Investigating the genetics of thermal tolerance and adaptation to temperature amongst populations of Australian barramundi (*Lates calcarifer*).

Thesis submitted by

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Statement on the contributions of others

At the time of thesis submission, two manuscripts had been accepted for publication in peer-reviewed academic journals and one remained under review. The contributions of co-authors to the respective manuscripts are outlined below:

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KRZ – Project design, data generation, data analysis, review of final manuscript.

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Abstract

Australian barramundi (*Lates calcarifer*), are distributed over much of the northern and north-eastern coast where they inhabit rivers, estuaries and near coastal waters spanning some 16 degrees of latitude (10°S - 26°S). Over their distribution, populations of barramundi experience differences in thermal environmental conditions that vary from warmer and more consistent tropical conditions at northern latitudes (mean yearly range of 23.2 – 32 °C), to cooler and more variable conditions at southern latitudes (mean yearly range of 18.5 – 27.7 °C). Australian barramundi populations show strong genetic structuring and this, coupled with exposure to varying thermal environments, may have led to temperature tolerance differences among these populations indicative of local adaptation. As barramundi are currently cultured to some degree within all mainland states of Australia, the replication of optimal culture conditions, at significant cost to farmers, is necessary to ensure the success of primary breeding objectives. Identifying the underlying genetic mechanisms contributing towards peak growth and survival under a range of culture temperatures in barramundi would therefore be of significant benefit to the aquaculture industry. Research described in this thesis aimed to identify whether or not barramundi populations at the extreme of their Australian distribution exhibited evidence for local phenotypic thermal adaptation and, if so, whether underlying genetic differences could be established.

To demonstrate evidence of local adaptation, initial experiments aimed to differentiate between two genetically distinct populations of barramundi based upon their tolerance to increased water temperatures. To determine this, loss of swimming equilibrium (LOSE) was used as a predictor of upper thermal tolerance in fish from a warm-water adapted (Darwin, Northern Territory) and cool-water adapted (Gladstone, Queensland) population. Barramundi from both populations were simultaneously exposed to an increase in water temperature from 28 °C to 40 °C at the rate of 2 °C/h. LOSE was recorded as the time taken for individual fish to demonstrate loss of swimming equilibrium after water heating began. Significant differences in upper thermal tolerance, suggestive of local adaptation, were evident between the two populations with warm adapted, northern barramundi demonstrating a significantly longer time until LOSE at 40 ºC than cool adapted, southern barramundi (518.5 ± 8.0 min and 452.8 ± 8.0 min respectively, ANOVA; $F_{1, 22} = 7.86$, $P \leq 0.01$). However, as LOSE challenge tests are not practical to the identification of commercial broodstock; the response to temperature was also evaluated within dissociated caudal fin cells as a means of providing a sensitive and non-invasive method with which to determine upper thermal tolerance in whole animals. Prior to measurements of LOSE, small fin clips were taken from each fish and enzymatically digested to produce ‘free’ caudal fin

cells. Cells were incubated at 40 °C for 1 h as a thermal stress, before cell staining with Propidium Iodide (stains dead cells) and Calcein AM (stains live cells) was used, allowing for the determination of a dead/live cell ratio. Thermal tolerance results generated from dissociated caudal fin cells strongly correlated cell viability with LOSE measurements (average r = 0.69), confirming that this method can be used to discriminate between populations with different thermal tolerances without having to directly thermally challenge valuable broodfish. In doing so, these results provide strong evidence that thermal tolerance differences amongst barramundi populations arise due to significant contribution from differences at the genetic level.

Having demonstrated that divergent populations of barramundi show strong evidence for genetic adaptation to temperature, the expression of a group of genes likely to be involved in this species' response to an acute heat stress was examined. The acute heat shock response, as indicated by the expression of genes within the cellular stress (*Hsp90α, Hsp90β, Hsc70, Hsp70*), metabolic (*CiSy*, *CcoII*, *Ldh*) and growth (*Igf1*, *Mstn1*) related pathways, was examined following an increase in water temperature from 28 °C to 36 °C over 30 min. Barramundi were maintained at the acute stress temperature of 36 °C for 1 hr before being returned to 28 °C and allowed to recover at this temperature for a further 2 weeks. Muscle tissue sampling over the experimental period allowed for the expression quantification of stress, metabolic and growth related genes via real time quantitative PCR (RT-qPCR), where a robust and reliable normalization approach identified both *α-tub* and *Rpl8* as appropriate genes for the analysis of gene expression in response to an acute heat stress. *Hsp90α* and *Hsp70* of the inducible heat-shock response pathway showed a massive up-regulation of gene expression in response to heat stress, whilst the constitutive heat shock genes *Hsp90β* and *Hsc70* showed no change over the course of the experiment and a small increase after 2 weeks of recovery respectively. Of the three genes representing the metabolic pathway (*CiSy*, *CcoII* and *Ldh*) only *CcoII* changed significantly showing a decrease in gene expression which may suggest a small suppression of aerobic metabolism. *Igf1* of the growth pathway showed no significant differences in response to an acute heat stress, whilst *Mstn1* increased at the beginning of the heat stress, but returned to basal levels soon after. Overall, the results demonstrate that an acute heat stress in *L. calcarifer* caused a significant increase in the expression of genes from the cellular stress response pathway along with a potential decrease in aerobic metabolism and only relatively minor changes to the growth pathway. These results highlight the hardy nature of *L. calcarifer* and demonstrate the importance of an adaptive gene expression response in coping with the sudden temperature changes routinely encountered on a daily basis within its natural environment.

Having identified key genes from temperature responsive pathways, differences in the phenotypic performance of barramundi populations to temperature (as highlighted in *Chapter*

2), were interpreted using gene expression data. Following on from the analysis of gene expression in response to an acute heat stress, key genes from the cellular stress, metabolic and growth pathways were analysed via RT-qPCR in both a warm (Darwin, Northern Territory) and cool (Gladstone, central Queensland) water adapted barramundi population reared at either hot (36 °C), control (28 °C) or cool (22 °C) temperatures for 106 days. Growth indicators were periodically measured during the growth trial and white muscle tissue was also sampled at day 0 (T=0), day 3 (T=3), day 9 (T=9) and day 106 (T=106) for gene expression analysis. At a rearing temperature of 22 °C, a higher final weight in cool adapted barramundi over warm adapted barramundi (145.9 ± 11.1 g and 89.9 ± 3.5 g, respectively) was underpinned by a significantly faster induction of the cellular stress response and greater expression of *Hsp90α*. Conversely, no changes in heat shock protein (*Hsp*) gene expression were observed in barramundi reared at either 36 °C or 28 °C. Genetically regulated adaptation to cool temperatures in barramundi therefore seems to be correlated with changes to the cellular stress response pathway. Regulation of metabolic and growth associated genes to temperature were consistent between populations and were not affected by a control (28 °C) or a cool (22 °C) rearing temperature. At 36 °C, both warm and cool adapted barramundi exhibited a significant decrease in *CcoII* expression consistent with expectations associated with alterations in aerobic capacity, however, *CiSy* expression remained unchanged. The impaired growth of both populations reared at 36 °C was accompanied by a decrease in the expression of *Igf1* and an increase in the expression of *Mstn1*. As such the expression of both genes can be reliably used to indicate the growth status of barramundi at high temperatures, however, long term control of growth at cool temperatures seems to be under the control of alternate gene pathways, as no significant differences in the expression of these two growth related genes was observed.

To further investigate population differences, the underlying transcriptome profile of barramundi reared over a long term period was examined via Illumina mRNA deep sequencing as a means of determining the major contributing gene categories giving rise to the phenotypic differences in population growth. White muscle tissue from warm and cool adapted barramundi reared for 106 days was sampled and used for pathway expression analysis in conjunction with the phenotypic data collected previously. Gene ontology (GO) analysis revealed enrichment in categories relating to the regulation of peptidase activity as well as microtubule, cytoplasmic and cellular metabolic based processes. Further analysis of the GO category "microtubule based process" with associated genes from the "response to stress" category revealed an apparent re-organisation of cytoskeletal elements in response to an induced cold stress in northern barramundi reared at 22 °C, when compared with northern barramundi reared at 36 °C. Between southern barramundi and northern barramundi reared at 36 °C, an analysis of the "endopeptidase inhibitor activity" GO category, in conjunction with stress genes, indicated a

suppression of the immune complement system in southern barramundi, along with an increase in the cellular stress response. As southern populations of barramundi from a cooler environment grew significantly faster at 22 °C than northern barramundi populations from a warm environment; the results of the present study show that southern populations of barramundi exhibit underlying molecular adaptation to cooler water temperatures, but still retain a tolerance for warm water temperatures. Furthermore, GO profiling has revealed groups of genes that underlie population differences in temperature tolerance as a means to prioritize the analysis of differential gene expression in studies of local adaptation in the future.

The results of this thesis demonstrate the occurrence of local adaptation to environmental temperature amongst Australian populations of barramundi from significantly different environments. Both phenotypic and genetic indicators reveal the hardy and adaptable nature of barramundi to adverse temperatures in accordance with the variable characteristics of estuarine environments. Specifically, barramundi from the northern end of the species distribution range show a greater tolerance to short, but significant spikes, in water temperature compared with barramundi from southern populations. However, gene expression analysis and growth data reveal that southern populations of barramundi show adaptive traits leading to better growth at cooler temperatures when compared with northern populations. The performance of southern barramundi at cool temperatures was accompanied by significant differences in the expression of heat shock genes and other associated stress responsive genes, suggesting that faster induction and higher expression of heat shock genes aids the continuation of growth at cooler temperatures. The expression of nominated growth related genes was only affected at high rearing temperatures and it is therefore likely that growth at cooler temperatures is under the influence of alternate mechanisms, which could prove interesting for future research.

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Chapter 1.0 General Introduction

1.1 The effects of temperature on aspects of fish biology.

Temperature affects every level of biological organisation from the expression of genes and proteins through to changes in organism physiology and behaviour. At its most fundamental, temperature affects the kinetic energy of molecules and modifies diffusion rates, enzymatic activity, interactions between molecules, as well as the properties of cell membranes. In doing so temperature can significantly affect cellular processes and by extension alter the metabolic and physiological activities of tissues and organs where even changes on the order of 1 °C may cause the rates of biological reactions to change by almost 10% (Huey & Bennet, 1990). By virtue of these influences, ambient temperature is therefore a major determinate of growth, reproduction, immune-competence and many other important physiological processes in poikilothermic organisms such as fish (Guderley, 2004). Moreover, the external and hence internal thermal environment of fish is not constant but continually changes throughout both time and space. Consequently, many physiological processes must be able to function at the range of temperatures commonly encountered within the organism's natural thermal environment.

The influence of temperature on the performance of physiological traits is described using thermal performance curves which specify the relative performance of a trait over a range of body temperatures (Huey & Kingsolver, 1989). According to these curves, as temperature increases, a rise in physiological trait performance occurs up to a maximum value (optimal temperature). If temperature continues to increase a rapid decrease in trait performance quickly follows. On the other hand, a reduction in temperature below a trait optimum results in a slower, more gradual loss of trait performance (Fig. 1.1) (Huey & Bennet, 1990). Many physiological processes in fish, such as growth, follow this general pattern. For instance, in cultured barramundi (*Lates calcarifer*) it was found that growth efficiency was optimal at temperatures between 27 °C and 33 °C, whilst at rearing temperatures significantly above and below this range (39 °C and 21 °C respectively) growth rate, whilst still positive, was significantly reduced (Katersky & Carter, 2007; Katersky & Carter, 2005). Growth, food efficiency and physiological status were similarly affected by temperature in juvenile turbot (*Scophthalmus maximus*) and found to be optimal at temperatures between 17 °C and 20 °C. The lower limit for growth in turbot was shown to be around 14 °C with a significant depression of growth occurring at temperatures between 8 °C and 11 °C, and at temperatures of 23 °C and above (Burel et al., 1996; Pichavant et al., 2000). Temperature is also known to be the principal environmental cue initiating changes in immune function within a variety of fish species, affecting both the innate and acquired immune responses. In sockeye salmon (*Oncorhynchus nerka*) a larger role of the innate immune response was observed in fish reared at 8 °C compared with fish reared at 12 °C

(Alcorn et al., 2002), whilst channel catfish (*Ictalurus punctatus*) and tench (*Tinca tinca*) showed an increase in macrophage respiratory burst activity at low temperatures in response to pathogenic bacteria (Ainsworth et al., 1991; Bowden et al. 2007; Collazos et al., 1994). Conversely, aspects of acquired immunity such as the production of antibodies have been shown to respond to an increase in temperature, whilst low temperatures seem to suppress the acquired immune system (Bly & Clem, 1992). Bromage et al., (2004) examined the effects of cold water (19 °C) on the acquired immune response in barramundi following exposure to attenuated *Streptococcus iniae* and found that the humoral response was significantly diminished and extremely variable compared with fish maintained at higher culture temperatures. Furthermore, in Nile tilapia (*Oreochromis niloticus*) plasma IgM levels increased with rising temperatures in fish reared at 18.4 °C, 23 °C and 28 °C, but decreased in fish reared at 33 °C, indicative of the thermal range for antibody production in this species (Dominguez et al., 2004). Temperature is also one of the most important extrinsic factors determining muscle performance in ectotherms and may indirectly affect the swimming performance of a developing fish by influencing size at hatching, body shape, fin morphology and fibre development (Koumoundouros et al., 2002).

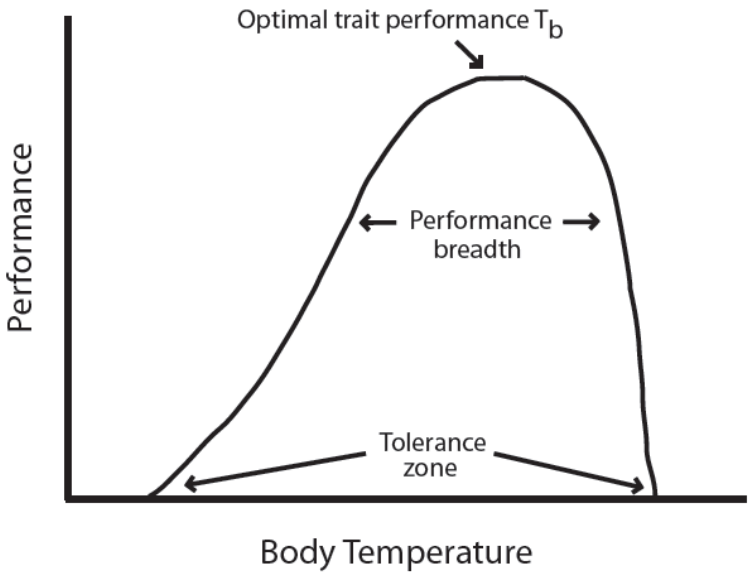

Fig. 1.1 Thermal tolerance curve of trait performance**.** Thermal tolerance curves are used to indicate a temperature range at which trait performance will be above a certain threshold (performance breadth), the temperature at which trait performance will be optimal (optimal trait performance) and the absolute upper and lower permissible temperatures for a trait (tolerance zone) (Huey & Bennett, 1990).

1.1.1 Temperature stress and the physiological stress response.

Episodes of acute or prolonged exposure to temperatures outside of an organism's physiological 'norm' can threaten homeostasis causing stress and the activation of behavioural, physiological and cellular responses that aim to restore equilibrium and limit damage from the stressor. These generalised responses to stress can be broadly grouped into three main categories; the primary, secondary and tertiary responses, although these classifications are flexible due to a high degree of overlap between categories (Barton, 2002). The primary stress response involves recognition of an altered or stressed state and the induction of the neuroendocrine system. This response activates two major pathways; the hypothalamic-pituitary-interrenal (HPI) axis and the sympathetic-chromaffin (SC) system (Iwama et al., 1999). Induction of the HPI axis causes the inter-renal tissue of the head kidney to release the stress hormone cortisol into the circulation whereas stimulation of the SC system causes catecholamine (CA) release (most notably adrenaline) from the chromaffin tissue as an immediate response to almost any severe acute stress (Wendelaar Bonga, 1997). Following from the primary response, the secondary stress response is mediated in part by the actions of stress hormones and includes changes in biochemical, physiological and immunological parameters. CAs have a major effect on the circulatory and respiratory systems in fish. Following CA release a significant increase in oxygen uptake by gill tissue occurs due to increased ventilation, flow rate and diffusion. The capacity for blood to carry oxygen is also enhanced as CA's stimulate the production of erythrocytes and cause them to swell resulting in an increase in hematocrit. In rainbow trout (Barton, 2002), CAs also affect erythrocyte pH levels, increasing the affinity of haemoglobin for oxygen (Wendelaar Bonga, 1997). The hyperglycaemic state associated with stress in fish can also be attributed in part to the affects of CA secretion. Increased levels of circulating glucose are due to the effects of CAs on glycogenolysis and to a lesser extent gluconeogenesis. However, the role of gluconeogenesis in maintaining plasma glucose levels probably increases with a prolonged stress and the depletion of glycogen stores (Randall & Perry, 1992). Although the functions of CAs and cortisol are similar, the rise in circulating cortisol is slower, more sustained, and aimed at either facilitating or moderating the effects of CAs (Brown, 1993). Cortisol primarily targets the gill, liver and intestine to regulate hydro-mineral balance and energy metabolism but is also responsible for growth inhibition and the suppression of reproductive and immune functions. Cortisol is most widely used as an indicator of stress in fish as levels rise rapidly in response to a stress, reaching peak levels after only a few minutes following an acute heat stress. If the stress is chronic, cortisol levels may persist at levels above basal for hours or even days (Brown, 1993). If a stressor is severe enough or sufficiently prolonged, the tertiary response may follow resulting in whole animal and population level changes. Following the failure of a fish to acclimate to a stress, the consequences of continual

energy partitioning to supply the metabolic demands of the stress response may have adverse consequences for anabolic processes such as growth and reproduction. In addition to this, overall immune function can be affected as well as behaviour and ultimately the survival of the organism. At the population level a prolonged and sufficient stress can impair recruitment and productivity and eventually alter the species abundance and diversity (Pottinger & Pickering, 1992). Generally speaking, the actions of stressors are two-fold. Initially, disturbance of an organism's homeostasis elicits a coordinated set of compensatory and/or adaptive behavioural and physiological responses intended to overcome a threat. However, if a stressor is chronic or overly persistent then the stress response may lose its adaptive properties and become dysfunctional, causing a reduction in growth, reduced fecundity and impaired immunity to disease (Iwama et al., 1999; Wendelaar Bonga, 1997).

1.1.2 Temperature stress and the cellular stress response.

The cellular stress response is a universal response that essentially protects the cell from damage done to macromolecules. Some aspects of the cellular stress response are non-stressor specific and are activated purely upon detection of macromolecule damage whilst other aspects of the response, such as those directed at re-establishing homeostasis, are highly stressor specific and variable across taxa (Kultz, 2005). The relatively recent application of broad scale genomic techniques to the study of stress in fish, such as microarray and next generation transcriptome profiling, have gone a long way to unravelling the complexity of the cellular stress response at the molecular level. The response to temperature stress involves many facets of the transcriptome, however, changes to transcriptional and translational regulation are common. In response to an acute heat shock in the goby (*Gillichthys mirabilis*), transcriptional activity was increased via the regulation of genes such as *Creb2*, *C/Bep* and *Stat3*, which are important effectors of gene expression (Buckley et al., 2006). The increased demand for heat tolerant proteins, as well as a rise in the expression of molecular chaperones such as heat shock proteins, is thought to be a main driving force behind the heat induced increase in translational and transcriptional activity (Gasch et al., 2000). However, genes involved in transcriptional regulation, RNA splicing and translation, such as RNA polymerase II activators and numerous ribonucleoproteins, were also increased in carp and channel catfish in response to cold stress (Gracey et al., 2004; Ju et al., 2002). This is most likely due to an increase in RNA secondary structure at cold temperatures and also demonstrates a key concept of cold acclimation; the cell synthesises more enzymes to offset the rate limiting effects that cold temperatures have on biochemical performance (Gracey et al., 2004). Temperature stress also has a significant effect on carbohydrate metabolism. In response to both heat and cold exposure the expression of numerous genes involved in glycolysis, gluconeogenesis, the tri-carboxylic acid cycle and the

electron transport chain has been repeatedly shown. In the gill of acute heat stressed goby the expression of ATP-producing genes such as glucose-6-phosphate, lactate dehydrogenase of the glycolytic pathway, and various cytochrome subunits from the electron transport chain were observed (Buckley et al., 2006). The expression profile of cold exposed carp also suggested a large number of changes to mitochondrial metabolism and the production of ATP molecules in line with the high energy demands of coping with stress (Gracey et al., 2004). Not surprising then is the suppressive effect of adverse temperatures on the cell cycle and growth pathway, as resources are diverted away from these processes to combat stress.

Cold temperatures have been shown to suppress the expression of genes involved in muscle contraction and calcium binding such as calcium binding proteins, parvalbumin genes and slow twitch troponins. In cooled carp muscle, the expression of ubiquitin ligases were detected which are responsible for targeting proteins for destruction and have been used as reliable indicators of muscle atrophy (Gracey et al., 2004). The pattern of gene expression in response to heat stress suggests a suppression of the cell cycle, owing to a reduction in growth factor 6 and *Mapk3* genes which are involved in cell cycle regulation and the promotion of cell growth. In addition the transcriptional regulators, *Hd9* and *Mef2*, were down regulated and these are linked to cell proliferation (Gracey et al., 2004). Perhaps one of the best known responses to heat stress, however, involves the expression of heat shock proteins (Hsp). Hsps play a vital role in the cellular stress response through the maintenance of protein integrity, aiding the translocation of proteins around the cell, preventing premature folding and aggregation of proteins, and in mediating steroid and receptor binding (Iwama et al., 1999). As an example of how heat shock protein genes respond to heat stress, both the gill and muscle tissue of the goby showed a significant up regulation of both inducible heat shock proteins Hsp70 and HspP90. An increase in the smaller Hsp27 was also detected and may reflect a particular emphasis on protecting cytoskeletal protein function (Buckley et al., 2006). Conversely, Hsp70 was induced only briefly in channel catfish in response to cold, whilst the expression of cold inducible RNA binding protein (Cirbp), which is commonly regulated during cold exposure, showed expression levels that were proportional to the degree of cold stress in carp (Gracey et al., 2004; Ju et al., 2002). Also demonstrated in response to cold stress was an increase in the metabolism of lipids. This has been shown to occur in both the brain and the liver of cold stressed fish where genes involved in fatty acid metabolism and cholesterol synthesis were expressed in the liver, and genes involved in long chain fatty acid synthesis were expressed in the brain (Ju et al., 2002). These expression changes are a distinctive characteristic of the response of cells to cold, where adaptive changes are made to the cellular membrane through the alteration of lipid composition in order to maintain membrane fluidity and function (Ju et al., 2002). Furthermore, the effects of both hot and cold temperature stress commonly results in protein damage, as evidenced by the

expression of numerous ubiquitin units that conjugate to proteins and target them for digestion. It is thought that the induction of the protein degradation pathway may occur when, despite an increase in chaperone activity, the cell incurs significant protein damage. It may also be that proteolytic activation occurs as part of an adaptive response where temperature intolerant molecules are recycled in favour of more robust isoforms. Following this, significant cellular damage as a result of temperature stress can result in apoptosis or programmed cell death (ref).

1.1.3 Temperature compensation in fish.

Upon exposure to a relatively long term and persistent temperature challenge, compensatory changes are initiated that allow fish to achieve optimal physiological and biochemical rates despite disparate internal body temperatures (Hazel & Prosser, 1974). Compensatory changes occur in response to seasonal changes in environmental temperature and hence may be quite common. This process is customarily referred to as acclimation, where changes occur within the lifetime of the organism and generally take several weeks to transpire. Compensation may also occur over evolutionary time spans where a species adapts to a specific thermal environment over many generations. This process is referred to as adaptation and results in organisms from widely different thermal environments exhibiting very similar rates of physiological activity (Hazel & Prosser, 1974). At the molecular level, mechanisms that maintain homeostasis (physiological acclimation) and/or that act upon heritable differences within or between species (evolutionary adaptation) often involve altering the activity of specific enzymes via changes in kinetic rate constants, or active enzyme concentrations (Hochachka & Somero, 1968). For example, populations of the fish *Fundulus heteroclitus* are distributed along the eastern coast of northern America and are exposed to clinal variations in temperature of nearly 1 °C change per 1 degree of latitude. At the southern end of the species distribution, summer temperatures often reach 35 °C, whereas northern populations seldom experience temperatures greater than 25 °C. Between northern and southern populations of *F. heteroclitus*, RNA transcription and associated heart-specific lactate dehydrogenase B (Ldh-B) protein levels correlated with clinal variation in temperature (Crawford & Powers, 1992). Ldh is important as it catalyses the reversible reaction of lactate to pyruvate during anaerobic respiration and thus plays an essential role in maintaining energy balances within the cell. Locally adapted populations of *F.heteroclitus* were shown to possess two different alleles encoding for different Ldh isozymes with different kinetic characteristics. These isozymes are thought to be evolutionarily important to the adaptation of *F.heteroclitus* to different environments as they are associated with differences in swimming performance, developmental rates, metabolic change and survival at high temperatures (Di Michele et al., 1991; Paynter et al., 1991; Powers et al., 1983). Further studies looking for adaptive differences between populations of *F.heteroclitus* revealed that southern

populations recorded a critical thermal maximum (CTMax) that was on average 1.5 °C higher than northern populations across a range of acclimation temperatures. This 1.5 °C difference was also maintained for critical thermal minima (CTMin) in all but the three lowest acclimation temperatures (Fangue et al., 2006). At the same time, the relationship between whole organism thermal tolerance and the regulation and sequence of heat shock protein genes (*Hsp's*) was examined. Although no fixed differences in the amino acid sequence of these proteins was detected between populations, there were significant differences in the onset temperature for gene expression and in mRNA expression levels in response to a common heat shock protocol (Fangue et al., 2006; Schulte, 2007).

In adapting to new thermal environments and acquiring new characteristics to aid in performance, fish often experience a correlated decline in other traits or functions, termed a 'trade-off'. A trade-off can be defined as a decline in non-selected characters that accompany adaptation to new selective conditions and can best be described using examples from cold water species (Portner et al., 2006). The loss of haemoglobin and myoglobin expression in the icefish (Family *Channichthyidae*) are well known examples of traits lost during the evolution of these fish to extremely cold water temperatures (Cocca et al., 1997; Montgomery & Clements, 2000; Sidell et al., 1997). The loss of these respiratory proteins can be linked to the absence of positive selection for their function due to the high solubility of oxygen at low temperatures and the relatively sluggish swimming behaviour of these fish (Hofmann et al., 2000). Antarctic notothenoid fishes (to which the *Channichthyidae* belong) are distinguished by a number of other physiological adaptations to extreme temperatures such as the presence of antifreeze glycoproteins that prevent freezing of tissues at sub-zero temperatures, enzymes that exhibit high activity to offset the effects of low temperatures on metabolic rates (Hofmann et al., 2000), and in some species the lack of a heat shock response. In hepatocytes isolated from the Antarctic fish *Trematomus bernacchii*, the inducible heat shock response to an acute temperature stress was not detected. Western blot analysis detected the constitutive Hsc70 protein, but was not able to detect the synthesis of any size class of inducible heat shock protein after hepatocytes were incubated at a stress temperature of 10 °C (Buckley et al., 2004). The inducible heat shock response has been lost in this species as a result of years of evolution at stable sub zero temperatures (Hofmann et al., 2000). Compensatory changes to metabolism are also commonly demonstrated during adaptation of fish to different thermal environments. As the primary producers of ATP, adjustments to mitochondrial rates of proliferation and degradation are crucial in mitigating the decelerating effects of cold on metabolism. This was demonstrated in a comparison of North Sea and Barents Sea populations of cod (*Gadus morhua*) by examining the expression of key mitochondrial enzymes in white muscle. In both populations, cold acclimation caused an increase in the activity of both citrate synthase (Cisy) and cytochrome c oxidase (Cox), however, enzyme activity was more pronounced in the Arctic

adapted Barents Sea population (Lucassen et al., 2006). In cod in general, but particularly in Barents Sea cod, the negative effects of cold on aerobic capacity are compensated for by an increase in mitochondrial densities and an increase in enzymatic activity. Similarly, in Antarctic fish mitochondrial volume densities are amongst the highest known for vertebrates and are associated with cold compensation of aerobic metabolic pathways, improved recovery from exhaustive exercise and enhanced lipid stores (Portner, 2002). The mechanical properties of muscle are also highly temperature dependant and adaptation to different thermal environments often involves major changes to the contractile mechanisms of fish muscle. Some species of fish have the ability to rebuild their myofibrillar system by expressing a different set of myosin contractile genes at low temperatures. This enables fish acclimated to low environmental temperatures to develop more force and power output than those acclimated to warm environmental temperatures. The myofibrils of Antarctic fish have been shown to have a greater specific ATPase at low temperatures, but at the expense of reduced thermostability (Goldspink, 1995). Pond fish such as carp are also able to undergo seasonal adaptation to environmental temperature by changing the activity and thermal stability of their myofibrillar ATPase to better cope with seasonal fluctuations and periods of extreme temperatures such as drought (Goldspink, 1998).

1.1.4 The future impacts of climate change on estuarine environments.

Estuarine environments are characterised by the presence of multiple interacting abiotic stressors and are considered 'high stress' environments due to frequent variation in factors such as temperature, salinity and dissolved oxygen (Bianchi, 2006). Climate change is predicted to affect estuaries predominantly through an exacerbation of current stressors, including rising temperatures (Gillanders et al., 2011). However, the warming of estuarine waters will affect tropical and temperate species differently depending upon whether a species resides at the extremes of it distribution range and temperature tolerance (Gillanders et al., 2011). For example, rising ocean temperatures are predicted to result in the expansion of tropical and low latitude species towards polar regions with significant secondary effects upon predator/prey interactions, whilst species from colder climates may experience a reduction in habitat size and increased competition for resources. Welch et al., (1998) used fisheries and oceanographic data to predict that by the year 2090 the temperature of the Pacific Ocean will have risen to such an extent as to be virtually uninhabitable to stocks of Pacific salmon. Their distribution, along with that of other salmonids, will be restricted to marginal seas such as the Bering Sea and the Sea of Okhotsk. For other species, particularly estuarine species, certain habitat requirements may prevent or limit migration. In some instances mobile fish and fish at older life stages may be able to migrate, however, many species will be unable to travel suitable distances to locate

favourable conditions (Scavia et al., 2002). These species may have specific habitat requirements, such as the need for brackish or fresh water at early life stages or during spawning periods, swamp and marsh environments for shelter or foraging, or dependence upon local food types that preclude their survival elsewhere. As a result, these species will be forced to adapt and those already existing near their upper thermal tolerance limits will show impaired growth and behavioural characteristics during periods of warming (Gillanders et al., 2011). An increase in temperature may also have a positive impact for species living within their thermal tolerance limits. Increased growth rates as a result of a rise in temperature may reduce the susceptibility of some species to predation at early life stages and lead to earlier migration of anadromous fish out to sea. Although these effects may only be realised if temperature does not deleteriously affect other factors such as swimming speed and food resources. Additional complications to predicting the future impacts of climate change in fish assemblages arise from the fact that the effects of climate change are expected to vary geographically. For example, although climate models predict a general warming of Australian environments over the next century, warming will occur to a lesser extent towards the south of the continent especially over winter months, whilst the rate of precipitation is expected to decrease in southern regions, but increase in northern regions (Gillanders et al., 2011). This information together with the fact that basic biology and life history information is lacking for many estuarine species suggests that further research into estuarine fish, particularly tropical estuarine species, is needed with an emphasis on how temperature affects their biology and inherent capacity to adapt to a changing environment.

1.2 Barramundi (*Lates calcarifer*) as a likely candidate for the study of temperature adaptation.

Barramundi (*Lates calcarifer*) inhabit tropical estuaries and lagoons over Southeast Asia and throughout much of northern Australia. Within Australia itself, barramundi range from the Ashburton River in Western Australia (22° 30' S), across the north of the country, and down the east coast as far as the Noosa River in Central Queensland (26° 30' S). This range covers some 16 degrees of latitude and encompasses a wide range of environmental temperature regimes. For example, at the northern end of the species distribution range in Darwin (Northern Territory), temperatures fluctuate on a mean yearly average from 23.2 °C in colder months to 32 °C during summer, whilst at the southern end of the species distribution near Gladstone (Central Queensland), mean yearly temperatures vary between 18.5 °C in winter to around 27.7 °C in summer (Bureau of Meteorology; http://www.bom.gov.au). In general, northern populations of barramundi experience significantly warmer and more consistent temperatures than populations towards the southern end of the species distribution where temperatures are cooler and much

more variable. Across this thermal cline, barramundi has been shown to exhibit high levels of genetic structuring, with protein and genetic studies identifying a total of 16 discrete genetic stocks (Keenan, 1994; Salini & Shaklee, 1988). In addition to this, barramundi are euryhaline and a catadromous species and require estuarine and in-shore marine habitats to breed. However, after the eggs hatch juvenile barramundi migrate upstream to freshwater river systems away from river mouths (Pusey et al., 2004) and on the basis of recorded tagged fish movements it is believed that the migration of individuals between adjacent river-mouths more than 100 km apart is unlikely (Keenan, 1994). Therefore, adjacent populations exchange little genetic information by way of individual migration. Taken together, these observations give rise to the question as to whether or not the high levels of genetic structure in barramundi have translated into functional genetic adaptation to local environmental stressors, including that of temperature. However, an examination of the current barramundi stock structure in Australia through biogeographical studies suggests that phenotypic differences arising between populations from genetic differences should be relatively small. This is due in part to the relatively recent establishment of the current population structure (~17,000 years ago) and evidence for substantial migration and hybridization between historical eastern and western populations during the last glacial maxima (Keenan, 2000). Nonetheless, studies have shown that populations of barramundi from varying thermal environments respond differently under thermal stress for traits such as survival and growth. Two Queensland strains of barramundi (from Cairns in northern Queensland and Burrum River, central Queensland) were co-reared in open freshwater cage culture where it was found that similar growth rates were recorded for both populations, however, bacterial infections caused greater mortality during cold temperature snaps in the more northerly derived Cairns strain (Rogers & Bloomfield, 1993). As temperatures cooled with the onset of winter, Burrum River fish were observed to have higher feed rates whilst Cairns fish had lower appetite, lower condition factor, reduced growth during winter and higher mortality rates. These findings were symptomatic of the unique adaptation of Cairns and Burrum River strains to local thermal conditions lending support to the argument that Australian barramundi may in fact show evidence of local adaptation to temperature (Rogers & Bloomfield, 1993).

The presence of local adaptation within barramundi populations poses important considerations for aquaculture. In Australia, particularly in Queensland, barramundi supports significant commercial and recreational fisheries. The current value of barramundi aquaculture in Queensland was valued at $21.4 million in 2008-2009, however, barramundi are now cultured to some degree in all mainland states of Australia with significant contributions arising from their culture in Western Australia ($4.8 million), New South Wales ($1.3 million) and the Northern Territory ($4.2 million) (Pham, 2010). As a result of the expanding base of

barramundi farming, significant costs to heating and the maintenance of minimal temperature standards required for growth are incurred in farms located outside of the species natural distribution range suggesting that there may be advantages to identifying stocks with broader thermal tolerances. Coupled with the high level of genetic structuring observed between populations of Australian barramundi and the resulting probability of local adaptation, aquaculture farms may currently fail to realise the growth and fitness potential of their stock due to the introduction of genetic constraints on temperature tolerance. Further research is needed to determine the response of barramundi to temperature and to ascertain whether or not populations show evidence of adaptation to local environment.

1.3 Thesis aims and structure

The current thesis aimed to determine whether local adaptation to temperature had occurred within Australian populations of barramundi and to investigate the gene expression response to temperature between any such adapted populations. In doing so this research aimed to identify genetic mechanisms likely to underpin adaptation and tolerance to temperature in these populations to provide the foundation for future investigation into temperature tolerance in this species. The structure and aims of the current thesis are outlined as follows.

Firstly, *Chapter 2* determined whether or not two populations of barramundi from opposite ends of the species distribution range exhibited differences in their response to high temperatures indicative of local adaptation to environment. The response to temperature in each population was determined using a measure of whole animal upper thermal tolerance and correlated with a novel approach to establish the thermal tolerance of individual barramundi using a homogenate of caudal fin cells from each fish tested. *Chapter 2* reports on the differences in upper thermal tolerance between a Darwin and a Gladstone population of barramundi and demonstrates a correlation between measurements of upper thermal tolerance in whole fish, and ratios of live to dead cells in free cell assays as a way of non-invasively ranking temperature tolerance in populations of barramundi.

Chapter 3 of this thesis measured the expression of genes from the general stress, metabolic and growth pathways using RT-qPCR to reliably indicate the response of barramundi to an acute heat stress. Genes from the general stress response included the inducible heat shock proteins (*Hsps*) *Hsp70* and *Hsp90α*, as well as their constitutive counterparts *Hsc70* and *Hsp90β*. Gene expression was examined for citrate synthase (*CiSy)*, cytochrome c oxidase subunit II (*CcoII*) and lactate dehydrogenase B (*LdhB*) from the metabolic pathway, whilst insulin like growth factor 1 (*Igf1*) and myostatin 1 (*Mstn-1*) were used as representatives of the general growth

pathway. In doing so all *Hsps* from the general stress response pathway as well as *CiSy* were characterised in barramundi for the first time and a robust normalization approach for the analysis of gene expression in fin fish under temperature treatment was established from a pool of eight potential reference genes.

Chapter 4 analyses temporal changes in gene expression amongst two populations of barramundi (Darwin and Gladstone) throughout an extended rearing period at cool, control and warm water temperatures. Gene expression is measured using RT-qPCR to quantify the expression of genes previously characterised and analysed in *Chapter 3* and is used in conjunction with phenotypic growth data gathered from both populations at all three rearing temperatures to identify potential differences in gene expression and growth performance resulting from the adaptation and specialisation of barramundi to temperature.

Chapter 5 builds upon the results of *Chapter 4* and uses next-generation transcriptome profiling to provide a snap shot of gene expression in two populations of barramundi reared during a long term exposure to both warm and cool rearing temperatures. This chapter identifies expression differences between a warm adapted northern population and a cool adapted southern population of barramundi (Darwin and Gladstone, respectively) and identifies major areas of the transcriptome that will enable the expansion of future research into the analysis of adaptive mechanisms for coping with temperature.

Each data chapter (2-5) of this thesis is presented with a stand-alone introduction, material and methods, results and discussion section. At the time of thesis submission all experimental data chapters were either submitted or accepted for publication into scientific journals and are thus presented here in publication form with only minor modifications.

Chapter 2.0 Thermal tolerance varies on tropical and sub-tropical populations of barramundi (*Lates calcarifer*) consistent with local adaptation.

2.1 Introduction

Barramundi (*Lates calcarifer*) are a eurythermal finfish species that inhabit tropical estuaries and freshwater lagoons throughout northern Australia and Southeast Asia (where it is known as Asian sea bass). Barramundi are exposed to a broad range of thermal conditions across the 16 degrees of latitude in which they occur within Australia and this, coupled with significant genetic stock structure (Keenan, 1994; Salini & Shaklee, 1988; Shaklee et al., 1993; Shaklee & Salini, 1985), may have resulted in localized adaptation to temperature. Barramundi is an important aquaculture species in Australia and is increasingly being seen as an emerging global aquaculture species in North America, Asia and Europe. The majority of barramundi aquaculture in tropical Australia occurs outdoors in large ponds, raceways, or cages, where temperatures fluctuate freely on a daily and seasonal basis and in summer often reach 35 °C. In more southern (temperate) regions, farmers rear barramundi in intensive indoor re-circulation systems where the cost of water heating is often high and the minimum water temperatures for fast growth are utilized (Rimmer, 1995). Relatively few hatcheries supply the whole Australian industry and as a result barramundi seedstock from the same hatchery are often reared under different thermal conditions and exposed to temperatures that frequently reach the species' upper and lower thermal tolerance limits (Katersky & Carter, 2005). Therefore the aquaculture industry would significantly benefit from the identification of broodstock source populations that are thermally tolerant and better adapted to being reared over a broader range of culture temperatures and conditions.

Despite the presence of significant stock-structure genetically correlated differences in the thermal tolerance of barramundi have yet to be conclusively demonstrated (Keenan, 1994; Salini & Shaklee, 1988; Shaklee et al., 1993; Shaklee & Salini 1985). To date studies investigating the impact of temperature on barramundi have been confined to associating temperature and feeding/immune competence within single genetic stocks (Bromage & Owens, 2009; Katersky & Carter, 2005). For example, Katersky and Carter (2007) showed that high temperatures (39 °C) caused a decrease in food intake and an increase in metabolic rate that together significantly decreased growth in barramundi. However, these results were from experimental fish gathered from just one hatchery with a presumable genetic diversity confined to differences between related families. Rogers and Bloomfield (1993) communally reared barramundi originating from two different stocks within Queensland and found that the two stocks showed differences in feed consumption, condition (K) factor, growth rate and mortality across seasons suggestive of unique adaptation to local environments. However, fish stocks were relatively geographically and genetically close compared to what exists within the distribution of the species and no reciprocal genotype by environment experiments were

conducted to try to elucidate how the differences seen in growth and survival were due to genetic-based thermal adaptation. Consequently, whilst the existence of distinct genetic stocks of Australian barramundi is accepted there remains some question as to whether functional genetically-based differences have also arisen and whether this genetic diversity arose due to founder effects or local adaptation. In fact Keenan (1994), suggested that the genetic diversity between Australian stocks, although significant, was overall small, and that the genetic differences present would have little biological relevance to physiological traits like thermal tolerance.

Estimating or identifying the upper thermal tolerance limits of finfish is usually accomplished by exposing fish to near lethal temperatures and measuring the time to loss of swimming equilibrium (LOSE) (Rajaguru, 2002). Whilst the LOSE experimental method is effective at estimating upper thermal tolerance it exposes commercially valuable fish to an extreme stress rendering them unsuitable for subsequent aquaculture use, therefore this approach cannot be used to identify thermally tolerant broodstock. Recently a non-invasive estimate of a fish's upper thermal tolerance has been suggested based upon the temperature tolerance of dissociated caudal fin cells. This dissociated caudal fin cell assay has been successfully applied to both ayu (*Plecoglossus altivelis*) and Japanese flounder (*Paralichthys olivaceus*), where strong correlations were observed between the thermal tolerance of caudal fin cells and LOSE in individual fish (Sakamoto et al., 2005). A strong correlation between the response to temperature in dissociated caudal fin cells and LOSE in individual fish suggests a substantial underlying genetic component of temperature tolerance and this assay could be used to look for phenotypic differences in barramundi suggestive of adaptive genetic differences.

In this study we used loss of swimming equilibrium (LOSE) to examine if two Australian barramundi stocks from a tropical and subtropical environment showed differences in their upper thermal tolerances. The potential of dissociated caudal fin cell assays as a non-destructive predictor of whole organism upper thermal tolerance was also evaluated.

2.2 Materials and Methods

2.2.1 Experimental fish

Barramundi fingerlings (between 14.2-25.6 cm in total body length) representing a tropical (Darwin, Northern Territory 12 ° 27’ S, 130 °50’ E) and subtropical (Gladstone, Queensland, 23 ° 50 ’ S, 151 ° 15 ’ E) population (Fig. 2.1) were obtained from hatcheries and transported to James Cook University, Townsville, Queensland, where they were communally maintained within their separate populations in two 5000 L re-circulating freshwater tanks. Population identity was maintained by dividing the fish into separate floating tubs within each 5000 L tank. Before experiments all fish were allowed to acclimate under the same water conditions to 28 °C for one month and were fed formulated barramundi pellets (Ridley Aquafeed; http://www.agriproducts.com) once a day to satiation.

