Stability of an evolutionary relevant behavioural trait in fish: impact of environmental and genetic variation on behaviour and physiology

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Contents

Acknowledgments

weirdness and bringing a smile to my face, and to my old mates from Bangor, Swansea and home who've kept me going.

Thanks, finally, to all my lovely rainbow trout. I'm going to miss you guys.

Abbreviations

$5HT_{1A}$	5-hydroxytryptamine (serotonin) receptor 1A
AANAT	Aralkylamine-*N*-acetyltransferase
ACTH	Adrenocorticotrophic hormone
ASR	Aquatic surface respiration
AVT	Arginine vasotocin
CaM	Calmodulin
CaN	Calcineurin
CRF	Corticotrophic releasing factor
Epd	Ependymin
$GABA_A$	γ-aminobutyric acid A
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
$Hb\alpha4$	Haemoglobin alpha 4 subunit
HPA	Hypothalamo-pituitary-adrenal
HPI	Hypothalamo-pituitary-interrenal
$MC2R/MC_2$	Melanocortin 2 receptor
MCH	Melanin concentrating hormone
Mel_{1A}	Melatonin receptor 1A
MHCI	Major histocompatability complex I
MR	Mineralocorticoid receptor
MSH	Melanocyte stimulating hormone
POMC	Proopiomelanocortin
RBP	Retinol binding protein
UI	Urotensin I

1 General Introduction

1.1 Abstract

Personality in animals describes a suite of correlated behavioural traits that distinguishes one individual from another. One important personality measure is boldness, which is related to intraspecific differences in responses to novelty, levels of activity and aggressiveness, propensity for exploration, and capacity for learning and memory. Personality and boldness have previously been linked with physiological responses to stress, termed coping styles, wherein lower stress responsiveness often correlates with bolder behaviour. Variation in these traits has important implications for how animals respond to environmental challenges. Personality and stress responsiveness are both also partly heritable; a greater understanding of the genetic control of behavioural and physiological traits is therefore necessary to understand how individual differences are maintained in the face of natural selection, and how genes can control changes in behaviour and physiology. Boldness and behavioural plasticity was therefore examined in the rainbow trout, *Oncorhynchus mykiss* Walbaum, which provides an excellent model since the behaviour and physiology of this economically important species has been well-studied. Throughout, boldness was assessed using novel object tests, where latency to approach to within 5 cm of an object was the primary determinant. Trout which approached within 180 s were considered bold, those which did not approach within 300 s as shy, and the remainder as intermediate which were discarded from analysis. Boldness was consistent over time in two lines of rainbow trout bred for a divergent response to stress, but no correlation was found between boldness and either stress responsiveness or gene expression, in contrast to expectations. Stress responsiveness was, however, strongly linked with gene expression: a suite of candidate genes was uniformly upregulated in low stress-responding trout compared to high-responders, suggesting fine control of hormones and receptors throughout the stress response may occur downstream of gene expression. Outbred bold rainbow trout placed into a group of either completely bold or completely shy trout tended to become shyer, whereas initially shy trout did not display any behavioural plasticity. Likewise, bold trout exposed to predation threat in combination with different levels of feed availability modified their behaviour seemingly dependent upon the combination of risk level and internal state. Shy trout, again, were less labile. This is in contrast to coping style theory which suggests shy, reactive animals have more

behavioural flexibility than bold, proactive individuals. When exposed to variable abiotic factors - increased temperature and reduced dissolved oxygen content - both bold and shy fish exhibited some behavioural change dependent upon the combination of factors. When exposed to these stressors and challenges, plasma cortisol levels more closely matched behavioural profiles such that shy trout generally had a greater stress response than bold trout, and this could be linked to exposure to threat or temperature change. Furthermore, gene expression profiles corresponded with predation threat: genes coding for corticotrophin releasing factor (CRF), ependymin and γ-aminobutyric acid A ($GABA_A$) increased in expression under increasing threat levels, indicating these genes were involved in the response to this particular challenge. These results thus show the existence of personality in rainbow trout, linking responses towards novelty with levels of activity and identifying physiological and genetic correlates to these behavioural traits. Boldness was shown not to be a fixed trait but, instead, dependent upon social, environmental, nutritional and energetic state and on intensity of risk. Bold trout generally altered their behavioural strategy in an adaptive manner according to both context and state yet shy individuals remained shy. These empirical data highlight the importance of taking individual personality into account when assessing molecular, physiological and behavioural responses to stimuli. Furthermore, these data provide new insights into intraspecific variation within a variety of contexts that may be used to fuel theoretical models of the evolutionary and ecological significance of animal personalities.

1.2 - Introduction

1.2.1 Personality & Boldness

Behaviour can vary considerably both inter- (Bergman and Kitchen, 2009; Seferta et al., 2001; Yoshida et al., 2005) and intraspecifically, either within or between groups or populations (Carere and Eens, 2005; Wilson et al., 1994), and even in standard conditions. Whilst such variation has fundamental importance for adaptability to different environmental challenges, the functional significance of behavioural variation remains poorly understood (Réale et al., 2010; Wilson et al., 1994). Initially, behavioural variation was considered random noise around an adaptive mean, but recent studies suggest that, instead, variation describes alternative behavioural strategies which may still be adaptive (Brown et al., 2007; Dall et al., 2004). Often these different strategies may be linked to individual differences in intrinsic characteristics, such as age, size or sex, or states such as hunger (Dall et al., 2004; Wilson et al., 1994), but may also be linked with variation in environmental variables (Réale et al., 2010; Sih et al., 2004a).

