Long-term exposure to elevated temperature in Atlantic salmon

(Salmo salar)

By

Jared John Tromp

BEnvSc(Hons)

Submitted in the fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

July, 2018
DEAKIN UNIVERSITY
CANDIDATE DECLARATION

I certify the following about the thesis entitled (10 word maximum)

Long-term exposure to elevated temperature in Atlantic salmon (*Salmo salar*)

submitted for the degree of Doctor of Philosophy

a. I am the creator of all or part of the whole work(s) (including content and layout) and that where reference is made to the work of others, due acknowledgment is given.

b. The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

c. That if the work(s) have been commissioned, sponsored or supported by any organisation, I have fulfilled all of the obligations required by such contract or agreement.

d. That any material in the thesis which has been accepted for a degree or diploma by any university or institution is identified in the text.

e. All research integrity requirements have been complied with.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: JARED JOHN TROMP

Signed: [Signature Redacted by Library]

Date: 28/07/2018
I am the author of the thesis entitled

Long-term exposure to elevated temperature in Atlantic salmon (*Salmo salar*)

submitted for the degree of Doctor of Philosophy

This thesis may be made available for consultation, loan and limited copying in accordance with the Copyright Act 1968.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: JARED JOHN TRUMP

Signed: [Signature Redacted by Library]

Date: 28/07/2018
“For with God nothing will be impossible”
Luke 1:37 NKJV
Acknowledgements

Firstly I would like to extend my appreciation to my supervisory team Associate Professor Luis Afonso, Dr. Paul Jones, Dr. Craig Sherman, Dr. Mark Richardson and Professor John Donald, who have given an enormous amount of time to the completion of this project. This research was supported by Tassal Group and funding from the Centre of Integrative Ecology. I would like to thank Deakin University for giving me the opportunity to further my scientific career through this PhD and scholarship.

To the members of the fish physiology lab, Christian van Rijn and Morgan Brown, thank you for your help, availability, advice and friendship. You have enriched my time here at Deakin. Additionally I would like to thank my fellow colleagues in the HDR office for all of your comments and discussions. A huge thank you to everyone that was involved in sampling during the experiment, in particular Chris Henagan and Anthony Tumbarello. Without you the data presented in this thesis would not exist. In addition, I’m grateful for the technical assistance provided by Bob Collins, Amber Chen and Zoe Robertson at Deakin’s Aquaculture Futures Facility.

To God for challenging, stretching, growing me and providing abundantly, thank you for always being there. To everyone at Extreme Life Church for all the encouragement, especially WhLarni home group.

To my family, Ken, Sharon, Laura, Jonathan, Gilbert, Rachael, Jarred, Manuel, Julie, Joseph, Michelle, Rowan, Micaiah and Ayva, thank you for your endless support, encouragement and understanding of missed social events. Also, to Jordan Bryne for helping me enjoy life outside of the PhD.
Finally to my gorgeous wife Jahzeel, for all the times that you have helped me overcome my disappointments and frustrations and celebrated my successes, for supporting me, and for creating, carrying and caring for our baby Jeddah, a far greater achievement than anything outlined in this thesis. I could not have done this without you and I love you both.
Keywords

*Salmo salar*

Atlantic salmon

RNA-Seq

Thermal stress

Bimodal growth
Abstract

Fish are subjected to a variety of stressors under common aquaculture conditions. Stress elicits a suite of physiological responses that enable the organism to return to their pre-stressed state. While short-term exposures to a stressor results in an adaptive response, to deal with and overcome the stress, repeated or long-term exposure to stress can be maladaptive. A major consequence of global climate change is that weather events are becoming more frequent and longer in duration. One issue pertaining to sea cage aquaculture is the prevalence of increased sea surface temperatures during the summer months. Temperatures that are either outside or close to the physiological limits of a species are likely to pose a significant challenge to survival and growth in intensive sea cage culture. This highlights a key need to increase our knowledge regarding physiological responses in fish chronically exposed to elevated water temperatures.

In this thesis I examined growth, survival, physiological (plasma cortisol, glucose, cholesterol levels and eye darkening) and hepatic transcriptome responses in juvenile Atlantic salmon (*Salmo salar*), maintained under a chronic (99 days) thermal (12 °C, 16 °C and 20 °C) regime. Samples were collected prior to the increase in temperature on day 1, then at day 8, 37, 69 and 99. The development of a bimodal growth distribution was identified at the end (day 99) of the study, where fish with a fork length ≤ 240 mm were categorised as lower mode and > 240 mm as the upper mode of growth. Therefore, I also described growth, physiological and transcriptomic responses for both modes. In my first chapter, I present a literature review of the relevant fields of study relating to physiology, growth and transcriptomics. In my second chapter, I described the growth and physiological responses. In chapters three, four, and five, I described the transcriptomic responses...
of fish maintained at 12 °C, 20 °C, and fish categorized as the upper and lower mode, respectively.

Fish body mass was not significantly different amongst temperature treatments after 99 days. By day 99, plasma cortisol levels and eye darkening had increased significantly in the group exposed to 16 °C when compared with 12 °C. Plasma glucose and cholesterol were not significantly different amongst the temperatures throughout the study. Plasma cortisol and glucose levels were not significantly different between the upper mode and lower mode, however plasma cholesterol was significantly lower in the lower mode compared with the upper mode. This study shows a clear physiological stress response (elevated levels of cortisol) and eye darkening in fish maintained at 16 °C but not at 12 °C or 20 °C, suggesting that the physiological responses available to deal with long-term stress are affected by temperature.

Through a comparative RNA sequencing approach I was able to determine 342 genes that were differentially expressed in the liver over the 99 day culture period in fish maintained at 12 °C. I identified differentially expressed genes with significant temporal changes in the metabolism, homeostasis, cellular transport, sexual maturation and immune response. This provided the first transcriptomic resource for determining temporal change in gene expression for juvenile pre-smolt Atlantic salmon under a normal rearing temperature of 12 °C.

This thesis also highlights important genes that are responsive in juvenile Atlantic salmon chronically exposed to an elevated water temperature of 20 °C. I identified 378 differentially expressed genes between juvenile Atlantic salmon maintained at 20 °C compared with 12 °C at day 8 and day 99. In fish cultured at higher temperatures, we found an upregulation of genes involved in immune and
heat shock responses and inflammation, and a downregulation of genes involved in homeostasis, glucose and fatty acid metabolism and cell cycle at 20 °C. I identified several candidate genes which had highly conserved expression patterns over the sampling period, which are presented to be used in the development of potential biomarkers for thermal stress.

This thesis has developed the first RNA sequencing study to characterise bimodal growth in Atlantic salmon. From the differential gene testing there were 2,339 and 2,147 genes differentially expressed between the high (upper mode) and low (lower mode) growth groups at 12 °C and 20 °C, respectively. With the aims of finding genes that were consistently differentially expressed for both temperatures between the upper and lower mode, expression profiles were combined between temperatures resulting in 872 differentially expressed genes. In total these genes enriched 592 biological processes, with larval development, embryo development ending in birth or egg hatching, response to drug, small molecule metabolic process and reproduction containing the largest number of differentially expressed genes. I identified several genes that had a putative functional role in cellular function and remediation of protein damage in the lower mode. The genes presented in this study provide novel insights into the transcriptome level changes in Atlantic salmon under bimodal growth.

In summary, this thesis investigates the response of juvenile Atlantic salmon exposed to chronically elevated water temperatures. The data presented provides new insights into the transcriptome level response. Differentially expressed genes identified in this can be used to develop novel biomarkers for chronic thermal stress.
1. General introduction

1.1. Broad overview of the Atlantic salmon industry

1.2. Global warming

1.3. Atlantic salmon aquaculture and global warming

1.4. Changes around the east coast of Tasmania

1.5. Thermal tolerance

1.6. Stress physiology

1.7. Chronic stress

1.8. Chronic thermal stress

1.9. Bimodal growth

1.10. RNA Sequencing

1.11. Atlantic salmon genome.

1.12. Liver transcriptome

1.13. Thesis aims

1.14. Planned chapter submissions to academic journals

1.15. Conference contributions

1.16. References

2. Chronic exposure to increased water temperature reveals few impacts on stress physiology and growth responses in juvenile Atlantic salmon
2.2. Materials and methods

2.2.1. Animal husbandry

2.2.2. Chronic exposure to 12 °C, 16 °C and 20 °C water temperatures

2.2.3. Blood and tissue sampling

2.2.4. Growth parameters

2.2.5. Determination of plasma metabolites

2.2.6. Eye darkening determination

2.2.7. Statistical analysis

2.3. Results

2.3.1. Bimodal growth

2.3.2. Growth parameters

2.3.3. Longitudinal analysis of stress response

2.3.4. Responses by modal group at day 99

2.4. Discussion

2.5. References

3. Temporal transcriptomic analysis in juvenile Atlantic salmon maintained at 12 °C.

3.1. Introduction

3.2. Materials and methods

3.2.1. Experimental design

3.2.2. Total RNA extraction

3.2.3. RNA sequencing and quality control

3.2.4. Read mapping and expression quantification
3.2.5. Evaluation of differential expression testing and diagnostic performance 52
3.2.6. Differential gene expression 53
3.2.7. Gene ontology enrichment analysis 54

3.3. Results 55
3.3.1. Sequencing and read mapping 55
3.3.2. Differential gene expression 55
3.3.3. Putative function of differentially expressed genes 59
3.3.4. Heat maps 61

3.4. Discussion 72
3.4.1. Putative functional roles of the most differentially expressed genes 72
3.4.2. Functional roles of the selected GO terms 74
  3.4.2.1. GO:0042493 – Response to drug 74
    3.4.2.1.1. Solute Carrier family (SLC) 75
    3.4.2.1.2. Cellular mechanisms 76
    3.4.2.1.3. Fatty acid metabolism 77
  3.4.2.2. GO:0055114 – Oxidation-reduction process 80
    3.4.2.2.1. Vitamin D regulation 80
    3.4.2.2.2. Upregulated 81
    3.4.2.2.3. Downregulated 81
  3.4.2.3. GO:0000084 & GO:0006271 – Mitotic S phase and DNA strand elongation involved in DNA replication 82
    3.4.2.3.1. Unwinding DNA/replication fork 83
    3.4.2.3.2. GO:0007568 – Aging 84
4. Transcriptomic responses in juvenile Atlantic salmon (S. salar) subjected to long-term exposure to a thermal stress. 112

4.1. Introduction 112
4.2. Materials and methods 115
  4.2.1. Experimental design 115
  4.2.2. RNA Sequencing 116
4.3. Results 117
  4.3.1. Sequencing and read mapping 117
  4.3.2. Differentially expressed genes 118
  4.3.3. Assignment of differentially expressed genes to gene ontology terms 125
4.4. Discussion 129
4.5. Conclusions 137
4.6. References 138
4.7. Supplementary Material 153
  4.7.1. RNA sequencing summary and spike-in mix ratio 153
  4.7.2. Assessment of technical and diagnostic performance 159

5. Characterisation of differential gene expression patterns between fast and slow growing juvenile Atlantic salmon (S. salar). 162

5.1. Introduction 162
5.2. Materials and methods
   5.2.1. Experimental design
   5.2.2. Bimodal length frequencies
   5.2.3. RNA Sequencing
   5.2.4. Differential gene expression

5.3. Results
   5.3.1. Sequencing and read mapping
   5.3.2. Differential gene expression
   5.3.3. Putative function of differentially expressed genes

5.4. Discussion

5.5. Conclusions

5.6. References

5.7. Supplementary Material
   5.7.1. RNA sequencing summary and spike-in mix ratio
   5.7.2. Assessment of technical and diagnostic performance

6. Conclusions
   6.1. References
1. General introduction

1.1. Broad overview of the Atlantic salmon industry

Aquaculture is a rapidly developing intensive method for producing fish for human consumption. A recent FAO report has stated that aquaculture produces more than half of all fish consumed, with an estimated 20 kg per capita globally [1]. Aquaculture is expected to play a vital role in future food security, both in volume and nutrition, and to help sustain the global population which is expected to reach 9.7 billion by 2050 [1].

Atlantic salmon (*Salmo salar*) are a popular commercial aquaculture species with a production of over 2.3 million tonnes globally [2]. Recently in 2013, the share of salmonids overtook that of shrimp and prawns as the largest aquaculture commodity in world trade by value [1]. Whilst the culture of Atlantic salmon has its origins in the 19th century, the first commercial production was reported in 1985 [2]. The popularity of this species is likely linked to their relative ease of culture, ability to spawn in captivity, and the quality, texture and taste of their fillet. Globally the main producers of Atlantic salmon are North America, Europe, Chile and Australia, with European production being the largest [2].

Atlantic salmon were first cultured in Australia in the mid 1960s, where they were imported from the River Phillip in Nova Scotia, Canada [3]. The salmon were imported as eggs to New South Wales, and then during the mid-1980s, ova from New South Wales were transported to Tasmania, with the first commercial harvest in 1987 [4]. The development of this industry was financed through the Tasmanian government and Norwegian company, Noraqua [4,5].
1.2. **Global warming**

Due to global warming, environmental weather conditions are becoming more extreme and frequent. The global average combined land and ocean surface temperatures have already shown an average increase by 0.85 °C with maximum likelihoods between 0.65 – 1.06 °C [6]. Increased temperatures have already been demonstrated to have biological effects on species. For example, a meta-analysis conducted by Root et al. [7] has outlined a temperature dependent response across 587+ wild species populations, under an estimated average increase of 0.6 °C through global warming. Further increased temperatures have revealed an average range shift of 6.1 km per decade towards the poles across 99 different species [8]. North Sea fish populations have been shown to migrate further north to higher latitudes, inhabit deeper waters or both in response to warming temperatures [9]. Whilst wild populations are able to migrate towards the poles in response to increased temperature, there is no opportunity for cultured fish to do the same. The response to increased water temperatures can be mitigated slightly through vertical migration in a net pen, yet gross increases in water temperatures, poses a significant challenge for fish health and production. The ability for a fish to adapt to increased water temperatures in cultured species is therefore dependent on the species specific thermal range and phenotypic plasticity.

1.3. **Atlantic salmon aquaculture and global warming**

Atlantic salmon are commercially grown at latitudes of 40 °– 70 ° in the Northern hemisphere, and at 40 °– 50 ° in the Southern hemisphere [2], with temperatures between 15.9 °C – 18.7 °C considered to be optimal for growth [10,11]. The increase in sea surface temperature has already sparked research into the potential impacts that this will have on their physiology, as water temperatures
between 22.3 °C – 27.5 °C constitute the lower incipient thermal limit [12,13]. The implications of increasing sea surface temperature on Atlantic salmon have been investigated in Norway [14,15], Australia [16], Sweden [17] and Canada [12].

1.4. Changes around the east coast of Tasmania

Tasmania offers the only viable climate for commercial Atlantic salmon production in Australia. During winter and surrounding months, the water temperature is cool enough to facilitate growth, whereas in the summer months the water temperature can reach 18 °C at 5 m depth, and in extreme conditions up to 22 °C [5]. More recent data suggests that the average sea surface temperature during summer (Dec, Jan, Feb) from 2009 to 2014 is 17.3 ± 0.05 °C, yet the maximum temperature that can be reached is 19.9 °C (Tassal, pers. com., 2014, Figure 1.1). These temperatures are beginning to raise concern for commercial production as they are close to the upper thermal limit of Atlantic salmon.
Figure 1.1 Annual water temperature recorded daily on a commercial sea cage farm in Tasmania from June 2009 – March 2014 at a depth of 5 m.
Sea surface temperatures are predicted to rise by ~2°C in the Tasman Sea by 2060 [18]. Tasmania has already seen an increase in sea surface temperature over the past 20 years, off the eastern coast where it has increased ~0.6 °C per decade [19]. Furthermore, the east coast of Tasmania has seen the greatest rate of warming (0.20 °C decade $^{-1}$) of the Southwest Pacific boundary current [20]. One possible reason for this is, the extension of the east Australian current migrating south by ~350 km (Figure 1.2) [18,21]. While the farms are generally located within bays or sheltered coasts, the water temperature often reflect that of the adjacent oceans and regional atmospheric process, dismissing the notion that these farms will be buffered from increased sea surface temperatures that are present in higher energy environments [22]. Further, coastal waters are likely to have higher temperatures compared with offshore waters due to vertical mixing and reduced water depths [23]. The impacts of sea surface temperature increasing is not only an issue that is faced by the southern hemisphere aquaculture. In the summer, Norway can have peak sea surface temperature of 18 °C – 20 °C, and face similar problems concerning productivity and animal welfare [24]. Thus, increasing sea surface temperature poses a significant threat to the Atlantic salmon aquaculture industry.
Figure 1.2 Sea surface temperature anomaly in Tasmania (°C) during summer (February) derived from composite SST imagery from 1992 – 2006. A warm tongue is evident of the east coast due to the extension of the East Australian Current. Adapted from [21].

1.5. Thermal tolerance

Fish inhabit a diverse range of environmental temperatures, from -2.5 °C in the Antarctic [25] to 45.5 °C in the shallow hyper thermal mangrove ponds of Belize [26]. Temperature has been termed the central abiotic factor affecting physiological function in fish [15,24,27]. Fish typically maintain their internal body tissues within 1 °C of the surrounding water temperature [27]. As such the impact of temperatures outside of the optimum can have detrimental effects to structure and function of cellular components such as membranes [27], protein synthesis [28], immune function [29,30] and metabolism [31]. Further, all physiological and biochemical functions can be impacted by any change in environmental temperature [32]. Under
chronic thermal stress conditions, the organisms will aim to increase the production of protective mechanisms. Thermal changes in the environment through the process of global warming have the potential to affect basic physiological and biochemical processes in fish. This is of particular concern when there is an increase in temperature that is close to, or exceeds the thermal limits for a particular species [33].

Atlantic salmon are commonly cultured in sea cages in a sheltered coastal environment, with the preferred temperature ranging from 16 °C – 18 °C for Tasmanian culture [5]. Juvenile Atlantic salmon have been shown to be tolerant to a wide range of temperatures, where incipient (50% survival after 7 days) tolerance levels range from 0 – 28 °C, and ultimate lethal temperatures (survival for 10 minutes) ranges from -0.8 – 33 °C [13]. There is some discrepancy in the optimal temperature for growth of juvenile Atlantic salmon under a static thermal regime. In their review of the literature Elliot and Elliott [13] suggest that temperatures between 15.9 °C – 20 °C provide the maximum growth efficiency. However, Handeland et al., [34] report that the optimal temperature for growth of Atlantic salmon is 12.8 °C for 70 g – 150 g and 14 °C for 150 g – 300 g in post smolts. In light of this the optimal temperature for growth is suggested to be between 12.8 °C – 20 °C. Although water temperatures of 20 °C may initially be beneficial for growth performance, there is concern with long term growth performance at this temperature considering it is close to the lower incipient thermal limit of 22.3 °C – 27.5 °C [12,13]. Chronic exposure to increased water temperatures may result in reduced growth performance. Salmonids have shown behavioural strategies that enable them to conserve energy by selecting cooler waters [35]. As salmonids are ectothermic, their basal metabolic rate is determined by the environmental temperature. Increased
water temperatures, at sea cage sites can have the potential to cause salmon to function at increased metabolic rates for prolonged periods. Due to the diurnal fluctuation in water temperature, these periods are often intermittently prolonged over the summer period, rather than an acute 24 hr period. Prolonged exposure to temperatures that are close to the thermal limit of Atlantic salmon are likely to cause physiological stress.

1.6. Stress physiology

Stress elicits a suite of physiological changes in vertebrates and is an adaptive response used to regain homeostasis. In order to deal with the stress, a suite of physiological changes occur through the autonomic nervous system, hypothalamic pituitary adrenal (HPA) axis, cardiovascular, metabolic and immune systems [36]. This action may be initiated by real (environmental) or perceived stressors, perceived through the central nervous system [37]. Fish under culture conditions are commonly exposed to stress [38]. This can come through different forms, such as physical handling [39,40], disturbance to the cage/tank [41,42], changes in water quality [43] or temperature [44–46]. The extent to which an individual will mount a stress response, is dependent upon the frequency of exposure, habituation to the stressor [47] (which can be compounded by the type, duration, number and severity of the stress [38]) as well as the extent to which the species has been domesticated [48].

Stress physiology has been broadly categorised into three levels of the response [38]. The primary response involves the release of corticotrophin releasing hormone (CRH) in the hypothalamus, which acts as the agonist for the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland [37]. Circulating ACTH stimulates the production of corticosteroids in the interrenal cells of the head kidneys [49,50]. The main corticosteroid released in fish in response to stress is
cortisol, which is biosynthesised in the mitochondria from cholesterol [50]. The secondary response to stress is characterised by change in metabolic, haematological, hydro mineral and structural features [38]. Corticosteroids play a major role in increasing circulating levels of plasma glucose through gluconeogenesis [50]. Through this action cortisol preferentially catabolises lipid and protein stores over carbohydrate for energy production [51]. This provides immediate energy that can be used in the fight or flight response. Tertiary stress is categorised as a whole animal response and is commonly measured through factors affecting growth, health and reproduction [38]. Under acute stress, these responses actively function to regain homeostasis. Yet they require the production of hormones and metabolites in excess from what is needed for normal function. Therefore, maintaining an organised response to a stressor has a metabolic cost.

Until the stress is abated these systems are maintained beyond their normal range. The bio-energetic cost of maintaining these systems is referred to as the allostatic load [52]. Once the stress is over, the animal can recover. Acute stress is seen to have adaptive benefit for the organism, however if prolonged, there is a significant cost to maintain the stress response. At this transition chronic stress is seen to be a maladaptive, rather than adaptive [38].

1.7. Chronic stress

Chronic stress occurs when there is a significant energetic cost to the animal that has deleterious effects on their fitness. The cost of maintaining an organised stress response is revealed when the stressor is prolonged and is commonly referred to as chronic stress. The duration after which a stress is considered chronic is unclear and can range from days to months. Under the influence of chronic stress, it is not always possible to return to pre-stress physiological set points (homeostasis).
Exposure to a repeated stressor, can have dramatic effects on the function and survival of an organism, even at low or intermittent levels. This action is possibly driven through the continual stimulation, of the hypothalamic-pituitary-interrenal (HPI) axis in the presence of a persistent/prolonged stressor [53]. This can result in higher than normal levels of circulating plasma cortisol, mediated through a negative feedback loop. Chronic stress has been shown to adversely affect growth performance [51,53,54], immune function [39,44,55,56], metabolism [57], reproductive success [58], swimming performance and ultimately survival in fish [38,49]. Under chronic stress, the attenuation of this response can be limited over time, or with the addition of secondary stressors [37]. The difficulty in assessing chronic stress is that often the stressor is at low levels, over different developmental stages and over a long period of time [59]. While cortisol is understood to have adaptive physiological benefit, the continual expression of elevated cortisol is seen to be maladaptive. This can result in higher than normal levels of circulating plasma cortisol, which is seen to have a direct impact on growth through catabolic pathways on glycogen, lipids and protein [51,54,60]. For example, brown trout (Salmo trutta L.) with chronically elevated plasma cortisol through intraperitoneal implants (2 – 4 weeks) showed an increased susceptibility to disease and mortality rates [61]. In addition, chronically increased plasma cortisol levels in channel catfish (Ictalurus punctatus (Rafinesque)) fed exogenous cortisol for 10 weeks showed reduced growth, compared to the controls [51]. These studies demonstrate the pharmacological effects that exogenous cortisol can have. However, the physiological response of cortisol can show surprisingly different response to that seen in a pharmacological dose. Pérez-Casanova et al., [44] demonstrated that under a chronically increasing thermal profile, Atlantic cod didn’t have a correlated
increasing plasma cortisol profile. In fact the opposite was seen where plasma cortisol was highest at 16 °C then returned to basal levels (10 °C) even when exposed to the highest water temperature(19.1 °C) [44]. As such there remains some uncertainty about cortisol and its use in understanding the impact on growth under chronic stress [54]. There is a lack of experimental evidence for a consistent cortisol expression profile for wild animals under chronic stress [62]. The physiological effects of chronically elevated plasma cortisol are yet to be clearly demonstrated in vivo.

1.8. **Chronic thermal stress**

One form of chronic stress involves increased water temperatures that are close to the thermal limits of a species. Temperature is a persistent stressor and provides the ideal environmental stressor to investigate the chronic stress response. Water temperature that exceeds the thermal limits of fish will result in death, dependent upon the duration of the exposure [63]. Of particular interest for this thesis is the influence of water temperatures that are close to, but do not exceed the thermal limits of a species. Growth performance is significantly reduced when water temperatures exceed the species specific limit for optimal growth [14,15,64]. While the effects of chronic thermal stress can be observed through reduced growth performance, the timing at which chronic thermal stress begins to influence the animal is poorly understood. Acute thermal stress studies have demonstrated increased plasma cortisol after exposure to heat shock [29,46]. In addition, a few studies have investigated chronic thermal stress response in fish for their upper limits, for species such as Atlantic cod (*Gadus morhua*) [44], hapuku (*Polyprion oxygeneios*) [64] and Atlantic salmon [14]. While these studies provide an indication of the chronic thermal stress response in fish, they still lack information on the
timing and duration for which the effects of chronic stress first take effect. For example, Hevrøy et al. [14] exposed Atlantic salmon to 19 °C for 56 days and reported reduced growth, feed intake and utilization, but no significant change in plasma cortisol. In addition, Tromp et al. [64] cultured hapuku at 22 °C and showed reduced condition and growth compared to 18 °C after 14 weeks, but no significant change in plasma cortisol. The cumulative stress of chronically increased temperature (1 °C every 5 days) has shown that at 16 °C (after 30 days), Atlantic cod had increased plasma cortisol levels, yet these levels decreased at higher temperatures (19.1 °C) [44]. Taken together, while there is consensus of the effects of chronic thermal stress on growth, there is still research needed on the exact timing and magnitude when chronic stress results in a maladaptive effects. In addition, further research is needed to investigate the relationship between corticosteroids and their impact on growth performance.

Chronic thermal stress studies have shown that maintaining a response to a stressor has an energetic cost, as the organism focuses on mounting a defence. As seen in catfish exposed to a 12 °C increase in temperature for 100 hours, there was a significant increase in the expression of genes involved in protein folding, energy metabolism and molecule/ion transport, while genes involved in protein biosynthesis were significantly down regulated [65]. A similar study investigating the transcriptome of Atlantic salmon under a 45 day thermal stress (13 °C verses 19 °C), found genes related to oxygen transport and binding to be highly up regulated at 19 °C [15]. In Atlantic cod (Gadus morhua) under an 8 °C thermal increase (from 10 °C to 18 °C), genes associated with protein folding, signal transduction and immune response were highlighted as important strategies used in coping with thermal stress [29]. A microarray study looking at an 8 °C increase in gilthead bream (Sparus
aurata) found genes involved in nuclear transcription factors, oxidative metabolism, membrane structure, molecular chaperones, antioxidant enzymes, fusion/fission markers and apoptotic markers to be significantly up regulated in liver mitochondria [66]. Chronic thermal stress may also affect population structure, impacting upon the ability for Atlantic salmon brood stock to spawn through suppressing the endocrine system [16]. However life stage also plays an important role in thermal plasticity in the ability of the species to respond to the thermal stress [16].

1.9. Bimodal growth

Bimodal growth has been reported in Atlantic salmon populations in response to alternate smoltification strategies [67]. Numerous authors have reported the presence on two distinct cohorts that develop in 0+ parr in experimental populations [10,67–73]. This bimodality of growth, is apparent when the fish have not been subject to grading, which is a common practise in commercial Atlantic salmon hatcheries. Fish are often categorised as the upper mode or lower mode, depending on their position in the distribution of body length. The mechanisms behind the development of bimodality are unclear [67]. Thermal stress has not been demonstrated to influence feeding or survival between fish maintained for seven days at increased temperatures and categorised into upper and lower mode cohorts [74]. The authors have found no studies that have experimentally investigated the response progression of bimodal growth distributions in Atlantic salmon when exposed to a chronic thermal stress. The divergent growth response may provide valuable insight into alternate strategies in response to chronic thermal stress. Several studies have been able to demonstrate divergent response in plasma cortisol levels, which allowed fish to be categorized as high and low cortisol responders [75–78]. The correlation between high and low cortisol levels and growth performance, shows
conflicting results between reduced growth in the high responders [77,79,80] or increased growth [76,81,82]. Measurements based on the individual response may provide valuable insight into understanding the physiology of fish under stress. Taken together these studies show how complex the response to thermal stress can be in fish, and the need for further investigation into different levels of the organism’s structure and function, particularly at the cellular level. Investigating the transcriptional response in fish to environmental stressors, through RNA sequencing may help to develop our understanding of the stress response [83].

