



McLennan, Darryl (2016) *Life history & environmental effects on telomere dynamics in Atlantic salmon*. PhD thesis.

<http://theses.gla.ac.uk/7833/>

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Glasgow Theses Service

<http://theses.gla.ac.uk/>

theses@gla.ac.uk

LIFE HISTORY & ENVIRONMENTAL EFFECTS ON TELOMERE DYNAMICS IN ATLANTIC SALMON



Darryl McLennan

Zoology BSc (Hons), University of Glasgow

Submitted in fulfilment of the requirements for the degree of Doctor of
Philosophy

Institute of Biodiversity, Animal Health and Comparative Medicine
College of Medical, Veterinary and Life Sciences
University of Glasgow

ABSTRACT

While much of the study of molecular biology inevitably focuses on the parts of the genome that contain active genes, there are also non-coding regions that nonetheless play an essential role in maintaining genome integrity. One such region are telomeres, which cap the ends of all eukaryotic chromosomes and play an important role in chromosome protection. Telomere loss occurs at each cell division as a result of the ‘end replication problem’ and a relatively short telomere length is indicative of poor biological state.

Thus far, the majority of studies on the dynamics and role of telomeres have been biased towards certain taxa. Research to date has mostly focussed on humans, other mammals and birds. There has been far less research on the telomere dynamics of ectotherms. It is important that we do so, especially since ectothermic vertebrates do not seem to down-regulate telomerase expression in the same way as endotherms, suggesting that their telomere dynamics may be less predictable in the later life stages.

The main objective of this thesis was therefore to investigate how life history and environmental effects may influence telomere dynamics in Atlantic salmon *Salmo salar*. I carried out carefully designed experiments, both in the laboratory and in the wild, using a longitudinal approach where possible, in order to address a number of specific questions that are connected to this central theme.

In chapter 2, I demonstrate that there can be significant links between parental life history and offspring telomere dynamics. Maternal life history traits, in particular egg size, were most strongly related to offspring telomere length at the embryonic stages. Paternal life history traits, such as early life growth rate, had a greater association with offspring telomere dynamics in the later stages of development.

In chapter 3, using a wild Atlantic salmon population, I found that most individuals experienced a reduction in telomere length during the migratory phase of their life cycle; however the relative rate of telomere loss was dependent on sex, with males experiencing a relatively greater loss. Unexpectedly, I also found that juvenile salmon that had the shortest telomeres at the time of outward migration, had the greatest probability of surviving through to the return migration.

In chapter 4, again using a wild system involving experimental manipulations of juvenile Atlantic salmon in Scottish streams, I found that telomere length in juvenile fish was influenced by parental traits and by direct environmental effects. Faster-growing fish had shorter telomeres and there was a greater cost (in terms of reduced telomere length) if the growth occurred in a harsher environment. I also found a positive association between offspring telomere length and the growth history of their fathers (but not mothers), represented by the number of years that fathers had spent at sea.

Chapter 5 explored the hypotheses that oxidative DNA damage, catalase (CAT) antioxidant activity and cell proliferation rate are underlying mechanisms linking incubation temperature and telomere dynamics in salmon embryos. No evidence was found for any such effects, but telomere lengths in salmon embryos were found to be significantly affected by the temperature of the water in which they were living. There is also evidence that telomere length significantly increases during embryonic development.

In summary, this thesis has shown that a complex mix of environmental and parental effects appear to influence telomere dynamics in Atlantic salmon, with parental effects especially evident during early life stages. It also demonstrated that telomeres lengthen through the embryo stages of development before reducing once the fry begin feeding, indicating that the patterns of telomere loss commonly found in endotherms may differ in ectotherms. Reasons for this variation in telomere dynamics are presented in the final Discussion chapter of the thesis.

TABLE OF CONTENTS

Abstract	2
Table of Contents	4
Acknowledgements	5
Author's Declaration	7
Chapter 1. General Introduction	
1.1. Telomeres	8
1.2. Study species: the Atlantic salmon	14
1.3. Study site: the River Conon	17
1.4. Aims of the thesis	19
Chapter 2. The association between parental life history and offspring telomere dynamics in Atlantic salmon	
2.1. Abstract	21
2.2. Introduction	21
2.3. Methods.....	24
2.4. Results	33
2.5. Discussion	37
Chapter 3. The association between telomere dynamics, timing of seaward migration and the probability of marine survival in Atlantic salmon: a longitudinal study	
3.1. Abstract	42
3.2. Introduction	42
3.3. Methods.....	46
3.4. Results	53
3.5. Discussion	57
Chapter 4. Interactions between parental traits, environmental harshness and growth rate in determining telomere length in wild juvenile salmon	
4.1. Abstract	62
4.2. Introduction	62
4.3. Methods.....	66
4.4. Results	79
4.5. Discussion	85
Chapter 5. Cell proliferation and oxidative stress as possible mechanisms underlying incubation temperature effects on embryonic telomere dynamics in Atlantic salmon	
5.1. Abstract	91
5.2. Introduction	92
5.3. Methods.....	95
5.4. Results	109
5.5. Discussion	112
Chapter 6. General Discussion	
6.1. Considering my data chapters collectively	117
6.2. Project limitations	125
6.3. Future directions	129
Reference List	133

ACKNOWLEDGEMENTS

The majority of my thanks must go to my supervisors - Neil Metcalfe, Pat Monaghan, Winnie Boner, Simon McKelvey and John Armstrong. I am incredibly lucky to have had such an expert group of supervisors. Thank you for the informal manner in which you supported me, and for giving me such a vast amount of your time. I have come to learn that a good student-supervisor relationship should not be taken for granted, and as such, I am extremely thankful that each of you has shown me nothing but kindness and support. I will miss you all dearly when I leave. I must also thank my assessor Jane Robinson. Your external perspective has been extremely helpful throughout my PhD. Though not officially appointed, Lynn Brydon, along with Simon McKelvey, has been a huge support during the field seasons. I spent a total of 6 months in the field; during which you welcomed me into your home and made me a part of your family. I will always be grateful.

Everyone at the Cromarty Fisheries Trust was extremely helpful in all aspects of my fieldwork. Special thanks to Ed Rush for keeping me on my toes. I would also like to thank Natalie Young for the practical and dedicated support during the 6 weeks of PIT tagging in 2013. You have the special ability of turning a relatively mundane situation into something much more hilarious – I will always appreciate that. I would also like to extend a big thanks to everyone that assisted with the electrofishing experiment in 2014 – Keith Dean, Danielle Orrell, Ian Moodie, Matt Drysdale and Jason Henry. On the subject of field work, I would like to say a special thanks to Tim Burton. You took me under your ‘field’ wing from the very beginning, and were more help than you can ever know. Fieldwork probably shouldn’t have been that much fun. However, I’m extremely grateful that it was.

Special thanks to Dave Stewart from the Marine Scotland Freshwater Laboratory. Your expertise was invaluable to each of my data chapters; and your cooking skills are second to none. Thanks also to Mark Fordyce and Silvia Soares from the Marine Scotland Marine Laboratory. You turned the daunting task of immunohistochemistry analysis into something efficient and highly achievable.

Graham Law, Graeme Anderson, Ross Philips and Alastair Kirk - your help and assistance in Aquarium Room 6 was much appreciated. Also, thank you for just generally being great guys. I also owe a tremendous amount to Lab 316. To name just a few – Kate Griffiths, Robert Gillespie, Valeria Marasco, Sophie Reichert and Bart Adriaenssens. You have all been extremely helpful; from setting up the qPCR assay in the very beginning, to teaching me the wonders of qBASE at the very end. Also, thank you your patience with my Smooth Radio addiction. I’ll greatly miss working with you all.

I would like to say a special thanks to all of my office mates, both past and present from room 424 – Tim Burton, Katherine Herborn, Jose Noguera, Karine Salin, Sonya Auer, Ron Bassar and Antoine Stier. Apologies for being the annoying student in the corner (albeit the nice corner with the window). All of your knowledge, help and support was much appreciated; especially since I could not repay the favour for the most part. The mid-week beers were also extremely helpful.

In general, I would like to thank everyone in the Graham Kerr building. The friendly and supportive nature of the building is no secret, but definitely bears mentioning again. So many people have helped and supported me throughout this project. Special thanks to Aileen Adam, Elizabeth Kilbride, George Gow, Lorna Kennedy, Florence McGarrity and John Laurie. I have also been fortunate to make some great friends over the last four years, and it is largely due to them that I have enjoyed my PhD as much as I have. To name just a few – Alan Reynolds, Hans Recknagel, Hannah Trewby, James Buckley, Jess Duprez, Julie Nati and Laura Bergner – it's been amazing, thanks!

Lastly, I'd like to say a special thank you to all friends and family that have supported me through the last four years; in particular to my Mum, Dad and Uzma for bearing the brunt of my 'secret' grumpy side. I might not have said it enough, but you've all been great.

AUTHOR'S DECLARATION

The material presented in this thesis is the result of research conducted between October 2012 and September 2016. Throughout this time, I was under the supervision of Neil B Mectalfe, Pat Monaghan, Winnie Boner, John D Armstrong and Simon Mckelvey. This work has not been submitted as part of any other degree and is based, for the most part, on individual research carried out by myself.

In chapter 4, parental assignment by microsatellite analysis was conducted by Landcatch Natural Selection Ltd (Stirling, Scotland). In chapter 5, Immunohistochemistry was performed by the University of Glasgow Veterinary Diagnostic Services. Lastly, for each of the data chapters, scalimetry (where the circuli of the fish scale is analysed and used to determine age) was performed by Dave Stewart (Marine Scotland – Science, Freshwater Laboratory, Scotland).

Darryl McLennan

Chapter 1. General Introduction

1.1 TELOMERES

While much of the study of molecular biology inevitably focuses on the parts of the genome that contain active genes, there are also non-coding regions that nonetheless play an essential role in maintaining genomic integrity. One such region are the telomeres, which cap the ends of all eukaryotic chromosomes and play an important role in chromosome protection (for reviews see Blackburn 1991; Campisi *et al.* 2001; Haussmann & Vleck 2002; Monaghan 2010). The telomere ends are made up of the repetitive DNA sequence TTAGGG. The very ends of telomeres consist of a single stranded overhang, which folds back on itself to form a loop. It is this loop, along with associated shelterin proteins, that forms the protective telomere cap (Haussmann & Marchetto 2010). Telomeres play an essential role in chromosome maintenance and integrity in two ways. Firstly, they help differentiate between natural chromosome ends and double stranded DNA breaks (Haussmann & Marchetto 2010; Monaghan 2010). Secondly, they help to protect the central coding region of the chromosome from the ‘end replication problem’. This refers to the fact that DNA polymerase can only add nucleotides to a 3'-OH group, therefore, DNA synthesis can only proceed in a 5' to 3' direction. Because of this, the lagging strand of template DNA is unsuitable for continuous polymerisation and the new strand must be replicated by Okazaki fragments. Unlike in continuous polymerisation, Okazaki fragments are unable to fully replicate the lagging template strand. Therefore, each time a cell divides, the length of each chromosome decreases by a small number of bases (Haussmann & Marchetto 2010). Losing bases from the coding region of the chromosome (i.e. genomic DNA) could have disastrous phenotypic consequences. However, because telomere caps exist at the end of chromosomes, it is telomeric DNA, rather than genomic DNA which is lost. As a result, if no telomere restoration processes occur, telomeres will shorten at each cell division. After numerous rounds of cell division, telomeres may reach a critical length, when the telomere is too short to allow proper T-loop formation. At this stage, the cell may go into a state of senescence, or perhaps even die (Blackburn 2000).

Relatively short telomeres are indicative of cells in a relatively poor biological state, which may be linked to reduced longevity of the organism as a result of age-related deterioration and/or increased disease susceptibility (e.g. Ilmonen, Kotrschal & Penn 2008;

Bize *et al.* 2009; Heidinger *et al.* 2012; Stier *et al.* 2015; Fairlie *et al.* 2016). For example, Cawthon *et al.* (2003) reported that humans over the age of 60 with relatively short telomeres also had the poorest rate of survival. In addition, individuals with the shortest telomeres also had an increased mortality rate (> 3 x greater) from heart disease and an increased mortality rate from infectious disease (8 x greater), compared to individuals with the longest telomeres (Cawthon *et al.* 2003). In fact, studies in humans have reported links between short telomeres and a number of different diseases (Aubert & Lansdorp 2008; Haussmann & Treidel 2014). Using mice in a controlled laboratory setting, Ilmonen, Kotrschal and Penn (2008) found that individuals with longer telomeres were better able to recover from *Salmonella enterica* infection, suggesting that telomere length is not only an important factor in disease susceptibility, but also disease recovery. In one of the first studies to link telomere dynamics and survival in a wild population, Haussmann, Winkler and Vleck (2005) showed that 1 year old tree swallows *Tachycineta bicolor* with shorter telomeres had a reduced probability of annual survival, measured as returning to the same breeding site the following year. Subsequently, Angelier *et al.* (2013) reported that American redstarts *Setophaga ruticilla* with longer telomere lengths prior to their annual migration had a greater chance of returning from that migration. Studies such as these suggest that telomere length may be a good biological indicator in which to study individual fitness and probability of survival.

There is still much ongoing discussion as to whether the relationship between telomere length and individual fitness (e.g. lifespan and disease susceptibility) is causal or correlative (Simons 2015). It is known that senescent cells can have altered secretory profiles and may secrete more inflammatory compounds (Rodier *et al.* 2009). Thus, a combination of cell loss and an accumulation of senescent cells (as can be caused by telomere shortening) can contribute to the ageing process (Aubert & Lansdorp 2008; Monaghan 2014), and the ageing of the immune system has been linked to human longevity (Larbi *et al.* 2008). The underlying mechanisms linking telomere length and survival have been virtually untested out with human studies, however, Asghar *et al.* (2015b) studied the great reed warbler *Acrocephalus arundinaceus* and found that malaria-infected individuals experienced a greater rate of telomere attrition, as well as reduced lifespan. Similarly, Beirne *et al.* (2014) reported an age-related decline in a wild badger *Meles meles* population and found that individuals in the most advanced stages of bovine tuberculosis infection had relatively shorter telomeres.

There are several telomere restoration mechanisms whereby telomeres can be elongated. The enzyme telomerase is thought to be the most common means by which shortened telomere ends are restored (Hausmann & Marchetto 2010; Monaghan 2010). Studies - mostly in mammals thus far - indicate that telomere elongation occurs in the early stages of embryogenesis (Schaetzlein *et al.* 2004; Liu *et al.* 2007; Kalmbach *et al.* 2014). This is thought to be by alternative lengthening of telomere (ALT) pathways, as opposed to telomerase activity since it also occurs in telomerase-null mice (Kalmbach *et al.* 2014). Instead, upregulation of telomerase is thought to occur at the later stages of embryogenesis (Taylor & Delany 2000; Liu *et al.* 2007; Kalmbach *et al.* 2014) however, it has been suggested that this is to maintain, rather than elongate telomere length (Liu *et al.* 2007). Many endotherms appear to down-regulate telomerase expression in post-embryonic somatic tissues and instead use replicative ageing as a tumour suppression mechanism (Gomes, Shay & Wright 2010). Therefore, telomeres generally shorten with age, at least in humans, but also in many other mammal and bird species studied to date (Hausmann *et al.* 2003; Henriques & Ferreira 2012) In contrast, telomerase has been found to be expressed in post-embryonic tissue in reptiles, amphibians and fish (Gomes, Shay & Wright 2010), suggesting that ectothermic vertebrates have adopted an alternative strategy for telomere length regulation; and as a result, telomeric attrition with age seems not as general in ectotherms as in endotherms. For instance, Simide *et al.* (2016) found that around half, of the small number, of the fish telomere studies to date have reported either no telomere shortening with age, or even telomere elongation.

Growth rate, longevity & telomeres

There are many benefits to a relatively larger body size: increased reproductive success, evasion from predators etc. (Blanckenhorn 2000). However, individuals rarely achieve their maximum size, which suggests that there are associated costs (Blanckenhorn 2000). When comparing between species, there is generally a positive relationship between body size and longevity (Sacher 1959; Lindstedt & Calder 1976). In contrast, the opposite relationship is true within a species, with smaller individuals tending to live longer (Selman, Nussey & Monaghan 2013). The best known example of this is the domesticated dog. It has long been acknowledged that larger dog breeds tend to have shorter lifespans (Fleming, Creevy & Promislow 2011). There will likely be many underlying mechanisms which contribute towards this relationship. However, it is interesting that Fick *et al.* (2012) found a negative correlation between breed size and telomere length, with larger dog

breeds having shorter telomeres. In humans, there is a clear gender gap in life expectancy. This has often been attributed to lifestyle and socioeconomic effects. However, in recent times women and men have shared a more similar lifestyle, yet this has had no effect on the lifespan gap (Stindl 2004). As a result, there have been a number of suggestions that sexual size dimorphism may play an important role. Stindl (2004) argues that for men to reach a relatively larger size, they must undergo more rounds of cell division, thus leading to greater telomere attrition and reduced lifespan.

In fact, a growing number of studies have now reported a negative relationship between enhanced growth rate or body size and telomere loss (Fick *et al.* 2012; Herborn *et al.* 2014; Noguera *et al.* 2015; Pauliny *et al.* 2015; Ringsby *et al.* 2015). The relationship between growth rate and telomere loss has been demonstrated experimentally, when laboratory rats that have undergone a period of compensatory growth following a low birth weight also have relatively shorter telomeres (Tarry-Adkins *et al.* 2009). Also in the laboratory, Pauliny *et al.* (2015) showed that transgenic coho salmon *Oncorhynchus kisutch*, with an artificially increased growth rate, had a faster rate of telomere loss compared with maternal half sibs that grew at a rate more typical of wild fish. Looking at compensatory growth in the wild, Geiger *et al.* (2012) found that the initially smaller king penguin *Aptenodytes patagonicus* chicks in a cohort were able to grow faster to catch up in size, but did so at the cost of shorter telomeres. Again using a non-domesticated system, Turbill *et al.* (2013) found that actively growing sub-adult doormice *Glis glis* had an accelerated telomere loss rate compared to fully grown adult door mice, suggesting that growth and/or age may both be important determinants of telomere length.

Parental effects on telomere length

A number of studies have also reported a heritable component to telomere length across a range of vertebrates (Nordfjall *et al.* 2005; Njajou *et al.* 2007; Nordfjall *et al.* 2010; Horn *et al.* 2011; Olsson *et al.* 2011a; Broer *et al.* 2013; Eisenberg 2014; Asghar *et al.* 2015a; Reichert *et al.* 2015). These studies have mostly found a positive relationship: parents with relatively longer telomeres at the time of reproduction have tended to produce offspring with relatively longer telomeres. There is inconsistency within these studies as to whether the effect is stronger through the mother or the father; however patterns begin to appear when looking within a specific taxon, e.g. human studies mainly report stronger paternal

inheritance (e.g. Njajou *et al.* 2007; Nordfjall *et al.* 2010), while bird studies mostly report stronger maternal inheritance (e.g. Asghar *et al.* 2015a; Reichert *et al.* 2015).

A relationship has also been demonstrated between parental age at the time of conception and offspring lifespan for a number of organisms, with offspring from older parents often displaying reduced longevity (Gavrilov & Gavrilova 1997; Fox, Bush & Wallin 2003; Garcia-Palomares *et al.* 2009; Bouwhuis, Vedder & Becker 2015). It is therefore interesting that Heidinger *et al.* (2016) found an association between the age of both parents and the rate of early telomere loss in the offspring of wild European shags *Phalacrocorax aristotelis*. One suggestion from the authors is that older parents are less able to provide for offspring, which may alter physiological stress pathways. In contrast, human studies tend to find that offspring telomere length correlates positively with paternal age (e.g. Unryn, Cook & Riabowol 2005; De Meyer *et al.* 2007; Broer *et al.* 2013), and sperm telomere length has been shown to increase with donor age (Allsopp *et al.* 1992).

There are also possible ways in which the environment experienced by the parents may affect offspring telomere length (i.e. through parental effects). For example, Asghar *et al.* (2015b) studied the great reed warbler, and found that malaria-infected mothers produced offspring with shorter telomeres. There may also be links between a mother's physiological state and the quality of eggs that she produces (Blount *et al.* 2002; Tobler & Sandell 2009). Therefore, a mother may affect telomere dynamics in the early life of her offspring through the composition of her eggs. In support of this hypothesis, Noguera *et al.* (2016) found that ovulation order in zebra finches was negatively correlated with offspring telomere length, since the earlier-laid eggs in a clutch produced offspring with relatively longer telomeres. The authors suggested egg composition as one possible explanation for this relationship, since maternally derived antioxidants (which could protect against oxidative damage to telomeres – see next section) are known to decrease with laying order in birds (Royle, Surai & Hartley 2003).

Environmental effects on telomere length

There is also growing evidence that changes in telomere length are strongly influenced by environmental conditions (for review see Monaghan 2014). Growth rate and body size are both under the influence of various environmental factors (Metcalf & Monaghan 2001). This is especially true for ectotherms, such as fish, where fluctuations in environmental temperature can influence myogenic processes, morphological development, growth rate

and metabolism, all of which can have permanent long-term phenotypic effects (Johnston 2006; Jonsson & Jonsson 2011). Therefore, it is possible that the environmentally determined plasticity of growth rate may also have some influence on telomere dynamics. As mentioned, telomere loss occurs at each round of cell division as a result of the ‘end replication problem’, but the amount of loss is also influenced by levels of oxidative damage to the telomeric DNA (von Zglinicki 2002; Chan & Blackburn 2004; Haussmann & Marchetto 2010). This is partially because telomeric DNA has a high guanine content, which is particularly susceptible to oxidative damage and is hard to repair. Therefore, environmental stressors may also affect mean telomere lengths through their influence on the production of ROS within the organism (von Zglinicki 2002; Geiger *et al.* 2012; Kim & Velando 2015).

Environmentally-induced telomere attrition can start as early as embryonic development and has been linked to *in utero* stress (Haussmann *et al.* 2011; Marchetto *et al.* 2016). Marchetto *et al.* (2016) reported that human mothers with an elevated psychosocial stress score gave birth to babies with a relatively shorter telomere length. Similar processes are evident in wild animals: Watson, Bolton and Monaghan (2015) found that European storm petrel *Hydrobates pelagicus* eggs reared in unfavourable environmental conditions produced chicks that experienced significant telomere attrition during early life, while Herborn *et al.* (2014) found that altering stress exposure during early life (by stress hormone administration) increased telomere loss rate in the European shag *Phalacrocorax aristotelis*. Also looking at early life stages, Nettle *et al.* (2015) found that European starling *Sturnus vulgaris* chicks reared with relatively larger brood competitors (and so experiencing social stress) subsequently experienced increased telomere attrition. This effect is not confined to early life; Kotrschal, Ilmonen and Penn (2007) and Sohn *et al.* (2012) both reported a relationship between social crowding and accelerated telomere attrition; both of which involved experimental manipulations involving extreme differences in density (crowded vs non-crowded) at later life stages. Lastly, Olsson *et al.* (2010) reported that the anti-predator process of tail autotomy in sand lizards *Lacerta agilis* came at a detrimental cost to telomere length.

Current issues in telomere research

Thus far, the majority of telomere studies have been biased towards certain taxa. Research to date has mostly focussed on humans, but also birds and other mammal species. There

has been far less research on the telomere dynamics of ectotherms. It is important that we do so, especially since ectothermic vertebrates do not seem to down-regulate telomerase expression in the same way as endotherms (Gomes, Shay & Wright 2010) suggesting that telomere dynamics may be less predictable in the later life stages of ectotherms. In addition, given that there is accumulating evidence that changes in telomere length are strongly influenced by environmental conditions, it is important that we investigate how environmental temperatures affect telomere dynamics in ectotherms, given that fluctuations in temperature can influence their growth rate and metabolic processes, both of which have the potential to affect telomere dynamics. There is also a need for more longitudinal studies (in all taxa) to investigate how telomeres change in length within individuals. This can prove problematic when studying early life stages, since non-invasive sampling is required. It also poses a difficulty in wild populations, since the same individual must be caught and sampled at different time points. However, where possible, longitudinal analysis can help control for intraspecific variation in telomere length, and can rule out the potential complicating factor of the selective disappearance of individuals with short telomeres, both of which are problems for cross-sectional experiments.

In the hope of addressing a number of these issues, the main objective of my thesis is to investigate how life history and environmental effects may influence telomere dynamics in Atlantic salmon *Salmo salar*. More specifically, I have carried out carefully designed experiments, both in the laboratory and in the wild, using a longitudinal approach where possible, in order to address a number of specific questions that are connected to this central theme.

1.2 STUDY SPECIES: THE ATLANTIC SALMON

Atlantic salmon display some of the greatest within-population variation in life history strategies amongst vertebrates (for reviews see Fleming 1996; Klemetsen *et al.* 2003; Jonsson & Jonsson 2011). The species is widely distributed throughout the Atlantic regions of the northern hemisphere; within the UK many southern populations were lost through habitat degradation and over exploitations, but in Scotland salmon are found in nearly all suitable freshwater systems (Maitland 2007). In general, spawning occurs in late autumn in fresh, fast flowing water. Eggs are laid and fertilized in a cavity in the gravel of the stream bed, known as a redd, that is excavated by the female (Fig. 1.1). The embryos hatch ~ 3-4 months later, although the newly emerged alevins stay in the gravel substrate of the redd

until their yolk sac has been absorbed. They emerge from the redd as fry, usually in spring, at which stage they start feeding. At ~1 year old, they become parr and develop parr markings to help camouflage them from predators. Juveniles may spend up to 6 years in fresh water before transforming into the smolt stage and migrating to sea; however this period of freshwater residence tends to be 2-3 years in Scottish rivers (Maitland 2007). There are a number of factors affecting this variation in fresh water age. For example, it is generally accepted that growth rate and size are important factors determining when a salmon migrates to sea (Metcalf & Thorpe 1990; Økland *et al.* 1993). Sea migration allows better feeding opportunities and therefore greater growth and reproductive potential. However, there are also associated costs, such as an increased predation risk. Therefore, to maximise survival, an individual should migrate to sea at a size which best balances these trade-offs. Slower growing individuals will take longer to reach this size threshold and therefore remain in fresh water for longer.

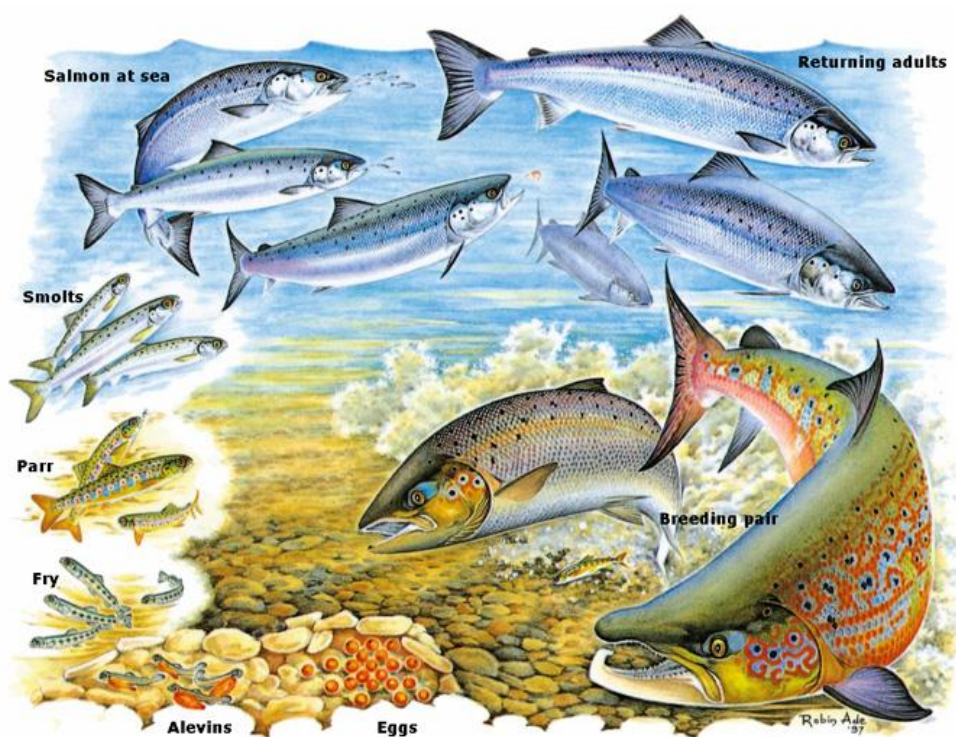


Fig. 1.1. Overview of the Atlantic salmon life cycle. Illustration courtesy of the Atlantic Salmon Trust and Robin Ade.

Atlantic salmon also vary in the number of years they spend at sea (sea age) before returning to their native river to reproduce. Again, there are a number of determinants at play (Gardner 1976; Fleming 1998; Barson *et al.* 2015). Broadly speaking, Scottish salmon populations fall into one of two categories: 1 sea winter (1SW) or multi sea winter (MSW).

1SW fish migrate to the waters surrounding the Faroe Islands and then return to fresh water after one winter in the ocean. MSW fish migrate much further to the waters of south western Greenland and return to fresh water after 2 or more years and tend to be much larger in size (Trust 2012). In Atlantic salmon, there is a correlation between a female's body size and the average size of the eggs that she lays (Fleming 1996), and so MSW mothers, in general, produce significantly larger eggs than 1SW mothers.



Fig. 1.2. Photograph illustrating the extreme phenotypic variation between the different paternal life history strategies present in a single population during the breeding season. The largest individual is a MSW male, intermediate individual is a 1SW male and the smallest individual is a precocious parr. Their relative sizes are indicated by the metre-long measuring board in the background.

It is also possible for the parr stage to become precociously sexually mature, without migrating to sea (Fleming 1996; Baum *et al.* 2004). While precociously mature male salmon parr are found in most river systems, mature female parr are very uncommon (for an example see Hindar & Nordland 1989), most likely due to the greater reproductive investment required for females, which therefore benefit from the increased food resources at sea. The precocious male parr participate in spawning as sneaker males: they hide amongst the stones in the stream bed until the female is ready to deposit her eggs, at which point they dash into the redd to release their sperm alongside that of the much larger migrant males that aggressively compete to be closest to the spawning female (Fleming

1996). Therefore, within a given Scottish Atlantic salmon spawning population it is possible to distinguish, on the basis of size, two distinct maternal reproductive life-history variants (1SW and MSW) and three paternal variants (precocious parr, 1SW and MSW) (Fig. 1.2). However, within each of these life-history classes (e.g. 1SW) individuals will vary in the number of years spent in fresh water (and, therefore, total age).

The total reported catch of Atlantic salmon in the northeast Atlantic area has steadily decreased since the mid-1970s, with the lowest catch to date being reported in 2014 (ICES 2015). Although this may partially reflect a change in exploitation effort, it is also a clear indication of changes in stock size. In addition, the somatic condition of salmon has also decreased, with returning adults having a relatively lower body mass for a given body size than previous years (Todd *et al.* 2008). A number of studies have linked marine survival in Atlantic salmon to conditions experienced at sea (Jonsson & Jonsson 2004; Friedland *et al.* 2009; Trueman, MacKenzie & Palmer 2012). Fewer studies have linked this decline to conditions experienced in fresh water.

The Atlantic salmon is an ideal species in which to study the effects of life history and environmental factors on telomere dynamics. Their variable life history is easily quantified, as the circuli of fish scales can be analysed (referred to as scalimetry) and used to determine fresh water age, sea water age, frequency of spawning (i.e. whether or not the fish has spawned in a previous year) and even relative growth rate (Shearer 1992). This is an invaluable tool when studying life history effects in a wild system. Because salmon return to their native river system to reproduce, it is possible to catch the same individual before and after they have migrated to sea, providing an opportunity for longitudinal studies. Finally, the Atlantic salmon reproduces by external fertilisation and gametes can be easily extracted, and in large quantities. This provides an ideal system in which to study telomere inheritance, as it allows experimental *in vitro* fertilisation (and so controlled matings) and the simultaneous analysis of telomere lengths in parents, gametes and young.

1.3 STUDY SITE: THE RIVER CONON

The River Conon is located in northern Scotland (57° 60'N, 4°63'W) (Fig. 1.3). The Conon system has been highly impacted by the installation of hydroelectric schemes since the 1950s. As part of a compensation agreement, the Loch na Croic Atlantic salmon fish trap was constructed on the River Blackwater in the late 1950s, embedded in the dam at the upstream end of Loch na Croic. The trap is closed for the majority of the year. Migratory

adult Atlantic salmon return to the River Blackwater during the summer and autumn and congregate in Loch na Croic, downstream of the trap until it is opened in November/December. When the trap is opened, adult fish move upstream into the trap and are captured and stripped of gametes. These gametes are used for stocking purposes in the tributary streams upriver of the hydroelectric structures, which are now inaccessible to returning adult salmon. This stocking is done at the egg stage (no stocking is done of fry or older stages) and is at natural densities, and so the population lives in the wild except for the first few months of egg development.

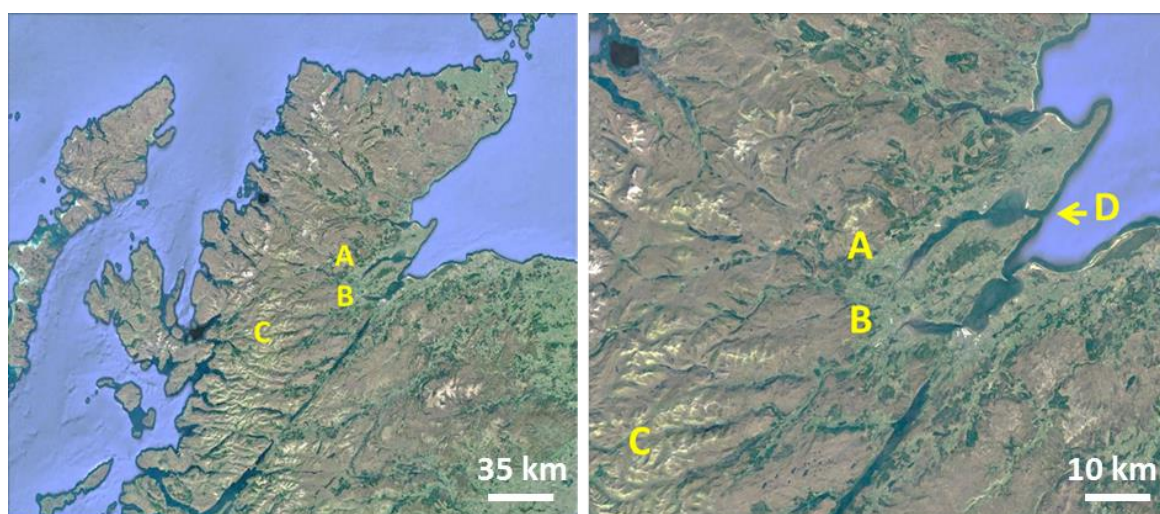


Fig. 1.3. Map outlining the location of the River Conon catchment, with specific reference to (A) Loch na Croic (and the associated fish trap); (B) the Allt Goibhre tributary; (C) the Upper Meig tributary and (D) the entrance to the Cromarty firth, which is the mouth of the River Conon catchment.

In a scientific context, the Loch na Croic trap provides an excellent opportunity to conduct longitudinal experiments in the wild. The mean survival of Scottish smolts returning to home waters (prior to coastal fisheries) is 6.1%, based on data from the last 10 years (ICES 2015), and the trap allows hands-on access to the majority of adult salmon returning to the River Blackwater (once they have passed any coastal fisheries); the annual catch in the trap averages over 1790 individuals per year (\pm s.d. 163; based on data from 1965-2012) with a slight majority being 1SW fish in most years. In addition to the permanent Loch na Croic trap, which is only suitable for capturing adult fish, I also had access to a rotary screw trap, which is ideal for capturing the smolt stage (Fig. 1.1) as they migrate towards the sea. I had access to the nearby broodstock holding facilities, as well as the SSE hatchery facilities in Contin, which provide excellent premises in which to hold adults and eggs respectively (Fig. 1.4).

Within the River Conon catchment, there are a number of tributaries where no Atlantic salmon spawning occurs, partly because it is impossible to artificially stock all tributaries affected by the hydroelectric schemes, but also because of natural barriers such as waterfalls. This provides an excellent opportunity to conduct wild experiments, since eggs can be planted out into these salmon-free streams and recaptured at a later date.



Fig. 1.4. Overview of the River Conon facilities: A) experimental space at the Loch na Croic broodstock facilities; B) SSE hatchery facilities in Contin; C) scales being sampled from a 1SW adult fish for subsequent scalimetry analysis and D) the rotary screw trap in location at Loch na Croic.

1.4 AIMS OF THE THESIS

The overall aim of the thesis is to examine how life history and environmental effects may influence telomere dynamics in Atlantic salmon. The thesis comprises four data chapters (thesis chapters 2-5). **Chapter 2** is based on a laboratory study and evaluates the relationship between parental life history (maternal investment in egg size, maternal/paternal age and years spent in fresh and sea water) and offspring telomere length. **Chapter 3** uses a longitudinal mark and recapture approach to examine whether telomere length is a good predictor of migration survival in a wild Atlantic salmon population. Firstly, I assess whether individuals experience a relative telomere change during the migration phase of their life cycle. Secondly, I assess whether an individual's state (measured in terms of size, weight and telomere length) at the smolt stage (prior to migration) affects their probability of surviving the migration. **Chapter 4** uses

experimental manipulations, both in the laboratory and in the wild, to examine parental and environmental effects on telomere dynamics. I assess: 1) whether faster growth is associated with increased telomere loss and 2) whether the magnitude of telomere loss for a given growth rate is greater in harsher conditions. In addition, I also simultaneously investigate the effect of parental life history and parental telomere length on offspring telomere length in the wild. **Chapter 5** investigates the relationship between incubation temperature and embryonic telomere dynamics in a controlled laboratory setting. In addition, cell proliferation and oxidative stress parameters are investigated as possible underlying mechanisms between temperature and telomere length. Finally, **Chapter 6** is a general discussion of the results from the previous chapters, placing the results in a broader context.

CHAPTER 2. The association between parental life history and offspring telomere dynamics in Atlantic salmon

2.1 ABSTRACT

The importance of parental contributions to offspring fitness is self-evident at a genomic level, but there are also many non-genetic pathways whereby parents can affect offspring development and subsequent performance. The life history strategy that an individual adopts may affect its pattern of investment in offspring and so have important consequences for offspring fitness, although the molecular mechanisms underlying these effects are little understood. However, recent research has highlighted links between telomere dynamics in early life and future viability and longevity, and has also shown that parents may influence offspring telomere lengths. I used an experimental split-brood IVF mating design to show that there can be significant links between parental life history and offspring telomere dynamics in Atlantic salmon. Maternal life history traits, in particular egg size, were most strongly related to offspring telomere length at the embryonic stage, but then became weaker through development, whereas paternal life history traits, such as early life growth rate, had a greater association in the later stages of offspring development. However, offspring telomere length was unrelated to either maternal or paternal age at the time of conception. This study demonstrates both the complexity and the importance of parental factors that can influence telomere dynamics in early life.

2.2 INTRODUCTION

The importance of parental contributions to offspring fitness is self-evident at a genomic level, but there are also many non-genetic pathways whereby parents can affect offspring development and subsequent performance (Rossiter 1996). Many species display non-genetic variation in a number of life history traits, such as age at reproduction; and the life history strategy that an individual adopts may in turn affect its pattern of investment in offspring and so have important consequences for offspring fitness. For example, the relationship between parental age at reproduction and offspring lifespan has been established for a number of organisms, with offspring from older parents often displaying reduced longevity (Gavrilov & Gavrilova 1997; Fox, Bush & Wallin 2003; Garcia-Palomares *et al.* 2009; Bouwhuis, Vedder & Becker 2015). Less is known about how offspring fitness could be influenced by variation in other parental life history traits, such

as parental body size at reproduction; moreover, the molecular mechanisms underlying these effects are little understood. However, recent research has identified the importance of telomeres in patterns of lifetime fitness and longevity (Monaghan 2010). With this in mind, telomere dynamics may prove as a useful indicator in which to further examine the relationship between various parental life history traits and offspring fitness.

Telomeres cap the ends of eukaryotic chromosomes and play an important role in chromosome protection (for reviews see Blackburn 1991; Campisi *et al.* 2001; Monaghan 2010). Telomere loss occurs at each cell division as a result of the ‘end replication problem’, but the amount of loss is also influenced by levels of oxidative damage to the telomeric DNA (Chan & Blackburn 2004), partially because telomeric DNA has a high guanine content, which is particularly susceptible to oxidative damage and is hard to repair. A relatively short telomere length is indicative of a poor biological state, which may be linked to reduced longevity as a result of age-related deterioration and/or increased disease susceptibility (Hausmann, Winkler & Vleck 2005; Aubert & Lansdorp 2008; Ilmonen, Kotrschal & Penn 2008; Heidinger *et al.* 2012; Stier *et al.* 2015). Therefore, an animal’s initial telomere length and subsequent rate of loss are both of potential importance to lifetime fitness and longevity.

There is considerable variation in telomere length, both among and within species. However, the determinants of this variation are still not fully understood. There is increasing evidence that telomere dynamics are partially under the influence of environmental conditions. Since telomeric DNA is susceptible to reactive oxygen species (ROS), environmental conditions that influence oxidative balance may also affect telomere attrition. For example, a number of studies have linked telomere dynamics to environmental stressors, such as *in utero* stress (Hausmann *et al.* 2011; Marchetto *et al.* 2016), disturbance (Herborn *et al.* 2014), social position (Nettle *et al.* 2015) and social crowding (Kotrschal, Ilmonen & Penn 2007; Sohn *et al.* 2012). Environmental effects on telomere dynamics may also be possible through the environmental plasticity of growth rate and body size, since there will likely be associated variation in cell proliferation rate.

There are also possible ways in which the environment experienced by a parent may affect the telomere length of its offspring (i.e. parental effects). For example, there are links between a mother’s physiological state and the quality of her eggs (and hence condition of her offspring) (Blount *et al.* 2002b; Tobler & Sandell 2009), which in turn may influence

the future telomere dynamics of those offspring. In support of this, Noguera *et al.* (2016) found that offspring telomere length in zebra finches was negatively correlated with ovulation order: the first laid eggs in a clutch developed into offspring with a relatively longer telomere length, while offspring from the last laid eggs had the shortest telomeres. This effect may be driven by variation in egg composition, since maternally-derived antioxidants in eggs are known to decrease with laying order in birds (Royle, Surai & Hartley 2003).

A number of studies have also reported a heritable component to telomere length (e.g. Njajou *et al.* 2007; Nordfjall *et al.* 2010; Olsson *et al.* 2011a; Asghar *et al.* 2015a; Reichert *et al.* 2015). These studies have mostly found a positive relationship: parents with relatively longer telomeres at the time of reproduction producing offspring with relatively longer telomeres. There is inconsistency within these studies as to whether the effect is stronger through the mother or the father; however patterns begin to appear when looking within a specific taxon, e.g. human studies mostly report stronger paternal inheritance (e.g. Njajou *et al.* 2007; Nordfjall *et al.* 2010), while bird studies mostly report stronger maternal inheritance (e.g. Asghar *et al.* 2015a; Reichert *et al.* 2015).

Atlantic salmon *Salmo salar* provide the opportunity to experimentally explore various parental effects on telomere dynamics in offspring. Their matings can be controlled using *in vitro* fertilisations (IVF), they produce large egg clutches, have no confounding effects of parental care and display extensive within-population variation in life history strategies, and hence parental state (for reviews see Fleming 1996; Klemetsen *et al.* 2003; Jonsson & Jonsson 2011). Eggs are laid in fresh water, where juveniles can spend up to 6 years (dependent on growth rate) before migrating to sea (Metcalf & Thorpe 1990; Økland *et al.* 1993). They then also vary in the number of years spent at sea (sea age, categorised as 1 sea winter (1SW) or multi sea winter (MSW)) before returning to their native river to reproduce. MSW fish have mostly spent 2 (occasionally 3 or more) years at sea and tend to be much larger in size at the time of spawning than 1SW fish (Trust 2012). There is a correlation between female body size and average egg size (Fleming 1996), therefore MSW mothers, in general, produce significantly larger eggs than 1SW mothers. It is also possible for the males (very rarely females) to become precociously mature as parr (the freshwater juvenile stage), prior to seaward migration. These precocious male parr adopt an alternative reproductive strategy of sneaking matings (Fleming 1996; Baum *et al.* 2004).

Here I evaluate the relationship between parental life history (maternal investment in egg size, maternal/paternal age and years spent in fresh and sea water) and offspring telomere length in Atlantic salmon. By using a split-brood IVF experimental design to create a diversity of parental crosses, and rearing the resulting families under standardised conditions, I demonstrate links between egg size and telomere length of the resulting offspring, as well as associations between both maternal and paternal early life history and offspring telomere dynamics.

2.3 METHODS

In Vitro Fertilisation

In vitro fertilisation (IVF) was conducted between November 28th and 30th 2012. All fish came from the River Blackwater system, northern Scotland (57° 60'N, 4°63'W). Wild anadromous (sea-migrating) 1SW and MSW parent fish were captured at the Loch na Croic fish trap whilst undertaking their return spawning migration. The trap was opened several weeks prior to the commencement of the IVF. Any captured, unripe fish were held at the trap site in dark circular tanks (4m diameter, 1.5m deep), supplied directly with water from the River Blackwater, until they had reached spawning condition (assessed by gently squeezing the abdomen to detect the presence of loose eggs within the body cavity in females, and of running sperm in males). Sexually mature male parr were captured each day of the IVF matings, by electrofishing tributaries of the River Blackwater.

The split-brood IVF design utilised all available parental life history types (Fig. 2.1). In each replication of the mating design, the clutches of two female fish (one 1SW and one MSW) were each divided into three equal portions, then each portion was fertilised with sperm from one of three male fish (one 1SW, one MSW and one precocious male parr) to produce six half-sib families with contrasting parental life histories. This design was replicated 10 times (using new fish each time) to produce 60 half-sib families in total. The anadromous parents were initially assigned to life-history categories on the basis of their body size. Analysis of the circuli of scales collected from the flank of each fish subsequently confirmed this categorisation, and also revealed the time spent in fresh water (FW) prior to seaward migration (which was either 1, 2 or 3 years in all cases).

