Death, A., et al. (2008). Characterization of a family of ice-active proteins from the ryegrass, *Lolium perenne*. *Cryobiology, 57*(3), 263-268.

Kurland, C. G. (2005). What tangled web: Barriers to rampant horizontal gene transfer. *Bioessays, 27*(7), 741-747.

Kurzik-Dumke, U., Kaymer, M., Gundacker, D., Debes, A., Labitzke, K. (1997). Gene within gene configuration and expression of the *Drosophila melanogaster* genes *lethal(2) neighbour of tid [l(2)not]* and *lethal(2) relative of tid [l(2) rot]*. *Gene, 200*(1-2), 45-58.

La Mesa, M., Eastman, J. T., Vacchi, M. (2004). The role of notothenioid fish in the food web of the Ross Sea shelf waters: A review. *Polar Biology, 27*(6), 321-338.

Lai, Z. W., Nice, E. C., Schilling, O. (2013). Glycocapture-based proteomics for secretome analysis. *Proteomics, 13*(3-4), 512-525.

Lauersen, K. J., Brown, A., Middleton, A., Davies, P. L., Walker, V. K. (2011). Expression and characterization of an antifreeze protein from the perennial ryegrass, *Lolium perenne*. *Cryobiology, 62*(3), 194-201.

Lee, D. K., Kwon, B. S., Ramamoorthy, A. (2008). Freezing point depression of water in phospholipid membranes: A solid-state NMR study. *Langmuir, 24*(23), 13598-13604.

Lee, J. H., Lee, S. G., Do, H., Park, J. C., Kim, E., Choe, Y.-H., et al. (2013). Optimization of the pilot-scale production of an ice-binding protein by fed-batch culture of *Pichia pastoris*. *Applied Microbiology and Biotechnology, 97*(8), 3383-3393.

Lee, J. K., Park, K. S., Park, S., Park, H., Song, Y. H., Kang, S.-H., et al. (2010). An extracellular ice-binding glycoprotein from an Arctic psychrophilic yeast. *Cryobiology, 60*(2), 222-228.

Leigh-Sharpe, W. H. (1916). *Platybdella anarrhichae*. *Parasitology 8*(3), 274 - 293.

Leiper, R. T., Atkinson, E. L. (1915). Parasitic worms, with a note on a free-living nematode. In Trustees of the British Museum (Ed.), *British Museum Natural History. British Antarctic ("Terra Nova") Expedition, 1910. Natural History Reports. Zoology. Collecting Stations, Mollusca, Brachiopoda, and Worms* (1 ed., Vol. 2, No. 3, pp. 19-60 plus five plates). London: British Museum.

Lesec, J., Quivoron, C. (1979). The use of polystyrene gels in preparative recycle-HPLC for the separation of structurally related molecules. *Journal of Liquid Chromatography, 2*(4), 467-484.

Levsen, K., Wipf, H.-K., McLafferty, F. W. (1974). Mass spectrometric studies of peptides - VI: Applications of metastable ion and collisional activation spectra. *Organic Mass Spectrometry, 8*(1), 117-128.

Levy, S. B., Marshall, B. (2004). Antibacterial resistance worldwide: Causes, challenges and responses. *Nature Medicine, 10*(12 Suppl), S122-129.

Li, N., Andorfer, C. A., Duman, J. G. (1998). Enhancement of insect antifreeze protein activity by solutes of low molecular mass. *Journal of Experimental Biology, 201*(15), 2243-2251.

Li, Y., Liu, H. T., Xu, Q. S., Du, Y. G., Xu, J. (2014). Chitosan oligosaccharides block LPS-induced O-GlcNAcylation of NF-kappa B and endothelial inflammatory response. *Carbohydrate Polymers, 99*, 568-578.

Liebscher, R. S., Richards, R. C., Lewis, J. M., Short, C. E., Muise, D. M., Driedzic, W. R., et al. (2006). Seasonal freeze resistance of rainbow smelt (*Osmerus mordax*) is generated by differential expression of glycerol-3-phosphate dehydrogenase, phosphoenolpyruvate carboxykinase, and antifreeze protein genes. *Physiological and Biochemical Zoology, 79*(2), 411-423.

Lin, Y., Duman, J. G., DeVries, A. L. (1972). Studies on the structure and activity of low molecular weight glycoproteins from an Antarctic fish. *Biochemical and Biophysical Research Communications, 46*(1), 87-92.

Liou, Y.-C., Thibault, P., Walker, V. K., Davies, P. L., Graham, L. A. (1999). A complex family of highly heterogeneous and internally repetitive hyperactive antifreeze proteins from the beetle *Tenebrio molitor*. *Biochemistry, 38*(35), 11415-11424.

Liou, Y. C., Daley, M. E., Graham, L. A., Kay, C. M., Walker, V. K., Sykes, B. D., et al. (2000). Folding and structural characterization of highly disulfide-bonded beetle antifreeze protein produced in bacteria. *Protein Expression and Purification, 19*(1), 148-157.

Littlepage, J. L. (1965). Oceanographic investigations in McMurdo Sound, Antarctica. In G. A. Llano (Ed.), *Antarctic Research Series. Biology of the Antarctic seas II* (Vol. 5, pp. 1-37). Washington, D. C.: American Geophysical Union.

Liu, J., Mushegian, A. (2003). Three monophyletic superfamilies account for the majority of the known glycosyltransferases. *Protein Science, 12*(7), 1418-1431.

Llewellyn, L. C. (1966). Pontobdellinae (Piscicolidae: Hirudinea) in the British Museum (Natural History) with a review of the subfamily. *Bulletin of the British Museum Natural History - Zoology, 14*(7), 389-439.

Loo, J. A., Edmonds, C. G., Smith, R. D. (1990). Primary sequence information from intact proteins by electrospray ionization tandem mass spectrometry. *Science, 248*(4952), 201-204.

Luyet, B. J., Gibbs, M. C. (1937). On the mechanism of congelation and of death in the rapid freezing of epidermal plant cells. *Biodynamica, 1*(25), 1-18.

Ma, J., Wang, J., Mao, X., Wang, Y. (2012). Differential expression of two antifreeze proteins in the desert beetle *Anatolica polita* (Coleoptera: Tenebriondae): Seasonal variation and environmental effects. *CryoLetters, 33*(5), 337-348.