1.10. RNA Sequencing

Next generation sequencing technologies collectively aim to determine the base order (i.e. sequence) from a population of fragmented nucleotide sequences (i.e. a cDNA library) [84]. This technology enables the production of enormous data sets in a very cost effective manner and allows us to understand changes in gene expression across individuals and tissues in response to a range of stressors or treatments [85]. Briefly the technology involves creating complementary DNA (cDNA) libraries that then are randomly fragmented into smaller transcripts. These transcripts are then sequenced based on instrument specific methods that identify individual nucleotides. Sequenced transcripts termed ‘reads’ are then filtered to remove poor quality reads that fail predetermined metrics, and are then either aligned to an existing genome, or a genome is created de novo. The breadth of analysis that can then be applied to this data includes differential expression testing, transcriptome mapping and genome annotation, novel transcript discovery, RNA splicing SNP discovery and more [86]. This information can provide a list of candidate genes that can potentially be developed as biomarkers associated with particular stressors.
1.11. Atlantic salmon genome

With the availability and cost of modern sequencing technology, molecular biologists are now able to investigate how the transcriptome changes under controlled experimental conditions. The vast majority of RNA sequencing work has focused on novel species, with the aim of discovering new genes and processes that have not previously been categorised.

The Atlantic salmon genome is publicly available through the NCBI website (https://www.ncbi.nlm.nih.gov/assembly/GCF_000233375.1/), and has individually labelled chromosomes. The Atlantic salmon genome underwent genome duplication 25 – 100 million years ago [87–89] and is comprised of ~50 000 genes, and is based on the European subspecies (S. salar europensis) [90]. There is some discussion about the differences between the Canadian and Norwegian strains of Atlantic salmon, with research suggesting that there are fewer chromosomal pairs in the Norwegian strain [91].

1.12. Liver transcriptome

The liver has a diverse functional role in carbohydrate metabolism, lipid storage, amino acid and prostaglandin metabolism, somatic growth regulation, immune response and detoxification [92–94]. In addition to these roles, the liver is also effected by environmental parameters, altering the structure, function and rate of metabolism. As such the liver has been commonly studied in fish under a variety of environmental stressors, such as temperature [15]. Further, the wide array of studies investigating hepatic responses allows for comparisons between this and those studies.
1.13. Thesis aims

Given the paucity of information currently available on the chronic thermal stress response in fish, this PhD aims to investigate the physiological and transcriptomal response of Atlantic salmon exposed to chronic high water temperature, and identify novel genes that can be developed as potential biomarkers to identify chronic thermal stress in fish. The main aims of this thesis are to:

i. Examine the effects of chronic exposure (99 days) to elevated temperatures (12 °C, 16 °C and 20 °C) on the stress response, growth, and eye darkening in juvenile Atlantic salmon. The chronic physiological stress response was studied by measuring total plasma cortisol, glucose, cholesterol levels and eye darkening. Growth was examined by determining mass, length, condition factor and specific growth rate.

ii. To compare the hepatic transcriptome profiles at three different time points (1, 8 and 99 days) to identify differentially expressed genes during under common culture conditions (12 °C). Further, we aim to describe key biological processes that show differential expression and elucidate putative gene function.

iii. To investigate the hepatic transcriptomal response of juvenile Atlantic salmon following chronic exposure to increased temperature of 20 °C for 1, 8 and 99 days. Further, we aim to characterise key biological processes that show differential expression and elucidate putative gene function.

iv. To investigate the hepatic transcriptomal response for fish categorised in the upper and lower mode. This study aimed to compare similar transcripts that were present in fish cultured at both 12 °C and 20 °C.
The research chapters in this thesis represent manuscripts that are presented in a format suitable for submission to a peer-reviewed academic journal. The data presented in chapters 3 – 5 was produced on the Illumina HiSeq 2500 platform covering two lanes of a flow cell, producing 2 x 125 bp paired-end reads. In addition the bioinformatics analytic workflow used a very similar approach through all three chapters. As such the methodology in subsequent chapters is written in a similar fashion with some aspects repeated, however there are minor changes for quality control metrics, workflow and output. Due to these minor differences between chapters and the overall structure of the thesis, a separate methods section was written for each chapter in favour of a single methods chapter. In addition, due to the size restrictions and overall length of the thesis I have been unable to include some of the supplementary material (HTseq count and RSEM gene expression estimates, edgeR raw expression and GO enrichment analysis) that will accompany these chapters at publication. These files will be made available electronically on request.

1.14 Planned chapter submissions to academic journals


Chapter 4 is in preparation for submission to the journal Genome Biology. Tromp, J.J., Richardson, M.F., Sherman, C.D.H, Jones, P.L., Donald, J.A., Afonso,
L.O.B. *Transcriptomic responses in juvenile Atlantic salmon (S. salar) subjected to long-term exposure to a thermal stress.* (lead contributor: Jared J. Tromp).

Chapter 5 is in preparation for submission to the journal *Genome Biology*.


### 1.15 Conference contributions

Research presented in this thesis has been presented with subsequent feedback to the following conference forums.


- Oral presentation at the Local Warrnambool Campus HDR Conference,


- Poster presentation at the Local Warrnambool Campus HDR Conference,


1.16 References


4. Treadwell R, Mckelvie L. Profitability of selected aquacultural species. 1991;


20. Shears NT, Bowen MM. Half a century of coastal temperature records reveal


28. McCarthy ID, Houlihan DF. The effect of temperature on protein metabolism in fish: The possible consequences for wild Atlantic salmon (Salmo salar L.) stocks in


59. Campbell WB, Emlen JM, Hershberger WK, Thermally WK. Thermally induced chronic developmental stress in coho salmon: Integrating measures of thermally


88. Hagen-Larsen H, Laerødahl JK, Panitz F, Adzhubei A, Høyheim B. An EST-based approach for identifying genes expressed in the intestine and gills of pre-smolt


Chronic exposure to increased water temperature reveals few impacts on stress physiology and growth responses in juvenile Atlantic salmon

Jared J. Tromp, Paul L. Jones, Morgan S. Brown, John A. Donald, Peter A. Biro, Luis O.B. Afonso

Deakin University, School of Life and Environmental Sciences, Centre for Integrative Ecology, Geelong, VIC, Australia

ABSTRACT

Fish are subjected to a variety of stressors under common cage aquaculture conditions. While short-term exposure to a stressor often results in an adaptive response to cope with stress, repeated and/or chronic exposure to stress can result in negative impacts on fish welfare and production. In fish, little is known about the impact of long-term exposure to stressors, including elevated water temperature. In this study we examined and developed temporal response profiles of physiological indicators of stress and growth in juvenile Atlantic salmon (Salmo salar) exposed to 12 °C, 16 °C, and 20 °C for 99 days. Five times throughout the study we quantified plasma cortisol, glucose and cholesterol levels, and growth. Fish body mass and fork length were not significantly different amongst temperatures after 99 days. Plasma cortisol was significantly elevated at 16 °C when comparing day 8 with 99, while at 12 °C plasma cortisol increased from day 1 to day 8, then returned to initial levels (day 1 and 8) after 99 days. Plasma glucose and cholesterol were not significantly different amongst the temperatures throughout the experiment. In addition, at the end of the experiment we quantified eye darkening, and identified the development of a bimodal growth distribution in all temperatures. Fish with a fork length ≤ 240 mm were categorised as lower mode (LM) and those with a fork length > 240 mm as in the upper mode (UM) of growth. Plasma cholesterol was significantly lower in the LM group in all three temperatures, but plasma cortisol and glucose levels did not differ between modes. Eye darkening also did not differ between modes, but increased significantly in the groups exposed to 16 °C and 20 °C when compared with 12 °C. This study showed a clear physiological stress response (elevated levels of cortisol) and eye darkening in fish maintained at 16 °C but not at 12 °C or 20 °C, suggesting that some aspects of the physiological responses available to deal with chronic stress are affected by temperature.

1. Introduction

Atlantic salmon (Salmo salar) aquaculture is an established industry in several countries, including Norway, Scotland, Ireland, the Faroe Islands, Canada, USA, Chile and Australia (Tasmania) (“Aquaculture topics and activities. Aquaculture resources. In: FAO Fisheries and Aquaculture Department,,”, 2015). The majority of the production areas are within latitudes 40–70° in the Northern Hemisphere, and 40–50° in the Southern Hemisphere (“Aquaculture topics and activities. Aquaculture resources. In: FAO Fisheries and Aquaculture Department,,”, 2015). When reared in freshwater or seawater phases, Atlantic salmon, can be restricted in their movement, and therefore, be exposed to several environmental stressors, including seasonal or abrupt changes in water temperature.

It has been demonstrated that Juvenile Atlantic salmon are tolerant to a wide range of temperatures, where incipient (50% survival after 7 days) and ultimate lethal tolerance (survival for 10 min) range from 0 to 28 °C and −0.8–33 °C respectively (Elliott and Elliott, 2010). However, reports concerning the optimal temperature for growth in juvenile Atlantic salmon when maintained under a static regime are conflicting. In their review, Elliott and Elliott (2010) suggested that 16–20 °C provided maximum growth efficiency in juvenile Atlantic salmon. Similarly, Jensen et al. (2015) demonstrated improved growth in juvenile Atlantic salmon maintained in saltwater for four weeks at 16 °C compared to 4 °C and 10 °C, respectively. In contrast, Handeland et al. (2008) reported that the optimal temperature for growth in post-smolt juvenile Atlantic salmon was 12.8 °C when 70–150 g, and 14 °C when 150–300 g.

The temperature range for which the onset of chronic thermal stress occurs in juvenile Atlantic salmon is not well understood. Under commercial aquaculture conditions, Atlantic salmon are confined to particular areas of the water column, and therefore, can be unwillingly
subjected to abrupt or seasonal changes in water temperature. It is well known that metabolism in fish is influenced by water temperature, and to some extent also health, stress response, growth and survival (Afonso et al., 2008; Dominguez et al., 2004; Pérez-Casanova et al., 2008a, 2008b; Tromp et al., 2016). Therefore, chronic thermal stress could have deleterious consequences on the overall fitness of fish. Most studies concerned with the metabolic effects of high water temperatures in Atlantic salmon were conducted for relatively short periods of time (Elliott and Elliott, 2010 (7 days); Hevrøy et al., 2012 (56 days); Olovik et al., 2013 (45 days)), and therefore, information regarding the effects of chronic exposure to high water temperatures is poorly known in this species.

Chronic effects of high water temperature are better known in other species (Pérez-Casanova et al., 2008b; Tromp et al., 2016). Acute exposure to stressors reveal that the immediate response to stress (increased plasma cortisol and glucose levels) helps the animal to cope with the stressor (Iwama et al., 2005). Information on the effects of chronic exposure to high temperatures on cortisol are important for understanding physiological processes, given its many effects on fish performance and production (Mommsen et al., 1999). For example, a chronic regime of increasing temperature led to elevated plasma cortisol, mortality, and expression of immune-related genes in Atlantic cod (Gadus morhua) when the temperature reached 16 °C, but this was only evident approximately 30 days after the beginning of the experiment (Pérez-Casanova et al., 2008b). On the other hand, in Atlantic salmon, constant exposure to 19 °C for 56 days led to reduced growth, but no significant change in plasma cortisol (Hevrøy et al., 2012). Similarly, culture of hapuku (Polyprion oxygeneios) at 22 °C for 98 days suppressed their gain in body mass, reduced their condition factor and specific growth rate, but did not significantly change plasma cortisol levels (Tromp et al., 2016). One explanation for the incidence of elevated cortisol in some studies where fish were subjected to chronic exposure to high water temperatures, yet not in others, may be an ability of some fish to acclimate to chronic stress, and thereby reduce elevated plasma cortisol levels back to a pre-stressed state (Barton and Schreck, 1987; Pickering and Pottinger, 1987).

In addition to a lack of studies on the effects long-term exposure to thermal stress on plasma cortisol levels, there is limited information about cholesterol levels in fish during stress. Cholesterol plays a central role in steroid hormone biosynthesis (Mommsen et al., 1999; Tokarz et al., 2015), including cortisol. The only study on plasma cholesterol levels in salmonids during exposure to high temperatures (from 10 °C to 20 °C) have shown decline in cholesterol values (Wedemeyer, 1973). In humans, low plasma cholesterol levels are described as hypcholesterolaemia, and affect immune and inflammation functions (Vyrubal et al., 2008). Obtaining information about cholesterol and cortisol levels during chronic exposure to high temperatures is important considering that cortisol is the principal corticosteroid in fish and exerts significant physiological roles in metabolic regulation, osmoregulation, growth and reproduction (Mommsen et al., 1999).

Throughout the last 30 years there has been considerable interest in studying the development of bimodal growth in juvenile Atlantic salmon during the freshwater phase. Most of these studies have been carried out in hatchery and laboratory-raised fish, and they have demonstrated that in their first year of growth, Atlantic salmon can be separated into two classes based on their length: upper mode (UM) and lower mode (LM) (Heggenes and Metcalf, 1991; Kristinsson et al., 1985; Nicieza et al., 1994; Simpson and Thorpe, 1976; Zydlewski et al., 2014) The larger fish (UM) have the potential to become smolts earlier than the LM fish. Most of these studies have examined the development of bimodal growth under normal or ambient temperatures for growth (Imsland et al., 2016; Kristinsson et al., 1985; Metcalf et al., 1988; Shrimpton et al., 2000; Shrimpton and McCormick, 1998). There are no studies that have investigated the effects of long-term exposure to elevated temperature on the bimodal growth. In addition a few studies have examined differences in physiological indicators between these two classes. For example, it has been shown that under normal hatchery conditions fish in the UM showed increased levels of classical indicators of smoltification (plasma cortisol, growth hormone, and gill Na⁺, K⁺-ATPase) well in advance than fish in the LM (Shrimpton et al., 2000; Shrimpton and McCormick, 1998).

Recently there have been few studies that investigate the development of rapid, non-invasive and reliable techniques for determining stress. The measurement of eye sclera colour changes, termed eye darkening (ED) has shown the potential to be used as an non-invasive indicator for stress in fish (Freitas et al., 2014; Suter and Huntingford, 2002; Vera Cruz and Brown, 2007; Volpato et al., 2003). The physiological processes that induce ED in fish are not well understood (Nilsson Sköld et al., 2013). However, there is evidence in sand goby (Pomatoschistus minutus) via an in vitro study that ED may be controlled by changes in the dispersal of eye chromatophores due to melanin concentrating hormone (MCH) and the adrenocorticotropic hormone (ACTH) (Sköld et al., 2015). Given the role of ACTH in cortisol production (Barton and Iwama, 1991), these findings suggest that ED may be controlled by hormones involved in the generalised stress response in fish.

Considering the paucity of studies investigating the long-term exposure of juvenile Atlantic salmon to elevated temperatures, we examined the effects of a chronic increase in water temperature for 99 days on this species stress response and growth. The chronic physiological responses were studied temporally by measuring total plasma cortisol, glucose, and cholesterol levels and eye darkening at the last sampling time. As we were able, at the end of the study, to identify bimodal growth, and therefore separate fish by size into upper and lower modes, we also measured the physiological and growth indicators in these groups. Investigation of the effects of long-term exposure to high temperature, including in fish categorised as fast or slow growers, may lead to a better understanding of their physiology, and identification of phenotypes that are better prepared to deal with elevated temperatures.

2. Materials and methods

2.1. Animal husbandry

Fish were held in three identical indoor recirculating aquaculture systems at the Deakin Aquaculture Futures Facility, and treated through physical, biological and UV sterilisation (Norambuena et al., 2016). Each system controlled for temperature at 12 °C and photoperiod (12:12D) prior to fish delivery. Juvenile Atlantic salmon (~70 g) were sourced from Mountain Fresh Trout and Salmon Farm, Victoria, Australia. Upon arrival, fish were distributed into two 2000 L circular polyethylene tanks, and held for 10 days. Following this, fish were randomly distributed into the three identical recirculating systems maintained at 12 °C, with each room containing five 1000 L circular tanks and 34 fish per tank. During the acclimation period (15 days), average dissolved oxygen concentration, percent saturation and temperature were 9.8 ± 0.03 mg L⁻¹, 89.7 ± 0.23% and 11.8 ± 0.05 °C, respectively. Fish were fed daily to apparent satiation using a commercial diet (Spirit Plus 100, Skretting). Water quality parameters (nitrite, nitrate, ammonia and pH) were measured twice weekly, using Aquarem test kits (Merck, Darmstadt, Germany), with all levels being maintained within the acceptable limits for Atlantic salmon in freshwater.

2.2. Chronic exposure to 12 °C, 16 °C and 20 °C water temperatures

To establish the thermal regimes to 16 and 20 °C, the water temperature was increased over seven days in two of the recirculating systems. There were five replicate tanks in each treatment (34 fish per tank). Average water temperatures in the experimental groups following the 7 day gradual increase interval were 11.6 ± 0.07 °C,
15.7 ± 0.03 °C and 19.3 ± 0.05 °C.

Fish sampling for length, mass and stress indicators began on day 1, prior to the increase in temperature (baseline levels in all groups at 12 °C) when 6 fish per tank were randomly sampled. Thereafter, 4 fish per tank were sampled at day 8, 37, 69 and 99. Thus, at least 20 fish in total were (lethally) sampled at each time point. At the last sampling time, the remainder of the fish in each tank (7–12) were also sampled to improve growth estimates.

2.3. Blood and tissue sampling

At each sampling time, fish were netted and immediately euthanised via a lethal dose of AQUI-S® (300 mg L⁻¹) in under two minutes, in order to determine baseline cortisol levels (Barton, 2002; Sumpter et al., 1986). Blood was collected from the caudal vein using 3 mL heparinised (200 U/mL) syringes with a 23 G × 32 mm needle. Blood was transferred to 2.0 mL centrifuge tubes and stored on ice prior to centrifugation. To obtain plasma, blood samples were centrifuged at 3000 x g for 15 min. The plasma aliquot was transferred to 1.5 mL microcentrifuge tube and immediately frozen in liquid nitrogen, and subsequently transferred to −80 °C until further analysis. In all cases, feeding was stopped 24 h before sampling events.

2.4. Growth parameters

At each sampling time fish body mass (g) and fork length (mm) were recorded. The following formula were used to calculate the indices reported: Fulton’s condition factor (K) = (10⁵ × body mass)/fork length³, specific growth rate (SGR) = (ln(final body mass) − ln(initial body mass))/days × 100, visceral somatic index (VSI) = visceral mass/body mass × 100.

2.5. Determination of plasma metabolites

Plasma cortisol was determined (in duplicate) using a commercially available enzyme immunoassay (ELISA) kit (Cayman Chemical Company, Ann Arbor, Michigan, USA), following the manufacturer’s specifications (Matsche, 2013). The plates were read at 412 nm, using a microplate reader (Molecular Devices Spectra Max M3) in conjunction with the software package, Softmax Pro v6.2.1. Plasma glucose and cholesterol samples were determined (in duplicate) using a commercially available reagent kit from SIEMENS© (Siemens Healthcare Diagnostics Inc., Newark, DE, USA) adapted for use in a 96-well microplate and read at 340 nm and 560 nm, respectively (Tromp et al., 2016). Intra-assay coefficient of variation recorded for cortisol, glucose and cholesterol was 15.07%, 2.00% and 2.01% respectively. Inter-assay coefficient of variation recorded for cortisol, glucose and cholesterol was 19.47%, 13.98% and 6.53%, respectively.

2.6. Eye darkening determination

The methodology used to assess eye darkening was adapted from Freitas et al. (2014). On the final sampling day (99) and after the fish had been euthanised (< 2 min) and blood collected, a photo of each fish was taken. A camera was mounted on a tripod 400 mm above the fish in order to capture an image of the eye. Photos were taken in a well-lit area using a Panasonic Lumix FT3 camera with a fixed zoom. A
was able to determine the area (mm²) for each component of the digital ruler, which was provided in each photo. By counting the number of pixels against the mm markings on the digital image of the darkened area was then calculated by standardising the number of pixels in separate layers of the eye. The area of each layer (pupil, sclera and iris) was also removed into a separate layer. This resulted in three digitally dissected eyes.

Photoshop (v2014.2.0), each eye was digitally dissected from the image, and copied into another layer in the program for analysis. The pixels per mm².

Each fish photo was randomised prior to analysis to avoid any bias of prior knowledge of treatments. Using inbuilt selection tools in Adobe Photoshop (v2014.2.0), each eye was digitally dissected from the fish image, and copied into another layer in the program for analysis. The pupil was then digitally removed, and the darkened portion of the sclera was also removed into a separate layer. This resulted in three separate layers of the eye. The area of each layer (pupil, sclera and darkened sclera) was then calculated by standardising the number of pixels in a straight line against the mm markings on the digital image of the ruler, which was provided in each photo. By counting the number of pixels that were present along the distance of the ruler, the software was able to determine the area (mm²) for each component of the digitally dissected eye.

2.7. Statistical analysis

All statistical analysis was performed through the R (v3.3.3) programming language (R Core Team, 2017), utilising the nlme package (v3.1-131) to create a linear mixed effects model (Pinheiro et al., 2017). A mixed effects model was used to account for differences between tanks that were measured repeatedly over time. We tested for temperature and time (and the interaction) effects on measures of growth and stress indicators, by specifying temperature and time as fixed effects and tank identity as a random (intercept) effect. Temperature and time were modelled as categorical variables. One exception to this was the cholesterol data, and in this instance we modelled time as a continuous variable to account for the smooth temporal trends in the data (unlike the other data). The same model was used for the comparisons between the UM and LM with mode replacing time as a factor. All assumptions for the statistical tests were checked visually for homogeneity of variance by examining the residual plotted against the predicted values and using q-q plots to see if the data was normally distributed. In addition formal hypothesis testing was performed through a Levene's test of equal variance and a Shapiro Wilk test of normality on the residuals. Planned post hoc comparisons were us to investigate significant differences with a false discovery rate used to account for multiple comparisons (Benjamini and Hochberg, 1995).

The experiment was conducted in accordance with animal care protocols approved (B30-2014) by the Animal Ethics Committee of Deakin University. All possible steps were taken to minimise negative impacts on animals.

3. Results

3.1. Bimodal growth

At the end of the study, a bimodal distribution was observed in all treatments groups (Fig. 2). Fork length was used for assessing bimodal growth, as previously it has been used as the standard method to categorise juvenile Atlantic salmon (Simpson and Thorpe, 1976; Thorpe et al., 1980). From plotting the distribution for all length measurements at all temperatures, it was determined that at a fork length of 240 mm, there was a clear visual separation between the two distributions, resulting in an upper and lower mode (Fig. 1). The bimodal distribution was consistently apparent at all temperatures (Fig. 1A, B, C), with the interval for the two modal groups being at a fork length of 240 mm. As such, fish that had a fork length of 240 mm or below were categorised as LM, while fish with a fork length > 240 were categorised as UM. In total there were 71 fish that were assigned to the LM and 153 fish in the UM. Separating this classification between temperatures, there were 21, 23 and 27 in the LM and 56, 57 and 40 in the UM for 12 °C, 16 °C and 20 °C, respectively. The LM appeared to be present in approximately one third of the fish, irrespective of the temperature regime that they were acclimated to. Therefore, the results are presented in two formats; prior to, and after categorization into the LM and UM.

3.2. Growth parameters

Over the 99 day study period, fish significantly increased in body mass (F4,465 = 35.78, p < .01) and fork length (F4,465 = 68.72, p < .01), with an average gain of 93.56 g (237.30%) and 60.13 mm (131.93%), respectively (Fig. 2A,B). There was no difference for the interaction term of mass (F4,465 = 1.76, p = .08) or length (F4,465 = 1.63, p = .11), or between temperatures of mass (F2,12 = 2.91, p = .09) or length (F2,12 = 1.90, p = .19). K was significantly affected (F4,465 = 4.21, p < .01) over time and between temperatures (Fig. 2C). This was seen through a decrease in initial condition at day 37 across all temperatures. After 99 days, fish at 16 °C and 20 °C had a K similar to day 1, while fish at 12 °C had lower K. Specific differences within time between temperatures were observed.

Fig. 2. Fish mass (g), fork length (mm) and condition factor (K), for juvenile Atlantic salmon held at three experimental water temperatures (12 °C, 16 °C and 20 °C) over a 99 day period. Fish were sampled on day 1 prior to the increase in water temperature then at 8, 37, 69, and 99 days post change to the water conditions. Values are presented as the mean ± S.E, represented by the symbols and lines. Different letters represent significant differences (p < .05) for the main effect of time (uppercase) or time within each temperature (lowercase). Different open (°) and closed (•) bullets indicate significant differences between temperatures within each time.
Table 1

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Mode</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LM</td>
<td>UM</td>
</tr>
<tr>
<td>Mass</td>
<td>12</td>
<td>190.68 ± 2.96a</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>198.17 ± 8.64a</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>224.8 ± 5.74a</td>
</tr>
<tr>
<td>Length</td>
<td>12</td>
<td>269.44 ± 1.73a</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>263.34 ± 3.17a</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>264.98 ± 1.52a</td>
</tr>
<tr>
<td>K</td>
<td>12</td>
<td>0.97 ± 0.01a</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1.05 ± 0.01a</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.2 ± 0.01a</td>
</tr>
<tr>
<td>SGR</td>
<td>12</td>
<td>1.14 ± 0.09a</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.97 ± 0.03a</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.19 ± 0.08a</td>
</tr>
<tr>
<td>VSI</td>
<td>12</td>
<td>11.23 ± 0.23a</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>10.55 ± 0.13a</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10.76 ± 0.13a</td>
</tr>
</tbody>
</table>

At day 8, the 16 °C treatment was significantly higher than 20 °C. However at day 69 and 99, 20 °C was significantly higher than 12 °C. SGR showed no change between temperatures (Table 1), and on average, fish increased in body mass by 0.87 ± 0.05% per day. Fish categorised as UM had significantly higher body mass, fork length, K, VSI and SGR when compared to fish in the LM (Table 1). Fish body mass and K had a significant interaction between modal group and temperature. In addition to being larger, there were specific temperature differences within modal group. For body mass, fish in the UM were significantly heavier at 20 °C. For K, fish in the UM had higher condition indices that increased with the temperature, were 20 °C had the highest K.

3.3. Longitudinal analysis of stress responses

There was a significant interaction \((F_{1,42} = 3.54, p < .01)\) between time and temperature in plasma cortisol, (Fig. 3A). At 12 °C plasma cortisol increased from day 1 to day 8, then returned to initial levels (day 1 and 8) after 99 days. At 16 °C, the only difference was observed between days 8 and 99. Plasma cortisol at 20 °C did not change over the duration of the study.

Plasma glucose was statistically different \((F_{4,272} = 6.54, p < .01)\) over the duration of the trial, with levels ranging from 65.38–88.61 mg dl\(^{-1}\) (Fig. 3B). Glucose levels increased after day 1, and remained elevated, with the highest values occurring at day 8 and lowest at day 37 and 99, respectively. There was no significant interaction for plasma glucose \((F_{4,272} = 1.57, p = .13)\) or for the main effect of temperature \((F_{2,12} = 3.31, p = .07)\).

For plasma cholesterol there was no significant interaction \((F_{4,272} = 1.76, p = .17)\), or main effect of time \((F_{1,272} = 2.97, p = .08)\) or temperature \((F_{2,12} = 0.63, p = .55)\) in plasma cholesterol, with levels ranging from 183.25–309.95 mg dl\(^{-1}\) (Fig. 3C).

3.4. Responses by modal group at day 99

Plasma cortisol \((F_{2,42} = 0.73, p = .49)\) and glucose \((F_{2,41} = 2.65, p = .08)\) levels were not significantly different for the interaction term (Fig. 4A, C). There was also no difference for the main effect of modal group for cortisol \((F_{2,42} = 1.25, p = .27)\) and glucose \((F_{2,42} = 0.28, p = .60)\), or the main effect of temperature for cortisol \((F_{2,12} = 3.25, p = .07)\) and glucose \((F_{2,12} = 0.69, p = .52)\). There was considerable variability in cortisol levels both within each temperature and between modal groups. Overall, average plasma cortisol levels in the LM and UM groups maintained at 12 °C were 2–4 fold lower (12.89 ± 7.31–19.40 ± 5.42 mg nl\(^{-1}\)) than fish maintained at 16 °C (47.51 ± 10.59–50.47 ± 8.44 mg nl\(^{-1}\)). Plasma cholesterol was significantly lower \((F_{1,42} = 27.92, p < .01)\) in the LM when compared to the UM (Fig. 4B). There was no significant interaction term \((F_{2,42} = 0.66, p = .52)\) or for the main effect of temperature \((F_{1,42} = 0.09, p = .92)\).

