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Abstract

Arctic, boreal and temperate latitudes are characterised by conspicuous shifts in photoperiod and temperature, which restricts ecosystem productivity and compels resident taxa to anticipate and coordinate physiological processes to the cyclic variability of environmental conditions. The increase in temperature of high latitude habitats due to climate change is likewise complicit in asserting a progressive phenological incongruence between photoperiod and temperature, the repercussions of which on circadian and seasonal rhythms remain veiled in uncertainty. An organism's capacity to physiologically adjust to a changing environment relies upon the induction of gene expression programmes quantifiable through steady-state mRNA level, which has long served as a proxy for transcription and functional inferences in ecophysiological studies, although it is ultimately determined by the integrated processes of nuclear transcription and nucleocytoplasmic degradation. There is consequently an inadequate appreciation of upstream and downstream regulatory elements that finely control gene expression. Pursuant to these considerations is the molecular regulation of heat shock proteins, which presents a suitable system to evaluate temperature inducible gene expression and the thermal and temporal relations in transcription, steady-state mRNA level and translation upon temperature acclimation and heat shock of ecologically and economically valuable salmonids. The studies comprising this thesis revealed temperature acclimation manifests in the alteration of whole organism upper thermal tolerance and ventricle morphology (**I**), which at the cellular level involved modifications to inducible hsp70 expression during chronic and acute temperature exposure (**II**). Although not directly measured, evidence for post-transcriptional regulation in the acclimation process and hsp70 expression is presented and discussed (**II** & **III**), whilst gaining fundamental insights in the final study (**IV**) assessing the effect of elevated temperature on gene transcription and its temporal relationship with steady-state mRNA level in a thermally sensitive salmonid at the forefront of climate change effects. These studies provide a detailed perspective on the capacity of salmonids to acclimate to novel habitat temperatures, and the potential practical application of molecular level measures that could be beneficial in the management and conservation of vulnerable wild and domesticated fish populations.

Tiivistelmä

Arktisille, boreaalisille sekä lauhkeille leveysasteille määritteleviä ominaisuuksia ovat huomiotaherättävä valojaksoisuus ja lämpötilanvaihtelu, jotka rajoittavat ekosysteemien tuottavuutta ja joiden johdosta alueilla elävien lajien on pystyttävä ennakoimaan syklisiä ympäristötekijöiden vaihteluita ja koordinoimaan fysiologisia prosesseja niiden tahdissa. Ilmastonmuutoksen aiheuttama kohonnut lämpötila korkeilla leveysasteilla sijaitsevissa elinympäristöissä on myös osallinen fotoperiodin ja lämpötilan välillä lisääntyvän fenologisen epäyhteneväisyyden, jonka seuraukset vuorokausi- ja vuodenaikaisrytmeille ovat vielä suurelta osin tuntemattomia. Organismien kapasiteetti säädellä fysiologiaansa muuttuvissa elinympäristöissä perustuu pohjimmiltaan geeniverkostojen ekspressiotasojen säätelyyn. Tasoja voidaan määrittää mittaamalla mRNA:n kokonaispitoisuuksia (engl. steady-state mRNA), joita on pitkään käytetty geenien ekspression mittarina ja funktionaalisten päätelmien tekemisen välineenä ekofysiologisissa tutkimuksissa, vaikkakin kokonais-mRNA-pitoisuuksiin vaikuttavat sekä geenituotteiden transkriptioprosessi tumassa että niiden nukleosytoplasminen degradaatioprosessi. Tästä seuraa sellaisten ylä- ja alavirran säätelyelementtien riittämätön arvostus, jotka hienovaraisesti kontrolloivat geenien ekspressiota. Lämpöshokkiproteiinien molekyylitason säätely on systeemi, jota tutkimalla voidaan arvioida lämpötilan indusoimaa geenien ekspressiota ja lämpötilan ja ajan suhdetta transkriptioon, lämpötila-akklimaation aiheuttamia muutoksia mRNA:n kokonaismäärässä ja translaatiossa sekä lämpöshokkireaktiota ekologisesti ja taloudellisesti arvokkaissa lohikaloissa. Tähän väitöskirjaan sisällytetyt tutkimukset paljastivat lämpötila-akklimaation seurauksena tapahtuvia muutoksia organismin lämmönsietokyvyn ylärajassa ja sydämen kammion morfologiassa (**I**), mikä solutasolla sisälsi hsp70-lämpöshokkigeenin ekspression käynnistymisen muutoksia kroonisen ja akuutin lämpöaltistuksen seurauksena (**II**). Vaikka transkriptionjälkeistä säätelyä ei tässä väitöskirjatyössä suoraan mitattu, esitetään ja keskustellaan myös tuloksista liittyen akklimaatioprosessin aikaiseen transkriptionjälkeiseen säätelyyn ja hsp70:n ekspressioon (**II** & **III**), sekä saavutetaan perustavanlaatuinen käsitys viimeisessä tutkimuksessa (**IV**), jossa arvioidaan kohonneen lämpötilan vaikutusta geenin transkriptioon ja sen ajallista suhdetta kokonais-mRNA-määrään lämpötilaherkällä lohikalalla, joka on ensimmäisten lajien joukossa kohtaamassa ilmastonmuutoksen vaikutukset. Tutkimukset tarjoavat yksityiskohtaisen näkökulman lohikalojen kapasiteettiin akklimoitua uudenlaisiin ympäristön lämpötiloihin, ja potentiaalisen käytännön sovelluksen molekyylitason mittauksille, jotka voivat olla hyödyksi haavoittuvien villien ja domestikoituneiden kalapopulaatioiden hoidossa ja suojelussa.

Original Publications

Whole organism thermal tolerance and tissue remodelling dataset in study **I** is derived from Anttila KA, Lewis M, Prokkola JM, Kanerva M, Seppänen E, Kolari I, Nikinmaa M (2015) Warm acclimation and oxygen depletion induce species-specific responses in salmonids. *Journal of Experimental Biology* **218**: 1471-1477.

The entire *hsp70* mRNA and hsp70 expression data in study **II** is derived from Lewis M, Götting M, Anttila K, Kanerva M, Prokkola JM, Seppänen E, Kolari I, Nikinmaa M (2016) Different relationship between *hsp70* mRNA and hsp70 levels in the heat shock response of two salmonids with dissimilar temperature preference. *Frontiers in Physiology* **7**: 511.

Acronyms and Abbreviations

~P	High energy phosphate bond
3Rs	Replacement, reduction, refinement
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary deoxyribonucleic acid
Cirbp	Cold inducible ribonucleic acid binding protein
Clock	Circadian locomotor output cycles kaput
CTMax / CTMin	Critical thermal maximum / minimum
DNA	Deoxyribonucleic acid
DnaK	Prokaryotic hsp70 orthologue
ECL	Enhanced chemiluminescence
EDTA / TAE	Ethylenediaminetetraacetic acid / tris-acetate EDTA
ELAV	Embryonic lethal abnormal visual system
FBS	Foetal bovine serum
FTO	Fat mass and obesity associated protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HDJ-1	Human DnaJ protein homologue 1 (hsp40)
HR-A / B / C	Heptad repeat domains A / B / C
HRP	Horseradish peroxidase
Hsc	Heat shock cognate
HSE	Heat shock element
HSF-1 / 2	Heat shock factor-1 / 2
Hsbp1	Heat shock binding protein 1

Hsp	Heat shock protein
HuR	Human antigen R
kDa	Kilo Dalton
kM	Michaelis constant
IPCC	Intergovernmental Panel on Climate Change
L:D	Light: dark cycle
LB	Lysogeny broth
lncRNA	Long non-coding ribonucleic acid
m^6A	N^6-methylation of adenosine
mM / μM	millimoles / micromoles per litre
miRNA	Microribonucleic acid
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
mTOR	Mammalian / mechanistic target of rapamycin
NCBI	National Centre for Biotechnology Information
NIH	National Institutes of Health
Nonidet	Non-ionising detergent
OCLTT	Oxygen- and capacity-limited thermal tolerance
PBS	Phosphate buffered saline
PMSF	Phenylmethane sulfonyl fluoride
RCF	Relative centrifugal force
rA/C/GTP	Ribonucleotide adenosine / cytidine / guanosine triphosphate
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
rRNA	Ribosomal ribonucleic acid
Rpb4 / 7	RNA polymerase II subunit B4 / B7
RT-qPCR / PCR	Real time / reverse transcriptase quantitative polymerase chain reaction
RVM	Relative ventricle mass
SAGA	Spt-ada-gcn5 acetyltransferase
SAPK	Stress activated protein kinase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
siRNA	Small interfering ribonucleic acid
TBP	TATA-binding protein
TFIID	Transcription factor II D
UTP	Uridine triphosphate
UTR	Untranslated region
UUILT	Ultimate upper incipient lethal temperature
YTHDF2	YTH N^6-methyladenosine RNA binding protein 2

Thermal Relations in Aquatic Ectotherms

The effect of temperature is comprehensive across all levels of biological organisation, from the molecule to the ecosystem. The cumulative occurrence of baseline biochemical reactions as temperature increases can be ascribed to a rise in the thermal energy of a system and the kinetic energy of molecules contained within. The enhanced random motion of molecules increases the propensity for intermolecular contacts required for reactions to occur, which accounts for only a small fraction of rate processes that increase concurrently with temperature, since reaction rates and physiological processes as defined by the Arrhenius equation (Arrhenius 1889, Peleg *et al.* 2012) and Q_{10} effect (temperature coefficient) (Rao *et al.* 1954), respectively, increase approximately exponentially for every 10°C increment within a biologically relevant temperature range. According to the statistical and physical principles of the Maxwell-Boltzmann probability distribution (Boltzmann 1872, 1877, Brush 2002, Maxwell 1860a, 1860b) and Arrhenius equation (Arrhenius 1889, Peleg *et al.* 2012), the proportion of molecules with sufficiently high kinetic energy and the likelihood of molecular collisions that exceed the activation energy of a reaction increases with the thermal energy of a system. Central to most biochemical reactions are the catalytic function of enzymes which lower the activation energy of highly specific reactions. The conformational organisation and functionality of enzymes and most proteins are typically dynamic and depend on a delicate balance between protein flexibility and stability, aptly described in the induced fit model of enzyme kinetics (Koshland 1958) and in the allosteric regulation of protein function, as depicted in the cooperative binding and dissociation of oxygen by tetrameric haemoglobin (Bohr *et al.* 1904, Perutz *et al.* 1987) and multi-subunit transcription factors to target sequences (Ackers *et al.* 1982, Krell *et al.* 2007, Ptashne *et al.* 1980).

The three-dimensional conformation and flexibility of proteins are conferred by structural characteristics such as dihedral angle rotations of the peptide backbone and steric hinderance, atomic forces responsible for van der Waals and hydrophobic interactions, hydrogen, ionic and covalent bonds. The hydrogen, ionic and van der Waals interactions imparting functionality and flexibility to secondary, tertiary and quaternary conformations of proteins (Dill 1990), as well as to nucleic acid structures, are relatively weak and are easily disrupted by temperature change. A polypeptide chain can adopt many possible conformations and an important driver of native structure formation is the collapse of the newly synthesised polypeptide facilitated by

hydrophobic effects that bury non-polar residues within the protein interior (Bowie 2005, Dill 1990, Hartl *et al.* 2011, Popot & Engelman 2000, Skach 2009), thereby bringing atoms of the polypeptide backbone and hydrophilic amino acid sidechains closer together to form the weak bonds necessary for structure, flexibility and function, whilst marginalising the net free energy required for protein stabilisation, estimated to range between 5-20 kcal mol^{-1}, which equates to a few electrostatic bonds (Dill 1990, Jaenicke 1991, 2000, Somero 1995).

The kinetic energy of molecules within a cell decreases in parallel with thermal energy and stabilises the weak bonds formed between amino acid atoms and the intracellular solvent. As cellular thermal energy increases, the transient interactions between amino acids and solvent constituents increase correspondingly, leading to progressive molecular agitation, destabilisation of labile bonds and eventual protein denaturation (Hochachka & Somero 2002). Elevated temperature disrupts van der Waals interactions, hydrogen and ionic bonds but stabilises hydrophobic effects, whilst the converse transpires at low temperature. The complex effect of temperature on different molecular characteristics, bonds and interactions explains the observed variations in temperature where protein stability is maximal and how low temperature can equally induce protein misfolding (Hochachka & Somero 2002, Somero 1995). As graphically illustrated by a thermal performance curve (Eliason *et al.* 2011, Schulte *et al.* 2011), all organisms and their protein building blocks have evolved to function optimally within a limited, species-specific thermal window (Hochachka & Somero 2002, Somero 1995), deviations from which increases the tendency of proteins to be rendered non-functional by denaturation, thus exposing the hydrophobic residues previously excluded from the hydrous environment, which are biochemically inclined to form maladaptive protein aggregates when exposed to the intracellular milieu. The dire consequences of aberrant protein folding and accumulation of protein aggregates is best exemplified in their implication as aetiological agents in a variety of zoonotic and human diseases, such as neurodegenerative spongiform encephalopathies (Casalone *et al.* 2004, Scott *et al.* 1999, Williams & Young 1980) and the formation of amyloid plaques in Alzheimer's disease, Parkinson's disease and type II diabetes (Conway *et al.* 2000, Masters *et al.* 2010, Oakley *et al.* 2006, Ramirez-Alvarado *et al.* 2000).

The Anthropocene is defined as the modern day epoch whereby human activity is the dominant force altering the environment and climate (Waters *et al.* 2016), causing the accumulation of ~93% of the solar energy retained in the biosphere by greenhouse gases in aquatic systems, and is the principle impetus accelerating the warming of the oceans (Cheng *et al.* 2017, 2019).

Global temperature has increased at an alarming rate over the last century and is anticipated to continue its exponential upsurge over the next century due to unwavering anthropogenic emissions (IPCC 2013, 2014), with the largest temperature increase and most pronounced temperature shifts currently taking place at high latitudes (Belkin 2009, Francis & Skific 2015, Manabe *et al.* 2011, Walsh *et al.* 2011, Wanishsakpong *et al.* 2016). The temperature of aquatic systems from temperate and more recently to polar latitudes can vary substantially at daily and seasonal timescales, and these cyclical fluctuations are further exacerbated by a rapidly changing climate, provoking the reduction in range and viability of marine, lacustrine and fluvial habitats (Deutsch *et al*. 2015, Edlund *et al.* 2017, Isaak *et al.* 2012a, 2012b), and pushing ambient temperature towards the upper thermal limits of molecular stability, where cellular function is progressively impaired and beyond which important macromolecules such as enzymes and other proteins become immediately and irreparably unravelled.

Although there are factions in society that still maintain denial of the existence of climate change (Bain *et al.* 2012) and that the Earth is in fact an oblate spheroid (Pigafetta 1922), they are few and far between, as the vast majority of the global scientific community are in unison in proclaiming this ominous climatic trend is a consequence of human activities and foreshadows catastrophe for biodiversity. Most animals are ectothermic and produce only a modicum of heat from a slower metabolism compared to endotherms, making the thermal energy they derive from the environment absolutely essential for their biological processes. The body temperature of aquatic ectotherms rarely deviates from that of their preferred environment as heat produced through metabolism is rapidly exchanged with the surrounding medium. Unlike the thermally heterogeneous terrestrial environment where ectotherms can thermoregulate behaviourally upon reaching an optimal body temperature (Cowles & Bogert 1944), aquatic environments are more thermally homogeneous and options for suitable refugia gradually decrease with increasing water temperature (Isaak *et al.* 2015). It is therefore critical for ectothermic fish in their everchanging habitats to acclimate to the novel and less favourable conditions, and regain homeostasis to improve chances for growth and ensure long term survival. The capacity of ectotherms to adjust to environmental variability relies upon plasticity of phenotype, facilitated by necessary modifications to the expression of their genes which must occur regardless of prevailing temperature. Since every level of ectotherm biology is influenced by temperature, it stands to reason that different stages of their gene expression would be profoundly affected by a change in environmental temperature.

Wild salmonid populations have been integral in maintaining the structure and balance of ecosystems, positively affecting aquatic and terrestrial chemistry through nutrient deposition, especially those derived from bountiful seas by anadromous confamiliars, and providing much needed nutrition to higher and lower trophic levels in environments comparatively limited in resources. Salmonids have displayed remarkable resilience and ability for colonisation in the face of dramatic climatic upheaval and the opening up of new frontiers, evidenced by their expansive territorial and evolutionary radiation as the northern hemisphere ice sheet retracted poleward at the end of the Pleistocene ~12,000 years ago; as it continues to do in the present day. Historical accounts describe a level of fecundity beneficial to the sustenance, spiritual and cultural identity of indigenous peoples for millennia (Watson 1999) and a vital food source for a rapidly growing global population. Bearing witness to such productivity in nature has unfortunately been relegated to the past due to decimation by overexploitation. However, these bygone observations are also indicative of the species' ability to bounce back from the brink of extinction with appropriate conservation and management measures.

Testament to the salmonids' robustness and significance to mankind is in their amenability to large-scale aquaculture farming. According to the International Salmon Farmers Association (ISFA Report 2018), annual global production generates 2.5 million tonnes of fish, US$ 15.4 billion in revenue, provides 132,600 jobs and 17.5 billion healthy meals rich in protein, vitamins, minerals and essential fatty acids, making the enterprise an irreplaceable cornerstone feeding and supporting communities worldwide through sustainable, energy efficient practices that produce a comparatively smaller carbon footprint and require considerably less space than conventional farming. For salmonids that have yet to be produced for global mass consumption, such as the circumpolar distributed anadromous Arctic charr *Salvelinus alpinus*, mostly confined north of the 10°C July isotherm where water temperature stays below 10°C throughout the year (Jørgensen & Johnsen 2014), opportunities abound for the discerning entrepreneur in an era where the less familiar can fetch a princely profit. As an example, taking advantage of their ability to grow rapidly within a limited timeframe, the warming seas and thawing of polar icecaps may be able to provide suitable higher latitude farming conditions for *S. alpinus* that have been reared in aquaculture, with smoltification induced through photoperiod and temperature manipulations (Randall *et al.* 1998, Sigholt *et al.* 1998, Solbakken *et al.* 1994, Taylor *et al.* 2005) prior to sea transfer. Although significant challenges in addressing environmental concerns remain in reconciling conservation efforts with the

demands of global consumption, what is clear as day is that the present state of affairs require immediate and drastic human intervention.

As species diversity teeters on the precipice of collapse, studies investigating the effects and possible ways to mitigate the effects of acute and chronic exposure to suboptimal temperature has increased awareness of the plight of stenothermal and eurythermal fish across the globe (Logan & Buckley 2015). Salmonids are keystone cold water species valuable economically and are important to terrestrial and aquatic habitats of European and North American marine and freshwater systems, and have a natural range encompassing the longitudinal breadth of the north Atlantic and Pacific Oceans where they are also extensively farmed; a practice that has since been extended to the temperate regions of the southern hemisphere (De Leaniz *et al.* 2010, Morgan *et al.* 2004). The latitudinal geographic boundaries and habitat preferences of salmonids and other fish are determined by their thermal requirements (Clarke 1987, 2003, Pörtner 2002, 2010, Pörtner & Knust 2007, Somero 2005) and as ambient temperature in temperate, boreal and Arctic regions continue to rise and gradually disrupt cellular biochemistry and homeostasis, and constrain their endemic range and ecosystem structure (Jonsson & Jonsson 2009, Kortsch *et al.* 2012), there is an imperative to shed light on the physiological mechanisms that underpin their ability to adapt, whether evolutionarily or non-evolutionarily, to new environmental challenges predicted to occur more frequently with advancing climate change.

Measures of Whole Organism Thermal Tolerance

Research on the temperature tolerance of fish has become increasingly important for evaluating the impact of a rapidly changing climate. The two primary methods developed for quantifying temperature tolerance are the static plunge or Fry method (Fry *et al.* 1942, Fry 1947), which measures the temperature where mortality begins to occur, termed the incipient lethal temperature, and the critical thermal methodology which typically uses the temperature that elicits loss of equilibrium as the measured endpoint (Becker & Genoway 1979, Kilgour & McCauley 1986). To determine the incipient lethal temperature, groups of fish are transferred to one of several experimental aquaria in a series of increasing and / or decreasing temperatures. Each aquarium is held at a constant temperature with one of the test temperatures at the higher and lower range having the upper incipient lethal temperature and lower incipient lethal

temperature, respectively. The incipient lethal temperature method gives the temperature where the mortality of half the individuals of the tested group is observed over time, and the determined incipient lethal temperature is regarded as the boundary between a species' zone of thermal tolerance and zone of thermal resistance (Beitinger & Bennett 2000, Fry *et al.* 1942, Fry 1947). Other studies have also utilised an ultimate incipient lethal temperature protocol (Lyytikäinen *et al.* 1997a) by exposing fish to the highest or lowest incipient lethal temperatures delineating the limits of a species' zone of thermal resistance, where survival time is markedly reduced (Beitinger & Bennett 2000). By contrast, measuring thermal limits using the critical thermal method is a dynamic approach involving the gradual increase or decrease in temperature until a critical point is reached. However, although both procedures yield important information regarding the margins and malleability of a species' temperature tolerance window, the endpoint values can differ because of inherent differences in exposure time and temperature change between the two methods.

The concept of critical thermal tolerance limits was first introduced in field and laboratory studies identifying the breadth of temperatures within which normal activity can still be observed in diurnal and nocturnal reptiles indigenous to the xeric environment of the Sonora desert in the southwest of the United States, where daily summer temperatures can rise to 55°C, winter evening temperatures can drop below 0°C and with frequent circadian temperature shifts of as much as 30°C (Cowles & Bogert 1944). The critical thermal maximum (CTMax) is defined as the temperature where locomotion is drastically affected that the animal becomes incapable of escaping the temperature that will promptly lead to its death; a definition that can also be used to adequately describe the parameter for assessing an organism's critical thermal minimum (CTMin). An important standard of the critical thermal methodology, particularly when considering the principles of bioethics and humane animal experimentation (3Rs: replacement, reduction, refinement) (Russell & Burch 1959), is that the animal can recover from the temperature induced incapacitation. Because prior acclimation (Anttila *et al.* 2015, Beitinger & Bennett 2000) and the rate of temperature change (Elliott & Elliott 1995b, Mora & Maya 2006, Moyano *et al.* 2017) are determining factors in defining the boundaries of a species' thermal tolerance, a constant rate of temperature change is used that is sufficiently fast yet slow enough to accommodate the equilibration of deep tissue temperature to the temperature of the external environment, and an appropriate, non-lethal, observable and repeatable endpoint that allows the investigator to immediately end the temperature trial in

order for the organism to recover, most often through the loss of dorsal-ventral orientation. A faster rate of temperature change may cause a delay in the adjustment of deep body relative to surface body temperature, especially in larger organisms, and it is also possible for partial or re-acclimation to develop from a slower rate of temperature change (Becker & Genoway 1979, Lutterschmidt & Hutchison 1997), leading to an overestimation of thermal tolerance limits in both scenarios. Alternatively, a slow rate of temperature change can result in underestimation of temperature tolerance limits because of prolonged exposure to suboptimal temperature (Peck *et al.* 2014). Despite these potentially confounding variables, very different rates of temperature change have been employed in previous studies, leading to discrepancies in measurements of critical thermal limits and highlights the need for standardisation of the methodology for comparative purposes (Beitinger *et al.* 2000, Lutterschmidt & Hutchison 1997, Mora & Maya 2006, Moyano *et al.* 2017).

The incipient lethal temperature is determined by chronically exposing an organism to a constant suboptimal temperature over a specified period, typically for seven days, whereas a critical temperature is reached through an acute increment or decrement in temperature and the procedure requiring only one to a few hours, depending on the species and prior acclimation. The relationship between temperature and time is therefore the main difference between the incipient lethal and critical thermal procedures, with both variables changing simultaneously in the latter method. In addition, permutations of the two main methods for determining a species' thermal limits have been utilised in previous studies, such as Elliott's hybrid temperature tolerance or chronic lethal method (Baroudy & Elliott 1994, Elliott 1981, 1991), which amalgamates the dynamic temperature change of the critical thermal protocol and constant test temperatures of the incipient lethal method, with either 50% mortality (Baroudy & Elliott 1994, Elliott 1981, 1991, Fangue *et al.* 2006) or time to loss of equilibrium (Galbreath *et al.* 2006) as the measured endpoint. Thus, providing experimental and acclimation conditions are the same, a species' CTMax obtained from temperature trials with a constant linear temperature increase typically overshoots its upper incipient lethal temperature obtained from prolonged exposure to a static temperature (Bennett & Beitinger 1997, Ford & Beitinger 2005). There is a sparsity of studies on CTMin compared to its inverse measurement but Beitinger *et al.* (2000) predicted that a species' CTMin would likely be at a lower temperature than its lower incipient lethal temperature, especially if the gradual decrement in temperature is somewhat free from the constraint of water's freezing point, because of a time lag between the rate of external temperature change and noticeable physiological effects. However,

although the thermal tolerance polygon (Figure 1) of the eurythermal sheepshead minnow *Cyprinodon variegatus* is larger when constructed from the critical thermal (1470°C^2) rather than the incipient lethal method (1380°C^2) (Bennett & Beitinger 1997), the CTMin of this heat tolerant species often found inhabiting shallow ephemeral tidepools is higher than its incipient lethal temperature. Compared to incipient lethal temperature measurements, the critical thermal methodology is becoming more widely used since conducting the assay is simpler, requires fewer animals, provides a faster acquisition of data and is more representative of temperature change that can occur in a natural setting.

Oxygen- and capacity-limited thermal tolerance (OCLTT) states the thermal tolerance limit of an organism is set at the highest level of physiological organisation, with the basal / standard rate of oxygen consumption observed at the optimal temperature, which increases concomitantly with temperature, reducing aerobic scope (Pörtner 2002, 2010, Pörtner & Knust 2007). As a consequence, the escalating metabolic requirement of an organism as temperature increases eventually surpasses the capacity of the organism's physiological makeup to supply sufficient oxygen levels systemically. In addition, a variety of biotic and abiotic factors can influence the thermal tolerance of fish, including evolutionary history (Eliason *et al.* 2011), recent thermal history (Kelly *et al.* 2014) and time of day (Healy & Schulte 2012, Prokkola & Nikinmaa 2018). One of the most important and not completely understood in terms of underlying physiological mechanisms is in acquired thermal tolerance arising from acclimation to a new temperature. This results in the adjustment of the range of temperatures a species can tolerate, which can occur within hours to days, as observed in *Caenorhabditis elegans* (Murray *et al.* 2007, Ohta *et al.* 2014, Okahata *et al.* 2016), but typically requiring weeks in higher eukaryotes with more multicellular and physiological complexity (Sandblom *et al.* 2014). Acclimation to warmer temperatures elevates both the upper and lower limits of temperature tolerance, signifying an increase in the capacity to tolerate higher temperatures and a decrease in tolerance of lower temperatures, whereas acclimation to colder temperatures decreases both upper and lower temperature tolerance limits and represents a loss of tolerance to higher temperatures and a gain of tolerance to lower temperatures (Beitinger *et al.* 2000, Beitinger & Bennett 2000, Bennett & Beitinger 1997, Brett 1952, Fangue *et al.* 2003, Fry *et al.* 1942, Fry 1947, Moyano *et al.* 2017). Thus, in measuring the temperature limits of an organism using either lethal or critical temperature endpoints from a series of acclimation temperatures, a two-dimensional graphic representation demarcated by thermal boundaries can be obtained by constructing a thermal tolerance polygon (Figure 1), from which a full spectrum of species-

specific and physiologically relevant acclimation temperature dependent thermal tolerance ranges can be interpolated. Moreover, whether a species is eurythermal or stenothermal can be determined by calculating the surface area ($°C^2$) of its thermal tolerance polygon, with eurythermal fish having a larger zone of thermal tolerance than stenothermal fish (Baroudy & Elliott 1994, Beitinger & Bennett 2000, Elliott 1981, 1991, 1995).

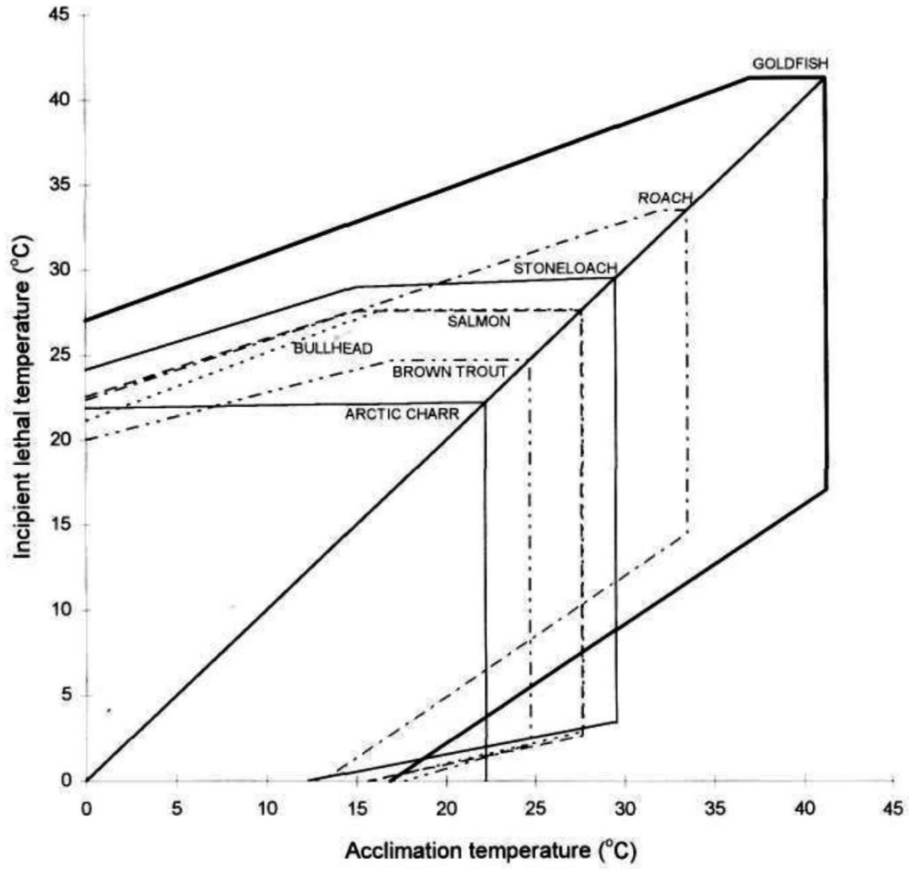

Figure 1 Incipient Lethal Thermal Tolerance Polygons for Arctic charr *Salvelinus alpinus* (Baroudy & Elliott 1995), brown trout *Salmo trutta* (Elliott 1981), Atlantic salmon *Salmo salar* (Elliott 1991), bullhead *Cottus gobio* (Elliott & Elliott 1995a), stone loach *Noemacheilus barbatulus* (Elliott *et al.* 1994), roach *Rutilus rutilus* (Cocking 1959) and goldfish *Carassius auratus* (Fry et al 1942) were compiled by and reprinted with permission from Dr Alex Elliott (Elliott 1995), Centre for Ecology and Hydrology, Natural Environment Research Council UK, and the publisher (The Freshwater Biological Association).

Molecular Regulation of Heat Shock Protein Expression

One of the most discernible features of the cellular response to temperature stress is the rapid synthesis of heat shock proteins (hsps), initially observed as chromosomal puffs in *Drosophila* cells inadvertently incubated at elevated temperature (Ritossa 1962), representing the formation of transcriptional complexes at several active gene loci (Ritossa 1963) and subsequent increase in protein synthesis (Tissieres *et al.* 1973), as an effort to maintain or re-establish cellular proteostasis and to cope with temperatures deleterious to cell survival (Lewis *et al.* 1975). Heat shock proteins are a highly conserved class of proteins grouped into families based on sequence similarity, function and approximate molecular mass (kDa), categorised as low molecular weight hsps, hsp40, hsp60 (chaperonins), hsp70, hsp90 and hsp100, with each family comprising several members that can differ in their expression levels, inducibility and intracellular localisation (Basu *et al.* 2002, Feder & Hofmann 1999, Iwama *et al.* 1998, Lindquist & Craig 1988). Heat shock proteins serve as molecular chaperones by interacting, stabilising and mediating the folding and refolding of proteins into their functional conformation in both stressed and unstressed states (Hartl *et al.* 2011). The cytosolic members of the hsp70 family are the best characterised of the heat shock proteins, consisting of constitutive (heat shock cognate 70, hsc70) and inducible isoforms (hsp70), with the former typically maintained at relatively constant levels in performing housekeeping duties, mainly facilitating the folding of nascent peptides and assisting in protein turnover in cells kept at optimal growth conditions, whereas the latter is induced by abiotic and biotic factors that cause cellular proteins to denature (Basu *et al.* 2002, Feder & Hofmann 1999, Iwama *et al.* 1998, Lindquist & Craig 1988, McKay 1993) . Two inducible *hsp70* paralogues (*hsp70-1* and *hsp70-2*) with similar patterns of induction but contrasting in tissue-specificity and magnitude of expression have been identified in *Fundulus heteroclitus*, *Gasterosteus aculeatus* (Metzger *et al.* 2016) and rainbow trout *Oncorhynchus mykiss* (Ojima *et al.* 2005).

Phylogenetic analysis of *hsp70-1*, *hsp70-2* and *hsc70* from 23 species of teleost fish with genomes available at Ensembl (www.ensembl.org) and NCBI (www.ncbi.nlm.nih.gov) places these genes, suggested to be likely present in all ray-finned fish, into distinct clades (Fangue *et al.* 2006, Metzger *et al.* 2016, Ojima *et al.* 2005, Yamashita *et al.* 2004, 2010). A search of salmonid genome annotations on NCBI identified several putative heat shock protein genes with molecular weights of ~70 kDa. There are at least two *hsc70* genes (chromosomes 4 and

20) and three *hsp70* genes (chromosomes 5 and 9) in *S. salar* (ID: 369), three *hsc70* genes (linkage groups 4, 22 and 23) and eight *hsp70* genes (linkage groups 4, 18, 20, 22, 23 and 25) in *S. alpinus* (ID: 12179) and three *hsc70* genes (chromosomes 10, 12 and 27) and three *hsp70* genes (chromosomes 12 and 13) in *O. mykiss* (ID: 196) (Dr Victoria Pritchard, personal communication). Some of the *hsp70* genes were identified in the same chromosomes or linkage groups, which might indicate synteny of paralogous genes. hsp70 is the most phylogenetically conserved among the highly conserved class of molecular chaperones, having a nucleotide and deduced amino acid sequence homology of approximately 50% between bacterial and mammalian (Hunt & Morimoto 1985, McKay 1993) and 75-85% between mammalian and piscine orthologues (Ojima *et al.* 2005, Yamashita *et al.* 2004, 2010).