Mace, T. F., Davis, C. C. (1972). Energetics of a host-parasite relationship as illustrated by the leech *Malmiana nuda*, and the shorthorn sculpin *Myoxocephalus scorpius*. *Oikos, 23*(3), 336-343.

Madsen, H. (1940). A study of the littoral fauna of Northwest Greenland *Meddelelser om Grønland. Natural History Expedition to Northwest Greenland 1936* (Vol. 124, no. 3, pp. 1-24). Copenhagen, Denmark: C. A. Reitzels Forlag.

Mager, S. M., Smith, I. J., Kempema, E. W., Thomson, B. J., Leonard, G. H. (2013). Anchor ice in polar oceans. *Progress in Physical Geography, 37*(4), 468-483.

Mahmudi-Azer, S., Lacy, P., Bablitz, B., Moqbel, R. (1998). Inhibition of nonspecific binding of fluorescent-labelled antibodies to human eosinophils. *Journal of Immunological Methods, 217*(1-2), 113-119.

Malm, A. W. (1863). *Svenska iglar, Disciferae, afbildade efter lefvande exemplar och beskrifna [Swedish leeches, Disciferae, depicted after living specimens and descriptions]* (Vol. 8). Göteborg: Göteborgs Kongliga Vetenskaps och Vitterhets Samhälles Handlingar [Royal Society of Sciences and Letters in Gothenburg].

Mao, X., Liu, Z., Li, H., Ma, J., Zhang, F. (2011). Calorimetric studies on an insect antifreeze protein ApAFP752 from *Anatolica polita*. *Journal of Thermal Analysis and Calorimetry, 104*(1), 343-349.

Mao, X., Liu, Z., Ma, J., Pang, H., Zhang, F. (2011). Characterization of a novel β-helix antifreeze protein from the desert beetle *Anatolica polita*. *Cryobiology, 62*(2), 91-99.

Marrack, J. (1934). Nature of antibodies. *Nature, 133*, 292-293.

Matveev, V., Okada, N. (2009). Retroposons of salmonoid fishes (Actinopterygii: Salmonoidei) and their evolution. *Gene, 434*(1-2), 16-28.

Maximov, N. A. (1914). Experimentelle und kritische Untersuchungen über das Gefrieren und Erfrieren der Pflanzen [Experimental and critical studies on the freezing and death by freezing of plants] (J. B. Kolb, Trans.). *Jahrbücher für wissenschaftliche Botanik, 53*, 327-420.

Mayr, E. (1942). *Systematics and the origin of species, from the viewpoint of a zoologist* (1 ed.). New York: Columbia University Press.

Mayr, E. (1963). *Animal species and evolution* (1 ed.). Cambridge: Belknap Press of Harvard University Press.

Meiser, H., Schulz, R. (2003). Detection and localization of two constitutive NOS isoforms in bull spermatozoa. *Anatomia Histologia Embryologia - Journal of Veterinary Medicine Series C, 32*(6), 321-325.

Mendel, G. J. (1866). Versuche über Pflanzen-Hybriden [Experiments on plant hybrids] (J. B. Kolb, Trans.). *Verhandlungen des naturforschenden Vereines in Brünn, 4*, 3-47.

Mengel, M., von Wasielewski, R., Wiese, B., Rüdiger, T., Müller-Hermelink, H. K., Kreipe, H. (2002). Inter-laboratory and inter-observer reproducibility of immunohistochemical assessment of the Ki-67 labelling index in a large multi-centre trial. *Journal of Pathology, 198*(3), 292-299.

Merritt, C., Robertso, D. H. (1967). The analysis of proteins, peptides and amino acids by pyrolysis-gas chromatography and mass spectrometry. *Journal of Gas Chromatography, 5*(2), 96-98.

Meyer, M. C., Burreson, E. M. (1990). Some leeches (Hirudinea: Piscicolidae) of the Southern Oceans. In L. S. Kornicker (Ed.), *Biology of the Antarctic Seas XXI* (Vol. 52, pp. 219-236). Washington, D. C.: American Geophysical Union.

Mihasan, M., Brandsch, R. (2013). pAO1 of *Arthrobacter nicotinovorans* and the spread of catabolic traits by horizontal gene transfer in gram-positive soil bacteria. *Journal of Molecular Evolution*, 1-9.

Modolo, L. V., Li, L., Pan, H., Blount, J. W., Dixon, R. A., Wang, X. (2009). Crystal structures of glycosyltransferase UGT78G1 reveal the molecular basis for glycosylation and deglycosylation of (iso)flavonoids. *Journal of Molecular Biology, 392*(5), 1292-1302.

Møller, P. R., Nielsen, J. G., Anderson, M. E. (2005). Systematics of polar fishes. In A. P. Farrell & J. F. Steffensen (Eds.), *The Physiology of Polar Fishes* (Vol. 22, pp. 25-78). San Diego: Elsevier Academic Press.

Monier, A., Pagarete, A., de Vargas, C., Allen, M. J., Read, B., Claverie, J. M., et al. (2009). Horizontal gene transfer of an entire metabolic pathway between a eukaryotic alga and its DNA virus. *Genome Research, 19*(8), 1441-1449.

Montero, F. E., Kostadinova, A., Raga, J. A. (2009). Development and habitat selection of a new sanguinicolid parasite of cultured greater amberjack, *Seriola dumerili*, in the Mediterranean. *Aquaculture, 288*(1-2), 132-139.

Moore, J. P. (1938). Leeches - Hirudinea of the Australasian Antarctic Expedition. *Australasian Antarctic Expedition 1911-14 Scientific Reports, Series C - Zoology and Botany, 10*(3), 1-16.

Mootz, R. (1936). Über den „Blutegelbazillus" [On the bacillus in the medicinal leech] (J. B. Kolb, Trans.). *Die medizinische Welt, 43*, 1552.

Morris, H. R., Paxton, T., Panico, M., McDowell, R., Dell, A. (1997). A novel geometry mass spectrometer, the Q-TOF, for low-femtomole/attomole-range biopolymer sequencing. *Journal of Protein Chemistry, 16*(5), 469-479.

