The effect of incubation temperature on early malformation, regionalisation and meristic characters of the vertebral column in farmed Chinook salmon (*Oncorhynchus tshawytscha*).

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ABSTRACT

Skeletal deformities are a recurrent problem in farmed Chinook salmon which limit production and have animal welfare impacts. Skeletal deformities of a variety of types are recognised especially when the external phenotype of the animal is affected. These types are well described in juvenile and adult stages of the production cycle. Which skeletal malformations affect early life stages in salmonids is less well known. Temperature is commonly manipulated in fish farming husbandry. High rearing temperatures are related to higher growth rates and in Atlantic salmon, elevated temperature has been inferred as a potential risk factor for skeletal deformities. In this thesis, malformations of the vertebral column in post-hatch to first feed life stages (500-900 degreedays) were studied in farmed Chinook salmon (*Oncorhynchus tshawytscha*) in New Zealand. Fish were reared at a constant 4°C, 8°C and 12°C, from fertilisation to juvenile stages. The effects of rearing fish at these temperatures on malformations of the vertebral column were studied in specimens whole-mount stained for cartilage and mineralised bone, and in histological sections. The external phenotype of post-hatch stages could be linked to internal skeletal malformations such as notochord malformations, chordacentra fusions and malformations of the associated elements. In all temperature groups, externally normal specimens could have internal malformations, predominantly fused chordacentra. Conversely, externally malformed fish usually displayed internal malformations. Specimens raised at 8°C had fewest malformations, followed by specimens of the 12°C group. Specimens raised at 4°C had the highest number of malformations. This study indicates that 8°C is the best incubation temperature of those tested. In addition, the effects of rearing temperature on morphological variation of skeletal elements such as vertebrae, vestigial ribs and vestigial elements in the caudal fin were studied. Six vertebral column regions were identified. The defining characters of each of these regions remained independent of the rearing temperature. Still, the postcranial, transitional and ural regions showed temperature sensitive meristic variation of the vertebrae, vestigial ribs, arches, epurals and uroneurals. Meristic variation can foreshadow skeletal malformations that emerge late in life and thus be significant for the early diagnosis of vertebral deformities.

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I was still in the full swing of writing the Ph.D. thesis when I first drafted this section. However, I am nearing the end and one question has been tumbling through my mind many times in the last six months: “Is it truly hard to do a Ph.D. project, and if yes, why is it so hard?” I can only answer this question from my own perspective and experience(s) and thus different people may have different answers or may ask the question differently.

From a scientific viewpoint my answer to the question is fairly straightforward and simple: “No, scientifically I find a Ph.D. project not hard to do.” I feel that when one is passionate about science, passionate about working in the lab, and is curious about the results of the experiments, a Ph.D. project is not so hard to do. One question that follows is: “Was the project not scientifically challenging?” The answer to this latter question is a firm: “No, it was challenging.” Not the difficult experimental methods or statistical analyses, but the scope, range and depth of the subject were challenging. Skeletal tissues have deep roots in evolution, are observable over hundreds of millions of years and have hundreds of books describing their anatomy in currently living animals. In addition, hundreds of outside and innate factors influence the skeleton. The width and depth of this subject take gargantuan proportions. The diversity in anatomy of skeletal tissues of currently living teleost fish alone is mindboggling. Moreover, one can happily spend an entire life studying the skeleton of a single fish species, for example the salmon. With passion, drive and curiosity the daily challenges while doing science are overcome. “Than why do PhD students suffer so much during their project?”

Many a time I heard from fellow Ph.D. students, friends and people close to me say that they are frustrated, stressed, burned-out, having health issues and being outright unhappy. “Are these ‘things’ also part of doing science?” Again, for me personally the answer is straightforward and simple: “No, the suffering is not a part of doing science.” The negative emotions and also positive emotion one experiences during a Ph.D. project is part of being human. This brings me to the ‘Why it is so hard to do a Ph.D. project’. For me, science is the absolute sustenance of my rationale. I eat, drink and breath science. But, above all I am still a human being, full of emotions and believes. However, being a human is sometimes hard. That is why doing a PhD project is so hard. For me, the project was a constant search for a balance. Distancing myself from feeling and believing during scientific activities, while feeling and believing during life experiences outside science. Searching for a balance, something we all struggle with is likely part of the real valuable lessons I have learned during this Ph.D. project. As humans, we have help. The amazing people who helped and supported me as scientist and human brings me to the true purpose of this section: ‘Saying thank you’.

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List of Symbols and Abbreviations

6m	six months (at sea)
°C	degrees centigrade
°d	degreedays
τ_s	Gorodilov's tau S
>	larger than
%	percent
±	plus or minus
<	smaller than
Σ	summation
antS	anterior spine
AB	Alcian blue
AC	alternating current
ALP	alkaline phosphatase
AB-PAS	Alcian blue-periodic acid Shiff
AR	Alizarin red
ARF	ADP-ribosylation factor
Atf	cyclic AMP-dependent transcription factor
Bbf	multifactor complex containing B element binding factor
BD	basidorsal
BMP	bone morphogenetic protein
BN	bent neck
BNA	base of neural arch
BTM	bent tail medium
BTS	bent tail severe
BV	basiventral
cm	centimetre
C	compression
C	compression and/or reduced intervertebral spaces
Cb	chordoblast(s)
CB	cancellous bone
CC	central canal
CHC	chordacentrum
Cltc	clathrin coated
CM	curly medium
Col	collagen

COP	coatomer protein
Creb	cyclic AMP response element-binding protein
CS	curly severe
CTM	critical thermal methodology
d	days
dd	degreedays
df	degrees of freedom
dpf	days post fertilisation
DEPC	diethyl pyrocarbonate
DIC	differential interference contrast microscopy
E	epural(s)
ECM	extracellular matrix
EEM	external elastic membrane
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
F	fertilisation
F	fusion
FF	first feed
Fig.	figure
FL	fork length
FW	freshwater
g	gram
glm	general linear model
h	hour(s)
H	hatching
HA	haemal arch
H_2O_2	hydrogen peroxide
Hox	homeobox
HS	haemal spine
HSP	heath shock protein
Hy	hypural(s)
ILT	incipient lethal temperature
IV	intervertebral space
K	kyphosis
KOH	potassium hydroxide
kV	kilovolt
l	left

logistf	Firth logistic regression
L	litre
L	lordosis
LKS	lordosis, kyphosis, scoliosis
LLT	lower lethal temperature
LRO	lysosome related organelles
μm	micrometre
m^3	cubic metre
mA	milliamps
min	minute
mm	millimetre
mM	millimolar
ml	millilitre
MET	mesenchymal-epithelial transition
Mfh	mesenchyme fork head
MMP	matrix metalloproteinase
nm	nanometre
nsh	notochord sheath
N	normal
NA	neural arch
NCH	notochord
NS	neural spine
NT	neural tube
NTC	notochord
NZKS	New Zealand King Salmon
opc	opistural cartilage
O.	*Oncorhynchus*
OPT	optimum temperature
pap	parapophyses
ppm	parts per million
pERK	phosphorylated extracellular signal-regulated kinase
pS6	phosphorylated S6
Pax	paired box
PBS	phosphate-buffered saline
PH	parhypural
PH	post-hatch
PHy	parhypural

PFA	paraformaldehyde
PIT	passive integrated transponder
PLP	paraformaldehyde-lysine-periodate
Pre-zyg	pre-zygapophysis
Pst-zyg	post-zygapophysis
PU	preural
r	right
R	region
R	rib
RAS	recirculating aquaculture system
sec	seconds
smolt	freshwater pre-smoltification stage
spp	species
Sox	sex-determining region Y-related high mobility group box
S	scoliosis
SC	scar tissue
SD	standard deviation
SHH	sonic hedgehog
SIG	salmon improvement group
SMM	spinal malformation medium
SMS	spinal malformation severe
ST	stegural
SW	seawater
SW-6	six months in seawater
SW-12	12 months in seawater
T	time
TEM	transmission electron microscopy
TL	total length
TM	tail malformation
TNL	TNL International Ltd
Total	total deformities
U	ural
ULT	upper lethal temperature
UN	uroneural
UPR	unfolded protein response
UV	ultraviolet
vbi	vertebrae imperfecta

V	vertebra(e)
VE	vertebral endplate
VNA/S	vestigial neural arch/spine
VS	vertical shift of the entire vertebral centrum
WT	wild type

CHAPTER 1: GENERAL INTRODUCTION

1.1. AQUACULTURE – AN EXPANDING INDUSTRY WITH CHALLENGES

1.1.1. A LUCRATIVE INDUSTRY

Aquaculture today is an important and fast growing global industry. Estimates of the production volume and value of aquaculture go as far back as the 1950's. However, reliable estimates are available from 1984 onwards (http://www.fao.org). The latest available estimates (2015) of some production categories are presented in Table 1. The global aquaculture production of all aquatic organisms, both freshwater and marine, increased from about 10.2 million tons in 1984 to about 106 million tons in 2015. The value in US Dollars increased from about $11 billion in 1984 to almost $163 billion in 2015. An increase in production volume also occurred in farming of salmonids. In 1984 the global salmonid production was 246 thousand tons and increased to 3.4 million tons in 2015. Atlantic salmon (*Salmo salar*) is the single most important farmed salmon species on a global scale. The main production occurs in Northern Europe, with Norway producing one million tons a year. Atlantic salmon is also produced in Chile, Australia and Canada. Global Atlantic salmon production in 1984, about 27 thousand tons, increased to almost 2.4 million tons in 2015. The farming of Chinook salmon (*Oncorhynchus tshawytscha*) now represents a small proportion of the global salmonid production and currently only occurs in New Zealand, whereas previously Chinook salmon was also produced in Chile and mainly in Canada. New Zealand Chinook salmon production in 1984 was about 135 tons, but has increased to almost 12.5 thousand tons in 2015.

Table1: Aquaculture production volume and value (estimates from 2015, http://www.fao.org)

Category	Volume (tons)	Value (1000 US$)	Value* (1000 NZ$)
Global (all)	106 004 184	162 974 582	236 639 093
Global salmonids	3 410 890	16 189 384	23 506 985
Global Atlantic salmon	2 381 576	11 945 146	17 344 351
Oceania salmonids	60 870	630 639	915 687
Australia salmonids	48 336	472 645	686 280
New Zealand Chinook salmon	12 474	157 233	215 093

* 1 US$ = 1.452 NZ$, 25th of October 2017 (https://xe.com)

1.1.2. INTRODUCTION AND FARMING OF CHINOOK SALMON IN NEW ZEALAND

The successful introduction of Chinook salmon occurred over a seven year period (1901-07) in which batches of 300 000 to 500 000 ova were transported to New Zealand. Some reports indicate that these eggs came from Battle Creek, a tributary of the Sacramento River, and probably also various other hatcheries in the Sacramento River basin (Haworth, 2010; McDowall, 1994). The eggs were brought

to the newly built hatchery on the Hakataramea River, a tributary of the Waitaki River in the South Island. The first reports of returning mature Chinook salmon were in 1906 and returns of a couple of hundred fish were reported in 1908. It was deducted at the time that these adult Chinook salmon originated from the 1901-07 releases. In the following decade the species spread into the rivers on the East Coast of the South Island of New Zealand (Quinn & Unwin, 1993) with currently major runs of Chinook salmon present in all the major rivers on the East Coast, minor and erratic runs in the rivers on the West Coast of the South Island and only occasional stray's into the rivers of the North Island (McDowall, 1994). The rivers supporting the largest populations of Chinook salmon in New Zealand are the Waitaki, Rangitata, Rakaia and Waimakariri River. These rivers are primarily fed by snow and ice melt from the Southern Alps flowing east to the South Pacific Ocean (Quinn et al., 2001).

Fishing for salmon in the rivers was part of the heritage and culture of the colonists who left their home countries behind to come to New Zealand. In addition, Chinook salmon was a prized game fish in California and was therefore selected for introduction in New Zealand. From 1900 until 1930 many small hatcheries in New Zealand produced post-hatching and juvenile stage Chinook salmon to stock rivers for anglers. From 1930 onwards these operations came under threat by the development of hydroelectric dams, which destroyed many natural runs to the original spawning grounds up-river. During the 1950's a battle ensued between the government, who wanted to commercialise salmon fishing, and the Acclimatisation Societies, who wanted to conserve salmon for sport fishing purposes. In the 1960's the Acclimatisation Societies and the Marine Department realised that more knowledge was necessary to save the Chinook salmon from local extinction due to the development of dams and the fishing in rivers and at sea. This led to the building of hatcheries as research facilities, for example the Glenariffe Salmon Research Station and the Silverstream Hatchery. In the 1970's a legislative battle started between the Acclimatisation Societies and the government for the approval of salmon and trout farming. Although in 1972 a salmon and trout farming bill was passed in parliament, opposing parties continued to battle in the 80's and 90's. Today only salmon is allowed to be farmed. By 1980 the Acclimation Societies recognised that farming salmon by ocean ranching methods would increase the number of salmon in the rivers and therefore the ability to sport fish. The ocean ranching farming method catches adult salmon in the rivers, fertilises the eggs and grows the embryos to juvenile stages. The juvenile salmon are released in the streams, which go to sea to grow and the adults are caught again when they return to the streams. A major issue with ocean ranching is the salmon by-catch at sea. This caused the rapid decline of returning salmon to the rivers and the marginal successes of ocean ranching methods. In 1983 a bill to legalise sea cage farming was proposed. This led to the development of the current aquaculture companies farming Chinook salmon in sea cages, amongst which New Zealand King Salmon is the largest with a production of about 8000 tons of salmon per year. A detailed history of the New Zealand salmon farming industry can be found in the book: 'Swimming Upstream' by Jennifer Haworth (2010).

1.1.3. CHALLENGES

1.1.3.1. An expanding industry

Although aquaculture is expanding worldwide, the industry also faces challenges. For example the growth in production in Nordic countries such as Denmark, Faroe Islands, Finland, Iceland and Sweden, was stagnant in 2010-2011. Changes in the natural environment, e.g. multiple years with lower seawater temperatures, together with legislative changes that limit access to freshwater and seawater sites, strict rules on waste water control and animal welfare can slow down the industry (Badiola et al., 2012; Dalsgaard et al., 2013). Moreover, environmental and legislative changes will become more relevant on a global scale in the future (Martins et al., 2010; Paisley et al., 2010). Easing some of these constraints, recirculating aquaculture systems (RAS) allow for the industry to further expand via land-based facilities and diminishing the need for extensive marine infrastructure. The grow-out of sea stage juvenile and adult animals (= post-smolts) takes place in sea cages, while traditionally the egg incubation and juvenile growth in freshwater (= pre-smolts) takes place in land based systems such as ponds, raceways and lakes (Bergheim et al., 2009; Terjesen et al., 2009). The RAS systems allow for incubation, juvenile growth and part of the adult grow-out stage to take place on land. While pre-smolt stages were grown to a size range of 30 to 170 g before going to sea, the RAS system allows to grow pre-smolt stages up to a size of 1000g before going to sea (Bergheim et al., 2009; Dalsgaard et al., 2013). The use of RAS systems reduces the water consumption and increases the control of waste water. Furthermore, the temperature and water quality parameters such as dissolved oxygen, carbon dioxide, nitrogen-products, pH, salinity and light, can be perfectly controlled allowing rearing conditions, growth rate and food utilisation to be optimised. The RAS systems also allow for a better year round production of salmon because seasonal effects of sea water temperature and light, and the time that the animal is in a sea cage, are reduced (Dalsgaard et al., 2013). Welfare of farmed animals has become a major topic over recent years. A RAS system allows for increased sustainability on the level of welfare issues (Badiola et al., 2012; Martins et al., 2010). Although current RAS systems are still sensitive to technical failures (Badiola et al., 2012), improvements in the engineering of RAS systems could provide a very stable environment, with temperatures and other water quality parameters under full control in all seasons. Thus, increasing the welfare of the farmed animal (Zhang et al., 2011).

1.1.3.2. Skeletal anomalies

Skeletal deformities present a major challenge to teleost aquaculture. The current knowledge of the aetiology, ontogeny, development and types of normal and abnormal skeletal tissues in farmed teleosts was reviewed by Boglione et al. (2013a; 2013b) and by Fjelldal et al. (2012) for Atlantic salmon. These authors made two important conclusions: (i) the underlying cause of skeletal anomalies

is multifactorial and (ii) the knowledge about how genetic and epigenetic factors cause skeletal anomalies needs to be further researched and extended. Many different terms define skeletal anomalies in scientific papers and books (Boglione et al., 2013b). However, throughout this thesis mainly two terms will be used consistently, i.e. (i) malformation, when a skeletal structure has an intrinsically abnormal development, and (ii) deformity, when a skeletal structure developed normally but acquired an abnormal phenotype in a later life stage.

The oldest written and illustrated reports of external or visual deformities in caught fish date back to the 1500s. Interestingly, salmon were the focus in two works by Pierre Belon (Belon du Mans, 1555; Cenomani Bellonii, 1553), showing the hooked lower jaw of an adult salmon. We now know this is not a deformity (Witten & Hall, 2002; Witten & Hall, 2003; Witten et al., 2005b). Therefore, Rondelet (1555), who showed a pug-nosed juvenile or adult carp (not specified), was the first to describe and illustrate a deformity. Reports of embryonic forms are much rarer and are only found later in the literature, in the late 1800's. Interestingly, the earliest accounts of malformations are again in salmonids. Moreover, the 'pug-nose' is the phenotype that is described in post-hatch salmon and trout (no species information) by Buckland (1877), Girdwoyn (1877) and Quatrefages (1888).

Skeletal deformities under farming conditions cause economic, biological and animal welfare issues. Deformed animals are less likely to be accepted by the consumer (Fjelldal et al., 2012; Gjerde et al., 2005). Normal looking animals with internal skeletal deformities may cause technical problems during the filleting process (Gjerde et al., 2005). Rainbow trout (*Oncorhynchus mykiss*) was the first species where skeletal anomalies were reported under farming conditions (Aulstad & Kittelsen, 1971). However, it was only in the mid 1990's that significant losses of farmed Atlantic salmon were attributed to skeletal deformities (Vågsholm & Djupvik, 1998). In addition, certain skeletal deformities can be present in high frequencies in externally normal animals (Gjerde et al., 2005). This means that skeletal deformities are only noticed in the harvest stage of the animal's life cycle.

Although all skeletal elements can be deformed, going from the lower jaw of 'screamer' deformities and upper jaw of 'pug-nose' deformities, to deformed fin rays in the caudal fin, this thesis focusses on the vertebral column. Three major categories of deformities of the vertebral column are distinguished based on studies of wild and farmed salmon species, i.e. (i) vertebral axis deviations, (ii) vertebral centra fusion and (iii) vertebral centrum deformity (Witten et al., 2009). These three major categories were further refined into 20 deformity types based on observations in farmed Atlantic salmon. Vertebral axis deviations include lordosis, kyphosis, and scoliosis. Vertebral centra fusions include a varying degree of compression, elongation and complete fusion of the vertebral centra. Finally, vertebral centrum deformities include radio-translucent and radio-dense centra, and centra symmetry deviations. Most of the 20 deformity types identified by Witten et al. (2009) are vertebral centrum deformities, with axis deviations (type 14, 15, 16) as a minor subset.

Relatively few of the twenty deformities identified in Atlantic salmon were observed in Pacific salmon. Gill and Fisk (1966) observed vertebral centra fusion, compression and vertical shift of centra, and displacement of vertebral arches in chum (*Oncorhynchus keta*), pink (*Oncorhynchus gorbuscha*) and sockeye salmon (*Oncorhynchus nerka*). They, however, did not consider displaced associated elements as a deformity. Seymour (1959) observed mainly fusion and compression and an occasional hyperdense vertebral centrum in four wild populations of Chinook salmon. Vertebral column axis deviations, such as lordosis, kyphosis and scoliosis, are the main type of deformities observed in free-living adult Chinook salmon in New Zealand. In addition, fusions, compression and vertical shifts of vertebral centra are present but are about three times less frequent compared to axis deviations (Davie et al., 2018). Similar deformities are observed in farmed Chinook salmon, with vertebral column curvatures (Lordosis, Kyphosis and Scoliosis = LKS) most prevalent followed by fusion, compression and vertical shift of vertebral centra. The deformity types can co-occur in a single vertebral column and can occur over its entire length (Perrott et al., 2018). Of the 20 deformity types described by Witten et al. (Witten et al., 2009), nine were very uncommon. These were types 4, compression without X-structure, 9 elongation, 10 widely spaced and undersized, 11 pronounced biconcave, 12 hyper-radiodense, 13 hyper-radiodense with flat endplates, 18 irregular internal structures, 19 internal dorsal or ventral shift, 20 severe multiple malformations. Together these nine deformity types made up four of the 327 deformities observed using the Witten system (Perrott et al., 2018). Interestingly, the vertebral column curvatures mostly occur in late sea life stages of farmed Chinook salmon, i.e. in the last three to nine months before harvest, and only rarely in pre-smoltification fresh water stages. The LKS deformity has been associated with unilateral perivertebral fibrosis of the vertical septum and adjacent muscle fibre bundles. The cause of the fibrosis and whether the fibrosis causes LKS remains to be elucidated (Munday et al., 2016).

Based on observations made on X-ray, an overall deformity prevalence of 4.3% was reported in farmed New Zealand Chinook salmon freshwater stages (Munday et al., 2018). Vertebral fusion was the most important deformity type with a prevalence of 2.7% followed by vertebral compression and vertical shift of the entire centrum, both with a prevalence of 1.3% (Munday et al., 2018). LKS deformities (0.1%) were shown to be very rare in freshwater stage Chinook salmon (Munday et al., 2018). For farmed New Zealand Chinook salmon at grading in sea and at harvest, respectively 0.9% and 4.6% of the animals were reported to have visual deformities (Perrott et al., 2018). The overall deformity prevalence at grading was reported to be 8.8% with vertebral fusion (5.6%) the most common deformity type, followed by vertebral compression (3.0%), vertical shift of the entire centrum (0.5%) and LKS (0.5%) (Perrott et al., 2018). At harvest the overall deformity prevalence was reported to be 38.4%, a large increase compared to the prevalence at grading. An increase in the prevalence of LKS (29.4%) and vertebral compression (22.0%) was responsible for most of the increased overall deformity prevalence at harvest (Perrott et al., 2018). Also vertebral fusion (7.6%)

was reported to be more prevalent at harvest. In contrast, vertical shift of the entire centrum had the same prevalence (0.5%) at harvest compared to grading (Perrott et al., 2018).

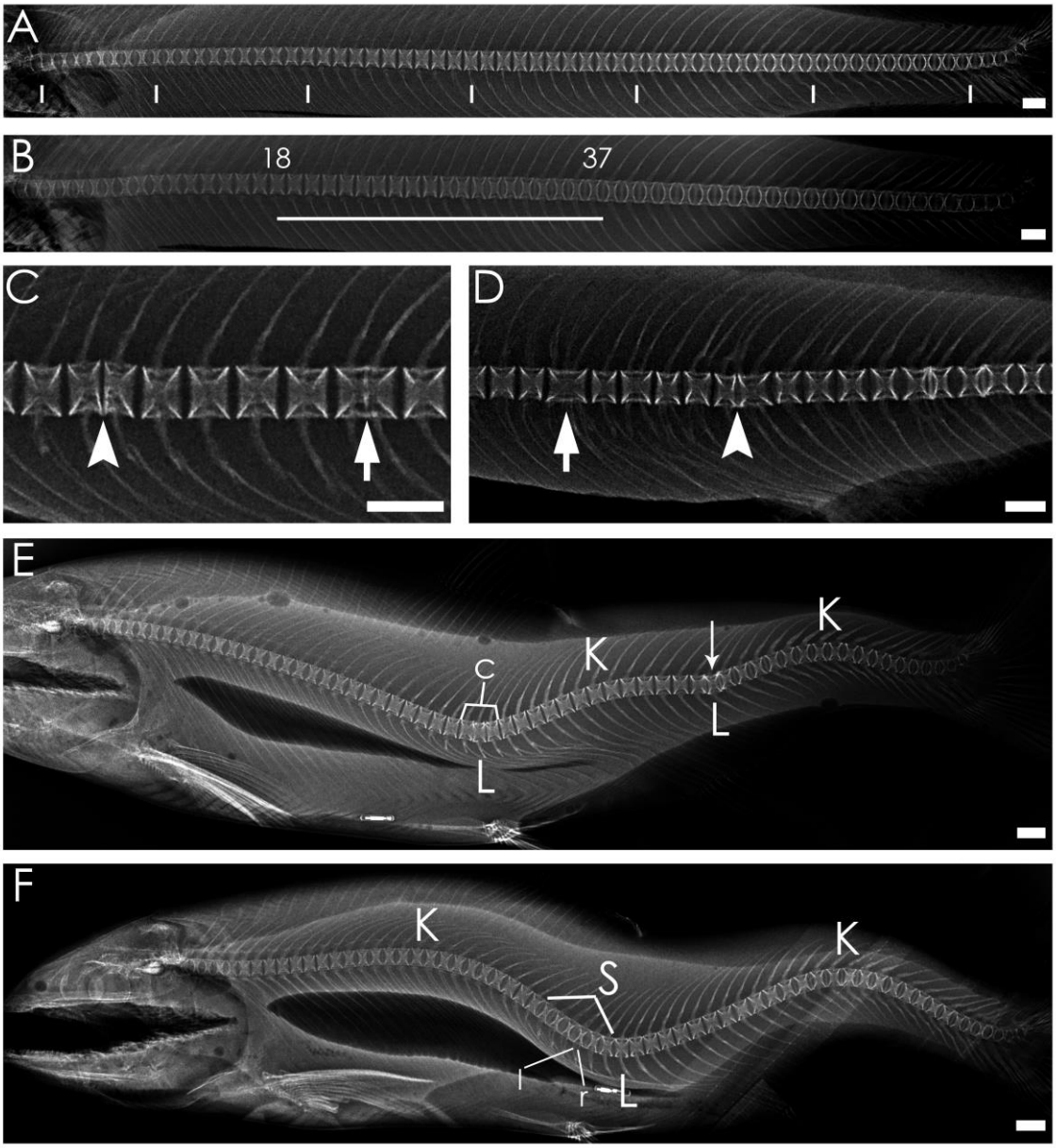


Figure 1: Deformities in New Zealand Chinook salmon

All images are digital X-ray images, oriented with anterior to the left, posterior to the right, dorsal at the top and ventral at the bottom. All scale bars are 1 cm. (A) Normal vertebral column in an adult animal. The left vertical bar indicates the first vertebral body. The subsequent white bars indicate every 10th vertebral body. This specimen has 65 vertebrae in total. (B) Entire vertebral column with a region of consistently compressed vertebrae. The compressed vertebrae (position 18 to 37) are indicated by the horizontal white bar. (C) Unilateral posterior (first vertebra) and anterior (second vertebra) compression (arrowhead) and fusion (block arrow) of adjacent vertebrae. (D) Fused and remodelled vertebrae (block arrow). Three neural and haemal arches indicate that three vertebrae are fused and remodelled. Compression is indicated by the white arrowhead, occurring between a single and double-remodelled vertebral body. The white arrow indicates a vertically shifted vertebral

centrum to the dorsal side. (E) Combination of lordosis (L) and kyphosis (K) with a vertically shifted vertebral centrum to the ventral side. The line indicates compression (C) of three vertebrae at the position of the downward bent. (F) Combination of lordosis (L), kyphosis (K) and scoliosis (S). Scoliosis is hard to observe on a lateral X-ray, however in this image both the left (l) and right (r) part of the haemal arches are visible.

Whether or not all these deformity types are truly deformities rather than malformations is currently unknown and represents a gap in the current knowledge. Early life stage malformations show a high prevalence in marine fish farming (Boglione et al., 2013b), but a far lower prevalence in salmon farming practices. This may explain why early life stage malformations in salmonids have not received much attention in the past. In addition, the literature on early life stage malformation in salmonids is sparse. Research on external malformations in salmonids increased after the Exxon Valdez oil spill in Alaska. These studies focussed on the relation between level of toxins in the water and prevalence of externally visible malformations (Finn, 2007; Johnson et al., 1998; Marty et al., 1997; Ron et al., 2000). One study investigated the metabolic effects of pesticides in egg and post-hatching stages of Chinook salmon (Viant et al., 2006). Deformities can occur in the three tissues that build the early life stage vertebral column, i.e. the notochord, the cartilaginous precursors of the neural and haemal arches and the early mineralised bone of the vertebral centrum and arches. Categorising deformities based on these tissue types in the adult vertebral column is challenging. The outer layer of the notochord, the notochord sheath, mineralises and appears to become bone. However, the chordacentrum is mineralised type II collagen and is more closely related to mineralised cartilage than to bone, which is mineralised type I collagen. The initially cartilaginous arches do become mostly bone. Also, the observed deformities have been mainly categorised in the adult vertebral column. Currently, knowledge about internal vertebral malformations in early life stages is sparse and needs to be expanded. Insights into the aetiology of deformities in later life stages will likely increase when more information is available about early malformations in salmonids.

1.2. TEMPERATURE: THE TOP EPIGENETIC FACTOR IN FISH

Temperature is one of the most important epigenetic factors affecting the physiology of most Osteichthyes. Not surprisingly, the effects of temperature on fish were first studied in the 1800's. The biggest incentive for investigating temperature effects on the physiology of fish came with the realisation that human activities had changed the temperature of water bodies (Belding, 1928). The proliferative use of once-through cooling systems of steam electric power plants in the 60's and 70's in the USA, resulted in the increase of temperature of the local water bodies due to the waste heat of this industry. Legislation by the US government, which classified heat as a pollutant, aimed to reduce the effect of human activity on local water bodies. In parallel, funds were made available for research on the effect of increasing temperatures on the physiology of fish. The modern founders of fish physiology related to temperature are F.E.J. Fry and J.R. Brett (Beitinger et al., 2000). Their work,

and that of many others, from early 1900's to the 1970's, established a deeper understanding of the multitude of effects temperature has on the physiology of temperate and coldwater teleosts (Brett, 1941; Brett, 1952; Brett, 1956; Brett, 1971; Brett et al., 1969; Fry, 1958; Fry et al., 1942; Fry et al., 1946). Interestingly, research on effects of increased water temperature on fish life found that warmer water in general was not lethal to fish. Rather, it was cold shock that killed fish (Beitinger et al., 2000).

A large number of studies investigate the temperature optima of teleosts and their temperature tolerance range. Temperature optima are often measured by survival rates of different life stages (Beitinger & Bennett, 2000; Brett, 1956). However, the most dramatic effect temperature can have on any aquatic life is killing the organism. The upper and lower lethal temperature limits of fish by either observing actual death (incipient lethal temperature, ILT; Bliss & Stevens, 1937) or by observing loss of equilibrium, i.e. loss of normal locomotion ('swimming belly-up'; critical thermal methodology, CTM; Becker & Genoway, 1979) provides information about the temperature tolerance. The CTM method allows estimating the lethal temperature limits without killing the fish and can therefore be used for endangered fish species. The upper and lower lethal temperature limits are affected by the acclimation temperature of the fish. This means that an animal acclimated to 20°C will have a higher upper lethal temperature limit compared to an animal of the same species acclimated at 15°C. Lethal temperature limits are expressed in a polygon where 'critical thermal limit' is plotted against 'acclimation temperature' (Fig. 2).

Since the effect of increased water temperature on cold water fish species was investigated in the 60's and 70's, the cause of death of fish was expected to be warm water. In contrast, cold temperatures are the most reported cause of temperature related death in fish. Four observations could explain the discrepancy in number of reported 'cold' versus 'heat' deaths. First, fish increased their tolerance of high temperatures more quickly than their tolerance of low temperatures (Brett, 1946; Davies, 1973; Doudoroff, 1945). Second, fish lost heat tolerance more slowly than cold tolerance (Davenport & Castle, 1895; Hathaway, 1928; Loeb & Wasteneys, 1912). Third, warm water temperatures increased the metabolism of fish and therefore the activity levels. In contrast, cold water temperatures slowed the fish metabolism down and caused lethargy. In addition, fish can acutely sense temperature changes and use behaviour to avoid or escape unfavourable temperatures. The higher activity levels of fish in warmer water allow a better avoidance of higher temperatures compared to colder temperatures (Coutant, 1975; Neill & Magnuson, 1974; Richards, 1977). Fourth, upper temperature tolerances of most fish were well above the ambient temperature they encountered in their natural environment (Mundahl, 1990).

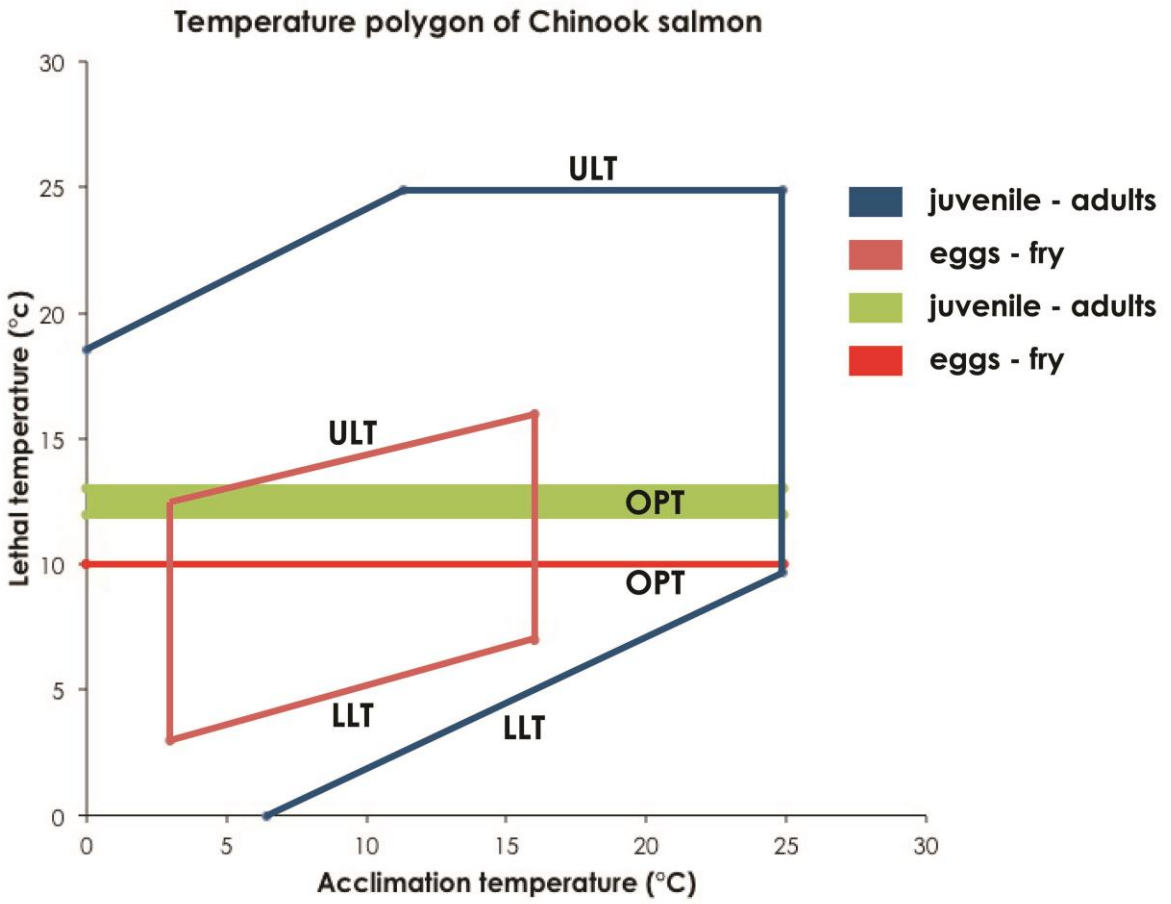


Figure 2: The temperature polygon of Chinook salmon

Critical temperature (°C) on the y-axis is plotted against acclimation temperature (°C) on the x-axis. The temperature polygon of adults is shown in blue, while the polygon for eggs and fry (post-hatch stages) is shown in pink. The optimal temperature (OPT) for juveniles and adults is shown in green (between 12-13°C), while the OPT for eggs and fry is shown in red (10°C). Further abbreviations: LLT, lower lethal temperature; ULT: upper lethal temperature. The temperature polygons are based on data in Alderdice & Velsen (1978) and Brett (1952).

1.2.1. TEMPERATURE RANGE OF CHINOOK SALMON

Salmonids are stenothermal meaning that they have a narrow range of temperature optima (Beitinger & Bennett, 2000). The temperature polygon of Chinook salmon is shown in Figure 2. The general avoidance of temperatures above 15°C for all *Oncorhynchus* species, in spite of acclimation to this level and to 20°C to 24°C, is marked. In addition, it seems that the range for a preferred freshwater and seawater temperature observed among Pacific salmon frequently is 8°C to 10°C (Brett, 1952). The optimal temperature for growth in Atlantic salmon smolts is 13°C in seawater, but growth still occurs at temperatures up to 19°C to 20°C (Handeland et al., 2003). The seawater surface temperatures in the Pacific Ocean native to Chinook salmon range from 9.9°C off the Adak Island (Andreanof Islands) to 24°C in the San Diego Bay (https://www.nodc.noaa.gov). Sea water temperatures are important for smoltification success of Pacific salmon. The likelihood of successful

smoltification and survival after smoltification decreases rapidly with increasing temperatures in rivers, estuaries and shore waters (McCullough, 1999).

The natural rivers in New Zealand have water temperatures from 8°C to 20°C (https://www.niwa.co.nz). The water temperatures of New Zealand rivers cited in research articles range between 6°C and 16°C (Davis & Unwin, 1989; Kinnison et al., 1998; Unwin, 1986; Unwin & Glova, 1996; Unwin et al., 2000). Temperatures are likely to be lower in High Country Canterbury rivers which are mainly glacial melt water fed (Quinn et al., 2001). Water temperatures flowing through hatcheries are very stable, for example 11.8°C and 12.4°C for two hatcheries belonging to New Zealand King Salmon Co Ltd The sea surface temperatures on the East coast of the South Island range between 9°C and 19°C. (https://www.seatemperature.org). Winter temperature regimes in the nearshore waters of the South Island's east coast are between 8 and 10°C (Greig et al., 1988). However, New Zealand summer temperatures are between 15 and 19°C. In the shallow waters of the Marlborough Sounds, where the sea cages of farmed Chinook salmon are located, even higher summer surface water temperatures (up to 23°C) have been measured (personal communication Mark Preece). The temperature profiles of rivers in the South Island of New Zealand, and its surrounding ocean, are ideal for Chinook salmon to thrive. However, shallow waters close to the shore line can approach the upper lethal temperature limit.

1.2.2. USE OF TEMPERATURE IN AQUACULTURE

Temperature is a widely used tool to create both ideal husbandry strategies and strategies for year round production (Fjelldal et al., 2012). In addition, aspects such as requirement of minerals, skeletal development and development of deformities of the vertebral column are affected by temperature (Boglione et al., 2013a; Boglione et al., 2013b; Dionísio et al., 2012; Takle et al., 2005). Unsurprisingly, a large number of studies investigating the effects of temperature, including temperature limits and temperature optima, exist for salmonids. *APPENDIX A* summarises some temperature studies in salmonids. Most studies focus on survival, metabolic performance, development, and growth performance. These studies provide knowledge to help improve farming practices and to aid conservation efforts of these species in their native ranges.

1.2.2.1. Early stage temperatures in farming practice

Changing the temperature during incubation in farming practices can be used for several reasons. Higher incubation temperatures will increase, while lower incubation temperatures will decrease the developmental rate (Hayes et al., 1953). Increasing or decreasing the developmental rate will change the time points at which certain developmental stages are reached (Mahon & Hoar, 1956; Murray & Beacham, 1989). Thus, the time of hatching and time that the first feed stage is reached can be shortened or prolonged (Crisp, 1981; Crisp, 1988; Jensen et al., 1989), which is useful in planning for

harvesting salmon year round. The 'wild' adult salmon prior to maturation, when they are ready to swim up-stream to their spawning grounds, is the life stage that an ocean ranch farmer aims to harvest. In the wild, Chinook salmon return to the rivers for spawning from early spring to late summer. If the life cycle of wild Chinook salmon is used, farmers would only be able to harvest over spring and summer. However, if farmers can shorten or prolong the incubation time and early freshwater stages, together with other husbandry changes in feeding regime and length of daylight during grow-out stages, salmon will go to the sea water stage over different periods during the year. Hence, the sea water grow-out stage will produce salmon ready to harvest throughout the year. Gradually increasing temperature during incubation is also used to synchronise the hatching of cold incubated eggs, after which the temperature is gradually lowered again. Although the effect of incubation temperature has been investigated in Chinook, chum, coho (*Oncorhynchus kisutch*), pink and sockeye salmon (Alderdice & Velsen, 1978; Beacham & Murray, 1990), it was unclear if the increased temperature was the underlying direct cause of hatching. Alternatively, a reduced level of oxygen in the incubation environment due to the temperature induced metabolic activity of the many embryos may cause the animals to hatch (DiMichele & Powers, 1984; Dimichele & Taylor, 1980). For example, a reduced level of oxygen at a constant 10°C caused premature hatching of chum salmon embryos (Alderdice et al., 1958). While temperature is the main tool and beneficial from a farm husbandry perspective, raising or lowering temperature too quickly in critically sensitive early stages is likely to induce adverse effects.

1.2.2.2. Temperature sensitive early stages

The effect of temperature on the skeleton has been mostly observed as variation in countable elements of the skeleton, such as vertebral bodies, gill rakers, branchiostegal rays and fin rays, i.e. meristic variation. Meristic variation in the skeleton follows temperature clines occurring in the natural habitat. The most famous of which is Jordan's rule which relates meristic variation to a large North-South latitudinal temperature scale (Jordan & Evermann, 1896). Meristic variation of skeletal elements in salmonids has been used mostly to identify stocks or populations, but proved to be complex since multiple skeletal elements need to be counted precisely (Beacham, 1990; Beacham et al., 1983; Beacham & Murray, 1986; Landrum, 1966). Only the trends of several skeletal elements combined gave significant results that could be used for clear stock identification. In addition, the counting of skeletal elements needs to follow consistent protocols to be able to compare results between studies.

Using temperature experiments to study meristic variation in several teleost species, such as medaka (*Oryzias latipes*), threespine stickleback (*Gasterosteus aculeatus*), sockeye, chum and pink salmon, and rainbow trout brought several authors to the conclusion that the magnitude and duration of exposure to changing temperatures are important in influencing skeletal variation (Batty et al., 1993; Beacham & Murray, 1986; Kwain, 1975; Lindsey, 1988; Murray & Beacham, 1989; Seymour, 1959).

Moreover, the developmental stages, at which temperatures are manipulated, affects meristic variation. In salmonids, investigations of temperature sensitive stages related to meristic changes were undertaken by Taning (1952) and Murray & Beacham (1989) in brown trout (*Salmo trutta*) and chum salmon respectively. The temperature sensitive developmental stages related to meristic variation are similar in both salmon species. The sensitive stages all occur before hatching and are: (i) the 16-cell stage, (ii) the early blastula stage, (iii) the late blastula stage, (iv) at completion of epiboly, (v) at completion of somitogenesis (also called initial eye pigmentation) and (vi) the eyed egg stage. Positive or negative temperature changes of 6°C were found to either or both decrease or increase the number of vertebrae and gill rakers (Murray & Beacham, 1989; Taning, 1952).

Temperature sensitive stages related to development of skeletal deformities were studied in Atlantic salmon (Takle et al., 2005; Wargelius et al., 2005). In these studies heat and cold shocks were used at specific developmental stages. Both authors found significant vertebral deformities in animals heat shocked at the 20th to 25th somite stages. Takle et al. (2005) used a one hour heat shock of 8°C (temperature increase) and a cold shock of 7°C (temperature decrease) at several early life stages, i.e. before and during the somitogenesis phase. Although vertebral deformities were observed in juvenile stages in almost all temperature treated groups (3-9%), including the control group at 8°C (7%), animals heat shocked at the 25th somite stage and at the completion of somitogenesis showed a significantly higher proportion of deformities. Cold shocked animals at the 45th somite stage and at the completion of somitogenesis showed a higher proportion of vertebral deformities compared to the control group. Wargelius et al. (2005) had a control group at 6°C and used a 6°C increase in temperature for 24 hours as a heat shock. Although no specific proportions were given, external malformations were observed in post-hatch stages. Juvenile specimens heat shocked at early gastrulation showed 34% vertebral deformities, 31% when heat shocked at the 1st somite stage, 34% when heat shocked at the 6th somite stage and 27% when heat shocked at the 15th to 20th somite stage. The control group also had vertebral deformities (3%). The deformities observed were mainly compression and fusions. Although temperature shocks are avoided in all forms of planned production it is clear that salmon are vulnerable to the effects of temperature perturbations and that early development is most temperature sensitive.

1.3. THE SALMON VERTEBRAL COLUMN

The vertebral column is a defining feature of vertebrates. The salmon vertebral column consists of amphicoelous (biconcave) vertebrae (Fig. 3 A-B, D-E) and intervertebral ligaments. The vertebral bodies have different functions within a single vertebral column. Attachment of the skull to the vertebral column, protection of the neural cord, providing attachment for blood vessels, intestines, swim bladder, reproductive organs and muscles, and transducing muscle forces are a few of their many functions.

Knowledge about the skeleton is mainly based on mammalian animal systems, where cartilage and bone can be clearly separated in two tissue type categories. In the last decades it became clear that teleost skeletal systems are not simply built of either cartilage or bone. Rather, cartilage and bone represent two ends of a continuum of skeletal tissues (Witten & Hall, 2015; Witten et al., 2010). Both cartilage and bone forming cells are controlled by a shared set of genes with *sox9* and *runx2* as regulators for cartilage and bone respectively (Eames et al., 2004).

1.3.1. THE SALMON VERTEBRAL BODY

The salmon vertebral body is introduced based on the detailed descriptions of salmonid vertebral bodies by Arratia & Schultze (1992). The vertebra depicted here (Fig. 3A-B, D-E) is found in late juvenile to adult Chinook salmon. Although the schematic representations are original work (Fig. 3C, F), the images and naming is adapted from Arratia & Schultze (1992). The combined chordacentrum, autocentrum and the cancellous bone layers are further referred to as the vertebral centrum (Fig. 3C, D-F). The cartilaginous bases of the associated elements, i.e. the basidorsals and basiventrals respectively, and the bony part sticking out of the vertebral centrum are further referred to as associated elements (Fig. 3A-C). The chordacentrum develops underneath the external elastic membrane of the notochord sheath (Fig. 3F). The chordacentrum is the only structure of the vertebra that develops within the notochord. All other structures of the vertebra develop perinotochordal, on the outside of the external elastic membrane of the notochord sheath. The autocentrum, consisting of a compact bone layer, develops on the outer surface of the external elastic membrane surrounding the notochordally derived chordacentrum (Fig. 3F). The anterior and posterior edges of the autocentrum are called the endplates (Fig. 3D). The compact bone layer of the autocentrum is observed as the typical X-structure in X-ray images (Witten et al., 2009; Fig. 1). The arcocentra, also outside of the notochord sheath but implanted at the level of the chordacentra, consist of cartilaginous parts and a bony part. The cartilaginous parts are called the basidorsals and basiventrals (Gadow & Abbott, 1895) which are the bases of the vertebral centrum associated elements (Fig. 3C, E). The bony part of the arcocentrum forms the cancellous bone fused to the autocentrum (Fig. 3E), which forms the round contour of the vertebral body (Fig. 3), and forms the bone of the associated elements (Fig. 3C). The associated elements are the neural arches, parapophyses, ribs, haemal arches and structures of the caudal fin skeleton.

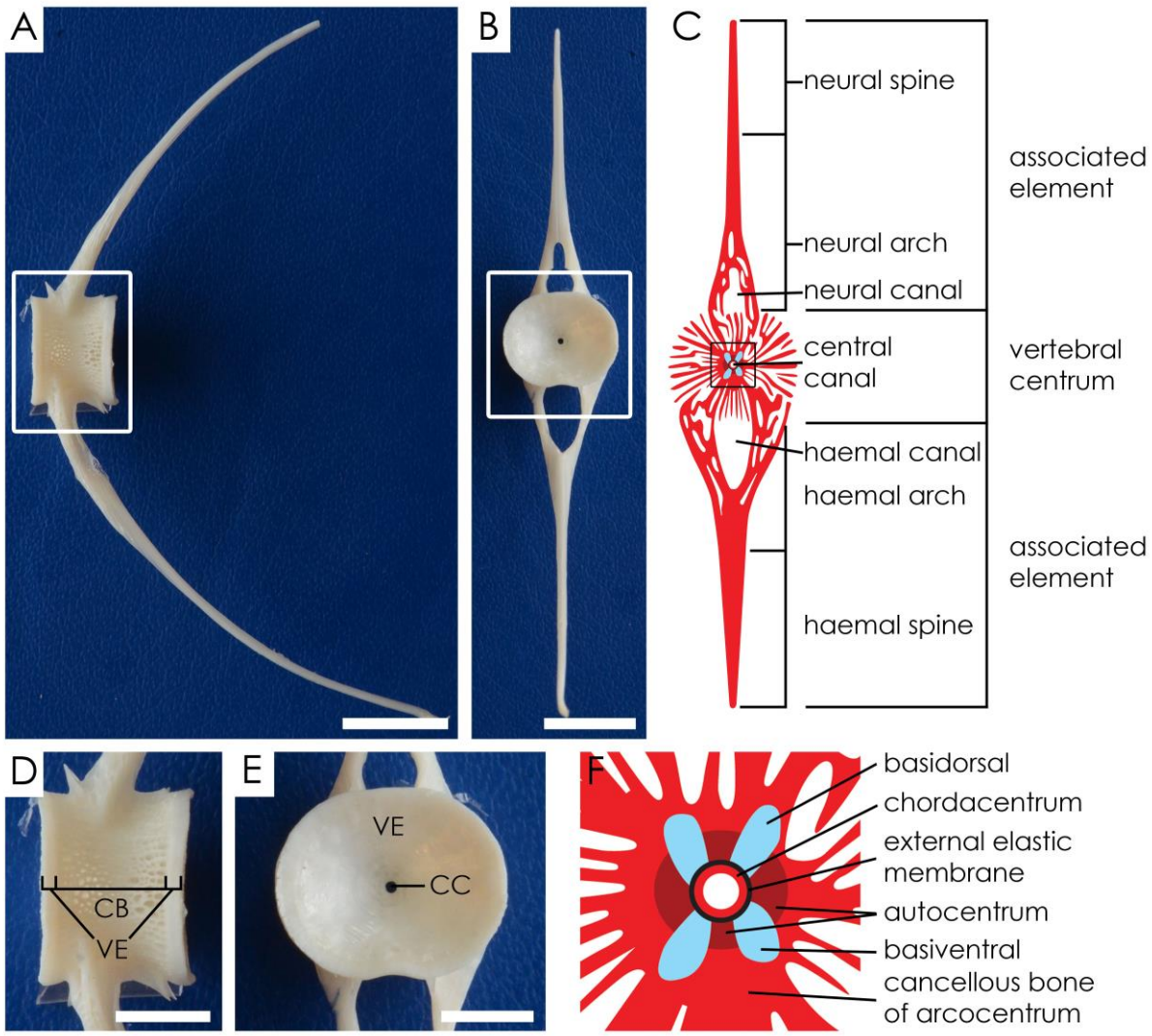


Figure 3: The Chinook salmon vertebral body

The images and schematics are the authors original work with naming adapted from Arratia & Schultze (1992). (A-C) The entire caudal vertebral body (vertebral centrum and associated elements), scale bars of (A) and (B) are 10 mm. (D-F) The vertebral centrum enlarged, scale bars of (D) and (E) are 5 mm. (A) The vertebra is represented in a side view (anterior to the left and posterior to the right). The box indicates the enlarged image (D). (B) Posterior view of the vertebra shown in (A). The box indicates the enlarged image (E). (C) A cross section through the vertebral body. The neural arch and spine, and the haemal arch and spine are collectively called the associated elements. The central part of the vertebral body to which the arches attach is called the vertebral centrum. Bone is indicated in red, cartilage is indicated in blue. The black box indicates the enlarged image (F). (D) Enlarged side view of the vertebral centrum [same orientation as (A)]. The vertebral endplates (VE), the anterior and posterior part of the autocentrum and the cancellous bone (CB) are indicated. (E) Enlarged posterior view of the vertebral centrum [same orientation as (B)]. The posterior vertebral endplate is indicated by VE and the central canal by CC. (F) Enlarged image of the cross section of the vertebral centrum. Notice that the chordacentrum lies underneath the external elastic membrane of the notochord sheath. All other vertebral centrum layers developed perinotochordal and thus outside of the external elastic membrane.

Deformities can occur in the three tissues that build the vertebral bodies and thus the vertebral column. These tissues are the notochord, the cartilaginous precursors of the neural and haemal arches and the early mineralised bone of the vertebral centrum and arches. Before vertebral deformities can be correctly interpreted on a tissue level, an in-depth knowledge of the three tissue types is necessary. Therefore the notochord, the cartilage structures associated with the axial skeleton as well as the bony elements of the vertebral column *per se* are introduced below.

1.3.2. NOTOCHORD

The notochord is the key characteristic of the phylum Chordata, including lancelets (Cephalochordata), tunicates and salps (Urochordata) and the craniates which, except for agnathans, have vertebral columns made of vertebrae in addition to skulls. The structure was first described by von Baer in chick embryos (von Baer, 1828). Alexander Kowalevsky (1867), a student of von Baer, was the first to describe the development of the notochord in detail, based on observations made in ascidians (tunicates) (Kowalevsky, 1867). Importantly, Kowalevksy (1867) suggested that the ascidians were closely related to the vertebrates. This hypothesis gave rise to several scenarios and theories homologising the notochord with ventral 'chord' structures in invertebrates such as annelids, nemerteans and hemichordates and is a current on-going discussion (Annona et al., 2015).

The notochord in vertebrates arises from the dorsal organiser (Harland & Gerhart, 1997; Spemann & Mangold, 1924). The equivalent of the dorsal organiser in teleosts is the embryonic shield (Oppenheimer, 1936). The notochord anlage is formed through chordamesodermal cell movements during gastrulation (Amacher & Kimmel, 1998; Fekany et al., 1999). Subsequently, through cell division and intercalating cell movements of chordamesodermal cells a 'single stack of coins' like notochord is formed (Grotmol et al., 2006; Kimmel et al., 1995). From these stacked cells two distinct cell types differentiate, (i) the chordocytes or inner large vacuolated cells and (ii) the chordoblasts or outer non-vacuolated epithelium-like cells that produce the notochord sheath (Coutinho et al., 2004; Grotmol et al., 2003; Kryvi et al., 2017; Parsons et al., 2002; Stemple, 2005). Genes such as *sneezy*, *happy* and *dopey* express coatomer complex components which were found to be important for the vacuolisation of the chordocytes (Coutinho et al., 2004), while *bashful*, *grumpy* and *sleepy* express laminin subunits important for the formation of the basement membrane of the notochord (Parsons et al., 2002). In addition, an elegant study on zebrafish (*Danio rerio*) showed that the vacuoles in the chordocytes are lysosome related organelles (LRO's) (Ellis et al., 2013). Although the chordocytes and chordoblasts appear to be two separate groups of cells with their own specific function, chordoblasts have been shown to transform into chordocytes in the Atlantic salmon notochord (Kryvi et al., 2017), which suggests that the notochord is a dynamic structure during the entire life cycle of salmonids.

The notochord has a dual function in embryonic and post-hatch bodies of vertebrates. It functions as the main axial skeleton and as a midline signalling centre. In embryos and post-hatch stages, the notochord is a hydrostatic rod to help locomotion (Satoh, 2003). For example, specific collagen lamellae in the notochord sheath of Atlantic salmon provide torsional strength and are interconnected to the surrounding connective tissue to direct forces from the muscles during swimming (Grotmol et al., 2006). In juvenile and adult vertebral columns, the notochord transforms into the intervertebral tissues (Wang, 2013). In the agnathans and some basal gnathostomes, such as dipnoans and sturgeons, the embryonic notochord is retained throughout juvenile and adult life (Arratia et al., 2001). In teleosts, although the chordocytes transform (Haga et al., 2009; Kryvi et al., 2017), the embryonic notochord is retained in the centre of the vertebra to form a small central canal (Fig. 3D, E). In addition, notochord is retained in the intervertebral spaces (Arratia et al., 2001; Grotmol et al., 2006; Kryvi et al., 2017). In adult mammals, the nucleus pulposus is a remnant of the notochord and is contained by an annulus fibrosus, and collectively better known as the intervertebral disc (Choi et al., 2008; Pattappa et al., 2012). Although the notochord tissue in the intervertebral spaces in salmonids is retained throughout life, the tissue structure changes over time in function of the forces elicited on the vertebral column (Kryvi et al., 2017).

Importantly the notochord has a major role in the patterning of the vertebral column in teleosts (Bensimon-Brito et al., 2012b; de Azevedo et al., 2012; Fleming et al., 2015; Grotmol et al., 2005). However, the underlying mechanisms are less well understood. First, the notochord has an inherent pattern that delineates the vertebral centrum anlage, i.e. the chordacentrum, representing the actual vertebral centrum proper (Arratia et al., 2001; Fleming et al., 2004; Grotmol et al., 2003; Grotmol et al., 2005; Nordvik et al., 2005; Wang et al., 2013). How the chordacentra pattern in the notochord arises is currently unknown, but evidence suggests that the cells responsible for the formation of the chordacentra have a notochord origin as laser ablation of notochord cells at specific repeated places resulted in the loss of chordacentra in zebrafish (Fleming et al., 2004; Fleming et al., 2015). In addition, the chordoblast cell-morphology changes and segmental expression of alkaline phosphatase (ALP) was found to exist in the chordoblasts lining the future chordacentra in Atlantic salmon and zebrafish (Bensimon-Brito et al., 2010; Fjelldal et al., 2005; Grotmol et al., 2003).

The notochord produces signalling molecules such as sonic hedgehog (SHH) that will signal to the sclerotome (Fig. 4A) in the somites (Fleming et al., 2004; Fleming et al., 2001; Gilbert, 2000). The somites are mesoderm derived and consist of two major multipotent cell populations. The sclerotome will form the skeleton producing cells (chondroblasts and osteoblasts) while the derma-myotome will form the dermis of the back and muscles inbetween the ribs, the deep back, the body wall, limb muscles and the tongue (Gilbert, 2000). Interestingly, analysis of cell differentiation and biochemical composition of extracellular material of notochord and cartilage showed that both tissues are related (Cooper, 1965; Miller & Mathews, 1974). Modern molecular tools also confirmed that notochord

tissue is closely related to cartilage based on shared expression of *type II* and *IX collagen*, *aggrecan* and *sox 9* (Domowicz et al., 1995; Grotmol et al., 2006; Ng et al., 1997; Zhao et al., 1997). However a major difference between notochord and cartilage exists. In notochord cells, specifically the chordocytes, hydrated materials are retained in large vacuoles to create hydrostatic pressure against the thick sheath (Adams et al., 1990; Grotmol et al., 2006; Koehl et al., 2000). In contrast, cartilage cells (chondroblasts and chondrocytes) secrete the hydrated materials into the extracellular matrix (ECM) (Knudson & Knudson, 2001).

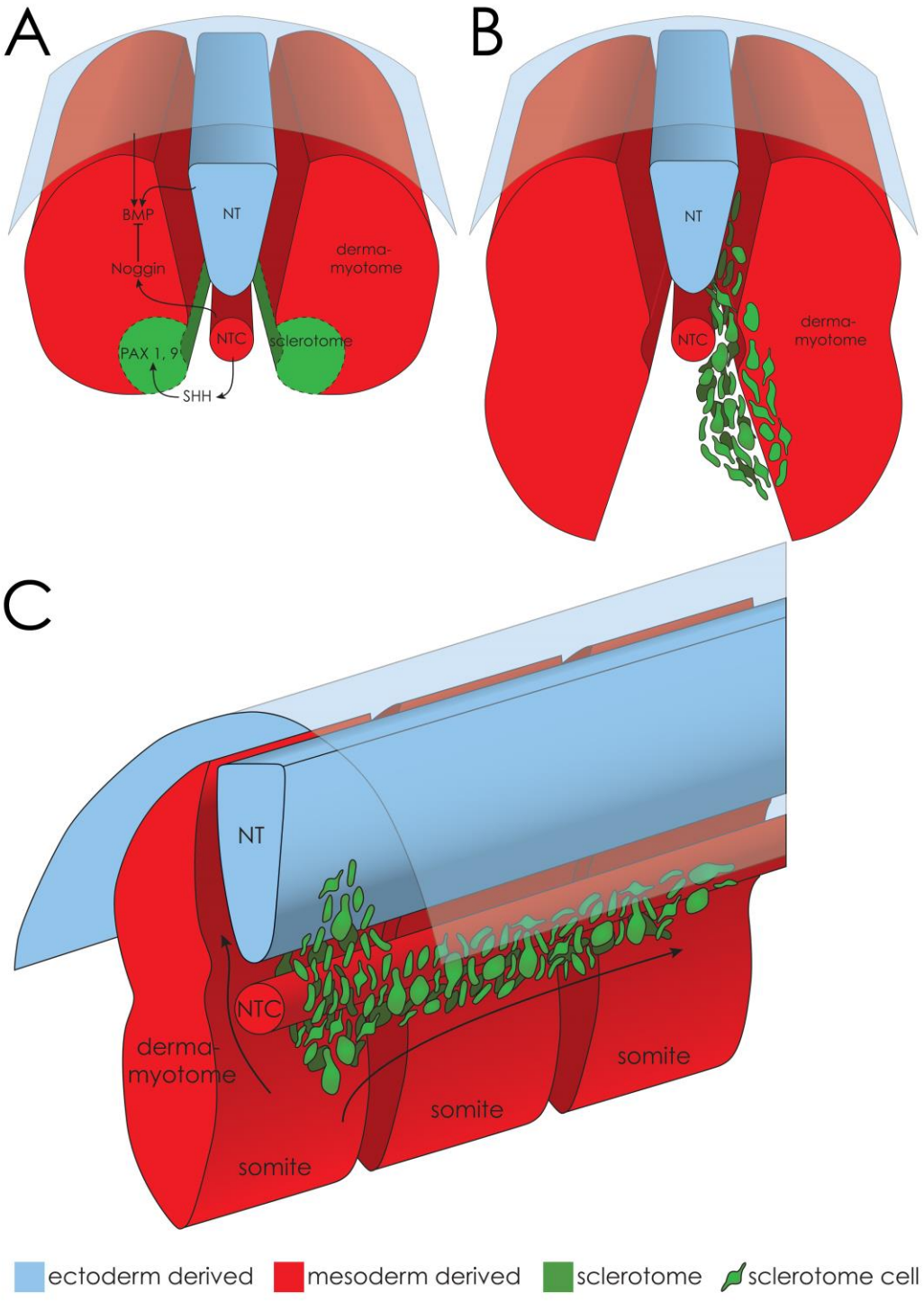


Figure 4: Somites and sclerotome in teleosts

The schematic representations are the authors' original work with naming adapted from Gilbert (2000) and Morin-Kensicki et al. (2002). (A) Anterior view on one pair of teleost somites in their epithelial state. Sonic hedgehog (SHH) signals from the notochord (NTC) to the somite induce the expression of *pax 1, 9* genes determining the sclerotomal fate of these cells. The region becoming sclerotome is shown in green. The ectoderm (blue transparent layer) and the neural tube (NT, shown in blue) provide bone morphogenetic protein (BMP) signals to the somites. BMP induces cells to become of derma-myotome (which is shown in red). The notochord also secretes Noggin, a major BMP antagonist, which helps the ventral SHH signal to induce *pax 1*

and *pax 9* in future sclerotomal cells. (B) Same view as in (A). Sclerotomal cells (shown in green) in their mesenchymal state have started migrating. The mesenchymal cells migrate upwards along the notochord and neural tube and downwards along the somite to form the future neural and haemal arches, and to provide osteo-progenitor cells which will ossify the perinotochordal vertebral centrum layers. The derma-myotome (shown in red) has changed into a more chevron like shape but has still an epithelial phenotype. (C) Anterior-side view of three somites (epithelial derma-myotome, shown in red) on the left side of the midline. The derma-myotome segments on the right side were removed to be able to show the sclerotome cell (shown in green) migration. Cell migration is shown by the arrows. Teleosts have leaky resegmentation, where sclerotome cells contribute to up to three body segments. Migration of sclerotome cells along the notochord (shown by bottom arrow) was shown in zebrafish (Morin-Kensicki et al., 2002).

1.3.3. CARTILAGE

Cartilage is best known as the text book mammalian hyaline cartilage and forms the basis of the embryonic endoskeleton. However, within vertebrates, in particular teleosts, a large range of different cartilaginous tissue types exist. Although some tissues intermediate between cartilage and bone are considered transient or pathological in mammals (especially humans), such tissues are part of the normal mature skeleton of teleosts and other actinopterygians (Hall & Witten, 2018; Witten et al., 2009). This section focuses on the development of cartilage in the vertebral column of teleosts giving rise to the associated elements, i.e. post-hatch neural and haemal arches, parapophyses, ribs and the caudal fin skeleton. The cartilaginous bases of the neural and haemal arches and parapophyses, the basidorsals and basiventrals, are the cartilaginous part of the arcocentra and remain cartilaginous until adult stages.

The cartilaginous associated elements in the vertebral column are sclerotome derived. During gastrulation, somitogenesis occurs in the embryo in an anterior to posterior direction. The number of somites varies between species but is relatively fixed within each species (Richardson et al., 1998; Ward & Mehta, 2014). The rate of somitogenesis can also vary within species (Schmidt & Starck, 2004; Schmidt & Starck, 2010). For example in zebrafish a pair of somites is formed about every 30 minutes (Schmidt & Starck, 2004; Schröter et al., 2008). Somites form through the interaction of complex molecular pathways. The most widely accepted model of somitogenesis is the ‘clock and wavefront’ mechanism (Cooke & Zeeman, 1976). Shortly after their formation, the mesenchymal cells of the somites become epithelial through mesenchymal-epithelial transition (MET). Two cell populations differentiate within the somite based on the signals the cells receive. The dermamyotome is determined by BMP signalling from the neural tube and ectoderm (Fig. 4A). The sclerotome differentiates upon receiving ventralising SHH and Noggin, which antagonises the dorsalising Bone Morphogenetic Protein (BMP) signals. These signals from the notochord induce *Pax1* (Paired box), *Pax9* and *Mfh1* (Mesenchyme fork head) expression in the sclerotomal cells (Fan & Tessier-Lavigne,

1994; Fleming et al., 2001; Furumoto et al., 1999; Hammerschmidt & McMahon, 1998; McMahon et al., 1998; Peters et al., 1995; Pourquié et al., 1993). In amniotes the sclerotome is the ventromedial portion of the somite (Fig. 4A). Interestingly, in zebrafish only a small group of cells found on the anterior ventromedial edge of the somite represent the sclerotome (Morin-Kensicki & Eisen, 1997). Upon receiving SHH signals from the notochord (Fig. 4A) the sclerotomal cell population will undergo epithelial-mesenchymal transition (EMT). The EMT allows the sclerotomal cells to migrate dorsally to surround the notochord and neural tube (Fig. 4B, C) (Morin-Kensicki & Eisen, 1997; Morin-Kensicki et al., 2002). Already in 1855 (Remak, 1855) an offset between the vertebral segment and the muscle segment was observed. Remak suggested that the vertebrae are formed by the posterior and anterior halves of consecutive somites. This theory is now recognised as somite resegmentation and was proven in later experimental work (Bagnall et al., 1988). How this resegmentation happens is still largely unknown. Resegmentation allows each muscle to insert on two consecutive vertebrae and allows the vertebral column to bend when muscles contract during locomotion. Interestingly, in zebrafish the sclerotomal cells of a somite were observed to migrate to the locations of a second and even third more posterior future vertebra (Fig. 4C). This observation suggests there is 'leaky resegmentation' in zebrafish (Morin-Kensicki & Eisen, 1997; Morin-Kensicki et al., 2002). Leaky resegmentation may be normal in teleosts in contrast to the one to one resegmentation relationship observed in mammals (Witten & Hall, 2015).

In Atlantic salmon, the sclerotomal cells surrounding the notochord have initially a fibroblast phenotype. The development of the post-hatch cartilaginous associated elements starts within the myosepta with fibroblasts forming a mesenchymal condensation (Fig. 5A) which later develops into hyaline cartilage (Fig. 5B) (Grotmol et al., 2006). Although remnants of the bases of the neural and haemal arches, basidorsals and basiventrals respectively, remain cartilaginous in the adult stages, most cartilage of the associated elements will be replaced by mineralised bone tissue during juvenile growth stages of salmon (Fig. 5C) (Arratia & Schultze, 1992).

1.3.4. BONE

Osteogenesis (= ossification) or bone formation involves two steps. First, a scaffold or matrix is laid down in the extracellular matrix, called ossification. Second, the bone matrix is fixed by the binding of a mineral component (see below), called mineralisation. Calcification is a term better reserved for processes that involve accumulations of calcium in the formation of shells in invertebrates. Bone in the salmon vertebral body is formed via three types of ossification, i.e. perichondral, endochondral and intramembranous ossification. The distinction between these types of ossification is based on where the bone matrix is deposited. In perichondral ossifications, bone matrix is deposited on the outside (peri = around) of a cartilaginous structure. All associated elements preformed in cartilage, are initially perichondrally ossified. The cartilage of the associated elements is eventually replaced by

bone via endochondral ossification. Here the cartilage is broken down and replaced by bone. There is no cartilaginous precursor in intramembranous ossification. The chordacentrum mineralises within the notochord sheath, while the autocentrum, the neural and haemal spines and vestigial skeletal elements, such as ribs and uroneurals, form via intramembranous ossification.

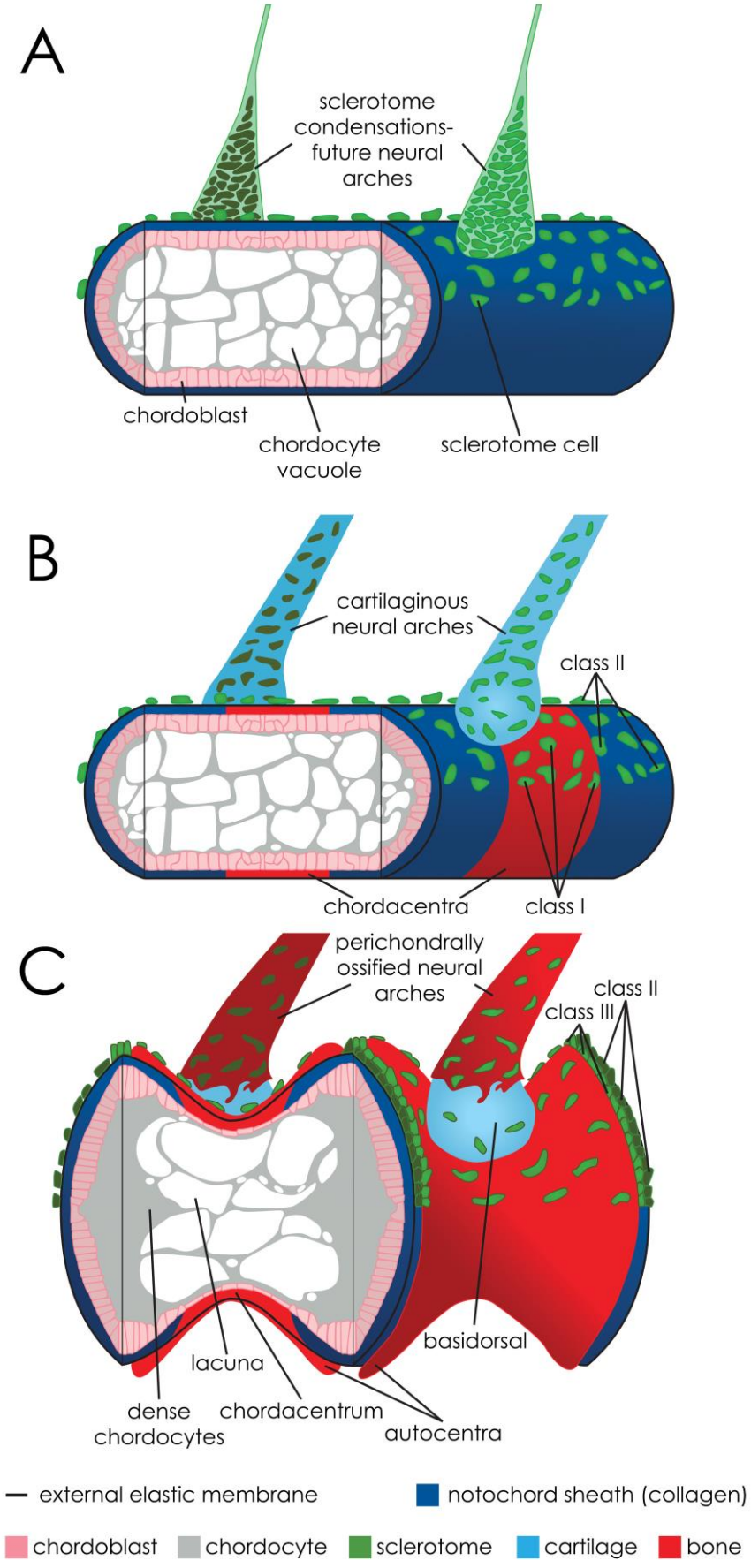
Two types of bone occur in teleosts, i.e. acellular bone and cellular bone. Acellular bone has no cells embedded within the bone tissue, while cells are embedded in the bone tissue in cellular bone. The cells producing acellular bone are positioned on the bone tissue surface. Acellular bone was first observed in teleost fish by Kölliker (1859). Acellular bone is a character of advanced teleosts and was used as a character in phylogenetic studies (Maisey, 1988; Parenti, 1986; Smith & Hall, 1990). A well-known example of a teleost with acellular bone in recent research is the model organism medaka. Salmon, have cellular bone (Witten & Hall, 2002). The cells that produce bone are called osteoblasts. Osteoblasts lay down the matrix (= scaffold) of the bone tissue called osteoid, which later mineralises to form mature bone tissue. Once these cells are themselves surrounded by bone matrix they are called osteocytes. The main component of the bone matrix is collagen type I. Non-collagenous components, such as osteopontin, support bone cell survival. Osteonectin is important in bone mineralisation and osteocalcin functions during bone remodelling (Hall, 2015a). The mineral component of bone in vertebrates is hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$) which is linked to the collagen matrix mediated by osteonectin. Bone is resorbed by three cell types, multi-nucleated osteoclasts, mono-nucleated osteoclasts and by osteocytes. Osteoclasts break down the mineral and matrix component of bone, a process called resorption. Osteocytes only remove the mineral component from the bone tissue, called osteocytic osteolysis. Together, the bone resorbing cells and bone producing cells form a highly active tissue where this tissue turnover provides great potential to repair and remodel when damage occurs.

The bony layers of the vertebral centrum external to the notochord in salmon, i.e. the autocentrum and the arcocentrum, are formed via intramembranous ossification. This perinotochordal bone is deposited directly on the outside of the external elastic membrane of the notochord sheath at the level of the chordacentrum (Fig. 5A) (Fleming et al., 2004; Grotmol et al., 2006; Inohaya et al., 2007). Three classes of sclerotomal derived cells have been recognised during the early stages of vertebral body formation (Fig. 5B, C). The first class of cells are non-mature osteoblasts scattered on the notochord elastic external membrane, outside the chordacentrum. These cells express ALP and COL10α1 and may contribute to perinotochordal ossification (Grotmol et al., 2005). The largest number of sclerotomal cells, class two, are clustered in the intervertebral region and differentiate into fibroblasts (Inohaya et al., 2007). Although these cells have no apparent osteoblast properties they secrete ECM molecules outside of the notochord sheath. Class three sclerotomal cells are a population of mature osteoblasts expressing *osterix*, an essential transcription factor for osteoblast differentiation (Nakashima et al., 2002). These cells are found at the anterior and posterior rims of the autocentrum, i.e. at the vertebral endplates, and were suggested to have differentiated from the class two cells in the

intervertebral region (Inohaya et al., 2007; Renn & Winkler, 2009; Spoorendonk et al., 2008; Willems et al., 2012). Class three cells also ossify the autocentrum and the bony part of the arcocentrum (ossification of the arches and the cancellous bone, Fig. 3C, F). Salmon bone, formed by different sclerotome cell types and different ossification types, is clearly complex but a dynamic tissue.

Figure 5: From notochord to vertebral body in salmonids (right)

The schematic representations are the authors' original work adapted from Fleming et al. (2004), Grotmol et al. (2005), Grotmol et al. (2006), Inohaya et al. (2007) and Wang (2013). (A-C) All figures have the same orientation, with anterior to the left, posterior to the right, dorsal at the top and ventral at the bottom. The first half shows a longitudinal section along the midline while the second half shows the entire external contour. Corresponding associated elements on the ventral side are not shown. (A) Mesenchymal condensations (shown in green) form the precursors of the basidorsals and cartilaginous neural arches [at ±400°d (degreedays) for Atlantic salmon]. These condensations implant on the outside of the external elastic membrane (black line) of the notochord sheath (see Fig. 3F, basidorsals and basiventrals). Sclerotomal cells are also scattered outside of the notochord sheath. (B) The mesenchymal condensations have developed into cartilaginous neural arches. Within the notochord sheath the chordacentra have mineralised and form closed rings (at ±750°d for Atlantic salmon). Sclerotomal cells occur scattered on the outer surface of the arches and the notochord. Class I sclerotomal cells occur perinotochordal at the level of the chordacentrum, while class II cells occur in the future intervertebral regions. (C) The neural arches have ossified perichondrally except for the basidorsals which remain cartilaginous. The perinotochordal autocentrum has also ossified. Sclerotomal cells and osteoblasts occur scattered on the neural arch and the autocentrum. Class III cells occur at the anterior and posterior rims of the autocentrum (indicated on the future posterior endplate), while class II cells (darker green) still occur in the intervertebral region.