There was no significant difference for the interaction term in ED \((F_{2,42} = 0.26, p = .77)\) or for the main effects of modal group \((F_{1,42} = 2.44, p = .13)\) or temperature \((F_{2,12} = 3.35, p = .07)\) (Fig. 4D). However by comparing the individual parameter summary of 16 °C against 12 °C from the linear model, there was a significant difference \((F_{2,12} = 2.53, p = .03)\) in ED levels. Through investigation with a post hoc comparison, we identified that 12 °C was significantly lower than 16 °C and 20 °C.

4. Discussion

In this study we reported the effects of chronic thermal stress on growth and stress physiology responses of Atlantic salmon. This study expanded on the current knowledge of growth profiles and cortisol, glucose and cholesterol levels in fish after exposure (99 days) to increased water temperature, and further investigated the temporal change within this period. We demonstrated that juvenile Atlantic salmon maintained at 16 °C over a period of 99 days had increased plasma cortisol levels at day 99 compared with day 8. In addition we showed the potential of using ED as a non-invasive indication of stress, which increased significantly and concomitantly with cortisol in fish maintained at 16 °C and 20 °C. Furthermore, we showed bimodality in the distribution of size, and how this may provide valuable information in chronic stress studies. Culturing of juvenile Atlantic salmon at 20 °C had no significant effect on body mass or fork length compared with 12 °C and 16 °C. Overall there was an effect of temperature seen through reduced K at 12 °C.

The relationship between body mass and fork length through K indicated that after 99 days, the fish at 12 °C were longer, but had a similar body mass in relation to 20 °C at the end of the study. The effects of chronic exposure to high water temperatures on growth in fish seems to vary with the duration of the study, species (including...
Atlantic salmon (Elliott and Hurley, 1997), the optimal temperature (~16°C) maintained in fresh water for 6 weeks had improved growth when compared to 18°C (Elliott and Hurley, 1997; Forseth et al., 2001). It is possible that during the juvenile life stage, Atlantic salmon are less sensitive to higher water temperatures.

The chronic exposure to high water temperatures resulted in elevated plasma cortisol levels in fish maintained at 16°C (~50 ng mL$^{-1}$) at the last sampling time, in comparison to the levels at day 8. However, exposure to 20°C did not result in elevated plasma cortisol levels at any time. It is unclear as to why in this study, only fish exposed to 16°C presented significantly elevated plasma cortisol at the last sampling time. An increase in plasma cortisol at 16°C has also been observed in juvenile Atlantic cod (Gadus morhua) subjected to acute and chronic increases in water temperature (Pérez-Casanova et al., 2008a, 2008b). These authors demonstrated that during an acute increase in water temperature; (from 10°C to 24°C, at a rate of 2°C h$^{-1}$), plasma cortisol levels were significantly elevated at 16°C in two size classes of juvenile Atlantic cod (10 and 50 g), and peaked at 22°C (Pérez-Casanova et al., 2008a). Over a chronic thermal stress regime (1°C every 5 days), plasma cortisol were not only elevated at 16°C but also peaked at this temperature, and by 18°C, plasma cortisol had returned to normal levels (Pérez-Casanova et al., 2008b). Taken together the current study and the cod experiments suggest that 16°C is stressful to both species. The finding in Atlantic cod that 16°C is stressful is supported by the fact that 16°C is the upper critical temperature for Atlantic cod (Björnsson et al., 2007; Björnsson et al., 2001; Pörtner et al., 2001). On the other hand, growth models for juvenile Atlantic salmon in the fresh water phase have demonstrated the ideal temperature for growth is between 15.9°C and 18.7°C (Elliott and Hurley, 1997; Förseth et al., 2001). It is possible that juvenile Atlantic salmon at 16°C had higher plasma cortisol levels in the days preceding the last sampling time, and at 99 days were either becoming less sensitive to high temperature or had increased cortisol clearance rates (Barton and Schreck, 1987; Redding et al., 1984). We cannot rule out that a similar pattern of increase and decrease in plasma cortisol could have also happened in fish maintained at 20°C, which probably did not coincide with sampling times. In addition, the differences in cortisol response between fish maintained at 16°C and 20°C, or the lack of cortisol response at 20°C, could be due to changes in the hypothalamus-pituitary-interrenal (HPI) axis and interrenal tissue biosynthesis capacity (Hori et al., 2012).

In this study, we showed a bimodality in the size distribution of juvenile Atlantic salmon, which was independent of temperature. This is in agreement with several studies in Atlantic salmon that have reported bimodality of growth within the first year in freshwater (Elliott and Hurley, 1997; Mctalfe et al., 1988; Shrimpton et al., 2000; Shrimpton and McCormick, 1998; Simpson and Thorpe, 1976; Thorpe, 1977; Thorpe et al., 1980; Wright et al., 1990). This bi-modality of growth is apparent when the fish have not been subjected to grading, and they are often categorised as UM or LM, depending upon their position in the distribution of body length. The mechanisms behind the development of bimodality are still unclear (Thorpe, 1977). It has been demonstrated that fish in the LM show reduced foraging behaviour (Simpson and Thorpe, 1976). The loss in appetite and concomitant reduction in food consumption may result in a depletion of energy reserves in different tissues, and therefore affect growth. In this study, the VSI in the LM fish was lower than that in the UM, suggesting a lower percentage of visceral fat in the fish. It has been demonstrated in hapuku (initial mean body mass of 1667–2080 g) that growth was significantly slower after 14 weeks at 22°C when compared to 18°C (Tromp et al., 2016). In addition, this study demonstrated that the gain in body mass was practically null in hapuku maintained at 22°C. On the other hand, hapuku averaging 47 g and acclimated to 12°C, 15°C, 18°C, 21°C and 24°C for four weeks had improved growth performance at 18°C and 21°C, when compared to 24°C (Khan et al., 2014). This clearly demonstrates that the effect of water temperature on growth varies with the developmental stage, and it is possible that during the juvenile life stage, Atlantic salmon are less sensitive to higher water temperatures.

![Fig. 3. Total plasma cortisol (ng mL$^{-1}$), glucose (mg dl$^{-1}$) and cholesterol (mg dl$^{-1}$) for juvenile Atlantic salmon held at three experimental water temperatures (12°C, 16°C and 20°C) over a 99 day period. Fish were sampled on day 1 prior to the increase in water temperature then at 8, 37, 69, and 99 days post change to the water conditions. Values are presented as the mean ± S.E., represented by the symbols and lines. An asterisk (*) denotes a significant (p < .05) slope in the model for the interaction between time and temperature.](image-url)

size, and stage of development. It has been reported that juvenile Atlantic salmon (77.0 ± 14.6 g) in the saltwater phase (post-smolt) had improved growth at 14°C, was reported when compared to 18°C (Handeland et al., 2008). In addition, Atlantic salmon of a larger size class (~1.6 kg) in saltwater, also had a reduced growth rate at 19°C when compared with 14°C, over a period of 56 days (Hervøy et al., 2012). On the other hand, pre-smolt juvenile Atlantic salmon (~162 g) maintained in freshwater for 6 weeks had improved growth when maintained at higher temperatures (20°C compared to 10°C) (Norambuena et al., 2016). Based on the growth model developed for Atlantic salmon (Elliott and Hurley, 1997), the optimal temperature for the growth of this species during the freshwater phase is 15.9°C. Our study, however, showed that fish maintained at 16°C grew in a similar fashion to fish at 12°C and 20°C. It is well established that temperature has a direct limiting effect on growth performance of teleost fish, due to the cost of maintaining an increased basal metabolic rate at higher temperatures (Clarke and Johnston, 1999). Typically, higher temperatures will result in a higher energy demand in the fish.
peritoneum cavity. Energy derived through fatty acid metabolism may have been increased in the LM fish due to fasting. On the other hand, it has been suggested that the increased growth in the UM is related to increased plasma thyroxine levels (Kristinsson et al., 1985; Simpson and Thorpe, 1976). While the physiological mechanisms that underpin bimodal growth remain unclear, the proportion of fish (~1/3) that were categorised in the LM was not affected by water temperature.

Our study demonstrated that there was no temporal significant changes in plasma glucose and cholesterol levels either within or amongst temperatures. However, plasma cholesterol levels were significant reduced in the lower mode fish. Independent of the temperatures tested, the range in plasma cholesterol levels reported in the LM of 123.97–166.12 mg dL⁻¹ is below the normal ranges in 3.5 kg Atlantic salmon (269.53–522.00 mg dL⁻¹; Braceland et al., 2016) while within the normal range in the UM: 293.96–349.95 mg dL⁻¹. Plasma cholesterol levels in the UM is also in agreement with the values observed in Atlantic salmon maintained in freshwater and saltwater (Farrell et al., 1986). In humans, optimal total plasma cholesterol levels are between 174 and 193 mg dL⁻¹ (4.5–5.0 mm). Low plasma cholesterol levels (100–135 mg dL⁻¹ or 2.6–3.5 mm) are described as hypocholesterolaemia (Vyroubal et al., 2008). This condition in humans has been used as a prognostic indicator of morbidity and mortality, and is usually associated with a range of pathological conditions (Vyroubal et al., 2008). The levels observed in the LM group fall within what is considered hypocholesterolaemia in humans. Our results also demonstrated that the LM group was significantly less heavy and smaller (Table 1) when compared with the UM fish. The mechanisms leading to low levels of cholesterol are not well understood. It could be a combination of changes in the synthesis and metabolism of cholesterol. The only study on low plasma cholesterol levels (< 260 mg dL⁻¹) in salmonids exposed to increased temperature have suggested that hypocholesterolaemia was caused by increased hepatic metabolism and biliary secretion (Wedemeyer, 1973).

This study has shown for the first time that ED is responsive to chronic thermal stress in Atlantic salmon. An in vitro study in sand goby, demonstrated that ACTH, a precursor to cortisol synthesis, caused dispersal of melanophores within the eyes of the fish, producing an ED response (Sköld et al., 2015). Although more evidence is needed, our findings and those of earlier studies, suggest that ED may be stimulated in conjunction with the production/release of stress hormones, particularly ACTH, which is a precursor to corticosteroid production (Barton, 2002). This is supported by the observation of a similar pattern that was observed in plasma cortisol and ED, although the main effects from both of these parameters was not significant for temperature (F₂,12 = 3.26, p = .07 and F₂,12 = 3.35, p = .07 for cortisol and ED respectively). It is also important to consider the putative role of the cortisol production pathway in the response of ED. Unfortunately, it was beyond the scope of this study to determine the mechanisms for ED differentiation. However the measurement of ED remains an interesting topic for future research. Finally, this study presents a digital photographic method that can be used to accurately calculate the area of ED, improving the reliability and reproducibility of the technique.

In conclusion, the lack of differences in growth or stress response to increased temperatures over a 99-day period in juvenile Atlantic salmon in freshwater, provide insight into how important it might be to carry out similar studies not only for longer periods but also at different developmental stages, including the adult phase in saltwater. Temperatures of 20 °C in freshwater do not appear severe enough to significantly reduce growth through mass or length. The identification of bimodality in growth studies should be investigated further to improve population growth modelling and response to stressors where individuals may behave quite differently. Future research should aim to investigate the effects of long-term exposure to higher water temperatures (> 20 °C) on physiological responses in Atlantic salmon in saltwater.

Acknowledgements

The authors would like to thank the tireless effort of Anthony Tumbarello, Chris Henaghan and all the other volunteers that donated their time. In addition we are grateful for the technical assistance provided by Bob Collins and Amber Chen at Deakin’s Aquaculture Futures Facility. This study was supported by grants from Tassal Group Limited.
Author contributions

Jared J. Tromp

Experimental design, conducted the sampling, implemented eye darkening (ED) measurements and analysis, statistical analysis, interpreted the analysis and wrote the manuscript.

Paul L. Jones

Experimental design, conducted sampling and comments on the manuscript.

Morgan S. Brown

Conducted sampling, implemented eye darkening (ED) measurements and analysis, comments on the manuscript.

Donald A. P. Biro

Statistical analysis, model design and comments on the manuscript.

Luis O.B. Afonso

Experimental design, conducted sampling and comments on the manuscript.

References


Reference text


## AUTHORSHIP STATEMENT

### 1. Details of publication and executive author

<table>
<thead>
<tr>
<th>Title of Publication</th>
<th>Publication details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic exposure to increased water temperature reveals few impacts on stress physiology and growth responses in juvenile Atlantic salmon</td>
<td>Published in Aquaculture</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of executive author</th>
<th>School/Institute/Division if based at Deakin; Organisation and address if non-Deakin</th>
<th>Email or phone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jared J. Tromp</td>
<td>School of Life and Environmental Sciences</td>
<td><a href="mailto:jjtro@deakin.edu.au">jjtro@deakin.edu.au</a>; 0430 369 700</td>
</tr>
</tbody>
</table>

### 2. Inclusion of publication in a thesis

<table>
<thead>
<tr>
<th>Is it intended to include this publication in a higher degree by research (HDR) thesis?</th>
<th>Yes / No</th>
<th>If Yes, please complete Section 3 If No, go straight to Section 4.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3. HDR thesis author’s declaration

<table>
<thead>
<tr>
<th>Name of HDR thesis author if different from above. (If the same, write “as above”)</th>
<th>School/Institute/Division if based at Deakin</th>
<th>Thesis title</th>
</tr>
</thead>
<tbody>
<tr>
<td>As above</td>
<td>School of Life and Environmental Sciences</td>
<td>Long-term exposure to elevated temperature in Atlantic salmon (<em>Salmo salar</em>)</td>
</tr>
</tbody>
</table>

If there are multiple authors, give a full description of HDR thesis author’s contribution to the publication (for example, how much did you contribute to the conception of the project, the design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)

I, Jared Tromp, contributed to the experimental design, conducted the sampling, implemented eye darkening (ED) measurements and analysis, statistical analysis, interpreted the analysis and wrote the manuscript.

I declare that the above is an accurate description of my contribution to this paper, and the contributions of other authors are as described below.

<table>
<thead>
<tr>
<th>Signature and date</th>
<th>Signature Redacted by Library</th>
</tr>
</thead>
<tbody>
<tr>
<td>22/06/18</td>
<td></td>
</tr>
</tbody>
</table>
4. Description of all author contributions

<table>
<thead>
<tr>
<th>Name and affiliation of author</th>
<th>Contribution(s) (for example, conception of the project, design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paul L. Jones</td>
<td>Contributed to the experimental design, conducted sampling and comments on the manuscript.</td>
</tr>
<tr>
<td>Deakin University</td>
<td></td>
</tr>
<tr>
<td>Morgan S. Brown</td>
<td>Conducted sampling, implemented eye darkening (ED) measurements and analysis, comments on the manuscript.</td>
</tr>
<tr>
<td>Deakin University</td>
<td></td>
</tr>
<tr>
<td>John A. Donald</td>
<td>Comments on the manuscript.</td>
</tr>
<tr>
<td>Deakin University</td>
<td></td>
</tr>
<tr>
<td>Peter A. Biro</td>
<td>Statistical analysis, model design and comments on the manuscript.</td>
</tr>
<tr>
<td>Deakin University</td>
<td></td>
</tr>
<tr>
<td>Luis O.B. Afonso</td>
<td>Contributed to the experimental design, conducted sampling and comments on the manuscript.</td>
</tr>
<tr>
<td>Deakin University</td>
<td></td>
</tr>
</tbody>
</table>
5. Author Declarations
I agree to be named as one of the authors of this work, and confirm:
i. that I have met the authorship criteria set out in the Deakin University Research Conduct Policy,
ii. that there are no other authors according to these criteria,
iii. that the description in Section 4 of my contribution(s) to this publication is accurate,
iv. that the data on which these findings are based are stored as set out in Section 7 below.

If this work is to form part of an HDR thesis as described in Sections 2 and 3, I further
v. consent to the incorporation of the publication into the candidate’s HDR thesis submitted to Deakin University and, if the higher degree is awarded, the subsequent publication of the thesis by the university (subject to relevant Copyright provisions).

<table>
<thead>
<tr>
<th>Name of author</th>
<th>Signature*</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paul L. Jones</td>
<td>Signature Redacted by Library</td>
<td>12-07-2018</td>
</tr>
<tr>
<td>Morgan S. Brown</td>
<td>Signature Redacted by Library</td>
<td>25-06-2018</td>
</tr>
<tr>
<td>John A. Donald</td>
<td>Signature Redacted by Library</td>
<td>12-07-2018</td>
</tr>
<tr>
<td>Peter A. Biro</td>
<td>Signature Redacted by Library</td>
<td>11-07-2018</td>
</tr>
<tr>
<td>Luis O.B. Afonso</td>
<td>Signature Redacted by Library</td>
<td>23-06-2018</td>
</tr>
</tbody>
</table>

6. Other contributor declarations
I agree to be named as a non-author contributor to this work.

<table>
<thead>
<tr>
<th>Name and affiliation of contributor</th>
<th>Contribution</th>
<th>Signature* and date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
* If an author or contributor is unavailable or otherwise unable to sign the statement of authorship, the Head of Academic Unit may sign on their behalf, noting the reason for their unavailability, provided there is no evidence to suggest that the person would object to being named as author.

7. Data storage

The original data for this project are stored in the following locations. (The locations must be within an appropriate institutional setting. If the executive author is a Deakin staff member and data are stored outside Deakin University, permission for this must be given by the Head of Academic Unit within which the executive author is based.)

<table>
<thead>
<tr>
<th>Data format</th>
<th>Storage Location</th>
<th>Date lodged</th>
<th>Name of custodian if other than the executive author</th>
</tr>
</thead>
<tbody>
<tr>
<td>xlsx</td>
<td>Afonso lab network share “~/shares.deakin.edu.au/school-les-g/Afonso-Lab/Raw-NGS-data-Jared’s PhD Thesis/CH2”</td>
<td>20/07/18</td>
<td>Luis O.B. Afonso</td>
</tr>
</tbody>
</table>

This form must be retained by the executive author, within the school or institute in which they are based.

If the publication is to be included as part of an HDR thesis, a copy of this form must be included in the thesis with the publication.

46
3 Temporal transcriptomic analysis in juvenile Atlantic salmon maintained at 12 °C.

3.1 Introduction

Similar to other vertebrates, fish go through different developmental stages ranging from embryonic stages through to sexual maturation. The progression of a species through their different developmental stages can provide unique requirements that can affect all levels of physiology, behaviour and morphology. For example, Atlantic salmon have a key developmental processes they undertake when they transfer from the freshwater to the salt water environment in a process commonly referred to as smoltification [1]. Key life history events characterised in Atlantic salmon include embryo fertilisation (embryo) through to completed egg sack absorption (alevin) [2], transition from freshwater parr to saltwater post-smolt (smoltification) [1], or the genetic basis for reproductive strategies from early life history [3]. The salmonid family has been intensively studied in areas of ecology, behaviour, physiology and genetics [4,5]. The extensive knowledge of this family has rapidly advanced their suitability and production in commercial aquaculture.

Most of the research in this species has focused largely on these key events (e.g. ontogeny, smoltification), however developmental stages outside of these events remains largely unexplored. The physiology prior to smoltification and subsequent saltwater migrate in juvenile Atlantic salmon is yet to be studied at the transcriptome level. This developmental period is significant as changes in growth outcomes can occur in Atlantic salmon during this time. The new approaches of ribonucleic acid sequencing (RNA-Seq) technologies affords the opportunity to identify changes in the expression of key genes during this stage. More so, Jeffries et al. [6] highlighted
the difficulty in identifying differentially expressed genes between experimental treatments and normal developmental processes, when using a transcriptome wide investigation. Therefore it is crucial to develop species specific transcriptome profiles to assist in differential gene expression studies.

The International Collaboration to Sequence the Atlantic Salmon Genome (ICSASG) is responsible for developing the first Atlantic salmon genome, based on the muscle tissue of a double haploid European sub species (\( S. \textit{salar europaeus} \)) \[4,7\]. Since its first inception, the genome has undergone three revisions (https://www.ncbi.nlm.nih.gov/assembly/GCF_000233375.1/) to produce the ICSASG_v2, which has annotation at the chromosome level. The development of an Atlantic salmon genome has provided an invaluable resource for furthering genetic research in Atlantic salmon. Previous transcriptome studies in Atlantic salmon have been based on expressed sequence tag (EST) analysis \[8–13\], however, due to the recent development of the Atlantic salmon genome this resource provides a new opportunity to carry out studies on gene expression during key developmental stages. Several gene expression studies in salmon have provided valuable insights into the genes underlying key developmental stages and the response of salmon to environmental stressors. For example, a study by Martin et al. \[8\] on the liver transcriptome of adult Atlantic salmon revealed 93 genes associated with primary liver functions of transport, acute phase response and blood clotting, with a further 12 genes that had functional homologues to those in the mammalian system. In addition, 510 identified ESTs were successfully mapped to 178 deoxyribonucleic acid (DNA) sequence markers for Atlantic salmon genes across six different tissues (liver, ovary, testis, brain, spleen and muscle) \[9\]. Other studies have screened for genes involved in key developmental periods such as the pre-smolt phase in Atlantic
salmon [11,12,14]. Sequence reads were transcribed from the liver due to this organ playing a dynamic role in carbohydrate, lipid, steroid, amino acid and prostaglandin metabolism [8].

The objective of this study were to, (1) determine temporal changes in transcriptomic responses between fish maintained at 12 °C for 8 and 99 days, (2) identify differentially expressed genes and their tissue function. The broader functional significance of these differentially expressed transcripts will be identified utilizing the biological processes available through the gene ontology project [15].

3.2 Materials and methods

3.2.1 Experimental design

The full experimental design follows that described in [16]. Briefly, juvenile Atlantic salmon (~70g) from the 1+ year class, were maintained at 12 °C in three identical recirculating aquaculture systems each containing five 1,000 L circular tanks at a stocking density of 34 fish per tank. Fish were sampled at the beginning of the experiment, day 1, 8 and 99. At the time of sampling, fish were quickly netted and placed in a lethal dose of AQUI-S® (300 mg/L), with stage 4 anaesthesia [17] observed in under 2 minutes. The use of AQUI-S® (isoeugenol) for anaesthesia in Atlantic salmon has been demonstrated not to affect RNA quality or transcription, except after prolonged sedation (>2 hours) [18]. After blood collection and biometric measurements, the distal portion of the liver was dissected and immediately flash frozen in liquid nitrogen. Liver samples were later transferred into a -80 °C freezer for long term storage. In total there were 30 liver samples collected for RNA-seq analysis from day 1 (n = 12), 8 (n=6) and 99 (n=12). Due to the relative high cost of sequencing, larger sample sizes were included at day 1 and 99, taken from available replicates in CH4 and CH5.
3.2.2 Total RNA extraction

Total RNA was extracted using an Aurum Total RNA Mini Kit (Bio-Rad, Gladesville, NSW, Australia). RNA was extracted following the manufacturer's specifications for the ‘Spin Protocol’ in animal tissue, with one amendment to step 6. A total of 10 μL of DNase I was reconstituted with 70 μL of DNase dilution solution, per sample, with an increased incubation time of 60 minutes at 37 °C to ensure that all genomic DNA (gDNA) was removed. RNA quality was assessed with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s specifications. Total RNA absorbance ratios of $A_{260}:A_{230}$ and $A_{260}:A_{280}$ nm were checked for each sample ($N = 48$), with averages of $2.21 \pm 0.01$ S.E. and $2.14 \pm 0.00$ S.E., respectively. These values met the acceptable ranges of $>1.8$ for RNA quality [19]. Additionally, all samples were visually checked on a 1% TAE agarose gel for the presence of 18S and 28S bands.

3.2.3 RNA sequencing and quality control

Total RNA for each sample was stored in RNAstable® tubes Biomatrca, Inc., San Diego, CA) following manufacturers procedure in to preparation for delivery to Macrogen (Seoul, Rep. of Korea). Briefly, the total amount of RNA in each sample was standardised to 2 μg. Each RNAstable® tube also contained a predetermined mix of 4 μL 1:100 External RNA Controls Consortium (ERCC) spike-in control Mix ratio pair RNA (Thermo Fisher Scientific, Waltham, MA, USA) (see Table S3.1). ERCC spike in mixes were distributed evenly between samples, to enable assessment of technical performance. The addition of external RNA controls serves as internal positive and negative controls, in order to allow for the determination of diagnostic performance [20]. The quantity and quality of the total RNA was checked upon arrival at Macrogen with a 2100 bioanalyser (Agilent, Santa Clara, CA, USA) after
rehydration, to ensure that the concentration was >1 μg (1.01 – 1.75) and had good RNA integrity numbers (6.7 – 9.1, see Table S3.1). Individual sample libraries were prepared using the TruSeq RNA Sample Prep Kit v2 (Illumina Inc., San Diego, CA, USA), following part #15026495 Rev. F generating individual libraries of ~302 bp fragments. The amplified cDNA libraries were sequenced on the Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA) covering two lanes of the flow cell, producing 2 x 125bp paired-end reads.

### 3.2.4 Read mapping and expression quantification

Raw reads were submitted to FastQC (v0.11.2) ([http://www.bioinformatics.babraham.ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) which was available through the Galaxy web server [21], to assess the quality of the raw sequences. The results suggested that there was an inherent bias in the first 13 bases due to the uneven distribution of kmer content, which can be caused under random hexamer priming on Illumina platforms [22]. Illumina adaptor sequences (TruSeq v3) were removed with cutadapt (v1.9) [23]. The first 13 and last 10 base pairs were removed using the fast_trimmer command within the FASTX Toolkit (v0.0.13) ([http://hannonlab.cshl.edu/fastx_toolkit/index.html](http://hannonlab.cshl.edu/fastx_toolkit/index.html)). Finally, any read that didn’t have an average score of 30 across 95% of the read was trimmed using fastq_quality_trimmer from the FASTX toolkit. Filtered reads were then matched through customised scripts in python (v2.7.5), to remove reads that had their mate pair filtered out. Processed reads where then aligned to the Atlantic salmon genome (ICSASG_v2: NCBI Accession; GCF_000233375.1) [4,7] using tophat2 (v2.0.13) [24] and bowtie2 (v2.2.4) [25]. Due to the need to match gene ontology terms in further downstream analysis, an additional identifier was added to the last column of each line in the GFF genome annotation file. These gene identifiers were derived
from the salmobase.org (http://salmobase.org/Downloads/Salmo_salar-annotation.gff3), which enabled matching of NCBI gene ids with gene ontology terms. HTSeq (v0.6.1) [26] was used to quantify expression at the gene level, based on these additional gene ids using the ‘intersection_nonempty’ method. In addition, the ERCC spike-ins were aligned to the bowtie index of the Atlantic salmon genome and their expression profile quantified using RSEM (v1.2.30) [27] as transcript level abundances are needed for this analysis.

### 3.2.5 Evaluation of differential expression testing and diagnostic performance

The ERCC have developed a library of 96 external RNA spike-in controls that can be used to evaluate the technical performance of RNA-Seq differential expression experiments [28]. Technical performance was analysed using erccdashboard (v1.4.0) [20] on all of the available RNA-Seq samples (N = 30). This consisted of 15 samples with ERCC mix 1 and 15 samples with ERCC mix 2. Erccdashboard provides four visual diagnostic performance figures which include receiver operating characteristic curves, area under the curve statistics, limit of detection of ratio estimates and expression ratio technical variability and bias [20]. Receiver operating characteristic curves and area under the curve statistics form the basis of determining if a gene is differentially expressed, by detecting true positive and negative ERCC control ratios [20]. An area under the curve statistic of 1 represents perfect diagnostic performance to determine differentially expressed at a specific ERCC ratio, while an area under the curve statistic of 0.5 represents no ability to determine differentially expressed beyond that of a random guess [20]. Limit of detection of ratio estimates the amount of information (gene counts) needed in order to call a gene transcript differentially expressed at a given fold change [20]. MA plots are used to investigate bias and variability for the ERCC control ratio
measurements by the distance between solid and dotted lines, where increasing disparity between the lines for each ratio indicates bias [20]. Finally the ERCC abundance should cover a dynamic range of $2^{20}$ where a loss in the dynamic range can be attributed to reduced sequencing depth [20]. Untransformed raw reads generated through RSEM of the ERCC sequences and ICSASG_v2 genes were used as input for erccdashboard, with all other parameters run under default conditions. The diagnostic performance results were used to inform differentially expressed parameters for log2-fold-change (LFC) and false discovery rate (FDR) correction thresholds.