Morris, H. R., Thompson, M. R., Osuga, D. T., Ahmed, A. I., Chan, S. M., Vandenheede, J. R., et al. (1978). Antifreeze glycoproteins from the blood of an Antarctic fish - the structure of the proline-containing glycopeptides. *Journal of Biological Chemistry, 253*(14), 5155-5162.

Mueller, E. F., Adams, L. H., Fairchild, F. O., Wensel, H. T. (1926). Laboratory technique - thermometry. In C. J. West, N. E. Dorsey, F. R. Bichowsky & A. Klemenc (Eds.), *International Critical Tables of Numerical Data, Physics, Chemistry and Technology* (1 ed., Vol. 1, pp. 415). New York: McGraw-Hill; Published for the National Research Council of the United States of America.

Müller-Erzbach, W. (1891). Die Widerstandsfähigkeit des Frosches gegen das Einfrieren [The resistance of the frog to freezing] (J. B. Kolb, Trans.). *Zoologischer Anzeiger, 14*(376), 383-384.

Müller-Thurgau, H. (1880). Über das Gefrieren und Erfrieren der Pflanzen I. [On the freezing and death by freezing of plants 1.] (J. B. Kolb, Trans.). *Landwirtschaftliche Jahrbücher, 9*, 133-189.

Mutimer, K. L., Banis, J. C., Upton, J. (1987). Microsurgical reattachment of totally amputated ears. *Plastic and Reconstructive Surgery, 79*(4), 535-540.

Nada, H., Furukawa, Y. (2012). Antifreeze proteins: Computer simulation studies on the mechanism of ice growth inhibition. *Polymer Journal, 44*(7), 690-698.

Nagasawa, K., Kikuchi, M., Arakane, Y., Yusa, S. (2009). New data on the hosts and attachment sites of the fish leech *Taimenobdella amurensis* (Hirudinida, Piscicolidae) *Biogeography, 11*, 3-8.

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N., et al. (1979). Nucleotide sequence of cloned cDNA for bovine corticotropin-β-lipotropin precursor. *Nature, 278*(5703), 423-427.

Near, T. J., Dornburg, A., Kuhn, K. L., Eastman, J. T., Pennington, J. N., Patarnello, T., et al. (2012). Ancient climate change, antifreeze, and the evolutionary diversification of Antarctic fishes. *Proceedings of the National Academy of Sciences of the United States of America, 109*(9), 3434-3439.

Neuman, S., Kaban, A., Volk, T., Yaffe, D., Nudel, U. (2001). The dystrophin/utrophin homologues in *Drosophila* and in sea urchin. *Gene, 263*(1-2), 17-29.

Nicholls, J. G., Kuffler, S. W. (1964). Extracellular space as a pathway for exchange between blood and neurons in the central nervous system of the leech: Ionic composition of glial cells and neurons. *Journal of Neurophysiology, 27*(4), 645-671.

Nickell, P. K., Sass, S., Verleye, D., Blumenthal, E. M., Duman, J. G. (2013). Antifreeze proteins in the primary urine of larvae of the beetle *Dendroides canadensis*. *Journal of Experimental Biology, 216*(9), 1695-1703.

Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., et al. (1982). Cloning and sequence analysis of cDNA for bovine adrenal preproenkephalin. *Nature, 295*(5846), 202-206.

Nonomura, H., Kato, N., Ohno, Y., Itokazu, M., Matsunaga, T., Watanabe, K. (1996). Indigenous bacterial flora of medicinal leeches and their susceptibilities to 15 antimicrobial agents. *Journal of Medical Microbiology, 45*(6), 490-493.

Norden, A. P., Strand, M. (1984). *Schistosoma mansoni, S. haematobium,* and *S. japonicum*: Identification of genus-, species-, and gender-specific antigenic worm glycoproteins. *Experimental Parasitology, 57*(1), 110-123.

Norton, A. J., Jordan, S., Yeomans, P. (1994). Brief, high temperature heat denaturation (pressure cooking) - a simple and effective method of antigen retrieval for routinely processed tissues. *Journal of Pathology, 173*(4), 371-379.

Nyame, K., Cummings, R. D., Damian, R. T. (1987). *Schistosoma mansoni* synthesizes glycoproteins containing terminal O-linked N-acetylglucosamine residues. *Journal of Biological Chemistry, 262*(17), 7990-7995.

Oguz, M. C., Heckmann, R. A., Cheng, C.-H. C., El-Naggar, A., Tepe, Y. (2012). Ecto and endoparasites of some fishes from the Antarctic region. *Scientia Parasitologica, 13*(3), 119-128.

Ohashi, T., Nakakita, S.-i., Sumiyoshi, W., Takegawa, K. (2010). Production of heterologous glycoproteins by a glycosylation-defective alg3och1 mutant of *Schizosaccharomyces pombe*. *Journal of Biotechnology, 150*(3), 348-356.

Osborn, H. F. (1902). The law of adaptive radiation. *American Naturalist, 36*(425), 353-363.

Osborn, H. F. (1910). *The age of mammals in Europe, Asia and North America* (1 ed.). New York: Macmillan.

Osuga, D. T., Feeney, R. E. (1978). Antifreeze glycoproteins from Arctic fish. *Journal of Biological Chemistry, 253*(15), 5338-5343.

Pan, Z. H., Zhu, C. D., Wu, C. S. (2013). A review of the genus *Monema* Walker in China (Lepidoptera, Limacodidae). *ZooKeys, 306*, 23-36.

Panchuk-Voloshina, N., Haugland, R. P., Bishop-Stewart, J., Bhalgat, M. K., Millard, P. J., Mao, F., et al. (1999). Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *Journal of Histochemistry and Cytochemistry, 47*(9), 1179-1188.

Papavramidou, N., Christopoulou-Aletra, H. (2009). Medicinal use of leeches in the texts of ancient Greek, Roman and early Byzantine writers. *Internal Medicine Journal, 39*(9), 624-627.

Pauling, L. (1953). *General chemistry - an introduction to descriptive chemistry and modern chemical theory* (2 ed.). San Francisco: W. H. Freeman.

Pearcy, W. G. (1961). Seasonal changes in osmotic pressure of flounder sera. *Science, 134*(3473), 193-194.

Peltier, R., Evans, C. W., DeVries, A. L., Brimble, M. A., Dingley, A. J., Williams, D. E. (2010). Growth habit modification of ice crystals using antifreeze glycoprotein (AFGP) analogues. *Crystal Growth and Design, 10*(12), 5066-5077.