Behavioural strategies can frequently be correlated across contexts and/or through time, and as such have been described as animal personalities, behavioural syndromes, behavioural types or temperament behaviours (Brydges et al., 2008; Groothuis and Carere, 2005; Sih et al., 2004a; Wilson et al., 1994); henceforth in this thesis the term 'personality' will be used to describe such a coherent set of correlated behavioural characteristics. Personality can have important consequences since traits adaptive in one context may not be adaptive in another (Bell and Stamps, 2004); personalities can therefore act as evolutionary or developmental constraints if linked behaviours cannot be uncoupled (Bell and Stamps, 2004). However, recent evidence suggests that individual behavioural traits or personalities may be context-specific: whilst an individual may consistently exhibit one set of behavioural characteristics in a foraging situation, for example, it may behave differently when confronted by a predator (Coleman and Wilson, 1998; Wilson and Stevens, 2005). Both domain-generality or context-specificity appear to be expressed amongst animals (Sih et al., 2004a), suggesting each may carry fitness advantages and disadvantages, but it is certainly necessary for behavioural studies to further probe the evolutionary and ecological implications of behavioural correlations.

One important personality is boldness, defined as the propensity for taking risks. Boldness exists along a continuum from bold to shy, where bold animals tend to be more aggressive, more active, more exploratory or dispersive, have a greater capacity for memory and learning, spend more time in the open and, in social animals, spend less time in groups than shy conspecifics (Fraser et al., 2001; Huntingford, 1976; Magnhagen and Staffan, 2005; Short and Petren, 2008; Smith et al., 2009; Sneddon, 2003; Sundström et al., 2004; Verbeek et al., 1996; Ward et al., 2004; Webster et al., 2009). The bold-shy continuum is not limited just to higher vertebrates, but has been demonstrated across most animal taxa including arthropods (arachnids, Johnson and Sih, 2007; crustaceans: Briffa et al., 2008; Vainikka et al., 2011; insects, Wilson et al., 2010), cephalopods (Sinn et al., 2008), reptiles (López et al., 2005; Short and Petren, 2008), birds (Carere et al., 2005; Cockrem, 2007), fish (Conrad et al., 2011), and mammals (Réale et al., 2000; Sluyter et al., 1996; Svartberg et al., 2005) including primates (Bergman and Kitchen, 2009) and humans (Wilson et al., 1994). Boldness is, therefore, an important animal personality with implications across a variety of contexts, yet animals which are bold in one context may not necessarily be bold in another (Coleman and Wilson, 1998; Wilson and Stevens, 2005; Wilson et al., 2010), nor are they necessarily consistently bold throughout ontogeny (Bell and Stamps, 2004; Sinn et al., 2008) since being bold or shy will not always be adaptive.

Animals therefore show a certain degree of behavioural plasticity, termed the reaction norm (Pigliucci, 1996), allowing them to modify their behaviour in response to internal and external cues; whilst some behavioural characteristics or personalities have a hereditary aspect (e.g. Bell and Sih, 2007; Drent et al., 2003), changes throughout ontogeny, along with learning and environmental influences also play important roles in defining individual behaviour (Robinson, 2004; Sih et al., 2004a). When unchallenged, behaviour is likely to remain stable (e.g. mice, *Mus musculus*: Benus et al., 1991; rainbow trout, *Oncorhynchus mykiss*: Frost et al., 2007; great tits, *Parus major*: Groothuis and Carere, 2005; blugill sunfish, *Lepomis macrochirus*: Wilson and Godin, 2009), but an acute environmental stress or stimulus may cause a temporary shift in behaviour; for example, individuals may reduce activity and foraging in response to a potential predator (particularly if from a predator-rich habitat; Carvalho and Del-Claro, 2004; Magurran, 1990) or increase activity as an

escape response from deleterious conditions such as extremes of hypoxia (Johansen et al., 2006; Section 1.3). Chronic or longer term challenges, which may arise through changes in the local environment through different life stages or after migrations, may require more fundamental behavioural adaptations. These may come about through learning, which occurs either through social transmission and copying of other animals (Galef and Laland, 2005; Heyes, 1993); or through individual experience and observing first-hand from the environment (Pigliucci, 1996). These long-term changes are often drawn from a pre-existing behavioural repertoire, and whilst gaining experience can be a slow process it can be facilitated and made more rapid by learning and observing others (Galef and Laland, 2005; Mery and Burns, 2010). However, the relative importance of the roles of learning and of asocial experience depend upon the sociality of the animals, both within and across age-groups (Galef and Laland, 2005). Furthermore, some behavioural changes come about naturally through ontogeny. For example, at some point in growth animals may reach a size-specific immunity from predation (e.g. Ioannou et al., 2008), at which point it becomes more adaptive to be active and spend more time foraging than taking shelter.