A
sclerotome condensations- future neural arches
chordoblast
chordocyte vacuole
sclerotome cell
B
cartilaginous neural arches
class II
chordacentra
class I
C
perichondrally ossified neural arches
class II
class III
basidorsal
lacuna
dense chordocytes
chordacentrum
autocentra
external elastic membrane
notochord sheath (collagen)
chordoblast
chordocyte
sclerotome
cartilage
bone

1.4. PACIFIC SALMON

"In spring the sea is filled with migrating fishes, some of them bound for the mouths of great rivers, which they will ascend to deposit their spawn. Such are the spring-run Chinook salmon coming in from the deep Pacific feeding grounds to breast the rolling flood of the Columbia, the shad moving into the Chesapeake and the Hudson and the Connecticut, the alewives seeking a hundred coastal streams of New England, the salmon feeling their way to the Penobscot and the Kennebec. For months or years these fish have known only the vast spaces of ocean. Now the spring sea and the maturing of their own bodies lead them back to the rivers of their birth." In 'The Sea Around Us' by Rachel Carson (1950)

Salmon are actinopterygian (ray-finned) fish and part of the infraclass Teleostei (from Greek complete bone). The order Salmoniformes belongs to the superorder Protacanthopterygii. Salmonids may be the best researched teleost fish family, but the phylogeny of the Salmoniformes clade remains debated (Shedko et al., 2013). The Salmonidae family contains three main lineages, the subfamilies Thymallinae, Coregoninae and Salmoninae. The Salmoninae count six genera, i.e. *Hucho*, *Brachymystax*, *Salmo* (including Atlantic salmon), *Parahucho*, *Oncorhynchus* (the Pacific salmon including Chinook salmon) and *Salvelinus*. The genus *Oncorhynchus* includes eight species of which Chinook salmon (*Oncorhynchus tshawytscha*), commonly known as king salmon in New Zealand, is the largest.

The distribution of native Pacific salmon species is the Northern part of the Pacific Ocean and in the Arctic Ocean. Pacific salmon occur from the Laptev Sea North of Russia to the Beaufort Sea North of Alaska in the Arctic Circle. The Southern-most border ranges from the Sea of Japan (35° north) to the Californian Coast line (28° north). Although most species of Pacific salmon have an overlapping native range, some species range very widely such as the Chum salmon, while others have a smaller range such as masu salmon (*Oncorhynchus masu*), ranging only in the Western Pacific Ocean.

1.4.1. CHINOOK SALMON DISTRIBUTION AND LIFE CYCLE

Different synonyms for Chinook salmon, such as king salmon, spring salmon and Quinnat salmon are used throughout the literature. The Chinook salmon native range starts 35° north in the West Pacific around Japan, and spreads over the Kamchatka peninsula to the East Siberian sea (72° north). In the East Pacific Chinook salmon range from the Bering Sea (57° north) in the North to the Californian peninsula about 28° north (http://www.fao.org).

Chinook salmon have an anadromous life cycle with reproduction in fresh water and juvenile to adult growth in seawater (Bradford, 1995). Adult male and female Chinook salmon enter the fresh water rivers typically in spring to late summer (cf. name spring salmon) but spawn mostly in autumn. The life cycle starts as fertilised eggs (one of the largest teleost eggs of about 7 mm across) in the gravel substrate of river beds. The eggs develop and alevins hatch from the eggs typically over the winter period (Phillips & Wright, 2006). The alevins remain within the gravel substrate while they absorb their yolk-sac. Once the yolk-sac is absorbed they are called fry and will emerge from between the gravel. In slow growth environments (e.g., cold rivers at higher latitudes and/or higher elevation) the juveniles tend to remain in streams, feeding chiefly on aquatic insects, for one year prior to seaward migration in the following spring. This type of life history called 'stream-type' (Unwin, 1986; Unwin & Lucas, 1993). Chinook salmon that migrate seaward in their first year, from spring through to early fall, and often feeding in estuaries for weeks or months, are called 'ocean-type' (Quinn & Unwin, 1993; Taylor, 1990). Once juveniles move to the sea they are called smolts. The physiological process by which juvenile salmon adapt to sea water is called smoltification. The smolts will form schools and travel to the open ocean to feed on small marine crustaceans, squid and small fish such as suary, anchovy and mackerel (Davis, 2003). After spending one to five years in the ocean the fully grown salmon home to the river they exited at smoltification. The animals stop feeding before swimming up-stream and start maturing once they start swimming up-stream. Some salmon swim more than a thousand kilometers (Hinch & Rand, 1998). Remarkably, the adult males and females will return to the exact place where they hatched (=homing or philopatry). Females select, prepare and guard nest sites and males compete for access to mature females. The female excavates a redd (=nest) into the gravel substrate of the river bottom by waving the pectoral fins and tail fin. The redd is an oval shaped depression with a length that can range from 2.4 to 10 meters and a width that can range from 1.2 to 9.5 meters (Deverall et al., 1993). The female can build several redds and will in total deposit around 7000-10000 eggs which are fertilised and buried by the parents. The female will guard the nest site as long as possible but will eventually die.

1.4.2. BATTLE CREEK CHINOOK SALMON

One native river system that deserves an introduction in this thesis is the Battle Creek watershed, a tributary of the Sacramento River (California, USA). First, the spawning Chinook salmon adults in this creek likely provided the progeny from which the entire current New Zealand stock is derived (Haworth, 2010; McDowall, 1994; see *SECTION 1.1.2*). Second, Chinook salmon in this river system currently arrive and spawn throughout the year, with major spring and winter runs. Runs occurring during the four seasons are unique. The water temperatures in Battle Creek range from 11 to 20°C (Ward & Kier, 1999). The local populations of salmonids in Battle Creek were declining due to human impact, such as flood control, irrigation, hydro-electric power and domestic water supply. The

unique year-round runs and the heritage value of this river system and its aquatic life were recognised in the early 2000's. A major government project by the U.S. Department of the Interior, Bureau of Reclamation (Mid-Pacific Region) and California State Water Recourse Control Board proposed the Battle Creek Salmon and Steelhead Restoration Project. This includes identifying problems that cause salmon populations to decline, monitor populations and changing the hydro-geological outlay of creeks and rivers to minimise the human impact and to allow adult salmon to go back to habitat that was previously closed off. The full documentation of this project is available on the following site: https://www.usbr.gov/mp/battlecreek/docs.html#deir.

1.5. GOALS AND OUTLINE OF THE THESIS

Chinook salmon have a well-studied biology, produce healthy fish for human consumption and salmon farming has a well-established social license to operate. Environmental factors such as temperature, oxygen, and light regime are adjusted to facilitate ideal farming conditions. Most important of these is temperature which is manipulated to change the growth rate of farmed animals in parts of the production cycle (Boglione et al., 2013a; Boglione et al., 2013b). For example, temperatures are increased to induce fast growth. Yet, fast growth is frequently associated with skeletal deformities (Fjelldal et al., 2012). Nutritional factors also affect deformity incidence (Boglione et al., 2013b) and complex interactions between epigenetics (= environment, nutrition, etc.) and genetics remain unelucidated.

Deformities of the axial skeleton are a severe and recurrent problem in salmon aquaculture. Vertebral column deformities and other skeletal deformities of the skull and fins affect growth and performance of the fish and lower the value of the fish product. Most of the limited data available about the pathogenesis of deformities in *Oncorhynchus* species comes from Rainbow trout (*Oncorhynchus mykiss)*. A large volume of information is available for Atlantic salmon, because this species is the primary farmed salmonid. However, Atlantic salmon is part of a different genus (*Salmo*) compared to the Pacific salmon species (*Oncorhynchus*) making extrapolation of knowledge sometimes difficult. Major classes of deformities, separately or in combination, present as a bending of the vertebral column, as a shortening of the body axis and as deformities of vertebrae themselves. In addition, skeletal deformities constitute a severe but rather poorly characterized problem for animal welfare (Witten et al., 2006). Current knowledge suggests that vertebral malformations can start to develop from early post-hatch life stages to late pre-harvesting stages (Fjelldal et al., 2012; Perrott et al., 2018). Yet, how and when exactly malformations and deformities start to develop remains poorly understood.

This research aims to help characterise skeletal deformities in early life stages of farmed Chinook salmon and to help understand the aetiology of skeletal malformations. To do so, we test the

hypothesis that incubation temperature affects the initiation and development of vertebral malformations. Control of skeletal deformities in New Zealand Chinook salmon to within acceptably low levels in commercial farm setting requires (a) identification of different skeletal anomalies, (b) identification of sensitive life stages at which skeletal anomalies start, (c) an in-depth understanding of the pathological processes underlying skeletal anomalies and (d) and in-depth understanding of how epigenetic or genetic factors influence the malformation process (Boglione et al., 2013a; Witten et al., 2009).

The current research focused on the following research tasks; outlined in *CHAPTER 2*:

1. **DIAGNOSTIC IMAGING.** Techniques such as whole-mount staining for cartilage and mineralized bone and serial histological sectioning of different life stages were implemented. In addition high-contrast and high-resolution X-ray imaging of the vertebral column of Chinook salmon of different sizes was used.

2. **IDENTIFICATION OF SENSITIVE LIFE STAGES.** Patterns of vertebral mineralisation as well as the development and progression of malformations were characterised. It is intuitive that initiation of malformations would occur before they become grossly evident. Therefore the task focussed on stages between hatching and first feed (= yolk sac absorbed and actively searching for food).

3. **IDENTIFICATION OF TEMPERATURE EFFECTS**. The effect of constant temperature on vertebral column development was investigated in post-hatch stages. In particular, the effect of temperature on the vertebral column regionalisation, meristic variation and the rate of malformation was of interest.

The temperature experiment provided the samples for each study of which the results are found in *CHAPTER 3-6*. These Chapters are formatted as manuscripts and are either published or prepared for submission to peer-reviewed journals. Post-hatch life stages were used to investigate if temperature influences the rate of malformations occurring in early life stages (*CHAPTER 3*, '**The external phenotype-skeleton link in post-hatch farmed Chinook salmon**', Journal of Fish Diseases, 2017). A close analysis of the juvenile vertebral column showed that six anatomical regions can be recognised in the Chinook salmon. The implications of the observed regionalisation are discussed from an evo-devo (evolutionary and developmental) perspective and provide evidence for an alternative regionalisation pattern compared to mammalians (*CHAPTER 4*: '**Vertebral column regionalisation in juvenile Chinook salmon (*Oncorhynchus tshawytscha*)**', Journal of Anatomy, 2017). The vertebral column regions are temperature sensitive and show temperature dependent meristic variation (*CHAPTER 5*: '**Temperature sensitive regions of the Chinook salmon vertebral column: Vestiges and meristic variation**', Journal of Morphology, 2018). Interestingly, the regional

variation is masked by a similar total number of vertebrae in each temperature group. Temperature also has a significant effect on the deformity prevalence of LKS, fusion, compression and vertical shift in freshwater smoltification and seawater stage Chinook salmon (*CHAPTER 6*: '**Lower incubation and freshwater grow-out temperatures protects Chinook salmon from subsequent vertebral deformities in seawater but not freshwater**', prepared for submission). High mortality after seawater transfer was highlighted, as well as notable deformities in 'runts', a category of under-performing animals with poorly understood reasons for retarded growth.

The thesis concludes with a general discussion (*CHAPTER 7*). Here, the results are integrated and further discussed in a developmental context. Furthermore, practical implications of the results, and the relationship of malformations and deformities are further explored. For example, constant temperature may influence the proportions of occurring malformations and deformities but is unlikely the direct cause of malformations. In addition, malformations in early life stages that survive in the farming environment may foreshadow deformities observed later in life. Finally, some recommendations for the salmon farming industry are made.

CHAPTER 2: MATERIAL AND METHODS

2.1. THE PROJECT FRAMEWORK

This PhD project is the result of close cooperation between two universities, New Zealand King Salmon Co Ltd (NZKS, 93 Beatty Street, Tahunanui, 7011 Nelson, New Zealand) and Skretting Australia (26 Maxwells Road, Cambridge, 7170 Tasmania, Australia). NZKS is the largest Chinook salmon producer in New Zealand and provided all experimental materials such as, experimental specimens, equipment and facilities. Skretting Australia is a branch of the aquaculture feed producer Skretting (Sjøhagen 15, 4016 Stavanger, Norway) and is part of the multinational animal and aquatic feed producer Nutreco N.V. (Prins Frederiklaan 4, 3818 KC Amersfoort, The Netherlands). The universities contributing to the research are the School of Veterinary Science (previously Institute of Veterinary, Animal and Biomedical Sciences) at Massey University (Tennent Drive, Turitea, 4474 Palmerston North, New Zealand) and the research group of Evolutionary Developmental Biology of Ghent University (Department Biology, K.L. Ledeganckstraat 35, 9000 Gent, Belgium).

This research took place parallel to a larger research effort undertaken by industry and research providers based on funding by the Ministry of Primary Industry (MPI). This Salmon Improvement Group (SIG-group) focussed on particular skeletal deformity issues occurring in late sea life stages (LKS-deformities in Munday et al., 2016; Perrott et al., 2018). Although the research in this PhD was not directly integrated into the research of the SIG-group, results were shared between the two parties. There is no conflict of interest.

2.2. BROOD STOCK AND CROSSES

Specific family crosses known to produce a high frequency of malformations were selected to provide off-spring for the temperature trial. The impact of incubation temperature on the frequency of malformations is more easily observed when the baseline malformation rate is high. This applies especially to malformation types that are less common.

Broodstock for the temperature experiment were provided by New Zealand King Salmon. Parents of the crosses chosen had a PIT-tag (Passive Integrated Tag) providing an individual ID to which life history, genetic background and information about X-ray based deformity traits were allocated. The genetic background and X-ray based deformity traits of the females and males provided information about which crosses would result in high malformation rates. Females were taken from parent stock used for production (called Strategy 3, which are slow growing) and were kept in freshwater facilities at Takaka (Tasman, New Zealand). Water of a constant 11.8°C feeds naturally into the hatchery

facilities originating from upwelling freshwater springs (Te Waikoropupu Springs). Eggs from 12 females were obtained by stripping individuals following aquaculture standards. Eggs were gently removed from the ovaries, washed and stored in a plastic bag together with the PIT-tag obtained from the body cavity. Bagged eggs were placed in polystyrene boxes at 10°C for transportation by plane to Tentburn Hatchery (Canterbury, New Zealand). These procedures were performed by NZKS staff and are part of the normal reproduction procedures used to generate commercially grown animals.

Male specimens were sex-reversed males and therefore had a female genetic background. Juvenile female specimens are treated with male hormone (17-α-methyltestosterone, Sigma-Aldrich, personal communication Mike Anderson). Consequently, the gonads developed as testes instead of ovaries. Male specimens, also Strategy 3 production salmon, were maintained at Tentburn hatchery (Canterbury, New Zealand). Females with high X-ray based deformity traits were crossed with males with low X-ray based deformity traits. Knowing the genetic background of the eggs of each stripped female, the genetic background allocated to the PIT-tag of each male was used to devise a list (Table 2). This list was created by Fiona Hely (AbacusBio, 442 Moray Place, 9016 Dunedin, New Zealand). The top six males best suited for the male x female crosses were selected for fertilising eggs. The PIT-tag of twelve males was scanned and specimens were tested for milt (sperm) production (see Table 2). The males are ready for reproduction when a white opaque fluid squirted from the anal pore after applying light pressure on the abdominal cavity. Three males, including the top two males failed to produce milt and one suggested male on the list was not present due to mortality.

Table 2: Male and females Chinook salmon: Males indicated in blue and all females with good egg batches were used for crosses

Males PIT-tag no.	Present on farm	Milt producer	
45379374	Yes	No	
29890286	No	No	
0A00147173	Yes	Yes	
107052590	Yes	Yes	
20581830	Yes	Yes	
107076810	Yes	Yes	
96577575	Yes	Yes	
0A00062039	Yes	Yes	
0A00134027	Yes	Yes	
29893351	Yes	Yes	
45573861	Yes	No	
29885588	Yes	Yes	
Females PIT-tag no.	100 eggs weight (g)	Total clutch weight (g)	Number of eggs
095812781	22.9	1964	8576
416363382	26.3	2076	7893
0A00152279	21.7	2042	9410
0A00154108	Bad eggs	Bad eggs	-
0A00145867	20.1	1772	8815
091108346	Not on list*	Not on list*	-
076892336	19.7	1430	7258
107039261	19.5	2296	11774
045578789	23.2	1942	8370
024265075	17.0	1858	10,929
021607021	24.7	2500	10,121
073379076	22.9	2300	10,043

* Specimens not on list provided by Abacus Bio Ltd (Dunedin, New Zealand)

Specimens with bad eggs and not on the list were not used for fertilisation

2.3. FERTILISATION

2.3.1. MILTING MALES

The six males selected from the list were caught with a landing net. Each salmon was held at the caudal end (just anterior of the caudal fin) with the non-dominant hand while the head was kept under water. Next, the male was lifted from the water and held against the body of the handler while placing the dominant hand on the upper abdominal region (the testis are located ventrally of the kidneys lying

ventral of the vertebral column). To extrude the sperm (= milt) hard pressure was applied on the abdominal cavity. The tail was gently moved left and right to increase the flow of sperm. Initially, translucent fluid (urine) squirted from the anal pore. Care was taken not to collect the urine because this will prematurely activate the sperm due to the reduced osmolality of the urine (Rosengrave et al., 2009). The first part of the white opaque fluid was not collected, because remnants of urine could still be present. Thereafter, the remaining milt was captured in a plastic container or bag labelled with the PIT-tag number to identify the male. Great care must be taken in handling adult males since these are not anaesthetised and can move vigorously while out of the water. After milting the males, milt quality was confirmed under a microscope. A streak of milt was placed on a microscope glass and one drop of coelomic fluid (1x concentration) was added. Coelomic fluid was made fresh and is based on ovarian fluid (composition see Table 3). Coelomic fluid activates sperm (Billard, 1983; Galvano et al., 2013; İnanan & Öğretmen, 2015), which was observed under the microscope as swirls occurring on the edge of the streak. Sperm of all males had activated after adding coelomic fluid and was used for the fertilisation process.

Table 3: Coelomic fluid recipe for 5L of 10x concentration

Chemical	Concentration (mM)	Molecular weight	Volume (g)
NaCl	155	58.44	452.910
KCl	3.1	74.55	11.555
$MgSO_4*7H_2O$	1.3	264.48	16.021
$CaCl_2*2H_2O$	3.4	147.02	24.993
Tris Base	20	121.1	105.720
Tris HCl	20	157.56	20.010

Personal communication Seumas Walker

2.3.2. EGG PREPARATION AND FERTILISATION

The unfertilised eggs (called green eggs) of twelve females arrived at the Tentburn hatchery site in plastic bags (20/05/2014). Each bag contained the egg clutch and PIT-tag of a single female. Eggs were transferred to a plastic container and the egg quality was confirmed by softly pressing fingers into the eggs. Good quality eggs feel smooth and silky. In contrast when eggs feel like hard solid tiny balls (like a bowl of tiny marbles) the eggs are not suitable for further fertilisation. Of the twelve egg clutches, one clutch had a PIT-tag which was not included on the list and one clutch had bad egg quality (hard eggs). These eggs were not used for fertilisation. Next, the entire clutch was weighed. Subsequently, 100 eggs were weighed. These two weights were used to estimate the number of eggs in each clutch (see Table 2). To randomise the genetic background of the crosses and thus all subsequent sampling strategies, all female gamete subsets were mixed prior to fertilisation (randomised). One tenth of the weight of each egg clutch was pooled into ten containers (*APPENDIX B*) and some coelomic fluid was added to keep the eggs moist. Subsequently, milt from the first three

males was divided over the first five containers of eggs with a transfer pipette (*APPENDIX B*, 1 ml milt from each male per container, 3 ml milt per container in total, 1 ml milt per 672 grams of egg weight). Milt from male four to six was divided over containers six to ten. Eggs and milt were gently mixed for 30 seconds with a gloved hand and were rested for three minutes for the fertilisation process to take place. After three minutes the eggs were gently washed two to three times in water of the same temperature of the eggs (10°C) by gently plunging a sieve with the eggs in consecutive bowls of water. The fertilised eggs from each of the ten containers were randomly divided over two incubation trays and their position in the incubation stack (Figure 6) also randomised. One tray received the acces of eggs that were not divided over the trays. Newly fertilised eggs are highly sensitive and die when rolled or moved too vigorously. Care was taken to avoid air bubbles that collect below the bottom mesh and may move upwards through the incubation tray suddenly causing mortality of the entire tray.

2.4. TEMPERATURE EXPERIMENT SET-UP

A temperature experiment with three constant temperatures, 4°C, 8°C and 12°C, during the incubation and freshwater grow-out period was devised. Subsequently, specimens would be transferred to sea cages at ambient temperatures. The experimental set-up, for incubation and freshwater grow-out, is schematised in Figure 6A. Large temperature steps were chosen to increase the likelihood of finding a significant impact of temperature on development. Bore water at a constant 12.4°C was pumped into two tanks, an ambient water tank and chilled water tank. The chilled water tank was connected to a chiller system cooling the water down to a range of 2.5 to 3.6°C. Water from each tank was pumped to three automated electromagnetic valves which mixed ambient water with chilled water (Fig. 6A). Each valve could be digitally set to provide a constant temperature (±0.1°C). Water mixed to the correct temperature was fed into three partitioned incubation stacks via a flow meter. One incubation stack per temperature had seven incubation trays. The upper part of the incubation stack contained three incubation trays and the lower part contained four incubation trays. Water was fed into the top tray of each partitioning of the incubation stack (Fig. 6A). An upper and lower partition of the incubation stack was created to avoid factors that cause mass mortalities to cascade through the entire stack (also called tank effect) because water passing through the eggs of one tray flowed into the next tray. The trays are designed to provide upwelling water flow to the eggs which lie on a bottom mesh (Fig. 6B, C). A top mesh prevents the eggs from flowing to the next tray. Water coming from the last tray was drained and not recirculated. To protect the sensitive developing eggs and yolk-sac larvae from light, the incubation stacks were covered with black plastic during the entire incubation period until first feed stage was reached and individuals were moved to the 420 L black circular tanks (Fig. 6D, 23A). Water mixed to the correct temperature also fed these 420 L black circular tanks to

maintain juvenile salmon at their designated water temperature. Two tanks per temperature were operated to avoid tank effects.

Monitoring the performance of the temperature set-up was crucial. The water level gauge in the ambient water tank, and the water level gauge and thermometer in the chilled tank were connected to electronic alarm systems (Fig. 6A, indicated by the electric symbol). Furthermore, the electromagnetic valves, all pumps, and the chiller system were also connected to electronic alarm systems. Alarm connected to thermometers such as in the chilled water tank, the electromagnetic valves and the chiller system were triggered when the temperature exceeded the programmed range. Alarms connected to pumps and the chiller compressor were also triggered when they failed or when a power cut occurred. A manual override to restart the chiller compressor was installed due to the old age of the chiller compressor. This manual override allowed for a reduction in restart procedures and reduced the impact of a chiller failure on the temperature in the cold water tank. Reset programmes and backup mechanisms for reserve pumps were in place to reduce the risk of a complete system failure. A major temperature spike during the sensitive incubation phase of development would have resulted in random and non-systematic errors. Temperature spikes would have created significant interpretation difficulties and was to be avoided at all costs.

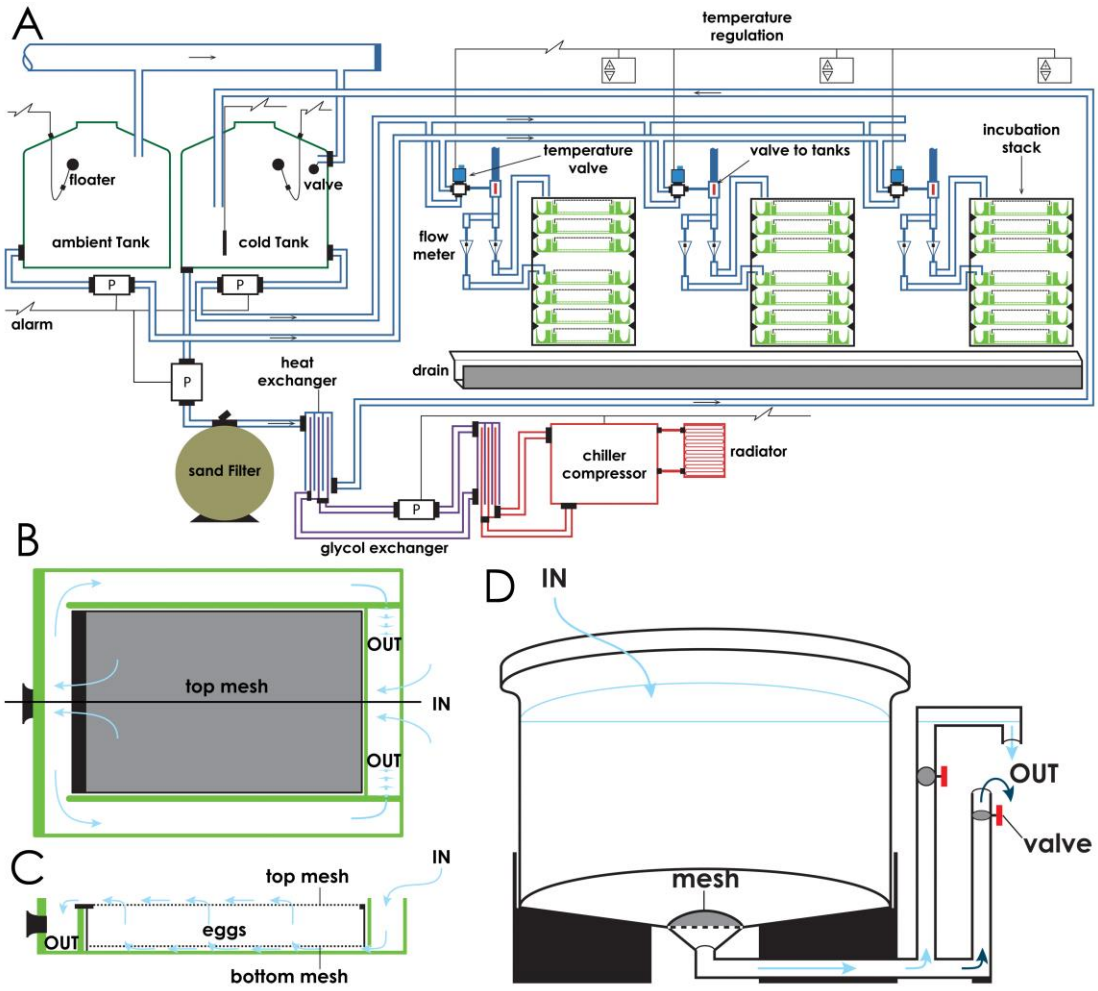


Figure 6: Temperature experiment set-up

The entire temperature experiment set-up is shown. The water flow is indicated by arrows in the blue pipes. The valve under the filled blue pipe section could be switched to provide tanks with water of the same constant water as in the incubation stacks. The glycol loop is shown with purple pipes and the chiller system is shown with red outlines. All the components connected to black lines with an electricity symbol indicate that they were connected to an alarm system. P stands for pump. (B) Top view of an incubation tray. The line indicates the cross section shown in (C). Eggs of Chinook salmon were placed under the top mesh. Water flow is indicated with blue arrows. The dotted arrow indicates were the water flows out of the tray and into the next tray below. (C) Cross section at the middle line of the incubation tray. Eggs of Chinook salmon were placed in-between the top and bottom mesh. The upwelling water flow is indicated by blue arrows. (D) Schematic representation (cross section) of a 420 L incubation tank. Post-hatch stages were placed in this tank at first feed stage (±900°d, fry stage) to grow-out to juveniles. Tanks were provided with the same constant temperature water as was provided to the incubation stacks [see filled blue pipe in (A)]. The mesh in the bottom of the tank prevented fish from escaping. The left outflow pipe valve was always open and was used to regulate the water level. The valve of the right outflow pipe was opened during cleaning, which increased the outflow of water and flushed waste feed from the bottom of the tank and outflow pipes.

The cold water pump (below the cold tank), the chiller circulation pump (above the sand filter), the glycol pump (in the purple loop) and the chiller (Fig. 6A) failed due to both power cuts and electrical failure. Only ambient water was pumped to the electromachnetic valves when the coldwater pump failed. The thermostats were not able to supply correct temperature water and sent an alarm signal. The coldwater pump was restarted by manual reset. When the chiller circulation pump failed, the chiller automatically shut down and sent an alarm signal. The automatic chiller shutdown was to avoid water in the water-glycol heat exchanger from freezing. A back-up chiller circulation pump was manually started until the first pump could be fixed or restarted. When the glycol pump failed, the chiller also automatically shut down and sent an alarm signal. The glycol pump was restarted by manual reset. The water temperature slowly increased in the incubation stack when the chiller circulation and glycol pumps, and/or the chiller failed because the water in the coldwater tank started to rise in temperature (Colt & Maynard, 2017). However, the 3000 L cold reservoir used here, was shown to only increase 1°C per 30 minutes (Colt & Maynard, 2017). Therefore, the failure of the chiller circulation and glycol pumps and/or the chiller did not produce a temperature shock. In addition, because the chiller system used here had a manual override restart procedure, it dramatically reduced the time to restart compared to chiller systems with automatic reboot functions (Colt & Maynard, 2017). Therefore, any chiller failure was resolved by a manual restart procedure and did not register in the temperature logs (personal observation).

2.5. FISH MAINTENANCE

2.5.1. FLOW PROFILE AND TEMPERATURE MEASUREMENT

The entire experimental set-up, incubation trays and tanks, was a complete flow through system. After fertilisation, the initial temperature was set at a constant 10°C for all three temperature groups. The water flow rate was set at 9 L/min for the first day post fertilisation. On the second day post fertilisation the flow rate was increased to 12 L/min and stayed at 12 L/min until smoltification. Temperature and flow rate were manually checked every hour on the first two days post fertilisation. On the third day the temperature was gradually adjusted to a constant 4°C, 8°C and 12°C for each temperature group at a rate of 0.2°C per hour, with a 2°C adjustment per day.

During the incubation the temperatures were constantly monitored by automated temperature data loggers which recorded the temperature every 15 minutes. The temperature data loggers were placed in the top tray of the upper partition and the bottom tray of the lower partition of the incubation stack. Temperature data was downloaded when first feed stage individuals were moved from incubation trays to the 420 L circular black tanks and when the individuals were moved to ambient water in the larger tanks. Temperature data of all temperature groups during the entire incubation period of the 4°C group is presented in Figure 7A, B, C. Irrespective of all the alarm and back-up systems in place,

temperature spikes due to pump and chiller compressor failures (see previous section) were still observed. However, the temperature spikes were of a very short nature, i.e. between 15 minutes and one hour, due to a quick response to each alarm and the use of back-up systems in place. The effects of these temperature changes are discussed in section *7.1.1 TEMPERATURE – SHOCKS AS POTENTIAL CAUSE OF MALFORMATIONS*.

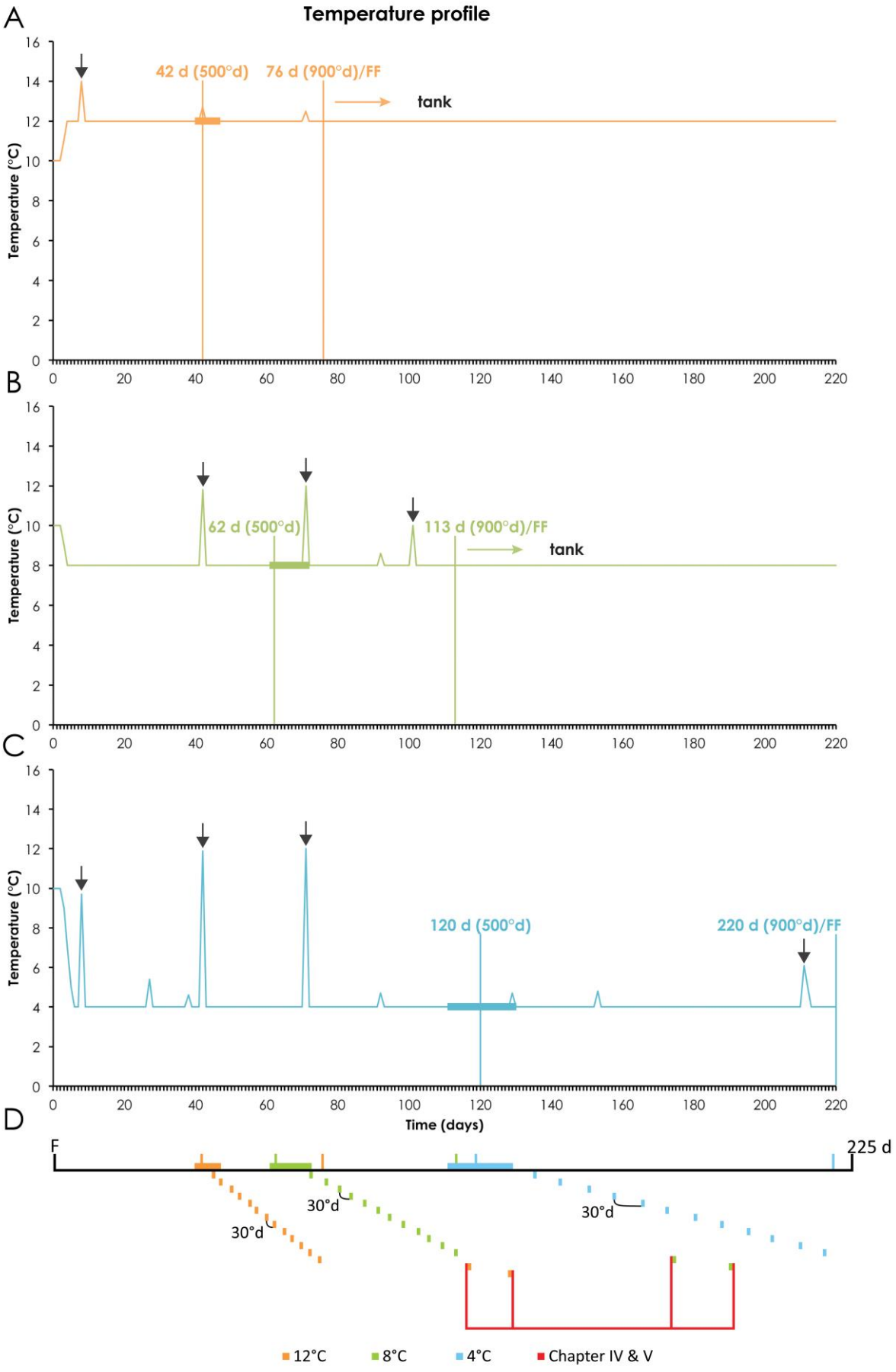

A
Temperature profile
42 d (500°d)
76 d (900°d)/FF
tank
Temperature (°C)
B
62 d (500°d)
113 d (900°d)/FF
tank
C
120 d (500°d)
220 d (900°d)/FF
Time (days)
D
F
225 d
30°d
30°d
30°d
12°C
8°C
4°C
Chapter IV & V

Figure 7: Temperature profile and sampling of post-hatch stages (left)

(A-C) Temperature profiles of the 12°C (orange), 8°C (green) and 4°C (blue) groups, with temperature (°C) on the y-axis and time in days on the x-axis. Timescale during incubation from fertilisation (F) to 225 days (d). The time axis starts at day 0 when eggs were fertilised [see F in (D)]. Accidental temperature shocks are indicated with black arrows. The hatching period is indicated with a coloured bar on the temperature profile of each temperature group. The standard hatching time points (500°d) and standard first feed (900°d/FF) time points are indicated in days (d) for each temperature group. When the 12°C and 8°C groups reached the first feed stage they were moved to tanks (coloured arrow). (D) Sampling scheme of post-hatch and juvenile stages. Samples were taken every 30°d from post-hatching to first-feed in each temperature group and are indicated by the dots below the timeline. The dots indicated between the red lines are samples of juveniles used in *CHAPTER 5*.

2.5.2. EMBRYO AND POST-HATCH STAGES

Since the newly fertilised eggs are very sensitive to any movement and UV-radiation, the eggs were left untouched and in the dark for the first 175°d (= degreedays) of their development. Dead individuals were then removed once a week to reduce the chance of fungal infections spreading. Between the 175°d and hatching (475°d) dead eggs were removed in the dark (with indirect light of a head torch) because they are still sensitive to UV-radiation. During hatching the mortality increases and the decomposing eggshells form ideal media for fungal growth. Therefore, dead individuals were removed twice a week during (475-590°d) and post-hatching (590°d-900°d). All dead individuals were counted. The 4°C group was not moved to tanks at first feed. The specimens in this temperature group reached the first feed stage (900°d) on 31/12/2014 at which time both the 8°C and 12°C groups were already PIT-tagged and in tanks at ambient temperature (Fig. 23A). If the 4°C specimens were kept at 4°C during their freshwater grow-out stage, they would have needed a year and ten months to reach a 3500°d smoltification stage. Even when the 4°C group, similar to the 8°C group, was transferred at 1700°d to ambient water and subsequently transferred to sea at 3500°d, the 4°C group would still need a year for the grow-out stage. If the number of salmon transferred to a sea cage is low, the cohort may experience of form of low density 'stress' and performed poorly. This could in turn further confound the results as well as risk losing many specimens due to unforeseen husbandry complications. New Zealand King salmon was aware of these risks and not prepared to organise experimental sea cages for the 4°C group to go to sea later than the 8°C and 12°C specimens. Therefore, the 4°C group was stopped at first feed stage and not transferred to 420 L tanks.

2.5.3. JUVENILES AND ADULTS

At first feed stage (900°d-950°d) 4000 specimens of the 8°C and 12°C groups were moved to two black 420L circular tanks (2000 individuals per tank) and the temperature regime continued (Fig. 23A). Dead individuals in the constant water temperature tanks were removed every day during

cleaning of the tanks. The dead individuals were counted and checked for external malformations. When cleaning tanks, care was taken to move slowly and deliberately, reducing the impact of stress on the specimens in the tank. The second outflow valve is opened and the bottom mesh in the tank is scrubbed and tapped to dislodge waste food and faeces. The nozzle of a water-blaster is placed on the bottom mesh and water is carefully squirted through the mesh to dislodge and remove sludge from underneath the mesh and from the outflow pipes. Next, water is also blasted in the second outflow pipe to clean out extra sludge. The second outflow tap is closed for the water level to rise. The brush and nozzle of the water-blaster is rinsed in a 3% Virkon®S (Antec International Ltd, DuPont, United Kingdom) solution. Fish were fed intensively for the first ten days. After the initial ten days the intensity of feeding per day was reduced and the size of the food pellet was increased. The feeding schedule can be found in Table 4. The 8°C group showed abraded fins due to fin nipping and had a fungal infection on the wounds of the fins. The fungal infection was successfully treated with 4.5 ppm Halamid solution (1.8g/tank, Agrivantage, Axcentive Asia Pte Ltd, Singapore) for one hour in the tank with a closed tap.

At 1400°d and 1530°d, 500 individuals were removed randomly in each temperature group to reduce crowding stress, reduce the risk of infections and to allow for growth. Of the 500 culled individuals, 200 were weighed (grams) and measured (fork length in cm) (Table 12). At 2500°d (15/12/2014, 209 days post fertilisation), 500 individuals of the 12°C group were PIT-tagged and moved to two 1000 L circular tanks (250 individuals/tank) at ambient water temperature (12.4°C) (Fig. 23A). On the same day the temperature of the 8°C fish was gradually increased to 10°C, and then to 12°C on the next day, at a rate of 0.2°C per hour. At 1700°d (17/12/2014, 211 days post fertilisation), 1000 individuals of the 8°C group, now acclimated to 12°C were also PIT-tagged and moved to four 420 L black circular tanks (250 individuals per tank) at ambient water temperature (12.4°C). During this grow out stage another 50 random individuals were culled in each tank of both temperature groups to reduce crowding in the tanks (Fig. 23A).

At 3348°d (29/04/2015, 344 days post fertilisation) 357 specimens of the 8°C and 376 specimens of the 12°C (4177°d) groups were X-rayed using Kodak Industrex M100 Ready Pack II films (Kodak Australia Pty Ltd, Victoria, Australia) (Fig. 23A, Table 12) and, after a week to recover from radiography at Tentburn hatchery, were moved to experimental sea cages (6x6x6, 216 m^3) on the Ruakaka farm in the Marlborough Sounds (Ruakaka Bay, Queen Charlotte Sound). No specimens were tested with a saltwater challenge to determine the freshwater smoltification status in order to avoid reducing the number of smoltification-stage salmon that would go to seawater and available for statistics. However, specimens were larger than the critical size for successful smoltification of Chinook salmon in New Zealand (> 7.0 g) and entered seawater in late autumn (06/05/2015, Fig. 23, Table 12) when Chinook salmon have a physiological smoltification window (Franklin, 1989). The feeding schedule for sea stages can be found in Table 4. Consecutive digital X-rays/radiographs were

taken of PIT-tagged specimens from the 8°C and 12°C groups on three further occasions, six-months-at-sea, 12-months-at-sea (Fig. 23B), at 15-months-at-see and at harvest (826 days post fertilisation). While 339, 8°C and 271, 12°C specimens were transferred to sea cages many failed to thrive and were culled when they were classified as runts or died. Following standard farming protocol, specimens were classified as runts when they weighed less than a fifth of the average weight of the cohort. Runts were euthanized with anaesthetic overdose of AquiS after they were X-rayed. Only twenty 8°C and seven 12°C specimens survived until harvest stage.

Table 4: Feeding schedule from first feed to smoltification

Food size	Food brand	Frequency per day	Time	
Powder meal	Str. 00, Golden Prima, Biomar	Every 20 minutes	5 days	FW
Crushed meal	Crumble, Golden Prima, Biomar	Every hour	5 days	FW
Crushed/0.8 mm	0.8 mm GAP Semi-Float, Biomar	3 times per day	4 weeks	FW
0.8/1.3 mm	GAP Semi-Float, Biomar	3 times per day	3 weeks	FW
1.3/1.5 mm	GAP Semi-Float, Biomar	3 times per day	1 week	FW
1.5 mm	GAP Semi-Float, Biomar	3 times per day	6 weeks	FW
2.0 mm	GAP Semi-Float, Biomar	3 times per day	16 weeks	FW
3.0 mm	Quinnat GAP Immune Transfer Sink, Biomar	Hand fed to satiation	24 weeks	SW
4.0 mm	Orient S 100 75 ppm Bulk, Skretting	Hand fed to satiation	10 weeks	SW
9.0 mm	Orient HT 2000 40as 1050 kg, Skretting	Hand fed to satiation	31 weeks	SW

FW = freshwater, SW = seawater

2.6. SAMPLING

2.6.1. POST-HATCH STAGES

Three independent 50 egg samples from randomly selected trays were taken to determine fertilisation rates. Fertilisation rates of 82-88% were observed. The first sample was taken after hatching (500°d). From 500°d until 900°d, 200 randomly selected specimens from four different randomly selected incubation trays were sampled every 30°d in each temperature group (Fig. 7D). Samples were fixed in paraformaldehyde (PFA) based fixatives, which are ideal for histology because these fixatives cross-link proteins and therefore mostly retain the inter- and intra-cellular structure of the tissue (Howat & Wilson, 2014; Thavarajah et al., 2012). RNase free PFA was made by treating the demineralised water with DEPC (diethyl pyrocarbonate, Sigma-Aldrich New Zealand Ltd, Auckland) and by using autoclaved phosphate buffered saline solution (PBS) to dissolve the PFA. Fifty specimens were fixed in 4% buffered paraformaldehyde (PFA), 100 specimens were fixed in 4% buffered RNase free PFA and 50 specimens were fixed in buffered paraformaldehyde-lysine-periodate (PLP; McLean & Nakane, 1974). Specimens fixed in 4% PFA and PLP were transferred gradually to 70% ethanol. Specimens fixed in 4% RNase free PFA were gradually transferred either to 100% acetone or 100%

methanol and were kept at -20°C (see Table 5). Specimens were fixed in PLP because this was shown to be a better fixative for embedding procedures aimed at electron microscopy. Specimens were fixed in RNase free PFA to have the option to do whole mount *in-situ* hybridization, immunohistochemistry and genotyping. Tissues were banked in a way to facilitate further research and depending on the outcome of more descriptive studies informing the origin of malformations

Table 5: Fixing and storage solution schedule of a single sample of 200 specimens

No specimens	Fixative		Solution 1		Solution 2		Storage	Temperature
	Type	Time (days)	Type	Time (days)	Type	Time (days)		
50	4% PFA	3	30% ethanol	3	50% ethanol	3	70% ethanol	Room temp
50	PLP	3	30% ethanol	3	50% ethanol	3	70% ethanol	Room temp
50	4% PFA Rnase -	3	50% acetone	3		3	100% acetone	-20°C
50	4% PFA Rnase -	3	30% methanol	3	70% methanol	3	100% methanol	-20°C

2.6.2. JUVENILES

One hundred juvenile individuals were randomly sampled at 1400°d and 1530°d from both the 8°C and 12°C groups, were fixed in 4% buffered PFA and were transferred gradually to 70% ethanol. The fixing steps and transfer to 30%, 50% and 70% ethanol took seven days each.

2.7. RADIOLOGY

2.7.1. RADIOGRAPHY

The X-rays images were obtained using an Atomscope HF80/15+ portable X-ray Unit (Mikasa, Tokyo). Freshwater smoltification-stage salmon were exposed to X-ray on Kodak Industrex M100 Ready Pack II films (Kodak Australia Pty Ltd, Victoria), while a 90-479 Sound Technologies Tru-DR flat panel amorphous silicon digital radiographic receptor (DLC Australia Pty Ltd, Melbourne) was used to obtain digital X-ray images of post-smoltification seawater stage salmon. The X-ray film was placed at a distance from the emitter of 53.5 cm with the emitter set at 50 kV, 0.25 mA/sec and 2.1 seconds exposure time. For digital X-ray images the distance from the emitter to the digital receptor was 50 cm in each case, with emitter settings at 80 kV and 0.45 mA/sec for fish at grading and at 80 kV and 0.60 mA/sec for harvest fish. Live fish at grading were anesthetised for radiography with 20 ppm Aqui-S (Aqui-S New Zealand Ltd, Aglionby St, Lower Hutt, New Zealand).

Exposed X-ray films were developed using Kodak Industrex single part developer (4x diluted in tap water) and Kodak Industrex LO fixative (5x diluted in tap water). X-ray were developed at room temperature (21°C) for seven minutes in the developer solution. The development process was stopped by using a 3% acetic acid solution for 15 seconds. The films were fixed for four minutes in the fixer solution. Subsequently, the X-ray films were vigorously rinsed in running tap water for 15 minutes and hung to dry. Developer and fixative were replenished according to manufacturer's recommendations.

2.7.2. ANALYSIS OF RADIOGRAPHS

Deformities were identified and scored on developed X-ray films that were placed on a light box while deformities on digital X-ray images were identified and scored by viewing the image in Photoshop software (see *CHAPTER 6*). Deformities were identified within vertebral column regions and scored following the protocol described in Perrott et al. (2018). The vertebral column was subdivided into four regions following Kacem et al. (1998): region 1 includes vertebrae 1 to 8 (V1-V8), region 2 includes vertebrae 9 to 31 (V9-V31), region 3 includes vertebrae 31 to 50 (V31-V50) and regions 4 includes vertebrae 51 to 63+ (V51-V63+). Variation in the number of vertebrae is present in region 4 and therefore the 63+ indication is used. The regions suggested by Kacem et al. (1998) have been well

established for the analysis of X-rays of the salmon vertebral column. Results obtained can therefore be more easily compared with other research when these regions are used.

Vertebral deformities were recognised based on the Witten et al. (2009) classification system of 20 types of vertebral deformities. A combination of lordosis, kyphosis and scoliosis (LKS; Witten et al., 2009, type 14, 15, 16), fusion (Witten et al., 2009, type 6, 7, 8), compression of vertebrae and or intervertebral spaces (Witten et al., 2009, type 1, 2, 3, 4, 5, further referred to as compression), and vertical shift within the entire vertebral centrum (Witten et al., 2009, type 17) were recognised and could be easily compared to other studies investigating deformities in farmed Chinook salmon. In addition to recognising the deformities, a severity score of 1 to 3 was assigned to each deformity. The score is based on the number of vertebrae involved in the deformity and the angle of the curvature in lordosis and kyphosis. Assessment of the severity of scoliosis was more subjective when it was not present in combination with lordosis and kyphosis.

- Severity 1: 1 to 2 vertebrae or 0 °to 20°
- Severity 2: 2 to 5 vertebrae or 20° to 40°
- Severity 3: 5+ vertebrae or >40°

The absence or presence of each deformity was scored per region by 0 or a 1, absent or present respectively. A parallel dataset was generated where the present deformities were given a severity score (1 to 3). The scoring protocol, its development and validation has been published in Perrott et al. (2018).

2.8. WHOLE MOUNT CLEARING AND STAINING

Alizarin red S and Alcian blue staining protocols were adapted from Taylor and Van Dyke (1985) and can be found in *APPENDIX D*. All specimens were weighed, measured and photographed (Nikon D7000 body, AF-S Micro NIKKOR 60 mm 1:2.8G ED lens). Subsequently the specimens stained for mineralised bone with Alizarin red S were gutted and the epidermis was removed. Specimens were rinsed in tap water and placed in acetone to remove fat to increase the clarity of tissues after clearing steps. Next, specimens were bleached, rinsed and placed in a neutralisation buffer. Specimens were stained in an Alizarin red S solution (0.5 mg/ml in 1% KOH), rinsed and cleared stepwise in glycerol/KOH solutions. A graded glycerol solution series was used to bring specimens to 100% glycerol for storage.

Specimens stained for cartilage with Alcian blue were gutted but the epidermis was not removed because it provides support for tissues during the trypsin treatment. Specimens were rinsed in tap water and fat was removed by placing the specimens in a 70% acetone solution. Subsequently, specimens were dehydrated in a graded ethanol series and stained in an Alcian blue 8GX solution (15

mg/ml) at pH 1.5-2. Next, specimens were rehydrated in a graded ethanol series, rinsed and treated with trypsin dissolved in the neutralisation buffer. Specimens were cleared in graded glycerol/KOH solutions and brought to 100% glycerol for storage with a graded glycerol series. The epidermis of specimens was removed for imaging.

The Alizarin red S whole mount stained and cleared close to first feed specimens were analysed for the presence of early malformations in chordacentra and mineralised associated elements (arches) (see *CHAPTER 3*). Juvenile specimens whole mount stained with Alizarin red S and cleared were analysed for vertebral morphology, vertebral meristic characters and vertebral column regionalisation (see *CHAPTER 4 AND 5*). Alcian blue whole mount stained and cleared post-hatch specimens were analysed for the presence of notochord malformations and malformation of the associated elements (see *CHAPTER 3*). Specimens were examined under stereomicroscopes (Leica M80 and AXIOzoom.V16, Zeiss) and images were taken with mid- and top-mounted cameras (Leica IC80 HD and AxioCam MRc, Zeiss). Images were processed in Photoshop CS5.

2.9. HISTOLOGY

To obtain a thorough understanding of tissue morphology, including early malformation processes in post-hatch stages, and of the juvenile vertebrae along the vertebral column, extensive efforts have been made to obtain the best quality histology. Serial sectioning was applied to all the tissues embedded, although the excess tissue was always trimmed. All specimens for histology were weighed, measured and photographed. For *CHAPTER 3*, three normal post-hatch and three normal first feed specimens per temperature group (4°C, 8°C and 12°C) were embedded (= 18 blocks). Also for *CHAPTER 3*, two specimens of each external malformation phenotype per temperature group were embedded, except for the rarer malformations such as tail malformation and bent neck for which one specimen per temperature group was embedded (= 42 blocks). Both for normal as for malformed phenotypes, the entire specimens were embedded to cut parasagittal sections. The excess tissue, until the first head of a rib observed under a binocular microscope with oblique light, was trimmed before serial sections from the entire vertebral column were taken. For *CHAPTER 4* juvenile specimens, the regions of interest were dissected out of the body of the specimens. Both at 1400°d and 1530°d, for both 8°C and 12°C specimens, one tissue block for the rib-bearing region and one tissue block for the transitional/caudal region were embedded for transverse sections (= 4 blocks). Although serial sections were made, the transverse orientation meant that a greater number of sections were necessary to observe multiple vertebrae. Therefore, both at 1400°d and 1530°d, for both 8°C and 12°C specimens, two tissue blocks per region of interest, i.e. postcranial, rib-bearing, transverse/caudal and preural/ural, were embedded for parasagittal sections (= 16 blocks). Again excess tissue was trimmed before making serial sections of the left half of the vertebral column.

All specimens and dissected tissue blocks were placed overnight to 24 hours in acetone to remove fat. Subsequently specimens and tissue blocks were rehydrated and decalcified in Morse solution [Morse, 1945; 22.5% formic acid (CH_2O_2, Merck KGaA, Darmstadt, Germany) and 10% sodium citrate ($C_6H_5Na_3O_7*2H_2O$, Merck KGaA, Darmstadt, Germany)]. Specimens and tissue blocks were rinsed, processed overnight (*APPENDIX C*) and embedded in paraffin. Tissue was either embedded to make parasagittal or transverse 4 μm sections on a Leica RM 2235 or Prosan MICROM HM360 microtome. Sections were mounted on glass slides (Grale HDS, Trajan Scientific Australia Pty Ltd, Victoria, Australia) and heated at 60°C to fix tissue sections to the glass slides. Sections were observed under a microscope fitted with DIC light (differential interference contrast) to determine which sections to stain, and therefore to reduce the cost and to reduce the number of sections for specific staining methods. Sections were stained with Haematoxylin and eosin (adapted from; Gill et al., 1974; see *APPENDIX E*), Masson's trichrome, Verhoeff-Van Gieson, AB-PAS (adapted from; Culling, 1974a; Culling, 1974b; see *APPENDIX E*) and Heidenhains AZAN trichrome (adapted from; Böck & Romeis, 1989; see *APPENDIX E*) methods to highlight collagen and elastin. Sections were mounted in Entellan® (Merck KGaA, Darmstadt, Germany) or DPX (Sigma-Aldrich Chemie Gmbh, Munich, Germany) and cover-slipped (Leica CV 5030). Sections were examined under a Zeiss Axiophot or Zeiss Axio Imager Z1 microscope and images were taken with top mounted cameras (Olympus DP72 and Zeiss AxioCam 503 color).

2.10. CONTRIBUTIONS

Although the eggs were stripped from the females by New Zealand King Salmon (NZKS) staff, following standard farming procedures, the males were milted and the eggs fertilised by Adelbert De Clercq under supervision of NZKS-staff (Michael 'Big Mike' Anderson). During the incubation period, Adelbert De Clercq responded to the alarms from equipment failures and power cuts. Dead specimens were removed, samples were taken, and first feed stages of the 8°C and 12°C groups were moved to tanks and fed by Adelbert De Clercq. The 8°C and 12°C specimens in tanks were further fed and the 4°C specimens sampled by Solenne Metayer, a French exchange student. Specimens were sampled and culled from the 8°C and 12°C group by Adelbert De Clercq and Solenne Metayer. Freshwater smoltification-stage specimens of the 8°C and 12°C group were X-rayed on the Tentburn hatchery by Matthew Perrott, Ben Wybourne, Bailey Lovett and NZKS-staff. The salmon were transported to sea cages by NZKS-staff in cooperation with TNL (TNL International Ltd, Hornby, Christchurch). The sea stages of the 8°C and 12°C group were X-rayed and harvested by Bailey Lovett, Seumas Walker and NZKS-staff.

CHAPTER 3: THE EXTERNAL PHENOTYPE-SKELETON LINK IN POST-HATCH FARMED CHINOOK SALMON

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3.1. ABSTRACT

Skeletal deformities in farmed fish are a recurrent problem. External malformations are easily recognised but there is little information on how external malformations relate to malformations of the axial skeleton: the external phenotype-skeleton link. Here this link is studied in post-hatch to first-feed life stages of Chinook salmon (*Oncorhynchus tshawytscha*) raised at 4°C, 8°C and 12°C. Specimens were whole mount stained for cartilage and bone, and analysed by histology.

In all temperature groups, externally normal specimens can have internal malformations, predominantly fused vertebral centra. Conversely, externally malformed fish usually display internal malformations. Externally curled animals typically have malformed haemal and neural arches. External malformations affecting a single region (tail malformation and bent neck) relate to malformed notochords and early fusion of fused vertebral centra. The frequencies of internal malformations in both externally normal and malformed specimens show a U-shaped response, with lowest frequency in 8°C specimens. The fused vertebral centra that occur in externally normal specimens represent a malformation that can be contained and could be carried through into harvest size animals. This study highlights the relationship between external phenotype and axial skeleton and may help to set the framework for the early identification of skeletal malformations on fish farms.

3.1.1. KEYWORDS

Atlantic salmon, early diagnosis, skeleton, temperature, zebrafish

3.2. INTRODUCTION

Skeletal deformities are a recurrent problem in farmed teleost species. The occurrence and causative factors of skeletal deformities have been reviewed for teleosts in general (Boglione et al., 2013a; Boglione et al., 2013b) and salmonids in particular (Fjelldal et al., 2012). However, salmonid literature focuses on juvenile and adult stages, emphasising that deformities can start to develop at juvenile or even adult life stages (Fjelldal et al., 2009a; Witten et al., 2005a). Yet, common deformities such as fusions and compressions might develop earlier, during embryonic stages or shortly after hatching. Early malformations might develop further and aggravate in juvenile stages or, conversely, might be contained with restoration of a functionally stable phenotype (Witten et al., 2006). Therefore research on early life stages can provide critical information that can help to understand the aetiology of malformations later in life (Koumoundouros, 2010).

Malformations in marine teleosts are extremely diverse and have been extensively studied (Boglione et al., 2013a; Boglione et al., 2013b; Jezierska et al., 2009; Rosenthal & Alderdice, 1976). For salmonids, most reports about malformations in early life stages describe external morphological

variations. Twinning, pug headedness, lower jaw malformations and axial deviations (lordosis, kyphosis and scoliosis) have been described (Eriksen et al., 2006; Fjelldal et al., 2016; Johnson et al., 1998; Leduc, 1978; Mahrosh et al., 2014; Quatrefages, 1888; Yamamoto et al., 1996). Few reports link external phenotypes with deformities of internal structures. One example is a study on pink salmon (*Oncorhynchus gorbuscha*, Walbaum), which describes histological changes related to external eye malformations (Marty et al., 1997). Another study on lake trout (*Salvelinus namaycush*, Walbaum) reports retinal degeneration and necrosis caused by the exposure to a dioxin (Spitsbergen et al., 1991). Such examples provide insights into the early onset of malformations that can lead to deformities later in life. Here, we focus on the relationship between external phenotype and internal malformations of the axial skeleton in farmed New Zealand Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum).

The teleost skeleton consists of many different types of cartilage and bone (Witten & Hall, 2015; Witten et al., 2010). The early structures that build up the vertebral column, are the notochord, the vertebral centra and their associated elements (Arratia et al., 2001). The tissues that constitute these skeletal structures, i.e. notochord, cartilage and bone, are liable to alterations under farming conditions (Boglione et al., 2013a). In teleosts the notochord is a permanent, continuous structure, while it is reduced to the nucleus pulposus in the mammalian intervertebral disc (Corallo et al., 2013; Stemple, 2005). The notochord functions as the mechanical support for the embryonic body axis and as a midline signalling centre (Anderson et al., 2007; Stemple, 2005; Wang, 2013). Vacuolated notochord cells (chordocytes) are surrounded by chordoblasts, which produce the notochord sheath (Kryvi et al., 2017; Stemple, 2005). The anlagen of the teleost vertebral centra arise by segmented mineralisation of the notochord sheath (Huxley, 1859; Kölliker, 1859), designated as chordacentra (Arratia et al., 2001). Elements associated with the vertebral centra are parapophyses, ribs, neural and haemal arches, neural and haemal spines, and the caudal fin skeletal elements (Arratia et al., 2001). Except neural and haemal spines which ossify intramembranously, associated elements derive from the somites (sclerotome), and in salmonids develop as cell-rich hyaline cartilaginous anlagen before perichondral and endochondral ossification starts (Arratia et al., 2001; de Azevedo et al., 2012; De Clercq et al., 2017b; Grotmol et al., 2003; Huxley, 1859; Kölliker, 1859; Schaeffer, 1967).

Temperature stands out among the many factors that influence skeletal development (Boglione et al., 2013a; Boglione et al., 2013b). Using optimal temperatures during fertilisation, embryonic and post hatching stages, is critical for normal development. At early stages temperature affects developmental rate (Alderdice & Velsen, 1978; Beacham & Murray, 1989; Hamor & Garside, 1976), mortality (Beacham & Murray, 1987b; Murray & Beacham, 1986; Tang et al., 1987), success of hatching (Beacham & Murray, 1987a; Hamor & Garside, 1976; Hayes et al., 1953) and also normal skeletal development (Cloutier et al., 2010; Fuiman et al., 1998; Grünbaum & Cloutier, 2010;

Koumoundouros et al., 2009; Koumoundouros et al., 2001). Elevating the temperature accelerates normal development but also increases the risk of abnormal development (Sfakianakis et al., 2004; Takle et al., 2005; Wargelius et al., 2005; Ytteborg et al., 2010a; Ytteborg et al., 2010b; Ytteborg et al., 2010c). While optimum temperatures for incubation of Chinook salmon are 8 to 10°C (Alderdice & Velsen, 1978; Brett, 1952), temperatures used in hatchery operations are usually above the optimum to accelerate growth (Fjelldal et al., 2012), i.e. 12°C in case of New Zealand Chinook salmon.

Here, the external phenotype-anatomy link is addressed through the study of post-hatch larvae raised until first feed at three different temperatures. Specifically this study asks (i) if malformation of internal skeletal elements occurs in externally normal specimens, (ii) if specimens with external malformations always have internally malformed skeletal structures, (iii) which skeletal structures are malformed (notochord, chordacentra or associated elements) and (iv) how malformations are influenced by rearing temperature.

3.3. MATERIAL AND METHODS

3.3.1. EXPERIMENTAL SET-UP AND SAMPLES

Eggs of ten females were randomised and fertilised with sperm of six males. Before hatching eggs were kept at a constant 10°C (temperature at stripping) and were fertilised at 10°C, according to the established farming practices (*APPENDIX B*). Specific family crosses known to produce a high frequency of external malformations were selected to provide off-spring (Gjerde et al., 2005). The impact of incubation temperature on the frequency of malformations is more easily observed when the baseline malformation rate is high. This applies especially to malformation types that are less frequent. Eggs were placed in incubation trays (±4000 eggs/tray). Initially the incubation trays were supplied with a constant 10°C water (until 3 days post-fertilisation = 30°d, degreedays). Subsequently the temperature was adjusted (0.2°C per hour) to constant 4, 8 and 12°C rearing temperatures. To protect the light sensitive developing eggs and larvae, the incubation stacks were covered from the light during the entire incubation period (fertilisation to first-feed stage).

Dead specimens were removed twice a week and not further analysed. During the hatching period dead specimens were removed every two days. Mortality from fertilisation to first feed stage was 64%, 29% and 23% for the 4, 8 and 12°C groups respectively. The mortality in the 8 and 4°C was higher than in the 12°C group due to mortality events of almost all eggs in one incubation tray in the 8°C group and two trays in the 4°C group. These mortality events occurred because the eggs were moved by air bubbles percolating through the eggs during the sensitive stage (from 0°d to 180°d). Eggs of these trays were not further sampled to avoid confounding of results. From the end of the

hatching period onwards (hatching at ±500°d) 200 specimens were sampled randomly every 30°d (Fig. 8) for the 8 and 12°C groups. For the 4°C group 80 specimens were sampled randomly every 30°d. The last sample was taken at ±900°d, also called first feed-stage (Fig. 8). For total number of samples per temperature group see Table 6 (Total number externally analysed). Specimens were euthanized by anaesthetic overdose of Aqui-S (New Zealand, Ltd). The specimens were fixed in 4% buffered paraformaldehyde (PFA) and stored in 60% ethanol. Experiments were approved by Animal Ethics Committee of Massey University: AEC protocol 14/32.

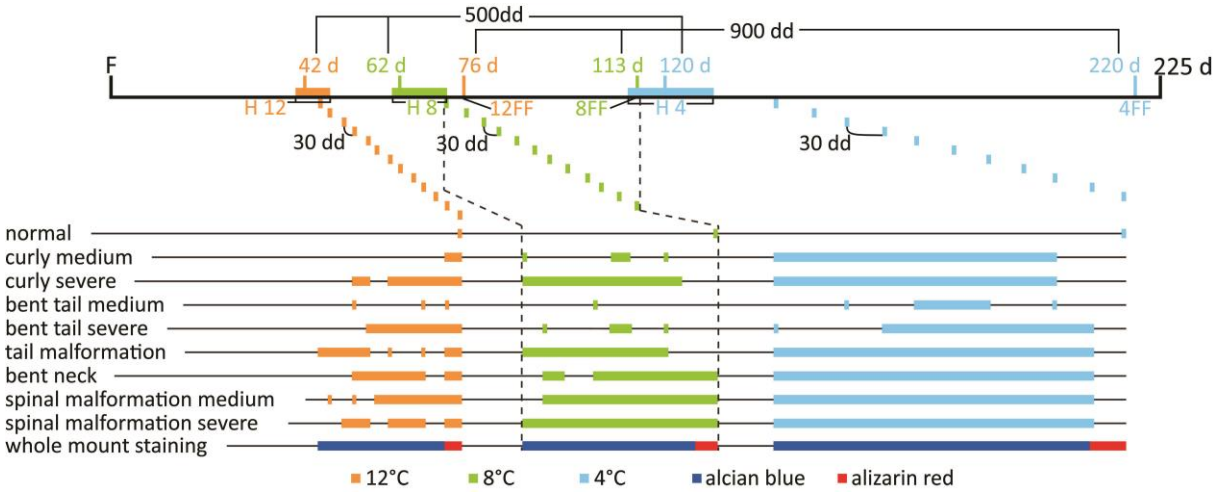

Figure 8: Incubation time scale and sampling schedule

Timescale during incubation from fertilisation (F) to 225 days (d). Orange, green and blue colours indicate the constant temperature groups: orange = 12°C, green = 8°C and blue = 4°C. The coloured strips on the time line delimit the hatching period of each temperature group indicated by H12, H8 and H4. Hatching occurs around 500°d, and the 500°d time point for each temperature is indicated in days. The incubation period ends at first-feed (FF) stage (absorption of the yolk sac) around 900°d (indicated by FF12, FF8 and FF4). The 900°d time point is also indicated in days for each temperature. Samples were taken every 30°d from post-hatching to first-feed in each temperature group and are indicated by the dots below the timeline.

The schematic below the time line and sample schedule indicates in which samples the external malformations (defined in Table 6) were observed. The start and end of the sampling period, at post-hatch and first-feed stages respectively, is indicated by a dotted line for the 8°C group. Normal phenotypes were studied at first-feed and therefore only sampled at first-feed stage. External body axis malformations occurred almost throughout the entire sample range from post-hatching to first-feed in all temperature groups, except curly mild, bent tail medium and bent tail severe. The bottom line indicates which samples were whole mount stained with Alcian blue or Alizarin red S.

3.3.2. EXTERNAL PHENOTYPE SCORING AND SELECTING AXIAL SKELETAL MALFORMATIONS

All samples of each temperature group were assessed for external body axis malformations. Different external malformations within a single specimen were recorded separately and are defined in Table 6. All assessments were made by the same person. Externally malformed specimens were stained whole

mount together with externally normal specimens (Table 7). Normal external phenotype specimens were selected at first feed stage and were stained with Alizarin red S for mineralised tissues (mostly bone) (Fig. 8). Malformed specimens were stained with Alcian blue except for the last two sampling points, which were stained with Alizarin red S due to the start of mineralisation of the skeleton (Fig. 8).

Table 6: External body axis malformation phenotypes in post-hatch to first feed stage Chinook salmon: percentages based on total number of specimens sampled per temperature group (samples taken every 30°d from post-hatch to first-feed and a definition for each malformation. If co-occurring within a single specimen, different external malformations were recorded separately.

Malformation type	4°C number (%)	8°C number (%)	12°C number (%)	Definition	Reference
Curly medium	25 (6.5)	6 (0.5)	5 (0.4)	Medium lordosis or kyphosis over the length of the entire body, specimen having the shape of half a circle	Mahrosh et al. (2014)
Curly severe	54 (14.1)	66 (5.4)	46 (3.3)	Very severe lordosis or kyphosis over the entire length of the body, ranging from the head touching the caudal fin to a complete spiral of the body (coiled tail)	Mahrosh et al. (2014)
Bent tail medium	9 (2.3)	1 (0.1)	4 (0.3)	Separate lordosis, kyphosis or scoliosis of the caudal region	Eriksen et al. (2006)
Bent tail severe	1 (0.3)	6 (0.5)	12 (0.9)	Severe separate lordosis, kyphosis or scoliosis of the caudal region, e.g. caudal region forming a single coil	Eriksen et al. (2006)
Tail malformation	6 (1.6)	16 (1.3)	24 (1.8)	Heavily malformed preural and ural region, ranging from stunted development to absence of caudal fin	Eriksen et al. (2006), Marty et al. (1997)
Bent neck	47 (12.3)	38 (3.1)	34 (2.6)	A bend in the postcranial region of the specimens body, includes ‘star gazer’ phenotype	Boglione et al. (2013a)
Spinal malformation medium	62 (16.2)	83 (6.8)	55 (4.2)	A combination of lordosis, kyphosis and scoliosis along the length of the specimens body in mild forms	Marty et al. (1997)
Spinal malformation severe	52 (11.0)	106 (8.7)	62 (4.7)	A combination of lordosis, kyphosis and scoliosis along the length of the specimens’ body in severe forms	Marty et al. (1997)

Total number of specimens analysed externally	383	1227	1313

3.3.3. WHOLE MOUNT STAINING AND ANALYSING INTERNAL MALFORMATIONS

Table 7 shows the number of larvae stained with Alcian blue 8GX (Acros organics, New Jersey, USA) for cartilage and stained for mineralised bone with Alizarin red S (Sigma-Aldrich, Steinheim am Albuch, Germany). The staining protocols were adapted from Taylor and Van Dyke (1985). Briefly, specimens for Alcian blue were rinsed and stained for 48 hrs in [15mg/ml] Alcian blue solution in 20% acetic acid/80% ethanol (pH = 1.5-2). Subsequently specimens were rehydrated, rinsed and cleared in a solution of [0.5 mg/ml] trypsin in 30% saturated borax (di-sodium tetraborate, $Na_2B_4O_7.10H_2O$) (pH = 9.4). Specimens were further cleared in a graded glycerol/KOH series, dehydrated in a graded glycerol series and stored in 100% glycerol. Specimens for Alizarin red S were rehydrated, bleached (3% H_2O_2 in 1% KOH, 1-1.5 hrs.), rinsed and placed for 24 hrs in a 30% saturated borax solution. Specimens were stained overnight in a [0.5 mg/ml] solution of Alizarin red S in 1% KOH. Specimens were cleared in a graded glycerol/KOH series, dehydrated in a graded glycerol series and stored in 100% glycerol. Specimens were analysed with a Zeiss Axiozoom.V16 microscope equipped with an AxioCam MRc, top mount camera (Carl Zeiss Microscopy, Oberkochen, Germany). Adobe Illustrator CS5 was used to create schematic representations.

Internal malformations were grouped into three phenotypes (Table 8): (i) notochord related malformations, (ii) fusions of chordacentra and (iii) malformations related to associated elements (further referred to as arches). When fusion of chordacentra occurred as a consequence of a malformed notochord (see white asterisk Fig. 12C), the malformation was recognised as a notochord malformation. Malformations of neural and haemal arches, ribs, neural and haemal spines of preural vertebrae and caudal fin skeletal elements (stegural, epurals, uroneurals and hypurals) were considered as malformations related to associated elements. Analysis and interpretation of vertebral centra follows Bensimon-Brito et al. (2012b).

Table 7: Number of post-hatch (PH) to first-feed (FF) externally normal and externally malformed fish stained with Alizarin red S (AR) and Alcian blue 8GX (AB)

External phenotype	Stain	Life stages sampled	4°C	8°C	12°C
Normal	AR	FF	50	50	50
Malformed	AB	From PH to FF	37	84	83
Malformed	AR	From PH to FF	9	9	34
Total number of malformed			46	93	117

3.3.4. HISTOLOGY

In total, 18 specimens with externally normal phenotype were selected, three post-hatch and three first-feed specimens per temperature group. In total, 64 specimens with externally malformed phenotypes were selected. For each externally malformed phenotype, three specimens were selected in the 8°C and 12°C temperature groups. In the 4°C temperature group two specimens for each externally malformed phenotype were selected due to lower sample numbers. Before processing the specimens were placed for 24 hours in acetone to remove fat, rinsed and subsequently decalcified for 48 hours in Morse solution (Morse, 1945) [22.5% formic acid (CH_2O_2, Merck KGaA, Darmstadt, Germany) and 10% sodium citrate ($C_6H_5Na_3O_7$*2 H_2O, Merck KGaA, Darmstadt, Germany)]. Finally, the specimens were rinsed and embedded in paraffin. Parasagittal sections were cut at 4 μm with a Leica RM 2235 microtome (Leica Microsystems, Wetzlar, Germany). Sections were stained with haematoxylin and eosin (protocol adapted from; Gill et al., 1974), Heidenhain's Azan trichrome, Masson's Trichrome and Verhoeff-Van Gieson methods to highlight collagen and elastin (Böck & Romeis, 1989; Culling, 1974a; Culling, 1974b). Sections were analysed with bright field under a Zeiss Axio Imager 0.Z.1 fitted with an Axiocam 503 colour top mount camera.

3.4. RESULTS

3.4.1. INTERNAL VERSUS EXTERNAL MORPHOLOGICAL PHENOTYPE

Table 8: Number and percentage (%) of specimens with internally normal and malformed phenotype for all external phenotypes. If co-occurring within a single specimen, different internal malformations were recorded separately. Associated elements = predominantly arches

External phenotype	Related figure	Internal phenotype	4°C (%)	8°C (%)	12°C (%)
Normal *	Figure 9A	No internal malformations	30 (60)	42 (84)	38 (76)
		Internal malformations	20 (40)	8 (16)	12 (24)
All external malformations *	Figure 9B	No internal malformation	10 (22)	47 (51)	57 (49)
		Internal malformations	36 (78)	46 (49)	60 (51)
Normal	Figure 10A	Total number of specimens	50	50	50
		Notochord	6 (12)	0 (0)	1 (2)
		Chordacentra fusion	16 (32)	8 (16)	8 (16)

		Associated elements	6 (12)	1 (2)	6 (12)
Curly medium	Figure 10B	Total number of specimens	7	13	16
		Notochord	0 (0)	0 (0)	1 (6)
		Chordacentra fusion	0 (0)	3 (23)	3 (19)
		Associated elements	4 (57)	6 (46)	4 (25)
Curly severe	Figure 10C	Total number of specimens	6	14	20
		Notochord	1 (17)	3 (21)	6 (30)
		Chordacentra fusion	1 (17)	2 (12)	5 (25)
		Associated elements	3 (50)	5 (36)	6 (30)
Bent tail medium	Figure 10D	Total number of specimens	4	8	13
		Notochord	2 (50)	3 (38)	2 (15)
		Chordacentra fusion	1 (25)	0 (0)	0 (0)
		Associated elements	4 (100)	3 (38)	4 (31)
Bent tail severe	Figure 10E	Total number of specimens	5	5	9
		Notochord	1 (20)	0 (0)	0 (0)
		Chordacentra fusion	1 (20)	1 (20)	2 (22)
		Associated elements	3 (60)	3 (60)	2 (22)
Tail malformation	Figure 10F	Total number of specimens	5	10	5
		Notochord	4 (80)	6 (60)	1 (20)
		Chordacentra fusion	3 (60)	4 (40)	2 (40)
		Associated elements	1 (20)	5 (50)	2 (40)
Bent neck	Figure 10G	Total number of specimens	5	13	13
		Notochord	5 (100)	6 (46)	8 (62)

		Chordacentra fusion	2	3	7
			(40)	(23)	(54)
		Associated elements	2	4	4
			(40)	(31)	(31)
Spinal malformation medium	Figure 10H	Total number of specimens	7	16	19
		Notochord	2	3	1
			(29)	(19)	(5)
		Chordacentra fusion	1	0	1
			(14)	(0)	(5)
		Associated elements	3	2	6
			(43)	(13)	(32)
Spinal malformation severe	Figure 10I	Total number of specimens	7	14	22
		Notochord	5	3	9
			(71)	(21)	(41)
		Chordacentra fusion	1	2	8
			(14)	(14)	(36)
		Associated elements	5	3	7
			(71)	(21)	(32)

***** Total number of normal and malformed specimens in Table 7.

3.4.1.1. NORMAL EXTERNAL PHENOTYPE

Alizarin red S staining of first-feed stage specimens with normal external phenotype revealed internal malformations in all three temperature groups (Fig. 9A, Table 8), with highest prevalence (40%) in the 4°C group and lowest (16%) in the 8°C group. Fusion of chordacentra is the most frequent malformation in specimens of all temperature groups, but its prevalence decreases in each temperature group from 4 to 8°C (Fig. 10A, Table 8). Malformation of the associated elements is the second most common phenotype in 4, 8 and 12°C specimens but has very low prevalence in the 8°C specimens. Malformation of the notochord is only present in 4 and 12°C specimens, with highest prevalence in the 4°C specimens.