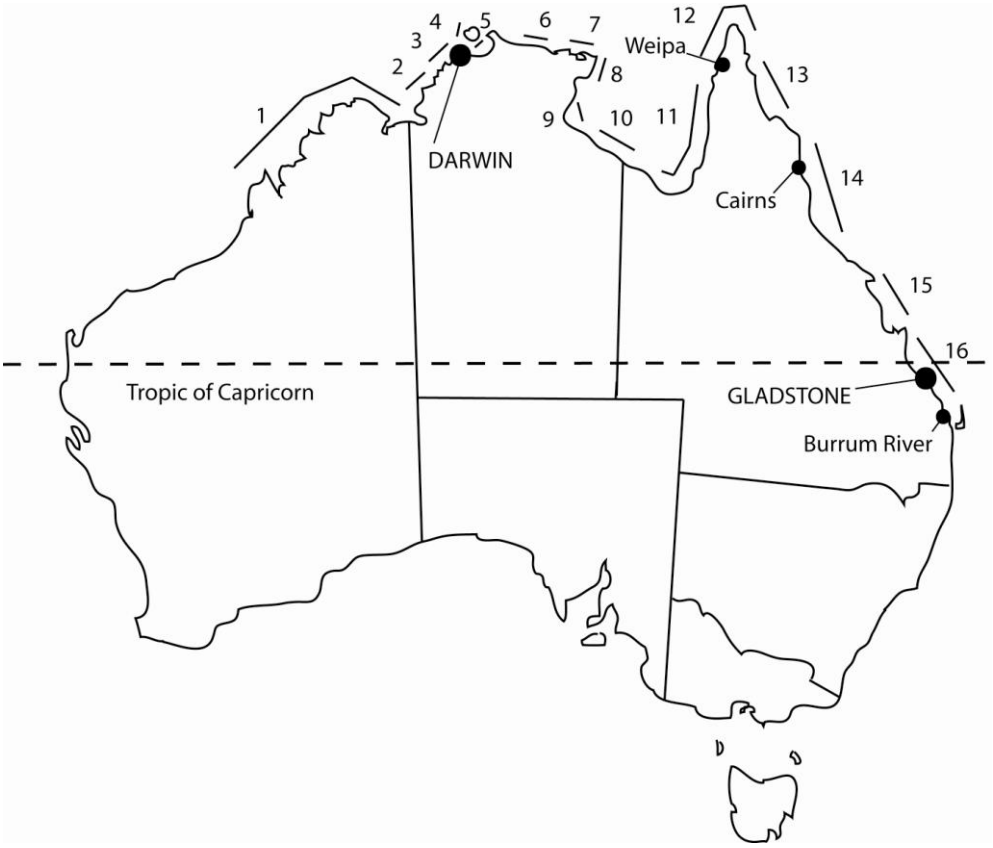

Fig. 2.1 Map of Australia showing known genetic stocks of barramundi (*Lates calcarifer*) based upon Keenan (2000). Tropical Darwin and sub-tropical Gladstone populations examined in the present study are indicated. Burrum River, Cairns and Weipa locations are also given, (see Discussion). Numbers refer to populations: 1, Fitzroy to Ord and Moyle Rivers; 2, Daly and Finniss Rivers; 3, Darwin Harbour and Shoal Bay; 4, Port Hurd; 5, Mary River, NT; 6, Goyder

River; 7, Buckingham Bay; 8, Blue Mud Bay; 9, Roper River; 10, McArthur River; 11, south-east Gulf of Carpentaria; 12, Cape York Tip; 13, east Cape York and Princes Charlotte Bay; 14, north east coast; 15 central east coast; 16, south east coast.

2.2.2 Temperature challenge of barramundi juveniles

Barramundi were divided into 12 replicate experimental groups that were temperature challenged on consecutive days due to time and space restrictions. Each replicate experiment consisted of eight individuals (four fish from each population selected at random each day) so that 96 fish were tested in total. For each experiment the eight selected fish were transferred to individual cages within a 500 L tank and allowed to acclimate overnight. After acclimation the water temperature was gradually raised at a rate of 2 °C ± 0.5 °C/h from 28 °C to 40 °C (± 0.1 ºC) where it was maintained. Dissolved oxygen was measured using a YSI-55 dissolved oxygen meter (John Morris Scientific; http://www.johnmorris.com.au) and aeration was supplied to the water at a constant rate to maintain dissolved oxygen above 4.9 mg/ml. LOSE was recorded as the time (min) between when the water was heated above 28 °C and the fish demonstrated a clear loss of swimming equilibrium (i.e. loss of righting response). As soon as LOSE was observed, fish were immediately euthanized in clove oil and humanely sacrificed in accordance with James Cook University animal ethics requirements. Standard anterior-posterior length (mm), weight (g) and dorsal-ventral breadth (mm) of each fish were also recorded for subsequent analysis of possible co-variates.

2.2.3 Dissociated caudal fin cell assay

Prior to LOSE measurements, a fin clip of approximately 1 cm^2 was taken from the caudal fin of each juvenile barramundi under anaesthesia. Dissociated caudal fin cells were prepared using a method modified from Sakamoto et al., (2002). Specifically, each fin clip was washed in sterile calcium and magnesium free phosphate buffered saline (CMF-PBS :0.01M PBS, 0.138M NaCl, 0.0027M KCl, pH 7.4) and dissected into pieces of approximately 1 mm^2. Dissected fin tissue was digested in 0.25 % Trypsin and incubated at room temperature with agitation for 25 min. The tissue digest was filtered (pore size of 53 µm) and centrifuged at 2100 g for 5 min to pellet cells. The cell pellet was washed twice with sterile CMF-PBS, centrifuged for 3 min and then cells were resuspended in Leibovitz-15 cell culture media (Cambrex Bio Science; http://www.cambrex.com), supplemented with 10 % foetal bovine serum (FBS) (Invitrogen; http://www.invitrogen.com).

The thermal tolerance of each cell suspension was tested by heating cells in a water bath at 40 ºC for 1 h. Cells were centrifuged (2100 g for 5 min) to remove cell culture medium and washed twice in CMF-PBS (removed by centrifugation at 2100 g for 3 min) before 30 µl of dye mix containing 1.5 µM Propidium Iodide (dead cell counts) and 2 µM Calcein-AM (live cell counts) (PromoKine; http://www.promokine.info) was added. Cells were incubated at room temperature for 30 min in the dye mix to enable dye binding before a 15 µl aliquot of the cell suspension was viewed on a standard microscope slide with cover slip under a fluorescence microscope (UVM-2^{TM} Ultraviolet Microscope, CRAIC Technologies^{TM}; http://www.microspectra.com) at 400 X magnification. The ratio of dead (red fluorescent) and live (green fluorescent) cells per sample was obtained from 10 randomly replicated counts per slide and was used as an indicator of caudal-fin cell thermal tolerance.

2.2.4 Measurements and statistical analysis

2.2.4.1 LOSE data

The use of 12 discrete experiments conducted on consecutive days to assess the temperature tolerance of barramundi stocks required an examination of the level of inter-experimental variation. Independent variables that described fish size, levels of dissolved oxygen and temperature were recorded for each experiment and their influence on LOSE measurements was determined. Two independent variables affecting the time to onset of LOSE that were not primarily of interest to the current study were identified with the application of a multiple regression model. Fish size (shown as 'breadth' and 'varbreadth' in the model) as well as temperature ramping rate (shown as 'T40' in the model) varied slightly between the 12 experiments. Using a backwards stepwise multiple regression model yielded the equation; $y = 207.695 + 1.421\ (T40) - 0.798\ (varbreadth) - 3.123\ (breadth)$. The effect of these variables on LOSE for each fish was taken into account using this equation to give an adjusted LOSE. Adjusted LOSE values were used for final analysis to ensure that inferences of whole fish thermal tolerance could confidently be attributed to their source population and underlying genetic stock differences. One-way ANOVA was used to demonstrate significant differences between adjusted group population means using an experiment-wise error rate (α) of ≤ 0.05.

2.2.4.2 Dissociated caudal fin cell data

In order to compile the dead/live cell ratio data all values were log transformed to fit a normal distribution plot before a univariate ANOVA with blocking was applied. The effect blocked for was the variation between experiments that arose due to different dye fluorescence intensity on

different days. Blocking in this way allowed us to explore the differences between individual fish and compare the relative thermal tolerance of the two populations. The ANOVA equation with blocking was adjusted to; $y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij}$ where y_{ij} is the j^{th} block on the i^{th} treatment, μ is the overall mean, τ_i is the effect of the i^{th} treatment and β_j is the effect of the j^{th} block. All statistical analyses were performed using SPSS version 16.0 (SPSS, 2006; Coakes et al., 2008).

2.3 Results

2.3.1 Whole body thermal tolerance (LOSE) assay

There were clear and significant differences in adjusted LOSE times between barramundi from tropical and sub-tropical locations (ANOVA; $F_{1, 22} = 7.86$ $P \leq 0.01$). Tropical fish had a significantly higher resistance to warmer water temperatures, with a mean LOSE of 518.5 ± 8 min (mean ± SE) compared to 452.8 ± 8 min for sub-tropical fish.

2.3.2 Dissociated caudal fin cell assay

Across all 12 dissociated caudal fin cell experiments the dead/live cell ratio of sub-tropical fish was consistently and on average 2.3 times greater than that for tropical fish, indicating greater sensitivity to high temperature in caudal fin cells from sub-tropical fish. This result was highly consistent across experiments (Fig. 2.2) despite some inter-experimental variation in ratio values caused by variable photo bleaching of the dye mix on different experimental days. The presence of significant population differences showing a higher dead/live cell ratio in sub-tropical fish than fish from the tropical location was demonstrated with a univariate ANOVA ($F_{1, 11} = 35.91$ $P \leq 0.001$).

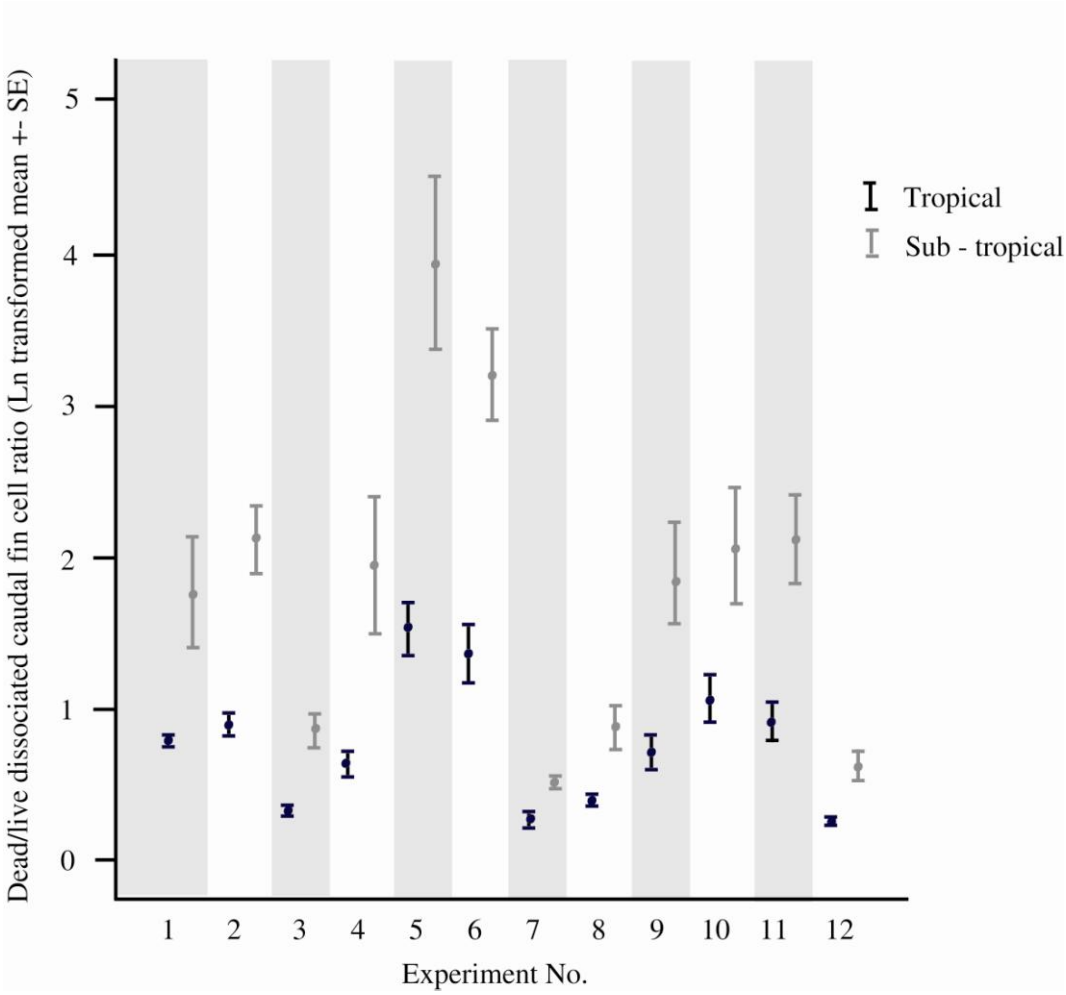

Fig. 2.2 Dead/live dissociated caudal fin cell ratios (Ln transformed) from tropical and subtropical *Lates calcarifer* across 12 replicate experiments. The dead/live cell ratio is expressed as the mean dead/live cell ratio ± 2 standard errors of the mean for each population within each experiment.

2.3.3 Dissociated caudal fin cell assay as a predictor of whole body thermal tolerance

The relationship between the upper thermal tolerance of each individual barramundi (LOSE) and the viability of caudal fin cells obtained from that fish and heat treated for 1 h was examined. Experiment by experiment correlations of the time until LOSE with the dead/live caudal fin cell ratio shows a clear and reproducible relationship (average r = 0.685) (Fig. 2.3). In each of the twelve comparisons a lower dead/live cell ratio coincided with a longer time until LOSE. There was a clear separation and distinction of fish from tropical and sub-tropical waters, with barramundi from the sub-tropical location having a higher dead/live cell ratio and succumbing to warm water temperatures sooner than tropical barramundi. This indicates that the dissociated caudal fin cell assay is a sensitive and useful predictive tool to determine the upper thermal tolerance limits of genetically divergent Australian barramundi populations.

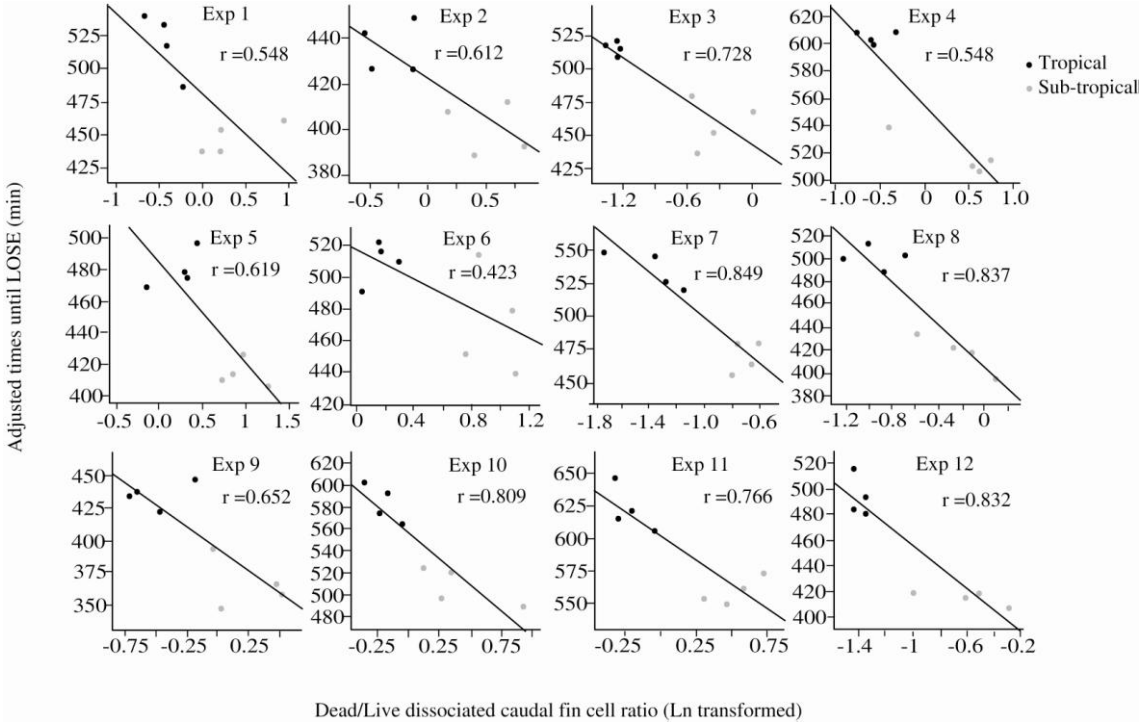

Fig. 2.3 Correlations between the adjusted time until LOSE (min) and the dead/live caudal fin cell ratio (Ln transformed) for *Lates calcarifer*, shown for each fish for all 12 replicate experiments. Black dots equal tropical population, grey dots equal subtropical population.

2.4. Discussion

Loss of swimming equilibrium and dissociated caudal fin cell assays show that genetically distinct barramundi populations at the tropical northern and sub-tropical southern extremes of the species distribution in Australia exhibit different upper thermal tolerances. Tropical barramundi lasted on average 66 min longer before they lost swimming equilibrium in water heated to 40 °C than sub-tropical barramundi, while caudal fin cells from sub-tropical fish exhibited higher ratios of dead/live cells indicative of a higher cell susceptibility to warmer temperatures. Barramundi from these two populations are geographically separated by 16 degrees of latitude over which substantial differences in mean maximum and minimum temperatures occur. Tropical Darwin populations are exposed to a mean annual temperature range of 23.2 °C -32 °C whilst subtropical Gladstone populations experience a mean annual range of 17.4 °C -26.6 °C (Bureau of Meteorology, http://www.bom.gov.au). These environments expose the barramundi stocks evaluated herein to different thermal conditions and impose a selective pressure upon local stocks that clearly has resulted in the development of population specific thermal tolerance to their localized thermal environment.

Australian barramundi are structured into at least 16 genetically distinct populations, many of which are exposed to different thermal regimes (Salini & Shaklee, 1988; Shaklee et al., 1993; Shaklee & Salini, 1985). Keenan (1994) proposed that these genetic differences were essentially neutral and developed primarily through isolation-by-distance and, as such, any differences seen in phenotypes between tropical and subtropical populations were a consequence of phenotypic responses to specific environments, not genetic adaptation. However, several laboratory studies, along with the present investigation, provide compelling evidence for genetic-based differences among populations in response to temperature. For example, Rogers and Bloomfield (1993) examined the performance of two genetic stocks of barramundi from Queensland (Burrum River and Cairns) when pond reared under identical field conditions. In these rearing trials fish from each population grew at similar rates until the onset of winter whereby fish from the subtropical Burrum River population were observed to maintain feeding, growth and exhibited fewer over-winter mortalities than fish originating from the tropical Cairns population. Rogers and Bloomfield (1993) subsequently conducted another growth experiment this time contrasting two tropical populations (Cairns and Weipa). Here they found that growth rates were highest in the more southern population (in this case the Cairns population) at temperatures between 21 – 26.5 °C, whilst the Weipa population had higher growth rates once water temperatures became warmer (28 °C) (Rogers & Bloomfield, 1993). These results are in accordance with what would be expected if there was strong selective pressure on local adaptation to temperature regimes and these studies, in conjunction with our direct estimates of the thermal tolerance of tropical and subtropical fish, imply that thermal tolerance in barramundi is under significant genetic control.

Demonstrating the presence of genetically determined differences in thermal tolerance is important to barramundi aquaculture for a number of reasons. Firstly, the few fish species where the genetic basis of thermal tolerance has been examined show that thermal tolerance is indeed under additive genetic influence. For example, heritability of upper thermal tolerance in salmon ranges from 0.41 - 0.48 (Perry et al., 2005), and in guppy (*Poecilia reticulate*) from 0.41 and 0.48 (Shah, 1986), while for cold tolerance in red drum (*Sciaenops ocellatus* L.) and tilapia (*Oreochromis niloticus*) heritability is 0.20 and 0.08, respectively (Eric et al., 2008). This suggests that additive genetic effects contribute significantly to variance in thermal tolerances among individuals and therefore the identification of individuals with increased thermal tolerances and the selection for this trait in breeding programs could lead to the development of more thermally tolerant strains of fish. The selection of more thermally tolerant fish should be advantageous for aquaculture production, as it would lower the incidence of temperature-induced stress which often restricts realisation of various trait performances.

Although the heritability of thermal tolerance in barramundi has not been measured, the fact that significant differences in upper thermal tolerance exist between geographically and genetically distinct populations of barramundi suggests a strong underlying genetic component. Current barramundi aquaculture uses different fish stocks farmed across a broad range of conditions with little consideration given to the origin of stocks. These common practices result in barramundi being cultured in conditions close to their upper and lower thermal limits with the failure to realise optimal trait performance a likely consequence. In addition, since the development of mass hatchery techniques and an interest in the enhancement of recreational fisheries, the aquaculture industry plays a key role in fish translocation for the restocking of rivers and lakes in northern Australia (Burrows, 2004). The introduction of translocated barramundi with functional genetic differences could result in the reduction of fitness components amongst native populations, particularly in the case where thermal regimes are vastly different. Consequently, genetic differences in thermal tolerance between barramundi populations should to be taken into consideration when sourcing broodstock or juveniles for culture purposes or restocking programs so that fish are cultured in their optimum thermal environment for growth.

Before hatcheries can detect and measure for optimal trait performance for the purposes of selective breeding they must first be able to predict the relative thermal tolerance of fish. For the first time in this species we have applied a simple dissociated caudal fin cell assay that differentiated tropical northern and sub-tropical southern populations of barramundi and that correlated with upper thermal tolerance. Caudal fin cells have also been used to successfully predict upper thermal tolerance in at least three other fish species, namely the ayu (*Plecoglossus altivelis*), Japanese flounder (*Paralichthys olivaceus*) and silver crucian carp (*Carassius langsdorfii*) (Sakamoto et al., 2005; Sakamoto et al., 2002). Consistent correlations between whole organism thermal tolerance measures and the dissociated caudal fin cell technique across these different species suggests that this technique has great potential to assist in the identification of thermally tolerant fish for the purposes of selective breeding for a diverse range of aquaculture species including barramundi.

Advancements toward selectively breeding thermally robust fish can be made for the barramundi aquaculture industry with the knowledge that differences in thermal tolerance exist at the population level in this species. These differences can be determined non-invasively in broodstock using the dissociated caudal fin cell method. Before thermal tolerance can be fully integrated as a breeding objective in selection programs, however, information is required on the heritability of this trait in barramundi and how thermal tolerance is genetically correlated

with other production traits. There is some evidence that thermal tolerance is positively genetically correlated with condition factor and growth in salmon and cutthroat trout (*Oncorhynchus clarkii*), however, this should be properly evaluated before thermal tolerance is selected in barramundi to avoid possible negative correlations with traits such as disease resistance etc (Perry et al., 2005, Robinson et al., 2008). Finally, whilst the dissociated caudal fin cell approach is robust in determining differences in population thermal tolerance, its ability to resolve finer-scale thermal tolerance differences at the family or individual level has not been proven and this should be investigated before it can be advocated as a predictive tool to estimate an individual's actual breeding value for this trait.

Chapter 3.0 The gene expression response of the catadromous perciform barramundi (*Lates calcarifer*) to an acute heat stress

3.1 Introduction

Temperature affects every level of biological organization from organism physiology to changes in protein and gene expression. Nowhere is this more apparent than in tropical aquatic estuarine environments which are generally considered to be the most variable aquatic environments on earth (Whitfield & Elliot, 2002). These environments are considered to be high stress environments due to the significant variation seen on a daily basis not only in temperature but also salinity, oxygen concentration, nutrient levels and pH. However, the biota denizen to these variable environments are well adapted to these conditions and may be considered more 'resilient' to such stressors than biota from alternate and less variable environments (Elliot & Quintino, 2007). The broader tolerance to environmental factors inherent in tropical estuarine species is important when considering how abiotic factors such as temperature influence the physiology of organisms from estuarine environments, but also when considering the effects of temperature on organism physiology in general. As the effects of temperature are so pervasive in aquatic environments there has been considerable research to understand how adverse temperatures are perceived and responded to by fish. Studies to date though have been largely restricted to temperate aquatic species and a more holistic picture of how a tropical estuarine species responds to an acute heat stress is not evident within the literature.

At the cellular level, temperature affects lipid membrane composition, cellular metabolism, the conformation and activity of proteins, as well as the expression of families of genes involved in maintaining homeostasis (Portner et al., 2006). However, at the gene level we are far from gaining a complete picture of the accompanying gene expression changes following a temperature stress and how these changes act to protect the organism and maintain/restore homeostasis. Of particular note is that most studies to date have focused only on the effect of temperature stress on one or two genes within a single defined gene pathway. Temperature stress, however, has the potential to impact on multiple genes across several metabolic pathways such as the general stress response, aerobic metabolism and growth.

Heat shock proteins (Hsps) are a highly conserved family of proteins belonging to the general stress response pathway. The role of Hsps is to maintain homeostasis through aiding the cell in protein synthesis, the folding and translocation of new proteins, as well as the assembly of large protein complexes. Hsps can be grouped into closely related families based upon molecular mass and contain amongst others, the 70 kDa and 90 kDa protein families known as Hsp70s and Hsp90s, respectively (Lund et al. 2006). The Hsp70 protein family consists of two different genes, a cognate or constitutive type (*Hsc70*) and an inducible type (*Hsp70*) whose chief roles are to act as molecular chaperones (Clark et al., 2007). The Hsp90 family also contains two

closely related genes as the result of a genome duplication event, resulting in an *α* (*aa*) and *β* (*ab*) form (Manchado et al., 2008). Despite a high level of similarity between these two genes at the molecular level, *Hsp90α* and *Hsp90β* exhibit different patterns of expression during both embryonic development and in response to stress (Hermesz et al., 2001; Manchado et al., 2008). As with *Hsc70* and *Hsp70, Hsp90β* is the more constitutive form whereas *Hsp90α* is strongly induced under stress temperatures and as such both families of Hsp lend themselves as likely candidates for the analysis of acute heat stress.

In response to adverse temperatures, changes in metabolic rate are also common as genes encoding various metabolic enzymes and mitochondrial proteins are regulated. In non-mammalian vertebrates, environmental temperatures can cause an increase in metabolism favouring changes in the rate of respiration in the muscle (Ekberg, 1958; Gillooly et al., 2001). Over the period of an acute heat stress, the length of this metabolic change may indicate the degree to which homeostasis is disrupted. Previous studies have reliably used measurements of key enzymes of the oxidative electron transport chain (i.e. cytochrome c oxidase subunit II (*CcoII*)) and of the citric acid cycle (i.e. citrate synthase (*CiSy*)) as key indicators of mitochondrial and aerobic activity in relation to temperature, most commonly in response to cold conditions (Hardewig et al., 1999; Lucassen et al., 2003; Lucassen et al., 2006; Speers-Roesch & Ballantyne, 2005). In addition, lactate dehydrogenase (*Ldh*) which converts lactate into pyruvate thus playing an important role in aerobic metabolism, has been shown to be temperature responsive after exercise (Edmunds et al., 2009; Smirnova et al., 2002) and can be used in conjunction with both *CcoII* and *CiSy* to provide a broad overview of energy metabolism in response to an acute heat stress.

It is well established that metabolic changes brought about by an acute heat stress will, over sustained periods, negatively impact the growth rate of an organism. After all, temperature is a major abiotic factor influencing growth (Gabillard et al., 2003). However, what is not understood is how quickly growth related genes respond to the acute heat stress and particularly whether changes are detectable during or immediately following the stress that might impede long-term growth processes. Identifying the pattern of response of growth related genes may help determine how long or short an acute heat stress event needs to be to significantly impact on the growth metabolic axis. Two genes which have been correlated with somatotropic and muscle growth in fish are insulin like growth factor 1 (*Igf1*) (Beckman et al., 2010) and myostatin-1 (*Mstn1*). *Igf1* is an integral gene associated with overall somatotropic growth, while *Mstn1* is a gene within the transforming growth factor β (Tgf- β) superfamily and a negative regulator of myogenesis (Lee & McPherron, 2001). Examining the expression profiles of these

two genes may therefore provide an indication of how soon sustained acute heat stresses manifest on genes within the growth pathway of tropical estuarine fish species.

As poikilotherms, fish are an ideal organism in which to study the effects of temperature and stress as their metabolic rate is strictly dependent upon the temperature of their environment, however, the majority of studies focusing upon gene regulation do so in temperate fishes and until now gene expression studies on tropical fish under stress have been few (Buentello et al., 2000; Glencross & Felsing et al., 2006; Mallekh & Lagardere, 2002). Barramundi *Lates calcarifer* (Bloch 1790) are an ideal species in which to study the gene expression response to an acute heat stress of a tropical estuarine species. In Australia, the distribution of *L. calcarifer* covers some 16 degrees of latitude incorporating a broad range of tropical and sub-tropical estuarine conditions that show high variability in environmental conditions, particularly temperature. The hardy nature of *L. calcarifer* is highly representative of tropical estuarine species in general and the study of an acute heat stress in this species will allow for a greater understanding of the scope for tolerance to environmental change in fish from naturally variable environments. Therefore, in this study the response to an acute heat shock in a tropical catadromous perciform fish, *L. calcarifer*, was examined across multiple metabolic pathways using nine genes linked to general stress, metabolism and growth. These nine genes were chosen based on reported variation to temperature within the literature, or were genes that had been identified as critical genes within each of these key metabolic pathways as previously outlined.

3.2 Materials and Methods

3.2.1 Animals and experimental design

Lates calcarifer of a standard length (~20 cm) were obtained from a local commercial hatchery and stocked communally in a 300 L freshwater tank at James Cook University (Townsville, Queensland). Fish were kept indoors in a temperature controlled room (~28 °C) with a 12 h light:dark photoperiod and fed a commercial diet to satiation (Ridley Aquafeed; http://www.agriproducts.com.au). Before the commencement of experiments fish were acclimated for a further 3 weeks at 28 °C; considered to be an optimal temperature for *L. calcarifer* growth (Katersky & Carter, 2005). Prior to the induction of acute heat shock and at a temperature of 28 °C, five fish (termed T = 0) were randomly selected as a control group and immediately, and humanely, euthanized in clove oil in accordance with James Cook University animal ethics requirements. White muscle tissue was immediately dissected from individual fish and snap frozen in liquid nitrogen. As an acute heat shock, water pre-heated to 36 °C was

gravity fed into the experimental tank to evenly raise the temperature from 28 °C to 36 °C over a period of 30 min. Fish (n = 5) were again sampled as soon as the temperature inside the experimental tank had reached 36 °C (termed T = 1) and after one hour at 36 °C (termed T = 2). To restore initial conditions water at a temperature of 28 °C was gravity fed into the experimental tank upon which time fish (n = 5) were sampled as soon as the temperature had reached 28 °C (termed T = 3) and again after 2 weeks at 28 °C (termed T = 4) to assess any long term alterations in gene expression (if any) as a result of the earlier acute heat stress. The acute heat shock protocol used in the current experiment is common to such experiments and also highly representative of conditions experienced by *L. calcarifer* in both the wild and under farming conditions. In estuarine environments tidal movements can influence water temperatures significantly and on a daily basis with short, rapid temperature spikes commonly occurring as water from shallow, sun baked salt flats enters estuaries as run-off. Furthermore, the culture of *L. calcarifer* in outdoor ponds is susceptible to significant increases in temperature that frequently reach the upper thermal tolerance limit for this species (~40 °C) (Katersky & Carter, 2005).

3.2.2 RNA isolation and cDNA synthesis

RNA extraction and cDNA synthesis were performed according to the protocol described in De Santis et al., (2011). Briefly total RNA was extracted by homogenizing frozen muscle tissues directly in Ultraspec RNA (Biotecx; http://www.biotecx.com) and precipitated in a solution containing 0.5 vol of RNA precipitation solution (1.2 M sodium chloride, 0.8 M disodium citrate (Sambrook & Russel, 2001) and 0.5 vol of isopropyl alcohol. RNA quality and quantity was verified using a Nanodrop spectrophotometer (Nanodrop technology; http://www.nanodrop.com) via examination of absorbance ratios at $OD_{260/280}$ (range 1.98-2.06) and $OD_{260/230}$ (range 1.96-2.07) and by the visual inspection of integrity of both the 18S and 28S ribosomal bands on a 1.5 % agarose gel. Samples were adjusted to a final concentration of 200 ng/µL and treated with a Turbo DNA-free kit (Ambion; http://www.invitrogen.com) followed by an ammonium acetate precipitation. First strand cDNA synthesis was performed on 5 µg of DNase treated total RNA from each individual sample (5 samples per time point × 5 time points) using a Super Script III first-strand synthesis supermix (Invitrogen; http://www.invitrogen.com) with 2.5 µM $oligo(dT)_{20}$ and 25 µM random hexamers (Resuehr & Spiess, 2003). Complete removal of contaminating DNA was verified by PCR amplification of RNA samples using gene specific primers as a no-amplification control (NAC) ($C_{q\ (NAC\ control)} - C_{q\ (cDNA\ synthesis)} > 10$). After cDNA synthesis, RNA template was digested with 1 µL of RNase cocktail mix (Ambion; http://www.invitrogen.com) within 20 µL of cDNA incubated at 37 °C for 30 min followed by enzyme deactivation at 70 °C for 10 min. Samples were purified using

Sepharose CL-6B spin columns (Sigma-Aldrich; http://www.sigmaaldrich.com) and cDNA was quantified and diluted to the same final concentration of 2 ng/µL prior to real time PCR analysis.

3.2.3 Gene characterization and primer design

Of the nine genes of interest, full or partial length *L. calcarifer* specific sequence was only available for *CcoII* (NC_007439.1), *Ldh* (FJ439509.1), *Igf1* (EU136176) and *Mstn1* (EF672685). Universal primers were designed for *CiSy*, *Hsc70*, *Hsp70*, *Hsp90α* and *Hsp90β* within conserved regions between other related fish species (Table 3.1.) using Perl-Primer v1.1.17 (Marshall, 2004). *Lates calcarifer* specific sequence was PCR amplified in a 25 µL reaction containing 1 x NH_4-based reaction buffer (Bioline; http://www.bioline.com), 2.0 mM $MgCl_2$, 200 µM dNTP mix, 0.2 µM of both forward and reverse primers, 1 u/µL of Biotaq DNA polymerase (Bioline; http://www.bioline.com) and approximately 4 ng of *L. calcarifer* muscle cDNA. Amplification was performed in an MJ research thermal cycler using standard conditions [3 min at 95 °C, 35 x (30 s at 95 °C, 30 s at primer specific annealing temperatures, 45 s at 72 °C), 10 min at 72 °C]. Each PCR reaction was visualized for the amplification of a single product on a 1.5 % agarose gel before being cloned into pGEM-T easy vector system (Promega; http://www.promega.com) and sequenced (Macrogen; http://www.macrogen.com) in both directions using M13 universal forward and reverse sequencing primers. Sequence specificity was confirmed via a comparison with known sequence in the BLASTn database (Altschul et al., 1990).

Table 3.1. Universal primers sequences used to isolate *L. calcarifer* specific sequences. Gene names are given in the abbreviated form; *Hsp* (heat shock protein) and *CiSy* (citrate synthase).

Candidate Gene of Interest	Universal primer pair 5`-3`	Accession numbers used in alignment	Primer annealing temperatures (°C)
Hsp90α	Alpha Fwd: ACAAGGGAGAAGGAGGTGGAC Alpha Rev: ACTGGCATGTCTTCATCAG	AY222612, AF170295, FJ426146, DQ202281, BT026666, NM005348, DQ662233, BT043623, AB367526, NM213973	60
Hsp90β	Beta Fwd: ATCATTCTGTAGATGCG Beta Rev: TTCTCCAAGAACATCAAGC	AF042108.1, AY395632, DQ662234, NM001123532.1, EU099575, AB367527	55
Hsc70	C70 Fwd: GCACCACCTACTCCTGTG C70 Rev: GGTGATGGAGGTGTAGAAG	AY762969.1, BC056709, NM001104800, DQ662231.1, AY436786.1, AB436486,	57

		AB062115	
Hsp70	P70 Fwd: ATGGCTCCAGCTAAAGGTG P70 Rev: CTGGGAGCCGCTTCCTGC	AY762970, AB092839, AY120894, NM131397, BC002453, EU884290, AJ001312, FJ213839, AY436787, AB062113, AB436470	56
CiSy	CiSy Fwd: GATCCTGAGGAGGGCATC CiSy Rev: GCTCTACTCCACACCAGC	BC166040, NM004077, AY461850, AY461852, AY461848, AY461851	55

To obtain the full length coding sequence an RNA ligase-mediated rapid amplification of both 5` and 3` cDNA ends (RLM-RACE) was performed on 5 µg of total RNA for *L. calcarifer* muscle using a GeneRacer™ Kit and gene specific RACE compatible primers as per the manufacturer's instructions (Invitrogen; http://www.invitrogen.com), (Table 3.2.). The full length coding region of each gene of interest was determined using MEGA-4 v4.0.2 (Tamura et al., 2007) for the assembly of gene fragments. To compare sequence similarity across multiple species a phylogenetic analysis of all heat shock proteins used the maximum composite likelihood method to construct a tree of the deduced *L. calcarifer* sequence with homologous sequences from other fish species using MEGA-4 v4.0.2 (Tamura et al., 2007). For both gene trees *Homo sapiens Trap1*, a heat shock protein homologue, (NM-016292) was used as the out-group.

Table 3.2. GeneRacer™ species compatible external and internal primers used to obtain full length *L. calcarifer* gene sequences. Gene names are given in the abbreviated form; *Hsp* (heat shock protein) and *CiSy* (citrate synthase).

Candidate Gene of Interest	RACE primer pair 5`-3`	Primer annealing *T* (°C)
Hsp90α	Rf1: GATTCCAGAGTATCTCAATTTCATCAAG Rr1: TGGTTTGGTCTTGTTCAACTCCTGAGC	55
	RfN1: TGCCTGGAACTTTTCACTGAACTT RfR1: TTCTCAGCAGCCTCTTTCTCAA	60
Hsp90β	Rf2: GAACGACCTGGAGATCAACCCTGAC Rr2: CATGTACAGGACCTCAAAGCCACGC	60
	RfN2: CTGCTGTCCTCAGGCTTCTCCC RrN2: AAAGGTATTCTGTGAGGGAGGTGGTC	58
Hsc70	Rf3: TGGAGATACTCATCTTGGTGGGGAAGT Rr3: TGTGATGACAGCGTTGTTGACAGTT	58
	RfN3: GATAATAAGAGAGCTGTCCGTCGT RrN3: CAACTGTGTCGTCAAACCTGCGG	60
Hsp70	Rf4: GAACATGAAGGGCAAAATTAG Rr4: ATTTTGGGCTTCCCTCCATCTGA	58
	RfN4: CTGGCTGATAAAGAGGAGTACC RrN4: GATCGCTACACCTTTAGCTGGAG	60
CiSy	Rf5: TACACCTGCCAGCGTGAATTTGCC Rr5: TGGCAGCACTGAACTGAGACATG	60
	RfN5: AGATTGTGCCCAATGTGCTCCTGG RrN5: TTGGACACCCAGTTCACCTGCTC	58

3.2.4 RT-qPCR primer design, validation and optimization

Newly isolated *L. calcarifer* specific gene sequences were characterized for *L. calcarifer* in order to design primer pairs for RT-qPCR assays using Perl-Primer v1.1.17 (Marshall, 2004). RT-qPCR primers for the closely related heat shock proteins *Hsc70* and *Hsp70*, and *Hsp90α* and *Hsp90β* were designed within unique regions of each gene whilst RT-qPCR primers and reaction conditions for the reference genes 18s ribosomal RNA (*18s*), alpha-tubulin (*α-tub*), cathepsin-d (*cat-d*), elongation factor 1-alpha (*elf1-α*), glyceraldehyde-3P-dehydroxenase (*gapdh*), parvalbumin (*parv*), ribosomal protein l8 (*rpl8*) and ubiquitin (*ubq*) were the same as those previously developed and utilized elsewhere (De Santis et al., 2011; Sampath et al., 2000; Xu et al., 2006). RT-qPCR reactions, carried out in a final reaction volume of 12 µL, consisted of 1 x SYBR GreenER qPCR Supermix Universal (Invitrogen; http://www.invitrogen.com), 2.5 µM of rox reference dye, 0.2 µM of each primer with 2 ng of cDNA template. All reactions were performed on an MJ research DNA engine with a Chromo 4 detector and utilizing Opticon Monitor 3.0 software (Biorad; http://www.bio-rad.com). Each reaction was amplified in triplicate and consisted of 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension for 30 s at primer specific

temperatures (Table 3.3.). Upon the completion of each run a melt curve analysis was performed to ensure amplification of the intended target only and the absence of primer dimer. A no-template control was included within every plate to ensure purity of reagents ($NTC_{(\alpha\text{-tub})}$ = 39.76; $NTC_{(\text{remaining genes})}$ > 40). The optimization of PCR conditions involved producing a $\log_{10}$ dilution series created by pooling undiluted cDNA from each animal used in the experiment. A standard curve was generated by plotting the quantification cycle (C_q) for each dilution point against the starting quantity of cDNA. Standard curves were run in triplicate on each plate run to estimate reaction efficiency (*E*) and reproducibility of each assay. *E* was determined by the equation [$E = 10^{(-1/\text{slope})}$] (Rasmussen, 2001) and ranged from 91.5 % to 107 %. Reproducibility and repeatability were represented by R^2 and standard deviation (SD) respectively and were in accordance with MIQE guidelines (Bustin et al., 2009). The expression of each gene of interest and each house keeping gene was analysed one at a time on a single plate that contained all samples from all time points as well as a standard curve for determining amplification efficiency and ensured that technical variation (inter-assay variability) was not confused with biological variation.

Table 3.3. *Lates calcarifer* RT-qPCR primers used to examine the expression of genes of interest in the current study. Gene names are given in the abbreviated form; *Hsp* (heat shock protein), *CiSy* (citrate synthase), *CcoII* (cytochrome oxidase C subunit two), *Ldh* (lactate dehydrogenase), *Igf1* (insulin like growth factor 1) and *Mstn1* (myostatin 1). E-1(%) gives the reaction efficiency as a percentage and is calculated using a standard curve with the equation $E = 10^{(-1/slope)}$.

Gene	Primer pair 5`-3` (RT-qPCR)[a]	Annealing Temp (°C)	Amplicon size (bp)	E-1(%)
Hsp90α	Qf1: AGAAAGAAGTGGACCTTGAG	59	118	104
	Qr1: CTTTGTGTCTTCATCCTCGT			
Hsp90β	Qf2: GAAGGAAGAGAAGGAGGATGG	61	126	98
	Qr2: CTGGTCGATGTACTTCTCCT			
Hsc70	Qf3: CATCAATGACAACACTCGCC	61	201	101
	Qr3: AGCATCCTTAGTAGCCTGAC			
Hsp70	Qf4: CAAGGTGATTTCAGATGGAGG	60	108	98
	Qr4: CTTCATCTTCACCAGGACCA			
CiSy	Qf5: CTCTACCTCACCATCCACAG	61	216	103
	Qr5: TCATCCTCTCATCAGACACC			
CcoII	Qf6: ATTCTTGAGCCGTACCATCC	62	116	98
	Qr6: AGATTTCAGAGCATTGTCCGT			
Ldh	Qf7: CAAAGACTACGCAGTGACAG	60	125	100
	Qr7: GGGATGATGGACTTGAAGAC			
Igf1	Qf8: CAGTGGCATTTATGTGATGTCTTC	55	41	105
	Qr8: TGAGGACGCACAGCAGTAG			
Mstn1	Qf9: ATGTAGTTATGGAGGAGGATG	58	84	103
	Qr9: CTTGGACGATGGACTCAG			

[a] Primers for MSTN1 are those used in De Santis et al., (2011).

3.2.5 Statistical analysis

The level of gene expression between treatment samples (T = 1, T = 2, T = 3, T = 4) and control (T = 0) were statistically compared by means of ANOVA and linear regression was used to examine the expression relationships between genes of interest. Homogeneity of variance was confirmed using a Levene's test and differences of $p < 0.05$ between time points were considered significant. All statistical analyses were performed using SPSS v.16.0 (IBM; http://www-01.ibm.com/software/analytics/spss/).

3.3 Results

3.3.1 Isolation and characterisation of L. calcarifer heat shock protein and citrate synthase cDNAs

PCR amplifications using degenerative primers yielded gene specific sequence for each previously uncharacterized *L. calcarifer* gene as confirmed by BLASTn analysis (Altschul et al., 1990). Two cDNAs were identified as members of the 90 kDa family of heat shock proteins. Their ORF's and deduced amino acid sequences, as well as a phylogenetic analysis with homologues from other fish allowed them to be classified into the constitutive *Hsp90β* and the inducible *Hsp90α* forms of the gene (Fig. 3.1). Both peptides in *L. calcarifer* contain the three conserved regions (1, 2 & 3) and four variable regions (A, B, C & D) characteristic of members of this family as identified by Chen et al., (2006). Moreover both peptides contain the highly conserved MEEVD C-terminal region which designates them as active cytosolic members that mediate inter-domain communication and peptide-binding capacity (Gupta, 1995), as well as other important residues involved in ATP hydrolysis (E43), ATP binding (D89), geldanamycin binding (K108), geldanamycin and p23 binding (G91, G128, G131, G133 and G179), ATPase activity (R392 and Q396), interdomain interaction (F361) and casein kinase II phosphorylation (S255) (Chen et al., 2006, Manchado et al., 2008).