Thus, within a species, there will be a large proportion of variation in behaviour driven by the local environment, causing between-population differences through pressure on individual populations throughout life- or evolutionary-history (Dall et al., 2004). Such behavioural variation, and therefore adaptability, is important considering environmental change at all time scales, and particularly important when considering anthropogenic influences on the environment which may expand environmental stressors outside of an animal's adaptive range (Jensen et al., 1993; Landman et al., 2005; Timmerman and Chapman, 2004). However, there are limits to the extent to which animals can modify their behaviour; for instance, plasticity is associated with the cost of maintaining up-to-date information about the local environment and adjusting physiological and behavioural parameters to suit, and phenotypic plasticity is only available over a finite range (DeWitt et al., 1998). Different personalities or coping styles may also show different propensities for behavioural plasticity (Koolhaas et al., 1999; Magnhagen and Staffan, 2005). Indeed, behavioural or phenotypic stability is often considered a trait in its own right (Kralj-Fiser et al., 2007).

1.2.2 Physiology & Coping Styles

When encountering a stressor, appropriate physiological changes can ameliorate or overcome negative effects of stress on the body. These effects occur at all structural levels, leading eventually to whole organism responses until the individual has escaped the stimulus (Pickering and Pottinger, 1995). However, these changes are only adaptive when countering acute stressors; long-term stressors which elicit chronic physiological changes are often detrimental to health, resulting in e.g. retardation of growth, immunosuppression and reducing reproductive capability, and eventually mortality (Pickering, 1993a, b; Pickering and Pottinger, 1995). This is particularly relevant in farming and aquaculture where fish may be exposed to stress as a matter of course (e.g. handling, overcrowding), and where output quality is of prime concern but may be adversely affected (Pottinger, 2001). It is therefore important to obtain a full understanding of the stress response, both physiological and behavioural, to improve standards and output in farming and aquaculture environments, and to understand some of the processes which shape behavioural responses to stressful stimuli.

Stress responses in animals occur in three phases: an initial change in endocrine activity, followed by the actions of these hormones on tissues and the mobilisation of energy resulting, finally, in whole organism effects such as on growth and immune responsiveness (Wendelaar Bonga, 1997). The primary hormonal response is coordinated through two key neuroendocrine pathways, beginning with immediate activation of the sympathetic-chromaffin pathway resulting in the release of catecholamines, principally adrenaline and noradrenaline (epinephrine and norepinephrine) from the chromaffin cells of the kidney/head kidney. This is shortly followed by activation of the hypothalamo-pituitary-interrenal (in fish, or –adrenal in other vertebrates; HPI/A) axis, resulting in adrenal or interrenal secretion of cortisol or corticosterone. Throughout vertebrate taxa different hormonal products of these systems appear to have a more prominent role than others, and in teleostean fish cortisol is the most important of these products (Pickering and Pottinger, 1995; Sumpter, 1997). In fish, the HPI axis (Figure 1.1) is activated by the release of corticotrophin-releasing factor (CRF) and urotensin I (UI) in the hypothalamus which stimulate the secretion of adrenocorticotrophic hormone (ACTH) in the anterior pituitary (Olivereau and Olivereau, 1988). ACTH is a cleavage product of

the precursor molecule proopiomelanocortin (POMC), of which other physiologically important hormones are derived such as the melanocyte stimulating hormones (α-, β- and γ-MSH; Rotllant et al., 2000). Arginine vasotocin (AVT) additively modulates or contributes to the CRF-stimulated secretion of ACTH (Olivereau and Olivereau, 1988), whilst MCH may inhibit the effects of CRF (Baker, 1994). The secretion of ACTH is also regulated through negative feedback by calcineurin (CaN), itself modulated by calmodulin (CaM; Antoni et al., 1994; Shipston et al., 1994), and by the neurotransmitter γ-aminobutyric acid (GABA; Kosaka and Mori, 1961; Makara and Stark, 1974). Binding of melatonin to the melatonin 1A receptor (Mel_{1A}) may also cause the attenuation of CRF release with corresponding effects on ACTH secretion (Konakchieva et al., 1997).