3.4.1.2. MALFORMED BODY AXIS EXTERNAL PHENOTYPES

The definition of each external body axis malformation phenotype, and the number and percentages per temperature group, are presented in Table 6. External body axis malformations are most common in 4°C specimens, except bent tail severe and tail malformation which shows the highest percentage in 12°C specimens (Table 6). Bent tail medium and tail malformation are least common in 8°C specimens (Table 6). In all temperature groups externally malformed specimens occur that have no apparent skeletal malformations (Table 8).

In almost more than 50% of the specimens, externally malformed fish displayed internal malformations as well (Table 8). The 4°C group showed the highest percentage (78%) of internally malformed specimens (Fig. 9C) and the 8°C group the lowest percentage (49%, Fig. 9B). In the 12°C group, 51.3% of the externally malformed specimens showed internal malformations (Fig. 9B). Below, internal malformations related to rearing temperature are briefly described for each of the external malformation phenotypes.

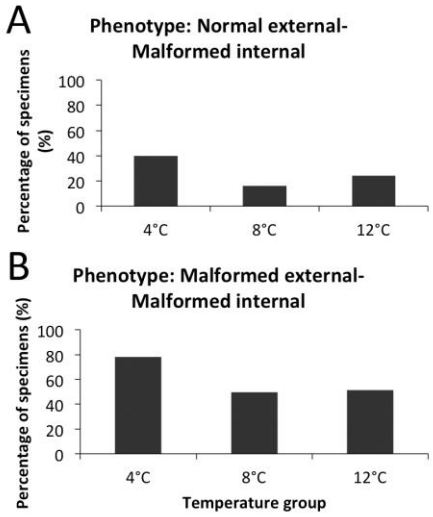

Figure 9: Proportion of specimens with internal malformations for externally normal specimens (A) and externally malformed specimens (B)

3.4.1.3. CURLY MEDIUM

In specimens with curly medium external phenotype, most internal malformations affect the associated elements, but the prevalence decreases in each temperature group from 4 to 12°C. Fusion of chordacentra occurs in 8 and 12°C specimens, while malformation of the notochord only appears in 12°C specimens (Fig. 10B).

3.4.1.4. CURLY SEVERE

In curly severe specimens the prevalence of malformed associated elements decreases in each temperature group from 4 to 12°C, as with curly medium. Malformations of the notochord are the second most common but increase in each temperature group from 4 to 12°C. Frequency of chordacentra fusions is lowest in 8°C specimens (Fig. 10C).

3.4.1.5. BENT TAIL MEDIUM

All 4°C specimens have malformed associated elements. The frequency of both associated elements and notochord malformations decreases in each temperature group from 4 to 12°C. Fusion of chordacentra is only present in 4°C specimens (Fig. 10D).

3.4.1.6. BENT TAIL SEVERE

In 12°C specimens, malformations of associated elements and fusion of chordacentra show equal prevalence. The 4 and 8°C specimens show equal prevalence of fused chordacentra and associated elements. Malformations of the notochord are only observed in 4°C specimens (Fig. 10E).

3.4.1.7. TAIL MALFORMATION

Malformation of the notochord and fusion of chordacentra both decrease in each temperature group from 4 to 12°C. Chordacentra fusions are most prevalent in 4°C specimens and show the lowest prevalence in 12°C specimens. In 12°C specimens fusion of chordacentra and malformation of associated elements show an equal prevalence (Fig. 10F).

3.4.1.8. BENT NECK

All specimens in the 4°C group have a malformed notochord; 4°C specimens furthermore show an equal prevalence of chordacentra fusions and malformed associated elements. Specimens of the 8°C group show the lowest frequency of notochord malformations and chordacentra fusions (Fig. 10G).

3.4.1.9. SPINAL MALFORMATION MEDIUM

Prevalence of malformations of the notochord decreases in each temperature group from 4 to 12°C. Associated elements are least affected by malformation in 8°C specimens. Fusion of chordacentra is most prevalent in 4°C specimens while absent in 8°C specimens (Fig. 10H).

3.4.1.10. SPINAL MALFORMATION SEVERE

Prevalence of malformations of the notochord and associated elements is highest in 4°C specimens and lowest in 8°C specimens. Prevalence of fusion of chordacentra is highest in 12°C specimens compared to 4°C and 8°C specimens (Fig. 10I).

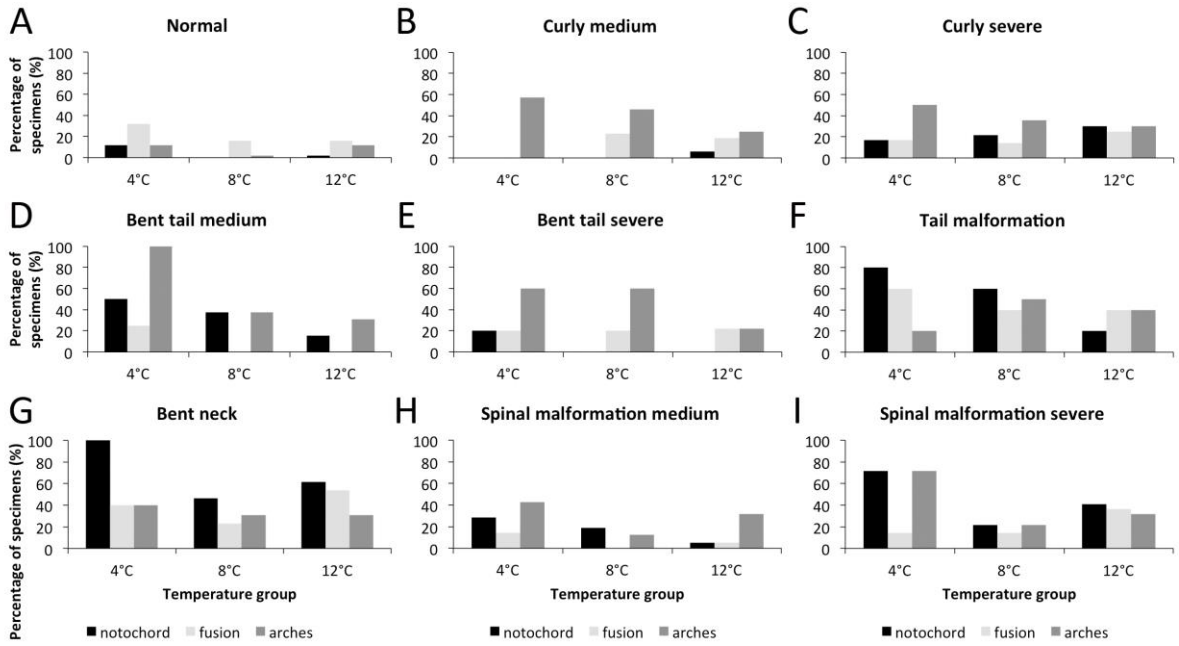

Figure 10: Proportion of internal malformation types per temperature for each of the external body axis phenotypes (normal and malformed) based on Table 8 (associated elements = arches)

3.4.2. AXIAL SKELETAL STRUCTURES

The elements of the axial skeleton (notochord, chordacentra and associated elements) are described based on Alizarin red S and Alcian blue whole mount stained specimens and serial sections, from post-hatch stage to first-feed stage.

3.4.2.1. NOTOCHORD

Two different types of notochord malformation occur in specimens of all temperature groups and often within a single malformed notochord (Fig. 11A). The most common malformation is a partial or complete pinching, where the external elastic membrane of the notochord sheath folds inward into the notochord. The inward fold ranges from a shallow pinch (Fig. 11A) to a completely pinched-off notochord (Fig. 11A, C-D). A pinch in the notochord often (but not necessarily) causes the notochord to either slightly bend or even make a severe deviation of the axis. When a shallow pinch occurs, the cartilaginous base of the anterior or posterior neural or haemal arch extends into the depression in the notochord sheath (Fig. 11A, D). When a deep depression occurs, the cartilaginous bases of the neural or haemal arches anterior and posterior of the pinch site fuse and extend into the deep pinch (Fig. 11B, F). Associated elements (neural and haemal arches, parapophyses and ribs) remain separate at the level of shallow or deep pinching sites. When the notochord is completely pinched-off, each of the two notochord ends is lined by external elastic membrane (Fig. 11A, C-D). The two external elastic membranes are closely apposed (Fig. 11A, D) or separated from each other (Fig. 11A, C). In the latter case, the cartilaginous bases of the neural and haemal arches fuse and fill the space between the

separated notochord ends (Fig. 11A, I). The associated elements (neural and haemal arches, parapophyses and ribs) are often fused at the level of separated notochord ends (Fig. 11I).

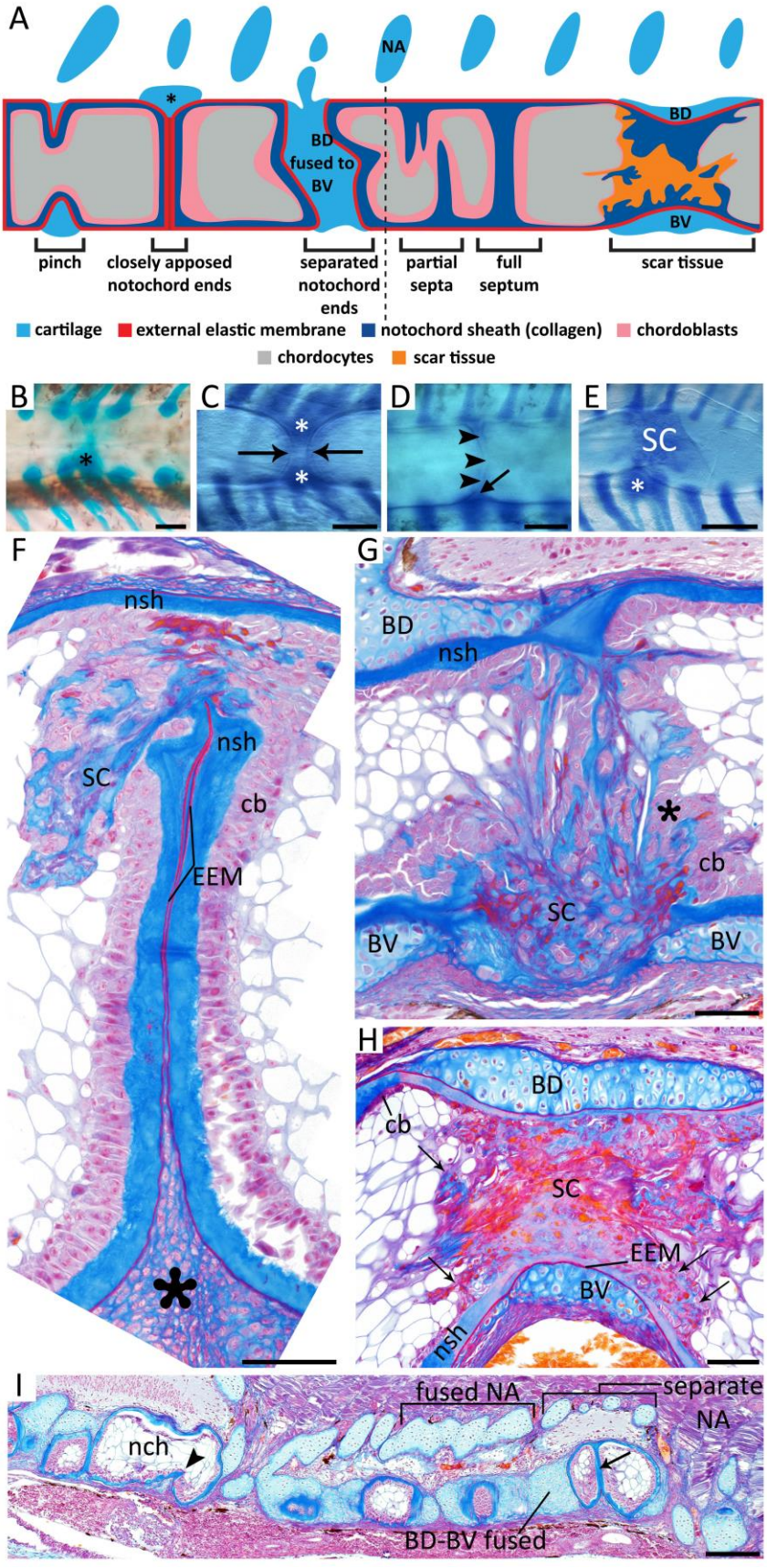

Figure 11: Notochord malformations (left)

All images use following orientation: anterior = left, posterior = right, dorsal = up and ventral = down. (A) Schematic of notochord malformation types based on Azan stained sections. Two types of notochord malformations exist. The first type (shown anterior of the dashed line) is characterised by folding of the external elastic membrane resulting in partial or complete pinching of the notochord. The depression in the external elastic membrane of a shallow pinch is filled with cartilage of the bases of neural and haemal arches. At the level of a complete pinching site, where the notochord ends remain closely apposed, cartilage of the fused bases of neural arches (indicated by the asterisk) fills a shallow depression in the external elastic membrane. When two notochord ends, each lined by external elastic membrane, are separated at a complete pinching site, the space in-between the notochord ends is filled with cartilage of the fused bases of neural (BD) and haemal (BV) arches. The second type of notochord malformation (shown posterior of the dashed line) is characterised by hyperplasia of chordoblasts resulting in partial or full septa and scar tissue. Scar tissue contained within the notochord replaces chordocytes. B-E. Alcian blue whole mount stained and cleared specimens. Scale bars = 250 μm. (B) Notochord pinched partially along the ventral side. The cartilaginous bases of the haemal arches positioned at the pinch site fuse and extend into the shallow depression of the external elastic membrane (asterisk). (C) Completely pinched notochord with two notochord ends separated (arrows). Each notochord end is lined by external elastic membrane. Cartilaginous bases of neural and haemal arches fuse and invade the space in-between the notochord ends (white asterisks). (D) Completely pinched notochord with the two notochord ends closely apposed to each other (arrowheads). A shallow depression at the pinch site on the ventral side is filled with cartilage of the base of the haemal arch (black arrow). (E) Scar tissue (SC) inside the notochord stains with Alcian blue. Ventrally the notochord is pinched at the level of the scar tissue. The cartilaginous bases of adjacent haemal arches are fused (white asterisk). (F-I) Parasagittal sections of notochord malformations. (F) Partial deep pinch along the ventral side of the notochord combined with hyperplastic chordoblasts starting to form scar tissue (SC). Cartilage (asterisk) fills the space of the inward fold of the external elastic membrane (EEM). Highly polarised (cylindrical) chordoblasts (cb) line the notochord sheath (nsh). Scale bar = 100 μm. (G) Hyperplastic chordoblasts (cb) produce collagenous notochord sheath material forming into scar tissue (SC). The notochord sheath (nsh) and external elastic membrane are interrupted by extruding scar tissue. Abbreviations: BD, cartilaginous base of neural arch; BV, cartilaginous base of haemal arch. Scale bar = 100 μm. (H) Advanced formation of scar tissue (SC) compared to (G). The scar tissue has replaced the chordocytes. The scar tissue is contained within the notochord sheath (nsh) and external elastic membrane (EEM). Abbreviations: BD, cartilaginous base of neural arch; BV, cartilaginous base of haemal arch. Scale bar = 100 μm. (I) Pinching malformations and hyperplasia of chordoblasts occurring in a single notochord. The arrowhead indicates a sharp pinch along the ventral side of the notochord. Neural arches (NA) fuse as a likely consequence of notochord (nch) malformations. The cartilaginous bases of neural arches (BD) fuse to the cartilaginous bases of haemal arches (BV) where the notochord is heavily pinched to the right side. The cartilage of fused bases fills the space created by the inward fold of the external elastic membrane. The arrow indicates a full septum. The neural arches (NA) dorsal of the septum are unaffected. Scale bar = 250 μm.

The second type of malformation of the notochord is dislocation of chordoblasts forming partial or complete septa and scar tissue (Fig. 11A, E-I). In contrast to pinching, the external elastic membrane of the notochord does not fold inwards when septa are present (Fig. 11A, I). These septa are lined by polarised (cylindrical) chordoblasts suggesting that these cells are actively producing notochord sheath material (Fig. 11A, I). The associated elements are not affected at the level of the notochord septa (Fig. 11A, I). The notochord is narrowed, shallowly pinched (Fig. 11A, E, H) or deeply pinched (Fig. 11F) at the level of the scar tissue and slightly bent or severely deflected. The scar tissue is contained within the notochord thereby replacing the chordocytes (Fig. 11A, H), or bulges out from the notochord, breaking through the external elastic membrane (Fig. 11G). Small chordocytes and highly polarised (indicated by asterisks in Fig. 11G) or flattened (indicated by arrows in Fig. 11H) chordoblasts line the scar tissue. While neural and haemal arches remain separate at the level of scar tissue, their cartilaginous bases often fuse (Fig. 11E). Septa and pinching malformations can occur at any point of the notochord contour. When pinching and dislocation of chordoblasts occur together (Fig. 4I), the notochord is folded like a concertina.

3.4.2.2. CHORDACENTRA

Fused chordacentra in Alcian blue stained specimens were identified if there was also the fusion of the cartilaginous bases of the neural or haemal arches. In Alizarin red S stained specimens, three patterns of fusions are observed when chordacentra start mineralising. First, the mineralisation of two or more adjacent chordacentra is continuous on the ventral side (Fig. 12A, B). Second, the chordacentra are fused on the dorsal side while remaining separate on their ventral side (Fig. 12A, C). This is often observed in preural vertebrae and co-occurs with separate haemal arches but fused haemal spine. Third, adjacent chordacentra are fused but no longer separately recognisable. The ventral side of the mineralisation has the width of two chordacentra (Fig. 12A). Each of these three fusion patterns can co-occur with neural and haemal arches that remain separated or that are fused at the level of their cartilaginous bases. In parasagittal sections chordacentrum fusions can be recognised before mineralisation of chordacentra. The notochord sheath is thicker in the future intervertebral regions and thinner where mineralisation of the notochord sheath (chordacentrum) will occur (Fig. 12D). In a normal notochord the intervertebral regions lie opposite to each other on the dorsal and ventral side (Fig. 12A). In case of fusion of chordacentra, this pattern is disturbed (Fig. 12A, E).

3.4.2.3. ASSOCIATED ELEMENTS

The most commonly observed malformation of the associated elements is misalignment along the notochord (Fig. 12F) and fusions of adjacent elements (Fig. 12G-I). Misaligned elements can be distinguished when the cartilaginous bases of neural or haemal arches insert in an alternating pattern left and right on the notochord (Fig. 12F). The distal ends of these neural and haemal arches are also

misaligned (Fig. 12F). Fusion of associated elements can occur in all positions along their proximal-distal axis (Fig. 12G-I). Associated elements can develop as small stunted cartilaginous elements (already mineralised in Fig. 12I) or are bent anteriorly or more posteriorly, but this occurs to a lesser degree compared to misalignments and fusions.

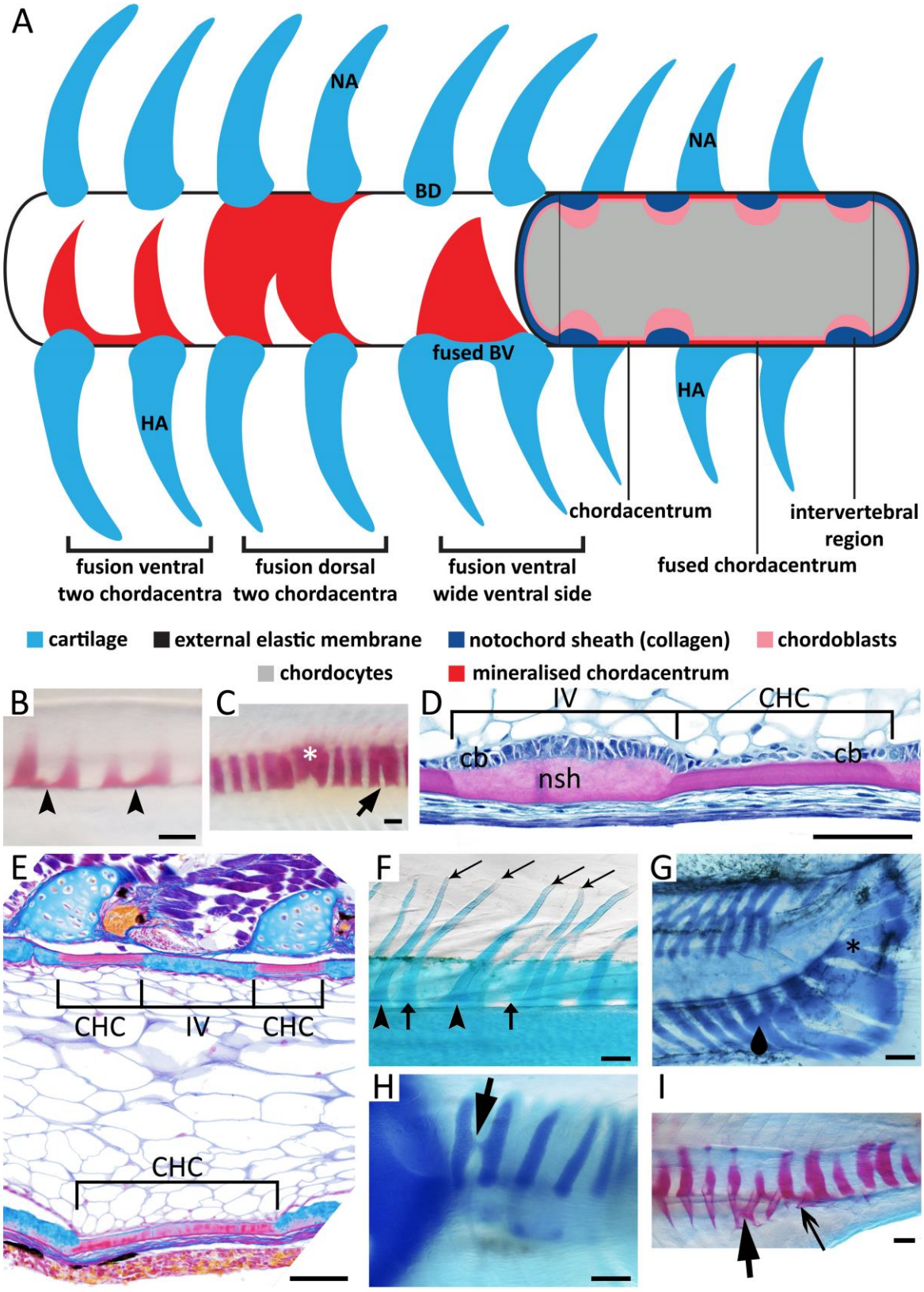

Figure 12: Chordacentra fusions and associated elements (= arches) malformations

All panel images use following orientation: anterior = left, posterior = right, dorsal = up and ventral = down. (A) Schematic of different chordacentra fusion patterns. From left to right, (i) chordacentra fused on the ventral side and (ii) dorsal side with the fusing chordacentra remaining visible, and (iii) fusion of chordacentra on the ventral

side but no longer separately recognisable. The latter is characterised by the wide ventral side of the chordacentrum. Fusion of the cartilaginous haemal arch bases (BV) can occur. The second part of the schematic shows how fused chordacentra are recognised in parasagittal sections. The notochord sheath is thicker in intervertebral regions and thinner in chordacentra. On the dorsal side, the normal situation is presented, where chordacentra alternate with intervertebral regions. On the ventral side, the alternating pattern is disturbed by fused chordacentra. Abbreviations: BD, cartilaginous base of neural arch; HA, haemal arch; NA, neural arch. (B-C) Alizarin red S stained specimens showing fused chordacentra. Scale bars = 250 μm. (B) Fusion of chordacentra on the ventral side with the fusing chordacentra remaining visible. The mineralisation of the notochord sheath between the two chordacentra on the ventral side is continuous (arrowheads). (C) Fusion of chordacentra on the dorsal side. The fusing chordacentra remain visible on the ventral side due to a gap in the mineralisation (block arrow). The white asterisk indicates a chordacentra fusion due to a notochord malformation (pinch along the ventral side) and is therefore recognised as a notochord malformation. (D-E) Parasagittal sections of the notochord showing normal and fused chordacentra. Scale bars = 100 μm. (D) A normal intervertebral region (IV) and mineralised chordacentrum (CHC). The chordoblasts (cb) in the intervertebral region are polarised (cylindrical), while the chordoblasts along the chordacentrum are flattened. The notochord sheath (nsh) is thicker in the intervertebral region and thinner in the chordacentrum. (E) On the dorsal side, a normal alternation of intervertebral region (IV) with mineralised chordacentra (CHC) can be recognised. On the ventral side, this pattern is disturbed by a fused chordacentrum spanning two adjacent chordacentra and the in-between intervertebral region. (F-I) Associated elements malformations: (F-H) Alcian blue stained. (I) Alizarin red S stained. Scale bars = 250 μm. (F) Misalignment of neural arches. The cartilaginous bases of neural arches have alternating positions on the left (arrowhead) and right side (block arrow) of the notochord. The distal ends of the neural arches are also misaligned (arrows). (G) Fusion of associated elements in the caudal fin skeleton. The rounded arrowhead indicates the distal fusion of two adjacent preural haemal arches. The asterisk indicates the proximal fusion of hypurals 2 and 3. (H) Medial fusion of two adjacent post-cranial neural arches (block arrow). (I) The block arrow indicates the medial fusion of two adjacent caudal haemal arches. The line arrow indicates the stunted development of a haemal arch.

3.5. DISCUSSION

This study aimed to link external body axis malformations in post-hatch Chinook salmon with internal malformations of axial skeletal structures, i.e. the notochord, the chordacentra and the associated elements (predominantly arches). In addition the effect of temperature on malformations is investigated. In all temperature groups, externally normal specimens with internal malformations showed mainly chordacentra fusions. Interestingly, specimens in all temperature groups with external malformations did not always display internal skeletal malformations. Yet, most animals' external malformations related to internal malformations.

A coherent diagnosing system for spinal malformations in salmonids is a prerequisite for linking a particular spinal malformation to a particular cause. Knowledge about causes will eventually enable

us to control and to avoid spinal malformations in farmed salmonids (Witten et al., 2009). The current study distinguishes between eight different external body axis malformations, including two severity classes in curly, bent tail and spinal malformations. The two severity classes can be distinguished based on the angle of the body axis curvature. The diagnosed external phenotypes (including normal) in the present study are represented in Figure 13. In addition this study looked more closely at the internal malformations, and found two types of notochord malformations (pinching and hyperplasia of chordoblasts), three patterns of chordacentra fusions and malformations of associated elements such as misalignment, fusion or stunted development.

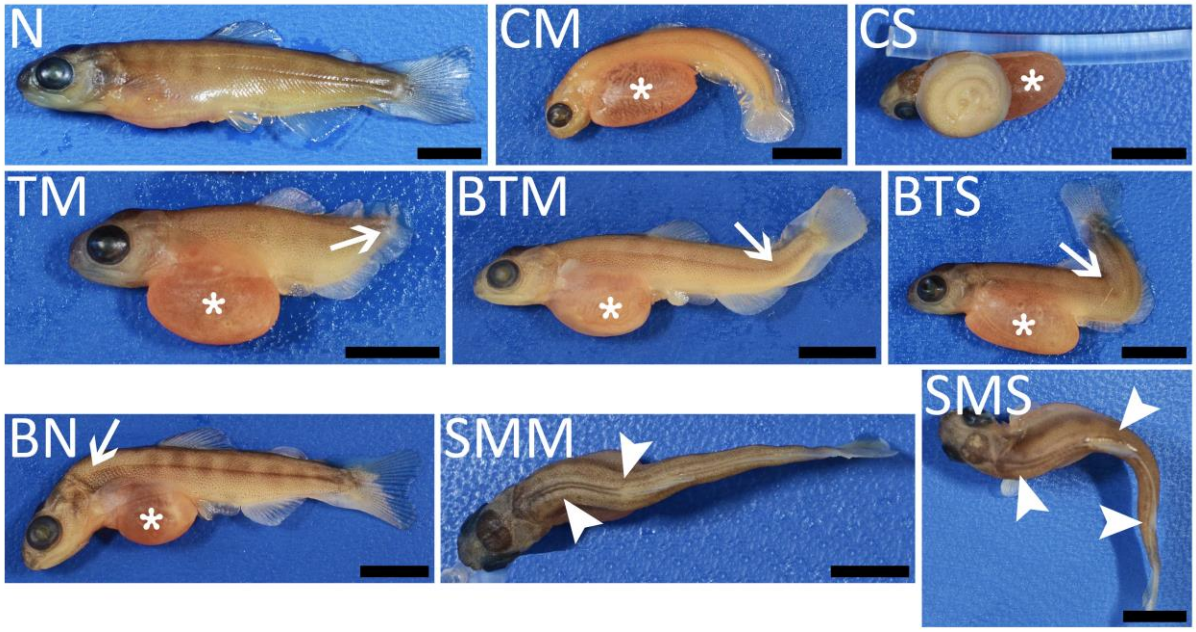

Figure 13: Diagnosing external phenotypes

All specimens represented are placed on their right side with anterior to the left and posterior to the right. A dorsal view is represented of the SMM and SMS specimens. Specimens range from post-hatch to first feed stages. Each specimen in this figure shows only one external malformation. However, multiple external malformations can occur in a single specimen. N, first feed externally normal specimen; TM, tail malformation, arrow indicates malformed tail and tail fin; BN, bent neck, arrow indicates a postcranial kyphosis; CM, curly medium; CS, curly severe, the kyphosis is so severe the body forms a spiral shape; BTM, bent tail medium, arrow indicates a bend (lordosis) in the caudal region of <40°; BTS, bent tail severe, arrow indicates a bend (lordosis) in the caudal region of >40°; SMM, spinal malformation medium, arrowheads indicate scoliosis of <40°; SMS, spinal malformation severe, arrowheads indicate scoliosis of >40° except the minor scoliosis occurring in the caudal region. Yolk sac indicated by asterisks. Scale bars: 5 mm.

Interestingly, the main internal malformation in externally normal specimens is chordacentra fusion. Fusion of caudal vertebral centra is a normal part of the urostyle formation in teleosts such as salmonids (Arratia & Schultze, 1992; Schultze & Arratia, 1989; Witten et al., 2009) and cyprinids (Bensimon-Brito et al., 2010; Bensimon-Brito et al., 2012a). Fusion of caudal centra in salmonids occurs in late juvenile stages where vertebral centrum structures external to the notochord sheath fuse

(Arratia & Schultze, 1992; Schultze & Arratia, 1988). Fusion in zebrafish (*Danio rerio*, Hamilton) occurs also within the notochord sheath by continuous mineralisation between adjacent chordacentra (Bensimon-Brito et al., 2012b). In gilthead sea bream (*Sparus aurata*, Linnaeus) pathological fusion of vertebral centra can occur in early life stages at the level of chordacentra, as is the case during zebrafish urostyle formation (Loizides et al., 2013). Fusions of chordacentra in the present study are assigned to two aetiologies, (i) fusion by continuous mineralisation between two or more centra, and (ii) fusion as a result of a notochord malformation such as pinching. How the pattern of chordacentra is established is currently unknown, however the notochord is thought to play an intrinsic part in the pattern formation (Fleming et al., 2004; Fleming et al., 2015). For example, expression of alkaline phosphatase (ALP) was detected in chordoblasts lining the mineralisation zones of Atlantic salmon (*Salmo salar*, Linnaeus) and zebrafish notochord (Bensimon-Brito et al., 2012b; Grotmol et al., 2005). Furthermore, the morphology of chordoblasts in Atlantic salmon is different in mineralising and non-mineralising zones of the notochord sheath (Grotmol et al., 2003; Grotmol et al., 2005). The most recent evidence for a notochord driven mineralisation of the notochord sheath comes from studies on medaka (*Oryzias latipes*, Temminck & Schlegel) *osterix/sp7* mutants. These animals have defective osteoblasts and bone formation is disrupted, yet the segmented mineralisation of the notochord sheath is unaffected (Yu et al., 2017). Possibly the early fusion of chordacentra is caused by a change in the behaviour of the chordoblasts that produce the notochord sheath.

Notochord malformations in this study occurred mostly in specimens with severe external malformations, and therefore, severe notochord malformation may cause external malformation. However, they also occurred in animals with less severe external malformations. Pinching of the notochord was observed in green sturgeon (*Acipencer medirostris*, Ayres) at yolk-sac depletion (Linares-Casenave et al., 2013). Shallow pinching, irregularities in the notochord sheath and distortions of the tip of the notochord were also observed in gilthead sea bream larvae (Koumoundouros et al., 1997; Santamaría et al., 1994). Andrades et al. (1996) showed a complete pinch of the notochord with each notochord end lined by notochord sheath. Connective tissue filled the space between the notochord ends. Interestingly, the observation in gilthead sea bream was made in an early stage where cartilaginous associated elements are not yet present (Andrades et al., 1996; Faustino & Power, 1998). The connective tissue between the notochord ends in the gilthead sea bream may be replaced by cartilage later in development. In cases of severe notochord malformation with dislocated chordoblasts and scar tissue the normal internal anatomy of the notochord is changed. Alternatively, severe notochord malformations occurring in a single site may not cause severe external malformations. Loizides et al. (2013) suggested that ectopic production of notochord sheath material, i.e. scar tissue, prevents mineralisation of the notochord sheath. In contrast, mineralisation of the notochord sheath and indications of mineralising ectopic sheath material (Fig. 11H) within the scar tissue was observed in the present study. Scar tissue could develop into a fusion if mineralisation

continues throughout post-hatch development. A fusion of two or three vertebral bodies can stabilise and remodel into a normal functioning vertebral centrum (Witten et al., 2006).

In the present study, notochord malformations, fusion of chordacentra and malformation of associated elements occurred separately in some specimens, confirming that the chordacentra and the associated elements represent two developmental modules (de Azevedo et al., 2012; Detwiler & Holtzer, 1956; Hall, 1977; Holtzer, 1951; Strudel, 1953a; Strudel, 1953b; To et al., 2015; Witten et al., 2007). Pathological malformations of associated elements (= arches) along the vertebral column, such as duplications, fusion, bending, dislocation and shortening, have been mainly observed in marine teleosts (Boglione et al., 2013b; Gavaia et al., 2002; Sfakianakis et al., 2004). In post-hatch Chinook salmon similar malformations of the associated elements are observed, with misalignment of the arches as the main malformation phenotype. Malformations of the associated elements are likely related to abnormal somite development. For example, left-right misalignment of somites occurs in retinoic acid deficient mouse, chick and zebrafish embryos (Kawakami et al., 2005; Vermot & Pourquie, 2005) and *Notch1* mutant zebrafish (Conlon et al., 1995). Misaligned somites may cause the associated elements (neural and haemal arches and parapophyses and ribs) to misalign along the notochord. Neural and haemal arches are malformed and fused in *fused somite* mutant zebrafish (Fleming et al., 2004; van Eeden et al., 1996). Also, fusion of associated elements has been observed in the caudal fin (preural and ural regions) of salmonids and zebrafish (Arratia & Schultze, 1992; Bensimon-Brito et al., 2012a) and confirms the results of this study. These fusions are likely part of the natural variation present in the teleost caudal fin skeleton (Arratia et al., 2001; Bensimon-Brito et al., 2010; Witten & Hall, 2015).

In addition to malformation of associated elements, abnormal somite development causes malformations of muscles (Uji et al., 2015; van Eeden et al., 1996). Abnormal muscle development could explain why specimens with external body axis malformations sometimes displayed normal skeletal structures in the present study (e.g. curly phenotype). Incubation temperatures are known to have immediate and long lasting effects on muscle development in teleosts (Finn, 2007; Johnston, 2006; Johnston et al., 2003). In Atlantic salmon, a higher temperature caused a higher rate of hypertrophy (increase in volume) compared to hyperplasia (increase in number) of muscle fibres (Johnston & McLay, 1997; Stickland et al., 1988). Temperature driven muscle plasticity was suggested as a possible underlying cause for haemal lordosis in European sea bass (*Dicentrarchus labrax*, Linnaeus) (Boglione et al., 2013b; Koumoundouros et al., 2009). Interestingly, when first feed stage Atlantic salmon incubated in 5°C water were moved to 10°C water muscle recovery and catch-up growth were observed (Nathanailides et al., 1995). This suggests that specimens with a normal axial skeleton and minor body axis malformations can recover during juvenile growth. Recovery from minor malformation phenotypes has also been suggested to occur in marine teleosts (Boglione et al., 2013b; Rosenthal & Alderdice, 1976).

In the present study the lowest frequency of 16% vertebral malformations occurred in the 8°C group. This can be considered as a high frequency. Certain year classes of adult Atlantic salmon also showed high frequencies (15.6% to 25.2%) of vertebral deformities (Gjerde et al., 2005). Although the heritability of vertebral anomalies in Atlantic salmon is low, parental animals with high frequencies of vertebral deformities produce off-spring with similar frequencies of vertebral deformities (Gjerde et al., 2005; Sullivan et al., 2007b). In addition, it seems that the heritability of vertebral deformities increases when there is a failure in husbandry practice, e.g. an unusually low or high incubation temperature. Moreover, this induces unusually high frequencies of skeletal deformities (Kause et al., 2007). The family crosses producing high frequencies of skeletal malformations together with the temperatures chosen in the present study may explain the high frequencies of vertebral malformations in all temperature groups. Different frequencies of both external and internal malformations were observed in the three temperature groups indicating that temperature affects the malformation rate in the present study.

Constantly elevated incubation temperatures during early life stages have been used to induce vertebral column anomalies in salmonids (Boglione et al., 2013b). A U-shaped response in the frequency of morphological malformations was found in brown trout (*Salmo trutta*, Linnaeus) reared at five different incubation temperatures, with the lowest frequency occurring at 8°C (Réalis-Doyelle et al., 2016). The frequencies of internal malformations in externally normal and malformed specimens in the present study also showed a U-shaped response, with lowest frequencies in 8°C. Chinook salmon were successfully transferred from Californian (6-16°C) waters to New Zealand Rivers (Kinnison et al., 1998; McDowall, 1994; Unwin & Glova, 1996) and are therefore acclimated to warmer temperatures. Perhaps it is not surprising that specimens incubated at the lowest temperature, 4°C, showed the highest incidence of external and internal malformations. The highest frequency of malformations (20%) was also observed in Arctic charr (*Salvelinus alpinus*, Linnaeus) reared at a constant 2.3°C (Jeuthe et al., 2013). Furthermore, Embody (1934) observed a high level of mortality in brook trout (*Salvelinus fontinalis*, Mitchill), lake trout, brown trout and rainbow trout (*Oncorhynchus mykiss*, Walbaum) incubated below 6°C. In contrast, very low mortalities were observed for brown trout and Arctic charr incubated at 2°C. However, high mortality occurred at incubation temperatures below 6°C for grayling (*Thymallys thymallus*, Linnaeus) and Danube salmon (*Hucho hucho*, Linnaeus) (Jungwirth & Winkler, 1984). Trends in mortality are similar for five species of Pacific salmon. While coho salmon and sockeye salmon survive incubation at 4°C well (Beacham & Murray, 1990; Murray & McPhail, 1988), Chinook salmon and chum salmon (*Oncorhyunchus keta*, Walbaum) show high mortality when incubated at 4°C (Beacham & Murray, 1985a; Beacham & Murray, 1985b; Beacham & Murray, 1987b; Murray & McPhail, 1988). Pink salmon does not survive incubation at 4°C (Beacham & Murray, 1990; Murray & Beacham, 1986; Murray & McPhail, 1988). In addition, the studies in Pacific salmon observed the following trends

during incubation in cold temperatures: (i) the highest mortality occurred before the completion of epiboly, (ii) higher mortality occurred before hatching and (iii) the mortality of alevins is lower in cold incubation temperatures compared to higher incubation temperatures. These trends in mortality, specifically lower mortality of alevins in cold temperatures, could explain why higher frequencies of malformations were observed in the 4°C group in the present study. Malformed embryos are more likely to die in the egg stages at 4°C, however, once hatched, malformed alevins are more likely to survive and therefore are more frequent in the 4°C group.

The mortality and malformations observed in previous studies and the present study suggests that constant cold incubation temperatures elicit more stress affecting normal development compared to warmer temperatures such as 12°C. Heat shock proteins (HSP) assist in stabilising and repairing altered proteins due to thermal stress (Basu et al., 2002; Iwama et al., 1998; Kiang & Tsokos, 1998). In Atlantic salmon, the presence of *HSP70* mRNA was shown to increase after both warm and cold thermal stress (Takle et al., 2005). A perturbation of the HSP mechanism may underlie the increased frequency of malformations in 4°C incubated Chinook salmon. The hydrostatic pressure of the vacuolated chordocytes against the notochord sheath provides the sturdiness of the notochord (Grotmol et al., 2006; Kryvi et al., 2017). The vacuolisation and myogenesis of muscle fibres start at a similar time point in trout (Killeen et al., 1999). However, while the myogenesis of muscle fibres was not delayed, the start of vacuolisation was found to be delayed in trout embryos incubated at 2°C (Killeen et al., 1999). Therefore, the muscle fibres which developed before the vacuolisation of the notochord may elicit damage to the notochord sheath resulting in notochord malformations. This could explain the higher frequency of malformations in the 4°C group in the present study.

The question as to when the pathological process(es) resulting in fusion start, remains to be answered. Studies using temperature induced skeletal deformities in Atlantic salmon have also examined the internal skeletal phenotype but did so in juvenile stages. Two studies used temperature shock to induce malformations in early stages and examined the deformities in juvenile parr stage animals (Takle et al., 2005; Wargelius et al., 2005). Ønsrud et al. (2004a) used two constant temperature regimes in combination with different vitamin A levels during the incubation period to investiagate the effect of temperature and vitamin A on vertebral column deformities in juvenile parr and pre- and post-smoltification stages. Also, two constant temperature regimens during incubation were used to study the expression levels of osteogenic and chondrogenic markers during fusion in juvenile parr and pre-smolt stages (Ytteborg et al., 2010a; Ytteborg et al., 2010b; Ytteborg et al., 2010c). The phenotypes of the vertebral deformities observed in these experiments are early, intermediate or final stages of the deformities (Ytteborg et al., 2010b; Ytteborg et al., 2010c). In addition, fused vertebral centra have been reported to develop normally before the deformation process starts (Fjelldal et al., 2007a; Nordvik et al., 2005; Witten et al., 2009). In the present study, fusion of chordacentra was observed to be present in early life stages. The literature reporting on temperature experiments as well

as the current study suggests that vertebral anomalies can start at completely different life stages. Importantly, this investigation demonstrates how distinct internal malformations can be characterised in the embryonic/post-hatch life stages of Chinook salmon. Investigation of the ontogeny and progression of compression and fusion in Atlantic salmon has previously led to the suggestion that vertebral anomalies may arise as early as embryonic stages (Kvellestad et al., 2000; Witten et al., 2006; Ytteborg et al., 2010a).

3.6. CONCLUSION

Although malformation frequencies were high due to specific family crosses, a temperature effect was observed. Results obtained in the current study suggest that incubation and early rearing at a constant 8°C is preferable to rearing at 12°C and indicates that rearing at 4°C results in unacceptable frequencies of body axis malformation (see Table 6). Interestingly, externally normal specimens showed fusions of vertebral anlagen which can grow up to harvest stages. The study shows that external malformations of the body axis are usually linked to internal malformations of the vertebral column. Externally curled specimens typically have malformed arches. Malformations affecting a single region relate to malformed notochords and fusions of vertebral centrum anlagen. Spinal malformations (medium and severe) relate to malformed notochords and arches. Apparent differences in the type and rate of malformations between the 4, 8 and 12°C groups provides the framework for future investigations. Specifically, it will be necessary to develop conditions in which externally normal specimens have at most mild internal malformations allowing the fish to grow beyond and stabilise early life malformations.

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CHAPTER 4: VERTEBRAL COLUMN REGIONALISATION IN CHINOOK SALMON (*ONCORHYNCHUS TSHAWYTSCHA*)

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4.1. ABSTRACT

Teleost vertebral centra are often similar in size and shape but vertebral associated elements, i.e., neural arches, haemal arches and ribs, show regional differences. Here we examine how presence, absence and specific anatomical and histological characters of vertebral centra associated elements can be used to define vertebral column regions in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). To investigate if the presence of regions within the vertebral column is independent of temperature, animals raised at 8 and 12°C were studied at 1400 and 1530 degreedays, in the freshwater phase of the life cycle. Anatomy and composition of the skeletal tissues of the vertebral column were analysed using alizarin red S whole mount staining and histological sections. Six regions, termed I to VI, are recognised in the vertebral column of specimens of both temperature groups. Postcranial vertebrae (region I) carry neural arches and parapophyses but lack ribs. Abdominal vertebrae (region II) carry neural arches and ribs that articulate with parapophyses. Elastic- and fibrohyaline cartilage and Sharpey's fibres connect the bone of the parapophyses to the bone of the ribs. In the transitional region (III) vertebrae carry neural arches and parapophyses change stepwise into haemal arches. Ribs decrease in size, anterior to posterior. Vestigial ribs remain attached to the haemal arches with Sharpey's fibres. Caudal vertebrae (region IV) carry neural and haemal arches and spines. Basidorsals and basiventrals are small and surrounded by cancellous bone. Preural vertebrae (region V) carry neural and haemal arches with modified neural and haemal spines to support the caudal fin. Ural vertebrae (region VI) carry hypurals and epurals that represent modified haemal and neural arches and spines respectively. The postcranial and transitional vertebrae and their respective characters are usually recognised but should be considered as regions within the vertebral column of teleosts because of their distinctive morphological characters. While the number of vertebrae within each region can vary, each of the six regions are recognised in specimens of both temperature groups. This refined identification of regionalisation in the vertebral column of Chinook salmon can help to address evolutionary developmental and functional questions and to support applied research into this farmed species.

4.1.1. KEYWORDS

Chinook salmon, Atlantic salmon, zebrafish, postcranial region, transitional region, vertebral column

4.2. INTRODUCTION

The teleost vertebral column is a complex structure comprising building blocks (vertebral centra) of relatively similar size and shape in most species. There is agreement about the presence of three major anterior-posterior regions within the vertebral column. Region one comprises the vertebral bodies from the occipital region of the cranium to the last rib-bearing vertebral body. Region two consists of

haemal arch-bearing vertebral bodies. Region three is characterised by the vertebral bodies of the caudal fin complex. Different anatomical terms have been used for these regions (Arratia et al., 2001; Bird & Mabee, 2003; Deschamps et al., 2008; Nybelin, 1963; Sallan, 2012). Terms used in the literature for region one are precaudal, abdominal, truncal or prehaemal. In this region some authors distinguish also postcranial (Deschamps et al., 2008) or cervical vertebral bodies (Fjelldal et al., 2013; Ford, 1937; Holley, 2007; Morin-Kensicki et al., 2002). Terms used for region two are caudal or haemal. Terms used for vertebrae of region three are postcaudal or ural (Britz & Conway, 2009; Cubbage & Mabee, 1996; Deschamps et al., 2008; Koumoundouros, 2010; Nybelin, 1963; Sallan, 2012). Several authors have recognised a region with transitional vertebral bodies between region one and two (Balfour & Parker, 1882; Bird & Mabee, 2003; Holley, 2007; Jawad et al., 2013; Nowroozi et al., 2012) but little or no attention has been paid to this region so far.

Teleosts have different elements associated with the vertebral centra, such as parapophyses, ribs and neural and haemal arches, of very different sizes and shapes (Arratia et al., 2001). The types of associated elements, as well as their development and homology have been extensively described and discussed in Balfour and Parker (1882), Gadow and Abbott (1895) and Arratia et al. (1992; 2001). Some authors have used the associated elements attached to the vertebral centra as characteristics to characterise the regions of the vertebral column. For *Oncorhynchus* species for example, Gill and Fisk (1966) subdivided the vertebral column of sockeye, pink and chum salmon (*O. nerka, O. gorbuscha, O. keta* respectively) into an abdominal and caudal region. In this system the presence of the first haemal arch defines the first caudal vertebra. In comparison, Seymour (1959) suggests a regionalisation of the vertebral column of Chinook salmon (*O. tshawytscha*) in which the presence of the first haemal spine defines the beginning of the caudal region. Other characteristics that display anterior to posterior alterations are skeletal tissue components and how the vertebrae-associated elements are connected to centra. Together these characters provide insight into regionalised vertebral column anatomy.

Regionalisation of the vertebral column is important from a functional, evolutionary and applied perspective. Which regions are recognised largely depends on the research approach used. To compare the structural and functional components of the vertebral column within teleosts (e.g. swimming modes of different fish species), a clear distinction of regions of the vertebral column is necessary. Ramzu and Meunier (1999) used morphometric characters of vertebral centra along the axis of rainbow trout (*Oncorhynchus mykiss*) to distinguish several functional regions within the vertebral column related to mode of swimming. In another approach, regions are recognised and interpreted based on elongation of fish throughout evolution. For example, the change in ratio of abdominal to caudal vertebrae is interpreted on an evolutionary time scale (Ward & Brainerd, 2007) and subdivides the vertebral column only into major anterior and posterior regions. A study investigating the meaning of the presence of vestiges, rudiments and fusions in the evolution of the

caudal fin skeleton of zebrafish (Bensimon-Brito et al., 2012a) focuses only on the posteriormost part of the axial skeleton. Understanding of aetiologies that cause deformities of the vertebral column in farmed fish is another application for a detailed vertebral column subdivision. Compression of vertebral centra in Atlantic salmon (*Salmo salar*) occurs in defined, and functionally relevant regions such as the caudal and ural regions (Fjelldal et al., 2009a; Grini et al., 2011; Wargelius et al., 2005). The occurrence of deformed vertebrae in the caudal and ural regions affects the swimming behaviour of farmed salmon (Fjelldal et al., 2012). Severely deformed fish constitute a problem for animal welfare and for animal growth (Fjelldal et al., 2012). A refined identification of vertebral column regions integrating different sets of morphometric, anatomical and molecular characteristics could help to better understand the normal development and development of deformities within the vertebral column of farmed salmonids and could provide better descriptions of how deformities progress through freshwater and marine life stages (Fjelldal et al., 2012; Grini et al., 2011; Witten et al., 2006). Molecular studies have found that subdivision of most parts of the mammalian vertebral column correspond to boundaries of *Hox*-gene expression (Asher et al., 2011; Burke et al., 1995; Galis, 1999). However, *Hox*-genes appear to be only partly responsible for the regionalisation of the vertebral column in zebrafish, i.e. the *Hox*-genes anterior expression boundaries are concentrated in the anteriormost part of the vertebral column (Holley, 2007; Morin-Kensicki et al., 2002).

Here the vertebral column of juvenile Chinook salmon, *Oncorhynchus tshawytscha* is studied. The species was introduced to New Zealand between 1901-1907 for game fishing (McDowall, 1994). Chinook salmon established a wild population on the east coast of New Zealand's South Island by 1915 and became a species of interest for commercial salmon farming in 1970 (Unwin & Glova, 1996). River water temperatures in New Zealand range from 6°C to 16°C (Davis & Unwin, 1989; Kinnison et al., 1998; Unwin & Glova, 1996; Unwin et al., 2000) with preferred incubation temperatures of Chinook salmon ranging from a minimum 3°C to a maximum 12°C and optimum temperature range from 8°C to 10°C (Alderdice & Velsen, 1978; Brett, 1952). Temperatures near the optimum and high end of the incubation range are more likely used in farming conditions to induce fast growth (Fjelldal et al., 2012). This study asks how the presence or absence, and specific anatomical and histological characters of vertebral centrum associated elements, can be used to define different regions in the vertebral column of Chinook salmon. To this end, region-specific changes of vertebral centrum associated elements along the vertebral column are characterised. Furthermore, to assess whether the characters of the regions within the vertebral column are temperature dependent, we use specimens raised at two temperatures (8°C and 12°C). This information is used to establish (i) six different vertebral types characteristic for six different regions and (ii) four vertebral subtypes.

4.3. MATERIAL AND METHODS

4.3.1. FISH MAINTENANCE AND SAMPLING

Approximately 60 000 Chinook salmon eggs obtained from 10 females were randomly divided over 10 batches (*APPENDIX B*). The batches were artificially fertilized (20/05/2014, *APPENDIX B*) with milt obtained from 6 males (brood stock provided by New Zealand King Salmon Co Ltd, Nelson, New Zealand). Fertilised eggs were placed into two incubation stacks. Each incubation stack (7 incubation trays/stack, ± 4000 eggs/incubation tray) was initially supplied with water at a constant 10°C from fertilisation to 3 days post-fertilisation (= 30°d, degreedays). Subsequently the temperature was gradually adjusted over 24 hours to constant 8°C and 12°C rearing temperatures. Dead individuals were removed once a week during incubation period before hatching (175°d-475°d), every two days during the hatching period (475°d-590°d) and twice a week after hatching (590°d-900°d) to avoid fungal infections and reduce mortalities. At first feed stage 4000 fish from the 8°C (933°d) and 12°C (943°d) groups were transferred to four 420 L circular tanks (two tanks per temperature). The fish were fed manually to satiation every hour during the day. Fish were randomly sampled at 1400°d and 1530°d for each temperature and euthanized by anaesthetic overdose of Aqui-S (New Zealand, Ltd). At sampling 50 specimens were taken from each of the four tanks, fixed in 4% buffered paraformaldehyde (PFA) and stored in 60% ethanol.

4.3.2. WHOLE MOUNT STAINING

In total 120 fish were stained for mineralised bone with Alizarin red S (N = 30 at 1400°d and N = 30 at 1530°d for 8 and 12°C groups) following an adapted protocol of Taylor and Van Dyke (1985). Briefly, after weighing and measuring fork length, specimens were rehydrated, bleached (1% KOH/3% H_2O_2, 1-1.5 hrs.), rinsed in demineralised water and placed for 24 hours in a 70% saturated borax solution (*di*-sodium tetraborate). Fish were stained overnight in a 0.5 mg/ml Alizarin red S/1% KOH solution and rinsed in demineralised water. Specimens were cleared in graded glycerol/KOH series, dehydrated in graded glycerol series and brought to 100% glycerol storage solution. Skeletal structures were analysed using a stereomicroscope (Leica M80).

4.3.3. HISTOLOGY

Four juvenile specimens were used for histology. Two specimens of 1400°d (8°C and 12°C, resp.) and two specimens of 1530°d (8°C and 12°C, resp.) were used. The 1400°d specimens weighed 1.38 and 3.09 grams and had fork lengths (FL) of 46.36 and 62.26 mm, respectively. The 1530°d specimens weighed 1.61 and 5.88 grams and measured 57.28 and 75.32 mm, respectively. Tissue blocks of the postcranial/abdominal region, the transitional region and the entire caudal fin were processed for

paraffin embedding and sectioning. Before processing the tissues were placed overnight in acetone to remove fat.

All tissue samples were rinsed in demineralised water for 24 hours and subsequently decalcified for 48 hours in Morse solution (Morse, 1945) [22.5% formic acid (CH_2O_2, Merck KGaA, Darmstadt, Germany)] and 10% sodium citrate ($C_6H_5Na_3O_7$*2 H_2O, Merck KGaA, Darmstadt, Germany) and rinsed again, processed overnight and embedded in paraffin. The tissue blocks were either embedded to make parasagittal or transverse sections of 4 μm on a Leica RM 2235 microtome. Sections were stained with haematoxylin and eosin (adapted from; Gill et al., 1974), Masson's Trichrome and Verhoeff-Van Gieson methods to highlight collagen and elastin (Culling, 1974a; Culling, 1974b). Sections were analysed with bright field under a Zeiss Axiophot microscope. Images were taken with an Olympus DP72 top mount camera. Illustrator CS5 was used to draw schematics.

4.3.4. TERMINOLOGY

Structures of the vertebral centrum and the vertebral centrum associated elements are defined in Table 9, following Schultze and Arratia (2013) and other authors listed in the table. Cartilages of the vertebral centra and associated elements were defined following the classification of Witten et al. (2010). The types of bone were defined following Hall (Hall, 2015a; p.4) and Sharpey's fibres following Hall (Hall, 2015b; p.140) and Witten and Hall (2002; 2003; 2015). Finally, following Hall (2003) a vestige is defined as a remnant of an ancestral feature that persists in adults, and a rudiment as an incomplete transitory structure that is only found in embryos.

Table 9: Definition of terms used

Term	Definition	Reference
Basidorsal	The dorsally positioned cartilaginous part of the neural arch resting with the base on the external elastic membrane of the notochordal sheath.	Gadow and Abbott (1895)
Basiventral	The ventrally positioned cartilaginous part of the haemal arch resting with the base on the external elastic membrane of the notochordal sheath.	Gadow and Abbott (1895)
Cancellous bone	Consists of different combinations of curved plates with holes of various sizes, and a latticework of rods of a variety of lengths and cross-sectional diameters. Trabecula is an individual structural element of cancellous bone tissue, whether plate-like or rod-like.	Parfitt (1988), Whitehouse (1974)
Epicentral	Intermuscular bone ligamentously connected to the cancellous layer of the vertebral centum.	Bird and Mabee (2003), Patterson and Johnson (1995)
Epineural	Mineralised intermuscular tendon.	Danos and Ward (2012)
Epural	Modified neural arch of ural vertebra.	Schultze and Arratia (2013)
Hypural	Modified haemal arch or spine of ural vertebra supporting caudal fin rays.	Stiassny (2000)
Parapophysis	Bony structure providing attachment to muscles and articulation for ribs.	Owen (1848)
Post-zygapophysis	Dorsal (neural) or ventral (haemal) extension from the anterior end of a neural or haemal arch. It articulates with the pre-zygapophysis of the preceding centrum.	Bird and Mabee (2003)
Preural vertebrae	Carry slightly modified haemal and neural arches that support caudal fin rays. Haemal arches enclose the caudal artery and vein.	Arratia and Schultze (1992), Nybelin (1963), Sanger and McCune (2002)
Pre-zygapophysis	Dorsal (neural) or ventral (haemal) extensions from the anterior end of a neural or haemal arch. They articulate with the post-zygapophyses of the preceding centrum.	Bird and Mabee (2003)
Rib	Ventral rib, pleurapophysis or haemapophysis are synonyms. Rod-like bony structure with a head that articulates with the parapophysis.	Bird and Mabee (2003), Britz and Bartsch (2003), Gadow and Abbott (1895), Owen, (1848)

Stegural	A modified uroneural positioned dorsally of preural 2 and 1 and ural 1 and 2.	Arratia and Schultze (1992)
Ural vertebrae	Carry modified neural arches and spines, and modified haemal arches.	Schultze and Arratia (2013)
Uroneural	Modified neural spine of ural vertebrae supporting caudal fin rays.	Schultze and Arratia (2013)

4.4. RESULTS

The same six regions, termed region I to VI, can be distinguished in the vertebral column in juvenile Chinook salmon (N = 120) of the two temperature groups: the postcranial, abdominal, transitional, caudal, preural and ural region (Table 10, Fig. 14-17). Temperature does not affect the anatomical subdivision of the regions or the total number of vertebrae (V) ranging from 62 to 68 in both temperature groups. The skeletal structures that show variation are briefly summed up at the end of each section describing the regions. Temperature has possibly a small effect on the number of vertebrae in different regions. A number of morphological characters are common for vertebrae in all regions. Each of these six regions is characterised by vertebrae with a specific array of associated elements (Table 10). Numbers of vertebrae within each region display minor variations. Vertebral centra in all regions have two basidorsals on the dorsal side and two basiventrals on the ventral side (Fig. 14). Basidorsals and basiventrals are composed of cell-rich hyaline cartilage and connect directly to the bone of the neural and haemal arches except in regions V and VI. All vertebral centra carry neural arches and neural spines dorsally, except in region VI (Fig. 14). Epineurals are attached to the base of neural arches of region I and II. At the level of the base of the neural and haemal arch, pre- and post-zygapophyses are present with specific shapes in regions I-II and III-IV. Ribs are present in regions II and III and are preformed in cartilage, except in region III (subtype IIId). Cartilaginous basidorsals and basiventrals are perichondrally ossifying except in regions V and VI. Vertebral centra associated elements such as parapophyses, ribs, neural arches of type I-IV and haemal arches of type IV vertebrae are completely ossified. Remnants of the cartilaginous precursor are still present in some parapophyses and ribs of type I, II and III vertebrae. The neural spines of type I-IV and haemal spines of type IV vertebrae are intramembranous ossifications. The cartilaginous neural and haemal arches and modified spines of type V vertebrae are perichondrally ossifying. The stegural, epurals and uroneurals dorsally and hypurals ventrally in region VI are perichondrally ossifying.

The description below for each of the six regions is based on a typical specimen with 64 vertebral centra.

Table 10: Definition of the vertebral column regions and types of vertebrae (+ indicates additional structure, > indicates fused structures)

Vertebral Type	Definition
Type I	Postcranial vertebrae: vertebral centrum + rudimentary or complete parapophyses + epineurals
Type II	Abdominal vertebrae: vertebral centrum + parapophyses + ribs + epineurals
Type III	Transitional vertebrae: four different subtypes
Type IIIa	Vertebral centrum > open haemal arch (ventrally extended parapophyses) + ribs
Type IIIb	Vertebral centrum > closed haemal arch + ribs
Type IIIc	Vertebral centrum > closed haemal arch > haemal spine + ribs
Type IIId	Vertebral centrum > closed haemal arch > haemal spine + rudimentary ribs
Type IV	Caudal vertebrae: vertebral centrum > neural arch > neural spine > haemal arch > haemal spine
Type V	Preural vertebrae: vertebral centrum + neural arch > modified neural spine + haemal arch > modified haemal spine
Type VI	Ural vertebrae: vertebral centrum + stegural + epurals + uronurals + hypurals

4.4.1. POSTCRANIAL VERTEBRAE

Postcranial vertebrae (two in total, V1-2) carry epineurals dorsally and parapophyses on the ventral side, and lack ribs (Table 10, Fig. 14).

Epineurals are attached to the base of the neural arches (not shown) and plate-like pre- and post-zygapophyses are present anterior and posterior of the base of the neural arches (indicated by arrows, Fig. 14). The first postcranial vertebra has a ventrolateral vestigial parapophysis consisting of a small cartilaginous element (Fig. 16A) recessed into the cancellous bone of the vertebral centrum. The second postcranial vertebra has a basiventral and parapophysis (Fig. 16B-C) similar to the basiventral-parapophysis configuration of abdominal vertebrae (Fig. 14). Possible variations include fusion of the first vertebra to the skull (basioccipital) and an additional postcranial vertebral centrum (V3).

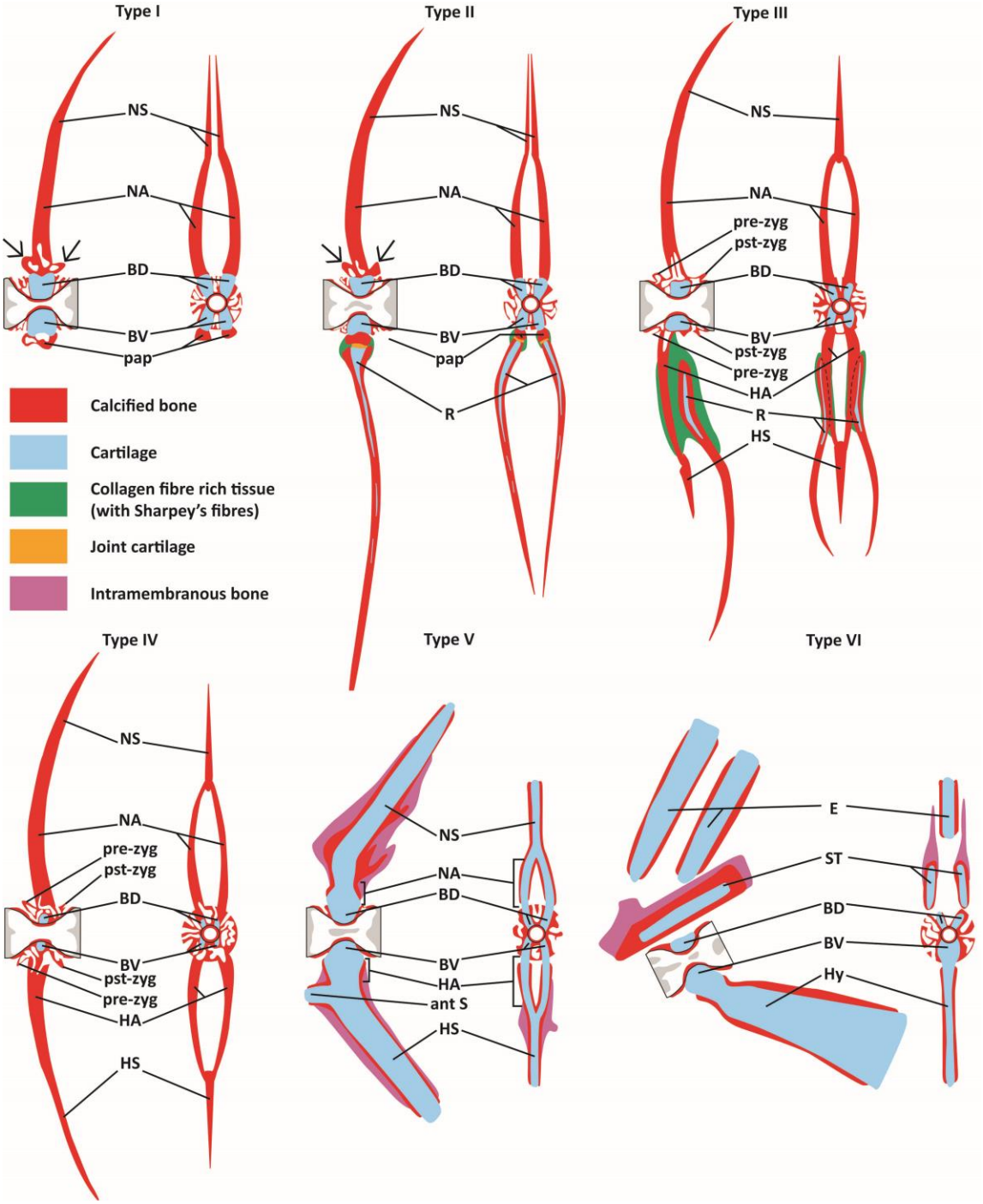


Figure 14: Schematic representation of vertebral centra and associated elements per vertebra type (Type I-VI)

Simplified scheme of vertebral centra types and vertebral centra associated elements in the six regions of the vertebral column of juvenile Chinook salmon (± 70 mm). Colours used for different tissue types are indicated in the key. Type I = postcranial, Type II = abdominal, Type III = transitional, Type IV = caudal, Type V = preural and Type VI = ural. Abbreviations: ant S, anterior spine, BD, basidorsal, BV, basiventral, E, epural, HA, haemal arch, HS, haemal spine, Hy, hypural, NA, neural arch, NS, neural spine, pap, parapophysis, pre-zyg, pre-zygapophysis, pst-zyg, post-zygapophysis, R, rib, ST, stegural. Arrows on type I and II point to pre- and post-zygapophyses.

4.4.2. ABDOMINAL VERTEBRAE

Abdominal vertebrae (V3-26) are rib-bearing and carry epineurals dorsally and parapophyses ventrally (Table 10, Fig. 14).

The rib of the first abdominal vertebra is half the length of the ribs of more posterior abdominal vertebral bodies (Fig. 15A). The abdominal vertebrae carry epineurals attached to the base of the neural arches (not shown). Like in postcranial vertebrae, plate-like pre- and post-zygapophyses are observed anterior and posterior of the bases of the neural arches (indicated by arrows, Fig. 14, 15B, 17A). Ventrally, abdominal vertebrae have basiventrals and parapophyses (Fig. 14, 15B, 17A). The cartilaginous part of the rib head is attached to the cartilages of the parapophyses by a cartilaginous joint containing two types of cartilage, elastic/cell-rich and fibro/cell-rich cartilage (Fig. 15B, 17A-E). The bone adjacent to the cartilage of the rib head is connected to the bone of the parapophysis by collagen fibre rich tissue and Sharpey's fibres which envelope the cartilaginous joint (Fig. 17A-C). The number of abdominal vertebrae can vary.

4.4.3. TRANSITIONAL VERTEBRAE

The transitional region (V 27-36, Table 10, Fig. 14, 15B) shows a stepwise anterior to posterior change from vertebrae with parapophyses and ribs anteriorly, over vertebrae with open haemal arches and ribs, to haemal arch and haemal spine-bearing vertebrae with vestigial ribs posteriorly. Anteriorly in the transitional region, the bases of the neural arches fuse progressively to the cancellous bone of the vertebral centra (Fig. 15B). Where fusion of the neural arch to the vertebral centrum is complete, the pre- and post-zygapophyses at the base of the neural arch have now a smaller, sharp cone-like shape (cf. Type IV vertebrae, Fig. 15B). The basidorsals gradually reduce in size anterior to posterior in transitional vertebrae. Each transitional vertebral subtype can vary in number. We have therefore distinguished four subtypes.

Subtype IIIa transitional vertebrae (V27-28) have parapophyses and ribs (Table 10, Fig. 15B). Dorsally the base of the neural arch is fusing to the cancellous bone of the vertebral centrum. Ventrally, the anterior part of the parapophysis gradually lengthens and extends ventrally (Table 10, Fig. 15B). In the anteriormost type IIIa vertebra the head of the rib is attached to the parapophysis with a cartilaginous joint (similar to abdominal vertebrae). In the posteriormost type IIIa centrum, the cartilaginous joint is lost and the rib is positioned posteroventrally of the parapophysis. The rib remains connected with collagen rich tissue and Sharpey's fibres (Fig. 15B).

Subtype IIIb vertebrae (V29-32) have haemal arches and ribs (Table 10, Fig. 15B). Dorsally the base of the neural arch is now completely fused to the cancellous bone of the vertebral centrum. The ventrally extending parapophysis represents an open haemal arch, which increases in length. The base of the open haemal arches fuses to the cancellous bone of the vertebral centrum. The left and right part of the haemal arches fuse distally via an intramembranous bony bridge. The head of each rib

remains connected posteriorly with collagen rich tissue and Sharpey's fibres (Fig. 17D-E) but gradually lies more ventrally relative to the haemal arches (Fig. 15B). The size of the ribs decreases antero-posteriorly (Fig. 15A).

Subtype IIIc vertebrae (V33-36) have haemal arches, haemal spines and ribs (Table 10, Fig. 14, 15B). A gradually longer haemal spine is fused to the distal bony bridge of the closed haemal arch. The ribs further decrease in size posteriorly.

Subtype IIId vertebrae (V35-36) have haemal arches, haemal spines and vestigial ribs (Table 10, Fig. 15B). Haemal pre- and post-zygapophyses and a full-sized haemal arch and spine are present. The vestigial ribs are often positioned more ventrally compared to the ribs in type IIIc vertebrae and remain connected to the haemal arch with collagen rich tissue and Sharpey's fibres (Fig. 15B). Vestigial ribs can have the shape of a miniature rib or be reduced to a small sliver of bone (Fig. 15B). Left-right asymmetry can occur in the shape, size and number of vestigial ribs.

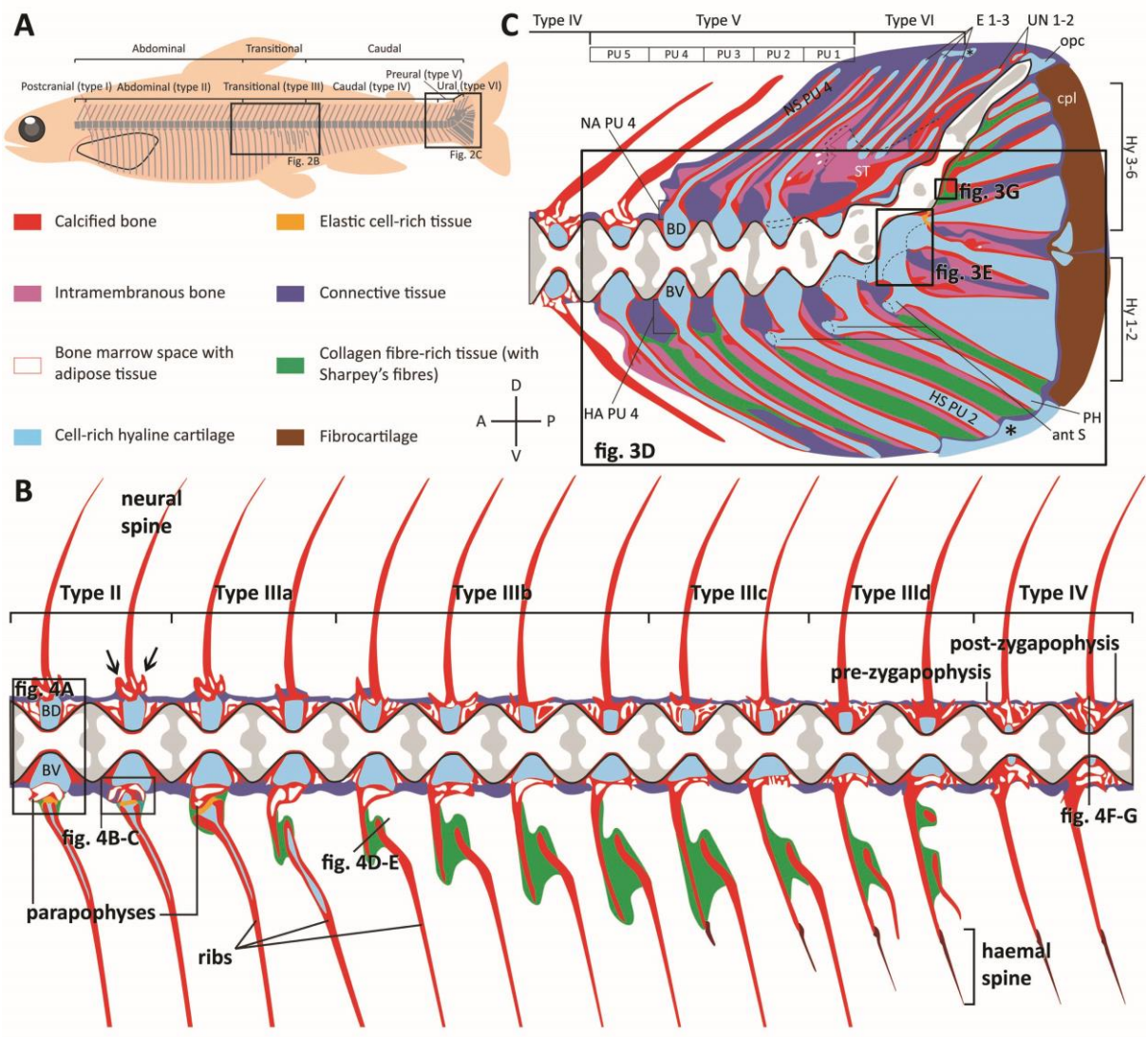
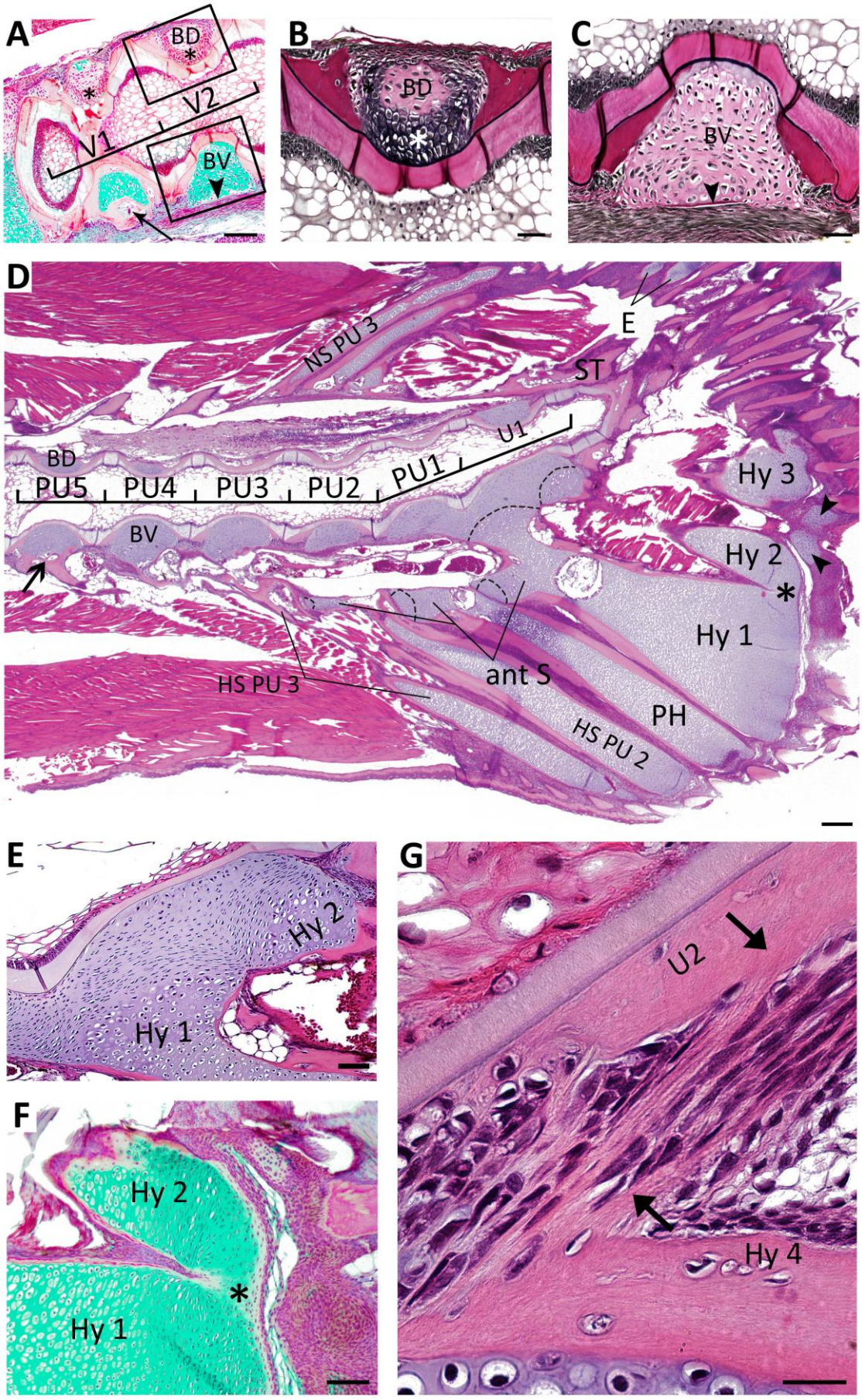


Figure 15: Chinook salmon vertebral column regionalisation, the transitional region and the caudal fin endoskeleton

(A) Simplified scheme of juvenile Chinook salmon vertebral column indicating six types of vertebrae (see Table 10). The transitional region (plus two abdominal vertebrae anteriorly and two caudal vertebrae posteriorly) and the caudal fin endoskeleton (preural and ural vertebral centra and associated elements) are highlighted in the black boxes and presented in more detail in (B) and (C), respectively. Images of areas enclosed in boxes labelled 16D, 16E and 16G are presented in Figure 16. Orientation of all drawings follows the anterior-posterior (A-P) and dorsal-ventral (D-V) axis indicated in the key. (B) Schematic representation of a typical transitional region based on mean number of vertebrae of 120 alizarin red stained specimens. Dorsally, no distinction is made between the neural arches and neural spines, which are fused to each other. The anteriormost vertebral centrum in the transitional region has a parapophysis that shows only small differences compared to parapophyses of the last abdominal (Type II) vertebrae. The ribs in these transitional vertebrae are attached in a similar way as on abdominal vertebrae (Type IIIa). Anteriorly, the parapophyses extend ventrally and form an open haemal arch (Type IIIa, Table 10). The ribs are loosely attached to the haemal arches and are in a more postero-ventral position. In Type IIIb haemal arches are closed and the bases fuse to the cancellous bone of the vertebral centra. The bases of the neural arches similarly fuse with the cancellous bone of the vertebral centrum. Type IIIc

vertebrae have an additional haemal spine which grows longer in each more posterior vertebral centrum. The rib size remains more or less the same. The ribs become vestigial in Type IIId (Table 10) vertebrae, and neural and haemal pre- and post-zygapophyses become apparent. Abbreviations: (BD) basidorsal, (BV) basiventral. (C) Schematic representation of the caudal fin endoskeleton, including one caudal vertebra (Type IV), preural vertebrae (PU, Type V), ural vertebrae (Type VI), and their respective associated elements. The cartilage of the basidorsals (BD) of the preural vertebrae is continuous with the cartilage of the neural arches (e.g. NA PU 4); likewise, the cartilage of the basiventrals (BV) is continuous with the haemal arches (e.g. HA PU 4), neural spines (e.g. NS PU 4) and haemal spines (e.g. HS PU 2). The stegural (ST) is positioned dorsolateral of the base of the neural spine of preural 2 (dashed line), dorsolateral of preural 1 and dorsal of urals 1 and 2, reaching posteriorly to a level opposite the anterior edge of hypural 6. The three epurals (E1-3) are positioned dorsal of the dermal bony outgrowth (dashed line) of the stegural. A cartilaginous plate is positioned dorsal of epurals 2 and 3 (*). A small opisthural cartilage (opc) is present at the tip of the notochord. Ventrally, the haemal spine of preural 2 (HS PU 2), the parhypural (PH) and hypural 1 carry proximally a cartilaginous anterior spine (ant S). Posterior of the hypurals (Hy) a fibrocartilaginous plate (cpl) is present. One or two rudimentary hyaline cartilages can be present in the fibrocartilaginous plate.