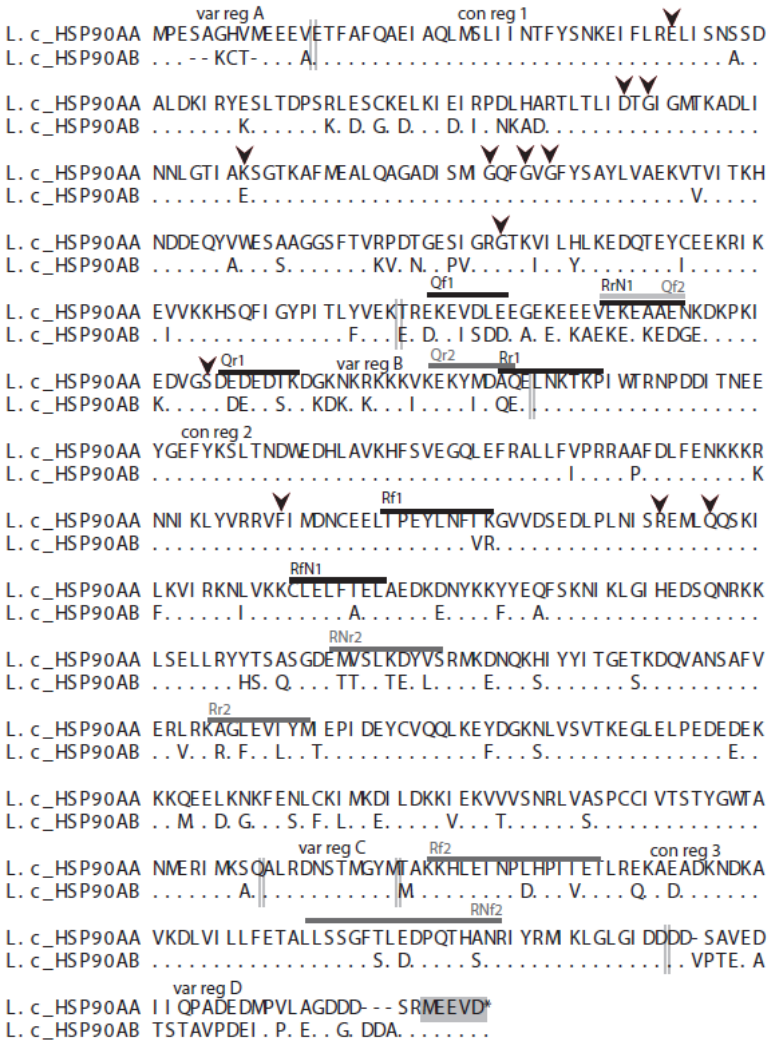

Fig. 3.1 Deduced amino acid sequence alignment of *L. calcarifer* heat shock protein 90α and β (*Hsp90AA* & *Hsp90AB*). The positions of primers used in this work are marked by a line over the appropriate sequence position and accompanied by the primer name. The conserved/variable regions are separated by '‖' with the corresponding name above the alignment. '▼' stands for functionally important residues (see text). The C-terminal MEEVD sequence characteristic of cytosolic Hsp90 members is shaded. *Hsp90α* (*AA*) is used as the reference sequence and only differences are shown for *Hsp90β* (*AB*); dots indicate identical amino acids and dashes indicate gaps.

Two more cDNAs belonging to the 70 kDa family of heat shock proteins were also identified in the current study. A phylogenetic comparison of these two sequences with known members of the 70 kDa family from other fish species grouped each cDNA sequence separately and identified them as the constitutive *Hsc70* and inducible *Hsp70* forms of the gene (Fig. 3.2). Two highly conserved motifs were detected within both sequences with putative roles as an ATP-

binding site and as a nuclear targeting signal respectively (Luft et al., 1996). Furthermore, despite a high sequence similarity at the peptide level, *Hsc70* and *Hsp70* could be differentiated at the C-terminal region. The constitutive *Hsc70* form of the gene is slightly longer than the inducible *Hsp70* and contains a repeated GG—P motif, as well as a signature SGPTIEEVD nonapeptide at the 3` end of the sequence, whereas *Hsp70* contains only one GG—P motif (Deane & Woo, 2005) (Fig. 3.2). Phylogenetic analysis of the *L. calcarifer Hsp* sequences by the maximum likelihood method clustered the *Hsp90α* and *Hsp90β* genes and *Hsp70* and *Hsc70* genes separately within distinct groups together with homologous genes from other fish species. In both cases bootstrapping results indicated strong support for the placement of genes into their respective clades (Fig. 3.3).

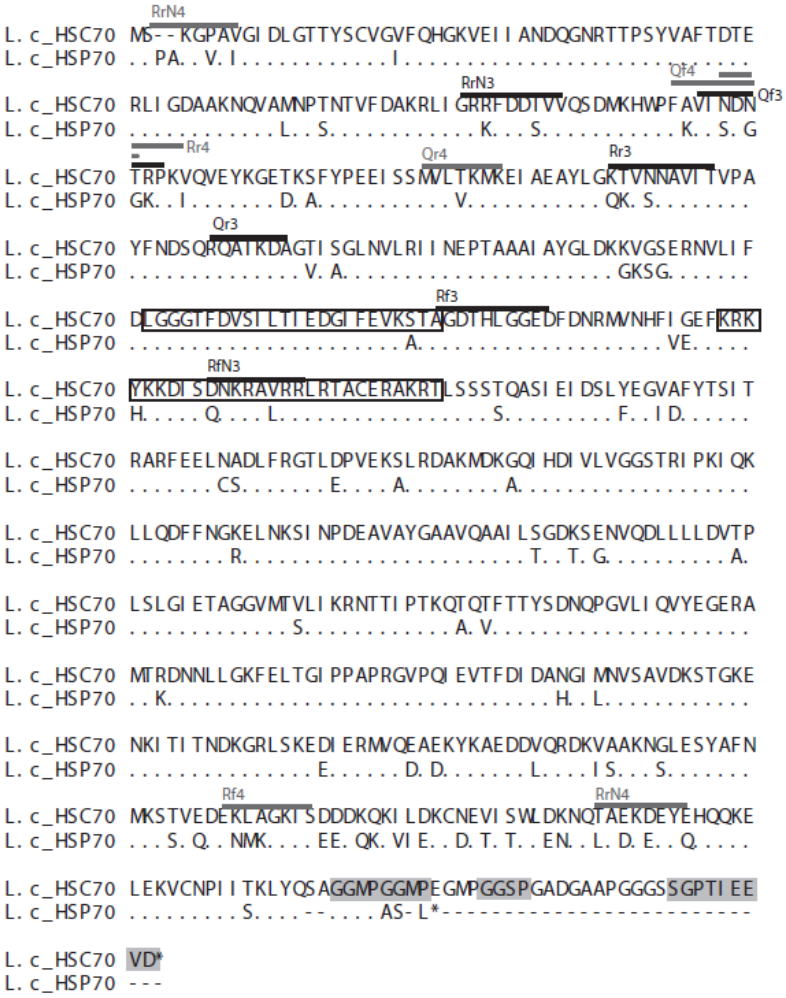

Fig. 3.2 Deduced amino acid sequence alignment of *L. calcarifer* heat shock cognate 70 and heat shock protein 70 (*Hsc70* & *Hsp70*). The positions of primers used in this work are marked by a line over the appropriate sequence position and accompanied by the primer name. Boxed residues indicate possible ATP-binding site and a nuclear targeting signal respectively. Shaded residues indicate GG—P motifs and the *Hsc70* signature nonapeptide. *Hsc70* is used as the reference sequence and only differences are shown for *Hsp70*; dots indicate identical amino acids and dashes indicate gaps.

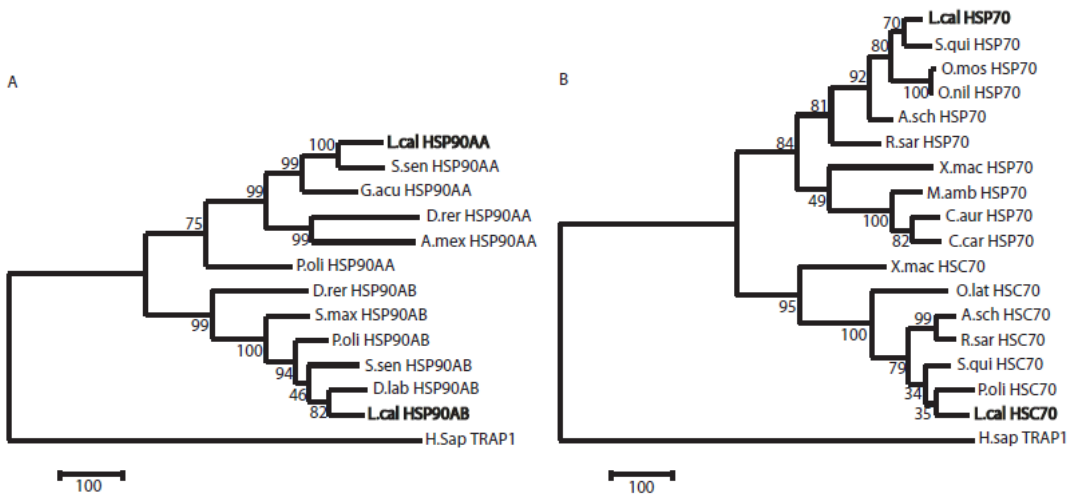

Fig. 3.3 Phylogenetic analysis of *L. calcarifer* heat shock proteins. Full length sequences were obtained from GenBank and aligned using the Maximum likelihood method. A) shows *L. calcarifer Hsp90α* and *Hsp90β* grouping separately into two different clades along with gene homologues from other fish species whilst B) shows the same trend for *L. calcarifer Hsc70* and *Hsp70*. *Homo sapiens Trap1* was used as the outgroup. Species abbreviations and full names are as follows; L.cal (*Lates calcarifer*), S.sen (*Solea senegalensis*), G.acu (*Gasterosteus aculeatus*), D.rer (*Danio rerio*), A.mex (*Astyanax mexicanus*), P.oli (*Paralichthys olivaceus*), S.max (*Scophthalmus maximus*), D.lab (*Dicentrarchus labrax*), H.sap (*Homo sapiens*), S.qui (*Seriola quinqueradiata*), O.mos (*Oreochromis mossambicus*), O.nil (*Oreochromis niloticus*), A.sch (*Acanthopagrus schlegeli*), R.sar (*Rhabdosargus sarba*), X.mac (Xiphophorus maculatus), M.amb (*Megalobrama amblycephala*), C.aur (*Carassius auratus*), C.car (*Cyprinus carpio*), O.lat (*Oryzias latipes*).

A final cDNA identified as citrate synthase (*CiSy*) was characterized in *L. calcarifer* and showed high sequence homology to citrate synthase in other fish species. Further confirmation of transcript identity was demonstrated by the presence of highly conserved α-helix zones (A-T) (Wiegand & Remington, 1986) as well as the presence of a hinge region involved in conformational change upon oxaloacetic acid (OAA) binding (G305 marked as # on Fig.3.4). *Lates calcarifer* citrate synthase was also revealed to contain other residues of importance such as multiple acetyl-CoA ligand binding sites (R76, K194, V344, V345, P346, G347, Y348, G349 and H350), oxaloacetate/citrate ligand binding sites (H268, H304, H350, R359, D408, R434, R451), catalytic residues (H304, H350 and D405) and other regions near binding sites or putatively involved in binding (H265, N272, D357, D405, D427) (Dalziel et al., 2005) (Fig. 3.4). The sequences encoding *L. calcarifer Hsp90α*, *Hsp90β*, *CiSy*, *Hsc70* and *Hsp70* have been deposited on GenBank under the following Accession numbers; HQ646105, HQ646106, HQ646107, HQ646108 and HQ646109 respectively.

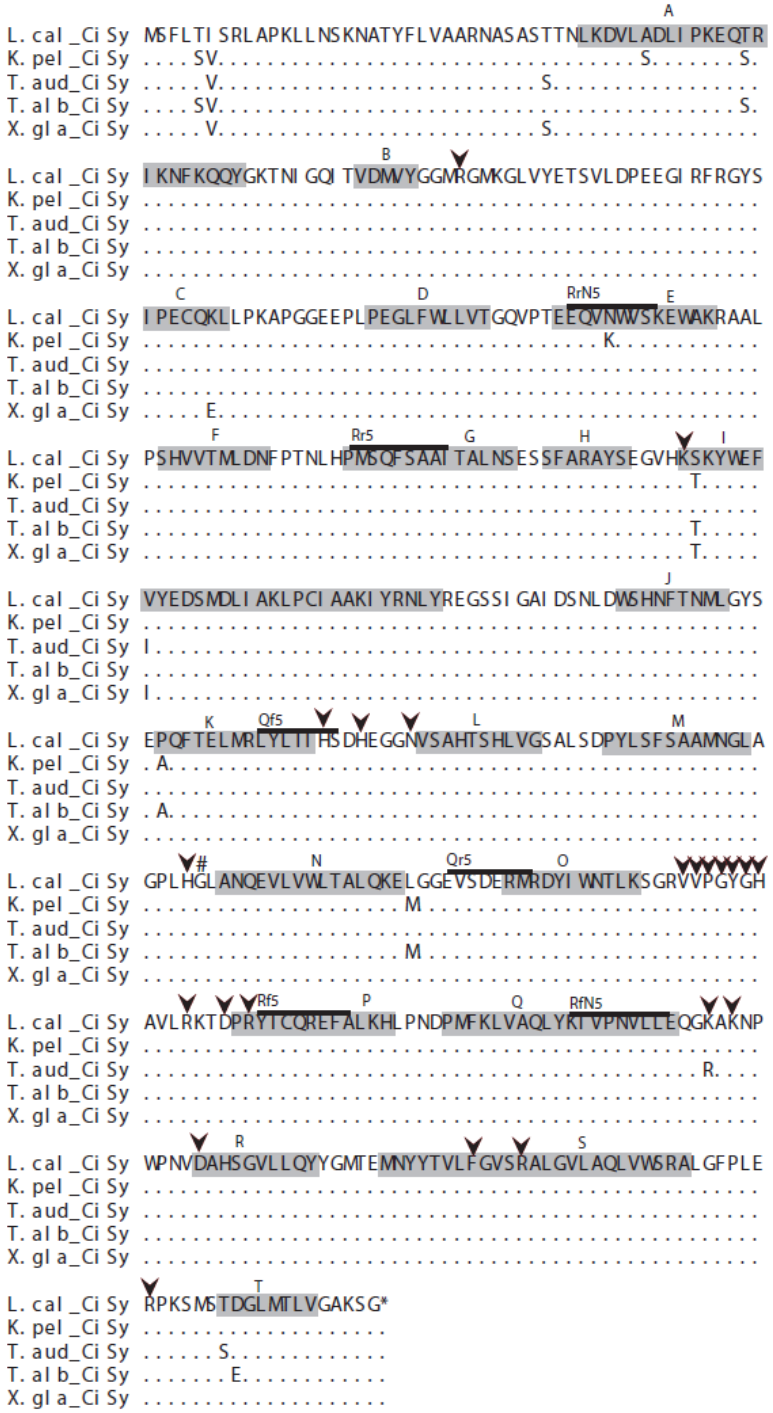

Fig. 3.4 Deduced amino acid sequences alignment of *L. calcarifer* citrate synthase (*CiSy*) with *CiSy* homologues from other fish species. The position of primers used in this work are marked by a line over the appropriate sequence position and accompanied by the primer name. Shaded residues indicate α-helices and are labelled A-T. '▼' stands for functionally important residues (see text). The hinge region involved in conformational change upon OAA binding is marked '#'. *Lates calcarifer CiSy* is used as the reference sequence and only differences for other aligned sequences are shown; dots indicate identical amino acids and dashes indicate gaps.

3.3.2 Candidate reference gene selection

18s, α-tub, cat-d, elf1α, gapdh, parv, rpl8 and *ubq* were all chosen as likely candidate reference genes due to their selection in other experiments (De Santis et al., 2011; Dheba et al., 2004; Dheba et al., 2005; Filby & Tyler 2007; Infante et al., 2008). The relative expression profile of each reference gene after an acute heat exposure was obtained by normalizing against the input amount of cDNA using the ΔC_q method ($Ratio_{(test/calibrator)} = E^{Cq\ (calibrator)\ -\ Cq\ (test)}$) with individual samples as the test and the average of all five control samples (T = 0) as the calibrator. Within all five time points (T = 0, T = 1, T = 2, T = 3, T = 4) no significant variation in gene expression was observed for *18s, α-tub, elf1α, rpl8* and *ubq*. As they showed the smallest variation in expression over the experimental time points, the geometric average of *α-tub* and *rpl8* (Fig. 3.5) was calculated using geNorm software (Vandesompele et al., 2002) and used to normalize the expression of all genes of interest. Henceforth both *α-tub* and *rpl8* are considered appropriate reference genes for examining gene expression in *L. calcarifer*, and possibly other fish species as a result of an acute heat stress.

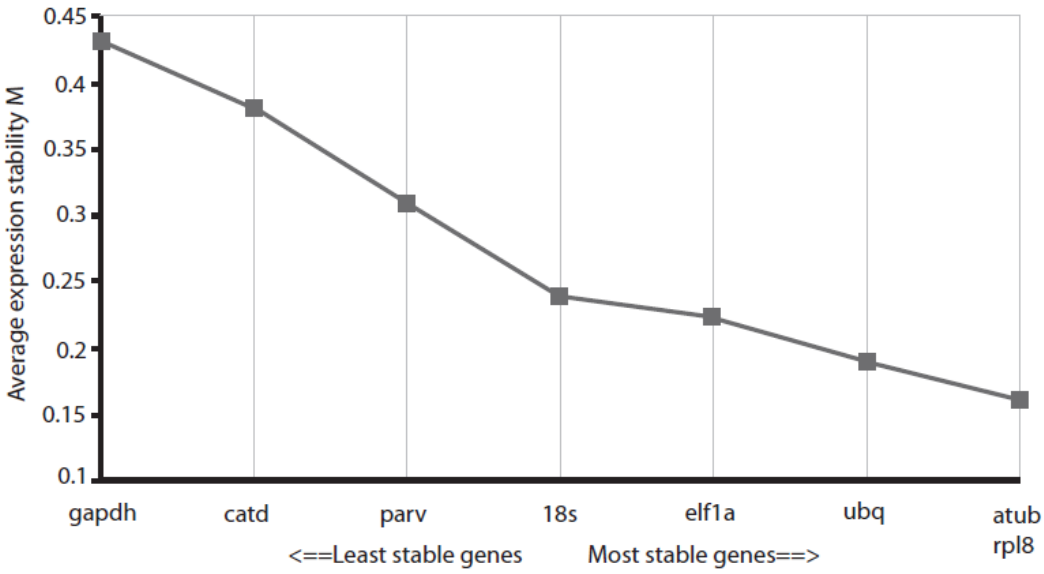

Fig. 3.5 Average expression stability (M) of all eight reference genes *Gapdh, Catd, Parv, 18s, Elf1α, Ubq, α-tub, Rpl8,* tested for suitability using geNorm software and plotted from least stable to most stable. A low *M* value suggests high stability and similarity between expression of genes, in this case geNorm selected and grouped together *α-tub* and *Rpl8* as the most stable reference genes with a pairwise variation estimate (V 2/3) of 0.063.

3.3.3 Gene expression response to acute heat stress

An increase in water temperature from 28 °C to 36 °C over the period of 30 min resulted in the rapid increase in gene expression of both *Hsp90α* and *Hsp70* as well as *Mstn1* (Fig. 3.6). Between T = 0 and T = 1, an increase in gene expression by ~6-fold (ANOVA; $F_{4, 19} = 12.412$, $p < 0.001$) and ~18-fold (ANOVA; $F_{4, 19} = 10.086$, $p < 0.001$) was observed for *Hsp90α* and *Hsp70* respectively, whilst *Mstn1* showed a ~2-fold increase in gene expression over the same time points (ANOVA; $F_{4, 20} = 4.686$, $p < 0.01$). After 1 h at 36 °C fish sampled at T = 2 showed a further increase in *Hsp90α* and *Hsp70* expression of ~55-fold ($p < 0.0001$) and ~450-fold ($p < 0.0001$) compared with expression levels at T = 1. Conversely *Mstn1*, after an initial increase between T = 0 and T = 1 dropped ~1.7-fold at T = 2 and was no longer significantly different from expression levels at T = 0, where it remained unchanged for the remainder of the experiment. *Hsp90α* and *Hsp70* expression levels were not significantly different between T = 2 and T = 3 despite a temperature return to 28 °C over a 30 min period. However *Hsp90α* expression decreased between T = 3 and T = 4 after 2 weeks at 28 °C and was not significantly different at this time from T = 0. Although *Hsp70* dropped significantly by ~430-fold gene expression remained significantly different at T = 4 from that measured at T = 0 ($p < 0.001$). Whilst the inducible forms *Hsp90α* and *Hsp70* showed large changes in gene expression over the course of the experiment, the constitutive forms, *Hsp90β* and *Hsc70* showed no significant changes in gene expression at any time between T = 0 and T = 3 over the period of an acute heat stress. At T = 4, *Hsc70* levels rose modestly but significantly by ~1-fold compared with levels at T = 0 (ANOVA; $F_{4, 20} = 4.143$, $p < 0.01$), however expression levels for *Hsp90β* again remained unchanged.

CcoII expression levels decreased as temperature rose from 28 °C to 36 °C (T = 0 to T = 1) but were not significantly different from T = 0 until a further decrease in gene expression at T = 2 (~0.6-fold from T = 0) (ANOVA; $F_{4, 20} = 5.290$, $p < 0.05$). *CcoII* expression remained stable between T = 2 and T = 3, but returned to levels similar to that of T = 0 after 2 weeks at 28 °C at T = 4 with a ~0.5-fold increase in expression.
Gene expression levels for *Ldh*, *Igf1* and *CiSy* did not change significantly at any time point over the course of the experiment despite small fluctuations (Fig. 3.6).

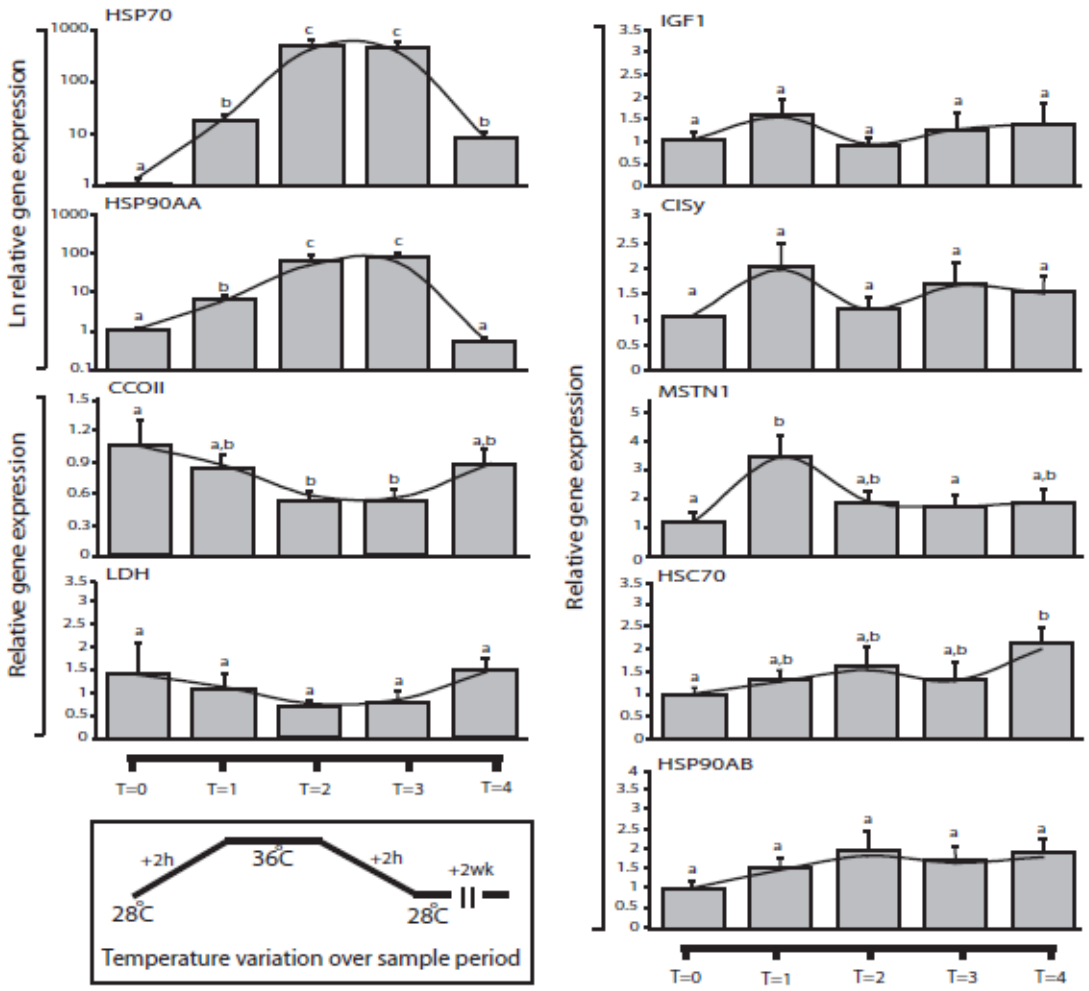

Fig. 3.6 Mean (± SEM) expression values of all nine genes of interest (*Hsp70*, *Hsp90α* (*AA*), *CcoII*, *Ldh*, *Igf1*, *CiSy*, *Mstn1*, *Hsc70*, *Hsp90β* (*AB*)), normalized by ΔC_q and geometric averaging of multiple reference genes (*α-tub* & *Rpl8*). Expression is represented as relative change in gene expression compared with control (T = 0). Expression levels marked with a different letter are statistically different ($p < 0.05$) whilst same letters indicate $p > 0.05$.

3.4 Discussion

In the present study the response in the muscle to an acute heat stress was closely examined in the hardy and temperature tolerant catadromous species *L. calcarifer*. To gain an accurate picture of the overall acute stress response in this species the expression of a number of genes representing different pathways of interest was examined.

3.4.1 Gene expression following an acute heat stress in L. calcarifer

3.4.1.1 The heat shock response

An acute temperature stress in *L. calcarifer* resulted in the rapid and extensive up-regulation of the inducible heat shock response pathway. The inducible forms *Hsp90α* and *Hsp70* responded immediately to an increase in temperature within the first 30 min of the experiment followed by a peak in gene expression after 1 h at 36 °C (Fig. 3.6), whereas the constitutive forms of both genes remained unchanged throughout the acute heat stress phase of the experiment. It is widely accepted that within the 90 kDa and 70 kDa families it is the inducible forms (*Hsp90α* and *Hsp70*) that are primarily associated with a fast acting stress-induced role in the maintenance of protein metabolism rather than the constitutive forms (*Hsp90β* and *Hsc70*) (Lindquist & Craig, 1988). Studies have shown heat induced increases in gene expression of the constitutive *Hsp90β* and *Hsc70;* however, these are not generally of the same magnitude as the inducible forms and may instead play a role in the long term adaptation of the cell to stress (Sreedhar et al., 2004). *Hsp90α* mRNA levels were rapidly up-regulated after heat shock injury in Senegalese sole but no change was detected in *Hsp90β* (Manchado et al., 2008). Similar results were found in silver sea bream (*Sparus sarba*) and in Ali et al., (2003). However, whilst the trend towards an increase in inducible heat shock gene expression is evident, the magnitude of gene expression reported in other fish species is far below that observed in *L. calcarifer* (Deane & Woo, 2005; Sreedhar et al., 2004). As estuarine habitats are prone to sudden changes in environmental conditions, it is essential that *L. calcarifer* possesses the ability to acclimate rapidly. In this instance the sensitivity of the heat shock response pathway and the magnitude of gene expression suggest that this response has become highly specialized in *L. calcarifer* toward coping with such variable environments.

3.4.1.2 The metabolic response

In *L. calcarifer*, the expression of *CcoII* showed an inverse correlation with temperature, whereas both *CiSy* and *Ldh* expression did not significantly change at any time point during the

experiment. *CcoII* expression remained low until after the recovery phase of the experiment upon which time expression levels mirrored those of the control (Fig. 3.6). This expression pattern may be indicative of a shift in metabolism and may potentially indicate a slight adjustment in the aerobic metabolism of *L. calcarifer* during the initial increase in temperature from 28 °C to 36 °C. Changes in the level of *CcoII* have also been shown to indicate impairment of, or damage to, the mitochondrial membrane caused by temperature stress (Kuzmin et al., 2004; Pandey et al., 2000). The RNA levels of *Cco* have routinely been used in various other fish species as reliable indicators of metabolism, for example in both *Zoarces viviparous* and *Danio rerio*, *Cco* mRNA expression increased in response to cold acclimation indicating enhanced aerobic capacity (Hardewig et al., 1999; Lucassen et al., 2003; McClelland et al., 2006). Perhaps not surprisingly though, when these markers are measured in fish subjected to an increase in temperature the patterns of gene expression are somewhat similar to those found in the current study and thus are likely to be appropriate for indicating the metabolic response over a range of temperatures and in a wide variety of fish species. For example, in the gill of *Gillichthys mirabilis* an acute heat stress resulted in the immediate induction of *CcoI* whilst in the muscle, however, it was observed that *CcoVIII* was down regulated (Buckley et al., 2006). Adjustments in metabolic activity are crucial in coping with an acute temperature shock as energy within the cell is accessed to fuel the stress response and mechanisms of cellular repair. The above results in *L. calcarifer* show similar findings for *Cco* fold change after heat stress to those reported in other fish species suggesting that *L. calcarifer* have become adapted to the highly variable nature of estuarine environments primarily through changes to other cellular pathways and not necessarily through significant alterations to the metabolic pathways.

3.4.1.3 The growth pathway

As temperature stress in fish can occur regularly both within natural and artificial environments it is important to understand whether these homeostatic disruptions impact upon the expression of genes involved in the growth pathway. As positive and negative regulators of muscle growth respectively, *Igf1* and *Mstn1* were used in this study to determine the effects of an acute heat stress on the growth potential of *L. calcarifer*. Throughout an acute heat stress *Igf1* levels fluctuated in *L. calcarifer*, however, no significant differences were evident at any time during the experiment. Alternatively, there was a modest but statistically significant increase in *Mstn*1 expression that corresponded with the increase in temperature from 28 °C to 36 °C. However, *Mstn1* quickly returned to control levels soon after and remained that way for the duration of the experiment (Fig. 3.6). As a potent negative regulator of muscle growth, *Mstn1* activity has been shown to increase in response to stress. In *Danio rerio* a near 600-fold increase in *Mstn1* levels

was observed in the skeletal muscle of individuals exposed to stocking stress (Helterline et al., 2007). Further evidence of the inverse relationship between growth and *Mstn1* levels has been shown during periods of muscle atrophy in mammals (Gonzalez-Cadavid & Bhasin 2004; Lalani et al., 2000). Despite no significant changes in the expression levels of *Igf1* in response to an acute heat stress in *L. calcarifer*, evidence does exist to suggest the influence of temperature on *Igf1* expression. In various salmonids it has been shown that fish exposed to higher temperatures exhibit higher *Igf1* levels (Beckman et al., 1998; Larsen et al., 2001; McCormick et al., 2000), in these cases due to a higher metabolism at near optimal growth temperatures. Alternatively, in *Oncorhynchus mykiss*, the quantity of *Igf1* receptor was significantly lower after a hot water rearing treatment (Gabillard et al., 2003). The results of this study suggest that although the growth pathway in *L. calcarifer* is sensitive to temperature change, in this instance, an exposure to 36 °C was not likely to be immediately inhibitory to growth. The initial increase in *Mstn1* at the beginning of the acute heat stress indicates the sensitivity of the growth pathway to acute heat stress, but the failure of *Igf1* to respond significantly at all in *L. calcarifer* suggests that other changes quickly brought about primarily by the heat shock response and perhaps to a lesser extent by the metabolic response were enough to maintain normal activity within the growth pathway.

In conclusion, the current study assessed for the first time the gene expression response of a tropical estuarine fish species from a highly variable environment to an acute heat stress. It was found that in *L. calcarifer*, an acute heat stress was perceived and responded to quickly by a considerable up regulation of the heat shock response far and above that of other fish species not from estuarine systems. The magnitude of this response probably underpins the success of *L. calcarifer* in such highly variable environments and may be true of the adaptation shown by other estuarine species. The expression of *CcoII* suggests that an acute heat stress caused a minor suppression of aerobic metabolism in the initial stress stage. Although the growth pathway seems to be highly sensitive to changes in cellular homeostasis, it is unlikely that anything other than a prolonged or repeated heat stress would cause a significant change in the growth potential of *L. calcarifer*.

Chapter 4.0 Analysis of genes involved in the adaptation of barramundi (*Lates calcarifer*) to warm and cool temperatures: A role for heat shock proteins.

4.1 Introduction

Elucidating the process of adaptation in aquatic organisms is crucial to understanding how organisms cope and respond to changes in their environment. Nowhere is this knowledge more applicable than in estuarine environments which are generally considered to be one of the most variable aquatic environments on the planet (Whitfield & Elliott, 2002). Estuarine environments are characterised by the presence of multiple interacting abiotic stressors that include fluctuations in temperature, salinity and oxygen concentration, subjecting the organisms which live in this environment to constantly changing conditions (Elliott & Quintino, 2007; Whitfield & Elliott, 2002). Furthermore, these already highly capricious environments suffer additional pressure from human coastal development, habitat destruction and the introduction of significant levels of nutrients and toxins into waterways causing algal blooms and episodes of aquatic hypoxia (Schulte, 2007; Conley et al., 2002; Rabalais et al., 2002). Climate change and associated sea level increases are also predicted to add further environmental stochasticity and stress (Scavia et al., 2002). The majority of research focusing on interactions between environmental variables and marine organisms is centred predominantly around temperate fish species and increasingly on aquatic organisms from tropical coral reefs which exist in comparatively stable environments. An understanding of how tropical estuarine fish have adapted and responded to changes in environmental temperature, particularly at the genetic level, is vital in predicting the impacts that future disturbances may bring.

Barramundi (or Asian sea bass) (*Lates calcarifer*) are one such tropical fish species ideally suited to studying processes of adaptation to an estuarine environment. Barramundi have a distribution which encompasses much of the tropical world from India, Southeast Asia, Australia and Papua New Guinea. In Australia, barramundi are distributed throughout coastal inshore and estuarine waters over 16 degrees of latitude, with populations subjected to a broad range of environmental clines across this distribution. At the northern terminus of the species distribution barramundi encounter warm tropical conditions where mean yearly average temperatures range from 23.2 – 32 °C (Darwin, Northern Territory), whilst towards the southern terminus of their distribution significantly cooler and far more variable temperate conditions are encountered with mean yearly average temperatures ranging 18.5 – 27.7 °C (Gladstone, central Queensland) (Bureau of Meteorology, www.bom.gov.au). Furthermore, barramundi show high levels of genetic structuring, with at least 16 discrete genetic populations identified across this species' Australian distribution (Keenan, 1994; Salini & Shaklee 1988; Shaklee & Salini, 1985). Evolution of the current barramundi stock structure in Australia suggests that northern populations from warmer tropical conditions were ancestral to southern populations where conditions are on average much cooler (Keenan 1994). Furthermore, *Chapter 2* (Newton et al.,

2010) showed that populations of barramundi from warm and cool adapted environments show strong evidence of local adaptation to temperature, displaying differences in their phenotypic response to a short-term chronic heat exposure. In addition to this, a number of genes from the cellular stress, metabolic and growth pathways have been recently identified in barramundi as useful candidates for examining the response of these fish to temperature (*Chapter3*; Newton et al., 2012). It is highly probable that locally adapted populations of barramundi show differential regulation of these genes in response to both hot and cool rearing temperatures. Barramundi are thus an excellent candidate to further study the process of adaptation and a valuable addition to current findings would come from the analysis of temporal changes in gene expression over both warm and cold temperatures. By utilising genes with an important biological function and an established link to the pervasive effects of temperature a better understanding of how barramundi have adapted over time to different environments and how populations might cope with environmental change can be achieved.

Heat shock proteins (Hsps) are one such group likely to be involved in the adaptation of barramundi to temperature. Hsps operate to maintain homeostasis through various cellular functions including protein synthesis, the folding and translocation of various new proteins, as well as the assembly of large protein complexes (Feder & Hofmann, 1999).
Despite its complex relationship, evidence exists for a functional relationship between *Hsp* expression and the general stress response suggesting that *Hsp* expression can be directly linked to physiological stress in vertebrates. In cultured trout hepatocytes, for example, adrenaline was shown to mediate *Hsp70* levels through actions of the hypothalamus-pituitary-interrenal (HPI) axis (Ackerman & Iwama, 2000). Furthermore, no *Hsp* response was detected following restraint stress in hypophysectomised rats, however, addition of adrenocorticotropic hormone (ACTH) to these same rats caused the expression of *Hsp70* in the adrenals (Blake et al., 1991).

Hsps can be classified into different families based upon their molecular weight and contain amongst numerous others the 70 kDa family which contains the stress inducible *Hsp70* gene, and the 90 kDa family which contains the stress inducible *Hsp90α* gene. Both *Hsp70* and *Hsp90α* are known responders to temperature stress in fish where their expression was detected upon heat stress in the muscle, liver and brain of *Danio rerio* (Murtha & Keller 2003). *Hsp90α* was also induced in the muscle, heart and skin following heat stress in *Solea senegalensis* (Manchado et al., 2008) and *Hsp70* increased in the liver of *Gillichthys mirabilis* after exposure to various high temperatures (Lund et al., 2006). Recently, an acute heat stress in barramundi induced a significant up-regulation of both inducible heat shock proteins, *Hsp70* and *Hsp90α,* whilst both constitutive forms remained unchanged until a two week recovery phase resulted in a small, but significant, up-regulation of *Hsc70* (*Chapter 3*; Newton et al., 2012). Accordingly,

the temporal analysis of heat shock protein gene expression over longer periods than an acute heat stress will be useful to understanding the adaptive process of barramundi to temperature.

The adaptation of organisms to new environments may also cause changes to metabolic pathways. Adjustments in mitochondrial gene and protein regulation are common in exothermic animals, particularly in response to cold adaptation. Studies have shown that mitochondrial densities increase with low temperatures and that respiration rates within the mitochondria fall (Johnston et al., 1998). Measurements of key enzymes of the oxidative electron transport chain (cytochrome c oxidase subunit II (*CcoII*)) and the citric acid cycle (citrate synthase (*CiSy*)) have been commonly used to monitor aerobic and mitochondrial capacities in response to adaptation in multiple cold water fish species. In the adaptation of *Zoarces viviparous* to acute cold, *CiSy* mRNA levels and protein activity increased in the liver (Lucassen et al., 2003), whilst the activity of both *CiSy* and *Cco* subunits increased after cold acclimation in *Danio rerio* concordant with greater cold tolerance (McClelland et al., 2006). As populations of barramundi have become locally adapted to cooler environmental temperatures, it is possible that adaptation has been aided through changes in metabolism reflected by differences in the expression of *CiSy* and *CcoII*.

Temperature is also a major abiotic factor influencing growth (Gabillard et al., 2003). In an estuarine environment, however, temperature can fluctuate significantly on a daily basis. As such, estuarine species are considered to be more resilient to variable temperature changes than organisms from less variable environments (Elliott & Quintino 2007). It is unclear though whether a greater tolerance to variable environment parameters leads to an improved ability for growth adaptation and how gene expression changes within the growth axis are altered as a result. Two genes which have been correlated with somatotropic and muscle growth in fish and which have been used successfully to assess growth potential are insulin like growth factor 1 (*Igf1*) and myostatin-1 (*Mstn1*). *Igf1* is an integral gene associated with overall somatotropic growth, while *Mstn1* is a gene within the transforming growth factor β (TGF- β) superfamily and a negative regulator of myogenesis (Beckman, 2010; Lee & McPherron 2001). In barramundi, an acute heat shock caused a brief reduction in *Mstn1* expression, but no change in the expression of *Igf1*, demonstrating the sensitivity of *Mstn1* to temperature stress and indicating that *Igf1* expression may only be influenced by exposure to sustained temperature extremes (*Chapter 3*, Newton et al., 2012). Examining the expression profiles of these two genes in locally adapted populations of barramundi will help determine whether changes to the growth pathway are important in the process of adaptation to warm and cool temperatures and what effect these changes have on growth.

Barramundi, as a widely distributed tropical estuarine species subjected to broad latitudinal-induced thermal variability, is an ideal species in which to elucidate deeper genetic mechanisms responsible for adaptation to changing environmental temperature. The current study therefore sought to identify changes to key genes of the cellular stress, metabolic and growth pathways essential for the long term adaptation of barramundi to temperature. To accomplish this, gene expression and growth were recorded over time in a warm and cool adapted barramundi population acclimated to warm, control and cool water temperatures.

4.2 Materials and Methods

4.2.1 Animals and experimental design

Barramundi (*Lates calcarifer*) fingerlings of the same age from two genetically distinct strains representing a warm water adapted (Darwin, Northern Territory, 12° 27' S, 126° 50' E) and cool water adapted (Gladstone, Queensland 23° 50' S, 151° 15' E) population were obtained from commercial hatcheries and housed in 6 x 3000 L saltwater aquaria facilities at James Cook University (Townsville, Australia). Fish were kept indoors in a temperature (~28 °C) and light cycle (12 h light:dark) controlled environment and fed a commercial pellet twice a day to satiation (Ridley Aquafeed; http://www.agriproducts.com.au). Warm and cool water adapted barramundi fingerlings at a standard length of approximately 10 cm and 50 g in weight were communally reared within floating mesh cages (2 cages per tank) within each of the six experimental tanks so as to maintain population identity without the need for tagging. All barramundi were acclimated to 28 °C for 2 months after which the temperature within two randomly selected tanks was either reduced to 22 °C (cool water temperature), maintained at 28 °C (control temperature), or increased to 36 °C (warm water temperature) at the rate of 1 °C/hr. Once water temperatures in tanks had reached their desired treatment levels they were maintained at this temperature (experimental temperature ± 1 °C) for the duration of the experiment. Water chemistry, dissolved oxygen (> 5 mg/ml), pH and salinity were routinely monitored and did not differ between treatments throughout the experiment.

Total weight (g) from five fish per tub (10 replicate fish per temperature per time point) was recorded as a measure of growth during the experiment at day 0 (T=0), day 1 (T=1), day 3 (T=3), day 9 (T=9), day 23 (T=23), day 50 (T=50) and day 106 (T=106), where after the growth trial was terminated. At time points T=0, T=3, T=9 and T=106 these five fish were also euthanized in (volume) clove oil and white muscle tissue collected and snap frozen in liquid nitrogen for gene expression analysis (again giving 10 replicate fish samples/temperature/time point).

4.2.2 RNA isolation and cDNA synthesis

RNA from frozen white muscle samples was extracted by homogenizing tissues directly in Ultraspec RNA (Biotecx; http://www.biotecx.com) followed by precipitation into a solution containing 0.5 vol of RNA precipitation solution (1.2 M sodium chloride, 0.8 M disodium citrate (Sambrook & Russel, 2001) and 0.5 vol of isopropyl alcohol. RNA quality and quantity was verified using a Nanodrop spectrophotometer (Nanodrop technology; http://www.nanodrop.com) via examination of absorbance ratios at $OD_{260/280}$ (range 1.97-2.04) and $OD_{260/230}$ (range 1.94-2.08) and by the visual inspection of integrity of both the 18S and 28S ribosomal bands on a 1.5 % agarose gel. Samples were adjusted to a final concentration of 200 ng/µL and treated with a Turbo DNA-free kit (Ambion; http://www.invitrogen.com), followed by an ammonium acetate precipitation. First strand cDNA synthesis was performed on 5 µg of DNase treated total RNA using a Super Script III first-strand synthesis supermix (Invitrogen; http://www.invitrogen.com) with 2.5 µM $oligo(dT)_{20}$ and 25 µM random hexamers (Resuehr & Spiess, 2003). Complete removal of contaminating DNA was verified by PCR amplification of RNA samples using gene specific primers as a no-amplification control (NAC) ($C_{q\ (NAC\ control)} - C_{q\ (cDNA\ synthesis)} > 10$). After cDNA synthesis, RNA template was digested with 1 µL of RNase cocktail mix (Ambion; http://www.invitrogen.com) within 20 µL of cDNA incubated at 37 °C for 30 min followed by enzyme deactivation at 70 °C for 10 min. Samples were purified using Sepharose CL-6B spin columns (Sigma-Aldrich; http://www.sigmaaldrich.com) and cDNA was quantified and diluted to the same final concentration of 2 ng/µL prior to real time PCR analysis.

4.2.3 Real time analysis of candidate gene expression

RT-qPCR primers and reaction conditions were the same as those previously described for heat shock proteins 70 and 90alpha (*Hsp70* & *Hsp90α* respectively), cytochrome c oxidase subunit II (*CcoII*), citrate synthase (*CiSy*), insulin like growth factor 1 (*Igf1*) and myostatin 1(*Mstn1*) (De Santis et al., 2011; *Chapter 3*; Newton et al., In press). All RT-qPCR reactions were performed in a final reaction volume of 12 µL using 1 x SYBR GreenER qPCR Supermix Universal (Invitrogen; http://www.invitrogen.com), 2.5 µM of Rox reference dye, 0.2 µM of each primer with 2 ng of cDNA template. All reactions were performed on an MJ research DNA engine with a Chromo 4 detector and utilizing Opticon Monitor 3.0 software (Biorad; http://www.bio-rad.com). Each reaction was amplified in triplicate and consisted of 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension for 30 s at primer specific temperatures. Routinely, a dissociation curve analysis was performed after each run and the product was cloned to ensure amplification specificity. Optimal PCR

conditions were ensured by calculating reaction efficiency (E) [$E = 10^{(-1/slope)}$] and reproducibility of each assay (Bustin et al., 2009; Rasmussen, 2001). The number of samples analysed necessitated that the expression of each gene be analysed over several plates. To avoid inter-assay variability a calibrator sample was run in triplicate on each plate as well as a standard curve for determining amplification efficiency, thereby ensuring that technical variation was not confused with biological variation.