ACTH finally binds with the melanocortin 2 receptor (MC2R or MC_2) to stimulate the release of cortisol in the interrenal (Metz et al., 2005). The effects of cortisol are accomplished through binding to corticoid receptors such as mineralocorticoid (MR) and glucocorticoid receptors (GR and GR2; Bury and Sturm, 2007). MR is known to be involved in regulation of the stress axis through negative feedback (Charmandari et al., 2005; De Kloet et al., 1998), but further roles in teleostean physiology are unknown. Binding of corticosteroids to GR likewise inhibits ACTH and CRF secretion under stress (Charmandari et al., 2005), but its other roles include acclimation to varying environmental hydromineral concentrations (Shaw et al., 2007). The serotonin pathway also has complex interactions with the HPI/A axes: serotonin acts on the anterior pituitary, and possibly the adrenal gland of mammals (or interrenal in fish), to regulate the release of ACTH and cortisol, and the synthesis of serotonin itself may be regulated by corticosteroids (Dinan, 1996; Kreke and Dietrich, 2008). However, the interaction of serotonin and its receptors with the HPI and sympathetic pathways is still not fully understood. The HPI system is thus an extremely complex web of interactions, only touched upon here.
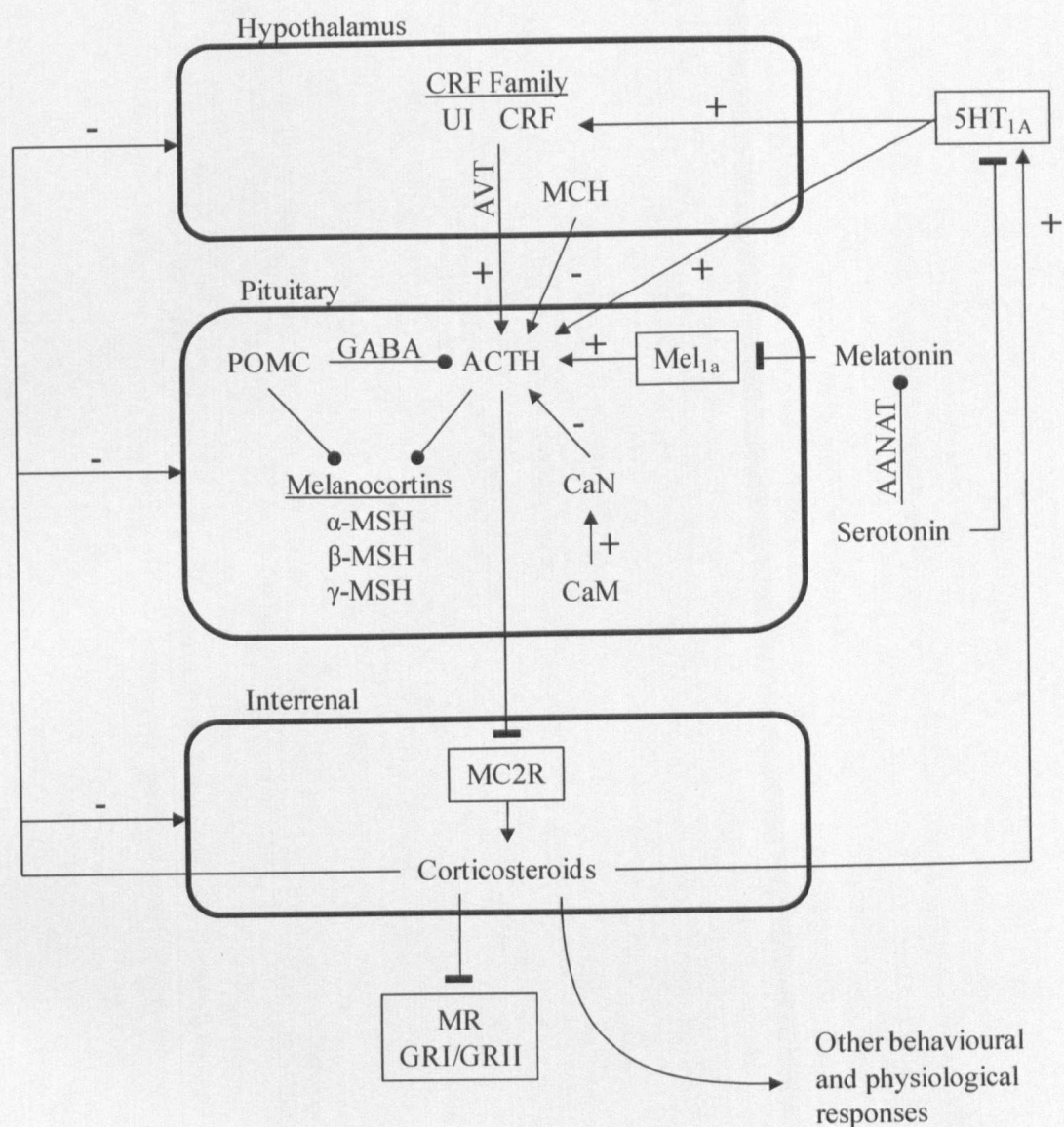
Figure 1.1: Schematic representation of the teleostean hypothalamo-pituitary-interrenal stress axis. Stimulatory and negative inhibitory control are indicated by + and − symbols respectively; flat-headed arrows indicate binding of a ligand to a receptor, and round arrowheads indicate a derivative or product. Boxed elements represent receptors. $5HT_{1A}$ = serotonin 1A receptor; AANAT = aralkylamine N-acetyltransferase; ACTH = adrenocorticotrophic hormone; AVT = arginine vasotocin; CaN = calcineurin; CaM = calmodulin; CRF = corticotrophin releasing factor; GABA = γ-aminobutyric acid; GR = glucocorticoid receptor; MC2R = melanocortin 2 receptor; MCH = melanin concentrating hormone; Mel_{1a} = melatonin receptor 1a; MR = mineralocorticoid receptor; MSH = melanocyte stimulating hormone; POMC = proopiomelanocortin; UI = urotensin I.