A
BD
V2
V1
BV
B
BD
C
BV
D
NS PU 3
E
ST
U1
BD
PU5
PU4
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BV
Hy 2
Hy 1
ant S
HS PU 3
PH
HS PU 2
E
Hy 2
Hy 1
F
Hy 2
Hy 1
G
U2
Hy 4

Figure 16: Postcranial vertebrae and the caudal fin endoskeleton: Key characteristics (left)

(A-G) = Parasagittal sections from regions indicated in Fig. 15C. All sections are oriented with dorsal to top (anterior to the left for parasagittal sections). (A-C) Postcranial vertebrae (Type I) have an elastic cartilaginous band in the basidorsals (BD, indicated by *), similar to abdominal vertebrae (Type II). Ventrally, the recessed parapophysis (arrow) and cartilaginous element are visible in vertebra 1 (V1). The parapophysis on vertebra 2 (V2) is indicated by a black arrowhead as a small sliver of bone in this plane of sectioning. The magnified images in (B) and (C) are indicated in the black boxes. (B) The basidorsal of V2 with the elastic cartilage band indicated by a white *. (C) The basiventral of V2 with the parapophysis indicated by the black arrowhead (A) = Masson's Trichrome, (B-C) = Verhoeff-Van Gieson. Scale bars = 100 μm. (D-G) The caudal fin endoskeleton: (D) Section through the caudal fin endoskeleton left of the midline of the specimen (see Fig. 15C). Not all structures are visible in this image due to the plane of sectioning. Dorsally, the basidorsals (BD) of preural (PU) 5-4 and ural 1 (U1) are visible. The neural spines of preurals 3 (NS PU 3) and 2, the stegural (ST) and the proximal part of the epurals (E) are visible. Ventrally, the basiventrals (BV) of preural 5-1 and ural 1 are visible. The bony bases of the haemal arches of preurals 5 (arrow) and 4 are fused to the basiventrals. A part of the arch and the distal part of the haemal spine of preural 3 (HS PU 3), the haemal spine of preural 2 (HS PU 2) and the parhypural (PH) are visible. Hypural 1 (Hy 1) and the basal and distal part of hypural 2 (Hy 2) are positioned ventrally of ural 1. Proximally, the anterior spine (ant S) of the preural haemal spines and hypural 1 are sectioned. Distally, the cartilage of hypural 1 and 2 is continuous (*) and the distal part of hypural 3 (Hy 3) is visible. Positioned distally from hypural 2 and 3, rudimentary cartilages (black arrowheads) embedded in the fibrocartilaginous plate can be observed. Haematoxylin and eosin. Scale bar = 200 μm. (E) Fused basiventrals of hypural 1 (Hy 1) and 2 (Hy 2) with each basiventral showing a proliferation zone. Haematoxylin and eosin. Scale bar = 200 μm. (F) Distally, the cartilage of hypurals 1 and 2 (Hy 1 and 2) are continuous (indicated by *). Masson's Trichrome. Scale bar = 100 μm. (G) Ural 2 (U2) and the bony base of hypural 4 (Hy 4). The bony base of hypural 4 is connected to ural 2 with collagen fibre rich tissue and Sharpey's fibres (black arrows). Haematoxylin and eosin. Scale bar = 50 μm.

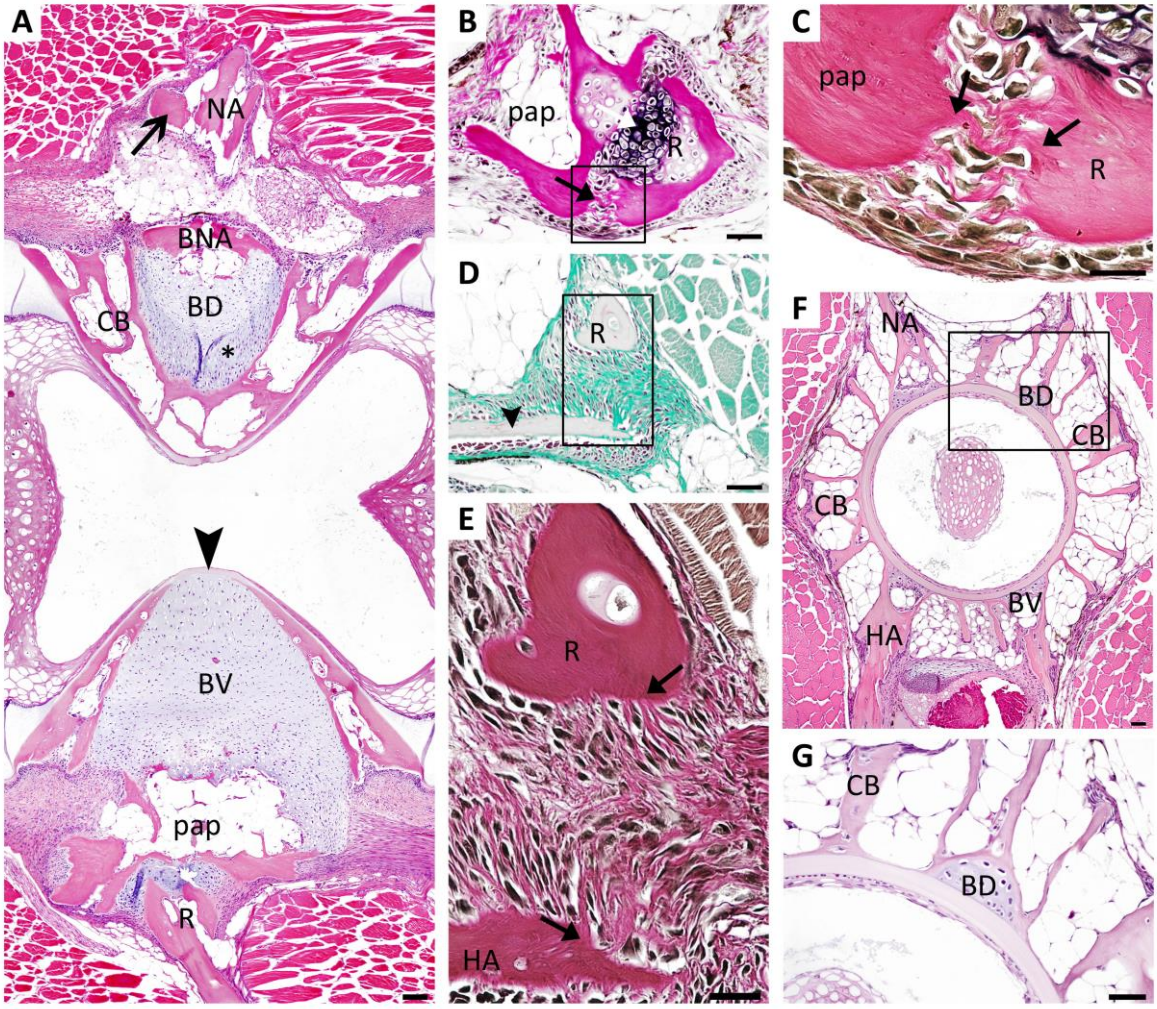

Figure 17: Connection of ribs and arches to the abdominal, transitional and caudal vertebral centra

(A-C) = Parasagittal sections from region indicated in Fig. 15B. All sections are oriented with dorsal to the top (anterior to the left for parasagittal sections). (A) Section through an abdominal vertebral centrum and associated elements. Dorsally, the anterior bony outgrowth at the base of the neural arch (arrow), the neural arch itself (NA) and the base of the neural arch (BNA) is visible. The basidorsal (BD) is fused to the base of the neural arch and an elastic cartilaginous band (indicated by *; stained darker) is positioned ventrally within the basidorsal. Ventrally the basiventral (BV) attaches to the mineralised notochord sheath (indicated by arrowhead) of the notochord and is fused to the parapophysis (pap) and distally joined with a rib (R). Scale bar = 100 μm. (B-C) The cartilage of the rib (R) is attached to the parapophysis (pap) with a cartilaginous joint. Elastic/cell-rich cartilage (white arrow) connects the hyaline cartilage of the rib to the hyaline cartilage of the parapophysis centrally. Fibro/cell-rich cartilage is positioned closely apposed to the elastic/cell-rich cartilage and Sharpey's fibres (black arrows) connect the bone of the rib to the bone of the parapophysis and line the joint. The magnified region in (C) is indicated in the black box. (B-C) = Verhoeff-Van Gieson. Scale bar (B) = 100 μm, (C) = 50 μm. (D-E) Sharpey's fibres (black arrows) connecting the rib (R) and the haemal arch [HA in (E)] in the transitional region (Type IIIb) are anchored both in the bone of the rib and the bone of the haemal arch. The section is at the level of the intramembranous bony bridge (indicated by arrowhead) connecting the left and right part of the haemal arch. The magnified region in (E) is indicated in the black box. (D) = Masson's Trichrome,

(E) = Verhoeff-Van Gieson. Scale bar (D) = 100 μm, (E) = 50 μm. (F-G) The basidorsals (BD) and basiventrals (BV) in caudal vertebrae are completely surrounded by the cancellous bone (CB) of the vertebral centrum. The base of the left neural arch (NA) and haemal arch (HA) are fused to the cancellous bone of the vertebral centrum. The magnified region in (G) is indicated in the black box. (F-G) = Haematoxylin and eosin. Scale bar = (F) = 100 μm, (G) = 50 μm.

4.4.4. CAUDAL VERTEBRAE

Caudal vertebrae (V37-58) have neural arches and neural spines dorsally, and haemal arches and haemal spines ventrally. Neural and haemal pre- and post-zygapophyses are present (Table 10, Fig. 14, 15B).

The basidorsals and basiventrals of caudal vertebrae are small and completely surrounded by cancellous bone of the vertebral centrum (Fig. 15B, 17F-G). The bases of the neural and haemal arches are solidly fused to the cancellous bone of the vertebral centrum. The number of caudal vertebrae can vary.

4.4.5. PREURAL VERTEBRAE

Preural vertebrae (V59-62, Table 10, Fig. 14, 15C) carry neural and haemal arches and modified spines to support caudal fin rays (Fig. 15A, C).

Preurals 4-2 have large basidorsals which are continuous with the cartilaginous neural arches (Fig. 14, 15C). The anterior part of the stegural is positioned directly dorsolateral of the basidorsals of preurals 2 and 1. The basidorsal of preural 1 is not fused to a neural arch (Fig. 15C). Ventrally, the cartilaginous haemal arches of preurals 5-2 are continuous with large cartilaginous basiventrals. Similarly, the cartilaginous parhypural on preural 1 is continuous with the basiventral and a semi-circular proliferation zone is visible (indicated by dashed line, Fig. 15C). The preural haemal spines and the parhypural have anterior and posterior intramembranous bony outgrowths and are interconnected with collagen fibre rich tissue and Sharpey's fibres. The haemal spine of preural 2 and the parhypural extend proximally into a perichondrally ossified anterior process. The anterior process has a distinct anterior cartilaginous proliferation zone (indicated by dashed line, Fig. 15C, 16D). Distal to of the haemal spines of preurals 3-1 a cartilaginous structure can be present (indicated by *, Fig. 15C). Possible variations include a varying number of preural vertebrae, the fusion of preural vertebrae and the presence (and left-right asymmetry) of a vestigial neural arch on preural 1. The neural arch of the anteriormost preural vertebra can be fused to the cancellous bone of the vertebral centrum. The cartilaginous structure distally of the haemal spines of preurals 3-1 can vary in shape and size.

4.4.6. URAL VERTEBRAE

The ural vertebrae (V63-64, Table 10) carry the stegural, epurals and uroneurals dorsally and hypurals ventrally. These neural and haemal elements are modified to support caudal fin rays (Fig. 15A, C).
Ural 1, but not ural 2, has a basidorsal (Fig. 15C). The medial and posterior part of the stegural is positioned immediately dorsal of the basidorsal of ural 1 and of ural 2 respectively. The medio-distal intramembranous bony outgrowth of the stegural overlaps with the proximal part of three epurals (Fig. 15C). Distally of epural 2 and 3 a cartilaginous structure is present (indicated by *, Fig. 15C). Uroneural 1 is positioned posteriorly of epural 3 and partly overlaps the dorsal end of the stegural. Uroneural 2 is positioned dorsally of uroneural 1 (Fig. 15C). Ventrally, ural 1 has a single very large basiventral, fused with the basiventrals of preural 1 through cartilaginous protrusions, which show a proliferation zone (indicated by dashed line, Fig. 15C). Within the basiventral of ural 1 itself, two semi-circular proliferation zones are present (indicated by dashed lines in Fig. 15C and 16D, magnified in Fig. 16E). The cartilage of these two zones is continuous with the cartilage of hypural 1 and 2 respectively (Fig. 15C, 16D). The bases of hypurals 1 and 2 are both fused to the basiventral of ural 1. Proximally, hypural 1 has a perichondrally ossified cartilaginous anterior process (Fig. 15C, 16D). Distally, the cartilage of hypurals 1 and 2 is continuous (Fig. 15C, 16D-F). Anterior and posterior intramembranous bony outgrowths are present on hypurals 1-3. The proximal part of hypural 3 is positioned at the level of the intervertebral region of ural 1 and 2. The proximal cartilaginous part of hypural 3 is connected to the proximal cartilaginous part of hypural 2 and the intervertebral region with elastin fibres. The perichondral bone at the base is connected with Sharpey's fibres to ural 2 (Fig. 15C). Ural 2 has no basiventral and the perichondrally ossified bases of hypurals 4 and 5 are connected with Sharpey's fibres to the bone of ural 2 (Fig. 15C, 16G). Hypural 6 is connected with collagen fibre rich tissue and Sharpey's fibres to hypural 5 (Fig. 15C). Possible variations include extra ural vertebral centra posterior of ural 2, the absence of epural 3 and/or presence of uroneural 3 and the presence of a seventh hypural. The shapes and sizes of the cartilaginous structure distally of epurals 2-3 can vary. Rudimentary hyaline cell-rich cartilages (one or two) can be present within the cartilaginous plate (Fig. 15C) and are positioned at the level of the gap between hypural 2 and 3.

4.5. DISCUSSION

Irrespective at which of the two temperatures specimens were raised, postcranial, abdominal, transitional, caudal, preural and ural regions were recognised within the vertebral column of Chinook salmon, based on distinct anatomical characters of vertebral centra and their associated elements. The recognition of the transitional region is new as a separate region in-between the abdominal and caudal region.

If one applies the new system of the recognition of six vertebral column regions to the salmonid vertebral column, in which anomalies frequently occur, most deformities are present in the posterior abdominal (Fjelldal & Hansen, 2010; Fjelldal et al., 2007a; Sullivan et al., 2007b) and caudal regions (Grini et al., 2011; Wargelius et al., 2005). Although the total number of vertebral centra of specimens in each temperature group is not related to temperature, the number of centra varies in each region. If variation in the number of vertebrae is related to temperature is currently investigated. The transitional region could be more temperature sensitive, e.g. showing variation in the number of skeletal elements such as vertebral centra and vestigial ribs related to rearing temperature. The variation of vertebrae within the transitional and other regions is likely related to variation of somite numbers in each region. This is supported by evidence found in threespine stickleback (*Gasterosteus aculeatus*), where the variation in the caudal, preural and ural regions is due to modulation of somite formation (Ahn & Gibson, 1999a; Lindsey, 1962). Alternatively, a homeotic shift could cause regional specific variation and was suggested as an underlying mechanism in actinopterygians (Ward & Brainerd, 2007; Ward & Mehta, 2010). Either variation of somite number or homeotic shift could cause regional variation in Chinook salmon, but also a synergistic effect of both mechanisms can drive regional variation (Ahn & Gibson, 1999b). In contrast, Ahn and Gibson (1999c) suggest that segmentation and axial regionalisation in teleosts are two separate developmental processes and is supported by evidence found in amniotes (Gomez & Pourquié, 2009; Müller et al., 2010). However, in the anterior part of the mammalian vertebral column a strong coupling between the number of vertebrae and the vertebral identity (vertebral column regions) appears evident. Occurring variation of skeletal elements such as vestigial ribs in the transitional region and, epurals and uroneurals in the caudal fin could be used as an indicator for developmental stress and stress caused by husbandry practices in fish farming (Allenbach, 2011; Leary & Allendorf, 1989; Leary et al., 1985; Parsons, 1992).

Vertebral centra have been previously recognised as “cervical” in dipnoans and teleosts. Postcranial vertebrae were recognised among other on the basis of lack of ribs in the Dipnoi *Neoceratodus forsteri* (Queensland lungfish; Johanson et al., 2005) and in the teleosts *Gadus morhua* (Atlantic cod) and *Danio rerio* (zebrafish) (Fjelldal et al., 2013; Holley, 2007; Morin-Kensicki et al., 2002). The first postcranial vertebra is known to fuse to the basioccipital in teleosts and is part of natural variation (Johnson & Britz, 2010). Moreover, fusion of vertebral centra to the base of the skull (occipital region) and to the urostyle (fusion of ural vertebrae) is a common non-pathological phenomenon in osteichthyans and occurs both in farmed and wild specimens (Arratia & Schultze, 1992; Arratia et al., 2001; Britz & Johnson, 2005; Johanson et al., 2005; Witten et al., 2009). The posterior end of the postcranial region co-aligns with the anterior expression boundary of the *Hoxc-6* in zebrafish (Burke et al., 1995; Holley, 2007; Morin-Kensicki et al., 2002). Whether the posteriormost postcranial centrum coincides with the anterior expression boundary of *Hoxc-6* in Chinook salmon is currently unknown. To our knowledge transitional centra have never been recognised as a separate region in the

literature before. Yet there are clearly recognisable anatomical characters that distinguish transitional vertebrae from abdominal vertebrae and caudal vertebrae and that support their identification as a separate transitional region: the presence of a haemal arch and ribs on the same vertebral centrum. Although a separate transitional region has never been defined before, vertebrae with transitional morphological characters have been described in basal actinopterygians of the genus *Lepidosteus* (Balfour & Parker, 1882) and in several teleost species, such as in Atlantic salmon, zebrafish, onion trevally (*Carangoides caeruleopinnatus*) and striped bass (*Morone saxatilis*) (Bird & Mabee, 2003; Götte, 1879; Holley, 2007; Jawad, 2015; Nowroozi et al., 2012). Transitional vertebrae are distinctive due to the presence and the anatomical shape of the parapophyses. For example, neoscopelid fishes have two series of parapophyses, i.e. a lateral and ventral series (Miyashita & Fujita, 2000). The parapophyses on the anterior transitional vertebrae of the onion trevally are lobe shaped, while long flange-like parapophyses are present on posterior transitional vertebrae (Jawad, 2015). In frigate tuna (*Auxias thazard*) the parapophyses on vertebral centra 8 to 12 have blade-like shapes with posteriorly bent distal ends (Jawad et al., 2013). Furthermore, parapophyses can have distinctive functions. For example, in black mackerel (*Scombrolabrax heterolepis*) the parapophyses bulge dorsolaterally to form hemispherical pockets or bullae. These bullae open ventrally to allow evaginations of the gas bladder to fit inside the pockets (Bond & Uyeno, 1981). In Atlantic salmon and zebrafish the parapophyses are connected with a bony bridge to form the haemal canal (Ford, 1937). Parapophyses in transitional region of Chinook salmon have likely a similar function.

A vertebral column consisting of five regions (cervical, thoracic, lumbar, sacral and caudal) is often considered typical for tetrapods. Interestingly, a tetrapod-like regionalisation was recognised in the early actinopterygian *Tarrasius problematicus* and could thus be an ancestral character of osteichthyans (Sallan, 2012). A vertebral column with distinct regions is also present in teleosts, however the morphology of the vertebral centrum and associated elements likely presents a highly derived state. For example the transitional region where haemal arches and ribs co-occur could be a derived character according to Sallan (2012), who discussed that the tetrapod sacral region is anteriorised in teleosts causing ribs and haemal arches to be present on a single vertebral centum. It is tempting to suggest that the ‘lumbar’ and/or ‘sacral’ regions in *Tarrasius problematicus* are comparable to the transitional region described here. Specifically, the position of the first haemal arch (cf. Type IIIb vertebrae in Chinook salmon) and co-occurrence of haemal arches and ribs are common to *O. tshawytscha* and *T. problematicus*. Moreover, the stepwise anterior to posterior change of parapophyses into haemal arches is also observed in other actinopterygians and even sarcopterygians (Balfour & Parker, 1882; Jawad et al., 2013; Nowroozi et al., 2012; Sallan, 2012).

Although regions, such as postcranial, abdominal, transitional, caudal, preural and ural, in the actinopterygian vertebral column have been described, the literature is still dominated by the use of a dichotomous subdivision: an anterior and posterior region, often referred to as abdominal and caudal

respectively (Gill & Fisk, 1966; Grande & Bemis, 1998; Seymour, 1959). The anterior region consists of postcranial (Type I), abdominal (Type II) and transitional vertebrae (Type III) and the posterior region of caudal (Type IV), preural (Type V) and ural vertebrae (Type VI). Initially the abdominal-caudal subdivision of the vertebral column was based on anatomical characters. For example, in the vertebral column of *Oncorhynchus spp.* the first caudal vertebral body was recognised as the vertebral centrum with a closed haemal arch and median spine as associated elements (Canagaratnam, 1959; Clothier, 1950; Gill & Fisk, 1966; Seymour, 1959). Such a definition would include our transitional region in the anterior region. Recently the abdominal-caudal subdivision gained support in evolutionary developmental studies, investigating which mechanisms underlie vertebral column subdivision. Ward and Brainerd (2007) hypothesised that the actinopterygian vertebral column contains two axial modules. Several studies on development and evolution of body shape and elongation of fish have collected evidence in favour of a separate evolution of the anterior and posterior axial modules (Maxwell & Wilson, 2013; Schilling & Long Jr, 2014; Ward & Brainerd, 2007; Ward & Mehta, 2010; Ward & Mehta, 2014). For example most actinopterygian fish evolve elongated bodies mainly by adding vertebral centra rather than by increasing the length of individual vertebrae. The increase in number of vertebral centra occurs either in the abdominal or in the caudal region. Moreover, when vertebral centra are added in the abdominal region little change occurs in the caudal region and vice versa. Ward and Metha (2010) suggested that a homeotic shift could change the number of vertebral centra in the anterior or posterior vertebral column regions. Such an identity switch could be caused by changes in *Hox*-gene expression patterns. In mammalian vertebral columns the *Hox*-genes anterior expression boundaries correspond to sharp changes in vertebral morphology. However, the transitional region in Chinook salmon shows a gradual anterior to posterior change of the parapophyses to haemal arches and the regression of ribs to vestigial ribs. The gradual change of structures in the transitional region challenges the correspondence of *Hox*-genes expression patterns to sharp morphological boundaries. Only the *Hoxc-6* expression co-aligns with the postcranial-abdominal border in zebrafish. In contrast, the *Hoxd12a* anterior expression boundary is located in the middle of the transitional region and is not co-aligned with any anatomical boundary (Burke et al., 1995; Holley, 2007; Morin-Kensicki et al., 2002; Ward & Brainerd, 2007). Except for the *Hoxc-6* anterior expression boundary, there is currently no strong support for co-alignment of *Hox*-gene expression domains and vertebral column regions in actinopterygians. Regionalisation of the anterior part of the actinopterygian vertebral column might be controlled by a different, and currently unknown, mechanism that facilitates homeotic shifts and meristic changes (Ward & Mehta, 2010). Also, a combination of homeotic and meristic mechanisms could underlie axial variation in teleost species. Studies on the threespine stickleback species complex support a synergistic effect of both mechanisms on axial variation (Ahn & Gibson, 1999a; Ahn & Gibson, 1999b; Ahn & Gibson, 1999c).

Examples of exceptions to the anterior and posterior axial modules suggested by Ward and Brainerd (2007) exist in literature. The position of the anus and the anterior insertion point of the anal fin (Mabee et al., 2002) have been associated with the border of the abdominal and caudal axial modules (Maxwell & Wilson, 2013). However, in a study on the vertebral column of 'ambush predator' type teleosts it was suggested that the abdominal/caudal regional border dissociates from the anal fin insertion point. This represents a first example of an exception of the anterior and posterior axial modules. Specifically, the position of the anal fin moves posterior and consequently up to nine haemal arch bearing vertebrae are positioned anterior of the anal fin (Maxwell & Wilson, 2013). A second example is represented by the Weberian apparatus in zebrafish (Bird & Mabee, 2003), a modification of the five anterior postcranial vertebral centra, that evolved in the abdominal region. The postcranial vertebral centra and connected elements comprising the Weberian apparatus show marked growth rate differences compared to other vertebral centra in Ostariophysi (Bird & Hernandez, 2009) supporting the idea about the separate evolution of this region of the vertebral column. The dissociation of the position of the anal fin in 'ambush predators' and the different growth rate of the Weberian apparatus in Ostariophysi both show that more than two anterior-posterior modules can exist in the vertebral column of teleosts.

Another way of explaining the regionalisation of the vertebral column in teleosts and other vertebrates is to look at centra and associated elements (neural and haemal arches) as separate developmental modules (de Azevedo et al., 2012; Detwiler & Holtzer, 1956; Hall, 1977; Holtzer, 1951; Strudel, 1953a; Strudel, 1953b; To et al., 2015; Witten et al., 2007). The development of the teleost vertebral centrum starts with the mineralisation of the notochord sheath without the presence of a cartilaginous precursor. Arratia et al. (2001) designates the teleost vertebral centrum anlage as a chordacentrum. Conversely, the arches are somitic (sclerotome) derived and develop from cartilage precursors (Arratia et al., 2001; de Azevedo et al., 2012; Grotmol et al., 2003; Huxley, 1859; Kölliker, 1859; Schaeffer, 1967). Investigation of 'fused somite' mutant zebrafish shows that the pattern of neural and haemal arches is disrupted yet the vertebral centra develop normally (Fleming et al., 2004). A recent study on medaka (*Oryzias latipes*) shows that the ablation of somite-derived osteoblasts has no influence on the mineralisation of vertebral centra within the notochord sheath (Yu et al., 2017). Furthermore, in farmed Atlantic salmon the neural and haemal arches stay separate when vertebral centra fuse (Witten et al., 2006). These studies show the separation of vertebral centra and associated elements and support the idea that centra and associated elements are developmental modules. Interestingly, also for the Chinook salmon vertebral column the associated elements rather than the vertebral centra themselves are the anatomical structures that determine the regional identity of the centrum and its associated elements.

In conclusion, six anatomical regions, including a new transitional region, represent a refined regionalisation of the vertebral column of juvenile Chinook salmon. The recognition of this refined

regionalisation is based on the associated elements of vertebral centra rather than the centra themselves. Studying vertebrate taxa using this refined identification of regionalisation can help to address evolutionary developmental and functional questions related to the gnathostome vertebral column. Understanding of such fundamental questions supports applied research into farmed fish species like Chinook salmon.

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4.7. AUTHOR CONTRIBUTIONS (IN CREDIT TERMS)

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P.S. Davie: Conceptualisation, review and editing of manuscript
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P.E. Witten: Conceptualisation, methodology, review and editing of manuscript

CHAPTER 5: TEMPERATURE SENSITIVE REGIONS OF THE CHINOOK SALMON VERTEBRAL COLUMN. VESTIGES AND MERISTIC VARIATION

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5.1. ABSTRACT

Variation of vertebral centra numbers is common in vertebrates. Likewise the number of associated elements such as ribs and neural and haemal arches can vary and affect all regions of the vertebral column. In mammals, only the number of cervical vertebrae is invariable. Variation of total vertebral centra numbers is well documented in teleost fish, often related to temperature. Less information is available about which part of the vertebral column and which associated elements are liable to variation. Here variation in number of vertebral centra and associated elements is studied in Chinook salmon in six distinct anatomical regions. Animals are raised at 8°C and 12°C to ask if vertebral centrum numbers, the pattern and the frequency of variation in particular regions are temperature dependent. No significant difference concerning the total number of vertebrae was found but regional differences occurred between the 8°C and 12°C groups. Twelve specimens out of sixty of the 12°C group had three postcranial vertebrae compared to only one specimen in the 8°C group. The number of transitional vertebrae is significantly different in 8°C and 12°C specimens. Fewer transitional vertebrae occur in more anterior positions in 8°C specimens. Most specimens of both temperature groups had two ural centra, however 17 specimens out of sixty of the 12°C group had up to five ural centra. Specimens of the 12°C group show more variation in the presence of the vestigial ribs associated with transitional vertebrae. Clearly, the postcranial, transitional and ural regions are temperature sensitive. This study shows that non-significant differences in the total number of vertebrae can mask significant regional variation. Variation of vertebral numbers could be the consequence of loss or gain of vertebral centra and/or a change in the identity of the associated element on the vertebral centrum.

5.1.1. KEYWORDS

Atlantic salmon, regions, transitional, vertebrae, zebrafish

5.2. INTRODUCTION

Observing variations in the vertebral body numbers Darwin emphasised that, “it seems to be a rule that when a part or organ is repeated many times in the same individual the number is variable” (Darwin, 1859). The variation of discrete morphological features, such as scales, fin rays or vertebrae, is defined as meristic variation (Fowler, 1970) and has been studied in many teleost species. Several epigenetic factors are related to meristic variation, such as oxygen concentrations, salinity, light regime and temperature during incubation (Brander, 1979; Canagaratnam, 1959; Fowler, 1970; Gabriel, 1944; Garside, 1966a; Jordan & Evermann, 1896; McDowall, 2008; Tester, 1938; Witten & Hall, 2015).

A well-recognised temperature effect on meristic variation of vertebrae is known as Jordan's rule, stipulating a geographical tendency for populations of the same fish species from higher latitudes to have an increased number of vertebral centra (Jordan & Evermann, 1896). Indeed temperature is considered as a main epigenetic factor that influences teleost development (Beitinger et al., 2000; Brett, 1972). It is frequently manipulated in fish farming practices to influence growth rates (Fjelldal et al., 2012) and in laboratory studies to investigate meristic variation (Beacham, 1990; Beacham & Murray, 1986; Fowler, 1970; Gabriel, 1944; Hubbs, 1922; Sfakianakis et al., 2011; Taning, 1952). Apart from inducing meristic variation, temperature has been reported to affect a large number of parameters, such as embryonic developmental rate and hatching time (Alderdice & Velsen, 1978; Beacham & Murray, 1990; Boyd et al., 2010; Ojanguren & Braña, 2003; Tang et al., 1987), somatic growth rate and animal size (Beacham & Murray, 1990; Georgakopoulou et al., 2010), requirement for minerals (Boglione et al., 2013a) and skeletal growth (Dionísio et al., 2012).

Embody (1934) was the first to carefully review and study the relationship between temperature and development of salmonid eggs. The relationship between temperature and development is often expressed as degreedays or daydegrees. A degreeday is the average amount of thermal energy an ectothermal organism accumulates on a single day (Chezik et al., 2013). Degreedays are used in salmon farming practices to classify developmental stages (Bergheim et al., 2009; Gjedrem & Gunnes, 1978; Grotmol et al., 2005; Nordvik et al., 2005) so that developmental stages of fish reared at different temperatures can be compared. Although degreedays provides an approximate measure of development as a function of temperature and time, this metric does not reveal the effect of temperature on development directly. For example, development in specimens with equal degreeday values might have progressed differently within the measured time period resulting in a different length. Thus, for zebrafish (*Danio rerio*) length has been suggested to be a better predictor of development (Bird & Mabee, 2003). Another attempt for a more accurate estimation of early development as a function of temperature and time is Gorodilov`s Tau-somite (τ_s) unit. This unit represents the time it takes to develop one pair of somites at a given temperature. If salmonids develop at a certain temperature the time interval during somite formation is relatively equal and therefore τ_s is a relatively good estimation (Gorodilov, 1996; Gorodilov, 1995; Gorodilov & Melnikova, 2003). However, to estimate τ_s accurately a strictly constant temperature during development is necessary. The rate of somitogenesis (a constant rate of 2.4 somites per hour) was calculated for zebrafish developing at a constant 27.4°C (Schmidt & Starck, 2004). The direct effects of temperature on development are not easy to measure. Meristic characters, on the other hand, are easily observable and quantifiable, and are also affected by temperature (Fowler, 1970).

Studying meristic variation of vertebral centra in salmonids has provided insights into how temperature affects early freshwater stages, since the number of vertebral centra is established early in development (Fowler, 1970). For example, chum salmon (*Oncorhynchus keta*) and sockeye salmon

(*Oncorhynchus nerka*) have the highest number of vertebrae and other meristic characters such as gill rakers, reared at cold water temperatures (Beacham, 1985; Beacham & Murray, 1986). Likewise, rainbow trout (*Oncorhynchus mykiss*) have a higher number of vertebrae, gill rakers and fin rays when reared at lower temperatures (Kwain, 1975; Lindsey et al., 1984).

Studies that addressed regional meristic variation in teleosts have subdivided the vertebral column into just an anterior and posterior part. Ward and Brainerd (2007) examined 867 actinopterygian and elasmobranch species and showed that vertebral numbers, when compared between species, change independently in the anterior (abdominal) and posterior (caudal) part of the vertebral column. In threespine stickleback (*Gasterosteus aculeatus*), females and males have the same number of total vertebrae but females have more abdominal vertebrae and males more caudal vertebrae (Aguirre et al., 2014). Since these studies subdivide the vertebral column into just two major regions, abdominal and caudal, there is no information on potential temperature-induced meristic variation within these broadly defined regions. It is however known that zebrafish and other teleosts show a high degree of variation in regions of the vertebral column that support the caudal fin, i.e. the ural region (Bensimon-Brito et al., 2012a).

Here meristic variation is studied within the vertebral column of farmed Chinook salmon, raised at 8°C. The results are compared with specimens raised at 12°C. The usage of 8°C is a standard farming practice (Alderdice & Velsen, 1978; Brett, 1952). The second temperature, 12°C, is considered to be a high incubation temperature for Chinook salmon eggs. Although in some teleost species a negative linear relation between temperature and number of vertebrae was shown (Jordan & Evermann, 1896), a U-shaped response with the lowest number of meristic characters related to the median temperature has been shown in other teleost species (Fowler, 1970). Notably, this U-shaped response was also shown to occur in salmonids by Seymour (1959) and Taning (1952). A wider span of temperature in Chinook salmon could therefore result in a U-shaped response of meristic characters, such as number of vertebrae, resulting in animals incubated in colder and warmer temperatures having similar variation. Therefore, a wider temperature span could reduce the differentiation of meristic characters between specimens rather than increase it. Based on observations for these two temperatures, this study asks which regions are particularly temperature sensitive. Attention is paid to variation in vestigial skeletal elements, such as the vestigial ribs in the transitional region and the vestigial neural arches and spine in the preural and ural regions.

5.3. MATERIAL AND METHODS

5.3.1. SAMPLES

Juvenile Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum 1792) from farmed New Zealand stocks were raised from eggs under strictly controlled temperature conditions (started at 20/05/2014). Briefly, eggs were stripped from broodstock at 10°C, fertilised (*APPENDIX B*) and placed in incubation trays supplied with water of a constant 10°C until 3 days post-fertilisation (= 30°d, degreedays). Keeping the eggs three days at 10°C to settle in the incubation trays is standard farming practice. Subsequently temperature was gradually changed (0.2°C per hour) to a constant 8°C and 12°C to avoid temperature shock effects. At first-feed stage, fish from the 8°C (933°d) and 12°C (943°d) groups were transferred to 420L tanks and further maintained at 8°C and 12°C. Specimens from both temperature groups were randomly sampled at 1400°d and 1530°d and euthanized by anaesthetic overdose (Aqui-S, New Zealand Ltd, Lower Hutt, New Zealand). The specimens were fixed in 4% buffered paraformaldehyde (PFA) and stored in 60% ethanol. Experiments were approved by Animal Ethics Committee of Massey University: AEC protocol 14/32.

5.3.2. SYSTEM OF VERTEBRAL COLUMN SUBDIVISION

This study subdivides the vertebral column into six regions (region I-IV) to analyse the effect of temperature on meristic characters. These regions have been described in detail previously (De Clercq et al., 2017b), and are shown in Figure 18. Briefly, the postcranial (I) region comprises the first vertebrae that lack ribs. The abdominal (II) region is characterised by rib-bearing vertebrae. The transitional (III) region is subdivided into four smaller regions (subtype IIIa-d) defined by the stepwise change of parapophyses into haemal arches carrying a haemal spine. Subtype IIIa transitional vertebrae have ventrally extended parapophyses. Subtype IIIb vertebrae have haemal arches, while subtype IIIc have haemal arches and stepwise longer haemal spines. Finally, subtype IIId vertebrae have haemal arches and full sized haemal spines. Ribs are associated with each transitional vertebral subtype and change stepwise from full sized ribs of subtype IIIa vertebrae into vestigial ribs of subtype IIId. A vestige is defined as a persisting remnant of an ancestral feature (Hall, 2003). The caudal (IV) region has fused haemal arches, carrying haemal spines. In the preural (V) region the neural and haemal spines are modified to support caudal fin rays. The ural (VI) vertebrae carry hypurals and epurals also supporting caudal fin rays.

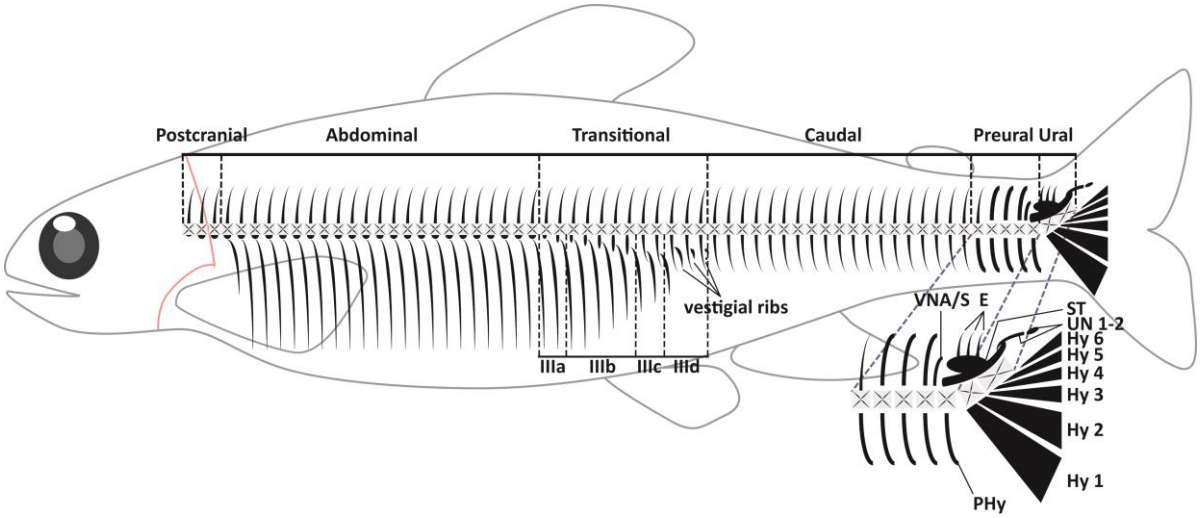

Figure 18: Regionalisation of the Chinook vertebral column

Schematic representation of the vertebral column regions in Chinook salmon described in De Clercq et al. (2017b). The subtype transitional vertebrae are abbreviated with IIIa, IIIb, IIIc and IIId. The preural and ural regions have been enlarged to show the caudal fin skeletal elements in more detail. The purple lines indicate the boarders of the preural and ural regions. Abbreviations: E, epurals; Hy 1, hypural 1; Hy 2, hypural 2; Hy 3, hypural 3; Hy 4, hypural 4; Hy 5, hypural 5; Hy 6, hypural 6; PHy, parhypural; ST, stegural; UN, uroneurals; VNA/S, vestigial neural arch/spine.

5.3.3. WHOLE MOUNT STAINING AND COUNTING VERTEBRAE

In total, 120 fish were stained for mineralized tissues with Alizarin red S (protocol adapted from Taylor & Van Dyke, 1985). For each temperature group, 30 fish from 1400°d and 30 fish from 1530°d were grouped, resulting in N = 60 for each temperature group. The range and average of the total lengths and weights of specimens at 1400°d and 1530°d are represented in Table 11. For whole mount staining, specimens were rehydrated, bleached (1% KOH/3% H_2O_2, 1-1.5 hrs), rinsed in demineralised water and placed for 24 hrs in a 70% saturated borax solution (di-sodium tetraborate). Specimens were stained overnight in a 0.5 mg/ml Alizarin red S/1% KOH solution and subsequently rinsed in demineralised water. Specimens were cleared in a graded glycerol/KOH series, dehydrated in a graded glycerol series and stored in 100% glycerol. Specimens were placed on their right side. The total number of vertebral centra (both with and without ural centra) was counted using a stereomicroscope (Leica M80) and the centra assigned to regions. When fused vertebrae were observed the number of associated elements (neural arches/spines, ribs or haemal arches/spines) were counted to assess the original number of centra (Witten et al., 2006). When a postcranial vertebral centrum was fused to the basioccipital, it was counted as a separate vertebra (Johnson & Britz, 2010). Likewise, the vestigial ribs of subtype IIId transitional vertebrae, the vestigial neural arch and spine on the second preural vertebra, the epurals and uroneurals were counted. A vertebral anlage, also designated as chordacentrum, is defined as a ring shaped mineralisation within the notochord sheath (Arratia et al., 2001). Illustrator CS5 was used to draw schematic representations.

Table 11: Total length (TL) in millimetres and weight in grams of samples (N = 60) analysed at 1400°d and 1530°d (SD = standard deviation)

	8°C		12°C	
1400°d	TL (mm)	Weight (g)	TL (mm)	Weight (g)
Range	40.15-59.15	0.49-2.07	40.45-66.60	0.68-3.50
Average (±SD)	51.30 ±5.38	1.24 ±0.44	58.10 ±7.66	2.11 ±0.80
1530°d				
Range	48.20-70.25	0.57-3.86	53.55-81.45	1.76-6.29
Average (±SD)	59.05 ±6.78	1.75 ±1.02	69.40 ±6.99	3.97 ±1.27

5.3.4. STATISTICS

For each temperature group, the median number of vertebrae for the total spine, and for each region separately, was calculated. Medians were compared with the non-parametric Kruskall-Wallis rank sum test to account for the non-continuous, discreet nature of the count data generated from the number of vertebral centra in the specimens. The data was not normally distributed and a Poisson regression was not utilized due to the low number of samples available. An alpha value (P) of less than 0.05 was considered statistically significant. Statistics were calculated using RStudio Team (2015, Integrated Development for R, RStudio, Inc., Boston).

5.4. RESULTS

Specimens raised both at 8°C and 12°C showed variation in the number of vertebrae in each region. In addition, 12°C specimens showed different variation compared to 8°C specimens. To compare and illustrate how extensive regional meristic variation between 8°C and 12°C specimens is, an 8°C specimen with a total of 64 vertebrae and a 12°C specimen with a total of 66 vertebrae are represented in Figure 19.

5.4.1. TOTAL NUMBER OF VERTEBRAE

The total number of vertebrae in the 8°C group ranged from 62 to 67 (median = 64) while in the 12°C group it ranged from 63 to 68 vertebrae (median = 65). Total number of vertebrae in 8°C specimens was not significantly different from 12°C specimens (p-value = 0.128) (Figure 20a). The number of vertebral centra in 8°C specimens, not counting ural centra, ranged from 60 to 65, with a median of 62 vertebrae (Figure 20b). The number of vertebrae in 12°C specimens, not counting ural centra, ranged from 61 to 65 with a median of 63 vertebrae (Figure 20b), and was not significantly different from 8°C specimens (p-value = 0.377).

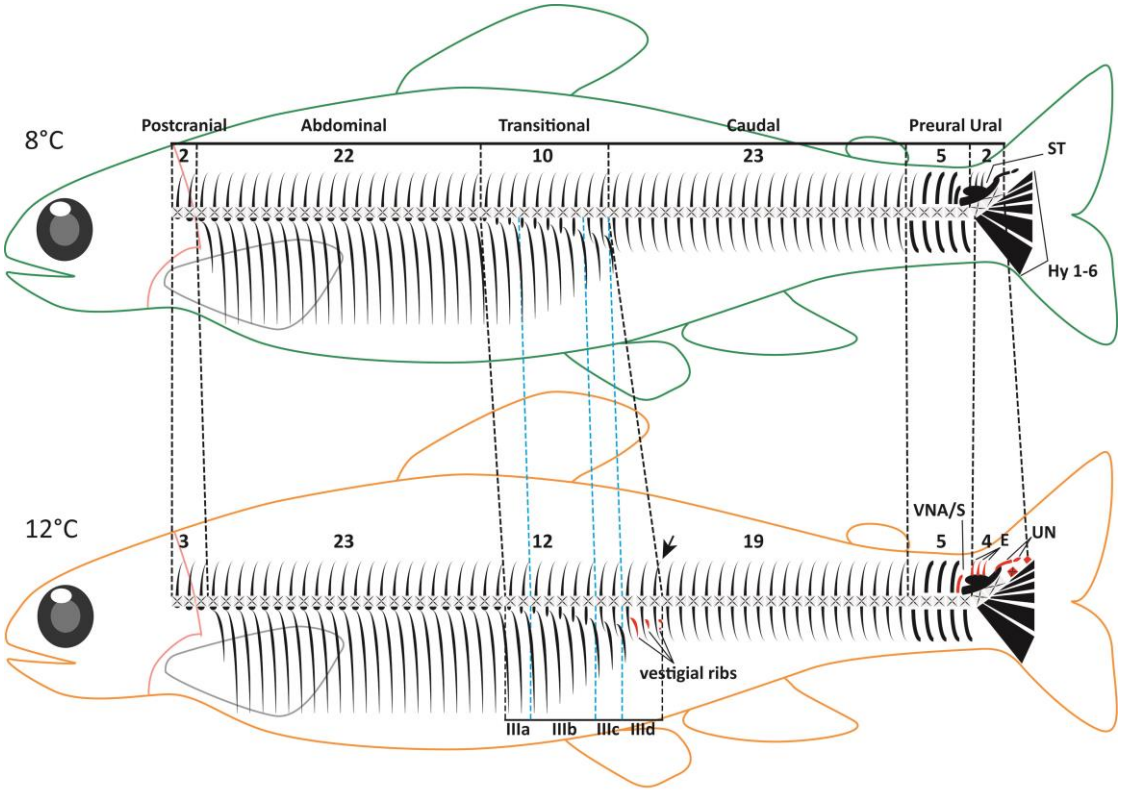

Figure 19: Comparison of the vertebral column regions in an 8°C and 12°C specimen

The 8°C group is represented by a juvenile Chinook salmon with 64 vertebrae (green outlay) and the 12°C group is represented by a juvenile Chinook salmon with 66 vertebrae (orange outlay). The black dotted lines denote the vertebral column regions and the blue dotted lines denote the transitional vertebrae subtypes. The arrow in the bottom specimens indicates the vertebral centrum magnified in figure 21. Ural centra that develop posterior of the two ural centra depicted in the 8°C specimen and the vestigial skeletal elements are shown in red. Abbreviations: E, epural; Hy, hypurals; ST, stegural; UN, uroneurals; VNA/S, vestigial neural arch/spine.

5.4.2. POSTCRANIAL VERTEBRAE

The median number of postcranial vertebrae in the 8°C specimens was two. Only one specimen had three postcranial vertebrae. The median number of postcranial vertebrae in the 12°C specimens was also two. However, 12 specimens had three postcranial vertebrae (Figure 20c). The number of postcranial vertebrae was not significantly different between the 8°C and 12°C specimens (p-value = 0.617).

5.4.3. ABDOMINAL VERTEBRAE

In the 8°C specimens the number of abdominal vertebrae ranged from 22 to 26 with a median of 24 vertebrae. The number of abdominal vertebrae in the 12°C specimens had the same range (22 to 26 vertebrae) and median (24 vertebrae) as in 8°C specimens. The number of abdominal vertebrae was not significantly different between the 8°C and 12°C specimens (p-value = 0.743).

5.4.4. TRANSITIONAL VERTEBRAE

The number of transitional vertebral centra in 12°C specimens was significantly different from 8°C specimens (p-value = 0.025). The distribution of vertebrae showed distinctive peaks with 10 and 11 transitional vertebrae for the 8°C and 12°C groups, respectively (Figure 20d). In the 8°C group, the number of transitional vertebrae ranged from 8 to 12 with a median of 10 vertebrae (Figure 20d). Specimens of the 12°C group had a median of 11 transitional vertebrae, and ranged from 9 to 12 vertebrae. A greater proportion of 8°C specimens had transitional vertebrae in more anterior positions (vertebrae 25-28) compared to the 12°C specimens (Figure 20e). A lower proportion of 8°C specimens had transitional vertebrae in more posterior positions (vertebrae 35-38) compared to the 12°C specimens.

More subtype IIIa transitional vertebrae were observed in specimens of the 8°C group compared to specimens of the 12°C group, but the number of subtype IIIa vertebrae was not significantly different (p-value = 0.052). The number of subtype IIIa transitional vertebrae ranged from 1 to 5 in both the 8°C and 12°C groups. However the median number of subtype IIIa transitional vertebrae was 3 and 2.5 in the 8°C and 12°C groups, respectively.

The number of subtype IIIb vertebrae ranged from 3 to 6 in the 8°C specimens and from 2 to 6 in 12°C specimens. The median was 4.5 and 5 for the 8°C and 12°C specimens, respectively (p-value = 0.458). The number of subtype IIIc vertebrae ranged from 1 to 3 in the 8°C specimens and from 0 to 4 in 12°C specimens. The median is 1.5 and 1 for 8°C and 12°C specimens, respectively (p-value = 0.124). The number of subtype IIId vertebrae ranged from 0 to 3 in the 8°C specimens and from 0 to 3 in 12°C specimens. The median was 1 and 2 for the 8°C and 12°C specimens, respectively (p-value = 0.366). A greater proportion of 12°C specimens had subtype IIId transitional vertebrae in more posterior positions (vertebrae 35 to 38).

5.4.5. CAUDAL VERTEBRAE

The number of caudal vertebrae ranged from 18 to 23 with a median of 22 vertebrae in the 8°C specimens. The 12°C specimens had the same range (18 to 23), but a median of 21 vertebrae. A greater proportion of 8°C specimens had more caudal vertebrae in more anterior positions (vertebrae 35-38). The number of caudal vertebrae was not significantly different between the 8°C and 12°C specimens (p-value = 0.455).

5.4.6. PREURAL VERTEBRAE

The number of preural vertebrae ranged from 4 to 5 with a median of 5 vertebrae in the 8°C specimens. The number of preural vertebrae in the 12°C specimens ranged from 4 to 6 with the same

median number of vertebrae as the 8°C specimens. The number of preural vertebrae was not significantly different between the 8°C and 12°C specimens (p-value = 0.131).

5.4.7. URAL VERTEBRAE

Nearly all specimens (N = 59) in the 8°C group had two ural vertebrae with only one specimen having a third ural vertebral anlage (i.e. chordacentrum) (Figure 20f). The number of ural vertebrae ranged from 2 to 5 in 12°C specimens. Extra vertebral anlagen (i.e. chordacentra) in addition to the two ural vertebrae were observed in 17 specimens: 13 specimens had three ural centra, three specimens had 4 ural centra (cf. Figure 19) and one specimen had 5 ural centra. Although the distribution of ural vertebrae of 12°C specimens was skewed to the right (Figure 20f), the median of 2 vertebrae was the same for both the 8 and 12°C groups. Also, the number of ural vertebrae was not significantly different between the 8°C and 12°C specimens (p-value = NA (not applicable); due to skewed distribution in the 12°C group the p-value cannot be calculated).

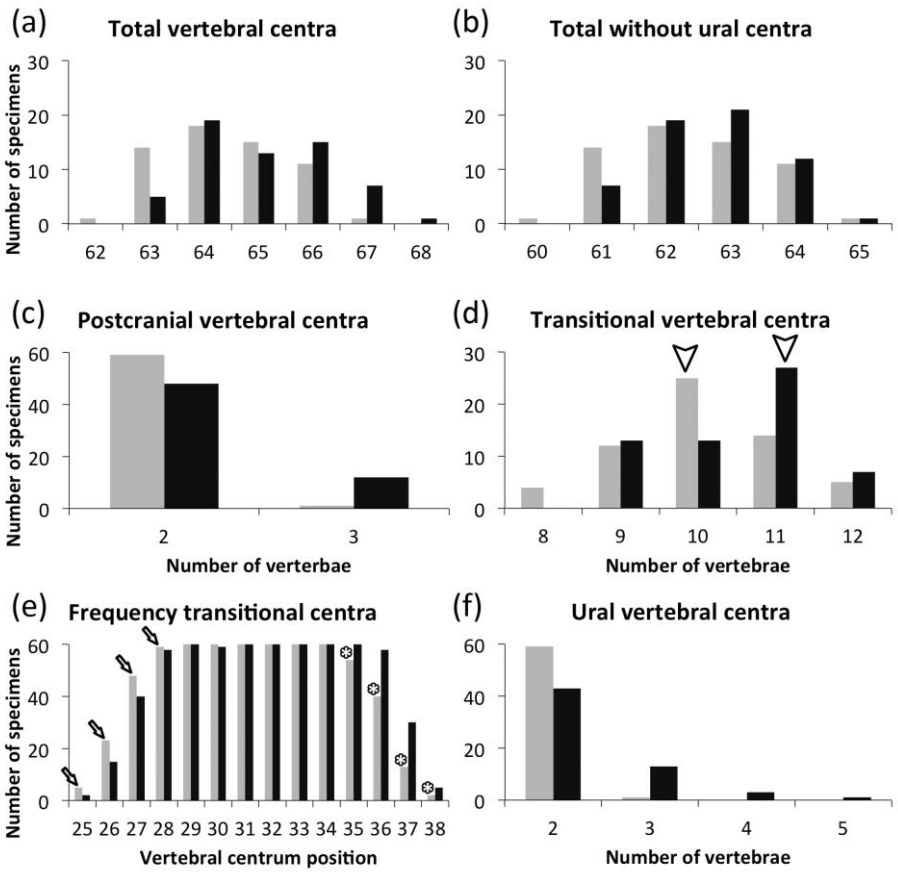

Figure 20: Comparison of the number of vertebral centra between specimens of the 8°C and 12°C groups
Grey bars depict the 8°C group and black bars the 12°C group. The y-axis shows number of specimens, note that in (c), (e) and (f) the range is 0 to 60 specimens. X-axis shows the number of vertebrae except in (e) where the x-axis shows the vertebral centrum position of transitional vertebrae, e.g. vertebra 25 shows the number of specimens in the 8°C (grey) and 12°C (black) with a transitional vertebral centrum in this position along the

vertebral column. Arrowheads in (d) show the significantly different medians of transitional vertebrae in the 8°C group (10) and 12°C group (11). Arrows and asterisks in (e) show a greater proportion of 8°C specimens have transitional vertebral centra in a more anterior position of the vertebral column.

5.4.8. VESTIGES

As in *Salmo salar* (Patterson & Johnson, 1995) the ribs of subtype IIIa to IIIc transitional vertebrae are preformed in cartilage and subsequently ossify perichondrally (Figure 21a, b). The vestigial ribs in subtype IIId transitional vertebrae are formed by intramembranous ossification within the myosepta tissue and are sometimes only a speck of bone (Figure 21b, c, d). Variation in the number of these vestigial ribs was observed as presence or absence of elements on the left and right side of the body (Figure 22). Also the position and the size of the elements varied (Figure 22). In the 8°C group, 29 specimens showed variation of the vestigial rib number on the left and right side. The vestigial ribs associated with subtype IIId vertebrae showed a higher level of variation in 12°C specimens, where 37 specimens showed variation in the number on the left and right side.

In the caudal fin, a number of skeletal elements showed variation: the presence or absence of the vestigial neural arch and spine on preural two, fusion of epurals and variation of the number of uroneurals. Variation in the presence of the vestigial neural arch and spine, fusions of epurals and number of uroneurals on the left and right side was very similar in 8°C and 12°C specimens. The vestigial neural arch and spine were present in 36 specimens of the 8°C group, fusion of epurals occurred in 13 specimens and 17 specimens showed variation in the number of uroneurals on the left and right side. The vestigial neural arch and spine were present in 35 specimens of the 12°C group, fusion of epurals occurred in 11 specimens and 18 specimens showed variation in the number of uroneurals on the left and right side.

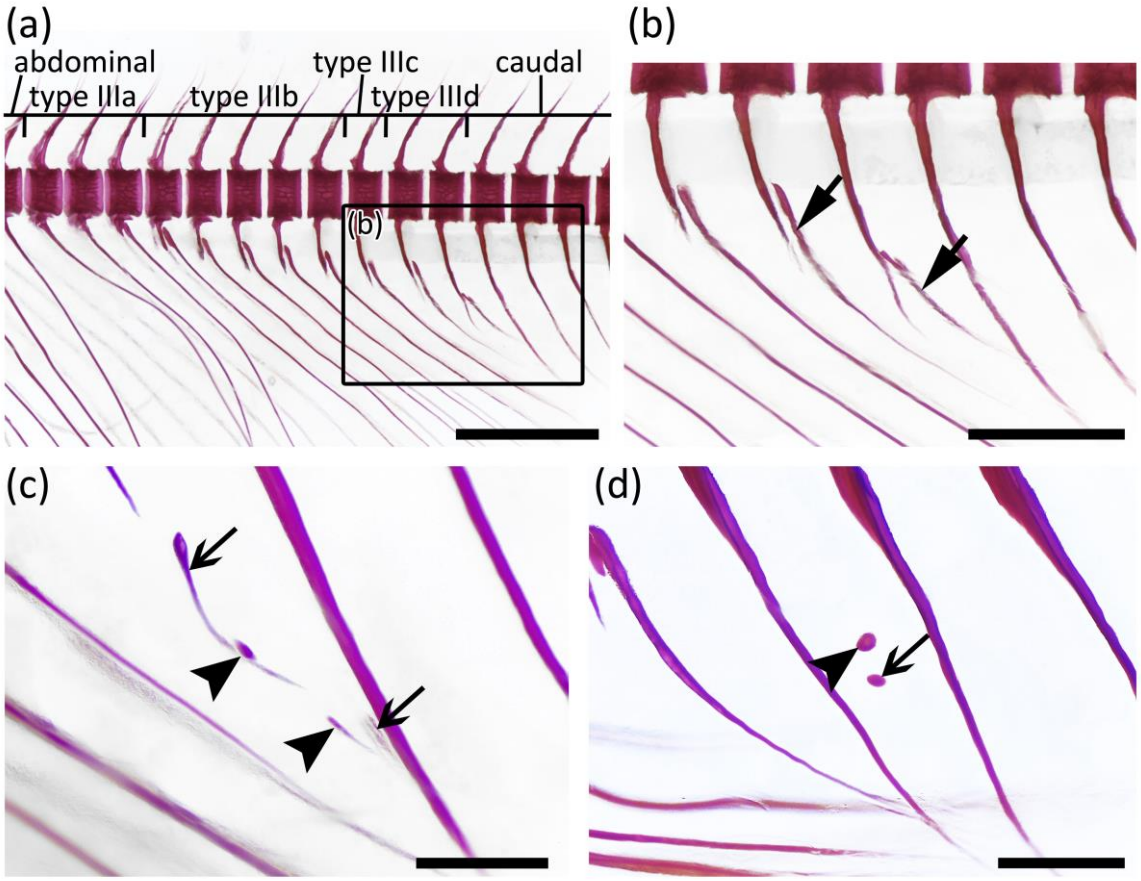

Figure 21: The transitional region, vestigial ribs and specks of bone *in situ*

All images show Alizarin red whole mount cleared and stained Chinook salmon oriented with anterior to the left, posterior to the right. Scale bars of (a) and (b) are 200 μm and for (c) and (d) 50 μm. The entire transitional region is shown in (a), with anteriorly an abdominal vertebra and posteriorly caudal vertebrae. Subtype transitional vertebrae are indicated, with the shown specimen having 3 type IIIa, 5 type IIIb, 1 type IIIc and 2 type IIId vertebrae. The box in (a) indicates the vestigial ribs of type IIId transitional vertebrae shown enlarged in (b). The arrows in (b) indicate the vestigial ribs on the left side of the specimen. The arrowheads in (c) and (d) show the vestigial ribs on the left side while the line arrows show the vestigial ribs on the right side of the specimens. The dorsal-most vestigial rib on the left side in (c) and the vestigial ribs in (d) are spherical specks of bone. The ventral most vestigial ribs in (c) are elongated specks of bone.

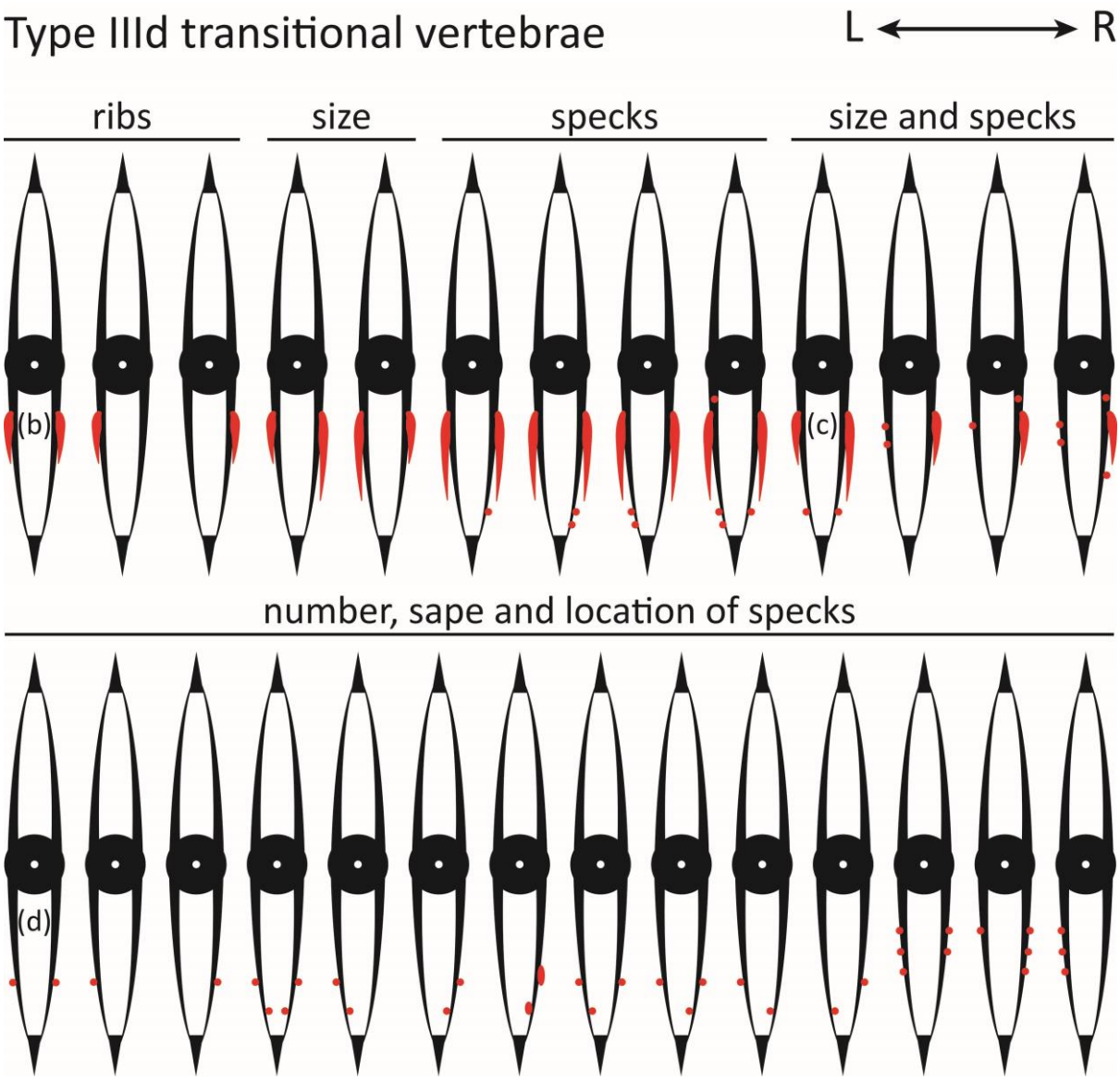

Figure 22: Schematised caudal view of posterior-most transitional vertebral centrum

Variation occurs mainly in the left-right presence or absence of the vestigial ribs, but also in rib size (shown in the `size` section). The presence of spherical vestigial specks of bone can vary in combination with ribs (shown in the `specks` and `size and specks` sections). Vestigial specks of bone also occur without vestigial ribs and vary in number, location and shape (shown in red in bottom row). The shape of vestigial specks varies from spherical to elongated. The orientation is indicated in the top right of the figure: L = left, R = right. The observed variations of vestigial ribs and specks in Figure 21 are indicated by (b) = Figure 21b, (c) = Figure 21c, (d) = Figure 21d.

5.5. DISCUSSION

In this study variation of the total number of vertebral centra was observed within each temperature group, but was not significantly different when compared between the two temperature groups. A similar total number of vertebrae however may mask significant differences in the number of transitional vertebrae as well as variation in the postcranial and ural regions. These differences would

go unnoticed if only total vertebral count was compared between the two temperature groups. Importantly, all regions of the vertebral column of Chinook salmon in the 8 and 12°C groups show meristic variation.

Variation of total number of vertebrae (not counting ural vertebrae) has been related to temperature and has been studied in salmonids (Beacham, 1985; Beacham & Murray, 1986; Fleming, 2013; Fraser et al., 2015; Kwain, 1975; Lindsey et al., 1984), threespine stickleback (Ahn & Gibson, 1999a; Lindsey, 1962), paradise fish (*Macropodus opercularis*; Lindsey, 1954) and zebrafish (Ackerly & Ward, 2016; Sfakianakis et al., 2011). In the current study, the total vertebral body number ranges from 62 to 68. Seymour (1959) reported the range for the total number of vertebrae (not counting ural centra) to be from 63 to 70 in Chinook salmon from the Sacramento River (California, USA), stock from which the New Zealand Chinook salmon originated in the early 1900's (McDowall, 1994). Given similar water temperatures, the difference in total vertebrae range (lower in current study) could be due to factors other than water temperature.

Sacramento River Chinook salmon were found to have a higher level of genetic variation (more alleles per locus, polymorphic loci and greater mean heterozygosity) than New Zealand Chinook salmon (Quinn & Unwin, 1993). A higher level of microsatellite variation was also found in Chinook salmon from Battle Creek, a tributary to the Sacramento River (Kinnison, 1999). These observations indicate a modest bottleneck effect may have occurred in New Zealand Chinook salmon. However, Quinn et al. (2001) argued that the bottleneck effect was not great enough to prevent phenotypic divergence among the New Zealand populations.

Life history traits such as average age, mean length and weight at maturity, timing of entry into fresh water and arrival at spawning grounds were shown to differ between populations living in New Zealand rivers and the native Sacramento River (Quinn & Unwin, 1993). This indicates that Chinook salmon are, phenotypically and genetically, locally adapted and therefore different from their source populations in California (Quinn & Unwin, 1993).

An apparent difference between Chinook salmon investigated by Seymour (1959) and those reported here is that the fish in current study were propagated from farmed stocks. It was previously suggested that rearing conditions could produce changes in meristic variations of skeletal structures (Boglione et al., 2001). For example, the number of vertebrae, and dorsal, pectoral, anal and lower principal caudal fin rays was reported to show higher meristic variation in hatchery-reared compared to wild-caught juveniles of gilthead sea bream (*Sparus aurata*; Boglione et al., 2001). A study in rainbow trout showed that increased domestication levels reduced the total number of vertebrae and the number of dorsal and anal pterygiophores and rays (Pulcini et al., 2015). Zebrafish maintained at different temperatures but with a similar total number of vertebrae show variation in caudal, and to a lesser degree in abdominal, vertebrae (Ferreri et al., 2000). In wild and laboratory reared zebrafish the

number of transitional vertebrae showed significant differences (Ferreri et al., 2000). Wild zebrafish (30°C) were shown to have a higher proportion of specimens with 0 to 1 transitional vertebrae, while reared zebrafish (27.5°C) had a higher proportion of specimens with 1 to 2 transitional vertebrae (Ferreri et al., 2000). This observation, with fewer transitional vertebrae in warmer water is in contrast to what is observed in Chinook salmon in the present study, where fewer transitional vertebrae occurred in the colder 8°C group specimens.

Variation in the total and regional number of vertebrae can be seen as phenotypic plasticity, that is, a single genotype expressing different phenotypes in response to the environment (Pigliucci, 2001). For example, in threespine stickleback, the vertebrae between the second dorsal spine pterygiophore and first anal fin pterygiophore were found to be most variable with respect to temperature and salinity (Lindsey, 1962). Interestingly these vertebrae have parapophyses changing stepwise from anterior to posterior into haemal arches, recently defined as transitional vertebrae (De Clercq et al., 2017b). Important to note is that this region corresponds to the only region found in the present study to show significant variation. Meristic variation of vertebrae in salmonids can also relate to the genetic background of different populations (Daisei et al., 2008; Vernon, 1957). Since the current study investigates animals with a homogenous genetic background and it focuses on an environmental factor, i.e. temperature. However, it is hard to speculate about the effects of the genetic background on the present observations. Moreover, an interaction between genotype and environment can be expected, in line with a suggestion made by Beacham (1985) and Beacham & Murray (1987a). These authors suggested that natural selection produced stocks of sockeye and chum salmon in the rivers of the North American West Coast (USA, Canada and Alaska) that are adapted to the different environments and that a genotype x environment interaction underlies the observed meristic variation of vertebrae, gill rakers and branchiostegal rays.

The vertebral centra anlagen in teleosts are formed as a result of the early metameric mineralisation of the notochord sheath, called the chordacentra (Arratia et al., 2001; Bensimon-Brito et al., 2012b; Grotmol et al., 2003; Grotmol et al., 2005; Huxley, 1859; Kölliker, 1859). There is increasing evidence that this process is independent from sclerotome-derived osteoblasts (Fleming et al., 2004; Fleming et al., 2015; Lleras Forero et al., 2018; Pogoda et al., 2018; Yu et al., 2017). In addition, the number of vertebrae can be modified only in early embryogenesis (Lindsey & Ali, 1965). Taning (1952) described three temperature sensitive time periods in sea trout, i.e. (i) the late blastula stage, (ii) the closure of blastopore (at the end of epiboly) and (iii) the time period when the last somites form. Murray and Beacham (1989) found that moving chum salmon specimens to colder water temperatures at the completion of epiboly stage and initial eye pigmentation time points can change the number of vertebral centra.

From a mechanistic viewpoint, variation in the postcranial, transitional and ural regions may be also explained in one of two other ways: either the number of vertebrae in a specific region changes, i.e. regions expand or reduce in numbers, or vertebral number in a specific region remains essentially unchanged, but the associated elements undergo a vertebral identity change (homeotic transformation) leading to change in identity.

Vertebral identity changes (homeotic transformations) could also underlie the variation observed in the transitional region. The borders at which the morphological identity of vertebrae changes in the mammalian vertebral column is mostly aligned with the anterior border of *Hox*-gene expression domains (Asher et al., 2011; Burke et al., 1995; Galis, 1999). However in teleosts, *Hox*-gene expression patterns do not strictly align with morphological changes of associated elements along the longitudinal axis. For example in zebrafish, the anterior boundary of *Hoxc-6* expression aligns with the anatomical postcranial-abdominal border, while the *Hoxd12a* anterior expression boundary is located in the middle of the transitional region and is not aligned with any distinct anatomical border (Burke et al., 1995; Holley, 2007; Morin-Kensicki et al., 2002). Recent research in the mouse showed that *Hox*-cluster micro-RNA's of the miR-196 family can independently modulate the number of vertebrae in the thoracic region. Moreover, miR-196 family members are evolutionary conserved in vertebrates (Wong et al., 2015). The more anterior position and higher number of subtype IIIa transitional vertebrae in the specimens raised at 8°C could be the result of changes in the identity of the associated elements rather than a change in the number of vertebrae.

Other than forming the anatomical transition between the abdominal and caudal vertebrae, the role of (each subtype of) transitional vertebrae in Chinook salmon is currently unknown. In black mackerel the parapophyses of transitional vertebrae form bullae which house evaginations of the gas bladder (Bond & Uyeno, 1981). In Atlantic salmon and zebrafish the parapophyses of transitional vertebrae are connected with a bony bridge to from the haemal canal (Ford, 1937). The parapophyses in the transitional region of Chinook salmon likely have a similar function to parapophyses in Atlantic salmon and zebrafish (De Clercq et al., 2017b).