4.2.4 Statistical analysis and data normalization

Growth differences between barramundi populations reared at different temperatures were analysed at all seven time points (10 replicate fish/population/temperature/time point at T=0, T=1, T-3, T=9, T=23, T=50 and T=106) by means of ANOVA, with Bonferroni post hoc testing. Samples were considered significantly different based upon differences of $p < 0.05$.

The relative expression of all six genes of interest was obtained by normalizing against the input amount of cDNA via the ΔC_q method ($Ratio_{(test/calibrator)} = E^{Cq\,(calibrator)\,-\,Cq\,(test)}$) with individual samples as the test and the average of all eight fish reared at 28 ºC at each time point for each population as the calibrator. The level of gene expression between experimental samples (T=0, T=3, T=9 and T=106) were then statistically compared by means of ANOVA. Homogeneity of variance was confirmed using a Levene's test and differences of $p < 0.05$ between samples were considered significant. All statistical analyses were performed using SPSS v19.0 (SPSS, 2010).

4.3. Results

4.3.1 Growth response of barramundi (Lates calcarifer) to hot and cool rearing temperatures

At the final time point, T=106, growth differences were significantly different between warm and cool adapted barramundi reared at 22 °C only. At this time point cool adapted barramundi had an average weight of 145.9 ± 11.1 g (mean ± SE) and were 62.2 % heavier than warm adapted barramundi (89.9 ± 3.5 g) (ANOVA; $F_{5, 54} = 43.213$, $p<0.0001$) (Fig. 4.1). No significant differences in growth between warm and cool adapted barramundi reared at 36 °C and 28 °C were evident at any time during the growth trial. In general though, both warm and cool adapted barramundi attained a significantly heavier end weight when reared at 28 °C than when reared at either 22 °C or 36 °C. At the final time point (T=106, ANOVA; $F_{5, 36} = 8.752$), warm adapted barramundi reared at 28 °C grew to an average weight of 233.2 ± 13.2 g, which was significantly higher than when the same population was reared at either 36 °C (124.0 ± 7.5 g, $p < 0.0001$), or 22 °C (90.0 ± 3.5 g, $p < 0.0001$). Similarly for cool adapted barramundi, fish

reared at 28 °C recorded the heaviest end weight at T=106 (237.2 ± 11.4 g), which was significantly heavier when compared within the same population reared at 36 °C (138.0 ± 7.0 g, p <0.0001) and 22 °C (145.9 ± 11.1 g, p <0.0001). No significant differences in growth were recorded when comparing cool adapted barramundi reared at 36 °C and 22 °C, although warm adapted fish obtained a significantly heavier weight when reared at 36 °C over 22 °C (p <0.05). Figure 4.1 shows the growth pattern and gradual divergence in weight of the two barramundi populations reared at the three temperatures over time.

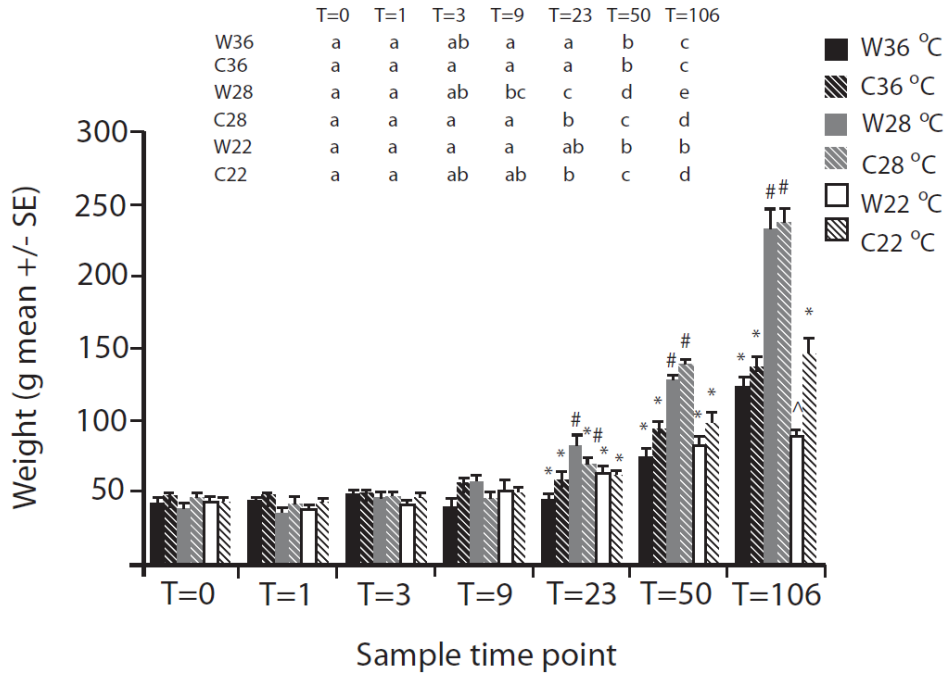

Fig. 4.1 Growth data for both warm (W) and cool (C) adapted barramundi groups reared at 36 °C (W36 °C & C36 °C respectively), 28 °C (W28 °C & C28 °C respectively) and 22 °C (W22 °C & C22 °C respectively) at each sample time point (T=0 through to T=106). Growth data is presented as average weight in (g) ± 1 SE. Symbols directly above columns indicate comparisons between each group within a time point. Letters arrayed within the table indicate group comparisons across time points. Common symbols/lettering indicates no significant differences whilst different letters indicate significant differences between those groups. Groups without symbol/lettering are not significantly different.

4.3.2 Gene expression response of barramundi (Lates calcarifer) to hot and cold rearing temperatures

4.3.2.1 The heat shock response

Throughout the experiment *Hsp70* and *Hsp90α* expression varied significantly between time points within both warm and cool adapted barramundi reared at 22 °C only. Neither heat shock gene showed any significant changes in gene expression over time in fish reared at 36 °C or at control 28 °C (Fig. 4.2). In cool adapted barramundi, *Hsp70* and *Hsp90α* first showed significant increases in gene expression above control temperatures at T=3 (ANOVA; $F_{5, 41}$ = 4.886 and 5.337, $p < 0.05$ for both genes). *Hsp70* and *Hsp90α* gene expression increased further at T=9 compared with control levels in cool adapted barramundi by ~145 fold and ~13 fold respectively (ANOVA; $F_{5, 42}$ = 10.923 and 9.158, $p < 0.01$ for both genes) (Fig. 4.2). By T=106, the expression of both *Hsp70* and *Hsp90α* in cool adapted barramundi had returned to basal levels that was not significantly different from control temperatures.

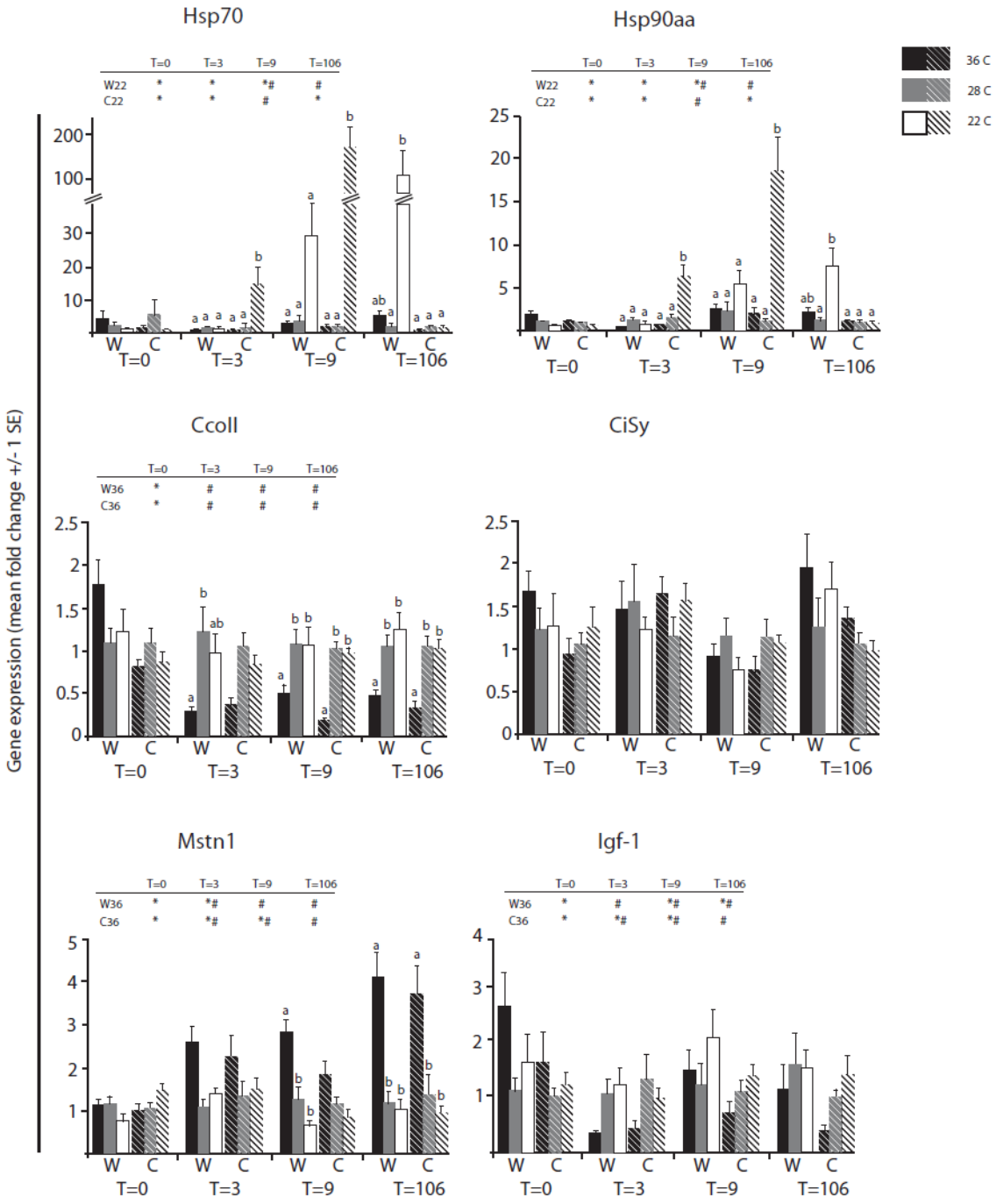

Fig. 4.2 Gene expression data for *Hsp70* and *Hsp90α* (*aa*) of the cellular stress response pathway, *CcoII* and *CiSy* of the metabolic pathway and *Mstn1* and *Igf1* of the growth pathway. Gene expression graphs depict gene expression at time points T=0, T=3, T=9 and T=106 at 36 °C, 28 °C and 22 °C rearing temperatures in both warm adapted (W - solid colours) and cool adapted (C - broken colours) barramundi. Symbols above columns represent comparisons between groups at each time point whilst letters in tables represent group comparisons across time points. Common symbols/lettering indicates no significant differences whilst different

letters indicate significant differences between those groups. Groups without symbol/lettering are not significantly different.

In warm adapted barramundi, the expression of *Hsp70* and *Hsp90α* showed a similar trend to that observed within cool adapted barramundi with the exception that expression was somewhat delayed and not generally of the same magnitude. No significant differences in heat shock gene expression were evident in warm adapted fish until T=106 when expression of both heat shock genes was significantly higher in fish reared at 22 °C compared with fish reared at control 28 °C (ANOVA; $F_{5, 41}$ = 3.285 and 3.229, $p < 0.05$ for both genes). Despite a large increase in the expression of both heat shock genes, expression in fish reared at 22 °C was not significantly different from expression levels recorded in fish reared at 36 °C (Fig. 4.2). Again, no significant change in *Hsp70* or *Hsp90α* expression was observed at any time in warm adapted fish reared at either 36 °C or at control 28 °C.

When comparing populations, differences in *Hsp* gene expression were first evident at T=3. At this time point both *Hsp70* and *Hsp90α* expression was significantly higher in cool adapted barramundi compared with warm adapted barramundi at 22 °C (ANOVA; $F_{5, 41}$ = 4.886 and 5.337, $p < 0.01$ for both genes). Differences in the expression of heat shock genes between cool and warm adapted barramundi were again evident at T=9 as the expression of both *Hsp70* and *Hsp90α* was again significantly higher in cool adapted barramundi reared at 22 °C (ANOVA; $F_{5, 42}$ = 10.923, $p < 0.0001$ and ANOVA; $F_{5, 42}$ = 9.158, $p < 0.001$ respectively) (Fig. 4.2). By T=106, *Hsp* gene expression in cool adapted barramundi had returned to basal levels whilst both *Hsp70* and *Hsp90α* had increased and were significantly higher in warm adapted barramundi reared at 22 °C (ANOVA; $F_{5, 41}$ = 3.285 and 3.229, $p < 0.01$ for both genes) (Fig. 4.2).

4.3.2.2 The metabolic response

Changes in the metabolic gene *CcoII* were most evident in barramundi reared at 36 °C (Fig. 4.2). In both warm and cool adapted populations of fish reared at 36 °C, *CcoII* expression showed an immediate decrease of ~1.5 fold (ANOVA; $F_{3, 27}$ = 17.544, $p < 0.0001$) and ~0.5 fold (ANOVA; $F_{3, 28}$ = 16.899, $p < 0.0001$) respectively, from T=0 to T=3. From then on, *CcoII* expression remained significantly reduced at T=9 and at T=106 for both populations reared at 36 °C (Fig. 4.2). No significant differences in *CcoII* gene expression occurred within either population reared at 22 °C or control 28 °C (Fig. 4.2). Expression of the metabolic gene *CiSy* fluctuated consistently throughout the experiment, but showed no significant changes in gene expression between rearing temperatures within any time point for either population (Fig. 4.2).

4.3.2.3 The growth response

Differences in the expression of the growth genes *Igf1* and *Mstn1* showed variation in fish reared at 36 °C only (Fig. 4.2). At this temperature, warm adapted barramundi exhibited a significant decrease in *Igf1* expression from T=0 to T=3 (AN OVA; $F_{3, 25} = 5.082$, $p < 0.01$). At time points T=9 and T=106, gene expression remained low, but had returned to levels that were not significantly different between time points (Fig. 4.2). In cool adapted barramundi reared at 36 °C, *Igf1* expression gradually decreased throughout the experiment, but did not become significantly lower than levels recorded at T=0 until T=106 (ANOVA; $F_{3, 28} = 3.559$, $p < 0.05$). At no time point were differences in the expression of *Igf1* present between warm and cool adapted populations (Fig. 4.2).

Mstn1 gene expression increased over time in both warm and cool adapted barramundi reared at 36 °C. By T=106 *Mstn1* gene expression was significantly greater than at T=0 in both warm and cool adapted fish reared at 36 °C (ANOVA; $F_{3, 24} = 7.046$ and 5.786, $p < 0.01$ for both populations) (Fig. 4.2). At T=9 (ANOVA; $F_{5, 42} = 7.281$), warm adapted barramundi had significantly higher *Mstn1* expression when reared at 36 °C than at either 22 °C ($p < 0.0001$) or control 28 °C ($p < 0.05$). By T=106 (ANOVA; $F_{5, 41} = 5.333$), both warm and cool adapted barramundi showed higher *Mstn1* gene expression when reared at 36 °C than when reared at 22 °C (p 0.01 for both populations), or 28 °C ($p < 0.01$ for both populations) (Fig. 4.2).

4.4. Discussion

In the current study temporal changes in gene expression were analysed in response to rearing temperature between warm adapted and cool adapted barramundi. In conjunction with phenotypic growth data, the analysis of key genes from the cellular stress, metabolic and growth pathways illustrated mechanisms by which barramundi have become adapted to environmental temperature. These results may also demonstrate in broader terms the degree of plasticity inherent in other estuarine species and hence provide some indication of their ability to adapt to environmental changes and the mechanisms by which they may do so.

4.4.1 Adaptation of the cellular stress response in Lates calcarifer

Both warm and cool adapted barramundi showed a significant induction of the cellular stress response when reared at 22 °C. In both populations, *Hsp70* and *Hsp90α* showed a continual increase in expression at this temperature over the course of the experiment. However, induction of the cellular stress response at 22 °C was much faster in cool adapted barramundi than in

warm adapted barramundi. In cool adapted barramundi both *Hsp70* and *Hsp90α* gene expression was significantly higher at 22 °C compared to 28 °C at T=9, whilst in warm adapted barramundi the same differential response were not seen until much later at T=106. In addition to this, cool adapted barramundi induced *Hsp90α* expression to a far greater magnitude than warm adapted barramundi (Fig. 4.2) and were significantly heavier at the end of the experiment due to a superior growth rate when reared at 22 °C (Fig. 4.1). These findings may suggest that cool adapted barramundi may have been aided in their adaptation to reduced temperatures through changes in heat shock protein (HSP) expression associated with changes to the cellular and general stress responses (Iwama et al., 1999). These results are interesting, as although the heat shock response in fish has been well characterised, the most cohesive results to date come from studies looking at the induction of the heat shock response after an increase in temperature. It is well known that Hsps function primarily as molecular chaperones to stabilise the folding of proteins, prevent protein denaturation and non-specific protein binding. However, these occurrences are mostly attributed to the adverse effects of high temperatures within the cell, whilst the primary effects of a reduction in temperature are usually the slowing of enzymatic reaction rates and alterations to membrane fluidity (Donaldson et al., 2008; Iwama et al., 1998; Sonna et al., 2002). Interestingly though, it has also been suggested that many of the cellular changes occurring within a cell due to the effects of cold exposure are similar to those due to heat exposure, including alterations to protein synthesis and the cell cycle, alterations to membrane permeability and the structure of the cytoskeleton, as well as protein denaturation (Sonna et al., 2002). There has also been direct evidence to suggest that alterations to membrane fluidity stimulates Hsp production and therefore could stimulate *Hsp* gene expression in response to either hot or cold temperatures (Vigh et al., 1998). Consequently, a role for *Hsps* in preventing damage from cellular stress in fish has been found at both high and low temperatures (Basu et al., 2002; Nakano & Iwama 2002; Werner et al., 2006). Accordingly, a faster response within the cellular stress pathway combined with superior growth in cool adapted barramundi reared at 22 °C may indicate an important function for *Hsp70* and *Hsp90α* in the adaptation of barramundi to cooler temperatures. Thus far, few studies have shown the prolonged expression of *Hsps* due to cold exposure and evidence within the literature for the role of *Hsps* in conferring cold tolerance within tropical estuarine species is rare.

At a rearing temperature of 36 °C, however, the expression of *Hsp70* or *Hsp90α* was not altered at any stage in either warm or cool adapted barramundi (Fig. 4.2), although the growth rate of barramundi was significantly reduced in both populations at 36 °C compared with growth at 28 °C (Fig. 4.1). Growth trials in barramundi have shown that feed intake and standard growth rate (SGR) increase with increasing temperature up until a maximum threshold of approximately 36 °C, beyond which growth and feed efficiency decrease significantly (Katersky & Carter, 2005).

The current results are in agreement with these findings and further show that the inhibition of growth is not brought about by changes in the expression of genes of the cellular stress response pathway, at least not over a long term rearing period as *Chapter 3* (Newton et al., 2012) has previously shown that barramundi exposed to a rapid *acute* heat stress of 36 ° C do elicit a very strong heat shock response. After 1 hour at 36 °C, *Hsp70* and *Hsp90α* expression were found to have increased dramatically by ~450 fold and ~55 fold respectively in barramundi compared to control fish (*Chapter 3*, Newton et al., 2012). The fact that no population differences in *Hsp70* or *Hsp90α* expression was evident in the current study between locally adapted barramundi can be explained by the fact that the current study looked for Hsp expression in response to a long term temperature stress *after* a suitable acclimation period. In addition, it is probable that southern, cool adapted populations of barramundi have retained a tolerance to warmer waters owing to the environment from which they historically originate. In barramundi it seems likely that at high temperatures, induction of heat shock genes occurs during the acute phase of the stress and is not prolonged, whereas at cold temperatures there is a more gradual and sustained increase in gene expression.

4.4.2 The metabolic and growth response

Gene expression within the metabolic and growth pathways responded in warm and cool adapted barramundi reared at 36 °C only, with no significant changes to gene expression occurring in barramundi reared at either 28 °C or 22 °C. The metabolic gene citrate synthase (*CiSy*) fluctuated throughout the course of the experiment within both populations, but at no time were there significant differences in gene expression. In contrast, Cytochrome c oxidase subunit II (*CcoII*) from the electron transport chain was immediately suppressed in both warm and cool adapted barramundi reared at 36 °C and remained that way for the duration of the experiment, suggesting a reduction in metabolic processes. In both *Zoarces viviparous* and *Danio rerio*, the expression of *CcoII* increased in response to cold acclimation and was indicative of enhanced aerobic capacity (Hardewig et al., 1999; Lucassen et al., 2003). Furthermore, at 32 °C in the gill and white muscle of *Gillichthys mirabilis* an increase in ATP producing genes, including an increase in various cytochrome subunits, was recorded due to an increased demand on cellular energy resources aimed at mitigating the effects of heat stress (Buckley et al., 2006). As the expression of *CcoII* in these studies was used to indicate alterations in aerobic and mitochondrial activity due to temperature stress, it can be surmised that a decrease in *CcoII* expression in barramundi at 36 °C is possibly the result of a suppression of these processes. In both warm and cool adapted barramundi reared at 36 °C there was also a simultaneous increase in the negative growth regulator myostatin 1 (*Mstn1*), and a concurrent decrease in the positive growth regulator insulin-like growth factor 1 (*Igf1*). Again, neither

representative of the growth pathway showed significant changes in gene expression within either population at 28 °C or 22 °C rearing temperatures. As the growth of barramundi at 36 °C was significantly lower than growth at 28 °C it is possible that the discordant regulation of the two growth regulating genes may be indicative of a general reduction within the growth axis. High rearing temperatures have been shown to stunt growth consistent with a reduction in *Igf1* mRNA expression in a number of species. In *Paralichthys lethostigma* fish reared at 23 °C recorded higher *Igf1* expression and also grew to be 65-83% larger than fish reared at 28 °C (Luckenbach et al., 2007). Similarly, muscle *Igf1* expression in *Oncorhynchus mykiss* was lower in fish after 2 weeks at 16 °C compared with fish held at 8 °C (Gabillard et al., 2003). The concurrent decrease in *Igf1* expression accompanied by reduced growth in barramundi reared at 36 °C suggests that *Igf1* is indeed an accurate determinate of growth in this species. However, although studies report the expression response of *Mstn1* to numerous stimuli such as feeding regimes, overcrowding stress and cortisol, comparatively little is known about *Mstn1* expression in response to heat stress and the results that do report on *Mstn1* expression due to temperature fail to provide a cohesive picture. Bertotto et al., (2011) described the response of developing *Dicentrarchus labrax* larvae to heat shock by measuring the expression of cortisol, *Hsp70*, *Igf1* and *Mstn*. Heat shock resulted in an increase in cortisol levels and *Hsp70* expression and caused a reduction in *Igf1* mRNA, but did not significantly affect the expression of *Mstn1*. In Channel catfish, however, *Mstn* transcript levels were abundant during a period of slow muscle growth at cold rearing temperatures (Weber & Bosworth, 2005). Although a decrease in *Mstn1* was evident at high temperatures in barramundi it appears that an abundance of *Mstn1* transcript may accompany a reduction in growth at both high and low stress temperatures. To date decreased growth due to *Mstn1* expression has been linked to an abundance of stress stimuli and although the current study suggests a role for *Mstn1* in the reduction of growth at high temperatures, further work is needed to characterise its role in response to temperature in general.

In the current study the expression of key genes from the cellular stress, metabolic and growth pathways were analysed in response to alternate rearing temperatures in a long-term growth experiment in both a warm and a cool adapted barramundi population. The results of the current study suggest that adaptation of barramundi to cool water temperatures has arisen in part due to significant alterations in the cellular stress response. Cool adapted barramundi were able to express *Hsp70* and *Hsp90α* heat shock proteins faster and in some instances to a greater magnitude than warm adapted barramundi when reared at 22 °C. As a result, cool adapted barramundi showed significantly higher end weights after a long term rearing experiment. Alterations in the expression of genes from the metabolic and growth pathways occurred at 36 °C rearing temperatures only, with no differences between populations and it is unlikely that

these genes were directly involved in the adaptive process. The results do show that cool adapted barramundi still maintain a tolerance to warmer waters, although a decrease in the metabolic gene *CcoII* suggests an increased demand upon aerobic capacity at this temperature in both populations. Furthermore, a reduction in the expression of *Igf1* and an increase in *Mstn1* expression coinciding with poor growth at 36 °C suggest that *Igf1* expression and possibly *Mstn1* expression are good indicators of growth in barramundi. As barramundi lend themselves as an important species for gauging the adaptive ability of other tropical estuarine inhabitants, it is likely that the cellular stress response and the genes analysed from the growth pathway are important to environmental adaptation within a broad range of tropical estuarine organisms. The analysis of these genes within barramundi suggests that expression may have significant implications in the preparation of an organism for climate change and not just in the response of cells to immediate stress.

Chapter 5.0 Next-generation transcriptome profiling reveals insights into genetic factors contributing to growth differences and temperature adaptation in Australian populations of barramundi (*Lates calcarifer*)

5.1 Introduction

The effect of temperature on poikilothermic organisms is felt at every level of biological organization, from animal behaviour and physiology, to the cellular expression of genes and proteins (Huey & Bennet, 1990). For tropical estuarine species such as barramundi (*Lates calcarifer*), coping with fluctuations in environmental temperature is paramount to their survival as estuarine water temperatures vary significantly on a daily and seasonal basis. Climate change is expected to further exacerbate these already frequent variations in environmental conditions, and is thus likely to pose a significant challenge for resident barramundi populations in the near future (Bianchi, 2006). Australian populations of barramundi (*Lates calcarifer*) are distributed from the Ashburton River (22 ° 30 ' S) in Western Australia, across the tropical north of the country, and down the eastern Queensland coast to the Noosa River (26 ° 30 ' S). Throughout this distribution barramundi inhabit fresh, estuarine and near coastal waters over some 16 degrees of latitude that encompass a wide range of environmental temperatures. At the northern and southern end of their Australian distribution, mean yearly average temperatures differ significantly and range from 23.2 – 32 °C in Darwin, Northern Territory, to 18.5 – 27.7 °C in Gladstone, central Queensland, respectively (Bureau of Meteorology, http://www.bom.gov.au). As a species, barramundi experience significantly warmer and more consistent temperatures at lower latitudes whilst encountering cooler and less consistent temperatures at higher latitudes. Across this thermal cline barramundi has also been shown to exhibit significant genetic structuring, with up to 16 discrete genetic stocks identified (Keenan, 1994; Salini & Shaklee, 1988) (Fig. 5.1). In addition to this, barramundi are a euryhaline and catadromous species and require estuarine and in-shore marine habitats to breed. However, after eggs hatch, juvenile barramundi migrate upstream to freshwater river systems away from river mouths (Pusey, 2004) and on the basis of recorded tagged fish movements it is believed that the migration of individuals between adjacent river-mouths more than 100 km apart, while it does occur, is relatively rare (Keenan, 1994). Therefore, adjacent populations exchange little genetic information by way of individual migration. Taken together, these observations have prompted speculation as to whether the high levels of genetic structure in barramundi have translated into functional genetic adaptation to local environmental stressors, including that of temperature.

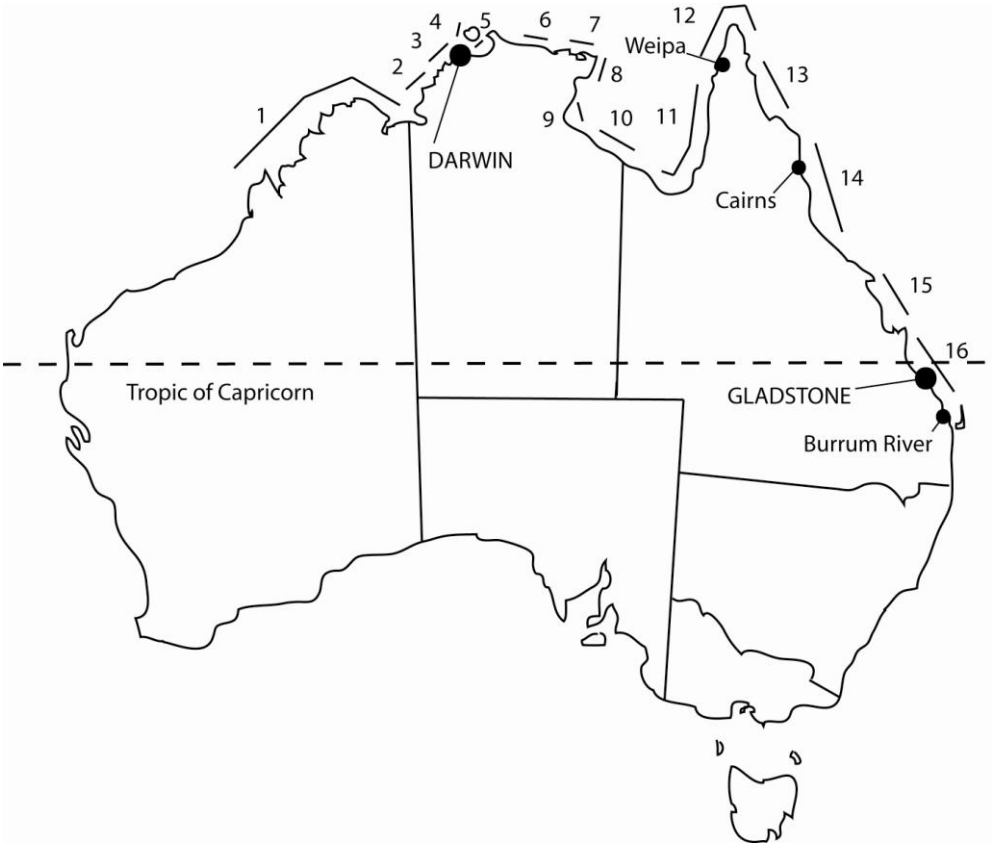

Fig. 5.1 Map of Australia showing genetic stocks of barramundi (*Lates calcarifer*). Taken from Newton et al., (2010). Australian populations of barramundi based upon Keenan (2000). 1, Fitzroy to Ord and Moyle Rivers; 2, Daly and Finniss Rivers; 3, Darwin Harbour and Shoal Bay; 4, Port Hurd; 5, Mary River, NT; 6, Goyder River; 7, Buckingham Bay; 8, Blue Mud Bay; 9, Roper River; 10, McArthur River; 11, south-east Gulf of Carpentaria; 12, Cape York Tip; 13, east Cape York and Princes Charlotte Bay; 14, north east coast; 15 central east coast; 16, south east coast. The locations of Darwin, Gladstone, Cairns and Burrum River are also shown.

Examination of the current barramundi stock structure in Australia through biogeographical studies suggests that phenotypic differences arising between populations from genetic differences should be relatively small. This is due in part to the relatively recent establishment of the current population structure (~17,000 years ago) and evidence for substantial migration and hybridization between historical eastern and western populations during the last glacial maxima (Keenan, 2000). However, studies by Rogers & Bloomfield (1993) and later Newton et al., (2010) show that populations from different thermal environments respond differently under thermal stress for traits such as survival, growth and upper thermal tolerance. Rogers & Bloomfield (1993) reared two Queensland strains of barramundi (from Cairns, northern Queensland and Burrum River, central Queensland – see Fig. 5.1) in open freshwater cage culture whilst recording environmental conditions and the performance of both fish populations.

Over the entire culture period both populations exhibited similar growth rates, however, bacterial infections caused greater mortality during cold weather periods in the northern Cairns strain. As temperatures cooled with the onset of winter, Burrum River fish were observed to have higher feed rates, whilst Cairns fish had lower appetite, lower condition factor, reduced growth during winter and higher mortality rates. The authors suggested that their findings were indicative of the unique adaptation of Cairns and Burrum River strains to local thermal conditions (Rogers & Bloomfield, 1993). Newton et al., (2010), using thermal challenge experiments showed that the upper thermal tolerance of barramundi populations from the extreme latitudinal ranges of the species Australian distribution significantly differed, with barramundi from the lower latitude (warmer conditions) exhibiting greater tolerance to high water temperatures than fish from a higher latitude (colder conditions). These results lend strong support to the argument that Australian barramundi do in fact show evidence of local adaptation to temperature.

The relationship between local environment and thermal tolerance in fish has also been revealed in a few other species. In common killifish (*Fundulus heteroclitus*) critical thermal maxima and minima were shown to be different between northern and southern populations over a range of acclimation temperatures. The underlying genetics revealed differences in Ldh-B concentration (Crawford & Powers, 1992) and heat shock protein (*Hsps*) expression between populations, showing that killifish thermal tolerance limits have a substantial genetic basis and vary in a direction consistent with what is predicted for fish that have undergone localized adaptation to environment (Fangue et al., 2006). A genetic analysis looking at the effects of acclimation to various cold water temperatures in carp (*Cyprinus carpio*) found a large body of genes underlying this response. Specifically, in muscle many genes were found to be involved in the remodeling of the contractile apparatus, hence improving physiological performance at low temperatures. With such a large volume of genetic information generated, gene ontology (GO) categories were constructed revealing that the acclimation of carp to cold temperatures had significant contributions from genes involved in transcription regulation, RNA splicing and translation (Gracey et al., 2004). Furthermore, adjustments in the mitochondrial aerobic properties of cod (*Gadus morhua*) at the gene level were shown to be crucial in seasonal acclimatization as well as in evolutionary adaptation to Arctic cold (Lucassen et al., 2006). Exploring the underlying genetics of temperature adaptation in fish species has helped identify a multitude of mechanisms by which various fish species cope with different environments. It has also helped to explain the depth and biogeographical distribution of fish populations and has enabled researchers to predict the likely impacts of climate change on many marine ectotherms. Despite this, a holistic understanding of the gene expression differences underlying fish populations adapted to different environments is lacking. In addition to this there have been no

studies looking at the underlying genetic mechanisms of temperature adaptation in a tropical estuarine species such as barramundi.

Next-generation RNA sequencing (e.g., Illumina mRNA- seq) allows for the profiling of large quantities of expression data from many samples simultaneously, where individual genes or entire ontologies can be identified and examined in response to an experimental hypothesis. This methodology is ideal for examining temperature adaptation in fish populations as numerous genes and pathways are likely to be involved and RNA sequencing allows for examination of the entire transcriptome. In the current study the transcriptomic differences underlying growth differences due to temperature adaptation were examined in two populations of barramundi from different thermal environments (warm-adapted Darwin and cool-adapted Gladstone) using next generation sequencing data (Illumina GAIIx) and GO analysis, in conjunction with growth experiments.

5.2 Materials and methods

5.2.1 Animals and experimental design

Two genetically distinct stocks of barramundi (*Lates calcarifer*) representing a northern, warm-adapted (Darwin, Northern Territory, 12 ° 27 ' S, 126 ° 50 ' E) and southern, cool-adapted (Gladstone, Queensland 23 ° 50 ' S, 151 ° 15 ' E) population were obtained from commercial fish hatcheries at a standard length (~125 mm) and weight (48 g) and reared communally in 5000 L tanks at James Cook University. Fish were kept indoors in a temperature controlled room (~25 °C) with a 12 h light:dark photoperiod and fed a commercial diet twice a day to satiation throughout the experiment (Ridley Aquafeed; http://www.agriproducts.com.au). Juvenile fish from each population were divided evenly into replicates of three groups and introduced to either a cool 22 °C, a control 28 °C or a hot 36 °C water temperature at the rate of 1 °C/h. A control temperature of 28 °C was selected as it is generally considered to be the optimal temperature for growth in barramundi. Population identity was maintained at all times through the separation of populations into floating mesh tubs within the same tank so that northern barramundi (N) could be distinguished from southern barramundi (S) at cool (N^{22} from S^{22}), control (N^{28} from S^{28}) and hot (N^{36} from S^{36}) temperatures. During growth trials fish were reared for a period of approximately 3.5 months (106 days), whilst at all times water chemistry, dissolved oxygen (> 5 mg/ml), pH, and temperature (experimental conditions ± 1 °C) were rigorously maintained. After this time, a total of 32 fish from both the warm-adapted and cool-adapted populations were humanely sacrificed in accordance with James Cook University animal ethics requirements and their weight recorded as a measure of growth over the rearing

experiment. White muscle tissue was chosen for gene expression analysis due to its growth responsiveness and high metabolic rate and was immediately dissected from each fish and snap frozen in liquid nitrogen.

5.2.2 RNA isolation and sequencing preparation

Total RNA was extracted by homogenizing frozen muscle tissue in Ultraspec solution (Biotecx; http://www.biotecx.com) using a PRO200 homogenizer (PRO scientific Inc; http://www.proscientific.com). RNA was precipitated in a solution containing 0.5 vol of RNA precipitation solution (1.2 M sodium chloride, 0.8 M disodium citrate, (Sambrook and Russell, 2001) and 0.5 vol of isopropyl alcohol. RNA quality and quantity was verified using a Nanodrop spectrophotometer (Nanodrop technology;http://www.nanodrop.com) via examination of absorbance ratios at $OD_{260/280}$ (range 1.98-2.06) and $OD_{260/230}$ (range 1.96-2.07) and by the visual inspection of the 18S and 28S ribosomal bands (and possible DNA contaminants) on a 1.5 % agarose gel. After Nanodrop quantification, four RNA pools were constructed by combining individual fish RNA samples representing northern barramundi reared at 22 °C and 36 °C, and cool-adapted barramundi reared at 22 °C and 36 °C. Each sample pool consisted of 5 ug of total RNA from a total of eight separate individuals so that any potential variation between individual fish could be captured. Each RNA pool was then treated with a Turbo DNA-free kit (Ambion; http://www/invitrogen.com) as a precaution to eliminate trace DNA contamination before being sent for further processing including sample quality and quantity verification on an Agilent RNA Bioanalyzer chip directly prior to sequencing on an Illumina Genome Analyzer IIx (Macrogen Inc; http://www.macrogen.com).

5.2.3 Sequence analysis and de novo contig assembly

Four mRNA-seq libraries were constructed representing pooled samples from northern and southern populations of barramundi reared at 22 °C and 36 °C incorporating unique bar-coding for each pool library. Illumina transcriptome mRNA pair-end sequencing (101 bp reads) was performed using standard protocols and reagents according to manufacturer's recommendations (Illumina Inc; http://www.illumina.com). Following sequencing, read data were quality checked with any adaptors and contamination removed using the GALAXY analysis package (Giardine et al., 2005). Sequences were then assembled into contigs using the OASES sequence assembly software (Schultz et al., 2012). OASES Kmer lengths of between 49 and 59 were evaluated to determine the optimal contig size. Contig ID was determined using a stand-alone BLASTx search against the Ensembl zebrafish protein database (version Zv8.59, E-value < $1e^{-10}$) and

contigs that could not be assigned to zebrafish transcripts, splice variants or non-conserved regions of known proteins were eliminated from further analysis. The zebrafish proteome was chosen for identification of contigs as despite the fact that databases for species more closely related to barramundi are available (i.e. *Takifugu rubripes*, *Tetraodon nigrviridis*), they are not as thoroughly annotated and did not return as high a number of BLASTx matches to known proteins. Sequence reads were then mapped to annotated contigs using Novacraft software (Li et al., 2009) with count data recorded for each annotated gene within each sample pool of interest.

5.2.4 Statistics and transcriptome analysis

Weight differences between northern and southern barramundi reared at 36 °C, 28 °C and 22 °C were statistically compared by means of ANOVA. Homogeneity of variance was confirmed using a Levene's test and differences of $p < 0.05$ between time points were considered significant. All ANOVA testing was performed using SPSS v 16.0 (SPSS, 2006). To detect differentially expressed genes between all four experimental comparisons (N^{22} vs. N^{36}, N^{22} vs. S^{22}, N^{36} vs. S^{36} and S^{22} vs. S^{36}) the edgeR package (Robinson et al., 2010) was used in conjunction with R software and customized script commands. Program estimated method of dispersion was generated and applied to the data with a false discovery rate (FDR) cutoff of ≤ 0.05. Gene ontology analysis was then performed upon contigs identified as differentially expressed using the goseq R Bioconductor package (Young et al., 2010) to retrieve information relating to cellular components, biological processes and molecular functions.

5.3 Results

5.3.1 Barramundi (Lates calcarifer) population growth

Weight data was recorded for both southern and northern barramundi populations reared for ~3.5 months (106 days) at 22 °C, 28 °C and 36 °C as a measure of growth and to compare the performance of each population at different temperatures (ANOVA; $F_{5, 54} = 33.857$) . At a rearing temperature of 22 °C southern barramundi showed significantly higher growth after 106 days than northern barramundi ($p < 0.001$) (Table 5.1.). As expected, at the control rearing temperature of 28 °C there was no significant growth differences between southern and northern barramundi and there were also no recorded growth differences in the final weights of both southern and northern barramundi grown at 36 °C (Table 5.1.). Within populations, southern barramundi showed significantly higher end weights at 28 °C than at either 22 °C or 36 °C ($p < 0.001$ for both comparisons), similarly northern barramundi also showed significantly better growth at 28 °C than at either 22 °C or 36 °C ($p < 0.001$ for both comparisons). In addition to

this, northern barramundi showed a preference for warmer water with significantly better end weight when reared at 36 °C compared with 22 °C ($p < 0.01$). However, there was no difference in the weight of southern barramundi grown at either 36 °C or 22 °C (Table 5.1.).

Table 5.1. Weight data of both northern and southern populations of barramundi grown at 22 °C, 28 °C and 36 °C water temperatures. Weight is given as the mean of each group in g ± SE.

	22 °C	28 °C	36 °C
Northern barramundi	90.0 ± 7.0 g	233.2 ± 13.0 g	124.00 ± 7.5 g
Southern barramundi	145.9 ± 11.1 g	237.2 ± 11.4 g	138.0 ± 7.0 g

5.3.2 Contig assembly

Following removal of contaminated or poor quality sequences, a total of 133,357,102 pair-end reads (average quality score of 31) were available for contig assembly and mapping. After contig construction using OASES, a minimum contig size threshold of ≥ 300 bp was chosen (44,361 contigs with a maximum size of 62440 bp and a N50 of 1048 bp) for sequence mapping and annotations as this cutoff captured the majority of unique contigs whilst minimizing poor or low informative assemblies. Putative gene identification of all retained contigs was performed using BLASTx and the complete zebrafish sequence/protein database which identified ~22,310 significant hits. Since contig length is generally shorter than the corresponding full cDNA, multiple contigs were found to map to the same gene. In this case the count data for all contigs returning the same BLAST hit were collapsed and summed to give a final result of 9019 unique annotated contigs with count data.

5.3.3 Gene ontology analysis

One thousand five hundred and twenty three differentially expressed genes were detected between all four experimental comparisons using edgeR and an FDR cutoff of $p \leq 0.05$ (See Appendix). Seven hundred and twelve significantly differentially expressed genes were found between N^{36} and S^{36}, of these, 82 had higher levels of expression in N^{36} and 630 had higher expression levels in S^{36} demonstrating large differences between the responses to high temperature between the two populations (Fig. 5.2). The second largest number of differentially expressed genes was found in a comparison between N^{22} and N^{36} where a total of 521 genes were found to be differentially expressed. From these differentially expressed genes, eight had higher levels of expression in N^{36} and 513 had higher expression levels in N^{22} indicating the

necessity for large changes in gene expression in response to cooler temperatures amongst this population (Fig. 5.2). To reduce the complexity of analysing such a large number of individual differentially expressed (DE) genes GO analysis was performed to highlight biologically meaningful processes and pathways of significance using GOseq.