Pertaya, N., Celik, Y., DiPrinzio, C. L., Wettlaufer, J. S., Davies, P. L., Braslavsky, I. (2007). Growth-melt asymmetry in ice crystals under the influence of spruce budworm antifreeze protein. *Journal of Physics - Condensed Matter, 19*(41), 12.

Pertaya, N., Marshall, C. B., Celik, Y., Davies, P. L., Braslavsky, I. (2008). Direct visualization of spruce budworm antifreeze protein interacting with ice crystals: Basal plane affinity confers hyperactivity. *Biophysical Journal, 95*(1), 333-341.

Petit, J. R., Jouzel, J., Raynaud, D., Barkov, N. I., Barnola, J. M., Basile, I., et al. (1999). Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature, 399*(6735), 429-436.

Petzel, D. H., Reisman, H. M., DeVries, A. L. (1980). Seasonal variation of antifreeze peptide in the winter flounder, *Pseudopleuronectes americanus*. *Journal of Experimental Zoology, 211*(1), 63-69.

Pickford, G. E., Grant, F. B. (1967). Serum osmolality in the coelacanth, *Latimeria chalumnae*: Urea retention and ion regulation. *Science, 155*(3762), 568-570.

Pimm, S. L. (2012). Biodiversity: Not just lots of fish in the sea. *Current Biology, 22*(23), R996–R997.

Pörtner, H. O., Lucassen, M., Storch, D. (2005). Metabolic biochemistry: Its role in thermal tolerance and in the capacities of physiological and ecological function. In A. P. Farrell & J. F. Steffensen (Eds.), *The Physiology of Polar Fishes* (Vol. 22, pp. 79-154). San Diego: Elsevier Academic Press.

Poulin, R. (1993). The disparity between observed and uniform distributions: A new look at parasite aggregation. *International Journal for Parasitology, 23*(7), 937-944.

Power, H. (1664). *Experimental philosophy, in three books: Containing new experiments microscopical, mercurial, magnetical: With some deductions, and probable hypotheses, raised from them, in avouchment and illustration of the now famous atomical hypothesis* (1 ed.). London: T. Roycroft for John Martin and James Allestry, at the Bell in St. Pauls Churchyard.

Pütter, A. (1907). Der Stoffwechsel des Blutegels (*Hirudo medicinalis* L.). 1. Teil [The metabolism of the bloodsucker (*Hirudo medicinalis* L.). Part 1] (J. B. Kolb, Trans.). *Zeitschrift für Allgemeine Physiologie, Teil 1*(6), 217-286.

Pütter, A. (1908). Der Stoffwechsel des Blutegels (*Hirudo medicinalis* L.). 2. Teil [The metabolism of the bloodsucker (*Hirudo medicinalis* L.). Part 2] (J. B. Kolb, Trans.). *Zeitschrift für Allgemeine Physiologie, Teil 2*(7), 16-61.

Rahm, G. F. (1924). Weitere physiologische Versuche mit niederen Temperaturen (ein Beitrag zur Lösung des Kälteproblems) [Further physiological experiments at lower temperatures (a contribution to the solution of the chilling problem)] (J. B. Kolb, Trans.). *Verhandlungen der Deutschen Zoologischen Gesellschaft eingetragener Verein, 1*, 106-111.

Ramsay, A. D. (2013). 30 years of lymph node pathology: Biomarkers and other advances. *Applied Immunohistochemistry and Molecular Morphology, 21*(2), 103-109.

Ramsay, J. A. (1964). The rectal complex of the mealworm *Tenebrio molitor*, L. (Coleoptera, Tenebrionidae). *Philosophical Transactions of the Royal Society of London Series B - Biological Sciences, 248*(748), 279-314.

Ramsay, J. A., Brown, R. H. J. (1955). Simplified apparatus and procedure for freezing-point determinations upon small volumes of fluid. *Journal of Scientific Instruments, 32*(10), 372-375.

Raymond, J. A., Christner, B. C., Schuster, S. C. (2008). A bacterial ice-binding protein from the Vostok ice core. *Extremophiles, 12*(5), 713-717.

Raymond, J. A., DeVries, A. L. (1977). Adsorption inhibition as a mechanism of freezing resistance in polar fishes. *Proceedings of the National Academy of Sciences of the United States of America, 74*(6), 2589-2593.

Raymond, J. A., Janech, M. G. (2003). Cryoprotective property of diatom ice-active substance. *Cryobiology, 46*(2), 203-204.

Raymond, J. A., Janech, M. G. (2009). Ice-binding proteins from enoki and shiitake mushrooms. *Cryobiology, 58*(2), 151-156.

Raymond, J. A., Janech, M. G., Fritsen, C. H. (2009). Novel ice-binding proteins from a psychrophilic Antarctic alga (Chlamydomonadaceae, Chlorophyceae). *Journal of Phycology, 45*(1), 130-136.

Raymond, J. A., Kim, H. J. (2012). Possible role of horizontal gene transfer in the colonization of sea ice by algae. *Plos One, 7*(5), 9.

Raymond, J. A., Knight, C. A. (2003). Ice binding, recrystallization inhibition, and cryoprotective properties of ice-active substances associated with Antarctic sea ice diatoms. *Cryobiology, 46*(2), 174-181.

Raymond, J. A., Wilson, P., DeVries, A. L. (1989). Inhibition of growth of nonbasal planes in ice by fish antifreezes. *Proceedings of the National Academy of Sciences of the United States of America, 86*(3), 881-885.

Raymond, M. R., Wharton, D. A. (2013). The ability of the Antarctic nematode *Panagrolaimus davidi* to survive intracellular freezing is dependent upon nutritional status. *Journal of Comparative Physiology B - Biochemical Systemic and Environmental Physiology, 183*(2), 181-188.

Réaumur, R.-A. F. d. (1736). Suite de l'histoire des chenilles et des papillons; et l'histoire des insectes ennemis des chenilles [Following the history of caterpillars and butterflies; and the history of insect predators of caterpillars] (J. B. Kolb, Trans.). In R.-A. F. d. Réaumur (Ed.), *Mémoires pour servir à l'Histoire des Insectes* (1 ed., Vol. 2). Paris: L'Imprimerie Royale.