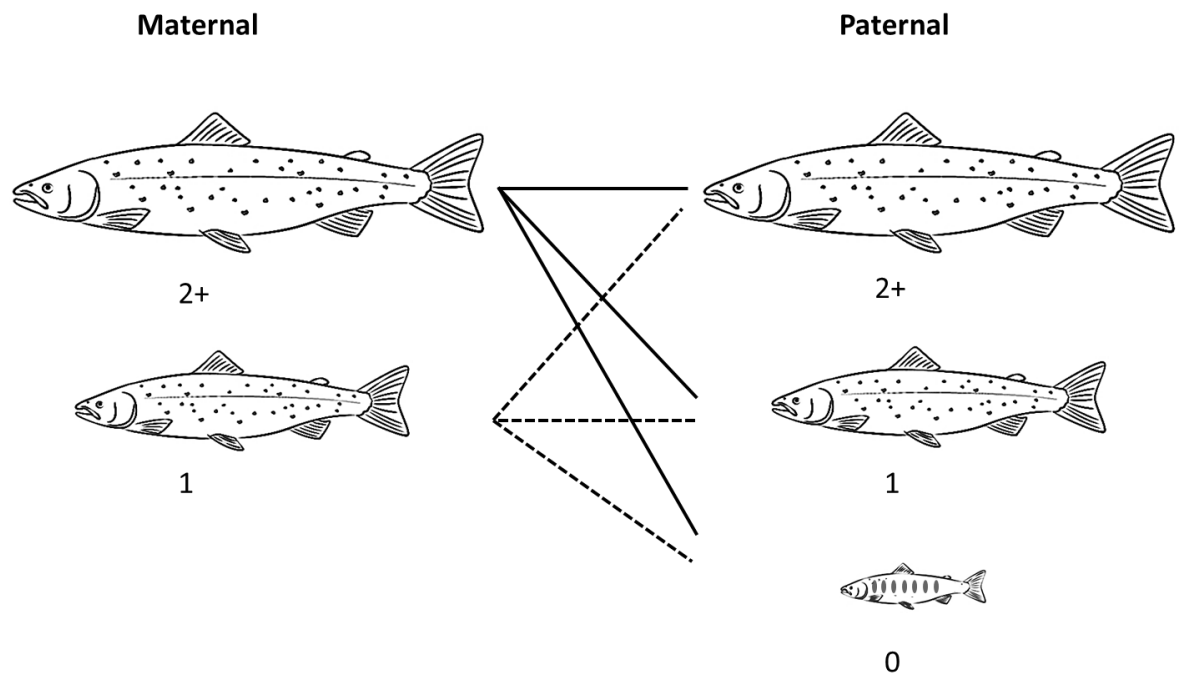


Fig. 2.1. A schematic diagram of the split-brood *in vitro* fertilisation design, utilising all possible parent types with respect to time spent in sea water. 1 = 1 year spent in sea water, 2+ = 2+ years spent in sea water and 0 = parr males that matured in FW and spent 0 years in sea water.

Prior to the stripping of gametes, all parent fish were anaesthetised, using a 5% benzocaine solution. The two female fish in each mating group (1SW and MSW) were blotted dry and manually stripped of their clutch of eggs. The eggs were drained of ovarian fluid and then weighed (to 0.01g) to obtain a clutch mass. Each clutch was then divided into three approximately equal subsets. The three male fish (1SW, MSW and precocious parr) were blotted dry and manually stripped of sperm. The sperm of each male was divided in two, and each half was used to fertilise one subset of eggs from each of the two hens. Each batch of eggs was left for several minutes, to allow sufficient time for fertilisation to occur. Afterwards, the eggs were washed in fresh water to remove any remaining sperm. The fertilised eggs were then placed in fresh water and remained at the field site for at least 1 hour, to promote water hardening of the eggs (the stage at which the eggs absorb water and become firm). After being stripped of eggs/sperm, each parent fish was measured (fork length to 0.5cm; somatic body mass to 0.1 g). A small sample of tissue was taken from the adipose fin of each parent, and flash frozen for subsequent analysis of parental telomere length. In addition, a small sample of sperm was taken from each of the males and flash frozen for subsequent analysis of paternal gamete telomere length. A small sub sample of eggs was also taken for each of the females, with the aim of determining maternal gamete telomere length. However, I was unable to recover sufficient nucleic DNA from the eggs,

most likely because each egg possessed only one nucleus. These egg samples were still used to calculate average egg size per family.

All parent fish used in the crosses were then returned to the river, with adult salmon being returned just above the trap and precocious parr returned to the section of stream from which they had been electrofished (which was not fished in subsequent days to avoid recapturing the same fish). In addition to the samples taken from each parent fish used in my split-brood IVF design, I also sampled supplementary parental tissue from an experiment that was being simultaneously conducted at the same field site (for outline of experiment see Burton *et al.* 2016) For each of the supplementary fish; I recorded body measurements (fork length to 0.5cm; body mass to 0.1 g), fin tissue was sampled for telomere analysis and a sample of scales was taken for subsequent scalimetry analysis. All parent fish from the supplementary experiment were 1SW, but they differed in weight, length, sex and years in fresh water.

Hatchery and Aquarium Conditions

Several hours after fertilisation, eggs were transferred to the nearby SSE hatchery at Contin, Scotland. They remained in separate family groups at the hatchery, under ambient water temperatures ($3.82 \pm \text{s.d. } 0.69^{\circ}\text{C}$) until they had reached the more stable ‘eyed egg’ stage of development (~ 2 months old). During this time, they were checked daily and any dead eggs were removed. Mortality was minimal for all families. On March 5th 2013, when all eggs had passed the eyed stage and so were safe to move, a small sample of eggs (n=50) was counted for each family and transferred to the aquarium facilities at the University of Glasgow. Five eggs per family were immediately sampled and stored in 95-100% ethanol for subsequent telomere analysis of the embryo stage. The remainder were held as separate family groups in 2L compartments of a recirculating stream system, initially at 6°C to match the ambient temperature at the initial hatchery facilities, but then slowly raised to 13°C over the first 30 days, as would occur in the wild.

Eggs hatched into alevins from March 25th – April 3rd. From this point onwards, a small proportion of water was changed daily to maintain water quality within the stream system. The alevins reached the first-feeding stage (when the majority of the yolk sac had been utilised and they began feeding on exogenous food) over the period April 15th-22nd. Fry were then fed to satisfaction several times per day with commercial pelleted food (EWOS Ltd, stage 1). Food was left in each family compartment for 30-60 minutes, after which any

uneaten food was removed. Fry remained in the stream system until 8 weeks post first feeding. At this stage (June 4th 2013) 10 fry per family were euthanized, their fork length and body mass measured, and preserved in 95-100% ethanol for subsequent telomere analysis of the fry stage.

Telomere analysis

For the telomere analysis, DNA was extracted from all tissue types using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturers protocol, with minor modifications to the lysis step for each of the tissue types as follows. For the parental adipose fin tissue, a small sample of tissue was dissected and placed in 180µl Buffer ATL + 20µl proteinase K (20 mg/ml) solution. Samples were incubated at 56°C until the tissue was fully lysed. For the eyed embryo tissue, five embryos per family were dissected from the surrounding lipid mass, pooled and homogenised in 1ml of 1 x PBS in a 2ml tube containing a ceramic bead. 100µl of the homogenate was then added to 80µl Buffer ATL + 20µl proteinase K. Samples were incubated at 56°C until the tissue was fully lysed. For the fry tissue, the adipose fin was carefully dissected from the 10 fry per family, pooled and added to 180µl Buffer ATL + 20µl proteinase K. Samples were incubated at 56°C until the tissue was fully lysed. For the sperm analysis, sperm was diluted 1/200 with sterile PBS solution. 100µl of the diluted sperm solution was added to 100µl of buffer X2 (20mM Tris-Cl pH 8.0, 20mM EDTA, 200mM NaCl, 80mM DTT, 4% SDS, 250µg/ml proteinase K). Samples were incubated at 55°C until tissue was fully lysed. Each set of DNA extractions conducted also included a negative control which contained all of the reagents, but without any tissue. This was used to check for contamination during the lysis and extraction steps. DNA concentration and purity was measured spectrophotometrically using a Nanodrop 8000.

Telomere length was measured in all samples using the quantitative PCR method described by Cawthon (2002). Real-time PCR calculates a cycle threshold (Ct) for each sample: how many cycles it takes for the accumulating fluorescence of that sample to cross a given threshold. There is a linear negative relationship between Ct and the amount of starting DNA, which makes it suitable for calculating relative quantities. The Cawthon method provides a relative measure of telomere length (RTL) and is calculated as a ratio (T/S) of telomere repeat copy number (T) to a control, single copy gene number (S). The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was chosen as the single copy

gene. The Atlantic salmon GAPDH sequence (Genbank accession number: NM_001123561) was used to design primers. Using the Genbank blast tool, the gene was compared to the Atlantic salmon genome to confirm that it was present at a single location. The following forward and reverse GAPDH primers successfully amplified a single amplicon, as determined by melt curve analysis, and were subsequently used for the single copy gene assay:

SalGAP8-F 5'-GTAAGACAGGATTGAGGCATCTC-3' and

SalGAP8-R 5'-CCGAATCCATTGACACCTACTT-3'

For amplification of telomeric repeats, the universal primers designed by Cawthon (2002) were used:

Tel1b 5'-CGGTTTGTGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and

Tel2b 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'

A primer optimisation matrix was used to determine the optimal concentration of the primers and five different samples of Atlantic salmon DNA were tested to confirm primer performance and specificity. Telomere PCR conditions were 15 min at 95°C followed by 27 cycles of 15 secs at 95°C, 30 secs at 58°C and 30 secs at 72°C. This was followed by the melt curve profile. GAPDH PCR conditions were 15 min at 95°C followed by 40 cycles of 15 secs at 95°C, 30 secs at 60°C and 30 secs at 72°C. This was followed by the melt curve profile. PCRs were performed on a Mx3005P qPCR system (Agilent).

The telomere (T) and single copy gene (S) assays were performed on separate 96 well plates, with each sample run in triplicate for each assay. In addition to the samples, each plate also included a six-fold serial dilution of a reference sample (1.25 - 40 ng/well), a 'golden reference' sample and a non-target control (NTC). The DNA for the serial dilution was a pool of 60 samples drawn from all life stages (embryo, fry and adult). The serial dilution was used to generate a standard curve and calculate assay efficiencies. The 'golden standard' was a pool of DNA from 20 samples and included all life stages and was used as a reference sample. The NTC contained all reaction components apart from DNA and were included on each plate (in triplicate) to check for non-specific binding and potential contamination between sample wells. Each reaction contained 12.5µl 2x Absolute Blue

qPCR SYBR Green Mix low ROX (Fisher Scientific), forward and reverse primers and DNA (wells containing sample, standards, gold reference) or water (wells containing NTC) in a total volume of 25 μ l. Both T and S assays were performed using 10ng of DNA (equivalent to 6 μ l of the diluted samples). Primer concentrations were 500nM for the telomere assay (Tel1b and Tel2b) and 200nM for the GAPDH assay (salGAP8-F and salGAP8-R). The mean assay efficiencies for the telomere and GAPDH were 101.0 and 100.7 respectively, well within the acceptable range (85-115). The average intraplate variation of the Ct values was 1.04 for the telomere assay and 0.81 for the GAPDH assay. The average interplate variation of the Ct values was 1.85 for the telomere assay and 0.90 for the GAPDH assay. qPCR raw data was analysed using qBASE software for windows (Hellemans et al., 2007). I used the software to control for differences in amplification efficiency between plates (assessed from the standard curve of each plate). This controlled for differences in amplification efficiency between plates (assessed from the standard curve of each plate) and produced a relative quantity (RQ) of T and S for each sample. Results were scaled to the average across all unknown samples for each target (i.e. telomere and GAPDH). In addition, by including three inter-run calibrators (the gold reference and two points from the standard curve) I corrected for further inter-run variation. Finally, I used the software to normalise each telomere RQ by the GAPDH RQ for that sample. Therefore, for each sample, the qBASE software produced a calibrated normalized relative quantity (CNRQ). This is similar to the T/S ratio described by Cawthon (2002) but with greater control of inter-plate variation.

Data analysis

I measured/calculated the following four variables, which were used as dependent variables in the analyses; note that in all cases a mean value per half-sib family was used: 1 - parental relative telomere length (subsequently referred to as *parental RTL*), measured at the time of spawning, 2 – embryonic relative telomere length (*embryo RTL*), measured at the eyed egg stage, 3 – fry relative telomere length (*fry RTL*), measured 8 weeks after first feeding and 4 – relative telomere change, calculated as the difference between a family's mean embryo RTL and its mean fry RTL (*RTL change*).

I used Pearson correlation coefficient matrices to assess potential collinearity between explanatory variables (with problematic collinearity being defined as a coefficient > 0.8). Fry weight and fry length were highly collinear (Pearson $r = 0.95$, $p < 0.001$); therefore

only fry weight was used in analyses. Paternal total age was not independent from the number of years each father had spent in FW (Pearson $r = 0.83$, $p < 0.001$) and at sea (Pearson $r = 0.90$, $p < 0.001$), and so for each dependent variable I ran two models; one using total ages of each parent and another separating these total ages into years spent in fresh and sea water.

Factors affecting variation in parental RTL (using the supplementary parent data in addition to the parent data from the split-brood IVF experiment) was assessed by general linear models (GLM, $n=92$) which included sex, weight and age (either total age or separated years spent in FW and SW). Variation in embryo RTL, fry RTL and RTL change was assessed by linear mixed models (LME, $n=60$) that included maternal ID and paternal ID as random factors to control for non-independence of half siblings, along with the following independent variables, where appropriate: maternal and paternal years in FW and SW (or total age, in the alternative models), maternal relative telomere length (maternal RTL), paternal relative telomere length (paternal RTL), sperm relative telomere length for each father (sperm RTL), average egg weight for each family (egg weight) and average 8-week-old fry weight for each family (fry weight).

Details of the full LME models containing all considered main effects and interactions are given in Table 2.1. The models were then simplified using the Akaike Information Criterion (AIC), with variables being removed from a model if this resulted in a reduction of the AIC score. Because the offspring model involved repeated measures of telomere length, the relationship between embryo RTL and offspring RTL loss could be confounded by a statistical phenomenon known as regression to the mean (RTM) (Barnett, van der Pols & Dobson 2005; Verhulst *et al.* 2013). To overcome this, an initial model was run, using offspring RTL loss data which had been RTM-corrected; see Verhulst *et al.* (2013) for calculations. Embryo RTL did not significantly contribute to this model. Therefore, I was confident in using the conventional telomere loss data (fry RTL – embryo RTL) as a dependent in the final model, but excluding embryo RTL as a covariate. All statistical analyses were carried out using IBM SPSS 22 for Windows.

Table 2.1. Summary of the six full linear mixed-effects models explaining variation in: embryo telomere length, fry telomere length and telomere change (between embryo and fry stage).

Model	Fixed factors	Covariates	Interactions	Random factors
Embryo RTL 1		Maternal age Paternal age Maternal RTL Paternal RTL Sperm RTL Egg weight	Maternal age x paternal age	Mother ID Father ID
Embryo RTL 2	Maternal years in FW Maternal years in SW Paternal years in FW Paternal years in SW	Maternal RTL Paternal RTL Sperm RTL Egg weight	All possible 2-way interactions between maternal and paternal years in FW and SW	Mother ID Father ID
Fry RTL 1		Maternal age Paternal age Maternal RTL Paternal RTL Sperm RTL Embryo RTL Egg weight Fry weight	Maternal age x paternal age	Mother ID Father ID
Fry RTL 2	Maternal years in FW Maternal years in SW Paternal years in FW Paternal years in SW	Maternal RTL Paternal RTL Sperm RTL Embryo RTL Egg weight Fry weight	All possible 2-way interactions between maternal and paternal years in FW and SW	Mother ID Father ID
RTL Change 1		Maternal age Paternal age Maternal RTL Paternal RTL Sperm RTL Egg weight Fry weight	Maternal age x paternal age	Mother ID Father ID
RTL Change 2	Maternal years in FW Maternal years in SW Paternal years in FW Paternal years in SW	Maternal RTL Paternal RTL Sperm RTL Egg weight Fry weight	All possible 2-way interactions between maternal and paternal years in FW and SW	Mother ID Father ID

Notes: Terms were then sequentially removed if not significant or if not contributing to significant interactions. Mother ID and Father ID were included as random effects to control for non-independence of siblings. See Table 2.2 for summary of the final models.

Table 2.2. Summary of the six final linear mixed-effect models explaining variation in: (A) embryo telomere length, (B) fry telomere length and (C) telomere change (between embryo and fry stage).

	Including parental ages					Including parental years in FW & SW				
	Independent variable	Num. df	Den. Df	F	p	Independent variable	Num. df	Den. Df	F	p
A	Egg weight	1	6.48	22.69	0.003	Mat. Years in FW	1	13.40	0.79	0.390
	Maternal RTL	1	9.05	5.55	0.043	Pat. years in SW	2	16.62	0.26	0.773
						Mat. Years in FW x Pat. years in SW	2	26.87	3.83	0.034
						Egg weight	1	8.55	12.67	0.007
B	Embryo RTL	1	47.03	17.07	<0.001	Pat. years in FW	2	43.62	5.01	0.011
	Fry weight	1	47.61	6.76	0.012	Embryo RTL	1	46.48	14.14	<0.001
						Fry weight	1	41.64	7.23	0.010
C	Egg weight	1	33.11	4.90	0.034	Pat. years in FW	2	35.71	3.86	0.030
	Fry weight	1	42.17	2.62	0.113	Egg weight	1	29.87	5.25	0.029
						Fry weight	1	35.23	3.34	0.076

Notes: The first column shows models which used parental ages (both maternal (Mat.) and paternal (Pat.)) as independent variables. The second column shows models which instead divided these ages into years spent in FW and SW. The main effects and interactions initially included in each of the models are outlined in Table 2.1. Terms were sequentially removed if not significant or if not contributing to significant interactions. Mother ID and Father ID were included as random effects to control for non-independence of half-siblings. See Methods for definitions.

2.4 RESULTS

Parental RTL

I did not find a significant association between parental RTL and any of the independent variables included in the GLMs - sex, weight and age (either total age or separated years spent in FW and SW).

Embryo RTL

Embryo RTL was positively correlated with initial egg weight: heavier eggs resulted in offspring (embryos) with relatively longer telomeres (Table 2.2A, Fig. 2.2). When running the version of the model that considered parental total age (rather than separated into time spent in FW and SW), neither maternal nor paternal age had any significant effect but there was an effect of maternal RTL: mothers with relatively longer telomeres produced embryos with a relatively longer telomere length (Table 2.2A, Fig. 2.3). In the alternative model that used parental years in FW and SW, there was a significant paternal SW age x maternal FW age interaction (Table 2.2A). When crossed with females that had spent 3 years in FW, precocious parr fathers (0 SW years) and MSW fathers (2+ SW years) produced offspring with relatively longer telomeres. However, this was not the case when they were crossed with females that had spent only two years in FW (Fig. 2.4).

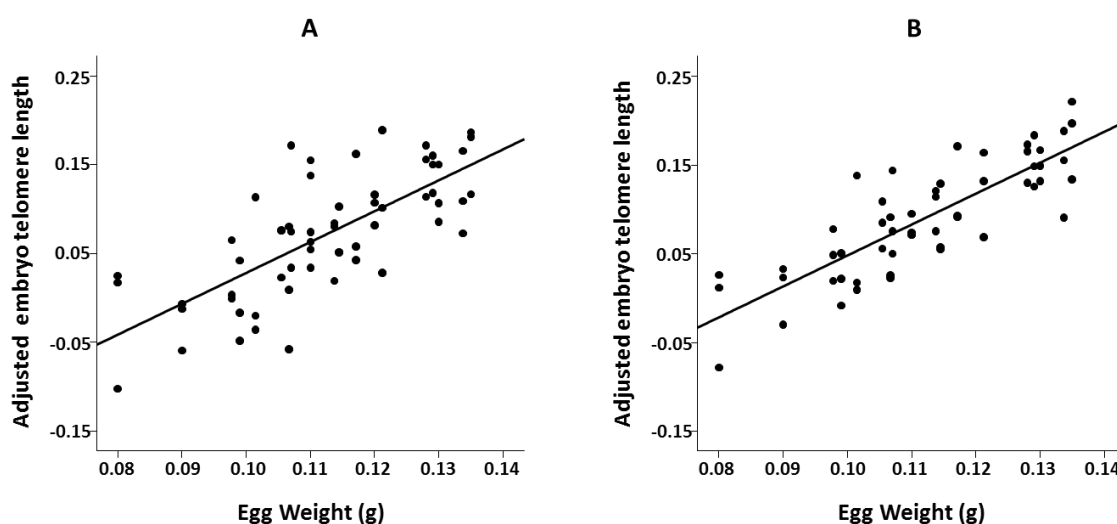


Fig. 2.2. The relationship between egg weight (g) and embryo relative telomere length. Embryo telomere lengths have been plotted as partial residuals as a function of egg weight when all other independent variables = 0; when (A) parental age is included in the model as an independent variable and (B) when parental years in FW and SW are included in the model as independent variables. Each data point is for a separate family.

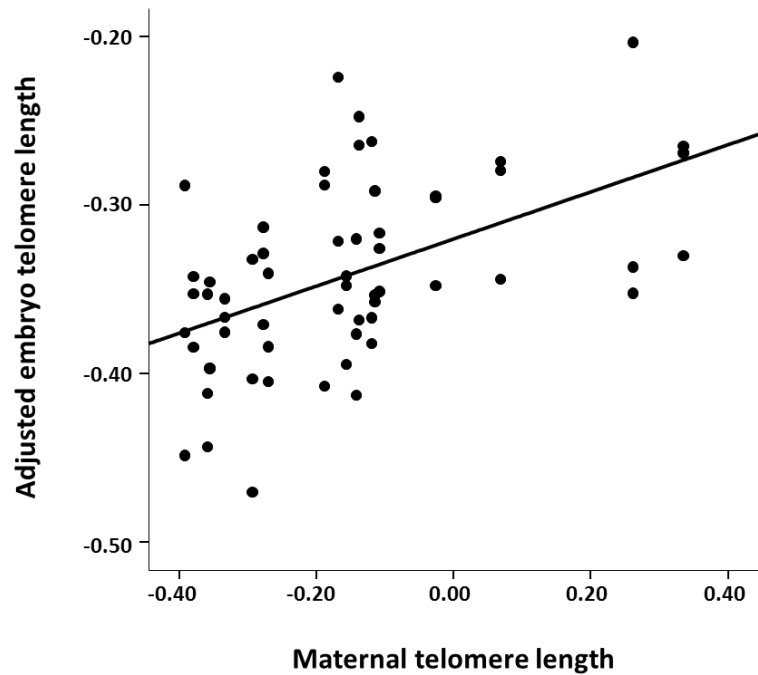


Fig. 2.3. The relationship between maternal relative telomere length and embryo relative telomere length. Embryo telomere lengths (one data point per family) have been plotted as partial residuals as a function of maternal telomere length when all other independent variables = 0.

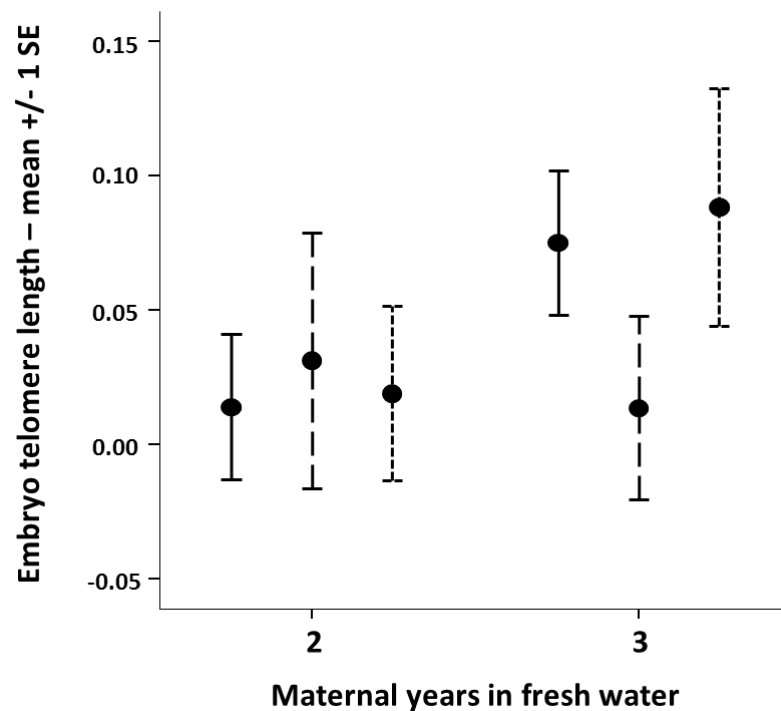


Fig. 2.4. The relationship between maternal years in fresh water and embryo relative telomere length. Solid lines represent offspring from fathers that had spent 0 years at sea (i.e. precocious parr fathers which had not yet migrated), large dashed lines represent offspring from fathers that had spent 1 year at sea and the small dashed line represents offspring from fathers that had spent multiple years at sea.

Fry RTL

Fry RTL was negatively correlated with fry body weight, with larger fry having shorter telomeres (Table 2.2B, Fig. 2.5). At a family level, fry RTL was positively related to embryo RTL, since families with a relatively greater telomere length at the embryo stage produced fry with longer telomeres (Table 2.2B, Fig. 2.6). There was also a significant negative effect of paternal years in FW (Table 2.2B), with offspring (fry) from fathers that had spent the least time in fresh water having the longest telomeres (Fig. 2.7).

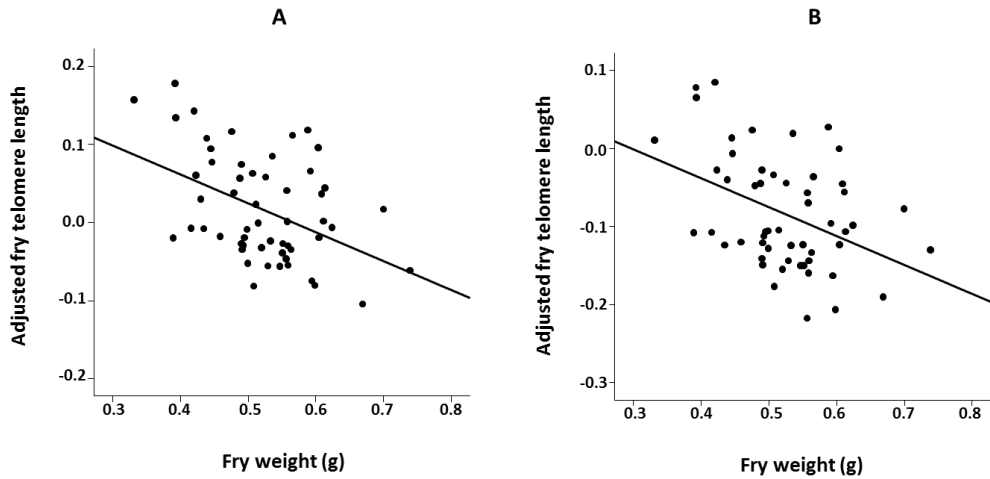


Fig. 2.5. The relationship between the average fry weight and average fry relative telomere length for a given family. Fry telomere lengths (one data point per family) have been plotted as partial residuals as a function of fry weight when all other independent variables = 0; when (A) parental age is included in the model as an independent variable and (B) when parental years in FW and SW are included in the model as independent variables.

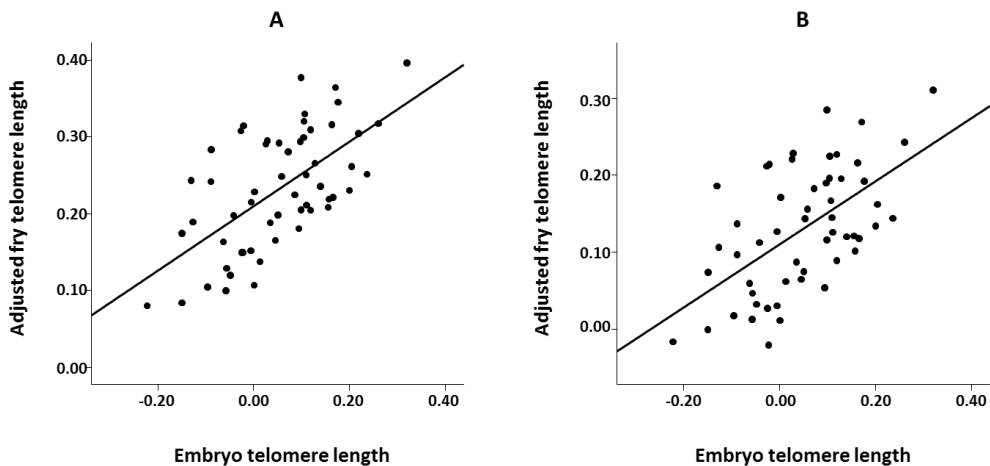


Fig. 2.6. The relationship between the average embryo relative telomere length and average fry relative telomere length for a given family. Fry telomere lengths have been plotted as partial residuals as a function of embryo telomere length when all other independent variables = 0; when (A) parental age is included in the model as an independent variable and (B) when parental years in FW and SW are included in the model as independent variables.

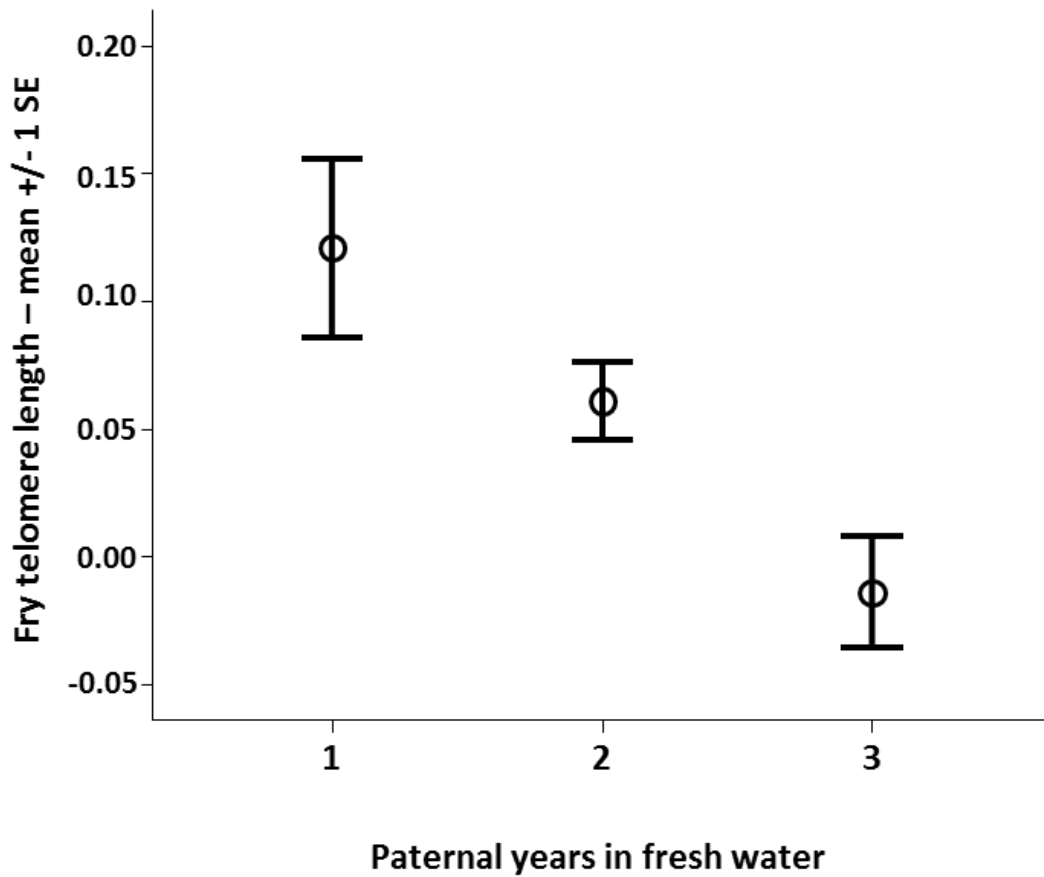


Fig. 2.7. The relationship between paternal years in fresh water and fry relative telomere length.

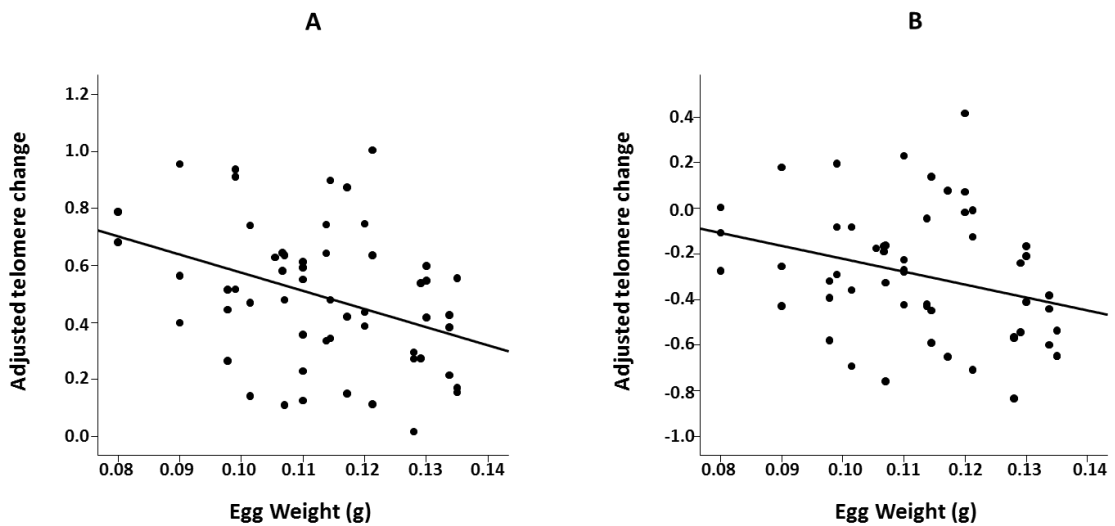


Fig. 2.8. The relationship between the average egg weight and average relative telomere change for a given family. Telomere changes have been plotted as partial residuals as a function of egg weight when all other independent variables = 0, when (A) parental age is included in the model as an independent variable and (B) when parental years in FW and SW are included in the model as independent variables.

Telomere change

The rate of telomere loss (between the embryo and fry stage in each family) was negatively correlated with its mean egg weight (Table 2.2C). Offspring from families in which eggs were large experienced a greater mean rate of telomere loss (Fig. 2.8). Although fry weight was not significant, the model fitted better when fry weight was included as an independent variable. Neither maternal nor paternal total age had any effect on offspring RTL loss, however, as with fry telomere length, paternal years in FW did have a significant effect (Table 2.2C), since fathers that had spent only 1 year in fresh water produced offspring with the lowest mean rate of telomere loss (Fig. 2.9).

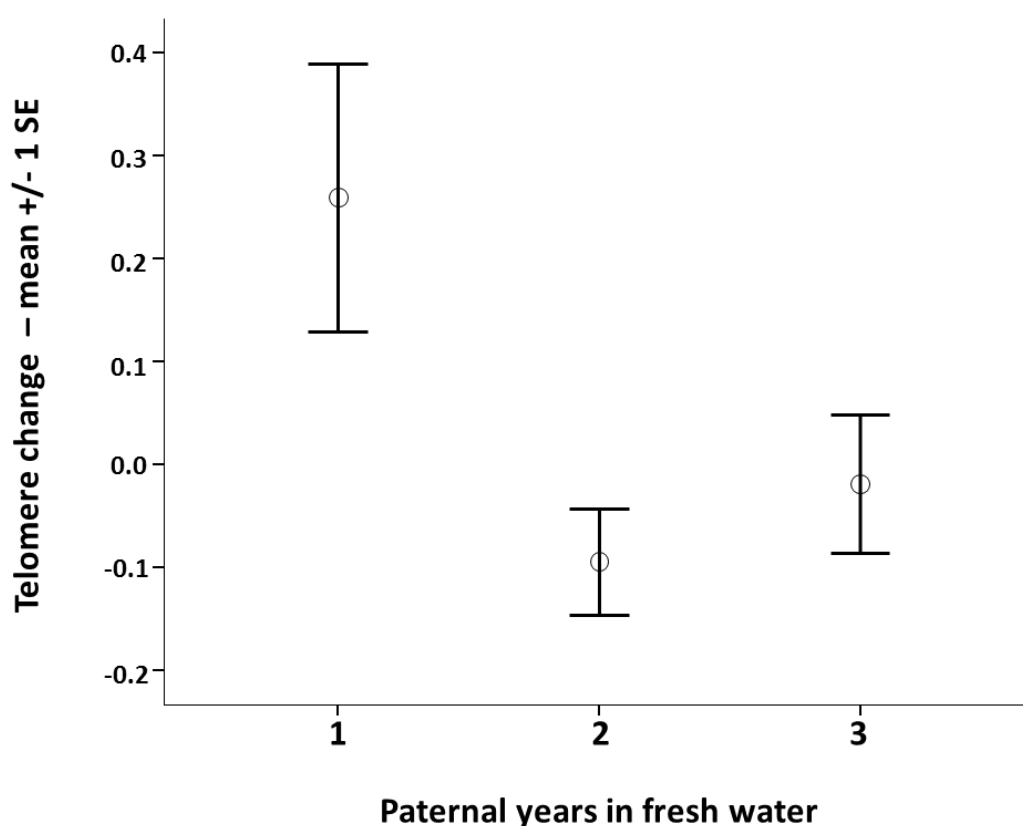


Fig. 2.9. The relationship between paternal years in fresh water and relative telomere change between the embryo and fry stage for each family.

2.5 DISCUSSION

This study demonstrates that there can be significant links between parental life history and offspring telomere dynamics. Maternal life history traits, in particular egg size, were most significant at the embryonic stages. Paternal life history traits, such as early life growth

rate, had a greater association in the later stages of development. However, although parental age has previously been found to be one of the most pervasive parental effects on offspring fitness across a range of taxa, I did not find any significant link between parental age and offspring telomere length in salmon.

Embryo telomere length correlated positively with egg size. This significant egg size effect was present irrespective of whether parental ages were expressed as total age or as separate years spent in fresh and sea water. Neither the paternal nor the maternal age had a significant effect on embryo telomere length, but there was a relationship with maternal telomere length, since mothers with a relatively longer telomere length at the time of reproduction produced offspring with relatively longer telomeres. However, the effects of maternal telomere length and egg size may share a common cause, since telomere length is considered to be an index of an individual's physiological fitness and there are links between a mother's fitness and the quality of her eggs (Blount *et al.* 2002a; Tobler & Sandell 2009).

Both the effect of egg weight and maternal telomere length had disappeared by the time the offspring had reached the fry stage, which suggests that these effects arose mostly through variation in egg provisioning (Donelson, Munday & McCormick 2009; Van Leeuwen *et al.* 2015). Non-genetic maternal effects arising through differential egg provisioning have been reported in a number of species (Royle, Surai & Hartley 2003; Tobler & Sandell 2009; Costantini 2010; Noguera *et al.* 2016). Variation in egg proximate composition (i.e. percent of water, lipid, protein and carbohydrate) is fairly limited in fish (Kamler 1992), therefore larger eggs will generally have more of all components than small eggs. This is supported by the fact that larger salmon eggs generally result in larger offspring at emergence (for reviews Fleming 1996; Jonsson & Jonsson 2011). The relationship between egg size and antioxidant content is not well known, however it is reasonable to assume that larger eggs could contain a greater reserve of antioxidants. Oxidative damage is one of the main factors affecting telomere loss and antioxidants have been shown to help mitigate rates of telomere loss during development (Kim & Velando 2015; Noguera, Monaghan & Metcalfe 2015). Therefore, it may be that mothers are influencing the telomere dynamics of their offspring through the provisioning of their eggs (e.g. with antioxidants).

I found that offspring telomere length was influenced by a significant interaction between maternal years in fresh water and paternal years in sea water: mothers that had spent an extra (3rd) year in fresh water produced offspring with longer telomeres, but only when crossed with precocious parr fathers (0 SW years) or MSW fathers (2+ SW years). Salmon

that spend an extra year in fresh water generally do so because they have grown relatively slowly and so failed to reach a minimum size threshold by the previous year (Metcalf & Thorpe 1990; Økland *et al.* 1993). Previous studies have found that this is associated with the subsequent production of relatively larger eggs (while controlling for maternal size at reproduction) (Thorpe, Miles & Keay 1984; Burton *et al.* 2013); this may be an adaptive maternal effect if mothers are preparing their offspring for a relatively adverse freshwater environment. Longer offspring telomeres might be part of this intergenerational effect, although the reason for the complex interaction with paternal years at sea is unclear.

There was a positive relationship between embryo telomere length and fry telomere length. Families which had relatively longer telomeres at the embryo stage also produced fry with a relatively longer telomere length. Families were reared in stable laboratory conditions, with similar food rations and conditions. As mentioned, one of the main pathways by which telomere length can change is by cell proliferation (i.e. growth). However, because food rations were similar between groups, I saw relatively little variation in body size by the fry stage, compared to what would be observed in the wild. The second main pathway by which telomere length can change is by variation in oxidative damage. Again, rearing conditions were relatively stable (constant temperature, low constant water flow, high food availability) so that oxidative stress might have been relatively low and similar among families. Therefore, although I have identified a strong relationship between embryo telomere length and fry telomere length, I would expect this relationship to be weakened in the wild, with much greater variation in environmental and individual condition.

In contrast to the positive relationship between embryo and fry telomere length, there was a negative relationship between fry weight and fry telomere length. Therefore, for a given embryo telomere length, families which produced larger fry had relatively shorter telomeres at the fry stage. Fry may achieve an increase in body size by cell division or cell growth (or both) (Arendt 2007). While cell growth does not cause any change in telomere length, cell division will lead to telomere shortening, so an increase in cell proliferation rate (to achieve a larger body size) could have a detrimental effect on telomere length, which may help explain this correlation. Although egg size had no effect on fry telomere length, it was associated the relative rate of telomere change (between embryo and fry): offspring from families in which eggs were large experienced a greater mean rate of telomere loss. It should be noted that egg size and fry size were positively correlated (Pearson's $\rho = 0.56$, $p < 0.001$, $n = 59$, based on mean values per family). Therefore, egg size had contrasting effects on telomere length, dependent on the developmental stage.

Although it may be advantageous to originate from a larger egg with respect to embryo telomere length, individuals from larger eggs are also more likely to be larger at the fry stage, which may have negative consequences for telomere length. Of course this may differ in wild, when fry growth will be under the influence of various other factors.

Fry telomere length (and associated telomere change from the embryo stage) was significantly associated with the number of years the father had spent in fresh water. Fathers that had spent longer in fresh water prior to spawning produced fry with shorter telomere lengths, while fathers that had spent only 1 year in fresh water produced fry with the longest telomeres. While the number of years a father had spent in fresh water had no apparent effect on his weight by the time of spawning, it is possible that this link between freshwater residence time and offspring telomeres is related to the growth rate of the fathers in early life. Early (precocious) maturation in Atlantic salmon has been linked to fast early growth rate (Whalen & Parrish 1999; Aubin-Horth & Dodson 2004), and so all of the 1 year old fathers are likely to have experienced a relatively fast growth rate. In contrast, migratory males which remain in fresh water for 3 years prior to migration do so because their slow growth rate prevents migration after 2 years (Metcalf & Thorpe 1990; Økland *et al.* 1993). It is known that environmental conditions experienced in early life can affect not only the individual that is experiencing them but also the generations that follow (Burton & Metcalfe 2014). The number of years that Atlantic salmon spend in fresh water and the associated implications that this can have for offspring phenotype has been relatively well studied, though mostly in the context of the mother (Thorpe, Miles & Keay 1984; Burton *et al.* 2013; Burton *et al.* 2016). For example, it has been shown that offspring of mothers with slower early growth rates often display slower early growth rate themselves (Burton *et al.* 2013).

However, it is unclear why early paternal growth rate might influence offspring telomere length. One possible explanation could be telomerase expression: the enzyme telomerase is capable of causing an elongation of telomere length (Blackburn 2005) but its expression is often down-regulated in many types of somatic cells, probably to suppress the formation of tumours (Collins 2006). However, a number of fish studies have found telomerase activity to correlate positively with cell proliferation (Yap *et al.* 2005; Peterson, Mok & Au 2015) and it is possible that fathers who have a slower growth rate in early life also experience a down-regulation in telomerase expression, which could affect germline telomere length and therefore the telomere length of their offspring. This explanation contrasts slightly with the relationship I found at the fry stage; with fry somatic weight correlating negatively

with fry telomere length. However, it may be the case that the relationship between telomerase activity and growth rate (i.e. the rate of cell proliferation) is in turn dependant on developmental stage.

This study demonstrates both the complexity and the importance of parental factors that can influence telomere dynamics in early life. I have shown that maternal effects, possibly mediated through egg provisioning, are important in the embryonic and to a lesser extent the later fry stages in Atlantic salmon. The links between paternal life history and offspring traits have received much less attention, in comparison with maternal effects. However, I have shown that the environmental conditions experienced by fathers in early life are associated with lasting effects on offspring telomere dynamics. For this study, I chose a controlled laboratory set-up, which allowed us to identify trends that may not be detectable in the wild. However, a sensible next step would be to examine how parental effects may influence offspring telomere dynamics in the wild, where offspring would also be under the influence of many different environmental factors.

CHAPTER 3. The association between telomere dynamics, timing of seaward migration and the probability of marine survival in Atlantic salmon: a longitudinal study

3.1 ABSTRACT

Many animal species are migratory between different geographical areas and or habitats. While there can be much to gain from migration, it can be energetically demanding and a cause of significant mortality. Performance during migration (and associated probability of survival) may be influenced by an individual's physiological state prior to the start of migration. For example, the likelihood of surviving migration has been linked to pre-migration body size and condition. However, although a larger size may increase the probability of migration survival, it may come at a physiological cost elsewhere. For example, studies have linked an increase in body size (and associated growth rate) to increased oxidative stress and reduced longevity. With this in mind, focusing simply on body size and condition as an indicator of fitness is perhaps not the best approach when assessing pre-migration physiological state. Telomere length may be a useful additional indicator of individual state, when examining the link between pre-migration physiological state and subsequent likelihood of surviving migration. Telomeres cap the ends of eukaryotic chromosomes and play an important role in chromosome protection. A relatively short telomere length is indicative of poor biological state, including poorer tissue and organ performance, reduced longevity and increased disease susceptibility. Using a wild Atlantic salmon population, I found that most individuals experienced a reduction in telomere length during the marine migratory phase of their life cycle. The relative rate of telomere loss varied with sex, with males experiencing a relatively greater loss. In contrast to my hypothesis, I found that juvenile salmon that had the shortest telomeres at the time of outward migration had the greatest probability of surviving through to the return migration. I also found that migration survival was significantly influenced by the timing of outward smolt migration, with the smolts migrating earliest in the spring having the highest probability of return.

3.2 INTRODUCTION

Many animal species are migratory between different geographical areas and or habitats. While there can be much to gain from migration, it can be energetically demanding and a

cause of significant mortality (Gauthreaux 1980; Dingle 2014). Performance during migration (and the associated probability of survival) may be influenced by an individual's physiological state prior to the start of migration. For example, the likelihood of surviving migration has been linked to pre-migration body size and condition (Owen & Black 1989; Tinbergen & Boerlijst 1990; Schmutz 1993; Crossin *et al.* 2004; Mitchell *et al.* 2011) and pre-migration levels of stress hormones (Angelier, Holberton & Marra 2009; MacDougall-Shackleton *et al.* 2009). It can be argued that body condition should be the most important physiological determinant of migration success, since animals in poor body condition may be carrying fewer energy reserves, and therefore may be more likely to run out of energy during the migration.

However, using a simple index of pre-migratory 'body condition' can be problematic, since it may depend on migration distance and the likelihood of feeding en route (Piersma 1998). Moreover, although a larger size may increase the probability of migration survival, it may come at a physiological cost elsewhere. For example, studies have linked an increase in body size (and associated growth rate) to increased oxidative stress (Alonso-Álvarez *et al.* 2007; Nussey *et al.* 2009) and reduced longevity (Speakman 2005; Selman, Nussey & Monaghan 2013) which may partially explain why individuals rarely maximise their growth rates (Metcalf & Monaghan 2003). With this in mind, focusing simply on an index of body size and/or condition is perhaps not the best approach when assessing pre-migration physiological state.