Physiological responses to stress vary intraspecifically, with individuals consistently having a low or a high physiological response to a stressor (Pottinger et al., 1992; von Borell and Ladewig, 1992). These physiological differences are linked with personality traits and termed coping styles (Koolhaas et al., 1999). The proactive coping style is typified by a low physiological response to stress combined with increased activity and aggression but a general inflexibility of behaviour; in contrast, reactive coping styles are characterised by a higher physiological response, with passive and submissive temperaments but generally greater behavioural flexibility (Koolhaas et al., 1999). Coping styles have been well characterised in mammals, particularly rodents (Benus et al., 1991; Sgoifo et al., 1996) and also to some extent in birds (Cockrem, 2007), but less so in teleosts; indeed, the association between stress and behaviour in rainbow trout, whilst apparent, is often weak or indeterminate, suggesting the mechanisms are not yet fully understood in these animals (Koolhaas et al., 2010; Schjolden et al., 2005). However, coping styles provide an informative tool to further our understanding of the relationship between behavioural and physiological responses to stressors or challenges.

Individual variation in physiological stress responsiveness is a hereditary trait; early work focussed on poultry which showed divergence in hormonal expression and duration of the response between two inbred lines (Edens and Siegel, 1975), and a similar procedure followed for fish of aquaculture importance. Cortisol proves a useful target for selection in fish due to its importance in the teleostean stress response and its role in a range of deleterious effects associated with stress (Pottinger and Pickering, 1997), and in rainbow trout post-stress cortisol concentrations and response dynamics consistently differ between strains (Pottinger and Moran, 1993). Selection studies found that low and high cortisol responses to a stressor were moderately heritable in this species; lines were successfully bred for their divergent cortisol response to stress, with a heritability, h^2, of 0.41 - 0.73 across four generations (Øverli et al., 2002b; Pottinger and Carrick, 1999, 2001b). These differences were also linked with divergence in serotonin and sympathetic pathway reactivity to stress, indicating heritability of coping style as a whole (Schjolden and Winberg, 2007). Despite this, behavioural analyses of these trout lines have thus far proved inconclusive as to the presence of coping styles (Ruiz-Gomez et al., 2008; Schjolden et al., 2005). However, breeding stress-resistant fish has multiple benefits:

survivability and output (flesh quality and yield) would be improved along with the general health and well-being of the fish (Pottinger and Pickering, 1997). Associated with this would be reduced costs of upkeep in terms of feed wastage and healthcare. The utility of breeding animals for low stress-responsiveness when they will be exposed to stressful environments as a matter of course is therefore obvious.

Moreover, many of the hormones and neurotransmitters involved in stress axes have been linked with roles outside of the physiological stress response itself. For example, arginine vasotocin (AVT; mammals: vasopressin, AVP) is a neuropeptide which modulates a wide variety of social and non-social behaviours (Goodson and Bass, 2001), including those related to memory and learning (Engelmann et al., 1996), a behaviour itself linked to boldness. AVT is, like cortisol, also involved in teleostean osmoregulatory processes (Perrott et al., 1991), and is itself potentially modulated by stress and stress-related glucocorticoids (DeVries et al., 1996). Calmodulin acts as a second messenger, combining with calcium ions to form a Ca^{2+}/CaM complex with a wide variety of functions. It is principally involved in immune system regulation by activation of the Ser-Thr kinases (Racioppi and Means, 2008), or by regulating dendritic cells and the subsequent expression of MHC Class II (Herrmann et al., 2007), which is heavily involved in control of the vertebrate immune response (Götze, 1977). Therefore, a complete understanding of the full stress axis and the roles of the molecules involved may provide a more comprehensive understanding of the behavioural responses to individual stressors.

1.2.3 Behavioural & Physiological Responses to Environmental Stressors
The behavioural and physiological mechanisms described above allow for adaptive responses to the environment. In many instances, however, these mechanisms are still not fully understood, and some responses to environmental challenges are thus described below.

Predation threat is a significant environmental pressure, yet there does not appear to be a single correct behavioural response; instead, the strategy depends on a variety of other factors including state, temporal variations in threat (Dall et al., 2004; Lima and Bednekoff, 1999; Metcalfe et al., 1987), habitat stability (Brydges et al., 2008)

17

and age and size (Magnhagen and Borcherding, 2008; Werner et al., 1983). For instance, if threat is predictable then it would be adaptive to behave shyly and reduce activity at times when threat is high, only resuming activity when the danger has passed (Carvalho and Del-Claro, 2004; Lima and Bednekoff, 1999). However, if threat is unpredictable or prolonged, then individuals must weigh the risk of exposure and activity against the costs of reduced time spent foraging or performing other activities, perhaps becoming bolder and taking risks to do so (Dall et al., 2004; Lima and Bednekoff, 1999). As such, environments exhibiting consistently low or high predation threat often contain populations of animals skewed in their behaviour either towards being mostly bold or mostly shy (Brown et al., 2007; Giles and Huntingford, 1984; Magnhagen and Borcherding, 2008). Predation threat can also drive the development of personality, especially that of a bold-aggressive syndrome (Bell and Sih, 2007; Brick and Jakobsson, 2001).