Réaumur, R.-A. F. d. (1738). Histoire des gallinsectes, des progallinsectes, et des mouches, à deux aîles [History of gall insects, progall insects and flies with two wings] (J. B. Kolb, Trans.). In R.-A. F. d. Réaumur (Ed.), *Mémoires pour servir à l'Histoire des Insectes* (1 ed., Vol. 4). Paris: L'Imprimerie Royale.

Regan, C. T. (1914a). Fishes. In Trustees of the British Museum (Ed.), *British Museum Natural History. British Antarctic ("Terra Nova") Expedition, 1910. Natural History Report. Zoology* (1 ed., Vol. 1, Vertebrata, No. 1, pp. 1-54 plus eight text figures and 13 plates). London: British Museum.

Regan, C. T. (1914b). II. - Diagnoses of new marine fishes collected by the British Antarctic ('Terra Nova') expedition. 1. Antarctic fishes. *Annals and Magazine of Natural History, 13*(73), 11-17.

Rivier, J., McClintock, R. (1983). Reversed-phase high-performance liquid chromatography of insulins from different species. *Journal of Chromatography, 268*(1), 112-119.

Roberts, R. G., Bobrow, M. (1998). Dystrophins in vertebrates and invertebrates. *Human Molecular Genetics, 7*(4), 589-595.

Robinson, H. W. (1945). Robert Hooke, M.D., F.R.S., with special reference to his work in medicine and biology. *Proceedings of the Royal Society of Medicine - London, 38*(9), 485-489.

Rocka, A. (2002). Nematodes of fishes in the Weddell Sea (Antarctic). *Acta Parasitologica, 47*(4), 294-299.

Rogers, M. A., Roos, Y. H., Goff, H. D. (2006). Structural heterogeneity and its effect on the glass transition in sucrose solutions containing protein and polysaccharide. *Food Hydrocolloids, 20*(6), 774-779.

Romanenko, L. A., Uchino, M., Mikhailov, V. V., Zhukova, N. V., Uchimura, T. (2003). *Marinomonas primoryensis* sp nov., a novel psychrophile isolated from coastal sea-

ice in the Sea of Japan. *International Journal of Systematic and Evolutionary Microbiology, 53*(3), 829-832.

Royuela, M., Hugon, G., Rivier, F., Fehrentz, J. A., Martinez, J., Paniagua, R., et al. (2001). Variations in dystrophin complex in red and white caudal muscles from *Torpedo marmorata*. *Journal of Histochemistry and Cytochemistry, 49*(7), 857-865.

Royuela, M., Hugon, G., Rivier, F., Paniagua, R., Mornet, D. (2001). Dystrophin-associated proteins in obliquely striated muscle of the leech *Pontobdella muricata* (Annelida, Hirudinea). *Histochemical Journal, 33*(3), 135-139.

Royuela, M., Paniagua, R., Rivier, F., Hugon, G., Robert, A., Mornet, D. (1999). Presence of invertebrate dystrophin-like products in obliquely striated muscle of the leech, *Pontobdella muricata* (Annelida, Hirudinea). *Histochemical Journal, 31*(9), 603-608.

Rózsa, L., Reiczigel, J., Majoros, G. (2000). Quantifying parasites in samples of hosts. *Journal of Parasitology, 86*(2), 228-232.

Salt, R. W. (1961). Principles of insect cold-hardiness. *Annual Review of Entomology, 6*, 55-74.

Salt, R. W. (1964). Terrestrial animals in cold: Arthropods. In D. B. Dill & E. F. Adolph (Eds.), *Handbook of Physiology - Section 4: Adaptation to the Environment* (pp. 349-355). Washington, D.C.: American Physiological Society.

Santos, I. A., Castellano, G. C., Freire, C. A. (2013). Direct relationship between osmotic and ionic conforming behavior and tissue water regulatory capacity in echinoids. *Comparative Biochemistry and Physiology Part A - Molecular and Integrative Physiology, 164*(3), 466-476.

Sawyer, R. T. (1986a). *Leech biology and behaviour. Vol. I. Anatomy, physiology, and behaviour* (1 ed. Vol. 1). Oxford: Clarendon Press.

Sawyer, R. T. (1986b). *Leech biology and behaviour. Vol. II. Feeding biology, ecology, and systematics* (1 ed. Vol. 2). Oxford: Clarendon Press.

Sawyer, R. T. (1986c). *Leech biology and behaviour. Vol. III. Bibliography* (1 ed. Vol. 3). Oxford: Clarendon Press.

Sayre, R. M. (1964). Cold-hardiness of nematodes. I. Effects of rapid freezing on the eggs and larvae of *Meloidogyne incognita* and *M. hapla*. *Nematologica, 10*(1), 168-179.

Schander, R., Schaffnit, E. (1918). Untersuchungen über das Auswintern des Getreides [Studies on the hibernation of crop] (J. B. Kolb, Trans.). *Landwirtschaftliche Jahrbücher, 52*, 1-66.

Schiaparelli, S., Hopcroft, R. R. (2011). The census of Antarctic marine life: Diversity and change in Southern Ocean ecosystems. *Deep-Sea Research Part II - Topical Studies in Oceanography, 58*(1-2), 1-4.

Schjoldager, K. T.-B. G., Clausen, H. (2012). Site-specific protein O-glycosylation modulates proprotein processing - deciphering specific functions of the large polypeptide GalNAc-transferase gene family. *Biochimica et Biophysica Acta, 1820*(12), 2079-2094.

Schmidt-Nielsen, K. (1997). *Animal physiology: Adaptation and environment* (5 ed.). Cambridge: Cambridge University Press.

Scholander, P. F., Flagg, W., Hock, R. J., Irving, L. (1953). Studies on the physiology of frozen plants and animals in the Arctic. *Journal of Cellular and Comparative Physiology, 42*(Suppl. 1), 1-56.

Scholander, P. F., van Dam, L., Kanwisher, J. W., Hammel, H. T., Gordon, M. S. (1957). Supercooling and osmoregulation in Arctic fish. *Journal of Cellular and Comparative Physiology, 49*(1), 5-24.