Within the primary structure of hsp70, the highest sequence conservation is at the amino-terminal ATP binding domain responsible for providing energy for ATP consuming foldase and degradase reactions, whereas most of the sequence variation is at the carboxy-terminal peptide binding domain (Figure 2), possibly indicating a level of substrate recognition specificity (Pierpaoli *et al.* 1998). The substrate binding domain of hsp70 recognises a short sequence motif estimated to occur every 30 to 40 amino acids in most proteins, composed of four to five hydrophobic amino acid residues, typically leucine, isoleucine, valine, phenylalanine and tyrosine, flanked by regions enriched with basic amino acid residues and exposed to the cellular environment in proteins not in their native conformation (Mayer 2013, Pierpaoli *et al.* 1998, Rüdiger *et al.* 1997). hsc70 is structurally and functionally homologous to hsp70 (e.g. *O. mykiss hsc70* (NM_001124232.1) is 79% identical to *hsp70-1* (NM_001124228.1) and *hsp70-2* (NM_001124745.1) at the nucleotide level), and congruent with the difference in sequence conservation between the N- and C-terminal is a thermostable ATPase activity and a more temperature sensitive peptide binding domain across ecologically relevant temperatures, as revealed in an *in vitro* study of the thermal properties of hsc70 from the eurythermal *Gillichthys mirabilis* (Place & Hofmann 2001). The high level of conservation of hsp70 and its cognates over the course of evolution at the sequence, structural and functional level, and their ubiquity in Archaea, Bacteria and Eukarya, is a robust indication of the vital cytoprotective role they perform in all three domains of life.

Figure 2 Salmonid hsp70-2 (NCBI: NM_001124745.1, NP_001118217.1 and UniProt: Q5KT34) modelled with I-TASSER TM-align structural alignment program (Roy *et al.* 2010, Yang & Zhang 2015) and modified in PyMol (DeLano 2002) to highlight coiled (green), α-helix (red) and β-sheet (yellow) secondary structures. The N-terminal ATP-binding domain forming the globular head on the upper part of the image is composed of β-sheets and α-helices, and the C-terminal substrate binding domain forming the tail-like protrusion at the bottom of the image is composed of three α-helices and a partial α-helix interrupted by coils. The epitope to which the utilised hsp70 antibody (Agrisera, Sweden) in the thesis binds is within the final 24 amino acids of the C-terminal. The three-dimensional model of salmonid hsp70-2 is based on homologous heat shock protein crystallographic structures from *Homo sapiens* (5E84, 4J8F, 3FE1, 3UIC), *Chaetomium thermophilum* (5TKY, 4GNI), *Saccharomyces cerevisiae* (2QXL), *Rattus norvegicus* (4J8F, 2V7Z), *Bos taurus* (1YUW) and *Mycoplasma genitalium* (5OBU) available in the Protein Data Bank (PDB) (www.rcsb.org).

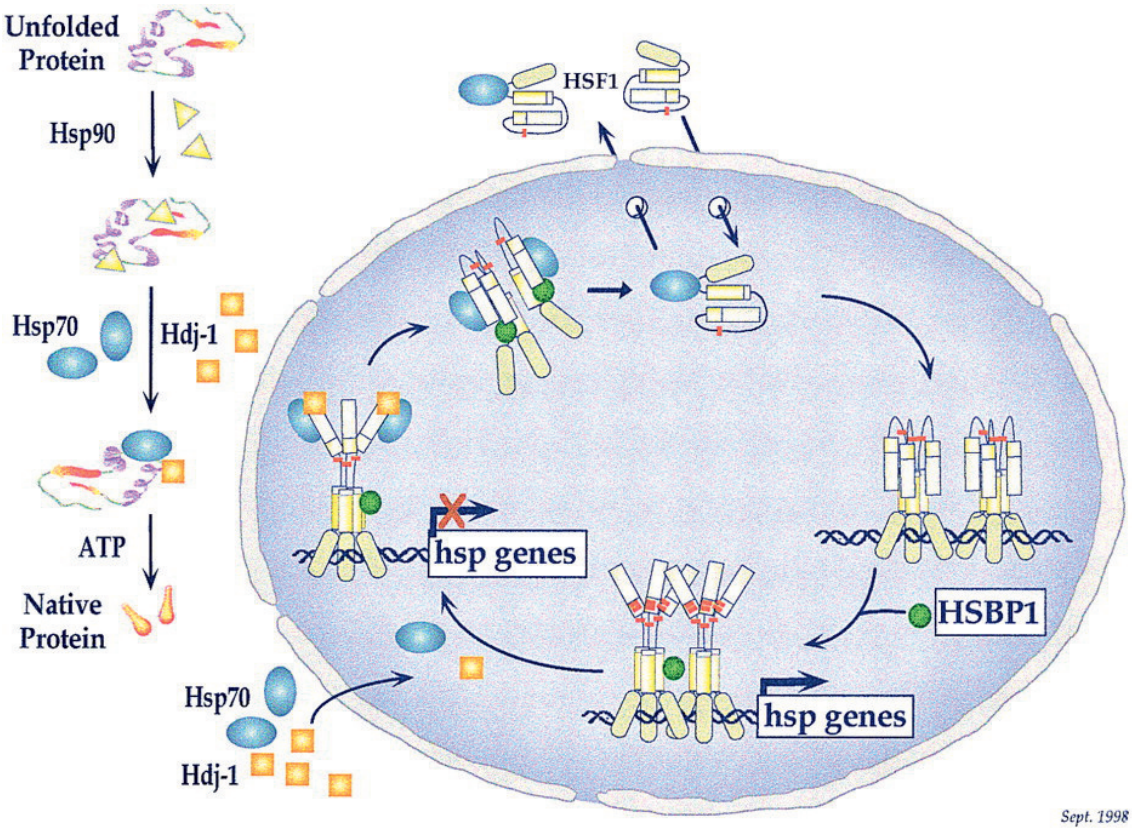

Figure 3 The Cellular Thermometer Model depicting the molecular mechanism by which cells sense thermal stress and upregulate heat shock protein synthesis through transcriptional induction mediated by HSF-1, and the negative regulation of transcriptional activity by standing stock levels of hsp90, hsp70, hsp40 (aka hdj-1) and heat shock binding protein 1 (hsbp1). The schematic diagram was obtained and reprinted with permission from Professor Richard Morimoto (Morimoto 1998), Northwestern University, Illinois USA, and the publisher (copyright by Cold Spring Harbor Laboratory Press, New York USA).

A complete understanding of the molecular processes that underlie the capacity of cells to sense thermal stress and control the expression of heat shock proteins remains to be elucidated, however the general consensus is that transcriptional regulation mediated by a heat inducible transcription factor is the primary mechanism governing the induction of heat shock proteins outlined by the 'cellular thermometer' model (Figure 3) (Ananthan *et al.* 1986, Craig & Gross 1991, DiDomenico *et al.* 1982, Morimoto 1993, 1998). As is the case with hsp70, the principal

transcriptional activator of heat shock protein genes, heat shock factor-1 (HSF-1), is a highly conserved transcription factor with an overall amino acid sequence homology of 60 to 70% between *H. sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus*, *Drosophila*, and *Danio rerio* (Råbergh *et al.* 2000). HSF-1 is composed of an amino-terminal DNA binding domain with a winged helix-turn-helix motif (Harrison *et al.* 1994) which is the most conserved within its primary structure, followed by hydrophobic heptad repeat leucine zipper-like domains HR-A/B required for trimer formation (Peteranderl & Nelson 1992, Peteranderl *et al.* 1999), and HR-C adjacent to the carboxy-terminal transactivation domain functions as a negative regulator via an intramolecular coiled-coil interaction with HR-A/B to impede transcription factor oligomerisation, whereas the regulatory domain between HR-A/B and C inhibits the activity of the transactivation domain (Anckar & Sistonen 2011, Rabindran *et al.* 1993). HSF-1 subunits are kept as inactive monomers in the cytoplasm or nucleus, with hsp70, hsp40 (Abravaya *et al.* 1992, Baler *et al.* 1992, Shi *et al.* 1998), hsp90 (Ali *et al.* 1998, Wang *et al.* 2006, Zou *et al.* 1998) and heat shock binding protein 1 (hsbp1) (Satyal *et al.* 1998) functioning as transcriptional repressors under non-stressed conditions by complexing with HSF-1. It has also been demonstrated in a recent study that HSF-1 *per se* acts as a thermal sensor through a temperature induced conformational change that unfolds the transactivation domain and negates the intramolecular inhibition by HR-C, whilst simultaneously stabilising the intermolecular contacts mediated by HR-A/B (Hentze *et al.* 2016).

The accumulation of misfolded proteins as a consequence of subjection to detrimental temperatures recruits available heat shock proteins, including those bound to HSF-1 monomers, thus releasing HSF-1 from inhibition to form intermolecular coiled-coil trimeric quaternary structures that recognise and bind to a pentanucleotide heat shock element (HSE) motif (5′-nGAAn-3′) found in multiple, contiguous and palindromic iterations upstream of heat shock protein genes (Airaksinen *et al.* 1998, Anckar & Sistonen 2011, Fernandes *et al.* 1994, Wu 1995). Post-translational modifications such as phosphorylation of specific serine residues on the DNA bound trimer provides additional control of HSF-1 transcriptional activity by either repression (Chu *et al.* 1996,1997, Kline & Morimoto 1997, Wang *et al.* 2006) or activation (Holmberg *et al.* 2002), although HSF-1 activation can also be uncoupled from its phosphorylation status (Budzynski *et al.* 2015). Phosphorylation of serine 230 (Holmberg *et al.* 2001), serine 326 (Guettouche *et al.* 2005) and hyperphosphorylation of the regulatory domain between HR-A/B and HR-C (Xia & Voellmy 1997) have been shown to activate the transcriptional machinery for the rapid transcription of heat shock protein genes, followed

closely by the preferential translation of the produced transcripts (DiDomenico *et al.* 1982, Lindquist 1981). Abatement of the stress signal, a decline in denatured proteins and the persistence of elevated heat shock protein levels attenuates the transcriptional activity of HSF-1 via a stoichiometry favouring heat shock protein binding and disaggregation of the HSF-1 trimer into inert monomers. The cellular thermometer model is an elegant portrayal of a cellular negative feedback loop system revolving around key hydrophobic interactions between substrate, chaperone and transcriptional machinery components, functioning as a thermostat to ensure a finely tuned and highly regulated response to denaturing stress, whilst maintaining sufficient heat shock protein levels and appropriately managing the energetic cost associated with heat shock protein expression. The rapid synthesis of heat shock proteins in the heat shock response is a prime example of inducible gene expression regulated by changing environmental parameters and is observed in most organisms as a fundamental strategy for cells to cope with deleterious temperatures. The molecular schematic detailed in this section is the foundation on which most heat shock protein expression analysis is based and is the product of ~60 years of meticulous enquiry on the biochemical properties of heat shock proteins.

Temperature Acclimation and Heat Shock Proteins

One of the most important phenotypic changes that affects the ability of fish to tolerate suboptimal temperature is in their capacity for temperature acclimation, which at the whole organism level manifests in the adjustment of thermal limits to better cope with the new temperature and to buffer the effects of further temperature change that would have been lethal prior to the acclimation (Underwood *et al.* 2012). Temperature acclimation entails substantial physiological modifications, including changes to vascularisation and the remodelling of organs involved in oxygen uptake and transport (Anttila *et al.* 2015, Keen *et al.* 2016, Klaiman *et al.* 2011). Acclimatory mechanisms are likewise observed at the cellular level through the induction of alternative gene expression programmes (Logan & Somero 2010, Vornanen *et al.* 2005, Windisch *et al.* 2014) that result in major alterations to the proteome (Jayasundara *et al.* 2015), changes to mRNA, protein and mitochondrial abundance and activity (Bouchard & Guderley 2003, Dhillon & Schulte 2011, Foster *et al.* 1992, Hardewig *et al.* 1999, Kraffe *et al.* 2007), and the expression of enzymes that modify cell membrane fluidity and stability (Hazel 1984, Tiku *et al.* 1996, Trueman *et al.* 2000), for the purpose of improving whole organism performance under the new conditions (Alderman *et al.* 2012, Schulte *et al.* 2011).

The expression of heat shock proteins has become a frequently used biomarker for assessing an organism's temperature stress levels and its characterisation often conducted by eliciting a strong heat shock response that occurs within minutes of temperature stress (Boehm *et al.* 2003), with a magnitude that is typically proportional to the stress intensity and duration (Buckley & Hofmann 2004) and protein damage (Hofmann & Somero 1995). The function of heat shock proteins in conferring protection or delaying the cytotoxic effects of acute thermal stress is well established, however heat shock proteins also safeguard the cell from chronic exposure to suboptimal temperature where the tendency for aberrant protein folding increases considerably because of a protracted exposure time. In aquatic vertebrates and invertebrates, the threshold induction temperature for enhanced synthesis and the temperature for maximal expression of heat shock proteins is modifiable by temperature acclimation or seasonal acclimatisation (Barua & Heckathorn 2004, Buckley *et al.* 2001, Dietz 1994, Dietz & Somero 1992, Kelly *et al.* 2018, Lund *et al.* 2006, Tomanek & Somero 1999). This indicates that although there are aspects of the heat shock response that appear genetically fixed and heritable in a manner that reflects the genetic and evolutionary history and ecological niche of a species (Dietz & Somero 1993, Heredia-Middleton 2008, Stitt *et al.* 2014, Tomanek & Somero 1999), the regulation of heat shock protein expression also displays plasticity in relation to the individual's recent thermal history.

Associated with acclimation temperature dependent variations in the onset temperature for heat shock protein induction is the altered DNA binding kinetics of HSF-1 (Buckley & Hofmann 2002, Lund *et al.* 2006) and the elevated steady-state level of heat shock proteins and gene transcripts in organisms acclimated to suboptimal temperatures (Buckley *et al.* 2001, Deane & Woo 2005, Lund *et al.* 2006, Oksala *et al.* 2014, Stitt *et al.* 2014, Tomanek & Somero 2002). The inhibition or delay in the initiation of the heat shock response because of changes to HSF-1 activity and elevated heat shock protein level is thus concordant with the cellular thermometer model describing the molecular regulation of heat shock protein expression in both acute and chronic exposure to temperature stress. However, there are also heat shock protein studies in fish that appear not to conform with this consensus paradigm, whereby the threshold induction temperature for heat shock protein synthesis is unaffected by acclimation temperature (Koban *et al.* 1987) or when it is lowered at a higher acclimation temperature (Currie *et al.* 2000), possibly due to differences in the *in vitro* and *in vivo* conditions of the experiments and / or additional complexity in the regulation of heat shock protein expression.

Thermal Relations in Transcription and Translation and Bioenergetic Costs

Gene expression is an intricate sequence of events interspersed with regulatory control points that a cell can utilise to balance an appropriate response to external / internal stimuli and conservation of cellular resources. Transcription is the primary and arguably the most important control step in gene expression as it ensures superfluous intermediates are not synthesised by the cell, illustrated in its extensive regulation requiring the spatiotemporal coordination of a myriad of factors before a gene can be transcribed (Amin *et al.* 1987, Bintu *et al.* 2005, Boehm *et al.* 2003, Dvir *et al.* 1996, Fuda *et al.* 2009, Sims *et al.* 2004). For a finer precision in controlling gene expression, post-transcriptional mechanisms including splicing and processing of pre-mRNA to mRNA, nucleocytoplasmic transport and localisation of mRNA, mRNA stability, translational and post-translational regulation such as peptide folding, subunit assembly, covalent modifications to proteins, protein stability, etc., are also necessary (Schaefke *et al.* 2018). Among the many genes induced in the cellular stress response, the relationship between temperature, transcription and translation has been thoroughly studied and best characterised in the rapid expression of hsp70 in response to heat shock, the preferential transcription and translation of which is believed to reduce competition for RNA polymerases and ribosomes, respectively, whilst inhibiting the synthesis of other proteins that will inevitably misfold and aggravate a worsening situation. Since there is a limited number of heat shock protein genes in a genome, an important control step in the transcription of *hsp70* is by stalling transcriptionally engaged polymerases at the promoter-proximal region of the gene, which can then be rapidly and synchronously reactivated for a robust response to an otherwise lethal stimulus (Rougvie & Lis 1988).

Irreversibly denatured proteins must also be replaced to maintain cell function and to prevent cytotoxic protein aggregates that may cause further harm at a greater cost than the loss and replacement of damaged proteins, and this is achieved through the involvement of heat shock proteins in the ubiquitin-proteasomal degradation (Morozov *et al.* 2017, Qian *et al.* 2006, Valenzuela *et al.* 2018) and chaperone mediated autophagy pathways (Reggiori & Klionsky 2002, Yabu *et al.* 2011). Quantifying the exact bioenergetic costs of temperature stress and increased hsp70 expression can be challenging, however it is evident that the synthesis and ATP consuming function of heat shock proteins superimposes additional energy demands on the cell. Trade-offs associated with gratuitous hsp70 expression on organism physiology can

be far-reaching, as revealed in the arthropod model organism *Drosophila melanogaster* genetically engineered to possess extra copies of *hsp70*, which although providing additional protection from the immediate harm associated with temperature stress, incurs a substantial cost to physiological performance, growth, development, survival to adulthood and reproductive success (Hoekstra & Montooth 2013, Krebs & Feder 1997, Silbermann & Tatar 2000). Moreover, constitutive *hsp70* expression in transfected *Drosophila* cells at a normal growth temperature has been shown to substantially reduce growth rate (Feder *et al.* 1992), while in the piscine model organism *D. rerio* microinjected with an *hsp70-GFP* gene construct under the control of an *hsc70* promoter, extensive apoptosis in embryos and morphological deformities in individuals that developed into adults were observed as a consequence of hsp70 overexpression (Yamashita & Hojo 2004).

At a global perspective, genome-wide gene expression has been revealed to be tightly regulated through an interplay of antagonistic intracellular signalling networks that control resource allocation and utilisation between growth related activities and the cellular stress response, by diverting energy used for biosynthesis and replication towards cell protection and survival during environmental challenge. The coordination and integration of growth, mRNA and protein synthesis and degradation has been scrupulously investigated in the prokaryotic gram-negative bacteria *Escherichia coli* (Esquerre *et al.* 2014) and eukaryotic fungi *S. cerevisiae* (Haimovich *et al.* 2013, Garcia-Martinez 2015) cultured in chemostat bioreactors to accurately control growth conditions, offering indispensable insights that may likewise be applicable to multicellular organisms on their ability to keep in tune with external conditions. The contrasting and preferential expression of growth related and stress related genes is achieved through the transduction of environmental and physiological cues via conserved intracellular signalling networks such as the mechanistic target of rapamycin (mTOR) and stress activated protein kinase (SAPK) pathways, respectively (Bahn *et al.* 2007, Wullschleger *et al.* 2006). These signal transduction pathways provide the means to alternate gene expression between growth and stress responses, by facilitating the formation of the transcriptional machinery on gene promoters through the initial binding of multi-subunit transcription activator complexes with contrasting but overlapping activities. The modification of chromatin to permit access to gene promoters is mediated by Transcription Factor II D (TFIID) and Spt-Ada-Gcn5 acetyltransferase (SAGA), which require the TATA-binding protein (TBP) as a key regulatory subunit. TBP is delivered by SAGA to TATA box motifs present in the core promoters of stress response genes such as inducible *hsp70* (Metzeger *et al.* 2016), but not growth-related genes

(Basehoar *et al.* 2004, Zanton & Pugh 2004). TFIID and SAGA are crucial control components in the genome-wide transcriptional regulation of gene expression, with TFIID being responsible for the expression of ~90% of housekeeping genes, whereas SAGA predominates the expression of ~10% of mostly stress inducible genes (Huisinga & Pugh 2004). This antagonistic management of global transcription provides a highly specific mechanism for launching contrasting gene expression programmes and offers additional comprehension of the *quid pro quo* relationship between growth and stress responses that takes place during adaptation to environmental variability.

Since mRNAs contain more building blocks than proteins, the combined precursor synthesis and polymerization cost estimate for mRNA is deduced to be more than for proteins, with average values of 46 to 49.3 high energy phosphate bonds (~P) nucleotide^{-1} (Lynch & Marinov 2015, Wagner 2005). Amino acids are more diverse in structure and synthesis costs compared to nucleotides, varying from 12 to 74 ~P amino acid^{-1} depending on the amino acid (Akashi & Gojobori 2002) and averaged at 30.3 ~P amino acid^{-1} (Wagner 2005). However, cellular proteins are generally more abundant than mRNAs, with an approximate protein to mRNA ratio of 4800:1 in *S. cerevisiae* (Ghaemmaghami *et al.* 2003) and 2800:1 in mammalian cells (Schwanhäusser *et al.* 2011). A conservative assessment of the energetic costs of gene expression from a compilation of available data conducted by Wagner (2005) in *S. cerevisiae*, for which genomic and a vast number of gene expression resources are available, reveal the relative cellular energy investment for mRNA synthesis to be approximately 6.69 x 10^5 ~P second^{-1}, whereas protein synthesis is one to two orders of magnitude greater, ranging from 6.22 x 10^6 ~P second^{-1} to 1.55 x 10^7 ~P second^{-1} depending on methodological considerations. The former calculated cost is based on a two-dimensional gel electrophoresis average protein half-life estimate (~31.51 h) (Pratt *et al.* 2002), whereas the latter is obtained from ribosomal occupancy estimate of protein synthesis rates based on mRNA bound ribosomal density data (Arava *et al.* 2003). The energetic demands for mRNA and protein synthesis are relevant for prokaryotes and eukaryotes in most cases and are suggested to increase with cell size and multicellularity (Lynch & Marinov 2015).

Although it has long been established that a hierarchy exists in the energy usage of cellular processes, with translation followed by transcription being the most energy consuming in endothermic mammalian (Buttgereit & Brand 1995) and ectothermic piscine cells

(Krumschnabel *et al.* 1997, Mark *et al.* 2005, Wieser & Krumschnabel 2001), these prudent estimations provide a valuable quantitative understanding of the bioenergetic cost of gene expression in the molecular unit of currency for energy transfer of the cell. Based on the above values, the cost of transcription and translation of hsp70 can be accurately estimated. Using NCBI reference nucleotide sequences for *O. mykiss hsp70-1* (NM_001124228.1) and *hsp70-2* (NM_001124745.1), the energy needed to synthesise a 5′-capped and poly-A tailed *hsp70-1* and *hsp70-2* mRNA (excluding intron(s) and other processing costs) is at least 103,000 and 129,000 ATPs, respectively, and these nucleotide sequences correspond to 644 amino acids (*hsp70-1*: NP_001117700 and *hsp70-2*: NP_001118217.1) at a cost of ~19,500 ATPs for every hsp70 protein synthesised. The energy invested in synthesising one mRNA molecule is generally higher than one protein molecule, but because many proteins can be synthesised from one template the total cost of protein synthesis goes well beyond the total cost for mRNA synthesis. The ATP consuming function of hsp70 is more economical however; as established in an *in vitro* study using *E. coli* hsp70 (aka DnaK), one molecular chaperone to unfold a misfolded protein into an intermediate which upon chaperone disassociation would spontaneously refold into the correct conformation, requires five ATPs; a cost that is a thousand times less than the expected cost of protein degradation and re-synthesis (Sharma *et al.* 2010).

There is paucity of available data on the effect of temperature and bioenergetic costs on different control steps of gene expression in ectothermic fish, although some insights can be derived from the studies of extreme stenothermal fish from the Antarctic, which have received much scientific interest (Almroth *et al.* 2015, Hardewig *et al.* 1999, Windisch *et al.* 2011, 2014) due to their increasing susceptibility to perturbations in their previously remarkably stable environments (Turner *et al.* 2005). By inhibiting transcription and translation and measuring changes in cellular respiration rates over a range of temperatures, Mark *et al.* (2005) showed approximately a quarter to a third of oxygen consumption, and thus cellular energy usage, is assigned to RNA / DNA and protein synthesis, which together consume 50-66% of the total energy budget. Basal rates of cellular respiration were observed at temperatures deemed optimal for the different notothenioid and zoarcid species, which increased in parallel with temperature, indicating a higher aerobic metabolism and ATP turnover at suboptimal temperatures. The proportion of allocated energy for each of the two major steps of gene expression remained relatively uniform across temperature treatments despite the higher energetic demands at elevated temperatures, which although suggesting a negligible disruption

of gene expression, provides further evidence for the considerable energetic burden imposed by transcriptional and translational processes in piscine cells, that is amplified by efforts to maintain function at unfavourable temperatures. However, it is less apparent how transcription, translation and the cellular energy budget allocated to these processes change with acclimation.

Gene transcription has rarely been measured directly in ectothermic fish, which must remain active across a range of body temperatures. Salmonids for example maintain physiological function and activity between ~0 to >30°C depending on the species, and there is currently no evidence to provide an indication of how the first step of gene expression is regulated over a temperature range relevant to their physiology and environment. However, some endotherms also undergo similar shifts in body temperature when entering hibernation, since maintaining a constant and elevated body temperature requires an energetically demanding high metabolic rate, which can have negative consequences during periods of limited resources, such as during the depths of winter. To escape the energetic demands of homeothermy, hibernating mammals downregulate activity and metabolism to permit their body temperatures to approach equilibrium with ambient temperature. Using *in vivo* labelling, Bocharova *et al.* (1992) showed that although intracellular transport of nucleotide precursors remained unaffected, mRNA synthesis was markedly depressed in hibernating ground squirrel *Citellus suslicus*. This was later determined in torpid golden-mantled ground squirrel *Spermophilus lateralis* to be due to a reduction in transcription rate and transcript elongation, measured using nuclear run-on transcription (van Breukelen & Martin 2002). The attenuation of transcription initiation and transcript elongation at hibernation body temperatures of 5 to 7°C was reversed by bouts of arousal to transcriptional levels comparable to euthermic conspecifics.

While it is evident that the rate of *in vitro* nascent transcript elongation is influenced by the thermodynamic conditions of the nuclear run-on assay, it is less certain whether the change in the rate of transcription was due to the direct effects of temperature on enzyme kinetics and / or other intrinsic genetic mechanisms. In *C. suslicus*, mRNA synthesis increased within hours of arousal, but did not reach peak levels observed in animals that have been awakened two days prior, therefore a rise in body temperature alone was reasoned not to entirely explain full transcriptional upregulation (Bocharova *et al.* 1992). A similar temperature response was also observed for translation in *S. lateralis*, but with a degree of uncoupling between translation initiation and peptide elongation that suggests the involvement of both passive and active

temperature response mechanisms in translational control (van Breukelen & Martin 2001). Thus, hibernation has provided a suitable physiological system to evaluate temperature relationships in transcription and translation in mammals capable of temporal heterothermy ranging from ~0 to 37°C. However, these investigations were conducted in endotherms exhibiting significant inactivity and metabolic depression at the lower temperature and are physiologically active with a high metabolic rate at the normal body temperature. Since metabolic and thermoregulation strategies have divergently evolved in poikilotherms and hibernating homeotherms, with the former needing to maintain activity across a similar thermal window, inferences made on the thermal relations of transcription and translation in aquatic ectotherms based on comparative observations of torpid and active homeotherms may be somewhat erroneous, and underscores the need for further inquiries on the understudied question of how a change in environmental temperature will affect transcriptional and translational processes in ectothermic fish at the frontline and most vulnerable to the repercussions of a rapidly changing climate.

Molecular Measures in Temperature Acclimation and Rhythmicity of Gene Expression

One of the pertinent topics in the thermal biology of fish is whether an enhanced understanding of physiological and molecular mechanisms underpinning thermal response and tolerance can improve predictions on their ability to acclimate / adapt to climate change (McCullough *et al.* 2009), although the ecophysiological relevance of molecular level responses can be difficult to reconcile. For example, despite the near universal observation of heat shock protein upregulation in thermally stressed organisms, heat shock proteins and their use as a biomarker in fish is still interpreted with caution. Investigators have pointed to inconsistencies between physiological and cellular stress responses, tissue-, ontogeny- and heat shock protein-specific regulation and a cornucopia of stress variables that can confound experimental observations (Iwama *et al.* 2004, Rendell *et al.* 2006). Indeed, stress hormones produced in the primary generalised stress response (Iwama 1998) have been shown to have contrasting effects on the magnitude of heat shock protein expression, with catecholamines enhancing the response (Currie *et al.* 2008, Templeman *et al.* 2014) and glucocorticoids having a dampening effect (Basu *et al.* 2001). Adding to the incongruencies in the physiological regulation of heat shock proteins is the observation that in *O. mykiss* selectively bred to have either a high or low cortisol

response yet have the same tolerance to thermal stress, the induction of the heat shock response was greater for individuals with a high cortisol response (LeBlanc *et al.* 2012). Other confounding factors include the diversity of heat shock proteins that may have similar and divergent roles in short- (Airaksinen *et al.* 1998, Quinn *et al.* 2011a) and long-term (Quinn *et al.* 2011b) responses to thermal stress, whereas some species have completely lost the ability to mount a heat shock response (Hofmann *et al.* 2005). There are also circumstances when acquired thermal tolerance can develop independently from heat shock protein induction and / or when heat shock protein expression is an insensitive biomarker for acute and chronic temperature stress exposure (Lee & Dewey 1988, Lindquist & Craig 1988, Watson *et al.* 1984, Zakhartsev *et al.* 2005), suggesting the involvement of other physiological mechanisms and that alterations to whole organism thermal tolerance is not solely reliant upon the molecular chaperoning activity of heat shock proteins. Moreover, although the expression of heat shock proteins, recent thermal history and whole organism thermal tolerance can exhibit tight correlation (Dalvi *et al.* 2012, Fangue *et al.* 2011), the relationship can differ in the expressed heat shock protein isoforms between tissues and populations (Fangue *et al.* 2006, Healy & Schulte 2012, Stitt *et al.* 2014).

Quantifying gene expression from changes in mRNA level has become more widespread in recent times with the development and improved accessibility of high-throughput sequencing technologies, and although the transcriptome provides a wealth of information on expression pathways that might be integral to the plasticity of physiological responses, it does not provide insights on the fundamental mechanisms that regulate gene expression. As a matter of fact, the measured steady-state mRNA is often described as transcription in literature (e.g. Prokkola *et al.* 2018, Wellband & Heath 2017) despite being ultimately defined by the rate of transcription of a gene and processes involved in determining mRNA stability. The initial step of gene expression, transcription, allows an organism to promptly respond and adapt to variations in its environment and has been suggested to be a more informative parameter for determining functional relatedness and the dynamic regulation of biochemical networks than measuring mRNA level (Hayles *et al.* 2010). Indeed, the direct role of transcription in response to acute temperature stress (Sidaway-Lee *et al.* 2014, Vasquez *et al.* 1993) and in temperature adaptation over evolutionary time (Crawford & Powers 1992) has been documented and exemplifies the significance of transcriptional regulation in two of the three major timeframes to which ectotherms physiologically respond. Transcription may be the most important control point in the expression of many genes, however it is also one of the most complex and tightly

regulated stages of gene expression. It is therefore a rational deduction to make that the complexity of transcriptional regulation would be highly sensitive to temperature change and imposes an energetic burden on the cell that is compounded at suboptimal temperature, and since metabolic demands increase with temperature, it is not entirely clear whether transcription is the defining control point in the molecular adjustments that typifies acclimation to a new temperature.

Transcription is quantified using the nuclear run-on transcription assay, which measures the density of actively transcribing polymerases bound to a gene. Previous nuclear run-on protocols were typically qualitative or semi-quantitative and laborious, requiring considerably more preparatory time, processing steps and the use of a radioactive label, which might explain the limited use of the nuclear run-on assay compared to measuring steady-state mRNA level in gene expression studies. More recently, the development of biotin-streptavidin systems has become indispensable in a wide range of biochemical applications, including the nuclear run-on method (Patrone *et al.* 2000), which when coupled with the amplification power of polymerase chain reaction, reduces the complexity and increases the sensitivity of transcriptional induction quantification. The most commonly used assay temperature for measuring transcription is 30°C (Sambrook & Russell 2001), even with *S. salar* (Bemanian *et al.* 2004) and *O. mykiss* (Sadar *et al.* 1996); a temperature virtually never experienced by these temperate fish. To date only a single study has conducted transcription rate measurements at a range of assay temperatures (van Breukelen & Martin 2002), which showed temperature effects on transcription that reflect metabolic adjustments associated with torpid and active physiological states of a temporally heterothermic homeotherm. Although previous studies on *F. heteroclitus* and *O. mykiss* have measured transcription at 20 (Crawford & Powers 1992, Götting & Nikinmaa 2017a, Levine & Oris 1999) and 26°C (Flouriot *et al.* 1996, Pakdel *et al.* 1997), these studies have not addressed whether the assay temperature used for measuring transcription *in vitro* has a significant effect on the stability of engaged polymerases and transcript elongation in species with different thermal requirements.

The Arctic, boreal and temperate environments of salmonids are characterised by large fluctuations in daily and seasonal irradiance, with constant light above the Arctic circle in the summer and no sunlight during winter. From 66°N downwards, annual changes in photoperiod are less extreme but with only 1 to 8 hours of light during the depths of winter and the same

amount of darkness at the height of summer. The limitations imposed by such pronounced environmental variations on ecosystem productivity compels organisms to anticipate and coordinate their biological activities to the periodicity of the light-dark cycle. This is epitomised in Arctic Ocean trophic interactions at 70°N, where diel vertical migration of Arctic cod *Boreogadus saida* and its zooplankton prey influences the predatory activity of ringed seals (Benoit *et al.* 2010). Along the same latitude, anadromous *S. alpinus* overwintering in ice covered lakes accurately keep track of the duration of surface level illumination throughout the year based on their melatonin profiles (Strand *et al.* 2008). The circadian and circannual predictability of photoperiod makes it the primary cue by which behaviour, physiology and life history events of salmonids are synchronised to the environment, as demonstrated in phase-shifts in the timing of reproduction of *O. mykiss* adjusted by exposure to continuous light at different times of the year (Randall *et al.* 1998). The duration of the light cycle has been shown to be a major influence on *O. mykiss* growth rate, however correlations between water temperature, growth and growth-related hormone levels were also noted (Taylor *et al.* 2005). Photoperiod is evidently the most predictable and important entrainment signal of rhythmic biological processes, but for ectothermic fish ambient temperature also calibrates physiological responses that arise with changes to environmental conditions. This is displayed in the growth and development of *S. salar* smolt characteristics, accelerated by the combination of elevated temperature and increasing photoperiod influencing the timing of their seaward migration (Sigholt *et al.* 1998, Solbakken *et al.* 1994), whereas low temperature has an opposing effect (McCormick *et al.* 2000).