Telomere length may be a useful additional indicator of individual state, which can be used to examine the link between pre-migration physiological state and subsequent likelihood of surviving migration. Telomeres cap the ends of eukaryotic chromosomes and play an important role in chromosome protection (for reviews see Blackburn 1991; Campisi *et al.* 2001; Monaghan 2010). Telomere loss occurs at each round of cell division as a result of the 'end replication problem'. Consequently, telomeres often shorten with age and may eventually reach a 'critical length', at which point cells may enter a state of replicative senescence. Senescent cells can have altered secretory profiles and may secrete more inflammatory compounds (Rodier *et al.* 2009). Thus a combination of cell loss and an accumulation of senescent cells can contribute to the ageing process (Aubert & Lansdorp 2008; Monaghan 2014). Therefore, a relatively short telomere length is indicative of relatively poor biological state and is linked to age-related deterioration and/or increased

disease susceptibility (Kotrschal, Ilmonen & Penn 2007; Aubert & Lansdorp 2008; Ilmonen, Kotrschal & Penn 2008; Stier *et al.* 2015).

In addition to the effect of cell proliferation, the rate of telomere loss may also be influenced by levels of oxidative damage (Chan & Blackburn 2004) since telomeric DNA has a high guanine content, which is particularly susceptible to oxidative damage from reactive oxygen species (ROS). ROS production may occur as a result of environmental stress, and a number of studies have linked telomere loss to the exposure to various environmental stressors (for several recent examples see Herborn *et al.* 2014; Nettle *et al.* 2015; Watson, Bolton & Monaghan 2015). Partly because of the association with ageing and disease susceptibility, a relatively short telomere length has also been linked to reduced survival, both in the laboratory (Heidinger *et al.* 2012) and in the wild (Hausmann, Winkler & Vleck 2005; Barrett *et al.* 2013; Watson, Bolton & Monaghan 2015) with possible implications for individual fitness. Angelier *et al.* (2013) reported that American redstarts (*Setophaga ruticilla*) with a longer telomere length pre-migration had a greater chance of returning from that migration, suggesting that telomere length may also be a good biological indicator in which to study individual fitness and the probability of successful migration. However, Bauch, Becker and Verhulst (2013) found a different pattern when examining post-migration telomere dynamics in the common tern (*Sterna hirundo*), since better performing individuals (in relation to timing of migration) also had shorter telomeres post-migration. This counter-intuitive relationship may have arisen as a consequence of a trade-off between investment in migration (and associated migration success) versus investment in somatic maintenance (and associated telomere dynamics).

In order to examine the relationship between telomere length and likelihood of surviving a migration, it is necessary to use a system in which there is likely to be variation in telomere length at the time of migration, and where a significant proportion of individuals fail to complete the migration (and the means to detect which animals have survived). Atlantic salmon *Salmo salar* can potentially fulfil these requirements. The strong tendency for migrants to return to their natal river to spawn can provide the opportunity to monitor survival rates when combined with fisheries management practices in which all returning fish are captured in the river. They display significant within-population variation in life history strategy (for reviews see Fleming 1996; Klemetsen *et al.* 2003; Jonsson & Jonsson 2011) that is linked to migratory behaviour. Salmon are born in fresh water but then usually migrate to sea (at the smolt stage), where they spend either one or two

(occasionally three or more) winters, prior to returning to their natal river to spawn (described as one sea winter (1SW) or multi sea winter (MSW) fish respectively). 1SW fish generally migrate to the waters surrounding the Faroe Islands, whereas MSW fish migrate further to the waters of south western Greenland and tend to be much larger in size by the time of spawning (Trust 2012). There are thought to be a number of determinants, both genetic and environmental, which influence how many years an individual will spend at sea (Gardner 1976; Fleming 1998; Barson *et al.* 2015).

Survival rates from the out-migrating smolt stage to the returning breeding adult stage among Scottish smolts (prior to coastal fisheries) is 6%; based on data from the last 10 years (ICES 2015). The total reported catch of Atlantic salmon in the northeast Atlantic area has steadily decreased since the mid-1970s, with the lowest catch to date reported in 2014 (ICES 2015). In addition, the somatic condition of adult salmon has also decreased, with returning adults having a relatively lower body mass for a given body size than previous years (Todd *et al.* 2008). A number of studies have linked marine survival in Atlantic salmon to conditions experienced at sea (Jonsson & Jonsson 2004; Friedland *et al.* 2009; Trueman, MacKenzie & Palmer 2012). However, conditions experienced in early life can also have long term effects on phenotypic fitness (Burton & Metcalfe 2014). Prior to undertaking the seaward migration (when they are described as smolts), juvenile salmon may spend up to 6 years in fresh water, depending on their growth rate (Metcalf & Thorpe 1990; Økland *et al.* 1993) however this tends to be 2-3 years in Scottish rivers (Maitland 2007). Therefore, variability in their early life conditions and growth performance prior to migration could also affect their probability of successfully completing the return migration.

In this study I examined whether telomere length was a good predictor of migration survival in a wild Atlantic salmon population. Using a longitudinal mark and recapture approach, I measured body size, weight and telomere length in the same individuals, both before and after migration, in order to address two questions. Firstly, I assessed whether individuals experienced a relative telomere change during the migration phase of their life cycle. Secondly, I assessed whether an individual's state (measured in terms of size, weight and telomere length) at the smolt stage (prior to migration) affected their probability of surviving the return migration. By addressing both questions, I hoped to better understand the extent to which telomere dynamics may influence individual fitness and migration

survival. To my knowledge, this study is the first longitudinal analysis of telomere dynamics and survival in a wild migratory fish species.

3.3 METHODS

Study system

The study was conducted at the River Blackwater, which is part of the larger River Conon system, northern Scotland (57° 60'N, 4°63'W). The River Conon has been highly impacted by the installation of hydroelectric schemes since the 1950s. As part of a compensation agreement, the Loch na Croic Atlantic salmon trap was constructed on the River Blackwater in the late 1950s, just upstream of Loch na Croic (Fig. 3.1)

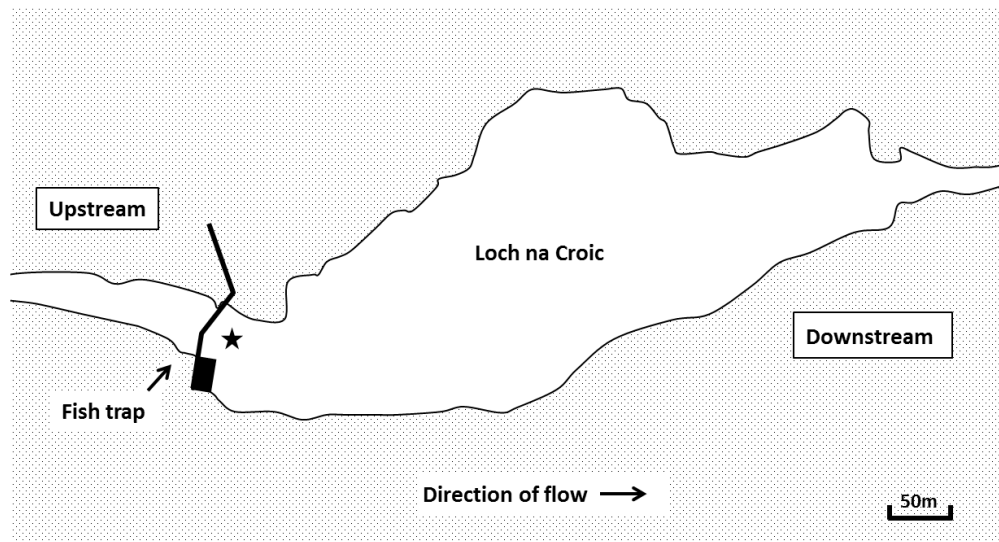


Fig. 3.1. Diagrammatic outline of Loch na Croic (situated on the River Blackwater) and its associated fish trap. The star outlines approximate location of the temporary rotary screw trap, used for the purposes of this experiment.

The trap is closed for the majority of the year. Migratory adult Atlantic salmon return to the River Blackwater during the summer and autumn and congregate in Loch na Croic until the trap is open in November/December. When the trap is opened, adult fish move upstream into the trap and are captured and stripped of gametes; used for stocking purposes upstream of the hydroelectric structures. All fertilised eggs from these fish are incubated in a nearby hatchery for a few months (until the eyed stage) and are then planted throughout the catchment; at natural densities and in typical salmon spawning habitat.

The resulting Atlantic salmon smolts (the freshwater stage at which they physiologically prepare themselves for life in salt water) from these planted eggs, were captured during their seaward migration in April – May 2013. A temporary rotary screw trap (Thedinga *et al.* 1994; Riley *et al.* 2010) was placed at the upstream entrance of Loch na Croic; just below the permanent Loch na Croic fish trap (approximate location of rotary screw trap designated by the star in Fig. 3.1). The permanent trap prevents adults from moving upstream, however, the smaller juvenile smolt stage is able to pass freely downstream through the trap and into the loch. The rotary screw trap was checked daily from 30th April 2013 until 30th May 2013 and any captured smolts removed. In total, 1806 smolts were captured for this study (~ 5% of the estimated total smolt migration on the River Blackwater, S. McKelvey pers. comm.). Daily catch numbers were lowest at the beginning and end of the capture period and followed a normal distribution, peaking on May 17th (Julian 137) and then declining steeply, indicating that the trap was in operation for the majority of the smolt run (Fig. 3.2).

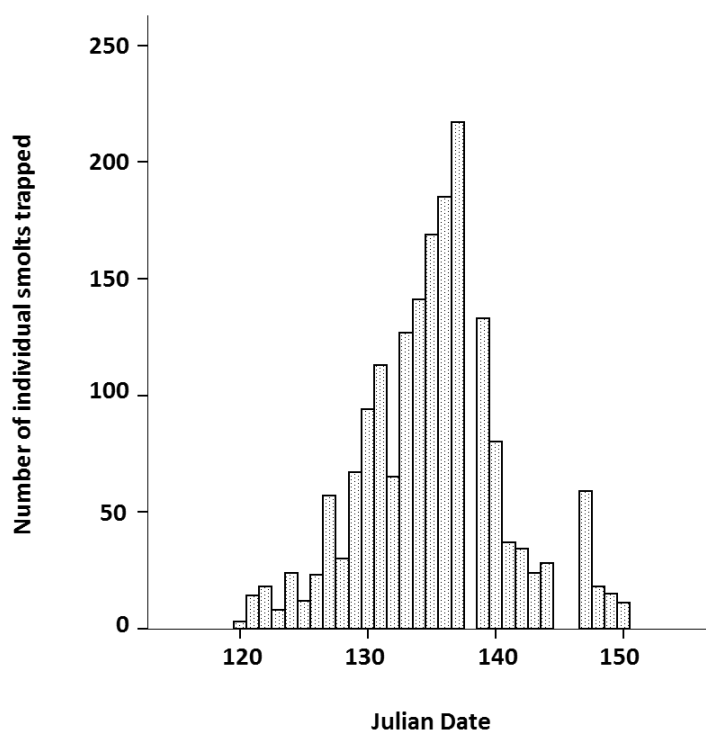


Fig. 3.2. Frequency of individual smolts captured each day in the rotary screw trap.

Captured smolts were then transferred to a holding tank at the nearby broodstock facilities and were tagged with a passive integrated transponder (PIT) tag on the day of capture. Each tag had a unique code which allowed for subsequent identification, should the same individual be recaptured as an adult. For PIT tagging, individuals were anaesthetised using

a 5% benzocaine solution and the tag was inserted into the abdominal cavity. Fish were measured (fork length, mm; body mass, to 0.1 g). A number of scales were sampled for subsequent scalimetry analysis (Shearer 1992) where the circuli of the fish scale can be used to determine age, prior to seaward migration. A small sample of tissue was taken from the adipose fin and stored in 100% ethanol for subsequent telomere analysis. Tagged fish were then placed in a recovery tank, supplied directly with water from the adjacent river, for a maximum of 4 hours until fully recovered, and then released several metres downstream of the initial capture site.

Since anadromous Scottish salmon generally spend either one (1SW) or two years at sea (MSW), surviving tagged smolts were expected to return to the River Blackwater to spawn in either 2014 or 2015. All returning adult fish captured in the Loch na Croic salmon trap in November-December 2014 and 2015 were scanned for the presence of a PIT tag. Any tagged individuals were held separately at the broodstock facilities. These fish were then anaesthetised, using a 5% benzocaine solution and measured (fork length to 0.5cm; body mass to 0.1 g). A second small sample of tissue was taken from the adipose fin and stored in 100% ethanol for telomere analysis. All tagged fish were then stripped of gametes as part of the fisheries management programme and released back into the river, upstream of the trap.

Telomere analysis

For the telomere analysis, DNA was extracted from the fin tissue (of both smolts and adults) using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturers protocol. Each set of DNA extractions also included a negative control, which contained all of the reagents, but without tissue. This was used to check for contamination during the lysis and extraction steps. DNA concentration and purity was measured spectrophotometrically using a Nanodrop 8000.

Telomere length was measured in all samples using the quantitative PCR method described by Cawthon (2002), which provides a relative measure of telomere length (RTL) and is calculated as a ratio (T/S) of telomere repeat copy number (T) to a control, single copy number (S) (here the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene). The *Salmo salar* GAPDH sequence (Genbank accession number: NM_001123561) was used to design primers. Using the Genbank blast tool, the gene was compared to the Atlantic salmon genome to confirm that it was present at a single location. The following forward

and reverse GAPDH primers successfully amplified a single amplicon; as determined by melt curve analysis and were subsequently used for the GAPDH analysis:

SalGAP8-F 5'-GTAAGACAGGATTGAGGCATCTC-3' and

SalGAP8-R 5'-CCGAATCCATTGACACCTACTT-3'

For amplification of telomeric repeats, the universal primers designed by Cawthon (2002) were used:

Tel1b 5'-CGGTTTGTGGTTGGGTTGGGTTGGGTTGGGTTGGGTT-3' and

Tel2b 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'.

A primer optimisation matrix was used to determine the optimal concentration of the primers and five different samples of Atlantic salmon DNA were tested to confirm primer performance and specificity. Telomere PCR conditions were 15 min at 95°C followed by 27 cycles of 15 secs at 95°C, 30 secs at 58°C and 30 secs at 72°C . This was followed by the melt curve profile: temperature was slowly increased from 58°C to 95°C at a rate of 0.2°C /sec. GAPDH PCR conditions were 15 min at 95°C followed by 40 cycles of 15 secs at 95°C, 30 secs at 60°C and 30 secs at 72°C. Again this was followed by the melt curve profile (same as before). PCRs were performed on a Mx3005P qPCR system (Agilent).

The telomere (T) and single copy gene (S) assays were performed on separate 96 well plates, with each sample run in triplicate for each assay. In addition to the samples, each plate also included a six-fold serial dilution of a reference sample (1.25 - 40 ng/well) and a non-target control (NTC). The DNA for the serial dilution was a pool of 24 samples and included both life stages (smolt and adult). The serial dilution was used to generate a standard curve and calculate assay efficiencies. The NTC contained all reaction components apart from DNA and was included on each plate (in triplicate) to check for non-specific binding and potential contamination between sample wells. Each reaction contained 12.5µl 2x ABsolute Blue qPCR SYBR Green Mix low ROX (Fisher Scientific), forward and reverse primers and DNA (for wells containing sample or standards) or water (for wells containing NTC) in a total volume of 25µl. Both T and S assays were performed using 10ng of DNA (equivalent to 6µl of the diluted samples). Primer concentrations were 500nM for the telomere assay (Tel1b and Tel2b) and 200nM for the GAPDH assay

(salGAP8-F and salGAP8-R). The mean assay efficiencies for the telomere and GAPDH were 103.5% and 99.2% respectively and within the acceptable range (85-115%). The average intraplate variation of the Ct values was 1.15 for the telomere assay and 0.59 for the GAPDH assay, respectively. The average interplate variation of the Ct values was 2.59 for the telomere assay and 1.86 for the GAPDH assay, respectively. qPCR raw data were analysed using qBASE software for Windows (Hellemans *et al.* 2007). This controlled for differences in amplification efficiency between plates (assessed from the standard curve of each plate) and produced a relative quantity (RQ) of T and S for each sample. Results were scaled to the average across all unknown samples for each target (i.e. telomere and GAPDH). In addition, by including three inter-run calibrators (three points from the standard curve) I corrected for further inter-run variation. Finally, I used the software to normalise each telomere RQ by the GAPDH RQ for that sample. Therefore, for each sample, the qBASE software produced a calibrated normalized relative quantity (CNRQ). This is similar to the T/S ratio described by Cawthon (2002) but with greater control of inter-plate variation.

Data analysis

The following variables were measured/calculated, to be included in statistical models as dependent/independent variables as appropriate: the downstream migration date of the smolts, i.e. the Julian date on which an individual was captured and PIT tagged (subsequently referred to as *timing of smolt migration*), whether or not each individual was recaptured as an adult post-migration (*migration survival* – note that the great majority of Atlantic salmon smolts return to the same river that they emigrated out of as smolts (estimated 6% straying rate; Jonsson, Jonsson & Hansen 2003) and so the recaptures could be used to estimate survival rates during the marine phase), the fresh body weight of smolts at capture (*smolt weight*), fresh body weight of adults at recapture (*adult weight*), percent body weight change between smolt and adult stage (*percent weight change*), the total age of each individual, as determined by scalimetry (*total age*), the number of years that each individual had spent in fresh water prior to migration (*FW age*), the sex of each returning individual (*sex*), the smolt and adult relative telomere lengths (*smolt RTL* and *adult RTL* respectively), the time point at which relative telomere length was measured (i.e. whether at the smolt or adult stage; subsequently referred to as *time point*) and relative telomere change, calculated for each returning individual as the difference between smolt RTL and adult RTL (*RTL change*).

Using the entire sample of smolts (n=1806), I analysed migration survival in relation to smolt weight and timing of smolt migration using binary logistic regression. Variation in adult RTL and RTL change was assessed in relation to sex, total age, adult weight, percent weight change and timing of smolt migration by general linear models (GLM). I analysed RTL in relation to time point by GLM, to test whether or not telomere length significantly changed over time within an individual. Lastly, to test the hypothesis that smolt RTL predicts migration survival, I also compared relative telomere lengths in the fish that did return, to a comparable sample of smolts which did not return (returned individual n= 23, non-returned individual n = 46). To control for variation in the timing of smolt migration, I selected non-returners that had been tagged on the same days as returned individuals. For each returner I therefore selected two smolts (the fish that had been tagged immediately prior to and after the returned individual). If the returned fish was the first/last individual to be tagged that day, the subsequent/prior two fish were used instead. Because of the association between growth rate and telomere dynamics (e.g. Pauliny *et al.* 2015) I controlled for possible smolt weight effects by conducting a GLM with weight as the dependent variable and return status as a fixed factor (return vs non-returned). There was no significant difference in smolt weight between the fish that returned and the matched sample of fish that undertook the smolt migration over the same time period ($F_{1,67} = 0.221$, $p = 0.640$), indicating that my matched samples were equivalent in body size at the time of outward migration. I then analysed migration survival in relation to smolt RTL and FW age using binary logistic regression.

I used Pearson correlation coefficient matrices to assess potential collinearity between explanatory variables (with a cut-off coefficient of 0.8). Body weight and body length were highly collinear at the smolt stage (Pearson $r = 0.96$, $p < 0.001$) and the adult stage (Pearson $r = 0.97$, $p < 0.001$); therefore only body weight was used in statistical models. Because the RTL change model involved repeated measures of telomere length, the relationship between smolt RTL and RTL change could be confounded by regression to the mean (RTM) (Barnett, van der Pols & Dobson 2005; Verhulst *et al.* 2013). To overcome this, an initial model was run using RTL change values which had been RTM-corrected; see Verhulst *et al.* (2013) for calculations. The relationship between smolt RTL and RTL change was not significant after correction for regression to the mean. Therefore, for the final analysis I was confident in using the conventional telomere change data (adult RTL – smolt RTL) as a dependent in the final model, but excluding smolt RTL as a covariate. All statistical analyses were carried out using IBM SPSS 22 for Windows.

Table 3.1. Summary of the parameters measured in returning adult fish that had been tagged as outgoing smolts in 2013.

	Male		Female		Difference between sexes		
	Mean	SE	Mean	SE	t	d.f.	p
Adult Length (mm)	571.3	11.5	571.0	17.3	-0.11	19	0.99
Adult Weight (g)	1528.6	89.3	1413.2	143.3	-0.64	19	0.53
Adult Age	3.8	0.1	4.0	0.3	0.95	19	0.35
Timing of smolt migration	132.5	0.9	131.2	2.6	-0.59	19	0.56
Smolt length (mm)	122.8	1.6	117.2	2.4	-1.80	19	0.09
Smolt weight (g)	17.4	0.7	15.5	0.8	-1.4	19	0.18

Notes: only adults captured in 2014 were used for the purposes of this summary, since only 1 male and 1 female were captured in 2015. I used Independent-Sample t tests to analyse the difference between sexes with respect to length (at the smolt and adult stage), somatic weight (at the smolt and adult stage), total age (the numbers of years spent in fresh water and sea water) and timing of smolt migration (i.e. the Julian date that smolts were captured and tagged)

3.4 RESULTS

Summary of characteristics of returning fish

Of the smolts that were tagged in the spring of 2013, 21 were recaptured as 1SW adults at the Loch na Croic salmon trap while on return spawning migration in early winter 2014 (16 males and 5 females). A further two individuals were recaptured as MSW adult fish in the next year's spawning migration (1 male and 1 female). In total, this represents 1.3% of the total number of individuals tagged as outgoing smolts. A comparison of these tagged returning fish showed that males and females did not differ in body length or weight (at the smolt or returning adult stage), nor did they differ in age or the timing of smolt migration (Table 3.1).

Outlier analysis

Telomere values at the adult stage were extremely large for two of the individuals (Fig. 3.3) and were identified as outliers by applying the outlier labelling rule (Hoaglin, Iglewicz & Tukey 1986). These two individuals were subsequently excluded from all analyses that included adult telomere length or telomere change.

The effect of migration on telomere dynamics

Telomere length significantly changed between the smolt and adult stage ($F_{1, 42} = 30.88$, $p < 0.001$). The majority of fish experienced a relative telomere loss (85.7% of the 21 returned individuals); all of these fish showing a decline in telomere length were 1SW fish (Fig. 3.3). Only three fish experienced a relative increase in telomere length, and two of these were the fish that had spent multiple years at sea. However, because there were only two of these individuals that had spent more than 1 winter at sea, I did not include time at sea as a factor in the analysis.

Variation in adult RTL and RTL change was assessed in relation to sex, total age, adult weight, percent weight change and timing of smolt migration. (Note: years in SW significantly affected somatic weight ($F_{1,21} = 118.8$, $p < 0.001$) with MSW fish being much larger than 1SW fish. Therefore, to be able to test the effect of weight on adult telomere dynamics independently of this effect of time at sea, only adult fish that had spent 1 year at sea were included in the model, thus removing the two individuals that had spent multiple

years at sea). Total age (3-5 years), adult weight, percent weight change (between smolt and adult stage) and timing of smolt migration were not significantly associated with telomere dynamics (either adult RTL or RTL change). However, adult telomere length and the rate of telomere change (between smolt stage and adult stage) both significantly differed between the sexes (Adult RTL $F_{1,17} = 11.78$, $p=0.003$; RTL change $F_{1,17} = 4.85$, $p=0.04$). Males experienced a relatively greater rate of telomere loss while at sea and as a result had significantly shorter telomeres at the returning adult stage (Fig. 3.4).

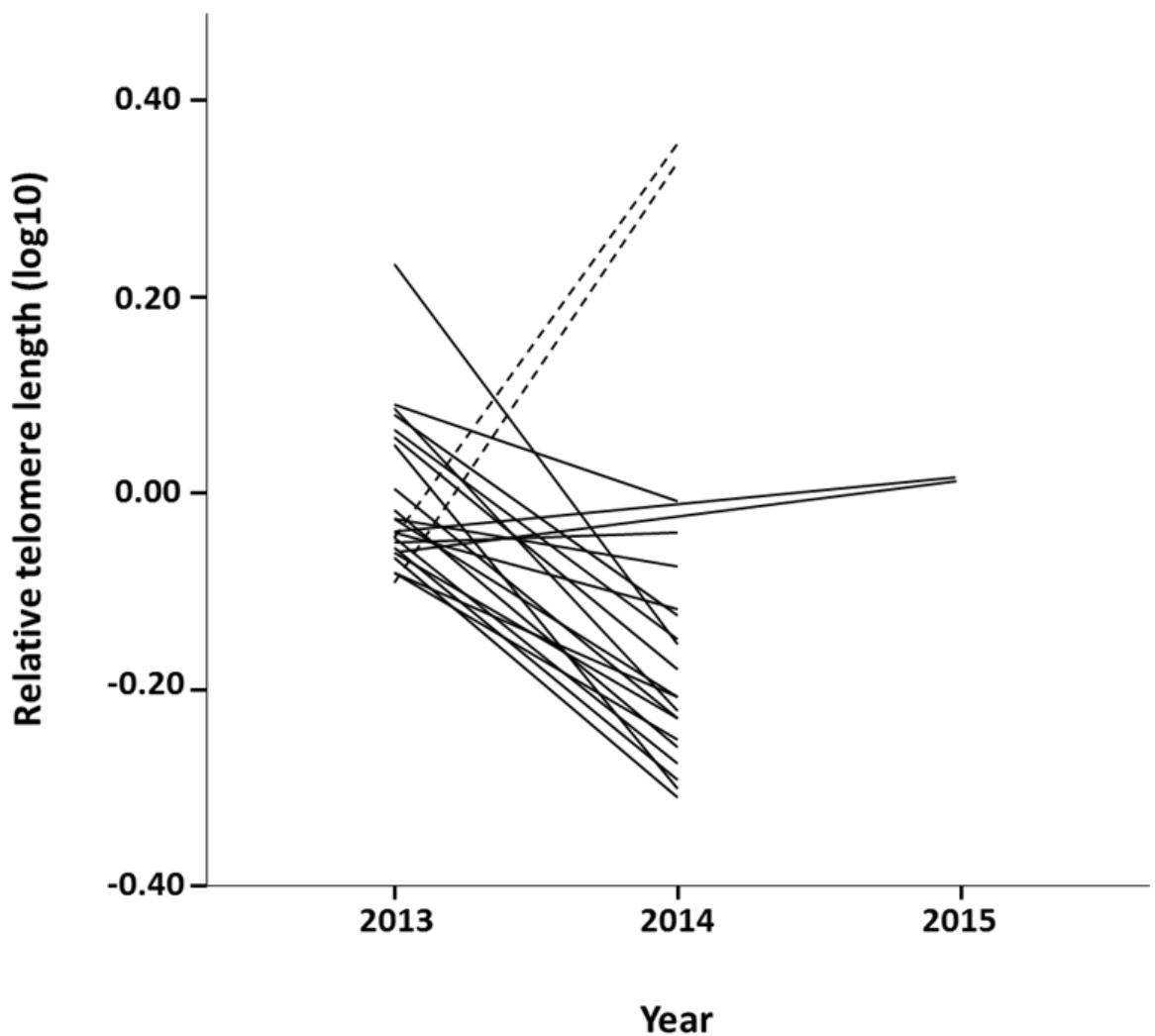


Fig. 3.3. The relative change in telomere length during sea migration, plotted for each individual. Dashed lines represent the two individuals that were identified as outliers. They are included here for presentation purposes, however, they were subsequently removed from all analyses that included telomere length or telomere change

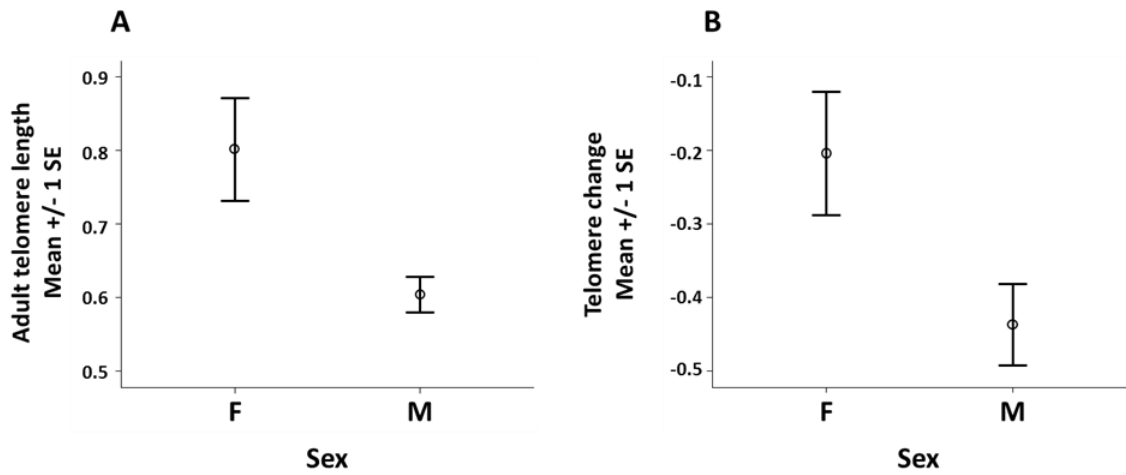


Fig. 3.4. The relationship between sex (F = female, M = male) and (A) adult relative telomere length and (B) relative change in telomere length between the smolt and adult stage. Data plotted as means \pm 1 SE

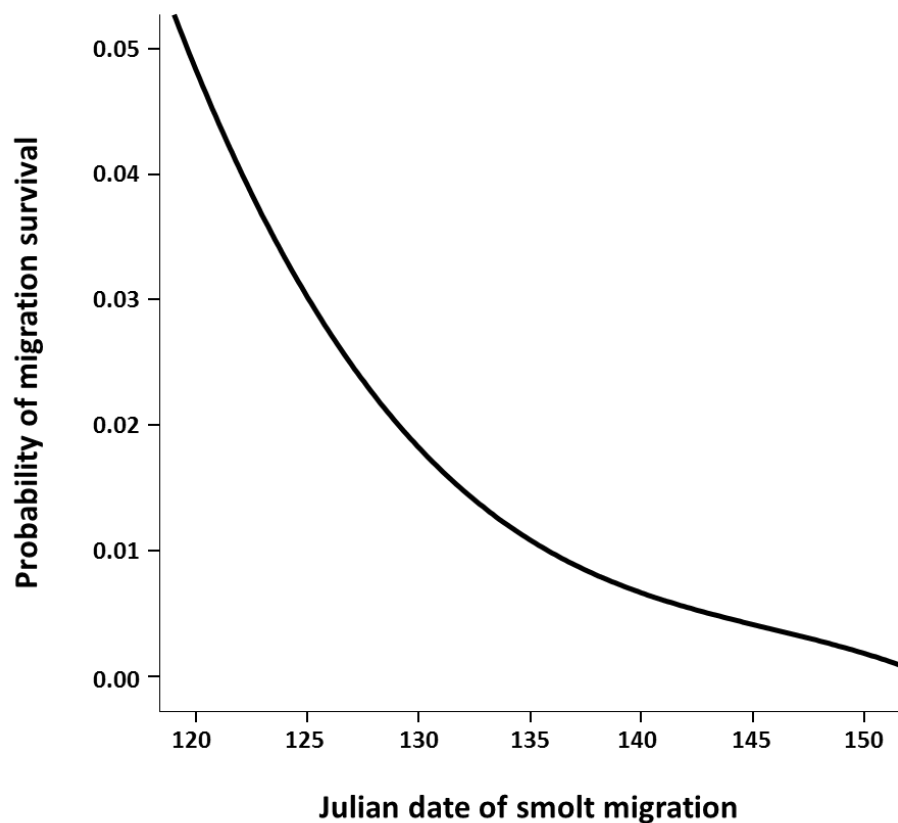


Fig. 3.5. Logistic regression curve showing the relationship between timing of smolt migration (Julian date) and the probability of surviving the marine phase and migrating back to the river ('migration survival').

Migration survival

When using the entire sample of smolts ($n=1806$), migration survival (i.e. the probability of an outgoing smolt returning as a spawning adult and being caught in the trap) was not significantly affected by weight of the smolts on their outward migration. However, it was significantly influenced by the date of smolt migration (Logistic regression, Wald statistic = 6.99, $df = 1$, $p = 0.008$). Migrating to sea later in the smolt run was associated with reduced migration survival (Fig. 3.5). When comparing those tagged individuals that had successfully returned to a sub-sample of individuals that did not return (while controlling for smolt weight and timing of migration), FW age and smolt weight did not significantly affect the probability of surviving the migration. However, smolt RTL did have a significant effect on migration survival (Logistic regression, Wald statistic = 4.08, $df = 1$, $p = 0.043$), but the effect was the opposite to that expected, since individuals with shorter telomeres were more likely to return from migration (Fig. 3.6).

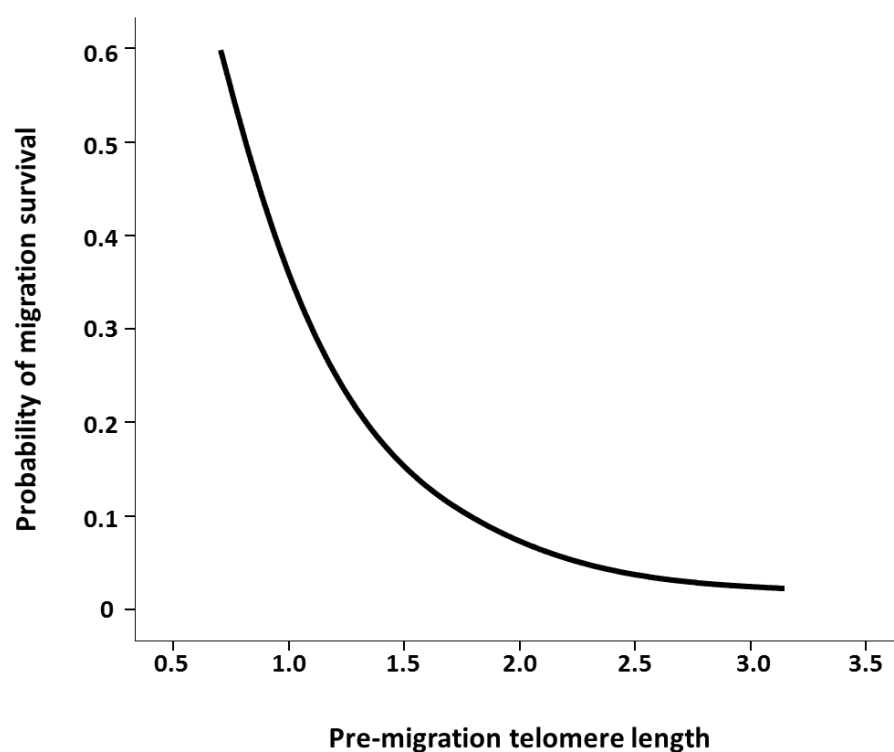


Fig. 3.6. Logistic regression curve showing the relationship between smolt relative telomere length and the probability of surviving the marine phase and migrating back to the river ('migration survival'). Note that these probabilities are relative (and are much higher than the true survival probabilities) since the subsample of fish on which this analysis was based only included two non-returning fish for each returner, in order to make the sample size for telomere analysis manageable - see text for selection criteria and analysis.

3.5 DISCUSSION

To my knowledge, this study is the first longitudinal analysis of telomere dynamics and survival in a wild migratory fish species. Using a wild Atlantic salmon population, I found that migration survival (i.e. the likelihood of completing the return migration) was significantly influenced by the timing of outward smolt migration, with the smolts migrating earliest in the spring having the highest probability of return. I found that most individuals experienced a reduction in telomere length during the migratory phase of their life cycle; however the relative rate of telomere loss was dependent on sex, with males experiencing a relatively greater loss. In contrast to my hypothesis, I also found that juvenile salmon that had the shortest telomeres at the time of outward migration had the greatest probability of surviving through to the return migration.

In general, telomeres have been found to shorten with age, at least in many mammal and bird studies to date (Hausmann *et al.* 2003; Henriques & Ferreira 2012). Many mammal and bird species appear to down-regulate telomerase expression in post-embryonic somatic tissue and instead use replicative ageing as a tumour suppression mechanism (Gomes, Shay & Wright 2010). In contrast, telomerase expression has been detected in post-embryonic somatic tissue in reptiles, amphibians and fish (Gomes, Shay & Wright 2010) suggesting that ectothermic vertebrates have adopted an alternative strategy. As a result, telomere attrition with age is not as common in ectotherms. Simide *et al.* (2016) conducted a comprehensive review of the literature with respect to telomere dynamics in fish, and reported that around half of the fish telomere studies to date have reported telomere shortening with age, while the rest reported no effect, or even telomere elongation.

I found that most individuals migrating to sea for 1 year experienced a relative telomere loss during their time at sea (18 out of 19 individuals). The two fish that experienced the greatest increase in telomere length were also the only two returning individuals that had remained at sea for multiple winters. 1SW fish and MSW fish generally migrate to different regions at sea (Trust 2012) and may therefore experience differences in food availability and local thermal environment, which in turn may affect telomere dynamics. In addition to spending an extra year at sea, perhaps the most striking difference between the two life history strategies is the body size that the fish attain. These different sizes and associated growth rates may be associated with differing levels of telomerase expression. It has been reported that telomerase expression in fish is higher in actively-dividing cells

(Yap *et al.* 2005; Peterson, Mok & Au 2015). In fish, muscle fibre recruitment continues beyond embryogenesis (Weatherley, Gill & Lobo 1988), and active cell proliferation is still detectable in Atlantic salmon at least 6 months after the smolts have migrated to sea water (Johnston *et al.* 2003) Therefore, it is possible that the more sustained growth of MSW fish results in a greater expression of telomerase, which in turn may cause a relative increase in telomere length in fish that spend multiple years at sea.

Telomere length in the returning adults differed significantly between the sexes, with males undergoing a greater rate of telomere loss and having significantly shorter telomeres in adulthood, when compared with females of the same age. To measure telomere length, I sampled tissue from the adipose fin of the returning adults (for both sexes). The size of the male's adipose fin (but not that of the female) is known to increase during sexual maturation and reaches its maximum size at spawning. It is therefore possible that cell proliferation rates differ between male and female adipose fin tissue. Some telomere loss inevitably occurs at each cell division as a result of the 'end replication problem' (Chan & Blackburn 2004) and a number of studies have reported a relationship between enhanced growth rate (i.e. rate of cell proliferation) and telomere loss (Fick *et al.* 2012; Herborn *et al.* 2014; Noguera *et al.* 2015; Pauliny *et al.* 2015). However, this relationship is less well documented in fish, and as mentioned, telomerase expression is known to positively correlate with cell proliferation in fish (Yap *et al.* 2005; Peterson, Mok & Au 2015). An alternative approach to understanding why telomere dynamics might differ between the sexes is to compare their relative reproductive investment or energy expenditure. Olsson *et al.* (2011b) found that female sand lizards *Lacerta agilis* had longer telomeres than males. They also found that telomere length was a predictor of lifetime reproductive success in females (but not males) suggesting that there is a greater selection pressure to maintain telomere length in females. Gonadal volume, often used as a proxy for reproductive investment (Stearns 1992), is much larger in female Atlantic salmon than in males (Jonsson & Jonsson 2003). However, Jonsson, Jonsson and Hansen (1997) found reproductive energy expenditure to be similar between the sexes: though females invest more in growth of the gonads, males expend more energy through activities such as fighting and nest defence. In fact, survival over the spawning period is higher in females than males (Mills 1989; Jonsson & Jonsson 2011), which is mostly attributed to the effort exerted by males fighting rivals (and consequent lack of investment in somatic maintenance). This may also help provide an evolutionary explanation as to why males have shorter telomeres. The disposable soma theory (Kirkwood 1990) predicts that

organisms will alter somatic repair mechanisms in accordance with their expected life history trajectory. Therefore, higher extrinsic mortality rates in one sex may also cause a relative reduction in the somatic maintenance (e.g. telomere length) of that sex (Jemielity *et al.* 2007). In birds, mammals and XY-sex determined reptiles, adult lifespan tends to be higher in the heterogametic sex (Liker & Székely 2005; Barrett & Richardson 2011), as does telomere length (Barrett & Richardson 2011). In salmon, it is males that are the heterogametic sex (Davidson *et al.* 2009), so this pattern also holds. Hormonal differences between sexes may also contribute to the difference in telomere length (Barrett & Richardson 2011). For example, oestrogen is known to activate a promoter of telomerase (Kyo *et al.* 1999) and can contribute to telomerase differences between the sexes (Leri *et al.* 2000).

In contrast to my hypothesis, I found that juvenile salmon (pre-migration) with the shortest telomeres had an increased probability of surviving migration. This is somewhat surprising, since a relatively short telomere length has also been linked to reduced survival, both in the laboratory (Heidinger *et al.* 2012) and in the wild (Hausmann, Winkler & Vleck 2005; Barrett *et al.* 2013; Watson, Bolton & Monaghan 2015), and Angelier *et al.* (2013) reported a correlation between longer telomere length pre-migration and a greater chance of migration survival in American redstarts (*Setophaga ruticilla*). That being said, Bauch, Becker and Verhulst (2013) found that better performing individuals (in relation to migration arrival date) also had shorter telomeres at the end of migration, perhaps as a trade-off between investment in migration (and associated migration success) and somatic maintenance (and associated telomere dynamics). Therefore, it may also be the case in this study that we are witnessing a trade-off, with a greater investment in migration preparation coming at an increased cost (in terms of telomere attrition).

The process of smolting requires many metabolic and biochemical changes, in preparation for life at sea (Jonsson & Jonsson 2011). There are also associated changes in circulating hormone levels during smolting, such as increased levels of cortisol, growth hormone and insulin-like growth factors. These hormones have positive effects on smolt development (Hoar 1988). For example, increased cortisol is known to increase gill $\text{Na}^+\text{-K}^+2\text{Cl}^-$ cotransporter abundance (Pelis & McCormick 2001) and lipid mobilisation (Sheridan 1986), both of which are known to aid smolting. Smolts that have properly undergone the necessary physiological changes are believed to have greater migration success than those that are less prepared (Moser, Olson & Quinn 1991; Schreck *et al.* 2006). Therefore, an

increase in hormone levels (such as cortisol) may be advantageous with respect to increased marine survival. However, there is also mounting evidence of an association between glucocorticoids (e.g. cortisol) and telomere attrition (for review see Haussmann & Marchetto 2010). Therefore, investing properly in the smolting process may be beneficial in terms of survival over the period of smolt migration, but come at a cost to telomere length. Somewhat in support of this, several studies have found telomere length to be negatively associated with investment in reproduction (Bauch, Becker & Verhulst 2013; Gao & Munch 2015) with reproductive success being achieved at the expense of telomere length.

There may also be a behavioural basis to the relationship between juvenile salmon telomere length and subsequent migration survival. Studies have found that a reduced telomere length is associated with altered behaviours, such as elevated impulsive foraging (Bateson *et al.* 2015) and enhanced foraging and aggression (Adriaenssens *et al.* 2016). It is arguable that these behaviours require a greater activity level and a higher metabolic rate, which in turn may lead to greater exposure to oxidative stress and telomere attrition (Chan & Blackburn 2004). That being said, behaviours such as boldness and aggression are generally found to positively correlate with food intake and growth (for review see Biro & Stamps 2008) and there is a strong link between growth and survival in Atlantic salmon (Friedland *et al.* 2000). Therefore, it is plausible that we are again witnessing a trade-off between individual fitness and telomere dynamics: juvenile salmon that display bolder and more aggressive behaviour may then go on to have a better chance of survival at sea, but do so at the expense of telomere length.

I found that migration survival (i.e. the likelihood of completing the return migration) was also significantly influenced by the timing of outward smolt migration, with the smolts migrating earliest in the spring having the highest probability of return. Mortality is thought to be high in salmon smolts, both during the movement to the mouth of the river and when they first reach the sea (Thorstad *et al.* 2012). Many populations of smolts must pass through lakes during their downstream migration, and a previous study on the River Conon found that the chances of migrating successfully across Loch Meig depended on the timing of entry to the upstream end of the loch, with the earliest migrating individuals having the best chance of completing their migration through the loch (E. Rush & S. Mckelvey, unpubl. data). Predation is one of the biggest natural causes of smolt mortality during downstream migration, and it may be that predators (such as pike *Esox Lucius*) take

time to cue in on the arrival of smolts, thus giving early migrants an advantage. There may also be a difference in physiological fitness between early and late migrants. The transformation into the migratory smolt stage appears to be triggered only when the fish reach a minimum size (Metcalf & Thorpe 1990; Økland *et al.* 1993). It is possible that relatively fitter individuals, having achieved a faster growth rate in the preceding months, are able to start migrating early in the run, while smaller, potentially less fit individuals remain on their feeding territories in fresh water for as long as possible, prior to starting migration. A further factor linking timing of smolt migration to survival is the timing of entry to the sea (McCormick *et al.* 1998). Reports suggest that ocean temperature at the time of sea entry is one of the key determinants of survival (Friedland 1998; Friedland *et al.* 2000). Therefore, it may be that the earlier migrating smolts from this study were met with more favourable sea-entry conditions. Of course, predicting sea conditions will be difficult for smolts, and it is possible that annual fluctuations in sea temperature may favour either early or late migrants, depending on the conditions in a particular year.

Although my sample size was relatively small, I have identified a clear association between early life telomere dynamics and subsequent survival over the migratory part of the Atlantic salmon life cycle. The mechanisms underlying this relationship are currently unclear. However, I have shown that indicators of fitness other than size or body condition can be taken into consideration when assessing the relationship between an individual's physiological state and the likelihood of successful migration.

CHAPTER 4. Interactions between parental traits, environmental harshness and growth rate in determining telomere length in wild juvenile salmon

A version of this chapter is published as - McLennan, D., Armstrong, J.D., Stewart, D.C., McKelvey, S., Boner, W., Monaghan, P. & Metcalfe, N.B. (2016) Interactions between parental traits, environmental harshness and growth rate in determining telomere length in wild juvenile salmon. *Molecular Ecology*, DOI: 10.1111/mec.13857.

4.1 ABSTRACT

A larger body size confers many benefits, such as increased reproductive success, ability to evade predators and increased competitive ability and social status. However, individuals rarely maximise their growth rates, suggesting that this carries costs. One such cost could be faster attrition of the telomeres that cap the ends of eukaryotic chromosomes and play an important role in chromosome protection. A relatively short telomere length is indicative of poor biological state, including poorer tissue and organ performance, reduced potential longevity and increased disease susceptibility. Telomere loss during growth may also be accelerated by environmental factors, but these have rarely been subjected to experimental manipulation in the natural environment. Using a wild system involving experimental manipulations of juvenile Atlantic salmon *Salmo salar* in Scottish streams, I found that telomere length in juvenile fish was influenced by parental traits and by direct environmental effects. I found that faster-growing fish had shorter telomeres and there was a greater cost (in terms of reduced telomere length) if the growth occurred in a harsher environment. I also found a positive association between offspring telomere length and the growth history of their fathers (but not mothers), represented by the number of years that fathers had spent at sea. This suggests that there may be long term consequences of growth conditions and parental life history for individual longevity.