Schotte, M., Kensley, B. F., Shilling, S. (1995 onwards). *World list of marine, freshwater and terrestrial crustacea isopoda*. Washington, D.C.: National Museum of Natural History Smithsonian Institution: http://invertebrates.si.edu/isopod/

Schrag, J. D., DeVries, A. L. (1983). The effect of freezing rate on the cooperativity of antifreeze glycopeptides. *Comparative Biochemistry and Physiology Part A - Physiology, 74*(2), 381-385.

Schrag, J. D., O'Grady, S. M., DeVries, A. L. (1982). Relationship of amino acid composition and molecular weight of antifreeze glycopeptides to non-colligative freezing point depression. *Biochimica et Biophysica Acta, 717*(2), 322-326.

Schwartz, S. A., Reyzer, M. L., Caprioli, R. M. (2003). Direct tissue analysis using matrix-assisted laser desorption/ionization mass spectrometry: Practical aspects of sample preparation. *Journal of Mass Spectrometry, 38*(7), 699-708.

Seeley, E. H., Caprioli, R. M. (2008). Molecular imaging of proteins in tissues by mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America, 105*(47), 18126-18131.

Selensky, W. (1914). Über einige auf Arthropoden schmarotzende Ichthyobdelliden [About some parasitic Ichthyobdellids on arthropods] (J. B. Kolb, Trans.). *Zoologischer Anzeiger, 44*, 270-282.

Senchenkov, A., Jacobson, S. R. (2013). Microvascular salvage of a thrombosed total ear replant. *Microsurgery, 33*(5), 396-400.

Shier, W. T., Lin, Y., DeVries, A. L. (1972). Structure and mode of action of glycoproteins from an Antarctic fish. *Biochimica et Biophysica Acta, 263*(2), 406-413.

Shier, W. T., Lin, Y., DeVries, A. L. (1975). Structure of the carbohydrate of antifreeze glycoproteins from an Antarctic fish. *FEBS Letters, 54*(2), 135-138.

Shumway, S. E. (1977). The effects of fluctuating salinities on four species of asteroid echinoderms. *Comparative Biochemistry and Physiology Part A - Physiology, 58*(2), 177-179.

Siddall, M. E. (2002). Phylogeny of the leech family Erpobdellidae (Hirudinida : Oligochaeta). *Invertebrate Systematics, 16*(1), 1-6.

Siddall, M. E., Burreson, E. M. (1998). Phylogeny of leeches (Hirudinea) based on mitochondrial cytochrome c oxidase subunit I. *Molecular Phylogenetics and Evolution, 9*(1), 156-162.

Simon, W., Giacobbo, H. (1965). Thermische Fragmentierung und Strukturbestimmung organischer Verbindungen [Thermal fragmentation and the determination of the structure of organic compounds]. *Chemie Ingenieur Technik, 37*(7), 709-714.

Singh, A. P. (2010). Medicinal leech therapy (Hirudotherapy): A brief overview. *Complementary Therapies in Clinical Practice, 16*(4), 213-215.

Sloan, N. A., Bower, S. M., Robinson, S. M. C. (1984). Cocoon deposition on three crab species and fish parasitism by the leech *Notostomum cyclostoma* from deep fjords in northern British Columbia. *Marine Ecology - Progress Series, 20*, 51-58.

Smith, K. E., Bradley, R. L. (1983). Effects on freezing point of carbohydrates commonly used in frozen desserts. *Journal of Dairy Science, 66*(12), 2464-2467.

Somero, G. N., DeVries, A. L. (1967). Temperature tolerance of some Antarctic fishes. *Science, 156*(3772), 257-258.

Sorhannus, U. (2012). Evolution of type II antifreeze protein genes in teleost fish: A complex scenario involving lateral gene transfers and episodic directional selection. *Evolutionary Bioinformatics, 8*, 535-544.

Stan, C. A., Schneider, G. F., Shevkoplyas, S. S., Hashimoto, M., Ibanescu, M., Wiley, B. J., et al. (2009). A microfluidic apparatus for the study of ice nucleation in supercooled water drops. *Lab Chip, 9*(16), 2293-2305.

Stephenson, J. (1922). The Oligochæta of the Oxford University Spitzbergen expedition. *Proceedings of the Zoological Society of London, 92*(4), 1109-1138.

Stone, J. V., Mordue, W., Batley, K. E., Morris, H. R. (1976). Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilisation during flight. *Nature, 263*(5574), 207-211.

Strous, G. J. A. M. (1979). Initial glycosylation of proteins with acetylgalactosaminylserine linkages. *Proceedings of the National Academy of Sciences of the United States of America, 76*(6), 2694-2698.

Stuckey, I. H., Curtis, O. F. (1938). Ice formations and the death of plant cells by freezing. *Plant Physiology, 13*, 815-833.

Tatarenko, D. E., Poltaraus, A. B. (1993). Affiliation of sea urchin *Pseudocentrotus depressus* to the family *Strongylocentrotidae* and description of a new genus *Mesocentrotus* belonging to the group based on DNA-DNA hybridization and comparative morphological data. *Zoologicheskii Zhurnal [Russian Journal of Zoology], 72*(2), 61-72.

Ten Hagen, K. G., Fritz, T. A., Tabak, L. A. (2003). All in the family: The UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases. *Glycobiology, 13*(1), 1R-16R.

Theede, H. (1972). Vergleichende ökologisch-physiologische Untersuchungen zur zellulären Kälteresistenz mariner Evertebraten [Comparative ecological and physiological studies on the cellular cold resistance in marine invertebrates] (J. B. Kolb, Trans.). *Marine Biology, 15*(2), 160-191.

Theede, H., Schneppenheim, R., Béress, L. (1976). Frostschutz-Glykoproteine bei *Mytilus edulis*? [Antifreeze glycoproteins in *Mytilus edulis*?] (J. B. Kolb, Trans.). *Marine Biology, 36*(2), 183-189.

Thomashow, M. F. (1998). Role of cold-responsive genes in plant freezing tolerance. *Plant Physiology, 118*(1), 1-7.

Thomson, J. J. (1913). On the appearance of Helium and Neon in vacuum tubes. *Science, 37*(949), 360-364.

Timm, R. W. (1971). Antarctic soil and freshwater nematodes from the McMurdo Sound region. *Proceedings of the Helminthological Society of Washington, 38*(1), 42-52.