An increase in photoperiod is usually accompanied by an increase in temperature and this photoperiod-temperature relationship is expected to increase in severity in temperate and boreal regions, and upend the comparatively stable seasonal temperatures of polar latitudes because of climate change. Milder winters, warmer summers, anomalous weather and prolonged elevation of autumn temperatures and early warming of spring temperatures are expected to occur more frequently, thereby creating a disparity in the timing of photoperiod and temperature shifts, with likely deleterious outcomes on the seasonality of high latitude fish that use photoperiod and temperature as proximate environmental cues (Prokkola & Nikinmaa 2018). For instance, in studies of anadromous *S. alpinus* in northern Norway (70°N), early elevation of spring temperatures although accelerating growth (Bottengård & Jorgensen 2008), impaired development of hypoosmoregulatory competence required for transitioning to marine

life (Aas-Hansen *et al.* 2003). It has been proposed that the significance of temperature as an entrainment signal decreases with increasing latitude (Pankhurst & Porter 2003) and this might be especially true for *S. alpinus*, whose ecological niche and geographic boundaries are defined by the rather limited temperature breadth it can tolerate compared to other salmonids (Anttila *et al.* 2015, Elliott & Elliott 2010). For this reason, the pronounced warming of the climate at high latitudes in the last two decades has led to the recent decline of *S. alpinus* abundance (Svenning *et al.* 2016), and the expected trajectory of predicted temperatures places this stenothermal salmonid more than any member of the Salmonidae family at the forefront of climatic effects that threaten their survival, therefore an improved understanding of their ability to adapt to future environmental conditions is highly warranted.

The significance of rhythmic gene expression in coordinating physiological responses to the cyclical environment has been gradually brought to light in the last few decades. Studies in ectothermic and endothermic organisms have revealed a significant proportion of the transcriptome mediating transcription and metabolism fluctuate in a circadian and tissue-specific manner concurrently with gene transcripts encoding the *clock* transcription factor (Amaral & Johnston 2012, McCarthy *et al.* 2007, Miller *et al.* 2006). Mutation of *clock* has been shown to drastically alter the expression of many rhythmic and non-rhythmic transcripts in the model organism *M. musculus* (McCarthy *et al.* 2007, Miller *et al.* 2006) and is testimony to the importance of keeping mRNA expression in time with changing habitat conditions to organism fitness. In non-model ectotherms such as temperate fish which inhabit highly changeable thermal and photoperiodic environments, overt circadian expression of core clock and metabolism transcription factors and enzymes have been characterised in nervous and peripheral tissues at the mRNA (Betancor *et al.* 2014, Huang *et al.* 2010a, 2010b) and enzyme level (Gomez-Milan & Sanchez-Muros Lozano 2007), further validating that the coupling of transcriptional and metabolic processes to environmental cues is a fundamental necessity for species survival and fecundity. In high latitude fish, direct evidence for the synchronisation of gene transcription to the most predictable environmental signal is in the suggested transcriptional control of myosin gene expression by the endogenous clock transcription-translation feedback network in Atlantic cod *Gadus morhua*, which possess clock related transcription factor binding sites at putative promoters of myosin genes that display mRNA rhythmicity (Lazado *et al.* 2014). The protein products of myosin genes in fish similarly exhibit photic plasticity and are altered in composition during temperature acclimation to enhance

swimming performance at the new temperature (Cole & Johnston 2001, Nagasawa *et al.* 2012, Watabe *et al.* 1994, 2002).

Transcription is a key control point in the expression of genes and it is an important element in the timing, entrainment and precision of the self-sustaining endogenous clock (Khalsa *et al.* 1996, Lahiri *et al.* 2005), however biological rhythms can also persist independently of transcription (Dibner *et al.* 2009, O'Neill *et al.* 2011, O'Neill & Reddy 2011). Many studies describing circadian gene expression at the mRNA level have only indirectly measured and often infer transcriptional induction, despite evidence that the transcriptional and mRNA rhythms of a gene can differ (Le Martelot *et al.* 2012), thus there is uncertainty as to whether transcription is the primary contributing factor in observable mRNA oscillations, since it is equally probable that the rate of mRNA degradation plays a prominent role in determining mRNA level, particularly during stress and / or resource limitations. Studies on model unicellular organisms have revealed mRNA stability to be an important post-transcriptional mechanism regulating global gene expression (Esquerre *et al.* 2014, Garcia-Martinez *et al.* 2015), whereas evidence for the contribution of mRNA stability in controlling steady-state mRNA level in non-model organisms is less extensive and typically limited to a few genes, as demonstrated in the increase in mRNA stability upon hormonal stimulation of *O. mykiss* cells (Flouriot *et al.* 1996, Götting & Nikinmaa 2017a) and in the acclimation of *C. auratus* to suboptimal temperature (Bremer & Moyes 2014).

mRNA expression and rhythmicity can be regulated post-transcriptionally by various cis- and trans-acting elements that control transcript stability, the latter of which include ~30 families of ribonucleases (Mitchell & Tollervey 2000, Schaefke *et al.* 2018) that require successive cycles of ATP binding and hydrolysis to unwind and degrade RNA (Hossain *et al.* 2016), and while the ATP cost of proteasomal degradation has been empirically determined to be 60 $ATPs^{-min}$ at basal level and ~50 to 160 ATPs depending on the ubiquitin-protein conjugate (Peth *et al.* 2013), the exact bioenergetic cost of mRNA degradation remains elusive. However, increasing mRNA stability whether dependently or independently from gene transcription, is intuitively a cost-effective strategy as the ATP invested in the synthesis of pre-existing transcripts is maximised by inhibiting ATP consuming degradative pathways. Increasing gene transcription alone would compound the bioenergetic cost of transcription with mRNA degradation, whereas a concomitant increase in transcript stability would require less *de novo* transcript synthesis to attain the required steady-state mRNA level. qPCR, microarray and

RNA-Seq methodologies have provided important information on the possible physiological mechanisms and pathways that permit an organism to interact with and adapt to its environment, and although measuring steady-state mRNA level is still the gold standard for evaluating gene expression, it does not provide insights on the involvement of different control steps that regulate transcript level. Transcriptional regulation of functionally important genes is crucial to the adaptation of fish to environments with different temperature profiles (Crawford and Powers 1992) and provides the means to promptly respond to toxicant exposure (Bemanian *et al.* 2004, Levine & Oris 1999, Sadar *et al.* 1996), glucose (Ehrman *et al.* 2004) and hormonal stimulation (Bemanian *et al.* 2004, Flouriot *et al.* 1996, Flouriot *et al.* 1998, Götting & Nikinmaa 2017a, Nelson & Sheridan 2006, Pakdel *et al.* 1997), making the first step of gene expression a principal determinant in immediate responses to environmental perturbations, physiological regulation and in temperature adaptation over evolutionary time.

Thesis Objectives

Assessing how close species are to their thermal limits and their capacity to acclimate to novel thermal conditions are essential considerations for understanding the consequences of increasing habitat temperature on aquatic ectotherm biology. Temperature acclimation is an important adaptive response serving as a reliable gauge of a species' susceptibility to contemporary and future conditions. However, the mechanistic intricacies of physiological processes that confer phenotypic plasticity and underlie temperature acclimation, and how they may be disrupted by elevated temperature and the developing discrepancy in the photoperiod-temperature relationship at polar to temperate latitudes, remain to be fully elucidated. Given these particulars, heat shock proteins as a thermal stress biomarker present a suitable system to evaluate temperature inducible gene expression and the thermal and temporal relations in transcription, steady-state mRNA level and translation. The primary objective of this thesis is to provide additional insights that can improve awareness of the relationship of aquatic ectotherms with their increasingly thermally altered environment, by investigating upper thermal tolerance limits, the effects of acute and chronic temperature exposure on key regulatory stages of gene expression and their rhythms, in three species from the Salmonidae family with different thermal optima but overlapping thermal breadths and geographic distribution. The thesis is composed of four separate experiments in a top-down arrangement to examine physiological responses to low and elevated temperatures at multiple levels of

biological organisation, from the whole organism to the tissue and molecular level, within the remit of the following summarised objectives:

I) Determine the upper critical thermal limits of a stenothermal and eurythermal salmonid acclimated to 8 and 15°C, and how the morphological characteristics of their oxygen supply and delivery system may be modified by temperature acclimation.

II) Evaluate the extent of thermal stress experienced by a stenothermal and eurythermal salmonid acclimated to 8 and 15°C through their *hsp70* mRNA and hsp70 steady-state circadian profiles, and to expound upon the transcriptional and translational relationship of hsp70 expression as a function of time upon mild but environmentally relevant acute temperature increase.

III) Assess the effects of acclimation to optimal and suboptimal temperatures on gene transcription and its correlation with steady-state mRNA level in a eurythermal salmonid cell line.

IV) Quantify the circadian rhythmicity of transcription and steady-state mRNA level in a stenothermal salmonid acclimated to 8 and 15°C.

Study	Experimental Design	Species	Biological Organisation	Tissue
I	CTMax and histological measurements of upper thermal tolerance and tissue remodelling, respectively, after four weeks of *in vivo* acclimation.	Arctic charr and Atlantic salmon	Whole organism to tissue	Gills and heart
II	Circadian expression quantification of *hsp70* mRNA and protein level after four weeks of *in vivo* acclimation and heat shock.	Arctic charr and Atlantic salmon	Tissue to cellular	Gills and liver
III	Transcriptional and mRNA level determinations after two weeks of *in vitro* acclimation of a commercially available cell line.	Rainbow trout	Cellular to nuclear	Gill epithelial cells
IV	Diel transcriptional induction and mRNA expression quantification after four weeks of *in vivo* acclimation.	Arctic charr	Cellular to nuclear	Erythrocytes

Table 1 Precis of Experiments in sequence and denoted with Roman numerals throughout the thesis.

Material and Methods

Temperature Acclimation Experimental Design (**I** & **II**)

Experiments were conducted at the Natural Resources Institute Finland in Enonkoski, south eastern Finland, from 1st of July to 10th of August 2013. All procedures were approved by the Finnish Animal Experiment Board (ESAVI/4068/04.10.07/2013). *S. alpinus* and *S. salar* originated from lake Saimaa (62°04′ N; 28°33′ E) and were reared under a natural photoperiod (the photoperiod was ~17:7 L:D during sampling) at the Natural Resources Institute Finland hatchery for one and three generations, respectively. Juvenile (~1-year-old) *S. alpinus* and *S. salar* were kept separately in 320 L cylindrical (90 cm diameter) tanks with constantly flowing, filtered, aerated and temperature controlled water from Lake Pahkajärvi. Fish were fed commercial fish pellets (Raisio Group, Finland) *ad libitum* and both species were acclimated to 8 and 15°C for four weeks, a period considered to be adequate for acclimation to take place and is also close to the longest period of time that water temperature can be expected to remain constant in nature. Water temperature and oxygen concentration measurements were performed daily and at different times of the day to ensure constancy. Feeding was ceased 24

h prior to the CTMax and heat shock experiments, then fish were sacrificed in 200 ppm tricaine methanesulfonate (MS-222, Sigma-Aldrich USA) buffered with sodium bicarbonate.

Whole Organism Thermal Tolerance and Tissue Level Measurements (**I**)

The CTMax protocol was conducted according to Fangue *et al.* (2006) between 1000 to 1300 h to obviate any possible circadian variations in upper thermal tolerance limits (Healy & Schulte 2012), and fish of comparable length and size were used since size can affect thermal tolerance. Fifteen fish per species and acclimation group were transferred from the acclimation tank into an experimental tank (100 L) with the same water temperature as the corresponding acclimation temperature. Fish were provided 1 h to acclimate to the conditions of the experimental tank, then the water temperature was increased at a constant rate of 0.3°C min^{-1} up to 24°C and 0.1°C min^{-1} thereafter until loss of equilibrium. The former rate of temperature change is the suggested standard for the methodology and often used for measuring the thermal tolerance of eurythermal species with a wider thermal breadth than cold water adapted salmonids, hence the use of the latter and slower rate of change as the test temperature approached the upper limits for the species, so as not to overshoot critical thermal endpoints and to ensure individual recovery. A circulating 2500 W heater (RC6 Lauda, Lauda-Königshofen, Germany) and two submersible aquarium heaters (Theo 100 W, Hydor, Bassano del Grappa (VI), Italy) were used to control water temperature. Aquarium pumps were used to circulate water and prevent thermal stratification, and oxygen saturation was maintained above 80% using submersible air pumps. Fish were immediately removed from the experimental tank upon loss of dorsal-ventral orientation and placed in individual recovery tanks (10 L) at the acclimation temperature.

Gill and ventricle histological procedure and measurements were conducted by Anttila *et al.* (2015) and reanalysed statistically to determine acclimation induced tissue remodelling in a subset of fish. In brief, ten additional fish per species and acclimation group that have not undergone CTMax trials were sacrificed to acquire gill tissue unaltered by the acute temperature increase of the CTMax protocol. Ventricles were excised from CTMax fish then weighed and divided along a mid-sagittal plane. Tissues were fixed in 4% formalin in PBS and subsequently dehydrated in an ethanol and UltraClear (Mallinckrodt Baker Inc, PA USA) series of increasing ethanol concentrations, followed by embedding in paraffin wax and serial sectioning (5 µm) with a microtome. Sections were mounted on glass slides, dewaxed with

UltraClear and rehydrated with an alcohol and distilled water series. Ventricle sections were stained with amylase-periodic acid-Schiff whereas cross sections of secondary lamellae were stained with Mayer's haemotoxylin (RAL Diagnostics, Martillac France), then rinsed and dehydrated with an ethanol and UltraClear series. Microscopy was performed to obtain relative compact myocardium thickness, measured by dividing compact myocardium thickness by the cross sectional area of the ventricle, and capillary density was determined by quantifying the number of capillaries within a ~4000 μm^2 surface area then divided by four to obtain capillary density per 1000 μm^2 of compact layer. Relative ventricle mass was quantified by dividing ventricle mass by fish mass and relative secondary lamellar height by dividing lamellar height by fish mass.

Inducible hsp70 Rhythms in Steady-State and Heat Shock (**II**)

One hundred fish from each species and acclimation temperature were used for the experiment, with 40 fish for steady-state level measurements and 60 fish for evaluating the heat shock response. Gills and liver were chosen since the former is in immediate contact with the external environment whilst the latter is metabolically active. Fish mass and length were obtained prior to tissue excision and flash freezing in liquid nitrogen at 1, 8, 16 h after the start of the light cycle and at 21 h, 4 h into the dark cycle. The remaining 60 fish were subjected to a non-lethal +7°C heat shock for 30 min at the start of the light period and organs were excised 1, 2, 4, 8, 16 and 24 h post-heat shock. Steady-state and heat shock induced *hsp70* mRNA expression levels were quantified using salmonid-specific RT-qPCR primers that recognise both inducible *hsp70-1* and *hsp70-2* isoforms, the details of which are described in the succeeding section. To quantify hsp70 levels, frozen tissues were weighed and homogenised in 5 volumes of lysis buffer (62.5 mM Tris-HCl, 1µg/ml leupeptin, pepstatin, antipain and 1mM PMSF) using a TissueLyser (Qiagen, USA) at 30 shakes / sec for 2 min. Lysates were kept on ice for 30 min prior to +4°C centrifugation at 10000 RCF for 30 min and supernatant storage at -80°C. Protein concentrations were determined using the Bradford method (Bradford 1976) and a protein assay dye reagent (Bio-Rad, Germany), with a serial dilution of bovine serum albumin (1 mg / ml) as a standard.

Spectrophotometric measurements were performed at 595 nm using a Wallac EnVision 2103 Multilabel Reader (PerkinElmer, Finland). An equal amount (20 µg) of protein per sample was mixed with 5X Laemmli buffer (Laemmli, 1970) and denatured for 5 min at 95°C, then loaded

onto an SDS-PAGE gel comprised of 10% polyacrylamide. Gels were placed in a Mini-Protean 3 electrophoresis module (Bio-Rad, USA) and the proteins separated by size, first at 100 V for 30 min then 150 V for 1 h. Proteins were transferred onto a nitrocellulose membrane (Perkin Elmer, USA) at 100 V for 1 h at +4°C and incubated in PBS blocking solution containing 3% non-fat powdered milk and 0.3% Tween for 1 h. Membranes were incubated overnight simultaneously with a rabbit polyclonal anti-salmonid inducible hsp70 (AS05061A) primary antibody (1:10000) (Agrisera, Sweden), and a rabbit polyclonal anti-β-actin (ab8227) primary antibody (1:5000) (Abcam, UK) in PBS-Tween with 3% milk at +4°C. Thereafter, membranes were incubated in PBS-Tween with 3% milk with an HRP-conjugated anti-rabbit secondary antibody (1:2500) (Sigma-Aldrich, USA) for 1 h at room temperature, then washed and immersed in Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, UK), followed by exposure to x-ray film. A short exposure (~5 sec) for β-actin and a longer exposure (~2 min) for hsp70 was used to acquire a quantifiable signal in steady-state measurements, while a short exposure (~5 sec) was used for both β-actin and hsp70 for heat shock measurements. Densitometry was performed using ImageJ 1.48v (NIH, USA) and relative quantities were obtained by normalising hsp70 values to β-actin.

Gene Cloning, Sequence Validation and Primer Design (**II, III** & **IV**)

Primers used to amplify genes of interest were designed based on alignments of salmonid nucleotide sequences available at NCBI (www.ncbi.nlm.nih.gov). *hexokinase* primers to amplify a 483-nucleotide sequence (For-CGTGTGCATATGAAGAGCCC, Rev-GTCAAGGAGGTATGCAGCAC) were based on AY864082.1 (*O. mykiss*). Primers to amplify a 60-nucleotide *gapdh* sequence (For-CCTGGTATGACAATGAGTTTGG, Rev-GCATGTACAGCAACAGGTCAG) were based on NM_001124246.1 (*O. mykiss*) and primers to amplify a 463-nucleotide *enolase* sequence (For-TGCGCTTGTACGGCAGAA, Rev-CTCTGGAGCTGCTGAAGGAA) were based on HM100654.1 (*O. mykiss*), NM_001139894.1 and FN812741.1 (*S. salar*). Primers to amplify a 1128 *β-actin* sequence (For-ATGGAAGATGAAATCGCCGCAC, Rev-TTAGAAGCATTTACGGTGGACG) were based on mRNA sequences with accession numbers AB196465.1 (*O. mykiss*), AB111057.1 (*Oncorhynchus nerka*), JR540730.1, KU885450 (*S. alpinus*), NM_001123525.1 and KU885449 (*S. salar*). Primers used to amplify an 812-nucleotide *inducible hsp70* sequence (For -CCTCTACATTCATAAACTGCAACT, Rev-CTGGCTGATGTCCTTCTTGTGT) were designed from NM_001124228.1, NM_001124745.1 and AB062281.1 (*O. mykiss*),

KF783199.1 (*Salvelinus fontinalis*), KU885452 (*S. alpinus*), OUT35064 (*Oncorhynchus tschawytscha*), AJ632154.1, KU885451 and BT059361 (*S. salar*).

The isolated gene sequences from salmonid cells were validated from PCR products obtained from cDNA reverse transcribed from 1 µg of RNA template. RNA isolation and cDNA synthesis protocols are detailed in succeeding sections. Genes of interest were amplified using a KAPA HiFi HotStart PCR Kit (KAPA Biosystems, USA) with the following thermal cycling parameters: 1 cycle of initial denaturation for 3 min at 95°C, then 30 cycles each of second denaturation at 98°C for 20 sec, annealing at 60°C for 15 sec and extension at 72°C for 60 sec / kb. PCR products were size-separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide, followed by gel extraction using a NucleoSpin gel and PCR clean up kit (Macherey-Nagel, Germany). Gene fragments were ligated onto a pJET1.2 / blunt cloning vector with a CloneJet PCR Cloning kit (ThermoScientific, USA), propagated in $CaCl_2$ competent DH5α *E. coli* and screened on LB-agar containing ampicillin. Positive colonies were selected for further propagation then purified with a NucleoSpin Plasmid EasyPure Kit (Macherey-Nagel, Germany). Sequencing was done on purified plasmids at the European Custom Sequencing Centre (GATC Biotech AG, Köln Germany). The obtained sequences were aligned and confirmed with homologous sequences using BioEdit (Ibis Biosciences, USA) and NCBI BLAST. Phylogenetic analysis of *hsp70* gene sequences obtained from *S. alpinus* and *S. salar* were conducted according to Metzger *et al.* (2016) and confirmed to be inducible isoforms, although the analysis did not differentiate between which clade (*hsp70-1* and *hsp70-2*) the isolated sequences should be grouped.

Gene-specific Taqman qPCR primers and fluorescent probes were designed from cloned sequences using the Universal Probe Library Assay Design Centre (Roche Diagnostics) to amplify a 60 - 88 base amplicon (*β-actin* For-CCAAAGCCAACAGGGAGA**,** Rev-GTACATGGCAGGGGTGTTG, probe # 115 cat. no. 04693493001; *hsp70* For-AGCTAAAGGCCCGTCTATCG, Rev-AACACCCCCACACAGGAGTA, probe # 104 cat. no. 04692225001; *hexokinase* For- AGAAGATGTGTAGCGGCATGT, Rev-TCAGGTCAATCAGGATGTTCC, probe # 14 cat no. 04685130001; *gapdh* For-CCTGGTATGACAATGAGTTTGG, Rev-GCATGTACAGCAACAGGTCAG, probe # 83 cat. no. 04689062001; *enolase* For-CCACAACCTGAAGAACGTCAT, Rev-CCTCGTCTCCCACGTTAGTG, probe # 162 cat no. 04694490001). mRNA expression quantification in study **II** was performed on inducible *hsp70* normalised to *β-actin* mRNA

levels, whereas transcription and mRNA measurements in studies **III** and **IV** were conducted on genes central to metabolism (*hexokinase*, *gapdh*, *enolase*) and proteostasis (*hsp70*), all of which were expected to be essential in the temperature responses of salmonids (Kieffer *et al.* 1998, Lewis *et al.* 2016, Vornanen *et al.* 2005). Transcription and mRNA levels were also measured for the abundant transcripts of the commonly used reference gene *β-actin* in studies **III** and **IV** to ascertain if transcript synthesis and mRNA level would remain relatively constant with changes in acclimation temperature.

Cell Culture, Nucleus Isolation and Nuclear Run-On Transcription (**III**)

The acclimation experiments were conducted using an available rainbow trout (*O. mykiss*) gill epithelial cell line (RTgill-W1) from the American Type Culture Collection (ATCC CRL 2523), as the species is known to tolerate a broad temperature range (Threader & Houston 1983), and a single cell type was preferred to minimise potential variations in transcription and mRNA levels that are likely to arise from a heterogeneous population of cells. RTgill-W1 cells with a recommended growth temperature of 18-20°C as per the ATCC guidelines (https://www.lgcstandards-atcc.org/Products/All/CRL-2523.aspx#culturemethod), were cultured as a monolayer in 75 cm^2 tissue culture flasks with Leibovitz L15-medium, supplemented with 10% FBS, 20 mM L-glutamine and 1000 U/ml of penicillin and streptomycin. Growth medium was replaced twice a week and confluent cells were detached from the substrate with 0.25% trypsin and passaged once a week. Cells were initially cultured at 18°C in a temperature controlled incubator with air as the equilibration gas for a minimum of three weeks, followed by a two week acclimation to 14°C and 22°C.

Cells were washed with PBS and detached with trypsin and a cell scraper after the acclimation period. Cell number was determined using a haemocytometer and were pelleted by centrifugation at 500 x RCF for 5 min at +4°C. Cells, nuclei and buffers were kept on ice and centrifuged at +4°C throughout the experimental procedure. The cell pellet was washed with PBS, centrifuged then re-suspended in PBS and aliquoted into 1.5 x 10^7 cells per replicate. After resuspension, cells were again pelleted by centrifugation, re-suspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl,) with 1% Nonidet P-40 and incubated for 5 min. Lysates were briefly centrifuged (~5 sec) to pellet cell debris, which was removed prior to centrifugation at 1000 x RCF for 5 min to pellet isolated nuclei. The nuclear pellet was

re-suspended in lysis buffer without Nonidet P-40 and centrifuged at 1000 x RCF for 5 min. The supernatant was aspirated, and the nuclear pellet suspended in 100 µl glycerol storage buffer (50 mM Tris-HCl, pH 8.0, 30% glycerol, 2 mM $MgCl_2$, 0.1 mM EDTA) and stored at -80°C.

Nuclear run-on transcription determinations were performed on isolated nuclei according to Patrone *et al.* (2000) with modifications and at three *in vitro* temperatures; a temperature within the range for optimal cell culture of RTgill-W1 (20°C) and previously used in transcription measurements of *O. mykiss* erythrocytes and hepatocytes (Götting & Nikinmaa 2017a, Levin & Oris 1999, respectively), a temperature 6°C above the optimal cell culture temperature and previously used in transcription measurements of *O. mykiss* primary hepatocyte cultures (26°C) (Flouriot *et al.* 1996, Pakdel *et al.* 1997), and a temperature close to the thermal preference of *O. mykiss* (Alanärä 1994, Bear & McMahon 2007, Ehrman *et al*. 2004), which is 6°C below the optimal cell culture temperature and coincides with the lowest acclimation temperature used in the study (14°C). The transcriptional activity of stalled polymerases in isolated nuclei was resumed *in vitro* with the inclusion of ribonucleotide substrates and a biotin label, which permits the separation of newly synthesised transcripts from total nuclear RNA. An equal volume (100 µl) of 2X transcription buffer (10 mM Tris-HCl, pH 8, 300 mM KCl, 5 mM $MgCl_2$) with 4 mM of rATP, rGTP and rCTP (Promega, USA), was mixed with nuclei and 10 µl of 10X biotin-16-UTP RNA labelling mix (11685597910, Roche Diagnostics, USA) and incubated at 14, 20 and 26°C using a temperature controlled water bath (RC6 RCS Lauda, Germany). The nuclear run-on assay incubation time was 1.5 h, during which samples were intermittently and gently tapped to promote nucleotide incorporation. *In vitro* transcription was terminated with the addition of 6 µl DNase I (1 U/µl) (Promega, USA), 6 µl 250 mM $CaCl_2$ and a 10 min incubation at the respective *in vitro* transcription reaction temperature.

Experimental Design, Nucleus Isolation and Nuclear Run-On Transcription (**IV**)

All procedures were approved by the Finnish Animal Experiment Board (ESAVI/4068/04.10.07/2013). The experiment was conducted at the Natural Resources Institute Finland in Enonkoski, eastern Finland, from 23rd of July to 23rd of August 2015. *S. alpinus* originated from Lake Saimaa (62°04′ N; 28°33′ E) and were initially reared indoors under a natural photoperiod at the Natural Resources Institute Finland hatchery. Juvenile (~1-

year-old) *S. alpinus* were transferred to an acclimation room with a constant 18:6 L:D photoperiod using artificial overhead illumination evenly distributed throughout the acclimation room. One hundred fish per acclimation group were acclimated to 8 and 15°C for four weeks in 320 L cylindrical (90 cm diameter) tanks with constantly flowing, filtered, aerated and temperature adjusted water from Lakes Pahkajärvi and Ylä-Enonvesi. Fish were anesthetised in 70 ppm tricaine methanesulfonate (MS-222, Sigma-Aldrich USA) buffered with sodium bicarbonate and weights and lengths were obtained pre- and post-acclimation to determine whether acclimation affected growth. Fish were fed once a day at random times to prevent entrainment and feeding was ceased 24 h prior to sampling. Six fish per acclimation temperature were sacrificed in 200 ppm MS-222 (Sigma-Aldrich USA) buffered with sodium bicarbonate and sampled at the start (1 h), middle (9 h) and towards the end (17 h) of the light cycle.

Blood was extracted from fish using a syringe injected through the caudal vessel and subsequently transferred into two separate tubes for transcription and steady-state mRNA measurements. All procedures thereafter were performed on ice and centrifugation kept at +4°C. Erythrocytes were isolated from plasma for transcription measurements and whole blood composed mostly of erythrocytes was used for mRNA measurements. Erythrocytes were chosen as cells were already individually separated in plasma suspension, making the isolation of nuclei and ribonucleotide incorporation steps of the nuclear run-on protocol simpler and more effective, respectively, whilst circumventing tissue processing steps, such as collagenase treatment required to degrade the extracellular matrix of bulk tissue samples. Whole blood was immediately frozen in liquid nitrogen for steady-state mRNA measurements and erythrocytes were separated from plasma by centrifugation for 5 min at 500 x RCF for the nuclear run-on assay. Plasma was discarded and erythrocytes re-suspended in sterile PBS then centrifuged several times to remove potential plasma derived contaminants. Cell number was determined using a light microscope and a haemocytometer and aliquoted into 5 x 10^7 red blood cells per replicate. Cells were pelleted by centrifugation then re-suspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 10 mM NaCl,) with 1% Nonidet P-40 and incubated for 5 min. Lysates were centrifuged at 500 x RCF for 5 min to pellet isolated nuclei, the supernatant was aspirated and the nuclear pellet re-suspended in lysis buffer without Nonidet P-40, followed by a 5 min centrifugation. The supernatant was removed, the nuclear pellet re-suspended in 100 µl glycerol storage buffer (50 mM Tris-HCl, pH 8.0, 30% glycerol, 2 mM MgCl2, 0.1 mM

EDTA) and flash frozen in liquid nitrogen prior to transport to the University of Turku, south western Finland.

The nuclear run-on transcription assay was performed similarly to study **III** at the University of Turku according to Patrone *et al.* (2000) with modifications. An equal volume (100 µl) of 2X transcription buffer (10 mM Tris-HCl, pH 8, 300 mM KCl, 5 mM $MgCl_2$) supplemented with 4 mM of rATP, rGTP and rCTP (Promega, USA) was mixed with nuclei and 10 µl of 10X biotin-16-UTP RNA labelling mix (11685597910, Roche Diagnostics, USA) and incubated for 1.5 h at 20°C using a temperature-controlled water bath (RC6 RCS Lauda, Germany). *In vitro* nascent transcript elongation was conducted at a temperature close to the upper end of the temperature tolerance range of *S. alpinus*, however the reaction temperature provided comparatively less variations in transcription rate determinations between replicates and acclimation temperatures in study **III** and was estimated to provide a balance between sufficient polymerase stability and molecular kinetic energy for nucleotide incorporation.
The nuclear suspension was intermittently and gently agitated throughout the incubation period to promote nucleotide incorporation. *In vitro* transcription was terminated with the addition of 6 µl DNase I (1 U/µl) (Promega, USA), 6 µl 250 mM $CaCl_2$ and a 10 min incubation at 20°C.

RNA Isolation and Quality Assurance (**II, III** & **IV**)

Total RNA from study **II** was extracted from tissues using the phenol guanidine isothiocyanate method (Chomczynski and Sacchi 1987) with TRI Reagent (Molecular Research Centre, USA), according to the manufacturer's instructions with additional purification steps. Frozen tissues were placed in TRI Reagent and homogenised mechanically with a TissueLyser (Qiagen, USA) at 30 shakes / sec for 2 min. Phase separation of RNA was performed using 1-bromo-3-chloropropane, followed by isopropanol precipitation, washing with 75% ethanol, then the RNA was dissolved in RNase free water. To remove residual genomic DNA contamination, DNase I (Promega, USA) was added (1 µg) in solution to an aliquot of RNA and incubated for 10 min at 37°C, followed by another round of phase separation, precipitation and washing. The purified RNA was stored overnight at +4°C in 75% ethanol to ensure the thorough removal of potential contaminants, then centrifuged at 7500 RCF for 5 min, subsequently air-dried and re-dissolved in RNase free water. RNA concentration and purity

were measured using a Nanodrop 2000 spectrophotometer (ThermoScientific, USA). Only samples with an A260/280 ratio of ≥ 1.8 were used in downstream applications.

Extraction of RNA in studies **III** and **IV** was conducted using the phenol guanidinium thiocyanate method (Chomczynski & Sacchi 1987) with NucleoZol (Macherey-Nagel, Germany), according to the manufacturer's instructions with additional purification steps. To obtain a measure of steady-state mRNA level in study **III**, monolayers of adherent RTgill-W1 cells were washed with PBS prior to lysis with NucleoZol and the addition of RNase-free water to precipitate contaminants. For steady-state mRNA measurements in study **IV**, whole blood was mixed with NucleoZol and lysed mechanically with a TissueLyser (Qiagen USA) at 30 shakes / sec for 2 min followed by the addition of RNase-free water to the lysate. RNA was precipitated out of solution using isopropanol then pelleted by centrifugation. The supernatant was discarded, and RNA pellet washed with 75% ethanol and re-dissolved in RNase-free water. Residual genomic contamination was removed by adding DNase I (Promega, USA) (1 µg) in solution to an aliquot of RNA and incubated for 10 min at 37°C, followed by another round of phase separation, precipitation and washing. Purified RNA was washed several times with 75% ethanol and stored overnight at +4°C to promote the thorough removal of contaminants, then centrifuged at 7500 x RCF for 5 min and re-dissolved in RNase-free water. RNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (ThermoScientific, USA). Steady-state samples with an A260 / 280 ratio of ≥ 1.8 were used for qPCR.

Nuclear run-on assay biotin-tagged transcripts in studies **III** and **IV** were extracted from nuclei with the addition of NucleoZol to disrupt the nuclear lamina, as described above but without mechanical lysis, DNase I treatment and additional purification. To separate biotin labelled RNA from total nuclear RNA, streptavidin magnetic particles (50 µl per replicate) (11641786001, Roche Diagnostics, USA) were washed with binding buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl) and magnetically separated from solution with a MagJET Separation Rack (MR02, ThermoScientific, USA). Magnetic particles were re-suspended in binding buffer and biotin-labelled RNA was added and incubated with continuous shaking for 15 min at room temperature. Streptavidin bound biotin-RNA was separated from the binding buffer and washed with washing buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 M NaCl), followed by resuspension in RNase-free water, 10 min incubation at 80°C and magnetic separation. The reconstituted RNA was transferred to a sterile Eppendorf tube and RNA purity and concentration determined with Nanodrop.