Studies of the caudal fin skeleton showed that a tail with two ural centra, also called a diural tail, evolved from a polyural tail via regressive evolution (Bensimon-Brito et al., 2012a; Eastman, 1980; Gosline, 1961; Wiley et al., 2015), i.e. skeletal elements were reduced, modified and fused. Salmonids have a diural tail (Arratia & Schultze, 1992) and have a high degree of natural variation of vertebrae and vestigial elements, i.e. epurals and uroneurals in the ural region. Compared to the 8°C group, where almost all specimens had two ural centra, specimens of the 12°C group showed more variation in the number of ural centra. Arratia and Schultze (1992) stated that the presence of two ural vertebrae is typical for hatchery reared Pacific salmon, including Chinook salmon. Interestingly, more than two ural centra, up to five, were almost consistently observed in animals reared at 12°C which is 4°C

above the Chinook salmon optimum temperature. More than two ural vertebral anlagen (i.e. chordacentra) have been observed before in salmonids (Arratia & Schultze, 1992); (Arctic charr (*Salvelinus alpinus*), Grünbaum & Cloutier, 2010). Vestiges and rudiments of ural centra have also been found in zebrafish and have been considered atavistic rather than an anomaly (Bensimon-Brito et al., 2012a).

It has been well established that increased mechanical loads transmitted to the bone tissue by muscle forces promote bone formation and accelerates bone mineralisation in teleost fish, as is generally the case in vertebrates (Hall & Witten, 2018; Huysseune et al., 1994; Witten & Hall, 2015). Muscle activity was shown to induce the earlier appearance of cartilaginous structures, such as parapophyses, dorsal and anal fin pterygiophores, preural haemal and neural arches and hypurals in zebrafish (Fiaz et al., 2012). In addition, muscle activity was also shown to induce changes in timing and ossification rate of vertebral centra and their associated elements in zebrafish and Arctic charr (Cloutier et al., 2010; Fiaz et al., 2012; Grünbaum et al., 2012). The vestigial ribs in Chinook salmon are located in the myosepta of the antero-ventral caudal muscle tissue bordering the abdominal cavity. Interestingly, variation in the presence and absence of vestigial ribs was more prominent in the 12°C compared to 8°C specimens. Higher variation in the 12°C group could be explained by the combined effect of the elevated temperature regime and the increased activity at 12°C. Variation of vestigial transitional ribs could be useful to assess asymmetry, as an index of developmental instability, together with other documented skeletal elements showing fluctuating asymmetry such as branchiostegal rays, fin rays and gill rakers (Allenbach, 2011; Dongen, 2006; Leary & Allendorf, 1989; Parsons, 1990).

In conclusion, regional meristic variation is present even when the total number of vertebrae is not significantly different. Importantly, different skeletal elements showing meristic variation do not always respond in the same way to environmental changes (Fowler, 1970; Murray & Beacham, 1986; Taning, 1952). The present study lends further support to the view that vertebral centra and associated elements represent developmental modules of the vertebral column (de Azevedo et al., 2012; Detwiler & Holtzer, 1956; Hall, 1977; Holtzer, 1951; Strudel, 1953a; b; To, Witten, Huysseune & Winkler, 2015; Witten, Takle, Baeverfjord & Huysseune, 2007). The regional meristic variation could be caused by a change in the number of centra anlagen (i.e. chordacentra) and therefore a change in the notochord module may occur. Alternatively, a change in the identity of the centrum due to a change of the element associated with the centrum could cause vertebral variation. This would indicate plasticity in the somite derived sclerotome module. A synergistic effect of both the notochord derived chordacentra and sclerotome derived associated elements may also underlie regional variation.

5.6. ACKNOWLEDGEMENTS

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5.7. AUTHOR CONTRIBUTIONS (IN CREDIT TERMS)

A. De Clercq: Conceptualisation, methodology, investigation, writing – original draft preparation, and visualisation

M.R. Perrott: Conceptualisation, writing – review and editing, supervision and funding acquisition

P.S. Davie: Conceptualisation, supervision and funding acquisition

M.A. Preece: Resources and methodology

M.A.G. Owen: Formal analysis, writing – review and editing

A. Huysseune: Writing – review and editing, supervision and funding acquisition

P.E. Witten: Conceptualisation, writing – review and editing, supervision and funding acquisition

CHAPTER 6: LOWER INCUBATION AND FRESHWATER GROW-OUT TEMPERATURES PROTECTS CHINOOK SALMON FROM SUBSEQUENT VERTEBRAL DEFORMITIES IN SEAWATER BUT NOT IN FRESHWATER

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6.1. ABSTRACT

Vertebral anomalies are a severe and recurrent problem in both Atlantic and Chinook salmon farming. Temperature is often manipulated in salmon farming to influence growth rates and to obtain a year-round production. However, temperature is also a risk factor for vertebral deformities. In salmonids, vertebral deformities can develop *de novo* in every life stage, both in freshwater and in seawater growth stages. Here PIT-tagged Chinook salmon, of crosses that produced off-spring with high deformity rates, were incubated and grown in freshwater at a constant 8°C and 12°C before being transferred to sea cages. Salmon were X-rayed at three life stages; the freshwater smoltification stage, the six-months-at-sea-stage and the 12-months-at-sea stage. Radiographs of salmon were evaluated for the presence of four deformity categories: a combination of lordosis, kyphosis and scoliosis (=LKS), fusion, compression and/or reduced intervertebral space, and vertical shift. Deformities were also scored from one (mild) to three (severe). The mortality in both temperature groups was highest between the freshwater smoltification and six-months-at-sea stages. The 8°C group had a higher prevalence of deformities and average severity score in the freshwater stage but a lower prevalence of deformities and average severity score in the seawater stages compared to the 12°C group. LKS was not present in freshwater stage specimens of either temperature group. Specimens that were culled six months after seawater entry had significantly more deformities and LKS in the 12°C group only. Both temperature groups had a significantly higher prevalence of deformities and LKS after the 12-months-at-sea stage compared to after six-months-at-sea. In the current study we highlight that the mortality is high after the seawater transfer stage, and that deformity is also notable in runts, a category of under-performing animals with poorly understood reasons for retarded growth. These phenomena should be better studied since the causes of mortality at this stage are still unknown. Here we also show that incubation and freshwater rearing temperatures can influence the deformity prevalence in subsequent seawater stages.

6.1.1. KEYWORDS

Smoltification, seawater stage, deformities, Chinook salmon, Atlantic salmon

6.2. INTRODUCTION

Vertebral anomalies are a severe and recurrent problem in salmon farming (Fjelldal et al., 2012). It has been established that the underlying cause of vertebral anomalies is multifactorial (Boglione et al., 2013a; Boglione et al., 2013b; Fjelldal et al., 2012), i.e. a plethora of genetic and epigenetic factors which often interact with each other, underlie vertebral anomalies. Husbandry factors such as diet, light, temperature, and vaccination are some of the most frequently manipulated factors in farming conditions (Boglione et al., 2013b; Fjelldal et al., 2012). Often several of these factors are changed

simultaneously to obtain fast or slow growing salmon, a necessity for a year-round production. For example, a combination of more frequent feeding, longer light photoperiods and higher rearing temperatures will lead to faster growth of freshwater juvenile stages. Faster growing salmon go into seawater earlier in the year than slower growing salmon and therefore can be harvested earlier in the year. Importantly, changes to specifications in the diet (Ørnsrud et al., 2004a; Ørnsrud et al., 2004b), photoperiod (Fjelldal et al., 2004; Fjelldal et al., 2016), and fast growth juvenile production strategies due to elevated temperatures (Fjelldal & Hansen, 2010; Fjelldal et al., 2006), are all factors known to induce vertebral anomalies. Because several factors are manipulated at once, it is hard to ascertain which factor is responsible for causing vertebral anomalies. In contrast, a single factor, i.e. the lack of dietary phosphorous, could be linked to a single vertebral deformity phenotype (Witten type 10), undersized vertebrae with increased intervertebral spaces (Witten et al., 2009), in Atlantic salmon (*Salmo salar*) that were four months in seawater (Witten et al., 2016). Moreover, many one-factor drivers for skeletal deformity have been mitigated via improvements to the diet.

Among the many environmental factors, temperature stands out as one of the master factors in fish biology (Beitinger et al., 2000). Temperature influences developmental rate (Beacham & Murray, 1989), mortality (Beacham & Murray, 1987a; Murray & Beacham, 1986), success of hatching (Hamor & Garside, 1976; Hayes et al., 1953) and normal skeletal development (Koumoundouros et al., 2001) in early stages of development. Also in later stages of development temperature is important for normal skeletal development (Cloutier et al., 2010; Grünbaum & Cloutier, 2010). Elevated rearing temperature reduces the production time in salmon farming but increases the risk of abnormal vertebral development (Fjelldal et al., 2006; Grini et al., 2011; Ytteborg et al., 2010a; Ytteborg et al., 2010b) and can induce a wide variety of vertebral deformity phenotypes (Witten et al., 2009). Fast growing Chinook salmon (*Oncorhynchus tshawytscha*) eggs and freshwater juveniles in New Zealand are incubated at ~12°C, close to the upper temperature limit of 13°C for early incubation (Brett, 1952). This upper temperature limit, and lower temperature limit, in Chinook salmon was determined by lethal temperature experiment on wild caught and laboratory reared salmon of different populations along North-South latitude acclimated to different temperatures (Alderdice & Velsen, 1978; Brett, 1952). The same experiments showed that the preferred temperature range for incubation and freshwater grow-out of Chinook salmon was between 8°C and 10°C (Alderdice & Velsen, 1978; Brett, 1952).

Vertebral deformities in salmonids are often diagnosed and studied using radiography (Fjelldal et al., 2012; Gill & Fisk, 1966; Witten et al., 2009; Witten et al., 2016). Based on radiographic images of Atlantic salmon vertebral columns, vertebral deformity phenotypes have been classified into 20 types (Witten et al., 2009). Within the 20 types of vertebral deformities a further subdivision into three major categories can be made, i.e. (i) vertebral column curvatures, (ii) vertebral centra compressions and fusions, and (iii) vertebral centrum deformities (Witten et al., 2009). Vertebral axis deviations

include lordosis (ventral bending), kyphosis (dorsal bending) and scoliosis (sideways bending). Vertebral centra compressions and fusions include varying degrees of compression, fusions and/or complete fusions along the antero-posterior axis of the vertebrae. Vertebral centrum deformities include radio-translucent and radio-dense centra, and deviations from the autocentrum symmetry within the vertebral centrum. Vertebral deformities such as fusion and compression are most common in Atlantic salmon (Fjelldal et al., 2012; Witten et al., 2005a; Witten et al., 2006), while vertebral column curvatures have been observed but are less frequent (Fjelldal et al., 2004; Gil Martens et al., 2012; Sullivan et al., 2007b). In Chinook salmon, fusion was reported as the most common vertebral deformity in freshwater juveniles (Seymour, 1959). In other Pacific salmon species, i.e. sockeye, pink and chum salmon (*O. nerka*, *O. gorbuscha* and *O. keta*, respectively) a varying degree of compression and fusion was reported in juvenile and adult life stages (Gill & Fisk, 1966).

It has been stated in literature that vertebral deformities can develop at any salmonid life stage (De Clercq et al., 2017a; Fjelldal et al., 2012; Witten et al., 2006). Temperature has been found to be a risk factor in both early life stages, where elevated temperature and temperature shocks induce a range of notochord and vertebral malformations (De Clercq et al., 2017a; Takle et al., 2005; Wargelius et al., 2005), and late life stages (Fjelldal et al., 2006; Grini et al., 2011). More recently in studies of Chinook salmon the development of certain vertebral deformities appeared to be life-stage dependent. While the commonly occurring vertebral deformities such as fusion, compression and vertical shift of the entire vertebral centrum can develop both in freshwater pre-smoltification and seawater post-smoltification stages, vertebral column curvatures only appear to develop in late seawater stages (Munday et al., 2016; Munday et al., 2018; Perrott et al., 2018).

In the current study we ask if hatchery rearing at 12°C predisposes Chinook salmon to higher deformity rates overall, whether or not specific deformity phenotypes are more prevalent and if salmon are more prone to deformity at certain life stages, compared to specimens raised at a more optimum temperature of 8°C. The deformity types considered in the current study have been previously described in Chinook salmon by Perrott et al. (2018), i.e. LKS (a combination of lordosis, kyphosis and scoliosis), fusions, compression and/or reduced intervertebral spaces and vertical shift of the entire vertebral centrum. The juvenile and adult life stages considered here span pre-smoltification (~12 months rearing in freshwater), and post-smoltification, a period of known transition and stress. Specimens were followed and evaluated after six months and 12 months of exposure to a seawater husbandry environment.

6.3. MATERIAL AND METHODS

6.3.1. BROODSTOCK

Commercial slow growing Chinook salmon adults were used to set up crosses that produced offspring with high deformity rates (Table 2, *SECTION 2.2*). Broodstock were kept at a constant 11.8°C (Takaka Hatchery, Takaka, New Zealand). Females with high X-ray based deformity rates were crossed with sex-reversed males (juvenile females treated with 17-α-methyltestosterone, Sigma-Aldrich) with low X-ray based deformity. Adults which provided offspring with high deformity rates were chosen to increase the chance of less frequent deformities occurring in the experimental specimens. Eggs from ten females were fertilised with milt from six males at 10°C. The gametes of both the females and males were randomised to avoid any effect of the genetic background of specific crosses confounding the constant temperature experiment (*APPENDIX B*).

6.3.2. EXPERIMENT

To avoid aberrant effects of potential temperature shocks after fertilisation, eggs were placed in incubation trays (±4000 eggs/tray) and were supplied with water at a constant 10°C until 3 days post-fertilisation. Subsequently, the temperature was adjusted (0.2°C per hour) to a constant 8°C and 12°C for rearing. Dead specimens were removed every two days during the hatching period and twice a week before and after the hatching period until specimens reached first feed stage. At first feed stage (±900°d) 4000 specimens of each temperature group, i.e. the 8°C and 12°C groups, were moved to two black 420 L circular tanks (2000 specimens per tank) (Fig. 23). The 8°C and 12°C temperature regimes were continued in the tanks. Once dead individuals were removed, the tanks were carefully cleaned every day to reduce the impact of stress on the specimens. The removal of waste feed from the bottom of the tank and the filter mesh was to avoid infections. At 1400°d (cull 1) and 1500°d (cull 2), 250 individuals were randomly removed from each tank in order to reduce crowding stress, allow for growth and to further reduce the risk of infections (Table 12, Fig. 23). One hundred of the 250 culled specimens of each tank were measured and weighed. A fungal infection of the fins was successfully treated for 1 hour (water flow taps closed) with a 4.5 ppm Halamid solution (1.8g/tank, Agrivantage, Axcentive Asia Pte Ltd, Singapore) before PIT-tagging. Due to fin nipping (biting) some specimens had abraded and sometimes infected tail fins at the end of the freshwater stage, but remained untreated before transfer to seawater.

At 2500°d, 500 specimens of the 12°C group were PIT-tagged (Avi-Tag 162e, Avid Identification Systems Inc, Norco, CA, USA) and moved to 1000 L circular tanks (250 salmon/tank) at ambient temperature (12.4°C, Table 12, Fig. 23) When the 12°C specimens were transferred to bigger tanks the temperature of the 8°C specimens was raised to 12°C over two days (0.2°C per hour during the

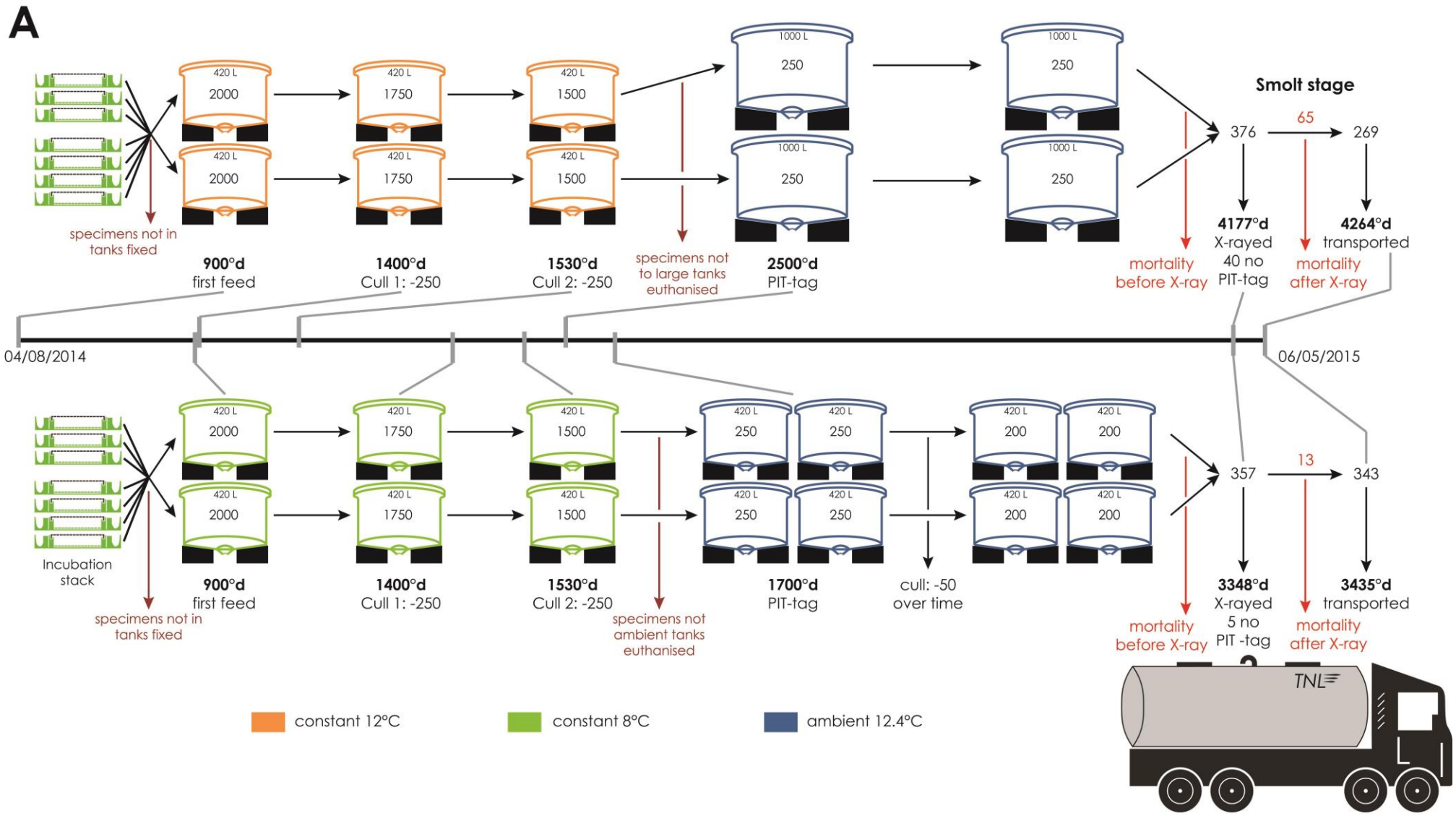
day). Subsequently, 1000 specimens of the 8°C group at 1700°d, now acclimated to 12°C were also PIT-tagged and moved to four 420 L black circular tanks (250 specimens/tank) at ambient 12.4°C (Table 12, Fig. 23). Another 50 specimens per tank were randomly removed to allow for growth and reduce crowding stress after PIT-tagging (Fig. 23). Smoltification-stage salmon (based on size and age) from both temperature groups, 343 specimens of the 8°C and 269 specimens of the 12°C group, were transported at the same time to an experimental sea cage (6x6x6, 216 m^3) at Ruakaka farm in the Marlborough Sounds (Ruakaka Bay, Queen Charlotte Sound). No specimens were tested with a saltwater challenge to determine the freshwater smoltification status in order to avoid reducing the number of animals that would go to seawater and available for statistics. However, specimens were larger than the critical size for successful smoltification of Chinook salmon in New Zealand (> 7.0 g, Table 12) and entered seawater in late autumn (06/05/2015, Table 12, Fig. 23) when Chinook salmon have a physiological smoltification window (Franklin, 1989). Specimens that were classified as 'runts' (animals that failed to thrive, weigh less than one fifth of the average weight of the cohort) at the six-months-in-sea X-ray event were culled and euthanized with anaesthetic overdose (AquiS, AquiS New Zealand Ltd, Lower Hutt, New Zealand).

Smoltification-stage Chinook salmon were X-rayed prior to transportation to sea cages, 357 specimens of the 8°C (3348°d) and 376 specimens of the 12°C (4177°d) (Table 12, Fig. 23). Specimens were anaesthetised for radiography with 20 ppm AquiS and placed on their right side on the film (Kodak Industrex M100 Ready Pack II, Kodak Australia Pty Ltd, Victoria) or on the digital receptor. For films, the X-ray emitter was placed on a distance of 53.5 cm and set at 50 kV, 0.25 mA/sec and 2.1 seconds exposure time. Exposed X-ray films were developed at 21°C for seven minutes, using Kodak Industrex developer and Kodak Industrex LO fixative following manufacturers recommendations. After a week to recover from radiography, the salmon were moved to the experimental sea cage. Digital X-rays were captured using an amorphous silicon digital receptor (90-479 Sound Technologies Tru-DR, DLC Australia Pty Ltd, Melbourne) with the digital receptor focus distance of 50 cm and X-ray settings of 80 kV and 0.45 mA/sec. Individually identified salmon from each temperature group were radiographed at six months and 12 months following seawater transfer (Table 12, Fig. 23), and at harvest (15 months). The X-ray source for both film and digital radiography was an Atomscope HF80/15+ Portable Unit (Mikasa, Tokyo).

Figure 23: Schematised flow diagram of the 8°C and 12°C specimens from freshwater to seawater. (below)

(A) The representative timeline is shown by a black line between the 12°C group trajectory (top, orange) and the 8°C group trajectory (middle, green). At the left of both temperature group trajectories an incubation stack is shown, from which 4000 specimens were moved to tanks (2000 per tank) at first feed (900°d). First feed is indicated on the timeline as the first grey vertical bar. The °d-stage and event is given below the tanks of the freshwater stages. Grey vertical bars on the time line indicate the time points of these stages (see Table 12).

Tanks containing water with ambient temperature are indicated in blue. The number of specimens at each stage is shown in the tanks, underneath the volume of the tank. The stages where specimens were euthanized or died (mortalities) are indicated in red. The exact number of mortalities, if known, is indicated above the arrows in the freshwater stages (see also Table 13). Smolt-stage animals (smoltification-stage) of both temperature groups were transported together (truck) to an experimental sea cage (see also B, barge and truck). (B) The timeline now runs above the sea cages starting at the transfer date. The number of specimens of each temperature group that were transferred is repeated (indicated by 'transported'). The repeatedly measured specimens are indicated by 'repeated measure' (above arrow), in first instance specimens that survived to six-months-at-sea and in second instance specimens that survived to 12-months-at-sea. The number of animals in the sea cage at the two X-ray events (i.e. six-months-at-sea and 12-months-at-sea) is shown in bold letters. The number of mortalities (red) is indicated above and below the arrows in the seawater stages (see also Table 13). The number of runts is indicated between the two sea cages after six-months-at-sea.


A
420 L
2000
1750
1500
1000 L
250
200
specimens not in tanks fixed
900°d
first feed
1400°d
Cull 1: -250
1530°d
Cull 2: -250
specimens not to large tanks euthanised
2500°d
PIT-tag
Smolt stage
376
65
269
mortality before X-ray
4177°d
X-rayed
40 no
PIT-tag
mortality after X-ray
4264°d
transported
04/08/2014
06/05/2015
Incubation stack
specimens not in tanks fixed
900°d
first feed
1400°d
Cull 1: -250
1530°d
Cull 2: -250
specimens not ambient tanks euthanised
1700°d
PIT-tag
cull: -50
over time
357
13
343
mortality before X-ray
3348°d
X-rayed
5 no
PIT -tag
mortality after X-ray
3435°d
transported
TNL
constant 12°C
constant 8°C
ambient 12.4°C

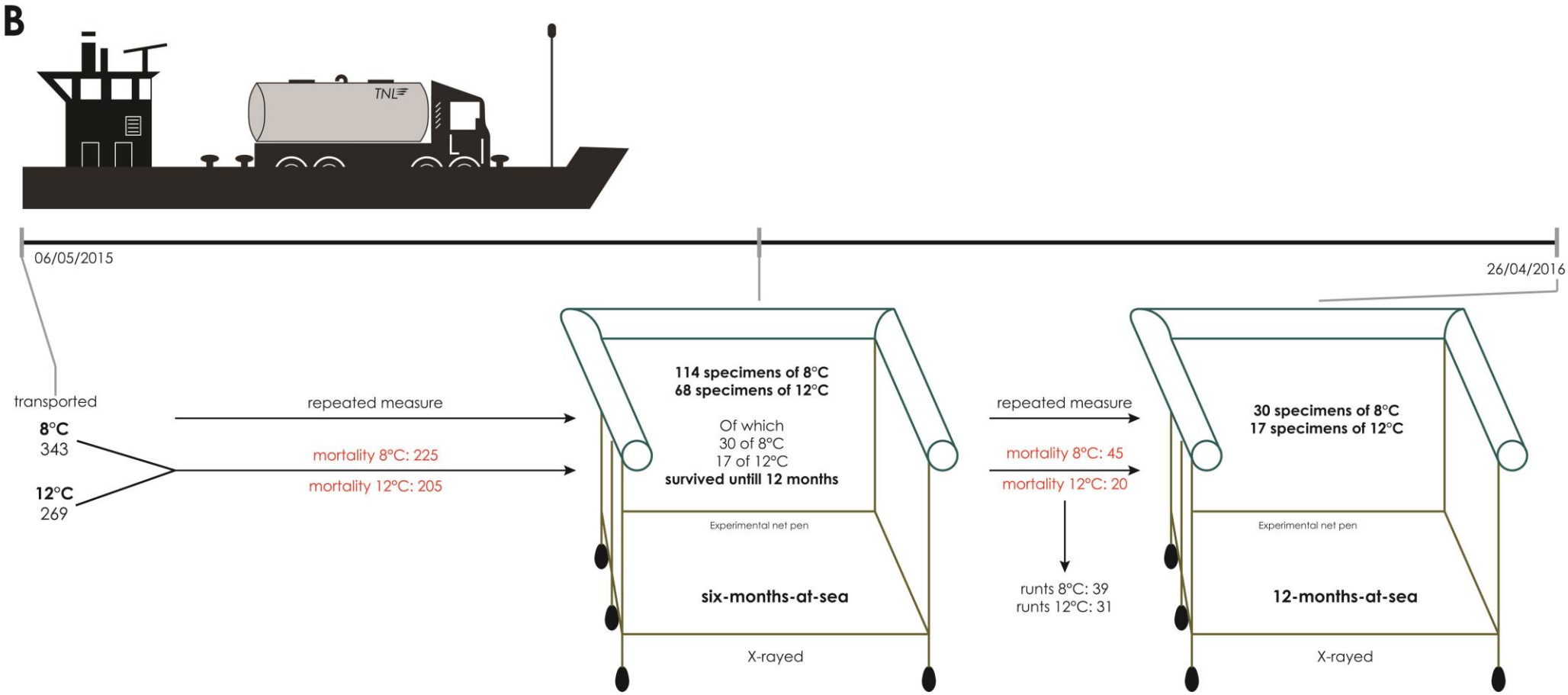

B
TNL
06/05/2015
26/04/2016
transported
8°C
343
12°C
269
repeated measure
mortality 8°C: 225
mortality 12°C: 205
114 specimens of 8°C
68 specimens of 12°C
Of which
30 of 8°C
17 of 12°C
survived untill 12 months
Experimental net pen
six-months-at-sea
X-rayed
repeated measure
mortality 8°C: 45
mortality 12°C: 20
runts 8°C: 39
runts 12°C: 31
30 specimens of 8°C
17 specimens of 12°C
Experimental net pen
12-months-at-sea
X-rayed

Table 12: Summary data of Chinook salmon life stages during the constant temperature experiment in freshwater and in sea.

°d	Temperature	Date	Days	Event	Fork length (cm)	Weight (g)
0	8°C and 12°C	20/05/14	0	Fertilisation		
1400	8°C	11/11/14	175	Cull 1	4.5 (±0.04)	1.5 (±0.03)
1400	12°C	13/09/14	116	Cull 1	5.7 (±0.03)	2.2 (±0.04)
1500	8°C	27/11/14	191	Cull 2	5.4 (±0.04)	1.7 (±0.05)
1500	12°C	25/09/14	128	Cull 2	6.9 (±0.04)	4.0 (±0.06)
1700	8°C	17/12/14	211	PIT-tagging		
2500	12°C	15/12/14	209			
3348	8°C	29/04/15	344	X-ray (film)	16.8 (±0.05)	64.1 (±0.54)
4177	12°C				21.1 (±0.06)	151.6 (±1.53)
3435	8°C	06/05/15	351	Transfer		
4264	12°C					
5543	8°C	23/10/15	521	6-months-sea X-ray (digital)	27.7 (±0.67)	407.1 (±24.86)
6372	12°C				30.6 (±0.88)	501.6 (±43.44)
7849	8°C	26/04/16	707	12-months-sea X-ray (digital)	44.3 (±0.68)	1229.0 (±65.60)
8678	12°C				44.4 (±0.74)	1064.4 (±64.18)

Developmental stage in degreedays (°d). The "Event" column indicates what happened at the corresponding age. The average (±standard error) of the fork length (cm) and weight (g) is given for the cull 1, cull 2, freshwater X-ray, six-months and 12-months-at-sea time points. The three X-ray datasets that were available for analyses are shown in blue.

6.3.3. FEEDING REGIME

First feed specimens of the 8°C and 12°C groups were fed intensively for the first ten days, five days with powder meal (Str. 00, Golden Prima, Biomar) and five days with crushed meal (Crumble, Golden Prima, Biomar). Subsequently, during the freshwater grow-out period, the growing animals were fed three times a day with food pellets of increasing size (GAP Semi-Float, Biomar). After transfer to the sea cage, specimens were hand fed to satiation with commercially available salmon feeds, the first six months with 3.0 mm size pellets comprising 48% protein, 21% oil and 0.9% available P; the subsequent ten weeks with 4.0 mm size pellets comprising 50% protein, 22 % oil and 0.8% available P; finally with 9.0 mm pellets until harvest comprising 39% protein, 26% oil and 0.6% available P.

6.3.4. DATA COLLECTION AND STATISTICS

Deformities were identified within vertebral column regions and scored following the procedure in Perrott et al. (2018). Deformities were identified and scored on developed X-ray film that was placed

on a light box while deformities in digital X-ray images were identified and scored by viewing the image in Photoshop software.

The vertebral column was subdivided into four regions following Kacem et al. (1998): region 1 includes vertebrae 1 to 8 (V1-V8), region 2 includes vertebrae 9 to 31 (V9-V31), region 3 includes vertebrae 31 to 50 (V31-V50) and region 4 includes vertebrae 51 to 63+ (V51-V63+). Variation in the number of vertebrae is present in region 4 and therefore the 63+ indication is used.

Vertebral deformities were recognised based on Witten et al. (2009) classification of 20 types of vertebral deformities. In the present study, a combination of lordosis, kyphosis and scoliosis (Witten et al., 2009, type 14, 15, 16), fusion (Witten et al., 2009, type 6, 7, 8), compression of vertebrae and or intervertebral spaces (Witten et al., 2009, type 1, 2, 3, 4, 5, further referred to as compression), and vertical shift within the entire vertebral centrum (Witten et al., 2009, type 17) were recognised. These specific deformity types were chosen because they can be compared to other studies investigating deformities in farmed Chinook salmon.

In addition to recognising the deformities, a severity score of 1 to 3 was assigned to each deformity. The score is based on the number of vertebrae involved in the deformity and the angle of the curvature in lordosis and kyphosis. Assessment of the severity of scoliosis was more subjective when it was not present in combination with lordosis and kyphosis.

- Severity 1: 1 to 2 vertebrae or 0 °to 20°
- Severity 2: 2 to 5 vertebrae or 20° to 40°
- Severity 3: 5+ vertebrae or >40°

The absence or presence of each deformity was scored per region by 0 or a 1, absent or present respectively. For total deformity, a score of 1 was allocated when the specimen had a deformity of any type in any region, even when different types of deformities in multiple regions along the vertebral column occurred. In addition, a parallel dataset was generated in which each of the present deformities were given a severity score (1 to 3). All statistical analyses were performed in R-studio Team (2015, R-studio Inc., Boston). Figures were constructed with ggplot2 package (R-studio) and Photoshop CS5.

Fork length and weight were measured at two freshwater culling events (cull 1 and cull 2), at the pre-transfer X-ray event and at the sea water X-ray events at six and 12 months at sea (Table 12). The average fork length and weight of the 8°C and 12°C specimens were tested for significant differences with the non-parametric Wilcoxon rank sum test. This was due to the fact that the data were not normally distributed and that the variances were unequal.

Two datasets from the freshwater smoltification-stage X-ray scoring were constructed. The first dataset contained the scoring of all smoltification-stage salmon (dataset 1). The second dataset contained only the scoring of smoltification-stage salmon that survived to six-months-at-sea (dataset 2). A general linear model (glm, outcome variable binomial, Fisher exact method) was used to test for statistically significant differences between the proportion of deformities (prevalence) of the 8°C and 12°C specimens at freshwater smoltification-stage (dataset 1 and dataset 2), and at six-months-at-sea (all specimens). To increase the robustness of the glm-model the fork length and weights of each individual were added as variables to the model. Fork length and weights of a number of freshwater smoltification-stage salmon were missing due to a random set of specimens that had no PIT-tag. Multiple imputation (5 iterations, 50 times, assuming missing data completely at random) on the fork length and weight variables in the freshwater dataset was applied (mice package) to fill the missing data points. For the six-months-at-sea dataset, a culling variable (whether or not a specimen was removed after X-ray, 'runts') was also added. Because a low prevalence was observed for fusion, compression and vertical shift in the seawater datasets, resulting in 2x2 contingency tables with low values, the Firth logistic regression (logistf package) was used to test for significant differences in the prevalence of deformities between the 8°C and 12°C groups on all datasets. The statistical output values reported in the results are based on the Firth logistic regression except when indicated otherwise. The Wald test indicates the fit of model. To test for significant differences in the prevalence of total deformity proportion and LKS proportion from the freshwater to the 12-month-at-sea stage (see repeated measure Fig. 23), a conditional logistic regression for matched paired binary data (survival package) was used.

The severity score data for the freshwater smoltification-stage (dataset 1 and dataset 2), and at six-months-at-sea (all specimens) was not distributed normally and had unequal variances. The average of the score data was tested for significant differences between the 8°C and 12°C groups with the non-parametric Wilcoxon test. The average score was calculated on the sum of scores, for total deformity:

$$\Sigma_i(\text{score } i^{th} \text{ deformity type } (\Sigma_i(\text{score } j^{th} \text{ region})))/\text{number of specimens}$$

The average score for each deformity type:

$$\Sigma_i(\text{score } j^{th} \text{ region})/\text{number of specimens}$$

Table 13. Attrition of Chinook salmon specimens raised at a constant 8°C and 12°C available for X-ray exposure at different life stages.

Life stage	X-rayed	No PIT-tag	Mortality	Culled
Pre-seawater transfer 8°C	357	5	238 (225 + 13)	
Pre-seawater transfer 12°C	376	40	270 (205+65)	
6-months-at-sea 8°C	114		45	39
6-months-at-sea 12°C	68		20	31
12-months-at-sea 8°C	30		10 (8 + 2)	
12-months-at-sea 12°C	17		10 (7 + 3)	
15-months-at-sea 8°C	20			
15-months-at-sea 12°C	7			

The "Mortality" column shows the total of specimens that died (died between life stages + died at time of X-ray). The "Culled" columns shows the specimens removed due to growth arrest (runting).

6.4. RESULTS

6.4.1. ATTRITION FROM FRESHWATER SMOLTIFICATION TO 15-MONTHS-AT-SEA STAGE

The highest attrition occurred in the 12°C group where only 1.9% of the specimens survived, compared to 5.6% in the 8°C group, from the freshwater smoltification-stage until 15 months at sea (harvest, Table 13). More specimens in the 12°C group (40) compared to the 8°C group (5) lacked a PIT-tag and were culled. Also in the 12°C group more specimens (65) died during the X-ray event compared to the 8°C group (13). The culling of specimens due to an arrest in growth (runting) only occurred at the six-months-at-sea X-ray event, with 39 specimens culled in the 8°C group and 31 in the 12°C group. The largest number of mortalities occurred between the X-ray time points in both temperature groups, especially between the freshwater X-ray time point and the six-months-at-sea X-ray time point, with a larger attrition rate in the 12°C group (71.8%), compared to the 8°C group (66.7%). Due to high mortality rates in specimens of both temperature groups, only a few animals were left to X-ray at 15-months-at-sea and at harvest (Table 13). No biologically meaningful statistical analyses could be made with these very small sample sizes. Thus, the 15-months sample point is not included in this report.

6.4.2. LENGTH AND WEIGHT

Although specimens of the 12°C group had a higher average fork length and weight compared to the 8°C group in freshwater, their average fork length and weight appeared to be no longer different at six months in seawater (Fig. 24, *APPENDIX C*). In freshwater, the average (±SE) fork length and weight of 200 specimens during both culling events (cull 1 and cull 2) are given in Table 12. Specimens raised

at 12°C had significantly greater fork lengths (p-value < 0.0001) and weights (p-value < 0.0001) compared to 8°C specimens during both culling events (Fig. 24A). Smoltification-stage juveniles of the 12°C group also had significantly greater lengths (p-value < 0.0001) and weights (p-value < 0.0001) at the pre-transfer X-ray time point (Fig. 24B). In seawater, at the six-months-at-sea stage the 12°C salmon were still significantly larger (p-value = 0.00038) and heavier (p-value = 0.0023) although the difference was less significant compared to freshwater stages (Fig. 24C). At 12-months-at-sea the 8°C and 12°C specimens had no significant differences in their length and weight, however the sample number at this life stage was low (Fig. 24C). After the six-months-at-sea stage X-ray event 39 specimens in the 8°C group and 31 specimens in the 12°C group were culled due to runting (Table 13). Both the fork lengths (p-value < 0.0001) and the weights (p-value < 0.0001) of runts were significantly smaller compared to the not culled specimens (Fig 24D).

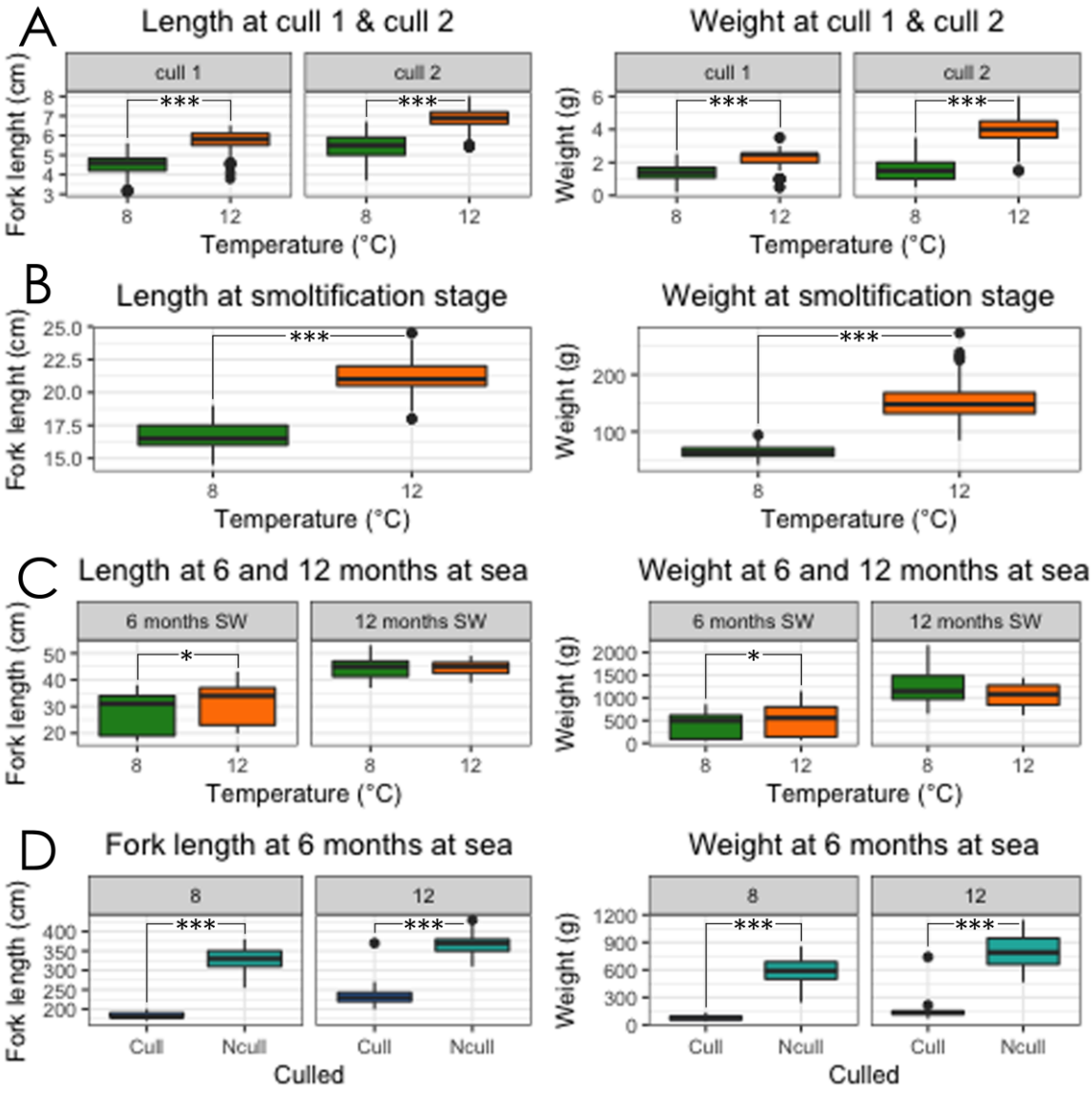


Figure 24: Comparison of fork length and weight of the 8°C and 12°C groups at each life stage.

Boxplot of fork length in centimetre and weight in gram for (A) cull 1 (1400°d, Table 12) and cull 2 (1530°d, Table 12), (B) freshwater smoltification-stage (indicated as 'at smoltification stage'), (C) six- and 12-months-at-sea. The 8°C group is coloured in dark green and the 12°C group in orange. Asterisks indicate significant differences. (D) culled (runts) and not culled (Ncull) animals.

6.4.3. PREVALENCE OF DEFORMITIES

6.4.3.1. *FRESHWATER SMOLTIFICATION-STAGE SPECIMENS (TABLE 14, FIG. 25A)*

Before transferring the Chinook salmon from fresh water to seawater, the 8°C (357) and 12°C (376) specimens were X-rayed. Temperature was found to be a statistically significant predictor of deformity outcome (glm, 2947df, p-value < 0.0001). The total deformity prevalence was significantly higher in specimens of the 8°C group (glm, 2947df, p-value < 0.0001), compared to specimens of the 12°C group. Specimens of the 8°C group had a higher but non-significant prevalence of fusion,

compression and vertical shift compared to specimens of the 12°C group. No LKS was present in freshwater stage Chinook salmon of either temperature group.

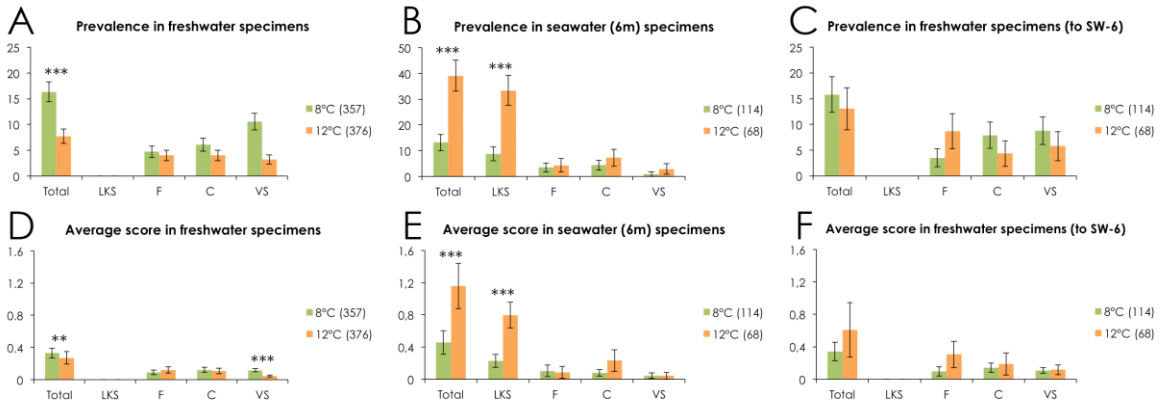


Figure 25: Prevalence and average severity score of deformities in Chinook salmon from the 8°C and 12°C groups.

Freshwater smoltification-stage (A, D), six-months-at-sea (6m) stage (B, E) and freshwater specimens that survived to six-months-at-sea (C, F, see Fig. 23 repeated measure). The prevalence plots show the percentage of deformity (y-axis). The average score plots show the average severity score (y-axis). Error bars indicate standard error (based on average score). The number of specimens in each temperature group is indicated in the legend of each bar plot. Asterisks indicate significant differences. Abbreviations: Total = total deformity (any deformity type in any region), LKS = lordosis, kyphosis, scoliosis, F = fusion, C = compression, VS = vertical shift, SW6 = six-months-at-sea.

6.4.3.2. SIX MONTHS IN SEAWATER SPECIMENS (TABLE 14, FIG. 25B, 26A, B)

At six months in seawater, 114 specimens of the 8°C group and 69 specimens of the 12°C group were X-rayed. Temperature (p-value < 0.0001) and 'runting' (p-value < 0.0001) were found to be statistically significant predictors for deformity outcome (Wald test 6df, p-value = < 0.0001). Specimens of the 12°C group had a higher prevalence of total deformity and of each deformity type, including LKS, compared to the 8°C group (Fig. 25A). Total deformity (p-value < 0.0001), LKS (p-value < 0.0001) and vertical shift (p-value = 0.032) were significantly more prevalent in specimens of the 12°C group compared to the 8°C group.

Culled specimens of the 8°C group (Fig. 26A) had a higher prevalence of total deformity and LKS, and a lower prevalence of fusion. Compression and vertical shift deformity types occurred only in specimens that were in the not-culled category. The prevalence of deformity types between culled and not-culled specimens was not significantly different.

Culled specimens of the 12°C group (Fig. 26B) had a significantly higher prevalence of total deformity (p-value < 0.0001) and LKS (p-value < 0.0001). Fusion and compression also had a higher

prevalence in culled specimens but this was not statistically significant. Vertical shift occurred only in culled specimens.

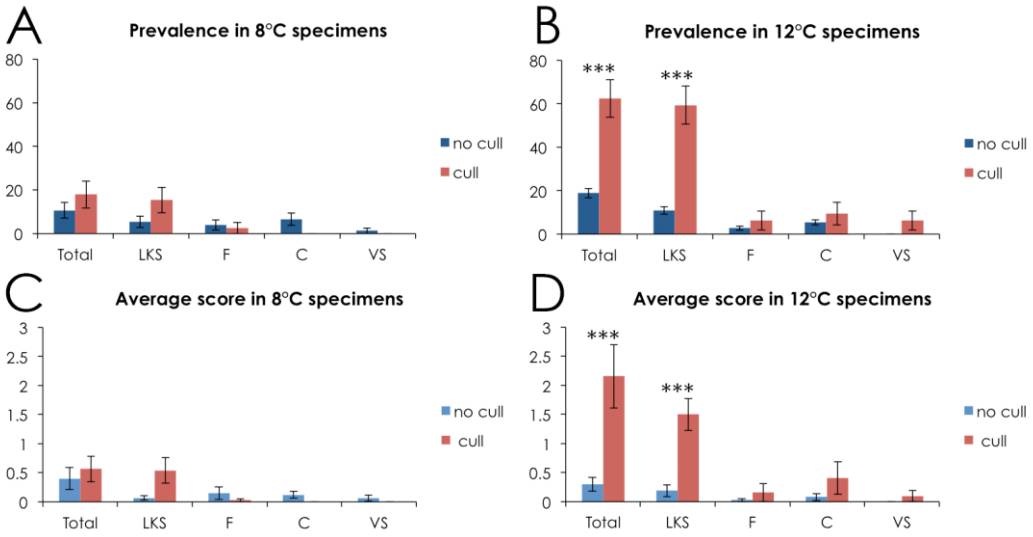


Figure 26: Deformity prevalence and average severity score in not-culled versus culled specimens. Specimens of the 8°C (A, C) and 12°C groups (B, D) at the six-months-at-sea (6m) stage. Specimens were culled due to 'runting'. The prevalence plots show the percentage of deformity (y-axis). The average severity score plots are also shown on the y-axis. Error bars indicate standard error (based on average score). The 8°C group contained 114 specimens, while the 12°C group contained 68 specimens. Asterisks indicate significant differences. Abbreviations: Total = total deformity (any deformity type in any region), LKS = lordosis, kyphosis, scoliosis, F = fusion, C = compression, VS = vertical shift.

6.4.3.3. FRESHWATER SMOLTIFICATION-STAGE SALMON THAT SURVIVED TO SIX-MONTHS-AT-SEA (TABLE 14, FIG. 25C)

Both temperature (p-value < 0.0001) and weight (p-value < 0.0001) were found to be statistically significant predictors of deformity outcome (Wald test 6df, p-value < 0.0001) in this subset of freshwater specimens of both temperature groups. Specimens of the 8°C group had a lower prevalence of fusion, a higher total deformity prevalence and a higher prevalence of compression and vertical shift compared to 12°C group. The differences in prevalence were not statistically significant for any deformity type.

6.4.3.4. PREVALENCE OF TOTAL DEFORMITY AND LKS FROM FRESHWATER TO 12-MONTHS-AT-SEA IN REPEATEDLY MEASURED SPECIMENS (TABLE 16, FIG. 27A)

Only total deformity and LKS were considered when comparing prevalence between life stages because only these deformity types were present in specimens, 30 in the 8°C group and 17 in the 12°C group, that lived in every life stage (see repeated measure Fig. 23). In these specimens, the 12°C group had no deformity of any type at the freshwater smoltification-stage (Fig. 27A). The prevalence

of total deformities was lower in repeatedly measured specimens of the 12°C group at six-months-at-sea but higher at 12-months-at-sea. LKS did not occur in freshwater (Fig. 27B, D). The prevalence of LKS was higher in specimens of the 12°C group at both six-months- and 12-months-at-sea. Temperature was not a significant predictor of either total deformity and LKS. The incidence (prevalence at different life stages) both for total deformity and LKS increased significantly from six-months-at-sea to 12-months-at-sea (p-value < 0.0001, Wald test 3df, p-value < 0.0001). LKS went from a low prevalence at six-months-at-sea to a high prevalence at 12-months-at-sea in specimens of both temperature groups.

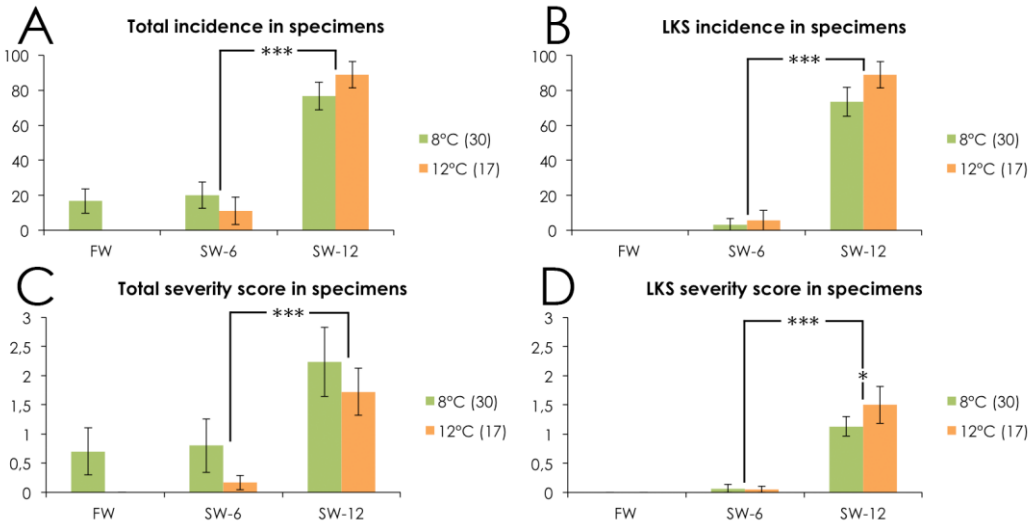


Figure 27: Prevalence and severity score of total deformity (A, C) and LKS (B, D) compared between specimens of the 8°C and 12°C groups repeatedly measured and X-rayed at three different life stages

The prevalence plots show the percentage of deformity (y-axis). The average score plots show the average severity score (y-axis). Error bars indicate standard error (based on average score). The number of specimens included in each life stage was determined by the number of specimens which survived to 12-months-at-sea (see repeated measure Fig. 23), i.e. 30 specimens for the 8°C group and 17 specimens for the 12°C group (indicated in the plot legend). The asterisks connected by lines at the top indicate the significance level between six-months- and 12-months-at-sea stages (*** p-value < 0.001). The asterisk in D above the SW-12 bars indicates the significance level of the average severity score between the temperature groups (* p-value < 0.05). Abbreviations: FW = freshwater smoltification-stage, SW-6 = repeated measure of the same specimens at the six-months-at-sea stage, SW-12 = repeated measure of the same specimens at the 12-months-at-sea-stage.

6.4.4. CULLED SPECIMENS AT SIX MONTHS IN SEAWATER (FIG. 28)

At the six-months-at-sea grading time point, specimens were culled based on their weight, i.e. less than one fifth of the average weight of the cohort. The culled animals were also significantly shorter compared to the not-culled specimens (Fig. 24D). Based on X-ray, culled specimens had either, (i) no vertebral deformities at the freshwater smoltification-stage but vertebral deformities after six months in seawater (Fig. 28A, A'), or (ii) no vertebral deformities both at the smoltification-stage and after

six months in seawater (Fig. 28B, B'), or (iii) vertebral deformities both at the smoltification-stage and after six months in seawater (Fig. 28C, C'). If specimens that were culled at six-months-at-sea had vertebral deformities at the smoltification-stage, then the deformities present at the six-months-at-sea stage were either stable, had progressed or had arisen *de novo*, i.e. extra deformities developed (Fig. 28C, C').

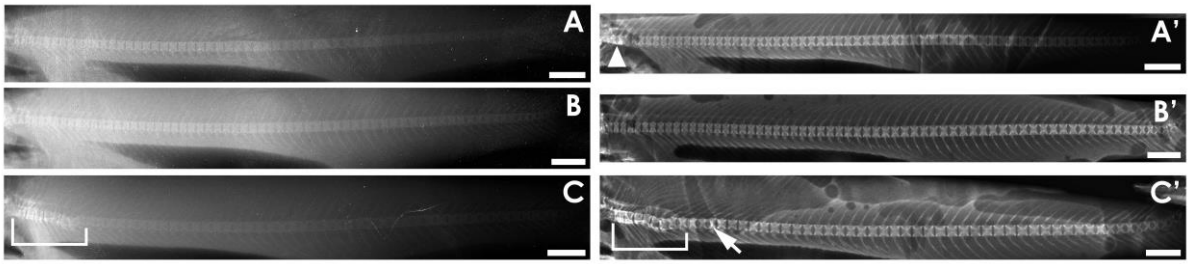


Figure 28: X-rays of culled specimens of the 12°C group.

(A-C) Freshwater smoltification-stage scanned X-ray exposures on film. Specimens in A and B had no vertebral deformities, while the specimen in C has fusion, compression of postcranial and abdominal vertebrae (R1, R2) and lordosis in the abdominal region (R2) as indicated by the white bracket. (A'-C') Digital X-ray of the same specimens as in A-C at six-months-at-sea (grading). The specimen in A' has developed fusion in postcranial vertebrae (R1, arrowhead) after transfer to seawater because pre-transfer no fusion was visible (A). Although the fusion, compression and lordosis complex (bracket) stayed stable in specimen C', an extra compression (in combination with hyper-density, arrow) has developed after transfer to seawater because this was not present pre-seawater transfer (C). All scale bars are 1 cm.

6.4.5. DEFORMITY SEVERITY SCORES

6.4.5.1. *FRESHWATER SMOLTIFICATION-STAGE SPECIMENS (TABLE 15, FIG. 25D)*

Specimens of the 8°C group had a significantly higher average severity score for total deformity (p-value = 0.0011) and vertical shift (p-value < 0.0001) compared to the 12°C group. For compression, specimens of the 8°C group had a higher average severity score and for fusion a lower average severity score compared to the 12°C group, however not significantly.

6.4.5.2. *SIX MONTHS IN SEAWATER SPECIMENS (TABLE 15, FIG. 25E, 26C, D)*

At six months in seawater, specimens of the 12°C group had a significantly higher average severity score for total deformity (p-value = 0.00023) and LKS (p-value < 0.0001), and a non-significantly higher average severity score for compression compared to the 8°C group (Fig. 25E). For fusion, specimens of the 12°C group had a lower average severity score and nearly equal average severity score for vertical shift compared to the 8°C group.

Culled specimens of the 8°C group had a higher average severity score for total deformity and LKS (Fig. 26C). For the fusion deformity type the average severity score was lower in culled animals. The differences in average severity score were not significant.

Culled specimens of the 12°C group had a significantly higher average severity score for total deformity (p-value < 0.0001) and LKS (p-value < 0.0001) and a higher average severity score for fusion and compression (Fig. 26D) that was not significant. Vertical shift only occurred in culled animals.

6.4.5.3. FRESHWATER SMOLTIFICATION-STAGE SALMON THAT SURVIVED TO SIX-MONTHS-AT-SEA (TABLE 15, FIG. 25F)

Specimens of the 12°C group in this subset of freshwater smoltification-stage juveniles had a higher average severity score for each deformity type compared to the 8°C group, but none of the differences were significant.

6.4.5.4. TOTAL DEFORMITY AND LKS FROM FRESHWATER TO 12-MONTHS-AT-SEA IN REPEATEDLY MEASURED SPECIMENS (TABLE 17, FIG. 27C, D)

The average severity score for total deformity was equal for the repeatedly measured specimens of the 8°C group at the freshwater smoltification-stage and at six-months-at-sea (Fig. 27C). Both at six-month-at-sea and 12-months-at-sea the average severity score of total deformity was higher for the repeatedly measured specimens of the 8°C group compared to the 12°C group. No significant differences between the temperature groups were found. For LKS, the average severity score at six-months-at-sea was slightly higher but not significant in specimens of the 8°C group compared to the 12°C group (Fig. 27D). At 12-months-at-sea the average severity score was significantly higher in specimens of the 12°C group (p-value = 0.033, indicated by single asterisks). Both for total deformity (p-value < 0.001) and LKS (p-value < 0.001) the average severity score increased significantly from six-months-at-sea to 12-months-at-sea in both temperature groups.

Table 14. The prevalence (proportion in % (±standard error)) of deformities in Chinook salmon at different life stages.

Group	Temperature/cull	Total	LKS	Fusion	Compression	Vertical shift
Smoltification-stage	8°C (357)	16.3 (±1.95)	0	4.7 (±1.12)	6.1 (±1.26)	10.5 (±1.62)
	12°C (376)	7.7 (±1.38)	0	4.0 (±1.01)	4.0 (±1.01)	3.2 (±0.91)
6-months-at-sea	8°C (114)	13.2 (±3.19)	8.8 (±2.67)	3.5 (±1.74)	4.4 (±1.93)	0.9 (±0.88)
	12°C (69)	39.1 (±6.00)	33.3 (±5.80)	4.3 (±4.30)	7.2 (±3.30)	2.9 (±2.10)
6-months-at-sea 8°C specimens (114)	No cull (75)	10.7 (±3.59)	5.3 (±2.61)	4.0 (±2.28)	6.7 (±2.90)	1.3 (±1.33)
	Cull (39)	17.9 (±6.23)	15.4 (±5.85)	2.6 (±2.56)	0	0
6-months-at-sea 12°C specimens (69)	No cull (38)	18.2 (±2.16)	10.8 (±1.71)	2.7 (±0.90)	5.4 (±1.25)	0
	Cull (31)	62.5 (±8.70)	59.4 (±8.82)	6.3 (±4.35)	9.4 (±5.24)	6.3 (±4.35)
Freshwater specimens that survived to 6-months-at-sea	8°C (114)	15.8 (±3.43)	0	3.5 (±1.73)	7.9 (±2.54)	8.8 (±2.66)
	12°C (69)	13.0 (±4.08)	0	8.7 (±3.42)	4.3 (±2.47)	5.8 (±2.83)

Number of specimens analysed are given between bracket in both the "Group" and "Temperature/cull" columns. The standard error is given in percentage between brackets in the deformity type columns. The "Total" column shows the prevalence of any type of deformity or multiple deformities that may occur in a single specimen.

Table 15. The average severity score (±standard error) of deformities in Chinook salmon at different life stages.

Group	Temperature/cull	Total	LKS	Fusion	Compression	Vertical shift
Smoltification-stage	8°C (357)	0.33 (±0.06)	0	0.09 (±0.03)	0.12 (±0.03)	0.12 (±0.02)
	12°C (376)	0.27 (±0.08)	0	0.12 (±0.04)	0.11 (±0.03)	0.05 (±0.01)
6-months-at-sea	8°C (114)	0.46 (±0.15)	0.23 (±0.08)	0.11 (±0.07)	0.08 (±0.04)	0.04 (±0.04)
	12°C (69)	1.16 (±0.28)	0.80 (±0.16)	0.09 (±0.07)	0.23 (±0.14)	0.04 (±0.04)
6-months-at-sea 8°C specimens (114)	No cull (75)	0.40 (±0.19)	0.07 (±0.03)	0.15 (±0.11)	0.12 (±0.06)	0.07 (±0.05)
	Cull (39)	0.56 (±0.22)	0.54 (±0.22)	0.03 (±0.03)	0	0
6-months-at-sea 12°C specimens (69)	No cull (38)	0.30 (±0.12)	0.19 (±0.10)	0.03 (±0.03)	0.08 (±0.06)	0
	Cull (31)	2.16 (±0.55)	1.50 (±0.28)	0.16 (±0.16)	0.41 (±0.28)	0.09 (±0.09)
Freshwater specimens that survived to 6-months-at-sea	8°C (114)	0.34 (±0.11)	0	0.10 (±0.06)	0.14 (±0.06)	0.11 (±0.03)
	12°C (69)	0.61 (±0.34)	0	0.30 (±0.16)	0.19 (±0.14)	0.12 (±0.06)

Number of specimens analysed are given between brackets in both the "Group" and "Temperature/cull" columns. The standard error is given between brackets in the deformity type columns. The average severity score in the "Total" column is based on the sum of scores of each deformity type in each vertebral column region. The average severity score in the deformity type columns is based on the sum of scores of each vertebral column region.

Table 16. Incidence of total deformity and LKS, prevalence (±standard error) in %, of Chinook salmon at consecutive life stages.

Deformity type	Temperature	Smoltification-stage	6-months-at-sea	12-months-at-sea
Total	8°C (30)	16.7 (±6.92)	20.0 (±7.43)	76.7 (±7.85)
	12°C (17)	0	11.1 (±7.83)	88.9 (±7.62)
LKS	8°C (30)	0	3.3 (±3.33)	73.3 (±8.21)
	12°C (17)	0	5.6 (±5.72)	8.89 (±7.62)

The number of specimens per temperature group is indicated between brackets in the "Temperature" column.

Table 17. The average severity score (±standard error) of total deformity and LKS of Chinook salmon at consecutive life stages.

Deformity type	Temperature	Smoltification-stage	6-months-at-sea	12-months-at-sea
Total	8°C (30)	0.70 (±0.40)	0.80 (±0.46)	2.23 (±0.59)
	12°C (17)	0	0.18 (±0.12)	1.72 (±0.40)
LKS	8°C (30)	0	0.07 (±0.07)	1.13 (±0.17)
	12°C (17)	0	0.06 (±0.06)	1.50 (±0.32)

The number of specimens per temperature group is indicated between brackets in the "Temperature" column.

6.4.6. COMPARING PREVALENCE (PROPORTIONS) WITH AVERAGE SEVERITY SCORE

Although prevalence and average severity score represented largely the same results, some differences were noticeable. For example, although not significant, the prevalence of fusion was higher but the average severity score lower in freshwater smoltification-stage specimens of the 8°C group compared to the 12°C group (Table 18, Fig. 25A, D). At six-months-at-sea the deformity prevalence and average severity score showed the same pattern for the deformity types of both temperature groups except for fusion that was more prevalent, but less severe, in the 12°C group compared to the 8°C group (Table 18, Fig. 25B, E). For the freshwater smoltification-stage specimens that survived to six-months-at-sea (repeated measure Fig. 23, Fig. 25C, F) the differences between deformity prevalence and average severity score were most noticeable. While specimens of the 8°C group had a higher prevalence of total deformity, they showed a lower average severity score for total deformity compared to the 12°C group, i.e. a reversal of the results. A similar reversal of results was present for compression and vertical shift. Also a reversal of the results was present between the prevalence of total deformity at 12-months-at-sea compared to the average severity score at 12-months-at-sea (Fig. 27A, C). No pronounced differences between the prevalence and average severity score pattern of culled and not-culled specimens of both temperature groups at six-months-at-sea were observed (Fig. 26).

Table 18. Comparing prevalence (proportion) with average severity score

Group	Deformity type	8°C		12°C	
Freshwater smoltification	Fusion	4.7	>	4.0	Prevalence
		0.09	<	0.12	Severity score
Six-months-at-sea	Fusion	3.5	<	4.3	Prevalence
		0.11	>	0.09	Severity score
Freshwater specimens that survived to six-months-at-sea	Total	15.8	>	13.0	Prevalence
		0.34	<	0.61	Severity score
	Compression	7.9	>	4.3	Prevalence
		0.14	<	0.19	Severity score
	Vertical shift	8.8	>	5.8	Prevalence
		0.11	<	0.12	Severity score
12-months-at-sea	Total	76.7	<	88.9	Prevalence
		2.23	>	1.72	Severity score

6.5. DISCUSSION

6.5.1. MORTALITY

The substantial attrition, 91.6% loss in the 8°C group and 95.5% in the 12°C group from freshwater smoltification to 12-months-at-sea stage (Table 13), makes direct comparison of some deformity types at different time points very difficult based on numbers. The number of specimens available for comparison of deformities between life stages, determined by the number of salmon surviving to 12-months-at-sea (see repeated measure Fig. 23), was very low in both temperature groups, i.e. 30 specimens in the 8°C group and 17 specimens in the 12°C group. In addition, the 8°C and 12°C groups experienced different stresses during the later phase of growth in freshwater. The 8°C group underwent treatment for fungal infections during the earlier stages of freshwater growth, while the 12°C group had fungal infections of the tailfin due to fin nipping at the end of the freshwater growth phase. Experiments with the anaesthetic AquiS on bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), lake trout (*Salvelinus namaycush*), rainbow trout (*Oncorhynchus mykiss*), walleye (*Sander vitreus*) and yellow perch (*Perca flavescens*) showed that induction to sedation time and recovery time decreased with decreasing temperature (Stehly & Gingerich, 2001). These results show that induction and recovery timing needs to be followed closely when animals acclimated to warm water are anaesthetised to minimise stress. The specimens reared in 12°C may be more vulnerable to anaesthetic and therefore handling stress. Indeed, 65 specimens of the 12°C group died at X-ray compared to 13 specimens in the 8°C group (Table 13). The results from the current study are therefore interpreted cautiously.

The most notable period of attrition for both temperature groups occurred after seawater entry, i.e. 225 specimens (63%) in the 8°C group and 205 specimens (55%) in the 12°C group (Table 13). Also in commercially grown Chinook salmon the highest attrition occurs after seawater transfer, with a loss of 63% at grading (four-months-at-sea; Perrott et al., 2018). Survival of smoltification-stage salmonids in the seawater environment depends on the degree of smoltification (Clarke & Shelbourn, 1985; Mahnken Conrad & Waknitz, 1979). Freshwater rearing temperatures and time of transfer have been stated in literature to be the most important factors regulating seawater adaptability (McCullough, 1999). It has been reported for Chinook salmon that the maximum freshwater rearing temperature for successful smoltification is 12°C (Wedemeyer et al., 1980). If indeed 12°C is close to the maximum temperature for successful smoltification, specimens reared at 8°C could be expected to adapt better to the marine environment and therefore have a lower attrition rate. Yet in the current study the 8°C group showed higher attrition rate after seawater transfer compared to the 12°C group. The smoltification success of 8°C specimens may have been reduced because they were moved to ambient rearing temperatures four months before seawater transfer.

Seawater temperatures subsequent to the smoltification process are also important for smoltification success (McCullough, 1999). For Chinook salmon, the temperature of seawater at entry should be a few degrees colder than the freshwater temperature, with the highest smoltification success occurring when freshwater at 13.8°C is followed by seawater at 10.2°C (Clarke & Shelbourn, 1985). The temperatures reported in Clarke and Shelbourn's research are from coastal seawaters around river mouths in Canada. However, the coastal seawater around New Zealand is known to be warmer, up to 19°C (https://www.seatemperature.org). Moreover, coastal surface water temperatures can rise to 23°C (personal communication Mark Preece). In experiments where two seawater temperatures were tested in relation to smoltification success of sockeye and Chinook salmon, 13°C was found to be preferable compared to 19°C for both species (Franklin, 1989). It appears that New Zealand Chinook salmon prefer cool seawater but are adapted to higher seawater temperatures than Chinook salmon populations that occur in Canada. Smoltification success is also affected by other stress factors such as transportation from hatcheries or predation in main river stems in the wild (Price & Schreck, 2003). Clearly, smoltification is an essential life stage that has to be negotiated successfully for salmonids to thrive well in seawater.

The large scale attrition after seawater transfer in Chinook salmon is often caused by a 'failure to thrive', called 'runting' by farm staff, and characterised by a growth arrest or significantly slower growth of a subset of salmon in a cohort. The 'runting' observed in cultured New Zealand Chinook salmon is not unique. In cultured coho salmon (*Oncorhynchus kisutch*) two phenotypes of 'runt'-like salmon have been described. The first type was called 'stunt', early freshwater juvenile salmon (parr-stage) that were transported to seawater too early and did not go through the endocrinological changes necessary for successful smoltification (Clarke & Nagahama, 1977; Duan et al., 1995; Folmar et al.,

1982). The fate of 'stunt' juveniles in seawater was always death (Folmar et al., 1982). The second type was called 'parr-revertant', freshwater smoltification-stage juveniles that were transported to seawater too early or too late and underwent desmoltification (Folmar et al., 1982). Often the larger freshwater smoltification-stage juveniles went through desmoltification, i.e. they reacquired parr-marks but retained many characteristics of smoltification-stage salmon, and went through smoltification the following year (Folmar et al., 1982). In contrast, the smaller 'parr-revertant' salmon performed badly in the early stages of seawater and eventually died (Folmar et al., 1982), similar to the Chinook salmon 'runts' in the current study. Notably, the 'runts' do not reacquire parr-marks and remain 'silver' (personal observation).

The endocrinological changes in 'stunt' and 'parr-revertant' coho salmon are well described (Clarke & Nagahama, 1977; Duan et al., 1995; Folmar et al., 1982; Gray et al., 1990). In contrast, the endocrinological status of Chinook salmon 'runts' is unknown. However, the phenotypic differences between 'stunt' and 'parr-revertant' coho salmon, and 'runt' Chinook salmon in New Zealand, may indicate that different mechanisms are involved in cohorts which fail to thrive. Also, no skeletal abnormalities were mentioned in the phenotype of coho salmon 'stunt' or 'parr-revertant' animals, while skeletal abnormalities are obvious in Chinook salmon 'runts'. In the current study, at the six-months-at-sea stage 39 specimens (34% of X-rayed specimens at six months) of the 8°C group and 31 (46% of X-rayed specimens at six months) specimens of the 12°C group were culled because of runting (Table 13). Also in commercially farmed Chinook salmon, 73 individuals were classified as 'runts' during a grading event (four-months-at-sea; Perrott et al., 2018). It would be interesting to investigate the endocrinological status of Chinook salmon shortly after transfer to seawater in a systematic way. Currently, it is unknown if 'runts' fail to start feeding, or start and subsequently stop feeding at sea. Mitigation of excess early attrition in seawater would avoid both unnecessary wastage of feed and co-morbidities that add to the ethical cost of salmon farming and may also shed light on *de novo* deformities, for example LKS. The early seawater stages are critical and appear to represent a poorly understood period of development in commercial New Zealand Chinook farming operations. This study highlights a future potential research focus for Chinook salmon farmers.

6.5.2. LENGTH AND WEIGHT

The fork length and weight differed significantly between specimens of the 8°C and 12°C groups at almost all life stages except at 12-months-at-sea, with 12°C specimens being longer and heavier at respective life stages (Table 12, Fig. 24) due to faster growth in the warmer rearing conditions. At six-months-at-sea the difference in fork length and weight was less marked between the two temperature groups but still significant. It appears that the 8°C group was catching up in length and weight to the 12°C group (Fig. 24C). At 12-months-at-sea, the 8°C specimens had a higher average weight compared to 12°C specimens, although not significant (Table 12, Fig. 24C). This could indicate that

by harvest, even if specimens at early and juvenile life stages are reared in cooler freshwater temperatures, they catch up in length and weight to specimens initially reared in warmer freshwater temperatures. However, the number of specimens available for comparison at 12-months-at-sea was very low (30 specimens in 8°C and 17 specimens in 12°C groups). As a result the following discussion is mostly limited to the individuals that survived to six months in seawater.

Compared to commercially grown Chinook salmon, which had an average weight of 432 grams at four-months-at-sea and 3700 grams at 13-months-at-sea (Perrott et al., 2018), the Chinook salmon in the current study had lower average weights at similar stages (Table 12). Specifically, at the 12-months-at-sea stage the Chinook salmon in the current study weighed on average much less compared to commercially grown salmon, i.e. 1229 grams for the 8°C group and 1064 for the 12°C group (Table 12) compared to 3700 grams for commercially grown salmon (Perrott et al., 2018). The low weight of the specimens in both temperature groups at 12-months-at-sea could be explained by their high deformity rate, especially the high prevalence of LKS at this life stage. Deformed Chinook salmon have been reported to weigh significantly less compared to normal salmon at harvest (Perrott et al., 2018). In a study reporting on the occurrence of perivertebral fibrosis associated with LKS in Chinook salmon, the specimens with LKS had a significantly lower weight compared to specimens without vertebral deformities (Munday et al., 2018). For example, Chinook salmon with LKS weighed on average 1210 grams, similar to the specimens in the 8°C group at 12-months-at-sea in the current study (Table 12), compared to Chinook salmon without LKS weighing 3325 grams (Munday et al., 2016). Although no significant differences in fork length and weight could be found between specimens of the 8°C and 12°C groups at 12-months-at-sea, the presence of vertebral deformities, particularly LKS, has a detrimental effect on the growth of seawater stage Chinook salmon.

The weight cut-off for culling runts at grading was reported as one fifth of the overall average weight of the cohort (Perrott et al., 2018). In the current study the runts that were culled had a significantly smaller average fork length and weight in both temperature groups (Fig. 24D). Although the average weight of the runts in the 8°C group was less than the one fifth of the overall average weight cut-off value, the runts of the 12°C had a greater average weight than the one fifth cut-off value. The greater weight in the 12°C runts was because farm staff also treated severely deformed salmon as runt, since these animals were unlikely to thrive.

6.5.3. DEFORMITY PREVALENCE

Temperature was found to be a significant predictor of deformity outcome both in freshwater smoltification-stage and in seawater life stages. This is in agreement with previous reports for Atlantic salmon stating that temperature is a risk factor for vertebral deformities (Fjelldal et al., 2012; Fjelldal et al., 2006; Fraser et al., 2015; Grini et al., 2011; Ørnsrud et al., 2004a). Although in the current

study parent crosses were made to obtain offspring with high deformity rates, all possible measures were taken to have no background genetic effect. These results are likely to show a direct effect of rearing temperature on vertebral deformity rates. The caveats due to early rearing stressors and a very high rate of attrition remain. While, the prevalence of deformities in each temperature group is difficult to directly compare with commercially grown Chinook salmon, comparisons are possible and some consistent trends are worth nothing.

The crosses aimed for offspring with high deformity rates, may also explain why the total deformity and separate deformity type prevalence reported in the current study is higher compared to other reports in Chinook salmon. An overall prevalence of 4.3% has been reported for freshwater smoltification-stage Chinook salmon (Munday et al., 2018), which is lower than to the 7.7% and 16.3% respectively for the 8°C and 12°C groups reported here (Table 14). While fusion, at 2.5%, has been reported to be the most common vertebral deformity in commercially farmed smoltification-stage juveniles (Munday et al., 2018), fusion, compression and vertical shift have a prevalence of about 4% in the current study. Vertical shift, especially in the 8°C group (10.5%, Table 14), has a surprisingly high prevalence in the current study, compared to the 1.3% in commercially grown smoltification-stage juveniles (Munday et al., 2018). No LKS was observed in the freshwater stage salmon in the current study, which is in concordance to the very rare presence of LKS deformity in farmed freshwater Chinook salmon, with a reported five cases out of 3736 analysed freshwater juveniles (Munday et al., 2018).