3.2.6 Differential gene expression

We tested for differential gene expression with edgeR (v3.12.1) [29], which is a Bioconductor package that is available through the R programming language [30]. Firstly, all gene counts were filtered to only keep genes that where present in at least six of the samples (as this constitutes the lowest level of replication) and had a normalised expression value over 1 count per million [31]. Transcript counts are generally proportional to the level of the gene expression, gene length and the sequencing depth of the library, where larger libraries can have a higher abundance of genes compared to smaller libraries [32]. Normalising genes by counts per million ensures that differences in gene counts are keep relative to the library size, and allow for comparisons between libraries of different sizes without bias [32]. All libraries were then normalised to control for the potential bias of gene counts such as large counts of unique or highly expressed genes that could be limited to one experimental condition [33], and the inherent heteroscedastic nature of probability distribution where there are larger variances for larger transcript counts [32]. The default method of normalisation for edgeR was adopted, trimmed mean of M-values, which
estimates a scaling factor between samples [33]. Multidimensional scaling analysis was used to compare similarity between samples by their positional distance. This was used to visually inspect the variation between days (1, 8 and 99). As this study was designed to look at multiple factors, we used the Cox-Reid profile-adjusted likelihood method to estimate common dispersions [34]. We fitted a generalised linear model to test for any differences between time, where differential expression of genes was determined by a FDR < 0.05 in at least one of the times (day 8 or 99), and a LFC ≥ 1. [35].

3.2.7 Gene ontology enrichment analysis

Differentially expressed genes were then analysed using the R package GoSeq (v1.22.0) [36] which controls for the inherent length bias (longer reads having higher expression). It is important to control for length bias in these tests, as the expected gene counts are proportional to transcript expression multiplied by the gene length [36]. Gene ontology terms where matched to NCBI gene identifiers utilising Ssa.RefSeq.db (v1.2) (https://github.com/FabianGrammes/Ssa.RefSeq.db) and gene ontology enrichment analysis followed the standard pipeline described in Young et al. [36]. Gene lengths were determined using Ssa.RefSeq.db, by taking the difference between the start and end gene coordinates. The probability weighting function was used to determine length bias. The probability weighting function estimates the probability that a gene will be called differentially expressed, based on length information alone [36]. This value is then used to inform the Wallenius approximation to test for gene ontology term enrichment, where a term was called to be enriched or under represented with a FDR value < 0.05. Gene ontology terms that were significantly enriched were imported into reduce and visualise gene ontology
REVIGO (http://revigo.irb.hr/) for visualisation. REVIGO groups gene ontology terms based on their semantic similarity [37].

3.3 Results

3.3.1 Sequencing and read mapping

Sequencing of cDNA libraries yielded between 17.9 – 35.6 million reads per sample (Table S3.2). From these raw reads the adaptor sequences and low-quality base reads (phred score < 30) were removed, retaining only those with a paired-end counterpart; we kept between 8.0 – 18.7 million pair end reads per sample (Table S3.2). The Atlantic salmon genome contains 57,783 genes based on the entrez records [38]. Of these, we were able to identify 42,003 gene counts, matched through HTSeq-count. For differential expression analysis, each sample was normalised by counts per million to enable comparisons between different sized libraries [32], resulting in the sample library gene counts ranging from 0 – 52,619 counts per million. Genes that weren’t present at a level of 1 count per million in at least 6 samples (lowest level of replication), were excluded from the analysis, resulting in the use of 23,189 of the 42,003 genes for the differential expression analysis. Performance diagnostics from the ERCC ratio analysis revealed that there was perfect diagnostic performance to detect differential gene expression at the 2- and 4-fold levels and good diagnostic power at 1.5 fold (Figure S3.1). Yet the results indicate that there was some bias between the ratio control measurements and reduced LDOR estimates under the 4-fold levels. Based on these performance estimates of the ERCC spike-ins, the minimum requirements to call a gene differentially expressed was set at a fold change of 2 and a FDR threshold < 0.05.

3.3.2 Differential gene expression
A generalised linear model approach was taken in edgeR, to compare the differential expression of genes over the 99 days. A total of 342 genes were found to be differentially expressed. Figure 3.1 shows the number of genes that were differentially expressed at day 8 and 99. The majority of genes are seen to be differentially expressed at 99 days (Figure 3.1). For genes that were upregulated, there were 19 genes that were consistently differentially expressed at day 8 and day 99. Furthermore, 45 genes were consistently downregulated at day 8 and day 99. Although these genes had a consistent up/downregulated expression profile; these genes were not always expressed at the same magnitude. Only 8 genes had a significant expression profile that differed between day 8 and 99. In all instances genes were shown to be significantly downregulated at day 8, while being upregulated at day 99. In summary, there was a greater number of genes that were downregulated compared to upregulated, with day 99 showing the largest number of differentially expressed genes (Figure 3.1). At day 8 there were 25 genes that were significantly (FDR <0.05) upregulated and 60 genes that were downregulated when compared to day 0. Day 99 had 175 and 154 genes determined to be up or downregulated, respectively. The top 10 most differentially expressed genes based on FDR value, are presented in Table 3.1. All the genes at 99 days are significantly expressed at a LFC > 1, while only 4 genes at day 8 have a significant expression profile (Table 3.1). Despite the high number of differentially expressed genes, there were few genes that had a consistent expression profile across the duration of the trial. Of the 342 differentially expressed genes, 36 genes had no known annotation of function within the Atlantic salmon genome.
Figure 3.1 The number of differentially expressed (DE) genes for juvenile Atlantic salmon (*S. salar*) maintained at 12 °C for 99 days. The open bars (□) represent the total number of genes that were upregulated and the closed bars (■) represent the total number of genes that were downregulated. Genes represented in this graph are all above a log₂-fold-change of 1, and have been determined to be significantly expressed (FDR <0.05) by a GLM model in the Bioconductor package edgeR. Positive values represent an increase in relative gene abundance, while negative values represent a decrease.
Table 3.1 Top 10 most significantly expressed genes from the liver transcriptome of juvenile Atlantic salmon (*S. salar*) held at a water temperature of 12 °C and sampled at day 0, 8 and 99. Significant differential expression (FDR <0.05) was assigned using a GLM approach with the initial day (1) as the intercept, and determined to be biologically relevant at log2-fold-change (LFC) < 1. Positive fold change values are considered to be upregulated, while negative fold change values are downregulated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day 8 LFC</th>
<th>Day 99 LFC</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>uncharacterized LOC106611397</td>
<td>0.22</td>
<td>2.29</td>
<td>2.62E-11</td>
</tr>
<tr>
<td>cholesterol desaturase daf-36-like</td>
<td>-0.08</td>
<td>1.56</td>
<td>2.62E-11</td>
</tr>
<tr>
<td>C-type lectin domain family 4 member D-like</td>
<td>0.18</td>
<td>2.27</td>
<td>4.59E-11</td>
</tr>
<tr>
<td>vitellogenin</td>
<td>-4.17</td>
<td>-8.07</td>
<td>3.39E-10</td>
</tr>
<tr>
<td>slit homolog 3 protein-like</td>
<td>1.13</td>
<td>2.64</td>
<td>7.51E-09</td>
</tr>
<tr>
<td>C type lectin receptor B</td>
<td>0.38</td>
<td>1.89</td>
<td>7.51E-09</td>
</tr>
<tr>
<td>leukocyte cell-derived chemotaxin-2-like</td>
<td>1.25</td>
<td>2.90</td>
<td>2.85E-08</td>
</tr>
<tr>
<td>alpha amylase</td>
<td>0.28</td>
<td>1.14</td>
<td>3.03E-08</td>
</tr>
<tr>
<td>haptoglobin-like</td>
<td>1.11</td>
<td>2.40</td>
<td>3.03E-08</td>
</tr>
<tr>
<td>SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 3-like</td>
<td>-0.48</td>
<td>-1.25</td>
<td>4.76E-08</td>
</tr>
</tbody>
</table>
3.3.3 Putative function of differentially expressed genes

Of the 342 genes that were identified as differentially expressed, 257 genes were successfully matched to 2,990 unique GO terms. After enrichment analysis, we maintained 630 of these terms. The terms were divided between biological processes (BP), molecular function (MF) and cellular component (CC), with each category containing 428, 51 and 151 GO terms, respectively. Of the 428 BP that were submitted to the REVIGO web server, 231 terms were kept. The most highly represented BP GO terms based on the highest number of genes that where differentially expressed were “response to drug”, “oxidation-reduction process”, “mitotic S phase”, “aging” and “DNA strand elongation involved in DNA replication” with a total of 22, 19, 14, 13, and 11 genes annotated in each term, respectively (Table 3.2). Genes normalised to library size (counts per million) are presented for the top five most represented biological processes at the individual fish level (Figure 3.2 – 3.6). At day 8, the most five highly represented BP GO terms that were up regulated were “response to drug” (3), “positive regulation of fibroblast proliferation” (2), “chloride transmembrane transport” (2), “regulation of response to stress” (2) and “intrinsic apoptotic signalling pathway in response to DNA damage” (1) while the top five GO terms that were down regulated were “DNA replication initiation” (5), “mitotic S phase” (5), “DNA strand elongation involved in DNA replication” (4), “cellular response to xenobiotic stimulus” (3) and “regulation of transcription involved in G1/S transition of mitotic cell cycle” (3). The profile of BP changed in response at day 99. The top five GO terms that were up regulated were “response to drug” (14), “oxidation-reduction process” (9), “aging” (9), “response to lead ion” (5) and “response to progesterone” (5). The top five GO terms that were down regulated at day 99 were “mitotic S phase” (14), “DNA strand elongation
Table 3.2 Top five gene ontology (GO) terms enriched in significant differentially expressed (DE) genes in Atlantic salmon (*S. salar*) held at a water temperature of 12 °C and sampled at day 1, 8 and 99.

<table>
<thead>
<tr>
<th>GO term ID</th>
<th>Description</th>
<th>Day 8</th>
<th>Day 99</th>
<th>Total DE</th>
<th>Total in term</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0042493</td>
<td>response to drug</td>
<td>3</td>
<td>14</td>
<td>22</td>
<td>709</td>
<td>3.25E-04</td>
</tr>
<tr>
<td>GO:0055114</td>
<td>oxidation-reduction process</td>
<td>0</td>
<td>2</td>
<td>9</td>
<td>569</td>
<td>6.96E-04</td>
</tr>
<tr>
<td>GO:0000084</td>
<td>mitotic S phase</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>14</td>
<td>216</td>
</tr>
<tr>
<td>GO:0007568</td>
<td>aging</td>
<td>0</td>
<td>1</td>
<td>9</td>
<td>13</td>
<td>413</td>
</tr>
<tr>
<td>GO:0006271</td>
<td>DNA strand elongation involved in DNA replication</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>11</td>
<td>33</td>
</tr>
</tbody>
</table>

3.3.4 Heat maps
GO:0042493
response to drug

sodium- and chloride-dependent GABA transporter 2-like
cyclin E2
RAD51 recombinase
high affinity nerve growth factor receptor-like
thymidylate synthetase
solute carrier family 12 member 7-like
G protein–coupled receptor kinase 6–like
solute carrier family 1 (neutral amino acid transporter), member 5
receptor tyrosine–protein kinase erbB–3–like
haptoglobin–like
haptoglobin–like
long–chain–fatty–acid–CoA ligase 1–like
transforming growth factor beta–2–like
sterol regulatory element–binding protein 1–like
solute carrier family 12 member 5–like
endothelial lipase–like
transcriptional regulator Myc
purine nucleoside phosphorylase–like
sterol regulatory element–binding protein 1–like
purine nucleoside phosphorylase–like
haptoglobin–like
catechol O–methyltransferase–like
Figure 3.2 Heat map of differentially expressed genes between 1, 8 and 99 days, categorised through the Gene Ontology (GO) term for biological process, response to drug (GO:0042493). Moderated log2 counts per million (CPM) was used as input into the heat map for each individual transcript, z-scores were then used to represent differences with blue representing lower expression and red higher expression.
GO:0055114
oxidation–reduction process

retinol dehydrogenase 7–like
1,25–dihydroxyvitamin D(3) 24–hydroxylase, mitochondrial–like
ferritin, middle subunit
dihydrofolate reductase
L–lactate dehydrogenase B chain
Ribonucleoside–diphosphate reductase subunit M2
Ribonucleoside–diphosphate reductase large subunit
minichromosome maintenance complex component 3
chloride intracellular channel 2
gamma–butyrobetaine dioxygenase–like
mitochondrial amidoxime–reducing component 1–like
phenylalanine–4–hydroxylase–like
dimethylglycine dehydrogenase
1,25–dihydroxyvitamin D(3) 24–hydroxylase, mitochondrial–like
cystathionine beta–synthase–like
cystathionine beta–synthase–like
cystathionine beta–synthase–like
thioredoxin–like
vitamin D 25–hydroxylase–like
**Figure 3.3** Heat map of differentially expressed genes between 1, 8 and 99 days, categorised through the Gene Ontology (GO) term for biological process, oxidation-reduction process (GO:0055114). Moderated log2 counts per million (CPM) was used as input into the heat map for each individual transcript, z-scores were then used to represent differences with blue representing lower expression and red higher expression.
GO:0000084
mitotic S phase

cell division cycle 45
proliferating cell nuclear antigen–like
GINS complex subunit 1 (Psf1 homolog)
DNA replication complex GINS protein SLD5
Zygotic DNA replication licensing factor ncm6–B
minichromosome maintenance complex component 3
replication factor C (activator 1) 5
replication factor C (activator 1) 2
primase, DNA, polypeptide 1 (49kDa)
proliferating cell nuclear antigen
replication protein A 32 kDa subunit–like
primase, DNA, polypeptide 2 (58kDa)
polymerase (DNA directed), alpha 2, accessory subunit
replication protein A3, 14kDa
Figure 3.4 Heat map of differentially expressed genes between 1, 8 and 99 days, categorised through the Gene Ontology (GO) term for biological process, mitotic S phase (GO:0000084). Moderated log\textsubscript{2} counts per million (CPM) was used as input into the heat map for each individual transcript, z-scores were then used to represent differences with blue representing lower expression and red higher expression.
GO:0007568

aging

neurabin-1-like
high affinity nerve growth factor receptor-like
neural cell adhesion molecule 1-like
thymidylate synthase
suppressor of cytokine signaling 2-like
suppressor of cytokine signaling 2-like
transforming growth factor beta-2-like
sterol regulatory element-binding protein 1-like
metalloproteinase inhibitor 3-like
sterol regulatory element-binding protein 1-like
mitochondrial uncoupling protein 2-like
P2Y purinoceptor 1-like
reticulon-1-A-like
Figure 3.5 Heat map of differentially expressed genes between 1, 8 and 99 days, categorised through the Gene Ontology (GO) term for biological process, aging (GO:0007568). Moderated log$_2$ counts per million (CPM) was used as input into the heat map for each individual transcript, z-scores were then used to represent differences with blue representing lower expression and red higher expression.
GO:0006271
DNA strand elongation involved in DNA replication

cell division cycle 45
GINS complex subunit 1 (Psfl homolog)
DNA replication complex GINS protein SLD5
Zygotic DNA replication licensing factor ncm6–B
minichromosome maintenance complex component 3
replication factor C (activator 1) 2
primase, DNA, polypeptide 1 (49kDa)
replication protein A 32 kDa subunit–like
primase, DNA, polypeptide 2 (58kDa)
polymerase (DNA directed), alpha 2, accessory subunit
replication protein A3, 14kDa
**Figure 3.6** Heat map of differentially expressed genes between 1, 8 and 99 days, categorised through the Gene Ontology (GO) term for biological process, DNA strand elongation involved in DNA replication (GO:0006271). Moderated log₂ counts per million (CPM) was used as input into the heat map for each individual transcript, z-scores were then used to represent differences with blue representing lower expression and red higher expression.
3.4 Discussion

We conducted the first comparative study of the transcriptome of Atlantic salmon prior to smoltification. Our study identified differentially expressed genes in the hepatic tissue in fish maintained at 12 °C for 99 days. The characterisation of differentially expressed genes under normal culture conditions provides critical information on the biological processes that progress in juvenile Atlantic salmon. Significant changes in the hepatic transcriptome were observed after 8 days with 85 differentially expressed genes. A further 329 genes were significantly expressed at 99 days. The total profile of 342 genes indicates that there is significant temporal change in the transcriptome of Atlantic salmon under normal rearing conditions. This data provides an important baseline that experimental studies can compare against to identify genes relating to their experimental treatments, over and above those genes that change through time due to developmental changes.

3.4.1 Putative functional roles of the most differentially expressed genes

Nine out of the ten genes that had the highest significance values for differential expression have previously been annotated for the Atlantic salmon genome. The majority of these genes were found to be upregulated at both day 8 and day 99. While we were unable to discern with an appropriate level of confidence (LFC > 1) the expression profile of all the genes at day 8, it is clear that all the genes at day 99 had a differentially expressed profile. In light of the fact that the majority of genes were upregulated, we have discussed these genes first followed by the ones that were down regulated.

Cholesterol desaturase daf-36-like has been listed as one of the genes involved in the steroidogenesis pathway, from a transcriptome study in shrimp (Macrobrachium rosenbergii) [39]. In addition, DAF-36 has been demonstrated to
regulate aspects of developmental timing and longevity of *C. elegans* [40]. More broadly, DAF-36 is likely involved as a component of hormone metabolism [41]. C-type lectins (CLEC) are involved in cell-cell interactions and immune response [42,43], with two CLECs being identified (C-type lectin domain family 4 member D-like and C type lectin receptor B). Slit genes (slit homologue 3 protein-like) have been predominantly shown to be expressed in the brain, spinal cord and thyroid where they are involved in the formation and maintenance of the nervous and endocrine systems [44]. Leukocyte cell-derived chemotaxin 2 (LECT2) was initially identified for its involvement in neutrophil chemotaxis [45], further investigation has also shown this chemotactic protein to be involved in glucose metabolism [46] and homeostasis of natural killer T (NKT) cells [47]. α-amylase was significantly upregulated at 99 days and one of its functions is to hydrolyse starch into derivatives such as glucose [48]. Haptoglobin acts to bind free plasma haemoglobin and can assist in the innate immune response such as bacterial infection [49]. Vitellogenin is produced in the liver of females, under the control of estrogen and transported through the blood to the ovaries where it is converted into the yolk proteins lipovitellin and phosvitin [50]. The differential expression of vitellogenin shows the largest value LFC, with both day 8 and 99 being significantly down regulated. SWI/SNF complexes have been suggested to be involved in developmental functions in mammals, and are involved in transcriptional activities in yeast [51]. The most consistently expressed function that these genes encode for involve aspects of the immune response. Other functions that had multiple representative genes where hormone and glucose metabolism. The majority of these genes were up regulated, indicating that the expression of these genes was increased over time. The down regulation of vitellogenin may indicate a preference for somatic growth over the
development of gonads. The presence of multiple genes that are involved in the immune response is intriguing, and further investigating is required to confirm the consistent expression profile of these genes.

3.4.2 Functional roles of the selected GO terms

Due to the high number of significantly enriched (p<0.05) GO terms filtered through REVIGO (139), we are not able to discuss all of the BP identified. As such a subset of BP that contain the most differentially expressed genes will be discussed. The five GO terms that contained the largest number of genes are listed in Table 3.1.

3.4.2.1 GO:0042493 - Response to drug

Genes identified under the biological process GO:0042493, have been characterised as transcriptional changes at the cellular or organism level, due to a drug substance that is involved in the diagnosis, treatment or prevention of a disease [52]. As there were no signs of disease during this trial, and thus no subsequent remedial treatment, it is intriguing that this process was identified with the greatest number of differentially expressed genes. This process has previously been identified in rats exposed to a two hour consecutive heat stress (+15 °C) over 10 days, yielding 6 differentially expressed genes from the intestine [53]. Additionally this BP was identified in insulin-resistant severely obese human subjects, two hours into a hyperinsulinemic euglycemic clamp [54]. This term has also been listed as the top 3 BP upregulated in the blood and cortex in patients with Parkinson's disease [55]. Yi et al., [55] suggests that the identification of this term is a possible artefact of the use of drugs by the patients. However, in light of the previous studies, it may be possible that the genes under the “response to drug” may form additional cellular functions to their involvement in drug interactions. These genes are also seen to be involved in
metabolic processes, and therefore their action is likely to mimic biological processes consistent with returning or maintaining homeostasis.

3.4.2.1.1 Solute carrier family (SLC)

Of the 22 genes that were identified within the BP process, there were four genes that were identified to belong to the solute carrier (SLC) family. Solute carrier proteins are involved in active or passive transport of crucial compounds in and out of the cell [56]. These genes have an important functional role, facilitating cell homeostasis through cell transport and it is estimated that ~5% of all the human genes are transport related [56]. The human SLC family is comprised of 55 gene families that contain at least 362 genes, which form various functions from ATP-powered pumps, ion channels and transporters [57]. Amongst the teleost fish, there have been 50 SLC families that have been reported, with a total of 338 putatively functional protein coding genes [58]. Most of the genes that have been identified have come from zebrafish, which have 304 out of the 338 SLC genes found [58]. Atlantic salmon comprise the next largest teleost group with 53 genes [58]. This study has identified two genes form the SLC that were significantly upregulated at day 8. These genes were solute carrier family 1 member 5 (SLC1A5) and solute carrier family 12 member 5 (SLC12A5). Genes from the solute carrier family 1 (SLC1) are involved in cellular transport, specifically in glutamate and neutral amino acid transport [57,59]. The putative function of this gene, is more specifically involved in transport of C/Na⁺ and amino acids [59]. Interestingly this response was not maintained over time (day 99), with SLC1A5 falling below a LFC that could be called biologically significant (LFC -0.74). The human SLC12 family contains 9 members, and is involved in the electroneutral cation/Cl⁻ co-transporter family [57]. In teleost fish, there has been 13 members reported for the SLC12 family, however
only one of the members has been tested for its putative function [58]. This study identified two genes from the SLC12 family, both of which that had significant differential expression for both day 8 and 99. SLC12A5 was significantly upregulated and is specifically involved in the potassium or ammonium chloride transport [58]. SLC12A7 was downregulated and the human SLC12A7 gene encodes for the protein KCL cotransporter family (KCC) member 4 which acts as component of epithelial cell ionic and osmotic homeostasis [60]. Specifically KCC4 is involved in KCL transport activated through swelling [60]. Downregulation of this gene is likely attributed to no swelling or cellular stress on the hepatocytes. Sodium and chloride dependent GABA transporter 2-like (SLC6A13), is one of the 19 genes in the SLC6 family of proteins, that are involved in the symport of Na⁺ and Cl⁻ dependent neurotransmitters (gamma-aminobutyric acid (GABA), serotonin, dopamine and norepinephrine) [57].

3.4.2.1.2 Cellular mechanisms

Haptoglobin was significantly upregulated at day 8 (GeneID:106598822) and 99 (106598822, 106587252 and 106594032). The expression profile of these genes all showed a consistent upregulation at day 99. While it is logical that these genes all form a similar putative function, the expression profile of haptoglobin-like (Gene ID:106594032) is different to the other two genes as it is significantly upregulated at day 8, and has the highest LFC (2.40). While this could be an artefact of the sequencing and a misrepresentation, it could also be that this haptoglobin-like gene is either being preferentially expressed, or is forming some other function that the other two haptoglobin genes. The involvement of haptoglobin genes has been seen to be involved in developmental and acute-phase responses in rat livers [61]. The putative role of haptoglobins in the acute-phase response has also been reported in rainbow
trout (*Oncorhynchus mykiss*) livers [62]. The remainder of the genes that were upregulated under the GO term, “response to drug” were only differentially expressed at 99 days. The transcriptional regulator Myc, is one of the key regulators for cell proliferation, which if deregulation occurs, has contributed to the development of many human tumours [63]. In addition, receptor tyrosine kinases are also reported to in signalling of cell proliferation and differentiation [64].

Transforming growth factor beta (TGF-β) has a multitude of functions that it has been attributed to, in general terms it is associated with limiting cell growth [65]. Purine nucleoside phosphorylase (PNP) is an enzyme involved in the catabolic pathway of purine, where its major role is to phosphorylate guanosine and inosine to guanine and hypoxanthine, respectively [66]. The disruption in the expression and the production of PNP, can result in severe immune and neurological dysfunction [66]. Increased expression of PNP may be indicative of increased metabolism of adenine and guanine. The increased expression of these genes may be a result of increased growth rate at 99 days compared to the initial time. Increased gene expression for processes that involve cell proliferation and genomic building blocks.

### 3.4.2.1.3 Fatty acid metabolism

The majority of genes involved in cellular metabolic processes are seen to have a maintenance role needed to remove metabolites that could cause damaging aggregations within the cell. The greatest upregulation at 99 days, was seen in the catechol O-methyltransferase-like gene. The function of Catechol-O-methyltransferase (COMT), is to remove biologically active or toxic catechols and some hydroxylated metabolites [67]. COMT has been linked with agonistic behaviour [68] and ovarian developmental processes [69]. Three other genes were recorded that are involved in fatty acid metabolism, which is the precursor to all
hormone production, including steroid hormones. There was an upregulation (LFC = 1.64) of the endothelial lipase-like gene at 99 days. Endothelial lipase (EL) is a member of the triglyceride lipase gene family along with lipoprotein lipase, pancreatic lipase and hepatic lipase [70]. This family of lipases are involved in intestinal lipid absorption, energy homeostasis, plasma lipoprotein metabolism and atherosclerosis; with all having the capacity to act as triglyceride lipase and phospholipase to varying degrees [70]. Specifically EL is involved in the metabolism of high density lipoproteins (HDL), through catabolic pathways [71]. HDL levels in EL inactivated mice has been reported to increase, which is seen to have positive health benefits for the cardio-vascular system [72]. Thus the upregulation of EL may have health risks with the associated reduction in HDL. Long-chain acyl-CoA synthases (LACS) are a group of enzymes that catalyse the production of acyl-CoA from fatty acids, ATP and CoA [73,74]. Acyl-CoA is an important component of lipid synthesis (de novo) and β-oxidation [73]. Sterol regulatory element binding protein (SREBPs) are involved in the synthesis of cholesterol and its uptake from LDL [75]. The inability to regulate this protein can result in reduced cholesterol uptake, or an inability to clear cholesterol from the cell, which can have lethal consciences [76]. Knock out trials on mice have reported that in the absence of SREBP1, 50 – 85% died in utero, while the remaining mice were able to compensate cholesterol metabolism through SREBP2 [75], hence the production of SREBP can be seen to be essential for development.

Of the 22 genes that were discovered under the GO term “response to drug”, three were found to be significantly downregulated at both days 8 and 99, which were cyclin E2, SLC12 member 7-like and G protein-coupled receptor kinase 6-like. These genes were all consistently downregulated, expressed at a LFC > 1. Including
the three already mentioned, there were seven genes that down regulated after 99 days, with the highest down regulated gene being sodium- and chloride-dependent GABA transporter 2-like. G protein-coupled receptor kinase (GKK) are a group of seven serine/threonine protein kinases, that phosphorylate G protein-coupled receptors [77]. The main action of these protein kinases is to desensitize G protein coupled receptors (GPCRs), through GRK-mediated receptor phosphorylation [77]. The influence that these GKK proteins can have, is through their effect on GPCRs. GPCRs are a major family of proteins that act as cell surface receptors, for a large range of chemical or sensory stimuli [78]. Cyclin E2 is an important kinase, in the transition from G1 to S phase, in the cell cycle [79]. Cyclin E2 is involved in the phosphorylation of histone H1 and retinoblastoma protein with cyclin-dependent kinase 2 [79]. Reduced expression of the cyclin E2 gene is influences the rate of mitotic division, which will affect cellular growth. Increased hepatocyte growth not apparent for this stage in the Atlantic salmon’s life history. Energy for growth may be diverted to other aspects of cellular growth, such as muscle growth. RAD51 recombinase is involved the repair of double-stranded DNA breaks [80]. Double stranded DNA breaks occur due to a variety of factors including, oxidative damage, ionizing radiation, mechanical stress and through the action of DNA endonucleases [80]. High affinity nerve growth factor receptors are responsible for mediating the effects of nerve growth factor [81]. Nerve growth factors are essential for the survival, development, function and plasticity of neuronal cells [82]. Thymidylate synthase is an enzyme that catalyses the reductive methylation of dUMP through CH2H4 folate to dTMP and H2 folate [83,84]. The synthesis of dTMP is an essential building block for the formation of DNA [85]. Taken together, the genes that were downregulated are mainly involved in cellular responses.
3.4.2.2 GO:0055114 – Oxidation-reduction process

The GO term for oxidation-reduction process (GO:0055114) represents metabolic processes that are involved in the removal or addition of one or more electrons to or from a substance; in addition this term can also represent concomitant removal or addition of protons [52]. This process was enriched by the differential expression of 19 genes, which include two genes down regulated at day 8, 10 genes down regulated at day 99 and the remaining genes up regulated at day 99. Vitamin D 25-hydroxylase-like had the highest upregulation at 99 days, while retinol dehydrogenase 7-like was the highest downregulated gene. The expression of one of these genes, minichromosomal maintenance complex component 3, is absent from this part of the discussion as its functional role will be reported below. Briefly this gene is involved in the DNA replication fork during mitosis.