RNA integrity of steady-state samples was confirmed by agarose gel electrophoresis using sodium hypochlorite as a denaturant, as described previously (Aranda *et al.* 2012). An aliquot of RNA (600 ng) from each sample was mixed with 10X loading buffer (1.9 mM xylene cyanol, 1.5 mM bromophenol blue, 25% glycerol) and pipetted onto a gel comprised of 1% agarose, and 1% commercial bleach (Kiilto, Finland) containing 6% sodium hypochlorite and stained with ethidium bromide. RNA samples were separated by size in 1 x TAE at 100 V for 35 min. To test for genomic contamination, PCR was performed without reverse transcription on each steady-state RNA sample in triplicate in a final reaction volume of 10 µl per well, including 1 and 2 ng of RNA for study **III** and **IV**, respectively, 0.3 µM of *β-actin* forward and reverse primers, 0.1 µM of probe # 115 and 5 µl 2X KAPA Probe Fast qPCR kit master mix (KAPA Biosystems, USA). Thermal cycling parameters are the same as in the qPCR methodology detailed below. Samples which did not amplify after 40 cycles were deemed free of genomic DNA and samples which amplified were re-treated with DNase I and purified as described above.

cDNA Synthesis and Real-Time Quantitative Polymerase Chain Reaction (**II, III** & **IV**)

An aliquot of RNA (100 ng) from each sample from study **II** was used for cDNA synthesis using a PTC-150 MiniCycler (MJ Research, USA), with a DyNAmo cDNA synthesis kit (ThermoScientific, USA) according to the manufacturer's instructions, in a final reaction volume of 20 µl inclusive of random hexamers, reverse transcription buffer with dNTP mix and $MgCl_2$, M-MuLV RNase H+ reverse transcriptase and the following thermal cycling parameters: primer extension at 25°C for 10 min, cDNA synthesis at 37°C for 1 h and reaction termination at 85°C for 5 min, and cDNAs were subsequently stored at -20°C. RT-qPCR was conducted using a 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) for the steady-state data and QuantStudio 12K Flex Real Time PCR System (Applied Biosystems, USA) for the heat shock data, in a final reaction volume of 10 µl, with 1 ng of cDNA, 0.3 µM forward and reverse primers, 0.1 µM probe and 5 µl 2X KAPA Probe Fast qPCR master mix (KAPA Biosystems, USA), with the following thermal cycling parameters: Stage 1 (enzyme activation) at 50°C for 2 min. Stage 2 (denaturation) at 95°C for 10 min, and 40 cycles of Stage 3 at 95°C for 15 sec, then 60°C for 1 min (annealing and extension). Temperature changes were kept at a constant 1.6°C / sec. Target and reference gene reaction quantities were determined from a standard curve generated from a 1:2 (steady-state data) and a 1:5 (heat shock

data) serial dilution of randomly chosen and pooled samples, and *hsp70* values were normalised to *β-actin* to obtain relative quantities.

In studies **III** and **IV**, an aliquot of RNA (50 and 100 ng for study **III** and **IV**, respectively) from each sample was used for cDNA synthesis using a PTC-150 MiniCycler (MJ Research, USA), with a SensiFAST cDNA synthesis kit (Bioline, UK) according to the manufacturer's instructions, in a final reaction volume of 20 and 25 µl (for study **III** and **IV**, respectively) inclusive of 5X TransAmp reverse transcription buffer, random hexamers and reverse transcriptase, with the following thermal cycling parameters: primer annealing at 25°C for 10 min, reverse transcription at 42°C for 15 min and reaction termination at 85°C for 5 min. Resultant cDNAs were stored at -20°C for downstream use. RT-qPCR was conducted using the QuantStudio 12K Flex Real Time PCR System (Applied Biosystems, USA) in a final reaction volume of 10 µl containing 0.5 ng cDNA for study **III** and 0.8 ng cDNA for study **IV**, 0.3 µM forward and reverse primers, 0.1 µM probe and 5 µl 2X KAPA Probe Fast qPCR master mix (KAPA Biosystems, USA), with the following thermal cycling parameters: Stage 1 (enzyme activation) at 50°C for 2 min followed by Stage 2 (denaturation) at 95°C for 10 min, and 40 cycles of Stage 3 at 95°C for 15 sec followed by 60°C for 1 min (annealing and extension). Temperature changes were kept at a constant 1.6°C / sec. Transcript copy numbers were determined from standard curves generated from a 1:10 serial dilution of plasmids containing the appropriate inserts. All primers were tested for efficiency and amplification signals obtained were within the quantifiable range of primer efficiencies (90 - 110%).

Statistics

SigmaPlot 13 (SyStat Software, USA) was used for statistical comparisons for studies **I** - **IV** and $p < 0.05$ was accepted as statistically significant. The stability and suitability of *β-actin* and *gapdh* as consistent housekeeping reference genes and β-actin as a reference protein were validated with pair-wise correlation analysis in studies **II** and **IV** using BestKeeper (Pfaffl *et al.* 2004), however *β-actin* mRNA and protein levels were only used as references in study **II**. A time series statistical analysis (www.circadian.org) fitted with a cosine wave function (Cornelissen 2014) was used to determine circadian rhythmicity of transcription, mRNA and protein level data in studies **II** and **IV**.

I) Normality (Shapiro-Wilk) and equal variance (Brown-Fosythe) tests were performed on CTMax data, which did not meet the assumptions of normality and homogeneity of variance before and after log transformation, therefore Kruskal-Wallis test on ranks was conducted and independent factors species and acclimation temperature tested separately. Two-way ANOVA was conducted to re-analyse tissue level measurements from fish that have undergone CTMax trials (Anttila *et al.* 2015), with species and acclimation temperature as independent factors. *Post hoc* pairwise multiple comparisons (Dunn's test) were performed whenever significant effects were identified.

II) Most of the mRNA and protein level data did not conform with the assumptions of normality (Shapiro-Wilk) and equal variance (Brown-Fosythe) despite log transformation, therefore one-way ANOVA was used for parametric data (p) and Kruskal-Wallis test on ranks for non-parametric data (n-p), with independent factors species and acclimation temperature tested separately. When statistically significant effects were identified, *post hoc* pairwise multiple comparisons (Holm-Sidak or Dunn's test for parametric (p) and non-parametric (n-p) steady-state data, respectively, and Dunnett's test for the heat shock data) were conducted. *T*-test or Mann-Whitney *U*-test (for parametric (p) and non-parametric (n-p) data, respectively) was used to identify differences between fish weight post-acclimation and differences in steady-state mRNA and protein levels between acclimation temperatures at the same timepoint.

III) The majority of the data were log transformed to meet the assumptions of normality (Shapiro-Wilk) and homogeneity of variance (Brown-Forsythe). Two-way ANOVA was conducted on transcription data with acclimation temperature and *in vitro* reaction temperature as independent factors. One-way ANOVA or Kruskal-Wallis test on ranks (for parametric (p) and non-parametric (n-p) data, respectively) was conducted on mRNA data with acclimation temperature as the independent factor. *Post hoc* pairwise multiple comparisons (Holm-Sidak or Dunn's test for parametric (p) and non-parametric (n-p) data, respectively) were performed whenever significant effects were identified.

IV) Transcription and mRNA data were tested for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe), but since most of the data did not meet the assumptions of normality and homogeneity of variance after log transformation, one-way ANOVA or Kruskal-Wallis test on ranks (for parametric (p) and non-parametric (n-p) data, respectively) was conducted with independent factors acclimation temperature and timepoint tested separately. *Post hoc* pairwise

multiple comparisons (Holm-Sidak or Dunn's test for parametric and non-parametric data, respectively) were performed whenever significant effects were identified. *T*-test or Mann-Whitney *U*-test (for parametric (p) and non-parametric (n-p) data, respectively) was used to identify differences between fish weight pre- and post-acclimation and steady-state mRNA levels between acclimation temperatures at the same timepoint.

Results

(**I**) *Whole Organism Thermal Tolerance and Tissue Remodelling*

To minimise size and time lag effects, an attempt was made to ensure the size of fish were similar for the CTMax trials through visual estimation, although there was still a statistically significant difference in fish weight between acclimation groups ($p = 0.019$ and $p < 0.001$ for *S. alpinus* and *S. salar*, respectively) post-acclimation, with an average of 19 g ± 2.31 (SEM) in weight and 13.24 cm ± 0.47 in length for *S. alpinus* acclimated to 8°C and 27 g ± 2.96 and 14.67 cm ± 0.45 for *S. alpinus* acclimated to 15°C. The average *S. salar* size at 8°C and 15°C was 22.33 g ± 1.13 and 12.65 cm ± 0.22 and 37.14 g ± 3.93 and 15.12 cm ± 0.66, respectively. Kruskal-Wallis test on ranks also identified statistically significant differences in critical thermal maxima between acclimation temperatures within a species and between species within an acclimation temperature after four weeks at 8 and 15°C (Figure 4). The critical thermal maximum for *S. alpinus* at the lower acclimation temperature was 26.71°C ± 0.09, which increased by 1.28°C to 27.99°C ± 0.05 at the higher acclimation temperature ($H_1 = 48.85$, $p < 0.001$). The critical thermal maximum for *S. salar* at the lower acclimation temperature was 27.6°C ± 0.08, which increased by 2.17°C to 29.77°C ± 0.10 at the higher acclimation temperature ($H_1 = 53.75$, $p = < 0.001$). Between species, *S. salar* had a critical thermal maximum that was 0.89°C higher than *S. alpinus* at 8°C ($H_1 = 58.24$, $p < 0.001$) and 1.78°C higher at 15°C ($H_1 = 44.18$, $p < 0.001$).

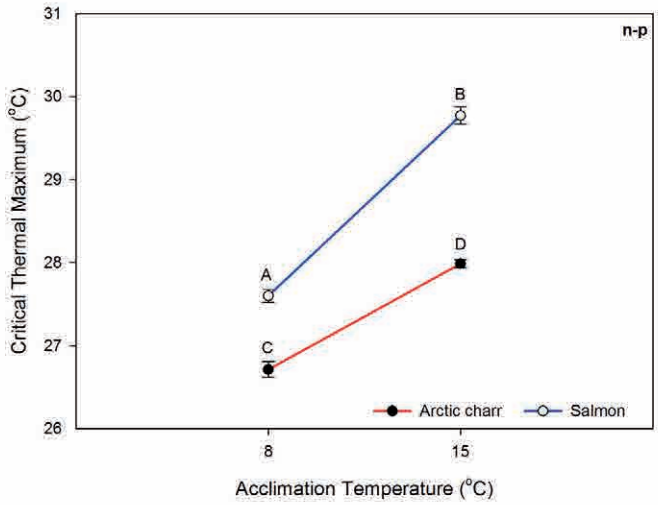

Figure 4 Arctic charr *S. alpinus* and Atlantic salmon *S. salar* Critical Thermal Maxima at acclimation temperatures 8 and 15°C. Significant differences between acclimation temperatures and species are denoted by different letters. Statistical significance ($p < 0.05$) was determined by two-way ANOVA or Kruskal-Wallis on ranks (parametric (p) or non-parametric (n-p), respectively) and subsequent *post hoc* analysis (Holm-Sidak (p) or Dunn's test (n-p)). Mean ± SEM; n = 15 per acclimation (total = 60).

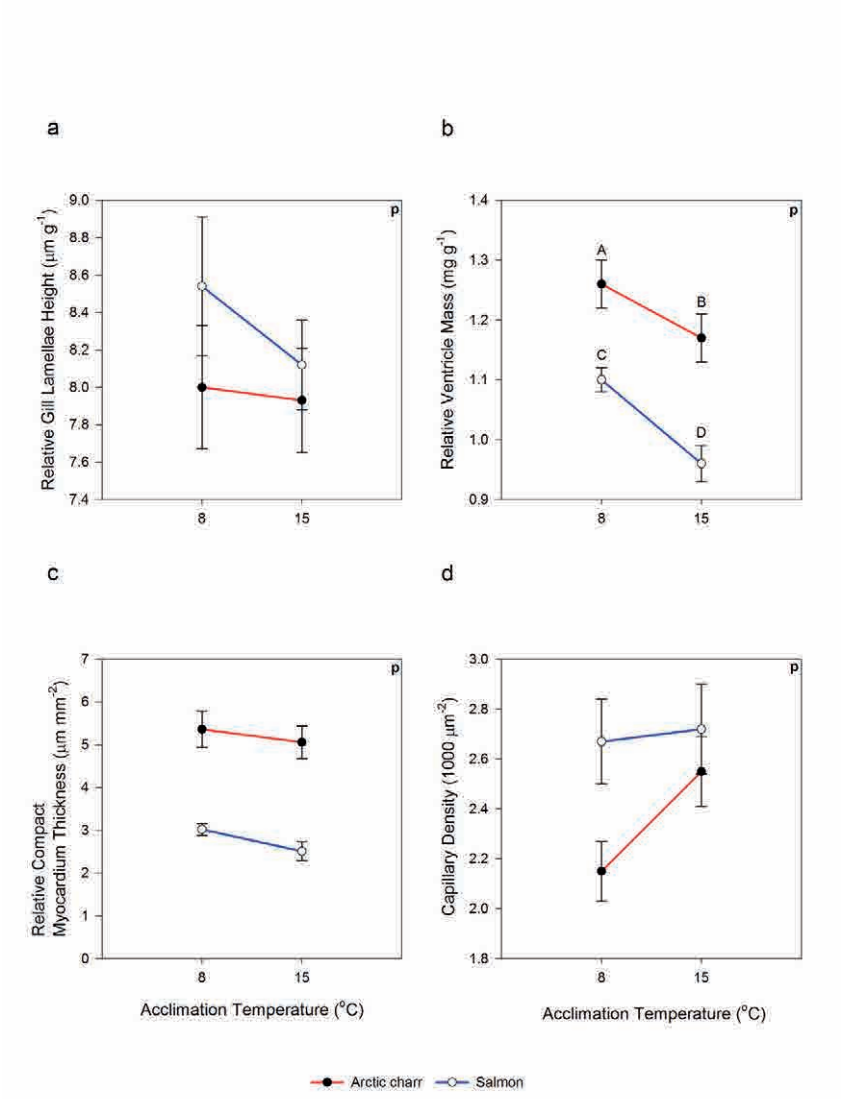

Figure 5 Temperature Acclimation Induced Tissue Remodelling showing no significant difference in gill morphology (**a**) but a significant difference between species in relative compact myocardium thickness (**c**) ($p < 0.001$) and compact myocardium capillary density (**d**) ($p = 0.042$). Significant differences in relative ventricle mass (**b**) between species and acclimation temperatures are denoted by different letters. Statistical significance ($p < 0.05$) was determined by two-way ANOVA or Kruskal-Wallis on ranks (parametric (p) or non-parametric (n-p), respectively) and subsequent *post hoc* analysis (Holm-Sidak (p) or Dunn's test (n-p)). Mean ± SEM; n = 15 per acclimation (total = 60).

At the tissue level, two-way ANOVA was not able to identify a significant difference in relative gill lamellae height between species (F_1 = 1.145, p = 0.295) and acclimation temperatures (F_1 = 0.522, p = 0.477), which at 8°C was 8 μm g^{-1} ± 0.33 for *S. alpinus* and 8.54 ± 0.37 for *S. salar*, and at 15°C was 7.93 ± 0.28 for *S. alpinus* and 8.12 ± 0.24 for *S. salar* (Figure 5a). There were significant differences in the cardiovascular structure of both species after the four-week acclimation period. *S. alpinus* relative ventricle mass was significantly heavier than *S. salar* (F_1 = 24.812, p < 0.001), with values of 1.26 mg g^{-1} ± 0.04 for *S. alpinus* and 1.10 mg g^{-1} ± 0.02 for *S. salar* at 8°C (Figure 5b). The increase in acclimation temperature significantly altered the relative ventricle mass of both species (F_1 = 10.792, p = 0.002), which decreased to 1.17 mg g^{-1} ± 0.04 and 0.96 mg g^{-1} ± 0.03 for *S. alpinus* and *S. salar*, respectively. The compact myocardium of *S. alpinus* was significantly thicker (F_1 = 59.975, p < 0.001) than *S. salar* at both acclimation temperatures (Figure 5c), with values of 5.36 mm^2 ± 0.42 for *S. alpinus* and 3.02 mm^2 ± 0.14 for *S. salar* at 8°C. The thickness of the compact layer appears to have decreased slightly at 15°C for both species to 5.06 mm^2 ± 0.38 for *S. alpinus* and 2.51 mm^2 ± 0.22 for *S. salar*, but the difference between acclimation temperatures (F_1 = 1.674, p = 0.212) and the interaction between species and acclimation temperature (F_1 = 0.107, p = 0.747) was statistically insignificant. Capillary density of the compact myocardium (Figure 5d) was significantly different between species (F_1 = 4.634, p = 0.042), which was 2.15 1000 μm^{-2} ± 0.12 for *S. alpinus* and 2.67 1000 μm^{-2} ± 0.17 for *S. salar* at 8°C. Capillary density seems to have slightly increased at 15°C to 2.55 1000 μm^{-2} ± 0.14 for *S. alpinus* and 2.72 1000 μm^{-2} ± 0.18 for *S. salar*, but the effect of acclimation temperature (F_1 = 2.032, p = 0.167) and species-acclimation temperature interaction (F_1 = 1.183, p = 0.288) was not statistically significant.

(**II**) *Species- and Acclimation-Specific Transcription and Translation of Inducible hsp70*

S. alpinus was significantly heavier (p = 0.011) at 8°C (26.6 g ± 1.3 (SEM) mass and 14.5 cm ± 0.2 length) than at 15°C (22.9 g ± 1.0 and 13.8 cm ± 0.2), whereas *S. salar* was significantly heavier (p < 0.001) at 15°C (27.5 g ± 1.0 and 13.6 cm ± 0.2) than at 8°C (22.8 g ± 0.6 and 12.8 cm ± 0.1) (Figure 6). The difference in fish mass between acclimation temperatures was not mirrored in the condition factor of fish (Fulton's condition factor, K = 100 x weight/$length^3$), which were 0.809 ± 0.17 and 0.808 ± 0.08 for 8 and 15°C-acclimated *S. alpinus*, and 1.064 ± 0.09 and 1.060 ± 0.11 for 8 and 15°C-acclimated *S. salar*, respectively. Using one-way ANOVA / Kruskal-Wallis test on ranks, steady-state *hsp70* mRNA (H_1 = 29.598, p < 0.001

and $H_1 = 7.222$, p = 0.007 for gills and liver, respectively) and hsp70 levels ($H_1 = 15.726$, p < 0.001 and $H_1 = 5.491$, p = 0.019 for gills and liver, respectively) were determined to be significantly elevated in 15°C- than in 8°C-acclimated *S. alpinus* (Figure 7). In contrast, significantly higher *hsp70* mRNA ($H_1 = 4.129$, p = 0.042 and $H_1 = 23.846$, p < 0.001 for gills and liver, respectively) and hsp70 levels ($F_1 = 19.921$, p < 0.001 and $H_1 = 5.962$, p = 0.015 for gills and liver, respectively) were observed in 8°C- than in 15°C-acclimated *S. salar* (Figure 8). Between acclimation temperatures at the same timepoint, mRNA level was significantly higher at 1, 8 and 21 h in the gills (p < 0.05) and at 16 h in the liver (p = 0.008), and protein level was significantly higher at 16 and 21 h in the gills (p < 0.04) and at 1 h in the liver (p = 0.003) of 15°C-acclimated *S. alpinus*. For 8°C-acclimated *S. salar*, mRNA level was significantly higher at 1, 8 and 16 h in the liver (p ≤ 0.003) and at 1, 8 and 16 h for protein level in gills (p < 0.022).

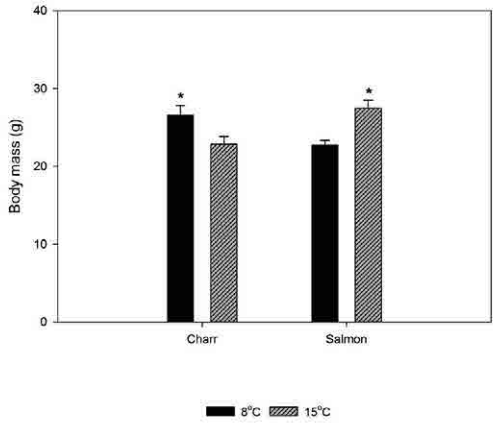

Figure 6 *S. alpinus* and *S. salar* Body Mass after four weeks of acclimation to 8 and 15°C. Fish were fed daily *ad libitum* throughout the acclimation and *t*-test was used to determine statistical significance (p < 0.05) between 8 and 15°C within the same species. Mean weight (g) ± SEM; n = 100 per acclimation (total = 400). * denotes a significant difference between acclimation temperatures within species.

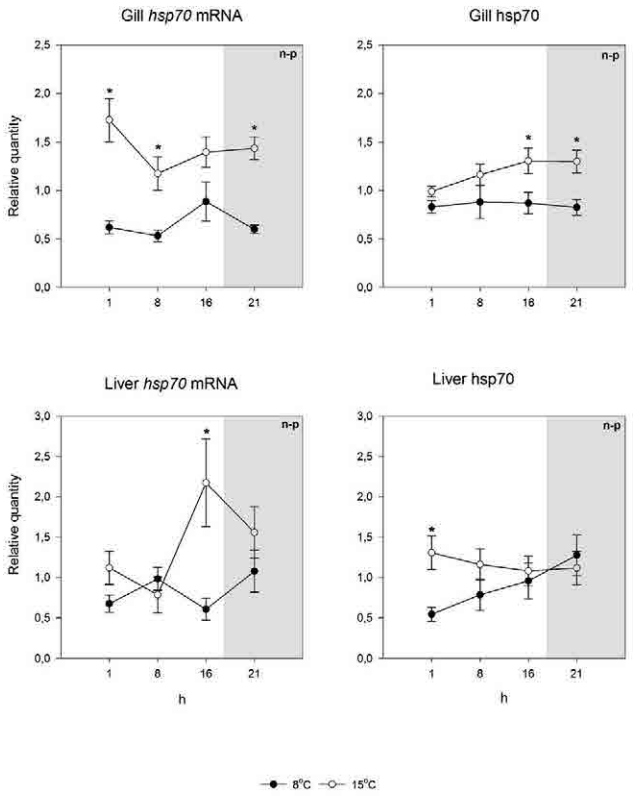

Figure 7 *S. alpinus* Steady-State *hsp70* mRNA and hsp70 Circadian Expression under an 18:6 L:D photoperiod. *hsp70* and hsp70 levels in gills ($p < 0.001$ and $p < 0.001$, respectively) and liver ($p = 0.007$ and $p = 0.019$, respectively) were significantly elevated at 15 than at 8°C. Statistical significance ($p < 0.05$) between acclimation temperatures and between timepoints within an acclimation temperature was determined by one-way ANOVA or Kruskal-Wallis on ranks (parametric (p) or non-parametric (n-p), respectively) and subsequent *post hoc* analysis (Holm-Sidak (p) or Dunn's test (n-p)). * denotes significant differences between acclimation temperatures within the same timepoint as identified by *t*- or Mann-Whitney *U*-test, and the significant *hsp70* rhythmicity in 15°C-acclimated liver ($p = 0.024$) was resolved using the cosinor periodogram (circadian.org). Mean relative quantity ± SEM; n = 7 per timepoint (total = 56). The dark period is indicated by grey shading.

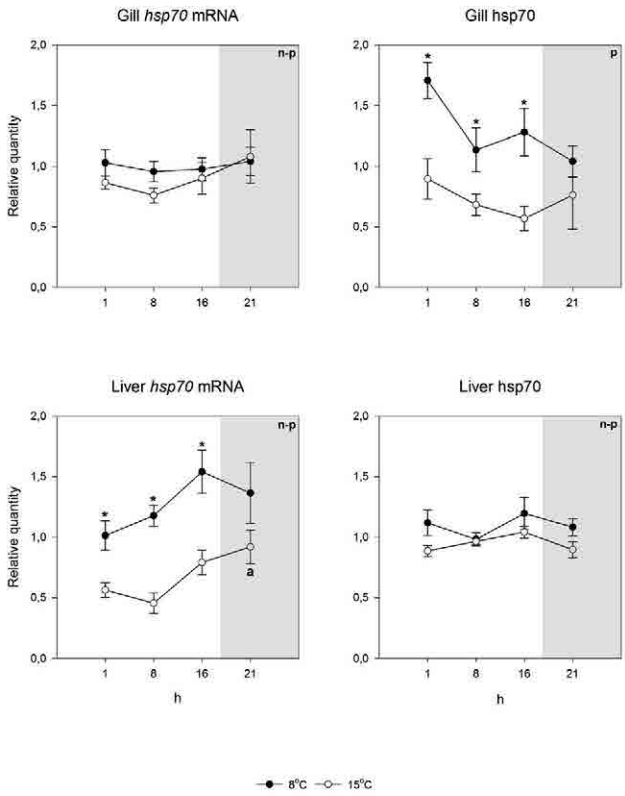

Figure 8 *S. salar* Steady-State *hsp70* mRNA and hsp70 Circadian Expression under an 18:6 L:D photoperiod. *hsp70* and hsp70 levels in gills ($p = 0.042$ and $p < 0.001$, respectively) and liver ($p < 0.001$ and $p = 0.015$, respectively) were significantly elevated at 8 than at 15°C. Statistical significance ($p < 0.05$) between acclimation temperatures and between timepoints within an acclimation temperature was determined by one-way ANOVA or Kruskal-Wallis on ranks (parametric (p) or non-parametric (n-p), respectively) and subsequent *post hoc* analysis (Holm-Sidak (p) or Dunn's test (n-p)), with the letter indicating a significant difference in mRNA level between 21 and 8 h ($p = 0.01$) within the same acclimation temperature. * denotes significant differences between acclimation temperatures within the same timepoint as identified by *t*- or Mann-Whitney *U*-test, and the significant *hsp70* rhythmicity in 15°C-acclimated liver ($p = 0.007$) was resolved using the cosinor periodogram (circadian.org). Mean relative quantity ± SEM; $n = 7$ per timepoint (total = 56). The dark period is indicated by grey shading.

ANOVA / Kruskal-Wallis test on ranks was initially used but was unable to identify significant mRNA and protein level rhythms within the chosen sampling intervals and photoperiod regime, with the exception of the mRNA level in 15°C-acclimated *S. salar* liver which increased significantly during the dark phase at 21 h compared to 8 h ($H_3 = 11.339$, $p = 0.01$), whereas the expression of *hsp70* mRNA in the liver and hsp70 in the gills of 15°C-acclimated *S. alpinus* was close to statistical significance ($F_3 = 2.954$, $p = 0.053$ and $H_3 = 6.336$, $p = 0.096$, respectively). Since ANOVA / Kruskal-Wallis test on ranks was not designed to determine circadian rhythmicity, a time series cosinor (circadian.org) statistical analysis fitted with a cosine wave function was used to test for steady-state mRNA and protein level rhythmicity, which confirmed the significant *hsp70* mRNA rhythm in the liver of 15°C-acclimated *S. alpinus* (Figure 7) ($F_{2.15} = 4.293$, $p = 0.024$, mesor (midline estimating statistic of rhythm) = 1.364, amplitude = 0.733 acrophase = 16 h 56 min) and *S. salar* (Figure 8) ($F_{2.15} = 5.995$, $p = 0.007$, mesor = 0.649, amplitude = 0.243, acrophase = 18 h 36 min), whereas the rhythmicity of *hsp70* mRNA at 8°C and hsp70 at 15°C in *S. salar* liver was close to significance ($F_{2.15} = 2.688$, $p = 0.086$, mesor = 1.275, amplitude = 0.292, acrophase = 15 h 35 min and $F_{2.15} = 3.017$, $p = 0.066$, mesor = 0.958, amplitude = 0.091, acrophase = 13 h 16 min, respectively).

A 30 min +7°C shift in temperature from 8 to 15°C did not elicit an increase in mRNA and protein expression in cold acclimated *S. alpinus*, although a shift from 15 to 22°C resulted in a significant increase in *hsp70* mRNA at 1, 2 and 4 h post-heat shock (Figure 9), before subsiding to pre-heat shock baseline values by 8 until 24 h in the gills ($H_6 = 43.236$, $p < 0.001$) and liver ($H_6 = 43.485$, $p < 0.001$) of warm acclimated fish. The increase in *hsp70* in 15°C-acclimated *S. alpinus* was followed by an increase in hsp70, reaching peak levels at 4 h in the gills ($H_6 = 34.504$, $p < 0.001$), already at 2 h in the liver ($F_6 = 4.776$, $p < 0.001$) and with protein levels remaining elevated up to 24 h. Exposure of 8°C-acclimated *S. salar* to 15°C for 30 min neither induced an increase in *hsp70* mRNA nor hsp70 in both gills and liver (Figure 10). In fact, gill hsp70 was significantly higher in 8°C-acclimated *S. salar* prior to the heat shock (0 h) than at subsequent timepoints ($F_6 = 12.987$, $p < 0.001$). An increase in temperature from 15 to 22°C resulted in a significant increase in *hsp70* mRNA at 1 and 2 h in gills ($H_6 = 41.431$, $p < 0.001$) and liver ($H_6 = 41.431$, $p < 0.001$) before returning to basal levels by 4 h post-heat shock, although the increase in mRNA was not accompanied by an increase in liver hsp70, and as observed in cold acclimated *S. salar*, gill hsp70 was significantly higher prior to the heat shock (0 h) ($H_6 = 15.205$, $p = 0.019$) than at 1 and 8 h post-heat shock.

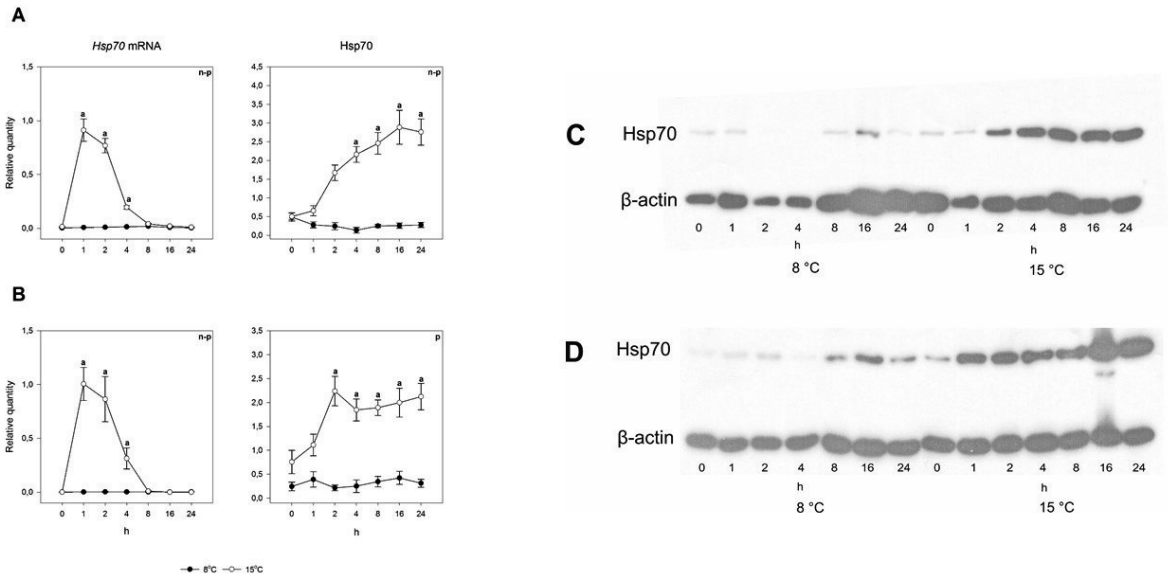

Figure 9 Time Course of Heat Shock Induced *hsp70* mRNA and hsp70 Synthesis in *S. alpinus* under an 18:6 L:D photoperiod. *hsp70* and hsp70 levels in gills (**A**) and liver (**B**) were significantly elevated by a 30 min +7°C exposure of 15°C-acclimated fish. Statistical significance ($p < 0.05$) between timepoints within an acclimation temperature was determined by one-way ANOVA or Kruskal-Wallis on ranks (parametric (p) or non-parametric (n-p), respectively) and subsequent *post hoc* analysis (Dunnett's method). Letters indicate a significant difference between the level at the timepoint and the level prior to the heat shock. Representative examples of western blots for gills (**C**) and liver (**D**) are provided. Mean relative quantity ± SEM; n = 7 per timepoint (total = 98).

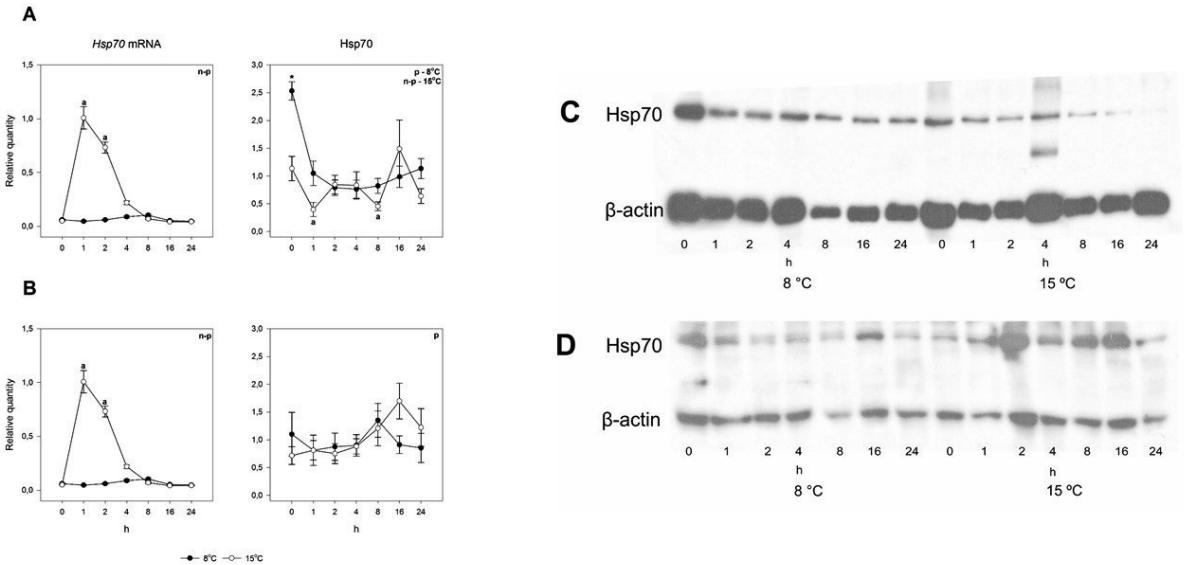

Figure 10 Time Course of Heat Shock Induced *hsp70* mRNA and hsp70 Synthesis in *S. salar* under an 18:6 L:D photoperiod. *hsp70* in gills (**A**) and liver (**B**) were significantly elevated by a 30 min +7°C exposure of 15°C-acclimated fish. hsp70 levels were significantly higher in the gills (**A**) of 8- and 15°C-acclimated fish prior to the heat shock. Statistical significance ($p < 0.05$) between timepoints within an acclimation temperature was determined by one-way ANOVA or Kruskal-Wallis on ranks (parametric (p) or non-parametric (n-p), respectively) and subsequent *post hoc* analysis (Dunnett's method). Letters indicate a significant difference between the level at the timepoint and the level prior the heat shock, whereas * denotes a significant difference before the heat shock than at succeeding timepoints. Representative examples of western blots for gills (**C**) and liver (**D**) are provided. Mean relative quantity ± SEM; n = 7 per timepoint (total = 98).