4.2 INTRODUCTION

A larger body size has many benefits, such as increased reproductive success, ability to evade predators and increased competitive ability and social status (Blanckenhorn 2000; Dmitriew 2011). Attaining a large size requires either prolonged or faster growth. However there is evidence that individuals rarely maximise their growth rates, suggesting that there are costs associated with rapid growth (Metcalfe & Monaghan 2003). It is becoming

clearer that one such cost may be reduced longevity. Within species, even when comparing adults of the same size, those with faster growth rates earlier in life often exhibit faster senescence and/or shorter lifespans (Metcalf & Monaghan 2003; Geiger *et al.* 2012). There are likely to be multiple underlying mechanisms linking growth rate and faster senescence. Several studies have found growth rate to correlate positively with levels of oxidative damage, in both the laboratory and in the field (Alonso-Álvarez *et al.* 2007; Nussey *et al.* 2009; Carney Almroth *et al.* 2012) and oxidative damage levels are considered to be important influences on longevity and senescence (Halliwell & Gutteridge 2015). In addition, growth rate may alter trade-offs between body growth and body maintenance (i.e. antioxidant production and protein repair). McCarthy, Houlihan and Carter (1994) reported such a trade-off in rainbow trout (*Oncorhynchus mykiss*), with faster growing individuals undergoing increased protein synthesis (growth) and reduced protein turnover (maintenance). The costs of a particular pace of growth may also, in turn, be partly dependent on environmental factors. For example, Stier *et al.* (2014a) found that faster growth in coal tits *Periparus ater* was more costly (in terms of DNA oxidative damage) when they were reared under environmentally harsher conditions. Therefore, it may not only be the rate at which you grow, but also the environment in which you do so that has an effect on the costs and benefits of different growth trajectories.

Telomere length might be a good indicator of these costs. Telomeres cap the ends of eukaryotic chromosomes and play an important role in chromosome protection (for reviews see Blackburn 1991; Campisi *et al.* 2001). Some telomere loss inevitably occurs at each cell division as a result of the ‘end replication problem’ (Chan & Blackburn 2004), so telomere dynamics are affected by the pattern and rate of cell division. In addition to this, telomere loss may also be accelerated by the effects of reactive oxygen species (ROS). Telomeric DNA is vulnerable to oxidative damage for a number of reasons, particularly its high guanine content (Hausmann & Marchetto 2010). While the enzyme telomerase is capable of restoring telomere length, telomerase expression is down-regulated in many types of somatic cells, which is thought to be linked to tumour suppression (Collins 2006). A relatively short telomere length is indicative of poor biological state e.g. reduced potential longevity and increased disease susceptibility (Hausmann, Winkler & Vleck 2005; Imonen, Kotrschal & Penn 2008; Calado & Young 2009; Heidinger *et al.* 2012).

The determinants of variation in telomere length among and within species are still not fully understood. This is especially the case for animals living in the wild, as telomere

dynamics and their consequences have mainly been studied in stable laboratory conditions. An increasing number of studies have reported a heritable component to telomere length. These studies have mostly focussed on humans (for example Nordfjall *et al.* 2005; Njajou *et al.* 2007); however other taxa have also been studied, including lizards (Olsson *et al.* 2011a) and many bird species (Horn *et al.* 2011; Asghar *et al.* 2015a; Reichert *et al.* 2015). Results to date are inconsistent as to whether the inheritance is strongest through the father or mother (Eisenberg 2014).

Changes in telomere length are also strongly influenced by environmental conditions. As mentioned above, telomere attrition is related to the dynamics of cell proliferation and a number of studies have reported a relationship between enhanced growth rate or body size and telomere loss (Fick *et al.* 2012; Herborn *et al.* 2014; Noguera *et al.* 2015; Pauliny *et al.* 2015). These studies are mainly correlational. However, using transgenic coho salmon *Oncorhynchus kisutch* with an artificially increased growth rate, Pauliny *et al.* (2015) found that these fast growing fish showed a faster telomere loss compared with maternal half sibs that grew at a rate more typical of wild fish. On the other hand, Näslund *et al.* (2015) found that wild brown trout *Salmo trutta* induced to undergo compensatory growth did not show increased telomere loss. There have also been suggestions that telomere length may help explain the previously-mentioned relationship between growth rate and longevity (Stindl 2004; Fick *et al.* 2012). Growth rate and body size are both under the influence of various environmental factors (Metcalf & Monaghan 2001) This is especially true for ectotherms, such as fish, where fluctuations in temperature can influence myogenic processes, morphological development, growth rate and metabolism, all of which can have permanent long-term phenotypic effects (Johnston 2006; Jonsson & Jonsson 2011). Therefore, it is also possible that the environmentally conditioned plasticity of growth rate may also have some influence on telomere dynamics. Environmental stressors may also affect telomere length, most notably through their influence on the production of ROS (von Zglinicki 2002; Geiger *et al.* 2012; Kim & Velando 2015). A number of experimental studies have linked telomere dynamics to environmental stressors such as *in utero* stress (Hausmann *et al.* 2011; Marchetto *et al.* 2016), stress hormone administration and disturbance (Herborn *et al.* 2014), social position (Nettle *et al.* 2015) and social crowding (Kotrschal, Ilmonen & Penn 2007; Sohn *et al.* 2012). Therefore, if growth rate does have an effect on telomere dynamics, its impact may also in turn be dependent on environmental conditions.

In order to gain a better understanding of telomere length as a possible link between environmental conditions, growth rate and individual fitness, it is also important that we study these processes in animals living in the wild. Using free-living animals to tease apart parental and environmental effects can prove challenging, due to the logistics of accessing and obtaining data for parents and offspring alike. However, by utilising managed populations of wild Atlantic salmon *Salmo salar*, it is possible to examine environmental effects on telomere dynamics, while simultaneously investigating potential parental effects. Atlantic salmon exhibit intra-population variation in their life histories (for reviews see Fleming 1996; Klemetsen *et al.* 2003). They migrate to sea and undergo a substantial increase in body mass during this time, remaining there for either one sea winter (1SW) or multiple sea winters (MSW) before returning to fresh water to reproduce. MSW fish are generally much larger than 1SW fish when they return to freshwater (Klemetsen *et al.* 2003). There is a correlation between female body size and average egg size (Fleming 1996) and therefore MSW mothers, in general, produce significantly larger eggs than 1SW mothers. It is also possible for males to become precociously mature as parr, prior to sea-migration, and thus have a much smaller body size than males that go to sea to complete their growth (Fleming 1996; Baum *et al.* 2004). Salmon will also vary in the number of years spent in fresh water prior to seaward migration, with faster growing individuals reaching the minimum-size threshold required for sea migration earlier and thus migrating to sea at a younger age (Metcalf & Thorpe 1990; Økland *et al.* 1993). Therefore, within a typical Atlantic salmon population there are two distinct maternal reproductive life-history variants with respect to time spent at sea (1SW and MSW) and three such variants in males (1SW, MSW and precocious parr) and within a given sea age, individuals will vary in the number of years spent in freshwater prior to migration. Since the rate of offspring growth is highly dependent on water temperature (Jonsson *et al.* 2001) spatial variation in water temperatures within a river catchment will mean that the exact location in which an egg is laid will influence the growth rate, and hence potentially the telomere dynamics, of the resulting offspring.

I used this system to examine parental and environmental effects on telomere dynamics in Atlantic salmon. I placed eggs from controlled matings of wild Atlantic salmon in contrasting streams within the same river system in order to test two hypotheses: 1) that faster growth is associated with increased telomere loss, 2) that the magnitude of telomere loss for a given growth rate is greater in harsher conditions. In addition, I was also able to

simultaneously investigate the effect of parental life history and parental telomere length on offspring telomere length in the wild.

4.3 METHODS

In vitro fertilisation

The experiment was conducted at the River Blackwater, which is part of the larger River Conon catchment, northern Scotland (57° 60'N, 4°63'W) (Fig. 4.1). Fish movements in the River Conon have been highly impacted by the installation of hydroelectric schemes since the 1950s. In particular, this has prevented fish from reaching many of the traditional spawning areas in tributary streams. The Loch na Croic Atlantic salmon trap was constructed on the River Blackwater in the late 1950s to maintain an Atlantic salmon population in the upper regions of the River Conon (i.e. above the hydroelectric schemes) by artificial spawning. This is done by collecting returning wild adults in the trap (1790 individuals per year \pm s.d. 163; based on data from 1965-2012), conducting random crosses and then planting out developing eggs (mixed at random) into tributaries throughout the upper region of the catchment. No other stocking takes place in the Conon system. Since all parent fish are collected from the same trap and their eggs are mixed thoroughly before being spread among the streams in the catchment, there is no reason to expect any genetic spatial differentiation in the catchment that would complicate this study.

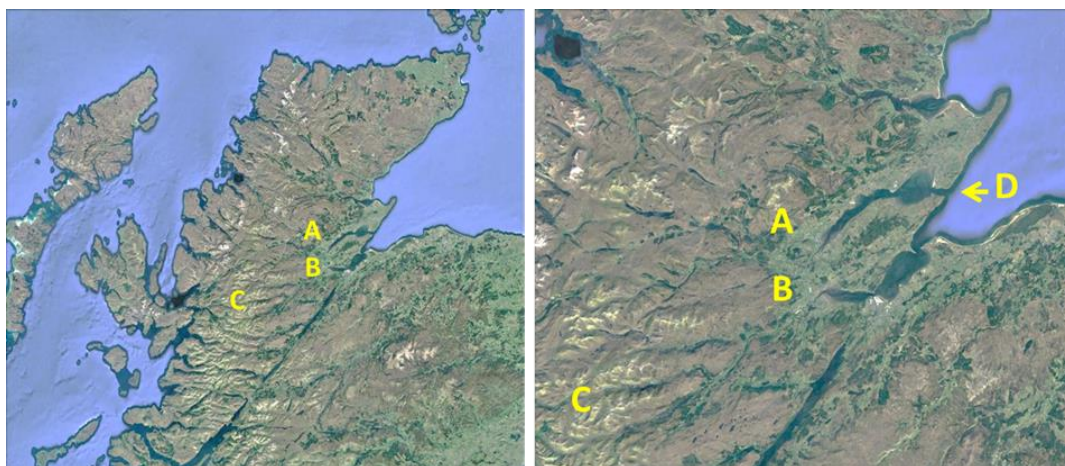


Fig. 4.1. Map outlining the location of (A) the Loch na Croic fish trap; (B) the Allt Goibhre (benign) tributary; (C) the Upper Meig (harsh) tributary and (D) the entrance to the Cromarty firth, which is the mouth of the River Conon catchment in which the two tributaries and trap are located.

This set up allowed us to access large numbers of sexually mature wild fish, which were used to generate experimental families by *in vitro* fertilisation (IVF). The experimental design (Fig. 4.2) was to first incubate the resulting eggs under contrasting aquarium temperatures (to examine the effect on telomeres of early embryonic conditions), then plant the eggs in contrasting tributary streams: a lower altitude stream with moderate temperature and predator density and, in contrast, a harsher high altitude stream with a relatively colder temperature and greater predator density (as a consequence of differences in stocking with salmon eggs in previous years). In both streams the mean temperature was below the optimum for growth (16 °C; (Elliott & Hurley 1997)) but it was furthest from the optimum in the harsher high altitude stream. The juvenile salmon were subsequently recaptured at a later date and parental assignment was established by microsatellite analysis. This allowed the measurement of relative telomere length in parents and offspring (embryos and fry), all within a wild system.

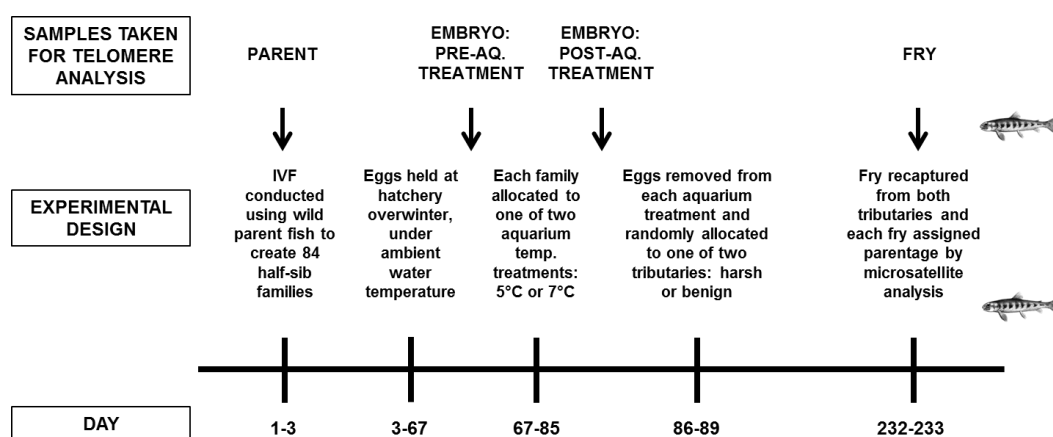


Fig. 4.2. Outline of the experimental design. Day 1 = 3rd December 2013.

The fish trap was first opened on 14th November 2013. The IVF matings for this experiment were all conducted between 3-5th December, using predominantly fish captured the same day (and always within one week of capture). Unripe fish that could not be used in IVF crosses on the day of capture were held at the trap site in dark circular tanks (4m diameter, 1.5m deep), supplied directly with water from the adjacent river, until they had reached spawning condition. It is unknown if fish of different life history strategies (i.e. 1SW and MSW) arrived at the loch downstream of the trap at different times. However, 1SW and MSW fish were both present by the time the trap was first opened, were treated in a similar way and were stripped over the same narrow time window. Sexually mature precocious male parr (i.e. small sneaker males, that had matured without going to sea)

were captured daily between 3-5th December, by electrofishing various tributaries of the River Blackwater. All captured parr were assessed for sexual maturity, and precocious males (indicated by the production of milt after abdominal squeezing) were retained for use that day in the IVF crosses.

In this experiment I used a split-brood IVF design. In each replication of the mating design the eggs of two female fish (one 1SW and one MSW) were fertilised with sperm from three male fish (one 1SW, one MSW and one precocious male parr) to produce six half-sib families which represented all possible parental crosses (Fig. 4.3). This design was repeated 14 times (using new fish each time) to produce 84 families in total. The life history of each of the anadromous parents was initially determined by size (MSW fish are generally much larger than 1SW fish (mean mass (g): MSW females = $3125 \pm \text{SE } 101$; 1SW females = $1368 \pm \text{SE } 48$; MSW males = $4199 \pm \text{SE } 158$; 1SW males = $1606 \pm \text{SE } 123$; male parr = $23 \pm \text{SE } 2$). This was subsequently confirmed by analysis of the scales taken from the flank of each fish (below the adipose fin) (Shearer 1992). The scale analysis also allowed us to retrospectively determine the number of years each parent fish had spent in fresh water prior to seaward migration, and confirmed that all parent fish were virgin spawners. There was not a strong correlation between the number of years that parents had spent in FW and SW (Pearson $r = 0.47$, $p < 0.001$). Prior to the stripping of gametes, all parent fish were anaesthetised, using a 5% benzocaine solution. The two female fish in each mating group (1SW & MSW) were blotted dry and manually stripped of their clutch of eggs. The eggs were drained of ovarian fluid and then weighed (to 0.01g) to obtain a clutch mass. For each female, a small sample of eggs (~15) was stored in 100% ethanol, for subsequent calculation of dry egg weight after overnight drying at 60°C.

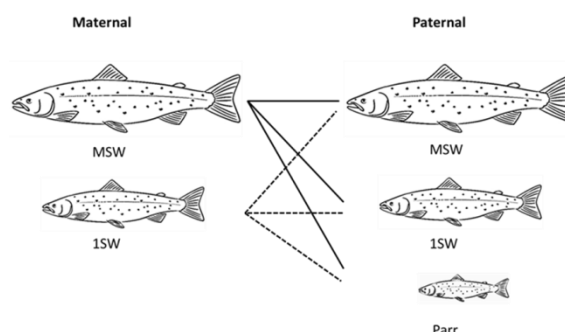


Fig. 4.3. A schematic diagram of the split-brood *in vitro* fertilisation design, utilising all possible parent types with respect to time spent in sea water. 1SW = 1 year spent in sea water, MSW = 2+ years spent in sea water and Parr = males that matured in FW and spent 0 years in sea water.

Each clutch was then divided into three approximately equal subsets. The three male fish (1SW, MSW and precocious parr) were blotted dry and manually stripped of sperm. The sperm of each male was divided in two and each half was used to fertilise one subset of eggs from each of the two females. Each batch of eggs was left for several minutes to allow sufficient time for fertilisation to occur. Afterwards, the eggs were washed in fresh water to remove any remaining sperm. Each parent fish was measured (fork length to 0.5cm; body mass to 0.1 g) and a small sample of tissue was taken from the adipose fin and stored in 100% ethanol for subsequent telomere analysis. All parent fish used in the crosses were then returned to the river, with adult salmon being returned just above the trap (so as not to be mixed up with previously un-trapped individuals) and precocious parr returned to the section of stream from which they had been electrofished (which was not fished in subsequent days to avoid recapturing the same fish).

Hatchery conditions and aquarium manipulation

Eggs were transferred to the nearby SSE hatchery at Contin where they were held for two months in separate family groups under ambient water temperatures ($3.90 \pm \text{s.d. } 0.91^{\circ}\text{C}$) until they had reached the more stable 'eyed egg' stage of development. On February 5th 2014, 10 eggs were sampled from each family for subsequent analysis of embryo telomere length, prior to the aquarium temperature treatments (see Fig. 4.2). A large and a small sub-sample of eggs ($n = 500$ and 30 eggs respectively) was then counted from each family. The small sub-samples were held as separate families in organza pouches to allow later collection of family-specific data on telomere dynamics at the embryo stage, since the larger sub-samples were to be subsequently mixed together. On February 7th 2014 each large sub-sample of eggs was allocated to one of two temperature treatments, depending on which family it belonged to: families with odd numbers (i.e. family 1, family 3 etc.) were assigned to the 5°C group and families with even numbers were assigned to the 7°C group. This resulted in each of the 84 families being assigned randomly to only one temperature treatment, so that the aquarium temperature treatment of a fish could subsequently be known by using genotyping to determine its family. For each treatment group, the large sub-samples of eggs were mixed together to create two batches of pooled eggs (~21,000 eggs per batch). The two large batches of eggs (along with the small sub-samples of each family in the organza pouches) were then transferred to the aquarium facilities at the University of Glasgow, where they were held in egg baskets in two separate dark tanks (100cm X 100cm X 40cm). The small sub-samples were allocated to their corresponding

tank (families with odd numbers to the 5°C group and families with even numbers to the 7°C group) and remained in their pouches throughout the treatment. Both tanks were initially held at 5°C, to match the ambient temperature at the hatchery facilities. The temperature in the 7°C group was slowly raised to 7°C over a two day period. Eggs were exposed to these temperature treatments for 18 days. On February 25th the small sub-samples of eggs were removed and stored in ethanol for subsequent analysis of embryo telomere length at the end of the aquarium temperature treatment period. The mixed eggs in the baskets of the two temperature treatment tanks were combined and mixed thoroughly to create a single large batch of ~42,000 eggs. This was then transferred back to the SSE hatchery at Contin, where the eggs were kept for several days under ambient water temperature conditions until being planted in the appropriate treatment streams.

Field manipulation

Two tributaries (Upper Meig and Allt Goibhre) of the River Blackwater were selected to represent a harsh and a benign stream respectively (Fig.4.1). While it is difficult to produce an absolute definition of ‘harsh’ and ‘benign’, I use these as relative terms to describe the conditions for growth in the two streams: the selected streams provided similar spawning substrate and water flow (Table 4.1), but while both showed a seasonal variation in temperature, the harsher high altitude stream (Upper Meig) was consistently colder and so further from the optimum temperature for growth (mean difference between streams = $0.72 \pm \text{SE } 0.04$ °C based on recordings logged every 4h over the duration of the experiment; see Fig. 4.4).

In addition, higher altitude tributaries often show reduced primary productivity, compared to relatively lower altitude tributaries of the same system (Elliott *et al.* 1998; Nislow, Armstrong & McKelvey 2004). Both streams contained resident brown trout, and the Upper Meig also contained older juvenile Atlantic salmon that had been stocked as eggs in previous years. Both trout and older juvenile salmon predate Atlantic salmon fry (Henderson & Letcher 2003) and are hereafter combined and referred to as ‘predators’. Subsequent electrofishing showed that the harsher Upper Meig tributary had a ~ 3.6 x greater density of these predators than the Allt Goibhre tributary (Table 4.1). There was no natural salmon spawning in either stream due to migration barriers, so the only salmon fry present were those introduced as part of the experiment.

Table 4.1: Summary of the SFCC general electrofishing habitat survey results for the two experimental streams.

	Harsh		Benign		Difference between sites		
	Mean	SE	Mean	SE	T	d.f.	p
Mean temp. (°C)	8.48	0.63	9.20	0.65	4.880	19	<0.001
Fry density (fry/m ²)	0.75	0.08	0.75	0.08	0.029	29	0.977
Parr density (parr/m ²)	0.11	0.02	0.03	0.01	3.476	25	0.002
% Depth (cm)							
0-10	34.5	4.59	27.73	6.99	0.841	29	0.408
11-20	40	4.63	48.64	4.91	-1.192	29	0.243
21-30	22.3	3.47	19.55	5.82	0.426	29	0.673
31-40	3.0	1.05	4.09	2.00	-0.533	29	0.598
41-50	0.25	0.25	0.00	0.00	0.736	29	0.468
% Substrate							
Gravel	5.5	0.80	5.9	0.91	-0.320	29	0.751
Pebble	40.25	4.61	22.27	2.06	3.560	25	0.001
Cobble	44.0	4.22	47.27	2.64	-0.540	29	0.593
Boulder	10.25	2.16	24.55	2.82	-3.987	29	<0.001
% Flow							
Riffle	41.58	6.19	45.90	8.52	-0.416	28	0.680
Run	12.89	4.59	0.91	0.91	2.560	19	0.019
Glide	44.47	5.77	50.91	8.50	-0.646	28	0.523
Pool	1.05	0.61	2.27	1.04	-1.084	28	0.288

Definitions: Gravel = inorganic particles 2-16mm. Pebble = inorganic particles 16-64mm. Cobble = inorganic particle 64-256mm. Boulder = inorganic particle >256mm. Riffle = fast, broken waves, audible. Run = fast, unbroken waves, silent. Glide = moderate/fast, smooth, silent. Pool = slow, eddy, pool. I used a Paired-Sample t test to analyse the difference in temperature at each time point between the two streams. For all other habitat variables, I used Independent-Sample t-tests to analyse the difference between streams.

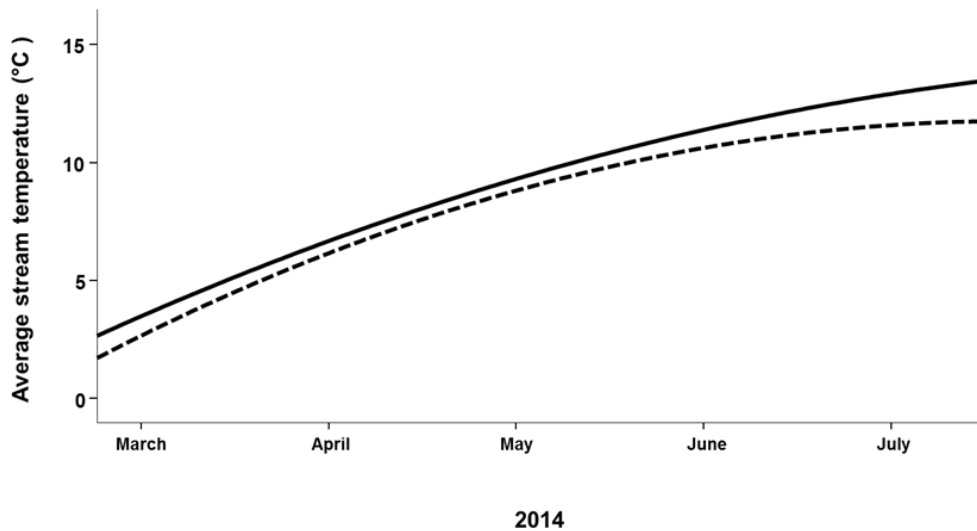


Fig. 4.4. Summary of the average temperatures in the two tributary streams over the course of the experiment. HOBO temperature data loggers were placed in each stream at the time and site of egg deposition and programmed to record data every 4h. For presentation purposes, data have been fitted to a quadratic regression line and individual data points have been removed. Solid line = Allt Goibhre (benign) stream, in which eggs were planted on February 26th and fry were recaptured on 23rd July. Dashed line = Upper Meig (harsh) stream, in which eggs were planted on March 1st and fry recaptured on July 22nd.

The pooled batch of eggs was mixed again and then divided into two new batches (~21,000 eggs per batch), with each batch assigned to one of the study streams. As a result, eggs from each of the aquarium temperature treatments were equally represented in each batch, and subsequently stream. The eggs were then planted in the benign and harsh streams on February 26th 2014 and March 1st 2014, respectively. The eggs were planted in pairs of artificial nests of c.500 eggs at 20 different sites along a section of each stream; sites were 10-20m apart, depending on stream width and the availability of suitable spawning substrate and water flow. Artificial nests were made by excavating a hole (at least 20cm deep) in clean well oxygenated substrate. A pipe (10cm diameter) was placed in the centre of the hole and substrate was replaced around the pipe, to form the basic structure of the nest. Eggs were then poured down the pipe, into the centre of the nest. The pipe was then carefully removed and more substrate was added to cover the nest. These artificial nests were similar in structure to natural nests (Aas *et al.* 2011; Jonsson & Jonsson 2011). A Vibert box (containing c.100 eggs) was used at the first, middle and last site, in addition to the two artificial nests, to assess hatching success on each river. The Vibert boxes were recovered in May 2014 and any unhatched eggs were counted. No unhatched eggs were present in any of the Vibert boxes, which indicate an excellent hatching rate for each of the streams.

Both streams were then electrofished between 22nd and 23rd July 2014 to collect samples for telomere analysis and determine fry survival and growth rates. Beginning downstream of the treatment section, successive 10 m stretches were measured out along the stream. Each section was sampled with a single electrofishing pass. All Atlantic salmon fry (which by definition were experimental fish) were euthanized with a 10% benzocaine solution and stored in 100% ethanol for subsequent body size measurements and tissue sampling in the lab. All non-experimental fish (brown trout and older Atlantic salmon) were briefly anaesthetised, measured and then returned to the stream. For each 10 m section a number of habitat parameters were measured using the SFCC general electrofishing habitat survey (see Table 4.1). Consecutive sections were electrofished and analysed in this manner until ~400 experimental fry had been caught at each site. Fry density and predator density was calculated for each electrofishing site by dividing the total number of experimental fry or predatory salmonids (older brown trout and salmon) by the surface area of that site. All preserved experimental fry were weighed (0.001g), and their fresh weight (mg) estimated from the following equation: $M_{B1} = 1.51M_{B2} + 70.69$, where M_{B1} and M_{B2} are the fresh and ethanol preserved values, respectively (equation from Burton *et al.* 2013).

Parental assignment and telomere analysis

The caudal fin was removed from each fry and sent to Landcatch Natural Selection Ltd (Stirling, Scotland) for microsatellite analysis of parentage. Genotyping was conducted using a panel of markers that they had customised for internal use. DNA was extracted from the fin clips of all parental fish and recaptured offspring using an E-Z 96 tissue DNA Tissue kit (Omega Bio-Tek, Georgia, USA) following the manufacturer's protocol. Genotyping was performed using three iplex panels, which equalled 106 informative SNPs scattered across the genome. A Sequenom MassARRAY platform (Sequenom Inc., California, USA) was used for the genotyping of the samples. Analysis for parentage assignment by exclusion was carried out with the programme Vitassign 8.3 (Vandeputte, Mauger & Dupont-Nivet 2006), with some modifications to allow the analysis of more than 100 markers. From a total of 843 fry, 822 (>97%) were uniquely assigned to a single set of parents, 8 had a Single Parental Assignment (0.95%) and 13 failed to assign, mostly due to low allele calls.

For the telomere analysis, DNA was extracted from all tissue types using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturers protocol, with a minor

modification to the lysis step for each of the tissue types. For the parental adipose fin tissue, a small sample of tissue was dissected and placed in 180µl Buffer ATL + 20µl proteinase K (20 mg/ml) solution. Samples were incubated at 56°C until the tissue was fully lysed. For the eyed embryo tissue (both pre- and post- aquarium temperature treatment), 5 embryos per family were dissected from the surrounding lipid mass, pooled and homogenised in 1ml of 1 x PBS in a 2ml tube containing a ceramic bead. 100µl of the homogenate was then added to 80µl Buffer ATL + 20µl proteinase K. Samples were incubated at 56°C until the tissue was fully lysed. For the fry tissue, the adipose fin was carefully dissected from each fry and added to 180µl Buffer ATL + 20µl proteinase K. Samples were incubated at 56°C until the tissue was fully lysed. Each set of DNA extractions conducted also included a negative control which contained all of the reagents, but without any tissue. This was used to check for contamination during the lysis and extraction steps. DNA concentration and purity was measured spectrophotometrically using a Nanodrop 8000.

Telomere length was measured in all samples using the quantitative PCR method described by Cawthon (2002), which provides a relative measure of telomere length (RTL) and is calculated as a ratio (T/S) of telomere repeat copy number (T) to a control, single copy number (S) (here the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene). The *Salmo salar* GAPDH sequence (Genbank accession number: NM_001123561) was used to design primers for the single copy gene (S) assay. Using the Genbank blast tool, the gene was compared to the Atlantic salmon genome to confirm that it was present at a single location. The following forward and reverse GAPDH primers successfully amplified a single amplicon; as determined by melt curve analysis and were subsequently used in the S assay:

SalGAP8-F 5'-GTAAGACAGGATTGAGGCATCTC-3' and

SalGAP8-R 5'-CCGAATCCATTGACACCTACTT-3'

For amplification of telomeric repeats (T assay), the universal primers designed by Cawthon (2002) were used:

Tel1b 5'-CGGTTTGTGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and

Tel2b 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'.

A primer optimisation matrix was used to determine the optimal concentration of the primers (for both S and T assay) and five different samples of Atlantic salmon DNA were tested to confirm primer performance and specificity. Telomere PCR conditions were 15 min at 95°C followed by 27 cycles of 15 secs at 95°C, 30 secs at 58°C and 30 secs at 72°C. This was followed by the melt curve profile: temperature was slowly increased from 58°C to 95°C at a rate of 0.2°C /sec. GAPDH PCR conditions were 15 min at 95°C followed by 40 cycles of 15 secs at 95°C, 30 secs at 60°C and 30 secs at 72°C. Again this was followed by the melt curve profile (same as before). PCRs were performed on a Mx3005P qPCR system (Agilent).

The telomere (T) and single copy gene (S) assays were performed on separate 96 well plates, with each sample run in triplicate for each assay. In addition to the samples, each plate also included a six-fold serial dilution of a reference sample (1.25 - 40 ng/well), a 'golden reference' sample and a non-target control (NTC). The DNA for the serial dilution was a pool of 60 samples and included all life stages (adult, embryo and fry). The serial dilution was used to generate a standard curve and calculate assay efficiencies. The 'golden standard' was a pool of DNA from 20 samples, including all life stages, used as a reference sample. The NTC contained all reaction components apart from DNA and was included on each plate (in triplicate) to check for non-specific binding and potential contamination between sample wells. Each reaction contained 12.5µl 2x Absolute Blue qPCR SYBR Green Mix low ROX (Fisher Scientific), forward and reverse primers and DNA (for wells containing sample, standards, gold reference) or water (for wells containing NTC) in a total volume of 25µl. Both T and S assays were performed using 10ng of DNA (equivalent to 6µl of the diluted samples). Primer concentrations were 500nM for the telomere assay (Tel1b and Tel2b) and 200nM for the GAPDH assay (salGAP8-F and salGAP8-R). The mean assay efficiencies for the telomere and GAPDH were 103.5% and 99.2% respectively and within the acceptable range (85-115%). The average intraplate variation of the Ct values was 1.15 for the telomere assay and 0.59 for the GAPDH assay, respectively. The average interplate variation of the Ct values was 2.59 for the telomere assay and 1.86 for the GAPDH assay, respectively. qPCR raw data was analysed using qBASE software for Windows (Hellemans *et al.* 2007). This controlled for differences in amplification efficiency between plates (assessed from the standard curve of each plate) and produced a relative quantity (RQ) of T and S for each sample. Results were scaled to the average across all unknown samples for each target (i.e. telomere and GAPDH). In addition, by including three inter-run calibrators (the golden reference and two points from the standard

curve) I corrected for further inter-run variation. Finally, I used the software to normalise each by the GAPDH RQ for that sample. Therefore, for each sample, the qBASE software produced a calibrated normalized relative quantity (CNRQ). This is similar to the T/S ratio described by Cawthon (2002) but with greater control of inter-plate variation.

Data analysis

I measured/calculated the following four dependent variables: A) fresh weight of each fry, calculated from the ethanol preserved weight values (*fry weight*); B) the percent of fry recaptured in July, calculated separately for each family in both streams (subsequently referred to as *fry survival rate*); C) embryo relative telomere length, calculated as the mean for each family based on a pooled sample of 10 embryos (*embryo RTL*) and D) fry relative telomere length, for each individual fry (*fry RTL*). All four of the dependent variables were assessed by linear mixed models (LME) that included maternal ID and paternal ID as random effects to control for non-independence of siblings.

I measured/calculated the following variables to be included as factors/covariates in analyses, where appropriate: the time point at which the embryo stage was sampled (i.e. whether before or after the aquarium temperature treatment, and subsequently referred to as *embryo time point*), the aquarium temperature treatment each family was kept under (*aquarium temperature*), the relative telomere length of mother and father (*maternal RTL* and *paternal RTL*), the number of years each parent spent in fresh water (*maternal FW age* and *paternal FW age*) and at sea (*maternal SW age* and *paternal SW age*; note that total age = FW age + SW age), average dry egg weight for each family (*egg weight*), which experimental stream a fry was reared in (*stream*), fry density for each electrofishing section within a stream (*fry density*) and predator density for each electrofishing section within a stream (*predator density*).

The Akaike Information Criterion (AIC) was used during model fitting and variables were only removed from a model if this resulted in a relative reduction of the AIC score. I used Pearson correlation coefficient matrices to assess potential collinearity between explanatory variables (with a cut-off coefficient of 0.8); however, none of the explanatory variables were identified as being collinear. I also used Pearson's correlation coefficient to assess the relationship between predator density and fry density. All statistical analyses were carried out using IBM SPSS 22 for Windows.

Table 4.2. Summary of the four full linear mixed-effects models explaining variation in: fry weight, fry survival, embryo telomere length and fry telomere length.

Model	Dependent variable	Fixed Factors	Covariates	Interactions	Random Factors
A	Fry weight	Maternal FW age Paternal FW age Maternal SW age Paternal SW age Stream Aquarium temperature	Embryo RTL Fry density Predator density Egg weight	Maternal SW age x egg weight Stream x egg weight Stream x fry density Stream x predator density	Mother ID Father ID
B	Fry survival	Maternal FW age Paternal FW age Maternal SW age Paternal SW age Stream Aquarium temperature	Embryo RTL Fry density Predator density Egg weight		Mother ID Father ID
C	Embryo RTL	Maternal FW age Paternal FW age Maternal SW age Paternal SW age Aquarium temperature Time point	Maternal RTL Paternal RTL Egg weight		Mother ID Father ID
D	Fry RTL	Maternal FW age Paternal FW age Maternal SW age Paternal SW age Stream Aquarium temperature	Maternal RTL Paternal RTL Embryo RTL Fry weight Fry density Predator density Egg weight	Fry weight x stream Fry weight x fry density Fry weight x predator density	Mother ID Father ID

Notes: Terms were then sequentially removed if not significant or if not contributing to significant interactions. Mother ID and Father ID were included as random effects to control for non-independence of siblings. See Table 2.2 for summary of the final models.

Table 4.3. Summary of the four final linear mixed-effect models explaining variation in: fry weight, fry survival, embryo telomere length and fry telomere length.

Model	Explanatory variable	Numerator df	Denominator df	F	p
A	Stream	1	763.12	1199.89	<0.001
	Fry density	1	765.17	11.34	0.001
	Egg weight	1	50.08	20.44	<0.001
B	Egg weight	1	18.53	11.66	0.003
C	Embryo time point	1	100.84	22.37	<0.001
	Paternal RTL	1	41.58	4.38	0.042
D	Stream	1	733.96	2.52	0.113
	Fry weight	1	732.79	1.22	0.269
	Fry density	1	733.14	14.53	<0.001
	Predator density	1	736.60	14.98	<0.001
	Paternal SW age	3	106.11	3.859	0.012
	Fry weight x stream	1	728.81	13.02	<0.001
	Fry weight x fry density	1	731.55	8.51	0.004

Notes: The main effects and interactions initially included in each of the models are outlined in Table 4.2. Terms were sequentially removed if not significant or if not contributing to significant interactions. Mother ID and Father ID were included as random effects to control for non-independence of half-siblings. See Methods for definitions.

4.4 RESULTS

Fry weight and fry density

As expected, fry weight (i.e. size achieved by late July, approximately two months after first feeding) differed between the two streams, being significantly greater in the more benign (and warmer) stream (Table 4.3A, Fig. 4.5). There was also a significant correlation between fry weight and fry density, with relatively smaller fry being present in areas of increased fry density (Table 4.3A, Fig. 4.6). Both fry weight and survival were positively related to initial egg weight (Table 4.3A and 4.3B, Fig. 4.7 and 4.8). There was no significant correlation between predator density and fry density, either within streams or across streams (Pearson $r < 0.30$, $p > 0.17$).

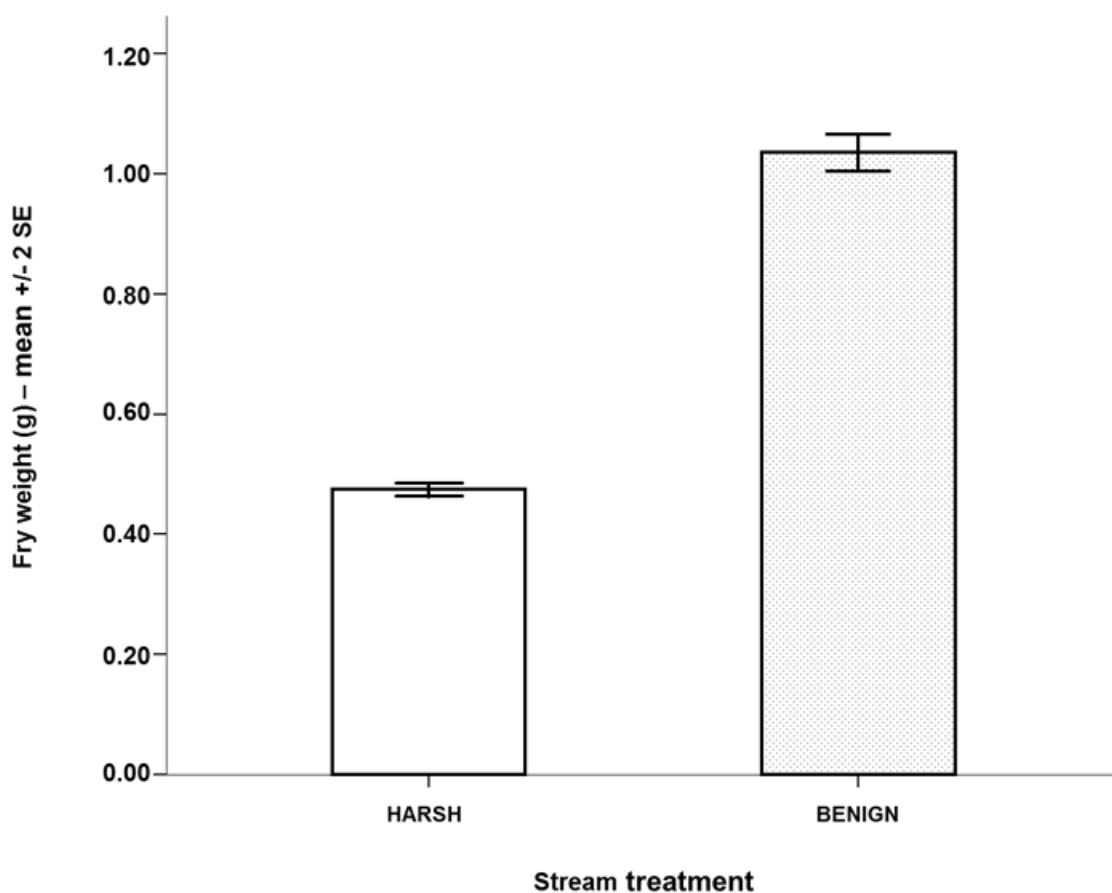


Fig. 4.5. Comparison of the mean fry weight (g) in the two streams at the time of recapture, approximately two months after first feeding.

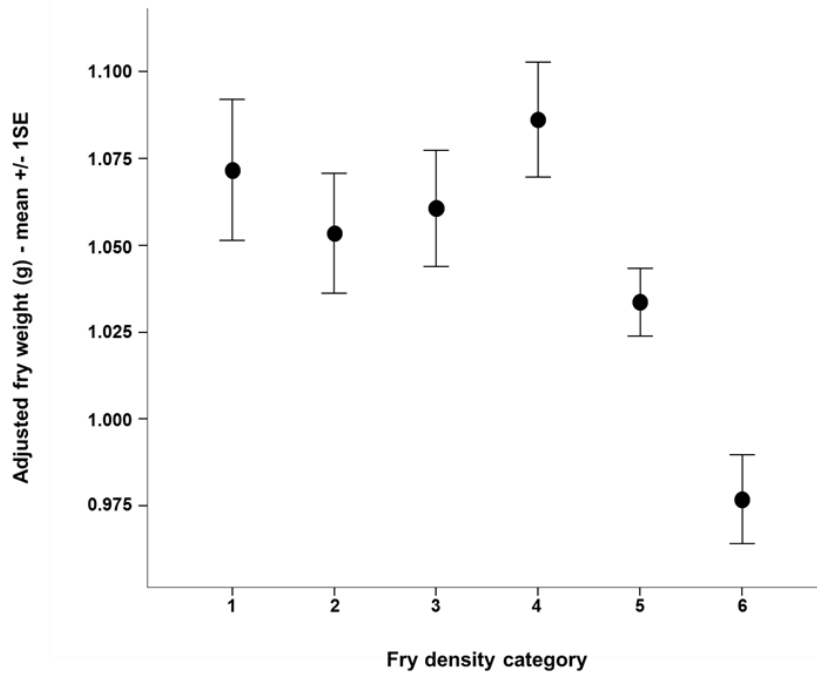


Fig. 4.6. The relationship between fry density and fry weight (g). Fry weight has been plotted as partial residuals as a function of fry density, evaluated when all other independent variables = 0. Definition of density groups (fry/m²): group 1 = <0.2, group 2 = 0.21-0.40, group 3 = 0.41-0.60, group 4 = 0.61-0.80, group 5 = 0.81-1.00 and group 6 = >1.01.

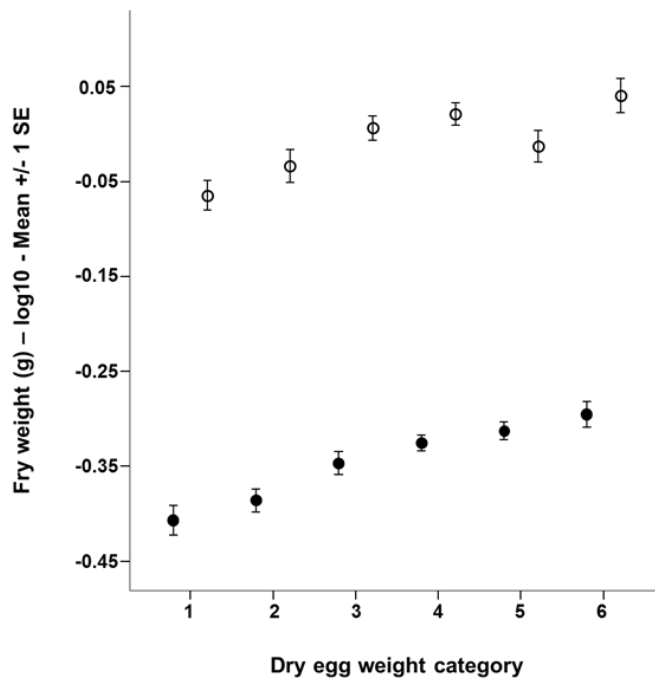


Fig. 4.7. The relationship between average dry egg weight per family (g) and subsequent fry weight (g) at the time of recapture. Closed circles = harsh stream, open circles = benign stream. Definition of dry egg weight categories (g): group 1 = 0.030-0.035, group 2 = 0.036-0.040, group 3 = 0.041-0.045, group 4 = 0.046-0.050, group 5 = 0.051-0.055, group 6 = 0.056-0.060.

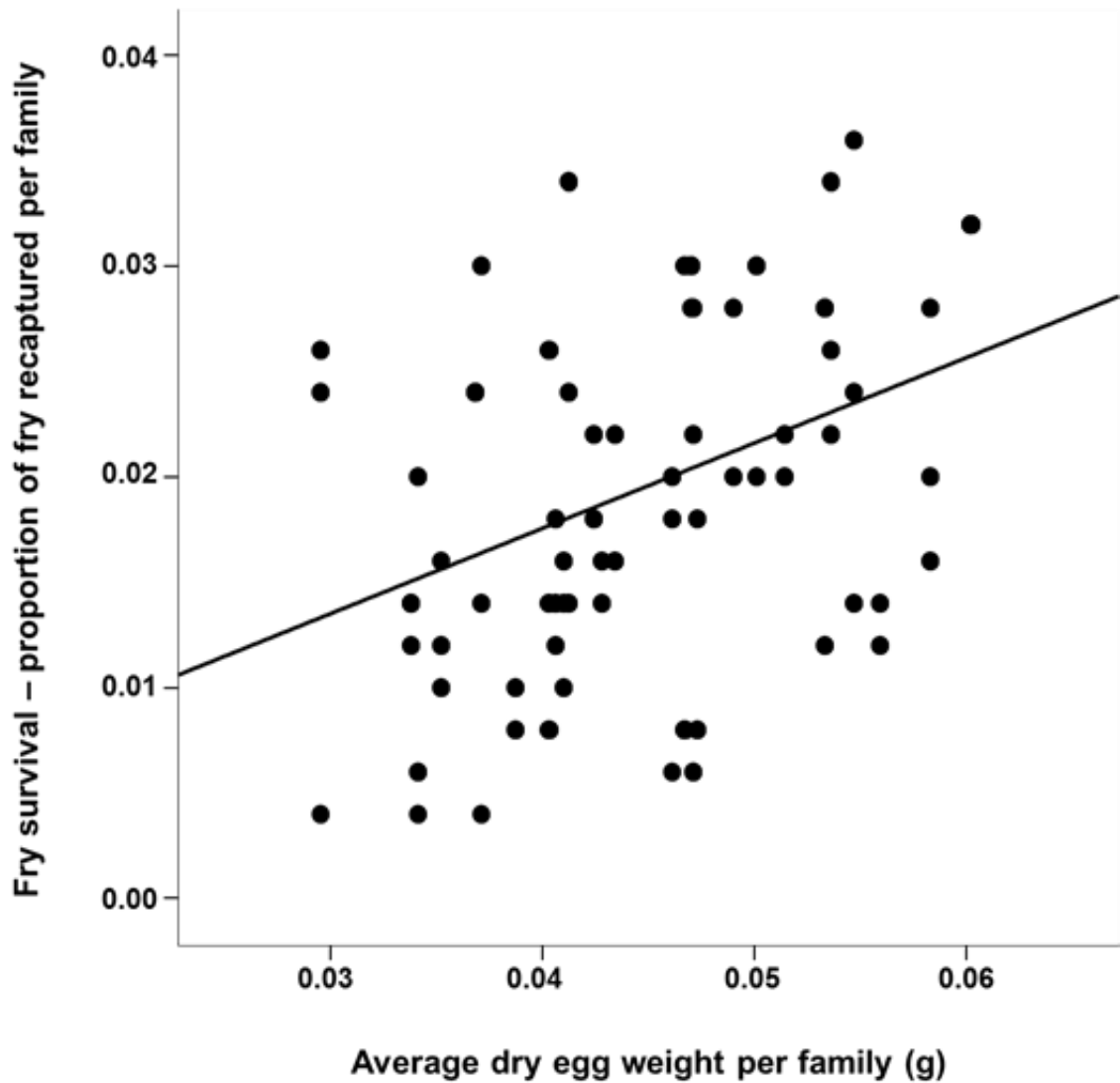


Figure 4.8. The relationship between average dry egg weight per family (g) and subsequent fry survival. Fry survival = proportion of fry recaptured in comparison to the initial number of eggs planted in the stream.