An overall deformity prevalence of 8.8% has been reported in Chinook salmon that were four months in seawater (Perrott et al., 2018). While this is lower than the total deformity prevalence reported here, 13.2% and 39.1% respectively for the 8°C and 12°C groups (Table 14), the Chinook salmon in the current study were six months in seawater. Fusion was also the most common deformity (5.6%) after four months in seawater for Chinook salmon reported by Perrott et al. (2018), which is higher than the 3.5% and 4.3% fusion in the 8°C and 12°C groups, respectively, observed here (Table 14).

Compression and vertical shift show a higher prevalence in the 8°C and 12°C groups (Table 14) compared to the farmed Chinook salmon that were four-months-at-sea (3.0% and 0.5% respectively; Perrott et al., 2018). However, the prevalence of compression and vertical shift in the current study (Table 14) includes the observations on the runts that were culled after X-raying, where the reported prevalence of compression and vertical shift in Perrott et al. (2018) excludes the runts. Nonetheless, compression and vertical shift still had a higher prevalence in not-culled specimens of the 8°C and 12°C groups, except for vertical shift that was not present in the not-culled specimens of the 12°C group. Moreover, counter-intuitively compression and vertical shift was not present in culled specimens of the 8°C group.

One major difference with the Chinook salmon that were four-months-at-sea (Perrott et al., 2018), is the very low prevalence of LKS (0.5%) in comparison to the respectively 8.8% and 33.3% prevalence of LKS in specimens of the 8°C and 12°C group reported here. Also at 12-months-at-sea total deformity and LKS prevalence were higher in the current study (Table 16) compared to commercially grown Chinook salmon (38.4% for overall deformity prevalence and 29.4% for LKS; Perrott et al., 2018). The exact time of onset of LKS in seawater stage Chinook salmon remains unknown but Perrott et al. (2018) discussed that LKS starts to develop within nine months before harvest. The Chinook salmon in the current study were harvested after 15 month in seawater meaning that, following the time of onset suggested for LKS by Perrott et al. (2018), LKS should only start developing after nine months in seawater. However, in the current study a high prevalence of LKS was already observed at six-months-at-sea. In order to be so readily observable at six-months-at-sea the onset period would certainly be earlier.

The LKS deformity in late seawater stage Chinook salmon is suggested to be a secondary bone deformity as a reaction to asymmetric muscle forces caused by unilateral fibrosis in the perivertebral muscle (Munday et al., 2016). Therefore, the onset of the bone phenotype, i.e. vertebral column curvature (=LKS), is likely dependent on the onset of the muscle fibrosis. The apparent earlier development of LKS in salmon in the current study could be due to different sampling times after seawater transfer, assuming that unilateral perivertebral fibrosis underlies LKS also in the current study. If changes in the muscle leading to fibrosis that cause LKS and fibrosis takes time to develop then LKS may not have been easily detected after four months in seawater (Perrott et al., 2018). The current study, with LKS established at the six-months-at-sea stage, suggests that either LKS was initiated between four and six months after seawater transfer or that initiation was at an earlier stage and requires more than four months to become detectable by X-ray. Since the exact cause of the muscle fibrosis remains unknown (Munday et al., 2016), there is room to specifically add hypotheses which would fit with a driver for soft tissue changes soon after salmon are introduced to the seawater environment. Since many specimens of both temperature groups died after seawater transfer, the surviving specimens may carry evidence of the stresses felt during the freshwater-seawater transition period. That 70-90% of the specimens had LKS at 12-months-at-sea suggests that the specimens of both temperature groups were highly vulnerable to deformity from a combination of genetic and epigenetic factors such as the rearing temperature, grow-out husbandry and, transition to and on-going stresses in seawater. Thus, it could be hypothesised that the onset of fibrosis, and therefore LKS, may be associated to how well Chinook salmon transfer from freshwater to seawater. This study suggests that transition to seawater (optimisation of smoltification) and management of the early sea stage should be further investigated.

The prevalence of deformity types was consistently higher in 8°C freshwater specimens (Fig. 25A) but consistently lower in this group at six-months-at-sea (Fig. 25B). These results seem to imply that

incubation at a constant 8°C protects Chinook salmon against the development of deformities in subsequent seawater stages but, with the exception of fusion deformities, not during the freshwater stages. Incubation at 12°C appears especially detrimental for the development of LKS in seawater stages. The prevalence of fusion, compression and vertical shift was reduced in the six months at seawater stage (Fig. 25B) compared to the freshwater stage (Fig. 25A).

The prevalence of vertical shift in the freshwater specimens that survived to six-months-at-sea (see repeated measure Fig. 23) of the 8°C group (Fig. 25C) was heavily reduced by the six-months-at-sea stage (Fig. 25B). This reduction in prevalence may be explained in more than one way. First, vertical shift could become integral to the LKS deformity that occurs at six-months-at-sea, such that a mild vertical shift deformity may no longer be observable at six-months-at-sea. Second, similar to fusion and compression (Gil Martens et al., 2005), a vertical shift of the entire vertebral centrum is likely the consequence of aberrations in the intervertebral space (notochord tissue) or the vertebral ligament that connects adjacent vertebrae. The intervertebral tissue was described to be dynamic throughout the life cycle of Atlantic salmon (Kryvi et al., 2017). During the freshwater juvenile stages of Atlantic salmon the intervertebral space consists of dense almost un-vacuolated chordocytes. In smoltification and seawater stage Atlantic salmon, proliferating chordoblasts give rise to a new population of vacuolated chordocytes in the intervertebral regions (Kryvi et al., 2017; Nordvik et al., 2005). The intervertebral tissue changes morphology during the smoltification process in Atlantic salmon, which could also happen in Chinook salmon. Assuming a vertical shift remains stable from the point of detection, either an allometric or isometric growth pattern, together with changes in the morphology of the intervertebral space, would allow this deformity phenotype to revert toward a normal radiographic phenotype. Furthermore, the average severity score of vertical shift in the freshwater specimens that survived to six months is low (Fig. 25F). The presence of one or two vertically shifted vertebrae is more likely to be resolved compared to a situation involving severe vertical shift deformities.

Under farming conditions, and after approximately six-months-at-sea, salmon that are underperforming in their growth are removed. This ‘grading for runt removal’ ensures that cohorts remain largely uniform and that stocking densities can be evaluated and adjusted. Farmed Chinook salmon that are removed at grading have not been systematically investigated or reported on for the presence of skeletal deformities identifiable using X-rays. The types of vertebral column deformities that are present in farmed Chinook salmon ‘runts’ are likely to be the same as those previously described (Perrott et al., 2018) but their prevalence is largely unknown. While all types of deformities were present in culled specimens of the 12°C group, only LKS and fusion were present in the culled 8°C group. More importantly, all deformity types were more prevalent in culled specimens from the 12°C group compared to the 8°C group, with significantly more LKS in culled specimens of the 12°C group. Although a clear difference was observed in the prevalence of LKS not-culled/culled between the two temperature groups, it has to be noted that the prevalence of deformities could be high due to

specific parental crosses and maybe also because of stresses during the freshwater-seawater transfer period. Interpretation of the current results should be made carefully because the crosses used to create experimental deformity are not used in real farming practices. The observations are nonetheless of potential importance since they provide preliminary data on the prevalence of deformities in runting salmon and further highlight the importance of successful smoltification for further seawater growth.

6.5.4. PREVALENCE AND SCORE COMPARED

Except for a few instances, the average severity score reflected the prevalence results. In freshwater smoltification-stage, specimens in the 8°C group had more fusions (Fig. 25A), but of lesser severity (Fig. 25D), compared to the 12°C group. At six-months-at-sea the exact reverse situation was found for fusions, in that 8°C specimens had fewer fusions but of a higher severity. The difference in prevalence and average severity score was most clear in the subset of freshwater specimens that survived to six-month-at-sea (repeated measure Fig. 23, Fig. 25C, F). Total deformity, compression and vertical shift were more prevalent in specimens of the 8°C group (Fig. 25C) but had a lower severity (Fig. 25F), compared to the 12°C group. At 12-months-at-sea, the total deformity prevalence was lower in specimens of the 8°C group (Fig. 26A), but more severe (Fig. 26C), compared to the 12°C group.

The prevalence and average severity score results in general mirrored each other within temperature groups. Yet, care must be taken to interpret either prevalence or a measure of severity in context of farming conditions. For example, about 5% of the freshwater smoltification-stage specimens of the 8°C group had fusions (Table 14). Although a 5% deformity prevalence for fusions would be considered high for farming standards, the average severity score for fusion in freshwater was low, i.e. a value of 0.09 (Table 15). This means that specimens with fusions have on average only a few vertebrae involved in the deformity. A few fused vertebrae can remodel into a larger vertebra with a near similar function as a normal vertebra (Witten et al., 2006), which likely has a low impact on the welfare of the animal, growth performance and harvesting process. Indeed, only Chinook salmon with LKS had a significantly lower weight (slower growth) compared to salmon with fusions, compressions and vertical shift deformities (Munday et al., 2016; Perrott et al., 2018). A 5% prevalence of fusion with low severity may therefore be acceptable for a salmon farmer. Importantly, results about the prevalence of deformities are best represented alongside a measure of the severity of the deformity so that a salmon farmer can correctly interpret the impact of a certain deformity on the growth and welfare of the production stock.

In conclusion, this study clearly observed a temperature effect on deformity prevalence in New Zealand Chinook salmon. Results obtained in the current study suggest that incubation and freshwater

grow-out temperatures of 8°C are preferable to rearing at 12°C and 'protect' Chinook salmon from subsequent vertebral deformities in seawater but not freshwater stages. This study also shows that when either prevalence or severity scores are reported, interpretation of the results should be made carefully. It is best to report the prevalence and severity scores together. Finally, this study highlights that the mortality is high in the life stages after seawater transfer and that deformity is also notable in runts. Importantly, mortality after seawater transfer and deformities in runts were identified as important future research focuses for Chinook salmon farmers.

6.6. ACKNOWLEDGEMENTS

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CHAPTER 7: GENERAL DISCUSSION

7.1. EARLY LIFE MALFORMATIONS – INCUBATION TEMPERATURE

Results in *CHAPTER 3* showed that internal malformations, such as notochord malformations, chordacentra fusions and malformations of the associated elements can occur early in the post-hatch stages of farmed Chinook salmon. Moreover, the proportions of these internal malformations were linked to incubation temperature, with fewest malformations occurring in the 8°C group, followed by the 12°C group and 4°C group. Although the experimental methods in this thesis aimed for an absolute constant temperature experiment, perturbations in the temperature profile occurred due to power cuts and electrical failure of pumps and the chiller system. A temperature change of at least 2°C occurring over a one hour period can be considered as a temperature shock (Takle et al., 2005; Wargelius et al., 2005). The temperature increases that occurred in the main experiment of the thesis are represented in Table 19. The temperature profile is also indicated in Figure 29. Only five temperature increases occurred during the entire incubation period across all temperature groups, with one temperature increase in the 12°C group, three in the 8°C group and four in the 4°C group (Fig. 29A-C). Although most temperature increases observed were more than 2°C (Table 19), they were of a very short nature, i.e. between 15 minutes and one hour (see section *2.5.1 FLOW PROFILE AND TEMPERATURE MEASUREMENT*). Therefore these temperature changes may not have been severe enough to cause developmental aberrations. In addition, it makes the interpretation of the effect of the various temperature changes on development difficult. Moreover, because the experiment did not aim to dissect the effect of temperature shock on skeletal development, the temperature changes were not discussed in the results chapters. However, the possible implications of the temperature increases can be discussed here.

Temperature, as the causative factor for vertebral deformities, was shown to affect early temperature sensitive stages during the development of Atlantic salmon (Takle et al., 2005; Wargelius et al., 2005). The critical stages observed were the late blastula stage, the early gastrulation stage, the complete epiboly stage (other terms used are blastopore closure, the germ ring together with the embryo have enclosed the entire egg yolk, or 15th to 20th somite stage), the completion of somitogenesis (another term used is initial eye pigmentation) and the eyed egg stage (another term is complete eye pigmentation). In addition to the effect of constant temperature on early development, the observation of temperature changes during critical stages may have influenced the development and may have confounded the observed differences in *CHAPTER 3*.

Table 19. Temperature shocks during the temperature experiment.

Age (days)	4°C group		8°C group		12°C group	
	Age (°d)	ΔT	Age (°d)	ΔT	Age (°d)	ΔT
8	52	+6			91	+2
42	188	+8	341	+4		
70	300	+8	565	+4		
101			813	+2		
211	864	+2				

7.1.1. TEMPERATURE – 'SHOCKS' AS POTENTIAL CAUSE OF MALFORMATION

The highest proportion of malformations was observed in the 4°C group (*CHAPTER 3*). Compared to the 8°C and 12°C groups, the 4°C group had the three most severe temperature perturbations (Table 19) during the sensitive stages from fertilisation until hatching (Fig. 29C). There was only one temperature change in the 12°C group with a 2°C increase (Fig. 29A), two moderately severe and one less severe temperature change before hatching in the 8°C group with a 4°C and 2°C increase in temperature respectively (Fig. 29B). Although both the 8°C and 12°C groups had mostly a constant temperature during the pre-hatching incubation period, the 12°C rearing regime is closer to the upper lethal temperature limit and therefore a smaller temperature increase may have a greater effect. Conversely, 4°C is close to the lower lethal temperature limit. Although no negative temperature changes occurred, an 8°C increase may seriously perturb normal development. Because the 8°C is part of the optimal temperature range for Chinook salmon embryos, a 4°C increase from the set temperature may have been relatively tolerated (Alderdice & Velsen, 1978; Brett, 1952). This may explain why the 12°C group had lower frequencies of vertebral column malformations compared to the 4°C group, but higher than the 8°C group (*CHAPTER 3*). Moreover, a constant 8°C can be suggested as the best incubation temperature.

7.1.1.1. Temperature change day 8

The first temperature increase occurred in the 4°C and 12°C groups on day eight. A 6°C increase was observed for the 4°C group (Table 19, Fig. 29C). At 52°d the embryos are in the late blastula stage of development, defined as a temperature sensitive stage, where the fates of the embryonic cells are already determined to a certain extent (Ballard, 1973a; Ballard, 1973b; Gilbert, 2000). For example, the cells that will become future notochord are already delineated in the embryos of rainbow trout (Ballard, 1973b). However, these cells remain pluripotent and thus, their fates can be changed. A temperature perturbation in the late blastula stage, e.g. in the 4°C group, may affect the cells determined to become notochord and therefore affect the normal development of this structure.

Also on day eight, a 2°C increase was observed for the 12°C group (Fig. 29A). At 91°d the embryos are in the gastrulation stage, between the 6th and 10th somite stages (Gorodilov, 1996; Gorodilov & Melnikova, 2003). At gastrulation stage, the three germ layers (ectoderm, endoderm and mesoderm) are formed from which the mesoderm will eventually form the notochord and somites. High levels of cell movement and cell mixing occurs during early gastrulation (Ballard, 1973a; Ballard, 1973c; Gilbert, 2000). Fortunately for the group closest to the upper lethal temperature limit (12°C), a 2°C increase is the only temperature shock that occurred. This relatively small temperature increase of short duration is unlikely to have had the same impact as the 8°C temperature increase that occurred in the 4°C group.

7.1.1.2. Temperature change day 42

In the 4°C group a severe temperature change with an increase in temperature of 8°C occurred (Table 19, Fig. 29C). At 188°d the embryos have reached the completion of somitogenesis stage. The maximum somite number in Chinook salmon (72-73) differs from the maximum somite number of Atlantic salmon (66-68) (Gorodilov & Melnikova, 2003). Interestingly, in Pacific salmon species such as Chinook, cherry (*Oncorhynchus masu*) and pink salmon, the complete epiboly stage corresponds to the presence of 56-62 pairs of somites (Gorodilov & Melnikova, 2003) and thus is close to the completion of somitogenesis stage time point. Completion of somitogenesis corresponds to the appearance of pigmentation in the eye. First eye pigmentation was recognised in Atlantic salmon to be around 186°d to 189°d, in coho salmon around 200°d and in Chinook salmon around 211°d (Boyd et al., 2010; Gorodilov, 1996; Zeitoun & Tack, 1974). A temperature perturbation at 188°d in post-hatch Chinook salmon may impact the normal formation of the posterior somites. Abnormal somite formation later in development may lead to malformed associated elements as was observed in post-hatch Chinook salmon (*CHAPTER 3*).

In the 8°C group, also on day 42, a temperature increase of 4°C was observed (Table 19, Fig. 29B). At 341°d the embryos have reached the appropriately named eyed egg stage. At this stage the eye is completely covered by pigment and can be observed through the eggshell. This stage is also the final temperature sensitive stage of the pre-hatching developmental period. Although the eyed egg stage is reached at 236°d to 239°d in Atlantic salmon (Gorodilov, 1996), pigmentation of the eye was not observed until 298°d in Chinook salmon (Boyd et al., 2010), closer to the temperature shock at 341°d in the 8°C group. In Atlantic salmon the notochord has reached its full length and is vacuolised up to somite 25 to 26 at this stage (Gorodilov, 1996). In contrast, the notochord has just reached full length after the eyed egg stage in coho salmon (Zeitoun & Tack, 1974). A temperature change at this stage could perturb the start or on-going notochord vacuolisation process. Improper vacuolisation of the notochord could lead to kinking and pinching of the notochord, malformations that have been observed in post-hatch Chinook salmon (*CHAPTER 3*).

Interestingly, in all temperature groups visually malformed specimens occurred without apparent internal skeletal malformations (*CHAPTER 3*). During the eyed egg stage, the myotomes develop and the first developing myofibres can be distinguished (Zeitoun & Tack, 1974). Early muscle development is influenced by incubation temperature (Campos et al., 2013; Johnston & McLay, 1997; Johnston et al., 1999; Macqueen et al., 2008; Stickland et al., 1988). Hypertrophied muscles (few but large volume muscle cells) because of high incubation temperatures could cause post-hatch salmon stages to experience excess of asymmetric forces on their notochord. This could likely result in the bending of the notochord and could explain why a proportion of visually malformed post-hatch Chinook salmon had no skeletal malformations (*CHAPTER 3*). The bending of the notochord would be due to anomalies in muscle, or other soft tissue, development rather than vertebral malformations *per se*.

7.1.1.3. Temperature change day 70

A third temperature change occurred on day 70 in the 4°C and 8°C groups. An 8°C increase occurred in the 4°C group and a 4°C increase in the 8°C group (Table 19, Fig. 29B, C). At 300°d the 4°C group experienced a temperature increase at a similar stage as the 8°C group did on day 42, i.e. the eyed egg stage (341°d). The mesenchymal condensations that will form the associated elements (see Fig. 5A) in Atlantic salmon start forming before 420°d (Grotmol et al., 2006), between 300°d and 390°d (Grotmol et al., 2003). Although in general the rate of development of Chinook salmon is slower than that of Atlantic salmon (Gorodilov & Melnikova, 2003) it is possible that the migration of mesenchymal cell (sclerotome cells, see Fig. 4) could be perturbed. Also the condensation process proper could be perturbed later in development. The mesenchymal cell migration and condensation process is likely to vary between individuals of the same temperature group and therefore these events could occur over a 90°d to 100°d developmental time period. Malformed associated elements, as observed in post-hatch stage Chinook salmon (*CHAPTER 3*), could have arisen due to a temperature shock.

At day 70, the 8°C group was at a 565°d stage, i.e. during or after hatching. The notochord at hatching stage has reached the full length of the embryo and vacuolisation of the chordocytes has taken place throughout the notochord. The notochord will keep increasing in length and diameter from hatching to first feed (Grotmol et al., 2006). Shortly after hatching (around 500°d), a shift in the spatial arrangement of the collagen type II fibrils in the notochord sheath occurs (Grotmol et al., 2006). The fibrils shift from a concentric orientation to a helical orientation along the axis of the notochord. Several of these helical layers, shifting from left- to right-handed coils will later be embedded in the chordacentra, which are not yet present at hatching. It was suggested that the collagenous coils increase the flexural stiffness and elastic recoil which are necessary during locomotion of post-hatch stages (Grotmol et al., 2006). A temperature change could perturb the process of correctly orienting

the collagen fibrils and reduce the strength and resistance of the notochord to kinking under load. A bent and kinked notochord was particularly a feature of post-hatch Chinook salmon tail malformation and bent neck phenotypes in which high proportions of notochord malformations, including pinching, were observed (*CHAPTER 3*). Also, in bent tail medium, spinal malformation medium and spinal malformations severe phenotypes notochord malformations were the second most common internal malformations (*CHAPTER 3*).

7.1.1.4. Temperature changes day 101 and 211

The fourth temperature increase only occurred in the 8°C group at 813°d (Table 19, Fig. 29B) while the fifth temperature increase only occurred in the 4°C group at 864°d (Table 19, Fig. 29C). Both these temperature changes are close to the 900°d first feed stage which is not considered as a temperature sensitive stage. Close to the first feed stage the skeletal elements of the caudal fin and the chordacentra start mineralising (personal observation). Temperature perturbations may interfere with the normal mineralisation of the chordacentra and lead to chordacentra fusions. Temperature has been shown to have no significant effect on the sequence of mineralisation but increases the phenotypic variation in timing of the start of mineralisation (Cloutier et al., 2010; Mabee et al., 2000). In addition, temperature modulates to a small degree where mineralisation starts (Cloutier et al., 2010; Mabee et al., 2000). Atlantic salmon incubated at high temperatures showed the downregulation of matrix metalloproteinase (MMP) 9 and MMP13 (Ytteborg et al., 2010b). These MMP's are important proteases that help break down the cartilage matrix during ossification (Engsig et al., 2000; Vu et al., 1998). Moreover, the absence of MMP's in the cartilage matrix delays endochondral ossification (Stickens et al., 2004). Although, chordacentra fusion was the most common internal malformation observed in visually normal post-hatch Chinook salmon (*CHAPTER 3*), the start of ossification of the associated elements could be delayed by high incubation temperatures resulting in ossification defects of the cartilaginous elements. Irregular ossification of associated elements, especially caudal fin skeletal elements which experience forces during swimming, may expose these skeletal elements to deformation processes in early juvenile stages (>1000°d).

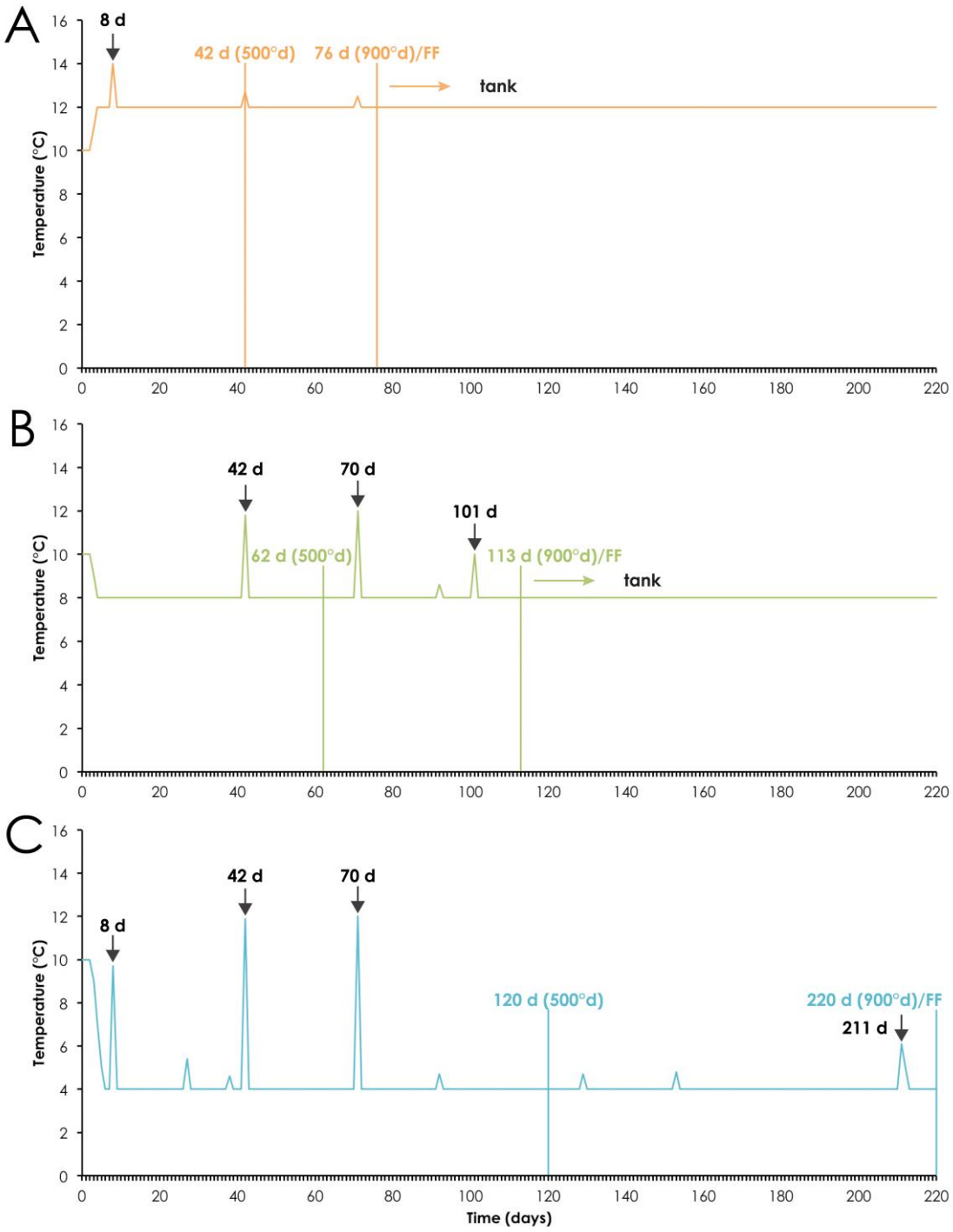

Figure 29: Temperature shocks during the incubation period of Chinook salmon

Graphic representation of the temperature profiles of the 12°C (A, orange), 8°C (B, green) and 4°C (C, blue) groups, with temperature (°C) on the y-axis and time in days on the x-axis. Timescale runs from incubation (day 0) to day 225. Each accidental temperature shock is indicated with a black arrow and on which day the temperature shock occurred. The day of the expected hatching time at 500°d is indicated by a vertical line. The expected day the specimens reach first feed (FF) at 900°d is also indicated with a vertical line. The specimens of the 12°C (A) and 8°C (B) were transferred to tanks (indicated by arrow) after first feed stage.

7.1.1.5. Temperature change – Proposed mechanisms

Scar tissue was one of the notochord malformations observed in post-hatch Chinook salmon (*CHAPTER 3*). In this malformation, chordocytes could be damaged as a consequence of temperature perturbations. A positive temperature change increases the activity levels of fish. For example, salmonids are more active in warmer water and show avoidance behaviour towards high sublethal and lethal temperatures (Beitinger et al., 2000; Graham, 1949). The embryos prior to hatching also show activity inside the egg (Johnston et al., 1999; Knight, 1963; Proctor et al., 1980), which is higher in water with warmer temperatures (personal observation). A temperature increase could have momentarily increased the number of movements made by the embryo inside the egg. Erratic movements due to high temperature could have caused notochord lesions, ultimately resulting in scar tissue in Chinook salmon (*CHAPTER 3*). This process is likely initiated by the collapse and rupture of chordocytes when excess mechanical force occurred over a small area. At a cellular level, the plasma membrane and its associated caveolae must directly absorb cell stretching by forces on the plasma membrane and thus protect the notochord against damaging deformation. Caveolae (illustrated in *APPENDIX F*) are small plasma membrane pits (60-80 nm) providing a reserve of plasma membrane for when the cell is stretched. Hence, the cells are protected against rupturing (Bastiani & Parton, 2010; Garcia et al., 2017; Lim et al., 2017). When the eggshells of zebrafish caveolae mutants (*cavin1b*$^{-/-}$) were bleached, they became harder necessitating increased movements of the embryo to hatch, a higher number of notochord lesions were observed compared to the control group (Lim et al., 2017). In contrast, when the zebrafish embryos were anaesthetised, reducing the movements in the egg, and dechorionated, a lower number of notochord lesions were observed compared to the control group (Lim et al., 2017). These observations showed that notochord lesions developed by the movement of embryos before hatching. A similar mechanism that causes notochord lesions in zebrafish may happen in Chinook salmon. Movements of pre-hatch stage or hatch stage Chinook salmon could have been increased due to a positive temperature change and may have caused notochord lesions, where chordocytes collapse and consequently scar tissue develops. Therefore, a temperature shock on day 70 at 565°d for the 8°C group, could have increased the rate of notochord lesions.

The pinching and kinking of the notochord sheath was another notochord malformation observed in post-hatch Chinook salmon (*CHAPTER 3*). At deep pinching sites and at the level of scar tissue the collagenous middle layer of the notochord appeared thinner and contorted (Fig. 11F, G; *CHAPTER 3*). A temperature increase could cause the proto-collagens, which are produced in the endoplasmic reticulum (ER) and exported to the notochord sheath, to fold incorrectly. These proteins remain in the ER because they cannot be exported to the Golgi-complex, and causes ER stress (Mori, 2009; Walter & Ron, 2011). The ER stress will induce an unfolded protein response (UPR) response (illustrated in

APPENDIX F) which results in the up-regulation of heat shock protein (HSP) chaperones that help to correctly fold proteins. The HSP family members were reported to be present in high levels in the notochord of which HSP 70 operates in the ER (Sitia & Braakman, 2003). The heat shock protein was shown to be up-regulated in Atlantic salmon post-hatch stages receiving a heat shock (Takle et al., 2005). Mutations in the UPR response regulators *Atf6α/β* and *Bbf2h7* in medaka and *feelgood* (*Creb3l2*) in zebrafish (*APPENDIX F*) cause an incorrect UPR response and have been reported to show abnormally developed notochords (Ishikawa et al., 2013; Ishikawa et al., 2017; Melville et al., 2011). The notochord in these mutants appears bent and kinked, similar to what is observed in Chinook salmon post-hatch stages (*CHAPTER 3*). In the *Bbf2h7* medaka mutant the chordoblasts were reported to appear detached from the basement membrane of the notochord. Also the chordoblasts in post-hatch stage Chinook salmon in the present study appeared dislocated at the level of deep pinching sites and scar tissue (*CHAPTER 3*). Malformed associated elements observed in the post-hatch stages (*CHAPTER 3*) could develop by a similar mechanism or be affected by bent and pinched notochord malformations. Also in the cartilage cells, incorrectly folded proto-collagens due to a heat shock remain in the ER and are not transported to the extracellular matrix. The *Atf6α/β* and *Bbf2h7* medaka and *feelgood* (*Creb3l2*) zebrafish mutants were indeed reported to have abnormal cartilage formation in the skull (Ishikawa et al., 2013; Ishikawa et al., 2017; Melville et al., 2011). The cartilaginous skull bones appeared thin and underdeveloped with little extracellular matrix present between the cartilage cells, suggesting that the collagens that build the cartilage matrix are not transported to the extracellular space. Thus a temperature increase may cause both malformations of the notochord and associated elements and may explain the co-occurrence of these internal malformations observed in post-hatch Chinook salmon phenotypes such as curly severe, bent tail medium and spinal malformation severe (*CHAPTER 3*).

7.1.2. TEMPERATURE – A MODULATOR OF DEVELOPMENT

Six regions were characterised in the juvenile Chinook salmon vertebral column (*CHAPTER 4*). A detailed regionalisation of the vertebral column is necessary to investigate regional variation of skeletal elements such as vertebrae, ribs and vestigial elements. Regional meristic variation of skeletal structures, such as vertebrae, vestigial ribs in the transitional region, the vestigial neural arch and spine on preural 2 and the uroneurals was observed in the vertebral column of juvenile Chinook salmon (*CHAPTER 5*). Although a temperature shock at critical stages can cause malformation of the vertebral column, a temperature change can modulate its normal development. For example, meristic variation of vertebrae, fin rays, branchiostegal rays and gill rakers was observed when populations of the Pacific salmon species living at different latitudes were compared (Murray & Beacham, 1989; Taning, 1952). The variation of vertebrae in teleost populations occurring along environmental latitudinal lines, of which temperature variation is most important, was stipulated as Jordan's rule

(Jordan & Evermann, 1896). Research in zebrafish and Atlantic salmon found that the rate of somitogenesis is changed by incubation temperature and slows down near the caudal end (Gorodilov, 2004; Holley, 2006; Schröter et al., 2008). For example, zebrafish embryos raised in higher temperatures (30°C) had more somites compared to embryos raised in colder temperatures (27°C) (Schmidt & Starck, 2010). Furthermore, Gorodilov (2004) and Moriyama et al. (2012) observed the presence of pseudo-somites in the posteriormost end of the caudal region in coho salmon and medaka. These observations indicate that the processes forming the last few somites are less well controlled and may therefore be more prone to perturbations. Somites may fuse, could regress or extra somites may develop into vestigial skeletal structures (Witten & Hall, 2015). The presence and variation in occurrence of vestigial elements has been shown in the zebrafish caudal fin (Bensimon-Brito et al., 2012a) and in Chinook salmon in the present study (*CHAPTER 5*).

The vestigial ribs in the transitional region, the vestigial neural arch on preural 2 and the uroneurals in Chinook salmon are bilateral paired skeletal elements, and variation in their left and right presence was observed (*CHAPTER 5*). Elevated constant temperatures, such as 12°C for Chinook salmon, can cause chronic stress, which can be observed as variation in the symmetry of bilateral skeletal elements such as fin rays, branchiostegal rays and gill rakers (Allenbach, 2011; Dongen, 2006; Leary & Allendorf, 1989; Leary et al., 1985; Van Valen, 1962). The deviation of symmetry in these skeletal elements is mostly assigned as fluctuating asymmetry, defined as the equal random variation of a character on the left and right side of the individual (Leary & Allendorf, 1989; Leary et al., 1985; Van Valen, 1962). Fluctuating asymmetry in the number of paired fin rays, scales, gill rakers and branchiostegal rays was studied in chum salmon, sockeye salmon and coho salmon (Beacham, 1985; Beacham, 1990; Campbell et al., 1998). Also teeth are part of the skeleton and in zebrafish variation in the asymmetry of the dentition on the fifth ceratobranchial was observed (Woltmann et al., 2018). Significant asymmetry in skeletal structures could be used as an indicator for unstable development (Allenbach, 2011), which occurs in non-ideal rearing environments. However, consistent observation of several skeletal elements needs to be taken into account to make precise interpretations of the observed asymmetry. The value of using asymmetry of skeletal structures as an indicator for abnormal development is still heavily debated (Allenbach, 2011). Although in juvenile Chinook salmon, significant variation in the number of vertebrae was observed between the 8°C and 12°C groups, the number of vestigial ribs, the arch on preural 2, and the uroneurals between the two temperature groups was very similar (*CHAPTER 5*). The meristic variations of the vertebrae observed in the present study could be a consequence of the accidental temperature shocks that occurred during incubation, rather than the consequence of a constant elevated temperature (e.g. 12°C). The lack of variation in vestigial skeletal elements could be explained by the absence of accidental temperature shocks during their development in late first feed stages. Alternatively, 12°C may not have been high enough to cause chronic developmental stress and therefore significant variation of paired vestigial skeletal elements.

7.2. EARLY LIFE MALFORMATIONS – A GENETIC BACKGROUND

The results in *CHAPTER 3* show a link between the visual phenotype and the malformed skeletal structures for the first time in a salmon species. Although visual phenotypes, such as curly medium and severe, bent tail, lordosis, kyphosis and scoliosis also have been observed in early stages of Atlantic salmon, chum salmon, trout and brook trout (Eriksen et al., 2006; Johnson et al., 1998; Leduc, 1978; Mahrosh et al., 2014; Quatrefages, 1888; Yamamoto et al., 1996), malformation of the underlying skeletal structures, such as notochord, chordacentra and associated elements have not been reported in salmonids. The specific crosses between parental Chinook salmon used in the present study (*APPENDIX B*) were chosen to produce offspring with more malformations and many different types of vertebral column malformation. This means that the malformed phenotypes of post-hatch Chinook salmon in the present study, apart from being caused by differences in the incubation temperature, may also have a genetic basis and may be the result of mutations. Malformations of the notochord, chordacentra and associated elements have been observed in several different zebrafish and medaka mutants which have malfunctions in the cellular vesicular transport mechanisms and the caveolae (summarised in *APPENDIX F*). Where in the cell, and which cellular components the mutations affect in the chordocytes, the chordoblasts and the notochord sheath, are shown in *APPENDIX F*. Salmonids share these universally conserved intracellular mechanisms for post-translational transport of proteins. Below, malformation phenotypes observed in post-hatch stage Chinook salmon in *CHAPTER 3* are compared and discussed with malformation phenotypes observed in mutant zebrafish and medaka. It is emphasised here that hypotheses of potential mechanisms are only discussed in light of possible future research areas.

At shallow pinching sites, the post-hatch Chinook salmon notochord sheath was bent but was otherwise unaffected by contortions, indicating that only the chordocytes were affected (*CHAPTER 3*). The vacuoles in chordocytes were collapsed and the notochord appeared kinked in the *Cltca* zebrafish mutant, which have clathrin coated vesicles with an impaired function (Ellis et al., 2013). Clathrin coated vesicles are important in the protein transport from the plasma membrane to intracellular vesicles such as lysosomes (*APPENDIX F*). The vacuole in chordocytes was shown to be a lysosome related organelle (LRO) and therefore clathrin mediated vesicle transport was suggested to be important in the maintenance of the vacuole (Ellis et al., 2013). In the *sneezy*, *happy* and *dopey* zebrafish mutants the COP I vesicle coat components are truncated and impair correct COP I mediated transport functions (*APPENDIX F*). In these mutants vacuoles in the chordocytes fail to form (Coutinho et al., 2004; Odenthal et al., 1996; Stemple, 2005). The collapse of a few chordocytes in the post-hatch Chinook salmon notochord could explain a shallow pinching of the notochord sheath (*CHAPTER 3*).

At deep pinching sites and at the level of scar tissue in post-hatch Chinook salmon, the notochord sheath and external elastic membrane was thinner, contorted and perturbed (Fig. 11F-H; *CHAPTER 3*). A thinner and fragmented elastin membrane and a change in the notochord sheath collagen architecture were observed in fusions in parr stage Atlantic salmon (Ytteborg et al., 2010a). The collagenous middle layer of the notochord in *sneezy*, *happy* and *dopey* COP I mutants was observed to be thinner or even absent resulting in a bent notochord (Coutinho et al., 2004; Odenthal et al., 1996; Stemple, 2005). In zebrafish laminin mutants, *bashful, grumpy and sleepy*, the formation of the basal lamina is perturbed (Parsons et al., 2002; Stemple, 2005). A correctly formed basement membrane is necessary for cell differentiation, attachment and function (Bornstein & Sage, 2002; Corallo et al., 2013; Coutinho et al., 2004; Parsons et al., 2002). If the chordoblasts cannot attach properly to the basement membrane, the production of the collagenous sheath material is likely also compromised. Grotmol et al. (2006) concluded that normal notochord sheath organisation is dependent on an intact basal lamina. Indeed, in the *bashful, grumpy and sleepy* mutants, the formation of a perturbed basal lamina resulted in the disorganisation of the collagen in the notochord sheath and of elastin layers of the external elastic membrane (Parsons et al., 2002; Stemple, 2005). Displaced and hyperplastic chordoblasts were also observed at deep pinching sites and at the level of scar tissue in post-hatch Chinook salmon notochords (*CHAPTER 3*), where the notochord sheath and external elastic membrane appeared disorganised.

Kinking of the notochord as was observed in in post-hatch Chinook salmon (*CHAPTER 3*). Mutations in the *emilin3a* and *emilin3b* could cause a malformed notochord sheath and result in a bent notochord. These genes were found to have an important role in organising the collagen II fibres in the medial layer of the zebrafish notochord (Corallo et al., 2013). Mutant fish showed disorganised collagen II fibres in the medial layer and a shortened and bent notochord. However, the basement membrane and outer notochord sheath layer were unaffected (Corallo et al., 2013).

Chordacentra fusions were observed mostly in externally normal specimens but also in externally malformed post-hatch Chinook salmon (Fig. 10; *CHAPTER 3*). The organisation of the collagen fibres in the notochord sheath has been shown to be important for the correct mineralisation of chordacentra, which starts in the collagen below the external elastic membrane in Atlantic salmon (Grotmol et al., 2006). Incorrectly organised collagen fibres in the notochord sheath could explain the occurrence of chordacentra fusions observed in Chinook salmon post-hatch stages (*CHAPTER 3*). The *vbi* (*vertebra imperfect*) medaka mutant has a mutation in the SEC24D component of the COP II coated vesicles (*APPENDIX F*). In this mutant the mineralisation of chordacentra is delayed and irregular. Adjacent chordacentra fused ventrally, and the neural and haemal arches can develop malformed (Ohisa et al., 2010). The zebrafish mutant *bulldog* has also mutation in the SEC24D COP II component (*APPENDIX F*). This mutant exhibits craniofacial malformations, defects in chondrocyte maturation and delayed mineralisation (Sarmah et al., 2010). In post-hatch Chinook salmon ventrally fused chordacentra (Fig.

12A-C), irregular mineralisation of chordacentra (Fig. 12I) and malformed arches (Fig. 12F-H) were observed (*CHAPTER 3*) similar to the internal vertebral column phenotypes of the *vbi* and *bulldog* mutants. The high baseline number of malformations occurring in the offspring of the selected crosses in the present research may be the consequence of the genetic background and accidental temperature shocks. However, the differences in proportions of malformations observed between the three temperature groups (*CHAPTER 3*) is more likely a consequence of the three different constant incubation temperatures rather than genetic background or accidental temperature shocks.

7.3. EARLY LIFE MALFORMATIONS FORESHADOW LATE LIFE DEFORMITIES

The results in *CHAPTER 3* showed that notochord malformations, vertebral fusions and malformations of the associated elements can already be present in post-hatch stages of Chinook salmon that are visually malformed. The results in the current research thus confirm that malformations of the vertebral column can occur in early life stages of salmon. Early life stage malformations must be sublethal and carried through the freshwater and seawater stages in order to be present as later life stage deformities. When malformations in the vertebral column of salmonids first appear and which malformations of the vertebral column occur in early life stages represented a gap in the current knowledge. In contrast, late and final stage deformities, such as compressions, fusions and bending of the vertebral column (lordosis, kyphosis and scoliosis) are well studied in farmed freshwater pre-smoltification stage salmonids (Fjelldal et al., 2007a; Grini et al., 2011; Kvellestad et al., 2000; Munday et al., 2018; Sullivan et al., 2007a; Takle et al., 2005) and in both wild and farmed adult salmonids (Deschamps et al., 2009; Deschamps et al., 2008; Fjelldal et al., 2009a; Fjelldal et al., 2009b; Fjelldal et al., 2007b; Gil Martens et al., 2005; Munday et al., 2016; Perrott et al., 2018; Seymour, 1959; Sullivan et al., 2007b; Witten et al., 2009). Importantly, Kvellestad et al. (2000), Fjelldal et al. (2012) and Witten et al. (2005a) have suggested that deformities such as compression and fusions observed in juvenile or adult life stages could have developed very early in life. However, the phenotype of later deformities may mimic or be different from early life stage malformations, which potentially foreshadow later deformities.

Most of the severely malformed (externally and internally) animals will not survive past the first feed stage. Additionally, salmon farmers remove malformed individuals and aim to grow visually normal salmon. Surprisingly, visually normal specimens in each temperature group, investigated in *CHAPTER 3*, showed internal malformation of the vertebral column. The main internal malformation observed in normal phenotype post-hatch Chinook salmon was fusion of chordacentra. The chordacentrum is the foundation of the vertebral centrum (Arratia et al., 2001) with the perinotochordal autocentrum and bony arcocentrum forming around and connected to the chordacentrum. Therefore, the autocentra and the bony arcocentra of adjacent vertebrae are likely to fuse during growth when their underlying chordacentra are fused in early developmental stages. The cartilaginous arcocentra, i.e. the basidorsals

and basiventrals, remain separate since fused vertebrae have two neural and haemal arches. Indeed, fused chordacentra with separate basidorsals and basiventrals have been observed in post-hatch Chinook salmon (Fig. 12A; *CHAPTER 3*). In addition, early fusions do not affect the visual appearance of the animal and are likely survivable. Fusions that originate in post-hatch stages could therefore be carried through to the harvest stages. It appears that a deformity, such as a fusion, in late life stages can already exist in early life stages. This answers the question: "Are some late life deformities foreshadowed by early life stage malformations?"

While vertebral fusions can start to develop in salmonid post-hatch stages (*CHAPTER 3*), they can also start to develop in both juvenile pre-smoltification stages and adult post-smoltification stages (Fjelldal et al., 2012; Munday et al., 2018; Perrott et al., 2018). However, the exact moment when malformations and deformities start to develop remains to be elucidated. Understanding when malformations and deformities develop may help identify malformation and deformity prone stages. Once these sensitive stages are identified changes in the husbandry strategies could be made to prevent the development of malformations and deformities. Suggested measures could be a change in incubation temperature, changing the timing of grading events and changes in the nutrition. Whether or not deformities observed in juvenile and adult stages are early life stage malformations that were carried through the development and growth of the animals is unknown. The experiment in the current research aimed to follow early life stage specimens, reared at two different incubation and freshwater grow-out temperatures, into their juvenile and adult life stages. The results would link malformations in early life stages to deformities observed in late life stages. However, due to runting (= stop in growth) in early sea stages, and malnutrition and mortality events in later seawater stages, the current project failed to directly link early malformations to deformities that are observed in juvenile and adult stages. However, results in *CHAPTER 6* do show that early incubation and freshwater grow-out temperatures significantly affect the prevalence of deformities in freshwater versus seawater stages. Moreover, it appears that compared to 12°C, incubating at a cooler 8°C protects Chinook salmon against the development of deformities, especially LKS, in seawater stages. Together, the results in *CHAPTER 3 AND 6* show that a changing of the incubation temperature can indeed be useful to reduce the number of malformations and deformities that occur in farmed Chinook salmon.

Currently there is information about the skeletal phenotype of early malformations in Chinook salmon (*CHAPTER 3*) and information about skeletal phenotype of late and final stage deformities in Chinook salmon (Perrott et al., 2018) and Atlantic salmon (Witten et al., 2009; Witten et al., 2016). Information about the start or early stages of deformities is currently still lacking. Linking malformations to deformities is difficult but careful hypotheses can be postulated. For example, Witten et al. (2006) proposed that two scenarios can occur during deformation of vertebral centra based only on X-ray data of juvenile and adult Atlantic salmon. First, the containment scenario occurs when two or three vertebrae progress from fusion, in which two or three vertebrae can be recognised,

to a single larger vertebral centrum typically recognised by the presence of two or three neural and haemal associated elements. It was suggested that this 'new' larger vertebral centrum had a close to normal function (Witten et al., 2006). Second, is the aggravation scenario, where over time adjacent vertebral centra become sequentially involved in the vertebral deformity. Now, two new scenarios, which link early malformations observed in post-hatch Chinook salmon (*CHAPTER 3*) to late deformities observed in X-ray data in Atlantic and Chinook salmon (Perrott et al., 2018; Witten et al., 2009) can be postulated. These scenarios are a true containment scenario and a stabilisation scenario.

7.3.1. THE CONTAINMENT SCENARIO – 'SET IN BONE'

Figure 30 shows a hypothesised containment scenario of a pinched notochord observed in post-hatch Chinook salmon (Fig. 11A; *CHAPTER 3*) into an 'internal dorsal or ventral shift' deformity (type 19; Witten et al., 2009). Currently, a containment scenario is considered when two fused vertebral centra are remodelled into one functional vertebral body (Witten et al., 2009; Witten et al., 2006). Still, the shape of the internal structures of a vertebral centrum, i.e. the chordacentrum, could be retained from post-hatch to adult life stages. The basidorsals of the neural associated elements are fused at the level of the pinch (Fig. 30A) as was observed in post-hatch Chinook salmon (Fig. 11B; *CHAPTER 3*). The chordacentra start to mineralise within the notochord sheath on the ventral side of the notochord. The mineralisation progresses and the chordacentra become ventrally fused at the pinching site (Fig. 30B) similar to observations in Chinook salmon (Fig. 12A, B; *CHAPTER 3*). The fusion between the adjacent mineralised chordacentra at the pinching site has further progressed when the chordacentra form dorsally closed rings (Fig. 30C). Subsequently, the perinotochordal autocentra start to ossify and fuse to likely form a single autocentrum (Fig. 30D). The pinch of the notochord is now 'set in bone' with the fused chordacentra retaining the pinched shape of the notochord sheath. The autocentrum ossifying around the pinch likely further contains the shape (indicated by blue dotted line Fig. 30D). The cancellous bone of the arcocentrum ossifies around the autocentrum (Arratia & Schultze, 1992), firmly connecting the double arches to the autocentrum (only neural arches depicted Fig. 30E). On X-ray images, the compact bone of the perinotochordal autocentra is visible as the characteristic X-shapes in vertebrae of salmonids (Witten et al., 2009). The autocentrum covers the notochord sheath. Thus, if the chordacentrum (within the notochord sheath) is mineralised in the shape of the pinched notochord sheath, the perinotochordal autocentrum will have the shape of the pinched notochord. Due to appositional bone growth of the bony arcocentrum the overall contour of the vertebral centrum is normal even though the autocentrum forming around a pinch may appear on X-ray images as a deformity type 19 observed by Witten et al. (2009). The internal X-structure of the vertebral body is affected, meaning the centrum of the X is in a more dorsal or ventral position. Although type 19 deformities were not reported in Chinook salmon previously (Perrott et al., 2018), they have been subsequently seen in juvenile and adult Chinook salmon (personal observation).

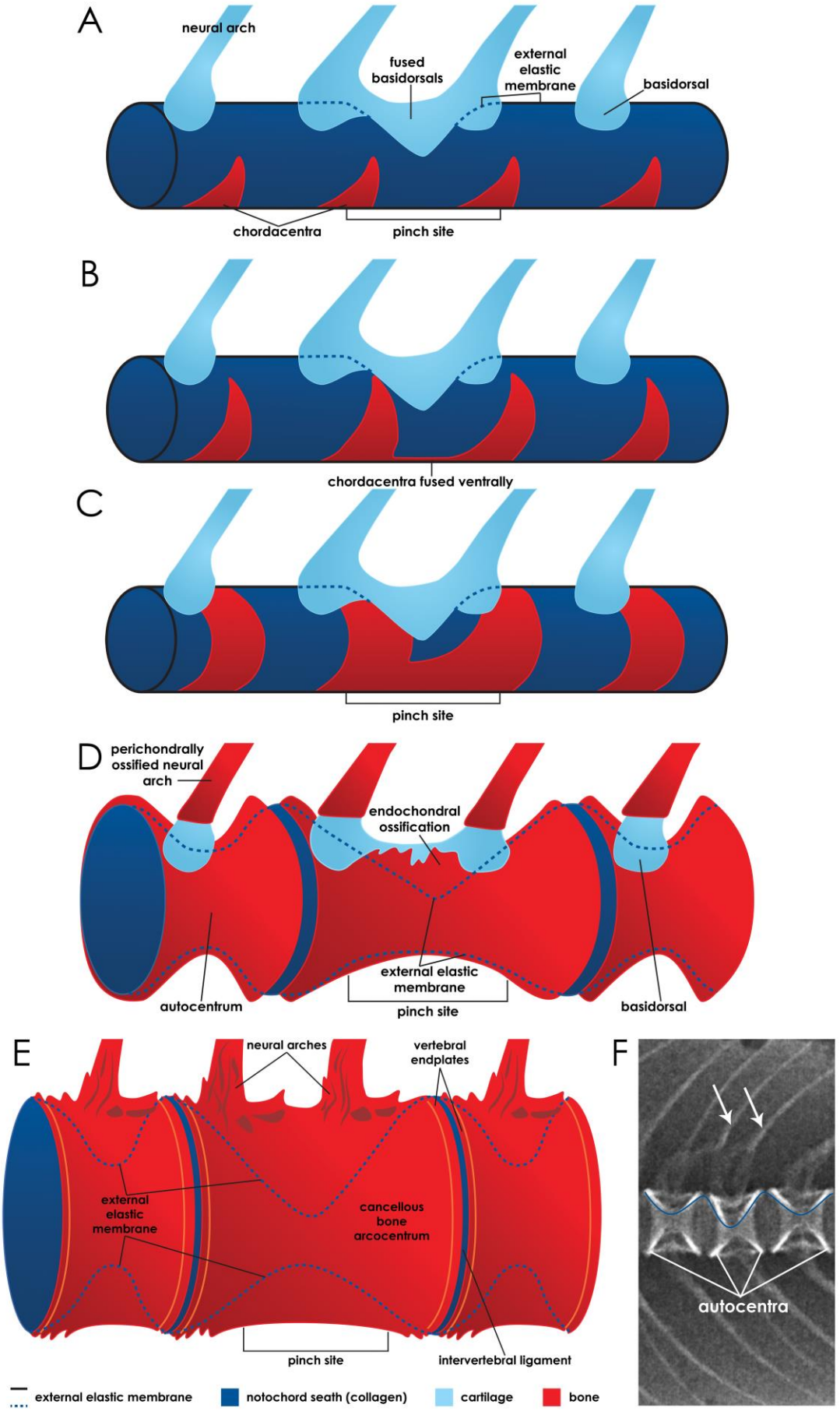
A
neural arch
fused basidorsals
external elastic membrane
basidorsal
chordacentra
pinch site
B
chordacentra fused ventrally
C
pinch site
D
perichondrally ossified neural arch
endochondral ossification
external elastic membrane
autocentrum
pinch site
basidorsal
E
neural arches
vertebral endplates
external elastic membrane
cancellous bone arcocentrum
pinch site
intervertebral ligament
external elastic membrane
notochord seath (collagen)
cartilage
bone
F
autocentra

Figure 30: A proposed containment scenario

Schematic representation of the different developmental steps: a pinch in the notochord develops into a type 19 deformity (internal dorsal or ventral shift), where the internal X-structure of the vertebral body is shifted dorsally or ventrally (Witten et al., 2009). All images are oriented with anterior to the left, posterior to the right, dorsal at the top and ventral at the bottom. Associated elements are only shown on the dorsal side to better illustrate the layers on the ventral side. (A-C) first feed stages (850°d-900°d). (D) Juvenile parr stage (1000-1400°d). (E-F) Juvenile pre- and post-smoltification and adult stages (>1500°d). The internal structure of the adult vertebral body follows the shape of the external elastic membrane (indicated in solid black and dotted blue lines) which retains its shape throughout development because the chordacentra (A-C), develops first as a mineralisation of the notochord sheath, followed by bone deposition onto the mineralised notochord sheath and its outer elastic membrane, the autocentra (D) and bony arcocentra (E). All bony layers have been given the same colour (red) because they are mineralised, although essentially chordacentra are mineralised type II collagen and autocentra and bony arcocentra are mineralised type I collagen. (A) Basidorsals fuse at the level of the pinch and space created by the pinch is filled with cartilage. On the ventral side the chordacentra (within the notochord sheath) start to mineralise. (B) The adjacent chordacentra at the level of the pinch fuse ventrally during their mineralisation. (C) The chordacentra have formed closed mineralised rings within the notochord sheath. (D) The autocentra have ossified around the external elastic membrane fixing the shape of the pinch in place. The neural arches have perichondrally ossified. (E) The arcocentra have ossified around and connected to the autocentra. Appositional growth of the arcocentra (more growth on the dorsal side compared to the ventral side) results in the normal external contour of the vertebral centra. The yellow lines mark the vertebral endplates. (F) X-ray of adult Chinook salmon. The white X-structure apparent on the X-ray are the vertebral autocentra. The course of the internal external elastic membrane (dorsal side) in the vertebral centra is indicated with a blue line. The internal X-structure of the middle vertebral centrum is shifted ventrally. Notice the occurrence of a double arch on the middle vertebral centrum (indicated by white arrows). This vertebra is a fusion product of two vertebrae.

7.3.2. THE STABILISATION SCENARIO

A combination of notochord pinching, bending, dislocation and hyperplasia of chordoblasts and scar tissue was one of the severe internal malformation phenotypes observed in post-hatch Chinook salmon (Fig. 11I; *CHAPTER 3*). This resulted in the marked collapse of the notochord towards a concertina shape and a shorter body axis (personal observation). The basidorsals and basiventrals are fused in many places which could be the consequence of a restriction in space along the notochord. Inside the notochord, the chordocytes, chordoblasts and notochord sheath are affected. At the level of a notochord lesion, scar tissue replaces the chordocytes and the chordoblast monolayer is dislocated. The dislocated chordoblasts produce ectopic notochord sheath material at the scar tissue site (*CHAPTER 3*). The notochord sheath is folded, pinched and the external elastic membrane can be disrupted. A proposed contour and internal structure for the collapsed notochord malformation are schematically represented in Figure 31A (contour, based on Fig. 11A) and Figure 31B (internal

structure, based on Fig. 11I). Similar to the observations in Chinook salmon (*CHAPTER 3*), a collapse of the notochord with pinching of the notochord sheath and the occurrence of scar tissue was observed in a new type of vertebral compression and fusion in the caudal region of gilthead sea bream (*Sparus aurata*) (Loizides et al., 2013).

A collapsed and severely malformed notochord likely provides less mechanical support and must in some way be stabilised. The malformed notochord in post-hatch Chinook salmon could have been stabilised by the cartilage of the fused basidorsals and basiventrals lining the notochord (Fig. 31A, B). In post-hatch Chinook salmon (*CHAPTER 3*), the cartilage of the fused basidorsals and basiventrals filled the spaces where the notochord was folded inwards, folded towards the left or right or folded towards the dorsal or ventral side of the midline axis (Fig. 31A, B). This is shown histologically on a parasagittal section of a collapsed notochord in Figure 11I. How this collapsed but stabilised notochord further develops is currently unknown. Zebrafish with mutations in the caveolae components, resulting in severe scar tissue lesions in the notochord, survived to adult stages (Garcia et al., 2017; Lim et al., 2017). Moreover, although scar tissue in the notochord impaired the swimming capacity of early post-hatch zebrafish stages, normal locomotor capacity was observed by 7 dpf when vertebral centra started to form (Lim et al., 2017). Mineralisation of chordacentra was observed in the severely malformed notochord of post-hatch Chinook salmon (*CHAPTER 3*). This result suggests that post-hatch Chinook salmon with a collapsed notochord and shorter body axis could be able to swim when chordacentra form. When the malformed salmon can swim, it can feed and survive under farming conditions. The survival of these malformed post-hatch Chinook salmon until pre-smoltification stages also means that the perinotochordal autocentra and bony arcocentra develop. It is conceivable that, similar to basidorsals and basiventrals which likely develop fused due to a shortened notochord, adjacent chordacentra fuse during mineralisation due to restriction in space. Once chordacentra are mineralised, the perinotochordal bone starts to ossify. The ossification of this perinotochordal bone likely further stabilises the mechanically weaker notochord. If chordacentra develop fused in an abnormally shaped notochord, both the internal structure and the perinotochordal vertebral centra can be expected to develop heavily compressed and contorted. The progression of the collapsed notochord into a large fusion centre could result in the known external phenotype ‘pumpkin-seed’ in Chinook salmon. An example of a pumpkin-seed phenotype is shown in Figure 31C and D (C = visual appearance, D = X-ray image). Scar tissue observed in *CHAPTER 3* (Fig. 11E, G, H) can also occur in a single site and likely forms a weak part of the notochord. Thus, the stabilisation of the notochord could also occur locally rather than over the entire vertebral column length. The scar tissue site can be as small as the length of two chordacentra. The adjacent chordacentra and perinotochordal vertebral centra likely fuse during growth to further stabilise the notochord resulting in vertebral fusion that in later life stages may be remodelled to a new larger unit.

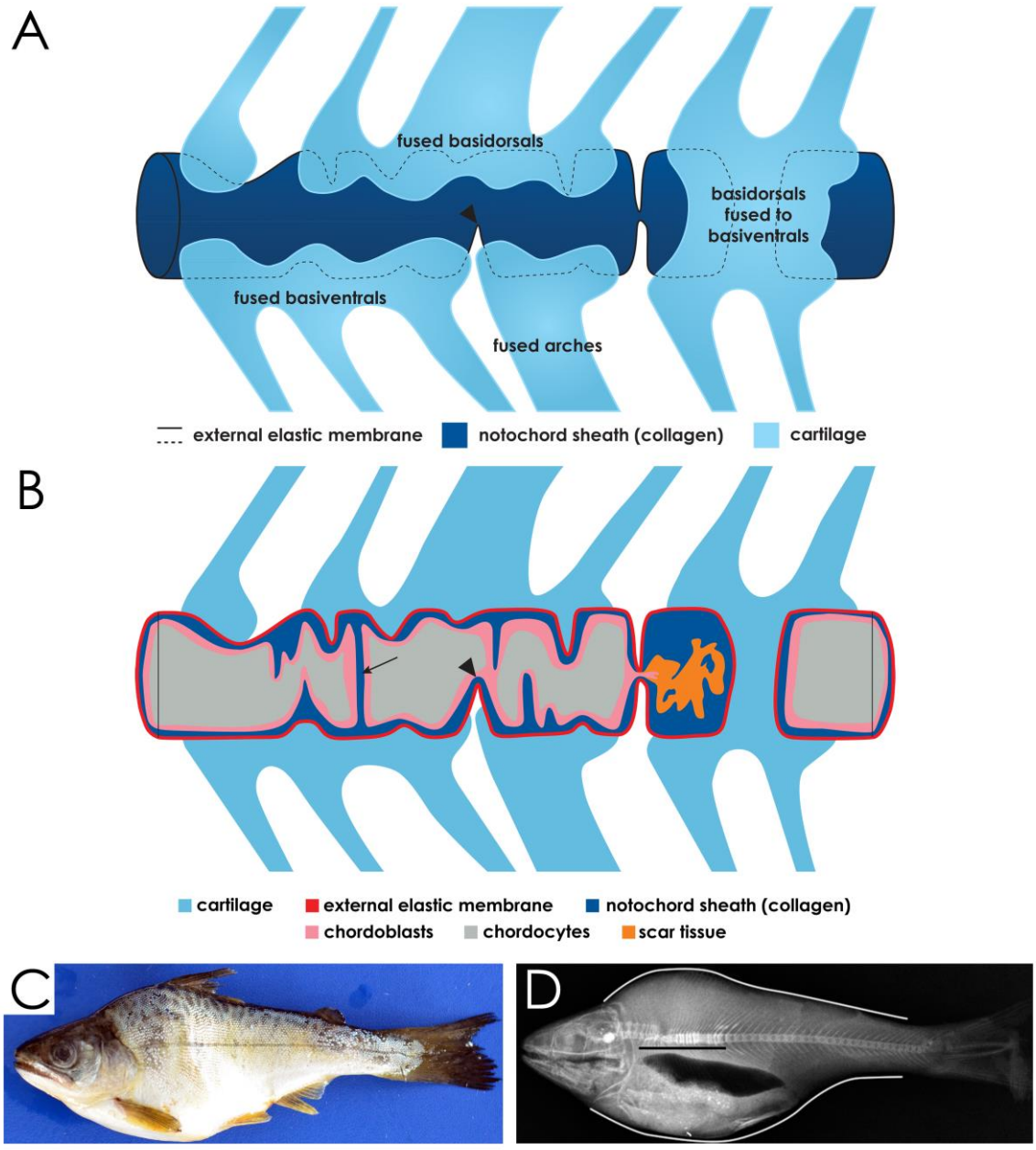

Figure 31: Stabilisation scenario of a heavily malformed notochord

(A-D) All images are oriented with anterior to the left, posterior to the right, dorsal at the top and ventral at the bottom. (A) Schematic representation of the outside contour of a collapsed notochord in post-hatch stage Chinook salmon (500°d to 800°d). The notochord external elastic membrane (black solid and dotted line) is pinched and folded inwards to separate two notochord ends. Similar to the basidorsals and basiventrals the arches remain separate or are fused. On the far right the basidorsals are fused to the basiventrals. (B) A longitudinal section of the notochord in (A). Internally the notochord shows partial and complete septa (arrow) and scar tissue. The arrowhead shows a pinch of the external elastic membrane. (C) Pre-smoltification stage juvenile Chinook salmon with external 'pumpkin-seed' phenotype. (D) X-ray of the specimen shown in (C). The external shape is indicated in white lines. Notice the fusion centre in the abdominal region. The fusion of the vertebrae could be the consequence of a collapsed notochord or a mixture of early perturbations that could not be contained but that eventually were stabilised.

7.4. RECOMMENDATIONS AND PERSPECTIVES

7.4.1. THE BEST INCUBATION TEMPERATURE IS A CONSTANT 8°C

Results from *CHAPTER 3* showed that the lowest frequency of internal malformations occurred in the constant 8°C temperature group. Results in *CHAPTER 5* showed that juvenile Chinook salmon reared at this temperature have more vertebral centra in the caudal region of the vertebral column, which is possibly advantageous for swimming and likely improves the ability to feed. Also, specimens of the 8°C group showed less variation in the left-right presence of skeletal elements indicating that development may be more stable at 8°C (Allenbach, 2011; Leary & Allendorf, 1989). Although in *CHAPTER 6*, freshwater smoltification-stage salmon of the 8°C group had a significantly higher prevalence of total deformities, this group had a significantly lower prevalence of total deformities and LKS at the six-months-at-sea-stage. Moreover, fewer specimens were classified as runts in the 8°C group and had a lower prevalence of total deformities and LKS compared to the 12°C group. Also, the 8°C group had a lower attrition rate during and between each life stage compared to the 12°C group. Therefore, it appears that incubation at 8°C and a period of growth in fresh water at 8°C protects Chinook salmon against deformities in early sea stages. Given the greater number of unexpected temperature changes in the 4°C and 8°C groups and the superior outcomes for the 8°C group, it is likely that 8°C is the best incubation temperature for farmed salmon in New Zealand. It may be that the actual difference between the 8°C and 12°C groups is underrepresented as the 12°C group had the fewest temperatures changes.

In Chinook salmon, 8°C is at the lower end of the optimal temperature range for embryos and post-hatch stages, from 8°C to 10°C (Alderdice & Velsen, 1978; Brett, 1952). The optimal temperature range reported by these authors, is based on experiments investigating developmental rates, and mortality and survival rates related to upper and lower lethal temperatures in Pacific salmon egg stages. In the current study, early mortality was only reported briefly in *CHAPTER 3* and developmental and growth rates were not reported. Condition factor, another important measure of animal quality and welfare for salmon farmers (Jones et al., 1999; Segner et al., 2012), was also not reported. Early mortality, developmental rates, growth rates and condition factor related to temperature in farmed Chinook salmon should be further investigated as datasets built on these measures could corroborate that 8°C is indeed close to an optimal incubation temperature. However, only three temperatures were tested here due to the cost of the large-scale experiment. Although clearer differences in results were likely obtained between the temperature groups due to the large temperature steps, the temperatures in-between 4°C, 8°C and 12°C would have to be tested in future experiments to refine the trend indicated in this thesis. For example, repeating the 8°C incubation and adding smaller temperature steps on either side of 8°C. Since the existing optimal temperature range for Chinook salmon

incubation is between 8°C and 10°C, the best results with respect to mortality rates, developmental rates, condition factor and malformation rates, may in theory be obtained at any temperature between 8°C and 10°C. On the other hand, optimum temperature ranges for each of these metrics may be slightly different from one another. Finding significant differences for these measures, when using smaller temperature increments of ±1°C or < 1°C may be challenging.

For Chinook salmon, an incubation temperature of 8°C may be ideal in experimental conditions (Alderdice & Velsen, 1978; Brett, 1952; current research), but may not be feasible in farming operations. Due to the need for year round harvest, salmon farmers often influence husbandry factors such as temperature, light regime and feeding regime to grow salmon faster or slower. Although light and feeding regime can be changed in the seawater environment (Fjelldal et al., 2009b; Fjelldal et al., 2005), temperature can only be easily influenced in the freshwater environment. The differentiation between fast and slow growing salmon is more easily made by changing freshwater husbandry factors which, in case of temperature, is likely to be a change to being above or below the optimal range for robust skeletal development and growth. An example of how the industry manipulates temperature and influences development, based on personal observations, is given below: *The cohort of salmon that was harvested in the first part of the year, and thus the faster growing group, was fertilised at 10°C and brought to 12°C for incubation and during the freshwater grow out stages. This cohort received a '16 hours light-8 hours dark' cycle and was fed frequently during the day with automated hoppers. The cohort of salmon that was harvested in the second part of the year (slow growing group) was also fertilised at 10°C but instead brought to 6°C for the incubation period until first feed stage. Hatching for this group was synchronised by gradually raising the temperature to 12°C and then returning it to 6°C. These animals had a natural day-night cycle and were fed less frequently compared to the fast growing cohort*. Thus, both the fast and slow growing groups had substantial periods of early development at temperatures not considered to be optimal for rearing Chinook salmon. Especially the temperature rise from 6°C (below optimal) to 12°C (above optimal) could be considered stressful for early stages and may result in deformities that develop in seawater life stages (*CHAPTER 6*).

Freshwater grow-out facilities provide space for early juveniles stages to swim. Sustained swimming occurring in the freshwater grow-out system will increase the bone mineral content and osteocyte density, as was observed for Atlantic salmon and rainbow trout (Deschamps et al., 2009; Totland et al., 2011). Healthy bone, defined as a well-balanced deposition of bone by osteoblasts and resorption by osteoclasts, decreases the risk of developing deformities during fast growth in freshwater stages. However, for healthy bone formation to be sustained throughout the farm life cycle, the chordacentra as the template of the vertebrae and the cartilaginous precursors of the neural and haemal arches, i.e. the post-hatch stage associated elements, need to be perfectly formed in the pre- and post-hatch stages

of the salmon. The requirements for commercially dictated growth rates and the use of temperature manipulation to achieve these results puts normal developmental processes at risk.

One thing that has become clear is the strong likelihood that to form a normal vertebral column and in order to avoid as many malformations and deformities possible, low to moderate developmental rates are preferred. Close to ideal development is largely driven by constant temperatures, and ideally no fluctuations or temperature manipulations for synchronised hatching. In order to farm efficiently, aquaculture must find a balance between close to ideal development and optimal growth for commercial targets. These goals are likely to remain in conflict and a healthy dialogue that weighs developmental science, aquaculture commerce and aquaculture social licence is critical to maintain. It is likely that performance in seawater and freshwater is linked (*CHAPTER 6*) but that these linkages are not well understood. Communication between the freshwater and seawater operations is therefore equally, if not more, critical to maintain. Only through robust monitoring and commitment to track performance of cohorts that experience different husbandry strategies during the early stages of development will further clues of 'optimal' rearing identified.

7.4.2. KNOWLEDGE OF POST-HATCH SALMONID STAGES NEEDS TO BE INCREASED

As was shown in *CHAPTER 3*, the study of early malformations is useful, providing knowledge about how early malformations develop, how they progress and about their regional occurrence. The deformities of salmonids in later life stages, especially harvest stages, represent a cost to the farmer (Fjelldal et al., 2012). However, studying early malformations can help prevent the presence of deformities later in life and therefore make the farming process more efficient. In addition, malformations and deformities represent challenges from an ethical and animal welfare perspective. Decreasing early malformations that potentially foreshadow late life deformities, thus decreasing deformities at harvest, will improve animal welfare. Improving farmed animal welfare is of concern to the consumer, who has become more aware of ethical and animal welfare issues. In turn the salmon farmers will have to focus more resources on maintaining social license and animal welfare.

The importance of accurate studies of skeletal structures in early life stages of teleosts, in order to recognise and identify skeletal abnormalities, has been recognised for a long time (McMurrich, 1883). Still, the lack of research and knowledge of post-hatch salmonid stages is remarkable. Boglione et al. (2013a) noted that a frequency of about 20% severely malformed marine farmed teleosts at the end of the hatchery phase can be considered as a good, but a rare result. In contrast, the frequency of malformations at the end of the freshwater stage in salmon farming is lower compared to marine teleost farming in general. Therefore, early malformations have never been a major issue for salmon farmers and may explain why studies on early malformations in salmonids are limited. However, the results in *CHAPTER 3* clearly show that early skeletal malformations are present even if they are not

obvious. Internal malformations that are externally invisible may represent a good research target to help avoid such malformations becoming costly vertebral deformities in harvest stages.

7.4.3. SALMON AS MODEL ORGANISM

Scar tissue observed in *CHAPTER 3* looks very similar to scar tissue (notochord lesions) observed in zebrafish (Garcia et al., 2017; Lim et al., 2017). In addition, scar tissue in post-hatch stage Chinook salmon looks remarkably similar to chordoma tissue. A chordoma is a rare, but aggressive notochord cell tumour that can occur in the bones of the human skeleton (Choi et al., 2008; Kitamura et al., 2013; Presneau et al., 2011; Sun et al., 2015). Zebrafish with an HRASV12 mutation had notochords with histological characteristics that were highly similar to the chordomas observed in human patients. In the zebrafish mutant, cells were found to be plump, had focally prominent nuclear pleomorphism and hyperchromasia, and grew in nests and cords with both a focal and a solid pattern. The tumor matrix was found to be variable, ranging from dense eosinophilic to myxoid with vacuolated spaces, which is also typical of human chordoma. Large cells with a vacuolated cytoplasm were observed in zebrafish, which is also reminiscent of physaliferous cells of human chordomas (Burger et al., 2014). Several markers that are used to diagnose chordoma in humans, i.e. Brachyury, Cytokeratin, pErk and pS6, were immunohistochemically shown to be present in zebrafish chordoma tissue (Burger et al., 2014). However, the HRAS mutation in zebrafish has not been reported in humans and the chordoma in zebrafish has a rapid onset (3 dpf onwards) compared to the slow growing human tumor, which often takes 5 years for symptoms to appear (Burger et al., 2014). It is possible that the notochord scar tissue observed in Chinook salmon could be used to study chordoma cancer in humans.

The use of fish as a model organism is not a new concept (Witten et al., 2017). Studies on bone diseases, such as Osteogenesis Imperfecta, Cranio-lenticulo-sutural dysplasia and Spondyloepiphysial dysplasia tarda, have been undertaken in zebrafish and medaka (Gistelinck et al., 2016a; Gistelinck et al., 2018; Gistelinck et al., 2016b; Kondylis et al., 2009; Lang et al., 2006; Ohisa et al., 2010). Although Chinook and Atlantic salmon do not have translucent eggs, embryos and post-hatch stages or a reasonably fast life cycle, their eggs are large and are produced in high numbers per single female. In addition, the early developmental stages of Chinook and Atlantic salmon are larger than zebrafish stages and may be easier to handle in that specimens can be cleared and whole mount stained. Techniques, such as whole mount staining and clearing with Alcian blue and Alizarin red used in the present research, are well established. In addition, compared to the acellular bone in the model organism medaka, the bones of salmonids are composed of cellular bone and provide another advantage when comparisons and extrapolations to human bone physiology studies are required. Early stages of Chinook salmon could therefore represent a useful research organism for:

(i) aquaculture purposes to gather knowledge about vertebral malformations and deformities (*CHAPTER 3, 6*)

(ii) fundamental studies in the field of evo-devo to gather knowledge about the development and evolution of the vertebral column (*CHAPTERS 4, 5*)

(iii) potentially studying human skeletal diseases

CHAPTER 8: REFERENCES

Ackerly KL, Ward AB (2016) How temperature-induced variation in musculoskeletal anatomy affects escape performance and survival of zebrafish (*Danio rerio*). *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology,* **325,** 25-40.

Adams DS, Keller R, Koehl MA (1990) The mechanics of notochord elongation, straightening and stiffening in the embryo of *Xenopus laevis*. *Development,* **110,** 115-130.

Aguirre WE, Walker K, Gideon S (2014) Tinkering with the axial skeleton: Vertebral number variation in ecologically divergent threespine stickleback populations. *Biological Journal of the Linnean Society,* **113,** 204-219.

Ahn D, Gibson G (1999a) Axial variation in the threespine stickleback: Genetic and environmental factors. *Evolution & Development,* **1,** 100-112.

Ahn D, Gibson G (1999b) Axial variation in the threespine stickleback: Relationship to *Hox* gene expression. *Development Genes and Evolution,* **209,** 473-481.

Ahn D, Gibson G (1999c) Expression patterns of threespine stickleback *Hox* genes and insights into the evolution of the vertebrate body axis. *Development Genes and Evolution,* **209,** 482-494.

Alderdice D, Velsen F (1978) Relation between temperature and incubation time for eggs of Chinook salmon (*Oncorhynchus tshawytscha*). *Journal of the Fisheries Research Board of Canada,* **35,** 69-75.

Alderdice DF, Wickett WP, Brett JR (1958) Some effects of temporary exposure to low dissolved oxygen levels on Pacific salmon eggs. *Journal of the Fisheries Research Board of Canada,* **15,** 229-250.

Allenbach DM (2011) Fluctuating asymmetry and exogenous stress in fishes: A review. *Reviews in Fish Biololgy and Fisheries,* **21,** 355-376.

Altimiras J, Axelsoon M, Claireaux G, Lefrançois C, Mercier C, Farrell AP (2002) Cardiorespiratory status of triploid brown trout during swimming at two acclimation temperatures. *Journal of Fish Biology,* **60,** 102-116.

Amacher SL, Kimmel CB (1998) Promoting notochord fate and repressing muscle development in zebrafish axial mesoderm. *Development,* **125,** 1397-1406.