3.4.2.2.1 Vitamin D regulation

The uptake and mobilisation of minerals from the diet is an important physiological mechanism for normal function. Vitamin D plays an integral role, by regulating the calcium and phosphate metabolism through acting as an agonist on the vitamin D receptor [86]. However, vitamin D must first be converted to the active ligand by 25-hydroxylation in the liver [86,87]. The human vitamin D 25-hydroxylase (CYP2R1) has been demonstrated to play an important physiological role in vitamin D hydroxylation [88]. The gene transcript for vitamin D 25-hydroxylase-like was upregulated in Atlantic salmon at 99 days. The inferred function of this gene indicates an increase in vitamin D regulation. Further evidence in the increased function of vitamin D regulation can be seen by the differentially expressed of the two transcripts (106583685 and 106565076) which encode for 1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial-like protein. This gene is
involved in in the catalysing the conversion of the circulating and hormonal form of vitamin D into 24- and 23-hydroxilated products which will be targeted for excretion [89]. Paradoxically these two putative gene transcripts are showing both up and down regulation.

3.4.2.2 Upregulated

Thioredoxin is involved in DNA synthesis through its action of hydrogen donation for ribonucleotide reductase [90,91]. In human carcinoma cell lines exposed to ionizing radiation, there was increased AP-1 DNA binding [92]. Cystathionine β-synthase (CBS) is an enzyme that catalyses the conversion of serine and homocysteine to cystathionine and water [93]. Deficiencies in CBS has been linked to a variety of diseases and physiological defects [93]. In humans, dimethylglycine dehydrogenase is involved in choline degradation, one-carbon metabolism and mitochondrial respiratory chain [94]. Phenylalanine hydroxylase (PAH) catalyses the conversion of L-phenylalanine to L-tyrosine [95]. PAH plays a dynamic role in the liver by removal of excess L-phenylalanine which can have neuro-toxic effects, while also ensuring that there is enough L-phenylalanine to act as a proteinogenic amino acid [95]. Mitochondrial amidoxime-reducing component 1 (MARC1) acts as a for the N-reduction of a variety of N-hydroxylated substances [96].

3.4.2.2.3 Downregulated

Retinoids play an important role in embryonic development, reproduction, postnatal growth differentiation and maintenance of various epithelia, immune responses, and vision [97], with retinol dehydrogenases acting in retinoid metabolism. Ferritin stores iron in a soluble, non-toxic and readily available form [98]. Dihydrofolate reductase plays an important role in metabolism and cellular growth through its action on converting dihydrofolate acid into tetrahydrofolate acid,
which then the synthesis of essential metabolites such as amino acids, lipids, pyrimidines and purines [99]. In humans L-lactate dehydrogenase is involved in the removal and production of L-lactate primarily in the cytosol [100]. L-lactate is a stereoisomer of lactate (2-hydroxypropanoate) which involved in the metabolic response to exercise [100]. The balanced production of deoxynucleoside triphosphates (dNTP) are required for DNA replication and repair [101]. Ribonucleotide diphosphates provide the building blocks for dNTPs through their reduction by the enzyme ribonuclease reductase family [102]. Specifically the ribonucleoside-diphosphate reductase subunit M2 is involved in the p53 checkpoint for repair of damaged DNA [103]. In addition, another ribonucleoside-diphosphate reductase was present (100195484). The chloride intracellular channel (CLIC) family that plays a role in kidney function, cell division and bone resorption [104]. Only one gene from the CLIC family was differentially expressed, which was chloride intracellular channel 2. The human CLIC2 has been identified to be involved in chloride channels, with its activity increased at low pH or greatly reduced in the presence of a reducing agent [104]. Gamma-butyrobetaine dioxygenase is involved in the cellular energy production, by catalysing the biosynthesis of L-carnitine which is a component required for long-chain fatty acids [105].

3.4.2.3 GO:0000084 & GO:0006271 - Mitotic S phase and DNA strand elongation involved in DNA replication

Due to the high number of identical genes between mitotic S phase (GO:0000084) and DNA strand elongation involved in DNA replication (GO:0006271), where mitotic S phase contained all the same transcripts (11) the two terms will be discussed together. The biological process, DNA strand elongation involved in DNA replication is used to identify the process of DNA replication.
where a DNA strand is synthesised by the addition of nucleotides to the 3’ end of the nascent DNA strand, catalysed by polymerase [52]. Mitotic S phase describes the cell cycle phase where DNA synthesis takes place following the G1 phase in the mitotic cell cycle [52]. Of the 14 individual genes contained within both of these categories, 5 genes were downregulated at day 8 and all 14 genes were downregulated at 99 days. Down regulation of all genes within this term indicates that there is a significant reduction in both mitotic S phase and DNA strand elongation involved in DNA replication needed in the hepatic tissue at 99 days. The expression of mitotic S phase has previously been demonstrated to be down regulated in flies (Drosophila melanogaster) fed a caloric restricted diet, which slows down the normal rate of aging [106].

3.4.2.3.1 Unwinding DNA/replication fork

DNA replication through the progression of DNA replication forks are an essential component mitosis, cell function and survival [107]. Part of cell mitosis involves mitotic S phase, which proceeds the gap 1 phase. There were a total of 14 genes that were differentially expressed that all play a role in DNA replication forks. The high number of genes involved in the same biological process and the consistency of expression, where all the genes are down regulated warrants further investigation. Cell division cycle 45 (CDC45) and MCM2-7 act together to form an active helicase that is able to unwind DNA during replication [107]. This complex then acts in tandem with the GINS, to maintain protein-protein interactions during the unwinding [107]. Replication factor C (RFC) acts to load proliferating cell nuclear antigen (PCNA) onto the unbound primed DNA [107]. PCNA then tethers the DNA polymerase to the chromosome and initiates DNA replication [107]. Two genes that code for proteins within the GINS complex were determined to be down
regulated by 99 days (100286577 & 100196511). In addition we discovered one gene that encoded for the protein CDC45 (100195156), two genes that encoded for the MCM complex (100380467 and LOC100195151), two genes for proliferating cell nuclear antigen (PCNA) (106580445 & 106585071), three genes involved in DNA polymerase (100195123, 100196854 & 100195137), two replication proteins (106605109 & 106588685) and two genes for RFC (100196285 & 100196545).

3.4.2.4 GO:0007568 - Aging

The GO term GO:0007568 is a biological term that encompasses aging, which involves genes that are responsible for deterioration [52]. Under the GO term, there were 13 genes that were found to be differentially expressed over a period of 99 days. There was only one gene (106600076) that was differentially expressed at day 8. This gene, along with three others was also significantly down regulated at 99 days. The remaining 9 genes were upregulated at 99 days with the highest LFC of 3.09. The GO term for aging shared five genes that occurred in the GO term for response to drug. The similarity in transcripts between the two may provide some preliminary evidence for a similar function between both terms. For a description of function for the similar transcripts (transforming growth factor beta-2-like, high affinity nerve growth factor receptor-like, sterol regulatory element-binding protein 1-like, sterol regulatory element-binding protein 1-like, thymidylate synthetase) between GO terms, see above. The remaining transcripts with the GO term aging, spanned an array of functions as follows. Reticulons are found in the endoplasmic reticulum were generally they influence endoplasmic reticulum-Golgi trafficking, vesicle formation and membrane morphogenesis [108]. Specifically the role of the mammalian isoform RTN1A, was involved in the mammalian endocytosis adaptor complex AP-2 [108]. P2Y receptors are a group of metabotropic receptors that plays
an important role in ADP-mediated platelet shape change [109]. Mitochondrial uncoupling proteins are an essential mitochondrial membrane protein, that play a specific role in thermogenic protein leak across the inner mitochondrial membrane [110]. Matrix metalloproteinases (MMP) are a family of zinc dependent endopeptidases that primarily are involved in the degradation of the extracellular matrix [111]. This action is formed under normal and abnormal conditions, such as embryonic development, bone and growth plate remodeling and wound healing [111,112]. Metalloproteinase inhibitors act to inhibit the action of MMP by forming reversible bonds [112]. Transforming growth factor-β (TGF-β) is involved in a vast array of cellular processes that include cell proliferation, lineage determination, differentiation, motility, adhesion, and death [113,114]. In general, the TGF family is comprised of a large number of transcripts that are related to polypeptide growth factors [113]. TGFβ-2 is seen as an import transcript for development, and distinctly different from other TGFβ isoforms (TGF-β1 and -3) [115]. As demonstrated in knock out mice, where TGF-β-null mice exhibited a wide range of developmental defects, with little to no compensatory efforts from the other isoforms [115]. The cellular response to growth is by signal transduction of growth hormone (GH), with suppressors of cytokine signaling (SOCS) acting through a negative feedback loop to inhibit the signaling cascade [116]. The SOCS protein family consist of eight proteins with conserved structural and functional domains [116]. There is some evidence that SOCS2 can act as both a positive and negative regulator of GH signaling, if over expressed in high levels [117,118]. The role that SOCS-2 plays in modulating growth is elucidated in knockout mice, were mice unable to express SOCS-2 gene grew significantly larger [119]. The last two genes were down regulated. Four of the 13 genes where downregulated at 99 days. The majority of
these genes are involved in the development of the peripheral nervous system (PNS) and one gene involved in the thymidylate synthase biosynthesis pathway. Neurabin is a ~180 kD neural specific actin that binds to, and inhibits the activity of protein phosphate 1 (PP1) [120–122]. Cell adhesion molecules represent a group of glycoproteins expressed on the cell surface that are linked in their role in the inflammatory response as well as neoplastic diseases [123]. This group contains four main families which are the integrin family, immunoglobulin super family, selectins and cadherins [123]. The function of neural cell adhesion molecules (NCAM) include neural development, regeneration and learning and memory formation [124]. Downregulation of neural cell adhesion molecule 1-like at 99 days is likely due to a reduced function of this gene in the hepatic tissue, as NCAM are generally expressed in large amounts in the nervous system [124]. In summary, the genes involved in the GO term aging are mostly involved in homeostatic processes, with difference in LFC attributed to temporal changes after 99 days.

3.5 Conclusions

This study has reported for the first time, the transcriptome level changes that occur in juvenile Atlantic salmon from the 1+ year class, cultured at 12 °C for a period of 99 days. GO analysis reported a wide range of biological process that had differentially expressed profiles. The processes that had the greatest number of genes present were those involved in the aging process as well as genes involved in mitosis, specifically in DNA replication forks, which is a common process during the mitotic S phase. The down regulation of genes during mitosis S phase may indicate the reduction in growth of hepatic cells during the pre-smolt phase. Further, there were significant temporal changes in the metabolism, homeostasis, cellular transport, sexual maturation and the immune response. The transcriptome profile presented in
this study will provide a valuable resource for future work as it provides a baseline of expression profiles in Atlantic salmon prior to smoltification and could be used to compare physiological events, such as thermal stress. Future work should aim to characterise changes at the transcriptome level for smolts and adult Atlantic salmon under normal and stressful conditions.
3.6 References


8. Martin SA, Caplice NC, Davey GC, Powell R. EST-based identification of genes


cDNA inserts (FLIcs) from Atlantic salmon (*Salmo salar*). BMC Genomics [Internet]. 2009;10:502. Available from:


16. Tromp JJ, Jones PL, Brown MS, Donald JA, Biro PA, Afonso LOB. Chronic exposure to increased water temperature reveals few impacts on stress physiology and growth responses in juvenile Atlantic salmon. Aquaculture. 2018;


19. Manchester KL. Use of UV methods for measurement of protein and nucleic acid concentrations. Biotechniques [Internet]. 1996;20:968–70. Available from:

http://www.nature.com/ncomms/2014/140925/ncomms6125/full/ncomms6125.html%3FWT.ec_id%3DNCOMMS-20141001?message-


56. Hediger MA, Romero MF, Peng J Bin, Rolfs A, Takanaga H, Bruford EA. The


115. Sanford LP, Ormsby I, Groot ACG, Sariola H, Friedman R, Boivin GP, et al. TGF β 2 knockout mice have multiple developmental defects that are non-overlapping with other TGF β knockout phenotypes. 1997;2670:2659–70.


123. Elangbam CS, Qualls CW, Dahlgren RR. Cell Adhesion Molecules—Update. Vet. Pathol. [Internet]. SAGE Publications; 1997;34:61–73. Available from:
3.7 Supplementary Materials

3.7.1 RNA sequencing summary and spike in mix ratio

Table S3.1 ERCC spike-in control ratio, and RNA integrity number for sequenced from Atlantic salmon (*S. salar*) livers, after exposure to 12 °C for a 99 day period.

<table>
<thead>
<tr>
<th>Day</th>
<th>Fish No.</th>
<th>ERCC</th>
<th>Ratio</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>ERCC1</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ERCC1</td>
<td></td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ERCC1</td>
<td></td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>ERCC2</td>
<td></td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>ERCC2</td>
<td></td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>ERCC2</td>
<td></td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>ERCC1</td>
<td></td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>ERCC1</td>
<td></td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>ERCC1</td>
<td></td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>ERCC2</td>
<td></td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>ERCC2</td>
<td></td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>ERCC2</td>
<td></td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>ERCC1</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>ERCC1</td>
<td></td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>ERCC1</td>
<td></td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>ERCC2</td>
<td></td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>ERCC2</td>
<td></td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>ERCC2</td>
<td></td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>Fish</td>
<td>Raw reads</td>
<td>Processed reads</td>
<td>Mapped reads</td>
</tr>
<tr>
<td>-----</td>
<td>------</td>
<td>-----------</td>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>25534720</td>
<td>10563236</td>
<td>10021166</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>20482780</td>
<td>7957142</td>
<td>7545094</td>
</tr>
<tr>
<td>3</td>
<td>24199178</td>
<td>10006484</td>
<td>9450432</td>
<td>97.00%</td>
</tr>
<tr>
<td>4</td>
<td>26624170</td>
<td>11196086</td>
<td>10725010</td>
<td>96.90%</td>
</tr>
<tr>
<td>5</td>
<td>22691972</td>
<td>9396010</td>
<td>8982156</td>
<td>96.70%</td>
</tr>
<tr>
<td>6</td>
<td>24236416</td>
<td>10107588</td>
<td>9689184</td>
<td>97.00%</td>
</tr>
<tr>
<td>7</td>
<td>24414730</td>
<td>10516146</td>
<td>9811144</td>
<td>94.50%</td>
</tr>
<tr>
<td>8</td>
<td>24128946</td>
<td>9992328</td>
<td>9418344</td>
<td>95.30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>----</td>
</tr>
<tr>
<td>9</td>
<td>26205874</td>
<td>10818398</td>
<td>10020954</td>
<td>93.80%</td>
</tr>
<tr>
<td>10</td>
<td>22369038</td>
<td>9243966</td>
<td>8844332</td>
<td>96.70%</td>
</tr>
<tr>
<td>11</td>
<td>19738480</td>
<td>8133438</td>
<td>7705454</td>
<td>95.70%</td>
</tr>
<tr>
<td>12</td>
<td>20966622</td>
<td>8776632</td>
<td>8343168</td>
<td>95.90%</td>
</tr>
<tr>
<td>13</td>
<td>29128438</td>
<td>11974576</td>
<td>11332464</td>
<td>95.80%</td>
</tr>
<tr>
<td>14</td>
<td>21911732</td>
<td>9417126</td>
<td>8938820</td>
<td>95.90%</td>
</tr>
<tr>
<td>15</td>
<td>26720588</td>
<td>10955328</td>
<td>10384522</td>
<td>95.90%</td>
</tr>
<tr>
<td>16</td>
<td>26106246</td>
<td>10695906</td>
<td>10236422</td>
<td>96.90%</td>
</tr>
<tr>
<td>17</td>
<td>26012174</td>
<td>11031690</td>
<td>10535146</td>
<td>96.70%</td>
</tr>
<tr>
<td>18</td>
<td>27348050</td>
<td>11316140</td>
<td>10798184</td>
<td>96.60%</td>
</tr>
<tr>
<td>19</td>
<td>22372768</td>
<td>12771782</td>
<td>12031390</td>
<td>95.10%</td>
</tr>
<tr>
<td>20</td>
<td>25251376</td>
<td>14148174</td>
<td>13096064</td>
<td>93.80%</td>
</tr>
<tr>
<td>21</td>
<td>26974470</td>
<td>14784388</td>
<td>14015190</td>
<td>96.00%</td>
</tr>
<tr>
<td>22</td>
<td>21150404</td>
<td>11554240</td>
<td>10898184</td>
<td>95.20%</td>
</tr>
<tr>
<td>23</td>
<td>27669388</td>
<td>15465434</td>
<td>14699588</td>
<td>96.30%</td>
</tr>
<tr>
<td>24</td>
<td>17865686</td>
<td>9812662</td>
<td>9286342</td>
<td>95.60%</td>
</tr>
<tr>
<td>25</td>
<td>23591924</td>
<td>13749540</td>
<td>12825204</td>
<td>94.20%</td>
</tr>
<tr>
<td>26</td>
<td>28992190</td>
<td>15129896</td>
<td>14390334</td>
<td>96.10%</td>
</tr>
<tr>
<td>27</td>
<td>26817454</td>
<td>15471814</td>
<td>14551666</td>
<td>95.00%</td>
</tr>
<tr>
<td>28</td>
<td>20970168</td>
<td>11827720</td>
<td>11160598</td>
<td>95.30%</td>
</tr>
<tr>
<td>29</td>
<td>35558590</td>
<td>18717354</td>
<td>17807922</td>
<td>96.20%</td>
</tr>
<tr>
<td>30</td>
<td>31533122</td>
<td>17210242</td>
<td>16474388</td>
<td>96.90%</td>
</tr>
</tbody>
</table>

**3.7.2 Assessment of technical and diagnostic performance**
Diagnostic and technical performance of ERCC spike-in ratios was measured utilising the erccdashboard [20] in the R statistical programming language [30]. Data was based on 30 individual libraries created from the distal portion of the liver in Atlantic salmon. The dynamic range of ERCC control measurements was determined at $\sim 2^{15}$ (Figure S3.1A). This range is reduced compared to the $2^{20}$ range that the ERCC should span [20]. One possible reason for the reduced range could be due to a loss in sequencing depth after the quality filtering. Diagnostic performance for the ERCC control ratios are recorded assessed through ROC curve analysis and AUC statistics. The resulting analysis showed that there was almost perfect (AUC = 1) diagnostic performance (AUC = 0.994 for 2- and 4-fold change) to determine differential expression at a 2- and 4-fold change, and good diagnostic power at the 1.5-fold change (AUC = 0.782), with between 13 – 15 of the ERCC ratio controls detected compared to the 23 that were originally spiked (Figure S3.1B). MA plots show that there is some bias concerning the control ratio measurements due to the disparity between solid and dotted lines (Figure S3.1C). Bias can occur during RNA handling and library preparation, causing an inefficient recovery of the ERCC transcripts for downstream analysis [20]. LODR graphs revealed that the minimum signal needed to call a 4-fold change was 22 gene counts, however there was insufficient information to determine counts below 2-fold (Figure S3.1D) Taken together this analysis revealed that there was a reduction in sequencing depth that likely affected the ability to determine smaller LODR estimates and caused some a small degree of bias in the ERCC ratios. However, there was perfect diagnostic ability to determine fold changes of 2 and greater, and that fold changes of 1.5 still had good diagnostic performance. The erccdashboard analysis was used to inform
differential expression testing parameters, where only gene comparisons that had an FDR value < 0.05 and fold change ≥ 2 were kept.

Figure S3.1 ERCC ratio technical and diagnostic plots produced through erccdashboard for Atlantic salmon (S. salar) that were cultured at 12 °C and 20 °C in freshwater for 99 days. (A) dynamic range of Atlantic salmon with coloured points representing different ERCC ratios and differently shaped points being the two different ratio mixes. Error bars represent the standard deviation of biological replicates (n=24). (B) ROC curves and the corresponding AUC statistics for the ERCC ratios, along with the table for the number of ERCC controls that were used in the analysis (‘detected’) and the total number that were
originally included (‘spiked’). (C) MA plot where filled circles indicate the ERCC ratios were above the LODR estimate and error bars for the standard deviation. Endogenous transcript ratios are represented by the grey points. The difference between samples ($r_m$) of the estimated mRNA fraction with weighted standard errors is provide in the table. Solid lines represent nominal ratios while the dashed lines represent the adjusted ratios informed from the weighted mean and standard error. (D) LODR estimates provides a table with the minimum signal (average counts) and bootstrap confidence intervals needed to determine if a transcript is differentially expressed at a given ERCC ratio and p value.
4 Transcriptomic responses in juvenile Atlantic salmon (*S. salar*) subjected to long-term exposure to a thermal stress.

4.1 Introduction

A major consequence of global warming is that weather conditions are becoming more extreme and increasing in frequency. The average global surface temperature has increased by 0.85 °C (range 0.65 °C – 1.06 °C) from 1880 – 2012 [1]. Sea surface temperatures in the subtropical western boundary currents have increased on average by 1.2 °C [2]. These increases may challenge the thermoregulation capacity of many ectothermic organisms, including fish. Species grown in commercial aquaculture are particularly vulnerable, as they are restricted from vertical migration due to the physical constraints of the sea cages. Atlantic salmon (*Salmo salar*) are commonly grown at latitudes of 40 ° – 70 ° in the Northern hemisphere, and at 40 ° – 50 ° in the Southern hemisphere [3]. The increase in sea surface temperature has already sparked research into the potential impacts that this will have on Atlantic salmon physiology, specifically on how they are able to deal with a prolonged exposure to high temperature. Studies reporting the effects of increasing water temperatures on Atlantic salmon have already been undertaken in Norway [4,5], Australia [6], Sweden [7] and Canada [8]. For example, maintaining adult Atlantic salmon at 19 °C for eight weeks altered the expression of genes and proteins in the cardiac tissue involved in the unfolded protein response, vascularization and remodelling of connective tissue [9]. In Australia, Tasmania offers the only suitable climate for commercial sea cage culture of Atlantic salmon. Water temperature during the summer months can reach 18 °C, at a depth of 5 m.
However during extreme conditions, the temperature has been reported as high as 22 °C [10]. Sea surface temperatures are predicted to rise by ~2 °C in the Tasman Sea by 2060 [11]. Tasmania has already seen an increase in sea surface temperature off the eastern coast over the past 20 years where it has increased ~0.6 °C in a decade [12]. [13]. While the farms are generally located within bays or sheltered coasts, the water temperatures often reflect that of the adjacent oceans and regional atmospheric processes, dismissing the notion that these farms will be buffered from sea surface temperature rise that are present in higher energy environments [14]. The increasing trend in sea surface temperature may introduce several challenges for the growth and production of Atlantic salmon [4–6].

The successful culture of Atlantic salmon relies upon providing a suitable environment that caters to the best growth performance of the animal. While there are many factors that can affect growth, temperature is one of the main abiotic factors to influence Atlantic salmon growth performance. It is therefore of vital importance to culture fish at temperatures that give the best growth outcomes without compromising fitness. For juvenile Atlantic salmon the optimal temperature for growth has been reported between 12.8 °C – 20 °C, dependent on the size and stage of development [15,16]. Growth performance measured through mass and fork length was not significantly different for juvenile Atlantic salmon, cultured at 12 °C, 16 °C and 20 °C [17]. Yet differences in growth performance have been demonstrated in UK (15.9 °C) and Norwegian populations (16.3 °C – 20.0 °C) [15,16]. It is unclear whether these differences in growth are the result of underlying genetic differences between populations (local adaptation) or phenotypic plasticity. Understanding the molecular mechanisms that underpin the physiological response of fish to increased water temperature is important. Transcriptomics, through
differential gene expression, allows for the identification of genes that change upon exposure to thermal stress. Identifying the genes involved in dealing with thermal stress, including long term exposure to high water temperature allows for the screening of variation across individuals, populations or region. As such the investigation of transcriptome profiles will give a better understanding of the molecular mechanisms underlying how fish modulate their physiology in response to temperature.

Thermal stress has been experimentally investigated in fish through a variety of regimes. These can include acute single pulse exposure and recovery [18], sub lethal thermal challenges [19], and prolonged (chronic) exposure to fluctuating [20,21] or constant temperatures [5,22]. These studies have shown that physiological and transcriptomic responses will vary with the duration of the thermal stress. However, there appears to be some commonalities between the different thermal stress regimes, such as the downregulation of genes involved in cell cycle/growth. This suggests that some aspects of the response to thermal stress are present between different thermal stress regimes.

Several studies have investigated the transcriptomic responses of Atlantic salmon [5,23] and more broadly in salmonids [24–27] exposed to short- and long-term increases in water temperature. These studies have shown increased production of immune and heat shock related genes at higher temperatures, and a down regulation in transcription of genes involved in protein biosynthesis. The difference in the transcriptomes of fish between short- and long-term exposure to increased water temperatures appears to be that short-term exposure upregulates protective mechanisms such as heat shock proteins, while long-term exposure repartitions energy away from key physiological processes such as the immune response or
transcription in order to respond to the thermal stress. Yet it is currently unclear what transcriptome changes occur during the transition from acute to chronic thermal stress. It is likely that transcriptome level changes during acute stress are very different from those under a chronic stress. This study investigated this hypothesis by comparing the hepatic transcriptomic response of Atlantic salmon maintained at constant ambient and elevated temperatures (12 °C and 20 °C).

The liver was selected as a target organ for transcriptome analysis as it has a diverse functional role in carbohydrate and lipid storage, amino acid and prostaglandin metabolism, somatic growth regulation, immune response and detoxification [28–31]. In addition to these roles, the liver is also affected by environmental parameters, altering the structure, function and rate of metabolism. As such the liver has been commonly studied in fish under a variety of environmental stressors, such as temperature [5]. Further, the wide array of studies investigating hepatic responses allows for comparisons between this and those studies.

The objectives of the present study were to, (1) determine the temporal changes in transcriptomic responses between fish groups maintained at 12 °C and 20 °C for 99 days, (2) determine whether there is consistent transcript responses between these different temperatures, and (3) identify differentially expressed genes and their tissue function. The broader functional significance of these differentially expressed transcripts will be identified utilizing the biological processes available through the gene ontology project [32].

4.2 Materials and methods

4.2.1 Experimental design

The full experimental design follows that described in [17]. Briefly, juvenile Atlantic salmon from the 1+ year class, were held at 12 °C in two identical
recirculating aquaculture systems each containing five 1000 L circular tanks at a stocking density of 34 fish per tank. Fish were sampled at the beginning of the experiment on day 1, then at day 8 and 99. The water temperature was increased after the initial sampling, and maintained for the duration of the trial at either 12 °C or 20 °C. At the time of sampling, fish were quickly netted and placed in a lethal dose of AQUI-S® (300 ppm), with stage 4 anaesthesia [33] observed in under 2 minutes. After blood collection and biometrics, the distal portion of the liver was dissected out and immediately flash frozen in liquid nitrogen. Liver samples were later transferred into a -80 °C freezer for long term storage. In total 48 liver samples collected for RNA-seq analysis. These samples represent fish maintained at two different temperatures (12 °C and 20 °C) over three sampling times (day 1, 8 and 99). Six samples were randomly selected from each temperature at day 1 and 8. 12 samples were randomly selected from each temperature at day 99 to account for variability in growth measurements [17].

4.2.2 RNA Sequencing

RNA samples were extracted, sequenced, filtered, trimmed, aligned and counted following methods previously described in chapter 3. Differential gene expression testing and gene ontology enrichment analysis was applied following the same methodology as in chapter 3.