Embryo RTL

Embryo telomere length significantly increased during the 18 days of the aquarium temperature manipulation, with no difference between the two treatment groups (Table 4.3C, Fig. 4.9). Embryo telomere length was negatively correlated with paternal (but not maternal) telomere length, a pattern that was evident both before and after the temperature manipulation (Table 4.3C, Fig. 4.10).

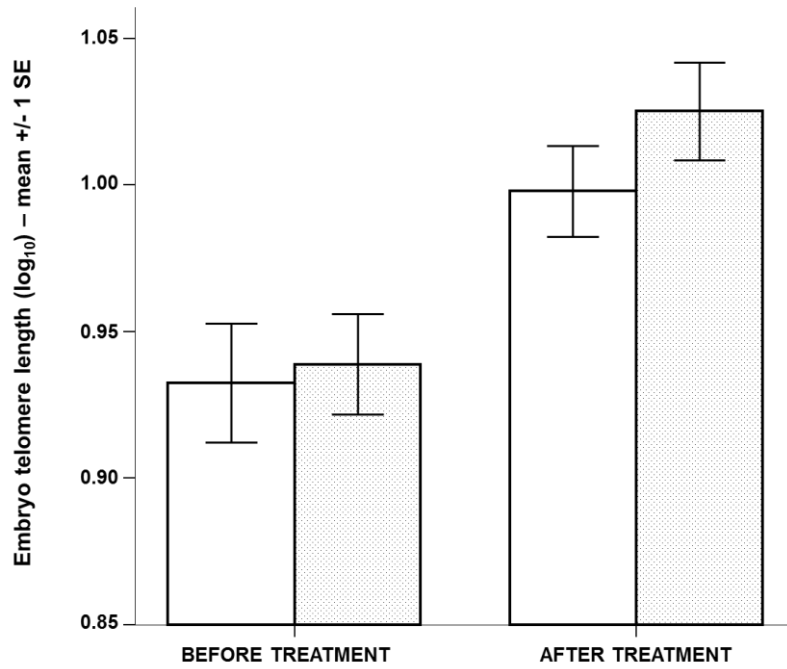


Fig. 4.9. The mean embryo relative telomere length before and after the aquarium temperature treatment. Blank columns represent the 5°C group and the patterned columns represent the 7°C group.

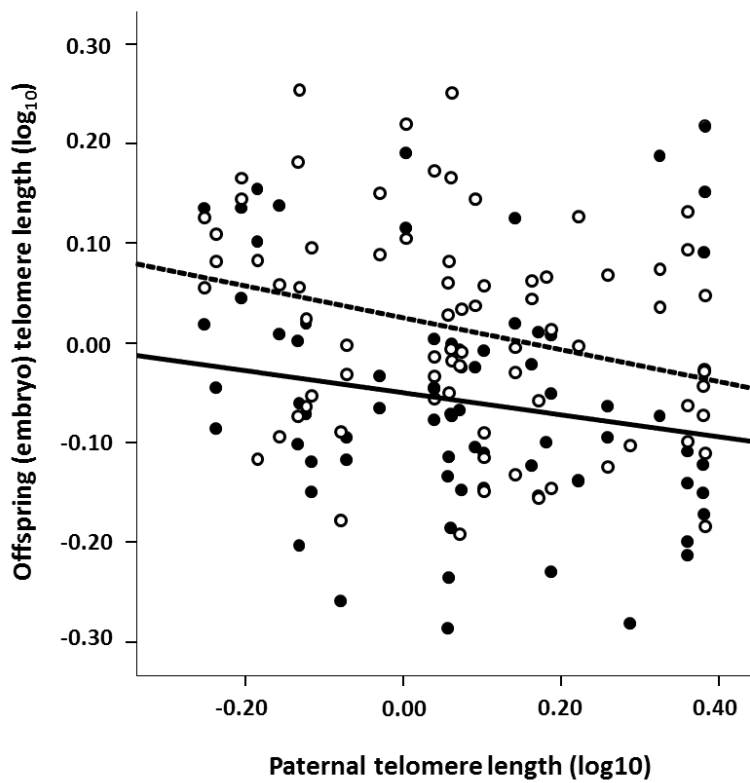


Fig. 4.10. The relationship between paternal relative telomere length and embryo relative telomere length. Solid circle and solid line = embryo telomere length prior to aquarium temperature treatment. Open circle and dashed line = embryo telomere length after aquarium temperature treatment.

Fry RTL

Telomere length in Atlantic salmon fry was negatively correlated with body size (i.e. growth rate), but this effect was dependent on the stream from which they came (significant fry body weight x stream interaction; Table 4.3D). Faster growing fry thus paid a disproportionate cost, in terms of reduced telomere length, in the harsher stream (Fig. 4.11). In addition to this, growth was also more costly in term of reduced telomere length when fry were at higher densities (significant fry body weight x fry density interaction; Table 4.3D, Fig. 4.12). In addition, fry with the longest telomeres were found in areas with the highest predator density (Table 4.3D, Fig. 4.13). Paternal SW age also had a significant effect on fry telomere length, with males that had spent longer at sea prior to reproduction producing fry with relatively longer telomeres (Table 4.3D, Fig. 4.14). However, fry telomere length was not related to either paternal FW age or maternal FW or SW ages.

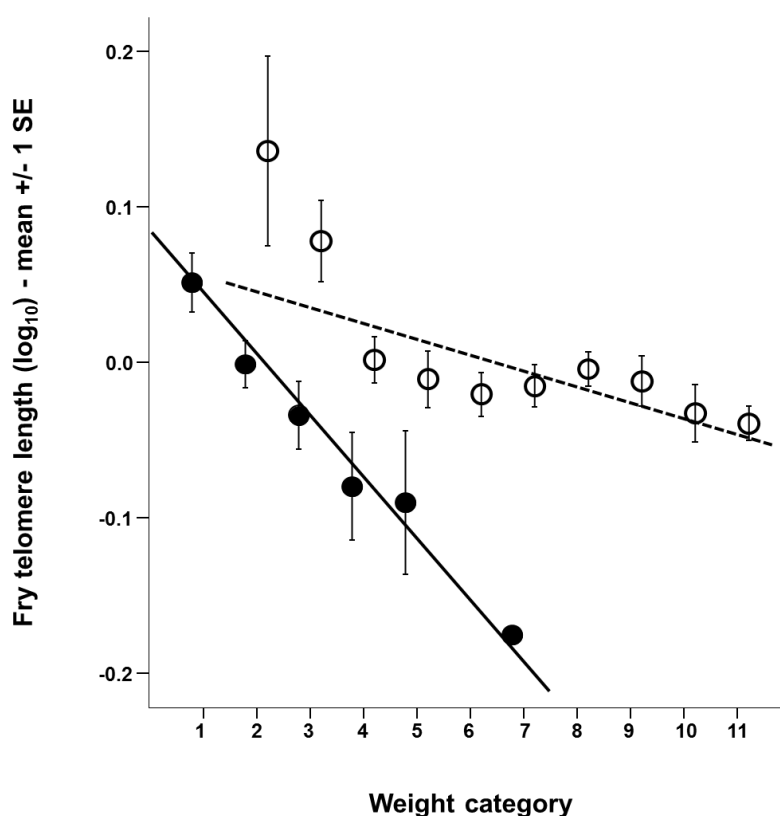


Fig. 4.11. The relationship between fry weight (g) and fry relative telomere length. Closed circles = harsh stream and open circles = benign stream. Weight data grouped for ease of presentation (analysis based on original data); definition of weight groups (g): group 1 = <0.40, group 2 = 0.41-0.50, group 3 = 0.51-0.60, group 4 = 0.61-0.70, group 5 = 0.71-0.80, group 6 = 0.81-0.90, group 7 = 0.91-1.00, group 8 = 1.01-1.10, group 9 = 1.11-1.20, group 10 = 1.21-1.30, group 11 = >1.31.

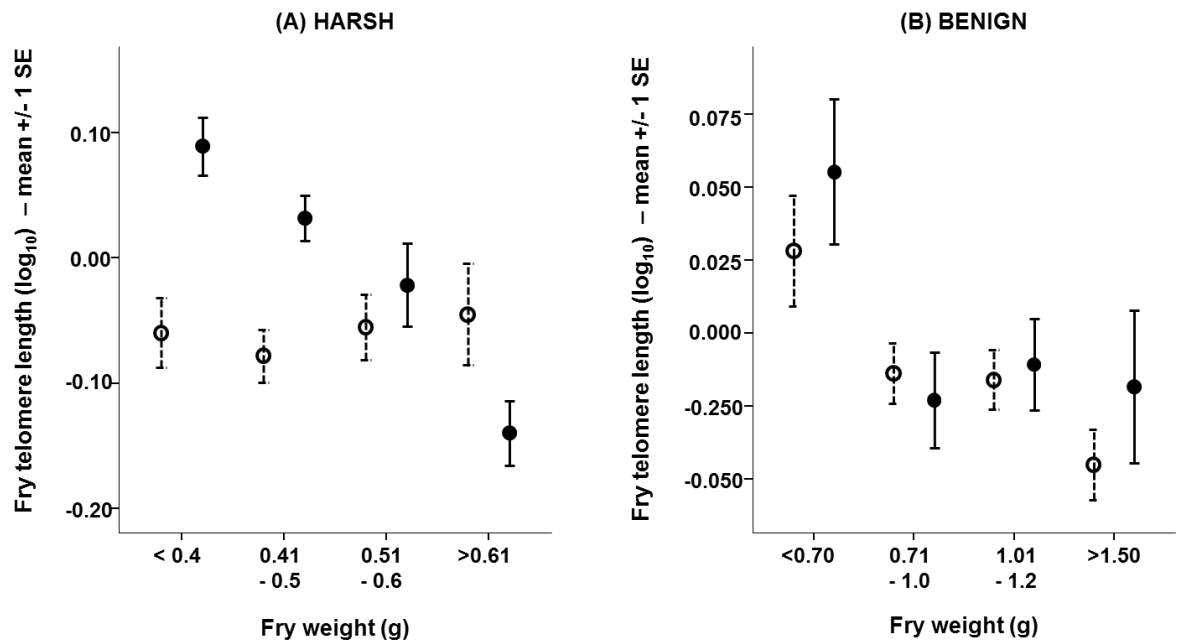


Fig. 4.12. The relationship between fry weight (g) and fry relative telomere length in (A) the harsh stream and (B) the benign stream. For presentation purposes, open circles represent fry that were captured in areas below the mean fry density (fry/m²) and closed circles represent fry that were captured in areas above the mean fry density, although analysis was based on original continuous data. Mean fry density was calculated separately for each stream.

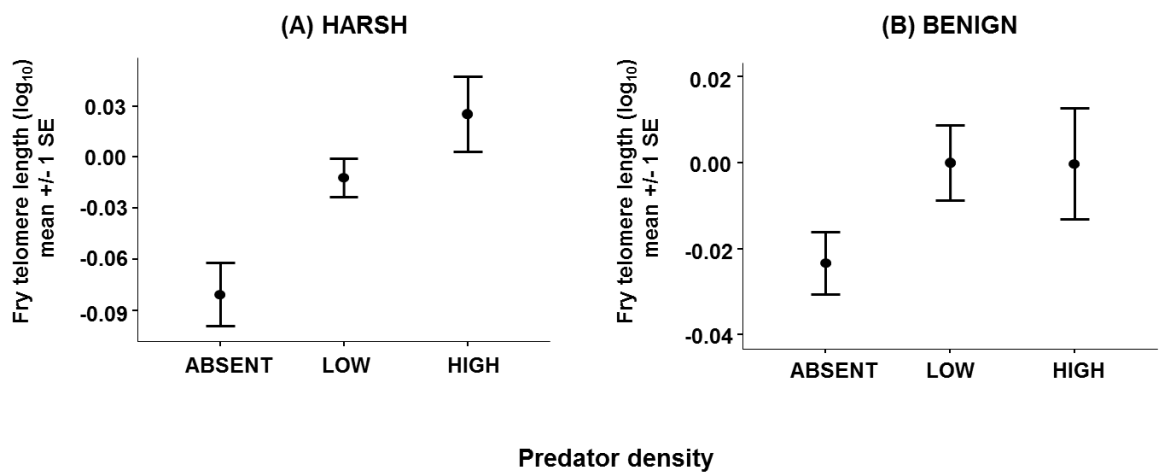


Fig. 4.13. The relationship between predator density and mean fry relative telomere length in (A) the harsh stream and (B) the benign stream. Predator density categorised for clarity of presentation, but treated as a continuous variable in the analysis. Definitions of predator density: ABSENT = 0, LOW = below the mean salmonid parr density (parr/m²), HIGH = above the mean parr density (calculated separately for each stream).

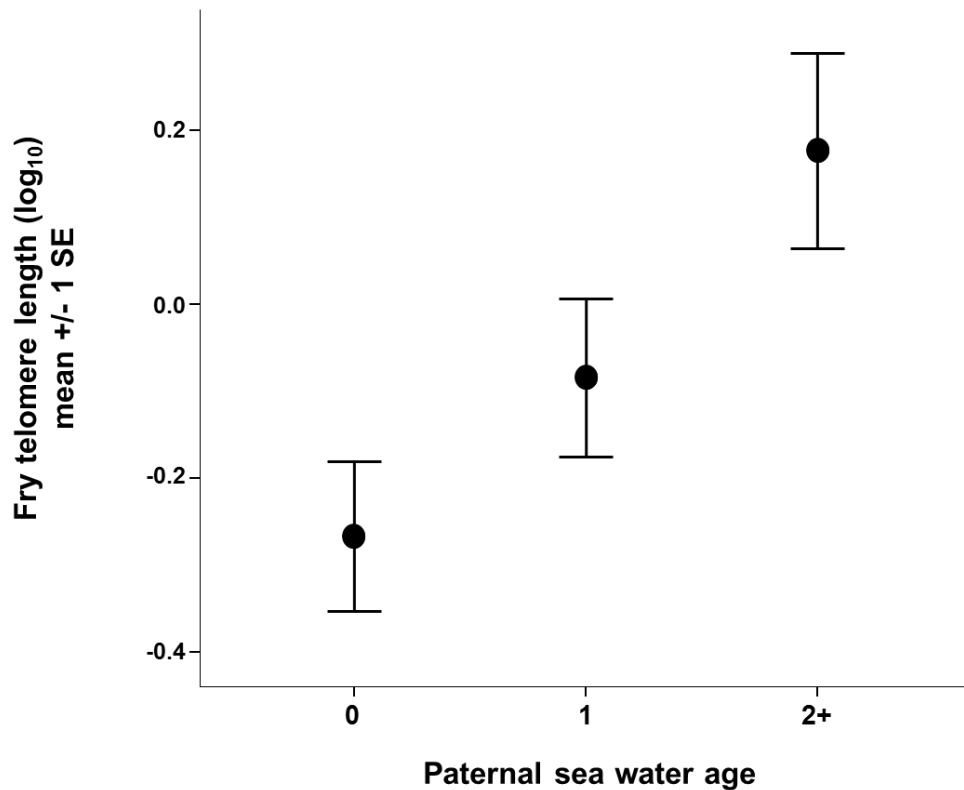


Fig. 4.14. The relationship between paternal sea water age and average fry relative telomere length. Paternal sea water age definitions: 0 = precocious parr fathers (which have not migrated to sea), 1 = 1SW fathers (which have spent 1 winter at sea), 2+ = MSW fathers (which have spent 2+ years at sea).

4.5 DISCUSSION

This study demonstrates the complexity of factors that can influence telomere dynamics in early life. I found that faster growth resulted in shorter telomeres but this effect depended on the relative harshness of the environment. I also found significant paternal effects, with males with longer telomeres producing embryos with shorter telomeres, and males that had spent longer at sea producing fry with relatively longer telomeres.

At the time of field site selection, I based the identification of the harsh and benign river systems on differences in altitude, with the rationale that a higher altitude stream would provide a harsher environment as a consequence of having a lower primary productivity (so lower invertebrate food supply for the fish) and being colder (so further from the optimal temperature for growth). The higher altitude Upper Meig tributary was colder, and also had a greater density of predators, than the Allt Goibhre tributary. Together, these

factors contribute to making the Upper Meig a harsher environment for growth for young salmon than the Allt Goibhre. As predicted, fry growth rate was faster in the more benign stream. It is possible that the difference in growth rate between the streams was also partially due to the difference in predator density, since an increased predator density may result in reduced foraging (Ward, Nislow & Folt 2011). Fry density had a negative effect on fry growth rate in both streams, as would be expected since density-dependent growth is well established in fish (for salmonid example see Jenkins *et al.* 1999).

Interestingly, embryo telomere length increased during the 18 day aquarium temperature manipulation, between the age of ~65 days and ~83 days post fertilization. Telomerase is often upregulated during various stages of embryogenesis (e.g. Mantell & Greider 1994; Schaetzlein *et al.* 2004), most likely due to the high cell proliferation rate at this stage (for review see Gomes, Shay & Wright 2010). This may also help explain why I did not see a difference in telomere length between the two temperature treatments, since a higher telomerase expression may help buffer any possible environmental effects. It may also be the case that the 18 day manipulation was too short a treatment period to produce any effect, especially if there is little embryonic variance in growth rate.

Telomere length in Atlantic salmon fry was negatively correlated with mass gain, but the effect was dependent on the stream they inhabited, with a shorter telomere length for a given mass gain in the harsher stream. This suggests that faster growing fry pay a higher cost in terms of telomere length in harsher environments. There are several mechanisms that might individually or collectively underlie this effect. Firstly, environmental temperature may affect trade-offs between cell division (hyperplasia) and cell growth (hypertrophy) in fishes (reviewed by Arendt 2007). Only hyperplasia has direct consequences for telomeres, since telomere loss occurs at each cell division. In Atlantic salmon, several studies have found warm-incubated hatchlings to have fewer but larger muscle fibres, indicating that hyperplasia is having a relatively smaller contribution towards growth when salmon develop in warmer conditions (Stickland *et al.* 1988; Usher, Stickland & Thorpe 1994). A possible cause of this effect is that dissolved oxygen is lower at warmer water temperature, which will limit an individual's capacity for cell proliferation (Matschak *et al.* 1997). Therefore, fish developing in warmer water may achieve somatic body growth most efficiently by increasing the size of their existing cells (hypertrophy).

Temperature may also affect the trade-off between growth and self-maintenance: individuals that allocate greater resources to growth will have a reduced allocation of resources to self-maintenance, e.g. tissue repair and antioxidant production (Zera & Harshman 2001). Temperature has been shown to affect components of the energy budget in the closely-related brown trout (*Salmo trutta*), with the amount of energy allocated to body materials (i.e. body growth and body maintenance) decreasing either side of the temperature at which growth is maximised (Elliott 1976). The optimal temperature for growth in juvenile Atlantic salmon is thought to be around 16°C (Elliott & Hurley 1997) which was only just reached by the warmer (benign) stream by mid-summer (the end of the experiment). Therefore, while neither stream provided ideal conditions for growth, the thermal conditions in the harsh stream were further from the optimum. For a given temperature (and food intake) body growth rate can only vary through an alteration of the relative amount of energy allocated to body growth vs body maintenance (McCarthy, Houlihan & Carter 1994). Therefore, achieving growth at a relatively colder temperature may come at a cost to body maintenance. A reduction in body maintenance (e.g. protein and DNA repair) could in turn affect telomere dynamics.

Ambient temperature may play an additional role in the telomere dynamics of ectotherms, since it can affect the exposure of telomeres to oxidative damage through its effect on mitochondrial functioning. Telomeric DNA is vulnerable to oxidative damage from Reactive Oxygen Species (ROS) and most ROS are produced in the mitochondria, which are affected by temperature in several ways. For example, it is often the case in endotherms that mitochondrial density increases as a phenotypic response to lowered temperature (for reviews see Guderley 2004; O'Brien 2011). In ectotherms mitochondrial respiration rate (and hence ATP production) decreases at lower temperatures (Schulte 2015), and it may be that an individual compensates by increasing mitochondrial density, thus helping to maintain ATP production (O'Brien 2011). An increase in mitochondrial density may also result in increased ROS production (Crockett 2008). Therefore, fish growing in colder environments might be exposed to more ROS and hence have more telomere attrition.

In addition to identifying a growth-environment effect on telomere length between the two streams, I also found growth-environment effects within streams. Electrofishing was conducted within 10m long sections and for each section I was able to calculate fry density. Although overall fry density did not differ significantly between the two streams, there was sufficient variation in density between the different sections within each stream

to reveal a significant growth-fry density effect on telomere length. In the benign stream, fry density had no significant effect on the relationship between fry weight and fry telomere length. However, in the harsh stream, it was beneficial in terms of telomere length for small, but not large fry to be at higher densities. The contrast between streams may reflect resource availability, with the harsher high altitude stream likely being more oligotrophic with reduced food availability. There were also more predators in the harsher stream. Therefore, while high densities may confer benefits to smaller fish from increased predator protection, one would expect increased intraspecific competition for resources (e.g. food) as individuals grow larger. Previous studies in other taxa have linked density to physiological stress (Montero *et al.* 1999; Trenzado, Morales & de la Higuera 2006) and telomere attrition (Kotrschal, Ilmonen & Penn 2007; Sohn *et al.* 2012). However, these studies have mostly involved experimental manipulations involving extreme differences in density (crowded vs non-crowded), whereas here the effect was found among naturally-occurring variation in density over relatively small spatial scales. Overall, the results are consistent with the hypothesis that growth in more challenging environments comes at a greater cost to telomere length.

There was also a significant predator density effect: areas of higher predator density within each stream were associated with increased fry telomere length. This is somewhat surprising since predation risk has previously been linked to a number of physiological stress parameters (Hawlena & Schmitz 2010; Clinchy, Sheriff & Zanette 2013) including oxidative stress (Slos & Stoks 2008; Janssens & Stoks 2013) and telomere loss (Olsson *et al.* 2010). However, predatory salmonid parr will exhibit greater movement within a stream compared to the limited movement of salmon fry. Therefore, my assessment of predatory parr density may only be a snapshot of parr distribution at the time of electrofishing. With this in mind, a more controlled laboratory manipulation is needed to elucidate potential predator effects on physiological stress pathways and telomere dynamics. The predator densities recorded here may actually have been an index of environmental quality. Predator distribution is partially dependent on prey abundance, and fry abundance would be expected to be greater in good quality habitat (e.g. plentiful shelter, food availability etc.). Therefore, better quality habitats within each stream may be simultaneously producing healthier offspring, with an associated reduction in telomere attrition, while at the same time attracting more predators due to the numbers of fry. Thus a greater local predator density could actually provide a more benign environment for surviving fry by reducing fry densities and hence intra-cohort competition, although the

situation is complicated by the evidence that fry and older fish may at times compete for the same resources (Kaspersson, Höjesjö & Bohlin 2012). Notwithstanding this possibility, I did not detect a significant correlation between predator density and fry density.

Offspring telomere length was not affected by any of the maternal traits included in the models. In contrast, it was significantly influenced by paternal telomere length and paternal life history. There was a negative relationship between paternal telomere length and offspring (embryo) telomere length. This effect was evident both before and after the temperature manipulation, but the reasons for it are unclear. Since there were no maternal effects, and I used an *in vitro* fertilisation design, assortative mating is not involved in generating this pattern, and it might possibly relate to some differences in telomerase activity during the embryo stage, but this would require further investigation.

There was a positive relationship between the number of years that fathers (but not mothers) had spent at sea (ranging between 0 and 2) and offspring (fry) telomere length. A number of studies report significant paternal inheritance of telomere length, mostly in humans (De Meyer *et al.* 2007; Njajou *et al.* 2007) but also in lizards (Olsson *et al.* 2011a). This effect is often attributed to paternal age, since telomere length has been shown to increase with age in human sperm cells (Allsopp *et al.* 1992). This may help explain the paternal effect in Atlantic salmon since, on average, MSW males will be the oldest and precocious parr will be the youngest. However, no effect was found of time spent by fathers in fresh water, and these paternal life history variants differ in many other ways than just age. Perhaps the most striking difference is in body size: MSW males used in this experiment were >2.5 times heavier than 1SW males and almost 200 times heavier than the precocious= parr. If the larger fathers produce offspring that grow faster, their offspring might have been expected to have shorter telomere lengths; this pattern of reduced telomere length in larger individuals has been reported, for example in birds (Ringsby *et al.* 2015). However, my results suggest the opposite pattern. This variation in size and associated growth rate may be associated with differing levels of telomerase expression. A number of fish studies have detected telomerase activity in various adult tissues (Klapper *et al.* 1998; Hatakeyama *et al.* 2008; Gomes, Shay & Wright 2010). It has also been reported that telomerase expression in fish is positively correlated with cell proliferation (Yap *et al.* 2005; Peterson, Mok & Au 2015). In fish, muscle fibre recruitment continues beyond embryogenesis (Weatherley, Gill & Lobo 1988) and active cell proliferation is still detectable in Atlantic salmon at least 6 months after transfer to sea water (Johnston *et al.*

2003). Therefore, it is possible that the higher growth rate of MSW fish results in a greater expression of telomerase, which in turn may affect germline telomere dynamics, and subsequently offspring telomere dynamics.

This study demonstrates the complexity of parental and environmental factors that can influence telomere dynamics in early life, but highlights the fact that paternal effects may be stronger than maternal, and that growth in harsher conditions has greater costs in terms of telomere length. It may be that individuals growing in harsher early environments are required to invest more in telomere maintenance at a later stage, if they are to avoid an acceleration of senescence. However, we now need to examine which features of a harsher environment have the greatest impact on telomere dynamics. Doing so will allow more accurate predictions of the effect of environmental conditions on patterns of senescence.

CHAPTER 5. Cell proliferation and oxidative stress as possible mechanisms underlying incubation temperature effects on embryonic telomere dynamics in Atlantic salmon

5.1 ABSTRACT

Environmental conditions experienced in early life can have permanent effects on individual phenotypes. In the case of ectotherms, such as fish, fluctuations in environmental temperature can influence myogenic processes, morphological development and growth rate. Growth is a consequence of cell division (hyperplasia) and/or cell growth (hypertrophy), and in ectotherms, the balance between them is known to be affected by environmental temperature. In addition, fluctuations in temperature may alter metabolic processes, and therefore the production of reactive oxygen species (ROS). Both cell division and ROS production can have negative consequences for telomeres that cap the end of eukaryotic chromosomes and play an important role in chromosome protection. A relatively short telomere length is indicative of poor biological state, including poorer tissue and organ performance, reduced potential longevity and increased disease susceptibility. In this study, I reared Atlantic salmon *Salmo salar* embryos at three water temperatures in order to test two hypotheses. Firstly, that variation in water temperature at the embryo stage of ectotherm development will have a significant effect on oxidative damage to DNA, antioxidant activity and cell proliferation dynamics. Secondly, that variation in levels of oxidative DNA damage, antioxidant activity and cell proliferation rate will partially explain the relationship between incubation temperature and telomere length in early stage Atlantic salmon. Telomere lengths in salmon embryos were found to be significantly affected by the temperature of the water in which they were living. Embryos reared at the coldest temperature (i.e. the least developed at the time of sampling) had the longest mean telomere length, while embryos reared at the warmest temperature (the most developed) had the shortest telomeres. However, there was no evidence to support the hypothesis that oxidative DNA damage, catalase antioxidant activity and cell proliferation rate are underlying mechanisms linking incubation temperature and telomere dynamics.

5.2 INTRODUCTION

Environmental conditions experienced in early life can have permanent effects on individual phenotypes (Lindström 1999; Monaghan 2008). Such effects might increase or decrease fitness, depending on whether the early life conditions were relatively poor or good. For example, relatively poor environmental conditions during growth have been linked to reduced reproductive performance (Ohlsson *et al.* 2002; Spencer *et al.* 2003; Auer *et al.* 2010; Lee, Monaghan & Metcalfe 2012), reduced adult body mass and dominance (Royle, Lindström & Metcalfe 2005; Douhard *et al.* 2013), as well as negatively impacting adult health (Gluckman *et al.* 2008). In addition, individuals that achieve early life growth in poorer environmental conditions may then experience reduced longevity and an accelerated pace of deterioration in later life (Metcalfe & Monaghan 2001; Metcalfe & Monaghan 2003; Tung *et al.* 2016) and studies have shown that telomere dynamics might be one route whereby this occurs (Monaghan & Haussmann 2006; Bize *et al.* 2009; Heidinger *et al.* 2012; Stier *et al.* 2014b).

Telomeres cap the ends of eukaryotic chromosomes and play an important role in chromosome protection (for reviews see Blackburn 1991; Campisi *et al.* 2001; Monaghan 2010). Within a species, variation in telomere length has been linked to physiological state. In general, a relatively short telomere length is indicative of relatively poor biological state, which may be linked to reduced longevity as a result of age-related deterioration and/or increased disease susceptibility (Haussmann, Winkler & Vleck 2005; Aubert & Lansdorp 2008; Ilmonen, Kotrschal & Penn 2008; Heidinger *et al.* 2012; Stier *et al.* 2015). Telomere loss occurs at each round of cell division as a result of the ‘end replication problem’, but the amount of loss is also influenced by levels of oxidative damage to the telomeric DNA (Chan & Blackburn 2004). This is partially because telomeric DNA has a high guanine content, which is particularly susceptible to oxidative damage and is hard to repair. The enzyme telomerase is thought to be the most common means by which shortened telomere ends are restored. However, many mammal and bird species appear to down-regulate telomerase expression in post-embryonic somatic tissue and instead use replicative ageing as a tumour suppression mechanism (Gomes, Shay & Wright 2010). In contrast, a number of studies in fish have detected somatic telomerase activity at post-embryonic stages (Klapper *et al.* 1998; Hatakeyama *et al.* 2008; Gomes, Shay & Wright 2010), suggesting that there can be taxonomic variation in telomere length regulation.

While there is evidence that telomere length is partially inherited (for examples see Nordfjall *et al.* 2005; Njajou *et al.* 2007; Horn *et al.* 2011; Olsson *et al.* 2011a; Asghar *et al.* 2015a; Reichert *et al.* 2015), it is also becoming clearer that changes in telomere length are strongly influenced by environmental conditions. In the case of ectotherms, such as fish, environmental temperature may play a significant role in telomere dynamics, since this affects many aspects of their biology. This is especially true during embryonic development, when fluctuations in temperature can influence myogenic processes, morphological development, growth rate and metabolism, all of which can have permanent long-term phenotypic effects (Johnston 2006; Jonsson & Jonsson 2011); although their impact on telomere dynamics is not known. With this in mind, I aim in this study to assess the relationship between temperature and telomere length during embryonic development in Atlantic salmon *Salmo salar*, while simultaneously exploring the potential underlying mechanisms.

Growth is a consequence of cell division (hyperplasia) and/or cell growth (hypertrophy), and in ectotherms the balance between them is known to be affected by environmental temperature (reviewed by Arendt 2007). Only hyperplasia could have potential consequences for telomeres, since telomere loss occurs at each cell division. In Atlantic salmon, several studies have found that incubating eggs at warmer temperatures produces hatchlings that have fewer but larger muscle fibres, indicating a shift in the balance from hyperplasia to hypertrophy (Stickland *et al.* 1988; Usher, Stickland & Thorpe 1994). A possible suggestion for this effect is that dissolved oxygen (DO) reduces with increasing water temperature, which will limit an individual's capacity for cell proliferation (Matschak *et al.* 1997). Therefore, embryos developing in warmer water appear to be achieving growth by disproportionately increasing the size of their existing cells (hypertrophy) – which might result in having longer telomeres for a given body size than conspecifics developing in colder water.

Temperature may also have an indirect effect on telomere dynamics, through its effect on Reactive Oxygen Species (ROS). ROS occur naturally as a by-product of mitochondrial activity. If left unquenched, ROS may cause oxidative damage of biological molecules, most importantly nucleic acids, proteins and lipids; referred to as oxidative stress (Halliwell & Gutteridge 2015). There is an antioxidant system in place to deal with the baseline production of ROS, but ROS levels may change with physiological state, work rate or levels of environmental stressors. An individual may respond to changing ROS

levels by altering the concentration or activity of key antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), found across the vertebrates, including fish (Rudneva 1997). However, levels of antioxidants may be insufficient to prevent ROS, causing oxidative damage (and consequent shortening) of telomeres; and it has been suggested that ROS act as the underlying mechanism that links environmental stressors and changes in telomere length (von Zglinicki 2002; Geiger *et al.* 2012; Kim & Velando 2015). Studies have also linked an increase in temperature to increased ROS production (Abele *et al.* 2002; Bae *et al.* 2016), higher antioxidant defences (Hemmer-Brepson *et al.* 2014) and variation in telomere length (Rollings, Miller & Olsson 2014).

Most ROS are produced in the mitochondria. Variation in temperature can influence the mitochondrial properties of ectotherms in several ways. For example, it is often the case that mitochondrial density increases as a phenotypic response to lowered temperature (for reviews see Guderley 2004; O'Brien 2011). This may be because mitochondrial respiration rate decreases at lower temperatures (Schulte 2015), and so an increased mitochondrial density may be necessary to maintain ATP production and hence aerobic respiration (O'Brien 2011). However, such temperature-related variation in mitochondrial density may also result in changing rates of ROS production (Crockett 2008).

Temperature may also affect the trade-off between growth and self-maintenance: for a given temperature, allocation of more resources to growth will potentially result in a reduced allocation of resources to self-maintenance, including antioxidant production (Zera & Harshman 2001). Elliott (1976) reported that the amount of energy allocated by fish to the soma (i.e. body growth and body maintenance) decreases with temperature. Therefore to achieve a given body size at relatively colder temperatures would require a shift in the resources allocated to growth vs maintenance (McCarthy, Houlihan & Carter 1994): achieving growth at a relatively colder temperature may come at a cost to body maintenance (i.e. antioxidant defences and oxidative repair), which may in turn affect telomere dynamics.

In this experiment I test two hypotheses. Firstly, that variation in water temperature at the embryo stage of ectotherm development will have a significant effect on oxidative damage to DNA, antioxidant activity and cell proliferation dynamics. Secondly, I hypothesise that variation in levels of oxidative DNA damage, antioxidant activity and cell proliferation rate will partially explain the relationship between incubation temperature and telomere

length in early stage Atlantic salmon. More specifically, I predict that siblings reared at a relatively colder temperature will experience increased cell proliferation and oxidative damage, both of which will cause greater reductions in telomere length. To test these hypotheses, I created IVF families from wild caught parent Atlantic salmon, which allowed me to rear sibling embryos at 3 contrasting temperatures within the natural range. Individuals were then sampled at two time points (the eyed embryo stage and the first feeding fry stage) in order to compare telomere lengths, levels of antioxidants/oxidative damage and rates of cell proliferation.

5.3 METHODS

In vitro fertilisation

In vitro fertilization (IVF) of the experimental fish was conducted on December 8th 2014. All parent fish came from the River Blackwater system, northern Scotland (57° 60'N, 4°63'W). Anadromous (sea-migrating) parent fish returning from sea were captured at the Loch na Croic fish trap whilst undertaking their return spawning migration. The trap was opened several weeks prior to the commencement of IVF. Any captured, unripe fish were held at the trap site in dark circular tanks (4m diameter, 1.5m deep), supplied directly with water from the River Blackwater, until they had reached spawning condition. Spawning condition was assessed in the female fish by gently squeezing the sides of the abdomen to detect the presence of loose eggs within the body cavity. All adult males had reached spawning condition (indicated by the production of milt after abdominal squeezing) at the time of capture. In this experiment I used a single-brood IVF design, utilising only adult anadromous parents that had spent 1 winter at sea (1SW) before returning to the river to spawn. The sea age of each selected parent fish was initially determined by size: 1SW fish are generally much smaller than those fish that migrate to sea for multiple winters (MSW). Sea age was subsequently confirmed by scalimetry, where the circuli of a fish scale are analysed and used to determine the various stages of ontogenic growth (Shearer 1992). The scalimetric analysis confirmed that putative 1SW parents had been correctly identified by their size, with all chosen parental fish having spent 1 winter at sea. Prior to the stripping of gametes, all parent fish were anaesthetised, using a 5% benzocaine solution. For each family, a female was blotted dry and stripped of her egg clutch. The eggs were drained of ovarian fluid and then weighed (to 0.01g) to obtain a clutch mass. A sample of eggs (corresponding to ~75ml, or approximately 150 eggs) was removed from each clutch of

eggs, to be used in the experiment. The rest of the clutch was used for stocking purposes by the hatchery. For each female, a randomly chosen 1SW male was then blotted dry and stripped of sperm to fertilise the experimental sample of eggs. This design was repeated 22 times (using new fish each time) to produce 22 independent full-sib families. After mixing the sperm and eggs, each batch of eggs was left for several minutes, to allow sufficient time for fertilisation to occur. Afterwards, the eggs were washed in fresh water (sourced from the loch) to remove any remaining sperm. The fertilised eggs were then placed in fresh water and remained at the field site for at least 1 hour, to promote water hardening (the stage at which the eggs absorb water and become firm). Each parent fish was measured (fork length to 0.5cm; body mass to 0.1 g). All parent fish used in the crosses were then returned to the river, just upriver of the Loch na Croic trap.

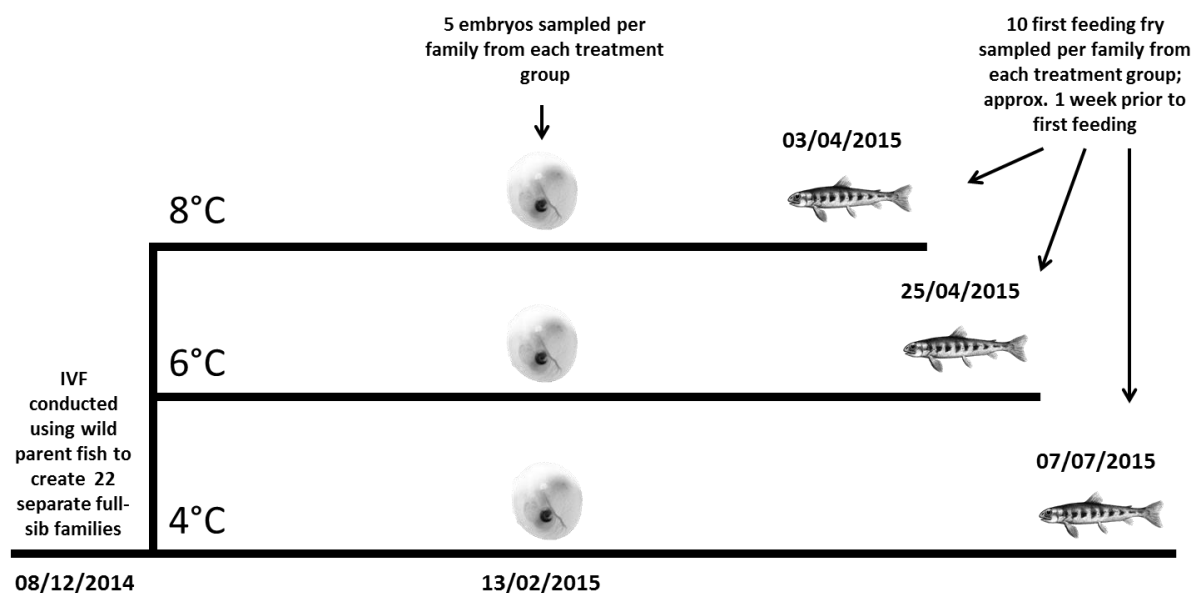


Fig. 5.1. Outline of experimental timeline. All experimental crosses were conducted on December 8th 2014.

Aquarium temperature treatment

Several hours after fertilisation, the experimental batches of eggs were transferred to the aquarium facilities at the University of Glasgow, Scotland. Each of the 22 families were divided into 3 equal batches (~25ml each) and each batch was allocated to a temperature treatment group (4°C, 6°C or 8°C). For each treatment group, eggs were held in egg baskets (one per family) distributed randomly within a tank (100cm X 100cm X 40cm). All treatment groups were initially held at 4°C, to represent ambient water temperature in the

native stream. The 6°C and 8°C group were then slowly increased from 4°C to their target temperature at a rate of 0.5°C/day. Water temperatures within each of treatment tanks were held at the required temperature using automated water chillers (Teco Tr25, Ravenna, Italy). During this time, both water temperatures and eggs were checked daily and any dead individuals were removed. On February 13th 2015, by which time all families had reached the eyed embryo stage (the stage at which the eyes of the developing embryo can be seen through the semi-transparent membrane; subsequently referred to as the *embryo* stage), five individuals were sampled per family from each of the 3 treatments and were flash frozen for subsequent analysis of embryo telomere length and oxidative stress (OS) markers. Due to the temperature manipulation, embryos were all the same chronological age (days since fertilisation) but at different developmental stages at the time of sampling, with the warmest treatment having the most advanced development (Fig. 5.1). The remaining eggs were allowed to continue development, through the alevin stage (at which they have hatched from the surrounding egg membrane but are still absorbing yolk from an attached yolk sac) until they reached the first feeding fry stage (subsequently referred to as the FF fry stage). This was identified by regularly checking the amount of remaining yolk sac until only a minimal amount remained, following the approach of Metcalfe and Thorpe (1992). When all families within a given treatment had reached the first feeding stage, 10 individuals per family were sampled, euthanized and fork length and body mass were measured. After measurement, five of these individuals were stored in 10% formaldehyde solution for subsequent immunohistochemical analysis and the remaining 5 were flash frozen for subsequent analysis of telomere length and OS markers. This happened for the 8°C, 6°C and 4°C group on 3rd April, 25th April and 7th July 2015 respectively. As a result, the FF fry samples were all at the same developmental stage, but differed in chronological age (due to the temperature manipulation), with the warmest treatment being the first to reach the first feeding stage (see Fig. 5.1). The samples fixed in 10% formaldehyde were transferred to 70% ethanol, three days after sampling, for long term storage at room temperature.

DNA and protein extractions

For the embryo and FF fry tissue, the flash frozen samples were slowly thawed in ice-cold PBS. Embryos were homogenised using a disposable tissue grinder and FF fry were homogenised in 1ml of ice cold 1 x PBS in a 2ml tube containing a ceramic bead. The homogenates of both embryo and FF fry were then passed through a 100µm mesh cell

strainer; along with ice-cold PBS to a total volume of 50ml. Samples were left for 1 minute, to allow larger aggregates of tissue to settle at the bottom of the flask. For each sample, 3ml of supernatant (containing the single cell suspension) was then transferred to a clean 15ml centrifuge tube. A further 5ml of supernatant was transferred to a second, clean 15ml centrifuge tube. Both samples per individual were then centrifuged at 500 x g for 10 minutes at 4°C. The supernatant was then discarded and both tubes containing pellets were held on ice until ready for extraction. For the protein extraction, the cell pellet derived from the 5 mls of cell suspension was re-suspended in 10x the volume of ice-cold protein extraction buffer (containing 1% Triton X-100, 0.4mM PMSF and complete protease inhibitors) and incubated on ice for 30 minutes, with periodic vortexing. The disrupted cell suspension was then centrifuged at 10,000 x g for 15 minutes at 4°C, to pellet any insoluble material. The supernatant (containing protein) was recovered to a clean, pre-chilled tube and snap frozen for storage at -80°C until assayed for catalase activity. DNA extraction was performed on the other cell pellet (derived from the 3 mls of cell suspension); to which 180 µl buffer ATL + 20 µl of proteinase K solution (20 mg/ml) was added. Samples were then incubated at 56°C until cells were fully lysed. DNA was then extracted from the lysate using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturers protocol. Each set of DNA extractions conducted also included a negative control which contained all of the reagents, but without any tissue. This was used to check for contamination during the lysis and extraction steps. DNA concentration and purity was measured spectrophotometrically using a Nanodrop 8000. Protein concentration was measured colourimetrically using a commercially available kit, following the manufacturer's protocol (Thermo scientific, item no. 23225).

Telomere analysis

Telomere length was measured in all samples using the quantitative PCR method described by Cawthon (2002). Real-time PCR calculates a cycle threshold (Ct) for each sample: how many cycles it takes for the accumulating fluorescence of that sample to cross a given threshold. There is a linear relationship between Ct and the amount of starting DNA, which makes it suitable for calculating relative quantities. The Cawthon method provides a relative measure of telomere length (RTL) and is calculated as a ratio (T/S) of telomere repeat copy number (T) to a control, single copy number (S). The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was chosen as the single copy gene. The *Salmo salar* GAPDH sequence (Genbank accession number: NM_001123561) was used to design

primers. Using the Genbank blast tool, the gene was compared to the Atlantic salmon genome, to confirm that it was present at a single location. The following forward and reverse GAPDH primers successfully amplified a single amplicon; as determined by melt curve analysis and were subsequently used in the S assay:

SalGAP8-F 5'-GTAAGACAGGATTGAGGCATCTC-3' and

SalGAP8-R 5'-CCGAATCCATTGACACCTACTT-3'

For amplification of telomeric repeats, the universal primers designed by Cawthon (2002) were used:

Tel1b 5'-CGGTTTGTGGTTGGGTTGGGTTGGGTTGGGTTGGGTT-3' and

Tel2b 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'.

A primer optimisation matrix was used to determine the optimal concentration of the primers and five different samples of Atlantic salmon DNA were tested to confirm primer performance and specificity. Telomere PCR conditions were 15 min at 95°C followed by 27 cycles of 15 secs at 95°C, 30 secs at 58°C and 30 secs at 72°C. This was followed by the melt curve profile. GAPDH PCR conditions were 15 min at 95°C followed by 40 cycles of 15 secs at 95°C, 30 secs at 60°C and 30 secs at 72°C. This was again followed by the melt curve profile. PCRs were performed on a Mx3005P qPCR system (Agilent).