Whilst threat varies temporally, thus limiting the times at which animals can perform other actions such as foraging, so might the availability of food or the requirements of feeding (Hughes, 1997). Often, patches of high food availability are also linked with higher incidence of predators and of competitors (Hughes, 1997; Vehanen, 2003), and thus potential prey species may feed at patches of inferior quality or quantity but at lower risk of being eaten themselves (Werner et al., 1983). Similarly, chances of food capture throughout the day depend on individual species and their adaptations to diurnal, nocturnal or crepuscular light levels (Hoar, 1942; Vehanen, 2003), and the relative foraging effort at any time of day will depend on the relationship between food capture efficiency and predation risk (Fraser and Metcalfe, 1997; Fraser et al., 1993). The location of greatest profitability is also liable to change, particularly with highly mobile food items, and the relative proportion of profitable to non-profitable food items within any feeding patch is also labile; there therefore exists a cost associated with maintaining up-to-date information regarding the most suitable places to feed and choosing the constituents of the diet (Hughes, 1997). But the value of food increases proportionately with hunger status and, as such, when food is unavailable individuals may increase risk-taking and competitiveness to increase their foraging success (Höjesjö et al., 1999).

Individuals will also be exposed to wide variations in abiotic factors across a range of temporal scales, from changes throughout the day to seasonal variety, and animals must account for these changes either through physiological mechanisms or through behavioural change. Water temperature varies widely on both a diurnal and seasonal basis, and is of significant physiological importance since it directly affects metabolism and related processes and, therefore, the general health and wellbeing of the animal (Evans, 1990). Small changes in temperature, or gradual changes over a certain period of time, are generally not detrimental to health: warmer temperatures generally result in increased metabolism and modifications to enzyme thermokinetics, with a resultant need for improved feed conversion efficiency but greater scope for activity (Evans, 1990; Weetman et al., 1998). Rapid changes, conversely, can be stressful, and most animals have upper and lower lethal limits to the temperatures they can tolerate; this may be particularly important in aquatic ectotherms, whose body temperature closely equates to ambient (Evans, 1990; Weetman et al., 1999). Physiological and behavioural coping mechanisms must therefore be highly efficient at ameliorating the effects of temperature change, particularly for those animals which live in habitats where temperature extremes are commonplace (e.g. rainbow trout living at lower latitudes of their range; Matthews and Berg, 1997). Initial physiological responses are with a rapid change in metabolic rate equivalent to the direction and proportionality of temperature change, followed by a short term stable phase, and finally an acclimation phase whereby metabolic rate is stabilised at an appropriate level for ambient temperature (Evans, 1990). Temperature has an important effect on anti-predator strategies in aquatic organisms, both as an environmental cue and as a metabolic constraint. Due to reduced metabolism, ectotherms are generally slower in low temperatures and therefore their escape responses are impaired (Fraser et al., 1993; Higgins and Talbot, 1985). In salmonids, the cooler temperatures associated with winter instigate a series of physiological changes resulting in increased nocturnal, rather than diurnal, activity, thereby reducing contact with diurnal endothermic predators (Fraser et al., 1993). At higher temperatures, fish will be faster, may increase predator inspection behaviour and, amongst social animals, increase schooling behaviour for safety; these enhanced antipredator activities may be a response to greater perceived threat due to a greater need for foraging to meet metabolic requirements, and the equally increased speed of aquatic predators (Weetman et al., 1998, 1999).

19

Hypoxia is another environmental challenge often encountered by fish, in which the dissolved oxygen content of the water is insufficient to support the energetic demands of the fish. Hypoxia may often be a seasonal problem when, for example, surface waters of lentic environments freeze, restricting the flow of oxygen from the air into the water or preventing light from reaching aquatic photosynthetic organisms (Bauer and Schlott, 2006). More problematic in agricultural areas, however, are eutrophication events, whereby phytoplanktonic blooms cause large diurnal variations in oxygen availability and, subsequently, oxygen depletion due to bacterial activity (Dean and Richardson, 1999; Herbert and Steffensen, 2005). Initial responses to low oxygen are inevitably behavioural, often involving an increase in activity as an escape response (Herbert and Steffensen, 2006; Johansen et al., 2006). Fish may alternatively reduce activity, thereby minimising energetic expenditure, utilising some energy instead to facilitate the movement of greater quantities of water across the gill epithelia (Dean and Richardson, 1999). Fish will further respond by performing a technique known as aquatic surface respiration (ASR) where possible, which involves ventilating the gills with surface water which, due to diffusion from the air, is relatively oxygen-rich (Shingles et al., 2005; Timmerman and Chapman, 2004). However, these behavioural responses are only useful when hypoxia is of short duration; where low dissolved oxygen is chronic fish often rely on physiological acclimation. These changes may include increased haemoglobin and red blood cell concentrations; increases in stress axis activity; an increase in lactate concentration in preparation for anaerobic respiration; and increased ventilation rates (Herbert and Steffensen, 2006; Johansen et al., 2006; Timmerman and Chapman, 2004; Vianen et al., 2001). Whilst in many species these adaptations mitigate the effects of hypoxia, the combined effects of physiological change along with some behavioural preferences, particularly ASR, can increase the likelihood of predation (Lefrancois et al., 2005; Shingles et al., 2005; Timmerman and Chapman, 2004). Furthermore, in some species at least (e.g. rainbow trout, *O. mykiss*), behavioural responses are divergent, and whilst there is utility in this phenomenon in, for example, determination of personality traits (Laursen et al., 2011), the different responses may have significant impacts on individual survivability (Vianen et al., 2001).