As we are interested in identifying differentially expressed genes between temperature treatments and how these varied over time, a generalised linear model approach was used to account for differences between individual fish maintained at two temperatures over time. We tested for the interaction by specifying temperature and time as fixed effects. A design matrix was created to determine an interaction at any time as outlined in the edgeR users guide (3.3.4) specifying day 1 as the
reference level [34]. The following model produced two interaction coefficients. The first coefficient tested for the effect of maintaining fish at an elevated temperature of 20 °C for 8 days compared to maintaining fish at 12 °C for 8 days relative to day 1. The second coefficient tested for the effect of maintaining fish at an elevated temperature of 20 °C for 99 days compared to maintaining fish at 12 °C for 99 days relative to day 1. As this was a time course experiment, we discretely split the two interaction coefficients so that we could better see patterns of expression across time. This approach was selected in favour of multiple pairwise comparisons (i.e. 12 °C vs 20 °C for day 1, 8 and 99, respectively) as we are able to detect temporal trends in genes that respond differently to 20 °C relative to 12 °C. Differentially expressed genes were determined by a FDR < 0.05 in at least one of the times (day 8 or 99), and a LFC ≥ 1. [35]. Soft clustering was then applied to the differentially expressed genes utilising the Mfuzz (v2.34.0) package available through the R programming language [36].

4.3 Results

4.3.1 Sequencing and read mapping

Sequencing of cDNA libraries yielded between 17.9 – 35.6 million reads per sample (Table S4.1). From these raw reads the adaptor sequences and low-quality base reads (phred score < 30) were removed retaining only those with paired-end counterparts; we kept between 8.0 – 19.6 million pair end reads per sample (Table S4.1). The Atlantic salmon genome contains 57,783 genes based on the entrez records [37], of these we were able to generate counts for 42,003 genes, matched through HTSeq-count with at least one count. For DE analysis, each sample was normalised by counts per million (CPM) to enable comparisons between different sized libraries [38], resulting in individual sample library gene counts ranging from 0
– 57,948 CPM. Genes that weren’t present at a level of 1 CPM in all 6 samples, were excluded from the analysis, resulting in 24,707 of the 42,003 genes being used the differential expression analysis. Performance diagnostics from the ERCC ratio analysis revealed that there was perfect diagnostic performance to detect differential expression at the 2- and 4-fold levels and good diagnostic power at 1.5-fold (Figure S4.1). Yet the results indicate that there was some bias between the ratio control measurements and reduced limit of detection of ratio estimates under the 4-fold levels. Based on the performance estimates of the ERCC spike-ins, the minimum requirements to call a gene differentially expressed was set at a fold change of 2 and a FDR threshold < 0.05.

4.3.2 Differentially expressed genes

We identified 378 differentially expressed genes between juvenile Atlantic salmon maintained at 20 °C compared with 12 °C for 8 and 99 days (Figure 4.1). Of these, 63 genes were uniquely expressed at day 8, with 31 genes upregulated and 32 down regulated (Figure 4.1). At day 99 there were 269 uniquely expressed genes with 113 genes upregulated and 156 downregulated (Figure 4.1). The top 5 genes upregulated by log2-fold-change (hereafter referred to as top) at day 8 and 99 included genes involved in cellular transport, transcriptional regulation, stress processes and immune response (Table 4.1). The top genes downregulated at day 8 and 99 included genes involved in immune response, the cell cycle, cell membrane receptors, mitosis, glucose metabolism and cellular respiration (Table 4.1).

There were 46 genes identified as consistently differentially expressed at both day 8 and 99, consisting of 19 genes upregulated, and 27 genes downregulated (Figure 4.1). Of the top upregulated genes, 4 were identified with known functions, 3 of which appear to be involved in stress responses (immune system, inflammation
and homeostasis) (Table 4.2). For the top downregulated genes, 3 were identified that are involved in lipid metabolism and cell division or DNA replication (Table 4.2). From the total number of differentially expressed genes, there were 43 genes that were classified as uncharacterised within the Atlantic salmon genome. In summary, there were a greater number of genes that were downregulated compared to upregulated between the temperature treatments, with day 99 showing the largest number of differentially expressed genes between the temperature treatments (Figure 4.1).
Figure 4.1 Venn diagram for the sum of differentially expressed genes that were up or downregulated in Atlantic salmon maintained at 20 °C compared to 12 °C at 8 and 99 days.
Table 4.1 Top 5 upregulated and top 5 downregulated genes ordered by log2-fold-change (LFC) between juvenile Atlantic salmon maintained at 12 °C and 20 °C for 8 and 99 days. Positive values represent genes that were upregulated while negative values represent genes that were downregulated.

<table>
<thead>
<tr>
<th>Day</th>
<th>Gene</th>
<th>Up-/down-regulation</th>
<th>LFC</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Multidrug resistance-associated protein 4-like</td>
<td>Up</td>
<td>4.23</td>
<td>Part of the ATP-binding cassette transporter superfamily, plays a role in protecting against xenobiotic substances [39].</td>
</tr>
<tr>
<td></td>
<td>Uncharacterized LOC106576772</td>
<td>Up</td>
<td>5.3</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2-like</td>
<td>Up</td>
<td>6.14</td>
<td>This gene may be involved in Ca\textsuperscript{2+} regulation, however it's role is relatively unknown [40]. Transport of secretory granules and Ca\textsuperscript{2+} binding proteins [41,42]</td>
</tr>
<tr>
<td></td>
<td>Secretagogin-like</td>
<td>Up</td>
<td>6.43</td>
<td>Essential role in cortical cytoskeleton organization, cell motility, adhesion and proliferation [43]</td>
</tr>
<tr>
<td></td>
<td>Radixin-like</td>
<td>Up</td>
<td>8.23</td>
<td>Innate immune response, possibly involved in platelet regulating in fish [44,45].</td>
</tr>
<tr>
<td></td>
<td>C-type lectin lectoxin-Thr1-like</td>
<td>Down</td>
<td>-11.21</td>
<td>Involved in the immune response [46].</td>
</tr>
<tr>
<td></td>
<td>Integrin beta-1-like</td>
<td>Down</td>
<td>-5.89</td>
<td>Involved in ciliogenesis, cell morphology and cell cycle [47]. C2 domains are also involved in cellular membrane signalling [48].</td>
</tr>
<tr>
<td></td>
<td>Coiled-coil and C2 domain-containing protein 1B-like</td>
<td>Down</td>
<td>-5.76</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Uncharacterized LOC106560374</td>
<td>Down</td>
<td>-5.36</td>
<td>Unknown</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Change</td>
<td>Log2 Fold Change</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>--------</td>
<td>-----------------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Protein Mis18-alpha-like</td>
<td>Down</td>
<td>-5.32</td>
<td>Involved in centromere/kinetochore structure and function, which is essential for chromosomal segregation during mitosis [49]</td>
<td></td>
</tr>
<tr>
<td>Uncharacterized LOC106578631</td>
<td>Up</td>
<td>5.1</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Zinc finger protein 14-like</td>
<td>Up</td>
<td>5.46</td>
<td>Diverse function from DNA/RNA binding, protein-protein interactions and membrane associations [50].</td>
<td></td>
</tr>
<tr>
<td>Uncharacterized LOC106563843</td>
<td>Up</td>
<td>5.56</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>E3 ubiquitin-protein ligase RNF213-like</td>
<td>Up</td>
<td>6.15</td>
<td>Involved in the immune response to infection [51,52].</td>
<td></td>
</tr>
<tr>
<td>Uncharacterized LOC106612301</td>
<td>Up</td>
<td>12.05</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Uncharacterized LOC106566266</td>
<td>Down</td>
<td>-5.79</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Glucokinase-like</td>
<td>Down</td>
<td>-4.86</td>
<td>Important role in the secretion of insulin, regulating glucose metabolism [53].</td>
<td></td>
</tr>
<tr>
<td>Carbonic anhydrase 4-like</td>
<td>Down</td>
<td>-4.63</td>
<td>Enzyme involved catalysing the carbon dioxide to carbonic acid [54].</td>
<td></td>
</tr>
<tr>
<td>Catechol O-methyltransferase-like</td>
<td>Down</td>
<td>-4.52</td>
<td>Remove biologically active or toxic catechols and some hydroxylated metabolites [55].</td>
<td></td>
</tr>
<tr>
<td>Nuclear receptor subfamily 0 group B member 2-like</td>
<td>Down</td>
<td>-3.74</td>
<td>Acts as a transcriptional regulator [56]</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2 Top 5 upregulated and top 5 downregulated genes across both day 8 and 99 ordered by log₂-fold-change (LFC) in juvenile Atlantic salmon maintained at 12 °C and 20 °C for 8 and 99 days. Positive values represent genes that were upregulated while negative values represent genes that were downregulated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Up-/down-regulation</th>
<th>Day 8</th>
<th>LFC</th>
<th>Day 99</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glandular kallikrein, prostatic-like</td>
<td>Up</td>
<td>4.16</td>
<td>3.99</td>
<td></td>
<td>Kallikreins are a subfamily of the serine proteases, and are involved in posttranslational modifications in polypeptides [57,58].</td>
</tr>
<tr>
<td>Alpha-kinase 2</td>
<td>Up</td>
<td>3.68</td>
<td>4.07</td>
<td></td>
<td>Kinases are involved in a broad range of cellular processes including protein translation, Mg²⁺ homeostasis, intracellular transport, cell migration, adhesion and proliferation through their catalytic actions on protein phosphorylation [59].</td>
</tr>
<tr>
<td>Trace amine-associated receptor 13c-like</td>
<td>Up</td>
<td>3.81</td>
<td>3.26</td>
<td></td>
<td>Trace amine-associated receptors interact with G-protein receptors were they can act as neural transmitters, however the role of trace amine systems in still relatively unknown [60].</td>
</tr>
<tr>
<td>Serpin H1-like</td>
<td>Up</td>
<td>3.77</td>
<td>3.4</td>
<td></td>
<td>Involved in maintain correct collagen conformation induced through heat stress [61].</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Expression</td>
<td>Log2 Fold Change</td>
<td>Description</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------------</td>
<td>-----------------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncharacterized LOC106573202</td>
<td>Up</td>
<td>3.75</td>
<td>3.65 Unknown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymidine kinase, cytosolic</td>
<td>Down</td>
<td>-3.27</td>
<td>-3.02 Involved in DNA replication during mitotic S phase [62].</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonucleotide reductase M2</td>
<td>Down</td>
<td>-3.08</td>
<td>-3.53 Involved as a rate limiting step in the synthesis of deoxyribonucleotides [63].</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acyl-CoA desaturase-like</td>
<td>Down</td>
<td>-3.77</td>
<td>-3.14 Important component of lipid synthesis (de novo) and β-oxidation [64].</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell division cycle 45</td>
<td>Down</td>
<td>-4.87</td>
<td>-3.35 Acts to form an active helicase that is able to unwind DNA during replication [65].</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear receptor subfamily 0 group B member 2-like</td>
<td>Down</td>
<td>-3.75</td>
<td>-3.74 Has a diverse range in function that is important in maintaining normal physiological state, which includes its involvement with development, reproduction and metabolism [66].</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.3 Assignment of differentially expressed genes to gene ontology terms

We were able to assign 505 gene ontology terms to 46 of the 63 differentially expressed genes at day 8. Enrichment of gene ontology terms provided no significant over-representation of differentially expressed genes after FDR correction. For the differentially expressed genes at day 99 we were able to match 1941 gene ontology terms to 213 of the 269 genes. One gene ontology term was significantly enriched, forebrain anterior/posterior pattern specification (GO:0021797). There were four genes that were differentially expressed under this term. Axin-2-like (Gene ID:106564887 and 106585623), protein Wnt-9a-like (106599874) and wingless-type MMTV integration site family member 9A (106569340). These genes are involved in the Wnt signalling pathway, which participates in embryonic developmental events as well as adult tissue homeostasis [67]. The expression of these genes may indicate a change in homeostasis in response to thermal stress. We were able to assign 363 gene ontology terms to 37 out of 46 differentially expressed genes that were commonly expressed between day 8 and 99. Enrichment identified 46 biological processes, 3 cellular components and 20 molecular function gene ontologies. The top five gene ontologies passed through REVIGO were positive regulation of interleukin-1 secretion (GO:0050716), male-specific defence response to bacterium (GO:0050831), DNA geometric change (GO:0032392), myoblast proliferation (GO:0051450) and plasmacytoid dendritic cell activation (GO:0002270). The enrichment of these GO terms was through the down regulation of three high mobility group proteins, which play an important role in DNA repair. These include; high-mobility group box 2 (100194613), high mobility group protein B3 (100195470) and high mobility group protein B2-like (106602389) all of which were downregulated across both days (8 and 99).
Differentially expressed genes were also grouped by soft clustering using Mfuzz, to examine common gene expression signals. Five clusters were identified and are presented in Figure 4.2. Genes with a high membership value (alpha-core of 0.7) were selected, of these the sum of genes in each cluster was 23, 25, 18, 10 and 1 from cluster 1 through to 5, respectively. Enrichment analysis was applied to each cluster, with cluster 3 being the only cluster to provide any significantly enriched GO terms, comprising of 52 terms. We were able to assign 194 gene ontology terms to 15 out of the 18 differentially expressed genes identified in cluster 3. Within the gene ontology terms there were 32 biological processes, 3 cellular components and 17 molecular functions. The top 5 gene ontology terms were response to UV (GO:0009411), hibernation (GO:0042750), negative regulation of nuclear-transcribed mRNA poly(A) tail shortening (GO:0060212), stress granule assembly (GO:0034063) and plasmacytoid dendritic cell activation (GO:0002270). There were 12 genes which were matched to these gene ontology terms (Table 4.3). These genes are involved in cellular respiration, gene expression, DNA replication and repair and cellular growth.
Figure 4.2 Soft clustering of differentially expressed genes in Atlantic salmon maintained at 12 °C and 20 °C for 99 days.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Up-/down-regulation</th>
<th>LFC</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate dehydrogenase (acetyl-transferring) kinase Isozyme 2, mitochondrial-like</td>
<td>Up</td>
<td>2.03</td>
<td>Play an essential role in the inactivation of pyruvate dehydrogenase complexes, which in turn oxidase pyruvate into the tricarboxylic cycle (citric acid or Krebs cycle) to produce adenosine triphosphate [68,69].</td>
</tr>
<tr>
<td>Polyadenylate-binding protein 4-like</td>
<td>Up</td>
<td>1.15</td>
<td>Important role in the regulation of gene expression [70].</td>
</tr>
<tr>
<td>PCNA-associated factor-like</td>
<td>Down</td>
<td>-3.81</td>
<td>Assist in DNA repair during DNA replication [71].</td>
</tr>
<tr>
<td>PCNA-associated factor</td>
<td>Down</td>
<td>-3.11</td>
<td></td>
</tr>
<tr>
<td>High mobility group protein B3</td>
<td>Down</td>
<td>-2.63</td>
<td>High mobility group proteins are involved in modulating DNA damage recognition and repair [72]. High mobility group proteins B family have a high affinity to bind with bent or distorted DNA [72].</td>
</tr>
<tr>
<td>High-mobility group box 2</td>
<td>Down</td>
<td>-1.92</td>
<td></td>
</tr>
<tr>
<td>High mobility group protein B2-like</td>
<td>Down</td>
<td>-1.82</td>
<td></td>
</tr>
<tr>
<td>High mobility group protein B3-like</td>
<td>Down</td>
<td>-1.43</td>
<td></td>
</tr>
<tr>
<td>Cold inducible RNA binding protein</td>
<td>Down</td>
<td>-2.36</td>
<td>The cold-shock response elicits a suite of genes that are involved in the synthesis of transcription and translation of proteins [73]. In mice these genes have been demonstrated to reduce growth rates and prolong G1 phase [74].</td>
</tr>
<tr>
<td>Cold-inducible RNA-binding protein B-like</td>
<td>Down</td>
<td>-1.86</td>
<td></td>
</tr>
<tr>
<td>Cold-inducible RNA-binding protein B-like</td>
<td>Down</td>
<td>-1.83</td>
<td></td>
</tr>
<tr>
<td>Cold-inducible RNA-binding protein B-like</td>
<td>Down</td>
<td>-1.73</td>
<td></td>
</tr>
</tbody>
</table>

*GO:0009411, GO:0034063, GO:0042750, GO:0060212 and GO:0002270.*
4.4 Discussion

In this study we reported on the transcriptome level changes in Atlantic salmon maintained under chronic thermal stress over 99 days. Understanding the molecular mechanisms that underpin the response of Atlantic salmon to such chronic thermal stresses may provide better management practices and preventative steps to improve fish welfare. In fish maintained at 20 °C, genes involved in immunity, heat shock and inflammation responses were upregulated, while genes involved in homeostasis, glucose and fatty acid metabolism and cell cycle were down regulated. The genes identified in this study may be used in the development of biomarkers for thermal stress.

Fish maintained at 20 °C downregulated genes at both day 8 and 99 which are involved in cell cycle and DNA replication, which are involved in growth and cellular maintenance. Downregulating these processes may be due to repartitioning energy away from cellular growth and/or maintenance (regeneration of new cells) through mitosis and into protective mechanisms at higher water temperatures. The effect of thermal stress on growth related transcripts including the reduction in cell cycle has been investigated in longfin and delta smelt [75], Chinook salmon [18], Cyprinidae (*Squalius torgalensis*) [76] and zebrafish [77]. Thermal stress in broiler chickens has also demonstrated the downregulation of genes involved in cell progression, specifically in the M and S phase [78]. The attenuation of neurogenesis has previously been shown through male Cobb chicks exposed to heat stress (37.5 or 40 °C), where there was an inhibitory effect of new cell generation in neural tissue [79]. The authors suggest that this action maybe the result of elevated corticosterone (cortisol in fish) through the stimulation of the hypothalamic-pituitary-adrenal axis [79]. As such
corticosterone, may play an inhibitory role on the progression of the cell cycle and subsequent growth. Interestingly the cortisol or glucose levels were not significantly increased at 20 °C when measured from the plasma of these fish, yet plasma cortisol was elevated at 16 °C as reported in [17]. It is not clear whether there is a link between the downregulation of growth related transcripts and the expression of plasma cortisol in this study. Through clustering we identified four genes from the cold-inducible RNA binding protein family which were also down regulated. The cold-shock response elicits a suite of genes that are involved in the synthesis of transcription and translation of proteins [73]. In mice these genes have been demonstrated to reduce growth rates and prolong G1 phase [74]. While transcriptome studies have demonstrated the reduction in growth related genes after exposure to thermal stress, it is unclear how the translation of these genes impacts upon growth. While there was a reduction in growth related genes in fish maintained at 20 °C, differences in body mass and fork length were not observed when compared to 12 °C [17].

After 99 days of exposure to high water temperature fish downregulated high mobility group proteins and upregulated heat shock proteins which suggests that the cells were putting in place mechanisms to deal with long term exposure to increased water temperature. High mobility group proteins which are involved in binding with high affinity to bent or distorted DNA and play an important role in modulating DNA damage recognition and repair [72]. This suggests that maintaining fish at elevated temperatures may have increased DNA damage. The downregulation of these proteins has also been demonstrated through proteomics in the liver of post-smolt Atlantic salmon maintained for 43 days at an elevated temperature of 21 °C [80]. The expression of high mobility group proteins may
form part of the generalised response to elevated temperature in fish and may be an early indication of potential negative consequences on fish health. Serpin H1-like (hsp47) was significantly upregulated in fish maintained at 20 °C across both day 8 and 99. Serpin H1-like is found in the endoplasmic reticulum and is specifically involved in maintaining correct collagen conformation induced through heat stress [61]. The study of molecular chaperones has commonly been reported in thermal stress studies [5,25,81,82]. Heat shock proteins are involved in maintaining protein conformation to avoid harmful aggregations of denatured proteins in the cell. Yet there has been some uncertainty in the use of this protein family as a single determinate to the generalised stress response in fish [83]. Nevertheless, several studies have reported the upregulation of serpin H1-like in response to thermal stress in a number of species e.g., Atlantic salmon [80], rainbow trout (Oncorhynchus mykiss) [84,85] and redband trout (Oncorhynchus mykiss gairdneri) [86]. For example post-smolt Atlantic salmon maintained at 21 °C had a 3 fold upregulation of serpin H1 after 43 days compared to fish maintained at 15 °C [80]. As such, serpin H1 appears to play a key regulatory role in maintaining collagen conformation during chronic thermal stress amongst teleost fish. Further serpin H1 has been demonstrated to be expressed under similar conditions that cause oxidative stress, which can often occur alongside thermal stress [85]. Serpin H1 has also been identified to be a key gene responsive to thermal stress in salmonids using meta-analysis approach covering Artic char (Salvelinus alpinus) and Pacific salmon (Oncorhynchus spp.) [87]. Therefore serpin H1 may constitute a promising biomarker for investigating the effects of climate change in fish populations.
We detected the differential expression of genes involved in the immune response, suggesting that immune function of Atlantic salmon was impacted at elevated temperatures. Thermal challenges have previously been demonstrated to elicit the expression of immune related genes in fish [75,88]. Maintaining an immune response has an associated energetic cost, where energy may be diverted away from growth. The ability to elicit an immune response has immediate adaptive benefit to the organism for survival, however the continual maintenance of the immune response may cause deleterious impacts if chronically stimulated. The highest change in expression was identified through a downregulation of C-type lectin lectoxin-Thr1-like (106597809). There is little information available on the function of this gene, however components of this gene, such as C-type lectins have been shown to be involved in the innate immune response [44]; while lectoxin-Thr1 has previously been described for its action in snake venoms on C-type lectin’s inhibition or activation of platelets [89]. When considering the function of this gene in Atlantic salmon, it is likely expressed due to its involvement in platelet regulation and their subsequent role in enhancing the adaptive immune response [45]. As such it may be that fish maintained at 20 °C have a reduced ability to maintain an immune response through platelet regulation. Multidrug resistance-associated protein 4-like (106574544) was upregulated, and these proteins are part of the ATP-binding cassette transporter superfamily [39]. Multidrug resistance proteins have been seen to play an important role in tissue defence in dealing with xenobiotic substances [39]. Integrin beta-1-like (106599515) was downregulated at 20 °C compared with 12 °C at day 8. This gene has been identified to be upregulated in the hard clam (Mercenaria mercenaria) when exposed to protistan parasite QPX (Quahog
Parasite Unknown) [46]. Glandular kallikrein, prostatic-like (106610912) was upregulated across both days, and has been shown to be a response to bacterial infection in Atlantic salmon, suggesting an immunological response similar to a bacterial infection for fish maintained at 20 °C for 99 days [90]. E3 ubiquitin-protein ligase RNF213-like (106597258) was upregulated at day 99. This gene was upregulated in response to a nervous necrosis viral infection in the epithelial cells in Asian seabass 24 hours post infection [51]. Isoform X1 of this gene was also identified to be upregulated in the gill tissue of channel catfish (Ictalurus punctatus) challenged with a bacteria (Flavobacterium. columnare) under two different diets [52]. The upregulation of these immune genes in 20 °C compared with 12 °C at day 99 indicates a similar response from thermal stress to bacterial and viral infection, and may have immunosuppressive effects. In summary these genes encompass a variety of functions within the immune response, and suggest that elevated temperature affects the expression of protective mechanisms, likely diverting energy away from growth and other basal metabolic functions.

In addition to immune related genes, we detected the differential expression of genes involved in ion regulation and transport, glucose and fatty acid metabolism, cell development and cellular structure; suggesting that cellular homeostasis of Atlantic salmon might be affected at elevated temperatures. The gene ontology term, forebrain anterior/posterior pattern specification (GO:0021797), was enriched in fish maintained at 20 °C for 99 days. Genes under this term are involved in the Wnt signalling pathway, which participates in embryonic developmental events as well as adult tissue homeostasis [67]. Wnt proteins are presented on the cell surface where they bind to receptors, which in turn, transduce signals to intracellular proteins such as Axin [67]. These signals
can inhibit degradation pathways and lead to an accumulation of β-catenin in the cytoplasm and nucleus [67]. Genes involved in the Wnt signalling pathway have previously been downregulated in the red sea coral (Stylophora pistillata) under a 13-day heat stress [91]. In addition this pathway has also been identified in smelt exposed to an acute thermal challenge [75]. The downregulation of genes in the Wnt signalling pathway expressed at 20 °C may highlight a reduced capacity for tissue homeostasis, and increased degradation pathways compared to fish at 12 °C. This indicates that fish maintained at 20 °C have the mechanisms to cope with higher temperatures, yet they may have to work harder and divert more energy to maintain homeostasis.

Several genes involved in energy production via the metabolism of glucose and fatty acid were downregulated at 20 °C compared with 12 °C. While circulating plasma glucose levels were not significantly different between temperatures after 99 days [17] the transcriptomic profile suggests that higher water temperatures may inhibit glucose specific energy production pathways and promote fatty acid metabolism. Pyruvate dehydrogenase kinases (PDK) play an essential role in the inactivation of pyruvate dehydrogenase complexes, which in turn oxidise pyruvate in the tricarboxylic cycle (citric acid or Krebs cycle) to produce adenosine triphosphate [68,69]. Under conditions where glucose is scarce, the conversion of pyruvate to acetyl-CoA is restricted by the suppression of pyruvate dehydrogenase complexes through pyruvate dehydrogenase kinases [69]. In turn the synthesis of malonyl-CoA is reduced, which is an inhibitor of fatty acid oxidation creating a switch from glucose to fatty acid oxidation [92]. The upregulation of pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 2 mitochondrial-like (106607477) may indicate a reduction in available glucose
and a switch to fatty acid oxidation to meet the energy demand due to increased metabolism at 20 °C compared with 12 °C. Pyruvate dehydrogenase kinases have been reported in European sea bass (Dicentrarchus labrax) that have been categorised by cortisol response (high and low) [93]. The expression of PDK2 was enriched in the fish categorised as high cortisol responders [93]. The expression of PDK may indicate a stressed state in the fish at 20 °C and may be useful as a biomarker for thermal stress in fish.

The greatest upregulation at 99 days, was seen in the catechol O-methyltransferase-like gene. The general function of Catechol-O-methyltransferase, is to remove biologically active or toxic catechols and some hydroxylated metabolites [55]. Alpha-kinase 2 (106568481) was upregulated across both days. Kinases are involved in a broad range of cellular processes including protein translation, Mg²⁺ homeostasis, intracellular transport, cell migration, adhesion and proliferation through their catalytic actions on protein phosphorylation [59]. The functional role of alpha-kinase 2 is not well established. There is some evidence for the function of this gene in cancer research in luminal apoptosis and DNA repair [94]. The increased expression of alpha-kinase 2 in fish maintained at 20 °C might indicate the response of damaging processes and cell death at higher temperatures. Two genes from the PCNA family were down regulated. PCNAs have been demonstrated to assist in DNA repair during DNA replication [71].

Nuclear receptor subfamily 0 group B member 2-like (106588612) had the highest down regulation that was consistent between both day 8 and 99. The nuclear receptor family has a diverse range of functions that are important in maintaining normal physiological state, which includes its involvement with
development, reproduction and metabolism [66]. Genes involved in the structural components of the cell were also expressed at 20 °C compared to 12 °C with the expression of radixin-like (106586613), which had the highest upregulation gene. This gene is thought to play an essential role in cortical cytoskeleton organization, cell motility, adhesion and proliferation [43]. The expression of this gene may affect the cortical cytoskeleton of the cell in fish at 20 °C. As such, various homeostatic processes such as glucose and fatty acid metabolism, DNA replication are affected in fish cultured under chronic thermal stress.

Several genes involved in calcium ion transport were differentially expressed after 8 days in fish maintained at 20 °C. Calcium signalling has previously been demonstrated to be responsive of thermal stress in fish [75]. Secretagogin-like (106569925) was upregulated in 20 °C compared with 12 °C at day 8. Broadly this gene belongs to the hexa EF-hand proteins which are involved in the sensor family of Ca^{2+} binding proteins [42]. Secretagogin has been found to interact with SNAP-25 which is involved in Ca^{2+} endocytosis [42]. Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2-like (106573734) was upregulated in 20 °C compared with 12 °C at day 8 and may play some role in calcium signalling [40]. Perhaps the role of this gene maybe sensitive to temperature in Ca^{2+} ion regulation. Calcium ion concentrations have been demonstrated to be effected under the influence of the unfolded protein response, when cellular stress occurs [95]. Calcium-mediated signalling has been linked to osmotic stress in fish through the role of osmosensory signal transduction in fish cells [96]. Further calcium ion concentrations were identified in juvenile chinook (Oncorhynchus tshawytscha) salmon in response to an acute thermal stress [18]. Calcium ion pathways through the expression of the
molecular chaperone FKBP10 have previously been highlighted in Pacific salmon to be responsive to thermal stress [97]. Calcium ion transport is an essential component of cellular signalling. The expression of genes involved in calcium ion pathways may indicate a response to increased temperature in fish maintained at 20 °C compared with 12 °C after 8 days.