The telomere (T) and single copy gene (S) assays were performed on separate 96 well plates, with each sample run in triplicate for each assay. In addition to the samples, each plate also included a six-fold serial dilution of a reference sample (1.25 - 40 ng/well) and a non-target control (NTC). The DNA for the serial dilution was a pool of 60 samples and included both life stages (embryo and FF fry). The serial dilution was used to generate a standard curve and calculate assay efficiencies. The NTC contained all reaction components, apart from DNA, and were included on each plate (in triplicate) to check for non-specific binding and potential contamination between sample wells. Each reaction contained 12.5µl 2x ABsolute Blue qPCR SYBR Green Mix low ROX (Fisher Scientific), forward and reverse primers and DNA (wells containing sample or standards) or water (wells containing NTC) in a total volume of 25µl. Both T and S assays were performed using 10ng of DNA (equivalent to 6µl of the diluted samples). Primer concentrations were

500nM for the telomere assay (Tel1b and Tel2b) and 200nM for the GAPDH assay (salGAP8-F and salGAP8-R). The mean assay efficiencies for the telomere and GAPDH were 91.9 and 94.5 respectively and within the acceptable range (85-115). The average intraplate variation of the Ct values was 1.43 for the telomere assay and 0.49 for the GAPDH assay, respectively. The average interplate variation of the Ct values was 1.88 for the telomere assay and 1.10 for the GAPDH assay, respectively. qPCR raw data was analysed using qBASE software for windows (Hellemans et al., 2007). This controlled for differences in amplification efficiency between plates (assessed from the standard curve of each plate) and produced a relative quantity (RQ) of T and S for each sample. Results were scaled to the average across all unknown samples for each target (i.e. telomere and GAPDH). In addition, by including three inter-run calibrators (three points from the standard curve) I corrected for further inter-run variation. Finally, I used the software to normalise each telomere RQ by the GAPDH RQ for that sample. Therefore, for each sample, the qBASE software produces a calibrated normalized relative quantity (CNRQ). This is similar to the T/S ratio described by Cawthon (2002) but with greater control of inter-plate variation.

Enzymatic antioxidant activity assay

To quantify enzymatic antioxidant defences, I analysed the activity of catalase (CAT) in the protein extract. I also considered measuring glutathione peroxidase (GPx) and superoxide dismutase (SOD); however the activity of these antioxidants was too low in my samples to allow quantification by the commercially available kits that I selected for the trial (Enzo: GPx activity kit item no. ADI-900-158, SOD activity kit item no. ADI-900-157). CAT is involved in the detoxification of hydrogen peroxide (H₂O₂) and is present in most aerobic cells. It is also known to be present at the embryonic stages of various fish species (Kalaimani et al. 2008; Díaz et al. 2010; Sharifi et al. 2013). To measure CAT activity, I used a commercially available kit, following manufacturer's protocol (Cayman Chemical, item no. 707002). In brief, the kit quantified the concentration of formaldehyde produced as a result of CAT's peroxidatic activity. To control for variation in initial protein concentration between samples, CAT activity (nmol/min/ml) was divided by the protein concentration of that sample (mg/ml) to calculate CAT activity as nmol/min/mg protein. All samples were run in duplicate; the intra class correlation coefficient between the two replicates was 0.96, indicating a high repeatability of the assay ($F_{114, 114} = 26.46$, $p < 0.001$).

Oxidative DNA damage assay

Oxidative damage to DNA was quantified by measuring levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG). 8-OHdG is a product of nuclear DNA oxidation and is widely accepted as a sensitive marker of oxidative DNA damage. I quantified 8-OHdG using a commercial kit, following the manufacturer's protocol (Epigentek, item no. P-6003). The same extract of DNA was used as for the telomere analysis. In brief, the colourimetric kit detected 8-OHdG by using capture and detection antibodies specific to 8-OHdG. From this, a relative quantification of 8-OHdG was calculated as the percentage of DNA in a sample that was damaged. All samples were run in duplicate, and again the replicate measurements were found to be consistent (intra class correlation coefficient = 0.821; $F_{131, 131} = 5.90$, $p < 0.001$).

Cell proliferation analysis

To quantify cell proliferation rate, I conducted an immunohistochemical analysis using an antibody stain specific to the Proliferative cell nuclear antigen (PCNA) protein. PCNA is essential in cell replication and is actively expressed in nuclei during the DNA synthesis phase of cell division (Yu, Woods & Levison 1992). The association of PCNA with cell replication led to it initially being used as the basis of an immunohistochemical assay for analysis of cell proliferation in mammals (Foley *et al.* 1991). However, it has since also been successfully used to measure cell proliferation rates in a variety of fish species (for examples see Ortego *et al.* 1994; Kilemade *et al.* 2002; Abdo *et al.* 2014; Peterson, Mok & Au 2015). Immunohistochemistry was performed by the University of Glasgow Veterinary Diagnostic Services. For each treatment, two individual FF fry per family (that had been preserved in formaldehyde and stored in ethanol) were embedded longitudinally in paraffin and sectioned at a thickness of 2.5 μ m. Antigen retrieval (i.e. the exposure of antigenic sites, allowing antibodies to bind) was employed using heat induced epitope retrieval (HIER) in a sodium citrate buffer pH6 for 10 minutes at 110°C. Sections were stained using a DAKO autostainer. The primary antibody was PCNA (Santa Cruz PCNA (FL-261) cat. No. sc-7907), dilution 1:100, incubated for 30 minutes. The secondary antibody (which binds to the primary antibody and assists in detection) was Dako EnVision+ System-HRP labelled polymer anti-rabbit, incubated for 30 minutes at room temperature. Visualisation was achieved using Dako + DAB Substrate and Chromogen. Sections were counterstained with the nuclei-specific Gills Haematoxylin for 27 seconds. Stained slides

were then scanned at the Marine Scotland Marine Laboratory in Aberdeen, using the Dot Slide virtual microscopy system version 2.5, at a resolution of 40x. In order to select the same body regions for analysis on all specimens, a reference line was drawn along the maximum longitudinal axis of the remaining yolk sac of each FF fry and the anterior-most point, the 1st quartile and the 2nd quartile point along this line were identified. The most dorsal region of myotome muscle was identified in relation to these 3 points and used for image capture (Fig. 5.2). Images were captured at 26x magnification (area = 0.03 mm²). Since there were two FF fry from the same family per temperature treatment, and 3 images were captured per FF fry, this gave, in total, 6 images per family from each treatment.

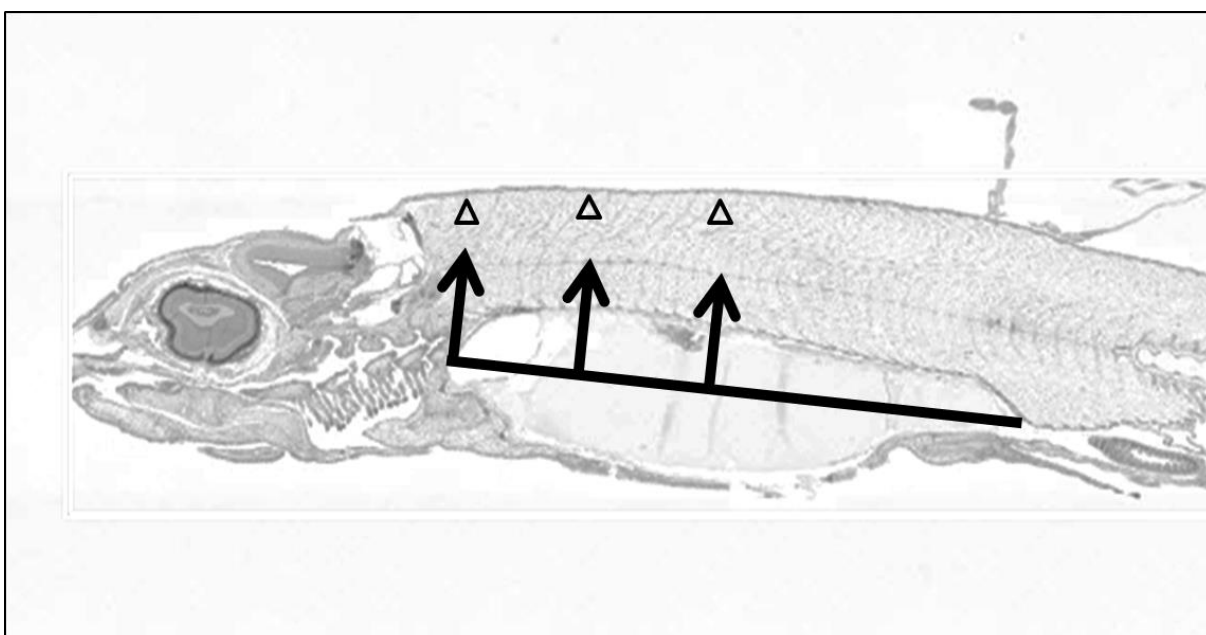
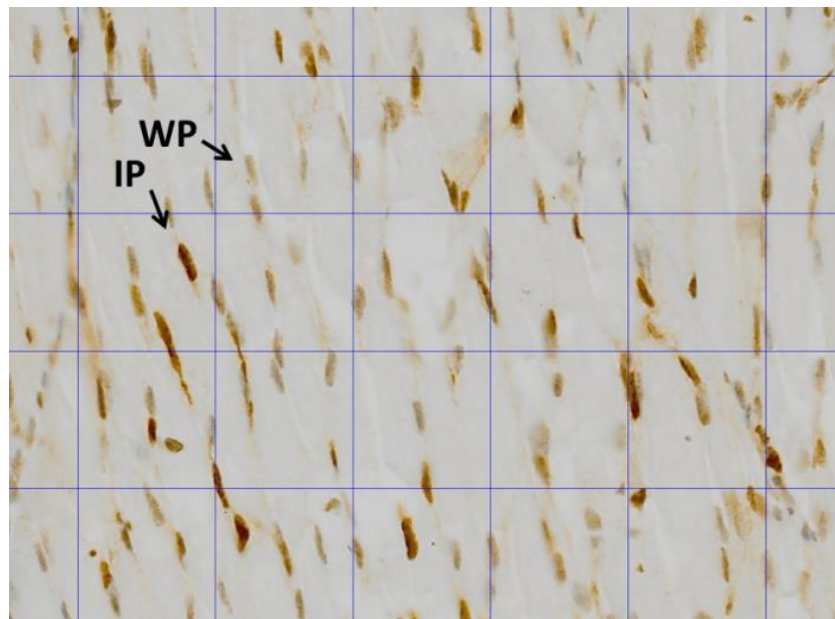


Fig. 5.2. Example of a scanned immunohistochemical slide, showing how the 3 regions of dorsal myotome muscle were selected and used for 26x image capture. The horizontal thick line is the maximum longitudinal axis of the remains of the yolk sac. The triangles demonstrate the 3 most dorsal regions in relation to the anterior-most point, the 1st quartile and the 2nd quartile point of the horizontal line. Triangles are also representative of the area size that was used for image capture.

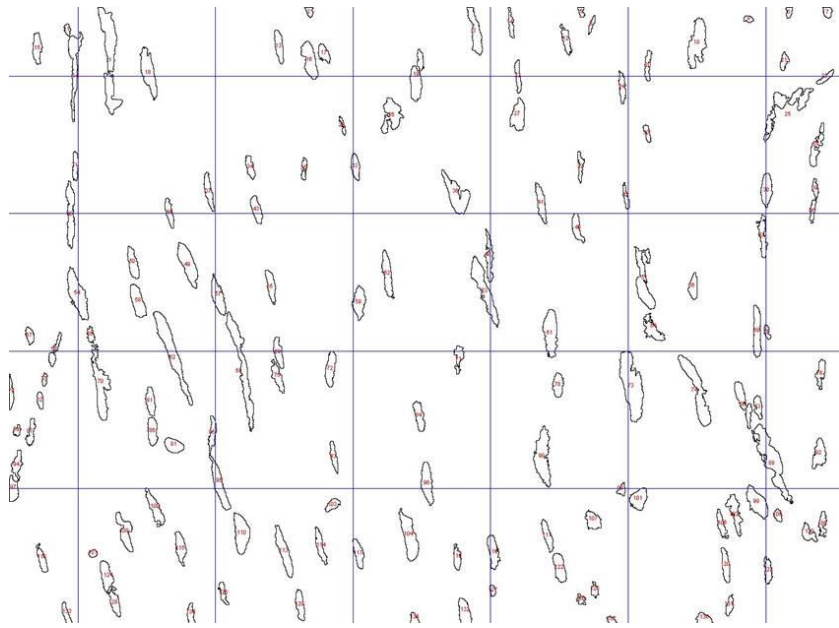
Previous studies have shown that PCNA has a relatively long half-life, meaning that residual PCNA may continue to be present in cells that have fully completed division (Scott *et al.* 1991). In addition, there is a relatively high cell turnover rate at the embryonic stage, which meant that most cells had incorporated some of the PCNA-specific stain. To overcome this, Kilemade *et al.* (2002) developed a method, based on image analysis, to distinguish between intensely PCNA positive (IP) nuclei (i.e. those actively dividing) and weakly PCNA positive (WP) nuclei (those no longer dividing). I adopted the same approach, using Image J to distinguish objectively and consistently which nuclei were

intensely PCNA positive. Firstly, I quantified the total number of nuclei in an image by conducting the following three steps: 1) the image was converted into an 8 bit grey scale format; 2) stained areas were identified by selecting all pixels that had an intensity between 0-165 (0 = black, 255 = white) and 3) the Analyse Particle tool was then used to differentiate between true nuclei (which I set as a minimum of 120 clustered pixels) and smaller randomly stained artefacts (see Fig. 5.3B for an example of the output from this process). The number of nuclei was then counted by Image J. Secondly, I again used Image J to analyse the same image, but this time to identify only nuclei that were intensely PCNA stained (IP); by repeating the above three steps, but this time increasing the 8 bit intensity threshold to <90 rather than <165 (Fig. 5.3C). Therefore, by using image J to separately identify the total number of nuclei (Fig. 5.3B) and the number of intensely PCNA stained nuclei (Fig 5.3C) from the same image, I was able to calculate the percentage of nuclei in that image that were actively undergoing cell replication at the time of sampling. Furthermore, since all images were captured at the same magnification (and were therefore of the same area), I was also able to generate a cell size index by counting the total number of nuclei per image (i.e. the higher the value, the smaller the cells). The inverse of the cell size values were calculated, so that relatively larger cells produced a higher value. For statistical analyses, the mean of the six images was calculated, for both cell proliferation rate and cell size index.

A



B



C

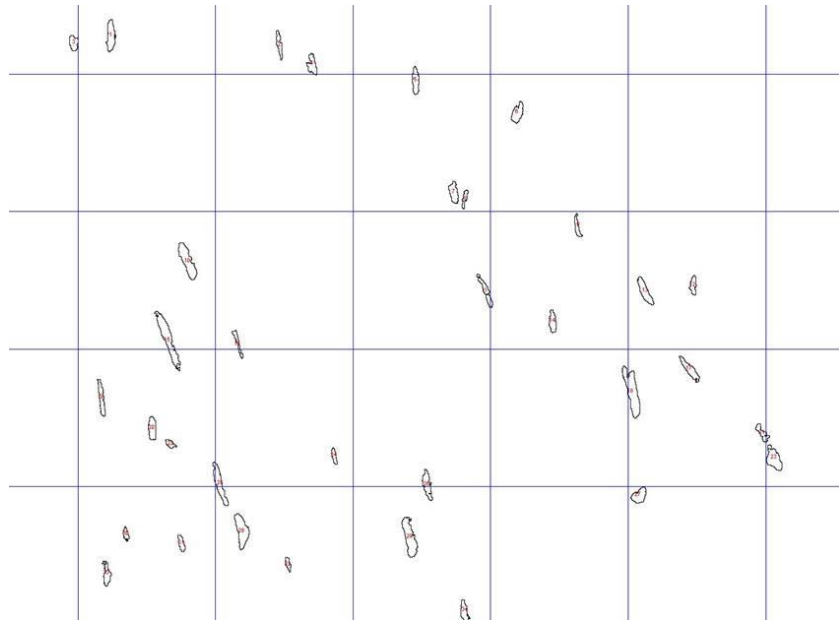


Fig. 5.3. Outline of Image J PCNA analysis: A) example of an original image showing intensely PCNA positive nuclei (IP) and weakly PCNA positive nuclei (WP), B) the same image filtered using Image J software to select all nuclei (IP and WP), and C) the same image filtered to now show only the IP nuclei.

Statistical analysis

In total, there were 22 samples for each of the three temperature treatments (corresponding to the 22 families) at both the embryo and the FF fry stage (i.e. 66 samples at the embryo stage and 66 samples the FF fry stage). Therefore, I had 132 data points for telomere length, CAT activity and oxidative DNA damage; for embryo and FF fry stage combined. And for the FF fry stage only, I had 66 data points for cell proliferation rate and cell size index. I measured/calculated the following variables to be used in statistical models, where appropriate: relative telomere length at the eyed embryo (*embryo RTL*) and first feeding fry stages (*FF fry RTL*); average fresh weight of first feeding fry, calculated as an average for each family (*FF fry weight*); the level of catalase activity at the embryo and FF fry stages (*embryo* and *FF fry CAT activity* respectively); the percentage of DNA that was damaged (i.e. % 8-OHdG) at the embryo and FF fry stages (*embryo* and *FF fry DNA damage* respectively); the percentage of intensely stained PCNA nuclei at the FF fry stage (*FF fry cell proliferation*); the inverse of the number of cells per unit area, as a proxy for cell size (*cell size index*); the life stage at which a sample was taken (i.e. whether at the embryo or FF fry stage, and subsequently referred to as *life stage*) and the aquarium temperature treatment (*temp. treatment*).

Firstly, I ran a simple model to assess if there was a significant change in telomere length between the two life stages (embryo and FF fry) irrespective of the other variables (model A). I then ran five models in order to test hypothesis 1: that variation in water temperature will have a significant effect on oxidative DNA damage at the embryo and FF fry stage and the relative change between the two stages (model B, model C and model D respectively), CAT activity at the embryo and FF fry stage (model E and model F) and cell proliferation at the FF fry stage only (model G). I also ran a model to assess the relationship between temperature treatment and FF fry weight, averaged for each family, at the time of first feeding (model H). I then ran two models in order to test hypothesis two: that variation in oxidative DNA damage, CAT activity and cell proliferation would, in turn significantly affect telomere length at the embryo stage (model I) and FF fry stage (model J). All ten of the dependent variables were assessed by linear mixed models (LME). Family ID was included as a random factor in the analyses to give a repeated measure design. For a full outline of the statistical models and the variables included in each, please see Table 5.1.

The Akaike Information Criterion (AIC) was used during model fitting and variables were only removed from a model if this resulted in a relative reduction of the AIC score. I used Pearson correlation coefficient matrices to assess potential collinearity between explanatory variables (with a cut-off coefficient of 0.8). FF Fry weight and length were identified as being highly collinear (Pearson $r = 0.81$, $p < 0.001$) and therefore only FF fry weight was used for subsequent analyses. All statistical analyses were carried out using IBM SPSS 22 for Windows.

Table 5.1. Summary of the ten initial linear mixed effect models, including all fixed factors, covariates, interactions and random factors, prior to model selection.

	Dependent variable	Fixed factors	Covariates	Interactions	Random factors
A	RTL (log10)	Temp. treatment Life stage	-	Temp. treatment * Life stage	Family ID
B	Embryo DNA damage	Temp. treatment	-	-	Family ID
C	Fry DNA damage	Temp. treatment	Cell prolif. Cell size Fry weight	Temp. treatment * Fry weight	Family ID
D	DNA damage change	Temp. treatment	Cell prolif. Cell size FF Fry weight	Temp. treatment * FF Fry weight	Family ID
E	Embryo CAT activity (log10)	Temp. treatment	Embryo DNA dam.	-	Family ID
F	Fry CAT activity (log10)	Temp. treatment	Cell prolif. Cell size Fry weight Fry DNA dam.	Temp. treatment * Fry weight	Family ID
G	Fry cell prolif. (log10)	Temp. treatment	Cell size Fry weight	Temp. treatment * Fry weight	Family ID
H	FRY weight	Temp. treatment	-	-	Family ID
I	Embryo RTL (log10)	Temp. treatment	Embryo CAT activity Embryo DNA damage	-	Family ID
J	Fry RTL (log10)	Temp. treatment	Cell prolif. Cell size Fry weight Fry CAT activity Fry DNA damage	Temp. treatment * Fry weight	Family ID

Table 5.2. Summary of the ten final linear mixed-effect models explaining variation in: A) overall RTL, B) embryo DNA damage, C) fry DNA damage, D) DNA damage change, E) embryo CAT activity, F) fry CAT activity, G) fry cell proliferation, H) fry weight, I) embryo RTL and J) fry RTL.

	Explanatory variable	Numerator df	Denominator df	F	p
A	Life stage x Temp. treatment	2	103.41	7.65	= 0.001
B	-				
C	-				
D	-				
E	Temp. treatment	2	42	33.66	<0.001
F	-				
G	Temp. treatment	2	41.71	10.88	<0.001
H	-				
I	Temp. treatment	2	42	11.41	<0.001
J	Fry CAT activity (log10)	1	33.11	8.58	=0.006

Notes: See Table 5.1 for the main effects and interactions initially included in each model. Terms were sequentially removed if not significant or if not contributing to significant interactions. Family ID was included as a random effect to control for any family effects. See Methods for full definitions.

5.4 RESULTS

Telomere dynamics

Telomere length significantly increased between the two life stages (embryo to FF fry) for each of the 3 temperature treatment groups. However, the relative amount of telomere change was dependent on the temperature treatment (significant life stage x temperature treatment interaction; Table 5.2A). Individuals reared in the coldest temperature had the longest telomere length when measured at the embryo stage (Fig. 5.4A) but then underwent the smallest change in telomere length, so that by the first-feeding fry stage the three temperature treatment groups had similar telomere lengths (Fig. 5.4B; note no effect of temperature treatment in Table 5.2J). Note that these effects must be interpreted with caution, since at the embryo stage, all the sampling was done on the same day so embryos from the three temperature treatments were the same chronological age, but were at different stages of development (warmer temperatures = more advanced). Whereas at the fry stage, all groups were at the same developmental stage (= completion of yolk absorption), but were of different chronological ages.

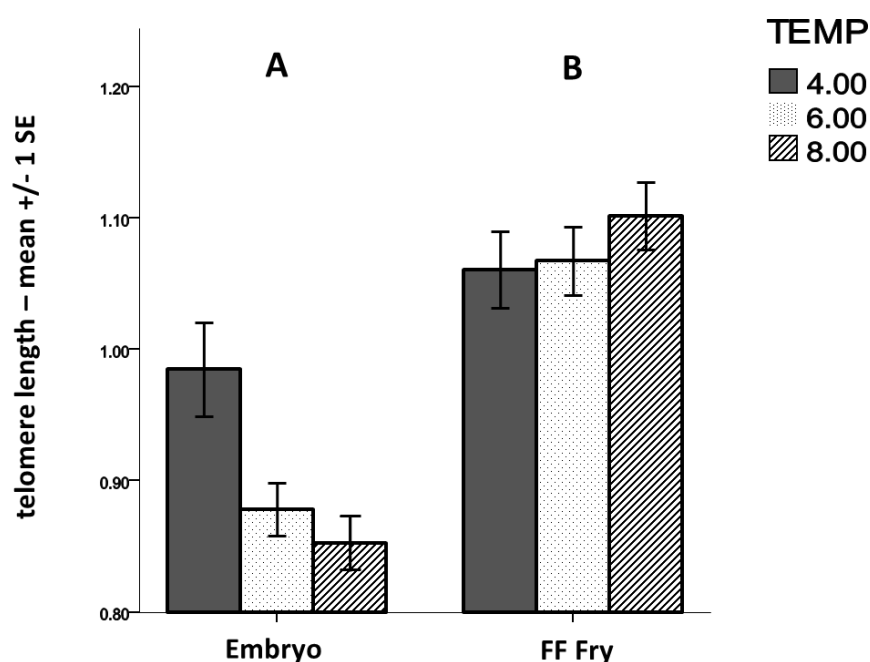


Fig. 5.4. The relative telomere length (\log_{10} plus a constant of 1, for presentation purposes) of the salmon at A) the embryo stage and B) the FF fry stage. Solid columns represent the 4°C group, dotted columns represent the 6°C group and the striped columns represent the 8°C group. Data plotted as means \pm SE, with each of 22 families contributing one data point per temperature per life stage.

CAT activity and oxidative DNA damage

Temperature had a significant effect on CAT activity levels at the embryo stage (Table 5.2E). CAT activity was lowest in the coldest group and highest in the warmest group (Fig. 5.5). However, the temperature treatment had no effect on CAT activity at the FF fry stage or on the levels of DNA damage (8-OHdG) at the embryo and FF fry stages. There was a significant negative relationship between CAT activity and FF fry telomere length (Table 5.2J), with relatively greater CAT activity being associated with shorter telomeres (Fig. 5.6).

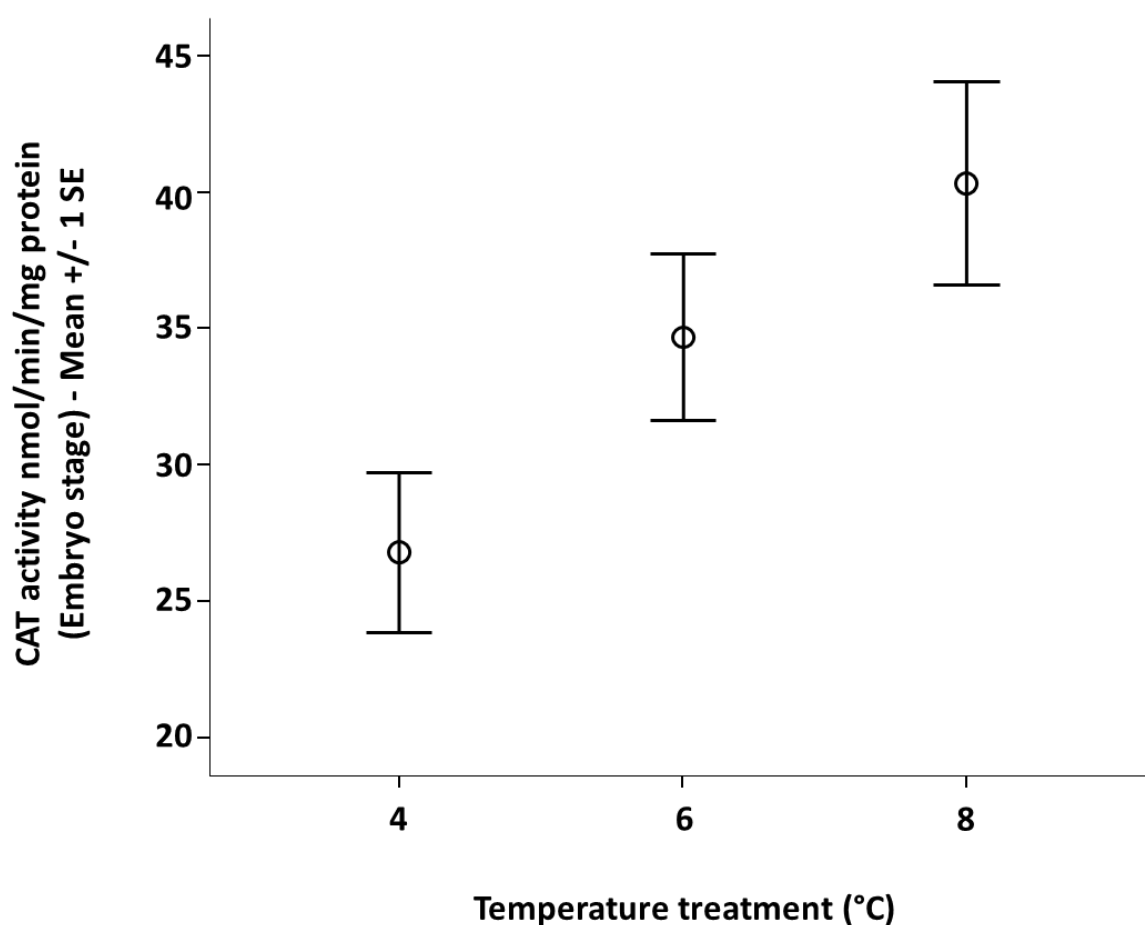


Fig. 5.5. The relationship between temperature treatment and CAT activity (nmol/min/mg protein) at the embryo stage. Data plotted as means \pm SE, with each of 22 families contributing one data point per temperature.

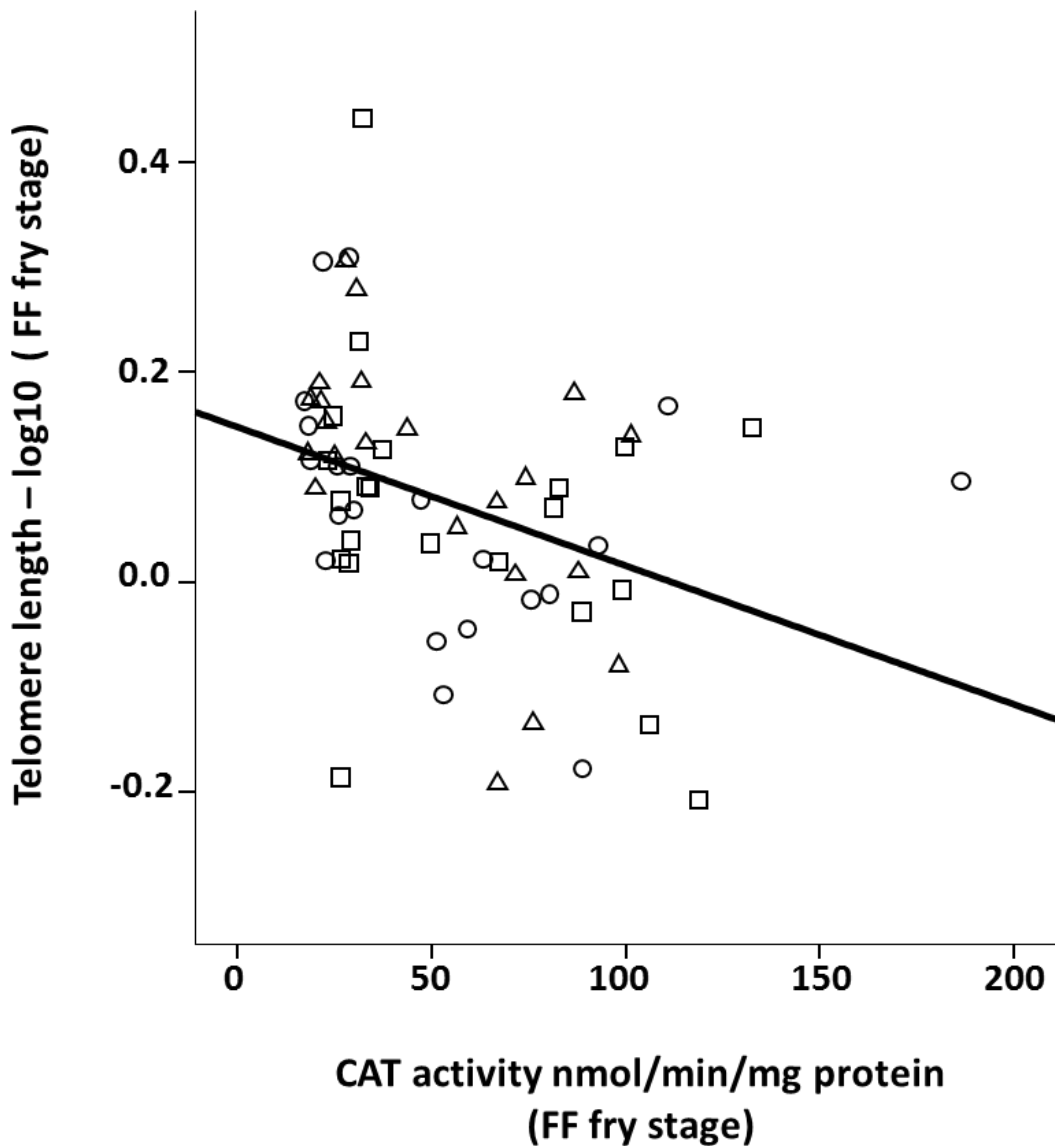


Fig. 5.6. The relationship between CAT activity (nmol/min/mg protein) and relative telomere length (log10) at the FF fry stage. Each of 22 families contributes one data point per temperature. Squares represent the 4°C group, circles represent the 6°C group and rectangles represent the 8°C group.

Cell proliferation rate

Temperature had a significant effect on the rate of cell proliferation in the anterior muscle of the FF fry (Table 5.2G). Individuals in the intermediate temperature group had the highest percentage of intensely stained PCNA nuclei; i.e. the highest rate of cell proliferation (Fig. 5.7). However, this variation in cell proliferation rate was not significantly associated with FF fry telomere length. Likewise, the inverse of the number of cells in a given area (as a proxy for cell size) did not significantly affect FF fry telomere dynamics.

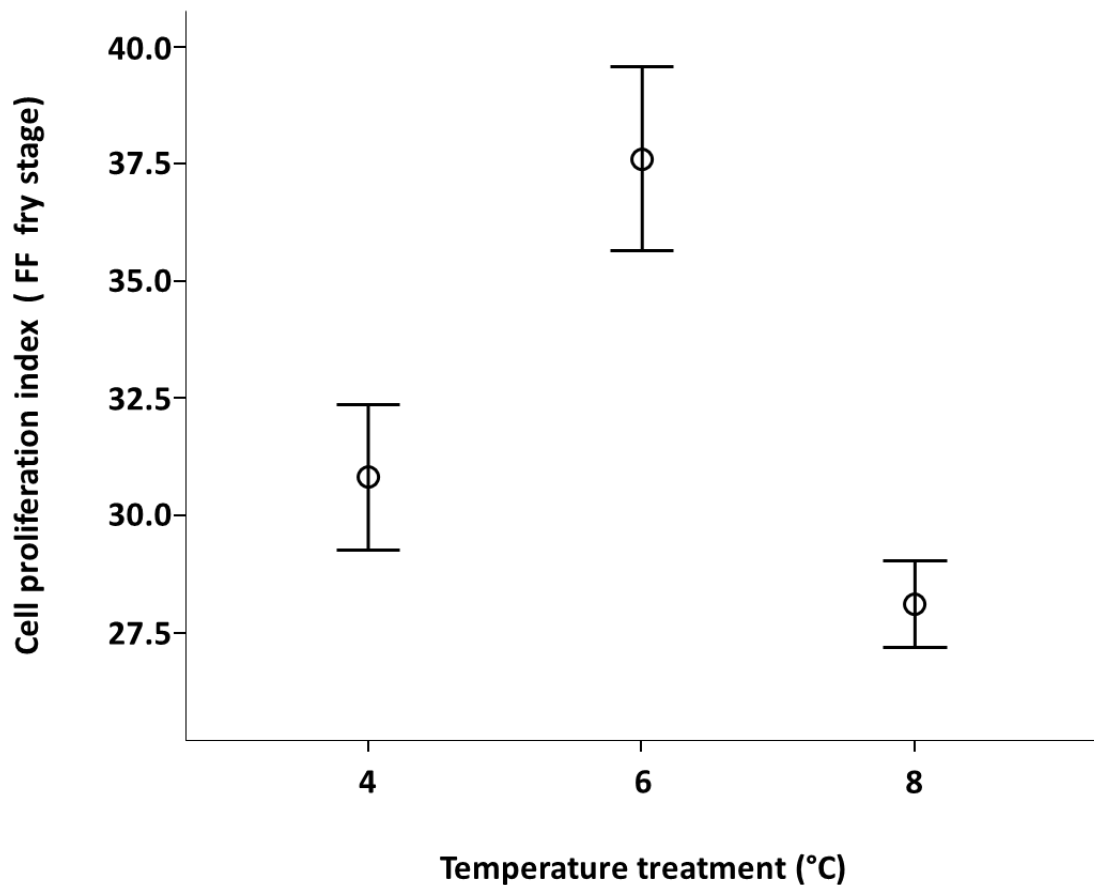


Fig. 5.7. The relationship between temperature treatment and the rate of cell proliferation (i.e. the percentage of intensely-stained positive PCNA nuclei) at the FF fry stage. Data plotted as means \pm SE, with each of 22 families contributing one data point per temperature.

5.5 DISCUSSION

This study showed that salmon embryo telomere length was significantly affected by the temperature of the water in which they were living. In other words, it would appear that there is a negative correlation between developmental rate and telomere length at the eyed embryo stage. All embryos were sampled on the same day, and were therefore all the same chronological age, but at different developmental stages. Embryos reared at the coldest temperature (i.e. the least developed at the time of sampling) had the longest mean telomere length, while embryos reared at the warmest temperature (the most developed) had the shortest telomeres.

This, however, contrasts with what I observed between the embryo and FF fry stage; where telomere length correlated positively with developmental stage. It may be that telomere length initially decreases during embryo development (e.g. as a result of rapid cell

proliferation), but this is rectified at a later embryonic stage. In support of this, there was no significant difference in telomere length between the three temperature treatment groups at the FF fry stage, when all groups were at the same developmental stage, but different chronological ages. In general, telomeres have been found to shorten with age, at least in many post-embryonic mammal and birds studied to date (Hausmann *et al.* 2003; Henriques & Ferreira 2012). Less is known about embryonic telomere dynamics. The few studies that exist (mostly in mammals thus far) indicate that telomere elongation occurs in the early stages of embryogenesis (Schaezlein *et al.* 2004; Liu *et al.* 2007; Kalmbach *et al.* 2014). This is thought to be by alternative lengthening of telomere (ALT) pathways, since this elongation step also occurs in telomerase-null mice (Kalmbach *et al.* 2014). Instead, upregulation of telomerase is thought to occur at the later stages of embryo development (Taylor & Delany 2000; Liu *et al.* 2007; Kalmbach *et al.* 2014), although it has been suggested that this is to maintain, rather than elongate, telomere length (Liu *et al.* 2007).

Much less is known about telomere dynamics during fish embryo development. In contrast to the situation in most endothermic vertebrates studied thus far, telomerase expression has been detected in post-embryonic somatic tissue in reptiles, amphibians and fish (Gomes, Shay & Wright 2010) suggesting that ectothermic vertebrates do not down-regulate telomerase expression to the same degree as endotherms in post-natal life. That being said, a number of reports have found that fish (including Atlantic salmon – see chapter 3) still experience telomere loss with age after the embryo stage. In reviewing this literature, Simide *et al.* (2016) reported that around half of the fish telomere studies to date report telomere shortening with age, but since none of the studies looked at the embryonic stages it is not possible to know at what life history stage telomere shortening in fish generally starts to occur. I have shown in this study that telomere length in Atlantic salmon is significantly longer at the FF fry stage (i.e. the end of embryo development) compared to the earlier eyed embryo stage. Therefore it may be that, in contrast to endotherms, telomerase expression in the later stages of ectothermic embryonic development is capable of not only maintaining telomere length, but also elongating it – or that a different mechanism for restoring telomeres (such as alternative lengthening of telomeres (ALT) (Henson *et al.* 2002) is at play.

The temperature manipulation had no significant effect on the levels of oxidative DNA damage at the embryo or FF fry stage, nor was variation in DNA damage levels associated with telomere length at either stage. There are complex DNA damage repair mechanisms

in place to repair oxidative DNA damage (Halliwell & Gutteridge 2015). Therefore, levels of 8-OHdG present at any one time will depend not only on the rate at which oxidative damage to DNA is occurring, but also on the relative rate of repair. Base excision repair (BER) corrects oxidative DNA damage at a single base level, and is the major pathway whereby 8-OHdG damage is repaired (Ménézo, Dale & Cohen 2010; Halliwell & Gutteridge 2015). BER pathways are known to be active in human (Ménézo, Dale & Cohen 2010) and fish embryos (Fortier *et al.* 2009). Therefore, even if the temperature treatment did influence rates of DNA damage, it may be that this was compensated for by repair mechanisms prior to analysis. It may also be the case that any possible link between oxidative damage and telomere length was buffered by the significant telomere elongation I observed later in embryo development.

The temperature treatment did have a significant effect on CAT activity at the embryo stage (but not at the FF fry stage). Embryos reared at the warmest temperature (i.e. the most developed embryos at the time of sampling) had the greatest CAT activity, while embryos in the coolest water (i.e. the least developed) had the lowest CAT activity. This is largely in keeping with other fish species, which have found an increase in CAT activity during embryonic development (Aceto *et al.* 1994; Peters & Livingstone 1996) but see also (Díaz *et al.* 2010). This suggests that there may be an increasing need to remove hydrogen peroxide (H₂O₂) as embryos develop, or that the embryo is gradually developing its own endogenous antioxidants, having earlier in development been reliant on maternal antioxidants deposited in the egg (Cowey *et al.* 1985). There was not a significant difference in CAT activity at the fry stage, suggesting that developmental stage, rather than chronological age, is most important in determining pro-oxidant and anti-oxidant activity in Atlantic salmon. This is somewhat in keeping with Hemmer-Brepson *et al.* (2014) who did not find a significant difference in CAT activity when rearing adult fish *Oryzias latipes* at two different (but non-stressful) temperatures.

There was a significant association between fry CAT activity and fry telomere length, in that fry with a relatively greater CAT activity had relatively shorter telomeres. An increasing number of studies are now linking physiological stress, oxidative stress and telomere dynamics (for review see Haussmann & Marchetto 2010). Therefore, it may be that this relationship is not causative, but rather an indication that stressed individuals have both shorter telomeres and a relatively greater CAT activity. The fry were reared in similar, stable laboratory conditions, however parental effects may cause variation in stress

hormone levels between families (Williams & Groothuis 2015) or even within families (Schmaltz, Quinn & Schoech 2016), which may in turn affect telomere dynamics. In addition, studies have also reported a heritable component to free radical production (Olsson *et al.* 2008) and oxidative defence (Kim *et al.* 2010).

The temperature treatment had a significant effect on the rate of cell proliferation in the FF fry (i.e. at the end of embryo development), with salmon reared at the intermediate water temperature (6°C) having the highest cell proliferation rate. The optimal temperature for growth in juvenile Atlantic salmon is thought to be around 16°C (Elliott & Hurley 1997). However, cell proliferation in Atlantic salmon is greatest at temperatures lower than the optimal growth temperature. For example, Usher, Stickland and Thorpe (1994) reared Atlantic salmon embryos at three different water temperatures (5, 8 and 10°C) and found the highest rate of embryonic cell proliferation in the 5°C group. Stickland *et al.* (1988) used a larger margin between temperatures (10°C versus an ambient water temperature of $1.6^{\circ}\text{C} \pm 0.2$) and found the proliferation rate to be greater at the cooler ambient temperature. Finally, MacQueen *et al.* (2008) reared salmon embryos at 2, 5, 8, and 11°C and then assessed their muscle phenotype in adulthood. Although salmon reared at the warmest temperature initially had the fastest growth, those reared at 5°C had the greatest final fibre number in adulthood, suggesting that incubation temperature may have long term effects on muscle structure through its effect on cell proliferation rates. Although each study (including this one) used slightly different experimental temperature regimes, together they suggest that cell proliferation rates may be maximised around 5°C in Atlantic salmon. It is reasonable to assume that this temperature is low enough that there will be a sufficient concentration of dissolved oxygen, since cell proliferation is oxygen demanding (Matschak *et al.* 1997).

While a trade-off between cell number (hyperplasia) and cell growth (hypertrophy) has been documented in fish (reviewed by Arendt 2007), I found no association between proliferation rate and cell size – possibly because the measure of proliferation rate reflects recent cellular activity whereas cell size is potentially a consequence of processes taking place over a longer period of development. I also did not find a significant association between cell proliferation rate and telomere length in FF fry.

Although individuals in the intermediate 6°C group had the highest cell proliferation rate, there was not a significant difference in fry telomere length between the three groups.

Again, it may be that the significant elongation of telomere length occurring late in embryonic development was buffering any possible effects. It would be interesting to look at this relationship in post-embryonic stages, such as fry and adults, when telomerase expression may be down-regulated. This is especially relevant in fish, where muscle fibre recruitment continues well beyond the embryo stage (Weatherley, Gill & Lobo 1988) – indeed, active cell proliferation in muscle tissue is still detectable in Atlantic salmon that have migrated to sea (Johnston *et al.* 2003).

This study does not support the hypothesis that oxidative DNA damage, catalase (CAT) antioxidant activity and cell proliferation rate are underlying mechanisms linking incubation temperature and telomere dynamics. Perhaps this is due to the significant telomere elongation that occurs prior to first feeding, which was not foreseen prior to conducting the experiment. With this in mind, it would be interesting to address these issues in experiments that include later developmental stages, when telomerase may be downregulated and growth and behaviour may have caused greater among-individual variation in physiological stress, oxidative stress levels and telomere dynamics.

CHAPTER 6. General discussion

6.1 CONSIDERING MY DATA CHAPTERS COLLECTIVELY

The main objective of my thesis was to investigate how life history and environmental factors influence telomere dynamics in Atlantic salmon. I carried out carefully designed experiments, both in the laboratory and in the wild, using a longitudinal approach where possible, in order to address a number of specific questions that are connected to this central theme. The use of IVF ensured that observed effects were not due to assortative mating. In chapter 2, I demonstrated that there can be significant links between parental life history and offspring telomere dynamics. Maternal life history traits, in particular egg size, were most significant at the embryonic stages. Paternal life history traits, which influence both early life growth rate and body size at reproduction, had more influence in the later stages of development.

In chapter 3, using a longitudinal approach in a wild Atlantic salmon population, I found that migration survival (i.e. the likelihood of completing the return migration) was significantly influenced by the timing of outward smolt migration, with the smolts migrating earliest in the spring having the highest probability of return. I found that most individuals experienced a reduction in telomere length during the migratory phase of their life cycle; however the relative rate of telomere loss was in turn dependent on sex, with males experiencing a relatively greater loss. In contrast to my hypothesis, I also found that juvenile salmon that had the shortest telomeres at the time of outward migration had the greatest probability of surviving through to the return migration.

In chapter 4, again using a wild system involving experimental manipulations of juvenile Atlantic salmon in Scottish streams, I found that telomere length in juvenile fish was influenced by parental traits and by direct environmental effects. Faster-growing fish had shorter telomeres and there was a greater cost (in terms of reduced telomere length) if the growth occurred in a harsher environment. I also found a positive association between offspring telomere length and the growth history of their fathers (but not mothers), represented by the number of years that fathers had spent at sea. Lastly, in chapter 5, I could not support the hypothesis that oxidative DNA damage, catalase (CAT) antioxidant activity and cell proliferation rate are underlying mechanisms linking incubation

temperature and telomere dynamics. However, I did show that salmon embryo telomere length was significantly affected by the temperature of the water in which they were living.

Each data chapter has addressed specific questions related to how life history and environmental effects may influence telomere dynamics in Atlantic salmon. Although the chapters had separate and specific aims, there are four main ‘themes’ (age effects, growth effects, parental effects and environmental effects) which, to a lesser or greater extent, run through each of the chapters. With this in mind, I will now discuss each of these themes, while considering my data chapters collectively.