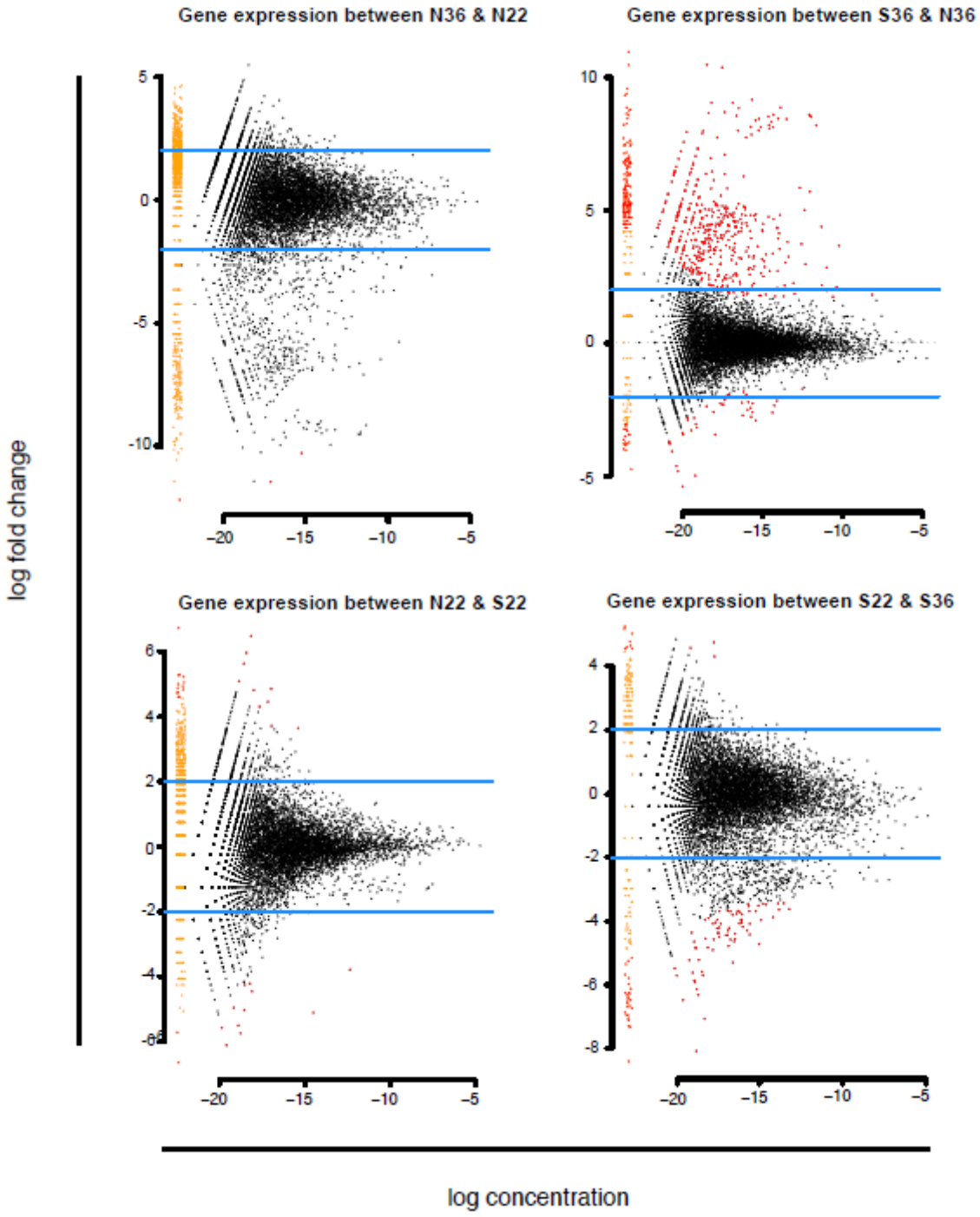

Fig. 5.2 Volcano plots displaying gene expression amongst comparisons of northern populations of barramundi (*Lates calcarifer*) reared at 36 °C and 22 °C (N^{36} and N^{22} respectively) and

southern populations of barramundi reared at 36 °C and 22 °C (S^{36} and S^{22} respectively). Volcano plots are plotted as log fold change as a function of log concentration. Solid blue horizontal lines represent the FDR cut off of 0.05.

Between N^{22} and N^{36}, 16 categories were found to be enriched by GO analysis and 26 categories were found to be enriched between N^{36} and S^{36}. These GO categories were largely representative of processes involving the regulation of peptidase activity ("endopeptidase inhibitor activity", "negative regulation of endopeptidase activity", "endopeptidase regulator activity", etc.), microtubule based processes and cell structural processes ("microtubule based movement", "cilium morphogenesis", "microtubule based process", etc.), as well as cell metabolism, membrane and cytoplasm activity ("intracellular membrane bound organelle", "cytoplasm", "cellular metabolic process", etc.) to name just a few (Table 5.2.). Between the remaining group combinations there were far fewer differentially expressed genes detected by comparison. Between N^{22} and S^{22}, 88 differentially expressed genes were detected. Of these, 32 had higher levels of expression in N^{22}, whilst the remaining 56 recorded higher expression levels in S^{22}. Finally, 202 genes were found to be differentially expressed between S^{36} and S^{22}, with 171 genes showing higher expression in S^{22} and 31 genes showing higher expression in S^{36}. Despite the detection of almost 300 significantly differentially expressed genes no GO categories showed enrichment in comparisons of either S^{22} and N^{22}, or S^{36} and S^{22} (Fig. 5.2).

Table 5.2. Enriched gene ontology (GO) categories. Columns shows GO categories significantly enriched in northern populations of barramundi reared at 36 °C (N^{36}) compared with northern populations of barramundi reared at 22 °C (N^{22}), and southern populations of barramundi reared at 36 °C (S^{36}) compared with northern populations of barramundi reared at 36 °C (N^{36}).

Enriched in $N^{36} \longrightarrow N^{22}$		Enriched in $S^{36} \longrightarrow N^{36}$	
"*GO:0007017*"	Microtubule based process	"*GO:0004866*"	Endopeptidase inhibitor activity
"*GO:0007018*"	microtubule based movement	"*GO:0010951*"	Negative regulation of endopeptidase activity
"*GO:0060271*"	Cilium morphogenesis	"*GO:0061135*"	Endopeptidase regulator activity
"*GO:0042384*"	cilium assembly	"*GO:0030414*"	Peptidase inhibitor activity
"*GO:0005737*"	cytoplasm	"*GO:0010466*"	Negative regulation of peptidase activity
"*GO:0016070*"	RNA metabolic process	"*GO:0061134*"	Peptidase regulator activity
"*GO:0043231*"	intracellular membrane bounded organelle	"*GO:0051346*"	negative regulation of hydrolase activity
"*GO:0043227*"	membrane bounded organelle	"*GO:0052548*"	regulation of endopeptidase activity
"*GO:0044425*"	Membrane part	"*GO:0052547*"	regulation of peptidase activity
"*GO:0016020*"	Membrane	"*GO:0060271*"	Cilium morphogenesis
"*GO:0009987*"	Cellular process	"*GO:0007017*"	microtubule-based activity
"*GO:0044464*"	Cell part	"*GO:0071844*"	Cellular component assembly at cellular level
"*GO:0005623*"	Cell	"*GO:0034622*"	cellular macromolecule complex assembly
"*GO:0005575*"	Cellular component	"*GO:0042384*"	cilium assembly
"*GO:0003674*"	Molecular Function	"*GO:0007018*"	microtubule-based movement
"*GO:0008150*"	Biological Process	"*GO:0044237*"	cellular metabolic process
		"*GO:0008152*"	metabolic process
		"*GO:0003824*"	catalytic activity
		"*GO:0009987*"	cellular process
		"*GO:0044444*"	cytoplasmic part
		"*GO:0005737*"	cytoplasm

	"*GO:0043231*"	intracellular membrane-bounded organelle
	"*GO:0043227*"	membrane-bounded organelle
	"*GO:0016020*"	membrane
	"*GO:0008150*"	biological process
	"*GO:0003674*"	molecular function

5.3.4 GO category gene expression

As "microtubule based process" (GO:0007017) and "endopeptidase inhibitor activity" (GO:0004866) where the most significantly enriched ontologies when comparing N^{36} with N^{22}, and S^{36} with N^{36} respectively, a breakdown of the genes comprising each ontology and an analysis of their likely role in the barramundi heat shock response was conducted. Three microtubule related genes, namely an α-tubulin like (*Tuba*) and 2 β-tubulin genes (*Tubb4b* and *Tubb2b*), (NM_001168287, NM_198809 and NM_213490 respectively) showed a -6.96, -6.52 and -5.76 fold expression difference respectively in N^{36} when compared to N^{22}. A fourth gene, the cytoplasmic motor protein constituent dynein (*DynII2a*, NM_200099), also showed a -6.05 fold gene expression difference in N^{36} compared with N^{22} (Fig. 5.3).

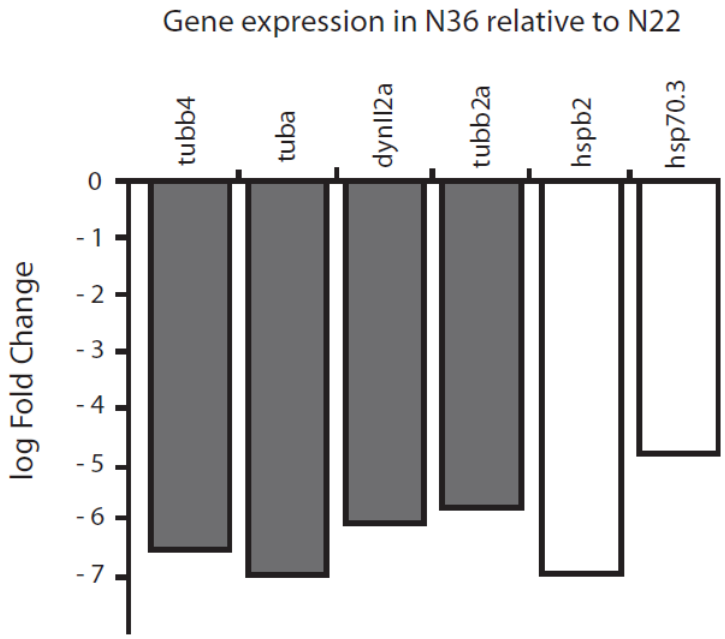

Fig. 5.3 Expression of genes from the gene ontology categories "Microtubule based process" (GO:0007017) and associated "Response to stress" (GO:0006950) in northern barramundi (*Lates calcarifer*) reared at 36 °C (N^{36}) relative to northern barramundi reared at 22 °C (N^{22}). Genes belonging to "Microtubule based process" are shaded in grey and include *Tubb4* (NM_198809), *Tuba* (NM_001168287), *DynII2a* (NM_200099) and *Tubb2a* (NM_213490). Genes belonging to "Response to stress" are shaded in white and include *Hspb2* (NM_001017744.1) and *Hsp70.3* (XP_003198158.1).

From "endopeptidase inhibitor activity", compliment component three (*C3 (9 of 9)*, *C3 (8 of 9)* and *C3 (2 of 9)*) (NM_001100020, NM_001100013 and XM_002660578 respectively) related

genes showed a decrease in expression when comparing S^{36} with N^{36} with a fold change of -1.44, -3.27 and -2.58 respectively (Fig. 5.4). Along with the genes from "microtubule based process" and "endopeptidase inhibitor activity", significantly differentially expressed genes belonging to the "response to stress" (GO:0006950) category were also analysed for the comparison of N^{36} with N^{22} and S^{36} with N^{36}. These genes were chosen for analysis despite no significant overall enrichment of the "response to stress" GO category as their response to heat stress in a wide range of organisms is well documented. Three out of the four 'response to stress genes' analysed were members of the heat shock protein family and consisted of *Hspb2* (heat shock protein, alpha crystalline related, b2), *Hsp90a.2* (heat shock protein 90, alpha (cystolic) class A member) and *Hsp70.3* (heat shock 70.3 kDa, protein-like), whilst the fourth gene from the 'response to stress' category was identified as *Pcna* (proliferating cell nuclear antigen). *Hspb2* (NM_001017744.1), *Hsp90a.2* (NP_001038538.1) and *Pcna* (NP_571479.1) were found to have a 5.12, 2.63 and 1.8 fold difference respectively in S^{36} compared with N^{36} barramundi. *Hspb2* and *Hsp70.3* (XP_003198158.1) were found to have a -6.93 and -4.81 fold expression difference in N^{36} compared with N^{22}, whilst *Hsp70.3* was also shown to have a -3.78 fold expression difference in S^{22} compared to N^{22} (FDR $p<0.0001$ in all seven genes).

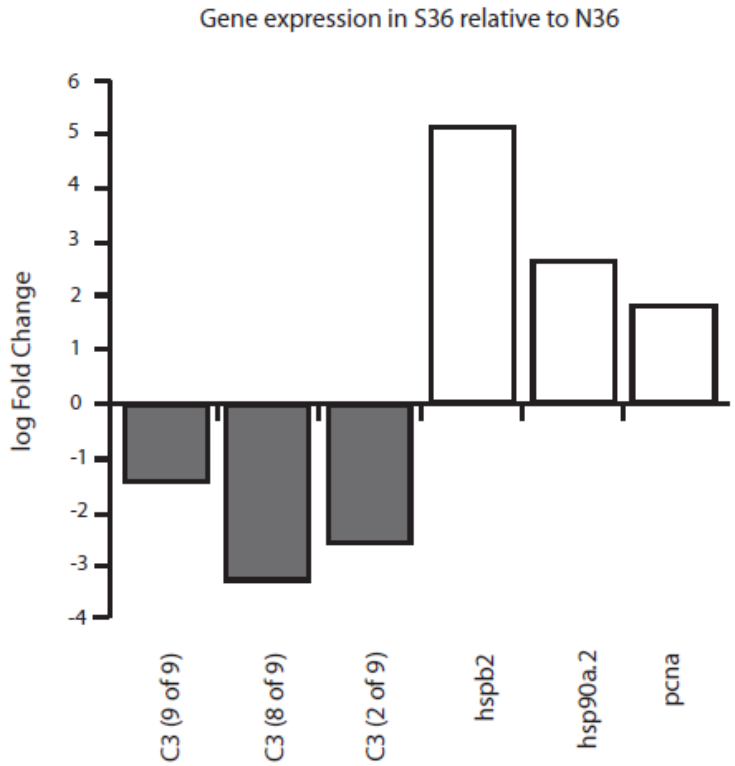

Fig. 5.4 Expression of genes from the gene ontology categories "Endopeptidase inhibitor activity" (GO:0004866) and associated "Response to stress" (GO:0006950) in southern barramundi (*Lates calcarifer*) reared at 36 °C (S^{36}) relative to northern barramundi reared at 36

°C (N^{36}). Genes belonging to "Endopeptidase inhibitor activity" are shaded in grey and include compliment component 3-like genes *C3 (9 of 9)* (NM_001100020), *C3 (8 of 9)* (NM_001100013) and *C3 (2 of 9)* (XM_002660578). Genes belonging to "Response to stress" are shaded in white and include *Hspb2* (NM_001017744.1), *Hsp90a.2* (NP_001038538.1) and *Pcna* (NP_571479.1).

5.4 Discussion

In the current study, mechanisms of local adaptation were examined by comparing the growth and underlying transcriptome response of distinct populations of barramundi reared at different temperatures. Gene ontology (GO) analysis was used to cluster large groups of related genes into broad functional groups for easy identification of important biological processes, and the expression of individual genes comprising "microtubule based process" and "endopeptidase inhibitor activity" ontologies were examined in conjunction with significantly differentially expressed stress genes from the "response to stress" GO category to better understand the transcriptome response of barramundi populations to temperature.

5.4.1 Barramundi growth at different temperatures

At a temperature of 22 °C, barramundi from a cooler, southern latitude showed far superior end weight (g) over a 3.5 month growth period than did barramundi from warmer, northern latitudes (145.90 ± 11.14 g and 89.99 ± 6.98 g (mean ± SE, $p < 0.001$) respectively), demonstrating that southern barramundi have adapted to grow better at the cooler temperatures encountered within their local environment. Like barramundi, adaptation to environment has occurred in other species where populations are distributed over clinal variations in temperature. Perhaps one of the most studied examples is that of the common killifish (*Fundulus heteroclitis*), where a steep thermal gradient over the species' large distribution range has resulted in the local adaptation of populations to environment both at the phenotypic and genetic level (Fangue et al., 2006; Schulte, 2007). Such changes promote better physiological performance and fitness at those temperatures most commonly encountered by the organism and thus it seems that the cooler average yearly temperatures encountered by barramundi at southern latitudes have prompted adaptation allowing for better growth in cooler waters.

Conversely, at 36 °C there were no significant growth differences between northern and southern barramundi, indicating that barramundi from lower latitudes do not seem to possess any growth advantages over their southern relatives at warmer temperatures. This seems contrary to popular theories of local adaptation that suggest a 'trade off' scenario in

performance characteristics whereby improved performance at one extreme results in a decrease in performance at the other extreme (Angilletta et al., 2002). In this scenario, barramundi from lower latitudes should perform best in warm water, but poorest in cool water and vice versa for barramundi from southern latitudes. Experimental evolution in *E. coli* was used to test for such tradeoffs, specifically a loss of fitness at high temperature coinciding with an evolutionary adaptation to cold temperature. While the majority of cultures in these experiments did show some evidence of tradeoffs, one third of cultures showed no reduction in fitness at high temperatures at all despite significant adaptation to cold (Bennett et al., 1992). This demonstrates that whilst tradeoffs in fitness may be common they are by no means universal (Portner et al., 2006). Furthermore, the evolution of the current population structure of Australian barramundi is only relatively recent. Southern populations of barramundi are believed to have been colonized by mid north-eastern populations where environmental temperatures are much closer to those experienced by barramundi from northern latitudes (Keenan, 1994). It is therefore possible that barramundi from southern latitudes have at this stage retained some tolerance of hot water temperatures owing to the environmental conditions from which they historically originate. However, this does not imply that southern populations of barramundi are best suited to all environmental conditions. The intensive culture of barramundi occasionally exposes individuals to temperatures reaching the upper thermal tolerance limit for this species (Katersky & Carter, 2005) and it has been previously demonstrated that under such conditions northern populations of barramundi have significantly higher upper thermal tolerance limits than southern populations of barramundi and would therefore encounter fewer mortalities during these temperature 'spikes'. Newton et al., (2010) shows that in response to an acute heat stress (exposure to 40 °C), barramundi from northern populations could survive for significantly longer before losing swimming equilibrium than barramundi from southern populations as average annual temperatures differ significantly for these populations at northern and southern latitudes.

5.4.2 GO category response

The transcriptomes of northern and southern barramundi were examined to identify the major biological features underpinning mechanisms of local adaptation to temperature. Gene ontology (GO) analysis revealed 42 unique categories amongst the comparison of populations across both hot and cool rearing temperatures. These 42 categories could be broadly grouped into 'parent' classes based upon their relatedness to common biological or molecular processes. The largest of these categories described processes involved in the regulation of peptidase activity such as 'endopeptidase inhibitor activity' (GO:0004866), 'negative regulation of endopeptidase activity' (GO:0010951), 'peptidase inhibitor activity' (GO:0030414), 'negative regulation of hydrolase

activity' (GO:0051346) and 'regulation of peptidase activity' (GO:0052547). Other significant 'parent' classes described processes involved in microtubule based processes and cell structure such as 'microtubule based process' (GO:0007017), 'microtubule based movement' (GO:0007018) and 'cilium assembly' (GO:0042384). A third 'parent' class contained a broad range of processes related to cell metabolism, membrane and cytoplasmic activity such as 'cellular metabolic process' (GO:0044237), 'cytoplasm' (GO:0005737), 'intracellular membrane bounded organelle' (GO:0043231), 'cellular process' (GO:0009987) and 'RNA metabolic process' (GO:0016070). Ontologies pertaining to the regulation of peptidase activity were enriched almost exclusively when comparing southern barramundi reared at 36 °C with northern barramundi reared at 36 °C and, therefore, demonstrate population based differences in the response to temperature. A large number of ontologies relating to metabolic, membrane and cytoplasmic processes were enriched when comparing southern barramundi with northern barramundi reared at 36 °C, but also when comparing northern barramundi reared at 36 °C with northern barramundi reared at 22 °C, whilst microtubule based and cell structural process were enriched in a comparison of northern barramundi reared at both 36 °C and 22 °C only. These ontologies most likely represent a more generalized response to temperature common to this species and independent of the origin of the population. The aforementioned GO categories are of particular interest as they identify some of the major areas of variation in response to temperature between the two barramundi populations, and it is likely that the variation in gene expression within these gene categories contributes significantly to the variation seen at the phenotypic level. In addition to this, GO profiling in Australian populations of barramundi has provided a focal point for further gene expression analyses by prioritizing biological processes and related genes specifically involved in the process of local adaptation to temperature.

5.4.3 Gene expression patterns

A breakdown of gene expression from the GO categories, "microtubule based process" and "endopeptidase inhibitor activity" was examined as these categories ranked as the most over-represented amongst a comparison of northern barramundi reared at 36 °C with northern barramundi reared at 22 °C, and southern barramundi reared at 36 °C with northern barramundi reared at 36 °C, respectively. The comprising genes were examined alongside differentially expressed genes from the "response to stress" GO category (despite no enrichment of this category amongst any population comparison) as their role in the heat stress response has been well documented in many fish species (Feidantsis et al., 2009; Hermesz et al., 2001; Manchado et al., 2008), and these genes are useful in obtaining a more meaningful understanding of the temperature response of barramundi.

Four genes from "microtubule based process" were shown to have significantly lower gene expression in N^{36} when compared to N^{22}. Three of these genes were tubulin genes, specifically tubulin beta 4b (*Tubb4b*), tubulin beta 2b (*Tubb2b*), and an uncharacterized tubulin-like gene similar to an alpha tubulin (*Tuba*), which function within the cell as major structural molecules in the makeup of microtubules. When reared at 22 °C, northern barramundi demonstrated a significantly higher expression, by comparison, of *Tubb4b*, *Tubb2b* and *Tuba* genes than northern barramundi reared at 36 °C. Similarly, the motor protein dynein (*DynII2a*) was also much lower in N^{36} barramundi than in N^{22}. The expression of these related genes suggests that in response to rearing at 22 °C, extensive remodelling of the cytoskeletal elements is necessary towards the adaptation of barramundi to cooler conditions, or that lower temperatures are damaging to these molecules and that new cytoskeletal proteins are required to replace them (Buckley et al., 2006). Osmotic stress in cells is known to induce remodelling of the cytoskeleton in order to modify cell volume and cytoskeletal proteins have previously been shown to be regulated in teleosts in response to temperature stress (Ju et al., 2002; Podrabsky & Somero, 2004; Sarmiento et al., 2000). Both of the above mentioned theories are credited by the expression of the "response to stress" genes, namely heat shock protein alpha crystalline related b2 (*Hspb2*) and heat shock 70.3 kDa protein like (*Hsp70.3*), which were both shown to exhibit lower expression in N^{36} barramundi compared with N^{22} (Fig. 5.3). Small heat shock proteins (such as *Hspb2*) are known to play important roles in the prevention of diseased states and in promoting resistance to environmental stressors. In *Danio rerio*, small heat shock proteins have been shown to express during embryonic development and in response to mild heat shocks (Elicker & Hutson, 2007). Small heat shock proteins have also been thought to protect cytoskeletal proteins in the muscle (Nakagawa et al., 2001) whilst the larger *Hsp70.3* is a known responder to temperature stress with a particular focus on molecular chaperoning (Buckley et al., 2006). The expression pattern of both heat shock proteins (*Hsps*) fits with the proposed theory that an increase in microtubule genes (*Tubb4b*, *Tubb2b* and *Tuba*) and the motor protein *DynnII2a*, demonstrates an adaptive response in northern barramundi towards coping with cooler temperatures through some form of cytoskeletal remodelling.

Through an analysis of genes from the "endopeptidase inhibitor activity" GO category, 3 complement component genes; complement component 3-like isoforms 1 precursor (*C3 9 of 9*), complement component 3-like precursor (*C3 8 of 9*) and predicted compliment C3 (*C3 2 of 9*), all showed a significant decrease in expression within southern barramundi reared at 36 °C in comparison to northern barramundi reared at 36 °C. In fish, the complement system is one of the main immune responses and causes lysis of target cells and the activation of phagocytosis (Boshra et al., 2006; Claire et al., 2002; Tort et al., 2004). The depression of all 3 *C3* related genes is suggestive of an immune suppression in cool adapted southern fish exposed to warmer

rearing temperatures in comparison to warm adapted northern fish. Stress-induced immunosuppression via a reduction in C3 has previously been shown in gilthead sea bream (*Sparus aurata*) following chasing stress whilst temperature was shown to have affected the lytic activity of compliment in rainbow trout (*Oncorhynchus mykiss*) (Nikoskelainen et al., 2004; Sunyer et al., 1995). Futhermore, 3 "response to stress" genes all showed an increase in expression in southern barramundi compared with northern barramundi reared at 36 °C, lending further support to an occurrence of heightened stress in southern barramundi resulting in a comparative decrease in immune efficacy (Fig. 5.4). *Hspb2* was again shown to be significantly differentially expressed, along with heat shock protein 90 alpha (cystolic) class A member (*Hsp90a.2*) and proliferating cell nuclear antigen (*Pcna*). The role of *Hsp90a.2* in protecting the cell during heat stress has been well documented and *Pcna* is known to play a crucial role in nucleic acid metabolism and has been shown to be involved in DNA repair as well as transcription, cell cycle regulation and hence growth (Feidantsis et al., 2009; Hermesz et al., 2001; Kelman, 1997; Manchado et al., 2008). The expression of these genes indicates that in southern barramundi reared at warmer temperatures an increase in perceived stress is accompanied by an increase in stress protein gene expression and that this incidence of stress likely results in the suppression of the compliment component of the innate immune system in barramundi.

The expression of genes from "microtubule based process" and "endopeptidase inhibitor activity" GO categories with supporting information from members of the "response to stress" GO category provides a more holistic picture of the phenotypic growth response of divergent barramundi populations to extremes in temperature. As many studies have demonstrated, the adaptive response of organisms, particularly that of fishes, is varied and not always consistent with what is predicted. Awareness of the underlying genetic mechanisms giving rise to the resulting phenotype would undoubtedly improve our knowledge of the nature of environmental adaptation and the various methods which it employs.

In the current study the growth of two genetically distinct populations of barramundi was compared at different temperatures and the major underlying genetic components of their growth response examined. Results show that southern populations of barramundi from a cool environment grow significantly better at cool water temperatures than northern populations of barramundi from a warmer environment, but that the reverse was not true of barramundi grown at warm temperatures. The underlying genetics of the response of these barramundi populations to temperature reveals significant differences in the regulation of peptidase activity, namely compliment component 3 genes, and cytoskeletal tubulin genes associated with microtubule based process as indicated by the enrichment of significant gene ontologies. The expression of

heat shock and stress related proteins provide further insight into the adaptation of barramundi to extremes in rearing temperature.

Chapter 6.0 General Discussion

6.1 Discussion

This research investigated temperature tolerance in Australian populations of barramundi (*Lates calcarifer*) to determine if local adaptation to environment had occurred within this species. In doing this, the underlying genetics associated with the adaptive response of barramundi populations to different environmental temperatures was examined and correlated with phenotypic growth data by measuring the expression of key genes at various stages of temperature exposure and by profiling the transcriptome of temperature challenged barramundi.

Chapter 2, (Newton et al., 2010), firstly establishes that genetically distinct barramundi populations from either end of the species Australian distribution exhibit significantly different phenotypic responses, as per measurements of upper thermal tolerance (UTT), when exposed to an acute heat shock. Consistent with the geography of this species, northern warm-adapted barramundi were able to withstand exposure to a 40 °C acute heat shock for a significantly longer period of time than were southern cool-adapted barramundi (as determined by recording the time until loss of swimming equilibrium – LOSE), indicating that populations of barramundi are likely to be adapted to local temperature regimes. These results challenge previous generalisations regarding the significance of genetic differentiation amongst established barramundi stocks. The relatively recent evolution of the current barramundi stock structure within Australia predicts that genetic differences between populations should be small (Keenan, 1994). Indeed, estimates of heterozygosity within Australian barramundi are less than half that of the average heterozygosity recorded within other marine and freshwater fish species (Keenan, 2000). Furthermore, the paucity of studies examining genotype-environment interactions within populations of barramundi to date have thus far fallen short of providing definitive evidence for growth differences between populations due to environmental factors such as temperature (see Rogers & Bloomfield, 1993). It was therefore assumed that genetic population differentiation was the result of neutral genetic drift as opposed to adaptive selection. However, by demonstrating differences in UTT between two genetically distinct barramundi populations from significantly different thermal environments, *Chapter 2* (Newton et al., 2010) provides solid evidence that barramundi do in fact display signs of local adaptation to temperature.

Currently, government policy allows for the translocation of fish stocks between genetically distinct populations for aquaculture purposes. However, when giving consideration to the optimal conditions needed to maximise productivity and growth, farms should now take into account the thermal history of fish stocks and question whether or not unique genetic populations are suited to local farming conditions. Within Australia, barramundi are now cultured to some degree in all mainland states and far outside their normal distribution range

(Katersky & Carter, 2005), where significant costs are often associated in maintaining optimal culture temperatures. It is therefore highly likely that improved growth performance and increased disease resistance could be achieved by selecting and stocking fish genetically suited to local farm conditions. With this consideration in mind, a correlation was established between the upper thermal tolerance limits of whole fish and the ratio of dead to live cells using a linear regression model in a novel and practical assay examining the thermal tolerance of 'free' caudal fin cells. The thermal tolerance of free caudal fin cells was in close agreement with whole fish measurements of UTT, where it was found that the dead to live cell ratio was on average 2.3 times greater in southern cool-adapted barramundi compared with northern warm-adapted barramundi. This non-destructive method of assessing the UTT of barramundi populations provides the initial framework required for the development of a method to assess temperature tolerance in valuable brood stock with the view towards implementing temperature tolerance traits into selective breeding programs. Further advances to this technique would be highly valuable to the aquaculture industry and should entail the following. Firstly, the UTT of all populations of Australian barramundi should be determined as stated above and correlated with the temperature tolerance of caudal fin cells. Secondly, efforts to further sensitise both whole body and caudal fin cell estimates of temperature tolerance would be beneficial by allowing within-population family differences, as well as within-family individual differences, in temperature tolerance to be determined. One way in which this could be achieved is by adapting the free caudal fin cell assay to a technique such as flow cytometry with an aim towards improving cell count numbers and generating a dead to live cell ratio with superior resolution. Furthermore, by exposing barramundi populations to temperatures nearing the species lower thermal tolerance limits and by examining the effect of cold temperatures of caudal fin cell viability, a more holistic view of temperature tolerance in this species can be achieved. A non-invasive and predictive tool that accurately estimates lower temperature tolerance has thus far not been developed, but would be invaluable for industry when combined with estimates of UTT to classify the thermal range of distinct populations and generate viable conditions for barramundi culture.

Following the premise established in *Chapter 2* (Newton et al., 2010) for the presence of an underlying genetic role in local adaptation, *Chapters 3* (Newton et al., 2012) *& 4* investigated the expression of key genes likely to be involved in the stress response to temperature of barramundi. In *Chapter 3* (Newton et al., 2012), a suite of genes from important biological pathways were selected as candidates for gene expression analysis based upon their functional role under temperature stress as reported within other fish species. Four heat shock protein (*Hsp*) family members, *Hsp70*, *Hsp90α*, *Hsc70* and *Hsp90β*, as well as the metabolic gene citrate synthase (*CiSy*) were isolated and characterised in barramundi for the first time as part of

this study. Sequence information for lactate dehydrogenase B (*LdhB*) and cytochrome c oxidase II (*CcoII*) from the metabolic pathway, as well as insulin-like growth factor 1 (*Igf1*) and myostatin 1 (*Mstn1*) from the growth pathway were previously available and thus obtained elsewhere (see De Santis et al., 2008; Drakenberg et al., 1997; Edmunds et al., 2009; Lin et al., 2006). Using RT-qPCR, the expression of all nine genes was analysed using a robust and reliable normalisation approach developed herein identifying two reference genes (*α-tub* and *rpl8*) ideally suited for use in temperature based experiments in fish. In response to an acute heat stress it was found that the expression of heat shock genes in barramundi was far above that reported following an acute heat stress in other fish species. For example, in *Sparus sarba*, an acute heat shock (+ 7 °C for 1 h) resulted in a comparatively modest 6.7 fold increase in *Hsp70* expression (Deane & Woo, 2005) when considering the 450 fold increase in *Hsp70* expression recorded for barramundi (+ 8 °C for 1 h). In *Solea senegalensis* a similar acute heat stress (+ 7.9 °C for 1 h) resulted in a 4.8 fold increase in inducible *Hsp90* gene expression (Manchado et al., 2008), but caused a 55 fold increase in the same gene in barramundi. As barramundi are an estuarine species it is likely that the magnitude of the *Hsp* response has evolved as an adaptation towards survival in such a variable environment where dramatic changes in environmental parameters can occur within hours on a daily basis. An absence of any large expression changes in genes of the metabolic and growth pathways further supports this hypothesis and highlights the hardy nature of this species. Furthermore, a similar response following an acute temperature stress is likely to be a common trait of estuarine species in general and further research into this group of fishes, especially tropical fish, may discover many more specialised adaptive mechanisms for coping with rapid environmental change.

Chapter 4 builds upon the results of *Chapter 3* (Newton et al., 2012) by examining the expression of heat shock, growth and metabolic genes in response to a long term stress in barramundi. In this chapter, the expression of these genes was analysed in response to both a long term hot and cold temperature stress and investigated within a northern, warm-adapted barramundi population and a southern, cool-adapted barramundi population, for which phenotypic growth data was also recorded. Surprisingly, it was found that warm adapted barramundi did not possess a growth advantage over cool-adapted barramundi when reared at a hot temperature. Under such circumstances it would be reasonable to expect that warm-adapted barramundi possess unique, specialised mechanisms tailored to improving growth at sustained high temperatures.

However, not only was growth the same between these two populations at a common high temperature, but the expression of *Hsp* was not significantly different between populations at any time point either. This can best be explained by examining the evolution of the current

population structure within Australia which suggests that north-eastern stocks, encompassing the Cairns and Burdekin regions, were ancestral to more southerly populations (Keenan, 1994). It seems that southern populations of barramundi (such as the current southern, cool-adapted population) where able to retain adaptive mechanisms allowing for sustainable growth during long term exposure to high rearing temperatures. However, it is important at this stage to mention that cool-adapted stocks are not interchangeable with warm-adapted stocks when reared under warm conditions. Although no difference in growth was evident over a long term period at hot rearing temperatures, *Chapter 2* (Newton et al., 2010) demonstrated that northern warm-adapted barramundi are much better adapted to surviving short, acute rises in temperature. Such temperature 'spikes' are a common occurrence in aquaculture farms due to abnormally hot days, or incidences of equipment failure, and if severe enough can result in mass mortalities amongst fish stock.

When both populations were reared at a cool temperature, cool-adapted barramundi were 62.2 % heavier over a long term rearing period than warm-adapted barramundi. This result was underpinned by significant differences between populations in the expression of *Hsp's*. When reared under low water temperatures, cool-adapted barramundi were able to express both *Hsp70* and *Hsp90α* significantly sooner than warm-adapted barramundi. In cool-adapted barramundi, *Hsp90α* was also expressed to a far greater degree than in warm-adapted barramundi. These results show the importance of the inducible heat shock pathway in the adaptation of barramundi (and possibly other tropical estuarine species) to cooler water temperatures. These results are significant in that they demonstrate a sustained role for heat shock protein expression in the adaptive response to a long term cold stress which to date has not been reported within the literature.

A small suppression of *CcoII* was evident in both barramundi populations reared at a hot temperature, however, results were not significantly different between populations. *CiSy* expression remained unchanged throughout the experiment. Results for the expression of cytochrome oxidases and other ATP producing genes following a rise in temperature is varied within the literature, with both increases and decreases in expression reported in diverse fish species (see Buckley et al., 2006; Hardewig et al., 1999; Lucassen et al., 2003; McClelland et al., 2006). These genes are commonly used to monitor the rate of respiration within muscle tissue in response to temperature challenge where it has been found that some species, particularly Arctic fish, demonstrate large changes in mitochondrial gene expression during cold exposure (Speers-Roesch & Ballantyne, 2005). However, due to the small reduction in *CcoII* expression and the lack of any significant differences in the expression of *CiSy* it can only be surmised that muscle respiration in a tropical estuarine fish such as barramundi is largely

unaffected by the temperatures employed within this study, or else these particular genes are more suited to monitoring gene expression in cold stenothermal species less likely to encounter wide variations in environmental parameters.

Of interest is the fact that both representatives of the growth pathway responded only in fish reared at a hot temperature. No differences in gene expression were recorded within either population when reared at control or cool temperatures, despite the presence of physical growth differences between these populations. The positive regulator of muscle growth, *Igf1*, displayed decreased gene expression in all fish reared under hot temperature conditions, whilst the negative regulator, *Mstn1*, showed a parallel increase in gene expression. Growth in barramundi reared under hot conditions was reduced when compared with barramundi reared under control conditions, suggesting that the suppression of growth at high temperatures is strongly linked with the expression of both *Igf1* and *Mstn1*. These results indicate that both *Igf1* and *Mstn1* are reliable indicators of growth performance in barramundi reared at hot temperature. However, the processes underpinning the regulation of growth at colder temperatures remain uncertain. In temperate fish species, both *Igf1* and *Mstn1* have at different times been shown to respond to cold temperatures (see Gabillard et al., 2003; Luckenbach et al., 2007; Weber & Bosworth, 2005), however, the current results suggest that in tropical species this may not be the case and further research should aim to correlate other genetic indicators of growth with cold stress in this group of fish.

Having established differences in the response to temperature between populations of barramundi through an analysis of candidate gene expression, *Chapter 5* explored the population response to temperature on a broader scale. This was achieved through an examination of the growth response of northern and southern barramundi populations to rearing at warm and cool temperatures, combined with an analysis of gene expression changes to whole pathways and biological processes using transcriptome profiling. From the *de novo* assembly and analysis of more than 44,000 barramundi contigs, 42 unique gene ontologies were revealed representing functional pathways underlying the population response to temperature within barramundi.

Between southern barramundi and northern barramundi reared at 36 °C, grouping of differentially expressed genes into ontological classes showed significant population differences in the activity of the immune system. A reduction in the expression of compliment component genes in the southern barramundi population was indicative of a temperature induced immune suppression upon exposure to 36 °C. This hypothesis is supported by a concordant increase in the expression of genes whose role it is to respond to cellular stress. At 36 °C, southern

barramundi populations were shown to have elevated levels of heat shock protein and proliferating cell nuclear antigen (*Pcna*), suggesting that the exposure of cool water adapted barramundi to rearing at 36 °C, whilst not detrimental to growth (as there were no significant differences in growth between populations), was enough to causes significant changes to immune function and induce a cellular stress response. The absence of growth differences between these two populations is explained by the evolution of the current population structure of barramundi. This indicates that southern populations originated from ancestral populations existing at latitudes similar to the northern population examined in the current study. It is therefore proposed that whilst adaptation to cooler temperatures has occurred within southern populations of barramundi, a degree of warm water tolerance has been retained such that the long term exposure of southern populations to the growth temperatures used in *Chapter 5* is not inhibitory to growth.

Further differences due to temperature were shown amongst northern populations of barramundi reared at 36 °C and at 22°C. In this instance, differences were uncovered as warm adapted northern barramundi exhibited a significantly reduced growth rate when reared at cooler temperatures. This was accompanied by changes to the expression of genes largely involved in microtubule based processes. The expression of tubulin genes and of a dynein motor protein was significantly higher in northern barramundi reared at 22 °C, suggesting necessary changes to cytoskeletal structure in response to low temperatures. Again, this finding was supported by the expression of heat shock proteins that were found to be comparatively higher in northern barramundi reared at 22 °C compared with northern barramundi reared at 36 °C. This shows a higher incidence of perceived stress amongst northern barramundi exposed to cooler rearing temperatures. These findings are lent further credence as the growth of northern barramundi at 22 °C was significantly lower than the growth of southern barramundi at 22 °C supporting the assumption that adaptation to temperature between northern and southern barramundi is, at least in part, underpinned by genetic differences.

Overall, the current analysis revealed 9019 unique contigs whose expression was correlated with the response to temperature in locally adapted populations of barramundi. The association of these contigs with the 42 unique GO categories identified in this study provides valuable information necessary for prioritising further analysis of the genes involved in the adaptive response of barramundi, and tropical estuarine fishes in general, to temperature. As the current technology advances, especially towards providing a greater annotation of transcriptomes from non-model organisms, a multitude of currently unidentified genes involved in the process of temperature adaptation can be mined from the existing data set with the aim of expanding the current knowledge base. *Chapter 5* was ultimately able to confirm that populations of

barramundi adapted to different thermal environments, respond to temperature challenge via the differential regulation of common molecular pathways. It was also shown that mechanisms of temperature compensation show a degree of temperature independence in that the response to high and low temperature extremes constitutes two separate responses.

6.2 General Conclusions

This study has explored thermal tolerance amongst Australian populations of barramundi (*Lates calcarifer*) and demonstrated for the first time that genetically distinct populations from opposite ends of the species distribution range have become locally adapted to environmental temperature. Northern warm-adapted barramundi populations possess a greater tolerance to short, but significant, instances of extreme temperature exposure compared to their southern, cool-adapted counterparts. In addition, southern, cool-adapted barramundi demonstrate better growth rates than northern, warm-adapted barramundi at cool water rearing temperatures and this growth difference corresponds with significant differences in the activation and expression of heat shock genes. However, at growth permissible temperatures slightly below those reaching the upper thermal tolerance limit for this species, no differences in growth were measured between warm and cool-adapted populations. Furthermore, whilst changes to the expression of *Igf1* and *Mstn1* appear to be linked to the universal suppression of growth at hot rearing temperatures, the control of growth in barramundi during exposure to cool water seems to be under the influence of alternate genes. The current research also explored the underlying transcriptome response associated with the adaptation of barramundi populations to different environmental temperatures. A number of gene ontologies were identified which were significantly enriched between barramundi populations reared at hot and cold temperatures corresponding to important biological processes such as the re-organisation of cytoskeletal elements, changes to the compliments component of the immune system and accompanying changes to the cellular stress response that will allow for the prioritising of genetic studies of adaptation if fish in the future.

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Appendix

Appendix 1. List of differentially expressed genes found between northern populations of barramundi reared at 22 °C compared with northern barramundi reared at 36 °C, northern barramundi reared at 22 °C compared with southern barramundi reared at 22 °C, northern barramundi reared at 36 °C compared with southern barramundi reared at 36 °C and southern barramundi reared at 36 °C compared with southern barramundi reared at 22 °C respectively. The Ensembl gene ID is given for each differentially expressed gene as well as the reported log concentration (logConc), log fold change (logFC), p value (PValue) and false discovery rate (FDR).