Anderson C, Bartlett SJ, Gansner JM, Wilson D, He L, Gitlin JD, Kelsh RN, Dowden J (2007) Chemical genetics suggests a critical role for lysyl oxidase in zebrafish notochord morphogenesis. *Molecular Biosystems,* **3,** 51-59.

Andrades JA, Becerra J, Fernández-Llebrez P (1996) Skeletal deformities in larval, juvenile and adult stages of cultured gilthead sea bream (*Sparus aurata* L.). *Aquaculture,* **141,** 1-11.

Annona G, Holland ND, D'Aniello S (2015) Evolution of the notochord. *EvoDevo,* **6,** 30-43.

Arratia G, Schultze H-P (1992) Reevaluation of the caudal skeleton of certain actinopterygian fishes: III. Salmonidae. Homologization of caudal skeletal structures. *Journal of Morphology,* **214,** 187-249.

Arratia G, Schultze H-P, Casciotta J (2001) Vertebral column and associated elements in dipnoans and comparison with other fishes: Development and homology. *Journal of Morphology,* **250,** 101-172.

Asher RJ, Lin KH, Kardjilov N, Hautier L (2011) Variability and constraint in the mammalian vertebral column. *Journal of Evolutionary Biology,* **24,** 1080-1090.

Atkins ME, Benfey TJ (2008) Effect of acclimation temperature on routine metabolic rate in triploid salmonids. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **149,** 157-161.

Aulstad D, Kittelsen A (1971) Abnormal body curvatures of rainbow trout (*Salmo gairdneri*) inbred fry. *Journal of the Fisheries Research Board of Canada,* **28,** 1918-1920.

Austreng E, Storebakken T, Åsgård T (1987) Growth rate estimates for cultured Atlantic salmon and rainbow trout. *Aquaculture,* **60,** 157-160.

Badiola M, Mendiola D, Bostock J (2012) Recirculating Aquaculture Systems (RAS) analysis: Main issues on management and future challenges. *Aquacultural Engineering,* **51,** 26-35.

Bagnall KM, Higgins SJ, Sanders EJ (1988) The contribution made by a single somite to the vertebral column: Experimental evidence in support of resegmentation using the chick-quail chimaera model. *Development,* **103,** 69-85.

Balfour FM, Parker WN (1882) On the structure and development of *Lepidosteus*. *Philosophical Transactions of the Royal Society of London,* **173,** 359-442.

Ballard WW (1973a) Morphogenetic movements in *Salmo gairdneri* Richardson. *Journal of Experimental Zoology,* **184,** 27-48.

Ballard WW (1973b) A new fate map for *Salmo gairdneri*. *Journal of Experimental Zoology,* **184,** 49-73.

Ballard WW (1973c) Normal embryonic stages for salmonid fishes, based on *Salmo gairdneri* (Richardson) and *Salvelinus fontinalis* (Mitchill). *Journal of Experimental Zoology,* **184,** 7-25.

Banks JL, Fowler LG, Elliott JW (1971) Effects of rearing temperature on growth, body form, and hematology of fall Chinook fingerlings. *The Progressive Fish-Culturist,* **33,** 20-26.

Bastiani M, Parton RG (2010) Caveolae at a glance. *Journal of Cell Science,* **123,** 3831-3836.

Basu N, Todgham AE, Ackerman PA, Bibeau MR, Nakano K, Schulte PM, Iwama GK (2002) Heat shock protein genes and their functional significance in fish. *Gene,* **295,** 173-183.

Batty RS, Blaxter JHS, Fretwell K (1993) Effect of temperature on the escape responses of larval herring, *Clupea harengus*. *Marine Biology,* **115,** 523-528.

Beacham T (1985) Variation in number of vertebrae and gill rakers of sockeye salmon, *Oncorhynchus nerka*, in North America. *Environmental Biology of Fishes,* **14,** 97-105.

Beacham TD (1990) A genetic analysis of meristic and morphometric variation in chum salmon (*Oncorhynchus keta*) at three different temperatures. *Canadian Journal of Zoology,* **68,** 225-229.

Beacham TD, Gould A, Stefanson A, Station PB (1983) Size, age, meristics, and morphometrics of chum salmon returning to southern British Columbia during 1981-1982. pp. 1-37. Nanaimo, British Columbia Department of Fisheries and Oceans, Fisheries Research Branch, Pacific Biological Station.

Beacham TD, Murray CB (1985a) Effect of female size, egg size, and water temperature on developmental biology of chum salmon (*Oncorhynchus keta*) from the Nitinat River, British Columbia. *Canadian Journal of Fisheries and Aquatic Sciences,* **42,** 1755-1765.

Beacham TD, Murray CB (1985b) Variation in length and body depth of pink salmon (*Oncorhynchus gorbuscha*) and chum salmon (*O. keta*) in Southern British Columbia. *Canadian Journal of Fisheries and Aquatic Sciences,* **42,** 312-319.

Beacham TD, Murray CB (1986) The effect of spawning time and incubation temperature on meristic variation in chum salmon (*Oncorhynchus keta*). *Canadian Journal of Zoology,* **64,** 45-48.

Beacham TD, Murray CB (1987a) Adaptive variation in body size, age, morphology, egg size, and developmental biology of chum salmon (*Oncorhynchus keta*) in British Columbia. *Canadian Journal of Fisheries and Aquatic Sciences,* **44,** 244-261.

Beacham TD, Murray CB (1987b) Effects of transferring pink (*Oncorhynchus gorbuscha*) and chum (*Oncorhynchus keta*) salmon embryos at different developmental stages to a low incubation temperature. *Canadian Journal of Zoology,* **65,** 96-105.

Beacham TD, Murray CB (1988) Variation in developmental biology of pink salmon (*Oncorhynchus gorbuscha*) in British Columbia. *Canadian Journal of Zoology,* **66,** 2634-2648.

Beacham TD, Murray CB (1989) Variation in developmental biology of sockeye salmon (*Oncorhynchus nerka*) and Chinook salmon (*O. tshawytscha*) in British Columbia. *Canadian Journal of Zoology,* **67,** 2081-2089.

Beacham TD, Murray CB (1990) Temperature, egg size, and development of embryos and alevins of five species of Pacific salmon: A comparative analysis. *Transactions of the American Fisheries Society,* **119,** 927-945.

Beacham TD, Varnavskaya NV (1991) Effect of parental heterozygosity on pink salmon (*Oncorhynchus gorbuscha*) embryonic and alevin survival and development at extreme temperatures. *Canadian Journal of Zoology,* **69,** 2485-2489.

Becker CD, Genoway RG (1979) Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environmental Biology of Fishes,* **4,** 245-256.

Beitinger T, Bennett W (2000) Quantification of the role of acclimation temperature in temperature tolerance of fishes. *Environmental Biology of Fishes,* **58,** 277-288.

Beitinger T, Bennett W, McCauley R (2000) Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environmental Biology of Fishes,* **58,** 237-275.

Belding DL (1928) Water temperature and fish life. *Transactions of the American Fisheries Society,* **58,** 98-105.

Belon du Mans P (1555) *La nature et diversité des poissons, Avec leurs pourtraicts, representez au plus pres du naturel,* Charles Estienne, Imprimeur ordinaire du Roy, A Monseigneur le Reverindiss Cardinal de Chastillon, Paris.

Bendiksen EÅ, Berg OK, Jobling M, Arnesen AM, Måsøval K (2003) Digestibility, growth and nutrient utilisation of Atlantic salmon parr (*Salmo salar* L.) in relation to temperature, feed fat content and oil source. *Aquaculture,* **224,** 283-299.

Benfey T, McCabe L, Pepin P (1997) Critical thermal maxima of diploid and triploid brook charr, *Salvelinus fontinalis*. *Environmental Biology of Fishes,* **49,** 259-264.

Benfey TJ, Sutterlin AM (1984) Triploidy induced by heat shock and hydrostatic pressure in landlocked Atlantic salmon (*Salmo salar* L.). *Aquaculture,* **36,** 359-367.

Bensimon-Brito A, Cancela ML, Huysseune A, Witten PE (2010) The zebrafish (*Danio rerio*) caudal complex – A model to study vertebral body fusion. *Journal of Applied Ichthyology,* **26,** 235-238.

Bensimon-Brito A, Cancela ML, Huysseune A, Witten PE (2012a) Vestiges, rudiments and fusion events: The zebrafish caudal fin endoskeleton in an evo-devo perspective. *Evolution & Development,* **14,** 116-127.

Bensimon-Brito A, Cardeira J, Cancela ML, Huysseune A, Witten PE (2012b) Distinct patterns of notochord mineralization in zebrafish coincide with the localization of Osteocalcin isoform 1 during early vertebral centra formation. *BMC Developmental Biology,* **12,** 28-42.

Bergheim A, Drengstig A, Ulgenes Y, Fivelstad S (2009) Production of Atlantic salmon smolts in Europe—Current characteristics and future trends. *Aquacultural Engineering,* **41,** 46-52.

Billard R (1983) Spermiogenesis in the rainbow trout (*Salmo gairdneri*). *Cell and Tissue Research,* **233,** 265-284.

Bird NC, Hernandez LP (2009) Building an evolutionary innovation: Differential growth in the modified vertebral elements of the zebrafish Weberian apparatus. *Zoology,* **112,** 97-112.

Bird NC, Mabee PM (2003) Developmental morphology of the axial skeleton of the zebrafish, *Danio rerio* (Ostariophysi: Cyprinidae). *Developmental Dynamics,* **228,** 337-357.

Bliss CI, Stevens WL (1937) The calculation of the time-mortality curve. *Annals of Applied Biology,* **24,** 815-852.

Böck P, Romeis B (1989) *Mikroskopische Technik.* Urban und Schwarzenberg, München.

Boglione C, Gagliardi F, Scardi M, Cataudella S (2001) Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared gilthead sea bream (*Sparus aurata* L. 1758). *Aquaculture,* **192,** 1-22.

Boglione C, Gavaia P, Koumoundouros G, Gisbert E, Moren M, Fontagné S, Witten PE (2013a) Skeletal anomalies in reared European fish larvae and juveniles. Part 1: Normal and anomalous skeletogenic processes. *Reviews in Aquaculture,* **5,** S99-S120.

Boglione C, Gisbert E, Gavaia P, Witten PE, Moren M, Fontagné S, Koumoundouros G (2013b) Skeletal anomalies in reared European fish larvae and juveniles. Part 2: Main typologies, occurrences and causative factors. *Reviews in Aquaculture,* **5,** S121-S167.

Bond CE, Uyeno T (1981) Remarkable changes in the vertebrae of perciform fish *Scombrolabrax* with notes on its anatomy and systematics. *Japanese Journal of Ichthyology,* **28,** 259-262.

Bornstein P, Sage EH (2002) Matricellular proteins: Extracellular modulators of cell function. *Current Opinion in Cell Biology,* **14,** 608-616.

Boyd JW, Oldenburg EW, McMichael GA (2010) Color photographic index of fall Chinook salmon embryonic development and accumulated thermal units. *PLoS ONE,* **5,** 1-10.

Bradford MJ (1995) Comparative review of Pacific salmon survival rates. *Canadian Journal of Fisheries and Aquatic Sciences,* **52,** 1327-1338.

Brander K (1979) The relationship between vertebral number and water temperature in cod. *Journal du Conseil,* **38,** 286-292.

Brett J (1967) Swimming performance of sockeye salmon (*Oncorhynchus nerka*) in relation to fatigue time and temperature. *Journal of the Fisheries Board of Canada,* **24,** 1731-1741.

Brett JR (1941) Tempering versus acclimation in the planting of speckled trout. *Transactions of the American Fisheries Society,* **70,** 397-403.

Brett JR (1946) Rate of gain of heat-tolerance in goldfish (*Carassius auratus*). *Publications of the Ontario Fisheries Research Laboratory,* **64,** 9-28.

Brett JR (1952) Temperature tolerance in young Pacific salmon, genus *Oncorhynchus*. *Journal of the Fisheries Research Board of Canada,* **9,** 265-323.

Brett JR (1956) Some principles in the thermal requirements of fishes. *The Quarterly Review of Biology,* **31,** 75-87.

Brett JR (1964) The respiratory metabolism and swimming performance of young sockeye salmon. *Journal of the Fisheries Research Board of Canada,* **21,** 1183-1226.

Brett JR (1971) Energetic responses of salmon to temperature. A study of some thermal relations in the physiology and freshwater ecology of sockeye salmon (*Oncorhynchus nerka*). *American Zoologist,* **11,** 99-113.

Brett JR (1972) The metabolic demand for oxygen in fish, particularly salmonids, and a comparison with other vertebrates. *Respiration Physiology,* **14,** 151-170.

Brett JR, Shelbourn JE, Shoop CT (1969) Growth rate and body composition of fingerling sockeye salmon, *Oncorhynchus nerka*, in relation to temperature and ration size. *Journal of the Fisheries Research Board of Canada,* **26,** 2363-2394.

Britz R, Bartsch P (2003) The myth of dorsal ribs in gnathostome vertebrates. *Proceedings of the Royal Society of London B: Biological Sciences,* **270,** 1-4.

Britz R, Conway KW (2009) Osteology of *Paedocypris*, a miniature and highly developmentally truncated fish (Teleostei: Ostariophysi: Cyprinidae). *Journal of Morphology,* **270,** 389-412.

Buckland F (1877) Snub-nosed Salmon. *Forest and Stream,* **VIII,** 96.

Burger A, Vasilyev A, Tomar R, Selig MK, Nielsen GP, Peterson RT, Drummond IA, Haber DA (2014) A zebrafish model of chordoma initiated by notochord-driven expression of HRASV12. *Disease Models & Mechanisms,* **7,** 907-913.

Burke AC, Nelson CE, Morgan BA, Tabin C (1995) *Hox* genes and the evolution of vertebrate axial morphology. *Development,* **121,** 333-346.

Campbell WB (2003) Assessing developmental errors in branchiostegal rays as indicators of chronic stress in two species of Pacific salmon. *Canadian Journal of Zoology,* **81,** 1876-1884.

Campbell WB, Emlen JM, Hershberger WK (1998) Thermally induced chronic developmental stress in coho salmon: Integrating measures of mortality, early growth, and developmental instability. *Oikos,* **81,** 398-410.

Campos C, Valente LMP, Conceição LEC, Engrola S, Fernandes JMO (2013) Temperature affects methylation of the *myogenin* putative promoter, its expression and muscle cellularity in *Senegalese sole* larvae. *Epigenetics,* **8,** 389-397.

Canagaratnam P (1959) The influence of light intensities and durations during early development on meristic variation in some salmonids. In *Department of Zoology*, pp. 1-124. Vancouver: Univeristy of British Columbia.

Carson R (1950) *The Sea Around Us,* Oxford University Press, New york.

Cenomani Bellonii P (1553) *De Aquatilibus, Libri duo Cum eiconibus ad viuam ipsorum effigiem, quoad eius fieri potuit expressis,* Apud Carolum Stephanum, Typographum Regium, Ad amplissimum Cardinalem Castillionoeum Parisiis (Paris).

Chezik KA, Lester NP, Venturelli PA (2013) Fish growth and degree-days I: Selecting a base temperature for a within-population study. *Canadian Journal of Fisheries and Aquatic Sciences,* **71,** 47-55.

Choi K-S, Cohn MJ, Harfe BD (2008) Identification of nucleus pulposus precursor cells and notochordal remnants in the mouse: Implications for disk degeneration and chordoma formation. *Developmental Dynamics,* **237,** 3953-3958.

Clarke WC, Nagahama Y (1977) Effect of premature transfer to sea water on growth and morphology of the pituitary, thyroid, pancreas, and interrenal in juvenile coho salmon (*Oncorhynchus kisutch*). *Canadian Journal of Zoology,* **55,** 1620-1630.

Clarke WC, Shelbourn JE (1985) Growth and development of seawater adaptability by juvenile fall Chinook salmon (*Oncorhynchus tshawytscha*) in relation to temperature. *Aquaculture,* **45,** 21-31.

Clarke WC, Shelbourn JE (1986) Delayed photoperiod produces more uniform growth and greater seawater adaptability in underyearling coho salmon (*Oncorhynchus kisutch*). *Aquaculture,* **56,** 287-299.

Clarke WC, Shelbourn JE, Brett JR (1981) Effect of artificial photoperiod cycles, temperature, and salinity on growth and smolting in underyearling coho (*Oncorhynchus kisutch*), Chinook (*O. tshawytscha*), and sockeye (*O. nerka*) salmon. *Aquaculture,* **22,** 105-116.

Clothier CR (1950) *A key to some Southern California fishes: Based on vertebral characters,* California State Print Office, California.

Cloutier R, Caron A, Grünbaum T, Le François NR (2010) Effect of water velocity on the timing of skeletogenesis in the Arctic charr, *Salvelinus alpinus* (Salmoniformes: Teleostei): An empirical case of developmental plasticity. *International Journal of Zoology,* **2010,** 1-15.

Colt J, Maynard D (2017) Impacts of chiller failure on temperature change in isolation incubators for salmonids. *Aquacultural Engineering,* **76,** 20-33.

Combs BD (1965) Effect of temperature on the development of salmon eggs. *The Progressive Fish-Culturist,* **27,** 134-137.

Combs BD, Burrows RE (1957) Threshold temperatures for the normal development of Chinook salmon eggs. *The Progressive Fish-Culturist,* **19,** 3-6.

Conlon RA, Reaume AG, Rossant J (1995) Notch1 is required for the coordinate segmentation of somites. *Development,* **121,** 1533-1545.

Cooke J, Zeeman EC (1976) A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. *Journal of Theoretical Biology,* **58,** 455-476.

Cooper GW (1965) Induction of somite chondrogenesis by cartilage and notochord: A correlation between inductive activity and specific stages of cytodifferentiation. *Developmental Biology,* **12,** 185-212.

Corallo D, Schiavinato A, Trapani V, Moro E, Argenton F, Bonaldo P (2013) Emilin3 is required for notochord sheath integrity and interacts with Scube2 to regulate notochord-derived Hedgehog signals. *Development,* **140,** 4594-4601.

Coutant C (1975) Temperature selection by fish - A factor in power-plant impact assessments. In *Environmental effects of cooling systems at nuclear power plants* (ed Agency IAE), pp. 575-597. Oslo: International Atomic Energy Agency, Vienna.

Coutinho P, Parsons MJ, Thomas KA, Hirst EMA, Saúde L, Campos I, Williams PH, Stemple DL (2004) Differential requirements for COPI transport during vertebrate early development. *Developmental Cell,* **7,** 547-558.

Crisp DT (1981) A desk study of the relationship between temperature and hatching time for the eggs of five species of salmonid fishes. *Freshwater Biology,* **11,** 361-368.

Crisp DT (1988) Prediction, from temperature, of eyeing, hatching and 'swim-up' times for salmonid embryos. *Freshwater Biology,* **19,** 41-48.

Cubbage CC, Mabee PM (1996) Development of the cranium and paired fins in the zebrafish *Danio rerio* (Ostariophysi, Cyprinidae). *Journal of Morphology,* **229,** 121-160.

Culling CFA (1974a) Chapter 14 - Carbohydrates. In *Handbook of Histopathological and Histochemical Techniques (Third Edition)* (ed Culling CFA), pp. 259-314. Wellington: Butterworth-Heinemann.

Culling CFA (1974b) Chapter 19 - Tissues Requiring Special Treatment or Techniques. In *Handbook of Histopathological and Histochemical Techniques (Third Edition)* (ed Culling CFA), pp. 405-466. Wellington: Butterworth-Heinemann.

Daisei A, Sh-ichi M, Nobuhisa K, Masamichi N (2008) Estimation of heritability and genetic correlation of number of abdominal and caudal vertebrae in masu salmon. *Fisheries Science,* **74,** 293-298.

Dalsgaard J, Lund I, Thorarinsdottir R, Drengstig A, Arvonen K, Pedersen PB (2013) Farming different species in RAS in Nordic countries: Current status and future perspectives. *Aquacultural Engineering,* **53,** 2-13.

Danos N, Ward AB (2012) The homology and origins of intermuscular bones in fishes: Phylogenetic or biomechanical determinants? *Biological Journal of the Linnean Society,* **106,** 607-622.

Darwin C (1859) *The Origin of Species*, The Harvard Classics, P F Collier and Son, New York.

Davenport CB, Castle WE (1895) Studies in morphogenesis, III. On the acclimatization of organisms to high temperatures. *Archiv für Entwicklungsmechanik der Organismen,* **2,** 227-249.

Davie PS, Walker SP, Perrott MR, Symonds JE, Preece M, De Clercq A, Munday JS (2018) Vertebral abnormalities in free-living Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum) in New Zealand. *New Zealand Journal of Marine and Freshwater Research,* **52,** 444-456.

Davies WD (1973) Rates of temperature acclimation for hatchery reared striped bass fry and fingerlings. *The Progressive Fish-Culturist,* **35,** 214-217.

Davis NCD (2003) Feeding ecology of Pacific salmon (*Oncorhynchus* spp.) in the central North Pacific Ocean and central Bering Sea, 1991-2000. In *Faculty of Fisheries*, pp. 1-206. Hakodate, Hokkaido, Japan: Hokkaido University.

Davis SF, Unwin MJ (1989) Freshwater life history of Chinook salmon (*Oncorhynchus tshawytscha*) in the Rangitata River catchment, New Zealand. *New Zealand Journal of Marine and Freshwater Research,* **23,** 311-319.

de Azevedo TP, Witten PE, Huysseune A, Bensimon-Brito A, Winkler C, To TT, Palmeirim I (2012) Interrelationship and modularity of notochord and somites: A comparative view on zebrafish and chicken vertebral body development. *Journal of Applied Ichthyology,* **28,** 316-319.

De Clercq A, Perrott MR, Davie PS, Preece MA, Huysseune A, Witten PE (2017a) The external phenotype–skeleton link in post-hatch farmed Chinook salmon (*Oncorhynchus tshawytscha*). *Journal of Fish Diseases,* **41,** 511-527.

De Clercq A, Perrott MR, Davie PS, Preece MA, Owen MAG, Huysseune A, Witten PE (2018) Temperature sensitive regions of the Chinook salmon vertebral column. Vestiges and meristic variation. *Journal of Morphology,* DOI:10.1002/jmor.20871, 1-11.

De Clercq A, Perrott MR, Davie PS, Preece MA, Wybourne B, Ruff N, Huysseune A, Witten PE (2017b) Vertebral column regionalisation in Chinook salmon, *Oncorhynchus tshawytscha*. *Journal of Anatomy,* **231,** 500-514.

Deschamps M-H, Labbé L, Baloche S, Fouchereau-Péron M, Dufour S, Sire J-Y (2009) Sustained exercise improves vertebral histomorphometry and modulates hormonal levels in rainbow trout. *Aquaculture,* **296,** 337-346.

Deschamps MH, Kacem A, Ventura R, Courty G, Haffray P, Meunier FJ, Sire JY (2008) Assessment of "discreet" vertebral abnormalities, bone mineralization and bone compactness in farmed rainbow trout. *Aquaculture,* **279,** 11-17.

Detwiler SR, Holtzer H (1956) The developmental dependence of the vertebral column upon the spinal cord in the urodeles. *Journal of Experimental Zoology,* **132,** 299-310.

Deverall KR, Kelso JRM, James GD (1993) Redd characteristics and implications for survival of Chinook salmon (*Oncorhynchus tshawytscha*) embryos in the Waitaki River, New Zealand. *New Zealand Journal of Marine and Freshwater Research,* **27,** 437-444.

Diaz NF, Iturra P, Veloso A, Estay F, Colihueque N (1993) Physiological factors affecting triploid production in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture,* **114,** 33-40.

DiMichele L, Powers DA (1984) The relationship between oxygen consumption rate and hatching in *Fundulus heteroclitus*. *Physiological Zoology,* **57,** 46-51.

Dimichele L, Taylor MH (1980) The environmental control of hatching in *Fundulus heteroclitus*. *Journal of Experimental Zoology,* **214,** 181-187.

Dionísio G, Campos C, Valente LMP, Conceição LEC, Cancela ML, Gavaia PJ (2012) Effect of egg incubation temperature on the occurrence of skeletal deformities in *Solea senegalensis*. *Journal of Applied Ichthyology,* **28,** 471-476.

Domowicz M, Li H, Hennig A, Henry J, Vertel BM, Schwartz NB (1995) The biochemically and immunologically distinct CSPG of notochord is a product of the *aggrecan* gene. *Developmental Biology,* **171,** 655-664.

Donaldson LR, Foster FJ (1941) Experimental study of the effect of various water temperatures on the growth, food utilization, and mortality rates of fingerling sockeye salmon. *Transactions of the American Fisheries Society,* **70,** 339-346.

Dongen SV (2006) Fluctuating asymmetry and developmental instability in evolutionary biology: Past, present and future. *Journal of Evolutionary Biology,* **19,** 1727-1743.

Doudoroff P (1945) The resistance and acclimatization of marine fishes to temperature changes. II. Experiments with *Fundulus* and *Atherinops*. *The Biological Bulletin,* **88,** 194-206.

Duan C, Plisetskaya EM, Dickhoff WW (1995) Expression of insulin-like growth factor I in normally and abnormally developing coho salmon (*Oncorhynchus kisutch*). *Endocrinology,* **136,** 446-452.

Dwyer WP, Piper RG (1987) Atlantic salmon growth efficiency as affected by temperature. *The Progressive Fish-Culturist,* **49,** 57-59.

Eames BF, Sharpe PT, Helms JA (2004) Hierarchy revealed in the specification of three skeletal fates by *Sox9* and *Runx2*. *Developmental Biology,* **274,** 188-200.

Eastman JT (1980) The caudal skeletons of catostomid fishes. *The American Midland Naturalist,* **103,** 133-148.

Eddy RM (1971) The influence of dissolved oxygen concentration and temperature on the survival and growth of Chinook salmon embryos and fry. In *Fisheries & Wildlife,* pp. 1-45. Corvallis, Oregon: Oregon State University.

Edeling MA, Smith C, Owen D (2006) Life of a clathrin coat: Insights from clathrin and AP structures. *Nature Reviews Molecular Cell Biology,* **7,** 32-44.

Edsall TA, Colby PJ (1970) Temperature tolerance of young-of-the-year cisco, *Coregonus artedii*. *Transactions of the American Fisheries Society,* **99,** 526-531.

Eliason EJ, Clark TD, Hague MJ, Hanson LM, Gallagher ZS, Jeffries KM, Gale MK, Patterson DA, Hinch SG, Farrell AP (2011) Differences in thermal tolerance among sockeye salmon populations. *Science,* **332,** 109-112.

Ellis K, Bagwell J, Bagnat M (2013) Notochord vacuoles are lysosome-related organelles that function in axis and spine morphogenesis. *The Journal of Cell Biology,* **200,** 667-679.

Embody GC (1934) Relation of temperature to the incubation periods of eggs of four species of trout. *Transactions of the American Fisheries Society,* **64,** 281-292.

Engsig MT, Chen Q-J, Vu TH, Pedersen A-C, Therkidsen B, Lund LR, Henriksen K, Lenhard T, Foged NT, Werb Z, Delaissé J-M (2000) Matrix metalloproteinase 9 and vascular endothelial

growth gactor are essential for osteoclast recruitment into developing long bones. *The Journal of Cell Biology,* **151,** 879-890.

Eriksen MS, Bakken M, Espmark Å, Braastad BO, Salte R (2006) Prespawning stress in farmed Atlantic salmon *Salmo salar*: Maternal cortisol exposure and hyperthermia during embryonic development affect offspring survival, growth and incidence of malformations. *Journal of Fish Biology,* **69,** 114-129.

Fan C-M, Tessier-Lavigne M (1994) Patterning of mammalian somites by surface ectoderm and notochord: Evidence for sclerotome induction by a hedgehog homolog. *Cell,* **79,** 1175-1186.

Faustino M, Power DM (1998) Development of osteological structures in the sea bream: Vertebral column and caudal fin complex. *Journal of Fish Biology,* **52,** 11-22.

Fekany K, Yamanaka Y, Leung T, Sirotkin HI, Topczewski J, Gates MA, Hibi M, Renucci A, Stemple D, Radbill A, Schier AF, Driever W, Hirano T, Talbot WS, Solnica-Krezel L (1999) The zebrafish *bozozok* locus encodes Dharma, a homeodomain protein essential for induction of gastrula organizer and dorsoanterior embryonic structures. *Development,* **126,** 1427-1438.

Ferreri F, Nicolais C, Boglione C, Bmertoline B (2000) Skeletal characterization of wild and reared zebrafish: Anomalies and meristic characters. *Journal of Fish Biology,* **56,** 1115-1128.

Fiaz AW, Léon-Kloosterziel KM, Gort G, Schulte-Merker S, van Leeuwen JL, Kranenbarg S (2012) Swim-training changes the spatio-temporal dynamics of skeletogenesis in zebrafish larvae (*Danio rerio*). *PLoS ONE,* **7,** 1-13.

Finn RN (2007) The physiology and toxicology of salmonid eggs and larvae in relation to water quality criteria. *Aquatic Toxicology,* **81,** 337-354.

Fjelldal PG, Glover KA, Skaala Ø, Imsland A, Hansen TJ (2009a) Vertebral body mineralization and deformities in cultured Atlantic salmon (*Salmo salar* L.): Effects of genetics and off-season smolt production. *Aquaculture,* **296,** 36-44.

Fjelldal PG, Grotmol S, Kryvi H, Gjerdet NR, Taranger GL, Hansen T, Porter MJR, Totland GK (2004) Pinealectomy induces malformation of the spine and reduces the mechanical strength of the vertebrae in Atlantic salmon, *Salmo salar*. *Journal of Pineal Research,* **36,** 132-139.

Fjelldal PG, Hansen T (2010) Vertebral deformities in triploid Atlantic salmon (*Salmo salar* L.) underyearling smolts. *Aquaculture,* **309,** 131-136.

Fjelldal PG, Hansen T, Breck O, Ørnsrud R, Lock EJ, Waagbø R, Wargelius A, Eckhard Witten P (2012) Vertebral deformities in farmed Atlantic salmon (*Salmo salar* L.) – Etiology and pathology. *Journal of Applied Ichthyology,* **28,** 433-440.

Fjelldal PG, Hansen T, Breck O, Sandvik R, Waagbø R, Berg A, Ørnsrud R (2009b) Supplementation of dietary minerals during the early seawater phase increase vertebral strength and reduce the prevalence of vertebral deformities in fast-growing under-yearling Atlantic salmon (*Salmo salar* L.) smolt. *Aquaculture Nutrition,* **15,** 366-378.

Fjelldal PG, Hansen TJ, Berg AE (2007a) A radiological study on the development of vertebral deformities in cultured Atlantic salmon (*Salmo salar* L.). *Aquaculture,* **273,** 721-728.

Fjelldal PG, Lock E-J, Grotmol S, Totland GK, Nordgarden U, Flik G, Hansen T (2006) Impact of smolt production strategy on vertebral growth and mineralisation during smoltification and the early seawater phase in Atlantic salmon (*Salmo salar,* L.). *Aquaculture,* **261,** 715-728.

Fjelldal PG, Nordgarden U, Berg A, Grotmol S, Totland GK, Wargelius A, Hansen T (2005) Vertebrae of the trunk and tail display different growth rates in response to photoperiod in Atlantic salmon, *Salmo salar* L., post-smolts. *Aquaculture,* **250,** 516-524.

Fjelldal PG, Nordgarden U, Hansen T (2007b) The mineral content affects vertebral morphology in underyearling smolt of Atlantic salmon (*Salmo salar* L.). *Aquaculture,* **270,** 231-239.

Fjelldal PG, Solberg MF, Hansen T, Vågseth T, Glover KA, Kryvi H (2016) Salmonid fish: Model organisms to study cardiovascular morphogenesis in conjoined twins? *BMC Developmental Biology,* **16,** 1-16.

Fjelldal PG, Totland GK, Hansen T, Kryvi H, Wang X, Søndergaard JL, Grotmol S (2013) Regional changes in vertebra morphology during ontogeny reflect the life history of Atlantic cod (*Gadus morhua* L.). *Journal of Anatomy,* **222,** 615-624.

Fleming A, Keynes R, Tannahill D (2004) A central role for the notochord in vertebral patterning. *Development,* **131,** 873-880.

Fleming A, Keynes RJ, Tannahill D (2001) The role of the notochord in vertebral column formation. *Journal of Anatomy,* **199,** 177-180.

Fleming A, Kishida MG, Kimmel CB, Keynes RJ (2015) Building the backbone: The development and evolution of vertebral patterning. *Development,* **142,** 1733-1744.

Fleming M (2013) Deformity prevalence and meristic characteristics in Atlantic salmon: The effect of ploidy, incubation temperature and hybridization. In *Centre of Ecological and Evolutionary Synthesis, Department of Bioscience*, pp. 1-35. Oslo: University of Oslo.

Folmar LC, Dickhoff WW, Mahnken CVW, Waknitz FW (1982) Stunting and parr-reversion during smoltification of coho salmon (*Oncorhynchus kisutch*). *Aquaculture,* **28,** 91-104.

Ford E (1937) Vertebral variation in teleostean fishes. *Journal of the Marine Biological Association of the United Kingdom,* **22,** 1-60.

Fowler JA (1970) Control of vertebral number in teleosts - An embryological problem. *The Quarterly Review of Biology,* **45,** 148-167.

Franklin CE (1989) Physiological stress, smoltification and seawater adaptation in New Zealand's sockeye and Quinnat salmon. In *Department of Zoology*, pp. 1-145. University of Canterbury: Univeristy of Canterbury.

Fraser TWK, Hansen T, Fleming MS, Fjelldal PG (2015) The prevalence of vertebral deformities is increased with higher egg incubation temperatures and triploidy in Atlantic salmon *Salmo salar* L. *Journal of Fish Diseases,* **38,** 75-89.

Fry F (1958) Temperature compensation. *Annual Review of Physiology,* **20,** 207-224.

Fry F, Brett J, Clawson G (1942) Lethal limits of temperature for young goldfish. *Revue Canadienne De Biology,* **1,** 50-56.

Fry F, Hart J, Walker K (1946) Lethal temperature relations for a sample of young speckled trout, *Salvelinus fontinalis*. *University of Toronto Studies, Biological Series, Publications of the Ontario Fisheries Research Laboratory,* **54,** 9-35.

Fuiman LA, Poling KR, Higgs DM (1998) Quantifying developmental progress for comparative studies of larval fishes. *Copeia,* **1998,** 602-611.

Furumoto T-a, Miura N, Akasaka T, Mizutani-Koseki Y, Sudo H, Fukuda K, Maekawa M, Yuasa S, Fu Y, Moriya H, Taniguchi M, Imai K, Dahl E, Balling R, Pavlova M, Gossler A, Koseki H (1999) Notochord-dependent expression of MFH1 and PAX1 cooperates to maintain the proliferation of sclerotome cells during the vertebral column development. *Developmental Biology,* **210,** 15-29.

Gabriel ML (1944) Factors affecting the number and form of vertebrae in *Fundulus heteroclitus*. *Journal of Experimental Zoology,* **95,** 105-147.

Gadow H, Abbott EC (1895) On the evolution of the vertebral column of fishes. *Philosophical Transactions of the Royal Society B: Biological Sciences,* **186,** 163-221.

Galbreath P, Adams N, Sherrill LWI, Martin T (2006) Thermal tolerance of diploid versus triploid rainbow trout and brook trout assessed by time to chronic lethal maximum. *Environmental Biology of Fishes,* **75,** 183-193.

Galis F (1999) Why do almost all mammals have seven cervical vertebrae? Developmental constraints, *Hox* genes, and cancer. *Journal of Experimental Zoology,* **285,** 19-26.

Galvano PM, Johnson K, Wilson CC, Pitcher TE, Butts IAE (2013) Ovarian fluid influences sperm performance in lake trout, *Salvelinus namaycush*. *Reproductive Biology,* **13,** 172-175.

Garcia J, Bagwell J, Njaine B, Norman J, Levic DS, Wopat S, Miller SE, Liu X, Locasale JW, Stainier DYR, Bagnat M (2017) Sheath cell invasion and trans-differentiation repair mechanical damage caused by loss of caveolae in the zebrafish notochord. *Current Biology,* **27,** 1982-1989.

Garside ET (1959) Some effects of oxygen in relation to temperature on the development of lake trout embryos. *Canadian Journal of Zoology,* **37,** 689-698.

Garside ET (1966a) Developmental rate and vertebral number in salmonids. *Journal of the Fisheries Research Board of Canada,* **23,** 1537-1551.

Garside ET (1966b) Effects of oxygen in relation to temperature on the development of embryos of brook rout and rainbow trout. *Journal of the Fisheries Research Board of Canada,* **23,** 1121-1134.

Gavaia PJ, Dinis MT, Cancela ML (2002) Osteological development and abnormalities of the vertebral column and caudal skeleton in larval and juvenile stages of hatchery-reared Senegal sole (*Solea senegalensis*). *Aquaculture,* **211,** 305-323.

Georgakopoulou E, Katharios P, Divanach P, Koumoundouros G (2010) Effect of temperature on the development of skeletal deformities in Gilthead seabream (*Sparus aurata* Linnaeus, 1758). *Aquaculture,* **308,** 13-19.

Gil Martens L, Fjelldal PG, Lock EJ, Wargelius A, Wergeland H, Witten PE, Hansen T, Waagbø R, Ørnsrud R (2012) Dietary phosphorus does not reduce the risk for spinal deformities in a model of adjuvant-induced inflammation in Atlantic salmon (*Salmo salar*) postsmolts. *Aquaculture Nutrition,* **18,** 12-20.

Gil Martens L, Obach A, Ritchie G, Witten PE (2005) Analysis of a short tail type farmed Atlantic salmon (*Salmo salar*). *Fish Veterinary Journal,* **8,** 71-79.

Gilbert SF (2000) *Developmental Biology,* Sinauer Associates, Inc, Sunderland, Massachusetts, USA.

Gill CD, Fisk DM (1966) Vertebral abnormalities in sockeye, pink, and chum salmon. *Transactions of the American Fisheries Society,* **95,** 177-182.

Gill G, Frost J, Miller K (1974) A new formula for a half-oxidized hematoxylin solution that neither overstains nor requires differentiation. *Acta Cytologica,* **18,** 300-311.

Girdwoyn M (1877) Pathology of fishes. Diseases, monstrosities, and anomalies of eggs and embryos. *Pamietnik Towarszystwa Nauk Scislych,* **Paryzu, IX, Pl. xi,** fig. 112.

Gistelinck C, Gioia R, Gagliardi A, Tonelli F, Marchese L, Bianchi L, Landi C, Bini L, Huysseune A, Witten PE, Staes A, Gevaert K, De Rocker N, Menten B, Malfait F, Leikin S, Carra S, Tenni R, Rossi A, De Paepe A, Coucke P, Willaert A, Forlino A (2016a) Zebrafish collagen type I: Molecular and biochemical characterization of the major dtructural protein in bone and skin. *Scientific Reports,* **6,** 1-13.

Gistelinck C, Kwon RY, Malfait F, Symoens S, Harris MP, Henke K, Hawkins MB, Fisher S, Sips P, Guillemyn B, Bek JW, Vermassen P, De Saffel H, Witten PE, Weis M, De Paepe A, Eyre DR, Willaert A, Coucke PJ (2018) Zebrafish type I collagen mutants faithfully recapitulate human type I collagenopathies. *Proceedings of the National Academy of Sciences,* **115,** E8037-E8046.

Gistelinck C, Witten PE, Huysseune A, Symoens S, Malfait F, Larionova D, Simoens P, Dierick M, Van Hoorebeke L, De Paepe A, Kwon RY, Weis M, Eyre DR, Willaert A, Coucke PJ (2016b) Loss of type I collagen telopeptide lysyl hydroxylation causes musculoskeletal abnormalities in a zebrafish model of Bruck Syndrome. *Journal of bone and mineral research,* **31,** 1930-1942.

Gjedrem T, Gunnes K (1978) Comparison of growth rate in Atlantic salmon, pink salmon, Arctic charr, sea trout and rainbow trout under Norwegian farming conditions. *Aquaculture,* **13,** 135-141.

Gjerde B, Pante MJR, Baeverfjord G (2005) Genetic variation for a vertebral deformity in Atlantic salmon (*Salmo salar*). *Aquaculture,* **244,** 77-87.

Gomez C, Pourquié O (2009) Developmental control of segment numbers in vertebrates. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution,* **312B,** 533-544.

Gomez-Navarro N, Miller EA (2016) COP-coated vesicles. *Current Biology,* **26,** 54-57.

Gorodilov Y (1996) Description of the early ontogeny of the Atlantic salmon, *Salmo salar*, with a novel system of interval (state) identification. *Environmental Biology of Fishes,* **47,** 109-127.

Gorodilov YN (1995) The relationship between the temperature and the duration of the embryogenesis in vertebrates may be described by a logarithmic parabola of the second order. *Animal Biology,* **4,** 145-152.

Gorodilov YN (2004) Studies of temporal and spatial peculiarities of somitogenesis in fish embryos. *Russian Journal of Developmental Biology,* **35,** 92-105.

Gorodilov YN, Melnikova EL (2003) Comparison of early ontogenesis of Atlantic and Pacific salmon species (genera *Salmo* and *Oncorhynchus*). In *International Conference Atlantic Salmon: Biology, Protection, and Breeding*, pp. 121-133. Petrozavodsk, Russia.

Gosline WA (1961) The Perciform caudal skeleton. *Copeia,* **1961,** 265-270.

Götte A (1879) Beiträge zur vergleichenden Morphologie des Skeletsystems der Wirbelthiere. *Archiv für mikroskopische Anatomie,* **16,** 117-152.

Graham J (1949) Some effects of temperature and oxygen pressure on the metabolism and activity of the speckled trout, *Salvelinus fontinalis*. *Canadian Journal of Research,* **27,** 270-288.

Grande L, Bemis WE (1998) A comprehensive phylogenetic study of amiid fishes (Amiidae) based on comparative skeletal anatomy. An empirical search for interconnected patterns of natural history. *Journal of Vertebrate Paleontology,* **18,** 1-696.

Gray ES, Young G, Bern HA (1990) Radioreceptor assay for growth hormone in coho salmon (*Oncorhynchus kisutch*) and its application to the study of stunting. *Journal of Experimental Zoology,* **256,** 290-296.

Greig MJ, Ridgway NM, Shakespeare BS (1988) Sea surface temperature variations at coastal sites around New Zealand. *New Zealand Journal of Marine and Freshwater Research,* **22,** 391-400.

Grini A, Hansen T, Berg A, Wargelius A, Fjelldal PG (2011) The effect of water temperature on vertebral deformities and vaccine-induced abdominal lesions in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases,* **34,** 531-546.

Grotmol S, Kryvi H, Keynes R, Krossøy C, Nordvik K, Totland GK (2006) Stepwise enforcement of the notochord and its intersection with the myoseptum: An evolutionary path leading to development of the vertebra? *Journal of Anatomy,* **209,** 339-357.

Grotmol S, Kryvi H, Nordvik K, Totland G (2003) Notochord segmentation may lay down the pathway for the development of the vertebral bodies in the Atlantic salmon. *Anatomy and Embryology*, **207,** 263-272.

Grotmol S, Nordvik K, Kryvi H, Totland GK (2005) A segmental pattern of alkaline phosphatase activity within the notochord coincides with the initial formation of the vertebral bodies. *Journal of Anatomy*, **206,** 427-436.

Grünbaum T, Cloutier R (2010) Ontogeny, variation, and homology in *Salvelinus alpinus* caudal skeleton (Teleostei: Salmonidae). *Journal of Morphology*, **271,** 12-24.

Grünbaum T, Cloutier R, Vincent B (2012) Dynamic skeletogenesis in fishes: Insight of exercise training on developmental plasticity. *Developmental Dynamics*, **241,** 1507-1524.

Gunnes K (1979) Survival and development of Atlantic salmon eggs and fry at three different temperatures. *Aquaculture*, **16,** 211-218.

Haga Y, Dominique V, III, Du S (2009) Analyzing notochord segmentation and intervertebral disc formation using the *twhh:gfp* transgenic zebrafish model. *Transgenic Research*, **18,** 669-683.

Hall BK (1977) *Chondrogenesis of the somitic mesoderm*, Springer-Verlag, Berlin Heidelberg.

Hall BK (2003) Descent with modification: The unity underlying homology and homoplasy as seen through an analysis of development and evolution. *Biological Reviews*, **78,** 409-433.

Hall BK (2015a) Chapter 1 - Vertebrate Skeletal Tissues. In *Bones and Cartilage (Second Edition)* (ed Hall BK), pp. 3-16. San Diego: Academic Press.

Hall BK (2015b) Chapter 9 - Tendon Skeletogenesis and Sesamoids. In *Bones and Cartilage (Second Edition)* (ed Hall BK), pp. 137-149. San Diego: Academic Press.

Hall BK, Witten PE (2018) Plasticity and variation of skeletal cells and tissues and the evolutionary development of actinopterygian fishes. In *Evolution and Development of Fishes* (eds Johanson Z, Underwood C, Richter M), pp. In press. Cambridge: Cambridge University Press.

Hammerschmidt M, McMahon AP (1998) The effect of pertussis toxin on zebrafish development: A possible role for inhibitory G-proteins in Hedgehog signaling. *Developmental Biology*, **194,** 166-171.

Hamor T, Garside ET (1976) Developmental rates of embryos of Atlantic salmon, *Salmo salar* L., in response to various levels of temperature, dissolved oxygen, and water exchange. *Canadian Journal of Zoology*, **54,** 1912-1917.

Hamor T, Garside ET (1977) Size relations and yolk utilization in embryonated ova and alevins of Atlantic salmon *Salmo salar* L. in various combinations of temperature and dissolved oxygen. *Canadian Journal of Zoology*, **55,** 1892-1898.

Hamor T, Garside ET (1979) Hourly and total oxygen consumption by ova of Atlantic salmon, *Salmo salar* L., during embryogenesis, at two temperatures and three levels of dissolved oxygen. *Canadian Journal of Zoology,* **57,** 1196-1200.

Handeland SO, Björnsson, Arnesen AM, Stefansson SO (2003) Seawater adaptation and growth of post-smolt Atlantic salmon (*Salmo salar*) of wild and farmed strains. *Aquaculture,* **220,** 367-384.

Handeland SO, Imsland AK, Stefansson SO (2008) The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. *Aquaculture,* **283,** 36-42.

Harland R, Gerhart J (1997) Formation and function of the Spemann's organizer. *Annual Review of Cell and Developmental Biology,* **13,** 611-667.

Hathaway ES (1928) Quantitative study of the changes produced by acclimatization in the tolerance of high temperatures by fishes and amphibians. *Bulletin of the U.S. Fish Commission,* **43,** 169-192.

Haworth J (2010) *Swimming Upstream: How Salmon Farming Developed in New Zealand,* Wily Publications, Christchurch, New Zealand.

Hayes FR, Pelluet D (1945) The effect of temperature on the growth and efficiency of yolk conversion in the salmon embryo. *Canadian Journal of Research,* **23d,** 7-15.

Hayes FR, Pelluet D, Gorham E (1953) Some effects of temperature on the embryonic development of the salmon (*Salmo salar*). *Canadian Journal of Zoology,* **31,** 42-51.

Heath AG, Hughes GM (1973) Cardiovascular and respiratory changes during heat stress in rainbow trout (*Salmo gairdneri*). *Journal of Experimental Biology,* **59,** 323-338.

Heath DD, Devlin RH, Heath JW, Iwama GK (1994) Genetic, environmental and interaction effects on the incidence of jacking in *Oncorhynchus tshawytscha* (Chinook salmon). *Heredity,* **72,** 146-154.

Heggberget TG, Wallace JC (1984) Incubation of the eggs of Atlantic salmon, *Salmo salar,* at low temperatures. *Canadian Journal of Fisheries and Aquatic Sciences,* **41,** 389-391.

Heming TA (1982) Effects of temperature on utilization of yolk by Chinook salmon (*Oncorhynchus tshawytscha*) eggs and alevins. *Canadian Journal of Fisheries and Aquatic Sciences,* **39,** 184-190.

Heming TA, McInerney JE, Alderdice DF (1982) Effect of temperature on initial feeding in alevins of Chinook salmon (*Oncorhynchus tshawytscha*). *Canadian Journal of Fisheries and Aquatic Sciences,* **39,** 1554-1562.

Hevrøy EM, Waagbø R, Torstensen BE, Takle H, Stubhaug I, Jørgensen SM, Torgersen T, Tvenning L, Susort S, Breck O, Hansen T (2012) Ghrelin is involved in voluntary anorexia in Atlantic salmon raised at elevated sea temperatures. *General and Comparative Endocrinology,* **175,** 118-134.

Hinch SG, Rand PS (1998) Swim speeds and energy use of upriver-migrating sockeye salmon (*Oncorhynchus nerka*): Role of local environment and fish characteristics. *Canadian Journal of Fisheries and Aquatic Sciences,* **55,** 1821-1831.

Hokanson KEF, Kleiner CF, Thorslund TW (1977) Effects of constant temperatures and diel temperature fluctuations on specific growth and mortality rates and yield of juvenile rainbow trout, *Salmo gairdneri*. *Journal of the Fisheries Research Board of Canada,* **34,** 639-648.

Holley SA (2006) Anterior–posterior differences in vertebrate segments: Specification of trunk and tail somites in the zebrafish blastula. *Genes & Development,* **20,** 1831-1837.

Holley SA (2007) The genetics and embryology of zebrafish metamerism. *Developmental Dynamics,* **236,** 1422-1449.

Holtzer H (1951) Morphogenetic influence of the spinal cord on the axial skeleton and musculature. *The Anatomical Record,* **109,** 373-374.

Howat WJ, Wilson BA (2014) Tissue fixation and the effect of molecular fixatives on downstream staining procedures. *Methods,* **70,** 12-19.

Hubbs CL (1922) Variations in the number of vertebrae and other meristic characters of fishes correlated with the temperature of water during development. *The American Naturalist,* **56,** 360-372.

Huntsman AG (1942) Death of salmon and trout with high temperature. *Journal of the Fisheries Research Board of Canada,* **5c,** 485-501.

Huxley TH (1859) Observations on the development of some parts of the skeleton of fishes. *Quarterly Journal of Microscopical Science,* **7,** 33-46.

Huysseune A, Sire JY, Meunier FJ (1994) Comparative study of lower pharyngeal jaw structure in two phenotypes of *Astatoreochromis alluaudi* (Teleostei: Cichlidae). *Journal of Morphology,* **221,** 25-43.

https://www.fao.org/fishery/species/2933/en

https://www.fao.org/fishery/statistics/global-aquaculture-production/en

https://www.niwa.co.nz/freshwater-and-estuaries/freshwater-and-estuaries-update/freshwater-update-33-may-2010/water-temperature-and-clarity

https://www.nodc.noaa.gov/dsdt/cwtg/spac_tmap.html

https://www.seatemperature.org/australia-pacific/new-zealand/

https://xe.com/currencycharts/?from=USD&to=NZD&view=1Y

Hyndman CA, Kieffer JD, Benfey TJ (2003) Physiology and survival of triploid brook trout following exhaustive exercise in warm water. *Aquaculture,* **221,** 629-643.

İnanan BE, Öğretmen F (2015) Determination of differences in the biochemical properties of sperm activating and non-activating ovarian fluids and their influences on sperm motility in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* **448,** 539-544.

Inohaya K, Takano Y, Kudo A (2007) The teleost intervertebral region acts as a growth center of the centrum: In vivo visualization of osteoblasts and their progenitors in transgenic fish. *Developmental Dynamics,* **236,** 3031-3046.

Irving L, Black EC, Safford V (1941) The influence of temperature upon the combination of oxygen with the blood of trout. *The Biological Bulletin,* **80,** 1-17.

Ishikawa T, Okada T, Ishikawa-Fujiwara T, Todo T, Kamei Y, Shigenobu S, Tanaka M, Saito TL, Yoshimura J, Morishita S, Toyoda A, Sakaki Y, Taniguchi Y, Takeda S, Mori K (2013) ATF6α/β-Mediated adjustment of ER chaperone levels is essential for development of the notochord in medaka fish. *Molecular Biology of the Cell,* **24,** 1387-1395.

Ishikawa T, Toyama T, Nakamura Y, Tamada K, Shimizu H, Ninagawa S, Okada T, Kamei Y, Ishikawa-Fujiwara T, Todo T, Aoyama E, Takigawa M, Harada A, Mori K (2017) UPR Transducer BBF2H7 allows export of type II collagen in a cargo- and developmental stage–specific manner. *The Journal of Cell Biology,* **216,** 1761-1774.

Iwama G, Thomas P, Forsyth R, Vijayan M (1998) Heat shock protein expression in fish. *Reviews in Fish Biology and Fisheries,* **8,** 35-56.

Jawad LA (2015) Study of the vertebral column of the onion trevally, *Carangoides caeruleopinnatus* (Teleostei: Carangidae) collected from the Sea of Oman. *Italian Journal of Zoology,* **82,** 41-47.

Jawad LA, Al-Hassani L, Al-Kharusi LH (2013) On the morphology of the vertebral column of the Frigate tuna, *Auxis thazard* (Lacepedea, 1800)(Family: Scombridae). *Acta Musei Nationalis Pragae,* **69,** 101-105.

Jensen AJ, Johnsen BO, Saksgård L (1989) Temperature requirements in Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*), and Arctic charr (*Salvelinus alpinus*) from hatching to initial feeding compared with geographic distribution. *Canadian Journal of Fisheries and Aquatic Sciences,* **46,** 786-789.

Jeuthe H, Brännäs E, Nilsson J (2013) Effects of egg size, maternal age and temperature on egg, viability of farmed Arctic charr. *Aquaculture,* **408,** 70-77.

Jezierska B, Ługowska K, Witeska M (2009) The effects of heavy metals on embryonic development of fish (a review). *Fish Physiology and Biochemistry,* **35,** 625-640.

Johanson Z, Sutija M, Joss J (2005) Regionalization of axial skeleton in the lungfish *Neoceratodus forsteri* (Dipnoi). *Journal of Experimental Biology part B: Molecular and Developmental Evolution,* **304B,** 229-237.

Johnson GD, Britz R (2010) Occipito-vertebral fusion in actinopterygians: Conjecture, myth and reality. Part 2: Teleosts. In *Origin and Phylogenetic Interrelationships of Teleosts.* (eds Nelson JS, Schultze H-P, Wilson MVH), pp. 111-121. München: Verlag Dr. Friederich Pfeil.

Johnson R, Tietge J, Jensen K, Fernandez J, Linnum A, Lothenbach D, Holcombe G, Cook P, Christ S, Lattier D, Gordon D (1998) Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to early life stage brook trout (*Salvelinus fontinalis*) following parental dietary exposure. *Environmental Toxicology and Chemistry*, **17,** 2408-2421.

Johnson SL (1988) The effects of the 1983 El Niño on Oregon's coho (*Oncorhynchus kisutch*) and Chinook (*O. tshawytscha*) salmon. *Fisheries Research,* **6,** 105-123.

Johnston IA (2006) Environment and plasticity of myogenesis in teleost fish. *Journal of Experimental Biology,* **209,** 2249-2264.

Johnston IA, Manthri S, Alderson R, Smart A, Campbell P, Nickell D, Robertson B, Paxton CGM, Burt ML (2003) Freshwater environment affects growth rate and muscle fibre recruitment in seawater stages of Atlantic salmon (*Salmo salar* L.). *Journal of Experimental Biology*, **206,** 1337-1351.

Johnston IA, McLay HA (1997) Temperature and family effects on muscle cellularity at hatch and first feeding in Atlantic salmon (*Salmo salar* L.). *Canadian Journal of Zoology*, **75,** 64-74.

Johnston IA, Strugnell G, McCracken ML, Johnstone R (1999) Muscle growth and development in normal-sex-ratio and all-female diploid and triploid Atlantic salmon. *Journal of Experimental Biology*, **202,** 99-115.

Jones RE, Petrell RJ, Pauly D (1999) Using modified length–weight relationships to assess the condition of fish. *Aquacultural Engineering,* **20,** 261-276.

Jordan DS, Evermann BW (1896) *A check-list of the fishes and fishlike vertebrates of North and Middle America,* US Government Printing Office, Washington.

Jungwirth M, Winkler H (1984) The temperature dependence of embryonic development of grayling (*Thymallus thymallus*), Danube salmon (*Hucho hucho*), Arctic charr (*Salvelinus alpinus*) and brown trout (*Salmo trutta fario*). *Aquaculture*, **38,** 315-327.

Kacem A, Meunier FJ, Baglinière JL (1998) A quantitative study of morphological and histological changes in the skeleton of *Salmo salar* during its anadromous migration. *Journal of Fish Biology*, **53,** 1096-1109.

Kause A, Ritola O, Paananen T (2007) Changes in the expression of genetic characteristics across cohorts in skeletal deformations of farmed salmonids. *Genetics, Selection, Evolution : GSE,* **39,** 529-543.

Kawakami Y, Raya A, Raya RM, Rodriguez-Esteban C, Belmonte JCI (2005) Retinoic acid signalling links left-right asymmetric patterning and bilaterally symmetric somitogenesis in the zebrafish embryo. *Nature*, **435,** 165-171.

Kiang JG, Tsokos GC (1998) Heat shock protein 70 kDa: Molecular biology, biochemistry, and physiology. *Pharmacology & Therapeutics*, **80,** 183-201.

Killeen JR, McLay HA, Johnston IA (1999) Temperature and neuromuscular development in embryos of the trout (*Salmo trutta* L.). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **122,** 53-64.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Developmental Dynamics*, **203,** 253-310.

Kinnison MT (1999) Life history divergence and population structure of New Zealand Chinook salmon: A study of contemporary microevolution. In *Graduate School*, pp. 6-165. Seattle, Washington: University of Washington.

Kinnison MT, Unwin MJ, Hershberger WK, Quinn TP (1998) Egg size, fecundity, and development rate of two introduced New Zealand Chinook salmon (*Oncorhynchus tshawytscha*) populations. *Canadian Journal of Fisheries and Aquatic Sciences*, **55,** 1946-1953.

Kitamura Y, Sasaki H, Kimura T, Miwa T, Takahashi S, Kawase T, Yoshida K (2013) Molecular and clinical risk factors for recurrence of skull base chordomas: Gain on chromosome 2p, expression of *brachyury*, and lack of irradiation negatively correlate zith patient prognosis. *Journal of Neuropathology & Experimental Neurology*, **79,** 816-823.

Knight AE (1963) The embryonic and larval development of the rainbow trout. *Transactions of the American Fisheries Society*, **92,** 344-355.

Knudson CB, Knudson W (2001) Cartilage proteoglycans. *Seminars in Cell & Developmental Biology*, **12,** 69-78.

Koehl MAR, Quillin KJ, Pell CA (2000) Mechanical design of fiber-wound hydraulic skeletons: The stiffening and straightening of embryonic notochords. *American Zoologist*, **40,** 28-041.

Kölliker A (1859) On the structure of the chorda dorsalis of the plagiostomes and some other fishes, and on the relation of its proper sheath to the development of the vertebrae. *Proceedings of the Royal Society of London*, **10,** 214-222.

Kondylis V, Pizette S, Rabouille C (2009) The early secretory pathway in development: A tale of proteins and mRNAs. *Seminars in Cell & Developmental Biology*, **20,** 817-827.

Koumoundouros G (2010) Morpho-anatomical abnormalities in Mediterranean marine aquaculture. In *Recent Advances in Aquaculture Research* (ed Koumoundouros G), pp. 125-148. Kerala, India: Transworld Research Network

Koumoundouros G, Ashton C, Sfakianakis DG, Divanach P, Kentouri M, Anthwal N, Stickland NC (2009) Thermally induced phenotypic plasticity of swimming performance in European sea bass *Dicentrarchus labrax* juveniles. *Journal of Fish Biology*, **74,** 1309-1322.

Koumoundouros G, Divanach P, Anezaki L, Kentouri M (2001) Temperature-induced ontogenetic plasticity in sea bass (*Dicentrarchus labrax*). *Marine Biology*, **139,** 817-830.

Koumoundouros G, Gagliardi F, Divanach P, Boglione C, Cataudella S, Kentouri M (1997) Normal and abnormal osteological development of caudal fin in *Sparus aurata* L. fry. *Aquaculture*, **149,** 215-226.

Kowalevsky A (1867) Entwicklungsgeschichte des *Amphioxus lanceolatus*. *Imperial Academy of Science Sint Petersburg (Ser VII),* **11,** 1-17.

Kryvi H, Rusten I, Fjelldal PG, Nordvik K, Totland GK, Karlsen T, Wiig H, Long JH (2017) The notochord in Atlantic salmon (*Salmo salar* L.) undergoes profound morphological and mechanical changes during development. *Journal of Anatomy,* **231,** 639-654.

Kullgren A, Jutfelt F, Fontanillas R, Sundell K, Samuelsson L, Wiklander K, Kling P, Koppe W, Larsson DGJ, Björnsson BT, Jönsson E (2013) The impact of temperature on the metabolome and endocrine metabolic signals in Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **164,** 44-53.

Kvellestad A, Høie S, Thorud K, Tørud B, Lyngøy A (2000) Platyspondyly and shortness of vertebral column in farmed Atlantic salmon *Salmo salar* in Norway-description and interpretation of pathologic changes. *Diseases of Aquatic Organisms,* **39,** 97-108.

Kwain W-h (1975) Embryonic development, early growth, and meristic variation in rainbow trout (*Salmo gairdneri*) exposed to combinations of light Intensity and temperature. *Journal of the Fisheries Research Board of Canada,* **32,** 397-402.

Lahnsteiner F (2012) Thermotolerance of brown trout, *Salmo trutta*, gametes and embryos to increased water temperatures. *Journal of Applied Ichthyology,* **28,** 745-751.

Landrum B (1966) Bilateral asymmetry in paired meristic characters of Pacific salmon. *Pacific Science,* **20,** 193-202.

Lang MR, Lapierre LA, Frotscher M, Goldenring JR, Knapik EW (2006) Secretory COPII coat component Sec23a is essential for craniofacial chondrocyte maturation. *Nature Genetics,* **38,** 1198-1203.

Leary RF, Allendorf FW (1989) Fluctuating asymmetry as an indicator of stress: Implications for conservation biology. *Trends in Ecology & Evolution,* **4,** 214-217.

Leary RF, Allendorf FW, Knudsen KL (1985) Inheritance of meristic variation and the evolution of developmental stability in rainbow trout. *Evolution,* **39,** 308-314.

Leduc G (1978) Deleterious effects of cyanide on early life stages of Atlantic salmon (*Salmo salar*). *Journal of the Fisheries Research Board of Canada,* **35,** 166-174.

Lee CG, Farrell AP, Lotto A, MacNutt MJ, Hinch SG, Healey MC (2003) The effect of temperature on swimming performance and oxygen consumption in adult sockeye (*Oncorhynchus nerka*) and coho (*O. kisutch*) salmon stocks. *Journal of Experimental Biology,* **206,** 3239-3251.

Levanduski MJ, Beck JC, Seeb JE (1990) Optimal thermal shocks for induced diploid gynogenesis in Chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture,* **90,** 239-250.

Lim Y-W, Lo HP, Ferguson C, Martel N, Giacomotto J, Gomez GA, Yap AS, Hall TE, Parton RG (2017) Caveolae protect notochord cells against catastrophic mechanical failure during development. *Current Biology,* **27,** 1968-1981.

Linares-Casenave J, Werner I, Van Eenennaam JP, Doroshov SI (2013) Temperature stress induces notochord abnormalities and heat shock proteins expression in larval green sturgeon (*Acipenser medirostris* Ayres 1854). *Journal of Applied Ichthyology,* **29,** 958-967.

Lindsey CC (1954) Temperature-controlled meristic variation in the paradise fish *Macropodus operculars* (L.). *Canadian Journal of Zoology,* **32,** 87-98.

Lindsey CC (1962) Experimental study of meristic variation in a population of threespine sticklebacks, *Gasterosteus aculeatus*. *Canadian Journal of Zoology,* **40,** 271-312.

Lindsey CC (1988) Three factors controlling meristic variation. *Fish Physiology,* **11,** 197-274.

Lindsey CC, Ali MY (1965) The effect of alternating temperature on vertebral count in medaka (*Oryzias latipes*). *Canadian Journal of Zoology,* **43,** 99-104.

Lindsey CC, Brett AM, Swain DP, Arnason AN (1984) Responses of vertebral numbers in rainbow trout to temperature changes during development. *Canadian Journal of Zoology,* **62,** 391-396.

Lleras Forero L, Narayanan R, Huitema LFA, VanBergen M, Apschner A, Peterson-Maduro J, Logister I, Valentin G, Morelli LG, Oates A, Schulte-Merker S (2018) Segmentation of the zebrafish axial skeleton relies on notochord sheath cells and not on the segmentation clock. *eLife,* **7,** [Epub ahead of print].

Loeb J, Wasteneys H (1912) On the adaptation of fish (*Fundulus*) to higher temperatures. *Journal of Experimental Zoology,* **12,** 543-557.

Loizides M, Georgiou AN, Somarakis S, Witten PE, Koumoundouros G (2013) A new type of lordosis and vertebral body compression in Gilthead sea bream, *Sparus aurata* L.: Aetiology, anatomy and consequences for survival. *Journal of Fish Diseases,* **37,** 949-957.

Mabee PM, Crotwell PL, Bird NC, Burke AC (2002) Evolution of median fin modules in the axial skeleton of fishes. *Journal of Experimental Biology,* **294,** 77-90.

Mabee PM, Olmstead KL, Cubbage CC (2000) An experimental study of intraspecific variation, developmental timing, and heterochrony in fishes. *Evolution,* **54,** 2091-2106.

MacGregor R, MacCrimmon H (1977) Evidence of genetic and environmental influences on meristic variation in the rainbow trout, *Salmo gairdneri* Richardson. *Environmental Biology of Fishes,* **2,** 25-33.

Macqueen DJ, Robb DHF, Olsen T, Melstveit L, Paxton CGM, Johnston IA (2008) Temperature until the 'eyed stage' of embryogenesis programmes the growth trajectory and muscle phenotype of adult Atlantic salmon. *Biology Letters,* **4,** 294-298.

Mahnken Conrad VW, Waknitz FW (1979) Factors affecting growth and survival of coho salmon (*Oncorhynchus kisutch*) and Chinook salmon (*O. tshawytscha*) in saltwater net-pens in Puget Sound. *Proceedings of the World Mariculture Society,* **10,** 280-305.

Mahon EF, Hoar WS (1956) The early development of the chum salmon, *Oncorhynchus keta* (Walbaum). *Journal of Morphology,* **98,** 1-47.

Mahrosh U, Kleiven M, Meland S, Rosseland BO, Salbu B, Teien H-C (2014) Toxicity of road deicing salt (NaCl) and copper (Cu) to fertilization and early developmental stages of Atlantic salmon (*Salmo salar*). *Journal of Hazardous Materials,* **280,** 331-339.

Maisey JG (1988) Phylogeny of early vertebrate skeletal induction and ossification patterns. *Evolutionary Biology,* **22,** 1-36.

Martins CIM, Eding EH, Verdegem MCJ, Heinsbroek LTN, Schneider O, Blancheton JP, d'Orbcastel ER, Verreth JAJ (2010) New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural Engineering,* **43,** 83-93.

Martins EG, Hinch SG, Patterson DA, Hague MJ, Cooke SJ, Miller KM, Lapointe MF, English KK, Farrell AP (2011) Effects of river temperature and climate warming on stock-specific survival of adult migrating Fraser River sockeye salmon (*Oncorhynchus nerka*). *Global Change Biology,* **17,** 99-114.

Marty GD, Heintz RA, Hinton DE (1997) Histology and teratology of pink salmon larvae near the time of emergence from gravel substrate in the laboratory. *Canadian Journal of Zoology,* **75,** 978-988.

Maxwell EE, Wilson LA (2013) Regionalization of the axial skeleton in the 'ambush predator' guild – Are there developmental rules underlying body shape evolution in ray-finned fishes? *BMC Evolutionary Biology,* **13,** 1-17.

McCullough DA (1999) A review on the synthesis of effects of alterations to the water temperature regime on freshwater life stages of salmonids, with special reference to Chinook salmon. (eds Rieman B, Thurow R, Beacham TD, Bumgarner J), pp. 1-252. Seatle, Washington, USA: Columbia River Inter-Tribal Fish Commission.

McDowall RM (1994) The origins of New Zealand's Chinook salmon, *Oncorhynchus tshawytscha*. *Marine Fisheries Review*, **56,** 1-7.

McDowall RM (2008) Jordan's and other ecogeographical rules, and the vertebral number in fishes. *Journal of Biogeography,* **35,** 501-508.

McLean IW, Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative a new fixative for immunoelectron microscopy. *Journal of Histochemistry & Cytochemistry,* **22,** 1077-1083.

McMahon JA, Takada S, Zimmerman LB, Fan C-M, Harland RM, McMahon AP (1998) Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes & Development,* **12,** 1438-1452.

McMurrich JP (1883) On the osteology and development of *Syngnathus peckianus* (Storer). In *A Treatise On Comparative Embryology* (ed Balfour FM), pp. 623. London: MacMillan and Co.

Melville DB, Montero-Balaguer M, Levic DS, Bradley K, Smith JR, Hatzopoulos AK, Knapik EW (2011) The *feelgood* mutation in zebrafish dysregulates COPII-dependent secretion of select extracellular matrix proteins in skeletal morphogenesis. *Disease Models & Mechanisms,* **4,** 763-776.

Mercier C, Axelsson M, Imbert N, Claireaux G, Lefrançois C, Altimiras J, Farrell AP (2002) In vitro cardiac performance in triploid brown trout at two acclimation temperatures. *Journal of Fish Biology,* **60,** 117-133.

Miller EJ, Mathews MB (1974) Characterization of notochord collagen as a cartilage-type collagen. *Biochemical and Biophysical Research Communications,* **60,** 424-430.

Miyashita T, Fujita K (2000) Two series of parapophyses in neoscopelid fishes (Teleostei: Myctophiformes). *Ichthyological Research,* **47,** 143-148.

Mori K (2009) Signalling pathways in the unfolded protein response: Development from yeast to mammals. *The Journal of Biochemistry,* **146,** 743-750.

Morin-Kensicki EM, Eisen JS (1997) Sclerotome development and peripheral nervous system segmentation in embryonic zebrafish. *Development,* **124,** 159-167.

Morin-Kensicki EM, Melancon E, Eisen JS (2002) Segmental relationship between somites and vertebral column in zebrafish. *Development,* **129,** 3851-3860.

Moriyama Y, Kawanishi T, Nakamura R, Tsukahara T, Sumiyama K, Suster Maximiliano L, Kawakami K, Toyoda A, Fujiyama A, Yasuoka Y, Nagao Y, Sawatari E, Shimizu A, Wakamatsu Y, Hibi M, Taira M, Okabe M, Naruse K, Hashimoto H, Shimada A, Takeda H (2012) The medaka zic1/zic4 mutant provides molecular insights into teleost caudal fin evolution. *Current Biology,* **22,** 601-607.

Morse A (1945) Formic acid-sodium citrate decalcification and butyl alcohol dehydration of teeth and bones for sectioning in paraffin. *Journal of Dental Research,* **24,** 143-153.

Mottley CM (1933) The effect of temperature during development on the number of scales in the Kamloops trout, *Salmo kamloops* Jordan. *Contributions to Canadian Biology and Fisheries,* **8,** 253-263.

Müller J, Scheyer TM, Head JJ, Barrett PM, Werneburg I, Ericson PGP, Pol D, Sánchez-Villagra MR (2010) Homeotic effects, somitogenesis and the evolution of vertebral numbers in recent and fossil amniotes. *Proceedings of the National Academy of Sciences,* **107,** 2118-2123.

Mundahl ND (1990) Heat death of fish in shrinking stream pools. *The American Midland Naturalist,* **123,** 40-46.

Munday JS, Perrott MR, Symonds JE, Walker SP, Lovett B, Preece MA, Davie PS (2016) Unilateral perivertebral fibrosis associated with lordosis, kyphosis and scoliosis (LKS) in farmed Chinook salmon in New Zealand. *Diseases of Aquatic Organisms,* **121,** 211-221.

Munday JS, Perrott MR, Symonds JE, Walker SP, Preece MA, Davie PS (2018) Prevalence of spinal abnormalities in Chinook salmon smolt and influence of early rearing temperature and growth rates. *Journal of Fish Diseases,* **41,** 1111-1116.

Murray CB (1980) Some effects of temperature on zygote and alevin survival, rate of development and size at hatching and emergence of Pacific salmon and rainbow trout. In *Department of Zoology*). Vancouver: University of British Columbia.

Murray CB, Beacham TD (1986) Effect of varying temperature regimes on the development of pink salmon (*Oncorhynchus gorbuscha*) eggs and alevins. *Canadian Journal of Zoology,* **64,** 670-676.

Murray CB, Beacham TD (1987) The development of Chinook (*Oncorhynchus tshawytscha*) and chum salmon (*Onchorhynchus keta*) embryos and alevins under varying temperature regimes. *Canadian Journal of Zoology,* **65,** 2672-2681.

Murray CB, Beacham TD (1989) Responses of meristic characters in chum salmon (*Oncorhynchus keta*) to temperature changes during development. *Canadian Journal of Zoology,* **67,** 596-600.

Murray CB, McPhail JD (1988) Effect of incubation temperature on the development of five species of Pacific salmon (*Oncorhynchus*) embryos and alevins. *Canadian Journal of Zoology,* **66,** 266-273.

Myers JM, Hershberger WK (1991) Early growth and survival of heat-shocked and tetraploid-derived triploid rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* **96,** 97-107.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation. *Cell,* **108,** 17-29.

Nathanailides C, Stickland NC, Lopez-Albors O (1995) Influence of prehatch temperature on the development of muscle cellularity in posthatch Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences,* **52,** 675-680.

Neill WH, Magnuson JJ (1974) Distributional ecology and behavioral thermoregulation of fishes in relation to heated effluent from a power plant at Lake Monona, Wisconsin. *Transactions of the American Fisheries Society,* **103,** 663-710.

Ng L-J, Wheatley S, Muscat GEO, Conway-Campbell J, Bowles J, Wright E, Bell DM, Tam PPL, Cheah KSE, Koopman P (1997) SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Developmental Biology,* **183,** 108-121.

Nicieza AG, Metcalfe NB (1997) Growth compensation in juvenile atlantic salmon: Responses to depressed temperature and food availibility *Ecology,* **78,** 2385-2400.

Nickel W, Brügger B, Wieland FT (2002) Vesicular transport: The core machinery of COPI recruitment and budding. *Journal of Cell Science,* **115,** 3235-3240.

Nordvik K, Kryvi H, Totland GK, Grotmol S (2005) The salmon vertebral body develops through mineralization of two preformed tissues that are encompassed by two layers of bone. *Journal of Anatomy,* **206,** 103-114.

Nowroozi BN, Harper CJ, De Kegel B, Adriaens D, Brainerd EL (2012) Regional variation in morphology of vertebral centra and intervertebral joints in striped bass, *Morone saxatilis*. *Journal of Morphology,* **273,** 441-452.

Nybelin O (1963) Zur morphologie und terminologie des Schwanzskelettes der Actinopterygier. *Arkiv för Zoologi*, **15,** 485-516.

Odenthal J, Haffter P, Vogelsang E, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Warga RM, Allende ML, Weinberg ES, Nusslein-Volhard C (1996) Mutations affecting the formation of the notochord in the zebrafish, *Danio rerio*. *Development*, **123,** 103-115.

Ohisa S, Inohaya K, Takano Y, Kudo A (2010) *sec24d* encoding a component of COPII is essential for vertebra formation, revealed by the analysis of the medaka mutant, *vbi*. *Developmental Biology*, **342,** 85-95.

Ojanguren A, Reyes-Gavilán F, Muñoz R (1999) Effects of temperature on growth and efficiency of yolk utilisation in eggs and pre-feeding larval stages of Atlantic salmon. *Aquaculture International*, **7,** 81-87.

Ojanguren AF, Braña F (2003) Thermal dependence of embryonic growth and development in brown trout. *Journal of Fish Biology*, **62,** 580-590.

Ojolick EJ, Cusack R, Benfey TJ, Kerr SR (1995) Survival and growth of all-female diploid and triploid rainbow trout (*Oncorhynchus mykiss*) reared at chronic high temperature. *Aquaculture*, **131,** 177-187.

Oliva-Teles A, Kaushik SJ (1990) Effect of temperature on utilization of endogenous energy reserves during embryonic development of diploid and triploid rainbow trout (*Salmo gairdneri* R.). *Aquaculture*, **84,** 373-382.

Olson PA, Foster RF (1957) Temperature tolerance of eggs and young of Columbia river Chinook salmon. *Transactions of the American Fisheries Society*, **85,** 203-207.

Oppenheimer JM (1936) Transplantation experiments on developing teleosts (*Fundulus* and *Perca*). *Journal of Experimental Zoology*, **72,** 409-437.

Ørnsrud R, Gil L, Waagbø R (2004a) Teratogenicity of elevated egg incubation temperature and egg vitamin A status in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases*, **27,** 213-223.

Ørnsrud R, Wargelius A, Sæle Ø, Pittman K, Waagbø R (2004b) Influence of egg vitamin A status and egg incubation temperature on subsequent development of the early vertebral column in Atlantic salmon fry. *Journal of Fish Biology*, **64,** 399-417.

