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

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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

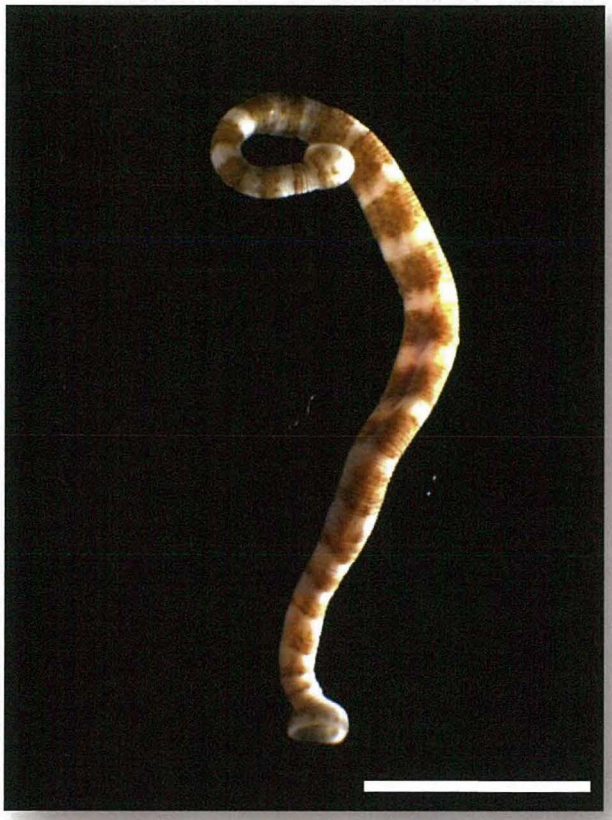

Image of *Cryobdella antarctica* (Epshtein, 1970), scale bar 5mm.

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.

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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

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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)