(**III**) *Transcription and Steady-State mRNA Level in the Temperature Acclimation of a Commercially Available Salmonid Cell Line*

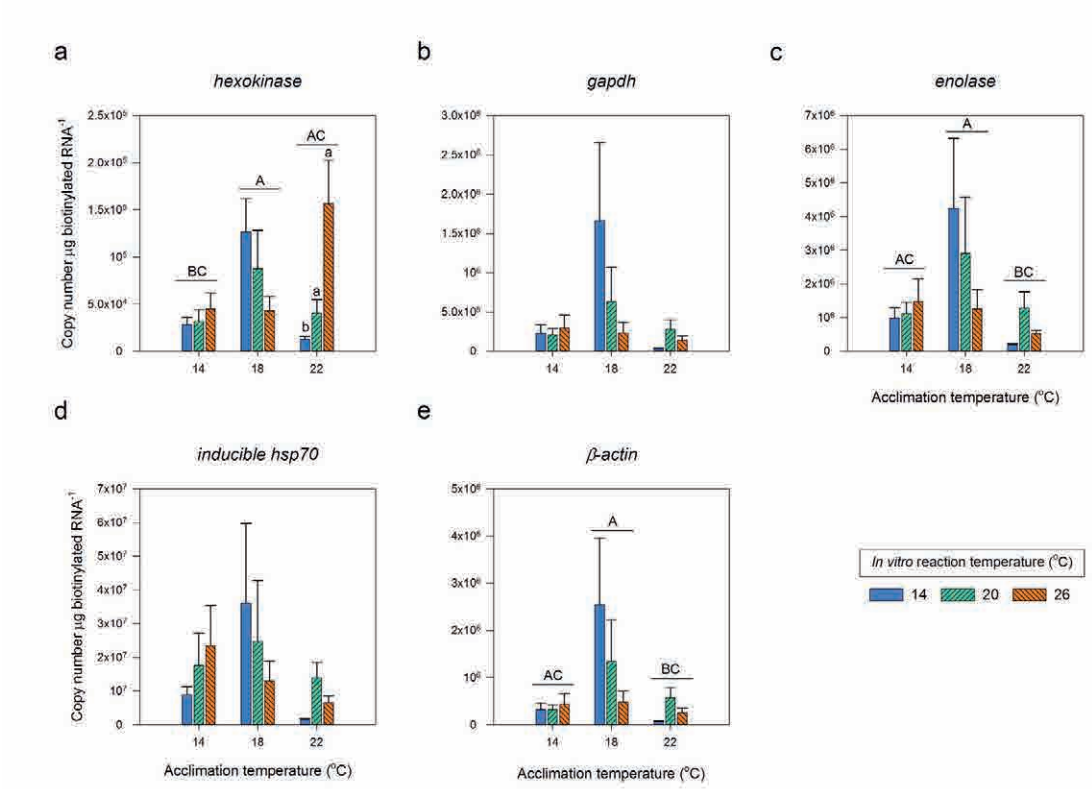

Figure 11 Gene Transcription in Rainbow Trout *O. mykiss* Cells Acclimated to Optimal and Suboptimal Temperatures and at three *in vitro* reaction temperatures. The transcription of genes encoding glycolytic enzymes (**a-c**), a molecular chaperone (**d**) and a cytoskeletal protein (**e**) in cells acclimated to optimal (18°C), cold (14°C) and warm (22°C) for two weeks. Statistical significance ($p < 0.05$) between acclimation temperatures and reaction temperatures was determined by two-way ANOVA and subsequent *post hoc* analysis (Holm-Sidak). Acclimation temperatures with dissimilar capital letters and reaction temperatures with dissimilar lower case letters are significantly different from each other. Mean copy number ± SEM; n = 5 per acclimation and reaction temperature (total = 45).

The results of a two-way ANOVA showed a statistically significant acclimation temperature effect in the transcription of *hexokinase*, *enolase* and *β-actin* ($F_2 = 3.415$, $p = 0.044$; $F_2 = 4.355$,

p = 0.020 and F_2 = 4.414, p = 0.019, respectively) and a close to significant effect in the transcription of *gapdh* and *hsp70* (F_2 = 2.450, p = 0.101 and F_2 = 2.718, p = 0.080, respectively) (Figure 11). *Post hoc* analysis determined the transcription of *hexokinase* was significantly higher at 18°C than at 14°C (p = 0.045) but not 22°C (p = 0.162), whereas the transcription of *enolase* and *β-actin* was significantly higher at 18°C than at 22°C (p = 0.017 and p = 0.022, respectively) but not 14°C (p = 0.916 and p = 0.072, respectively), although *β-actin* transcription at 18°C was close to being significantly more elevated than at 14°C. The effect of the *in vitro* assay temperature was not significant for most genes and neither was the interaction between acclimation temperature and assay temperature, with the exception of *hexokinase* (F_4 = 5.200, p = 0.002) which exhibited a parallel increase in transcription with assay temperature at 22°C, with a significantly higher transcription at the reaction temperature of 26°C than at 14°C (p < 0.001) but not 20°C (p = 0.064), although the intermediate reaction temperature followed the trend. In general, cells acclimated to 14 and 22°C were transcribing genes at lower levels and the negligible effect of assay temperature was evident, whereas the transcription of cells kept at 18°C was higher at the lowest assay temperature and which tended to decrease as the assay temperature increased, and although this change in transcription with reaction temperature was not statistically significant, the trend was the same for all five genes. Statistical testing was unable to definitively validate the noticeable homogeneous transcriptional pattern because of variations in the transcription measurements. The possible reasons for the variability and the observable trends are discussed in more detail in the succeeding section.

Cytoplasmic mRNA levels were in stark contrast to transcription (Figure 12), exhibiting significantly different transcript concentrations between acclimation temperatures (F_2 = 19.368, p < 0.001; F_2 = 34.980, p < 0.001; F_2 = 32.163, p < 0.001; F_2 = 56.392, p < 0.001 and H_2 = 12.500, p = 0.002, for *hexokinase*, *gapdh*, *enolase, β-actin* and *hsp70*, respectively), with the exception of *hexokinase* which had comparable mRNA levels at 14 and 22°C (p = 0.207). At the optimal acclimation temperature where transcription was generally higher, mRNA level was at their lowest. Conversely, at the suboptimal temperatures where transcription was generally lower, mRNA levels were at their highest. mRNA levels for genes encoding glycolytic enzymes (*hexokinase*, *gapdh* and *enolase*) and the cytoskeletal protein (*β-actin*) were highest in cells acclimated to 14°C and intermediate in cells acclimated to 22°C, whereas the level of mRNA for the gene encoding the temperature inducible chaperone (*hsp70*) was

highest in cells acclimated to 22°C and intermediate at 14°C. The elevated *hsp70* transcript levels at 14 and 22°C is further confirmation that the acclimation temperatures were not the ideal growth temperatures for RTgill-W1 cells.

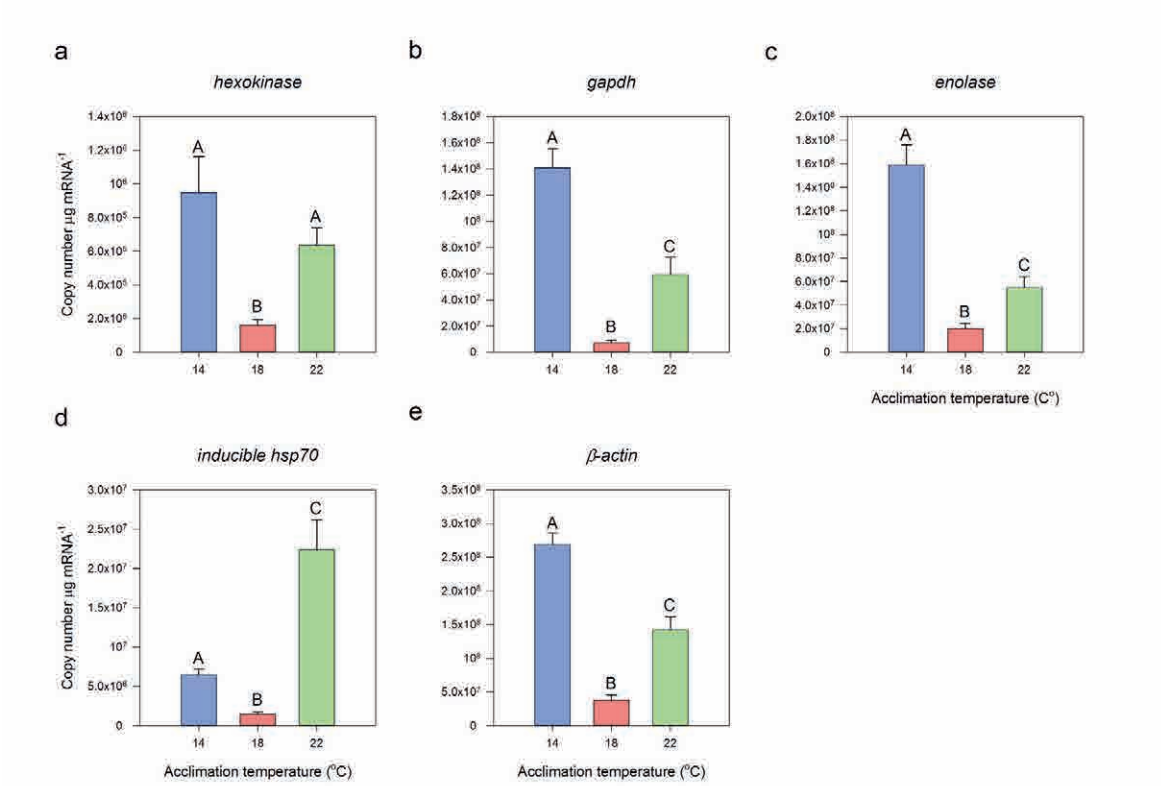

Figure 12 Steady-state mRNA Level in *O. mykiss* Cells Acclimated to Optimal and Suboptimal Temperatures for two weeks. mRNA levels of genes encoding glycolytic enzymes (**a-c**), a molecular chaperone (**d**) and a cytoskeletal protein (**e**) in cells acclimated to optimal (18°C), cold (14°C) and warm (22°C). Statistical significance ($p < 0.05$) between acclimation temperatures was determined by one-way ANOVA and subsequent *post hoc* analysis (Holm-Sidak). *hsp70* was tested using Kruskal-Wallis test on ranks followed by Dunn's test. Acclimation temperatures with dissimilar capital letters are significantly different from each other. Mean copy number ± SEM; n = 5 per acclimation temperature (total = 15).

(**IV**) *Transcriptional and Steady-state mRNA Rhythms in the Temperature Acclimation of S. alpinus Erythrocytes*

The size of *S. alpinus* between acclimation temperatures was not significantly different, nor was the size pre- and post-acclimation to 8 (29.04 g weight ± 1.78 (SEM) and 14.80 cm length ± 0.26; 29.93 g ± 2.14 and 15.33 cm ± 0.30, respectively) and 15°C (30.44 g ± 2.34 and 14.78 cm ± 0.35; 29.98 g ± 2.34 and 15.21 cm ± 0.38, respectively), but the nominal decrease in weight at the higher acclimation temperature may be indicative of a reduction in growth rate. As with study **III**, there was a fair amount of transcriptional variation in the present study but to a lesser extent, whereas steady-state mRNA level measurements were more consistent in study **III**, which is discussed in more detail in the succeeding section. Irrespective of the variation, the transcription of glycolysis and molecular chaperone genes was generally higher in erythrocytes acclimated to 15°C (Figure 13), with one-way ANOVA / Kruskal-Wallis on ranks identifying a significantly higher transcription for *gapdh* and *enolase* ($F_1 = 4.162$, $p = 0.049$ and $H_1 = 9.131$, $p = 0.003$, respectively). Following the trend is the close to significant increase in the transcription of *hexokinase* ($H_1 = 2.796$, $p = 0.094$) and inducible *hsp70* ($H_1 = 3.252$, $p = 0.071$) at 15°C, whereas the transcription of the cytoskeletal protein *β-actin* was comparable between acclimation temperatures ($H_1 = 0.100$, $p = 0.752$).

All five genes at the higher acclimation temperature exhibited a similar diurnal rhythm in transcriptional induction, starting at a baseline level 1 h after the start of the light period, peaking in the middle of the light period at 9 h before subsiding at 17 h to approximate baseline level 1 h before the onset of the dark phase, although statistical testing was only able to show a significant increase in the transcription of *hsp70* ($F_2 = 5.292$, $p = 0.018$) between 9 and 17 h ($p = 0.021$) and a close to significant increase between 9 and 1 h ($p = 0.066$). ANOVA / Kruskal-Wallis on ranks is not designed to determine biological rhythmicity, so a time series statistical analysis fitted with a cosine wave function was used to confirm gene transcription rhythms. According to the cosinor analysis (circadian.org), *hsp70* and *β-actin* display significant transcriptional rhythmicity at 15°C ($F_{2.15} = 4.789$, $p = 0.024$, mesor (midline estimating statistic of rhythm) = 463,049, amplitude = 581,121, acrophase = 8 h 40 min and $F_{2.15} = 4.054$, $p = 0.038$, mesor = 2,163,869, amplitude = 2,006,059, acrophase = 9 h 2 min, respectively). In contrast to the 15°C acclimation group, gene transcription in erythrocytes from fish acclimated to 8°C was comparatively lower and with noticeably dampened transcriptional

oscillations over the time course, with the possible exception of *hexokinase*, although a time series analysis showed the transcriptional rhythm for this gene was not statistically significant ($F_{2.15} = 0.989$, p = 0.603).

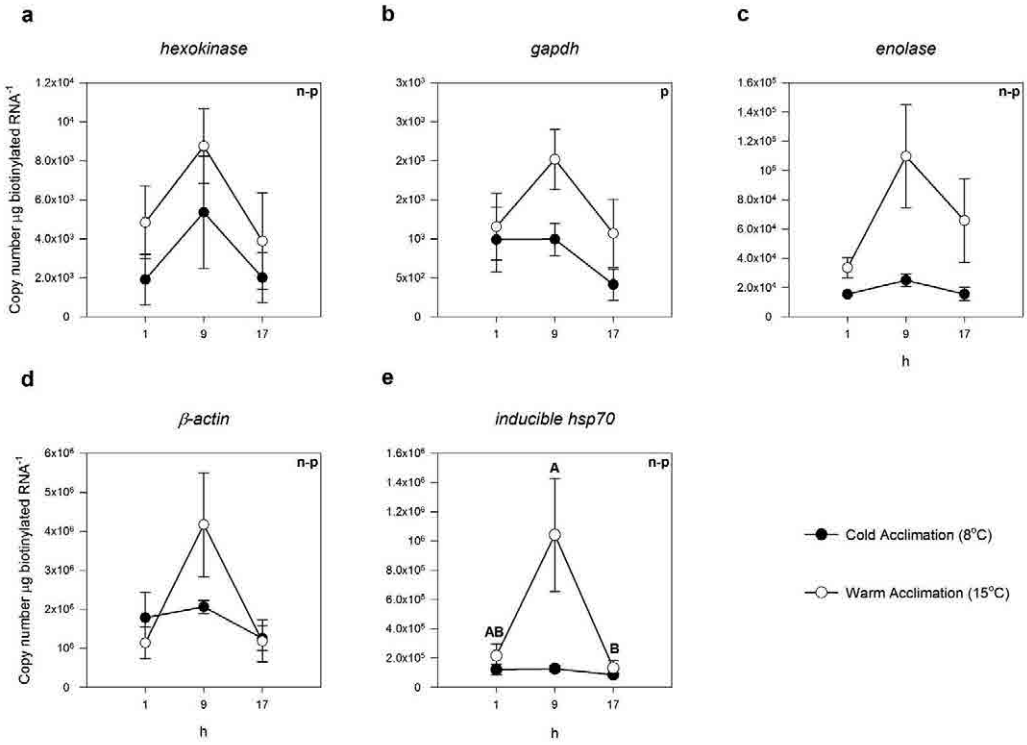

Figure 13 Diurnal Gene Transcription in *S. alpinus* Erythrocytes Acclimated to 8°C and 15°C under an 18:6 L:D photoperiod for four weeks. The transcription of genes encoding glycolytic enzymes (**a-c**), a cytoskeletal protein (**d**) and molecular chaperone (**e**) were quantified at three timepoints across the simulated light cycle. *gapdh* and *enolase* transcription was significantly higher at 15°C (p = 0.049 and p = 0.003, respectively). Statistical significance ($p < 0.05$) between acclimation temperatures and between timepoints within an acclimation temperature was determined by one-way ANOVA or Kruskal-Wallis on ranks (parametric (p) or non-parametric (n-p), respectively) and subsequent *post hoc* analysis (Holm-Sidak (p) or Dunn's test (n-p)). Timepoints within an acclimation temperature with dissimilar capital letters are significantly different from each other. The significant *hsp70* and *β-actin* transcriptional rhythm in 15°C-acclimated erythrocytes (p = 0.024 and p = 0.038, respectively) was resolved using the cosinor periodogram (circadian.org). Mean copy number ± SEM; n = 6 per timepoint (total = 36).

At the mRNA level (Figure 14), *hexokinase*, *enolase* and *hsp70* transcripts were significantly higher at 15 than at 8°C ($H_1 = 11.894$, $p < 0.001$, $H_1 = 12.782$, $p < 0.001$ and $H_1 = 14.656$, $p < 0.001$, respectively), suggesting the increase in transcription of these genes and / or the increase in transcription in middle of the light period contributes to the overall elevation of their respective mRNA pools. mRNA expression of the often-used reference genes *gapdh* and *β-actin* was not significantly different between acclimation groups ($H_1 = 0.361$, $p = 0.548$ and $H_1 = 0.842$, $p = 0.359$, respectively). mRNA expression did not exhibit strong diel fluctuations over the sampling period at 8 and 15°C, although ANOVA initially identified a significant increase in *enolase* mRNA at 15°C ($F_2 = 3.893$, $p = 0.043$) 1 h prior to the dark phase. *Post hoc* testing was less conclusive but did show the tendency for *enolase* mRNA to increase significantly at 17 h compared to starting and midday levels ($p = 0.081$ and $p = 0.059$, respectively). A time series cosinor analysis was also conducted on mRNA data, which confirmed the only significant mRNA rhythm in the expression of *enolase* at 15°C ($F_{2.15} = 3.893$, $p = 0.043$, mesor = 1,812,233, amplitude = 1,089,754, acrophase = 16 h 56 min). In addition, to identify differences in mRNA level between acclimation temperatures at the same timepoint, *t*- or Mann-Whitney *U*-tests were performed, which revealed significantly higher mRNA levels at 15°C for *hexokinase* at 9 h ($p = 0.002$), *gapdh* at 17 h ($p = 0.009$), *enolase* at 9 and 17 h ($p < 0.035$) and *hsp70* at 9 and 17 h ($p < 0.01$). Because of individual and temporal variations in the mRNA level of the genes often used as references in gene expression studies, pair-wise correlation analysis using BestKeeper (Pfaffl 2004) was also conducted on *β-actin* and *gapdh* cycle threshold values, which confirmed their suitability as housekeeping genes, but they were not used for normalisation of the mRNA data. Finally, steady-state mRNA levels in the cytoplasm were approximately one to two orders of magnitude higher than the number of transcripts produced in the nucleus of *S. alpinus* erythrocytes from both acclimation temperatures.

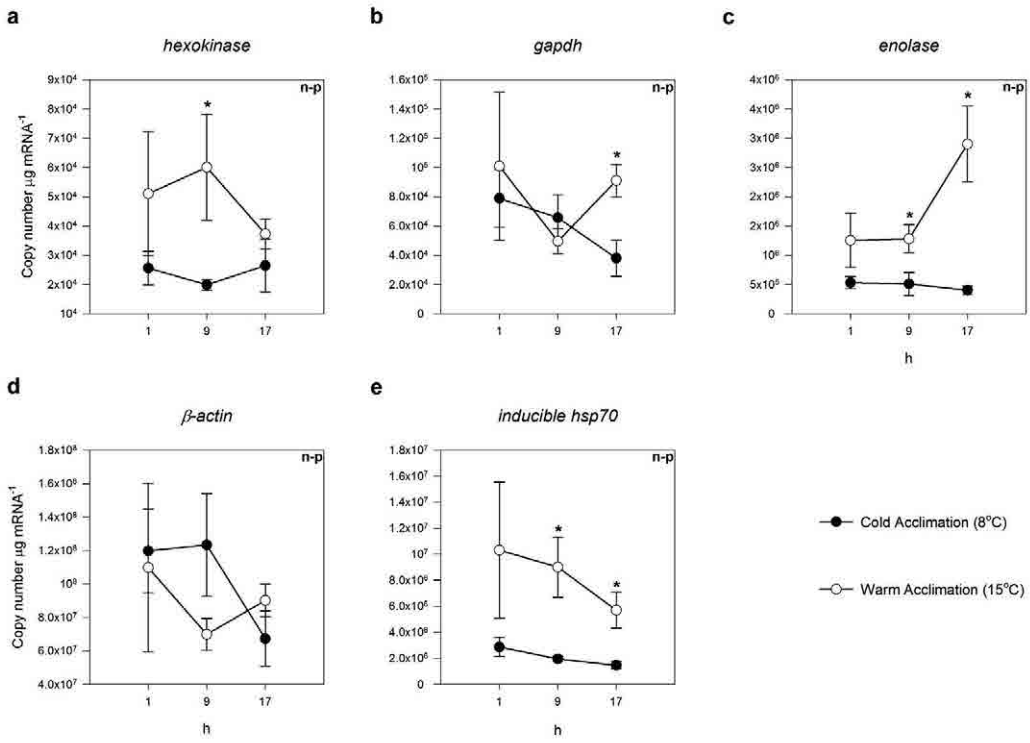

Figure 14 Diurnal Steady-State mRNA Expression in *S. alpinus* Erythrocytes Acclimated to 8°C and 15°C under an 18:6 L:D photoperiod for four weeks. The mRNA level of genes encoding glycolytic enzymes (**a-c**), a cytoskeletal protein (**d**) and molecular chaperone (**e**) were quantified at three timepoints across the simulated light cycle. The mRNA level for *hexokinase*, *enolase* and *hsp70* was significantly elevated at 15°C ($p < 0.001$). Statistical significance ($p < 0.05$) between acclimation temperatures and between timepoints within an acclimation temperature was determined by one-way ANOVA or Kruskal-Wallis on ranks (parametric (p) or non-parametric (n-p), respectively) and subsequent *post hoc* analysis (Holm-Sidak (p) or Dunn's method (n-p)). * denotes significant differences between acclimation temperatures within the same timepoint as identified by *t*- or Mann-Whitney *U*-test. The significant *enolase* mRNA rhythm in 15°C-acclimated erythrocytes ($p = 0.043$) was resolved using the cosinor periodogram (circadian.org). Mean copy number ± SEM; n = 6 per timepoint (total = 36).

Discussion

(**I**) *S. alpinus and S. salar Whole Organism Thermal Tolerance and Tissue Remodelling*

Whole organism thermal tolerance has been thoroughly studied previously in a variety of species of fish by chronically exposing them to constant test temperatures for the purpose of constructing a thermal tolerance polygon, which provides a complete summation of a species' thermal tolerance range across acclimation temperatures (Beitinger & Bennett 2000, Fry *et al.* 1942, Fry 1947). More recent studies have begun to contribute to the increasing number of polygons made from critical thermal measurements involving the gradual increment / decrement in temperature (e.g. Bennett & Beitinger 1997, Cheng et *al.* 2012, Dalvi *et al.* 2009, Fangue & Bennett 2003, King & Sardella 2017, Sarma 2008). There are still far fewer CTMax-CTMin thermal tolerance polygons available for fish however, and data that are available are often created using different rates of temperature change (Lutterschmidt & Hutchison 1997, Mora & Maya 2006), making accurate intraspecies, interspecies and interexperiment comparisons less feasible. A reconciliation of the two primary methods for determining whole organism thermal tolerance has been conducted (Kilgour & McCauley 1986) but comparing and contrasting results from dynamic and constant test temperature experiments can have an inherent methodological bias, specifically with regard to the relationship between time and temperature, that can confound correlations between incipient lethal and critical thermal endpoints. However, CTMax endpoints can be predicted from upper incipient lethal temperature resistance data and *vice versa* with a high degree of accuracy (Bennett & Beitinger 1997, Kilgour & McCauley 1986), suggesting a functional relationship and that some associations between the current and previous studies can be made for a detailed evaluation of thermal tolerance of similar and closely related species.

The CTMax results of the present study concur with previous observations that have demonstrated a lower capacity for *S. alpinus* to tolerate elevated temperatures compared to *S. salar* (Baroudy & Elliott 1994, Elliott 1991), which is consistent with their ecological niche and geographic distribution. An increase in acclimation temperature from 8 to 15°C resulted in a 1.28°C increase in CTMax for *S. alpinus*, signifying a 1°C gain in thermal tolerance for every 5.4°C increase in acclimation temperature, and a 2.17°C increase in CTMax for *S. salar* signifying a 1°C gain in thermal tolerance for every 3.23°C increase in acclimation temperature

(Figure 4). The relationship between CTMax and acclimation temperature when plotted on a graph produces a slope with a lesser incline for *S. alpinus* (1.27) than for *S. salar* (2.17). In the systematic analysis and quantification of thermal tolerance polygons by Beitinger & Bennett (2000), polygons constructed using incipient lethal temperatures consist of upper and lower slopes depicting an upward shift in thermal tolerance through acclimation, and upper and lower plateaus representing intrinsic physiological limits for acclimation, with thermal tolerance remaining constant irrespective of acclimation temperature. Therefore, the size of the thermal tolerance polygon increases as the slope delineating the upper incipient lethal temperature gets steeper and allows for its partitioning into acclimation dependent and independent zones, and the hexagonal or tetragonal shape of the polygon offers additional insight towards a species' capacity for temperature acclimation. For salmonids with available incipient lethal thermal tolerance polygons, the upper and combined upper and lower acclimation dependent zones were generally smallest for *Oncorhynchus* and *Salvelinus* species (Beitinger & Bennett 2000), with *S. fontinalis* having the smallest upper, lower and combined upper and lower acclimation dependent areas despite the largest thermal tolerance polygon among the five species. This indicates *S. fontinalis* has a limited ability to adjust thermal limits through acclimation compared to Pacific salmon *O. keta*, *O. kisutch*, *O. nerka* and *O. tshawytscha*, and conforms with the cold water stenothermal nature typical of the members of the *Salvelinus* genus.

To bring to the fore the similarities and differences in available thermal tolerance data, a potentially useful tool to quantify acclimation dependent acquisition of thermal tolerance is to measure the upper thermal tolerance slope, as has been done for incipient lethal thermal tolerance polygons, according to Beitinger & Bennett (2000) (slope = Δ thermal tolerance / Δ acclimation temperature). Only the two acclimation temperatures presented here were used to assess the shift in upper thermal tolerance, which produced a slope for *S. salar* (0.31) with a value close to that of eurythermal cyprinids such as *C. auratus* (0.39), *Notropis cornutus* (0.32) and *Catostomus commersoni* (0.32) (Beitinger & Bennett 2000), whereas the calculated slope for *S. alpinus* (0.18) was slightly steeper than the closely related brook charr (aka brook trout and speckled trout) *S. fontinalis* (0.13), but is further confirmation of the congeners' stenothermy and limited ability to adjust thermal limits, despite a difference in how whole organism thermal tolerance was determined. A compilation of thermal tolerance ranges across acclimation temperatures for 116 freshwater species, including several salmonids, exposed to dynamic changes in temperature (Beitinger *et al.* 2000), also provides a valuable data resource

from analogous experiments which can reduce possible variations in measurement endpoints stemming from differences in methodology. Thus, using a rate of temperature change approximately a magnitude slower (0.02-0.03°C^{-min}) than the present study, the 1.1°C increase in CTMax from an acclimation temperature of 10 to 20°C (28.7 to 29.8°C, respectively) for *S. fontinalis* (Lee & Rinne 1980) equates to a slope (0.11) similar to that obtained from its incipient lethal temperature polygon (0.13) (Beitinger & Bennett 2000, Fry 1947). Based on the current and previous experiments, the slope representing acquired thermal tolerance emerges as a suitable and additional measurement parameter that is relatively consistent between incipient lethal and critical thermal endpoints for quantifying the effect of acclimation temperature on a species' thermal limits, with the slope value increasing and decreasing depending on the level of eurythermy and stenothermy, respectively.

The most comprehensive evaluation of *S. alpinus* and *S. salar* thermal tolerance and how it is modified by acclimation temperature was conducted using a hybrid of the incipient lethal and critical thermal methodologies to preclude possible effects of handling stress (Baroudy & Elliott 1994, Elliott 1991). Temperature was increased / decreased at 1°C$^{-30\ min}$ (Baroudy & Elliott 1994) to 1°C^{-h} (Elliott 1991) until a test temperature was reached and fish were held at the constant test temperature and mortality monitored over seven days and ten minutes to obtain incipient lethal and ultimate incipient lethal temperatures, respectively. Despite differences in fish size, life stage, acclimation period and protocols used which could affect tolerance measurements (Lutterschmidt & Hutchison 1997, Mora & Maya 2006), the aforementioned together with earlier studies on other members of the Salmonidae family from the genus *Salvelinus*, *Oncorhynchus* and *Salmo* make available a useful index for comparing the thermal tolerance polygons of closely related species (Figure 1). The thermal tolerance polygon of 461°C^2 for *S. alpinus* parr (11-18 cm length) (Baroudy & Elliott 1994) is the smallest existing salmonid polygon, followed by 468-529°C^2 obtained for *Oncorhynchus* spp. fry (4-5 months old, 0.9-1.6 g weight) (Brett 1952). Next is 583°C^2 for *Salmo trutta* juveniles (10-185 g, 10-25 cm) (Elliott 1981), 625°C^2 for *S. fontinalis* juveniles (2-26 g) (Fry 1947) and the largest recorded polygon thus far for a salmonid is 708°C^2 for *S. salar* parr (5-10.2 cm, 1-11 g) (Elliott 1991). Noteworthy is the similar procedure, acclimation duration and fish size used in constructing the thermal tolerance polygons for *S. trutta*, *S. salar* and *S. alpinus*, which provides for a more precise comparison between the three species (Elliott 1995). The size range of fish in the present study was comparable between species at 9 g in weight and 10.6 cm in length to 52 g and 18 cm for *S. alpinus*, which is similar to that of Baroudy & Elliott (1994),

and 15 g and 10.2 cm to 48 g and 17.5 cm for *S. salar*, which is a slightly larger size range than the fish used by Elliott (1991). There was very little variation in the loss of equilibrium temperature within species and acclimation temperatures in the present study, indicating the size range was a negligible factor in the thermal tolerance measurements.

Although incipient lethal temperatures were obtained at a constant test temperature, *S. alpinus*, *S. trutta* and *S. salar* thermal tolerance polygons (Baroudy & Elliott 1994, Elliott 1981, 1991) were excluded from the analysis by Beitinger & Bennett (2000) but included in their study of fish exposed to dynamic changes in temperature (Beitinger *et al.* 2000). A plausible explanation is that the handling stress of the static plunge method and the gradual increase in temperature of 'Elliott's hybrid' / chronic lethal thermal tolerance protocol may have a significant and inverse effect on thermal tolerance measurements (Kilgour & McCauley 1986, Lutterschmidt & Hutchison 1997, Lyytikäinen *et al.* 1997a). The small difference in slope between the two stenothermal *Salvelinus* species may therefore be due in part to the constant temperature increase of CTMax trials for *S. alpinus*, which would likely go beyond the upper incipient lethal temperatures for *S. fontinalis* measured through the static plunge method (Fry 1947), as previously demonstrated for *C. variegatus* (Bennett & Beitinger 1997). It is also possible the slope difference indicates a higher capacity for *S. alpinus* to adjust its upper thermal tolerance limits compared to *S. fontinalis*, which has the second largest salmonid thermal tolerance polygon, although 556°C^2 or 89% of which is acclimation temperature independent (Beitinger & Bennett 2000), characteristic of a high intrinsic thermal tolerance, and the smallest acclimation dependent area indicative of a limited ability for temperature acclimation. However, the only available thermal tolerance polygon for *S. alpinus* was constructed using data from four resident populations in lake Windermere U.K. which produced a polygon resembling a square (Baroudy & Elliott 1994), suggesting that *S. alpinus* not only has a 164°C^2 smaller thermal tolerance polygon but also a lesser capacity for temperature acclimation than *S. fontinalis*. Indeed, the incipient lethal temperatures for Windermere *S. alpinus* juveniles acclimated to 5, 10 and 15°C were 21.5, 22.7 and 22.4°C, respectively (Baroudy & Elliott 1994), and since thermal tolerance polygons permit data interpolation, acclimation to 8°C would have produced an incipient lethal temperature of ~22.7°C and a slope between 8 and 15°C that is virtually horizontal (0.04).

This initial *S. alpinus* thermal tolerance study established a survival threshold of ~22-23°C, beyond which accelerated mortality is observed, and was corroborated by subsequent studies on the same species from higher latitude populations (Elliott & Elliott 2010, Elliott & Klemetsen 2002, Lyytikäinen et al 1997a, Thyrel *et al.* 1999). The result presented here showing a significant parallel increase in Saimaa *S. alpinus* thermal tolerance with acclimation temperature is thus contrary to these previous studies, although it should be reiterated that the 22-23°C setpoint was determined by exposing fish to the temperature for four (Lyytikäinen *et al.* 1997a) to seven days (Baroudy & Elliott 1994), whereas CTMax values were obtained within hours. For *S. salar* parr, incipient lethal temperatures of 24.8, 26 and 27.5°C for fish acclimated to 5, 10 and 15°C, respectively (Elliott 1991), and an interpolated incipient lethal temperature of ~25.5°C for an 8°C acclimation group, are 2.1 and 2.27°C lower than the CTMax values for 8- and 15°C- acclimated *S. salar*, respectively. This difference in *S. salar* incipient lethal and critical thermal endpoints may yet again be a reflection of a difference in suboptimal temperature exposure time, whereas the slope between 8 and 15°C (0.28) is close to the species' CTMax slope (0.31), which provides additional support to the proposition that the acquired thermal tolerance slope is somewhat comparable between the incipient lethal, chronic lethal and critical thermal procedures despite differences in suboptimal temperature exposure time.