Owen R (1848) *On the Archetype and homologies of the vertebrate skeleton*, Richard & John E. Taylor, Red Lion Court, Fleet street, London.

Paisley LG, Ariel E, Lyngstad T, Jónsson G, Vennerström P, Hellström A, Østergaard P (2010) An overview of aquaculture in the Nordic countries. *Journal of the World Aquaculture Society*, **41,** 1-17.

Parenti LR (1986) The phylogenetic significance of bone types in euteleost fishes. *Zoological Journal of the Linnean Society*, **87,** 37-51.

Parfitt AM (1988) Bone histomorphometry: Standardisation of nomenclature, symbols and units (summary of proposed system). *Bone*, **9,** 67-69.

Parsons MJ, Pollard SM, Saúde L, Feldman B, Coutinho P, Hirst EMA, Stemple DL (2002) Zebrafish mutants identify an essential role for laminins in notochord formation. *Development*, **129,** 313-3146.

Parsons PA (1990) Fluctuating asymmetry: An epigenetic measure of stress. *Biological Reviews*, **65,** 131-145.

Parsons PA (1992) Fluctuating asymmetry: A biological monitor of environmental and genomic stress. *Heredity*, **68,** 361-364.

Pattappa G, Li Z, Peroglio M, Wismer N, Alini M, Grad S (2012) Diversity of intervertebral disc cells: Phenotype and function. *Journal of Anatomy*, **221,** 480-496.

Patterson C, Johnson GD (1995) *The intermuscular bones and ligaments of teleostean fishes*, Smithsonian Institution Press, Washinton, DC.

Perrott MR, Symonds JE, Walker SP, Hely FS, Wybourne B, Preece MA, Davie PS (2018) Spinal curvatures and onset of vertebral deformities in farmed Chinook salmon (*Oncorhynchus tshawytscha*) in New Zealand. *Journal of Applied Ichthyology*, **34,** 501-511.

Peters H, Doll U, Niessing J (1995) Differential expression of the chicken *Pax-1* and *Pax-9* Gene: In situ hybridization and immunohistochemical analysis. *Developmental Dynamics*, **203,** 1-16.

Peterson R, Spinney H, Sreedharan A (1977) Development of Atlantic salmon (*Salmo salar*) eggs and alevins under varied temperature regimes. *Journal of the Fisheries Board of Canada*, **34,** 31-43.

Phillips B, Wright H (2006) COSEWIC assessment and status report on the Chinook salmon *Oncorhynchus tshawytscha* (Okanagan population) in Canada. pp. 7-41. Ottawa, Canada: Committee on the Status of Endangered Wildlife in Canada.

Pigliucci M (2001) *Phenotypic plasticity: Beyond nature and nurture*, Johns Hopkins University Press, Baltimore, Maryland.

Pogoda H-M, Riedl-Quinkertz I, Löhr H, Waxman JS, Dale RM, Topczewski J, Schulte-Merker S, Hammerschmidt M (2018) Direct activation of chordoblasts by retinoic acid is required for segmented centra mineralization during zebrafish spine development. *Development***,** DOI: 10.1242/dev.159418 [early view].

Pourquié O, Coltey M, Teillet MA, Ordahl C, Le Douarin NM (1993) Control of dorsoventral patterning of somitic derivatives by notochord and floor plate. *Proceedings of the National Academy of Sciences U.S.A.*, **90,** 5242-5246.

Presneau N, Shalaby A, Ye H, Pillay N, Halai D, Idowu B, Tirabosco R, Whitwell D, Jacques TS, Kindblom L-G, Brüderlein S, Möller P, Leithner A, Liegl B, Amary FM, Athanasou NN, Hogendoorn PCW, Mertens F, Szuhai K, Flanagan AM (2011) Role of the transcription factor

T (*brachyury*) in the pathogenesis of sporadic chordoma: A genetic and functional-based study. *The Journal of Pathology,* **223,** 327-335.

Price CS, Schreck CB (2003) Stress and saltwater-entry behavior of juvenile Chinook salmon (*Oncorhynchus tshawytscha*): Conflicts in physiological motivation. *Canadian Journal of Fisheries and Aquatic Sciences,* **60,** 910-918.

Proctor C, Mosse PRL, Hudson RCL (1980) A histochemical and ultrastructural study of the development of the propulsive musculature of the brown trout, *Salmo trutta* L., in relation to its swimming behaviour. *Journal of Fish Biology,* **16,** 309-329.

Pulcini D, Cataudella S, Boglione C, Russo T, Wheeler PA, Prestinicola L, Thorgaard GH (2015) Testing the relationship between domestication and developmental instability in rainbow trout, *Oncorhynchus mykiss* (Teleostei, Salmonidae). *Biological Journal of the Linnean Society,* **114,** 608-628.

Quatrefages AD (1888) *Mémoires sur la monstruosité double, chez les poisons*. Gauthier-Villars et Fils, Société Philomatique de Paris.

Quinn T, Kinnison M, Unwin M (2001) Evolution of Chinook salmon (*Oncorhynchus tshawytscha*) populations in New Zealand: Pattern, rate, and process. *Genetica,* **112,** 493-513.

Quinn TP, Unwin MJ (1993) Variation in life history patterns among New Zealand Chinook salmon (*Oncorhynchus tshawytscha*) populations. *Canadian Journal of Fisheries and Aquatic Sciences,* **50,** 1414-1421.

Ramzu M, Meunier FJ (1999) Descripteurs morphologiques de la zonation de la colonne vertébrale chez la truite arc-en-ciel *Oncorhynchus mykiss* (Walbaum, 1792) (Teleostei, Salmoniforme). *Annales des Sciences Naturelles - Zoologie et Biologie Animale,* **20,** 87-97.

Réalis-Doyelle E, Pasquet A, De Charleroy D, Fontaine P, Teletchea F (2016) Strong effects of temperature on the early life stages of a cold stenothermal fish species, brown trout (*Salmo trutta* L.). *PLoS ONE,* **11,** 1-17.

Refstie T, Stoss J, Donaldson EM (1982) Production of all female coho salmon (*Oncorhynchus kisutch*) by diploid gynogenesis using irradiated sperm and cold shock. *Aquaculture,* **29,** 67-82.

Remak R (1855) *Untersuchungen über die Entwickelung der Wirbelthiere,* G. Reimer, Berlin.

Renn J, Winkler C (2009) *Osterix*-mCherry transgenic medaka for in vivo imaging of bone formation. *Developmental Dynamics,* **238,** 241-248.

Richards FP (1977) Temperature preference studies in environmental impact assessments: An overview with procedural recommendations. *Journal of the Fisheries Board of Canada,* **34,** 728-761.

Richardson MK, Allen SP, Wright GM, Raynaud A, Hanken J (1998) Somite number and vertebrate evolution. *Development,* **125,** 151.

Robinson MS (2015) Forty years of clathrin-coated vesicles. *Traffic,* **16,** 1210-1238.

Ron AH, Stanley DR, Alex CW, Robert FB, Frank PT, John EJ, Jeffrey WS (2000) Delayed effects on growth and marine survival of pink salmon *Oncorhynchus gorbuscha* after exposure to crude oil during embryonic development. *Marine Ecology Progress Series,* **208,** 205-216.

Rondeletii G (1555) *Universae aquatilium Historiae pars altera, cum veris ipsorum Imaginibus,* Apud Matthiam Bonhomme, Lugduni (Lyon).

Rosengrave P, Taylor H, Montgomerie R, Metcalf V, McBride K, Gemmell NJ (2009) Chemical composition of seminal and ovarian fluids of Chinook salmon (*Oncorhynchus tshawytscha*) and their effects on sperm motility traits. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **152,** 123-129.

Rosenthal H, Alderdice DF (1976) Sublethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. *Journal of the Fisheries Research Board of Canada,* **33,** 2047-2065.

Rungruangsak-Torrissen K, Pringle GM, Moss R, Houlihan DF (1998) Effects of varying rearing temperatures on expression of different trypsin isozymes, feed conversion efficiency and growth in Atlantic salmon (*Salmo salar* L.). *Fish Physiology and Biochemistry,* **19,** 247-255.

Sadler SE, Friars GW, Ihssen PE (1986) The influence of temperature and genotype on the growth rate of hatchery-reared salmonids. *Canadian Journal of Animal Science,* **66,** 599-606.

Sallan LC (2012) Tetrapod-like axial regionalization in an early ray-finned fish. *Proceedings of the Royal Society of London B: Biological Sciences,* **279,** 3264-3271.

Sanger TJ, McCune AR (2002) Comparative osteology of the *Danio* (Cyprinidae: Ostariophysi) axial skeleton with comments on *Danio* relationships based on molecules and morphology. *Zoological Journal of the Linnean Society,* **135,** 529-546.

Santamaría JA, Andrades JA, Herráez P, Fernández-Llebrez P, Becerra J (1994) Perinotochordal connective sheet of gilthead sea bream larvae (*Sparus aurata,* L.) affected by axial malformations: An histochemical and immunocytochemical study. *The Anatomical Record,* **240,** 248-254.

Sarmah S, Barrallo-Gimeno A, Melville DB, Topczewski J, Solnica-Krezel L, Knapik EW (2010) Sec24D-Dependent transport of extracellular matrix proteins is required for zebrafish skeletal morphogenesis. *PLoS ONE,* **5,** 1-14.

Sato M, Kondo T, Yoshinaka R, Ikeda S (1983) Effect of water temperature on the skeletal deformity in ascorbic acid-deficient rainbow trout (*Salmo gairdneri*). *Bulletin of the Japanese Society of Scientific Fisheries,* **49,** 443-446.

Satoh N (2003) The ascidian tadpole larva: Comparative molecular development and genomics. *Nature Reviews Genetics,* **4,** 285-295.

Schaeffer B (1967) Osteichthyan vertebrae. *Zoological Journal of the Linnean Society,* **47,** 185-195.

Schilling N, Long Jr JH (2014) Axial systems and their actuation: New twists on the ancient body of craniates. *Zoology,* **117,** 1-6.

Schmidt K, Starck JM (2004) Developmental variability during early embryonic development of zebra fish, *Danio rerio*. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution,* **302B,** 446-457.

Schmidt K, Starck JM (2010) Developmental plasticity, modularity, and heterochrony during the phylotypic stage of the zebra fish, *Danio rerio*. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution,* **314B,** 166-178.

Schröter C, Herrgen L, Cardona A, Brouhard GJ, Feldman B, Oates AC (2008) Dynamics of zebrafish somitogenesis. *Developmental Dynamics,* **237,** 545-553.

Schultze H-P, Arratia G (1989) The composition of the caudal skeleton of teleosts (Actinopterygii: Osteichthyes). *Zoological Journal of the Linnean Society,* **97,** 189-231.

Schultze H-P, Arratia G (2013) The caudal skeleton of basal teleosts, its conventions, and some of its major evolutionary novelties in a temporal dimension. In *Mesosoic fishes 5 - Global diversity and evolution* (eds Arratia G, Schultze H-P, Wilson MVH), pp. 187-246. München: G. Verlag Dr. Friedrich Pfeil.

Schultze HP, Arratia G (1988) Reevaluation of the caudal skeleton of some actinopterygian fishes: II. *Hiodon*, *Elops*, and *Albula*. *Journal of Morphology,* **195,** 257-303.

Segner H, Sundh H, Buchmann K, Douxfils J, Sundell KS, Mathieu C, Ruane N, Jutfelt F, Toften H, Vaughan L (2012) Health of farmed fish: Its relation to fish welfare and its utility as welfare indicator. *Fish Physiology and Biochemistry,* **38,** 85-105.

Seymour A (1959) Effects of temperature upon the formation of vertebrae and fin rays in young Chinook salmon. *Transactions of the American Fisheries Society,* **88,** 58-69.

Sfakianakis DG, Koumoundouros G, Divanach P, Kentouri M (2004) Osteological development of the vertebral column and of the fins in *Pagellus erythrinus* (L. 1758). Temperature effect on the developmental plasticity and morpho-anatomical abnormalities. *Aquaculture,* **232,** 407-424.

Sfakianakis DG, Leris I, Laggis A, Kentouri M (2011) The effect of rearing temperature on body shape and meristic characters in zebrafish (*Danio rerio*) juveniles. *Environmental Biology of Fishes,* **92,** 197-206.

Shedko SV, Miroshnichenko IL, Nemkova GA (2013) Phylogeny of salmonids (Salmoniformes: Salmonidae) and its molecular dating: Analysis of mtDNA data. *Russian Journal of Genetics,* **49,** 623-637.

Sitia R, Braakman I (2003) Quality control in the endoplasmic reticulum protein factory. *Nature,* **426,** 891-894.

Smith MM, Hall BK (1990) Development and evolutionary origins of vertebrate skeletogenic and odontogenic tissues. *Biological Reviews,* **65,** 277-373.

Solar II, Donaldson EM, Hunter GA (1984) Induction of triploidy in rainbow trout (*Salmo gairdneri* Richardson) by heat shock, and investigation of early growth. *Aquaculture,* **42,** 57-67.

Solbakken VA, Hansen T, Stefansson SO (1994) Effects of photoperiod and temperature on growth and parr-smolt transformation in Atlantic salmon (*Salmo salar* L.) and subsequent performance in seawater. *Aquaculture,* **121,** 13-27.

Spemann H, Mangold H (1924) Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *Archiv für mikroskopische Anatomie und Entwicklungsmechanik,* **100,** 599-638.

Spitsbergen JM, Walker MK, Olson JR, Peterson RE (1991) Pathologic alterations in early life stages of lake trout, *Salvelinus namaycush*, exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin as fertilized eggs. *Aquatic Toxicology,* **19,** 41-71.

Spoorendonk KM, Peterson-Maduro J, Renn J, Trowe T, Kranenbarg S, Winkler C, Schulte-Merker S (2008) Retinoic acid and Cyp26b1 are critical regulators of osteogenesis in the axial skeleton. *Development,* **135,** 3765-3774.

Stehly GR, Gingerich WH (2001) Evaluation of AQUI-STM (efficacy and minimum toxic concentration) as a fish anaesthetic/sedative for public aquaculture in the United States. *Aquaculture Research,* **30,** 365-372.

Stemple DL (2005) Structure and function of the notochord: An essential organ for chordate development. *Development,* **132,** 2503-2512.

Stiassny MLJ (2000) Skeletal system. In *The laboratory fish* (ed Ostrander GK), pp. 109-118. San Diego, CA: Academic Press.

Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp-Kistner M, Angel P, Werb Z (2004) Altered endochondral bone development in matrix metalloproteinase13-deficient mice. *Development,* **131,** 5883-5895.

Stickland NC, White RN, Mescall PE, Crook AR, Thorpe JE (1988) The effect of temperature on myogenesis in embryonic development of the Atlantic salmon (*Salmo salar* L.). *Anatomy and Embryology,* **178,** 253-257.

Strudel G (1953a) Consequences de l' excision de troncons du tube nerveux sur la morphogène de l' embryon de poulet et sur la différenciation de ses organes: Contribution a la gene de l' orthosympathique. *Annales des Sciences Naturelles-Zoolgie et Biologie Animale,* **15,** 251-329.

Strudel G (1953b) Influence morphogenese du tube nerveux et de la corde sur la différenciation de la colonne vertebrale. *Comptes Rendus des Seances de la Societe de Biologie et des ses Filiales (Paris),* **47,** 132-133.

Sullivan M, Guy DR, Roberts RJ, Manchester NJ (2007a) The aetiology of spinal deformity in Atlantic salmon, *Salmo salar* L.: Influence of genetic factors on the frequency and severity in freshwater stages. *Journal of Fish Diseases,* **30,** 753-758.

Sullivan M, Hammond G, Roberts RJ, Manchester NJ (2007b) Spinal deformation in commercially cultured Atlantic salmon, *Salmo salar* L.: A clinical and radiological study. *Journal of Fish Diseases,* **30,** 745-752.

Sun X, Hornicek F, Schwab JH (2015) Chordoma: An update on the pathophysiology and molecular mechanisms. *Current Reviews in Musculoskeletal Medicine,* **8,** 344-352.

Takle H, Baeverfjord G, Lunde M, Kolstad K, Andersen Ø (2005) The effect of heat and cold exposure on HSP70 expression and development of deformities during embryogenesis of Atlantic salmon (*Salmo salar*). *Aquaculture,* **249,** 515-524.

Tang J, Bryant MD, Brannon EL (1987) Effect of temperature extremes on the mortality and development rates of coho salmon embryos and alevins. *The Progressive Fish-Culturist,* **49,** 167-174.

Taning ÅV (1952) Experimental study of meristic characters in fishes. *Biological Reviews,* **27,** 169-193.

Taylor EB (1990) Phenotypic correlates of life-history variation in juvenile Chinook salmon, *Oncorhynchus tshawytscha*. *Journal of Animal Ecology,* **59,** 455-468.

Taylor WR, Van Dyke G (1985) Revised procedures for staining and clearing small fishes and other vertebrates for bone and cartilage study. *Cybium,* **9,** 107-119.

Terjesen BF, Ulgenes Y, Fjæra SO, Summerfelt ST, Brunsvik P, Baeverfjord G, Nerland S, Takle H, Norvik OC, Kittelsen A (2009) RAS research facility dimensioning and design: A special case compared to planning production systems. In *Proceedings of Aquacultural Engineering Society's Fourth Issues Forum,* pp. 223-238. Roanoke, Virginia, USA.

Tester AL (1938) Variation in the mean vertebral count of herring (*Clupea pallasii*) with water temperature. *Journal du Conseil,* **13,** 71-75.

Thavarajah R, Mudimbaimannar VK, Elizabeth J, Rao UK, Ranganathan K (2012) Chemical and physical basics of routine formaldehyde fixation. *Journal of Oral and Maxillofacial Pathology,* **16,** 400-405.

To TT, Witten PE, Huysseune A, Winkler C (2015) An adult osteopetrosis model in medaka reveals the importance of osteoclast function for bone remodeling in teleost fish. *Comparative Biochemistry and Physiology Part C: Pharmacolgoy, Toxicology and Endocrinology,* **178,** 68-75.

Totland GK, Fjelldal PG, Kryvi H, Løkka G, Wargelius A, Sagstad A, Hansen T, Grotmol S (2011) Sustained swimming increases the mineral content and osteocyte density of salmon vertebral bone. *Journal of Anatomy,* **219,** 490-501.

Uji S, Suzuki T, Iwasaki T, Teruya K, Hirasawa K, Shirakashi M, Onoue S, Yamashita Y, Tsuji M, Tsuchihashi Y, Okuzawa K (2015) Effect of temperature, hypoxia and disinfection with ozonated seawater during somitogenesis on muscular development of the trunk in larval

seven-band grouper, *Epinephelus septemfasciatus* (Thunberg). *Aquaculture Research,* **46,** 2698-2706.

Unwin MJ (1986) Stream residence time, size characteristics, and migration patterns of juvenile chinook salmon (*Oncorhynchus tshawytscha*) from a tributary of the Rakaia river, New Zealand. *New Zealand Journal of Marine and Freshwater Research,* **20,** 231-252.

Unwin MJ, Glova GJ (1996) Changes in life history parameters in a naturally spawning population of Chinook salmon (*Oncorhynchus tshawytscha*) associated with releases of hatchery-reared fish. *Canadian Journal of Fisheries and Aquatic Sciences,* **54,** 1235-1245.

Unwin MJ, Lucas DH (1993) Scale Characteristics of Wild and Hatchery Chinook Salmon (*Oncorhynchus tshawytscha*) in the Rakaia River, New Zealand, and Their Use in Stock Identification. *Canadian Journal of Fisheries and Aquatic Sciences,* **50,** 2475-2484.

Unwin MJ, Quinn TP, Kinnison MT, Boustead NC (2000) Divergence in juvenile growth and life history in two recently colonized and partially isolated Chinook salmon populations. *Journal of Fish Biology,* **57,** 943-960.

Vacaru AM, Unlu G, Spitzner M, Mione M, Knapik EW, Sadler KC (2014) *In vivo* cell biology in zebrafish – Providing insights into vertebrate development and disease. *Journal of Cell Science,* **127,** 485-495.

Vågsholm I, Djupvik HO (1998) Risk factors for spinal deformities in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases,* **21,** 47-53.

van Eeden FJ, Granato M, Schach U, Brand M, Furutani-Seiki M, Haffter P, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Odenthal J, Warga RM, Allende ML, Weinberg ES, Nusslein-Volhard C (1996) Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development,* **123,** 153-164.

Van Valen L (1962) A study of fluctuating asymmetry. *Evolution,* **16,** 125-142.

Verhille C, Farrell AP (2012) The in vitro blood-O_2 affinity of triploid rainbow trout *Oncorhynchus mykiss* at different temperatures and CO_2 tensions. *Journal of Fish Biology,* **81,** 1124-1132.

Vermot J, Pourquie O (2005) Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos. *Nature,* **435,** 215-220.

Vernon EH (1957) Morphometric comparison of three races of kokanee (*Oncorhynchus nerka*) within a large British Columbia Lake. *Journal of the Fisheries Research Board of Canada,* **14,** 573-598.

Viant MR, Pincetich CA, Tjeerdema RS (2006) Metabolic effects of dinoseb, diazinon and esfenvalerate in eyed eggs and alevins of Chinook salmon (*Oncorhynchus tshawytscha*) determined by 1H NMR metabolomics. *Aquatic Toxicology,* **77,** 359-371.

Viant MR, Werner I, Rosenblum ES, Gantner AS, Tjeerdema RS, Johnson ML (2003) Correlation between heat-shock protein induction and reduced metabolic condition in juvenile steelhead

trout (*Oncorhynchus mykiss*) chronically exposed to elevated temperature. *Fish Physiology and Biochemistry,* **29,** 159-171.

von Baer KE (1828) *Über Entwickelungsgeschichte der Thiere, Beobachtung und Reflexion. Part I,* Bornträger, Königsberg.

Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z (1998) MMP-9/Gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell,* **93,** 411-422.

Wallace JC, Heggberget TG (1988) Incubation of eggs of Atlantic salmon (*Salmo Salar*) from different Norwegian streams at temperatures below 1°C. *Canadian Journal of Fisheries and Aquatic Sciences,* **45,** 193-196.

Walter P, Ron D (2011) The unfolded protein response: From stress pathway to homeostatic regulation. *Science,* **334,** 1081-1086.

Wang S (2013) Notochord development in Atlantic salmon (*Salmo salar* L.): Exploring molecular pathways and putative mechanism of segmentation. In *Department of Biology*, Norway: University of Bergen.

Wang S, Kryvi H, Grotmol S, Wargelius A, Krossøy C, Epple M, Neues F, Furmanek T, Totland GK (2013) Mineralization of the vertebral bodies in Atlantic salmon (*Salmo salar* L.) is initiated segmentally in the form of hydroxyapatite crystal accretions in the notochord sheath. *Journal of Anatomy,* **223,** 159-170.

Ward AB, Brainerd EL (2007) Evolution of axial patterning in elongate fishes. *Biological Journal of the Linnean Society,* **90,** 97-116.

Ward AB, Mehta RS (2010) Axial elongation in fishes: Using morphological approaches to elucidate developmental mechanisms in studying body shape. *Integrative and Comparative Biology,* **50,** 1106-1119.

Ward AB, Mehta RS (2014) Differential occupation of axial morphospace. *Zoology,* **117,** 70-76.

Ward MB, Kier WM (1999) Battle Creek Salmon and Steelhead Restoration Plan. (ed Group BCW). Sausalito, California: Kier Associates.

Wargelius A, Fjelldal P, Hansen T (2005) Heat shock during early somitogenesis induces caudal vertebral column defects in Atlantic salmon (*Salmo salar*). *Development Genes and Evolution,* **215,** 350-357.

Webb JH, McLay HA (1996) Variation in the time of spawning of Atlantic salmon (*Salmo salar*) and its relationship to temperature in the Aberdeenshire Dee, Scotland. *Canadian Journal of Fisheries and Aquatic Sciences,* **53,** 2739-2744.

Wedemeyer G, Saunders RL, Clarke CW (1980) Environmental factors affecting smoltification and early marine survival of anadromous salmonids. *Marine Fisheries Review,* **42,** 1-14.

Whitehouse WJ (1974) The quantitative morphology of anisotropic trabecular bone. *Journal of Microscopy,* **101,** 153-168.

Wiley EO, Fuiten AM, Doosey MH, Lohman BK, Merkes C, Azuma M (2015) The caudal skeleton of the zebrafish, *Danio rerio*, from a phylogenetic perspective: A polyural interpretation of homologous structures. *Copeia,* **103,** 740-750.

Willems B, Büttner A, Huysseune A, Renn J, Witten PE, Winkler C (2012) Conditional ablation of osteoblasts in medaka. *Developmental Biology,* **364,** 128-137.

Witten PE, Gil Martens L, Hall BK, Huysseune A, Obach A (2005a) Compressed vertebrae in Atlantic salmon *Salmo salar*: Evidence for metaplastic chondrogenesis as a skeletogenic response late in ontogeny. *Diseases of Aquatic Organisms,* **64,** 237-246.

Witten PE, Gil Martens L, Huysseune A, Takle H, Hjelde K (2009) Towards a classification and an understanding of developmental relationships of vertebral body malformations in Atlantic salmon (*Salmo salar* L.). *Aquaculture,* **295,** 6-14.

Witten PE, Hall BK (2002) Differentiation of kype skeletal tissues in the anadromous male Atlantic Salmon (*Salmo salar*). *International Journal of Developmental Biology,* **46,** 719-730.

Witten PE, Hall BK (2003) Seasonal changes in the lower jaw skeleton in male Atlantic salmon (*Salmo salar* L.): Remodelling and regression of the kype after spawning. *Journal of Anatomy,* **203,** 435-450.

Witten PE, Hall BK (2015) Teleost skeletal plasticity: Modulation, adaptation, and remodelling. *Copeia*, **103,** 1-13.

Witten PE, Hall BK, Huysseune A (2005b) Are breeding teeth in Atlantic salmon a component of the drastig alterations of the oral facial skeleton? *Archives of Oral Biology,* **50,** 213-217.

Witten PE, Harris MP, Huysseune A, Winkler C (2017) Small teleost fish provide new insights into human skeletal diseases. *Methods in Cell Biology,* **138,** 321-346.

Witten PE, Huysseune A, Hall BK (2010) A practical approach for the identification of the many cartilaginous tissues in teleost fish. *Journal of Applied Ichthyology,* **26,** 257-262.

Witten PE, Obach A, Huysseune A, Baeverfjord G (2006) Vertebrae fusion in Atlantic salmon (*Salmo salar*): Development, aggravation and pathways of containment. *Aquaculture,* **258,** 164-172.

Witten PE, Owen MAG, Fontanillas R, Soenens M, McGurk C, Obach A (2016) A primary phosphorus‐deficient skeletal phenotype in juvenile Atlantic salmon *Salmo salar*: The uncoupling of bone formation and mineralization. *Journal of Fish Biology,* **88,** 690-708.

Witten PE, Takle H, Baeverfjord G, Huysseune A (2007) Early and late vertebral fusion in Atlantic salmon (*Salmo salar*): Vertebral bodies and arches as developmental modules and the lifelong role of the notochord. *Journal of Morphology,* **268,** 1150.

Woltmann I, Shkil FN, De Clercq A, Huysseune A, Witten PE (2018) Supernumerary teeth in the pharyngeal dentition of slow developing zebrafish. *Journal of Applied Ichthyology,* **34,** 455-464.

Wong SFL, Agarwal V, Mansfield JH, Denans N, Schwartz MG, Prosser HM, Pourquié O, Bartel DP, Tabin CJ, McGlinn E (2015) Independent regulation of vertebral number and vertebral identity by microRNA-196 paralogs. *Proceedings of the National Academy of Sciences,* **112,** 4884-4893.

Yamamoto TS, Kobayashi W, Kuramoto T (1996) Twin malformation induced in chum salmon eggs by elevated water temperature, with a suggestion as to its mechanism. *Canadian Journal of Zoology,* **74,** 485-491.

Ytteborg E, Baeverfjord G, Torgersen J, Hjelde K, Takle H (2010a) Molecular pathology of vertebral deformities in hyperthermic Atlantic salmon (*Salmo salar*). *BMC Physiology,* **10,** 1-16.

Ytteborg E, Torgersen J, Baeverfjord G, Takle H (2010b) Morphological and molecular characterization of developing vertebral fusions using a teleost model. *BMC Physiology,* **10,** 1-15.

Ytteborg E, Torgersen JS, Pedersen ME, Baeverfjord G, Hannesson KO, Takle H (2010c) Remodeling of the notochord during development of vertebral fusions in Atlantic salmon (*Salmo salar*). *Cell and Tissue Research,* **342,** 363-376.

Yu T, Graf M, Renn J, Schartl M, Larionova D, Huysseune A, Witten PE, Winkler C (2017) A vertebrate specific and essential role for *sp7/osterix* in osteogenesis revealed by gene knock-out in the teleost medaka. *Development,* **144,** 265-271.

Zeitoun IH, Tack PI (1974) The embryology of the coho salmon, *Oncorhynchus kisutch* (Walbaum). *Transactions of the American Fisheries Society,* **103,** 371-375.

Zhang S-Y, Li G, Wu H-B, Liu X-G, Yao Y-H, Tao L, Liu H (2011) An integrated recirculating aquaculture system (RAS) for land-based fish farming: The effects on water quality and fish production. *Aquacultural Engineering,* **45,** 93-102.

Zhao Q, Eberspaecher H, Lefebvre V, de Crombrugghe B (1997) Parallel expression of *Sox9* and *Col2a1* in cells undergoing chondrogenesis. *Developmental Dynamics,* **209,** 377-386.

APPENDIX A: TEMPERATURE RESEARCH IN SALMONIDS

Table 20: Publications researching the effect of temperature in salmonids (search terms "salmon" and "temperature"; reviews not included). Order of species follows the phylogeny of Salmonidae of Shedko et al. (2013).

Species (common name)	Species (Latin name)	Temperature used in experiments	Other factors studied or associated with temperature	Reference
Grayling	*Thymallus thumallus*	Temperature groups	Embryonic development time and mortality	Jungwirth & Winkler (1984)
Cisco	*Coregonus artedii*	Acclimation temperature	Temperature tolerance	Beitinger & Bennett (2000)
		Acclimation temperature	High and low lethal temperature limits	Edsall & Colby (1970)
Danube salmon / Huchen	*Hucho hucho*	Temperature groups	Embryonic development time and mortality	Jungwirth & Winkler (1984)
Atlantic salmon	*Salmo salar*	Acclimation temperature	Routine metabolic rate	Atkins & Benfey (2008)
		Temperature range	Process of dying by temperature in rivers	Huntsman (1942)
			Time of morphogenesis	Hayes et al. (1953)
			Growth and efficiency of yolk conversion	Hayes & Pelluet (1945)
			Development time from hatching to first feed	Jensen et al. (1989)
			Time to hatch in geographical locations	Webb & McLay (1996)
			Model hatching time related to temperature	Crisp (1981)
			Muscle cellularity at hatching and post-hatching	Nathanailides et al. (1995)
			Family effects on muscle cellularity at hatching and first feed	Johnston & McLay (1997)
			Trypsin expression, feed conversion efficiency and growth	Rungruangsak-Torrissen et al. (1998)

		Catch-up growth in juveniles	Nicieza & Metcalfe (1997)
		Growth rate	Austreng et al. (1987)
	Constant temperature	Fertilisation to hatching time in very cold water	Wallace & Heggberget (1988)
	Temperature groups	Incubation time	Heggberget & Wallace (1984)
		Survival and development	Gunnes (1979)
		Oxygen and water exchange rate related to developmental rate and survival	Hamor & Garside (1976)
		Oxygen consumption by embryos in the egg	Hamor & Garside (1979)
		Yolk utilisation related to dissolved oxygen	Hamor & Garside (1977)
		Developmental rate and efficiency of yolk utilisation in embryos and alevins	Ojanguren et al. (1999)
		Myogenesis in embryonic development	Stickland et al. (1988)
		Temperature until eyed egg stage related adult growth and muscle phenotype	Macqueen et al. (2008)
		Teratogenicity (malformation rates) related to vitamin A in embryos and alevin	Ørnsrud et al. (2004a)
		Teratogenicity (malformation rates) related to vitamin A in fry	Ørnsrud et al. (2004b)
		Metabolome and endocrine metabolic signals	Kullgren et al. (2013)
		Growth, condition factor and smoltification related to photoperiod	Solbakken et al. (1994)
		Appatite at higher temperature	Hevrøy et al. (2012)
		Growth, feed intake, food conversion efficiency and evacuation rate of the stomach	Handeland et al. (2008)
		Growth rate and protein and fat digestibility at parr stage	Bendiksen et al. (2003)
		Growth efficiency	Dwyer & Piper (1987)
		Effect of vaccination lesions on vertebral deformity	Grini et al. (2011)
	Temperature transfer	Development and size of embryos and alevins	Peterson et al. (1977)
	Temperature shock	HSP70 expression and development of malformations in embryos	Takle et al. (2005)
		Malformations and expression of skeletal development markers	Wargelius et al. (2005)

			Heat shock for triploidisation	Benfey & Sutterlin, (1984)
Brown trout	*Salmo trutta*	Acclimation temperature	Embryonic growth and development until hatching	Ojanguren & Braña (2003)
			Cardiorespiratory status during swimming	Altimiras et al. (2002)
		Temperature range	Incubation period	Embody (1934)
			Development time from hatching to first feed	Jensen et al. (1989)
			Model hatching time related to temperature	Crisp (1981)
			Blood oxygen affinity and CO_2 effect	Irving et al. (1941)
		Temperature groups	Embryonic development time and mortality	Jungwirth & Winkler (1984)
			Developmental time, morphometrics and bioenergetics of embryos and fry	Réalis-Doyelle et al. (2016)
		Temperature groups/ temperature shocks	In vitro cardiac performance	Mercier et al. (2002)
			Thermotolerance of gametes, embryos and alevins	Lahnsteiner (2012)
Rainbow trout	*Oncorhynchus mykiss*	Temperature range	Incubation period	Embody (1934)
			Model hatching time related to temperature	Crisp (1981)
			Effect of ploidy on thermal tolerance	Galbreath et al. (2006)
			Blood oxygen affinity and CO_2 effect	Irving et al. (1941)
			Blood oxygen affinity of triploids in different CO_2 tension levels	Verhille & Farrell (2012)
			Development of the number of scales	Mottley (1933)
			Growth rate	Austreng et al. (1987)
			Cardiovascular and respiratory response to heat stress	Heath & Hughes (1973)
		Constant temperature	Ammonia and urea nitrogen excretion and oxygen consumption in diploids and triploids from fertilisation to fry stage	Oliva-Teles & Kaushik (1990)
			Survival and growth of all-female diploids and triploids	Ojolick et al. (1995)
		Temperature groups	Zygote and alevin survival, and rate of development to hatching and of alevin and fry	Murray (1980)
			Temperature at stripping affects triploidisation and survival of triploid embryos	Diaz et al. (1993)

			Embryonic development rate, growth rate and meristic variation related to light intensity	Kwain (1975)
			Oxygen related to temperature, effect on development	Garside (1966a)
			Oxygen, related to developmental rate and vertebral number	Garside (1966b)
			Effect of parentage on meristic variation	MacGregor & MacCrimmon (1977)
			Specific growth rate, mortality rate and yield of juveniles	Hokanson et al. (1977)
			Ascorbic acid deficiency related to skeletal deformities	Sato et al. (1983)
			HSP expression, metabolic condition and growth rates of juveniles	Viant et al. (2003)
			Juvenile growth rate	Sadler et al. (1986)
		Temperature shock	Early growth and survival of tetraploid derived triploids	Myers & Hershberger (1991)
			Optimisation of triploidisation	Solar et al. (1984)
Chinook salmon	*Oncorhynchus tshawytscha*	Acclimation temperature	Temperature tolerance	Beitinger & Bennett (2000)
		Critical temperature	High and low lethal temperature limits	Brett (1952)
		Temperature range	Incubation time	Alderdice & Velsen (1978)
			Model hatching time related to temperature	Crisp (1981)
			Temperature tolerance of eggs to fingerling stage	Olson & Foster (1957)
			Growth rate in sea and development of seawater adaptability	Clarke & Shelbourn (1985)
			Growth, body form and haematology	Banks et al. (1971)
			Developmental errors as indicator for chronic stress	Campbell (2003)
			Meristic variation	Seymour (1959)
			Effect of El Niño on average size and mortality	Johnson (1988)
		Constant temperature	Embryo and alevin survival, hatching and mergence time and length and weight of alevins and fry	Murray & McPhail (1988)
		Constant temperature/ Temperature groups	Egg weight to weight and length of alevin at hatching and fry at emergence / Length and weight of fry	Beacham & Murray (1990)

		Temperature groups	Survival to establish high and low threshold temperatures	Combs (1965)
			Mortality to establish high and low threshold temperatures	Combs & Burrows (1957)
			Zygote and alevin survival, and rate of development to hatching and of alevin and fry	Murray (1980)
			Alevin and fry survival and size and time of emergence related to spawning location	Beacham & Murray (1989)
			Dissolved oxygen on survival, hatching and growth of embryos and fry	Eddy (1971)
			Growth, development and survival of alevins	Heming (1982)
			Growth, survival and food utilisation in fry	Heming et al. (1982)
			Growth rate of smolts and smoltification	Clarke et al. (1981)
			Incidence of jacking	Heath et al. (1994)
		Temperature transfer	Egg and alevin survival, hatching and emergence time and length and weight of alevins and fry	Murray & Beacham (1987)
		Temperature shock	Production of triploid eggs and their survival	Levanduski et al. (1990)
Coho salmon	*Oncorhynchus kisutch*	Acclimation temperature	Temperature tolerance	Beitinger & Bennett (2000)
		Critical temperature	High and low lethal temperature limits	Brett (1952)
		Temperature range	Mortality and developmental rates of embryos and alevins	Tang et al. (1987)
			Swimming performance and metabolic rate	Lee et al. (2003)
			Mortality, early growth rate and developmental instability (meristic variation) as measures of chronic developmental stress	Campbell et al. (1998)
			Developmental errors as indicator for chronic stress	Campbell (2003)
			Effect of El Niño on average size and mortality	Johnson (1988)
		Constant temperature	Embryo and alevin survival, hatching and emergence time and length and weight of alevins and fry	Murray & McPhail (1988)
		Constant temperature/ temperature groups	Egg weight to weight and length of alevin at hatching and fry at emergence / Length and weight of fry	Beacham & Murray (1990)

		Temperature groups	Zygote and alevin survival, and rate of development to hatching and of alevin and fry	Murray (1980)
			Growth rate of smolts and smoltification	Clarke et al. (1981)
			Growth rate and sea water adaptability related to photoperiod	Clarke & Shelbourn (1986)
		Cold shock	Production of triploids with irradiated sperm	Refstie et al. (1982)
Sockeye salmon	*Oncorhynchus nerka*	Acclimation temperature	Temperature tolerance	Beitinger & Bennett (2000)
			Fatigue time and swimming performance	Brett (1967)
		Critical temperature	High and low lethal temperature limits	Brett (1952)
		Temperature range	Growth rate, mortality and food conversion rations of fingerlings	Donaldson & Foster (1941)
			Growth rate and body composition	Brett et al. (1969)
			Aerobic and cardiac capacity and heart rate for thermal optima in different populations	Eliason et al. (2011)
			Swimming performance and metabolic rate	Lee et al. (2003)
			Climate warming on stock specific survival of adults	Martins et al. (2011)
		Constant temperature	Embryo and alevin survival, hatching and emergence time and length and weight of alevins and fry	Murray & McPhail (1988)
		Constant temperature/ Temperature groups	Egg weight to weight and length of alevin at hatching and fry at emergence / Length and weight of fry	Beacham & Murray (1990)
		Temperature groups	Zygote and alevin survival, and rate of development to hatching and of alevin and fry	Murray (1980)
			Survival to establish high and low threshold temperatures	Combs (1965)
			Alevin and fry survival and size and time of emergence related to spawning location	Beacham & Murray (1989)
			Oxygen consumption related to swimming speed and fatigue level	Brett (1964)
			Growth rate of smolts and smoltification	Clarke et al. (1981)
Pink salmon	*Oncorhynchus*	Critical temperature	High and low lethal temperature limits	Brett (1952)

	gorbuscha	Temperature range	Survival, growth, time of emergence related to spawning time	Beacham & Murray, (1988)
		Constant temperature	Embryo and alevin survival, hatching and emergence time and length and weight of alevins and fry	Murray & McPhail, (1988)
		Constant temperature/ temperature groups	Egg weight to weight and length of alevin at hatching and fry at emergence / Length and weight of fry	Beacham & Murray, (1990)
		Temperature groups	Zygote and alevin survival, and rate of development to hatching and of alevin and fry	Murray, (1980)
			Embryo and alevin survival, hatching time and time of emergence related to heterozygosity	Beacham & Varnavskaya, (1991)
			Survival of families, egg weight, length and weight of alevins and fry	Murray & Beacham, (1986)
		Temperature transfer	Embryo survival	Beacham & Murray, (1987b)
			Egg and alevin survival, hatching and emergence time and length and weight of alevins	Murray & Beacham, (1986)
Chum salmon	*Oncorhynchus keta*	Acclimation temperature	Temperature tolerance	Beitinger & Bennett (2000)
		Critical temperature	High and low lethal temperature limits	Brett (1952)
		Constant temperature	Embryo and alevin survival, hatching and mergence time and length and weight of alevins and fry	Murray & McPhail (1988)
		Constant temperature/ temperature groups	Egg weight to weight and length of alevin at hatching and fry at emergence / Length and weight of fry	Beacham & Murray (1990)
		Temperature groups	Zygote and alevin survival, and rate of development to hatching and of alevin and fry	Murray (1980)
			Female size and egg size on developmental biology	Beacham & Murray (1985a)
			Survival of families	Beacham & Murray (1986)
			Spawning time and meristic variation	Beacham & Murray (1986)
			Meristic variations and fluctuating asymmetry	Beacham (1990)
			Occurrence of twinning malformation	Yamamoto et al. (1996)

		Temperature transfer	Embryo survival	Beacham & Murray (1987b)
			Meristic variation	Murray & Beacham (1987)
Brook charr	*Salvelinus*	Acclimation temperature	Temperature tolerance	Beitinger & Bennett (2000)
/ brook trout	*fontinalis*		Routine metabolic rate	Atkins & Benfey (2008)
		Critical temperature	Thermal maxima between diploid and triploid fish	Benfey et al. (1997)
		Temperature range	Process of dying by temperature in rivers	Huntsman (1942)
			Incubation period	Embody (1934)
			Model hatching time related to temperature	Crisp (1981)
			Blood oxygen affinity and CO_2 effect	Irving et al. (1941)
			Effect of ploidy on thermal tolerance	Galbreath et al. (2006)
		Constant temperature	Survival related to exhaustive exercise in triploids	Hyndman et al. (2003)
		Temperature groups	Oxygen related to temperature, effect on development	Garside (1966a)
			Oxygen, related to developmental rate and vertebral number	Garside (1966b)
			Oxygen uptake related to activity levels	Graham (1949)
			Juvenile growth rate	Sadler et al. (1986)
Lake trout	*Salvelinus*	Temperature range	Incubation period	Embody (1934)
	namaycush	Temperature groups	Oxygen related to temperature, effect on development	Garside (1959)
			Juvenile growth rate	Sadler et al. (1986)
Arctic charr	*Salvelinus alpinus*	Temperature range	Egg size and maternal age on egg viability at eyed egg stage	Jeuthe et al. (2013)
		Temperature groups	Embryonic development time and mortality	Jungwirth & Winkler (1984)

APPENDIX B: FERTILISATION SCHEDULE CHINOOK SALMON

Table 21: Fertilisation schedule of the temperature experiment with Chinook salmon.

Female	Containers									
Egg clutch	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
F1	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6
F2	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6
F3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6
F4	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6
F5	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6
F6	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6
F7	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6
F8	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6
F9	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6

F10	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M1, M2, M3	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6	10% eggs M4, M5, M6
Trays	Split over Two trays	Split over Two trays	Split over Two trays	Split over Two trays	Split over Two trays	Split over Two trays	Split over Two trays	Split over Two trays	Split over Two trays	Split over Two trays

The eggs of each female were divided over 10 containers, with 10% of the total egg weight per container (indicated by 10% eggs). The first five containers were fertilised with milt from the first three males (light orange), while container six to ten were fertilised with milt from male four to six (light blue). Each container received 1 ml milt from each male, e.g. M1, M2, M3 stands for 1 ml milt of male 1, 1 ml of male 2 and 1 ml of male 3 were added to that container. Male four to six are indicated as M4, M5, M6.

APPENDIX C: FORK LENGTH AND WEIGHT AT DIFFERENT LIFE STAGES

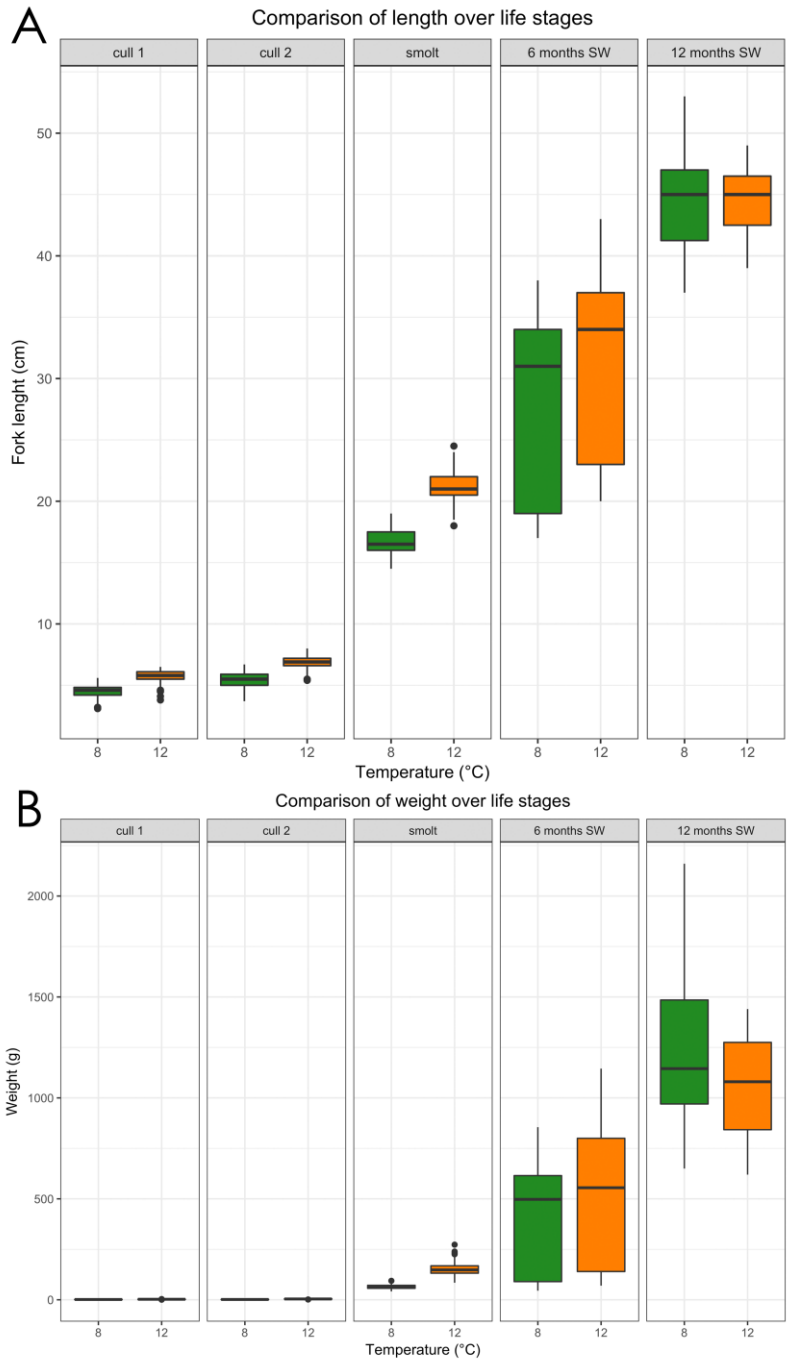

Figure 32: Fork length and weight of the 8°C and 12°C groups at different life stages.

Boxplot of fork length (A) in centimetres and weight (B) in grams for cull 1 (1400°d, Table 12), cull 2 (1530°d, Table 12), freshwater smoltification-stage (indicated as 'smolt'), six-months-at-sea and 12-months-at-sea. The 8°C group is coloured in dark green and the 12°C group in orange.

APPENDIX D: WHOLE-MOUNT STAINING PROTOCOLS

Table 22: Alizarin red S staining protocol, adapted from Taylor and Van Dyke (1985)

Step	Solution	Post-Hatch	First Feed	Juvenile
Remove epithelia and guts	Epithelium and guts removed in first feed stage specimens and juveniles	-	-	-
Rinse	Tap water	24 hrs	24 hrs	24 hrs
Remove fat	70-100% acetone solution (70% for post-hatch and first feed stage specimens)	12 hrs	24 hrs	48 hrs
Bleach pigment	1% KOH/3% H_2O_2	20 min	1hr	1:15 hrs
Rinse	Demineralised water	30 min	30 min	30 min
Neutralisation	Neutralisation buffer: 30% saturated borax ($Na_2B_4O_7$ sodium tetraborate)/70% demineralised water (pH 9.4)	12 hrs	12 hrs	12 hrs
Bone staining	Alizarin red solutions: 0.5 mg/ml alizarin red S in 1% KOH solutions	12 hrs	24 hrs	48 hrs
Rinse	Demineralised water	30 min	1-3 hrs	5 hrs
Clearing	20% glycerol in a 1% KOH solution	5 days	5 days	10 days
	40% glycerol in a 1% KOH solution	5 days	5 days	10 days
	60% glycerol solution	5 days	10 days	21 days
	80% glycerol solution	5 days	10 days	21 days
	90% glycerol solution	5 days	10 days	1 month
	95% glycerol solution	5 days	20 days	1 month
Storage	100% glycerol	-	-	-

Table 23: Alcian Blue 8GX staining protocol, adapted from Taylor and Van Dyke (1985)

Step	Solution	Post-Hatch	First Feed
Rinse	Tap water	24 hrs	24 hrs
Remove fat	70-100% acetone solution (70% for post-hatch and first feed stage specimens)	12 hrs	24 hrs
Dehydration	30% ethanol solution	48 hrs	48 hrs
	50% ethanol solution	48 hrs	48 hrs
	70% ethanol solution	24 hrs	24 hrs
Cartilage staining	15mg/ml Alcian blue in 20% acetic acid (100%) and 80% ethanol (100%), allow the solution to stabilise for two days before measuring and adjusting pH (pH ≤ 2.2 or 1.8 optimal)	48 hrs	48 hrs
Rehydration	70% ethanol solution	24 hrs	24 hrs
	50% ethanol solution	24 hrs	24 hrs
	30% ethanol solution	24 hrs	24 hrs
	15% ethanol solution	24 hrs	24 hrs
Rinse	Demineralised water	12 hrs	12 hrs
Neutralisation and clearing	Neutralisation buffer: 0.5mg/ml trypsin in 30% saturated borax ($Na_2B_4O_7$ sodium tetra borate)/70% demineralised water (pH 9.4)	8 hrs	24 hrs
Clearing	20% glycerol in a 1% KOH solution	12 hrs	5 days
	40% glycerol solution	5 days	5 days
	60% glycerol solution	5 days	10 days
	80% glycerol solution	5 days	10 days
	90% glycerol solution	5 days	10 days
	95% glycerol solution	5 days	20 days
Storage	100% glycerol	-	-

APPENDIX E: HISTOLOGICAL PROTOCOLS

Table 24: Processing for paraffin embedding

Step	Solution	Time (hours)	Temperature	Pressure
Dehydration	Ethanol 70%	0:30	Ambient	
	Ethanol 95%	1:00	40°C	
	Ethanol 100%	1:15	40°C	
	Ethanol 100%	1:15	40°C	
	Ethanol 100%	1:15	40°C	
	50% ethanol/50% xylene	1:20	40°C	
	Xylene 100%	0:45	40°C	
	Xylene 100%	0:45	40°C	Vacuum
Replacing with paraffin	Paraffin	1:20	60°C	Vacuum
	Paraffin	1:20	60°C	Vacuum
	Paraffin	1:20	60°C	Vacuum

Table 25: Haematoxylin and eosin staining protocol, adapted from (Gill et al., 1974)

Step	Solution	Time (minutes)
Paraffin removal	Xylene 100%	3:00
	Xylene 100%	2:00
	Ethanol 100%	1:00
Rehydration	Ethanol 100%	0:30
	Ethanol 70%	1:30
	Rinse (demineralised water)	1:00
Staining	Gill's Haematoxylin	5:00
	Rinse (demineralised water)	0:30
	Scott's tap water	0:30
	Rinse (demineralised water)	1:00
	Eoxin/Phloxine	2:00
	Rinse (demineralised water)	0:30
Dehydration	Ethanol 70%	0:10
	Ethanol 95%	0:15
	Ethanol 100%	0:45
	Ethanol 100%	1:00
	Xylene 100%	1:00
	Xylene 100%	1:00

Table 26: Masson's trichrome staining protocol, adapted from (Culling, 1974b)

Step	Solution	Time (minutes)
Paraffin removal	Xylene 100%	3:00
	Xylene 100%	2:00
	Ethanol 100%	1:00
Rehydration	Ethanol 100%	0:30
	Ethanol 70%	1:30
	Rinse (demineralised water)	1:00
Post fixation	Mordant in Bouin's fixative	Overnight
Stain	Rinse (demineralised water)	3:00
	Celestine blue	10:00
	Rinse (demineralised water)	0:10
	Mayer's Haematoxylin	10:00
	Rinse (demineralised water)	0:10
	Scott's tap water (differentiate nuclei)	2:00
	Rinse (demineralised water)	2:00
	Beiberich scarlet-acid fuschsin	2:00
	Rinse (demineralised water)	0:30
	Phosphotungstic acid 5%	15:00
	Rinse (demineralised water)	0:30
	Light green solution	1:00
	Rinse (demineralised water)	4 dips + drain
	Blot dry on filter paper (removal excess stain)	-
	Glacial acetic acid 1%	4 dips + drain
	Blot dry on filter paper (removal excess stain)	-
Dehydration	Ethanol 95%	10 dips + drain
	Ethanol 100%	10 dips + drain
	Ethanol 100%	10 dips + drain
	Xylene 100%	10 dips + drain
	Xylene 100%	10 dips + drain
	Xylene 100%	10 dips + drain

Table 27: Verhoeff-Van Gieson staining protocol, adapted from (Culling, 1974b)

Step	Solution	Time (minutes)
Paraffin removal	Xylene 100%	2:00
	Xylene 100%	2:00
	Ethanol 100%	1:00
Rehydration	Ethanol 100%	1:00
	Ethanol 95%	1:00
	Ethanol 70%	1:00
	Rinse (demineralised water)	1:00
Staining	Verhoeff's working solution (20 ml alcoholic haematoxylin + 8 ml 10% ferric chloride + 8 ml Verhoeff's iodine)	15:00
	Rinse (demineralised water)	0:30
	2% ferric chloride (differentiate elastin fibres)	1:00 + check
	Rinse (demineralised water)	0:30
	Rinse in ethanol 95% (removal iodine residue)	0:05
	Rinse (demineralised water)	5:00
	Van Gieson stain (counterstain)	3:00
	Blot dry on filter paper (removal excess stain)	-
Dehydration	Ethanol 95%	10 dips + drain
	Ethanol 100%	10 dips + drain
	Ethanol 100%	10 dips + drain
	Xylene 100%	10 dips + drain
	Xylene 100%	10 dips + drain
	Xylene 100%	10 dips + drain

Table 28: AB-PAS (Alcian Blue-Periodic Acid Shiff) staining protocol, adapted from (Culling, 1974a)

Step	Solution	Time (minutes)
Paraffin removal	Xylene 100%	2:00
	Xylene 100%	2:00
	Ethanol 100%	1:00
Rehydration	Ethanol 100%	1:00
	Ethanol 95%	1:00
	Ethanol 70%	1:00
	Rinse (demineralised water)	0:30
Staining	1% Alcian blue (pH 2.5)	10:00
	Rinse (demineralised water)	0:10
	0.5% periodic acid (oxidise)	5:00
	Rinse (demineralised water)	5:00
	Shiff's reagent (toxic!)	10:00
	Rinse (demineralised water)	10:00

	Gill's Haematoxylin (counterstain)	0:30
	Rinse (demineralised water)	0:30
	Scott's tap water	0:30
	Rinse (demineralised water)	1:00
Dehydration	Ethanol 70%	10 dips + drain
	Ethanol 95%	10 dips + drain
	Ethanol 100%	10 dips + drain
	Ethanol 100%	10 dips + drain
	Xylene 100%	10 dips + drain
	Xylene 100%	10 dips + drain
	Xylene 100%	10 dips + drain

Table 29: Heidenhains AZAN trichrome staining protocol (Böck & Romeis, 1989)

Step	Solution	Time (hours)
Paraffin removal	Ultraclear 100%	00:05
	Ultraclear 100%	00:05
	Ethanol 96%	00:04
	Ethanol 96%	00:04
Staining	Aniline alcohol (0.1% anilin in 95% ethanol)	00:05
	Rinse (demineralised water)	00:00:10
	Azocarmine	00:45
	Rinse (demineralised water)	00:00:10
	Aniline alcohol (0.1% anilin in 95% ethanol)	00:05
	Acetic acid alcohol 1%	00:01
	Rinse (demineralised water)	00:01
	Phosphotungstic acid 5%	03:00
	Rinse (demineralised water)	00:00:10
	Aniline blue-Orange G	01:00
	Rinse (demineralised water)	00:00:10
Dehydration	Ethanol 96%	00:02
	Ethanol 96%	00:03
	Isopropanol	00:03
	Ultraclear 100%	00:03
	Ultraclear 100%	00:03

APPENDIX F: MUTATIONS OF CELLULAR VESICULAR TRANSPORT

Table 30: Mutations affecting the normal chordocyte and chordoblast vesicular transport functions in zebrafish and medaka

Component (s)	Function	Mutation (s)	Species	Effects	Reference(s)
Unfolded Protein Response (UPR)	Folding proteins correctly in the ER lumen and evacuating proteins correctly when ER-stress occurs. This occurs when ER bulges due to the presence of to many proteins. Transcriptional regulation of COPII coat components (SEC23/SEC24/SAR1, SEC13/SEC 31, TANGO, SEDLIN, KLHL12, CUL3).	*Atf6α/β* mutants	Medaka	• ER-stress occurs • Reduced induction of ER chaperones • Chaperones: HSP90, GRP94, Lectins, HSP70, HSP40 family members	Ishikawa et al. (2013) Sitia & Braakman (2003)
	Bbf2h7 is a transducer of all COP II components (SEC23/SEC24/SAR1, SEC13/SEC31, TANGO, SEDLIN, KLHL12, CUL3) and is similar to the *feelgood* mutant in zebrafish.	*Bbf2h7* mutants	Medaka	• Cartilage in skull under develops • Short tail • Bended notochord • Incomplete bone formation • Detachment of chordoblasts from basal lamina • ER-stress due to build-up of proteins in the ER • No collagen type II secretion • No collagen type IV secretion • Downregulation of all COPII coat components	Ishikawa et al. (2017)
	Is similar to *Bbf2h7* mutant in medaka. However, *Creb3l2* only regulates a subset of COP II components, i.e. SEC23A/B and SEC24D.	*Feelgood* (*Creb3l2*) mutant	Zebrafish	• Cartilage in skull under develops • Short tail • Bended notochord	Melville et al. (2011)

				• ER-stress due to build-up of proteins in the ER • No collagen type II secretion • Downregulation of SEC23A/B and SEC24D COPII components	
Coatomer, COP I	COP I coated vesicles recycle membrane bound components form the Golgi-complex to the ER (retrograde transport). COP I coated vesicles have also been associated with limited anterograde transport and transport from the Golgi-complex to intra-cellular vacuoles. Coatomer components are P23, ARF, COP β, COP γ, COP δ, COP ζ, COP αβ'ε and are highly and consistently upregulated in chordocytes to develop and maintain the vacuoles.	*Sneezy* (*Cop α*) *Happy* (*Cop β*) *Dopey* (*Cop β'*) mutants	Zebrafish	• Aberrations in chordamesoderm differentiation • Vacuoles of chordocytes fail to form • ER and Golgi-complex disrupted • ER-stress due to build-up of proteins in the ER • Collagen layer in notochord sheath (middle layer) is thin and malformed	Coutinho et al. (2004) Kondylis et al. (2009) Nickel et al. (2002) Odenthal et al. (1996) Stemple (2005) Vacaru et al. (2014)
COP II	COP II coated vesicles (50-90 nm) transport proteins from the ER to the Golgi-complex (anterograde transport). COP II components are SEC23/SEC24/ SAR1, SEC13/SEC31 and are highly upregulated in chordoblasts and chondro-blasts (cells with high the demand for excretion to the ECM). Components such as TANGO, SEDLIN, KLHL12, CUL3 interact with the SEC proteins to build larger vesicles to cope with large proto-collagen type 2 molecules (300 nm).	*Sec23a* (*crusher*) mutant	Zebrafish	• Short tail • Skull underdeveloped and looks collapsed ('crushed') • Collagen and proteoglycan secretion impaired • ER-stress due to build-up of collagen type 2 in the ER • Higher proteasome activity • HSP70 (UPR-chaperone) is	Kondylis et al. (2009) Lang et al. (2006)

				upregulated	
	= *vbi* mutant in medaka	*Sec24d* (*bulldog*) mutant	Zebrafish	• Short tail • Skull underdeveloped • Bended notochord • Impaired chondrocyte maturation • ER-stress due to build-up of collagen type 2 in the ER of chondrocytes • UPR components are upregulated	Melville et al. (2011) Sarmah et al. (2010)
	= *bulldog* mutant in zebrafish	*Vbi* (*Vertebra imperfecta*) mutant	Medaka	• Centrum mineralisation delayed • Centrum mineralisation is irregular • Chordacentra fuse ventrally • Neural and haemal arches malformed • Irregular migration and position of osteoblasts lining the notochord • ER-stress due to build-up of collagen type 2 in the ER of chondrocytes • HSP47 (UPR-chaperone) is upregulated	Ohisa et al. (2010)
Clathrin coat complex	Clathrin coated vesicles transport membrane bound proteins and enzymes from the Golgi-complex to the	*Cltca* mutant	Zebrafish	• Bended in notochord • Vertebral centra form in shape of	Ellis et al. (2013)

	plasma membrane. These vesicles also transport extracellular signals to the Golgi complex or to other intra-cellular vesicles.			bended notochord • Vacuoles in the chordocytes collapse	
Caveolae	Plasma membrane pits of 60-80 nm which are 'cup'-shaped important in cellular signalling pathways (extra-cellular to intra-cellular signalling), endocytosis, cellular attachment and detachment, fatty acid regulation of the plasma membrane, mechanosensing and mechano-protection of cells. Caveolae are present in adipose cells and muscle cells. Highest number of Caveolae was recorded in zebrafish chordocytes. Components are Caveolin 1, 2, 3 and Cavin-1.	*Caveolin 1* (*Cav1*) *Caveolin 3* (*Cav3*) *Cavin-1b* mutants	Zebrafish	• Shorter fish • Compromised swimming Upon mechanical stress • Notochord lesions where most mechanical stress • Reduction in number of caveolae • Aberrant morphology of caveolae • Delamination of chordocyte • Collapse and fragmentation of vacuole • Cell rupture • Release of dense material in the ECM	Bastiani & Parton (2010) Garcia et al. (2017) Lim et al. (2017)

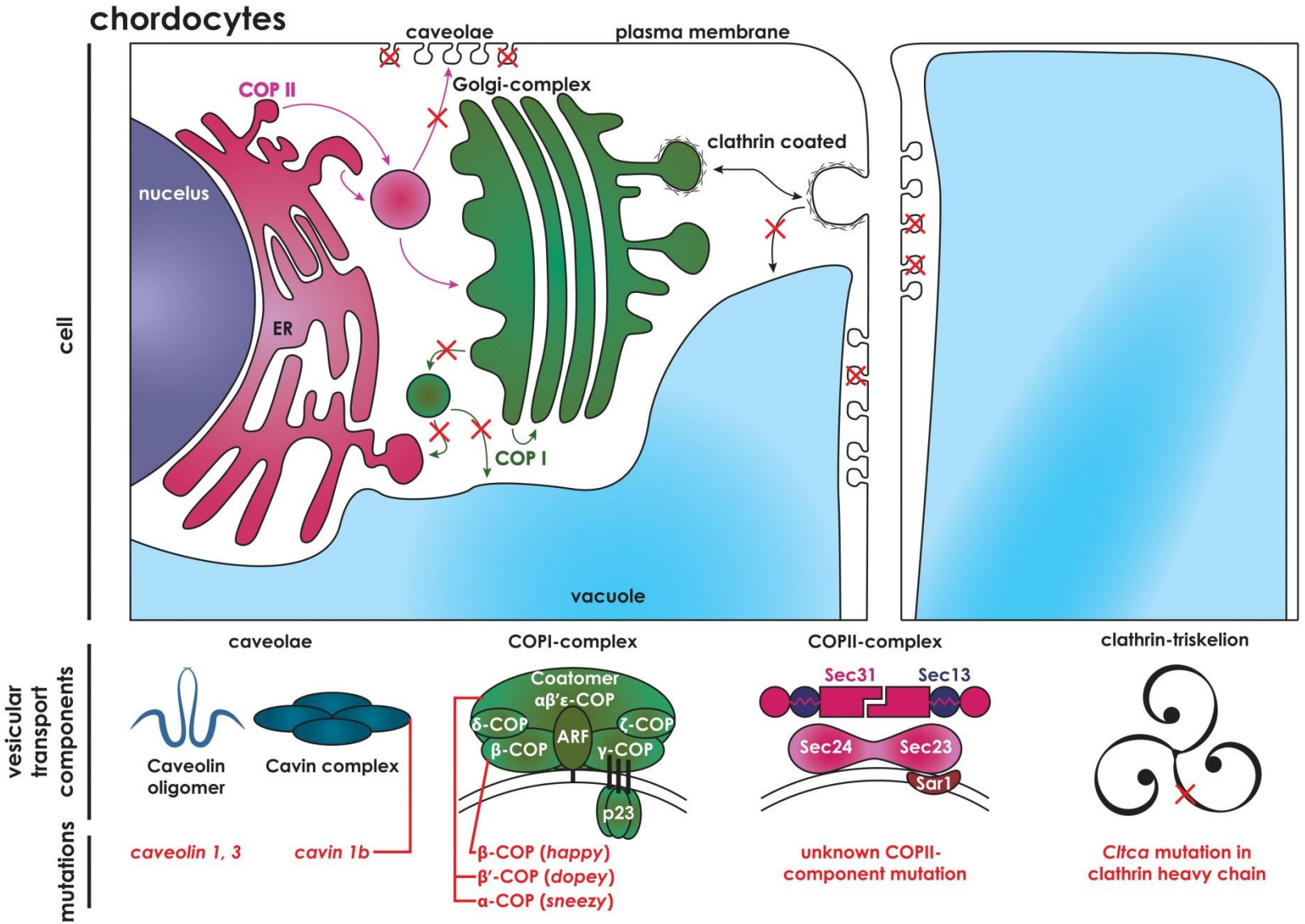
chordocytes
caveolae
plasma membrane
COP II
Golgi-complex
clathrin coated
nucelus
ER
COP I
vacuole
cell
caveolae
COPI-complex
COPII-complex
clathrin-triskelion
vesicular transport components
Caveolin oligomer
Cavin complex
Coatomer αβ'ε-COP
δ-COP
ζ-COP
β-COP
ARF
γ-COP
p23
Sec31
Sec13
Sec24
Sec23
Sar1
mutations
caveolin 1, 3
cavin 1b
β-COP (happy)
β'-COP (dopey)
α-COP (sneezy)
unknown COPII-component mutation
Cltca mutation in clathrin heavy chain

Figure 33: Mutations in cellular vesicular transport mechanisms in chordocytes

Schematic representation of two adjacent chordocytes (only part of the cell is shown). The top row shows the cells, the middle row the vesicular transport components and the bottom row shows the mutations that occur in the respective vesicular transport components (text in red). Within the cell, from left to right, the nucleus, the endoplasmic reticulum (ER), the Golgi-complex and a clathrin coated pit are shown. In the bottom part of the cell the vacuole (blue) is shown. The second cell only shows a vacuole, since the largest part of the chordocyte cell is filled with a vacuole. Caveolae pits are represented in the cell plasma membrane. The COP II dependent transport is indicated by purple arrows, while COP I dependent transport is indicated by green arrows. Clathrin coated vesicle transport is indicated by black arrows. Red crosses show which pathways or components (caveolae) fail when a mutation occurs. A side view of all transport vesicle components is shown, except for the clathrin-triskelion which represents a top view. Caveolae, (dark blue), COP I , COPII (purple) and Clathrin (black) components are adapted from Bastiani and Parton (2010), Nickel et al. (2002), Gomez-Navarro and Miller (2016), Edeling et al. (2006), Robinson (2015) respectively. The two bent black lines underneath the COP I and COP II components represents the plasma membrane phospholipid bilayer of the transport vesicle. The red cross on the clathrin-triskelion shows where the *Cltca* mutation forms a truncated protein (Edeling et al., 2006).

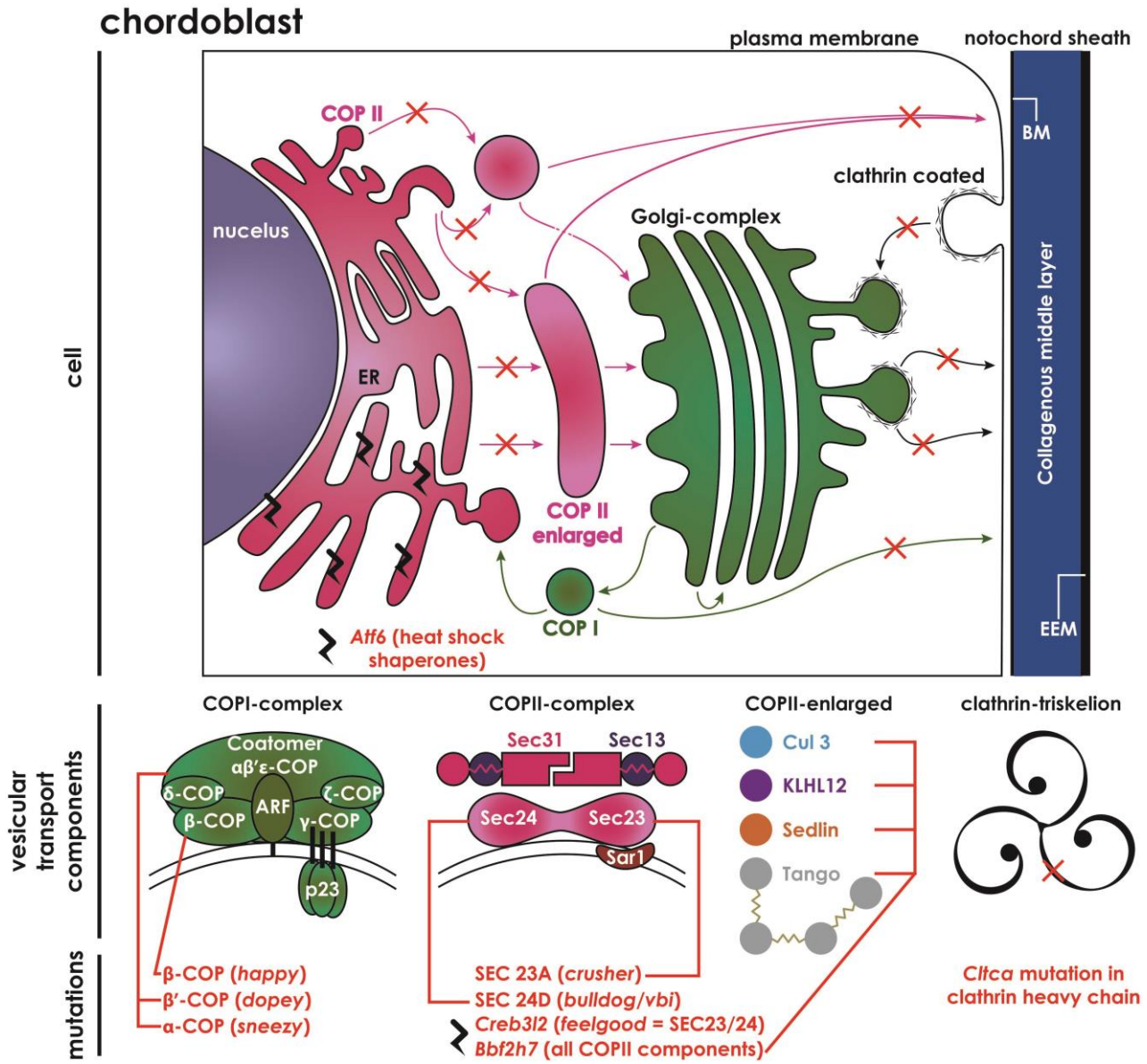
chordoblast
plasma membrane
notochord sheath
cell
COP II
nucelus
ER
clathrin coated
Golgi-complex
BM
Collagenous middle layer
COP II
enlarged
COP I
Atf6 (heat shock
shaperones)
EEM
vesicular
transport
components
COPI-complex
COPII-complex
COPII-enlarged
clathrin-triskelion
Coatomer
αβ'ε-COP
δ-COP
ARF
ζ-COP
β-COP
γ-COP
p23
Sec31
Sec13
Sec24
Sec23
Sar1
Cul 3
KLHL12
Sedlin
Tango
mutations
β-COP (happy)
β'-COP (dopey)
α-COP (sneezy)
SEC 23A (crusher)
SEC 24D (bulldog/vbi)
Creb3l2 (feelgood = SEC23/24)
Bbf2h7 (all COPII components)
Cltca mutation in
clathrin heavy chain

Figure 34: Mutations in cellular vesicular transport mechanisms in chordoblasts

Schematic representation of a chordoblast adjacent to the notochord sheath (only part of the cell is shown). The top row shows the cell, the middle row the vesicular transport components and the bottom row shows the mutations that occur in the respective vesicular transport components (text in red). Within the cell, from left to right, the nucleus, the endoplasmic reticulum (ER), the Golgi-complex and a clathrin coated pit are shown. To the far right the notochord sheath is shown, with the basement membrane directly opposed to the cell plasma membrane, the collagenous middle layer and the external elastic membrane (EEM) on the outside of the notochord sheath. The COP II dependent transport is indicated by purple arrows, while COP I dependent transport is indicated by green arrows. Clathrin coated vesicle transport is indicated by black arrows. Red crosses show which pathways fail when a mutation occurs. Black lightning bolts indicate where in the ER the unfolded protein response (UPR) is elicited by ER-stress. A side view of all transport vesicle components is shown. COP I , COPII (purple), COP II-enlarged (purple) and Clathrin (black) components are adapted from Nickel et al. (2002), Gomez-Navarro and Miller (2016), Ishikawa et al. (2017), Edeling et al. (2006) and Robinson (2015) respectively. The two bent black lines underneath the COP I and COP II components represents the plasma membrane phospholipid bilayer of the transport vesicle. The black bolt next to *Creb3l2* and *Bbf2h7* indicates that these mutations are involved in the unfolded protein response. The red cross on the clathrin-triskelion shows where the *Cltca* mutation forms a truncated protein (Edeling et al., 2006).

APPENDIX G: DRC 16 – V3 STATEMENT OF CONTRIBUTION

DRC 16

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STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Adelbert De Clercq

Name/Title of Principal Supervisor: Matthew Perrott / Snr. Lecturer in Histopathology

Name of Published Research Output and full reference:

De Clercq A, Perrott MR, Davie PS, Preece MA, Huysseune A, Witten PE (2017) The external phenotype-skeleton link in post-hatch farmed Chinook salmon (Oncorhynchus tshawytscha). J Fish Dis: In press

In which Chapter is the Published Work: Chapter 3

Please indicate either:

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  and / or

- Describe the contribution that the candidate has made to the Published Work:

  A De Clercq: conceptualisation, methodology, investigation, writing - original draft preparation, and visualisation.

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30/11/2017
Date

MRFPerrott
Principal Supervisor's signature

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Name/Title of Principal Supervisor: Matthew Perrott / Snr Lecturer in Histopathology

Name of Published Research Output and full reference:

De Clercq A, Perrott MR, Davie PS, Preece MA, Wybourne B, Ruff N, Huysseune A, Witten PE (2017) Vertebral column regionalisation in Chinook salmon, Oncorhynchus tshawytscha. J Anat 231: 500-514

In which Chapter is the Published Work: Chapter 4

Please indicate either:

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  and / or

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30/11/2017
Date

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Principal Supervisor's signature

03/12/2017
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Name/Title of Principal Supervisor: Matthew Perrott / Snr Lecturer in Histopathology

Name of Published Research Output and full reference:
De Clercq A, Perrott MR, Davie PS, Preece MA, Owen MAG, Huysseune A, Witten PE (2018) Temperature sensitive regions of the Chinook salmon vertebral column: Vestiges and meristic variation. J Morphol, DOI: 10.1002/jmor.20871

In which Chapter is the Published Work: Chapter 5

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Candidate's Signature — Date: 07/08/2018

MRFPerrott
Principal Supervisor's signature — Date: 10/09/2018

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