Age effects

Telomeres generally shorten with age, at least in humans, but also in many other mammal and bird species studied to date (Hausmann *et al.* 2003; Henriques & Ferreira 2012). In contrast, telomerase has been found to be expressed in post-embryonic tissue in reptiles, amphibians and fish (Gomes, Shay & Wright 2010), and in these taxa telomere length does not always decrease with age. For instance, Simide *et al.* (2016) found that around half of the fish telomere studies to date have reported either no telomere shortening with age, or even telomere elongation. Studies on zebrafish *Danio rerio* (Anchelin *et al.* 2011) and the frillneck lizard *Chlamydosaurus kingie* (Ujvari *et al.* 2016) both report a curvilinear relationship between age and telomere length, with telomere length generally increasing throughout life, but with a significant decrease in the latter stages. Studies of age effects on telomere length should ideally be conducted longitudinally, and should use the same tissue throughout the analysis. With respect to methodology, if using the Cawthon (2002) qPCR method for telomere analysis, telomere change with age can only be analysed within a given study, since the measurement of telomere length is only relative to the other samples in that experiment (Nussey *et al.* 2014). With this in mind, I have not focussed too heavily on age effects in the data chapters, with the exception of chapter 3, where a longitudinal approach was adopted. However, for the purposes of this discussion, it is perhaps interesting to collectively consider the results from my four data chapters and assess what the potential relationship between telomere length and age might be in Atlantic salmon.

It would appear that telomere length significantly increases during the embryonic stages of development in Atlantic salmon. In chapter 4, the two time points during embryo development at which telomeres were measured were only separated by 18 days. However,

this was sufficient to reveal a significant increase in telomere length with time. Chapter 5 also looked at telomere change at the embryonic stages, but this time between the eyed embryo stage and the alevin stage (i.e. ~ 1 week prior to first feeding). Again telomere length was found to increase between the two stages. Combined, these results suggest that telomeres lengthen throughout a large proportion of embryonic development. This is in contrast to mammals, where there is thought to be a single significant telomere elongation step in the early stages of embryo development (Schaezlein *et al.* 2004; Liu *et al.* 2007; Kalmbach *et al.* 2014). This is thought to be by alternative lengthening of telomere (ALT) pathways, as opposed to telomerase activity since it also occurs in telomerase-null mice (Kalmbach *et al.* 2014). Telomerase upregulation is thought to occur at the later stages of embryogenesis (Taylor & Delany 2000; Liu *et al.* 2007; Kalmbach *et al.* 2014), but Liu *et al.* (2007) suggested that this is to maintain, rather than elongate telomere length. The reasons why the telomeres of Atlantic salmon may increase in length throughout embryonic development is unclear, but could relate to the pattern of cell replenishment from stem cell pools.

The relationship between telomere length and age appears to be less straightforward once fry have started feeding. For example, in chapter 2, Atlantic salmon reared in stable laboratory conditions were sampled at two time points – the eyed embryo stage and 8 week old fry. 62.5% of the families had a shorter mean telomere length at the fry stage than at the embryo stage. Likewise, in chapter 4, 59.3% of recaptured fry (which had been feeding in the wild for several months) had a relatively shorter telomere length than the mean telomere length of their family at the late embryo stage. For both chapters, this assessment was based on a cross-sectional approach and, of necessity, used different tissue types (whole embryo vs fry fin tissue) and therefore any conclusions should be made with caution. That being said, this does suggest that telomere elongation mechanisms, which may be active during embryonic stages, are relatively reduced once fry start to feed.

In chapter 3, I studied changes in telomere length with age during the migratory (i.e. adult) phase of the Atlantic salmon life cycle. A longitudinal mark and recapture approach was used, with the same tissue (adipose fin) being sampled at each stage. Considering adult fish that returned from migration after 1 winter at sea (1SW), 94.7% of the individuals experienced a relative telomere loss. Only two fish returned after multiple winters at sea (MSW) and both of these fish had experienced a relative increase in telomere length between the smolt and adult stage. In that chapter I suggest that the more sustained growth

of MSW fish may result in a greater expression of telomerase. However, a more extensive study is needed.

In the review of fish telomere dynamics by Simide *et al.* (2016), only one of the previous studies had looked at telomere change over more than two time points. As a result, these studies reported only shortening, elongation or maintenance of telomere length with age. However, when collectively considering the results from my four data chapters, it would appear that changes in telomere length with age in fish are more complicated than a simple unidirectional change.

Growth effects

A growing number of studies have now reported a negative relationship between increased growth rate or body size and rate or extent of telomere loss (Tarry-Adkins *et al.* 2009; Fick *et al.* 2012; Geiger *et al.* 2012; Herborn *et al.* 2014; Noguera *et al.* 2015; Pauliny *et al.* 2015; Ringsby *et al.* 2015). With respect to fish, Näslund *et al.* (2015) found that wild brown trout induced to undergo compensatory growth did not show increased telomere loss. In contrast, Pauliny *et al.* (2015) reported that transgenic coho salmon with an artificially increased growth rate showed a faster telomere loss compared with maternal half sibs that grew at a rate more typical of wild fish. While these results initially suggested that natural variation in growth rate does not have a significant effect on telomere dynamics in fish, this was not backed up by my studies of growth rate variation in either laboratory or wild fish. In the earlier life stages, there appears to be a negative relationship between growth and telomere length. For example, in chapter 2 I reared Atlantic salmon fry in stable laboratory conditions and found that relatively larger fry had shorter telomeres. In chapter 4 (when wild fry were living in natural streams) there was again a negative association between body size and fry telomere length. However, the magnitude of this loss was dependent on the stream from which they came, with fry paying a higher cost, in terms of reduced telomere length, for a given amount of growth when the growth occurred in a relatively harsher stream. Combined, these results indicate that there may be a trade-off between body size and telomere length in the early life stages of Atlantic salmon. A larger body size has many benefits, such as increased competitive ability and social status (Blanckenhorn 2000; Dmitriew 2011). However, it would appear that attaining a relatively larger size comes at a cost to telomere length in Atlantic salmon fry, at least in the short term.

In chapter 3 I assessed whether there was a body size effect on telomere dynamics in adult salmon. When focussing on adults that had spent 1 winter at sea (1SW), I did not find a significant association between body size and telomere length in these 1SW adults. However, when looking at both 1SW and MSW adults, the two fish that returned after multiple winters at sea (MSW) were significantly larger than 1SW fish and had experienced a relative increase in telomere length between the smolt and adult stage. Again, further study with a larger sample size is needed.

Together, these results suggest that the relationship between growth (and body size) and telomere length may in turn be dependent on the developmental stage. Investing in growth at the early life stages comes at a cost to telomere length (perhaps due to lowered telomere repair mechanisms at that stage), whereas there is some evidence that investing in growth at the adult stage (as a result of spending more years at sea) may be beneficial to telomere length (perhaps due to increased telomere repair mechanisms at that stage).

Parental effects

Studies thus far have reported a heritable component to telomere length across a range of vertebrates (Nordfjall *et al.* 2005; Njajou *et al.* 2007; Nordfjall *et al.* 2010; Horn *et al.* 2011; Olsson *et al.* 2011a; Broer *et al.* 2013; Eisenberg 2014; Asghar *et al.* 2015a; Reichert *et al.* 2015). These studies have mostly found a positive relationship: parents with relatively longer telomeres at the time of reproduction have tended to produce offspring with relatively longer telomeres. However, there is inconsistency within these studies as to whether the effect is stronger through the mother or the father. There are also possible ways in which the environment experienced by the parents may affect offspring telomere length (i.e. through parental effects), although less is currently known about this (for one example see Noguera *et al.* 2016). By far the most studied parental effect on offspring lifespan is parental age at reproduction (Gavrilov & Gavrilova 1997; Fox, Bush & Wallin 2003; Garcia-Palomares *et al.* 2009; Bouwhuis, Vedder & Becker 2015), but in chapter 2 my capacity to disentangle parental age and time at sea was limited.

In chapter 2 I found that maternal life history traits, in particular egg size, had significant effects on embryonic telomere length: heavier eggs resulted in offspring (embryos) with longer telomeres. That being said, larger eggs led to larger size-at-age offspring at the fry stage, which I found in chapter 2 to have a negative consequence for fry telomere length. Therefore, egg size may have contrasting effects on telomere length, dependent on the

developmental stage. In chapter 4, when embryos were reared in the laboratory and fry were reared in wild streams, I did not find a significant effect of egg size on telomeres at either the embryo or fry stage. The reasons for this are not quite clear. It may be due to differences in protocols: chapter 2 was conducted under controlled laboratory settings at both the embryo and fry stage (i.e. controlled constant embryo temperature, similar food rations for all fry etc.) while chapter 4 involved temperature variation at both the embryo and the fry stage, as well as the fry being reared in the wild (with consequent variability in food supply). In chapter 2 I used fresh egg weight as the measure of egg size, whereas in chapter 4 I used dry egg weight; however this seems unlikely to be an important difference since both are frequently used as an index of egg mass (e.g. Einum & Fleming 2000; Heinimaa & Heinimaa 2004). Irrespective of mass, it may be that egg quality differed between the two experiments, since they were conducted in different years and environmental factors, such as diet (e.g. dietary antioxidants) and physiochemical condition of the water (e.g. temperature and pH) are known to affect egg quality (Brooks, Tyler & Sumpter 1997). It is possible that two cohorts of mothers experienced different conditions at sea, which influenced the relative quality of their eggs, with potential consequences for offspring telomere dynamics. In chapter 2, I also found maternal inheritance of telomere length at the embryo stage, since mothers with a relatively longer telomere length at the time of reproduction produced offspring (at the embryo stage) with relatively longer telomeres. I suggested in this chapter that the effects of maternal telomere length and egg size may share a common cause, since telomere length is considered to be an index of an individual's physiological fitness and there are links between a mother's fitness and the quality of her eggs (Blount *et al.* 2002a; Tobler & Sandell 2009). The effects of both egg weight and maternal telomere length had disappeared by the time the offspring had reached the fry stage, which further suggests that they arose mostly through variation in egg provisioning (Donelson, Munday & McCormick 2009; Van Leeuwen *et al.* 2015).

Paternal life history traits, such as early life growth rate, had a greater association in the later stages of development. In chapter 2, I found a significant association between paternal years in fresh water and offspring (fry) telomere length, with fathers that had spent the least time in fresh water producing fry with the longest telomeres. In slight contrast, I found in Chapter 4 that fry telomere length was significantly affected by paternal years in sea water (but not fresh water): males that had spent longer at sea prior to reproduction produced fry with relatively longer telomeres. However, the processes underlying this

inconsistency are slightly hard to disentangle. For both of these chapters I used a split brood IVF design, utilising all available parental life history types. Adopting this design has many advantages. For example, it offers a true representation of all possible parental life history crosses in a Scottish salmon population. That being said, this design may confound analyses that address questions related to parental years at sea, since all precocious parr fathers have (by definition) not migrated to sea. There is thus a potentially confounding effect of migration vs non-migration. For example, Fig. 6.1 shows the relationship between paternal SW age and offspring (fry) telomere length for chapter 2 (when paternal SW age was not significant) and chapter 4 (when paternal SW age was significant). For migratory fathers, the relationship is similar in both studies, with larger multi sea winter fathers producing offspring with relatively longer telomeres, compared to 1SW fathers. However, there are contrasting effects with respect to offspring from precocious parr fathers. In chapter 4 (where paternal years at sea was significant) precocious parr fathers produced offspring with the shortest telomeres, whereas in chapter 2 (where paternal years at sea was not significant) offspring from precocious parr fathers had a similar telomere length to offspring from multi sea winter fathers. In addition, these experiments were conducted in different years and thus used different cohorts of broodstock fish (and also different tributary streams as source populations). Therefore, it may be the case that parental effects are in turn dependent on temporal and/or spatial effects.

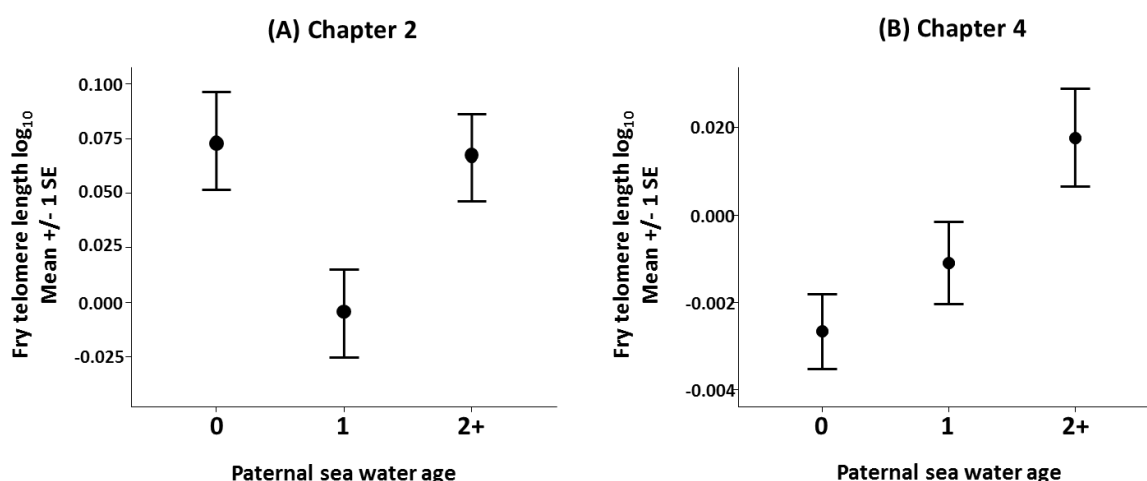


Fig. 6.1. The relationship between paternal years at sea and offspring (fry) telomere length in (A) chapter 2, and (B) chapter 4. Plotted telomere lengths are relative within each experiment and cannot be used to telomere lengths between the two experiments.

Environmental effects

There is evidence that changes in telomere length are strongly influenced by environmental conditions (for review see Monaghan 2014). For example, there is the possibility that environmentally determined plasticity of growth rate may have some influence on telomere dynamics. In addition, environmental stressors may also affect mean telomere lengths through their influence on the production of ROS within the organism (von Zglinicki 2002; Geiger *et al.* 2012; Kim & Velando 2015). A number of studies have linked telomere dynamics to environmental stressors, such as stress exposure, disturbance, social crowding and social status (Epel *et al.* 2004; Kotrschal, Ilmonen & Penn 2007; Haussmann *et al.* 2011; Blackburn & Epel 2012; Sohn *et al.* 2012; Herborn *et al.* 2014; Monaghan 2014; Nettle *et al.* 2015; Marchetto *et al.* 2016). However, to date this work has mostly focused on endothermic vertebrates. In chapter 4, I showed that faster growth in salmon fry resulted in shorter telomeres, but this effect depended on the relative harshness of the environment. Fry that grew in the harsher, colder stream paid a higher cost, in terms of telomere length. In that chapter I suggested several mechanisms that might individually or collectively underlie this effect, such as temperature induced trade-offs between cell division (hyperplasia) and cell growth (hypertrophy), temperature induced trade-offs between growth and self-maintenance (e.g. antioxidant production) and altered mitochondrial functioning, which has the potential to increase ROS production. In chapter 5, I tested a number of these hypotheses by rearing salmon embryos at three different temperatures within the natural range. Individuals were sampled at two time points (the eyed embryo and first feeding fry stages) and assays conducted of telomere lengths, oxidative stress and levels of cell proliferation. Although that chapter produced a number of interesting results, I did not find a direct link between embryo incubation temperature and telomere dynamics. This may have been because the focus was on the embryonic stages, and it was only later that I discovered that telomeres become longer through embryonic development, being largest at the first feeding fry stage. Therefore, any potential temperature effects may be confounded by this significant elongation. I did not anticipate this, since in studies to date (mostly mammals) it would appear that there is a single elongation step early in embryogenesis (Schaezlein *et al.* 2004; Liu *et al.* 2007; Kalmbach *et al.* 2014). In retrospect, it may have been better to continue the treatment until the fry were at a later stage (e.g. 2 months post-feeding).

In chapter 4, I also found that telomere length was influenced by a significant interaction between fry density and growth (i.e. body weight at age). In the benign stream, fry density had no significant effect on the relationship between fry weight and fry telomere length. However, in the harsh stream, it was beneficial in terms of telomere length for small, but not large fry to be at higher densities. The contrast between streams may reflect resource availability, with the harsher higher altitude stream likely being more oligotrophic with reduced food availability. There were also more predators in the harsher stream. Therefore, while high densities may confer benefits to smaller fish from increased predator protection, one would expect increased intraspecific competition for resources (e.g. food), especially as individuals grow larger. There was also a significant predator density effect: areas of higher predator density within each stream were associated with increased fry telomere length. This is somewhat surprising since predation risk has previously been linked to a number of physiological stress parameters (Hawlana & Schmitz 2010; Clinchy, Sheriff & Zanette 2013) including oxidative stress (Slos & Stoks 2008; Janssens & Stoks 2013) and telomere loss (Olsson *et al.* 2010).

While the field study suggested that there was a greater cost (in terms of reduced telomere length) to Atlantic salmon fry if growth occurred in a harsher and colder environment, the lab experiments did not enable me to identify which mechanisms underlie the relationship between environmental temperature and telomere dynamics. However, the experimental approach was somewhat limited, and therefore readdressing these issues could prove fruitful for future research. It is also important to establish how long these effects may persist, since it is becoming more apparent that telomere maintenance in ectothermic vertebrates differs from that of endotherms.

6.2 PROJECT LIMITATIONS

Methodological limitations

Wherever possible, it is good practise to use the same tissue for telomere length measurement, since cell proliferation rate and rate of telomere attrition may differ between different tissue types (Nussey *et al.* 2014). That being said, a growing number of studies are finding a significant correlation between the telomere length of different tissues; for example, in humans (Friedrich *et al.* 2000; Daniali *et al.* 2013), birds (Reichert *et al.* 2013; Asghar *et al.* 2016) and fish (Lund *et al.* 2009). This suggests that the use of different

tissue types may still provide useful scientific data, especially in correlative studies. However, to err on the side of caution, whenever possible I used the same tissue type for telomere analysis. For example, adipose fin tissue was used for all adult and fry analyses. However, it was not possible to sample adipose fin from embryos, and therefore telomere analysis at that life stage was conducted on whole embryo homogenate. I felt confident in using these data for correlative analysis, e.g. when examining whether parents with relatively longer telomeres produced embryos with relatively longer telomeres. However, I would have felt less confident comparing telomere length between different life stages, e.g. by analysing whether telomere length was relatively longer at the embryo (whole embryo homogenate) *versus* the fry stage (adipose fin tissue). Therefore, I did not focus too heavily on comparisons of telomere lengths between different developmental stages.

Throughout this thesis I used the Cawthon (2002) qPCR method for telomere analysis. This method is well established: at the time of writing this discussion, the Cawthon (2002) paper had been cited over 1,400 times. In addition, the qPCR method is the most efficient high throughput method currently available (Nussey *et al.* 2014): when run in triplicate, it was possible for me to analyse 75 samples per day. Moreover, it only requires small amounts of DNA (so is suitable for tissues with low DNA content such as fry adipose fins), in contrast to techniques such as the telomere restriction fragment (TRF) assay (Nussey *et al.* 2014). The qPCR method relies on identifying an appropriate single copy gene, which can prove relatively time-consuming when the method is first applied to a new study species. However, there were a large number of single copy genes already identified for Atlantic salmon (for several example see Olsvik *et al.* 2005), which made the validation of this method relatively straightforward. For each of the data chapters, raw data from each qPCR run was analysed using qBASE software for Windows (Hellemans *et al.* 2007). This controlled for differences in amplification efficiency between plates. In addition, by including three inter-run calibrators, I corrected for inter-run variation. The qBASE output is similar to the T/S ratio described by Cawthon (2002) but with greater control of inter-plate variation, which increases the precision and reproducibility of results.

There are however several drawbacks to the qPCR method. One is that the output is based on the average telomere length for a given sample, whereas TRF provides information on the range of telomere lengths (Nussey *et al.* 2014). Estimates based on average telomere lengths are useful in comparative analyses, but being able to calculate the range of telomere lengths is perhaps even more valuable, particularly since cellular senescence is

mostly associated with the critically short telomeres within a genome (Hemann *et al.* 2001). In addition, Bauch, Becker and Verhulst (2014) suggest that longer telomeres within a sample are more informative of telomere loss, since longer telomeres are larger targets for damage (e.g. oxidative damage). Another issue with the qPCR method is that it provides only relative values. It is therefore not possible to know actual telomere lengths (in kb), nor to compare values from separate studies. Lastly, the qPCR method also amplifies interstitial telomeric repeats, where the same telomeric DNA (TTAGGG) sequence occurs at points along the length of the chromosome, but plays no role in telomere functioning. Because the qPCR assay is not capable of differentiating these interstitial repeats from telomere DNA at the chromosome ends, variation in these interstitial repeats between individuals may confound results. There is substantial variation in the occurrence of interstitial repeats among species (Meyne *et al.* 1990), but they do not seem to be common in the Atlantic salmon genome (Abuin, Martinez & Sanchez 1996; Perez, Moran & Garcia-Vazquez 1999) so this may not have been an issue in the present study.

I also used various other methods for this thesis, mostly in chapter 5, where oxidative stress and cell proliferation analyses were conducted. The measurement of CAT antioxidant activity and DNA damage (8-OHdG) were conducted using commercially available kits, following the manufacturer's protocol, which was relatively straightforward. However, this was less the case with the analysis of cell proliferation, since there is currently no 'gold standard' or universally established method for the quantification of cell proliferation. I chose an immunohistochemical analysis based on an antibody stain specific to the protein PCNA, for several reasons. Firstly, PCNA is naturally expressed in nuclei during the DNA synthesis phase of cell division. Therefore, it was not necessary to administer any agents prior to quantification, as would be the case if measuring the incorporation of thymidine analogues such as 5-bromo-2'-deoxyuridine (BrdU) (Moore *et al.* 1994; Brennan *et al.* 2001; Abdo *et al.* 2014). A second reason was that it was routinely used by the University of Glasgow Veterinary Diagnostic Services and had been successfully applied to a variety of fish species (for examples see Ortego *et al.* 1994; Kilemade *et al.* 2002; Abdo *et al.* 2014; Peterson, Mok & Au 2015). One drawback to using PCNA is that it has a relatively long half-life, meaning that residual PCNA may continue to be present in cells that have fully completed division (Scott *et al.* 1991). In addition, because it was applied to embryos (which undergo a relatively fast cell turnover), I subsequently found that most cells had undergone some degree of staining. Therefore,

instead of comparing PCNA positive against PCNA negative nuclei, I instead had to develop a method which distinguished between intensely PCNA positive (IP) nuclei and weakly PCNA positive (WP) nuclei (similar to the method of Kilemade *et al.* 2002). I mostly felt confident in the results that this analysis produced. However, it is hard to know if the histological preparation may have added noise to the results. For example, it may be that cell proliferation rate varies slightly between different sections of the anterior muscle, and thus the exact point at which the longitudinal section was taken may have had an effect.

In general, each data chapter was influenced by financial and time limitations. It would have been interesting (but much more costly and time-consuming) to analyse telomere lengths of individual fish, rather than pooling siblings and analysing average family telomere lengths. Doing so would have allowed me to analyse variation in telomere dynamics within families, as well as between. Likewise, for the oxidative stress assays, I only looked at one marker of oxidative damage (8-OHdG) and one marker of antioxidant defence (catalase (CAT) activity). Using additional markers would have made for a more robust analysis, but would have required a much bigger financial and time investment.

Limitations of the study species: Atlantic salmon

The majority of *in vivo* longitudinal telomere studies, to date, have been based on analysis of blood samples, since nucleated red blood cells (e.g. as in birds and fish) are considered a good representative tissue for measuring telomere length (Nussey *et al.* 2014). However, one of the main disadvantages of using Atlantic salmon, or any fish species for that matter, for such studies is the difficulty in obtaining non-invasive blood samples from small fish (Le Vin *et al.* 2011). I initially considered using fish scales for telomere analysis, since the use of fish scales is becoming more common in molecular and genomic studies (Nielsen, Hansen & Loeschcke 1997; Finnegan *et al.* 2013; Johnston *et al.* 2013; Perrier *et al.* 2013; Johnsen *et al.* 2014). It is thought that DNA recovered from fish scales is from the dried dermic and epidemic cells on the outside of the scale (Nielsen, Hansen & Loeschcke 1999). However, I decided against the use of scales, partly because of the low DNA yields and the rapid decline in DNA quality (Nielsen, Hansen & Loeschcke 1999). Also, far less is known about scale DNA from early life stage fish, and from subsequent observations I do not think it would have been feasible to obtain sufficient scales from fry (at least non-invasively) to obtain a high enough yield of DNA for telomere analysis. In the end I

decided to use adipose fin clips for all of the post-embryonic telomere analyses. Clipping the adipose fin is a routine marking or sampling method in fisheries management and is thought to be relatively non-invasive (Dietrich & Cunjak 2006; Champagne *et al.* 2008; Bumgarner, Schuck & Blankenship 2009). From personal observation, removal of the adipose fin seemed to cause the fish little discomfort, whereas the removal of scales caused a greater reaction from the fish. One potential downside to fin clipping, with regards to telomere analysis, is the regeneration potential of fins, since studies have found a link between fin regeneration and telomere maintenance (Elmore *et al.* 2008; Anchin *et al.* 2011). However, Pauliny *et al.* (2015) found that while fin regeneration affected the telomere length of transgenic Coho salmon, in wild-strain salmon there was no difference between the telomere length of regenerated pelvic fins and that of pelvic fins that had not been previously clipped. In addition, the regeneration potential varies between the different fins of Atlantic salmon, and studies have found that the adipose fin shows the least regeneration (Stauffer & Hansen 1969; Weber & Wahle 1969; Johnsen & Ugedal 1988) making it suitable for marking fish (e.g. to give an external identifying mark to individuals that have been PIT tagged). Indeed, tissue regeneration would only be an issue in longitudinal studies, where the same fin would need to be sampled twice. In chapter 3, I did sample the same individual twice, both before and after sea migration, but used the upper lobe of the adipose fin for the first telomere measurement and the remaining half of the fin lobe for the second measurement. For adult fish that returned from migration after one winter at sea (1SW), 94.7% of the individuals experienced a relative telomere loss, suggesting that adipose fin may be a good choice of tissue for studying telomere change in wild Atlantic salmon.

6.3. FUTURE DIRECTIONS

A more detailed study of how telomere dynamics (including telomerase expression) changes with age in fish

Considering my study overall, it would appear that telomere elongation (possibly as a result of telomerase activity) is mostly related to developmental stage and growth. With this in mind, a sensible next step may be to better establish changes in telomere length and telomerase activity throughout the lifetime of fish such as Atlantic salmon. As I mentioned previously, most studies of fish telomeres to date have only looked at changes in telomere length between two time points, but it would appear that the relationship between age (i.e.

developmental stage) and telomere length is not a simple unidirectional relationship and so requires measurements at multiple time points. This could prove problematic for studies of wild fish, or for species that reach sizes difficult to house in typical laboratory aquaria. A solution may be to use farmed fish that could be followed and sampled throughout their lifetime. The aquaculture industry is perfectly set up to accommodate all life stages of species such as Atlantic salmon, and it may have facilities to retain broodstock fish over successive spawning seasons, which results in adult fish that are larger (and older) than may be present in a wild population. Lastly, post-reproductive mortality is relatively high in Atlantic salmon, with only around 5% of the UK population returning to spawn for a second time (Jonsson & Jonsson 2011). However, salmon aquaculture breeding programmes routinely ‘recondition kelts’, whereby fish which have spawned are fed and reared back up to spawning condition by the following year, so that they may go on to spawn multiple times. Therefore, in addition to age and growth effects, it may also be possible to study the effect of repeat spawning and reproductive investment on telomere/telomerase dynamics.

Consider alternative tissues for telomere analysis in fish

Fin tissue may not be the best option for studies that require multiple samples to be taken, since there is realistically only so many times you can sample a given fin. Recently there have been several publications discussing the potential of using mucus from the body or mouth cavity of fishes (Livia *et al.* 2006; Le Vin *et al.* 2011; Mirimin *et al.* 2011; Taslima *et al.* 2015). However, potential drawbacks with these methods are contamination and low DNA quality and quantity. For example, Taslima *et al.* (2015) found higher amounts of bacterial DNA in mucus DNA extraction from Nile tilapia *Oreochromis niloticus*, compared to the more traditional fin clip DNA extractions. Furthermore, most of these studies do not mention the tissue of origin of the DNA in the mucus samples. Presumably a proportion of the mucus is epithelial cells, but other cell types may also be present, leading to an unknown mixture of cell types within a single mucus sample, which could create noise in telomere measurements. Therefore, although skin mucus may prove to be a valuable DNA source for genomic studies, which are not so dependent on good quality, tissue-specific DNA, it seems less likely to be useful for telomere studies. Perhaps instead of trying to find alternative DNA sources to blood, it may be that techniques can be developed to allow easier blood sampling in the earlier/smaller developmental stages of fish. For example, Zang *et al.* (2015) recently developed a method for non-lethal blood

sampling in adult zebrafish, which are typically only 4.5cm in body length (Laale 1977) so previously considered too small for this procedure. That being said, an adult zebra fish is larger than the early life stage of Atlantic salmon – and it is possible that the protocol, while non-lethal, might still be stressful enough to influence telomere dynamics. Alternatively, it may be a better idea to shift the focus to a different ectothermic taxon. For example, Robert and Bronikowski (2010) studied a wild garter snake *Thamnophis elegans* population with two distinct ecotypes: a fast growing, early maturing ecotype with a relatively short lifespan; and a slow growing, late maturing ecotype with a relatively longer lifespan. In addition, Robert and Bronikowski (2010) found that the faster growing ecotype (with reduced lifespan) experienced a greater rate of ROS production and a reduced rate of DNA damage repair throughout its lifetime. There are several ways in which blood can be collected from snakes (Bush & Smeller 1978) and, although never applied to snakes, it may also be possible to take longitudinal samples by needle biopsy (Pesta & Gnaiger 2012; Ziak *et al.* 2015); though both procedures would require expertise and possibly anaesthesia. Combined, this suggests that this wild population of garter snake could provide a similar or perhaps even better option to Atlantic salmon, for studying longitudinal telomere dynamics in a wild ectothermic population.

Better establish what telomere dynamics tell us in fish

Lastly, one of the main future directions should be to establish what telomere dynamics in fish actually tell us. Telomeres are known to shorten with age in many long-lived mammal and bird species (Hausmann *et al.* 2003; Henriques & Ferreira 2012), and a relatively short telomere length in these endotherms is usually assumed to be indicative of relatively poor biological state, and linked to reduced longevity as a result of age-related deterioration and/or increased disease susceptibility (Hausmann, Winkler & Vleck 2005; Pauliny *et al.* 2006; Aubert & Lansdorp 2008; Ilmonen, Kotrschal & Penn 2008; Bize *et al.* 2009; Heidinger *et al.* 2012; Stier *et al.* 2015; Fairlie *et al.* 2016). Many mammal and bird species, especially longer lived species, appear to down-regulate telomerase expression in post-embryonic somatic tissue and instead use replicative ageing as a tumour suppression mechanism (Gomes, Shay & Wright 2010). However, studies have detected telomerase expression in post embryonic tissue in reptiles, amphibians and fish (Gomes, Shay & Wright 2010; Anchelin *et al.* 2011; Peterson, Mok & Au 2015), suggesting that ectothermic vertebrates do not use replicative ageing. Telomeres have been found in all vertebrates studied to date, as well as many other taxa, suggesting that telomeres are an

ancient, highly conserved means of genome protection (Gomes, Shay & Wright 2010). However, if fish have not adopted a replicative ageing strategy, can telomere dynamics actually tell us anything useful about lifespan in fish? Perhaps a more controlled experiment (e.g. using an aquaculture facility) could help address this question by monitoring telomere dynamics (telomere length and telomerase activity) continually throughout adult life, to see if it predicts lifespan as it appears to do in endotherms. Lifespan aside, another question is whether telomeres tell us anything about fitness in fish. In chapter 2 and 4 I showed that fry that invest more in growth did so at a cost to telomere length. Similarly, in chapter 3, I showed that smolts with the shortest telomeres had an increased probability of surviving migration, possibly as a result of investing more heavily in the smolting process. Similarly, Gao and Munch (2015) found telomere length to be negatively associated with investment in reproduction in a fish species, with reproductive success being achieved at the expense of telomere length. Therefore, it would appear that telomere length may prove useful as an additional indicator of life history trade-offs in fish, at least in the short term, but as yet there is no evidence that individual fish with shorter telomeres have a reduced life expectancy. Again, a longer term study, following telomere dynamics and life history strategy throughout an individual's life time could prove fruitful.

REFERENCE LIST

- Aas, Ø., Klemetsen, A., Einum, S. & Skurdal, J. (2011) *Atlantic salmon ecology*. Wiley-Blackwell, , Oxford, UK.
- Abdo, W., Ghattas, S., Sakai, H., Hirata, A. & Yanai, T. (2014) Assessment of proliferative activity by proliferative cell nuclear antigen (PCNA) and anti-bromodeoxyuridine (BrdU) immunolabeling in the tissues of japanese eels (*Anguilla japonica*). *Turkish Journal of Fisheries and Aquatic Sciences*, **14**, 413-419.
- Abele, D., Heise, K., Pörtner, H.O. & Puntarulo, S. (2002) Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. *Journal of Experimental Biology*, **205**, 1831-1841.
- Abuin, M., Martinez, P. & Sanchez, L. (1996) Localization of the repetitive telomeric sequence (TTAGGG)(n) in four salmonid species. *Genome*, **39**, 1035-1038.
- Aceto, A., Amicarelli, F., Sacchetta, P., Dragani, B., Bucciarelli, T., Masciocco, L., Miranda, M. & Ilio, C.D. (1994) Developmental aspects of detoxifying enzymes in fish (*Salmo iridaeus*). *Free radical research*, **21**, 285-294.
- Adriaenssens, B., Pauliny, A., Blomqvist, D. & Johnsson, J.I. (2016) Telomere length covaries with personality in wild brown trout. *Physiology & Behavior*, **165**, 217-222.
- Allsopp, R.C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E.V., Futcher, A.B., Greider, C.W. & Harley, C.B. (1992) Telomere length predicts replicative capacity of human fibroblasts. *Proceedings of the National Academy of Sciences, USA*, **89**, 10114-10118.
- Alonso-Álvarez, C., Bertrand, S., Faivre, B. & Sorci, G. (2007) Increased susceptibility to oxidative damage as a cost of accelerated somatic growth in zebra finches. *Functional Ecology*, **21**, 873-879.
- Anchelin, M., Murcia, L., Alcaraz-Pérez, F., García-Navarro, E.M. & Cayuela, M.L. (2011) Behaviour of telomere and telomerase during aging and regeneration in zebrafish. *PloS one*, **6**, e16955.
- Angelier, F., Holberton, R.L. & Marra, P.P. (2009) Does stress response predict return rate in a migratory bird species? A study of American redstarts and their non-breeding habitat. *Proceedings of the Royal Society of London B: Biological Sciences*, **276**, 3545-3551.
- Angelier, F., Vleck, C.M., Holberton, R.L. & Marra, P.P. (2013) Telomere length, non-breeding habitat and return rate in male American redstarts. *Functional Ecology*, **27**, 342-350.
- Arendt, J. (2007) Ecological correlates of body size in relation to cell size and cell number: patterns in flies, fish, fruits and foliage. *Biological Reviews*, **82**, 241-256.

- Asghar, M., Bensch, S., Tarka, M., Hansson, B. & Hasselquist, D. (2015a) Maternal and genetic factors determine early life telomere length. *Proceedings of the Royal Society of London B: Biological Sciences*, **282**, 20142263.
- Asghar, M., Hasselquist, D., Hansson, B., Zehtindjiev, P., Westerdahl, H. & Bensch, S. (2015b) Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. *Science*, **347**, 436-438.
- Asghar, M., Palinauskas, V., Zaghdoudi-Allan, N., Valkiūnas, G., Mukhin, A., Platonova, E., Färnert, A., Bensch, S. & Hasselquist, D. (2016) Parallel telomere shortening in multiple body tissues owing to malaria infection. *Proceedings of the Royal Society B: Biological Sciences*, **283**, 20161184.
- Aubert, G. & Lansdorp, P.M. (2008) Telomeres and aging. *Physiological Reviews*, **88**, 557-579.
- Aubin-Horth, N. & Dodson, J.J. (2004) Influence of individual body size and variable thresholds on the incidence of a sneaker male reproductive tactic in Atlantic salmon. *Evolution*, **58**, 136-144.
- Auer, S.K., Arendt, J.D., Chandramouli, R. & Reznick, D.N. (2010) Juvenile compensatory growth has negative consequences for reproduction in Trinidadian guppies (*Poecilia reticulata*). *Ecology Letters*, **13**, 998-1007.
- Bae, E., Samanta, P., Yoo, J. & Jung, J. (2016) Effects of multigenerational exposure to elevated temperature on reproduction, oxidative stress, and Cu toxicity in *Daphnia magna*. *Ecotoxicology and Environmental Safety*, **132**, 366-371.
- Barnett, A.G., van der Pols, J.C. & Dobson, A.J. (2005) Regression to the mean: what it is and how to deal with it. *International Journal of Epidemiology*, **34**, 215-220.
- Barrett, E.L., Burke, T.A., Hammers, M., Komdeur, J. & Richardson, D.S. (2013) Telomere length and dynamics predict mortality in a wild longitudinal study. *Molecular Ecology*, **22**, 249-259.
- Barrett, E.L.B. & Richardson, D.S. (2011) Sex differences in telomeres and lifespan. *Aging Cell*, **10**, 913-921.
- Barson, N.J., Aykanat, T., Hindar, K., Baranski, M., Bolstad, G.H., Fiske, P., Jacq, C., Jensen, A.J., Johnston, S.E. & Karlsson, S. (2015) Sex-dependent dominance at a single locus maintains variation in age at maturity in salmon. *Nature*, **528**, 405-408.
- Bateson, M., Brilot, B.O., Gillespie, R., Monaghan, P. & Nettle, D. (2015) Developmental telomere attrition predicts impulsive decision-making in adult starlings. *Proceedings of the Royal Society of London B: Biological Sciences*, **282**, 20142140.
- Bauch, C., Becker, P.H. & Verhulst, S. (2013) Telomere length reflects phenotypic quality and costs of reproduction in a long-lived seabird. *Proceedings of the Royal Society of London B: Biological Sciences*, **280**, 20122540.
- Bauch, C., Becker, P.H. & Verhulst, S. (2014) Within the genome, long telomeres are more informative than short telomeres with respect to fitness components in a long-lived seabird. *Molecular Ecology*, **23**, 300-310.

- Baum, D., Laughton, R., Armstrong, J.D. & Metcalfe, N.B. (2004) Altitudinal variation in the relationship between growth and maturation rate in salmon parr. *Journal of Animal Ecology*, **73**, 253-260.
- Beirne, C., Delahay, R., Hares, M. & Young, A. (2014) Age-related declines and disease-associated variation in immune cell telomere length in a wild mammal. *PloS one*, **9**, e108964.
- Biro, P.A. & Stamps, J.A. (2008) Are animal personality traits linked to life-history productivity? *Trends in Ecology & Evolution*, **23**, 361-368.
- Bize, P., Criscuolo, F., Metcalfe, N.B., Nasir, L. & Monaghan, P. (2009) Telomere dynamics rather than age predict lifespan in the wild. *Proceedings of the Royal Society B: Biological Sciences*, **276**, 1679-1683.
- Blackburn, E.H. (1991) Structure and function of telomeres. *Nature*, **350**, 569-573.
- Blackburn, E.H. (2000) Telomere states and cell fates. *Nature*, **408**, 53-56.
- Blackburn, E.H. (2005) Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. *FEBS Letters*, **579**, 859-862.
- Blackburn, E.H. & Epel, E.S. (2012) Telomeres and adversity: Too toxic to ignore. *Nature*, **490**, 169-171.
- Blanckenhorn, W.U. (2000) The evolution of body size: what keeps organisms small? *The Quarterly Review of Biology*, **75**, 385-407.
- Blount, J.D., Surai, P.F., Houston, D.C. & Møller, A.P. (2002a) Patterns of yolk enrichment with dietary carotenoids in gulls: the roles of pigment acquisition and utilization. *Functional Ecology*, **16**, 445-453.
- Blount, J.D., Surai, P.F., Nager, R.G., Houston, D.C., Møller, A.P., Trewby, M.L. & Kennedy, M.W. (2002b) Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. *Proceedings of the Royal Society of London B*, **269**, 29-36.
- Bouwhuis, S., Vedder, O. & Becker, P.H. (2015) Sex-specific pathways of parental age effects on offspring lifetime reproductive success in a long-lived seabird. *Evolution*, **69**, 1760-1771.
- Brennan, L.M., Boncavage-Hennessey, E.M., Wolfe, M.J., Toussaint, M.W., Dennis, W.E., Rosencrance, A.B. & Gardner, H.S. (2001) An *in vivo* method for using 5-bromo-2'-deoxyuridine (BrdU) as a marker of chemically-induced hepatocellular proliferation in the Japanese medaka (*Oryzias latipes*). *Toxicologic Pathology*, **29**, 387-397.
- Broer, L., Codd, V., Nyholt, D.R., Deelen, J., Mangino, M., Willemsen, G., Albrecht, E., Amin, N., Beekman, M., de Geus, E.J.C., Henders, A., Nelson, C.P., Steves, C.J., Wright, M.J., de Craen, A.J.M., Isaacs, A., Matthews, M., Moayyeri, A., Montgomery, G.W., Oostra, B.A., Vink, J.M., Spector, T.D., Slagboom, P.E., Martin, N.G., Samani, N.J., van Duijn, C.M. & Boomsma, D.I. (2013) Meta-analysis of telomere length in 19 713 subjects reveals high heritability, stronger

- maternal inheritance and a paternal age effect. *European Journal of Human Genetics*, **21**, 1163-1168.
- Brooks, S., Tyler, C.R. & Sumpter, J.P. (1997) Egg quality in fish: what makes a good egg? *Reviews in Fish Biology and Fisheries*, **7**, 387-416.
- Bumgarner, J.D., Schuck, M.L. & Blankenship, H.L. (2009) Returns of hatchery steelhead with different fin clips and coded wire tag lengths. *North American Journal of Fisheries Management*, **29**, 903-913.
- Burton, T., McKelvey, S., Stewart, D., Armstrong, J. & Metcalfe, N. (2013) Early maternal experience shapes offspring performance in the wild. *Ecology*, **94**, 618-626.
- Burton, T. & Metcalfe, N.B. (2014) Can environmental conditions experienced in early life influence future generations? *Proceedings of the Royal Society of London B: Biological Sciences*, **281**, 20140311.
- Burton, T., Robertsen, G., Stewart, D.C., McKelvey, S., Armstrong, J.D. & Metcalfe, N.B. (2016) Maternal age at maturation underpins contrasting behavior in offspring. *Behavioral Ecology*, **27**, 1280-1287.
- Bush, M. & Smeller, J. (1978) Blood collection & injection techniques in snakes. *Veterinary medicine, small animal clinician*, **73**, 211-214.
- Calado, R.T. & Young, N.S. (2009) Telomere diseases. *New England Journal of Medicine*, **361**, 2353-2365.
- Campisi, J., Kim, S.-h., Lim, C.-S. & Rubio, M. (2001) Cellular senescence, cancer and aging: the telomere connection. *Experimental Gerontology*, **36**, 1619-1637.
- Carney Almroth, B., Johnsson, J.I., Devlin, R. & Sturve, J. (2012) Oxidative stress in growth hormone transgenic coho salmon with compressed lifespan—a model for addressing aging. *Free radical research*, **46**, 1183-1189.
- Cawthon, R.M. (2002) Telomere measurement by quantitative PCR. *Nucleic Acids Research*, **30**, e47.
- Cawthon, R.M., Smith, K.R., O'Brien, E., Sivatchenko, A. & Kerber, R.A. (2003) Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet*, **361**, 393-395.
- Champagne, C.E., Austin, J.D., Jelks, H.L. & Jordan, F. (2008) Effects of fin clipping on survival and position-holding behavior of brown darters, *Etheostoma edwini*. *Copeia*, **2008**, 916-919.
- Chan, S.R.W.L. & Blackburn, E.H. (2004) Telomeres and telomerase. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359**, 109-121.
- Clinchy, M., Sheriff, M.J. & Zanette, L.Y. (2013) Predator-induced stress and the ecology of fear. *Functional Ecology*, **27**, 56-65.
- Collins, K. (2006) The biogenesis and regulation of telomerase holoenzymes. *Nature Reviews Molecular Cell Biology*, **7**, 484-494.

- Costantini, D. (2010) Complex trade-offs in the pigeon (*Columba livia*): egg antioxidant capacity and female serum antioxidant status in relation to diet quality. *Journal of Comparative Physiology B*, **180**, 731-739.
- Cowey, C.B., Bell, J.G., Knox, D., Fraser, A. & Youngson, A. (1985) Lipids and lipid antioxidant systems in developing eggs of salmon (*Salmo salar*). *Lipids*, **20**, 567-572.
- Crockett, E.L. (2008) The cold but not hard fats in ectotherms: consequences of lipid restructuring on susceptibility of biological membranes to peroxidation, a review. *Journal of Comparative Physiology B*, **178**, 795-809.
- Crossin, G.T., Hinch, S.G., Farrell, A.P., Higgs, D.A., Lotto, A.G., Oakes, J.D. & Healey, M.C. (2004) Energetics and morphology of sockeye salmon: effects of upriver migratory distance and elevation. *Journal of Fish Biology*, **65**, 788-810.
- Daniali, L., Benetos, A., Susser, E., Kark, J.D., Labat, C., Kimura, M., Desai, K.K., Granick, M. & Aviv, A. (2013) Telomeres shorten at equivalent rates in somatic tissues of adults. *Nature communications*, **4**, 1597.
- Davidson, W.S., Huang, T.K., Fujiki, K., von Schalburg, K.R. & Koop, B.F. (2009) The sex determining loci and sex chromosomes in the family salmonidae. *Sexual Development* **3**, 78-87.
- De Meyer, T., Rietzschel, E.R., De Buyzere, M.L., De Bacquer, D., Van Criekinge, W., De Backer, G.G., Gillebert, T.C., Van Oostveldt, P. & Bekaert, S. (2007) Paternal age at birth is an important determinant of offspring telomere length. *Human Molecular Genetics*, **16**, 3097-3102.
- Díaz, M.E., Furné, M., Trenzado, C.E., García-Gallego, M., Domezain, A. & Sanz, A. (2010) Antioxidant defences in the first life phases of the sturgeon *Acipenser naccarii*. *Aquaculture*, **307**, 123-129.
- Dietrich, J.P. & Cunjak, R.A. (2006) Evaluation of the impacts of Carlin tags, fin clips, and Panjet tattoos on juvenile Atlantic salmon. *North American Journal of Fisheries Management*, **26**, 163-169.
- Dingle, H. (2014) *Migration: The biology of life on the move*. By Oxford University Press, UK.
- Dmitriew, C.M. (2011) The evolution of growth trajectories: what limits growth rate? *Biological Reviews*, **86**, 97-116.
- Donelson, J.M., Munday, P.L. & McCormick, M.I. (2009) Parental effects on offspring life histories: when are they important? *Biology Letters*, **5**, 262-265.
- Douhard, M., Gaillard, J.-M., Delorme, D., Capron, G., Duncan, P., Klein, F. & Bonenfant, C. (2013) Variation in adult body mass of roe deer: early environmental conditions influence early and late body growth of females. *Ecology*, **94**, 1805-1814.
- Einum, S. & Fleming, I.A. (2000) Selection against late emergence and small offspring in Atlantic salmon (*Salmo salar*). *Evolution*, **54**, 628-639.