Tomchaney, A. P., Morris, J. P., Kang, S. H., Duman, J. G. (1982). Purification, composition, and physical properties of a thermal hysteresis "antifreeze" protein from larvae of the beetle *Tenebrio molitor*. *Biochemistry, 21*(4), 716-721.

Torlais, J. (1958). Chronologie de la vie et des œuvres de René-Antoine Ferchault de Réaumur [Chronology of the life and works of René-Antoine Ferchault de Réaumur] (J. B. Kolb, Trans.). *Revue d'histoire des sciences et de leurs applications, 11*(1), 1-12.

Torres, C. R., Hart, G. W. (1984). Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. *Journal of Biological Chemistry, 259*(5), 3308-3317.

Tyshenko, M. G., Doucet, D., Davies, P. L., Walker, V. K. (1997). The antifreeze potential of the spruce budworm thermal hysteresis protein. *Nature Biotechnology, 15*(9), 887-890.

Umminger, B. L. (1967). Sub-zero temperatures and supercooling in *Fundulus heteroclitus*. *American Zoologist, 7*(4), 731.

Umminger, B. L. (1975). Low temperature resistance adaptations in the killifish, *Fundulus heteroclitus*. In F. J. Vernberg (Ed.), *Physiological Ecology of Estuarine Organisms* (1 ed., pp. 59-71). Columbia: University of South Carolina Press.

Utevsky, A. Y. (1994a). A new genus of Antarctic piscicolid leeches (Hirudinea, Rhynchobdellida). *Vestnik Zoologii, 4*(5), 73-76 (In Russian with English summary and descriptive text for figures).

Utevsky, A. Y. (1994b). A new marine leech *Nototh eniobdella sawyeri* gen. et sp. n. from Antarctic seas (Hirudinea: Piscicolidae). *Zoosystematica Rossica, 1993, 2*(2), 237-240.

Utevsky, A. Y. (1995). A new Antarctic leech genus (Hirudinea, Piscicolidae). *Vestnik Zoologii, 5*(6), 3-12 (In Russian with English summary and descriptive text for figures).

Utevsky, A. Y. (1997). A new species of piscicolid leeches (Hirudinea, Piscicolidae) from Antarctic seas. *Vestnik Zoologii, 31*(1-2), 17-24 (In Russian with English summary and descriptive text for figures).

Utevsky, A. Y. (2005). An identification key to Antarctic fish leeches (Hirudinea: Piscicolidae). *УКРАЇНСЬКИЙ АНТАРКТИЧНИЙ ЖУРНАЛ [Ukrainian Antarctic Journal], 3*, 135-144.

Utevsky, A. Y. (2007). Antarctic piscicolid leeches. *Bonner zoologische Monographien, 54*, 1-80.

Utevsky, S. Y., Trontelj, P. (2004). Phylogenetic relationships of fish leeches (Hirudinea, Piscicolidae) based on mitochondrial DNA sequences and morphological data. *Zoologica Scripta, 33*(4), 375-385.

Utevsky, S. Y., Utevsky, A. Y., Schiaparelli, S., Trontelj, P. (2007). Molecular phylogeny of pontobdelline leeches and their place in the descent of fish leeches (Hirudinea, Piscicolidae). *Zoologica Scripta, 36*(3), 271-280.

Uy, R., Wold, F. (1977). Posttranslational covalent modification of proteins. *Science, 198*(4320), 890-896.

Van den Steen, P., Rudd, P. M., Dwek, R. A., Opdenakker, G. (1998). Concepts and principles of O-linked glycosylation. *Critical Reviews in Biochemistry and Molecular Biology, 33*(3), 151-208.

van Valen, L. M. (1973). A new evolutionary law. *Evolutionary Theory, 1*, 1-30.

Vandenheede, J. R., Ahmed, A. I., Feeney, R. E. (1972). Structure and role of carbohydrate in freezing point-depressing glycoproteins from an Antarctic fish. *Journal of Biological Chemistry, 247*(24), 7885-7889.

Venne, A. S., Kollipara, L., Zahedi, R. P. (2014). The next level of complexity: Crosstalk of posttranslational modifications. *Proteomics, 14*(4-5, SI), 513-524.

Vogt, T., Jones, P. (2000). Glycosyltransferases in plant natural product synthesis: Characterization of a supergene family. *Trends in Plant Science, 5*(9), 380-386.

Wägele, J.-W. (1988). Aspects of the life-cycle of the Antarctic fish parasite *Gnathia calva* Vanhöffen (Crustacea: Isopoda). *Polar Biology, 8*(4), 287-291.

Walk, T. B., Süßmuth, R., Kempter, C., Gnau, V., Jack, R. W., Jung, G. (1999). Identification of unusual amino acids in peptides using automated sequential Edman degradation coupled to direct detection by electrospray-ionization mass spectrometry. *Biopolymers, 49*(4), 329-340.

Wallace, A. R. (1869a). *The Malay Archipelago: The land of the orang-utan, and the bird of paradise. A narrative of travel, with studies of man and nature* (1 ed. Vol. 1). London: Macmillan.

Wallace, A. R. (1869b). *The Malay Archipelago: The land of the orang-utan, and the bird of paradise. A narrative of travel, with studies of man and nature* (1 ed. Vol. 2). London: Macmillan.

Waller, C. L., Worland, M. R., Convey, P., Barnes, D. K. A. (2006). Ecophysiological strategies of Antarctic intertidal invertebrates faced with freezing stress. *Polar Biology, 29*(12), 1077-1083.

Wang, J., Pansky, A., Venuti, J. M., Yaffe, D., Nudel, U. (1998). A sea urchin gene encoding dystrophin-related proteins. *Human Molecular Genetics, 7*(4), 581-588.

Wang, X. (2009). Structure, mechanism and engineering of plant natural product glycosyltransferases. *Federation of European Biochemical Societies Letters, 583*(20), 3303-3309.

Wang, X., DeVries, A. L., Cheng, C.-H. C. (1995). Genomic basis for antifreeze peptide heterogeneity and abundance in an Antarctic eel pout: Gene structures and organization. *Molecular Marine Biology and Biotechnology, 4*(2), 135-147.

Wang, Y. (2013). O-GlcNAc transferase and its inhibitors. *Acta Chimica Sinica, 71*(11), 1477-1487.

Ward, R. S., Pelter, A. (1974). The analysis of mixtures of closely related naturally-occurring organic compounds using high performance liquid chromatography. *Journal of Chromatographic Science, 12*(10), 570-574.