North22 vs North 36				
ENSMBL ID	**logConc**	**logFC**	**PValue**	**FDR**
ENSDARP00000058399	7.25832	-1.44E+08	2.11E-10	1.90E-06
ENSDARP00000102406	6.50117	-1.44E+08	9.91E-10	4.47E-06
ENSDARP00000097536	7.53978	-1.15E+01	3.51E-09	1.06E-05
ENSDARP00000109169	5.62612	-1.44E+08	5.91E-09	1.33E-05
ENSDARP00000107720	6.50499	-1.14E+01	9.56E-09	1.48E-05
ENSDARP00000110950	5.33943	-1.44E+08	1.06E-08	1.48E-05
ENSDARP00000110743	8.82865	-1.03E+01	1.23E-08	1.48E-05
ENSDARP00000053774	5.20016	-1.44E+08	1.41E-08	1.48E-05
ENSDARP00000104633	5.17558	-1.44E+08	1.48E-08	1.48E-05
ENSDARP00000106918	10.4213	-9.94E+00	1.91E-08	1.70E-05
ENSDARP00000110353	5.00937	-1.44E+08	2.08E-08	1.70E-05
ENSDARP00000109966	4.87336	-1.44E+08	2.74E-08	1.98E-05
ENSDARP00000124900	4.85284	-1.44E+08	2.86E-08	1.98E-05
ENSDARP00000019408	4.77863	-1.44E+08	3.32E-08	2.03E-05
ENSDARP00000109033	7.37591	-9.97E+00	3.38E-08	2.03E-05
ENSDARP00000025485	4.72318	-1.44E+08	3.72E-08	2.06E-05
ENSDARP00000110361	12.0062	-9.53E+00	4.09E-08	2.06E-05
ENSDARP00000053470	6.33844	-1.03E+01	4.13E-08	2.06E-05
ENSDARP00000111796	11.9081	-9.51E+00	4.33E-08	2.06E-05
ENSDARP00000104527	11.9952	-9.43E+00	5.05E-08	2.07E-05
ENSDARP00000108964	9.65407	-9.48E+00	5.05E-08	2.07E-05
ENSDARP00000090944	4.54278	-1.44E+08	5.37E-08	2.07E-05
ENSDARP00000113814	4.54278	-1.44E+08	5.37E-08	2.07E-05
ENSDARP00000110119	7.53142	-9.64E+00	5.54E-08	2.07E-05
ENSDARP00000107985	9.27902	-9.38E+00	6.30E-08	2.07E-05
ENSDARP00000106264	9.90132	-9.35E+00	6.36E-08	2.07E-05
ENSDARP00000106103	10.4054	-9.34E+00	6.36E-08	2.07E-05
ENSDARP00000111120	6.81317	-9.73E+00	6.43E-08	2.07E-05
ENSDARP00000022810	9.44331	-9.27E+00	7.80E-08	2.39E-05
ENSDARP00000107124	8.63812	-9.30E+00	7.96E-08	2.39E-05

ENSDARP00000109856	7.3364	-9.44E+00	8.27E-08	2.41E-05
ENSDARP00000107260	8.44484	-9.27E+00	8.72E-08	2.46E-05
ENSDARP00000110115	12.1903	-9.15E+00	9.00E-08	2.46E-05
ENSDARP00000117651	4.26071	-1.44E+08	9.51E-08	2.52E-05
ENSDARP00000002437	9.21143	-9.12E+00	1.06E-07	2.73E-05
ENSDARP00000107384	9.26499	-9.09E+00	1.13E-07	2.82E-05
ENSDARP00000105978	8.66533	-9.05E+00	1.28E-07	3.13E-05
ENSDARP00000086365	9.36974	-9.00E+00	1.34E-07	3.19E-05
ENSDARP00000109971	8.03723	-9.04E+00	1.43E-07	3.30E-05
ENSDARP00000105592	8.54083	-8.99E+00	1.46E-07	3.30E-05
ENSDARP00000063364	5.12654	-1.01E+01	1.61E-07	3.53E-05
ENSDARP00000070746	3.9098	-1.44E+08	1.93E-07	4.15E-05
ENSDARP00000118420	7.2076	-8.95E+00	2.04E-07	4.20E-05
ENSDARP00000007305	3.86952	-1.44E+08	2.10E-07	4.20E-05
ENSDARP00000029855	3.86952	-1.44E+08	2.10E-07	4.20E-05
ENSDARP00000116660	5.5151	-9.44E+00	2.23E-07	4.38E-05
ENSDARP00000111564	3.80691	-1.44E+08	2.38E-07	4.57E-05
ENSDARP00000101155	7.67734	-8.78E+00	2.50E-07	4.69E-05
ENSDARP00000043865	3.71898	-1.44E+08	2.84E-07	5.23E-05
ENSDARP00000108614	3.64932	-1.44E+08	3.27E-07	5.89E-05
ENSDARP00000099444	3.55089	-1.44E+08	3.98E-07	6.95E-05
ENSDARP00000111591	7.70263	-8.53E+00	4.03E-07	6.95E-05
ENSDARP00000088296	5.9138	-8.84E+00	4.09E-07	6.95E-05
ENSDARP00000088270	3.49904	-1.44E+08	4.42E-07	7.12E-05
ENSDARP00000089814	3.49904	-1.44E+08	4.42E-07	7.12E-05
ENSDARP00000113861	3.49904	-1.44E+08	4.42E-07	7.12E-05
ENSDARP00000098380	5.16171	-9.09E+00	4.61E-07	7.12E-05
ENSDARP00000094348	3.4724	-1.44E+08	4.66E-07	7.12E-05
ENSDARP00000101100	3.4724	-1.44E+08	4.66E-07	7.12E-05
ENSDARP00000076216	3.44526	-1.44E+08	4.92E-07	7.40E-05
ENSDARP00000016318	3.4176	-1.44E+08	5.20E-07	7.69E-05
ENSDARP00000118428	3.3894	-1.44E+08	5.50E-07	8.01E-05
ENSDARP00000068532	4.45248	-9.40E+00	6.36E-07	9.10E-05
ENSDARP00000104679	3.30135	-1.44E+08	6.56E-07	9.25E-05
ENSDARP00000067126	3.27076	-1.44E+08	6.98E-07	9.68E-05
ENSDARP00000109174	3.20757	-1.44E+08	7.91E-07	1.08E-04
ENSDARP00000094422	4.88686	-8.82E+00	8.09E-07	1.09E-04
ENSDARP00000115874	4.8355	-8.77E+00	8.99E-07	1.19E-04
ENSDARP00000103805	3.10729	-1.44E+08	9.66E-07	1.25E-04
ENSDARP00000108801	3.10729	-1.44E+08	9.66E-07	1.25E-04
ENSDARP00000086159	5.72439	-8.32E+00	1.01E-06	1.28E-04
ENSDARP00000065237	3.07226	-1.44E+08	1.04E-06	1.28E-04
ENSDARP00000118427	3.07226	-1.44E+08	1.04E-06	1.28E-04
ENSDARP00000111532	3.03636	-1.44E+08	1.11E-06	1.34E-04

ENSDARP00000114013	3.03636	-1.44E+08	1.11E-06	1.34E-04
ENSDARP00000110223	4.71562	-8.65E+00	1.15E-06	1.36E-04
ENSDARP00000108978	6.05294	-8.16E+00	1.16E-06	1.36E-04
ENSDARP00000117557	2.99954	-1.44E+08	1.20E-06	1.36E-04
ENSDARP00000121433	2.99954	-1.44E+08	1.20E-06	1.36E-04
ENSDARP00000087346	5.84962	-8.19E+00	1.21E-06	1.36E-04
ENSDARP00000105585	2.96176	-1.44E+08	1.29E-06	1.42E-04
ENSDARP00000109377	2.96176	-1.44E+08	1.29E-06	1.42E-04
ENSDARP00000054387	2.92297	-1.44E+08	1.39E-06	1.48E-04
ENSDARP00000085405	2.92297	-1.44E+08	1.39E-06	1.48E-04
ENSDARP00000099572	2.92297	-1.44E+08	1.39E-06	1.48E-04
ENSDARP00000116274	2.88311	-1.44E+08	1.51E-06	1.55E-04
ENSDARP00000121228	2.88311	-1.44E+08	1.51E-06	1.55E-04
ENSDARP00000036336	4.02742	-8.98E+00	1.51E-06	1.55E-04
ENSDARP00000113738	2.84212	-1.44E+08	1.64E-06	1.66E-04
ENSDARP00000117331	6.17089	-7.92E+00	1.73E-06	1.71E-04
ENSDARP00000068664	3.95228	-8.91E+00	1.76E-06	1.71E-04
ENSDARP00000021727	2.79994	-1.44E+08	1.78E-06	1.71E-04
ENSDARP00000105835	2.79994	-1.44E+08	1.78E-06	1.71E-04
ENSDARP00000119056	2.79994	-1.44E+08	1.78E-06	1.71E-04
ENSDARP00000071920	6.14581	-7.89E+00	1.82E-06	1.72E-04
ENSDARP00000104829	3.93287	-8.89E+00	1.83E-06	1.72E-04
ENSDARP00000071067	2.75649	-1.44E+08	1.94E-06	1.76E-04
ENSDARP00000075491	2.75649	-1.44E+08	1.94E-06	1.76E-04
ENSDARP00000113866	2.75649	-1.44E+08	1.94E-06	1.76E-04
ENSDARP00000013683	3.87301	-8.83E+00	2.06E-06	1.85E-04
ENSDARP00000078089	5.12222	-8.05E+00	2.08E-06	1.85E-04
ENSDARP00000096458	2.71169	-1.44E+08	2.12E-06	1.87E-04
ENSDARP00000049108	2.66547	-1.44E+08	2.32E-06	2.03E-04
ENSDARP00000017411	4.74002	-8.09E+00	2.43E-06	2.11E-04
ENSDARP00000059040	6.35175	-7.68E+00	2.54E-06	2.15E-04
ENSDARP00000110556	2.61773	-1.44E+08	2.55E-06	2.15E-04
ENSDARP00000119968	2.61773	-1.44E+08	2.55E-06	2.15E-04
ENSDARP00000070307	4.31177	-8.26E+00	2.62E-06	2.19E-04
ENSDARP00000125091	4.92992	-7.86E+00	3.08E-06	2.54E-04
ENSDARP00000108822	2.51724	-1.44E+08	3.10E-06	2.54E-04
ENSDARP00000111820	6.53515	-7.54E+00	3.19E-06	2.57E-04
ENSDARP00000113988	5.87268	-7.62E+00	3.20E-06	2.57E-04
ENSDARP00000111329	3.65343	-8.62E+00	3.22E-06	2.57E-04
ENSDARP00000090196	4.8902	-7.82E+00	3.34E-06	2.64E-04
ENSDARP00000054395	4.18621	-8.13E+00	3.38E-06	2.65E-04
ENSDARP00000109654	2.46426	-1.44E+08	3.44E-06	2.67E-04
ENSDARP00000025821	4.56188	-7.91E+00	3.50E-06	2.70E-04
ENSDARP00000033391	5.31703	-7.66E+00	3.61E-06	2.76E-04

ENSDARP00000021053	2.40927	-1.44E+08	3.83E-06	2.88E-04
ENSDARP00000103503	2.40927	-1.44E+08	3.83E-06	2.88E-04
ENSDARP00000108259	5.89876	-7.49E+00	3.99E-06	2.97E-04
ENSDARP00000117964	5.24011	-7.58E+00	4.23E-06	3.09E-04
ENSDARP00000016859	2.35212	-1.44E+08	4.28E-06	3.09E-04
ENSDARP00000041805	2.35212	-1.44E+08	4.28E-06	3.09E-04
ENSDARP00000125351	2.35212	-1.44E+08	4.28E-06	3.09E-04
ENSDARP00000124072	6.37887	-7.38E+00	4.40E-06	3.15E-04
ENSDARP00000010992	6.37528	-7.38E+00	4.43E-06	3.15E-04
ENSDARP00000105482	4.04868	-8.00E+00	4.47E-06	3.15E-04
ENSDARP00000106429	6.5135	-7.34E+00	4.70E-06	3.22E-04
ENSDARP00000029754	2.29263	-1.44E+08	4.80E-06	3.22E-04
ENSDARP00000046111	2.29263	-1.44E+08	4.80E-06	3.22E-04
ENSDARP00000076074	2.29263	-1.44E+08	4.80E-06	3.22E-04
ENSDARP00000116987	2.29263	-1.44E+08	4.80E-06	3.22E-04
ENSDARP00000104950	4.01215	-7.96E+00	4.82E-06	3.22E-04
ENSDARP00000120991	4.01215	-7.96E+00	4.82E-06	3.22E-04
ENSDARP00000108232	6.76936	-7.29E+00	4.99E-06	3.29E-04
ENSDARP00000017693	3.99354	-7.94E+00	5.00E-06	3.29E-04
ENSDARP00000106827	3.42248	-8.40E+00	5.12E-06	3.35E-04
ENSDARP00000086061	4.92173	-7.53E+00	5.24E-06	3.40E-04
ENSDARP00000034815	3.95557	-7.91E+00	5.40E-06	3.48E-04
ENSDARP00000065372	5.59231	-7.34E+00	5.70E-06	3.64E-04
ENSDARP00000106224	5.44131	-7.36E+00	5.74E-06	3.65E-04
ENSDARP00000118225	6.54045	-7.20E+00	6.05E-06	3.79E-04
ENSDARP00000091998	11.0107	-7.10E+00	6.10E-06	3.79E-04
ENSDARP00000088644	2.16579	-1.44E+08	6.14E-06	3.79E-04
ENSDARP00000111809	2.16579	-1.44E+08	6.14E-06	3.79E-04
ENSDARP00000110773	4.26857	-7.63E+00	6.37E-06	3.90E-04
ENSDARP00000071264	6.28304	-7.20E+00	6.40E-06	3.90E-04
ENSDARP00000052540	4.253	-7.61E+00	6.58E-06	3.98E-04
ENSDARP00000110583	6.80581	-7.13E+00	6.74E-06	4.05E-04
ENSDARP00000113090	2.09797	-1.44E+08	7.00E-06	4.15E-04
ENSDARP00000114168	2.09797	-1.44E+08	7.00E-06	4.15E-04
ENSDARP00000027071	5.17685	-7.29E+00	7.05E-06	4.16E-04
ENSDARP00000063033	3.81422	-7.77E+00	7.20E-06	4.19E-04
ENSDARP00000114169	3.81422	-7.77E+00	7.20E-06	4.19E-04
ENSDARP00000059417	3.21329	-8.20E+00	7.79E-06	4.47E-04
ENSDARP00000107795	5.43556	-7.19E+00	7.87E-06	4.47E-04
ENSDARP00000021453	2.02683	-1.44E+08	8.03E-06	4.47E-04
ENSDARP00000045970	2.02683	-1.44E+08	8.03E-06	4.47E-04
ENSDARP00000069088	2.02683	-1.44E+08	8.03E-06	4.47E-04
ENSDARP00000075712	2.02683	-1.44E+08	8.03E-06	4.47E-04
ENSDARP00000108906	2.02683	-1.44E+08	8.03E-06	4.47E-04

ENSDARP00000112972	3.74913	-7.71E+00	8.21E-06	4.54E-04
ENSDARP00000041381	3.72677	-7.69E+00	8.59E-06	4.72E-04
ENSDARP00000109536	4.10475	-7.47E+00	8.90E-06	4.86E-04
ENSDARP00000124390	3.70405	-7.66E+00	9.00E-06	4.89E-04
ENSDARP00000088159	5.93656	-7.04E+00	9.08E-06	4.90E-04
ENSDARP00000106523	1.95204	-1.44E+08	9.26E-06	4.90E-04
ENSDARP00000119943	1.95204	-1.44E+08	9.26E-06	4.90E-04
ENSDARP00000009320	7.00293	-6.96E+00	9.28E-06	4.90E-04
ENSDARP00000124531	4.38983	-7.33E+00	9.31E-06	4.90E-04
ENSDARP00000108836	4.6398	-7.25E+00	9.35E-06	4.90E-04
ENSDARP00000093124	7.79712	-6.92E+00	9.41E-06	4.91E-04
ENSDARP00000096034	9.0457	-6.89E+00	9.51E-06	4.93E-04
ENSDARP00000123948	7.06963	-6.93E+00	9.67E-06	4.98E-04
ENSDARP00000052062	7.23894	-6.90E+00	1.02E-05	5.22E-04
ENSDARP00000107533	5.5631	-7.02E+00	1.03E-05	5.22E-04
ENSDARP00000112810	6.58097	-6.90E+00	1.07E-05	5.37E-04
ENSDARP00000012411	1.87321	-1.44E+08	1.08E-05	5.37E-04
ENSDARP00000114392	1.87321	-1.44E+08	1.08E-05	5.37E-04
ENSDARP00000123640	1.87321	-1.44E+08	1.08E-05	5.37E-04
ENSDARP00000002893	3.04286	-8.03E+00	1.10E-05	5.43E-04
ENSDARP00000016806	5.4018	-7.00E+00	1.11E-05	5.48E-04
ENSDARP00000095092	3.58479	-7.55E+00	1.14E-05	5.53E-04
ENSDARP00000107713	3.58479	-7.55E+00	1.14E-05	5.53E-04
ENSDARP00000115789	3.58479	-7.55E+00	1.14E-05	5.53E-04
ENSDARP00000122502	7.48124	-6.83E+00	1.15E-05	5.53E-04
ENSDARP00000113979	9.99182	-6.79E+00	1.16E-05	5.55E-04
ENSDARP00000072678	7.7652	-6.81E+00	1.17E-05	5.58E-04
ENSDARP00000108512	5.0933	-7.02E+00	1.17E-05	5.58E-04
ENSDARP00000064864	5.22817	-6.98E+00	1.21E-05	5.67E-04
ENSDARP00000123096	6.34243	-6.86E+00	1.21E-05	5.67E-04
ENSDARP00000100078	4.25563	-7.20E+00	1.22E-05	5.72E-04
ENSDARP00000078928	3.93953	-7.30E+00	1.25E-05	5.75E-04
ENSDARP00000020894	5.78234	-6.89E+00	1.25E-05	5.75E-04
ENSDARP00000033381	1.78988	-1.44E+08	1.26E-05	5.75E-04
ENSDARP00000083878	1.78988	-1.44E+08	1.26E-05	5.75E-04
ENSDARP00000124313	1.78988	-1.44E+08	1.26E-05	5.75E-04
ENSDARP00000012437	4.48844	-7.10E+00	1.27E-05	5.78E-04
ENSDARP00000041589	5.19595	-6.95E+00	1.29E-05	5.78E-04
ENSDARP00000071629	6.02399	-6.85E+00	1.29E-05	5.78E-04
ENSDARP00000115180	3.91994	-7.28E+00	1.30E-05	5.78E-04
ENSDARP00000058628	4.22404	-7.16E+00	1.31E-05	5.80E-04
ENSDARP00000106507	5.65086	-6.87E+00	1.34E-05	5.92E-04
ENSDARP00000067578	5.90365	-6.82E+00	1.40E-05	6.17E-04
ENSDARP00000028542	3.48175	-7.45E+00	1.41E-05	6.17E-04

ENSDARP00000067199	3.45481	-7.42E+00	1.49E-05	6.47E-04
ENSDARP00000108845	1.70152	-1.44E+08	1.49E-05	6.47E-04
ENSDARP00000072394	4.96535	-6.89E+00	1.53E-05	6.59E-04
ENSDARP00000101978	3.42735	-7.40E+00	1.57E-05	6.74E-04
ENSDARP00000026811	5.3527	-6.81E+00	1.58E-05	6.74E-04
ENSDARP00000110408	10.5388	-6.63E+00	1.58E-05	6.74E-04
ENSDARP00000029276	3.81785	-7.19E+00	1.59E-05	6.75E-04
ENSDARP00000111333	5.65183	-6.76E+00	1.63E-05	6.89E-04
ENSDARP00000020257	6.48197	-6.69E+00	1.65E-05	6.92E-04
ENSDARP00000005284	3.39935	-7.37E+00	1.66E-05	6.92E-04
ENSDARP00000120683	5.2054	-6.80E+00	1.66E-05	6.92E-04
ENSDARP00000095098	6.47527	-6.68E+00	1.68E-05	6.93E-04
ENSDARP00000063356	5.19729	-6.79E+00	1.69E-05	6.97E-04
ENSDARP00000075344	5.52682	-6.74E+00	1.73E-05	7.09E-04
ENSDARP00000009077	1.60748	-1.44E+08	1.78E-05	7.20E-04
ENSDARP00000086805	1.60748	-1.44E+08	1.78E-05	7.20E-04
ENSDARP00000107046	1.60748	-1.44E+08	1.78E-05	7.20E-04
ENSDARP00000109090	6.81278	-6.63E+00	1.79E-05	7.22E-04
ENSDARP00000089064	6.00703	-6.67E+00	1.82E-05	7.31E-04
ENSDARP00000023131	4.51698	-6.86E+00	1.86E-05	7.44E-04
ENSDARP00000020804	6.70435	-6.61E+00	1.89E-05	7.50E-04
ENSDARP00000049419	2.76455	-7.77E+00	1.91E-05	7.53E-04
ENSDARP00000083250	4.85621	-6.78E+00	1.91E-05	7.53E-04
ENSDARP00000109190	5.75123	-6.66E+00	1.92E-05	7.53E-04
ENSDARP00000098032	4.49066	-6.84E+00	1.97E-05	7.62E-04
ENSDARP00000022077	8.24364	-6.54E+00	1.97E-05	7.62E-04
ENSDARP00000073091	4.27378	-6.89E+00	1.98E-05	7.62E-04
ENSDARP00000109129	3.31197	-7.28E+00	1.98E-05	7.62E-04
ENSDARP00000072746	4.01844	-6.96E+00	1.99E-05	7.62E-04
ENSDARP00000073753	5.64088	-6.65E+00	2.02E-05	7.72E-04
ENSDARP00000002175	9.05102	-6.52E+00	2.05E-05	7.81E-04
ENSDARP00000047470	1.507	-1.44E+08	2.15E-05	8.05E-04
ENSDARP00000047618	1.507	-1.44E+08	2.15E-05	8.05E-04
ENSDARP00000089375	1.507	-1.44E+08	2.15E-05	8.05E-04
ENSDARP00000109321	1.507	-1.44E+08	2.15E-05	8.05E-04
ENSDARP00000040327	3.6616	-7.03E+00	2.19E-05	8.15E-04
ENSDARP00000087875	11.7495	-6.47E+00	2.21E-05	8.19E-04
ENSDARP00000025885	6.33436	-6.54E+00	2.24E-05	8.26E-04
ENSDARP00000106777	6.48359	-6.53E+00	2.25E-05	8.26E-04
ENSDARP00000042477	5.29428	-6.63E+00	2.25E-05	8.26E-04
ENSDARP00000023459	3.63784	-7.01E+00	2.30E-05	8.38E-04
ENSDARP00000066212	7.129	-6.48E+00	2.38E-05	8.65E-04
ENSDARP00000002287	6.65283	-6.47E+00	2.50E-05	8.95E-04
ENSDARP00000016945	3.90351	-6.85E+00	2.51E-05	8.95E-04

ENSDARP00000078028	3.90351	-6.85E+00	2.51E-05	8.95E-04
ENSDARP00000110789	8.89156	-6.41E+00	2.53E-05	8.95E-04
ENSDARP00000090005	3.5891	-6.96E+00	2.53E-05	8.95E-04
ENSDARP00000026731	5.91681	-6.50E+00	2.54E-05	8.95E-04
ENSDARP00000108110	4.36594	-6.71E+00	2.54E-05	8.95E-04
ENSDARP00000099552	5.34043	-6.56E+00	2.54E-05	8.95E-04
ENSDARP00000091908	4.99684	-6.60E+00	2.56E-05	8.97E-04
ENSDARP00000041006	5.4274	-6.53E+00	2.60E-05	9.07E-04
ENSDARP00000024226	1.39916	-1.44E+08	2.63E-05	9.07E-04
ENSDARP00000083583	1.39916	-1.44E+08	2.63E-05	9.07E-04
ENSDARP00000110350	1.39916	-1.44E+08	2.63E-05	9.07E-04
ENSDARP00000087876	8.20381	-6.40E+00	2.63E-05	9.07E-04
ENSDARP00000105539	2.57769	-7.60E+00	2.76E-05	9.46E-04
ENSDARP00000106419	4.32185	-6.67E+00	2.78E-05	9.50E-04
ENSDARP00000002367	7.96863	-6.38E+00	2.80E-05	9.51E-04
ENSDARP00000062993	3.53866	-6.91E+00	2.81E-05	9.51E-04
ENSDARP00000108666	4.94931	-6.55E+00	2.82E-05	9.52E-04
ENSDARP00000114538	4.09324	-6.71E+00	2.86E-05	9.61E-04
ENSDARP00000026554	3.11959	-7.10E+00	2.92E-05	9.79E-04
ENSDARP00000025608	4.92986	-6.53E+00	2.93E-05	9.80E-04
ENSDARP00000049009	6.67487	-6.37E+00	3.05E-05	1.01E-03
ENSDARP00000111319	2.52696	-7.55E+00	3.05E-05	1.01E-03
ENSDARP00000024492	6.279	-6.38E+00	3.11E-05	1.03E-03
ENSDARP00000118073	3.08489	-7.07E+00	3.13E-05	1.03E-03
ENSDARP00000106411	1.2828	-1.44E+08	3.26E-05	1.06E-03
ENSDARP00000107225	1.2828	-1.44E+08	3.26E-05	1.06E-03
ENSDARP00000110533	1.2828	-1.44E+08	3.26E-05	1.06E-03
ENSDARP00000118733	1.2828	-1.44E+08	3.26E-05	1.06E-03
ENSDARP00000022287	5.85709	-6.37E+00	3.29E-05	1.06E-03
ENSDARP00000111379	5.31189	-6.42E+00	3.30E-05	1.06E-03
ENSDARP00000065036	8.84401	-6.28E+00	3.32E-05	1.07E-03
ENSDARP00000064008	3.04934	-7.03E+00	3.36E-05	1.07E-03
ENSDARP00000078740	3.04934	-7.03E+00	3.36E-05	1.07E-03
ENSDARP00000094438	2.47439	-7.50E+00	3.38E-05	1.07E-03
ENSDARP00000061114	5.96774	-6.35E+00	3.38E-05	1.07E-03
ENSDARP00000082574	5.77679	-6.36E+00	3.39E-05	1.07E-03
ENSDARP00000048816	6.58392	-6.32E+00	3.40E-05	1.07E-03
ENSDARP00000080636	4.84931	-6.45E+00	3.46E-05	1.08E-03
ENSDARP00000027138	6.00152	-6.32E+00	3.56E-05	1.11E-03
ENSDARP00000067164	3.01289	-7.00E+00	3.61E-05	1.12E-03
ENSDARP00000002618	5.24261	-6.35E+00	3.80E-05	1.18E-03
ENSDARP00000091646	4.4951	-6.42E+00	4.01E-05	1.24E-03
ENSDARP00000053859	7.85667	-6.20E+00	4.02E-05	1.24E-03
ENSDARP00000044558	1.15652	-1.44E+08	4.11E-05	1.26E-03

ENSDARP00000022929	5.74465	-6.26E+00	4.15E-05	1.27E-03
ENSDARP00000089247	2.36318	-7.40E+00	4.20E-05	1.28E-03
ENSDARP00000049044	3.64198	-6.60E+00	4.26E-05	1.29E-03
ENSDARP00000114945	3.64198	-6.60E+00	4.26E-05	1.29E-03
ENSDARP00000109807	4.97166	-6.30E+00	4.38E-05	1.32E-03
ENSDARP00000076839	4.58697	-6.34E+00	4.50E-05	1.35E-03
ENSDARP00000111575	2.8977	-6.89E+00	4.55E-05	1.36E-03
ENSDARP00000072603	4.07865	-6.43E+00	4.57E-05	1.36E-03
ENSDARP00000104920	7.05784	-6.15E+00	4.59E-05	1.36E-03
ENSDARP00000122171	6.23007	-6.18E+00	4.60E-05	1.36E-03
ENSDARP00000091840	6.12565	-6.17E+00	4.72E-05	1.39E-03
ENSDARP00000121109	2.30422	-7.34E+00	4.72E-05	1.39E-03
ENSDARP00000123131	2.30422	-7.34E+00	4.72E-05	1.39E-03
ENSDARP00000106068	3.56847	-6.52E+00	4.95E-05	1.45E-03
ENSDARP00000121183	6.4616	-6.11E+00	5.12E-05	1.49E-03
ENSDARP00000075677	5.70607	-6.15E+00	5.12E-05	1.49E-03
ENSDARP00000019643	1.01851	-1.44E+08	5.29E-05	1.51E-03
ENSDARP00000043945	1.01851	-1.44E+08	5.29E-05	1.51E-03
ENSDARP00000064106	1.01851	-1.44E+08	5.29E-05	1.51E-03
ENSDARP00000116318	1.01851	-1.44E+08	5.29E-05	1.51E-03
ENSDARP00000119942	1.01851	-1.44E+08	5.29E-05	1.51E-03
ENSDARP00000049686	2.81548	-6.81E+00	5.36E-05	1.53E-03
ENSDARP00000063949	4.18367	-6.31E+00	5.40E-05	1.54E-03
ENSDARP00000111236	4.86303	-6.20E+00	5.47E-05	1.55E-03
ENSDARP00000007503	6.02836	-6.07E+00	5.77E-05	1.63E-03
ENSDARP00000011797	3.94945	-6.30E+00	5.94E-05	1.67E-03
ENSDARP00000059357	6.55922	-6.03E+00	5.98E-05	1.68E-03
ENSDARP00000064110	6.76852	-6.02E+00	6.01E-05	1.68E-03
ENSDARP00000104643	6.14048	-6.04E+00	6.06E-05	1.69E-03
ENSDARP00000089057	6.00065	-6.04E+00	6.11E-05	1.70E-03
ENSDARP00000015065	5.78665	-6.05E+00	6.23E-05	1.72E-03
ENSDARP00000105987	5.41219	-6.07E+00	6.23E-05	1.72E-03
ENSDARP00000057697	2.72833	-6.73E+00	6.37E-05	1.76E-03
ENSDARP00000011150	4.26614	-6.20E+00	6.41E-05	1.76E-03
ENSDARP00000112726	4.97481	-6.08E+00	6.60E-05	1.81E-03
ENSDARP00000010852	5.80366	-6.01E+00	6.72E-05	1.84E-03
ENSDARP00000013307	5.62469	-6.01E+00	6.87E-05	1.87E-03
ENSDARP00000093342	0.86645	-1.44E+08	6.95E-05	1.88E-03
ENSDARP00000123458	0.86645	-1.44E+08	6.95E-05	1.88E-03
ENSDARP00000021244	2.68272	-6.69E+00	6.97E-05	1.88E-03
ENSDARP00000096709	8.70576	-5.90E+00	7.28E-05	1.96E-03
ENSDARP00000076347	4.34413	-6.10E+00	7.41E-05	1.99E-03
ENSDARP00000102848	7.02027	-5.89E+00	7.69E-05	2.06E-03
ENSDARP00000076298	4.66551	-6.00E+00	8.21E-05	2.19E-03

ENSDARP00000060257	4.95729	-5.96E+00	8.26E-05	2.20E-03
ENSDARP00000048886	4.41812	-6.02E+00	8.39E-05	2.22E-03
ENSDARP00000120135	4.6537	-5.99E+00	8.41E-05	2.22E-03
ENSDARP00000106234	6.90668	-5.85E+00	8.47E-05	2.23E-03
ENSDARP00000061820	3.52184	-6.15E+00	9.12E-05	2.40E-03
ENSDARP00000107191	2.53659	-6.55E+00	9.30E-05	2.40E-03
ENSDARP00000025514	0.6973	-1.44E+08	9.39E-05	2.40E-03
ENSDARP00000052351	0.6973	-1.44E+08	9.39E-05	2.40E-03
ENSDARP00000053010	0.6973	-1.44E+08	9.39E-05	2.40E-03
ENSDARP00000078846	0.6973	-1.44E+08	9.39E-05	2.40E-03
ENSDARP00000105148	0.6973	-1.44E+08	9.39E-05	2.40E-03
ENSDARP00000108456	0.6973	-1.44E+08	9.39E-05	2.40E-03
ENSDARP00000112281	0.6973	-1.44E+08	9.39E-05	2.40E-03
ENSDARP00000121981	0.6973	-1.44E+08	9.39E-05	2.40E-03
ENSDARP00000061148	12.5113	-5.76E+00	9.40E-05	2.40E-03
ENSDARP00000072564	2.48442	-6.50E+00	1.03E-04	2.63E-03
ENSDARP00000094171	5.36584	-5.81E+00	1.03E-04	2.63E-03
ENSDARP00000123852	6.41498	-5.76E+00	1.04E-04	2.65E-03
ENSDARP00000016376	6.11476	-5.76E+00	1.05E-04	2.65E-03
ENSDARP00000105396	3.85307	-5.98E+00	1.06E-04	2.67E-03
ENSDARP00000113163	4.30192	-5.90E+00	1.06E-04	2.67E-03
ENSDARP00000106016	4.15613	-5.91E+00	1.09E-04	2.72E-03
ENSDARP00000101346	3.16535	-6.13E+00	1.12E-04	2.79E-03
ENSDARP00000106586	4.59524	-5.81E+00	1.18E-04	2.91E-03
ENSDARP00000055324	2.82318	-6.23E+00	1.18E-04	2.91E-03
ENSDARP00000123185	2.82318	-6.23E+00	1.18E-04	2.91E-03
ENSDARP00000123631	2.82318	-6.23E+00	1.18E-04	2.91E-03
ENSDARP00000013189	3.13177	-6.10E+00	1.19E-04	2.94E-03
ENSDARP00000019915	7.27516	-5.66E+00	1.22E-04	3.00E-03
ENSDARP00000082978	1.80726	-6.89E+00	1.23E-04	3.02E-03
ENSDARP00000004830	3.93667	-5.87E+00	1.25E-04	3.07E-03
ENSDARP00000048622	3.09739	-6.07E+00	1.28E-04	3.11E-03
ENSDARP00000083450	3.09739	-6.07E+00	1.28E-04	3.11E-03
ENSDARP00000070898	2.78052	-6.19E+00	1.28E-04	3.11E-03
ENSDARP00000041624	0.50696	-1.44E+08	1.31E-04	3.15E-03
ENSDARP00000105950	0.50696	-1.44E+08	1.31E-04	3.15E-03
ENSDARP00000112216	0.50696	-1.44E+08	1.31E-04	3.15E-03
ENSDARP00000099646	4.97159	-5.71E+00	1.33E-04	3.19E-03
ENSDARP00000013778	4.71747	-5.72E+00	1.35E-04	3.24E-03
ENSDARP00000071819	5.35369	-5.67E+00	1.36E-04	3.24E-03
ENSDARP00000057576	5.82649	-5.64E+00	1.38E-04	3.29E-03
ENSDARP00000049102	5.16418	-5.61E+00	1.57E-04	3.72E-03
ENSDARP00000125019	5.75089	-5.56E+00	1.62E-04	3.82E-03
ENSDARP00000090958	2.64446	-6.06E+00	1.68E-04	3.97E-03

ENSDARP00000100904	2.9511	-5.93E+00	1.71E-04	4.03E-03
ENSDARP00000088110	5.45008	-5.54E+00	1.72E-04	4.05E-03
ENSDARP00000060687	4.26108	-5.60E+00	1.88E-04	4.40E-03
ENSDARP00000069198	0.28973	-1.44E+08	1.91E-04	4.41E-03
ENSDARP00000092403	0.28973	-1.44E+08	1.91E-04	4.41E-03
ENSDARP00000115068	0.28973	-1.44E+08	1.91E-04	4.41E-03
ENSDARP00000120786	0.28973	-1.44E+08	1.91E-04	4.41E-03
ENSDARP00000123477	0.28973	-1.44E+08	1.91E-04	4.41E-03
ENSDARP00000101110	4.11092	-5.57E+00	2.02E-04	4.67E-03
ENSDARP00000045815	6.45729	-5.41E+00	2.05E-04	4.71E-03
ENSDARP00000020968	1.52895	-6.64E+00	2.09E-04	4.78E-03
ENSDARP00000067188	1.52895	-6.64E+00	2.09E-04	4.78E-03
ENSDARP00000062934	6.13238	-5.35E+00	2.38E-04	5.43E-03
ENSDARP00000027914	3.03263	-5.68E+00	2.44E-04	5.56E-03
ENSDARP00000074127	3.24147	-5.61E+00	2.49E-04	5.66E-03
ENSDARP00000022576	4.41587	-5.42E+00	2.51E-04	5.69E-03
ENSDARP00000081101	3.86364	-5.47E+00	2.60E-04	5.88E-03
ENSDARP00000057097	5.55632	-5.32E+00	2.62E-04	5.92E-03
ENSDARP00000116378	3.84301	-5.45E+00	2.71E-04	6.10E-03
ENSDARP00000073778	9.03188	-5.23E+00	2.82E-04	6.33E-03
ENSDARP00000063605	0.03751	-1.44E+08	2.92E-04	6.46E-03
ENSDARP00000082791	0.03751	-1.44E+08	2.92E-04	6.46E-03
ENSDARP00000083241	0.03751	-1.44E+08	2.92E-04	6.46E-03
ENSDARP00000096230	0.03751	-1.44E+08	2.92E-04	6.46E-03
ENSDARP00000109876	0.03751	-1.44E+08	2.92E-04	6.46E-03
ENSDARP00000105398	4.981	-5.29E+00	2.92E-04	6.46E-03
ENSDARP00000112109	3.5094	-5.45E+00	2.99E-04	6.59E-03
ENSDARP00000096352	4.02506	-5.36E+00	3.04E-04	6.69E-03
ENSDARP00000073663	2.65324	-5.64E+00	3.10E-04	6.80E-03
ENSDARP00000092831	2.32705	-5.76E+00	3.14E-04	6.88E-03
ENSDARP00000065361	1.30927	-6.45E+00	3.15E-04	6.89E-03
ENSDARP00000091926	4.39005	-5.30E+00	3.16E-04	6.89E-03
ENSDARP00000097543	3.10976	-5.48E+00	3.25E-04	7.05E-03
ENSDARP00000060548	3.61464	-5.38E+00	3.27E-04	7.09E-03
ENSDARP00000123966	3.45612	-5.40E+00	3.33E-04	7.19E-03
ENSDARP00000114825	2.60524	-5.60E+00	3.41E-04	7.35E-03
ENSDARP00000096532	3.59011	-5.35E+00	3.44E-04	7.40E-03
ENSDARP00000052729	4.34673	-5.26E+00	3.45E-04	7.42E-03
ENSDARP00000077967	1.82436	-5.89E+00	3.72E-04	7.95E-03
ENSDARP00000103615	1.82436	-5.89E+00	3.72E-04	7.95E-03
ENSDARP00000120606	2.55561	-5.55E+00	3.76E-04	8.01E-03
ENSDARP00000092547	3.37235	-5.31E+00	3.94E-04	8.38E-03
ENSDARP00000027568	2.20383	-5.64E+00	4.00E-04	8.46E-03
ENSDARP00000107419	2.20383	-5.64E+00	4.00E-04	8.46E-03

ENSDARP00000113056	7.82746	-5.03E+00	4.19E-04	8.85E-03
ENSDARP00000094167	3.61884	-5.23E+00	4.28E-04	9.01E-03
ENSDARP00000030289	1.73856	-5.81E+00	4.38E-04	9.19E-03
ENSDARP00000077314	1.73856	-5.81E+00	4.38E-04	9.19E-03
ENSDARP00000011815	3.14981	-5.29E+00	4.39E-04	9.19E-03
ENSDARP00000109483	3.1159	-5.26E+00	4.70E-04	9.76E-03
ENSDARP00000014921	-0.2617	-1.44E+08	4.77E-04	9.76E-03
ENSDARP00000018505	-0.2617	-1.44E+08	4.77E-04	9.76E-03
ENSDARP00000046158	-0.2617	-1.44E+08	4.77E-04	9.76E-03
ENSDARP00000094626	-0.2617	-1.44E+08	4.77E-04	9.76E-03
ENSDARP00000103553	-0.2617	-1.44E+08	4.77E-04	9.76E-03
ENSDARP00000104946	-0.2617	-1.44E+08	4.77E-04	9.76E-03
ENSDARP00000112833	-0.2617	-1.44E+08	4.77E-04	9.76E-03
ENSDARP00000119608	-0.2617	-1.44E+08	4.77E-04	9.76E-03
ENSDARP00000125242	-0.2617	-1.44E+08	4.77E-04	9.76E-03
ENSDARP00000010986	5.89543	-4.99E+00	4.84E-04	9.87E-03
ENSDARP00000087122	3.78677	-5.12E+00	4.94E-04	1.01E-02
ENSDARP00000112232	3.40575	-5.17E+00	4.99E-04	1.01E-02
ENSDARP00000052079	1.64747	-5.73E+00	5.21E-04	1.05E-02
ENSDARP00000056187	1.64747	-5.73E+00	5.21E-04	1.05E-02
ENSDARP00000086813	1.64747	-5.73E+00	5.21E-04	1.05E-02
ENSDARP00000098603	1.64747	-5.73E+00	5.21E-04	1.05E-02
ENSDARP00000112654	1.64747	-5.73E+00	5.21E-04	1.05E-02
ENSDARP00000125522	3.37739	-5.14E+00	5.28E-04	1.06E-02
ENSDARP00000070502	4.44134	-5.02E+00	5.29E-04	1.06E-02
ENSDARP00000052861	6.73454	-4.91E+00	5.55E-04	1.11E-02
ENSDARP00000094053	4.01607	-5.02E+00	5.68E-04	1.13E-02
ENSDARP00000110074	1.9971	-5.45E+00	5.97E-04	1.19E-02
ENSDARP00000006457	3.67462	-5.01E+00	6.20E-04	1.23E-02
ENSDARP00000010413	8.70933	-4.82E+00	6.39E-04	1.26E-02
ENSDARP00000041710	1.9212	-5.38E+00	6.91E-04	1.36E-02
ENSDARP00000045762	2.71645	-5.10E+00	7.11E-04	1.40E-02
ENSDARP00000055263	2.89398	-5.04E+00	7.32E-04	1.44E-02
ENSDARP00000068317	3.79416	-4.90E+00	7.39E-04	1.45E-02
ENSDARP00000054063	9.1465	-4.74E+00	7.48E-04	1.46E-02
ENSDARP00000081302	3.05206	-5.00E+00	7.49E-04	1.46E-02
ENSDARP00000107314	2.48313	1.44E+08	7.55E-04	1.47E-02
ENSDARP00000090277	2.46117	-5.13E+00	7.59E-04	1.47E-02
ENSDARP00000124544	2.46117	-5.13E+00	7.59E-04	1.47E-02
ENSDARP00000090099	6.62231	-4.75E+00	7.61E-04	1.47E-02
ENSDARP00000075802	1.4466	-5.55E+00	7.62E-04	1.47E-02
ENSDARP00000013036	4.04054	-4.86E+00	7.63E-04	1.47E-02
ENSDARP00000104188	3.16169	-4.93E+00	8.15E-04	1.57E-02
ENSDARP00000107437	4.00387	-4.82E+00	8.22E-04	1.58E-02

ENSDARP00000058323	5.03645	-4.75E+00	8.30E-04	1.59E-02
ENSDARP00000066589	2.42104	1.44E+08	8.50E-04	1.62E-02
ENSDARP00000122818	3.90453	-4.81E+00	8.51E-04	1.62E-02
ENSDARP00000083463	-0.6262	-1.44E+08	8.53E-04	1.62E-02
ENSDARP00000009875	3.60716	-4.82E+00	8.82E-04	1.67E-02
ENSDARP00000063643	2.39357	1.44E+08	8.95E-04	1.70E-02
ENSDARP00000113136	2.57432	-4.96E+00	9.42E-04	1.78E-02
ENSDARP00000042085	1.75659	-5.23E+00	9.47E-04	1.78E-02
ENSDARP00000105058	2.08308	-5.10E+00	9.47E-04	1.78E-02
ENSDARP00000075688	2.90121	-4.85E+00	1.01E-03	1.90E-02
ENSDARP00000057261	5.21827	-4.63E+00	1.02E-03	1.91E-02
ENSDARP00000055618	8.09137	-4.59E+00	1.02E-03	1.91E-02
ENSDARP00000099032	2.01171	-5.03E+00	1.09E-03	2.03E-02
ENSDARP00000104315	7.64887	-4.51E+00	1.18E-03	2.20E-02
ENSDARP00000090943	2.8195	-4.77E+00	1.19E-03	2.22E-02
ENSDARP00000060133	2.18463	1.44E+08	1.33E-03	2.46E-02
ENSDARP00000122457	4.17884	-4.54E+00	1.34E-03	2.48E-02
ENSDARP00000102816	6.30757	-4.45E+00	1.38E-03	2.55E-02
ENSDARP00000122318	3.02892	-4.64E+00	1.40E-03	2.58E-02
ENSDARP00000093426	5.75846	-4.43E+00	1.47E-03	2.70E-02
ENSDARP00000114199	3.31173	5.49E+00	1.51E-03	2.78E-02
ENSDARP00000078009	1.46951	-4.96E+00	1.63E-03	2.99E-02
ENSDARP00000069565	6.11802	-4.36E+00	1.66E-03	3.04E-02
ENSDARP00000008048	6.89534	-4.33E+00	1.70E-03	3.10E-02
ENSDARP00000051529	1.77432	-4.81E+00	1.72E-03	3.10E-02
ENSDARP00000059242	1.77432	-4.81E+00	1.72E-03	3.10E-02
ENSDARP00000014024	-1.0851	-1.44E+08	1.73E-03	3.10E-02
ENSDARP00000066603	-1.0851	-1.44E+08	1.73E-03	3.10E-02
ENSDARP00000081883	-1.0851	-1.44E+08	1.73E-03	3.10E-02
ENSDARP00000098538	-1.0851	-1.44E+08	1.73E-03	3.10E-02
ENSDARP00000100455	-1.0851	-1.44E+08	1.73E-03	3.10E-02
ENSDARP00000109440	-1.0851	-1.44E+08	1.73E-03	3.10E-02
ENSDARP00000084713	2.03771	1.44E+08	1.74E-03	3.12E-02
ENSDARP00000078912	2.02612	-4.71E+00	1.77E-03	3.17E-02
ENSDARP00000068084	5.46302	-4.32E+00	1.83E-03	3.28E-02
ENSDARP00000051889	2.59273	-4.55E+00	1.87E-03	3.33E-02
ENSDARP00000062440	6.51444	-4.27E+00	1.93E-03	3.43E-02
ENSDARP00000074422	0.94031	-5.10E+00	1.94E-03	3.45E-02
ENSDARP00000111960	1.97682	1.44E+08	1.95E-03	3.45E-02
ENSDARP00000083057	5.82377	-4.25E+00	2.07E-03	3.67E-02
ENSDARP00000055151	6.61407	-4.21E+00	2.18E-03	3.85E-02
ENSDARP00000010130	2.31316	-4.48E+00	2.31E-03	4.06E-02
ENSDARP00000081395	1.86002	1.44E+08	2.41E-03	4.23E-02
ENSDARP00000052417	1.59206	-4.64E+00	2.43E-03	4.26E-02

ENSDARP00000071779	3.80216	-4.23E+00	2.54E-03	4.44E-02
ENSDARP00000003429	0.78237	-4.96E+00	2.59E-03	4.52E-02
ENSDARP00000104015	2.25257	-4.42E+00	2.59E-03	4.53E-02
ENSDARP00000008085	8.25861	-4.10E+00	2.64E-03	4.60E-02
ENSDARP00000116304	4.01371	-4.19E+00	2.64E-03	4.60E-02
ENSDARP00000098272	1.79175	-4.49E+00	2.78E-03	4.83E-02
ENSDARP00000030937	2.38217	-4.34E+00	2.82E-03	4.89E-02