The ultimate upper incipient lethal temperature (UUILT) is sometimes referred to as the critical thermal maximum and may be a more suitable metric for CTMax data comparisons, since the timeframe required for obtaining the UUILT is more analogous to the CTMax protocol and a species' CTMax typically overshoots its upper incipient lethal temperature (Bennett & Beitinger 1997, Billman *et al.* 2008). However, although Windermere *S. alpinus* UUILT of ~26.23°C at an acclimation temperature of 8-10°C and 26.36°C at an acclimation temperature of 15°C (Baroudy & Elliott 1994) are closer to the obtained CTMax values (26.71 and 27.99°C, respectively), it is quite evident that when assessing CTMax against UUILT, the acquisition of thermal tolerance from 8 to 15°C was still greater for Saimaa (+1.28°C) than for Windermere (+0.13°C) *S. alpinus*. To appropriately juxtapose the two methods used for determining *S. alpinus* thermal tolerance and to put the different tolerance values in the context of suboptimal temperature exposure time, the 1.63°C difference between the CTMax and UUILT of 15°C-acclimated Saimaa and Windermere *S. alpinus*, respectively, would require an additional 16.3 min (at a rate of $0.1°C^{-min}$) of exposing Saimaa *S. alpinus* to temperatures above the UUILT

for Windermere *S. alpinus*; an exposure time 163% longer than the time required to obtain the UUILT, thus lending credence to the inference that there is a difference in acclimation capacity and thermal tolerance between the two landlocked *S. alpinus* populations. Using a similar method of comparison, the UUILT of 29.9, 31 and 32°C for *S. salar* acclimated to 5, 10 and 15°C (Elliott 1991), respectively, and an interpolated UUILT of ~30.5°C for a theoretical 8°C-acclimation group, are 2.9 and 2.23°C higher than the CTMax values for 8- (27.6°C) and 15°C-acclimated (29.77°C) *S. salar*, respectively, whereas the UUILT slope is less steep (0.21) than the slopes quantified from incipient lethal (0.28) and CTMax (0.31) data. The 2.9°C and 2.23°C difference between UUILT and CTMax endpoints would require an additional 29 and 22.3 min, respectively, of exposing Saimaa *S. salar* to gradual increments in temperature to reach the UUILT for *S. salar* from northern England (Elliott 1991). These results suggest the *S. salar* brood stock from Saimaa is less tolerant of higher temperatures than the Windermere reared progeny of wild anadromous *S. salar* populations from rivers Leven in south Cumbria and Lune in north Lancashire, in keeping with the observation that the fitness of hatchery reared *S. salar* is diminished compared to wild conspecifics (Anttila *et al.* 2011, Besnier *et al.* 2015, Jonsson *et al.* 2003, McDonald *et al.* 1998).

There is a noticeable incongruence in thermal tolerance acquired through acclimation for Saimaa and Windermere *S. alpinus*, and although Baroudy & Elliott (1994) stated that the acclimation period was deemed sufficient based on previous experiments (Elliott 1981, 1991), the uniformity in UUILT and upper incipient lethal temperature boundaries could be the result of partial acclimation of Windermere *S. alpinus* because of the short acclimation period (5-7 days for parr) used in the study, which might also explain the similarities in thermal tolerance of the four Windermere populations with contrasting life histories and habitat preference (Baroudy & Elliott 1994). Alternatively, the difference in temperature acclimation capacity between *S. alpinus* from lakes Windermere (54°N) and Saimaa (62°N) may be due to adaptation to local conditions, which seems counterintuitive since Windermere is close to the southernmost margin of the latitudinal range of *S. alpinus*, where higher habitat temperatures are to be expected and higher latitudes being more typical of the species' preferred environment. The physiology of ectothermic fish is inextricably intertwined with their aquatic habitats which generally exhibit a temperature and photoperiodic gradient encompassing a latitudinal cline. In an attempt to explain an organism's relationship with the thermal characteristics of its environment, two contrasting hypotheses with supporting evidence have

been put forth to explicate how aquatic ectotherms counteract the physiological constraints imposed by the pronounced seasonality, lower temperatures and generally more hostile environments of higher latitudes. According to local thermal adaptation, populations have evolved to maximise performance at temperatures commonly experienced in their habitats (Angilletta *et al.* 2002, Levinton 1983, Somero 2002), exemplified in microcosm in variations in thermal tolerance of anadromous *O. nerka* populations that are consistent with historic temperatures of Fraser river estuaries from which they originated (Eliason *et al.* 2011). Therefore, at a larger geographic scale, local thermal adaptation would suggest higher latitude fish function better at lower temperatures and *vice versa*. In contrast, counter-gradient variation hypothesises higher latitude fish would prefer higher temperatures and perform better at any temperature than lower latitude conspecifics, as a compensatory adaptation to a shorter season for growth (Alvarez *et al.* 2006, Conover & Schultz 1995, Fangue *et al.* 2009, Marcil *et al.* 2006, Schultz *et al.* 1996).

Since thermal preference correlates well with thermal tolerance limits (Jobling 1981, Tsuchida 1995) and counter-gradient variation predicts higher latitude fish would prefer higher temperatures and perform better at any temperature (Alvarez *et al.* 2006, Fangue *et al.* 2009, Marcil *et al.* 2006, Schultz *et al.* 1996), such a theory might be an appealing rationalisation for the possible differences in thermal tolerance between Saimaa and Windermere *S. alpinus*. However, although counter-gradient variation has been reasoned to explain the higher thermal preference of *F. heteroclitus* from a northern population (Fangue *et al.* 2009), the higher thermal tolerance of *F. heteroclitus* from a southern population (Fangue *et al.* 2006), measured using the same CTMax protocol herein, refutes counter-gradient variation as sufficient clarification for the difference in thermal tolerance between the two geographically separated (8°N) landlocked *S. alpinus* populations. Contradictory to the predictions of both local adaptation and counter-gradient variation are investigations on the thermal biology of *S. alpinus* from Fjellfrøsvatn in Norway (69°N) (Elliott & Klemetsen 2002) and lake Inari in Finland (69-70°N) (Lyytikäinen *et al.* 1997a) above the Arctic circle, and *S. alpinus* from two Arctic (Hornavan at 66 and Råstojaure at 68°N) and two sub-Arctic (Torrön at 63 and Blåsjön at 64°N) Swedish lakes (Thyrel *et al.* 1999), which reveal thermal requirements for feeding and growth, and thermal tolerance as determined by Elliott's hybrid and static plunge procedures, do not vary significantly between the Arctic, sub-Arctic and temperate *S. alpinus* populations. These studies have suggested that despite their high phenotypic plasticity (Ahi *et al.* 2014, Baroudy & Elliott 1994, Janhunen *et al.* 2009, Jonsson & Jonsson 2001), *S. alpinus*

thermal tolerance is physiologically fixed regardless of geographic origin. A similar conclusion was reached in a more recent study of thermal tolerance and acclimation capacity of the progeny of four wild allopatric populations of stenothermal lake trout *Salvelinus namaycush* across Ontario Canada (45-48°N), which interestingly had CTMax values at acclimation temperatures 8 and 15°C (26-26.2°C and 28+°C, respectively) (Kelly *et al.* 2014) that are very similar to Saimaa *S. alpinus*, but with a steeper thermal tolerance slope (≥ 0.26) acquired through acclimation. These *S. alpinus* and *S. namaycush* thermal tolerance studies have determined that these salmonid populations exhibit a similar sensitivity to the effects of climate change, and although this assertion seems to be accurate for higher and lower latitude European *S. alpinus* populations (Baroudy & Elliott 1994, Elliott & Klemetsen 2002, Lyytikäinen *et al.* 1997a, Thyrel *et al.* 1999), it would appear Saimaa *S. alpinus* has the ability to tolerate higher temperatures than conspecifics because of an ability to adjust its thermal tolerance window and a vulnerability more akin to that of the progeny of its lower latitude north American congener from native populations (Kelly *et al.* 2014).

The hypothesis that Saimaa *S. alpinus* is more tolerant of higher temperatures than previously studied populations is not entirely consistent with its propagation however, which is solely dependent on an aquaculture rearing program using endangered brood stocks that exhibit a limited genetic diversity, reduced fitness, increased mortality and susceptibility to disease (Huuskonen *et al.* 2003, Piironen & Heinimaa 1998, Primmer *et al.* 1999). Since one of the main aims of the lake Saimaa restocking program was to preserve the population's genetic integrity at the expense of genetic diversity (Brunner *et al.* 1998, Huuskonen *et al.* 2003, Piironen & Heinimaa 1998, Primmer *et al.* 1999), and because of the recent availability of genomic resources for *S. alpinus* (Christensen *et al.* 2018), a prospect for a future study is to confirm whether Saimaa *S. alpinus* is indeed more thermotolerant by conducting the exact same whole organism assay across different *S. alpinus* populations acclimated to several temperatures covering the species' thermal range, and to identify traits that confer thermal tolerance to specific loci in Saimaa *S. alpinus*, which would build upon previous studies on other *S. alpinus* populations (Somorjai *et al.* 2003, Quinn *et al.* 2011a, 2011b), to further elucidate the genetic basis of thermal tolerance and acclimation capacity of a species facing the threat of extinction. If the premise that Saimaa *S. alpinus* has a higher capacity for temperature acclimation is proven to be correct, such a physiological trait would have beneficial consequences in breeding programs for populations less tolerant of future temperature increase.

A fundamental tenet in obtaining a well-rounded understanding of animal physiology is to attempt to link available information at different levels of biological organisation, as an integrative approach to reveal the mechanisms by which a function or physiological response, such as the capacity for temperature acclimation, is accomplished. Thus, as outlined by the OCLTT model (Pörtner 2002, 2010, Pörtner & Knust 2007), a progressive discrepancy in oxygen demand and supply as temperature increases is the unifying principle defining whole organism thermal tolerance, making the organs involved in the systemic supply and delivery of oxygen the primary physiological bottleneck setting a species' upper thermal limits. Consistent with OCLTT is the observation that variations in *S. salar* thermal tolerance is associated with hypoxia tolerance and ventricle size (Anttila *et al.* 2013). Indeed, the increase in *S. alpinus* and *S. salar* CTMax with acclimation temperature was accompanied by morphological changes to the oxygen supply and delivery system (Figure 5), for the likely purpose of enhancing oxygen uptake and metabolic by-product expulsion under conditions of elevated respiration seen in fish kept at suboptimal temperature (Brodte *et al.* 2006, Mark *et al.* 2005, Windisch *et al.* 2011). There was no significant change in gill morphology between species and acclimation temperatures after four weeks of acclimation under normoxic conditions (Figure 5a), however the height of the secondary lamellae increased significantly when *S. salar* was simultaneously exposed to cyclical overnight hypoxia throughout the acclimation to 15°C (Anttila *et al.* 2015). An increase in the surface area of gill secondary lamellae, the primary site of gas exchange between organism and environment, could help improve gas transport and increase hypoxia tolerance (Dhillon *et al.* 2013) and may be involved in hypoxia acclimation of *S. salar*, especially when combined with warm acclimation which would compound oxygen demand for the comparatively hypoxia intolerant salmonid (Anttila *et al.* 2015).

Aside from changes to oxygen utilisation, acute and chronic temperature change can affect the contractile properties of ventricles (Aho & Vornanen 1998, 1999, Alderman *et al.* 2012,) and an important tissue level response in the acclimation / acclimatisation of fish is the remodelling of the cardiovascular system to maintain function and activity as temperature changes with the season (Driedzic *et al.* 1996, Keen *et al.* 2016, Vornanen 1994). The relative ventricle mass (RVM) of *S. alpinus* and *S. salar* was heavier at 8°C and decreased for both species when acclimated to 15°C (Figure 5b), in agreement with observations of *O. mykiss* acclimated to cold and warm (Keen *et al.* 2016, Klaiman *et al.* 2011). In contrast to these previous reports however, temperature acclimation did not have a significant effect on the thickness of *S.*

alpinus and *S. salar* compact myocardium (Figure 5c), which suggests the decrease in RVM at 15°C was due to alterations in the structural properties of the spongy myocardium. The muscle bundle cross sectional area, myocardial fibrillar and connective tissue content of the spongy layer have all been shown to decrease with warm acclimation (Keen *et al.* 2016, Klaiman *et al.* 2011), concomitant to a downregulation of gene transcripts involved in muscle growth (Vornanen *et al.* 2005), collagen deposition and hypertrophy (Keen *et al.* 2016). Conversely, mRNA expression of genes involved in muscle growth, hypertrophy and collagen synthesis are upregulated in cold acclimation, and associated with increased fibrillar collagen deposition, myocyte bundle hypertrophy, hyperplasia and ventricle stiffness (Keen *et al.* 2016, Klaiman *et al.* 2011, Sun *et al.* 2009). Collagen and other extracellular matrix proteins provide structural support to cardiomyocytes and are crucial components in cardiac compliance during diastolic filling and systolic emptying of ventricles (Janicki & Bower 2002). A higher connective tissue content, increased hypertrophy and hyperplasia would therefore result in a heavier RVM for *S. alpinus* and *S. salar* acclimated to 8°C. These changes to organ structure are believed to enhance pressure generation to efficiently deliver blood to tissues, whilst protecting cardiomyocytes from increased haemodynamic load due to higher blood viscosity at lower temperatures (Klaiman *et al.* 2011, 2014). In contrast, the reduction in blood viscosity and higher intracellular diffusivity of gases at higher temperatures would require less systolic pressure to deliver the same volume of blood to tissues and less spongy layer surface area to oxygenate cardiomyocytes, respectively. These observations on the remodelling of ventricular phenotype demonstrate the remarkable plasticity of cardiac function in the temperature acclimation of salmonids, suggested to be the defining attribute in the capacity of *S. salar* to cope with future climate conditions (Anttila *et al.* 2014).

There was a significant difference in the tissue composition of ventricles between species, with *S. alpinus* having a heavier and thicker RVM and compact myocardium, respectively, than *S. salar* at either acclimation temperature. The difference in RVM is consistent with a comparative study of the two salmonids (Penney *et al.* 2014) and confirms a larger heart is characteristic of the *S. alpinus* body plan. The cardiomyocytes of most teleost fish obtain oxygen from luminal blood passing through the anastomosing lacunae of the spongy myocardium, whereas the ventricular pericardium of salmonids also consists of a compact layer supplied by well oxygenated blood from the coronary circulation. Some studies have reported the thickness of the compact myocardium and its vascularisation increase in parallel with acclimation temperature in salmonids (Anttila *et al.* 2015, Eggington & Cordiner 1997,

Klaiman *et al.* 2011), although a similar acclimation induced remodelling of the compact layer was not observed in the present study, which conducted statistical analysis on morphological changes to ventricles only on fish that underwent CTMax trials. It is possible the difference in tissue level measurements was due to a reduction in statistical power arising from fewer individuals used here compared to a closely related study (Anttila *et al.* 2015). However, the trade-off for assessing the relationship between CTMax and the oxygen supply and delivery system that comes at the expense of replicate number and statistical power was deemed necessary for a more precise evaluation of the role of morphological changes to key organs crucial in defining whole organism thermal tolerance. Notably, although the RVM and compact layer of *S. alpinus* ventricles was heavier and thicker, respectively, the compact layer of *S. salar* ventricles was significantly more vascularised (Figure 5d), particularly at the lower acclimation temperature. Indeed, the trend in gill and ventricle remodelling appears to be similar for the two species despite a divergence in their thermal tolerance, thermal optima and hypoxia tolerance (Antilla *et al.* 2015, Elliott & Elliott 2010), which suggests the morphological changes observed in response to warm acclimation is a general feature for both cold water species and that additional physiological factors may also be involved in determining the limits of their thermal and hypoxia tolerance (Sokolova *et al.* 2012).

The height of gill secondary lamellae, RVM and compact myocardium thickness while seeming to decrease with increasing acclimation temperature, the capillary density of the compact myocardium appears to increase in parallel with acclimation temperature in a manner reminiscent of the changes to the species' CTMax, leaving room for speculation that the vascularisation of the compact layer may be a good indicator of and correlates well with the thermal tolerance of the studied salmonids, potentially to reduce reliance on the low oxygen partial pressure of venous blood oxygenating the spongy myocardium and to ensure sufficient delivery of oxygen rich blood to the cardiomyocytes primarily responsible for ventricular contractile forces generating systolic and diastolic pressure (Axelsson & Farrell 1993, Gamperl *et al.* 1995, Poupa *et al.* 1974). Moreover, since *S. alpinus* is more tolerant of low oxygen levels but intolerant of high temperatures, whilst the reciprocal is true for *S. salar* (Anttila *et al.* 2015), the likelihood that a greater RVM and compact layer thickness may be more related to the species' hypoxia tolerance rather than thermal tolerance is a viable conjecture. In support of this is the observed effect of temperature increments ($2°C^{-h}$) on the cardiorespiratory response of seawater- and 9.5-10°C- acclimated *S. alpinus* and *S. salar* being related to their CTMax (23 and 26.5°C, respectively) (Penney *et al.* 2014), with the increase in oxygen consumption,

maximum metabolic rate and maximum heart rate significantly lower for *S. alpinus* (by ~27%, 35% and 13% or 116 vs 134 beats^{-min}, respectively). In addition, although basal oxygen consumption and heart rate were comparable between the two species in this study, the basal heart rate of *S. alpinus* was 8 beats^{-min} slower and statistically significant at 90% confidence, indicating that the species' larger ventricle accomplishes a similar cardiac output to *S. salar* but at a lower contraction frequency (Penney *et al.* 2014).

Bradycardia allows organisms to cope with hypoxia during extended periods of submersion, investigated in detail in diving marine mammals (Williams *et al.* 2015) and is a physiological mechanism similarly utilised by fish during low oxygen tension (Glass *et al.* 1991, Randall 1982, Smith & Jones 1982). Thus, the larger heart and lower heart rate associated with *S. alpinus*, in conjunction with a torpid-like behaviour when oxygen level is low (personal observation), may explain the species' tolerance of hypoxia which comes at a cost to thermal tolerance, as observed in *O. mykiss* (Roze *et al.* 2013). The high latitude lacustrine environments of overwintering anadromous and resident landlocked *S. alpinus* populations are typically enclosed by ice for a significant portion of the year (Strand *et al.* 2008, Jørgensen & Johnsen 2014), restricting not only the diffusion of atmospheric oxygen into the water column but also further reducing the level of illumination that can stimulate photosynthesis, which together with persistent respiration at every trophic level, albeit at a diminished rate, can result in prolonged highly hypoxic conditions. Conversely, to avoid the elevated temperatures of the thermally stratified limnetic and littoral zones of their habitat during the summer months, *S. alpinus* can behaviourally thermoregulate by residing in the profundal zone (Makkonen 1997, Seppovaara 1969) where temperature and oxygen tension are expected to be lower. Such circumstances and the historically low and comparatively stable temperatures of higher latitudes may have exerted strong selective pressure on *S. alpinus* evolution and could be a plausible explanation for the species' lower tolerance of elevated temperature and higher tolerance of hypoxia compared to *S. salar* (Anttila *et al.* 2015).

There has been a resurgence of interest pertaining to the relationship of ectotherms with their changing environment as global temperatures maintain their upward trajectory. The conclusion that *S. salar* has a higher capacity to tolerate elevated temperatures than *S. alpinus* is by no means a novel finding, as quantifying the thermal tolerance of fish was pioneered nearly 80 years ago and paved the way for successive studies of practical significance on a plethora of

species of considerable ecological and economic value. It was established then, as it has been now, that amongst the Salmonidae family, its northernmost distributed and least thermotolerant member displays the utmost susceptibility to temperature increase, especially as their high latitude habitats continue to experience the most pronounced shifts in temperature. Detailed thermal tolerance studies have been conducted in a variety of salmonids, including the stenothermal *S. alpinus* and eurythermal *S. salar* initiated approximately 30 years ago, and it would have been remiss to overlook these more comprehensive investigations as they present an opportunity to make reasonable comparative interexperimental assessments of closely related species and populations. On one hand, beneficial insights can be gleaned from available experimental data for an integrated view of the vulnerability of different species and populations to contemporary and forthcoming environmental challenges despite methodological differences, providing the relationship between temperature and exposure time in resolving whole organism thermal tolerance is sufficiently taken into account. On the other hand, the evaluation of current and previous thermal tolerance data further emphasises the need for standardisation of whole organism measurements for a more accurate comparative analysis, and in order to obtain a global view of interpopulation susceptibility to temperature increase. The ability to acclimate to a new temperature is an important determinant of an organism's survival and quantifying the slope delineating upper thermal tolerance acquired through acclimation may provide a concise and complementary metric for a more thorough appraisal of whole organism thermal tolerance. Ascertaining the proximity of species to their thermal limits and to what extent they are able to adjust their thermal range are essential for accurately predicting the effects of future temperatures, for an improved understanding of the repercussions of a rapidly changing climate and the necessary measures to be undertaken in the management and conservation of natural and domesticated fish populations.

(**II**) *Species- and Acclimation-Specific mRNA Expression and Translation of Inducible hsp70*

The steady-state level of *hsp70* mRNA and hsp70 was significantly more elevated in the gills and liver of 15°C-acclimated *S. alpinus* and 8°C-acclimated *S. salar*, in agreement with the specific thermal requirements of both species (Elliott & Elliott 2010), which despite overlapping in their geographic distribution and have dissimilar optimal thermal breadths wherein physiological performance and fitness are maximal (Jonsson *et al.* 2001, Larsson *et al.* 2005), are often found as sympatric populations in river and lake systems (Svenning *et al*. 2016). *S. alpinus* prefer and inhabit colder waters than *S. salar* (Elliott and Elliott 2010), and

in an aquaculture setting *S. alpinus* is typically reared at temperatures closer to 8°C (Baroudy & Elliott 1994, Huuskonen *et al.* 2003, Jobling *et al.* 1998, Lyytikäinen *et al.* 1997a) and *S. salar* closer to 15°C (Handeland *et al.* 2008), as is the case in the present and most likely all aquaculture facilities. Indeed, the lower temperature limit for feeding and growth and where *S. salar* begin to show signs of inactivity was previously determined to be ~7°C and ≤ 9°C, respectively (Allen 1940, 1941, 1969, Elliot 1991, Gibson 1978, Rimmer *et al.* 1983). This difference in thermal optima is reflected in *hsp70* and hsp70 levels, and the size of fish post-acclimation as a surrogate for growth (Figure 6). The increase in *hsp70* transcripts at 15°C is consistent with a previous observation (Quinn *et al.* 2011b) and the increase in hsp70 in the present study reconfirms that among the salmonids, *S. alpinus* is the least tolerant of high temperatures and most tolerant of low temperatures (Baroudy & Elliott 1994, Elliott 1995).

A decrease in temperature is generally thought to bring about an increase in molecular stability, primarily through a reduction in the Brownian motion of molecules (Einstein 1956, Feller *et al.* 1997), although evidence for protein denaturation at lower temperatures also exists (Dean & Woo 2005, Hochachka & Somero 2002, Teigen *et al.* 2015) and may be a rational explanation for the elevated hsp70 levels in 8°C-acclimated *S. salar*. The conformational stability and flexibility of proteins was selected for during the course of a species' evolution (Fields 2001) and designed to function within the boundaries of its thermal tolerance window, digressions from which increases the likelihood of protein misfolding. Whether the increase in *hsp70* and hsp70 levels in 8°C-acclimated *S. salar* was due to an increase in protein denaturation or an increase in the stability of *hsp70* transcripts and protein products, can be challenging to disentangle however. hsp70 expression is a tightly regulated process as it incurs a cost to organism bioenergetics and fitness, with unregulated levels that can be disadvantageous to physiology (Hoekstra & Montooth 2013, Feder *et al.* 1992, Krebs & Feder 1997, Silbermann & Tatar 2000, Yamashita & Hojo 2004), making it more plausible that the combination of increased protein denaturation and stability of *hsp70* and hsp70 accounts for the increase in mRNA and protein abundance observed in 8°C-acclimated *S. salar*, rather than passive thermodynamic effects on molecular stability alone.

Supporting this supposition is the increase in *cold inducible RNA binding protein* (*cirbp*) mRNA in cold acclimated *O. mykiss* (Rebl *et al.* 2013) and *Cyprinus carpio* (Gracey *et al.* 2004), which suggests the stabilisation of transcripts required at low temperatures also involves

active cellular mechanisms. Another potential justification for the elevation of heat shock proteins in 8°C-acclimated *S. salar* is the finding that cold acclimated fish increase protein synthesis, mitochondrial activity and proliferation (Bouchard & Guderley 2003, Dhillon & Schulte 2011, Foster *et al.* 1992, Hardewig *et al.* 1999, Kraffe *et al.* 2007) which may require enhanced chaperoning activity. It is also conceivable the increase in metabolism to offset the effect of low temperature elevates oxidative stress which can induce protein denaturation. The elevation of mRNA and protein levels at 15°C for *S. alpinus* and at 8°C for *S. salar* may therefore be an accurate indicator of an increase in protein misfolding. Furthermore, these results highlight a central role for the inducible isoform in the acclimation of the studied salmonids to suboptimal temperature and the improved sensitivity and utility of a salmonid-specific inducible hsp70 antibody as an ecophysiological biomarker for thermal stress, as previously demonstrated in wild *S. fontinalis* (Chadwick *et al.* 2015) and *S. namaycush* (Kelly *et al.* 2014) populations from various field sites experiencing different increases in habitat temperature.

The ability of extremophiles to thrive in severe environments on the edge of where life on Earth can exist has provided important insights on the evolutionary thermal adaptation of various cellular proteins. For example, 3-Isopropylmalate dehydrogenase in the thermophilic *Thermus thermophilus* and the mesophilic *E. coli* was determined to have a similar level of flexibility at their respective optimal growth temperatures of 65 and 37°C, although the enzyme from the thermal vent residing bacterium has a 17°C higher denaturing temperature and a higher conformational rigidity at room temperature than the *E. coli* homologue (Závodszky *et al.* 1998). This indicates increased conformational flexibility is a necessary adaptation to preserve *E. coli* enzyme activity at the lower temperature, whereas the increase in *T. thermophilus* enzyme thermostability allowed the bacterium to carve out an existence at elevated temperature that would otherwise have been disruptive to enzyme function. An identical observation was made in the comparative analysis of the thermal properties of lactate dehydrogenase A_4 from several Patagonian and Antarctic multicellular ectotherms, with amino acid substitutions that bestowed increased protein flexibility, whilst preserving catalytic activity, crucial for the survival of Antarctic notothenioid species at -1.86 to 2°C (Fields & Somero 1998). A more recent analysis of orthologous sequences from the extreme stenothermal Antarctic *Pachycara brachycephalum* and the temperate *Zoarces viviparus* also reveal in part the basis for the temperature adaptation of these confamiliar species at the DNA / RNA and protein level, which in *P. brachycephalum* involves a decrease in genomic guanosine and cytosine content; a more

thermostable base-pairing than its counterpart, an increase in the uncharged polar amino acid serine believed to increase solvent interaction, and a decrease in glutamic acid to reduce ionic interactions and the stabilising effect of salt bridges (Windisch *et al.* 2012).

S. alpinus has a high latitude and Holarctic distribution, which have aquatic habitats that, although at the vanguard of climate change effects, are characterised by low, relatively stable temperatures. This could have exerted strong selective pressure for protein constituents with amino acid substitutions that confer increased flexibility to offset the stabilising effects of low temperature (Fields 2001, Hochachka & Somero 2002, Teng *et al.* 2010, Závodszky *et al.* 1998). Such a trade-off is beneficial in environments with low temperatures as protein dynamism and function is retained, but detrimental as temperatures increase since a more flexible protein would become progressively agitated and have a higher propensity to denature. The finding that the *S. alpinus* genome has nearly three times more putative inducible *hsp70* genes than *S. salar* and *O. mykiss* (see introduction) is further support of this postulate. A species' genome only has a limited number of genes and having more *hsp70* copies would allow this stenothermal salmonid to mount a rapid and robust heat shock response to protect its thermally sensitive proteins upon encountering thermal stress. In contrast, a relatively eurythermal salmonid like *S. salar* has a higher optimal temperature and temperature preference, a broader thermal range (Elliott 1991), and as revealed in a variety of eurythermal and stenothermal fish (Fields & Somero 1998, Hochachka & Somero 2002, Windisch *et al.* 2012), a proteome most likely better suited for higher temperatures and across a wider thermal window compared to *S. alpinus*. The recent assembly of both *S. alpinus* (Christensen *et al.* 2018) and *S. salar* (Lien *et al.* 2016) genomes permits the validation of this inference, through the comparative analysis of both species' genetic makeup using a nucleotide and amino acid sequence-based algorithm, eScape (Gu & Hilser 2008), as previously conducted in thermophilic and mesophilic prokaryotes (Gu & Hilser 2009). The number of genomes in public repositories has gradually increased as sequencing technologies have become more accessible, making it possible to analyse a vast amount of sequence data from a diverse range of organisms from different thermal habitats, in order to shed light on the evolutionary process of thermal adaptation at a genome- and proteome-wide scale (Tekaia *et al.* 2002).

The physiology and behaviour of virtually all organisms evolved to keep in time with the periodicity of the light-dark cycle and accompanying changes in temperature, particularly for aquatic ectotherms which rely on ambient temperature for their thermal energy. Biological

rhythms are integral to the anticipatory and adaptability of organismal responses to the circadian and seasonal changes to photoperiod and temperature, which become more pronounced with increasing latitude, yet the interaction between environmentally dictated rhythms, the synchronicity of organism responses and how they may be disrupted is an underexplored subject (Prokkola & Nikinmaa 2018). Anthropogenic activities are accelerating the increase in habitat temperature at high latitudes and driving a progressive mismatch in the photoperiod-temperature relationship, the consequences of which on the temperature responses and the circadian and seasonal rhythms of resident taxa remain shrouded in much uncertainty. Upper thermal tolerance (CTMax) exhibits circadian rhythmicity concurrently with and which may be related to the steady-state level of inducible *hsp70* mRNA in *F. heteroclitus* (Fangue *et al.* 2006, Healy & Schulte 2012), whilst the possibility that different heat shock protein isoforms (i.e. *hsc70*) are involved and have contrasting roles in temperature responses may help explain thermal tolerance and heat shock response variations in closely related subspecies (Fangue *et al.* 2006).

The circadian regulation of inducible *hsp70* mRNA expression appears to be similar between the more distantly related salmonids however, as *hsp70* transcripts exhibited a significant rhythm in the liver of 15°C-acclimated *S. alpinus* (Figure 7) and *S. salar* (Figure 8), although peaking 1 h prior to the dark phase in the former and 4 h into the dark phase in the latter species. In a similar study, 2,080 genes or 5% of the transcriptome in 15°C-acclimated *S. alpinus* liver displayed accentuated circadian oscillations, with the largest increase in mRNA level also occurring 1 h prior to the dark phase (Prokkola *et al.* 2018). Provided that this increase in transcriptome rhythmicity is accompanied by protein synthesis, the pronounced *hsp70* rhythm and higher *hsp70* and hsp70 levels in 15°C-acclimated *S. alpinus* liver may be due to a heightened demand for inducible molecular chaperones, to accommodate the increase in the expression of other genes and / or the tendency for the produced proteins to denature at the acclimation temperature. Following the trend of enhanced rhythmicity are the levels of *hsp70* mRNA and hsp70 in the liver of 8- and 15°C-acclimated *S. salar*, respectively, which suggests gene expression may be highly rhythmic in this organ, in line with its prominent role in many physiological processes that exhibit circadian rhythms (Prokkola *et al.* 2018, Wang *et al.* 2018), whereas in 8°C-acclimated *S. salar* the close to significant rhythm could be attributed to the general increase in *hsp70* transcripts masking the undulating pattern of its expression, as previously determined in a terrestrial ectotherm (Vallone *et al.* 2007).

Investigations on the role and regulation of heat shock proteins in the temperature acclimation / acclimatisation of fish is scarce compared to their characterisation in the heat shock response, with earlier studies not being able to discriminate between constitutive and inducible isoforms (Dietz 1994, Dietz & Somero 1992, 1993) and / or having only assessed changes to mRNA (Currie *et al.* 2000, Jeffries *et al.* 2014, Quinn *et al.* 2011a, 2011b) or protein level (Chadwick *et al.* 2015, Currie & Tufts 1997, Kelly *et al.* 2014, 2018, Stitt *et al.* 2014, Tunnah *et al.* 2017). A few heat shock protein studies in fish have correlated changes to mRNA and protein level in the heat shock response (Airaksinen *et al.* 1998, Buckley *et al.* 2006, Fangue *et al.* 2006, Hofmann *et al.* 2005, Lund *et al.* 2002, 2006) but fewer still have assessed the temporal relationship between the transcription of a specific heat shock protein gene and the translation of the produced transcripts (Buckley *et al.* 2006), despite the widely held view in model organism cell biology (Greenbaum *et al.* 2003, Gygi *et al.* 1999, Schwanhäusser et al 2011), which is also becoming increasingly apparent in non-model organism studies (Buckley *et al.* 2006, Hofmann *et al.* 2005, Lund *et al.* 2002), that mRNA expression can be uncoupled from protein expression. Most studies on heat shock protein expression in homeotherms and poikilotherms were evaluated by acutely exposing cells or whole organisms to elevated temperatures rarely experienced in their natural environment, mostly for the purpose of eliciting an immediate, robust and measurable heat shock response.

Consequently, it is often assumed that an increase in *hsp70* is always accompanied by an increase in hsp70. In the present study, although *hsp70* mRNA and hsp70 expression correlated well overall, the relationship between transcript and protein production was not necessarily synchronous since an increase in mRNA was not always accompanied by an increase in protein at the same or succeeding timepoints; an observation exemplified in the liver of 15°C-acclimated *S. alpinus* which had a significant increase in *hsp70* mRNA at 17 h but hsp70 level remaining unchanged and even appearing to continue its gradual decline, raising the possibility that this rhythmicity in mRNA expression may be genetically programmed rather than a physiological response to protein denaturing stress. Nevertheless, this disconnect in *hsp70* mRNA and hsp70 expression has implications in the interpretation of qPCR, microarray and RNA-Seq data. In addition, the ratio of mRNA and protein within the same acclimation temperature and tissue of either species was also not consistent (i.e. the relationship is not 1:1) when comparing the difference in magnitude of expression between acclimation temperatures, although much of this is predicated upon the assumption that mRNA and protein levels for the reference β-actin are consistent across timepoints and acclimation temperatures.