However, the effects of such abiotic stressors on natural systems is difficult to predict since variations affect different organisms differently; for example, hypoxia induces marked intra- and interspecific differences in behavioural responses across a broad range of P_{O_2} (Dean and Richardson, 1999; Vianen et al., 2001), yet survival in some species may be improved due to the adverse effects on their predators (Rose, 2000). Furthermore, stressors are rarely encountered singly; rather, complex natural environments impose a variety of potential stressors that are likely to interact, and individuals must be able to meet the extra demands (Wendelaar Bonga, 1997); for instance, the respiratory performance of fish will come under strain under hypoxic conditions or at higher temperature, when more oxygen is required to feed metabolism and, even at saturation, water oxygen content reduced (Fernandes et al., 1995).

1.2.4 Outline & Aims of Thesis

This thesis is concerned with the expression of bold and shy behaviour in the rainbow trout, *Oncorhynchus mykiss*, measured as the response to a novel object, and how these behaviours may be modified by exogenous and endogenous cues. Boldness is linked with stress physiology, termed coping styles, and I investigated how bold and shy behaviour is correlated with stress physiology, both before and after the introduction of environmental challenges. This thesis is also concerned with the expression of candidate genes whose roles are linked with behaviour and stress physiology, and how the expression of these genes differs between animals of divergent behaviour or physiology, and how expression changes as these fish respond to environmental stress.

Chapter 2

Boldness was determined in individual rainbow trout to demonstrate the consistency of behavioural responses to novelty, and to attempt to correlate these behaviours with HPI axis reactivity to stress. In order to do this, two lines of trout bred for divergent responses to stress were utilised. The hypothesis was that low stress responding (LR) trout would show bold responses to a novel object; in contrast, high responding (HR) trout would have a shy approach to novelty. These individual

differences in behaviour and stress physiology would be linked with divergence in the expression of each of the candidate genes, selected for their roles in behaviour and the stress response.

Chapter 3

This chapter was a continuation of the work set out in Chapter 2. Whilst no correlations were observed between behaviour and gene expression, expression of these genes was strongly linked with divergence of the stress response in the two lines of trout. Here these links were further explored by examining a further eight candidate genes, selected primarily to provide a much more complete coverage of the HPI axis, with the aim of identifying any patterns in the expression of these genes to assist in determining the molecular control of physiological responses to stress.

Chapter 4

Here, the plasticity of boldness was examined in a social context. Individual outbred bold and shy rainbow trout were placed into groups of completely bold or shy conspecifics for one week. During this time, to reinforce social learning and the transmission of behaviours between members of the group and the focal individual (the fish under examination), the fish were exposed to three environmental challenges at random: a 2 minute net chase, an exposure to a novel object and a simulated predator attack. As such, the focal fish would be able to observe the other group members behaving boldly or shyly as a response. After the week the focal fish were replaced into their individual home tanks and retested for boldness. The hypothesis was that focal individuals would learn to behave more boldly or more shyly, and this would be manifest as a change in their behavioural response to a novel object. I was also interested in the plasticity of the physiological response to stress, and whether this would likewise shift to greater or lesser reactivity along with boldness, or if behavioural and physiological responses would be decoupled.

Chapter 5

Behavioural plasticity of boldness was again assessed but with asocial challenges, such that I investigated whether experience could drive behavioural change in these fish rather than learning from conspecifics. The environmental challenges consisted

of temporal variation in predation threat, simulated by striking into the water with a model heron head in combination with the addition of alarm substance into the tank, and also high or low availability of food. Time spent foraging in fish should correspond to a trade-off between ameliorating hunger and reducing the chances of being predated. Thus I expected that fish on low food availability would become bolder, taking more risks to capture food. This was also related to stress physiology and whether initial boldness or boldness after the challenges could be linked to circulating plasma cortisol. Furthermore I investigated the expression of three candidate genes linked with stress physiology and appetite regulation. The hypothesis was that individuals would alter risk-taking behaviour and levels of activity concordant with context (the perceived level of predation risk) and state (hunger): at higher risk individuals should become shyer, but this will be mediated by their need to forage. State would be reflected in plasma cortisol levels and the expression patterns of the candidate genes, and these would provide insight into the mechanisms driving behavioural change.

Chapter 6

In natural environments fish are likely to be exposed to a wide range of stressors, yet few studies look at behavioural and responses to multiple challenges. Here I addressed this by analysing a data set from a previous study on bold and shy rainbow trout in which the effects of temperature, dissolved water oxygen content and predation risk were examined. The hypothesis was that individuals would modify their behaviour to account for the effects of temperature and hypoxia on individual metabolism, thereby becoming bolder or shyer under prevailing predation threat in order to maintain healthy energetic status.