Weigmann, R. (1929). Die Wirkung starker Abkühlung auf Amphibien und Reptilien [The effect of strong cooling on amphibians and reptiles] (J. B. Kolb, Trans.). *Zeitschrift für wissenschaftliche Zoologie - Abteilung A, 134*, 641-692.

Wells, L., Vosseller, K., Hart, G. W. (2001). Glycosylation of nucleocytoplasmic proteins: Signal transduction and O-GlcNAc. *Science, 291*(5512), 2376-2378.

West, C. J., Dorsey, N. E., Bichowsky, F. R., Klemenc, A. (1926). International critical tables. In E. W. Washburn, G. K. Burgess, S. Dushman, J. Johnston, S. C. Lind, C. E. Mendenhall & R. B. Moore (Eds.), *International Critical Tables of Numerical Data, Physics, Chemistry and Technology* (1 ed., Vol. 1, pp. 415). New York: McGraw-Hill; Published for the National Research Council of the United States of America.

West, C. J., Dorsey, N. E., Bichowsky, F. R., Klemenc, A. (1928). Phase-equilibrium data - systems without the vapor phase. In E. W. Washburn, G. K. Burgess, S. Dushman, J. Johnston, S. C. Lind, C. E. Mendenhall & R. B. Moore (Eds.), *International Critical Tables of Numerical Data, Physics, Chemistry and Technology* (1 ed., Vol. 4, pp. 481). New York: McGraw-Hill; Published for the National Research Council of the United States of America.

Wharton, D. A., Ferns, D. J. (1995). Survival of intracellular freezing by the Antarctic nematode *Panagrolaimus davidi*. *Journal of Experimental Biology, 198*(6), 1381-1387.

Whitaker, I. S., Oboumarzouk, O., Rozen, W. M., Naderi, N., Balasubramanian, S. P., Azzopardi, E. A., et al. (2012). The efficacy of medicinal leeches in plastic and reconstructive surgery: A systematic review of 277 reported clinical cases. *Microsurgery, 32*(3), 240-250.

Whitaker, I. S., Rao, J., Izadi, D., Butler, P. E. (2004). Historical Article: *Hirudo medicinalis*: Ancient origins of, and trends in the use of medicinal leeches throughout history. *British Journal of Oral and Maxillofacial Surgery, 42*(2), 133-137.

White, J. D., Gall, C. M., McKelvy, J. F. (1986). Proenkephalin is processed in a projection-specific manner in the rat central nervous system. *Proceedings of the National Academy of Sciences of the United States of America, 83*(18), 7099-7103.

Whitlock, M. R., O'Hare, P. M., Sanders, R., Morrow, N. C. (1983). The medicinal leech and its use in plastic surgery: A possible cause for infection. *British Journal of Plastic Surgery, 36*(2), 240-244.

Wilkins, S. P., Blum, A. J., Burkepile, D. E., Rutland, T. J., Wierzbicki, A., Kelly, M., et al. (2002). Isolation of an antifreeze peptide from the Antarctic sponge *Homaxinella balfourensis*. *Cellular and Molecular Life Sciences, 59*(12), 2210-2215.

Williams, J., Burreson, E. (2005). Revision of the genus *Bdellamaris* (Hirudinea: Piscicolidae) including a new combination, *Bdellamaris manteri*. *New Zealand Journal of Marine and Freshwater Research, 39*(6), 1331-1337.

Williams, J. I., Burreson, E. M. (2006). Phylogeny of the fish leeches (Oligochaeta, Hirudinida, Piscicolidae) based on nuclear and mitochondrial genes and morphology. *Zoologica Scripta, 35*(6), 627-639.

Winder, S. J. (1997). The membrane-cytoskeleton interface: The role of dystrophin and utrophin. *Journal of Muscle Research and Cell Motility, 18*(6), 617-629.

Winnebeck, E. C., Millar, C. D., Warman, G. R. (2010). Why does insect RNA look degraded? *Journal of Insect Science, 10*(159), 1-7.

Wisztorski, M., Fatou, B., Franck, J., Desmons, A., Farre, I., Leblanc, E., et al. (2013). Microproteomics by liquid extraction surface analysis: Application to FFPE tissue to study the fimbria region of tubo-ovarian cancer. *Proteomics. Clinical applications, 7*(3-4), 234-240.

Wittenberg, J. B. (1957). The separation of the C_6–C_{12} fatty acids by reversed-phase partition chromatograph. *Biochemical Journal, 65*(1), 42-45.

WoRMS (2013). *Janusion nuda* (Richardson, 1970). Available from World Register of Marine Species at http://www.marinespecies.org/aphia.php?p=taxdetails&id=370632

Worthen, P. L., Gode, C. J., Graf, J. (2006). Culture-independent characterization of the digestive-tract microbiota of the medicinal leech reveals a tripartite symbiosis. *Applied and Environmental Microbiology, 72*(7), 4775-4781.

Wrabl, J. O., Grishin, N. V. (2001). Homology between O-linked GlcNAc transferases and proteins of the glycogen phosphorylase superfamily. *Journal of Molecular Biology, 314*(3), 365-374.

Wu, D. W., Duman, J. G., Cheng, C.-H. C., Castellino, F. J. (1991). Purification and characterization of antifreeze proteins from larvae of the beetle *Dendroides canadensis*. *Journal of Comparative Physiology B - Biochemical Systemic and Environmental Physiology, 161*(3), 271-278.

Xin, F., Radivojac, P. (2012). Post-translational modifications induce significant yet not extreme changes to protein structure. *Bioinformatics, 28*(22), 2905-2913.

Yang, T. (1987). Two marine leeches from an Antarctic fish of the genus *Notothenia*. *Acta Zoologica Sinica, 33*(4), 373-377.

Yates, J. R. (2000). Mass spectrometry - from genomics to proteomics. *Trends in Genetics, 16*(1), 5-8.

Zerbst-Boroffka, I. (1970). Organische Säurereste als wichtigste Anionen im Blut von *Hirudo medicinalis* [Organic acids as the most important anions in the blood of *Hirudo medicinalis*] (J. B. Kolb, Trans.). *Zeitschrift für vergleichende Physiologie, 70*(3), 313-321.

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