4.5 Conclusions

Elevated temperature elicited a greater response in the number of differentially expressed genes after 99 days compared to 8 days. In fish maintained at 20 °C we identified a reduction in cell cycle and DNA replication, expression of immune related genes and the expression of cellular protective mechanisms when compared to 12 °C. Exposure of juvenile Atlantic salmon to 20 °C does not appear to severely impact upon growth and survival. Yet it is important to consider that additional environmental stressors may compound the transcriptomic responses seen in this study, and should inform future multi-stress studies. While any of the differentially expressed genes identified in this study may be used for the development of biomarkers for chronic thermal stress in fish, candidate genes identified through soft clustering represent a group of genes that have highly conserved expression patterns. We suggest the consideration of these clusters for the development of biomarkers to chronic thermal stress.
4.6 References


6. Pankhurst NW, King HR, Anderson K, Elizur a., Pankhurst PM, Ruff N. Thermal impairment of reproduction is differentially expressed in maiden and repeat spawning Atlantic salmon. Aquaculture [Internet]. Elsevier B.V.;


12. Foster SD, Griffin D a., Dunstan PK. Twenty years of high-resolution sea


17. Tromp JJ, Jones PL, Brown MS, Donald JA, Biro PA, Afonso LOB. Chronic exposure to increased water temperature reveals few impacts on stress physiology and growth responses in juvenile Atlantic salmon. Aquaculture. 2018;


20. Coulter DP, Sepúlveda MS, Troy CD, Höök TO. Species-specific effects of


47. Zhou Q, Liu B, Sun Y, He CY. A coiled-coil- and C2-domain-containing protein is required for FAZ assembly and cell morphology in Trypanosoma


52. Zhao H. Impacts of feed additives on surface mucosal health and columnaris susceptibility in channel catfish, Ictalurus punctatus. 2015;


55. Mannisto PT, Kaakkola S. Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new


78. Zhang J, Schmidt CJ, Lamont SJ. Transcriptome analysis reveals potential
mechanisms underlying differential heart development in fast- and slow-growing broilers under heat stress. BMC Genomics [Internet]. BMC Genomics; 2017;18:295. Available from:
http://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-017-3675-9
http://dx.doi.org/10.1016/j.neuroscience.2014.07.047


91. Maor-Landaw K, Karako-Lampert S, Ben-Asher HW, Goffredo S, Falini G,


4.7 Supplementary Materials

4.7.1 RNA sequencing summary and spike-in mix ratio

Table S4.1 Summary of raw, processed, mapped reads and alignment for each individually sequenced fish cultured at 12 °C and 20 °C for 99 days.

<table>
<thead>
<tr>
<th>Fish No.</th>
<th>Day</th>
<th>Raw reads</th>
<th>Processed reads</th>
<th>Mapped reads</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>25534720</td>
<td>10563236</td>
<td>10021166</td>
<td>95.90%</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>20482780</td>
<td>7957142</td>
<td>7545094</td>
<td>95.80%</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>24199178</td>
<td>10006484</td>
<td>9450432</td>
<td>95.60%</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>26624170</td>
<td>11196086</td>
<td>10725010</td>
<td>96.90%</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>22691972</td>
<td>9396010</td>
<td>8982156</td>
<td>96.70%</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>24236416</td>
<td>10107588</td>
<td>9689184</td>
<td>97.00%</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>24414730</td>
<td>10516146</td>
<td>9811144</td>
<td>94.50%</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>24128946</td>
<td>9992328</td>
<td>9418344</td>
<td>95.30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>26205874</td>
<td>10818398</td>
<td>10020954</td>
<td>93.80%</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>22369038</td>
<td>9243966</td>
<td>8844332</td>
<td>96.70%</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>19738480</td>
<td>8133438</td>
<td>7705454</td>
<td>95.70%</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>20966622</td>
<td>8776632</td>
<td>8343168</td>
<td>95.90%</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>25318144</td>
<td>10622920</td>
<td>10130602</td>
<td>96.60%</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>21757992</td>
<td>8995120</td>
<td>8492948</td>
<td>95.30%</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>24129192</td>
<td>9217720</td>
<td>8789326</td>
<td>96.50%</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>24509378</td>
<td>9487584</td>
<td>8981174</td>
<td>95.80%</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>20991720</td>
<td>9297904</td>
<td>8805684</td>
<td>95.70%</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>24536266</td>
<td>10057370</td>
<td>9598718</td>
<td>96.50%</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>29128438</td>
<td>11974576</td>
<td>11332464</td>
<td>95.80%</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>21911732</td>
<td>9417126</td>
<td>8938820</td>
<td>95.90%</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>26720588</td>
<td>10955328</td>
<td>10384522</td>
<td>95.90%</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>26106246</td>
<td>10695906</td>
<td>10236422</td>
<td>96.90%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>26012174</td>
<td>11031690</td>
<td>10535146</td>
<td>96.70%</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>27348050</td>
<td>11316140</td>
<td>10798184</td>
<td>96.60%</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>19636312</td>
<td>11102952</td>
<td>10524782</td>
<td>95.70%</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>20839486</td>
<td>11930424</td>
<td>11209938</td>
<td>94.90%</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>28337116</td>
<td>16168588</td>
<td>15277764</td>
<td>95.60%</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>24773726</td>
<td>14262674</td>
<td>13451082</td>
<td>95.20%</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>20719760</td>
<td>12022408</td>
<td>11448116</td>
<td>96.10%</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>28190002</td>
<td>15754012</td>
<td>15108248</td>
<td>97.00%</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>22372768</td>
<td>12771782</td>
<td>12031390</td>
<td>95.10%</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>25251376</td>
<td>14148174</td>
<td>13096064</td>
<td>93.80%</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>26974470</td>
<td>14784388</td>
<td>14015190</td>
<td>96.00%</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>21150404</td>
<td>11554240</td>
<td>10898184</td>
<td>95.20%</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>27669388</td>
<td>15465434</td>
<td>14699588</td>
<td>96.30%</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>17865686</td>
<td>9812662</td>
<td>9286342</td>
<td>95.60%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>27538286</td>
<td>15737666</td>
<td>14967042</td>
<td>96.20%</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>26553458</td>
<td>14786964</td>
<td>14081456</td>
<td>96.30%</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>20202456</td>
<td>11483360</td>
<td>10916666</td>
<td>96.00%</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>19554758</td>
<td>11231730</td>
<td>10673252</td>
<td>95.90%</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>34934540</td>
<td>19645194</td>
<td>18822830</td>
<td>97.00%</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>32168224</td>
<td>18361154</td>
<td>17588580</td>
<td>96.90%</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>23591924</td>
<td>13749540</td>
<td>12825204</td>
<td>94.20%</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>28992190</td>
<td>15129896</td>
<td>14390334</td>
<td>96.10%</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>26817454</td>
<td>15471814</td>
<td>14551666</td>
<td>95.00%</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>20970168</td>
<td>11827720</td>
<td>11160598</td>
<td>95.30%</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>35558590</td>
<td>18717354</td>
<td>17807922</td>
<td>96.20%</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>31533122</td>
<td>17210242</td>
<td>16474388</td>
<td>96.90%</td>
<td></td>
</tr>
</tbody>
</table>
**Table S4.2** ERCC spike-in control ratio, and RNA integrity number for sequenced from Atlantic Salmon (*S. salar*) livers, after exposure to 12 °C and 20 °C over a 99 day period.

<table>
<thead>
<tr>
<th>Day</th>
<th>Fish No.</th>
<th>ERCC ratio</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>ERCC1</td>
<td>7.9</td>
</tr>
<tr>
<td>2</td>
<td>ERCC1</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ERCC1</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>ERCC2</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>ERCC2</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>ERCC2</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>ERCC1</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>ERCC1</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>ERCC1</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>ERCC2</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>ERCC2</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>ERCC2</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>ERCC2</td>
<td>8.7</td>
</tr>
<tr>
<td>14</td>
<td>ERCC2</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>ERCC2</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>ERCC1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>ERCC1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>ERCC1</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>ERCC1</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>ERCC1</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>ERCC1</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>ERCC2</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>ERCC2</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>ERCC2</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>ERCC1</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>ERCC2</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>ERCC1</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>ERCC2</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>ERCC1</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>ERCC2</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>ERCC2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>ERCC1</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>ERCC2</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>ERCC1</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>ERCC2</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>ERCC1</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>ERCC2</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>ERCC1</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>ERCC1</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>ERCC1</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>ERCC2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>ERCC2</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>ERCC1</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>ERCC1</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>ERCC2</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>
Diagnostic and technical performance of ERCC spike-in ratios was measured utilising the erccdashboard [98] in the R statistical programming language [99]. Data was based on 48 individual libraries created from the distal portion of the liver in Atlantic salmon. The dynamic range of ERCC control measurements was determined at $\sim2^{15}$ (Figure S4.1A). This range is reduced compared to the $2^{20}$ range that the ERCC should span [98]. One possible reason for the reduced range could be due to a loss in sequencing depth after the quality filtering. Diagnostic performance for the ERCC control ratios are recorded assessed through ROC curve analysis and AUC statistics. The resulting analysis showed that there was perfect diagnostic performance (AUC = 1) to determine differential expression at a 2- and 4-fold change, and good diagnostic power at the 1.5-fold change (AUC =0.846), with between 12 – 14 of the ERCC ratio controls detected compared to the 23 that were originally spiked (Figure S4.1B). MA plots show that there is some bias concerning the control ratio measurements due to the disparity between solid and dotted lines (Figure S4.1C). Bias can occur during RNA handling and library preparation, causing an inefficient recovery of the ERCC transcripts for downstream analysis [98]. LODR graphs revealed that the minimum signal needed to call a 4-fold change was 22 gene counts, however there was insufficient information to determine counts below 2-fold (Figure S4.1D) Taken together this analysis revealed that there was a reduction in sequencing depth that likely affected the ability to determine smaller
LODR estimates and caused some a small degree of bias in the ERCC ratios. However, there was perfect diagnostic ability to determine fold changes of 2 and greater, and that fold changes of 1.5 still had good diagnostic performance. The erccdashboard analysis was used to inform differential expression testing parameters, where only gene comparisons that had an FDR value < 0.05 and fold change ≥ 2 were kept.

**Figure S4.1** ERCC ratio technical and diagnostic plots produced through erccdashboard for Atlantic salmon (*S. salar*) that were cultured at 12 and 20 °C in
freshwater for 99 days. (A) Dynamic range of Atlantic salmon with coloured points representing different ERCC ratios and differently shaped points being the two different ratio mixes. Error bars represent the standard deviation of biological replicates (n=24). (B) ROC curves and the corresponding AUC statistics for the ERCC ratios, along with the table for the number of ERCC controls that were used in the analysis (‘detected’) and the total number that were originally included (‘spiked’). (C) MA plot where filled circles indicate the ERCC ratios were above the LODR estimate and error bars for the standard deviation. Endogenous transcript ratios are represented by the grey points. The difference between samples (r_m) of the estimated mRNA fraction with weighted standard errors is provide in the table. Solid lines represent nominal ratios while the dashed lines represent the adjusted ratios informed from the weighted mean and standard error. (D) LODR estimates provides a table with the minimum signal (average counts) and bootstrap confidence intervals needed to determine if a transcript is differentially expressed at a given ERCC ratio and p value.
Characterisation of differential gene expression patterns between fast and slow growing juvenile Atlantic salmon (*S. salar*).

5.1 Introduction

Salmonids represent the single largest commodity in commercial aquaculture by value in 2013 [1]. Extensive research in the ecology, behaviour, physiology and genetics of this family has promoted rapid development of their suitability for intensive aquaculture [2,3]. In particular, Atlantic salmon (*Salmo salar*) are an important commercial aquaculture species for food production around the world.

The development of bimodal growth frequencies in Atlantic salmon has been well documented [4–14]. Under artificial conditions during their first year of growth, juvenile Atlantic salmon can be separated by their position in the frequency distribution of length and mass, forming two distinct groups termed upper and lower mode, or fast and slow growers [12]. Indeed this is not limited to artificial rearing conditions, as wild populations may also develop bimodal growth frequencies [15].

This divergent growth is detectable when smolts reach a minimum length threshold, which coincides with the smoltification window. If the threshold is not reached, then the lower mode fish will remain in the streams and attempt to smolt in the following years, while the upper mode fish will continue their salt water migration. The size thresholds vary between populations, but has been reported from 80 mm to 160 mm [6,12,13,15,16].

It is not clear whether the changes in growth are driven by the morphological and physiological processes that occur during smoltification or if they develop independently. Some work has been done, investigating possible drivers for bimodal
growth [10,12]. One compelling hypothesis is that bimodal growth is a result of precocious maturation in fresh water remaining parr, where energy is diverted from growth and into gonadal development [10,12,13,17]. In this way lower mode fish are able to maintain a level of reproductive fitness in the population [17]. Differential gene expression between immature and mature parr that had significantly different weight, length and condition factor reported a downregulation of housekeeping genes in the brain of mature parr and differential expression of genes involved in the development of the maturing gonad in the testis [17]. While this response has been demonstrated through microarray expression in male gonads between immature and mature parr [17], it hasn’t been widely recognised in the literature for females, even though both sexes show a similar proportion of individuals in the bimodal distribution [10]. Therefore, it is likely that there are further drivers for the development of bimodal length frequencies. Indeed there is some evidence to suggest that stress induced growth suppression through the expression of cortisol in subordinate individuals may account for some of the variability in length frequencies [10]. However social hierarchies through the development of dominant and subordinate individuals do not appear to be a driver for bimodal growth [10]. The mechanisms that underpin the development of bimodal growth are currently unclear and require further investigation [10]. It is well established that temperature has a direct limiting effect on growth performance in teleosts, due to the cost of maintaining an increased basal metabolic rate at higher temperatures [18]. Yet there is no evidence that temperature has any limiting effects under short-term (7 days) thermal stress on either feed intake or survival between the upper and lower mode [19]. Currently no study has demonstrated the influence of elevated temperature on the bimodal growth development in Atlantic salmon.
Transcriptomic studies have previously investigated the differential gene expression between fast and slow growing individuals. Differential gene expression between fast and slow growing turbot (*Scophthalmus maximus*) revealed an upregulation of anaerobic glycolytic pathway and a downregulation of growth hormone receptor 2 in the muscle of fast growing fish [20]. In small and large rainbow trout (*Oncorhynchus mykiss*) examined through an Atlantic salmon microarray, there was an upregulation of insulin-like growth factor binding protein 1, immune and stress related genes and a downregulation of transcripts of genes related to blood and energy production in the small fish [21]. Slower growing rainbow trout had elevated transcripts related to mitochondrial and cytosolic creatine kinase, p53 and TSC2 while the faster growing fish had elevated expression of cytoskeletal gene component, glycogen metabolism cycling and higher PI3K levels [22]. Slow growing zebrafish (*Danio rerio*) upregulated transcripts involved in cell development and organisation, biological regulation and RNA metabolism while fast growers upregulated genes involved in sterol biosynthetic processes, cholesterol metabolic processes, generation of precursor metabolites and energy and in oxidation reduction [23]. Taken together these studies demonstrate that differences in growth within a population are in part affected by genes involved with growth, energy production, cellular development and the immune response.

Several studies have investigated whether cortisol responsiveness can affect growth in fish [24–26]. Studies in which fish have been separated into high and low cortisol responders have revealed conflicting results, including reduced growth in the high responders [27–29] or increased growth [24,25,30]. It appears that the relationship between growth and cortisol responsiveness varies with species, stressors and experimental conditions.
While there are many factors that can affect growth, temperature has been outlined to be the main abiotic factor of influence on Atlantic salmon growth performance. For juvenile Atlantic salmon, the optimal temperature for growth has been reported between 12.8 °C – 20 °C, dependent on the size and stage of smolt development [31,32]. Handeland et al., [31] provides specific temperatures for optimal growth of 12.8 °C for 70 g – 150 g and 14 °C for 150 g – 300 g. In addition, Elliott and Elliott, [32] provide optimal growth estimates across two different Atlantic salmon populations, one from the UK (15.9 °C) and the other from Norway (16.3 °C– 20.0 °C). In contrast, Tromp et al. [33] demonstrated that growth performance measured through body mass and fork length was not significantly different for juvenile Atlantic salmon, cultured at 12 °C, 16 °C and 20 °C. Elliott and Hurley [34] also reported that the growth data from fast and slow growing parr that were individually grown at 5 °C, 10 °C, 13 °C, 15 °C, 18 °C and 20 °C was combined to provide the best model fit, due to the parameter estimates not being significantly different. This indicates that the ideal temperature for the best growth performance is similar between the upper and lower mode. Temperatures below 6 °C are considered to inhibit growth in parr. Elliott and Hurley [4] found little difference in the growth rates of fast and slow-growing fish when maintained at 5 °C. As such, temperature has not been demonstrated to be a driving factor in the development of bimodal growth.

In this study, we used RNA-sequencing to identify differentially expressed transcripts in juvenile Atlantic salmon categorized as fast and slow growers and maintained at two temperatures (12 °C and 20 °C). The liver was targeted due to its involvement in carbohydrate, lipid storage, amino acid and prostaglandin metabolism, somatic growth regulation, immune response and detoxification [35–
Taking into account that temperature did not impact upon the development of divergent growth (bimodality), this study aims to compare similar transcripts that were consistently up or downregulated in fish maintained at both 12 °C and 20 °C. Transcriptome level changes are identified through differentially expressed transcripts between the modal groups at 12 °C and 20 °C. The broader functional significance of these differentially expressed transcripts will be identified utilising the biological processes available through the gene ontology project [39].

5.2 Materials and methods

5.2.1 Experimental design

The full experimental design follows that described in Tromp et al. [33]. Briefly, 1+ juvenile Atlantic salmon were held at 12 °C and 20 °C for 99 days in two identical recirculating aquaculture systems each containing five 1,000 L circular tanks at a stocking density of 34 fish per tank. At the time of sampling, fish were quickly netted and placed in a lethal dose of AQUI-S ® (300 ppm), with stage 4 anaesthesia [40] observed in under 2 minutes. After blood collection and biometrics, the distal portion of the liver was dissected out and immediately flash frozen in liquid nitrogen. Liver samples were later transferred into a -80 °C freezer for long term storage. In total 24 liver samples were used for RNA-Seq analysis. These samples represent fish maintained at two different temperatures (12 °C and 20 °C) after 99 days. Six samples were randomly selected from the five tanks for each temperature and bimodal classification at day 99.

5.2.2 Bimodal length frequencies

As previously demonstrated [33], the development of bimodal length frequencies after 99 days was observed in all treatments groups (Figure 5.1). Fork
length was used for assessing bimodal growth, as it has been used as the standard method to categorise juvenile Atlantic salmon [11,12]. From plotting the distribution of length measurements at all temperatures, it was determined that at a fork length of 240 mm there was a clear size separation between the two distributions, resulting in an upper and lower mode [33]. The bimodal distribution was consistently apparent in both temperatures (Figure 5.1A, B.), with the interval for the two modal groups being at a fork length of 240 mm. As such, fish that had a fork length of 240 mm or below were categorised as lower mode, while fish with a fork length greater than 240 mm were categorised as upper mode. In total there were 48 fish that were assigned to the lower mode and 96 fish in the upper mode. Separating this classification between temperatures, there were 21 and 27 in the lower mode and 56 and 40 in the upper mode for 12 °C, and 20 °C, respectively. From the fish randomly selected for RNA sequencing the average fork length at 20 °C was 263.17 ± 5.13 mm (n=6) and 208.83 ± 5.31mm (n=6) for the upper and lower mode, respectively. The average fork length at 12 °C was 274.17 ± 5.43 mm (n=6) and 208.5 ± 8.43 mm (n=6) for the upper and lower mode, respectively. Further information regarding the classification and growth performance for the upper and lower mode can be found in Tromp et al. [33].
Figure 5.1 Kernel density plots with smoothed lines showing the distribution of fork lengths for Atlantic salmon cultured at 12 °C (A) and 20 °C (B) for 99 days. Atlantic salmon were characterised as either upper (> 240 mm) or lower (≤ 240 mm) mode based on their fork length. Points represent the fork length of individual fish that were selected for sequencing.
5.2.3 RNA Sequencing

RNA samples were extracted, sequenced, filtered, trimmed, aligned and counted following methods previously described in chapter 3. Differential gene expression testing and gene ontology enrichment analysis was applied following the same methodology as in chapter 3.

5.2.4 Differential gene expression

A generalised linear model (GLM) was used to account for differences between individual fish maintained at two temperatures and categorised by modal group. We adopted the classic approach in edgeR to determine differentially expressed genes through pairwise comparisons between the upper and lower mode for each temperature. A design matrix was created following the GLM approach (3.2.3) specifying every group and no intercept, as outlined in the edgeR users guide [41]. Pairwise comparisons were identified within each temperature between the modal groups by specifying the contrast argument of the glmLRT function. The likelihood ratio test was then applied across the two pairwise comparisons to provide a likelihood ratio and p-value for each temperature. Differentially expressed genes were determined by a false discovery rate (FDR) < 0.05 and a log$_2$-fold-change (LFC) $\geq 1$ [42]. By comparing the gene expression profile of the upper mode against the lower mode for both temperatures we will be able to identify potential biomarkers for identifying bimodal growth in Atlantic salmon. Due to the aims of this study, the analysis will only focus on genes that are differentially expressed between the upper mode and the lower mode in both temperatures.

5.3 Results

5.3.1 Sequencing and read mapping
Sequencing of cDNA libraries yielded between 17.9 – 35.6 million reads per sample (Table S5.1). From these raw reads the adaptor sequences and low-quality base reads (phred score < 30) were removed, retaining only those with paired-end counterpart; and we kept between 9.8 – 19.6 million pair end reads (Table S5.1). These reads were then aligned to the Atlantic salmon genome, with an alignment rate between 93.8 – 97.0 %. The Atlantic salmon genome contains 57,783 genes based on the entrez records [43], of these we were able to identify 42,003 gene counts, matched through HTSeq-count. For differential expression analysis, each sample was normalised by counts per million (CPM) to enable comparisons between different sized libraries [44], resulting in sample library sizes ranging from 0 – 57,948 CPM. Genes that weren’t present at a level of 1 CPM in all 6 samples were excluded from the analysis, resulting in 22,248 of the 42,003 genes being used for differential expression analysis. Performance diagnostics from the External RNA Controls Consortium (ERCC) ratio analysis revealed that there was perfect diagnostic performance [45] to detect differential expression at the 2- and 4-fold levels and good diagnostic power at 1.5 fold (Figure S5.1). Yet the results indicate that there was some bias between the ratio control measurements and reduced limit of detection of ratio estimates under the 4-fold levels. Based on these performance estimates of the ERCC spike-ins, the minimum requirements to call a gene differentially expressed was set at a fold change of 2 and an FDR threshold < 0.05.

5.3.2 Differential gene expression

At 12 °C, a pairwise comparison was used to detect differentially expressed genes in the upper mode compared to the lower mode. A total of 2,339 genes were identified to be differential expressed, with 935 genes upregulated and 1,404 genes down regulated (Figure 5.2). At 20 °C, a pairwise comparison was used to compare...
differentially expressed genes in the upper mode compared to the lower mode. A total of 2,147 genes were identified to be differentially expressed, with 883 genes upregulated and 1,264 genes down regulated (Figure 5.2).

Due to the aims of this study we will be focusing only on the genes that are present in both temperatures, 12 °C and 20 °C, when comparing the upper mode to the lower mode (Figure S5.2). In total there are 872 genes that were differentially expressed in the upper mode compared to the lower mode at both 12 °C and 20 °C. There were 288 genes that were upregulated in the upper mode compared to the lower mode in both temperatures (Figure 5.2). Similarly, there were 577 genes that were consistently downregulated across both temperatures (Figure 5.2). Albeit while these genes had a consistent up/downregulated expression profile; it was not always the case that these genes were expressed at the same magnitude. Of the genes that had a similar expression profile between temperatures, 259 were upregulated and 527 were downregulated within 1 LFC across in the upper mode compared to the lower mode.

The majority of differently expressed genes between the upper mode and the lower mode had the same up/downregulation between the 12 °C and 20 °C. However, four genes were upregulated at 12 °C while being downregulated at 20 °C. In addition, three genes were downregulated at 12 °C while being upregulated in 20 °C. This switch in the regulation of the gene between temperatures may indicate an altered state of activity for the gene between the temperatures. In summary, there were more genes that were differentially expressed at 12 °C comparing the upper mode to the lower mode than at 20 °C, with a greater number of genes being downregulated than upregulated (Figure 5.2). There were 51 genes that were classified as uncharacterised when matched to the current Atlantic salmon genome.
The top 10 most differentially expressed genes based on LFC value, are presented in Table 5.1. These genes represent the most highly regulated processes between the upper and lower mode. Of these genes, 8 were downregulated and 2 were upregulated. These genes were involved in vitamin metabolism, glucose and lipid metabolism, growth and development.
Figure 5.2 The number of differentially expressed genes in juvenile Atlantic salmon (*S. salar*) with divergent growth responses comparing the upper mode to the lower mode when cultured at 12 °C and 20 °C. Genes represented in this figure are above a log2-fold-change (LFC) of 1, and have been determined to be significantly expressed (FDR <0.05) by a GLM model in the Bioconductor package edgeR. Positive values represent an increase in relative gene abundance, while negative values represent a decrease.
Table 5.1 Top 10 highest log₂-fold-change co-expressed genes from the liver transcriptome of upper mode compared to lower mode juvenile Atlantic salmon (*S. salar*) maintained at 12 °C and 20 °C. Significant differential expression (FDR < 0.05) was assigned using a pairwise comparisons for the upper and lower mode of growth and determined to be biologically relevant at log₂-fold-change (LFC) ≥ 1. Positive fold change values are considered to be upregulated, while negative fold change values are downregulated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Up-/down-regulation</th>
<th>LFC 12 °C</th>
<th>LFC 20 °C</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular serine/threonine protein kinase</td>
<td>Up</td>
<td>5.21</td>
<td>5.32</td>
<td>FAM20C kinases have been suggested to play a role in biomineralization, lipid homeostasis, wound healing, cell migration and adhesion [46].</td>
</tr>
<tr>
<td>FAM20C-like</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perlwapin-like</td>
<td>Up</td>
<td>4.70</td>
<td>3.88</td>
<td>Present in the nacreous layer of the shell of abalone (<em>Haliotis laevigata</em>), and is involved in the inhibition of calcium carbonate crystal growth [47]. Perlwapins contain three tandem whey acidic protein (WAP) domains [47], which have been linked to similar triple</td>
</tr>
</tbody>
</table>
WAP-domain-containing sequences in Atlantic salmon head kidney, however at current there is little known about the functional role of this gene [48,49].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression</th>
<th>Log2 Fold-down</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol dehydrogenase 7-like</td>
<td>Down</td>
<td>-5.03</td>
<td>Retinoids (vitamin A) are important for normal embryonic development, vision, cellular growth, differentiation and apoptosis [50]. Over expression induces enhanced skeletal muscle and glucose metabolism in transgenic mice [51].</td>
</tr>
<tr>
<td>Vitamin D 25-hydroxylase-like</td>
<td>Down</td>
<td>-5.03</td>
<td>Involved in the conversion of vitamin D into 25-hydroxyvitamin D, which is the main circulating form [52].</td>
</tr>
<tr>
<td>Phospholipase A2 inhibitor 31 kDa subunit-like</td>
<td>Down</td>
<td>-5.18</td>
<td>Phospholipase A2 is involved in the enzymatic hydrolysis of membrane phospholipids resulting in the production of free fatty acids such as arachidonic acid</td>
</tr>
</tbody>
</table>
The inhibition of phospholipase A2 can have an anti-inflammatory response [53].

Complement C1q-like protein 2 Down -5.54 -4.85 This gene has previously been identified in the spleen of miiuy croaker (Miichthys miiuy) as an immune related gene [54].

Hyaluronidase PH-20-like Down -7.55 -6.00 Hyaluronan has been implicated in many biological processes such as fertilization, embryonic development, cell migration and differentiation, wound healing, inflammation, growth and metastasis of tumour cells [55]. Hyaluronidase PH-20 has been identified to be homologous in bee and hornet venom, as well as mammalian spermatozoa [55].