North22 vs South22				
ENSMBL ID	**logConc**	**logFC**	**PValue**	**FDR**
ENSDARP00000090385	4.30829	1.44E+08	5.18E-09	4.67E-05
ENSDARP00000081302	2.76785	-1.44E+08	1.28E-08	5.79E-05
ENSDARP00000071278	7.01388	-5.11E+00	4.22E-08	1.27E-04
ENSDARP00000071204	4.08669	6.48E+00	2.97E-07	6.70E-04
ENSDARP00000031709	2.94593	-5.74E+00	7.17E-07	1.29E-03
ENSDARP00000068724	1.70467	-1.44E+08	1.03E-06	1.55E-03
ENSDARP00000110551	2.69003	-5.50E+00	2.16E-06	2.18E-03
ENSDARP00000052347	2.92112	1.44E+08	2.33E-06	2.18E-03
ENSDARP00000109654	2.25146	-6.11E+00	2.37E-06	2.18E-03
ENSDARP00000073932	2.90623	1.44E+08	2.47E-06	2.18E-03
ENSDARP00000072883	3.59324	5.96E+00	2.68E-06	2.18E-03
ENSDARP00000113772	2.8606	1.44E+08	2.98E-06	2.18E-03
ENSDARP00000048292	4.48129	4.85E+00	3.15E-06	2.18E-03
ENSDARP00000119078	2.82336	-5.02E+00	4.61E-06	2.97E-03
ENSDARP00000006884	2.69674	1.44E+08	5.79E-06	3.48E-03
ENSDARP00000015118	2.64358	1.44E+08	7.16E-06	4.04E-03
ENSDARP00000069926	2.58832	1.44E+08	8.92E-06	4.73E-03
ENSDARP00000077647	3.28563	5.63E+00	1.01E-05	5.04E-03
ENSDARP00000112756	2.53077	1.44E+08	1.12E-05	5.30E-03
ENSDARP00000010413	8.57114	-3.78E+00	1.21E-05	5.44E-03
ENSDARP00000112555	2.49104	1.44E+08	1.30E-05	5.60E-03
ENSDARP00000115340	3.02636	-4.45E+00	1.43E-05	5.86E-03
ENSDARP00000121241	4.11002	4.46E+00	1.67E-05	6.53E-03
ENSDARP00000119032	2.40798	1.44E+08	1.80E-05	6.75E-03
ENSDARP00000018378	0.91874	-1.44E+08	1.88E-05	6.77E-03
ENSDARP00000112999	2.38641	1.44E+08	1.95E-05	6.77E-03
ENSDARP00000030937	2.11313	-4.95E+00	2.36E-05	7.57E-03
ENSDARP00000008423	1.67081	-5.57E+00	2.40E-05	7.57E-03
ENSDARP00000104051	3.47933	4.81E+00	2.43E-05	7.57E-03
ENSDARP00000024380	0.78627	-1.44E+08	2.96E-05	8.44E-03
ENSDARP00000113607	2.27322	1.44E+08	2.99E-05	8.44E-03
ENSDARP00000121686	2.27322	1.44E+08	2.99E-05	8.44E-03
ENSDARP00000008879	2.2252	1.44E+08	3.58E-05	9.79E-03
ENSDARP00000102422	2.78634	-4.21E+00	3.98E-05	1.05E-02

ENSDARP00000083025	5.48962	3.65E+00	5.12E-05	1.32E-02
ENSDARP00000011179	2.4771	-4.25E+00	5.80E-05	1.45E-02
ENSDARP00000029406	3.56788	4.30E+00	6.07E-05	1.48E-02
ENSDARP00000106900	2.0702	1.44E+08	6.31E-05	1.50E-02
ENSDARP00000016261	2.04257	1.44E+08	6.97E-05	1.61E-02
ENSDARP00000022134	2.78119	5.09E+00	8.01E-05	1.81E-02
ENSDARP00000059399	2.38264	-4.16E+00	8.54E-05	1.83E-02
ENSDARP00000077859	2.38264	-4.16E+00	8.54E-05	1.83E-02
ENSDARP00000066589	1.95617	1.44E+08	9.47E-05	1.99E-02
ENSDARP00000007588	1.92611	1.44E+08	1.05E-04	2.16E-02
ENSDARP00000116733	2.5447	-3.97E+00	1.09E-04	2.18E-02
ENSDARP00000067513	1.71431	-4.57E+00	1.13E-04	2.18E-02
ENSDARP00000108210	3.4192	4.14E+00	1.14E-04	2.18E-02
ENSDARP00000123859	2.02522	-4.25E+00	1.24E-04	2.23E-02
ENSDARP00000077899	1.21964	-5.16E+00	1.29E-04	2.23E-02
ENSDARP00000081562	2.50038	-3.93E+00	1.30E-04	2.23E-02
ENSDARP00000055406	1.86393	1.44E+08	1.31E-04	2.23E-02
ENSDARP00000065664	1.86393	1.44E+08	1.31E-04	2.23E-02
ENSDARP00000064825	2.69043	-3.84E+00	1.31E-04	2.23E-02
ENSDARP00000103806	0.30917	-1.44E+08	1.40E-04	2.35E-02
ENSDARP00000073308	1.83174	1.44E+08	1.46E-04	2.39E-02
ENSDARP00000103553	3.99205	3.71E+00	1.51E-04	2.43E-02
ENSDARP00000096771	1.79876	1.44E+08	1.63E-04	2.58E-02
ENSDARP00000041769	3.00863	4.30E+00	1.72E-04	2.64E-02
ENSDARP00000107733	4.12034	3.61E+00	1.73E-04	2.64E-02
ENSDARP00000119137	1.76497	1.44E+08	1.83E-04	2.70E-02
ENSDARP00000121395	1.76497	1.44E+08	1.83E-04	2.70E-02
ENSDARP00000104580	1.1117	-5.06E+00	1.91E-04	2.77E-02
ENSDARP00000123477	4.22037	3.52E+00	2.09E-04	2.99E-02
ENSDARP00000087059	2.56676	-3.71E+00	2.18E-04	3.08E-02
ENSDARP00000075802	4.91038	3.30E+00	2.50E-04	3.46E-02
ENSDARP00000109853	2.90715	4.19E+00	2.57E-04	3.51E-02
ENSDARP00000101080	2.46962	4.75E+00	2.68E-04	3.60E-02
ENSDARP00000118918	3.20183	-3.41E+00	2.82E-04	3.73E-02
ENSDARP00000051230	1.46863	-4.34E+00	2.85E-04	3.73E-02
ENSDARP00000090658	1.62067	1.44E+08	2.95E-04	3.80E-02
ENSDARP00000026454	2.42789	4.70E+00	3.13E-04	3.98E-02
ENSDARP00000087484	1.58205	1.44E+08	3.34E-04	4.13E-02
ENSDARP00000103159	1.58205	1.44E+08	3.34E-04	4.13E-02
ENSDARP00000108265	2.38485	4.66E+00	3.68E-04	4.48E-02
ENSDARP00000002858	1.54229	1.44E+08	3.80E-04	4.50E-02
ENSDARP00000075369	1.54229	1.44E+08	3.80E-04	4.50E-02
ENSDARP00000109645	3.11712	3.81E+00	3.91E-04	4.58E-02
ENSDARP00000090050	1.99672	-3.77E+00	3.97E-04	4.59E-02

ENSDARP00000100455	3.35619	3.63E+00	4.15E-04	4.65E-02
ENSDARP00000111921	2.78131	4.05E+00	4.21E-04	4.65E-02
ENSDARP00000014968	1.50132	1.44E+08	4.32E-04	4.65E-02
ENSDARP00000115563	1.50132	1.44E+08	4.32E-04	4.65E-02
ENSDARP00000115979	1.50132	1.44E+08	4.32E-04	4.65E-02
ENSDARP00000052433	2.34041	4.61E+00	4.33E-04	4.65E-02
ENSDARP00000113601	2.31765	4.58E+00	4.71E-04	4.98E-02
ENSDARP00000000956	-0.1067	-1.44E+08	4.86E-04	4.98E-02
ENSDARP00000115525	-0.1067	-1.44E+08	4.86E-04	4.98E-02
ENSDARP00000124672	-0.1067	-1.44E+08	4.86E-04	4.98E-02

North36 vs South36				
ENSMBL ID	**logConc**	**logFC**	**PValue**	**FDR**
ENSDARP00000058399	6.18788	1.44E+08	2.24E-28	1.26E-24
ENSDARP00000106918	9.49949	8.86E+00	2.80E-28	1.26E-24
ENSDARP00000111796	11.1535	8.59E+00	8.43E-28	2.03E-24
ENSDARP00000110743	7.85562	9.15E+00	1.05E-27	2.03E-24
ENSDARP00000110361	11.1901	8.56E+00	1.13E-27	2.03E-24
ENSDARP00000104527	11.2349	8.51E+00	1.68E-27	2.52E-24
ENSDARP00000097536	6.60422	1.04E+01	1.95E-27	2.52E-24
ENSDARP00000108964	8.92106	8.59E+00	5.10E-27	5.75E-24
ENSDARP00000106103	9.64785	8.42E+00	8.53E-27	8.55E-24
ENSDARP00000106264	9.1482	8.44E+00	1.20E-26	1.08E-23
ENSDARP00000107985	8.57566	8.52E+00	1.42E-26	1.16E-23
ENSDARP00000102406	5.68297	1.44E+08	2.17E-26	1.63E-23
ENSDARP00000110115	11.3955	8.19E+00	2.73E-26	1.89E-23
ENSDARP00000022810	8.65727	8.32E+00	5.71E-26	3.68E-23
ENSDARP00000107124	7.93097	8.44E+00	8.95E-26	5.38E-23
ENSDARP00000002437	8.44992	8.20E+00	1.94E-25	1.05E-22
ENSDARP00000110119	6.88206	8.83E+00	1.97E-25	1.05E-22
ENSDARP00000107260	7.7076	8.37E+00	2.29E-25	1.15E-22
ENSDARP00000109033	6.59918	9.04E+00	2.49E-25	1.18E-22
ENSDARP00000086365	8.65632	8.12E+00	2.71E-25	1.22E-22
ENSDARP00000107384	8.41024	8.08E+00	5.48E-25	2.35E-22
ENSDARP00000107720	5.69873	1.05E+01	7.16E-25	2.93E-22
ENSDARP00000105978	7.89354	8.12E+00	8.95E-25	3.51E-22
ENSDARP00000109856	6.60029	8.55E+00	2.56E-24	9.64E-22
ENSDARP00000105592	7.70524	8.00E+00	3.15E-24	1.14E-21
ENSDARP00000109971	7.28019	8.13E+00	3.39E-24	1.18E-21
ENSDARP00000111120	5.92022	8.68E+00	2.99E-23	9.71E-21
ENSDARP00000111591	7.17289	7.84E+00	3.01E-23	9.71E-21
ENSDARP00000109169	4.79016	1.44E+08	6.58E-23	2.05E-20
ENSDARP00000118420	6.4001	7.99E+00	1.12E-22	3.37E-20
ENSDARP00000101155	6.78345	7.73E+00	1.64E-22	4.76E-20

ENSDARP00000053470	5.26696	9.04E+00	3.53E-22	9.95E-20
ENSDARP00000104633	4.59291	1.44E+08	3.80E-22	1.04E-19
ENSDARP00000110950	4.52854	1.44E+08	6.72E-22	1.78E-19
ENSDARP00000116660	4.87774	8.65E+00	1.15E-20	2.96E-18
ENSDARP00000110353	4.05422	1.44E+08	4.36E-20	1.09E-17
ENSDARP00000025485	3.9737	1.44E+08	8.80E-20	2.15E-17
ENSDARP00000113814	3.93878	1.44E+08	1.19E-19	2.83E-17
ENSDARP00000088296	4.96379	7.73E+00	1.62E-19	3.75E-17
ENSDARP00000109966	3.81332	1.44E+08	3.54E-19	7.99E-17
ENSDARP00000124900	3.76631	1.44E+08	5.32E-19	1.17E-16
ENSDARP00000108978	5.1296	7.09E+00	1.50E-18	3.22E-16
ENSDARP00000090944	3.62419	1.44E+08	1.81E-18	3.80E-16
ENSDARP00000115874	4.19134	7.98E+00	4.96E-18	1.02E-15
ENSDARP00000109174	3.42692	1.44E+08	9.78E-18	1.96E-15
ENSDARP00000090385	4.10576	7.89E+00	1.05E-17	2.06E-15
ENSDARP00000111564	3.3861	1.44E+08	1.38E-17	2.65E-15
ENSDARP00000108232	5.91572	6.27E+00	2.21E-17	4.16E-15
ENSDARP00000113979	9.21346	5.84E+00	4.73E-17	8.70E-15
ENSDARP00000110408	9.77614	5.70E+00	1.48E-16	2.67E-14
ENSDARP00000099032	4.17364	6.95E+00	1.74E-16	3.07E-14
ENSDARP00000113988	4.76077	6.35E+00	2.77E-16	4.81E-14
ENSDARP00000103805	2.85492	1.44E+08	1.18E-15	2.00E-13
ENSDARP00000094422	3.51537	7.32E+00	1.71E-15	2.85E-13
ENSDARP00000107713	3.50587	7.31E+00	1.85E-15	3.04E-13
ENSDARP00000108614	2.76265	1.44E+08	2.51E-15	4.04E-13
ENSDARP00000086159	3.99836	6.46E+00	3.11E-15	4.92E-13
ENSDARP00000111329	3.12048	7.94E+00	4.84E-15	7.45E-13
ENSDARP00000019408	2.68104	1.44E+08	4.88E-15	7.45E-13
ENSDARP00000016859	2.62981	1.44E+08	7.39E-15	1.11E-12
ENSDARP00000109377	2.61232	1.44E+08	8.51E-15	1.25E-12
ENSDARP00000121241	4.64645	5.82E+00	8.61E-15	1.25E-12
ENSDARP00000071204	2.55858	1.44E+08	1.31E-14	1.85E-12
ENSDARP00000117651	2.55858	1.44E+08	1.31E-14	1.85E-12
ENSDARP00000108110	3.93972	6.13E+00	1.74E-14	2.41E-12
ENSDARP00000098380	3.22285	7.04E+00	2.02E-14	2.76E-12
ENSDARP00000101346	3.57162	6.36E+00	3.11E-14	4.19E-12
ENSDARP00000091998	9.1093	5.00E+00	6.84E-14	9.08E-12
ENSDARP00000007305	2.27803	1.44E+08	1.22E-13	1.60E-11
ENSDARP00000013683	2.72334	7.56E+00	1.28E-13	1.64E-11
ENSDARP00000110583	5.08425	5.24E+00	1.43E-13	1.82E-11
ENSDARP00000009320	5.36927	5.15E+00	1.74E-13	2.14E-11
ENSDARP00000041805	2.23321	1.44E+08	1.74E-13	2.14E-11
ENSDARP00000114169	2.95703	6.78E+00	1.84E-13	2.23E-11
ENSDARP00000123948	5.43848	5.12E+00	1.86E-13	2.23E-11

ENSDARP00000108259	4.10672	5.55E+00	1.99E-13	2.36E-11
ENSDARP00000087346	3.61693	5.81E+00	2.74E-13	3.21E-11
ENSDARP00000075802	2.88591	6.71E+00	3.30E-13	3.82E-11
ENSDARP00000099444	2.13928	1.44E+08	3.61E-13	4.12E-11
ENSDARP00000002287	5.39402	5.03E+00	3.81E-13	4.29E-11
ENSDARP00000110789	7.48846	4.82E+00	4.50E-13	5.01E-11
ENSDARP00000061114	4.8654	5.08E+00	6.29E-13	6.92E-11
ENSDARP00000088270	2.039	1.44E+08	7.81E-13	8.29E-11
ENSDARP00000088644	2.039	1.44E+08	7.81E-13	8.29E-11
ENSDARP00000089814	2.039	1.44E+08	7.81E-13	8.29E-11
ENSDARP00000072678	5.99357	4.85E+00	8.00E-13	8.39E-11
ENSDARP00000087876	6.74819	4.76E+00	1.04E-12	1.08E-10
ENSDARP00000067199	2.73239	6.56E+00	1.15E-12	1.18E-10
ENSDARP00000119968	1.98621	1.44E+08	1.17E-12	1.19E-10
ENSDARP00000110773	2.93675	6.17E+00	1.34E-12	1.34E-10
ENSDARP00000093124	5.86447	4.80E+00	1.35E-12	1.34E-10
ENSDARP00000049108	1.95909	1.44E+08	1.44E-12	1.41E-10
ENSDARP00000122502	5.65213	4.82E+00	1.45E-12	1.41E-10
ENSDARP00000111333	4.20439	5.15E+00	1.60E-12	1.53E-10
ENSDARP00000029276	2.90834	6.14E+00	1.69E-12	1.60E-10
ENSDARP00000111532	1.93147	1.44E+08	1.77E-12	1.63E-10
ENSDARP00000116987	1.93147	1.44E+08	1.77E-12	1.63E-10
ENSDARP00000121433	1.93147	1.44E+08	1.77E-12	1.63E-10
ENSDARP00000071264	4.33375	5.08E+00	1.80E-12	1.64E-10
ENSDARP00000096034	7.01768	4.67E+00	1.92E-12	1.73E-10
ENSDARP00000052062	5.33614	4.81E+00	2.15E-12	1.92E-10
ENSDARP00000021453	1.90333	1.44E+08	2.20E-12	1.94E-10
ENSDARP00000020804	5.11289	4.84E+00	2.32E-12	2.03E-10
ENSDARP00000059417	2.35486	7.21E+00	2.44E-12	2.11E-10
ENSDARP00000059040	3.98917	5.16E+00	2.57E-12	2.20E-10
ENSDARP00000125351	1.87465	1.44E+08	2.73E-12	2.32E-10
ENSDARP00000041006	4.13211	5.07E+00	2.97E-12	2.50E-10
ENSDARP00000049102	4.62512	4.90E+00	3.24E-12	2.71E-10
ENSDARP00000063605	1.84541	1.44E+08	3.40E-12	2.77E-10
ENSDARP00000105950	1.84541	1.44E+08	3.40E-12	2.77E-10
ENSDARP00000111820	4.18973	5.03E+00	3.41E-12	2.77E-10
ENSDARP00000105482	2.59684	6.43E+00	3.44E-12	2.77E-10
ENSDARP00000065372	3.6568	5.25E+00	3.94E-12	3.15E-10
ENSDARP00000106224	3.5452	5.32E+00	4.04E-12	3.20E-10
ENSDARP00000076074	1.81559	1.44E+08	4.25E-12	3.30E-10
ENSDARP00000123477	1.81559	1.44E+08	4.25E-12	3.30E-10
ENSDARP00000045815	5.86261	4.64E+00	4.56E-12	3.52E-10
ENSDARP00000016806	3.72811	5.17E+00	5.05E-12	3.86E-10
ENSDARP00000118225	4.38414	4.87E+00	6.06E-12	4.59E-10

ENSDARP00000119056	1.7541	1.44E+08	6.72E-12	5.05E-10
ENSDARP00000123096	4.48448	4.83E+00	6.85E-12	5.11E-10
ENSDARP00000071920	3.58655	5.19E+00	7.13E-12	5.23E-10
ENSDARP00000054395	2.50531	6.35E+00	7.14E-12	5.23E-10
ENSDARP00000017411	2.7252	5.96E+00	7.48E-12	5.44E-10
ENSDARP00000052729	4.16288	4.91E+00	7.82E-12	5.64E-10
ENSDARP00000092403	1.72239	1.44E+08	8.50E-12	6.09E-10
ENSDARP00000082574	4.39198	4.81E+00	9.37E-12	6.66E-10
ENSDARP00000104643	4.94349	4.66E+00	1.02E-11	7.16E-10
ENSDARP00000105585	1.68999	1.44E+08	1.08E-11	7.55E-10
ENSDARP00000070898	2.67561	5.91E+00	1.11E-11	7.73E-10
ENSDARP00000002367	6.28135	4.49E+00	1.13E-11	7.74E-10
ENSDARP00000013307	4.53221	4.74E+00	1.13E-11	7.74E-10
ENSDARP00000087875	9.86791	4.38E+00	1.33E-11	9.03E-10
ENSDARP00000072883	1.65688	1.44E+08	1.38E-11	9.28E-10
ENSDARP00000026811	3.69949	5.00E+00	1.42E-11	9.50E-10
ENSDARP00000071629	4.13553	4.79E+00	1.75E-11	1.16E-09
ENSDARP00000099646	4.18111	4.75E+00	2.05E-11	1.35E-09
ENSDARP00000059357	5.23182	4.52E+00	2.11E-11	1.38E-09
ENSDARP00000073753	3.97247	4.81E+00	2.18E-11	1.41E-09
ENSDARP00000094729	1.5884	1.44E+08	2.27E-11	1.47E-09
ENSDARP00000002175	7.09581	4.35E+00	2.44E-11	1.56E-09
ENSDARP00000106586	3.80422	4.85E+00	2.49E-11	1.58E-09
ENSDARP00000063364	2.05418	6.93E+00	2.51E-11	1.59E-09
ENSDARP00000106777	4.70447	4.57E+00	2.82E-11	1.77E-09
ENSDARP00000048816	4.9611	4.51E+00	3.12E-11	1.94E-09
ENSDARP00000065036	7.09063	4.32E+00	3.15E-11	1.95E-09
ENSDARP00000095098	4.55791	4.58E+00	3.19E-11	1.96E-09
ENSDARP00000020257	4.5533	4.58E+00	3.32E-11	2.02E-09
ENSDARP00000025885	4.54868	4.57E+00	3.45E-11	2.09E-09
ENSDARP00000112810	4.44792	4.59E+00	3.60E-11	2.17E-09
ENSDARP00000078089	2.70158	5.52E+00	4.17E-11	2.49E-09
ENSDARP00000108512	3.24847	5.02E+00	4.78E-11	2.84E-09
ENSDARP00000066212	5.24623	4.40E+00	5.04E-11	2.97E-09
ENSDARP00000124072	3.86522	4.70E+00	5.40E-11	3.16E-09
ENSDARP00000028542	2.23679	6.09E+00	5.88E-11	3.42E-09
ENSDARP00000090005	2.45865	5.71E+00	6.27E-11	3.62E-09
ENSDARP00000060548	3.32082	4.92E+00	6.53E-11	3.75E-09
ENSDARP00000114013	1.44136	1.44E+08	6.57E-11	3.75E-09
ENSDARP00000058628	2.63402	5.45E+00	7.16E-11	4.06E-09
ENSDARP00000053859	6.11436	4.25E+00	8.19E-11	4.62E-09
ENSDARP00000109782	1.40226	1.44E+08	8.68E-11	4.83E-09
ENSDARP00000112833	1.40226	1.44E+08	8.68E-11	4.83E-09
ENSDARP00000060997	3.04756	5.02E+00	9.29E-11	5.14E-09

ENSDARP00000120135	3.55556	4.72E+00	9.97E-11	5.48E-09
ENSDARP00000109777	1.36213	1.44E+08	1.15E-10	6.30E-09
ENSDARP00000089057	4.53723	4.39E+00	1.19E-10	6.44E-09
ENSDARP00000022077	6.11044	4.20E+00	1.23E-10	6.66E-09
ENSDARP00000049009	4.83198	4.33E+00	1.28E-10	6.86E-09
ENSDARP00000086061	2.72705	5.21E+00	1.29E-10	6.88E-09
ENSDARP00000023459	2.35791	5.61E+00	1.38E-10	7.32E-09
ENSDARP00000088110	4.47278	4.38E+00	1.43E-10	7.52E-09
ENSDARP00000113056	7.11305	4.13E+00	1.50E-10	7.86E-09
ENSDARP00000029855	1.32092	1.44E+08	1.54E-10	7.99E-09
ENSDARP00000113866	1.32092	1.44E+08	1.54E-10	7.99E-09
ENSDARP00000007503	4.49463	4.35E+00	1.71E-10	8.79E-09
ENSDARP00000104920	5.33364	4.22E+00	1.72E-10	8.80E-09
ENSDARP00000010992	3.72512	4.56E+00	1.75E-10	8.91E-09
ENSDARP00000115789	2.0943	5.95E+00	1.76E-10	8.91E-09
ENSDARP00000026731	4.04376	4.45E+00	1.82E-10	9.18E-09
ENSDARP00000117964	2.82964	5.04E+00	1.85E-10	9.28E-09
ENSDARP00000022287	4.09225	4.43E+00	1.95E-10	9.72E-09
ENSDARP00000088159	3.63645	4.57E+00	1.97E-10	9.74E-09
ENSDARP00000117331	3.17329	4.77E+00	2.20E-10	1.08E-08
ENSDARP00000022929	4.0731	4.41E+00	2.29E-10	1.12E-08
ENSDARP00000110039	1.7417	6.64E+00	2.63E-10	1.28E-08
ENSDARP00000121183	4.74378	4.20E+00	3.64E-10	1.76E-08
ENSDARP00000123458	1.19018	1.44E+08	3.83E-10	1.84E-08
ENSDARP00000111379	3.55612	4.49E+00	3.83E-10	1.84E-08
ENSDARP00000078028	2.39032	5.21E+00	4.89E-10	2.32E-08
ENSDARP00000125091	2.39032	5.21E+00	4.89E-10	2.32E-08
ENSDARP00000109090	4.56294	4.18E+00	5.13E-10	2.42E-08
ENSDARP00000116274	1.14401	1.44E+08	5.26E-10	2.47E-08
ENSDARP00000099552	3.43633	4.48E+00	5.36E-10	2.50E-08
ENSDARP00000027071	2.82287	4.80E+00	5.70E-10	2.65E-08
ENSDARP00000107733	2.3698	5.19E+00	5.73E-10	2.65E-08
ENSDARP00000096352	3.3274	4.49E+00	6.57E-10	3.02E-08
ENSDARP00000020894	3.4808	4.42E+00	7.14E-10	3.27E-08
ENSDARP00000096709	6.96753	3.94E+00	7.18E-10	3.27E-08
ENSDARP00000114538	2.49962	4.99E+00	7.81E-10	3.54E-08
ENSDARP00000005284	1.88033	5.75E+00	8.83E-10	3.98E-08
ENSDARP00000106827	1.57492	6.48E+00	8.89E-10	3.99E-08
ENSDARP00000073091	2.48059	4.97E+00	9.06E-10	4.03E-08
ENSDARP00000118518	2.48059	4.97E+00	9.06E-10	4.03E-08
ENSDARP00000094171	3.96315	4.22E+00	9.14E-10	4.04E-08
ENSDARP00000096230	1.04729	1.44E+08	1.01E-09	4.44E-08
ENSDARP00000101100	1.04729	1.44E+08	1.01E-09	4.44E-08
ENSDARP00000017693	1.8513	5.72E+00	1.10E-09	4.78E-08

ENSDARP00000122171	4.34832	4.10E+00	1.19E-09	5.15E-08
ENSDARP00000012437	2.44177	4.93E+00	1.23E-09	5.29E-08
ENSDARP00000102848	5.28312	3.94E+00	1.45E-09	6.24E-08
ENSDARP00000036336	1.50271	6.42E+00	1.49E-09	6.35E-08
ENSDARP00000065361	1.50271	6.42E+00	1.49E-09	6.35E-08
ENSDARP00000091908	3.02972	4.47E+00	1.63E-09	6.87E-08
ENSDARP00000109807	3.21589	4.38E+00	1.63E-09	6.87E-08
ENSDARP00000110478	3.77828	4.18E+00	1.68E-09	7.05E-08
ENSDARP00000055618	7.50385	3.80E+00	1.89E-09	7.91E-08
ENSDARP00000072603	2.51029	4.72E+00	2.33E-09	9.67E-08
ENSDARP00000025821	1.98021	5.25E+00	2.48E-09	1.03E-07
ENSDARP00000068532	1.42689	6.35E+00	2.56E-09	1.05E-07
ENSDARP00000107795	2.86922	4.47E+00	2.56E-09	1.05E-07
ENSDARP00000024492	4.12133	4.02E+00	2.77E-09	1.13E-07
ENSDARP00000073455	0.88986	1.44E+08	2.89E-09	1.16E-07
ENSDARP00000083878	0.88986	1.44E+08	2.89E-09	1.16E-07
ENSDARP00000110556	0.88986	1.44E+08	2.89E-09	1.16E-07
ENSDARP00000118428	0.88986	1.44E+08	2.89E-09	1.16E-07
ENSDARP00000054063	8.3448	3.73E+00	3.06E-09	1.22E-07
ENSDARP00000023131	2.47228	4.69E+00	3.13E-09	1.24E-07
ENSDARP00000064864	2.83955	4.44E+00	3.24E-09	1.28E-07
ENSDARP00000104829	1.38753	6.31E+00	3.38E-09	1.33E-07
ENSDARP00000114168	0.83364	1.44E+08	4.17E-09	1.64E-07
ENSDARP00000061148	10.6574	3.67E+00	4.56E-09	1.78E-07
ENSDARP00000089064	3.58024	4.05E+00	5.26E-09	2.04E-07
ENSDARP00000106507	3.15125	4.19E+00	5.46E-09	2.12E-07
ENSDARP00000049044	2.07359	4.91E+00	5.53E-09	2.13E-07
ENSDARP00000072394	2.65529	4.43E+00	5.69E-09	2.18E-07
ENSDARP00000111236	3.05697	4.22E+00	5.88E-09	2.25E-07
ENSDARP00000113861	0.77533	1.44E+08	6.09E-09	2.32E-07
ENSDARP00000107437	3.4277	4.07E+00	6.44E-09	2.44E-07
ENSDARP00000025608	2.83283	4.27E+00	7.83E-09	2.96E-07
ENSDARP00000062993	1.80977	5.08E+00	8.79E-09	3.30E-07
ENSDARP00000053010	0.71477	1.44E+08	8.97E-09	3.33E-07
ENSDARP00000059197	0.71477	1.44E+08	8.97E-09	3.33E-07
ENSDARP00000118427	0.71477	1.44E+08	8.97E-09	3.33E-07
ENSDARP00000015065	3.82188	3.88E+00	1.05E-08	3.89E-07
ENSDARP00000027568	1.77939	5.05E+00	1.10E-08	4.04E-07
ENSDARP00000117273	5.57136	3.64E+00	1.12E-08	4.12E-07
ENSDARP00000112232	2.68153	4.28E+00	1.13E-08	4.12E-07
ENSDARP00000104950	1.52547	5.42E+00	1.17E-08	4.26E-07
ENSDARP00000074127	2.2881	4.51E+00	1.29E-08	4.68E-07
ENSDARP00000055263	2.42475	4.41E+00	1.30E-08	4.69E-07
ENSDARP00000106429	3.33471	3.97E+00	1.37E-08	4.91E-07

ENSDARP00000059359	1.74839	5.02E+00	1.38E-08	4.92E-07
ENSDARP00000105987	3.45808	3.93E+00	1.42E-08	5.07E-07
ENSDARP00000104315	6.88894	3.54E+00	1.60E-08	5.66E-07
ENSDARP00000057576	4.15273	3.75E+00	1.60E-08	5.66E-07
ENSDARP00000075344	3.0117	4.05E+00	1.67E-08	5.88E-07
ENSDARP00000076839	2.63064	4.23E+00	1.68E-08	5.89E-07
ENSDARP00000063949	2.3845	4.37E+00	1.77E-08	6.19E-07
ENSDARP00000098032	2.24388	4.46E+00	1.81E-08	6.27E-07
ENSDARP00000061246	1.91422	4.75E+00	1.81E-08	6.27E-07
ENSDARP00000123575	1.91422	4.75E+00	1.81E-08	6.27E-07
ENSDARP00000043865	0.58618	1.44E+08	2.01E-08	6.88E-07
ENSDARP00000051262	0.58618	1.44E+08	2.01E-08	6.88E-07
ENSDARP00000112216	0.58618	1.44E+08	2.01E-08	6.88E-07
ENSDARP00000105539	1.12744	6.07E+00	2.03E-08	6.92E-07
ENSDARP00000060257	3.14013	3.96E+00	2.10E-08	7.11E-07
ENSDARP00000068084	4.9408	3.60E+00	2.21E-08	7.47E-07
ENSDARP00000073663	1.88594	4.72E+00	2.23E-08	7.52E-07
ENSDARP00000123966	2.47488	4.25E+00	2.31E-08	7.75E-07
ENSDARP00000090196	1.85712	4.70E+00	2.76E-08	9.22E-07
ENSDARP00000076216	0.51775	1.44E+08	3.08E-08	1.02E-06
ENSDARP00000078740	1.37273	5.27E+00	3.43E-08	1.14E-06
ENSDARP00000063356	2.64028	4.08E+00	3.54E-08	1.17E-06
ENSDARP00000011797	2.12728	4.35E+00	4.35E-08	1.43E-06
ENSDARP00000033381	0.44624	1.44E+08	4.76E-08	1.54E-06
ENSDARP00000047618	0.44624	1.44E+08	4.76E-08	1.54E-06
ENSDARP00000103503	0.44624	1.44E+08	4.76E-08	1.54E-06
ENSDARP00000105835	0.44624	1.44E+08	4.76E-08	1.54E-06
ENSDARP00000114392	0.44624	1.44E+08	4.76E-08	1.54E-06
ENSDARP00000091646	2.37575	4.15E+00	4.93E-08	1.59E-06
ENSDARP00000067578	3.0974	3.82E+00	5.25E-08	1.69E-06
ENSDARP00000100904	1.76722	4.61E+00	5.31E-08	1.70E-06
ENSDARP00000057097	4.04246	3.59E+00	5.37E-08	1.71E-06
ENSDARP00000027138	3.56803	3.68E+00	5.55E-08	1.76E-06
ENSDARP00000109129	1.29026	5.19E+00	6.07E-08	1.92E-06
ENSDARP00000058964	6.38983	3.38E+00	6.28E-08	1.98E-06
ENSDARP00000091840	3.77615	3.60E+00	6.80E-08	2.14E-06
ENSDARP00000123697	2.64985	3.94E+00	7.05E-08	2.21E-06
ENSDARP00000106234	4.75677	3.45E+00	7.18E-08	2.24E-06
ENSDARP00000025514	0.37138	1.44E+08	7.46E-08	2.31E-06
ENSDARP00000043945	0.37138	1.44E+08	7.46E-08	2.31E-06
ENSDARP00000080636	2.53374	3.97E+00	8.04E-08	2.48E-06
ENSDARP00000008879	1.24731	5.15E+00	8.15E-08	2.50E-06
ENSDARP00000101978	1.24731	5.15E+00	8.15E-08	2.50E-06
ENSDARP00000060687	2.70917	3.87E+00	9.10E-08	2.78E-06

ENSDARP00000055151	5.95728	3.34E+00	9.33E-08	2.84E-06
ENSDARP00000014011	2.38773	-4.98E+00	9.73E-08	2.95E-06
ENSDARP00000114945	1.67165	4.52E+00	1.06E-07	3.19E-06
ENSDARP00000108836	1.84544	4.35E+00	1.10E-07	3.31E-06
ENSDARP00000049686	1.20313	5.11E+00	1.10E-07	3.31E-06
ENSDARP00000125019	3.91575	3.50E+00	1.12E-07	3.34E-06
ENSDARP00000061255	0.29285	1.44E+08	1.19E-07	3.53E-06
ENSDARP00000069088	0.29285	1.44E+08	1.19E-07	3.53E-06
ENSDARP00000120683	2.47747	3.91E+00	1.24E-07	3.67E-06
ENSDARP00000078009	1.43668	4.72E+00	1.27E-07	3.74E-06
ENSDARP00000073778	7.32555	3.26E+00	1.29E-07	3.81E-06
ENSDARP00000106419	1.97425	4.19E+00	1.35E-07	3.97E-06
ENSDARP00000016376	4.01309	3.43E+00	1.61E-07	4.72E-06
ENSDARP00000041769	2.32518	3.92E+00	1.74E-07	5.07E-06
ENSDARP00000019915	5.16764	3.29E+00	1.79E-07	5.20E-06
ENSDARP00000053774	0.21028	1.44E+08	1.92E-07	5.52E-06
ENSDARP00000103553	0.21028	1.44E+08	1.92E-07	5.52E-06
ENSDARP00000121981	0.21028	1.44E+08	1.92E-07	5.52E-06
ENSDARP00000070307	1.11082	5.02E+00	2.05E-07	5.88E-06
ENSDARP00000049166	2.48827	3.77E+00	2.43E-07	6.95E-06
ENSDARP00000071267	4.86327	3.27E+00	2.49E-07	7.10E-06
ENSDARP00000109190	2.8962	3.61E+00	2.54E-07	7.23E-06
ENSDARP00000014024	0.12325	1.44E+08	3.15E-07	8.77E-06
ENSDARP00000024226	0.12325	1.44E+08	3.15E-07	8.77E-06
ENSDARP00000045970	0.12325	1.44E+08	3.15E-07	8.77E-06
ENSDARP00000047470	0.12325	1.44E+08	3.15E-07	8.77E-06
ENSDARP00000054387	0.12325	1.44E+08	3.15E-07	8.77E-06
ENSDARP00000108845	0.12325	1.44E+08	3.15E-07	8.77E-06
ENSDARP00000119608	0.12325	1.44E+08	3.15E-07	8.77E-06
ENSDARP00000070502	3.12438	3.50E+00	3.29E-07	9.12E-06
ENSDARP00000034214	5.10139	3.20E+00	3.49E-07	9.66E-06
ENSDARP00000062440	5.63833	3.17E+00	3.63E-07	1.00E-05
ENSDARP00000040327	1.27483	4.57E+00	3.85E-07	1.05E-05
ENSDARP00000115180	1.27483	4.57E+00	3.85E-07	1.05E-05
ENSDARP00000057697	1.0127	4.93E+00	3.92E-07	1.07E-05
ENSDARP00000095092	1.0127	4.93E+00	3.92E-07	1.07E-05
ENSDARP00000041589	2.21534	3.81E+00	3.94E-07	1.07E-05
ENSDARP00000058323	3.82012	3.31E+00	4.34E-07	1.18E-05
ENSDARP00000042477	2.49897	3.65E+00	4.54E-07	1.22E-05
ENSDARP00000104188	2.19237	3.78E+00	4.67E-07	1.25E-05
ENSDARP00000106016	2.19237	3.78E+00	4.67E-07	1.25E-05
ENSDARP00000013778	2.7362	3.54E+00	4.94E-07	1.32E-05
ENSDARP00000035155	0.63161	5.61E+00	5.07E-07	1.35E-05
ENSDARP00000049419	0.63161	5.61E+00	5.07E-07	1.35E-05

ENSDARP00000124433	2.48004	3.63E+00	5.23E-07	1.37E-05
ENSDARP00000019643	0.03129	1.44E+08	5.27E-07	1.37E-05
ENSDARP00000044558	0.03129	1.44E+08	5.27E-07	1.37E-05
ENSDARP00000070746	0.03129	1.44E+08	5.27E-07	1.37E-05
ENSDARP00000082791	0.03129	1.44E+08	5.27E-07	1.37E-05
ENSDARP00000110533	0.03129	1.44E+08	5.27E-07	1.37E-05
ENSDARP00000113090	0.03129	1.44E+08	5.27E-07	1.37E-05
ENSDARP00000123640	0.03129	1.44E+08	5.27E-07	1.37E-05
ENSDARP00000024751	1.62539	4.13E+00	5.28E-07	1.37E-05
ENSDARP00000064110	4.21385	3.21E+00	5.80E-07	1.50E-05
ENSDARP00000112765	1.81998	-5.39E+00	5.82E-07	1.50E-05
ENSDARP00000048622	1.4223	4.27E+00	6.06E-07	1.56E-05
ENSDARP00000010986	4.33425	3.19E+00	6.12E-07	1.57E-05
ENSDARP00000091926	2.77656	3.48E+00	6.38E-07	1.63E-05
ENSDARP00000112109	2.03164	3.81E+00	6.39E-07	1.63E-05
ENSDARP00000020667	5.71361	3.08E+00	6.52E-07	1.66E-05
ENSDARP00000043451	2.14537	3.74E+00	6.61E-07	1.67E-05
ENSDARP00000022576	2.68738	3.49E+00	7.16E-07	1.81E-05
ENSDARP00000059651	7.05189	3.03E+00	7.21E-07	1.82E-05
ENSDARP00000064008	0.90804	4.83E+00	7.74E-07	1.94E-05
ENSDARP00000112972	0.90804	4.83E+00	7.74E-07	1.94E-05
ENSDARP00000016318	-0.0662	1.44E+08	8.98E-07	2.22E-05
ENSDARP00000070155	-0.0662	1.44E+08	8.98E-07	2.22E-05
ENSDARP00000081883	-0.0662	1.44E+08	8.98E-07	2.22E-05
ENSDARP00000121228	-0.0662	1.44E+08	8.98E-07	2.22E-05
ENSDARP00000009875	2.4908	3.51E+00	9.35E-07	2.31E-05
ENSDARP00000100377	8.27388	2.97E+00	9.85E-07	2.43E-05
ENSDARP00000014029	0.85298	4.78E+00	1.10E-06	2.68E-05
ENSDARP00000063033	0.85298	4.78E+00	1.10E-06	2.68E-05
ENSDARP00000110223	0.85298	4.78E+00	1.10E-06	2.68E-05
ENSDARP00000118073	0.85298	4.78E+00	1.10E-06	2.68E-05
ENSDARP00000033391	1.67889	3.90E+00	1.12E-06	2.73E-05
ENSDARP00000094263	1.15616	-1.44E+08	1.19E-06	2.88E-05
ENSDARP00000113136	1.64596	3.87E+00	1.42E-06	3.43E-05
ENSDARP00000058698	3.70534	3.14E+00	1.43E-06	3.45E-05
ENSDARP00000122818	2.66335	3.36E+00	1.51E-06	3.62E-05
ENSDARP00000009077	-0.1699	1.44E+08	1.56E-06	3.71E-05
ENSDARP00000066603	-0.1699	1.44E+08	1.56E-06	3.71E-05
ENSDARP00000083583	-0.1699	1.44E+08	1.56E-06	3.71E-05
ENSDARP00000108822	-0.1699	1.44E+08	1.56E-06	3.71E-05
ENSDARP00000113738	-0.1699	1.44E+08	1.56E-06	3.71E-05
ENSDARP00000075677	3.03692	3.25E+00	1.58E-06	3.72E-05
ENSDARP00000026554	0.79593	4.72E+00	1.58E-06	3.72E-05
ENSDARP00000120991	0.79593	4.72E+00	1.58E-06	3.72E-05

ENSDARP00000076298	2.31933	3.46E+00	1.73E-06	4.07E-05
ENSDARP00000106068	1.25935	4.11E+00	1.83E-06	4.29E-05
ENSDARP00000071962	9.54667	2.85E+00	2.39E-06	5.59E-05
ENSDARP00000029406	0.34916	5.35E+00	2.77E-06	6.32E-05
ENSDARP00000012411	-0.2806	1.44E+08	2.78E-06	6.32E-05
ENSDARP00000052351	-0.2806	1.44E+08	2.78E-06	6.32E-05
ENSDARP00000086805	-0.2806	1.44E+08	2.78E-06	6.32E-05
ENSDARP00000093342	-0.2806	1.44E+08	2.78E-06	6.32E-05
ENSDARP00000115068	-0.2806	1.44E+08	2.78E-06	6.32E-05
ENSDARP00000116318	-0.2806	1.44E+08	2.78E-06	6.32E-05
ENSDARP00000119942	-0.2806	1.44E+08	2.78E-06	6.32E-05
ENSDARP00000120786	-0.2806	1.44E+08	2.78E-06	6.32E-05
ENSDARP00000120937	-0.2806	1.44E+08	2.78E-06	6.32E-05
ENSDARP00000062323	5.69285	2.87E+00	2.80E-06	6.35E-05
ENSDARP00000123852	3.92849	2.99E+00	2.92E-06	6.62E-05
ENSDARP00000098272	1.36843	3.87E+00	3.10E-06	7.00E-05
ENSDARP00000101080	1.81011	3.58E+00	3.12E-06	7.04E-05
ENSDARP00000090099	5.03887	2.89E+00	3.15E-06	7.08E-05
ENSDARP00000116378	2.03624	3.46E+00	3.23E-06	7.25E-05
ENSDARP00000030289	0.67531	4.61E+00	3.35E-06	7.46E-05
ENSDARP00000034815	0.67531	4.61E+00	3.35E-06	7.46E-05
ENSDARP00000072564	0.67531	4.61E+00	3.35E-06	7.46E-05
ENSDARP00000087582	1.66617	3.64E+00	3.39E-06	7.54E-05
ENSDARP00000105626	2.89747	-3.44E+00	3.95E-06	8.75E-05
ENSDARP00000070561	5.16885	-2.90E+00	4.00E-06	8.84E-05
ENSDARP00000123131	0.27007	5.27E+00	4.38E-06	9.67E-05
ENSDARP00000109536	0.88991	4.19E+00	4.80E-06	1.06E-04
ENSDARP00000099211	4.72751	2.84E+00	4.94E-06	1.08E-04
ENSDARP00000021053	-0.3993	1.44E+08	5.05E-06	1.10E-04
ENSDARP00000083463	-0.3993	1.44E+08	5.05E-06	1.10E-04
ENSDARP00000103579	-0.3993	1.44E+08	5.05E-06	1.10E-04
ENSDARP00000107046	-0.3993	1.44E+08	5.05E-06	1.10E-04
ENSDARP00000112726	2.34346	3.24E+00	5.15E-06	1.12E-04
ENSDARP00000097543	1.43206	3.65E+00	6.15E-06	1.33E-04
ENSDARP00000052861	4.90982	2.79E+00	6.43E-06	1.39E-04
ENSDARP00000110049	6.44156	2.72E+00	6.81E-06	1.46E-04
ENSDARP00000093273	1.93016	3.35E+00	6.87E-06	1.47E-04
ENSDARP00000092547	1.68604	3.45E+00	7.41E-06	1.59E-04
ENSDARP00000052513	5.94585	2.72E+00	7.68E-06	1.64E-04
ENSDARP00000079617	1.79824	3.38E+00	7.91E-06	1.69E-04
ENSDARP00000110264	2.93022	2.99E+00	7.99E-06	1.70E-04
ENSDARP00000083057	4.61505	2.77E+00	8.26E-06	1.75E-04
ENSDARP00000004830	1.65339	3.42E+00	9.27E-06	1.96E-04
ENSDARP00000037865	-0.5272	1.44E+08	9.44E-06	1.97E-04