- Eisenberg, D.T.A. (2014) Inconsistent inheritance of telomere length (TL): is offspring TL more strongly correlated with maternal or paternal TL. *European Journal of Human Genetics*, **22**, 8-9.
- Elliott, J. & Hurley, M. (1997) A functional model for maximum growth of Atlantic salmon parr, *Salmo salar*, from two populations in northwest England. *Functional Ecology*, **11**, 592-603.
- Elliott, J.M. (1976) Body composition of brown trout (*Salmo trutta* L.) in relation to temperature and ration size. *Journal of Animal Ecology*, **45**, 273-289.
- Elliott, S.R., Coe, T.A., Helfield, J.M. & Naiman, R.J. (1998) Spatial variation in environmental characteristics of Atlantic salmon (*Salmo salar*) rivers. *Canadian Journal of Fisheries and Aquatic Sciences*, **55**, 267-280.
- Elmore, L.W., Norris, M.W., Sircar, S., Bright, A.T., McChesney, P.A., Winn, R.N. & Holt, S.E. (2008) Upregulation of telomerase function during tissue regeneration. *Experimental Biology and Medicine*, **233**, 958-967.
- Epel, E.S., Blackburn, E.H., Lin, J., Dhabhar, F.S., Adler, N.E., Morrow, J.D. & Cawthon, R.M. (2004) Accelerated telomere shortening in response to life stress. *Proceedings of the National Academy of Sciences, USA*, **101**, 17312-17315.
- Fairlie, J., Holland, R., Pilkington, J.G., Pemberton, J.M., Harrington, L. & Nussey, D.H. (2016) Lifelong leukocyte telomere dynamics and survival in a free-living mammal. *Aging Cell*, **15**, 140-148.
- Fick, L.J., Fick, G.H., Li, Z., Cao, E., Bao, B., Heffelfinger, D., Parker, H., Ostrander, E. & Riabowol, K. (2012) Telomere length correlates with life span of dog breeds. *Cell Reports*, **2**, 1530-1536.
- Finnegan, A.K., Griffiths, A.M., King, R.A., Machado-Schiaffino, G., Porcher, J.P., Garcia-Vazquez, E., Bright, D. & Stevens, J.R. (2013) Use of multiple markers demonstrates a cryptic western refugium and postglacial colonisation routes of Atlantic salmon (*Salmo salar* L.) in northwest Europe. *Heredity*, **111**, 34-43.
- Fleming, I.A. (1996) Reproductive strategies of Atlantic salmon: ecology and evolution. *Reviews in Fish Biology and Fisheries*, **6**, 379-416.
- Fleming, I.A. (1998) Pattern and variability in the breeding system of Atlantic salmon (*Salmo salar*), with comparisons to other salmonids. *Canadian Journal of Fisheries and Aquatic Sciences*, **55 Supplement 1**, 59-76.
- Fleming, J.M., Creevy, K.E. & Promislow, D.E. (2011) Mortality in north american dogs from 1984 to 2004: an investigation into age-, size-, and breed-related causes of death. *Journal of Veterinary Medical Science*, **25**, 187-198.
- Foley, J.F., Dietrich, D.R., Swenberg, J.A. & Maronpot, R.R. (1991) Detection and evaluation of proliferating cell nuclear antigen (PCNA) in rat tissue by an improved immunohistochemical procedure. *Journal of Histotechnology*, **14**, 237-241.
- Fortier, S., Yang, X., Wang, Y., Bennett, R.A. & Strauss, P.R. (2009) Base excision repair in early zebrafish development: evidence for DNA polymerase switching and standby AP endonuclease activity. *Biochemistry*, **48**, 5396-5404.

- Fox, C.W., Bush, M.L. & Wallin, W.G. (2003) Maternal age affects offspring lifespan of the seed beetle, *Callosobruchus maculatus*. *Functional Ecology*, **17**, 811-820.
- Friedland, K. (1998) Ocean climate influences on critical Atlantic salmon (*Salmo salar*) life history events. *Canadian Journal of Fisheries and Aquatic Sciences*, **55 Supplement 1**, 119-130.
- Friedland, K.D., Hansen, L.P., Dunkley, D.A. & Maclean, J.C. (2000) Linkage between ocean climate, post-smolt growth, and survival of Atlantic salmon (*Salmo salar* L.) in the North Sea area. *ICES Journal of Marine Science*, **57**, 419-429.
- Friedland, K.D., MacLean, J.C., Hansen, L.P., Peyronnet, A.J., Karlsson, L., Reddin, D.G., Maoileidigh, N.O. & McCarthy, J.L. (2009) The recruitment of Atlantic salmon in Europe. *ICES Journal of Marine Science*, **66**, 289-304.
- Friedrich, U., Griese, E.-U., Schwab, M., Fritz, P., Thon, K.-P. & Klotz, U. (2000) Telomere length in different tissues of elderly patients. *Mechanisms of ageing and development*, **119**, 89-99.
- Gao, J. & Munch, S.B. (2015) Does reproductive investment decrease telomere length in *Menidia menidia*? *PloS one*, **10**, e0125674.
- Garcia-Palomares, S., Navarro, S., Pertusa, J.F., Hermenegildo, C., Garcia-Perez, M.A., Rausell, F., Cano, A. & Tarin, J.J. (2009) Delayed fatherhood in mice decreases reproductive fitness and longevity of offspring. *Biology of Reproduction*, **80**, 343-349.
- Gardner, M.L.G. (1976) A review of factors which may influence the sea-age and maturation of Atlantic salmon *Salmo salar* L. *Journal of Fish Biology*, **9**, 289-327.
- Gauthreaux, S.A. (1980) *Animal migration, orientation, and navigation*. Academic Press, London.
- Gavrilov, L.A. & Gavrilova, N.S. (1997) Parental age at conception and offspring longevity. *Reviews in Clinical Gerontology*, **7**, 5-12.
- Geiger, S., Le Vaillant, M., Lebard, T., Reichert, S., Stier, A., Le Maho, Y. & Criscuolo, F. (2012) Catching-up but telomere loss: half-opening the black box of growth and ageing trade-off in wild king penguin chicks. *Molecular Ecology*, **21**, 1500-1510.
- Gluckman, P.D., Hanson, M.A., Cooper, C. & Thornburg, K.L. (2008) Effect of *in utero* and early-life conditions on adult health and disease. *New England Journal of Medicine*, **359**, 61-73.
- Gomes, N.M.V., Shay, J.W. & Wright, W.E. (2010) Telomere biology in Metazoa. *FEBS Letters*, **584**, 3741-3751.
- Guderley, H. (2004) Metabolic responses to low temperature in fish muscle. *Biological Reviews*, **79**, 409-427.
- Halliwell, B. & Gutteridge, J.M. (2015) *Free radicals in biology and medicine*. Oxford University Press, USA.

- Hatakeyama, H., Nakamura, K.I., Izumiya-Shimomura, N., Ishii, A., Tsuchida, S., Takubo, K. & Ishikawa, N. (2008) The teleost *Oryzias latipes* shows telomere shortening with age despite considerable telomerase activity throughout life. *Mechanisms of ageing and development*, **129**, 550-557.
- Hausmann, M.F., Longenecker, A.S., Marchetto, N.M., Juliano, S.A. & Bowden, R.M. (2011) Embryonic exposure to corticosterone modifies the juvenile stress response, oxidative stress and telomere length. *Proceedings of the Royal Society of London B: Biological Sciences*, **279**, 1447-1456.
- Hausmann, M.F. & Marchetto, N.M. (2010) Telomeres: linking stress and survival, ecology and evolution. *Current Zoology*, **56**, 714-727.
- Hausmann, M.F. & Treidel, L.A. (2014) Senescence: Integrating biology from cradle to the grave. *Integrative Organismal Biology*, pp. 257-275. John Wiley & Sons.
- Hausmann, M.F. & Vleck, C.M. (2002) Telomere length provides a new technique for aging animals. *Oecologia*, **130**, 325-328.
- Hausmann, M.F., Winkler, D.W., O'Reilly, K.M., Huntington, C.E., Nisbet, I.C.T. & Vleck, C.M. (2003) Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 1387-1392.
- Hausmann, M.F., Winkler, D.W. & Vleck, C.M. (2005) Longer telomeres associated with higher survival in birds. *Biology Letters*, **1**, 212-214.
- Hawlena, D. & Schmitz, Oswald J. (2010) Physiological stress as a fundamental mechanism linking predation to ecosystem functioning. *The American Naturalist*, **176**, 537-556.
- Heidinger, B.J., Blount, J.D., Boner, W., Griffiths, K., Metcalfe, N.B. & Monaghan, P. (2012) Telomere length in early life predicts lifespan. *Proceedings of the National Academy of Sciences, USA*, **109**, 1743-1748.
- Heidinger, B.J., Herborn, K.A., Granroth-Wilding, H., Boner, W., Burthe, S., Newell, M., Wanless, S., Daunt, F. & Monaghan, P. (2016) Parental age influences offspring telomere loss. *Functional Ecology*, **30**, 1531-1538.
- Heinimaa, S. & Heinimaa, P. (2004) Effect of the female size on egg quality and fecundity of the wild Atlantic salmon in the sub-arctic River Tenö. *Boreal Environment Research*, **9**, 55-62.
- Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. & Vandesompele, J. (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome biology*, **8**, R19.
- Hemann, M.T., Strong, M.A., Hao, L.-Y. & Greider, C.W. (2001) The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell*, **107**, 67-77.
- Hemmer-Brepson, C., Replumaz, L., Romestaing, C., Voituron, Y. & Daufresne, M. (2014) Non-stressful temperature effect on oxidative balance and life history traits in adult fish (*Oryzias latipes*). *Journal of Experimental Biology*, **217**, 274-282.

- Henderson, J.N. & Letcher, B.H. (2003) Predation on stocked Atlantic salmon (*Salmo salar*) fry. *Canadian Journal of Fisheries and Aquatic Sciences*, **60**, 32-42.
- Henriques, C.M. & Ferreira, M.G. (2012) Consequences of telomere shortening during lifespan. *Current Opinion in Cell Biology*, **24**, 804-808.
- Henson, J.D., Neumann, A.A., Yeager, T.R. & Reddel, R.R. (2002) Alternative lengthening of telomeres in mammalian cells. *Oncogene*, **21**, 598-610.
- Herborn, K.A., Heidinger, B.J., Boner, W., Noguera, J.C., Adam, A., Daunt, F. & Monaghan, P. (2014) Stress exposure in early post-natal life reduces telomere length: an experimental demonstration in a long-lived seabird. *Proceedings of the Royal Society of London B: Biological Sciences*, **281**, 20133151.
- Hindar, K. & Nordland, J. (1989) A female Atlantic salmon, *Salmo salar* L., maturing sexually in the parr stage. *Journal of Fish Biology*, **35**, 461-463.
- Hoaglin, D.C., Iglewicz, B. & Tukey, J.W. (1986) Performance of some resistant rules for outlier labeling. *Journal of the American Statistical Association*, **81**, 991-999.
- Hoar, W. (1988) 4. The physiology of smolting salmonids. *Fish physiology*, **11**, 275-343.
- Horn, T., Robertson, B.C., Will, M., Eason, D.K., Elliott, G.P. & Gemmill, N.J. (2011) Inheritance of telomere length in a bird. *PloS one*, **6**, e17199.
- ICES (2015) Report of the working group on North Atlantic salmon (WGNAS). ICES, Moncton, Canada.
- Ilmonen, P., Kotrschal, A. & Penn, D.J. (2008) Telomere Attrition Due to Infection. *PloS one*, **3**, e2143.
- Janssens, L. & Stoks, R. (2013) Predation risk causes oxidative damage in prey. *Biology Letters*, **9**, 20130350.
- Jemielity, S., Kimura, M., Parker, K.M., Parker, J.D., Cao, X.J., Aviv, A. & Keller, L. (2007) Short telomeres in short-lived males: what are the molecular and evolutionary causes? *Aging Cell*, **6**, 225-233.
- Jenkins, T.M.J., Diehl, S., Kratz, K.W. & Cooper, S.D. (1999) Effects of population density on individual growth of brown trout in streams. *Ecology*, **80**, 941-956.
- Johnsen, A., Brabrand, Å., Anmarkrud, J.A., Bjørnstad, G., Pavels, H. & Saltveit, S.J. (2014) Impact of human-induced environmental changes on genetic structure and variability in Atlantic salmon, *Salmo salar*. *Fisheries Management and Ecology*, **21**, 32-41.
- Johnsen, B. & Ugedal, O. (1988) Effects of different kinds of fin-clipping on over-winter survival and growth of fingerling brown trout, *Salmo trutta* L., stocked in small streams in Norway. *Aquaculture Research*, **19**, 305-311.
- Johnston, I.A. (2006) Environment and plasticity of myogenesis in teleost fish. *Journal of Experimental Biology*, **209**, 2249-2264.

- Johnston, I.A., Manthri, S., Alderson, R., Smart, A., Campbell, P., Nickell, D., Robertson, B., Paxton, C.G.M. & Burt, M.L. (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, S.E., Lindqvist, M., Niemelä, E., Orell, P., Erkinaro, J., Kent, M.P., Lien, S., Vähä, J.-P., Vasemägi, A. & Primmer, C.R. (2013) Fish scales and SNP chips: SNP genotyping and allele frequency estimation in individual and pooled DNA from historical samples of Atlantic salmon (*Salmo salar*). *BMC Genomics*, **14**, 1-13.
- Jonsson, B., Forseth, T., Jensen, A.J. & Næsje, T.F. (2001) Thermal performance of juvenile Atlantic salmon, *Salmo salar* L. *Functional Ecology*, **15**, 701-711.
- Jonsson, B. & Jonsson, N. (2004) Factors affecting marine production of Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences*, **61**, 2369-2383.
- Jonsson, B. & Jonsson, N. (2011) *Ecology of Atlantic salmon and Brown trout: habitat as a template for life histories*, 1st edn. Springer, Dordrecht, Netherlands.
- Jonsson, B., Jonsson, N. & Hansen, L. (2003) Atlantic salmon straying from the River Imsa. *Journal of Fish Biology*, **62**, 641-657.
- Jonsson, N. & Jonsson, B. (2003) Energy allocation among developmental stages, age groups, and types of Atlantic salmon (*Salmo salar*) spawners. *Canadian Journal of Fisheries and Aquatic Sciences*, **60**, 506-516.
- Jonsson, N., Jonsson, B. & Hansen, L.P. (1997) Changes in proximate composition and estimates of energetic costs during upstream migration and spawning in Atlantic salmon *Salmo salar*. *Journal of Animal Ecology*, **66**, 425-436.
- Kalaimani, N., Chakravarthy, N., Shanmugham, R., Thirunavukkarasu, A.R., Alavandi, S.V. & Santiago, T.C. (2008) Anti-oxidant status in embryonic, post-hatch and larval stages of Asian seabass (*Lates calcarifer*). *Fish Physiology and Biochemistry*, **34**, 151-158.
- Kalmbach, K., Robinson, L.G., Wang, F., Liu, L. & Keefe, D. (2014) Telomere length reprogramming in embryos and stem cells. *BioMed research international*, **2014**, 925121.
- Kamler, E. (1992) *Early Life History of Fish: An Energetics Approach*. Chapman & Hall, United Kingdom.
- Kaspersson, R., Höjesjö, J. & Bohlin, T. (2012) Habitat exclusion and reduced growth: a field experiment on the effects of inter-cohort competition in young-of-the-year brown trout. *Oecologia*, **169**, 733-742.
- Kilemade, M., Lyons-Alcantara, M., Rose, T., Fitzgerald, R. & Mothersill, C. (2002) Rainbow trout primary epidermal cell proliferation as an indicator of aquatic toxicity: an *in vitro/in vivo* exposure comparison. *Aquatic Toxicology*, **60**, 43-59.
- Kim, S.-Y. & Velando, A. (2015) Antioxidants safeguard telomeres in bold chicks. *Biology Letters*, **11**, 20150211.

- Kim, S.-Y., Velando, A., Sorci, G. & Alonso-Álvarez, C. (2010) Genetic correlation between resistance to oxidative stress and reproductive life span in a bird species. *Evolution*, **64**, 852-857.
- Kirkwood, T.B. (1990) The disposable soma theory of aging. *Genetic effects on aging II*, 9-19.
- Klapper, W., Heidorn, K., Kuhne, K., Parwaresch, R. & Krupp, G. (1998) Telomerase activity in 'immortal' fish. *FEBS Letters*, **434**, 409-412.
- Klemetsen, A., Amundsen, P.A., Dempson, J.B., Jonsson, B., Jonsson, N., O'Connell, M.F. & Mortensen, E. (2003) Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpinus* (L.): a review of aspects of their life histories. *Ecology of Freshwater Fish*, **12**, 1-59.
- Kotrschal, A., Ilmonen, P. & Penn, D.J. (2007) Stress impacts telomere dynamics. *Biology Letters*, **3**, 128-130.
- Kyo, S., Takakura, M., Kanaya, T., Zhuo, W., Fujimoto, K., Nishio, Y., Orimo, A. & Inoue, M. (1999) Estrogen activates telomerase. *Cancer Research*, **59**, 5917-5921.
- Laale, H.W. (1977) The biology and use of zebrafish, *Brachydanio rerio* in fisheries research. *Journal of Fish Biology*, **10**, 121-173.
- Larbi, A., Franceschi, C., Mazzatti, D., Solana, R., Wikby, A. & Pawelec, G. (2008) Aging of the immune system as a prognostic factor for human longevity. *Physiology*, **23**, 64-74.
- Le Vin, A.L., Adam, A., Tedder, A., Arnold, K.E. & Mable, B.K. (2011) Validation of swabs as a non-destructive and relatively non-invasive DNA sampling method in fish. *Molecular Ecology Resources*, **11**, 107-109.
- Lee, W.-S., Monaghan, P. & Metcalfe, N.B. (2012) The pattern of early growth trajectories affects adult breeding performance. *Ecology*, **93**, 902-912.
- Leri, A., Malhotra, A., Liew, C.-C., Kajstura, J. & Anversa, P. (2000) Telomerase activity in rat cardiac myocytes is age and gender dependent. *Journal of Molecular and Cellular Cardiology*, **32**, 385-390.
- Liker, A. & Székely, T. (2005) Mortality costs of sexual selection and parental care in natural populations of birds. *Evolution*, **59**, 890-897.
- Lindstedt, S.L. & Calder, W.A. (1976) Body size and longevity in birds. *The Condor*, **78**, 91-94.
- Lindström, J. (1999) Early development and fitness in birds and mammals. *Trends in Ecology & Evolution*, **14**, 343-348.
- Liu, L., Bailey, S.M., Okuka, M., Munoz, P., Li, C., Zhou, L.J., Wu, C., Czerwiec, E., Sandler, L., Seyfang, A., Blasco, M.A. & Keefe, D.L. (2007) Telomere lengthening early in development. *Nature Cell Biology*, **9**, 1436-1441.
- Livia, L., Antonella, P., Hovirag, L., Mauro, N. & Panara, F. (2006) A nondestructive, rapid, reliable and inexpensive method to sample, store and extract high-quality

- DNA from fish body mucus and buccal cells. *Molecular Ecology Notes*, **6**, 257-260.
- Lund, T.C., Glass, T.J., Tolar, J. & Blazar, B.R. (2009) Expression of telomerase and telomere length are unaffected by either age or limb regeneration in *Danio rerio*. *PloS one*, **4**, e7688.
- MacDougall-Shackleton, S.A., Dindia, L., Newman, A.E.M., Potvin, D.A., Stewart, K.A. & MacDougall-Shackleton, E.A. (2009) Stress, song and survival in sparrows. *Biology Letters*, **5**, 746-748.
- MacQueen, D.J., Robb, D.H.F., Olsen, T., Melstveit, L., Paxton, C.G.M. & Johnston, I.A. (2008) Temperature until the 'eyed stage' of embryogenesis programmes the growth trajectory and muscle phenotype of adult Atlantic salmon. *Biology Letters*, **4**, 294-298.
- Maitland, P.S. (2007) *Scotland's Freshwater Fish: Ecology, Conservation & Folklore*. Trafford Publishing (UK) Limited, Oxford.
- Mantell, L.L. & Greider, C.W. (1994) Telomerase activity in germline and embryonic cells of *Xenopus*. *The EMBO Journal*, **13**, 3211-3217.
- Marchetto, N.M., Glynn, R.A., Ferry, M.L., Ostojic, M., Wolff, S.M., Yao, R. & Haussmann, M.F. (2016) Prenatal stress and newborn telomere length. *American Journal of Obstetrics and Gynecology*, **215**, 94-95.
- Matschak, T.W., Stickland, N.C., Mason, P.S. & Crook, A.R. (1997) Oxygen availability and temperature affect embryonic muscle development in Atlantic salmon (*Salmo salar* L.). *Differentiation*, **61**, 229-235.
- McCarthy, I.D., Houlihan, D.F. & Carter, C.G. (1994) Individual variation in protein turnover and growth efficiency in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Proceedings of the Royal Society of London B: Biological Sciences*, **257**, 141-147.
- McCormick, S.D., Hansen, L.P., Quinn, T.P. & Saunders, R.L. (1998) Movement, migration, and smolting of Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences*, **55 Supplement 1**, 77-92.
- Ménézo, Y., Dale, B. & Cohen, M. (2010) DNA damage and repair in human oocytes and embryos: a review. *Zygote*, **18**, 357-365.
- Metcalf, N.B. & Monaghan, P. (2001) Compensation for a bad start: grow now, pay later? *Trends in Ecology & Evolution*, **16**, 254-260.
- Metcalf, N.B. & Monaghan, P. (2003) Growth versus lifespan: perspectives from evolutionary ecology. *Experimental Gerontology*, **38**, 935-940.
- Metcalf, N.B. & Thorpe, J.E. (1990) Determinants of geographical variation in the age of seaward-migrating salmon, *Salmo salar*. *Journal of Animal Ecology*, **59**, 135-145.
- Metcalf, N.B. & Thorpe, J.E. (1992) Early predictors of life-history events: the link between first feeding date, dominance and seaward migration in Atlantic salmon, *Salmo salar* L. *Journal of Fish Biology*, **41B**, 93-99.

- Meyne, J., Baker, R.J., Hobart, H.H., Hsu, T., Ryder, O.A., Ward, O.G., Wiley, J.E., Wurster-Hill, D.H., Yates, T.L. & Moyzis, R.K. (1990) Distribution of non-telomeric sites of the (TTAGGG)_n telomeric sequence in vertebrate chromosomes. *Chromosoma*, **99**, 3-10.
- Mills, D. (1989) *Ecology and management of Atlantic salmon*. Springer Science & Business Media, London.
- Mirimin, L., O'Keeffe, D., Ruggiero, A., Bolton-Warberg, M., Vartia, S. & FitzGerald, R. (2011) A quick, least-invasive, inexpensive and reliable method for sampling *Gadus morhua* postlarvae for genetic analysis. *Journal of Fish Biology*, **79**, 801-805.
- Mitchell, G.W., Guglielmo, C.G., Wheelwright, N.T., Freeman-Gallant, C.R. & Norris, D.R. (2011) Early life events carry over to influence pre-migratory condition in a free-living songbird. *PloS one*, **6**, e28838.
- Monaghan, P. (2008) Early growth conditions, phenotypic development and environmental change. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **363**, 1635-1645.
- Monaghan, P. (2010) Telomeres and life histories. The long and the short of it. *Annals of the New York Academy of Sciences*, **1206**, 130-142.
- Monaghan, P. (2014) Organismal stress, telomeres and life histories. *Journal of Experimental Biology*, **217**, 57-66.
- Monaghan, P. & Haussmann, M.F. (2006) Do telomere dynamics link lifestyle and lifespan? *Trends in Ecology & Evolution*, **21**, 47-53.
- Montero, D., Izquierdo, M., Tort, L., Robaina, L. & Vergara, J. (1999) High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. *Fish Physiology and Biochemistry*, **20**, 53-60.
- Moore, M.J., Leavitt, D.F., Shumate, A.M., Alatalo, P. & Stegeman, J.J. (1994) A cell proliferation assay for small fish and aquatic invertebrates using bath exposure to bromodeoxyuridine. *Aquatic Toxicology*, **30**, 183-188.
- Moser, M.L., Olson, A.F. & Quinn, T.P. (1991) Riverine and estuarine migratory behavior of coho salmon (*Oncorhynchus kisutch*) smolts. *Canadian Journal of Fisheries and Aquatic Sciences*, **48**, 1670-1678.
- Näslund, J., Pauliny, A., Blomqvist, D. & Johnsson, J.I. (2015) Telomere dynamics in wild brown trout: effects of compensatory growth and early growth investment. *Oecologia*, **177**, 1221-1230.
- Nettle, D., Monaghan, P., Gillespie, R., Brilot, B., Bedford, T. & Bateson, M. (2015) An experimental demonstration that early-life competitive disadvantage accelerates telomere loss. *Proceedings of the Royal Society of London B: Biological Sciences*, **282**, 20141610.

- Nielsen, E.E., Hansen, M.M. & Loeschcke, V. (1997) Analysis of microsatellite DNA from old scale samples of Atlantic salmon *Salmo salar*: a comparison of genetic composition over 60 years. *Molecular Ecology*, **6**, 487-492.
- Nielsen, E.E., Hansen, M.M. & Loeschcke, V. (1999) Analysis of applications DNA from old scale samples: technical aspects, and perspectives for conservation. *Hereditas*, **130**, 265-276.
- Nislow, K., Armstrong, J. & McKelvey, S. (2004) Phosphorus flux due to Atlantic salmon (*Salmo salar*) in an oligotrophic upland stream: effects of management and demography. *Canadian Journal of Fisheries and Aquatic Sciences*, **61**, 2401-2410.
- Njajou, O.T., Cawthon, R.M., Damcott, C.M., Wu, S.-H., Ott, S., Garant, M.J., Blackburn, E.H., Mitchell, B.D., Shuldiner, A.R. & Hsueh, W.-C. (2007) Telomere length is paternally inherited and is associated with parental lifespan. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 12135-12139.
- Noguera, J.C., Metcalfe, N.B., Boner, W. & Monaghan, P. (2015) Sex-dependent effects of nutrition on telomere dynamics in zebra finches (*Taeniopygia guttata*). *Biology Letters*, **11**, 20140938.
- Noguera, J.C., Metcalfe, N.B., Reichert, S. & Monaghan, P. (2016) Embryonic and postnatal telomere length decrease with ovulation order within clutches. *Scientific Reports*, **6**, 25915.
- Noguera, J.C., Monaghan, P. & Metcalfe, N.B. (2015) Interactive effects of early and later nutritional conditions on the adult antioxidant defence system in zebra finches. *Journal of Experimental Biology*, **218**, 2211-2217.
- Nordfjall, K., Larefalk, A., Lindgren, P., Holmberg, D. & Roos, G. (2005) Telomere length and heredity: Indications of paternal inheritance. *Proceedings of the National Academy of Sciences, USA*, **102**, 16374-16378.
- Nordfjall, K., Svenson, U., Norrback, K.-F., Adolfsson, R. & Roos, G. (2010) Large-scale parent-child comparison confirms a strong paternal influence on telomere length. *European Journal of Human Genetics*, **18**, 385-389.
- Nussey, D.H., Baird, D., Barrett, E., Boner, W., Fairlie, J., Gemmell, N., Hartmann, N., Horn, T., Hausmann, M. & Olsson, M. (2014) Measuring telomere length and telomere dynamics in evolutionary biology and ecology. *Methods in Ecology and Evolution*, **5**, 299-310.
- Nussey, D.H., Pemberton, J.M., Pilkington, J.G. & Blount, J.D. (2009) Life history correlates of oxidative damage in a free-living mammal population. *Functional Ecology*, **23**, 809-817.
- O'Brien, K.M. (2011) Mitochondrial biogenesis in cold-bodied fishes. *Journal of Experimental Biology*, **214**, 275-285.
- Ohlsson, T., Smith, H.G., Råberg, L. & Hasselquist, D. (2002) Pheasant sexual ornaments reflect nutritional conditions during early growth. *Proceedings of the Royal Society of London B: Biological Sciences*, **269**, 21-27.

- Økland, F., Jonsson, B., Jensen, A.J. & Hansen, L.P. (1993) Is there a threshold size regulating seaward migration of brown trout and atlantic salmon. *Journal of Fish Biology*, **42**, 541-550.
- Olsson, M., Pauliny, A., Wapstra, E. & Blomqvist, D. (2010) Proximate determinants of telomere length in sand lizards (*Lacerta agilis*). *Biology Letters*, **6**, 651-653.
- Olsson, M., Pauliny, A., Wapstra, E., Uller, T., Schwartz, T. & Blomqvist, D. (2011a) Sex differences in sand lizard telomere inheritance: paternal epigenetic effects increases telomere heritability and offspring survival. *PloS one*, **6**, e17473.
- Olsson, M., Pauliny, A., Wapstra, E., Uller, T., Schwartz, T., Miller, E. & Blomqvist, D. (2011b) Sexual differences in telomere selection in the wild. *Molecular Ecology*, **20**, 2085-2099.
- Olsson, M., Wilson, M., Uller, T., Mott, B., Isaksson, C., Healey, M. & Wanger, T. (2008) Free radicals run in lizard families. *Biology Letters*, **4**, 186-188.
- Olsvik, P.A., Lie, K.K., Jordal, A.-E.O., Nilsen, T.O. & Hordvik, I. (2005) Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Molecular Biology*, **6**, 1-9.
- Ortego, L.S., Hawkins, W.E., Walker, W.W., Krol, R.M. & Benson, W.H. (1994) Detection of proliferating cell nuclear antigen in tissues of three small fish species. *Biotechnic & Histochemistry*, **69**, 317-323.
- Owen, M. & Black, J.M. (1989) Factors affecting the survival of barnacle geese on migration from the breeding grounds. *Journal of Animal Ecology*, **58**, 603-617.
- Pauliny, A., Devlin, R.H., Johnsson, J.I. & Blomqvist, D. (2015) Rapid growth accelerates telomere attrition in a transgenic fish. *BMC Evolutionary Biology*, **15**, 159.
- Pauliny, A., Wagner, R.H., Augustin, J., Szep, T. & Blomqvist, D. (2006) Age-independent telomere length predicts fitness in two bird species. *Molecular Ecology*, **15**, 1681-1687.
- Pelis, R.M. & McCormick, S.D. (2001) Effects of growth hormone and cortisol on Na⁺-K⁺-2Cl⁻ cotransporter localization and abundance in the gills of Atlantic salmon. *General and Comparative Endocrinology*, **124**, 134-143.
- Perez, J., Moran, P. & Garcia-Vazquez, E. (1999) Cloning and physical mapping of Atlantic salmon (*Salmo salar* L.) telomeric sequences. *Heredity*, **82**, 409-414.
- Perrier, C., Guyomard, R., Bagliniere, J.-L., Nikolic, N. & Evanno, G. (2013) Changes in the genetic structure of Atlantic salmon populations over four decades reveal substantial impacts of stocking and potential resiliency. *Ecology and Evolution*, **3**, 2334-2349.
- Pesta, D. & Gnaiger, E. (2012) High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Mitochondrial bioenergetics: methods and protocols*, **810**, 25-58.
- Peters, L.D. & Livingstone, D.R. (1996) Antioxidant enzyme activities in embryologic and early larval stages of turbot. *Journal of Fish Biology*, **49**, 986-997.

- Peterson, D.R., Mok, H.O. & Au, D.W. (2015) Modulation of telomerase activity in fish muscle by biological and environmental factors. *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology*, **178**, 51-59.
- Piersma, T. (1998) Phenotypic flexibility during migration: Optimization of organ size contingent on the risks and rewards of fueling and flight? *Journal of Avian Biology*, **29**, 511-520.
- Reichert, S., Criscuolo, F., Verinaud, E., Zahn, S. & Massemin, S. (2013) Telomere length correlations among somatic tissues in adult zebra finches. *PLoS one*, **8**, e81496.
- Reichert, S., Rojas, E.R., Zahn, S., Robin, J.P., Criscuolo, F. & Massemin, S. (2015) Maternal telomere length inheritance in the king penguin. *Heredity*, **114**, 10-16.
- Riley, W.D., Ibbotson, A.T., Beaumont, W.R.C., Rycroft, P. & Cook, A.C. (2010) A portable, cost effective, pass-through system to detect downstream migrating salmonids marked with 12 mm passive integrated transponder tags. *Fisheries Research*, **101**, 203-206.
- Ringsby, T.H., Jensen, H., Pärn, H., Kvalnes, T., Boner, W., Gillespie, R., Holand, H., Hagen, I.J., Rønning, B., Sæther, B.-E. & Monaghan, P. (2015) On being the right size: increased body size is associated with reduced telomere length under natural conditions. *Proceedings of the Royal Society B: Biological Sciences*, **282**, 20152331.
- Robert, K.A. & Bronikowski, A.M. (2010) Evolution of senescence in nature: Physiological evolution in populations of garter snake with divergent life histories. *American Naturalist*, **175**, 147-159.
- Rodier, F., Coppe, J.-P., Patil, C.K., Hoeijmakers, W.A.M., Munoz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R. & Campisi, J. (2009) Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nature Cell Biology*, **11**, 973-979.
- Rollings, N., Miller, E. & Olsson, M. (2014) Telomeric attrition with age and temperature in Eastern mosquitofish (*Gambusia holbrooki*). *Naturwissenschaften*, **101**, 241-244.
- Rossiter, M. (1996) Incidence and consequences of inherited environmental effects. *Annual Review of Ecology and Systematics*, **27**, 451-476.
- Royle, N.J., Lindström, J. & Metcalfe, N.B. (2005) A poor start in life negatively affects dominance status in adulthood independent of body size in green swordtails *Xiphophorus helleri*. *Proceedings of the Royal Society of London B: Biological Sciences*, **272**, 1917-1922.
- Royle, N.J., Surai, P.F. & Hartley, I.R. (2003) The effect of variation in dietary intake on maternal deposition of antioxidants in zebra finch eggs. *Functional Ecology*, **17**, 472-481.
- Rudneva, I.I. (1997) Blood antioxidant system of Black Sea elasmobranch and teleosts. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology*, **118**, 255-260.

- Sacher, G.A. (1959) Relation of lifespan to brain weight and body weight in mammals. *Ciba Foundation colloquia on ageing*, pp. 115-133. John Wiley & Sons, London, UK.
- Schaetzlein, S., Lucas-Hahn, A., Lemme, E., Kues, W.A., Dorsch, M., Manns, M.P., Niemann, H. & Rudolph, K.L. (2004) Telomere length is reset during early mammalian embryogenesis. *Proceedings of the National Academy of Sciences, USA*, **101**, 8034-8038.
- Schmaltz, G., Quinn, J.S. & Schoech, S.J. (2016) Maternal corticosterone deposition in avian yolk: Influence of laying order and group size in a joint-nesting, cooperatively breeding species. *General and Comparative Endocrinology*, **232**, 145-150.
- Schmutz, J.A. (1993) Survival and pre-fledging body mass in juvenile emperor geese. *The Condor*, **95**, 222-225.
- Schreck, C.B., Stahl, T.P., Davis, L.E., Roby, D.D. & Clemens, B.J. (2006) Mortality estimates of juvenile spring–summer Chinook salmon in the lower Columbia River and estuary, 1992–1998: evidence for delayed mortality? *Transactions of the American Fisheries Society*, **135**, 457-475.
- Schulte, P.M. (2015) The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *Journal of Experimental Biology*, **218**, 1856-1866.
- Scott, R., Hall, P., Haldane, J., Van Noorden, S., Price, Y., Lane, D. & Wright, N. (1991) A comparison of immunohistochemical markers of cell proliferation with experimentally determined growth fraction. *The Journal of pathology*, **165**, 173-178.
- Selman, C., Nussey, Daniel H. & Monaghan, P. (2013) Ageing: It's a Dog's Life. *Current Biology*, **23**, R451-R453.
- Sharifi, S., Salati, A., Asadian, P., Gorjipor, E. & Moradian, H. (2013) Alterations to antioxidative enzymes in early life stages of rainbow trout (*Oncorhynchus mykiss*) reared in different densities. *Journal of Veterinary Research*, **68**, 389-395.
- Shearer, W.M. (1992) *Atlantic salmon scale reading guidelines*. International Council for the Exploration of the Sea Copenhagen, Copenhagen.
- Sheridan, M.A. (1986) Effects of thyroxin, cortisol, growth hormone, and prolactin on lipid metabolism of coho salmon, *Oncorhynchus kisutch*, during smoltification. *General and Comparative Endocrinology*, **64**, 220-238.
- Simide, R., Angelier, F., Gaillard, S. & Stier, A. (2016) Age and heat stress as determinants of telomere length in a long-lived fish, the Siberian sturgeon. *Physiological and Biochemical Zoology*, DOI: 10.1086/687378.
- Simons, M.J. (2015) Questioning causal involvement of telomeres in aging. *Ageing research reviews*, **24**, 191-196.
- Slos, S. & Stoks, R. (2008) Predation risk induces stress proteins and reduces antioxidant defense. *Functional Ecology*, **22**, 637-642.

- Sohn, S.H., Subramani, V.K., Moon, Y.S. & Jang, I.S. (2012) Telomeric DNA quantity, DNA damage, and heat shock protein gene expression as physiological stress markers in chickens. *Poultry Science*, **91**, 829-836.
- Speakman, J.R. (2005) Body size, energy metabolism and lifespan. *Journal of Experimental Biology*, **208**, 1717-1730.
- Spencer, K.A., Buchanan, K.L., Goldsmith, A.R. & Catchpole, C.K. (2003) Song as an honest signal of developmental stress in the zebra finch (*Taeniopygia guttata*). *Hormones and Behavior*, **44**, 132-139.
- Stauffer, T.M. & Hansen, M.J. (1969) Mark retention, survival, and growth of jaw-tagged and fin-clipped rainbow trout. *Transactions of the American Fisheries Society*, **98**, 225-229.
- Stearns, S.C. (1992) *The evolution of life histories*. Oxford University Press Oxford.
- Stickland, N.C., White, R.N., Mescall, P.E., Crook, A.R. & Thorpe, J.E. (1988) The effect of temperature on myogenesis in embryonic-development of the Atlantic salmon (*Salmo salar*). *Anatomy and Embryology*, **178**, 253-257.
- Stier, A., Delestrade, A., Zahn, S., Arrive, M., Criscuolo, F. & Massemin-Challet, S. (2014a) Elevation impacts the balance between growth and oxidative stress in coal tits. *Oecologia*, **175**, 791-800.
- Stier, A., Reichert, S., Criscuolo, F. & Bize, P. (2015) Red blood cells open promising avenues for longitudinal studies of ageing in laboratory, non-model and wild animals. *Experimental Gerontology*, **71**, 118-134.
- Stier, A., Viblanc, V.A., Massemin-Challet, S., Handrich, Y., Zahn, S., Rojas, E.R., Saraux, C., Le Vaillant, M., Prud'homme, O., Grosbellet, E., Robin, J.-P., Bize, P. & Criscuolo, F. (2014b) Starting with a handicap: phenotypic differences between early- and late-born king penguin chicks and their survival correlates. *Functional Ecology*, **28**, 601-611.
- Stindl, R. (2004) Tying it all together: telomeres, sexual size dimorphism and the gender gap in life expectancy. *Medical Hypotheses*, **62**, 151-154.
- Tarry-Adkins, J.L., Chen, J.H., Smith, N.S., Jones, R.H., Cherif, H. & Ozanne, S.E. (2009) Poor maternal nutrition followed by accelerated postnatal growth leads to telomere shortening and increased markers of cell senescence in rat islets. *FASEB Journal*, **23**, 1521-1528.
- Taslima, K., Davie, A., McAndrew, B.J. & Penman, D.J. (2015) DNA sampling from mucus in the Nile tilapia, *Oreochromis niloticus*: minimally invasive sampling for aquaculture-related genetics research. *Aquaculture Research*, **2015**, 1-6.
- Taylor, H.A. & Delany, M.E. (2000) Ontogeny of telomerase in chicken: Impact of downregulation on pre-and postnatal telomere length *in vivo*. *Development, growth & differentiation*, **42**, 613-621.
- Thedinga, J.F., Murphy, M.L., Johnson, S.W., Lorenz, J.M. & Koski, K.V. (1994) Determination of salmonid smolt yield with rotary-screw traps in the Situk River,

- Alaska, to predict effects of glacial flooding. *North American Journal of Fisheries Management*, **14**, 837-851.
- Thorpe, J.E., Miles, M.S. & Keay, D.S. (1984) Developmental rate, fecundity and egg size in Atlantic salmon, *Salmo salar* L. *Aquaculture*, **43**, 289-305.
- Thorstad, E.B., Whoriskey, F., Uglem, I., Moore, A., Rikardsen, A.H. & Finstad, B. (2012) A critical life stage of the Atlantic salmon *Salmo salar*: behaviour and survival during the smolt and initial post-smolt migration. *Journal of Fish Biology*, **81**, 500-542.
- Tinbergen, J.M. & Boerlijst, M.C. (1990) Nestling weight and survival in individual great tits (*Parus major*). *Journal of Animal Ecology*, **59**, 1113-1127.
- Tobler, M. & Sandell, M.I. (2009) Sex-specific effects of prenatal testosterone on nestling plasma antioxidant capacity in the zebra finch. *Journal of Experimental Biology*, **212**, 89-94.
- Todd, C.D., Hughes, S.L., Marshall, C.T., Maclean, J.C., Lonergan, M.E. & Biuw, E.M. (2008) Detrimental effects of recent ocean surface warming on growth condition of Atlantic salmon. *Global Change Biology*, **14**, 958-970.
- Trenzado, C.E., Morales, A.E. & de la Higuera, M. (2006) Physiological effects of crowding in rainbow trout, *Oncorhynchus mykiss*, selected for low and high stress responsiveness. *Aquaculture*, **258**, 583-593.
- Trueman, C.N., MacKenzie, K.M. & Palmer, M.R. (2012) Stable isotopes reveal linkages between ocean climate, plankton community dynamics, and survival of two populations of Atlantic salmon (*Salmo salar*). *ICES Journal of Marine Science*, **69**, 784-794.
- Trust, T.A.S. (2012) The Atlantic Salmon Trust. <http://www.atlanticsalmontrust.org/>.
- Tung, J., Archie, E.A., Altmann, J. & Alberts, S.C. (2016) Cumulative early life adversity predicts longevity in wild baboons. *Nature communications*, **7**, 11181.
- Turbill, C., Ruf, T., Smith, S. & Bieber, C. (2013) Seasonal variation in telomere length of a hibernating rodent. *Biology Letters*, **9**, 20121095.
- Ujvari, B., Biro, P.A., Charters, J.E., Brown, G., Heasman, K., Beckmann, C. & Madsen, T. (2016) Curvilinear telomere length dynamics in a squamate reptile. *Functional Ecology*, doi:10.1111/1365-2435.12764.
- Unryn, B.M., Cook, L.S. & Riabowol, K.T. (2005) Paternal age is positively linked to telomere length of children. *Aging Cell*, **4**, 97-101.
- Usher, M.L., Stickland, N.C. & Thorpe, J.E. (1994) Muscle development in Atlantic salmon (*Salmo salar*) embryos and the effect of temperature on muscle cellularity. *Journal of Fish Biology*, **44**, 953-964.
- Van Leeuwen, T.E., McLennan, D., McKelvey, S., Stewart, D.C., Adams, C.E. & Metcalfe, N.B. (2015) The association between parental life history and offspring phenotype. *Journal of Experimental Biology*, **219**, 374-382.

- Vandeputte, M., Mauger, S. & Dupont-Nivet, M. (2006) An evaluation of allowing for mismatches as a way to manage genotyping errors in parentage assignment by exclusion. *Molecular Ecology Notes*, **6**, 265-267.
- Verhulst, S., Aviv, A., Benetos, A., Berenson, G.S. & Kark, J.D. (2013) Do leukocyte telomere length dynamics depend on baseline telomere length? An analysis that corrects for 'regression to the mean'. *European Journal of Epidemiology*, **28**, 859-866.
- von Zglinicki, T. (2002) Oxidative stress shortens telomeres. *Trends in Biochemical Sciences*, **27**, 339-344.
- Ward, D.M., Nislow, K.H. & Folt, C.L. (2011) Seasonal shift in the effects of predators on juvenile Atlantic salmon (*Salmo salar*) energetics. *Canadian Journal of Fisheries and Aquatic Sciences*, **68**, 2080-2089.
- Watson, H., Bolton, M. & Monaghan, P. (2015) Variation in early-life telomere dynamics in a long-lived bird: links to environmental conditions and survival. *Journal of Experimental Biology*, **218**, 668-674.
- Weatherley, A.H., Gill, H.S. & Lobo, A.F. (1988) Recruitment and maximal diameter of axial muscle fibres in teleosts and their relationship to somatic growth and ultimate size. *Journal of Fish Biology*, **33**, 851-859.
- Weber, D. & Wahle, R.J. (1969) Effect of finclipping on survival of sockeye salmon (*Oncorhynchus nerka*). *Journal of the Fisheries Board of Canada*, **26**, 1263-1271.
- Whalen, K.G. & Parrish, D.L. (1999) Effect of maturation on parr growth and smolt recruitment of Atlantic salmon. *Canadian Journal of Fisheries and Aquatic Sciences*, **56**, 79-86.
- Williams, T. & Groothuis, T. (2015) Egg quality, embryonic development and post-hatching phenotype: an integrated perspective. *Nests, Eggs, and Incubation: New Ideas about Avian Reproduction*, pp. 113-126. Oxford University Press.
- Yap, W.H., Yeoh, E., Brenner, S. & Venkatesh, B. (2005) Cloning and expression of the reverse transcriptase component of pufferfish (*Fugu rubripes*) telomerase. *Gene*, **353**, 207-217.
- Yu, C.C.-W., Woods, A.L. & Levison, D.A. (1992) The assessment of cellular proliferation by immunohistochemistry: A review of currently available methods and their applications. *The Histochemical Journal*, **24**, 121-131.
- Zang, L., Shimada, Y., Nishimura, Y., Tanaka, T. & Nishimura, N. (2015) Repeated blood collection for blood tests in adult zebrafish. *JoVE (Journal of Visualized Experiments)*, **2015**, e53272.
- Zera, A.J. & Harshman, L.G. (2001) The physiology of life history trade-offs in animals. *Annual Review of Ecology and Systematics*, **32**, 95-126.
- Ziak, J., Krajcova, A., Jiroutkova, K., Nemcova, V., Dzupa, V. & Duska, F. (2015) Assessing the function of mitochondria in cytosolic context in human skeletal muscle: Adopting high-resolution respirometry to homogenate of needle biopsy tissue samples. *Mitochondrion*, **21**, 106-112.