Genome-scale quantification of mRNA and protein expression in model organisms reveal mRNA level only accounts for ~40% of the variation in protein level in mammalian cells (Schwanhäusser *et al.* 2011) and that protein abundance typically exceeds the level of their respective transcripts (Ghaemmaghami *et al.* 2003, Schwanhäusser *et al.* 2011). Despite an inability to explain 60% of the variation, the authors deemed this correlation to be better than previously thought, further bolstering the standpoint that functional protein level measurements should be conducted in conjunction with mRNA expression to be able to make accurate deductions regarding physiological responses. In terms of protein level exceeding mRNA level, while this appears to be true for cold acclimated *S. salar* gills, *hsp70* mRNA seems to be more abundant than hsp70 in warm acclimated *S. alpinus* gills and cold acclimated *S. salar* liver, and to some extent in warm acclimated *S. alpinus* liver. It should be noted that *hsp70* mRNA serves as a better proxy for hsp70 level in 8°C-acclimated *S. alpinus* and 15°C-acclimated *S. salar* tissues, which suggests steady-state mRNA and protein levels correlate better when the acclimation temperature is close to the optimal thermal requirements of the species and hsp70 expression is at basal levels. These preliminary steady-state observations hint at the possible involvement of post-transcriptional mechanisms in determining transcript and protein abundance of inducible hsp70 in the temperature acclimation of salmonids.

The transcription of heat shock protein genes has not been directly measured in fish and measuring the transcription of other inducible genes is rarely conducted, possibly due to technical issues associated with the nuclear run-on transcription assay (Sambrook & Russell 2001) and hurdles in quantifying transcription *in vivo*. For example, intact tissues need to be perfused, the extracellular matrix enzymatically degraded and the cells initially kept as primary cultures prior to experimental treatment and efficient isolation of nuclei (Bemanian *et al.* 2004, Fluoriot *et al.* 1996, 1998, Nelson & Sheridan 2006, Pakdel *et al.* 1997, Sadar *et al.* 1996), thus distancing them from *in vivo* physiological conditions. However, available data indicate that as with endothermic and ectothermic model organisms (Bohem *et al.* 2003, Morimoto 1993, 1998), heat shock protein expression in fish is primarily regulated at transcription (Airaksinen *et al.* 1998, Currie & Tufts 1997) and changes to transcript level are generally regarded as accurately mirroring changes to the level of the functional protein product, particularly for the most abundant and major inducible molecular chaperone hsp70. Although there is still a sizeable gap in the holistic understanding of the cellular mechanisms involved in heat shock protein regulation, especially in non-model organisms living in environments already

experiencing the effects of climate change, acclimation induced modifications to HSF-1 binding activity, changes to the threshold induction temperature for enhanced heat shock protein synthesis, the temperature at which heat shock protein synthesis is maximal and the steady-state level of heat shock proteins (Buckley *et al.* 2001, Buckley & Hoffmann 2002, Dietz 1994, Dietz & Somero 1992, 1993, Lund *et al.* 2006, Tomanek & Somero 2002), together provide evidence for the plasticity in the control of heat shock protein expression corresponding to the recent thermal history of aquatic organisms and fits well within the framework of the cellular thermometer model. This molecular mechanism mostly describes a negative feedback loop system, whereby the threshold for induction and repression of synthesis depends upon the level of pre-existing heat shock proteins in the cytosol and nucleus (Morimoto 1993, 1998). Aside from the function of hsp70, hsp40 and hsp90 as negative regulators of their own transcription (Abravaya *et al.* 1992, Ali *et al.* 1998, Baler *et al.* 1992, Shi *et al.* 1998, Wang *et al.* 2006, Zou *et al.* 1998), there is little evidence for the involvement of other post-transcriptional mechanisms in heat shock protein regulation in fish that could serve to fine-tune the cellular response to a stress stimulus. Alluding to the importance of post-transcriptional control of hsp70 expression however, is the identification of a 3´untranslated region (UTR) regulating *hsp70* mRNA degradation in the model ectotherm *Drosophila* (Petersen & Lindquist 1989) and endotherm *H. sapiens* (Moseley *et al.* 1993), and an element in the 5´ UTR of *H. sapiens hsp70* mRNA which enhances translational efficiency (Vivinus *et al.* 2001).

Temperature fluctuations provoke protein denaturation which in turn is expected to stimulate transcriptional and translational induction of heat shock proteins, with a small temperature increase eliciting minimal synthesis and a significant increase producing a greater heat shock response. A +7°C temperature change to 15°C did not cause an increase in mRNA and protein level in 8°C-acclimated *S. alpinus* and *S. salar*, but in keeping with the mechanistic regulatory paradigm is the canonical heat shock response observed in 15°C-acclimated *S. alpinus* exposed to 22°C (Figure 9); a temperature approximating the incipient lethal temperature (22-23°C) for Arctic, sub-Arctic and temperate *S. alpinus* populations (Baroudy & Elliott 1994, Elliott & Klemetsen 2002, Lyytikäinen *et al.* 1997a, Thyrel *et al.* 1999). The significant increase in *hsp70* and hsp70 after only 30 min of exposure to 22°C provides clarification at the subcellular level as to why at this temperature, feeding ceases for the Arctic and sub-arctic Swedish lake *S. alpinus* populations (Thyrel *et al.* 1999), and why lakes Inari, Windermere and Fjellfrøsvatn *S. alpinus* suffer 50% mortality after four (Lyytikäinen *et al.* 1997a) to seven days (Baroudy &

Elliott 1994, Elliott & Klemetsen 2002), respectively. Taken together, the general view that a concession to organism fitness is made in upregulating hsp70 expression, appears to have some bearing also for *S. alpinus*. The brief transfer of *S. alpinus* to 22°C resulted in a significant elevation of *hsp70* mRNA 1 h post-heat shock, followed closely by an increase in protein production reaching peak levels by 16-24 h in gills and 2 h in the liver, whilst transcript levels continued to decline from 2 h to basal levels by 8 h. This indicates that *S. alpinus* is already experiencing considerable thermal stress at 22°C and that hsp70 synthesis occurs faster in the more metabolically active hepatocytes, likely due to having a higher density of ribosomes required in their central role in many physiological and synthetic processes, including inducible detoxification (Hinton *et al.* 2008, Levine & Oris 1999, Sadar *et al.* 1996).

The time lag between the transcription and translation of a gene has previously been described for hsp70 in the heat shock response of *G. mirabilis* (Buckley *et al.* 2006) but remains inadequately characterised and taken into account in transcriptomic studies (Logan & Buckley 2015). Although the time lag between transcription and translation can be explained by ribosome and substrate availability, the biological significance of the time lag between peak *hsp70* mRNA level (1 h) and peak and subsequent persistence of hsp70 level (from 2-24 h in liver and 16-24 h in gills) is not entirely certain, as it seems to suggest that the signal for increased transcription is different from the reason for maximal hsp70 level. If the purpose of increased hsp70 synthesis is to negate the effect of acute thermal stress, then why do hsp70 levels continue to increase or remain elevated after the protein denaturing stress has long abated and mRNA having subsided to baseline levels hours prior? Aside from protection from immediate stress, it has been suggested that hsp70 also plays a role in the longer-term adaptation of an organism to its environment (Basu *et al.* 2002, Lindquist & Craig 1988, Sørensen *et al.* 2003), as the elevated steady-state *hsp70* and hsp70 levels in the present study would attest. It may be that the maintenance of elevated hsp70 levels 24 h after the mild heat shock serves a purpose trifecta: to ensure transcriptional inhibition of the heat shock response, as a buffering strategy in anticipation of possible further changes in temperature and conservation of invested and unspent cellular energy, the latter of which would otherwise be allocated to proteasomal degradation of hsp70 instead of growth related protein synthesis. Since a considerable amount of energy was invested in synthesising hsp70, this investment can be maximised fully by inhibiting degradative pathways and maintaining elevated hsp70 levels, the foldase activity of which only requiring approximately three orders of magnitude less ATP

needed to break down and resynthesise hsp70 from amino acid building blocks (Sharma *et al.* 2010).

As with *S. alpinus*, 15°C-acclimated *S. salar* was transferred to 22°C for 30 min, which happens to be the upper temperature limit for feeding for *S. salar* (Elliott 1991). After the +7°C exposure, *hsp70* mRNA increased significantly at 1 h and subsequently declined by 2 h and to basal levels by 4 h post-heat shock; half the time it took for *S. alpinus hsp70* mRNA level to reach pre-heat shock values (Figure 10). Although the mRNA level response was similar between the two warm-acclimated species, the decrease in mRNA level was faster in *S. salar*, indicative of an active role for *hsp70* mRNA degradation when the denaturing environment is manageable for the pre-existing level of hsp70 in the less thermally stressed salmonid; a conjecture supported by the observation that the 3′ UTR of *hsp70* mRNA in *Drosophila* and *H. sapiens* is an important determinant of transcript level by enhancing stability and facilitating translation of hsp70 upon heat shock, whilst promoting mRNA degradation during recovery (Moseley *et al.* 1993, Petersen & Lindquist 1989). Furthermore, the heat shock induced increase in mRNA was not accompanied by an increase in protein synthesis, with hsp70 being significantly more elevated prior to the +7°C temperature change in both 8- and 15°C-acclimated *S. salar*. The increase in mRNA and the lack of a protein level response indicates that the heat shock temperature of 22°C is a sufficient temperature increase for protein denaturation to occur and for the denatured proteins to compete with HSF-1 for available heat shock proteins, thus freeing the transcription factor from inhibition to upregulate *hsp70* transcription. It is also possible increased mRNA stability of newly synthesised and existing transcripts immediately after the heat shock is a contributing factor in elevating *hsp70* mRNA level (Moseley *et al.* 1993, Petersen & Lindquist 1989). However, the level of protein denaturation was not severe enough for translational induction, presumably because the pre-heat shock level of hsp70 was adequate to refold the proteins denatured by the 30 min 7°C temperature increase and to re-sequester HSF-1 soon after to terminate *hsp70* transcription, although this repression of transcription illustrated in the cellular thermometer model cannot account for the repression of protein synthesis and suggests additional downstream control mechanisms.

Post-transcriptional mechanisms regulating hsp70 synthesis have been elucidated in more detail in mammalian cells, with an element in the 5′UTR of *hsp70* mRNA that can form a stable secondary structure upon thermal stress to enhance translational induction (Vivinus *et*

al. 2001). In addition, trans-acting factors such as the RNA-binding protein human antigen R (HuR) which exhibits a high degree of conservation across distantly related taxa (Good 1995), has been shown to stabilise *hsp70* mRNA under protein denaturing conditions in mammalian cells (Amadio *et al.* 2008, Gallouzi *et al.* 2000), whereas the increase in the stability of *β-adrenergic receptor* transcripts due to *in vitro* β-adrenergic stimulation of *O. mykiss* erythrocytes (Götting & Nikinmaa 2017a) provides additional evidence that mRNA stability is an important gene expression control step in salmonid cells. At the translational level of hsp70 regulation, the nuclear translocation of YTHDF2 which consequently preserves the methylation state of specific adenosine residues (m^6A) in the 5′UTR of *hsp70* mRNA by inhibiting the demethylating activity of FTO during heat shock, has been shown to be primarily responsible for the preferential translation of newly synthesised *hsp70* transcripts (Zhou *et al.* 2015). These studies provide additional facets to the regulatory underpinnings of heat shock protein expression, and bearing in mind that heat shock proteins and the detailed mechanism of their induction and repression are without a doubt highly conserved in all three domains of life, it is very probable the transcriptional, post-transcriptional and translational regulation of hsp70 expression described in ectothermic and endothermic model organisms may also be applicable to non-model ectothermic species. Thus, the results obtained from the heat shock experiments provide important insights on post-transcriptional and translational control of inducible hsp70 synthesis, demonstrated in 15°C-acclimated and +7°C treated *S. alpinus* and *S. salar* as a positive and negative regulation of hsp70 expression, respectively, and extends from previous observations in ectotherms such as Miramichi *S. salar* (Lund *et al.* 2002), the Antarctic icefish *Notothenia angustata* (Hofmann *et al.* 2005) and in oocytes of the amphibian *Xenopus* (Bienz & Gurdon 1982).

Because of the relative simplicity and accessibility of quantifying mRNA expression using high resolution sequencing technologies, most gene expression studies have focused on steady-state mRNA level profiles serving as proxy for transcriptional regulation, whereas post-transcriptional mechanisms remain an understudied control step. For example, post-transcriptional regulation of mRNA level by miRNAs was discovered just before the turn of the current millennium (Lee *et al.* 1993) but has already opened up new and exciting avenues for important biological questions and the development of tools to investigate the diversity of post-transcriptional regulatory elements (Croce & Calin 2005). Mounting evidence has begun to reveal the significance of post-transcriptional mechanisms in contributing to the evolution

of multicellularity and developmental and behavioural complexity (Schaefke *et al.* 2018), such as in the function of miRNAs in caste determination in the Italian honey bee *Apis mellifera ligustica* (Guo *et al.* 2013). It has been suggested that because similar and closely related species have highly homologous genomes, and consequently proteomes to a certain degree, post-transcriptional regulation may be emerging as a significant player in influencing phenotypic divergence that cannot be explained by very similar genetic sequences, as observed between *H. sapiens* and *Pan troglodytes* (King & Wilson 1975). Furthermore, the observation that heat shock proteins can buffer the deleterious consequences of mutations suggests heat shock proteins may be a vital capacitor of morphological evolution and / or in preserving a tried and tested biological design (Rutherford & Lindquist 1998). With these in mind, investigations on phylogenetically close confamiliar species such as *S. salar* and *S. alpinus*, and especially more so between *S. alpinus* conspecifics renowned for their high phenotypic plasticity, may provide a suitable study system for illuminating the roles of heat shock proteins and post-transcriptional mechanisms in evolutionary processes such as speciation events. As genetic and proteomic resources improve and become more available for non-model organisms, research in this field is certainly to benefit from the ongoing and rapid development of tools for elucidating model organism post-transcriptional processes.

The heat shock protein expression data presented here is in accordance with the consensus model for the molecular regulation of heat shock proteins and suggests *S. alpinus* is experiencing thermal stress at 15°C and *S. salar* at 8°C. This deduction is supported by the differences in fish size as *S. alpinus* appears to have grown better at 8°C and *S. salar* at 15°C post-acclimation, congruent with their thermal requirements. Although the growth of fish was not precisely measured, studies in various endothermic and ectothermic organisms provide convincing evidence that the expression of heat shock proteins comes at a compromise to organism fitness, particularly when considering its preferential expression requires inhibiting the expression of other proteins involved in growth related processes. The results further suggest a vital role for inducible hsp70 in the temperature acclimation of *S. alpinus* and *S. salar*, on the basis of increased steady-state transcript and protein levels at suboptimal acclimation temperatures, which confirms their ecological relevance in ectothermic organisms. There was an evident correspondence in the elevation of mRNA and protein levels but the relationship was generally asynchronous, as demonstrated in the liver of both species acclimated to 15°C which exhibited a pronounced mRNA rhythm that was unaccompanied by enhanced protein synthesis. This uncoupling of mRNA expression from protein translation was

verified in the heat shock response, which was canonical for the stenothermal but negatively regulated at translation in the eurythermal salmonid, indicating an important role for post-transcriptional mechanisms in appropriately calibrating the level of heat shock proteins in the cell. *hsp70* mRNA is often used and presumed to be a suitable proxy for making protein level associations, and the observation that a considerable increase in transcripts need not be followed by upregulation of hsp70 synthesis has implications in the interpretation of mRNA level data, underscoring that physiological responses are more accurately represented at the protein level and its quantification should be conducted to be able to make appropriate functional inferences. Detailed information on the thermal and temporal relations of transcription and translation of heat shock proteins during acute and chronic exposure to suboptimal temperature builds upon the knowledgebase of aquatic ectotherm biology and the mechanistic intricacies of gene expression regulation in response to environmental perturbations, and provides indispensable insights on the position of species within their thermal tolerance windows, which could be utilised to improve predictions and management of future challenges to vulnerable taxa.

(**III**) *Temperature Acclimation Affects the Relationship Between Transcription and Steady-State mRNA Level in a Commercially Available Salmonid Cell Line*

The underlying assumption in many gene expression studies is that an increase in mRNA abundance is equivalent to an increase in the transcriptional induction of a gene, despite a myriad of regulatory mechanisms in place to precisely control mRNA level. The results presented here are thus contrary to this assumption as there was an inverse relationship between transcription and mRNA level in all the acclimation conditions. The relatively low transcription of genes in cells acclimated to 14 and 22°C was associated with elevated mRNA level, whereas the higher transcription at the optimal growth temperature corresponded with the lowest level of mRNA (Figure 11 & 12). The mRNA expression data presented here is consistent with the minimal and elevated mRNA levels in Antarctic fish *P. brachycephalum* acclimated to optimal and suboptimal growth temperatures, respectively (Windisch *et al.* 2014), whereas the higher rate of transcription and low transcript level at the optimal temperature indicates a faster mRNA turnover and a prominent role for post-transcriptional processes in maintaining appropriate mRNA levels, as previously observed in the genome-wide coordination of transcription and mRNA degradation in *Arabidopsis thaliana* (Sidaway-Lee *et al.* 2014) and *S. cerevisiae* (Garcia-Martinez *et al.* 2015). Additional support for the possible involvement of post-

transcriptional processes in explaining the contrast in high transcription and low mRNA level in cells growing at the optimal growth temperature, is the finding that factors promoting gene transcription in the nucleus of *S. cerevisiae* shuttle to the cytoplasm to also promote mRNA degradation (Haimovich *et al.* 2013), demonstrating the regulation and coordination of mRNA synthesis and degradation is a tightly integrated series that allows for a finer control of mRNA level during cell growth and fission.

The growth rate of *O. mykiss* cells was not directly measured, however the batch of cells cultured at the optimal growth temperature was passaged more frequently than those at the suboptimal temperatures, and since the recommended growth temperature for the cell line by the ATCC is 18-20°C, it is a sensible deduction to make that 18°C is closer to the optimal RTgill-W1 growth temperature than 14 and 22°C. This suggests increased transcription is associated with a high growth rate, as previously described in *E. coli* (Esquerre *et al.* 2014) and *S. cerevisiae* (Garcia-Martinez *et al.* 2015), whereas the low transcription and elevated mRNA level implies an increase in mRNA stability at the suboptimal acclimation temperatures. This hypothesis is reinforced by the observed increase in stability of oxidative phosphorylation and housekeeping gene mRNAs in *C. auratus* acclimated to a temperature lower than the species preference (Bremer & Moyes 2014). A decrease in molecular kinetic energy may partly account for the increase in mRNA stability in 14°C-acclimated cells, but the same reasoning does not explain the increase in stability at 22°C where higher molecular instability would be expected. A previous study identified an increase in the mRNA level of *cold inducible RNA binding protein* (*cirbp*) in cold acclimated *O. mykiss* gills that was subsequently validated in the same RTgill-W1 cell line used in the present study (Rebl *et al* 2013), which if accurately reflecting an increase in protein product, could bind and stabilise transcripts required in the acclimation of RTgill-W1 cells to the lower temperature.

Literature on gene expression is replete with data on elevated mRNA levels at elevated temperature in a variety of fish species (Jeffries *et al.* 2014, Logan & Somero 2010, 2011, Logan & Buckley 2015, Podrabsky & Somero 2004, Quinn *et al.* 2011a, 2011b), but whether increased transcription or mRNA stability is a contributing factor in these conditions remains to be clearly established. However, the increase in mRNA stability at lower growth rates in *E. coli* (Esquerre *et al.* 2014) and constant mRNA levels despite a reduction in transcription, mRNA degradation and growth in *S. cerevisiae* (Garcia-Martinez *et al.* 2015), conforms with the assertion that mRNA stability may be a key regulatory factor in modulating gene expression

of ectothermic piscine cells exposed to unfavourable temperatures. Moreover, inducible *hsp70* is known to be primarily regulated at transcription (Airaksinen *et al.* 1998, Boehm *et al.* 2003, Currie & Tufts 1997, Ritossa 1962, 1963, Vasquez *et al.* 1993), yet the transcription of this gene was markedly diminished at the suboptimal temperatures despite elevated mRNA levels, thus providing additional evidence for the role of mRNA stability in regulating mRNA abundance, particularly in response to chronic exposure to suboptimal temperature. An array of elements regulating mRNA stability, such as miRNAs, siRNAs, lncRNAs and RNA binding proteins, have been characterised in the last few decades and has improved the current understanding of post-transcriptional regulation of gene expression in homeostasis and disease (Feigerlova & Battaglia-Hsu 2017). While many of the non-coding RNAs and RNA binding proteins that have been identified thus far facilitate mRNA degradation and / or translational repression (Barreau *et al.* 2005, Wu & Brewer 2012), some like members of the Hu / ELAV family of binding proteins that are well conserved in invertebrates and vertebrates including fish (Good 1995), function to stabilise target mRNAs. The best characterised and ubiquitously expressed Hu protein, human antigen R (HuR), has been shown to increase the stability and consequent translation of anti-apoptosis and *hsp70* mRNAs in a variety of tissues, thereby promoting cell survival and cytoprotection during oxidative stress (Amadio *et al.* 2008), energy depletion (Ayupova *et al.* 2009) and heat shock (Gallouzi *et al.* 2000). It is therefore conceivable the preservation of mRNA integrity at the higher acclimation temperature, which requires the unabated expression of essential genes despite transcriptional downregulation, can be ascribed to an RNA binding protein homologous to HuR serving as a post-transcriptional and pre-translational regulatory control point in *O. mykiss* cells.

The use of reference genes in qPCR requires sufficiently consistent steady-state mRNA levels for accurate quantification of mRNA expression, and for this reason *gapdh*, *β-actin* and *18S* are often used for normalisation in different experimental conditions. *18S* ribosomal RNA (rRNA) abundance was not measured in the present study but *gapdh* and *β-actin* mRNA levels were significantly different between acclimation temperatures and precluded their use as reference genes. rRNAs are the most abundant RNA species in a cell as they are the primary components of ribosomes and their structural and catalytic functions are necessary for protein synthesis and cell growth. rRNA abundance in *S. cerevisiae* has been shown to decrease at slower growth rates and increase when cells are replicating optimally (Waldron & Lacroute 1975), which was shown more recently to be due to changes in transcription rate in conjunction

with a constant rRNA degradation rate, whilst mRNA levels remain constant because of alterations to the rate of mRNA decay (Garcia-Martinez *et al.* 2015). The relationship between growth rate and rRNA level in *S. cerevisiae* (Waldron & Lacroute 1975) has been suggested to be more relevant for unicellular than multicellular organisms (Johnson *et al.* 1974), however the possibility cannot be discounted that the increase in mRNA level for the studied genes at the suboptimal acclimation temperatures could be an artefact introduced by a relative decrease in rRNA concentration. Moreover, while the 2:1 *28S*:*18S* rRNA ratio was qualitatively assessed with gel electrophoresis to confirm RNA integrity, and although bands appeared to be consistent between samples, quantifying rRNA levels would have provided a more complete picture and help disentangle the roles of mRNA stability and rRNA concentration in determining the mRNA level of genes of interest. As for the reference genes that were measured, the effect of acclimation temperature on *β-actin* and *gapdh* mRNA levels was not observed in previous acclimation and thermal stress experiments using them as references (Lewis *et al.* 2016, Liu *et al.* 2012). It is possible the difference in reference gene mRNAs in RTgill-W1 cells could be due to the use of a single cell type, where temperature effects would be more discernible compared to an averaging effect on mRNA level likely to arise from the use of a heterogeneous cell population.

The use of clonal cells stemming from a discrete parental lineage can offer distinct advantages in gene expression studies as they are expected to be more homogeneous in their phenotype compared to the differentiated cell population of tissue samples. This deduction proved to be accurate for mRNA measurements which were comparatively uniform between replicates within an acclimation group (Figure 12). Transcription in contrast, was considerably more variable between replicates and statistical testing was not able to unambiguously confirm the significant differences in transcription for all genes and acclimation temperatures. There are potentially several sources of variation in the transcriptional measurements, including the use of an interconnected mesh-like monolayer of cells with neighbouring cells forming tight junctions via extracellular matrix components, alterations in cell membrane fluidity at different acclimation temperatures (Hazel 1984, Tiku *et al.* 1996, Trueman *et al.* 2000), variations in confluency and contact inhibition of adherent cells randomised by cell seeding prior to growth, and associated asynchronous cell cycle stages with dissimilar transcriptional programmes between multiplicative and quiescent cells, all of which can significantly affect downstream steps such as efficient cell detachment, suspension, isolation of nuclei and nascent transcript

elongation. Furthermore, *in vivo* visualisation and measurement of transcriptional kinetics in *E. coli* (Golding *et al.* 2005, So *et al.* 2011), *Dictyostelium* (Chubb *et al.* 2006)*, S. cerevisiae* (Zenklusen *et al.* 2008) and mammalian cells and tissue (Dar *et al.* 2012, Halpern *et al.* 2015, Suter *et al.* 2011) at single gene and genome-wide resolution, reveal transcriptional induction occurs in pulsatile bursts of different amplitudes with temporal irregularity, resulting in discontinuous transcript synthesis of even functionally related genes within and between cells. Thus, stochasticity of gene transcription appears to be an inherent and universal feature of biological systems and is a viable rationalisation of the observed transcriptional variations.

Transcription measurements were not significantly affected by the 6°C increments in assay reaction temperature used to incorporate nucleotides into nascent transcripts *in vitro*, although there was a notable and consistent trend in transcription decreasing with increasing reaction temperature for all five genes in cells cultured at the optimal growth temperature (Figure 11). Contradictory to this trend is the transcription of *hexokinase* in 22°C-acclimated cells which increased with assay temperature and resulted in a significantly higher transcription at 26°C than at 14°C. This transcription-reaction temperature interaction presents a conundrum as it seems to suggest *in vitro* nascent transcript elongation efficiency increases with reaction temperature for *hexokinase* but not for the other four genes in 22°C-acclimated cells. If this interaction was due to acclimation induced changes to the thermal properties of the bound transcriptional machinery, manifested as a more thermostable and efficient nucleotide incorporating RNA polymerase II at 26°C, then why is the same pattern not observed for the other genes? In an attempt to explain this observation, the parallel increase in *hexokinase* transcription with reaction temperature could hypothetically be construed as an increase in the number of engaged polymerases due to an increase in the kinetic energy of molecules in the *in vitro* system, thus promoting the rebinding of unbound polymerases and the reactivation of their transcriptional activity. It is unlikely however, that the *in vitro* conditions would significantly elicit the rebinding of disengaged polymerases and re-initiate transcription, since the assay is designed to promote only the extension of partially transcribed transcripts by the pre-engaged transcriptional complex in an elongating conformation.

Indicative of the tight regulation of the initial stage of gene expression is the step-wise process of transcription initiation and elongation, which in the former requires a varied set of general transcription factors (Sayre *et al.* 1992) and the oligomerisation of two additional

transcriptional coactivator subunits (rpb4 and rpb7) (Bushnell & Kornberg 2003, Meyer *et al.* 2006) to the core transcriptional assembly, which are not present in the latter elongation complex (Gnatt *et al.* 2001). It is of course possible that sufficient levels of regulatory factors are present in isolated nuclei, and that the probability of reinitiating transcription and transcript elongation is increased at the elevated reaction temperature. Alternatively, it could be argued that *hexokinase* transcription in 22°C-acclimated cells was an anomaly due to random variation and / or stochastic transcriptional bursting kinetics, as it was observed only for the one gene at one reaction temperature and acclimation temperature. In contrast and although not statistically significant, the transcription of genes of interest in cells cultured at the optimal temperature exhibited a pattern of increasing with decreasing assay temperature. A potential explanation for this trend is the size of the transcriptional machinery and its stability at different temperatures. RNA polymerase II is a relatively large complex (~550 kDa) (Young 1991) composed of up to 12 dissociable subunits in eukaryotes (Figure 15) (Bushnell & Kornberg 2003, Edwards *et al.* 1991, Meyer *et al.* 2006). Thus, the higher reaction temperature with a higher molecular kinetic energy being advantageous for transcript elongation and DNA template progression, would be disadvantaged by the decrease in the stability of polymerase quaternary structure, whereas the lower kinetic energy at the lower reaction temperature would preserve the active conformation of the transcriptional machinery at a cost to the rates of catalysis and transcript elongation.

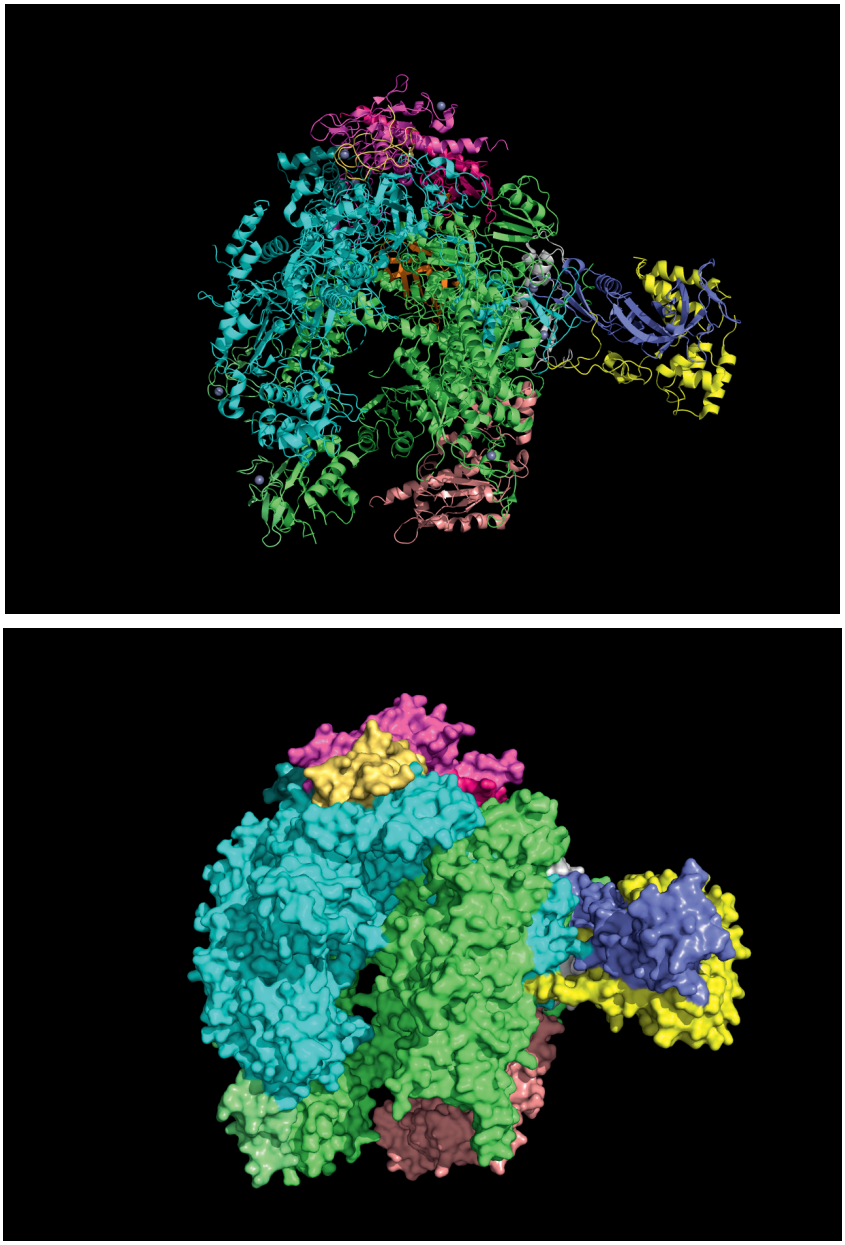

Figure 15 Twelve Subunit Eukaryotic RNA Polymerase II in an anterior orientation to highlight the cleft in which DNA and RNA interact with the transcriptional machinery. Cartoon (top) and surface fill (bottom) models rendered in PyMol (DeLano 2002) to highlight subunit interactions. Subunits rbp4 (yellow) and rpb7 (purple) are required for polymerase-DNA clamping and transcription initiation. The image is based on *S. cerevisiae* (2B8K) polymerase crystallographic structure (Meyer *et al.* 2006) available in the PDB (www.rcsb.org).

Two weeks of acclimation to suboptimal temperatures resulted in a significant increase in mRNA, whilst the lowest mRNA level was seen in cells cultured at the optimal temperature. Transcription in contrast was generally lower at the suboptimal temperatures than at the optimal growth temperature. The low level of mRNA combined with a higher transcription suggests mRNA synthesis and degradation are tightly coupled at the optimal temperature where cells are expected to be proliferating at a higher rate, and the proposed integration of transcript synthesis and degradation is necessary to finely control mRNA turnover and steady-state level, presumably to ensure appropriate transcript levels are retained in and transferred to parent and daughter cell, respectively. The association between growth, transcription and mRNA stability has previously been detailed in prokaryotic and eukaryotic model organisms, but it appears the interconnectedness of these biological processes can be extended to multicellular metazoans. The reduction in transcription in conjunction with elevated mRNA level hints at the role of mRNA stability in the acclimation of RTgill-W1 cells to suboptimal temperatures. However, although it is reasonable to assume that cell replication was more prolific at the recommended optimal growth temperature, differences in growth rate between acclimation temperatures and the role of mRNA stability in these conditions were not quantified empirically. Nevertheless, whether the increase in mRNA level was due to an increase in mRNA stability and / or a reduction in growth rate and rRNA abundance, the results presented here contradict the frequent assumption that the induction of gene transcription is the primary control point modulating steady-state mRNA level. These observations should be considered in the analysis of gene expression data from qPCR, microarray and RNA-Seq studies which do not always take into account relative changes in transcription, mRNA stability and growth, nor consider an increase in mRNA level may be a corollary to a decrease in rRNA. Cell growth, transcription and RNA stability as complementary measurements are therefore highly recommended to obtain a more complete understanding of the cellular processes governing changes to steady-state mRNA level. Based on these preliminary findings, it is equally plausible that transcription can be attenuated and post-transcriptional mechanisms recruited to control cytoplasmic mRNA level in the acclimation of ectothermic piscine cells to less than ideal temperatures.

From a methodological perspective, accurate quantification of transcription intuitively requires a suitable reaction temperature that reflects the optimal thermal breadth of the studied species, whilst finding a balance between the speed of nucleotide incorporation, template progression and the structural integrity of the polymerase, which after all evolved to function within an organism's thermal range. Since statistical testing was unable to unequivocally validate the

homogenous trend of increasing transcription with decreasing assay temperature at the optimal temperature, and because of the lone *hexokinase* transcription-reaction temperature interaction at the higher acclimation temperature confounding these results further, more detailed experiments and additional optimisation of the method must be conducted to elucidate the relationship between cell replication, transcription and steady-state mRNA level, as well as the thermal properties of salmonid RNA polymerase II stability and activity in the conditions of the nuclear-run on transcription assay. The variability in the transcription determinations prevent definitive conclusions from being made, but the results as discussed in the context of the statistical findings and observable trends could serve as a starting point for more comprehensive future experiments.