Insulin-like growth factor binding protein 1 paralog A1 Down -8.09 -5.45 The presence of insulin-like growth factor-binding protein-1 has been associated with relative muscle mass
in older women (55 – 85 years), where higher levels are associated with low relative muscle mass [56]. The effects of this protein appear to assist in growth.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Status</th>
<th>Fold Change 1</th>
<th>Fold Change 2</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoenolpyruvate carboxykinase 1 (soluble)</td>
<td>Down</td>
<td>-8.57</td>
<td>-7.79</td>
<td>Phosphoenolpyruvate carboxykinase is involved in the synthesis of glucose in the liver [57].</td>
</tr>
<tr>
<td>Mitochondrial 10-formyltetrahydrofolate dehydrogenase-like</td>
<td>Down</td>
<td>-10.95</td>
<td>-7.78</td>
<td>This gene is involved in folate metabolism where it converts 10-formyltetrahydrofolate (10-fTHF) to tetrahydrofolate and CO2 [58].</td>
</tr>
</tbody>
</table>
5.3.3 Putative function of differentially expressed genes

Of the 872 genes that were found to be differentially expressed, 717 were matched successfully to 3,952 unique GO terms. After enrichment analysis, we maintained 853 of these terms. The terms were divided between biological processes (BP) 592, molecular function (MF) 166 and cellular component (CC) 95. Of the 592 BP that were submitted to the REVIGO web server, 238 terms were kept. The aim of REVIGO is to cluster non-redundant gene ontology terms by finding a representative subset of terms, thus providing a reduced set of terms from an otherwise long unintelligible list. Four clusters were identified, with each cluster containing 49, 5, 139 and 45 biological processes. Based on the gene ontology term with the lowest dispensability value within each cluster, the four representative biological terms for each cluster are nematode larval development (GO:0002119), response to electrical stimulus involved in regulation of muscle adaptation (GO:0014878), reproduction (GO:0000003) and response to cadmium ion (GO:0046686). The top five gene ontology terms that contained the largest sum of differentially expressed genes are presented in Table 5.2.
Table 5.2 Gene ontology (GO) terms enriched in significant differentially expressed genes in upper mode compared against lower mode Atlantic salmon (*S. salar*) maintained at 12 °C and 20 °C for 99 days.

<table>
<thead>
<tr>
<th>GO Term ID</th>
<th>Description</th>
<th>Up</th>
<th>Down</th>
<th>Total DE</th>
<th>Total in term</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0002119</td>
<td>nematode larval development</td>
<td>3</td>
<td>57</td>
<td>60</td>
<td>739</td>
<td>1.18E-07</td>
</tr>
<tr>
<td>GO:0009792</td>
<td>embryo development ending in birth or egg hatching</td>
<td>4</td>
<td>54</td>
<td>58</td>
<td>910</td>
<td>3.21E-04</td>
</tr>
<tr>
<td>GO:0042493</td>
<td>response to drug</td>
<td>17</td>
<td>32</td>
<td>49</td>
<td>691</td>
<td>2.77E-05</td>
</tr>
<tr>
<td>GO:0044281</td>
<td>small molecule metabolic process</td>
<td>14</td>
<td>26</td>
<td>40</td>
<td>657</td>
<td>3.65E-03</td>
</tr>
<tr>
<td>GO:0000003</td>
<td>reproduction</td>
<td>3</td>
<td>36</td>
<td>39</td>
<td>491</td>
<td>3.50E-05</td>
</tr>
</tbody>
</table>
5.4 Discussion

Alternate growth strategies play an important ecological role in a species life history and understanding the mechanisms underlying these processes are crucial for successful commercial culture. The development of bimodal growth, first reported by Simpson and Thorpe, [12] in salmonid populations has lacked recent investigation into the physiological mechanisms that underpin the development of the upper and lower modes. As such we investigated the hepatic transcriptome in juvenile Atlantic salmon that had developed into a bimodal population after 99 days. The results from this study report significant changes in gene expression profiles between fish categorised to the upper or lower mode. Independent of temperature, 872 genes were identified to be differentially expressed in the upper mode when compared to the lower mode. We identified transcripts of genes involved in key biological processes including growth, biomineralisation, immune function, homeostasis, protein degradation and reproduction. This study suggests that these transcripts are important to understand the mechanisms involved in divergent growth in juvenile Atlantic salmon.

Insulin-like growth factor binding protein 1 paralog A1 (Gene ID: 101448057) was significantly down regulated in the upper mode compared to the lower mode. Further five other genes involved in growth were differentially expressed with a lower LFC in the upper mode compared to the lower mode. Insulin-like growth factor 1 (Gene ID:106609475 and 100136517) was significantly upregulated in the upper mode compared with the lower mode. Additionally insulin-like growth factor binding protein 1 (101448057, 100136518 and 101448065) was significantly downregulated in the upper mode compared to
the lower mode. Growth hormone receptor isoform 1 precursor (100136442) was significantly down regulated in the upper mode compared to the lower mode.

Insulin-like growth factor plays an important role in the neuroendocrine regulation of growth in fish [59]. Insulin-like growth factor is primarily secreted in the liver with insulin-like growth factor 1 being the major protein responsible for growth [60]. The action of insulin-like growth factors may also be inhibited or promoted by insulin-like growth factor binding proteins [60–62], however their action is mainly inhibitory [63]. In addition insulin-like growth factor binding protein 1 may also be involved in disrupting glucose homeostasis [63]. Due to the interaction between insulin-like growth factor 1 and insulin-like growth factor binding protein 1, it appears that the slower growing, lower mode fish have reduced growth compared to the upper mode due to an overexpression of insulin-like growth factor binding protein 1 resulting in an inhibition of insulin-like growth factor 1 action. In contrast insulin-like growth factor binding protein 1 (EF432856) was not present in Atlantic salmon that were transitioned from a restricted feeding regime to achieve zero growth, to a satiation feeding regime to achieve fast growth in skeletal muscle, but was found in the liver [64]. Conversely insulin-like growth factor binding protein related protein 1 (EF432866) was significantly down regulated at seven days after a transition to satiation feeding regime in the fast muscle [64]. The downregulation of insulin-like binding protein 1 and upregulation of insulin-like growth factor 1 observed in this study is in part consistent to the downregulation of insulin-like growth factor binding protein related protein 1 and upregulation of insulin-like growth factor 1 reported in Bower et al. [64] in fast growth fish. Insulin-like binding protein 1 has also been reported to be upregulated in the liver of small rainbow trout compared to the
larger fish [21]. The presence of insulin-like growth factor-binding protein-1 has also been associated with relative muscle mass in older women (55 – 85 years), where higher levels are associated with low relative muscle mass [56]. Thus, it appears that the production of this protein may inhibit the development of growth. In this context, downregulation of insulin-like growth factor binding protein 1 in the larger, upper mode fish is consistent with the higher growth rates in the upper mode compared to the lower mode. Where increased levels of insulin-like growth factor in the upper mode were associated with increased growth compared to the lower mode. Taken together, it appears insulin-like growth factor binding protein 1 plays an important role in the development of bimodal growth in Atlantic salmon through its action on the insulin-like growth factor system.

Several genes were identified to be involved in biomineralisation through their interaction with calcium. In this study the gene transcript vitamin D 25-hydroxylase-like (106587531), which is responsible for the conversion of vitamin D into 25-hydroxyvitamin D, into its main circulating form, was downregulated in the upper mode compared to the lower mode [52]. Two other gene transcripts which encode for 1,25-dihydroxyvitamin D(3) 24-hydroxylase mitochondrial-like protein which is involved in vitamin D metabolism through catalysing the conversion of circulating and hormonal form of vitamin D into 24- and 23-hydroxilated products targeted for excretion, were down regulated to a lesser extent in the upper mode independently at 12 °C (106565076) and 20 °C (106583685) [52]. Vitamin D plays a major role in the absorption of calcium in order to promote bone growth [65,66]. Extracellular serine/threonine protein kinase FAM20C-like was upregulated in the upper mode for both temperature pairwise comparisons. FAM20C kinases have been suggested to play a role in
biomineralisation, lipid homeostasis, wound healing, cell migration and adhesion [46]. The upregulation of this gene in the upper mode highlights that the fish have an increased capacity for biomineralisation compared with the lower mode fish. The gene perlwapin-like (106572146) was significantly upregulated in the upper mode compared with the lower mode in both temperatures. This gene has been reported in the nacreous layer of the shell of abalone (*Haliotis laevigata*), and is involved in the inhibition of calcium carbonate crystal growth [47]. Perlwapins contain three tandem whey acidic protein (WAP) domains [47], which have been linked to similar triple WAP-domain-containing sequences in Atlantic salmon head kidney, however at current there is little known about the functional role of this gene [48,49]. Considering the size difference between the upper and lower mode as well as the function of perlwapins in molluscs in shell formation through calcium carbonate crystal growth [48], this gene may have a functional role in modulating calcium carbonate growth in Atlantic salmon. When taken into consideration with the previous gene and it’s function in biomineralisation of bones and teeth, it is likely that deposition of calcium carbonate is impacted between the upper and lower mode [46]. Indeed abnormal vaterite formation, a deformity in the sagittal otoliths which are primary composed of calcium carbonate, have been reported in fast growing farmed Atlantic salmon and may suggest an increase in calcium carbonate metabolism [67]. Alternatively perlwapin-like (106572146) may be involved in the inflammatory response as suggested by Smith [48]. Structurally, the altered state in calcium metabolism between the upper and lower mode may result in reduced skeletal growth of the lower mode fish. Thus divergent of growth within a bimodal population may also reflect the ability of an individual fish to promote skeletal growth.
Several genes involved in the immune response were identified between the upper and lower mode. Retinol dehydrogenase 7-like was significantly downregulated in the upper compared to the lower mode. Retinoids (vitamin A) play an important role in embryonic development, reproduction, postnatal growth differentiation and maintenance of various epithelia, immune responses, and vision, with retinol dehydrogenases acting in retinoid metabolism [68]. Phospholipase A2 inhibitor 31 kDa subunit-like (106574371) was significantly downregulated in the upper compared to the lower mode. Phospholipase A2 is involved in the enzymatic hydrolysis of membrane phospholipids resulting in the production of free fatty acids such as arachidonic acid [53]. The inhibition of phospholipase A2 can have an anti-inflammatory response [53]. The downregulation of phospholipase A2 inhibitors may indicate an inflammatory response in the upper mode compared to the lower mode. Complement C1q-like protein 2 (106563105) was downregulated in the upper compared to the lower mode. This gene has previously been identified in the spleen of miiuy croaker (Miichthys miiuy) as an immune related gene [54]. Further Complement C1r subcomponent precursor (CA061305) has been identified to be upregulated in adult male and female coho salmon (Onchorhynchus kisutch) compared to smolts [69]. Hyaluronidase PH-20-like (106561030) was downregulated in the upper mode compared to the lower mode at both temperatures. Hyaluronan has been implicated in many biological processes such as fertilization, embryonic development, cell migration and differentiation, wound healing, inflammation, growth and metastasis of tumour cells [55]. Hyaluronidase PH-20 has been identified to be homologous in bee and hornet venom, as well as mammalian
spermatozoa [55]. It appears that the fish in the lower mode had a higher
expression in genes relating inflammation or anti-inflammatory processes.

Within the most significantly enriched GO terms, there were five genes
(106608168, 100195853, 106600937, 100380795 and 100196866) which were
significantly downregulated in the upper compared to the lower mode which are
involved in 26S proteasome. A further two genes (100194646 and 106588160)
involved in the 26S proteasome were also identified to be down regulated in the
upper compared to the lower mode. The 26S proteasome is essential for life
processes due to its function as a major cellular protease acting upon misfolded
and malfunctioning proteins [70,71]. Dysregulation of 26 proteasome can lead to
metabolic, oncogenic, neurodegenerative and cardiovascular disorders [70].
Under homeostatic conditions 26S proteasome is central to cell development,
protects against cellular aging and disease [70]. The enrichment of this gene
through the lower mode may indicate that these fish are under some form of
cellular stress, due to the role of 26S proteasomes in regulating the degradation of
ubiquitinated proteins [70].

There were many genes involved in the biological process of reproduction
and development. The majority of these genes were downregulated in the upper
mode. One explanation for the downregulation of genes in the upper mode may
refer back to a reproductive strategy suggested in the lower mode fish whereby
energy is diverted from growth and into gonadal development [10,13,17]. This
would suggest that lower mode juvenile Atlantic salmon have begun to invest
energy into the development of gonads over somatic growth.
5.5 Conclusions

By investigating the transcriptome between fish in a bimodal population we identified several key differences in growth, biomineralisation, expression of immune related genes, protein degradation and genes involved in reproduction and development. It is evident through both the biometric and transcriptomic profiles that fish in the upper mode focus more energy on growth, while fish in the lower mode appear to divert this energy into immune responses and sexual maturation. Further research should investigate the timing at which bimodality begins to develop within a population. The genetic profiling that this study has produced may be used to inform future studies on the transcriptomal response of juvenile Atlantic salmon under bimodal growth strategies.
5.6 References


22. Danzmann RG, Kocmarek AL, Norman JD, Rexroad CE, Palti Y. Transcriptome profiling in fast versus slow-growing rainbow trout across seasonal


33. Tromp JJ, Jones PL, Brown MS, Donald JA, Biro PA, Afonso LOB. Chronic exposure to increased water temperature reveals few impacts on stress physiology and growth responses in juvenile Atlantic salmon. Aquaculture. 2018;


CountsStats


of the miiuy croaker (Miichthys miiuy) by sequencing and bioinformatic analysis of ESTs. Fish Shellfish Immunol. [Internet]. Elsevier Ltd; 2010;29:1099–105. Available from: http://dx.doi.org/10.1016/j.fsi.2010.08.013


62. Fuentes EN, Antonio J, Molina A, Thrandur B. Regulation of skeletal muscle
growth in fish by the growth hormone – Insulin-like growth factor system.

http://link.springer.com/10.1007/s004670000347

http://jeb.biologists.org/cgi/doi/10.1242/jeb.024117


5.7 Supplementary Materials

5.7.1 RNA sequencing summary and spike-in mix ratio

Table S5.1 Summary of raw, processed, mapped reads and alignment for each individually sequenced fish cultured at 12 and 20 °C for 99 days and categorised into the upper mode or lower mode by their position in the length frequency distribution.

<table>
<thead>
<tr>
<th>Mode</th>
<th>Temperature (°C)</th>
<th>Raw reads</th>
<th>Processed reads</th>
<th>Mapped reads</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper mode</td>
<td>20</td>
<td>19636312</td>
<td>11102952</td>
<td>10524782</td>
<td>95.70%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>20</td>
<td>20839486</td>
<td>11930424</td>
<td>11209938</td>
<td>94.90%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>20</td>
<td>28337116</td>
<td>16168588</td>
<td>15277764</td>
<td>95.60%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>20</td>
<td>24773726</td>
<td>14262674</td>
<td>13451082</td>
<td>95.20%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>20</td>
<td>20719760</td>
<td>12022408</td>
<td>11448116</td>
<td>96.10%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>20</td>
<td>28190002</td>
<td>15754012</td>
<td>15108248</td>
<td>97.00%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>12</td>
<td>22372768</td>
<td>12771782</td>
<td>12031390</td>
<td>95.10%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>12</td>
<td>25251376</td>
<td>14148174</td>
<td>13096064</td>
<td>93.80%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>12</td>
<td>26974470</td>
<td>14784388</td>
<td>14015190</td>
<td>96.00%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>12</td>
<td>21150404</td>
<td>11554240</td>
<td>10898184</td>
<td>95.20%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>12</td>
<td>27669388</td>
<td>15465434</td>
<td>14699588</td>
<td>96.30%</td>
</tr>
<tr>
<td>Upper mode</td>
<td>12</td>
<td>17865686</td>
<td>9812662</td>
<td>9286342</td>
<td>95.60%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>20</td>
<td>27538286</td>
<td>15737666</td>
<td>14967042</td>
<td>96.20%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>20</td>
<td>26553458</td>
<td>14786964</td>
<td>14081456</td>
<td>96.30%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>20</td>
<td>20202456</td>
<td>11483360</td>
<td>10916666</td>
<td>96.00%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>20</td>
<td>19554758</td>
<td>11231730</td>
<td>10673252</td>
<td>95.90%</td>
</tr>
<tr>
<td>Mode</td>
<td>Temperature</td>
<td>No.</td>
<td>ERCC ratio</td>
<td>RIN</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-------------</td>
<td>-----</td>
<td>------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Lower mode</td>
<td>20</td>
<td>34934540</td>
<td>19645194</td>
<td>18822830</td>
<td>97.00%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>20</td>
<td>32168224</td>
<td>18361154</td>
<td>17588580</td>
<td>96.90%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>12</td>
<td>23591924</td>
<td>13749540</td>
<td>12825204</td>
<td>94.20%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>12</td>
<td>28992190</td>
<td>15129896</td>
<td>14390334</td>
<td>96.10%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>12</td>
<td>26817454</td>
<td>15471814</td>
<td>14551666</td>
<td>95.00%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>12</td>
<td>20970168</td>
<td>11827720</td>
<td>11160598</td>
<td>95.30%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>12</td>
<td>35558590</td>
<td>18717354</td>
<td>17807922</td>
<td>96.20%</td>
</tr>
<tr>
<td>Lower mode</td>
<td>12</td>
<td>31533122</td>
<td>17210242</td>
<td>16474388</td>
<td>96.90%</td>
</tr>
</tbody>
</table>

Table S5.2 ERCC spike-in control ratio, and RNA integrity number for sequenced from Atlantic salmon (*S. salar*) livers, after exposure to 12 °C and 20 °C and identified into two difference modes for growth.
<table>
<thead>
<tr>
<th>Mode</th>
<th>Value 1</th>
<th>Value 2</th>
<th>Value 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper</td>
<td>12</td>
<td>12</td>
<td>8.6</td>
</tr>
<tr>
<td>Lower</td>
<td>20</td>
<td>13</td>
<td>8.6</td>
</tr>
<tr>
<td>Lower</td>
<td>20</td>
<td>14</td>
<td>7.8</td>
</tr>
<tr>
<td>Lower</td>
<td>20</td>
<td>15</td>
<td>8.9</td>
</tr>
<tr>
<td>Lower</td>
<td>20</td>
<td>16</td>
<td>8.7</td>
</tr>
<tr>
<td>Lower</td>
<td>20</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Lower</td>
<td>20</td>
<td>18</td>
<td>7.8</td>
</tr>
<tr>
<td>Lower</td>
<td>12</td>
<td>19</td>
<td>8.1</td>
</tr>
<tr>
<td>Lower</td>
<td>12</td>
<td>20</td>
<td>7.1</td>
</tr>
<tr>
<td>Lower</td>
<td>12</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>Lower</td>
<td>12</td>
<td>22</td>
<td>7.4</td>
</tr>
<tr>
<td>Lower</td>
<td>12</td>
<td>23</td>
<td>8.3</td>
</tr>
<tr>
<td>Lower</td>
<td>12</td>
<td>24</td>
<td>8.6</td>
</tr>
</tbody>
</table>

5.7.2 Assessment of technical and diagnostic performance

Diagnostic and technical performance of ERCC spike-in ratios was measured utilising the erccdashboard [45] in the R statistical programming language [72]. Data was based on 48 individual libraries created from the distal portion of the liver in Atlantic salmon (S. salar). The dynamic range of ERCC control measurements was determined at ~2^{15} (Figure S5.1A). This range is reduced compared to the 2^{20} range that the ERCC should span [45]. One possible reason for the reduced range could be due to a loss in sequencing depth after the quality filtering. Diagnostic performance for the ERCC control ratios are recorded assessed through ROC curve analysis and AUC statistics. The resulting analysis showed that there was perfect diagnostic performance (AUC = 1) and good
diagnostic performance (0.989) to determine differential expression at a 2- and 4-fold change, respectively, and good diagnostic power at the 1.5-fold change (AUC =0.846), with between 13 – 15 of the ERCC ratio controls detected compared to the 23 that were originally spiked (Figure S5.1B). MA plots show that there is some bias concerning the control ratio measurements due to the disparity between solid and dotted lines (Figure S5.1C). Bias can occur during RNA handling and library preparation, causing an inefficient recovery of the ERCC transcripts for downstream analysis [45]. LODR graphs revealed that there was insufficient information to determine counts below 2- and 4-fold (Figure S5.1D) Taken together this analysis revealed that there was a reduction in sequencing depth that likely affected the ability to determine smaller LODR estimates and caused some a small degree of bias in the ERCC ratios. However, there was perfect diagnostic ability to determine fold changes of 2 and greater, and that fold changes of 1.5 still had good diagnostic performance. The erccdashboard analysis was used to inform differential expression testing parameters, where only gene comparisons that had an FDR value < 0.05 and fold change ≥ 2 were kept.
Figure S5.1 ERCC ratio technical and diagnostic plots produced through erccdashboard for Atlantic salmon (*S. salar*) that were cultured at 12 and 20 °C in freshwater for 99 days. (A) Dynamic range of Atlantic salmon with coloured points representing different ERCC ratios and differently shaped points being the two different ratio mixes. Error bars represent the standard deviation of biological replicates (n=24). (B) ROC curves and the corresponding AUC statistics for the ERCC ratios, along with the table for the number of ERCC controls that were used in the analysis (‘detected’) and the total number that were originally
included (‘spiked’). (C) MA plot where filled circles indicate the ERCC ratios were above the LODR estimate and error bars for the standard deviation. Endogenous transcript ratios are represented by the grey points. The difference between samples \( r_m \) of the estimated mRNA fraction with weighted standard errors is provide in the table. Solid lines represent nominal ratios while the dashed lines represent the adjusted ratios informed from the weighted mean and standard error. (D) LODR estimates provides a table with the minimum signal (average counts) and bootstrap confidence intervals needed to determine if a transcript is differentially expressed at a given ERCC ratio and p value.
Figure S5.2 Heat map of differentially expressed genes between Atlantic salmon categorised into the upper mode (UM) or lower mode (LM) for growth when cultured at 12 °C and 20 °C for 99 days. Moderated log2 counts per million (CPM) was used as input into the heat map for each individual transcript, z-scores were then used to represent differences with blue representing lower expression and red higher expression.
6 Conclusions

The response to thermal stress in Atlantic salmon (*Salmo salar*) has been studied extensively through physiological indicators [1–4] and more recently informed by varied RNA sequencing [5] and proteomic [6] approaches. However, currently there is a lack of information on the physiological processes that occur in fish exposed to chronic stress, and indeed, the overall impact of chronic stress has on fish at elevated temperatures.

In this thesis I investigated the growth and physiological response of juvenile Atlantic salmon maintained at three water temperatures (12 °C, 16 °C and 20 °C) for 99 days (chapter 2). Five times throughout the study we quantified plasma cortisol, glucose and cholesterol levels, and growth. Fish body mass and fork length were not significantly different amongst temperatures after 99 days. Plasma cortisol was significantly elevated at 16 °C when comparing day 8 with 99, while at 12 °C plasma cortisol increased from day 1 to day 8, then returned to initial levels (day 1 and 8) after 99 days. Plasma glucose and cholesterol were not significantly different amongst the temperatures throughout the experiment. In addition, at the end of the experiment we quantified eye darkening, and identified the development of a bimodal growth distribution in all temperatures. This study showed a clear physiological stress response (elevated levels of cortisol) and eye darkening in fish maintained at 16 °C but not at 12 °C or 20 °C, suggesting that some aspects of the physiological responses available to deal with chronic stress are affected by temperature. Fish with a fork length ≤ 240 mm were categorised as lower mode and those with a fork length > 240 mm as the upper mode of growth. Plasma cholesterol was significantly lower in the lower mode group in all three temperatures, but plasma cortisol and glucose levels did not differ between modes. Eye darkening also did not
differ between modes, but increased significantly in the groups exposed to 16 °C and 20 °C when compared with 12 °C at 99 days. The lack of differences in growth or stress response to increased temperatures over a 99-day period in juvenile Atlantic salmon in freshwater provides insight into how important it might be to carry out similar studies not only for longer periods but also at different developmental stages, including the adult phase in saltwater. Temperatures of 20 °C in freshwater do not appear severe enough to significantly reduce growth through mass or length. The identification of bimodality in growth studies should be investigated further to improve population growth modelling and response to stressors where individuals may behave quite differently. Differences in circulating plasma cholesterol may play an important role in the development of bimodal growth frequencies.

In chapter 3, I completed the first RNA sequencing study examining gene expression during over 1, 8 and 99 days in Atlantic salmon under normal rearing temperatures. This work aimed to further our understanding of key biological processes that underpin genetic change under normal culture conditions. In doing so this resource creates an essential step forward into normalising genomic change under chronic stress against temporal change under normal culture conditions. This study has reported for the first time the transcriptome level changes that occur in juvenile Atlantic salmon cultured at 12 °C for a period of 99 days. GO analysis reported a wide range of biological process that had differentially expressed profiles. The processes that had the greatest number of genes present where those involved in the aging process as well as genes involved in mitosis, specifically in DNA replication forks which function during the mitotic S phase. The down regulation of genes during mitosis S phase may indicate the reduction in growth of hepatic cells during the pre-smolt phase. The transcriptome profile presented in this study will
provide a valuable resource for future work as it provides a baseline expression in Atlantic salmon prior to smoltification and could be used to compare physiological events, such as thermal stress.

In chapter 4, I investigated gene expression through RNA sequencing, in juvenile Atlantic salmon maintained at 12 °C and 20 °C for 1, 8 and 99 days. Elevated temperature elicited a greater response after long term exposure to 20 °C compared with 12 °C. Changes in the transcriptome between fish maintained at 12 °C and 20 °C were observed through the upregulation of genes involved in the immune system, heat shock and inflammation response and a downregulation in genes involved in homeostasis, glucose and fatty acid metabolism and cell cycle. Exposure of juvenile Atlantic salmon to 20 °C does not appear to impact upon growth and survival. While there were few impacts on stress physiology and growth responses in juvenile Atlantic salmon (CH2), the transcriptomic profile presented in chapter 4 demonstrates a significant effect of chronic thermal stress. While some of the genes identified in this study may prove useful for the development of biomarkers for chronic thermal stress in fish, candidate genes identified through soft clustering represent a group of genes that have highly conserved expression patterns. We suggest the consideration of these clusters for the future development of biomarkers to chronic thermal stress.

By investigating the transcriptome between fish in a bimodal population (chapter 5) we identified several key differences in growth, biomineralisation, expression of immune related genes, protein degradation and genes involved in reproduction and development. As evident from both the biometric and transcriptomic profiles fish in the upper mode focus more energy on growth while fish in the lower mode appear to divert this energy into immune responses and sexual
maturation. Further research should investigate the timing at which bimodality develops as well as whether reduced growth persists in the lower mode fish or if they are able to compensate their growth at later stages. The transcriptome produced here may be used to inform future studies on the transcriptomal response of juvenile Atlantic salmon under bimodal growth strategies.

The research presented in this thesis outlines some aspects of the physiological and growth response of fish when exposed to chronically elevated water temperatures. While these results indicate that there was a significant stress response present in fish maintained at 16 °C through cortisol and eye darkening measurements, there was no significant reduction in the overall growth response measured at 20 °C. Conversely, the transcriptome level response highlights that for juvenile Atlantic salmon maintained in high water temperature (20 °C) presents a more subtle, but defined response that may over time cause significant dysregulation of homeostatic processes needed to maintain growth. This thesis presents a transcriptome level resource for the advancement of biomarkers into chronic thermal stress and bimodal growth in Atlantic salmon populations. In summary, given that few differences were observed between temperatures through growth and physiological responses, yet differentially expressed genes were identified in the liver transcriptome between 12 °C and 20 °C, it is likely that juvenile Atlantic salmon are sufficiently resilient to the immediate threat posed through increased sea surface temperatures. However the life stage, duration and severity of the stress as well as the involvement of additional stressors all play a crucial role in determining the survival and welfare of fish in sea cage aquaculture, and must be considered alongside the impacts of chromic thermal stress.
Future research should aim to progress the development of biomarkers at the gene and protein level in order to provide suitable and reliable indicators for identification of chronic stress in fish. It is essential to investigate different life stages and longer durations of chronic exposure to increased water temperatures, as this may provide crucial information about the absolute tolerance of Atlantic salmon.
6.1 References


response of pre-harvest Atlantic salmon following exposure to elevated temperature.