ENSDARP00000094348	-0.5272	1.44E+08	9.44E-06	1.97E-04
ENSDARP00000094626	-0.5272	1.44E+08	9.44E-06	1.97E-04
ENSDARP00000100455	-0.5272	1.44E+08	9.44E-06	1.97E-04
ENSDARP00000106900	-0.5272	1.44E+08	9.44E-06	1.97E-04
ENSDARP00000108456	-0.5272	1.44E+08	9.44E-06	1.97E-04
ENSDARP00000109654	-0.5272	1.44E+08	9.44E-06	1.97E-04
ENSDARP00000071819	2.90198	2.96E+00	9.87E-06	2.05E-04
ENSDARP00000028244	1.35368	3.57E+00	1.04E-05	2.15E-04
ENSDARP00000048394	4.8352	-2.77E+00	1.11E-05	2.30E-04
ENSDARP00000124531	0.9779	3.83E+00	1.15E-05	2.37E-04
ENSDARP00000055324	0.71741	4.02E+00	1.40E-05	2.89E-04
ENSDARP00000090976	1.29073	-4.81E+00	1.56E-05	3.20E-04
ENSDARP00000026065	5.37302	2.63E+00	1.56E-05	3.21E-04
ENSDARP00000021244	0.40287	4.35E+00	1.71E-05	3.49E-04
ENSDARP00000041381	0.40287	4.35E+00	1.71E-05	3.49E-04
ENSDARP00000041624	-0.666	1.44E+08	1.81E-05	3.67E-04
ENSDARP00000109321	-0.666	1.44E+08	1.81E-05	3.67E-04
ENSDARP00000117557	-0.666	1.44E+08	1.81E-05	3.67E-04
ENSDARP00000046442	6.09129	-2.61E+00	1.82E-05	3.68E-04
ENSDARP00000059795	1.89115	3.15E+00	1.86E-05	3.76E-04
ENSDARP00000076347	1.67341	3.25E+00	1.87E-05	3.76E-04
ENSDARP00000111319	0.0067	5.02E+00	1.90E-05	3.82E-04
ENSDARP00000055406	2.30446	2.98E+00	2.10E-05	4.21E-04
ENSDARP00000016228	4.29775	-2.71E+00	2.25E-05	4.51E-04
ENSDARP00000108666	1.75586	3.16E+00	2.30E-05	4.59E-04
ENSDARP00000051889	1.51548	3.27E+00	2.36E-05	4.70E-04
ENSDARP00000069565	4.64614	2.58E+00	2.65E-05	5.26E-04
ENSDARP00000090958	0.59115	3.90E+00	3.01E-05	5.93E-04
ENSDARP00000112999	0.59115	3.90E+00	3.01E-05	5.93E-04
ENSDARP00000011150	1.47901	3.23E+00	3.01E-05	5.93E-04
ENSDARP00000104015	1.33886	3.30E+00	3.08E-05	6.07E-04
ENSDARP00000103358	4.85723	-2.60E+00	3.11E-05	6.12E-04
ENSDARP00000073932	0.81589	3.67E+00	3.15E-05	6.17E-04
ENSDARP00000102656	5.47526	2.52E+00	3.18E-05	6.21E-04
ENSDARP00000106285	-0.0914	4.93E+00	3.20E-05	6.25E-04
ENSDARP00000094586	3.74515	2.62E+00	3.40E-05	6.62E-04
ENSDARP00000113163	1.6931	3.09E+00	3.53E-05	6.86E-04
ENSDARP00000021727	-0.8175	1.44E+08	3.58E-05	6.86E-04
ENSDARP00000069198	-0.8175	1.44E+08	3.58E-05	6.86E-04
ENSDARP00000077647	-0.8175	1.44E+08	3.58E-05	6.86E-04
ENSDARP00000104946	-0.8175	1.44E+08	3.58E-05	6.86E-04
ENSDARP00000106961	-0.8175	1.44E+08	3.58E-05	6.86E-04
ENSDARP00000111809	-0.8175	1.44E+08	3.58E-05	6.86E-04
ENSDARP00000113772	-0.8175	1.44E+08	3.58E-05	6.86E-04

ENSDARP00000061735	3.81224	2.60E+00	3.63E-05	6.93E-04
ENSDARP00000074099	1.44166	3.19E+00	3.85E-05	7.34E-04
ENSDARP00000090925	0.47765	-1.44E+08	4.11E-05	7.81E-04
ENSDARP00000050969	1.11857	-4.62E+00	4.12E-05	7.81E-04
ENSDARP00000067164	0.24729	4.19E+00	4.15E-05	7.82E-04
ENSDARP00000103615	0.24729	4.19E+00	4.15E-05	7.82E-04
ENSDARP00000125053	0.24729	4.19E+00	4.15E-05	7.82E-04
ENSDARP00000122457	2.64513	2.75E+00	4.39E-05	8.26E-04
ENSDARP00000110074	0.52412	3.83E+00	4.46E-05	8.38E-04
ENSDARP00000105058	0.75803	3.61E+00	4.48E-05	8.40E-04
ENSDARP00000096532	1.53766	3.10E+00	4.65E-05	8.70E-04
ENSDARP00000098698	0.43493	-1.44E+08	5.00E-05	9.34E-04
ENSDARP00000059917	3.81262	2.54E+00	5.21E-05	9.70E-04
ENSDARP00000107794	3.59717	2.55E+00	5.66E-05	1.05E-03
ENSDARP00000100078	0.69802	3.55E+00	6.43E-05	1.19E-03
ENSDARP00000096771	0.16357	4.11E+00	6.59E-05	1.22E-03
ENSDARP00000109878	4.51663	-2.50E+00	6.62E-05	1.22E-03
ENSDARP00000001256	3.15683	2.57E+00	7.04E-05	1.30E-03
ENSDARP00000110350	-0.9842	1.44E+08	7.32E-05	1.34E-03
ENSDARP00000119943	-0.9842	1.44E+08	7.32E-05	1.34E-03
ENSDARP00000124980	4.69048	-2.43E+00	9.08E-05	1.67E-03
ENSDARP00000071808	0.29791	-1.44E+08	9.24E-05	1.69E-03
ENSDARP00000001156	0.63569	3.48E+00	9.31E-05	1.69E-03
ENSDARP00000027536	0.63569	3.48E+00	9.31E-05	1.69E-03
ENSDARP00000110419	0.63569	3.48E+00	9.31E-05	1.69E-03
ENSDARP00000124561	0.63569	3.48E+00	9.31E-05	1.69E-03
ENSDARP00000025613	6.10776	-2.34E+00	9.64E-05	1.75E-03
ENSDARP00000057261	3.37114	2.47E+00	1.03E-04	1.87E-03
ENSDARP00000058066	0.24892	-1.44E+08	1.14E-04	2.06E-03
ENSDARP00000086117	0.24892	-1.44E+08	1.14E-04	2.06E-03
ENSDARP00000027914	0.79728	3.29E+00	1.18E-04	2.13E-03
ENSDARP00000105016	2.65659	2.55E+00	1.23E-04	2.20E-03
ENSDARP00000075083	7.26259	2.26E+00	1.30E-04	2.32E-03
ENSDARP00000065995	2.9915	2.48E+00	1.35E-04	2.41E-03
ENSDARP00000083250	1.24038	2.98E+00	1.41E-04	2.51E-03
ENSDARP00000033509	0.19807	-1.44E+08	1.42E-04	2.52E-03
ENSDARP00000068168	0.19807	-1.44E+08	1.42E-04	2.52E-03
ENSDARP00000099479	0.94288	3.14E+00	1.42E-04	2.52E-03
ENSDARP00000106101	2.5781	2.53E+00	1.50E-04	2.65E-03
ENSDARP00000075712	-1.1698	1.44E+08	1.55E-04	2.70E-03
ENSDARP00000083241	-1.1698	1.44E+08	1.55E-04	2.70E-03
ENSDARP00000102161	-1.1698	1.44E+08	1.55E-04	2.70E-03
ENSDARP00000106523	-1.1698	1.44E+08	1.55E-04	2.70E-03
ENSDARP00000108731	-1.1698	1.44E+08	1.55E-04	2.70E-03

ENSDARP00000110407	-1.1698	1.44E+08	1.55E-04	2.70E-03
ENSDARP00000118268	-1.1698	1.44E+08	1.55E-04	2.70E-03
ENSDARP00000124313	-1.1698	1.44E+08	1.55E-04	2.70E-03
ENSDARP00000052273	0.3047	3.61E+00	1.56E-04	2.70E-03
ENSDARP00000104051	0.3047	3.61E+00	1.56E-04	2.70E-03
ENSDARP00000011815	1.07536	3.02E+00	1.65E-04	2.85E-03
ENSDARP00000048292	3.91781	2.33E+00	1.66E-04	2.86E-03
ENSDARP00000114678	3.10931	2.42E+00	1.67E-04	2.87E-03
ENSDARP00000112897	2.99879	2.43E+00	1.69E-04	2.90E-03
ENSDARP00000115979	-0.4266	4.61E+00	1.73E-04	2.97E-03
ENSDARP00000052079	-0.0179	3.93E+00	1.74E-04	2.97E-03
ENSDARP00000065664	-0.0179	3.93E+00	1.74E-04	2.97E-03
ENSDARP00000112555	-0.0179	3.93E+00	1.74E-04	2.97E-03
ENSDARP00000103082	1.45111	2.83E+00	1.74E-04	2.97E-03
ENSDARP00000020993	1.70437	-3.17E+00	1.86E-04	3.17E-03
ENSDARP00000120798	4.78893	2.25E+00	1.95E-04	3.31E-03
ENSDARP00000016945	0.50338	3.35E+00	2.00E-04	3.40E-03
ENSDARP00000062934	3.47798	2.34E+00	2.02E-04	3.41E-03
ENSDARP00000106571	1.30907	2.85E+00	2.04E-04	3.45E-03
ENSDARP00000051064	9.02149	2.18E+00	2.05E-04	3.46E-03
ENSDARP00000065502	0.09012	-1.44E+08	2.21E-04	3.72E-03
ENSDARP00000108210	1.79869	2.64E+00	2.28E-04	3.83E-03
ENSDARP00000091498	4.75101	-2.26E+00	2.33E-04	3.91E-03
ENSDARP00000107618	0.22452	3.53E+00	2.42E-04	4.05E-03
ENSDARP00000008085	6.69058	2.17E+00	2.42E-04	4.05E-03
ENSDARP00000100767	2.01389	2.55E+00	2.60E-04	4.33E-03
ENSDARP00000111757	1.26754	2.81E+00	2.65E-04	4.41E-03
ENSDARP00000112046	6.22848	-2.16E+00	2.82E-04	4.69E-03
ENSDARP00000118396	1.40543	-3.27E+00	2.85E-04	4.72E-03
ENSDARP00000077967	-0.1166	3.83E+00	2.89E-04	4.78E-03
ENSDARP00000107673	-0.1166	3.83E+00	2.89E-04	4.78E-03
ENSDARP00000067094	2.84345	2.35E+00	2.92E-04	4.81E-03
ENSDARP00000093426	3.91134	2.23E+00	3.06E-04	5.04E-03
ENSDARP00000051892	9.91951	2.11E+00	3.11E-04	5.11E-03
ENSDARP00000119035	1.5676	2.65E+00	3.14E-04	5.15E-03
ENSDARP00000121109	-0.5553	4.48E+00	3.16E-04	5.18E-03
ENSDARP00000120304	4.45832	-2.22E+00	3.41E-04	5.56E-03
ENSDARP00000065237	-1.3791	1.44E+08	3.42E-04	5.56E-03
ENSDARP00000104679	-1.3791	1.44E+08	3.42E-04	5.56E-03
ENSDARP00000109993	4.63438	2.16E+00	3.44E-04	5.60E-03
ENSDARP00000120827	2.4697	-2.58E+00	3.48E-04	5.64E-03
ENSDARP00000011585	1.59701	-3.04E+00	3.48E-04	5.64E-03
ENSDARP00000015287	-0.0274	-1.44E+08	3.51E-04	5.68E-03
ENSDARP00000014491	1.33482	2.70E+00	3.64E-04	5.87E-03

ENSDARP00000106450	4.09083	-2.23E+00	3.76E-04	6.05E-03
ENSDARP00000101110	1.43708	2.65E+00	3.79E-04	6.10E-03
ENSDARP00000078928	0.14024	3.44E+00	3.80E-04	6.10E-03
ENSDARP00000010130	0.9253	2.86E+00	4.10E-04	6.57E-03
ENSDARP00000105398	2.37885	2.37E+00	4.12E-04	6.59E-03
ENSDARP00000090943	1.05874	2.78E+00	4.34E-04	6.92E-03
ENSDARP00000034658	6.20619	2.08E+00	4.42E-04	7.05E-03
ENSDARP00000002432	-0.0902	-1.44E+08	4.46E-04	7.08E-03
ENSDARP00000107615	-0.0902	-1.44E+08	4.46E-04	7.08E-03
ENSDARP00000013189	0.35944	3.19E+00	4.48E-04	7.09E-03
ENSDARP00000083450	0.35944	3.19E+00	4.48E-04	7.09E-03
ENSDARP00000107533	1.39902	2.61E+00	4.82E-04	7.61E-03
ENSDARP00000107191	-0.2216	3.72E+00	4.89E-04	7.72E-03
ENSDARP00000069744	0.55058	3.02E+00	4.98E-04	7.84E-03
ENSDARP00000027246	1.97723	2.41E+00	5.29E-04	8.31E-03
ENSDARP00000123867	0.61537	-4.06E+00	5.30E-04	8.31E-03
ENSDARP00000025062	0.99557	-3.44E+00	5.57E-04	8.72E-03
ENSDARP00000008648	5.98562	2.04E+00	5.67E-04	8.86E-03
ENSDARP00000030937	1.00993	2.72E+00	5.82E-04	9.06E-03
ENSDARP00000119144	1.00993	2.72E+00	5.82E-04	9.06E-03
ENSDARP00000016261	-0.6948	4.35E+00	5.93E-04	9.19E-03
ENSDARP00000082978	-0.6948	4.35E+00	5.93E-04	9.19E-03
ENSDARP00000112103	-0.6948	4.35E+00	5.93E-04	9.19E-03
ENSDARP00000081101	1.2523	2.61E+00	6.07E-04	9.39E-03
ENSDARP00000123715	2.90136	-2.32E+00	6.48E-04	1.00E-02
ENSDARP00000124045	2.97157	-2.30E+00	6.54E-04	1.01E-02
ENSDARP00000100241	6.16351	-2.02E+00	6.73E-04	1.04E-02
ENSDARP00000071301	3.06835	2.15E+00	7.13E-04	1.10E-02
ENSDARP00000019888	9.25185	1.97E+00	7.15E-04	1.10E-02
ENSDARP00000076803	0.48262	2.95E+00	7.28E-04	1.12E-02
ENSDARP00000025967	-0.2257	-1.44E+08	7.31E-04	1.12E-02
ENSDARP00000008048	4.97476	2.02E+00	7.39E-04	1.13E-02
ENSDARP00000111877	4.5951	2.03E+00	7.41E-04	1.13E-02
ENSDARP00000112139	0.93651	-3.37E+00	7.49E-04	1.14E-02
ENSDARP00000102862	2.78495	2.18E+00	7.49E-04	1.14E-02
ENSDARP00000104735	0.65913	2.83E+00	7.58E-04	1.15E-02
ENSDARP00000042828	0.81694	2.74E+00	7.75E-04	1.17E-02
ENSDARP00000109483	0.81694	2.74E+00	7.75E-04	1.17E-02
ENSDARP00000073353	1.51921	2.45E+00	7.78E-04	1.17E-02
ENSDARP00000067827	0.95961	2.67E+00	7.84E-04	1.18E-02
ENSDARP00000064106	-1.619	1.44E+08	7.87E-04	1.18E-02
ENSDARP00000086813	-0.3337	3.61E+00	8.42E-04	1.26E-02
ENSDARP00000038172	4.52178	2.00E+00	9.12E-04	1.37E-02
ENSDARP00000077546	-0.299	-1.44E+08	9.43E-04	1.41E-02

ENSDARP00000095567	-0.299	-1.44E+08	9.43E-04	1.41E-02
ENSDARP00000114964	-0.299	-1.44E+08	9.43E-04	1.41E-02
ENSDARP00000063643	2.76433	-2.26E+00	9.63E-04	1.43E-02
ENSDARP00000021771	2.69735	2.13E+00	1.04E-03	1.55E-02
ENSDARP00000099497	0.20176	3.02E+00	1.05E-03	1.55E-02
ENSDARP00000123755	0.20176	3.02E+00	1.05E-03	1.55E-02
ENSDARP00000089342	3.61935	2.02E+00	1.05E-03	1.55E-02
ENSDARP00000051611	1.39663	-2.81E+00	1.06E-03	1.56E-02
ENSDARP00000062382	0.90768	2.61E+00	1.06E-03	1.57E-02
ENSDARP00000111226	0.41175	2.87E+00	1.07E-03	1.58E-02
ENSDARP00000115942	0.41175	2.87E+00	1.07E-03	1.58E-02
ENSDARP00000091697	0.59595	2.76E+00	1.08E-03	1.59E-02
ENSDARP00000103660	3.03699	2.07E+00	1.09E-03	1.60E-02
ENSDARP00000110645	1.4465	2.37E+00	1.22E-03	1.78E-02
ENSDARP00000099827	2.14934	2.17E+00	1.31E-03	1.91E-02
ENSDARP00000092299	2.40449	2.12E+00	1.32E-03	1.93E-02
ENSDARP00000123307	2.98996	-2.13E+00	1.38E-03	2.01E-02
ENSDARP00000083836	2.02313	2.19E+00	1.39E-03	2.01E-02
ENSDARP00000095608	2.02313	2.19E+00	1.39E-03	2.01E-02
ENSDARP00000104469	7.22698	1.86E+00	1.41E-03	2.03E-02
ENSDARP00000121274	1.88504	2.20E+00	1.49E-03	2.15E-02
ENSDARP00000087847	3.97322	1.92E+00	1.55E-03	2.24E-02
ENSDARP00000109456	2.67379	2.04E+00	1.58E-03	2.28E-02
ENSDARP00000113212	0.33772	2.79E+00	1.60E-03	2.29E-02
ENSDARP00000123631	-0.1417	3.14E+00	1.60E-03	2.29E-02
ENSDARP00000077208	-0.4589	-1.44E+08	1.60E-03	2.29E-02
ENSDARP00000083553	-0.4589	-1.44E+08	1.60E-03	2.29E-02
ENSDARP00000093328	-0.4589	-1.44E+08	1.60E-03	2.29E-02
ENSDARP00000109134	0.36392	-3.78E+00	1.61E-03	2.30E-02
ENSDARP00000066947	0.77629	-3.19E+00	1.62E-03	2.31E-02
ENSDARP00000111864	1.97993	-2.37E+00	1.64E-03	2.33E-02
ENSDARP00000014290	1.06675	-2.88E+00	1.70E-03	2.41E-02
ENSDARP00000012691	4.27931	1.89E+00	1.70E-03	2.42E-02
ENSDARP00000093637	12.0482	1.82E+00	1.71E-03	2.42E-02
ENSDARP00000070225	4.25286	-1.93E+00	1.72E-03	2.43E-02
ENSDARP00000117352	2.396	2.05E+00	1.89E-03	2.67E-02
ENSDARP00000029754	-1.9006	1.44E+08	1.91E-03	2.68E-02
ENSDARP00000046158	-1.9006	1.44E+08	1.91E-03	2.68E-02
ENSDARP00000098538	-1.9006	1.44E+08	1.91E-03	2.68E-02
ENSDARP00000125242	-1.9006	1.44E+08	1.91E-03	2.68E-02
ENSDARP00000050204	5.4048	1.83E+00	1.93E-03	2.71E-02
ENSDARP00000055175	0.31696	-3.73E+00	1.96E-03	2.73E-02
ENSDARP00000099577	0.31696	-3.73E+00	1.96E-03	2.73E-02
ENSDARP00000077930	1.26398	2.30E+00	2.05E-03	2.86E-02

ENSDARP00000087215	2.42682	2.02E+00	2.07E-03	2.88E-02
ENSDARP00000115619	-0.5467	-1.44E+08	2.11E-03	2.93E-02
ENSDARP00000115183	0.63963	2.54E+00	2.11E-03	2.93E-02
ENSDARP00000076487	3.40807	1.90E+00	2.14E-03	2.96E-02
ENSDARP00000076580	3.05215	1.93E+00	2.19E-03	3.03E-02
ENSDARP00000121954	0.70638	-3.11E+00	2.23E-03	3.08E-02
ENSDARP00000094160	1.01053	-2.82E+00	2.24E-03	3.09E-02
ENSDARP00000067496	0.46185	2.61E+00	2.25E-03	3.10E-02
ENSDARP00000020968	-1.0146	4.02E+00	2.26E-03	3.10E-02
ENSDARP00000089247	-1.0146	4.02E+00	2.26E-03	3.10E-02
ENSDARP00000027770	4.19655	1.84E+00	2.32E-03	3.18E-02
ENSDARP00000052292	1.02535	2.35E+00	2.34E-03	3.20E-02
ENSDARP00000114221	1.02535	2.35E+00	2.34E-03	3.20E-02
ENSDARP00000046950	2.53364	1.98E+00	2.36E-03	3.22E-02
ENSDARP00000072334	2.64988	1.96E+00	2.37E-03	3.23E-02
ENSDARP00000034528	0.26827	-3.68E+00	2.39E-03	3.25E-02
ENSDARP00000078912	0.26025	2.70E+00	2.40E-03	3.25E-02
ENSDARP00000083020	0.26025	2.70E+00	2.40E-03	3.25E-02
ENSDARP00000070780	5.09405	1.80E+00	2.41E-03	3.26E-02
ENSDARP00000058289	2.48652	1.98E+00	2.46E-03	3.32E-02
ENSDARP00000073458	4.96667	1.79E+00	2.51E-03	3.39E-02
ENSDARP00000119517	6.51782	1.76E+00	2.51E-03	3.39E-02
ENSDARP00000073325	3.14094	1.89E+00	2.52E-03	3.39E-02
ENSDARP00000012808	0.89001	2.38E+00	2.52E-03	3.39E-02
ENSDARP00000015318	9.58352	1.75E+00	2.54E-03	3.40E-02
ENSDARP00000028515	0.02758	2.83E+00	2.54E-03	3.41E-02
ENSDARP00000030948	0.9815	-2.78E+00	2.58E-03	3.45E-02
ENSDARP00000028689	5.98813	1.76E+00	2.61E-03	3.48E-02
ENSDARP00000008668	0.66999	-3.06E+00	2.63E-03	3.51E-02
ENSDARP00000012969	-0.5832	3.35E+00	2.63E-03	3.51E-02
ENSDARP00000064901	1.22165	2.25E+00	2.64E-03	3.51E-02
ENSDARP00000052540	-0.2473	3.02E+00	2.65E-03	3.52E-02
ENSDARP00000065639	-0.2473	3.02E+00	2.65E-03	3.52E-02
ENSDARP00000087097	3.17212	-1.95E+00	2.73E-03	3.62E-02
ENSDARP00000073893	1.21157	-2.59E+00	2.76E-03	3.65E-02
ENSDARP00000018865	-0.6407	-1.44E+08	2.79E-03	3.67E-02
ENSDARP00000022543	-0.6407	-1.44E+08	2.79E-03	3.67E-02
ENSDARP00000111718	-0.6407	-1.44E+08	2.79E-03	3.67E-02
ENSDARP00000121464	-0.6407	-1.44E+08	2.79E-03	3.67E-02
ENSDARP00000020172	3.99117	1.81E+00	2.82E-03	3.70E-02
ENSDARP00000101957	1.10382	2.27E+00	2.85E-03	3.74E-02
ENSDARP00000088503	0.95183	-2.75E+00	2.97E-03	3.88E-02
ENSDARP00000011426	4.06304	-1.83E+00	2.97E-03	3.89E-02
ENSDARP00000066792	3.10475	-1.94E+00	2.98E-03	3.89E-02

ENSDARP00000114194	1.56378	-2.35E+00	3.22E-03	4.20E-02
ENSDARP00000100195	7.65357	-1.71E+00	3.23E-03	4.20E-02
ENSDARP00000052239	3.24716	-1.90E+00	3.23E-03	4.20E-02
ENSDARP00000031444	2.16673	1.97E+00	3.26E-03	4.22E-02
ENSDARP00000112091	6.55209	-1.72E+00	3.31E-03	4.29E-02
ENSDARP00000006457	1.17819	2.19E+00	3.40E-03	4.39E-02
ENSDARP00000087122	1.17819	2.19E+00	3.40E-03	4.39E-02
ENSDARP00000022947	1.81674	2.02E+00	3.42E-03	4.40E-02
ENSDARP00000099980	0.92148	-2.71E+00	3.42E-03	4.40E-02
ENSDARP00000036317	2.39034	-2.04E+00	3.42E-03	4.40E-02
ENSDARP00000072799	2.34711	1.92E+00	3.45E-03	4.44E-02
ENSDARP00000008466	5.07	-1.74E+00	3.51E-03	4.50E-02
ENSDARP00000113718	0.17902	2.61E+00	3.63E-03	4.66E-02
ENSDARP00000107543	0.59407	-2.98E+00	3.67E-03	4.69E-02
ENSDARP00000079658	1.0569	2.21E+00	3.73E-03	4.75E-02
ENSDARP00000063487	-0.7418	-1.44E+08	3.73E-03	4.75E-02
ENSDARP00000074288	-0.7418	-1.44E+08	3.73E-03	4.75E-02
ENSDARP00000111299	-0.7418	-1.44E+08	3.73E-03	4.75E-02
ENSDARP00000021046	0.68176	2.35E+00	3.79E-03	4.80E-02
ENSDARP00000083725	0.68176	2.35E+00	3.79E-03	4.80E-02
ENSDARP00000054055	2.99426	1.82E+00	3.86E-03	4.89E-02

South36 vs South22				
ENSMBL ID	**logConc**	**logFC**	**PValue**	**FDR**
ENSDARP00000072746	4.45822	-1.44E+08	1.69E-10	1.52E-06
ENSDARP00000068664	4.14283	-8.08E+00	9.29E-09	2.40E-05
ENSDARP00000096458	3.36401	-1.44E+08	9.72E-09	2.40E-05
ENSDARP00000107225	3.33974	-1.44E+08	1.06E-08	2.40E-05
ENSDARP00000108801	3.14339	-1.44E+08	2.17E-08	3.92E-05
ENSDARP00000109645	3.08618	-1.44E+08	2.67E-08	4.02E-05
ENSDARP00000056187	2.98028	-1.44E+08	3.92E-08	4.42E-05
ENSDARP00000099572	2.98028	-1.44E+08	3.92E-08	4.42E-05
ENSDARP00000071067	4.13417	-7.07E+00	4.62E-08	4.63E-05
ENSDARP00000067126	2.89959	-1.44E+08	5.24E-08	4.72E-05
ENSDARP00000059242	2.76031	-1.44E+08	8.63E-08	7.08E-05
ENSDARP00000006884	2.72329	-1.44E+08	9.85E-08	7.40E-05
ENSDARP00000085405	2.6853	-1.44E+08	1.13E-07	7.82E-05
ENSDARP00000069926	2.60618	-1.44E+08	1.49E-07	9.62E-05
ENSDARP00000118733	2.56494	-1.44E+08	1.73E-07	1.04E-04
ENSDARP00000046575	2.50078	-1.44E+08	2.17E-07	1.22E-04
ENSDARP00000067188	2.45638	-1.44E+08	2.54E-07	1.35E-04
ENSDARP00000048886	2.41057	-1.44E+08	2.98E-07	1.49E-04
ENSDARP00000106411	2.33901	-1.44E+08	3.83E-07	1.82E-04
ENSDARP00000052433	2.28927	-1.44E+08	4.55E-07	2.04E-04

ENSDARP00000003429	2.26374	-1.44E+08	4.98E-07	2.04E-04
ENSDARP00000113607	2.26374	-1.44E+08	4.98E-07	2.04E-04
ENSDARP00000075491	3.38258	-6.31E+00	7.37E-07	2.89E-04
ENSDARP00000077314	2.12889	-1.44E+08	7.94E-07	2.98E-04
ENSDARP00000045762	1.9802	-1.44E+08	1.32E-06	4.68E-04
ENSDARP00000010852	4.98294	-5.30E+00	1.36E-06	4.68E-04
ENSDARP00000090277	3.2059	-6.13E+00	1.40E-06	4.68E-04
ENSDARP00000094438	3.36798	-5.70E+00	2.50E-06	8.06E-04
ENSDARP00000015367	1.779	-1.44E+08	2.61E-06	8.12E-04
ENSDARP00000052347	2.98907	-5.92E+00	3.06E-06	9.20E-04
ENSDARP00000046111	2.53935	-6.48E+00	3.18E-06	9.26E-04
ENSDARP00000093777	2.95754	-5.89E+00	3.42E-06	9.52E-04
ENSDARP00000108906	2.94152	-5.87E+00	3.63E-06	9.52E-04
ENSDARP00000109876	2.94152	-5.87E+00	3.63E-06	9.52E-04
ENSDARP00000118396	3.25575	-5.59E+00	3.76E-06	9.52E-04
ENSDARP00000111299	1.66689	-1.44E+08	3.80E-06	9.52E-04
ENSDARP00000033391	6.29302	-4.72E+00	5.62E-06	1.37E-03
ENSDARP00000015118	2.69622	-5.62E+00	8.68E-06	2.03E-03
ENSDARP00000052540	4.48185	-4.79E+00	8.76E-06	2.03E-03
ENSDARP00000063937	1.31732	-1.44E+08	1.19E-05	2.62E-03
ENSDARP00000078928	4.75435	-4.64E+00	1.19E-05	2.62E-03
ENSDARP00000006015	3.59331	-4.91E+00	1.25E-05	2.68E-03
ENSDARP00000109744	1.7194	1.44E+08	1.33E-05	2.79E-03
ENSDARP00000074422	2.8887	-5.22E+00	1.40E-05	2.87E-03
ENSDARP00000070746	4.77155	-4.56E+00	1.49E-05	2.98E-03
ENSDARP00000125522	4.5068	-4.58E+00	1.60E-05	3.10E-03
ENSDARP00000024490	1.66054	1.44E+08	1.61E-05	3.10E-03
ENSDARP00000098698	1.21506	-1.44E+08	1.65E-05	3.10E-03
ENSDARP00000107533	5.59397	-4.42E+00	1.78E-05	3.27E-03
ENSDARP00000046158	3.24998	-4.83E+00	2.17E-05	3.92E-03
ENSDARP00000064081	1.10511	-1.44E+08	2.34E-05	4.13E-03
ENSDARP00000110478	3.60566	4.76E+00	2.60E-05	4.45E-03
ENSDARP00000094167	4.69115	-4.39E+00	2.61E-05	4.45E-03
ENSDARP00000116362	1.50179	1.44E+08	2.69E-05	4.50E-03
ENSDARP00000083250	5.3858	-4.30E+00	2.81E-05	4.58E-03
ENSDARP00000123631	4.259	-4.43E+00	2.84E-05	4.58E-03
ENSDARP00000105396	4.14616	-4.44E+00	3.00E-05	4.75E-03
ENSDARP00000054471	3.78811	-4.50E+00	3.17E-05	4.93E-03
ENSDARP00000104679	3.4954	-4.57E+00	3.26E-05	4.98E-03
ENSDARP00000113601	2.30394	-5.23E+00	3.41E-05	5.13E-03
ENSDARP00000074864	4.23794	-4.30E+00	4.29E-05	6.35E-03
ENSDARP00000049573	1.77188	-5.72E+00	4.43E-05	6.45E-03
ENSDARP00000002618	5.07222	-4.18E+00	4.52E-05	6.47E-03
ENSDARP00000124390	3.67629	-4.38E+00	4.75E-05	6.70E-03

ENSDARP00000099980	2.53027	-4.85E+00	4.94E-05	6.85E-03
ENSDARP00000119943	3.65678	-4.36E+00	5.10E-05	6.97E-03
ENSDARP00000018505	2.50871	-4.83E+00	5.32E-05	7.17E-03
ENSDARP00000113738	4.25601	-4.21E+00	5.50E-05	7.26E-03
ENSDARP00000011150	5.43989	-4.08E+00	5.56E-05	7.26E-03
ENSDARP00000029754	2.97879	-4.55E+00	5.70E-05	7.34E-03
ENSDARP00000119212	1.24491	1.44E+08	6.04E-05	7.66E-03
ENSDARP00000065502	0.78761	-1.44E+08	6.20E-05	7.66E-03
ENSDARP00000112281	0.78761	-1.44E+08	6.20E-05	7.66E-03
ENSDARP00000049419	4.80461	-4.08E+00	6.80E-05	8.29E-03
ENSDARP00000087122	4.94923	-4.05E+00	7.09E-05	8.53E-03
ENSDARP00000041589	6.07449	-3.97E+00	7.35E-05	8.72E-03
ENSDARP00000051529	3.65456	-4.20E+00	8.03E-05	9.40E-03
ENSDARP00000099275	3.52313	-4.22E+00	8.24E-05	9.52E-03
ENSDARP00000013036	4.88776	-3.98E+00	8.88E-05	1.00E-02
ENSDARP00000115525	1.11884	1.44E+08	8.89E-05	1.00E-02
ENSDARP00000064825	3.44386	4.32E+00	9.17E-05	1.01E-02
ENSDARP00000110223	4.87515	-3.97E+00	9.30E-05	1.01E-02
ENSDARP00000075344	6.7618	-3.87E+00	9.34E-05	1.01E-02
ENSDARP00000103159	1.53729	-5.48E+00	9.60E-05	1.02E-02
ENSDARP00000100857	0.63852	-1.44E+08	9.66E-05	1.02E-02
ENSDARP00000011815	4.91171	-3.95E+00	9.71E-05	1.02E-02
ENSDARP00000121686	2.3236	-4.64E+00	1.01E-04	1.04E-02
ENSDARP00000024380	1.07421	1.44E+08	1.02E-04	1.04E-02
ENSDARP00000121109	3.68159	-4.08E+00	1.10E-04	1.11E-02
ENSDARP00000105167	1.02812	1.44E+08	1.17E-04	1.16E-02
ENSDARP00000110479	1.02812	1.44E+08	1.17E-04	1.16E-02
ENSDARP00000099465	0.5579	-1.44E+08	1.22E-04	1.17E-02
ENSDARP00000114198	0.5579	-1.44E+08	1.22E-04	1.17E-02
ENSDARP00000114964	0.5579	-1.44E+08	1.22E-04	1.17E-02
ENSDARP00000123185	3.35182	-4.04E+00	1.52E-04	1.42E-02
ENSDARP00000020968	3.21487	-4.08E+00	1.52E-04	1.42E-02
ENSDARP00000058644	0.9312	1.44E+08	1.56E-04	1.43E-02
ENSDARP00000018865	0.47262	-1.44E+08	1.56E-04	1.43E-02
ENSDARP00000124544	0.47262	-1.44E+08	1.56E-04	1.43E-02
ENSDARP00000095092	4.85088	-3.78E+00	1.70E-04	1.53E-02
ENSDARP00000077647	3.42021	-3.95E+00	1.85E-04	1.65E-02
ENSDARP00000021244	4.28712	-3.81E+00	1.87E-04	1.65E-02
ENSDARP00000034815	4.51053	-3.76E+00	1.98E-04	1.72E-02
ENSDARP00000083025	5.63652	-3.68E+00	1.99E-04	1.72E-02
ENSDARP00000020894	7.01693	-3.64E+00	2.00E-04	1.72E-02
ENSDARP00000064110	7.62563	-3.62E+00	2.08E-04	1.76E-02
ENSDARP00000076347	5.25044	-3.69E+00	2.09E-04	1.76E-02
ENSDARP00000124531	4.6896	-3.72E+00	2.17E-04	1.82E-02

ENSDARP00000120991	4.55093	-3.68E+00	2.51E-04	2.07E-02
ENSDARP00000112654	2.05313	-4.36E+00	2.52E-04	2.07E-02
ENSDARP00000100863	2.13055	4.58E+00	2.56E-04	2.08E-02
ENSDARP00000059319	0.28572	-1.44E+08	2.65E-04	2.11E-02
ENSDARP00000077546	0.28572	-1.44E+08	2.65E-04	2.11E-02
ENSDARP00000103553	4.1064	-3.69E+00	2.81E-04	2.22E-02
ENSDARP00000047072	0.71489	1.44E+08	2.92E-04	2.29E-02
ENSDARP00000065237	2.85712	-3.91E+00	3.11E-04	2.42E-02
ENSDARP00000061820	2.67972	-3.96E+00	3.18E-04	2.44E-02
ENSDARP00000066932	3.92633	-3.67E+00	3.20E-04	2.44E-02
ENSDARP00000112765	1.15114	-5.09E+00	3.28E-04	2.49E-02
ENSDARP00000094053	2.98417	-3.84E+00	3.40E-04	2.51E-02
ENSDARP00000076298	5.72036	-3.51E+00	3.41E-04	2.51E-02
ENSDARP00000068557	0.65522	1.44E+08	3.45E-04	2.51E-02
ENSDARP00000121041	0.65522	1.44E+08	3.45E-04	2.51E-02
ENSDARP00000122224	0.65522	1.44E+08	3.45E-04	2.51E-02
ENSDARP00000111718	0.1826	-1.44E+08	3.52E-04	2.51E-02
ENSDARP00000121844	0.1826	-1.44E+08	3.52E-04	2.51E-02
ENSDARP00000092547	5.13797	-3.53E+00	3.53E-04	2.51E-02
ENSDARP00000086061	6.17596	-3.48E+00	3.62E-04	2.55E-02
ENSDARP00000080103	3.10093	-3.78E+00	3.66E-04	2.56E-02
ENSDARP00000105205	2.95256	-3.80E+00	3.80E-04	2.62E-02
ENSDARP00000100455	3.43805	-3.69E+00	3.80E-04	2.62E-02
ENSDARP00000108026	1.09484	-5.04E+00	3.91E-04	2.65E-02
ENSDARP00000124072	7.21294	-3.44E+00	3.92E-04	2.65E-02
ENSDARP00000027138	6.86997	-3.44E+00	3.94E-04	2.65E-02
ENSDARP00000122818	5.98242	-3.45E+00	4.03E-04	2.69E-02
ENSDARP00000019643	3.85966	-3.60E+00	4.05E-04	2.69E-02
ENSDARP00000083450	3.92448	-3.58E+00	4.18E-04	2.75E-02
ENSDARP00000061121	3.18193	-3.70E+00	4.28E-04	2.80E-02
ENSDARP00000070407	2.29926	4.15E+00	4.51E-04	2.93E-02
ENSDARP00000113136	5.0658	-3.45E+00	4.58E-04	2.95E-02
ENSDARP00000059419	1.0363	-4.98E+00	4.68E-04	2.97E-02
ENSDARP00000063477	1.0363	-4.98E+00	4.68E-04	2.97E-02
ENSDARP00000004016	0.07178	-1.44E+08	4.74E-04	2.97E-02
ENSDARP00000058066	0.07178	-1.44E+08	4.74E-04	2.97E-02
ENSDARP00000123891	2.36209	-3.90E+00	4.81E-04	2.99E-02
ENSDARP00000097277	0.52774	1.44E+08	4.92E-04	3.02E-02
ENSDARP00000104735	0.52774	1.44E+08	4.92E-04	3.02E-02
ENSDARP00000090527	1.92088	4.36E+00	5.09E-04	3.10E-02
ENSDARP00000075677	6.24976	-3.37E+00	5.21E-04	3.14E-02
ENSDARP00000110350	2.99632	-3.67E+00	5.26E-04	3.14E-02
ENSDARP00000060257	6.40288	-3.36E+00	5.27E-04	3.14E-02
ENSDARP00000054387	3.85772	-3.51E+00	5.30E-04	3.14E-02

ENSDARP00000042477	5.77317	-3.37E+00	5.42E-04	3.18E-02
ENSDARP00000107191	3.43549	-3.56E+00	5.44E-04	3.18E-02
ENSDARP00000013189	3.84915	-3.50E+00	5.46E-04	3.18E-02
ENSDARP00000040327	4.72926	-3.41E+00	5.57E-04	3.22E-02
ENSDARP00000106068	4.68771	-3.41E+00	5.61E-04	3.22E-02
ENSDARP00000124358	2.31325	-3.85E+00	5.68E-04	3.23E-02
ENSDARP00000091840	6.96113	-3.32E+00	5.70E-04	3.23E-02
ENSDARP00000031386	1.40567	4.85E+00	5.82E-04	3.28E-02
ENSDARP00000071779	4.45988	-3.41E+00	5.87E-04	3.28E-02
ENSDARP00000088018	0.4594	1.44E+08	5.92E-04	3.28E-02
ENSDARP00000123741	0.4594	1.44E+08	5.92E-04	3.28E-02
ENSDARP00000022543	-0.048	-1.44E+08	6.50E-04	3.57E-02
ENSDARP00000102816	5.68068	-3.31E+00	6.58E-04	3.60E-02
ENSDARP00000117964	6.09228	-3.29E+00	6.77E-04	3.68E-02
ENSDARP00000068317	3.92075	-3.41E+00	6.88E-04	3.70E-02
ENSDARP00000092831	2.91669	-3.58E+00	6.93E-04	3.70E-02
ENSDARP00000108836	5.15672	-3.31E+00	6.96E-04	3.70E-02
ENSDARP00000109090	7.70498	-3.25E+00	7.01E-04	3.70E-02
ENSDARP00000109483	4.09979	-3.39E+00	7.01E-04	3.70E-02
ENSDARP00000115027	0.38758	1.44E+08	7.18E-04	3.76E-02
ENSDARP00000074552	4.5329	-3.34E+00	7.26E-04	3.79E-02
ENSDARP00000022134	2.90023	-3.56E+00	7.33E-04	3.80E-02
ENSDARP00000016318	3.6111	-3.43E+00	7.39E-04	3.80E-02
ENSDARP00000113772	3.02365	-3.53E+00	7.41E-04	3.80E-02
ENSDARP00000078815	3.75142	-3.39E+00	7.70E-04	3.92E-02
ENSDARP00000094626	3.22962	-3.47E+00	7.87E-04	3.99E-02
ENSDARP00000089064	6.70872	-3.22E+00	8.05E-04	4.06E-02
ENSDARP00000010992	6.86418	-3.21E+00	8.33E-04	4.17E-02
ENSDARP00000081101	4.40111	-3.29E+00	8.50E-04	4.24E-02
ENSDARP00000053774	3.78569	-3.35E+00	8.74E-04	4.32E-02
ENSDARP00000069316	0.31189	1.44E+08	8.76E-04	4.32E-02
ENSDARP00000106234	7.76128	-3.16E+00	9.46E-04	4.64E-02
ENSDARP00000063364	5.32277	-3.20E+00	9.51E-04	4.64E-02
ENSDARP00000112726	5.43252	-3.20E+00	9.58E-04	4.65E-02
ENSDARP00000124424	1.92257	-3.79E+00	9.65E-04	4.66E-02
ENSDARP00000021053	3.26579	-3.38E+00	9.81E-04	4.69E-02
ENSDARP00000006457	4.21818	-3.26E+00	9.82E-04	4.69E-02
ENSDARP00000111809	2.92943	-3.43E+00	1.02E-03	4.81E-02
ENSDARP00000032722	0.77559	-4.72E+00	1.02E-03	4.81E-02
ENSDARP00000121228	3.51695	-3.32E+00	1.03E-03	4.81E-02
ENSDARP00000062934	6.35547	-3.15E+00	1.03E-03	4.81E-02
ENSDARP00000124947	3.43036	-3.33E+00	1.04E-03	4.81E-02
ENSDARP00000106107	3.58839	-3.31E+00	1.05E-03	4.81E-02
ENSDARP00000104051	3.65645	-3.29E+00	1.07E-03	4.81E-02

ENSDARP00000042900	0.23191	1.44E+08	1.08E-03	4.81E-02
ENSDARP00000085952	0.23191	1.44E+08	1.08E-03	4.81E-02
ENSDARP00000099455	0.23191	1.44E+08	1.08E-03	4.81E-02
ENSDARP00000100432	0.23191	1.44E+08	1.08E-03	4.81E-02
ENSDARP00000109467	0.23191	1.44E+08	1.08E-03	4.81E-02
ENSDARP00000111622	0.23191	1.44E+08	1.08E-03	4.81E-02