(**IV**) *Cold Acclimation Attenuates and Warm Acclimation Enhances the Diurnal Transcriptional Rhythm of S. alpinus Erythrocytes*

A single randomised feeding time per day was used in this study to prevent entrainment, and because the utilised indoor rearing environment and the overhead lighting was a substandard emulation of the prevailing natural summer photoperiod of the region, which might affect growth, the size of fish before and after the acclimation period was measured as a proxy for growth, but revealed no significant difference between 8 and 15°C. This may be due to the limited feeding period, the lower level of illumination and spectral quality of the artificial lighting affecting food conversion efficiency. Indeed, despite being highly adept at feeding in low light conditions, presumably as an adaptation to its natural environment and nocturnal predatory behaviour, *S. alpinus* feeding can be hindered by a decrease in light intensity (Elliott 2011). In addition to season (Jobling 1987, Mortensen *et al.* 2007), temperature and size influence the growth rate of fish, with under-yearling *S. alpinus* growing more efficiently at ~15°C (Lyytikäinen *et al*. 1997b) and one-year old fish preferring and growing optimally at ~11-12°C regardless of thermal history (Siikavuopio *et al.* 2014), and it may be that the acclimation temperatures used are on either side of the optimal growth temperature range for one-year old *S. alpinus*.

Biological rhythms are essential to the daily and seasonal adaptability of organisms, the disruption of which by changes to environmental parameters are gradually being brought to light, with a recent stream of publications conducting measurements from more than one

timepoint to take into account circadian fluctuations in mRNA expression that can obscure important physiological responses (Healy & Schulte 2012, Lewis *et al.* 2016, Lubiana *et al.* 2016, Prokkola *et al.* 2018, Prokkola & Nikinmaa 2018). However, circadian mRNA oscillations in qPCR, microarray and the rapidly growing number of RNA-Seq studies, are often conflated with transcription even though transcriptional rhythms have yet to be directly measured in fish. Thus, the aim of this study was to elucidate the correlation between transcription, mRNA expression and their possible rhythmicity across three timepoints spanning a simulated summer day (18:6 L:D), and how this relationship can be modified by temperature in a climate change scenario. Initial attempts were made to conduct transcriptional measurements from several tissues, although the amplification signals obtained from labelled nascent transcripts from gills and liver were very sporadic at best. It was then deduced that the disaggregation of cells from intact tissue and subsequent isolation of nuclei was inefficient and required further optimisation. Erythrocytes were chosen as a suitable cellular model for this reason, as they are already individually suspended in plasma and cell disaggregation was not necessary, making plasma separation simpler and isolation of nuclei and nascent transcript labelling more effective; although this choice of tissue comes at a compromise.

The nucleated erythrocytes of salmonids show significant reductions in mRNA, protein synthesis, metabolism and nuclear density as cells age (Götting & Nikinmaa 2017b, Lewis *et al.* 2010, Lund *et al.* 2000, Phillips *et al.* 2000). This decline in the transcriptional, translational and metabolic capacities of older erythrocytes results in an increased dependency on anaerobic energy production (Phillips *et al.* 2000) and is responsible for their inability to mount a heat shock response during thermal stress (Lund *et al.* 2000). An increase in temperature can also raise the oxygen carrying capacity of blood through increased red blood cell division, accelerated maturation of erythroblasts, the release of stored erythrocytes from the spleen and stimulation of erythropoiesis (Houston & Murad 1992, Lewis *et al.* 2010, 2012, Murad *et al.* 1990), thus bringing about a higher concentration of circulating juvenile red blood cells (Lewis *et al.* 2012). It is therefore possible that differences in transcription and mRNA level between acclimation temperatures was due to a shift in the composition of erythrocytes towards a younger population at the higher acclimation temperature. However, it was previously demonstrated that *S. alpinus* from the same brood stock and acclimated to similar temperatures as the present study, had comparable red blood cell concentrations at different times of the year before and after experimental anaemia (Lecklin & Nikinmaa 1998). Blood loss is a potent inducer of erythropoiesis in many animals including some salmonids (Lane 1979, Lecklin &

Nikinmaa 1998), but since blood composition appears to be similar between 8- and 14-16°C-acclimated *S. alpinus* and experimental anaemia ineffective at stimulating erythropoiesis and increasing the number of circulating juvenile erythrocytes (Lecklin & Nikinmaa 1998), it is likely that a mixed-age population of erythrocytes was obtained from both acclimation temperatures. Comparative analysis is thus possible as heterogeneity in cell age would be similar between the acclimation temperatures. Future transcriptional measurements using salmonid erythrocytes would be improved by separating the cells into age fractions via density gradient centrifugation to reduce data variability, as per Götting & Nikinmaa (2017b). Furthermore, *S. alpinus* is considered a habitat generalist but even sympatric populations exhibit a high degree of phenotypic plasticity at the behavioural, morphological and genetic level (Ahi *et al.* 2014, Baroudy & Elliott 1994, Janhunen *et al.* 2009), and are able to diversify into distinct populations and subspecies at a relatively fast rate, presumably due to the malleability in the expression of their underlying genetic makeup, which may have contributed to some of the transcription and mRNA level variability.

Despite the data variability, statistical analysis was able to confirm the transcription of *gapdh* and *enolase* was significantly higher in 15°C-acclimated erythrocytes, whereas the transcription of *hexokinase* and *hsp70* followed the same trend. The transcription of *β-actin* and *hsp70* was not significantly different between acclimation temperatures, but both genes exhibited significant transcriptional rhythmicity with an acrophase in the middle of the light period at 15°C (Figure 13). Although not statistically significant, this transcriptional pattern was also evident for the three genes encoding glycolytic enzymes in 15°C-acclimated fish, whilst the transcription of all five genes was markedly attenuated in fish acclimated to 8°C. This study is the first to directly measure the diel induction of transcription and how it can be modulated by acclimation temperature in ectothermic fish. Such a conspicuous temperature effect has not been observed previously for gene transcription but has been described for mRNA expression in hepatocytes of 15°C-acclimated *S. alpinus* (Prokkola *et al.* 2018). At the steady-state level (Figure 14), *hexokinase*, *enolase* and *hsp70* mRNA expression was significantly higher at 15°C, whereas *gapdh* and *β-actin* mRNA levels were similar between acclimation temperatures, as would be expected for reference genes. Interestingly, the acclimation temperature induced differences in reference gene mRNA levels in study **III** was conjectured to be due to the use of a single cell type (RTgill-W1), however the current study also used a single cell type yet reference gene mRNA levels were comparable between

acclimation temperatures. This dichotomy in reference gene expression levels, and transcriptional induction for that matter, could be attributed to fundamental differences in the *in vitro* and *in vivo* cellular environment. For the non-reference genes, the higher *enolase* transcription, *hexokinase* following the trend of higher transcription and the accentuated transcriptional rhythm for *hsp70* at 15°C appears to be contributing to the elevation of and correlates well with their respective mRNA pools. The only significant rhythm in mRNA expression at 15°C was for *enolase* which peaked 1 h before the dark phase, whereas the lower acclimation temperature diminished not only the transcription of all five genes but also their mRNA levels and rhythmicity, in agreement with the liver transcriptome data of 8°C- and 15°C-acclimated *S. alpinus* (Prokkola *et al.* 2018). The attenuation of transcriptional induction, mRNA expression and rhythmicity at 8°C and their enhancement at 15°C, as revealed in the present and a closely related study (Prokkola *et al.* 2018), indicates gene expression and its rhythmic induction in the stenothermal salmonid is markedly affected by temperature, with potentially far-reaching consequences on biological rhythms, seasonality and fitness of fish at high latitudes, where a progressive phenological mismatch between photoperiod and temperature is occurring.

Characterisation of acclimation induced transcriptomic signatures in the Antarctic zoarcid *P. brachycephalum*, reveal minimal changes to mRNA level is suggestive of a balanced steady-state in individuals at an optimal growth temperature, whereas acclimation to a lower and higher temperature results in the remodelling of the transcriptome through significant up and downregulation of mRNA expression (Windisch *et al.* 2014). This observation and the low *hsp70* and hsp70 levels in 8°C-acclimated *S. alpinus* (Lewis *et al.* 2016), provide ample support to the notion that the basal level of transcription and steady-state mRNA in 8°C-acclimated *S. alpinus* erythrocytes is a tell-tale sign of preferable conditions for the species. Reciprocally, based on elevated *hsp70* and hsp70 in the relatively moderate stenotherm *S. alpinus* acclimated to 15°C (Lewis *et al.* 2016), and the fact that acclimation of the extreme stenotherm *P. brachycephalum* to a higher temperature likewise results in an increase in heat shock protein, glycolytic and gluconeogenic enzyme gene transcripts (Windisch *et al.* 2011, 2014), and protein level changes that indicate a shift from lipid to glucose metabolism (Windisch *et al.* 2011), there is enough evidence to conclude the increase in transcription and / or mRNA level of genes of interest in 15°C-acclimated *S. alpinus* is symptomatic of thermal stress.

Biological rhythms have been observed at all levels of biological organisation and also persist independently of transcription (Dibner *et al.* 2009, O'Neill *et al.* 2011), such as in enucleated mammalian erythrocytes (O'Neill & Reddy 2011), and the nucleated *S. alpinus* erythrocytes which display circannual oscillations in ion content and adrenergic responses that can be influenced by previous temperature acclimation (Lecklin & Nikinmaa 1999a, 1999b). These studies underscore the fundamental importance of synchronising physiological processes with changing environmental conditions. However, although gene transcription in 15°C-acclimated erythrocytes exhibited an undulating diel pattern, the present study could have benefited from measuring transcription and transcript levels from more timepoints within the 24 h cycle to obtain a better resolution of the sinusoidal wave and periodicity typical of biological rhythms. Therefore, these results merely hint at transcriptional rhythmicity and are still preliminary, requiring further verification with a more rigorous experimental design, wherein temporal sampling is conducted over several days under a natural photoperiod to monitor transcriptional rhythms. Because of *S. alpinus* phenotypic plasticity which can manifest in diurnal or nocturnal phenotypes (Alanärä & Brännäs 2011), repeated sampling of cannulated fish as per Lewis *et al.* (2010) should preferably be conducted to reduce individual variability, although in the case of *S. alpinus* attention must be given to the size of fish and / or appropriate aliquoting of samples so as to avoid having bloodless fish before the experiment ends (Lecklin & Nikinmaa 1998).

There was an unambiguous correlation between low transcription and low mRNA level at 8°C and a higher transcription resulting in elevated mRNA level at 15°C. Aside from this positive transcription-mRNA level relationship, the consistent pattern of enhanced transcription in the middle of the light period for all five genes was not always accompanied by a concurrent increase in mRNA level at the same timepoint, suggesting transcriptional induction of a gene can be uncoupled from its steady-state mRNA level. The only potential evidence for the coupling of the measured parameters is the transcription and mRNA level of *hexokinase*, both of which exhibited an insignificant peak at 9 h in 15°C-acclimated erythrocytes. It could be argued that the higher transcription at 15°C for *gapdh* and *enolase* which followed the rhythmic trend by increasing at 9 h, and the enhanced transcriptional rhythm of *β-actin* which also peaked at 9 h, is responsible for the increase in their respective mRNA levels at 17 h because of an 8 h time lag. Such a delay is feasible considering a variety of nuclear control points are in place between transcription, RNA processing and nuclear export for the accurate temporal

and spatial control of gene expression (Stutz & Rosbash 1998, Xing *et al.* 1993, Zasloff 1983). Paradoxically, the mRNA level of the gene controlled primarily at transcription (*hsp70*) did not increase despite a significant transcriptional induction at 9 h. Steady-state *hsp70* mRNA expression has been shown to be rhythmic in the liver of 15°C-acclimated *S. alpinus*, but with the peak occurring 1 h before the dark phase and which was unaccompanied by an increase in hsp70 level (Lewis *et al.* 2016), thus highlighting the complexity of the transcription-steady-state mRNA level-translation relationship, especially for a gene considered to represent this sequence of intracellular events accurately. In contrast to the temporal induction of transcription, mRNA rhythms although statistically insignificant for the most part, were more heterogeneous and exhibited gene-specific peaks and troughs at 15°C. For instance, *gapdh* and *β-actin* mRNA levels seem to decline at 9 h, antiphase to the transcription of their genes, whereas mRNA expression for *hsp70*, *hexokinase* and *enolase* had their peaks at 1, 9 and 17 h, respectively. Then it is apparent that in the experimental conditions of the present study, transcription and mRNA expression are not as tightly coupled as often assumed, for temporal changes in transcription and mRNA abundance can occur synchronously or asynchronously, with other control mechanisms that can be independently recruited and must inevitably play a part between transcriptional and pre-translational regulation of gene expression.

A genome-wide analysis of circadian rhythms of transcription, mRNA level and the epigenetic landscape in *M. musculus* hepatocytes categorised genes exhibiting rhythmic expression into three classes: I) genes that are rhythmic in both transcription and mRNA accumulation, II) genes with rhythmic transcription but mRNA level remaining constant and III) genes that are transcribed constitutively but mRNAs accumulate in a rhythmic manner (Le Martelot *et al.* 2012). Although the expression of genes investigated here followed a similar transcriptional pattern, from a statistical standpoint the transcription at 15°C for *β-actin* and *hsp70* was rhythmic and falls within category II since there was no significant rhythm in their mRNA levels, whereas *enolase* seems to fit the description of category III as mRNA level exhibited rhythmicity and transcription was elevated but not necessarily rhythmic. *gapdh* and *hexokinase* on the other hand, did not fit the above categorisation as transcription and mRNA level at 15°C was determined not to be rhythmic. However, the transcription of *gapdh* was significantly elevated but not its mRNA level, whereas *hexokinase* transcription was close to being significantly elevated while its mRNA level was significantly elevated. *gapdh* is required for anaerobic glycolysis reactions and although its expression can be induced by hypoxia

(Higashimura *et al.* 2011, Yamaji *et al.* 2003), the gene is often regarded as constitutively expressed in many experimental conditions and its mRNA abundance regularly used as a reference gene (Almroth *et al.* 2015). This might be a plausible explanation for the comparable *gapdh* steady-state mRNA levels between acclimation temperatures and the increase in transcription at 15°C. It is tempting to arrive at the same conclusion for *hexokinase*, although out of the five tested genes, *hexokinase* was the second least expressed at the transcriptional and least expressed at the steady-state level, with many replicates not having an amplification signal, which is not the expected characteristic of constitutive gene expression. Moreover, transcription has been suggested to be a better parameter for evaluating functional relatedness and the structure of underlying biochemical networks in *S. cerevisiae* (Hayles *et al.* 2010). The similarity in the transcriptional rhythms of the glycolytic enzyme, molecular chaperone and cytoskeletal protein genes may be consistent with this assertion and hints at an even closer relationship in their involvement in ATP production, protein synthesis and folding, cell structural support and integrity, etc.

Four weeks of acclimation to 15°C resulted in an increase in transcription and / or mRNA level of genes of the glycolytic pathway in *S. alpinus* erythrocytes, with *enolase* exhibiting a positive correlation between gene transcription and mRNA level, whilst *hexokinase* followed the trend of concurrent increase in transcription and mRNA level, suggesting the first step of gene expression may be an important control point in regulating the steady-state mRNA level of these genes. In contrast, *gapdh* had a higher rate of transcription at 15°C but comparable mRNA levels between acclimation temperatures, which might indicate a higher mRNA turnover compared to the other glycolysis genes. The number of *de novo* transcripts produced by transcription was highest for *enolase*, intermediate for *hexokinase* and lowest for *gapdh*, whereas mRNA abundance was highest for *enolase*, intermediate for *gapdh* and lowest for *hexokinase*. The differences in the mRNA level of these genes that share the same functional pathway may be a reflection of the reversible (*enolase* and *gapdh*) and rate-limiting and ATP consuming (*hexokinase*) reactions their protein products perform. Lipids are the most important source of metabolic energy especially for carnivorous cold water adapted salmonids (Bailey & Driedzic 1993, Tocher 2003) which take advantage of higher ATP yields from β-oxidation of fatty acids and better oxygenation of cold water. Dietary lipids are readily stored and constitute a significant proportion of fish tissue, whereas a prolonged hyperglycaemic state after glucose administration has led to the conclusion that salmonids are glucose intolerant (Legate *et al.*

2001). However, since a crucial organ like the brain obtains metabolic fuel mostly from glucose, the unwavering mRNA and protein expression of gluconeogenesis enzymes despite glucose administration (Panserat *et al.* 2000, Panserat *et al.* 2001) suggests persistent hepatic glucose synthesis might explain the limited utilisation of exogenous glucose by salmonids.

Earlier studies that have assessed in detail the energy production in salmonid erythrocytes have been conducted on the relatively eurythermal *O. mykiss*, which show the capacity of nucleated red blood cells to harness energy from amino acids and lipids (Walsh *et al.* 1990), although glucose appears to be their primary metabolic fuel on the basis of a full complement and higher specific activities of glycolytic enzymes (Ferguson & Storey 1991), the linear uptake and metabolism of very high extra cellular glucose concentrations (Driedzic *et al.* 2013, Pesquero *et al.* 1992), non-saturation glycolytic enzyme kinetics despite high substrate levels and the majority of CO_2 production emanating from glucose catabolism (Walsh *et al.* 1990). Bearing in mind that stenothermal fish have been shown to transition from lipid based aerobic metabolism towards glucose derived anaerobic ATP production when acclimated to elevated temperature (Windisch *et al*. 2011, 2014), the increase in transcription and / or mRNA level of glycolysis genes, provided it is accompanied by translation into active enzymes, could be associated with a higher metabolic rate and / or an increased reliance on anaerobic metabolism in *S. alpinus* acclimated to 15°C, where oxygen demand and biochemical reaction rates are expected to be higher but where oxygen level is reduced. Conversely, the lower induction of transcription and mRNA level in 8°C-acclimated *S. alpinus* could be ascribed to less requirement for anaerobic metabolism and a balanced steady-state, as proposed for species that naturally thrive at lower temperatures (Lewis *et al.* 2016, Windisch *et al.* 2011, 2014). Maximal glycolytic enzyme activities of the studied genes in *O. mykiss* erythrocytes were shown to be highest for *gapdh*, followed by *enolase* and *hexokinase* (Ferguson & Storey 1991), and if glycolytic enzyme composition is similar in *S. alpinus* erythrocytes, an inference can be made that the low *hexokinase* transcription and mRNA level is highly representative of protein level, in keeping with its rate-limiting and ATP consuming duties, and its high substrate affinity (low kM) offsetting the need for abundant enzyme level (Ferguson & Storey 1991). The glycolytic pathway is fundamental to the biochemistry of most organisms and quantification of its highly conserved components at the protein level can be conducted with relative ease, but this unfortunately was not done. Although useful insights concerning the thermal and temporal relationship of transcription and steady-state mRNA level were revealed in the present study,

measuring enzyme activities would have complemented the acquired data at the functional level and provided a clearer resolution of the interrelationships between key stages of gene expression.

The predominantly transcriptional control of inducible *hsp70* in ectothermic cells was determined in the detailed studies of *D. melanogaster* (Boehm *et al.* 2003, Vasquez *et al.* 1993). The upregulation of *hsp70* transcription and mRNA level in warm acclimated erythrocytes is in accordance with this standard and a similar study that demonstrated *hsp70* mRNA level is a good indicator of hsp70 level in 15°C-acclimated *S. alpinus* (Lewis *et al.* 2016). Noteworthy is the observation that *hsp70* transcription follows a diurnal rhythm with an acrophase in the middle of light period. Although heat shock factors have been identified as circadian transcription factors putatively regulated by the circadian clock transcriptional network (*hsf-2*) in *D. rerio* (Amaral & Johnston 2012) and with rhythmic DNA binding activity (hsf-1) and influence over *M. musculus* endogenous timekeeping (Renke *et al.* 2008), this study is the first to directly show *hsp70* is transcribed in a circadian manner to maintain elevated mRNA and presumably protein levels during chronic temperature exposure. Moreover, the *hsp70* mRNA rhythm peaking at 17 h in 15°C-acclimated *S. alpinus* hepatocytes (Lewis *et al.* 2016) and the accentuated *hsp70* transcriptional rhythm of erythrocytes in the present study which peaked at 9 h, indicates the rhythmicity of this gene is tissue- and timepoint-specific, and can occur independently at the transcriptional and steady-state mRNA level. Another notable finding is the enhanced rhythmicity of *β-actin* transcription in 15°C-acclimated erythrocytes, yet comparable mRNA levels between acclimation temperatures. This suggests the increase in transcription in the middle of the light period is required to replenish mRNA level at the higher acclimation temperature, where there is potentially a higher turnover of molecules due to molecular instability and / or an increase in rate processes. Since *β-actin* mRNA and protein are assumed to correlate well in their levels and are commonly used as references in qPCR and western blotting, respectively, maintaining an appropriate amount of mRNA, and thus protein, may be for the purpose of preserving cell structure integrity at 15°C.

Photoperiod is the most predictable and important environmental cue of seasonal habitats, however changes in temperature are also used by fish for a fine-tuned orchestration of physiological transformations that precede life history events. Hormonal rhythms are some of the most studied circadian and seasonal outputs of fish and their levels also exhibit temperature

dependent modulation. For example, in salmonids an appropriate increase in temperature combined with endocrine mechanisms can promote reproductive readiness (Korsgaard *et al.* 1986, Mackay & Lazier 1993), accelerate growth, induce adaptations required for marine life and cue seaward migration (Sigholt *et al.* 1998, Solbakken *et al.* 1994). The best characterised hormone linked to the endogenous clock, melatonin, acts as a temporal conveyer of external photoperiodic information to peripheral tissues and is a central constituent of biological timekeeping and periodicity entrainment, such as in cueing reproduction (Vera *et al.* 2007, Clark *et al.* 2005). At the systemic level, pineal synthesis of melatonin and its release into the bloodstream is induced by the absence of light (Falcon 1999, Falcon *et al.* 1998, 2010, Huang *et al.* 2010a, Iigo *et al.* 2007, Strand *et al.* 2008) while ambient temperature affects the level in circulation (Clark *et al.* 2005, Porter *et al*. 2001, Vera *et al.* 2007). At the cellular level, the circadian clock transcriptional-translational feedback network is generally regarded as the transcriptional regulator of rhythmic and non-rhythmic expression of a sizeable portion of the transcriptome (Amaral & Johnston 2012, Davie *et al.* 2009, Huang *et al.* 2010a, Lazado *et al.* 2014, McCarthy *et al.* 2007, Miller *et al.* 2006, Prokkola *et al.* 2018, Wang *et al.* 2018).

Although the circadian signal transduction relationship between the systemic and cellular level of biological organisation is relatively inadequately characterised in aquatic ectotherms, it is evident that melatonin directly modulates clock gene expression in melatonin receptor expressing cells (Dinet & Korf 2007, Hiragaki *et al.* 2014), which display widespread distribution in fish (Falcon *et al.* 2007). The protein coding genes of the endogenous clock network are present in all cells and assumed to be involved to varying degrees in synchronising systemic signals with intracellular processes (Dinet & Korf 2007) to effectuate rhythmic gene expression and perpetuate biological rhythms in central and peripheral tissues, whether independently or interdependently from the master oscillator (Weger *et al.* 2011) surmised to be in the retina and pineal gland of salmonids (Iigo *et al.* 2007). The observation that mRNA oscillations are reduced in 8°C than in 15°C-acclimated *S. alpinus*, while some circadian clock genes have higher mRNA expression levels at the lower acclimation temperature (Prokkola *et al.* 2018), is noteworthy as it suggests divergent roles for different clock components in temperature acclimation and timekeeping, and that gene expression and its rhythmicity is affected by ambient temperature in a thermally sensitive species that has presumably rarely encountered substantial habitat temperature increase in its evolutionary history.

The hypothalamic-pituitary control of the endocrine system regulates many essential physiological functions, including growth, homeostasis, reproduction, ion and osmoregulation, metabolism and stress responses, and it appears melatonin is similarly involved in circadian regulation of various neuroendocrine functions, including the hypothalamus-pituitary axis in fish (Falcon *et al.* 2007). Glucocorticoids, growth factors and thyroid hormones, whether synergistically, permissively or antagonistically, mediate many processes including carbohydrate, protein and lipid metabolism, all of which display species-specific daily and seasonal rhythms in eurythermal and stenothermal fish (Cowan *et al.* 2017). Catecholamines and their effects likewise exhibit daily and seasonal changes, for example in *Anguilla anguilla* neural tissue (Le Bras 1978, Popek 1983) and in salmonid erythrocytes (Koldkjaer *et al.* 2004, Lecklin & Nikinmaa 1999a, 1999b). The intracellular pathways that result in hormone mediated gene transcription of metabolic and stress response genes have been elucidated mostly in endothermic mammalian systems (Bamberger *et al.* 1996, Wiley *et al.* 2016) and described in detail in cell biology textbooks (Alberts *et al.* 2008), but the fundamental mechanisms appear to be similarly conserved in ectothermic fish (Leung & Woo 2010, Mommsen *et al.* 1999, Reddy & Leatherhead 2003, Sathiyaa & Vijayan 2003, Vijayan *et al.* 1994a, 1994b). However, whether intracellular or systemic endogenous timekeeping mechanisms are primarily responsible for the transcriptional rhythms observed in the present study is indistinguishable at this juncture. It is equally conceivable that the endogenous clock and / or other temperature-, photoperiod- and stress-responsive signalling elements from different tissues are involved in the induction of rhythmic gene transcription. Thus, establishing the causal link between the endogenous clock, hormonal and transcriptional rhythms and how they can be modified by temperature would be able to provide perspective on upstream physiological signals regulating the first step of gene expression in salmonid cells.

The first novel finding of this study is that transcription and / or its rhythmicity in *S. alpinus* erythrocytes is enhanced at 15°C and occurs at a discrete point in the circadian period but is attenuated at 8°C. The second is the observed diel induction of *hsp70* transcription in the studied cold stenothermal species adds to the varied regulatory underpinnings of the cellular thermometer model and heat shock protein expression. The transcriptional induction of genes exhibited a positive correlation with their mRNA levels, but the temporal relationship between transcription and steady-state mRNA appears to be uncoupled for most genes, possibly due to post-transcriptional regulation. These results hence turn the spotlight to the often disregarded principle that steady-state mRNA level cannot be attributed only to transcription, as it is

determined by the integrated processes of nuclear transcription and nucleocytoplasmic RNA degradation. Moreover, it is apparent that 8°C is closer to the optimal temperature for *S. alpinus* and although the species can survive indefinitely at 15°C, the transcription and steady-state mRNA data at the higher acclimation temperature together with observations from closely related studies, provide validation to the deduction that the stenothermal salmonid is experiencing mild to moderate thermal stress, and presumably increased bioenergetic demands at the acclimation temperature.

Although the total cost of protein synthesis compared to mRNA is typically higher since many proteins can be produced from one transcript, the ATP invested in the synthesis of one mRNA molecule is typically more than three times that of a protein molecule due to a higher number of required building blocks (Lynch & Marinov 2015, Wagner 2005). This implies that even if translation does not occur (i.e. Lewis *et al.* 2016), a significant energetic cost is still incurred through increased mRNA synthesis and may be a plausible explanation as to why the first step of gene expression is a highly regulated control step. Moreover, the larger ATP investment that goes into the synthesis of a single transcript provides additional support to the proposal that post-transcriptional mechanisms are integral to the regulation of steady-state mRNA level and cellular bioenergetics. For instance, the recycling of nucleotides from transcripts not bound to ribosomes and undergoing translation is intuitively a cost effective strategy to reduce the amount of ATP required for nucleotide synthesis via the pentose phosphate pathway. A suitable illustration to emphasise the cost of transcriptional upregulation at 15°C is the significant increase in *hsp70* transcription at 9 h that is distinct from the low level of transcription at 1 and 17 h, which are both comparable to the level of transcription at 8°C (Figure 13e). In the context of the molecular unit of currency of the cell, the basal level of *hsp70* transcription (synthesis cost of ~103,000 ATPs per transcript) at the beginning (215,487 transcripts) and end (131,516 transcripts) of the light period, would have required an ATP investment of ~2.22 x 10^{10} and ~1.35 x 10^{10} ATPs, respectively. If *de novo* transcript synthesis was solely responsible for the increase in *hsp70* transcription in the middle of the light period (1,042,145 transcripts), this would have required a magnitude increase in investment to ~1.1 x 10^{11} ATPs. In light of the growing phenological mismatch between photoperiod and temperature at high latitudes, prolonged elevation of autumnal temperatures and early increase of spring temperatures may affect not only the seasonality of fish but also energy allocation, utilisation and cellular economics, through temperature induced transcriptional upregulation that may eventually become off tempo with physiological responses and transformations linked to proximate

environmental cues and prevailing habitat conditions, such as food availability, hydrodynamics, etc. Therefore, the elevated transcription and / or its rhythmicity and consequent mRNA expression lends further credence to the conclusion that the species is already experiencing thermal stress at 15°C and that considerable cellular energy is being allocated to transcript synthesis, ostensibly adding to the already high ATP demands required to preserve homeostasis at elevated temperature. These findings thus portend the liable consequences of increasing habitat temperatures to whole organism fitness, through alterations in transcriptional induction and energy utilisation in a thermally sensitive salmonid facing the threat of extinction due to anthropogenic activities.

Synthesis and Conclusion

The four studies in this thesis evaluated the effects of environmentally relevant acclimation temperatures and acute temperature increase on stenothermal and eurythermal representatives of the Salmonidae family. Starting from a higher level of organisational complexity in the first experiment (**I**), the consequence of a 7°C difference in acclimation temperature on whole organism thermal tolerance and its connection with morphological changes to tissues responsible for oxygen supply and delivery was investigated. It was conclusively demonstrated that both *S. alpinus* and *S. salar* exhibit ventricular plasticity and the capacity to adjust their thermal limits through acclimation, although the acquisition of thermal tolerance was more significant for the eurythermal than the stenothermal species. The second experiment was a cellular level analysis (**II**) of circadian *hsp70* and hsp70 expression at steady-state in two acclimation temperatures 7°C apart, and upon induction by a brief 7°C temperature increase. Temperature acclimation in this study was revealed to involve modifications to mRNA and protein expression of inducible hsp70, in a manner characteristic of its proposed function as a cellular thermostat capable of adjusting heat shock protein levels concurrently with chronic and acute temperature shifts as slight as 7°C, thus indicating a vital role for the molecular chaperone in the acclimation of *S. alpinus* and *S. salar* to suboptimal temperature. Furthermore, although *hsp70* and hsp70 expression displayed a positive correlation, the temporal relationship between transcript and protein level was generally asynchronous. The uncoupling of mRNA and protein expression at steady-state hinted at the possible involvement of post-transcriptional mechanisms, which was subsequently verified in the heat shock response of 15°C-acclimated *S. salar*, revealing an increase in *hsp70* level need not be accompanied by an increase in hsp70

and suggests translational inhibition, presumably in part through a decrease in mRNA stability. *S. alpinus* in contrast displayed the canonical induction of *hsp70* expression and consequent translation, signifying considerable thermal stress upon exposure to 22°C.

To establish how a 4°C difference in acclimation temperature influences gene transcription and steady-state mRNA level, an *in vitro* acclimation experiment (**III**) was conducted using a commercially available eurythermal *O. mykiss* cell line. It was determined that transcription may not be the primary control point regulating steady-state transcript level during acclimation, as there was an inverse relationship between transcription and mRNA level at the optimal and suboptimal temperatures. The results suggested that transcriptional upregulation is associated with low mRNA level, conceivably due to the integration of transcript synthesis and degradation at the optimal temperature, whereas the increase in mRNA level and reduction of transcription implies a central role for mRNA stability at the suboptimal temperatures, thus substantiating the deduction that post-transcriptional mechanisms are employed to regulate mRNA level. The final experiment was conducted using stenothermal *S. alpinus* cells obtained *in vivo* (**IV**) and examined how a 7°C difference in acclimation temperature affected gene transcription, steady-state mRNA level and their circadian rhythms. Transcription and / or its rhythmicity was found to be enhanced and that increased transcriptional induction occurs at a specific point in time when *S. alpinus* is acclimated to 15°C, whereas at 8°C the first step of gene expression is visibly attenuated. The increase in transcription was paralleled by an overall increase in mRNA level but the temporal induction of gene transcription appears to be disconnected from steady-state mRNA expression. Although the positive correlation between transcription and mRNA level is in stark contrast to the *in vitro* experiment and the conjecture that mRNA stability plays a prominent role in the acclimation process, it is quite apparent that transcription, mRNA level and their rhythmicity is markedly influenced by environmental temperature in the stenothermal salmonid, and that more cellular energy is allocated to gene transcription and maintaining elevated mRNA level at the higher acclimation temperature. The synchronicity of biological and environmental rhythms is of particular importance at high latitudes where photoperiod and temperature oscillations are pronounced and further exacerbated by recent climatic trends. The finding that mRNA level and transcriptional induction are affected by temperature may foretell how increasing habitat temperature and the progressive phenological discrepancy between photoperiod and temperature can disrupt circadian rhythms and seasonality, with likely deleterious outcomes on bioenergetics and

fitness of ecologically and economically valuable species in the frontline of climate change effects.

In summation, the data presented here collectively and unequivocally demonstrate small changes in temperature of no more than 4 to 7°C has a perceptible effect on salmonid physiology, from the whole organism, tissue, cellular and subcellular levels, and confirms the survival of *S. alpinus* is threatened and may be gradually outcompeted by *S. salar* as high latitude habitat temperatures continue their upward trajectory. From a methodological perspective, mRNA expression in conjunction with the improved utility of a salmonid-specific inducible hsp70 antibody as a sensitive biomarker for thermal stress, can incontrovertibly advance predictive capabilities concerning the effects of contemporary and future environmental perturbations. Fundamental insights were gained regarding the regulation of the first step of gene expression despite variations in transcriptional determinations, although steady-state mRNA expression remains a consistent and reliable proxy for transcription and gene expression analysis, especially as tools that permit protein level assessments are still lagging several steps behind nucleotide sequencing technologies. However, this is not to say that protein level measurements should be disregarded nor should transcriptional measurements be dismissed, as function is imparted at the protein level and further optimisation of the utilised nuclear run-on assay in combination with standard tools for evaluating gene expression, can provide a higher resolution perspective on physiological processes that allow an organism to sense and adapt to a changing environment. A detailed mechanistic understanding of the capacity of salmonids to adapt to novel conditions and the practical application of molecular level measures could substantially benefit the management and conservation of wild and domesticated fish stocks.

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