2 Physiological and genetic correlates of boldness: characterising the mechanisms of behavioural variation in rainbow trout, *Oncorhynchus mykiss*

2.1 Abstract

Boldness, the propensity to take risks, has previously been correlated with individual differences in the physiological response to stress: risk-taking bold animals display a proactive stress coping style whereas relatively shyer, risk-averse animals exhibit a reactive coping style. The aim of this study was to investigate whether differences in the expression of bold-type behaviour were evident within and between two lines of rainbow trout, *Oncorhynchus mykiss*, selectively bred for a low (LR) or high (HR) endocrine response to stress. Boldness was determined in individual fish over two trials by measuring the latency to approach a novel object. Differences in plasma cortisol concentrations and the expression of eight genes previously identified as being linked with divergent behaviours in trout were determined. Bold individuals were defined as approaching the object within 180 s and were significantly more active than shy fish that did not approach within 300 s. Plasma cortisol concentrations after exposure to a 1 minute emersion stress were significantly greater in the HR line compared with the LR line, and six of the eight tested genes were upregulated in the brains of LR fish compared with HR fish. Bold and shy phenotypes were identified within the lines, however, no direct relationship between boldness and either stress responsiveness or gene expression was found. Therefore, clear differences in stress physiology and gene expression could be identified between the lines. In contrast, discernable physiological and molecular responses did not correlate with behavioural variation within both lines, and highlight the complexity of the behavioural-physiological complex.

2.2 Introduction

Behavioural polymorphisms are a common feature of natural populations (Sih et al., 2004a). In some cases intraspecific variation in behaviour may be inherently necessary due to environmental changes, often corresponding with ontogenetic shifts (Slater, 1981), but for many complex behaviours the full adaptive significance of such variation generally is not fully understood. Despite this, recent studies have highlighted the underlying role of physiological and genetic factors in driving divergent behaviour, particularly differences in animal personality (e.g. Bell, 2007; Koolhaas et al., 1999; Korsten et al., 2010; Øverli et al., 2005). One emerging and fundamental personality trait is boldness. An individual's boldness is defined by its response to a novel challenge, with these responses regarded as an indicator of the amount of risk an animal is prepared to take in new circumstances (Koolhaas et al., 1999; Sih et al., 2004a; Sneddon, 2003; van Oers et al., 2005b). As such, boldness can directly influence an organism's fitness, with the direction depending upon the environmental context (Brown et al., 2007).

Boldness is not a discrete trait, but rather represents a continuous range of behavioural profiles from bold to shy (Cockrem, 2007). This bold/shy continuum describes a suite of correlated behaviours which are often considered consistent between contexts. In general, shy animals are more reclusive or unresponsive when faced with an unfamiliar situation, whilst bold organisms will act normally or even actively investigate novel environments (Beausoleil et al., 2008; Verbeek et al., 1994; Yoshida et al., 2005) or objects more readily under the same conditions (Carere and van Oers, 2004; Frost et al., 2007; Wilson et al., 1993). Bold animals are also relatively more aggressive, spend more time in the open, recover more quickly (e.g. from fear stimulation) and are able to learn more quickly than shy animals (Carere et al., 2005; Magnhagen, 2007; Sneddon, 2003; van Oers et al., 2005b; Verbeek et al., 1996). In combination, these consistent behavioural traits displayed by an individual can be used to differentiate or characterise their 'personality' or degree of boldness (Carere and Eens, 2005; Réale et al., 2000).

Behavioural phenotypes within a species have also been linked with the physiological response to a stressor, collectively comprising the individual's 'coping style' (Koolhaas et al., 1999). Stressors are defined as challenges to an individual's

homeostasis that result in a stress response: behavioural and neuroendocrine reactions that address the negative effects of that challenge (Wendelaar Bonga, 1997). Intraspecific differences in stress responsiveness reflect variation in the control of hormone release within the neuroendocrine stress axis. Consequently, the proactive (active) coping style, typified by aggression and territoriality, is characterised by a high adrenergic (noradrenaline) axis activity and low hypothalamo-pituitary-adrenal/interrenal (HPA/HPI) axis activity, whilst reactive (passive) behaviour, characterised by withdrawal and immobility, is linked with a higher HPI response (De Boer et al., 1990). These dichotomous behavioural strategies associated with coping style are often correlated with boldness (Koolhaas et al., 1999; Øverli et al., 2007).

Behavioural characteristics have a significant genetic component in many natural populations in several taxa (e.g. Álvarez and Bell, 2007; Benus et al., 1991; Fidler et al., 2007; Giles and Huntingford, 1984; Korsten et al., 2010; van Oers et al., 2004). In addition, physiological response to stress also appears to have a substantial underlying genetic basis. For example, it was possible to select two lines of rainbow trout, *Oncorhynchus mykiss*, for divergent endocrine response to a confinement stressor; across four generations, post-stress plasma cortisol concentrations remained significantly greater in high (HR) compared with low (LR) stress responding lines, with a moderate to high heritability ($h^2 = 0.41 - 0.73$) for HPI-reactivity to stress (Pottinger and Carrick, 1999; 2001b; T.G. Pottinger, unpublished data). Interestingly, these lines also exhibit characteristic and divergent behavioural traits which are linked with boldness: the LR line (bold) displays longer retention of a classically conditioned response than HR fish (shy), and also exhibits proactive behaviours such as enhanced aggression, social dominance, and rapid resumption of feed intake after exposure to a stressor (Øverli et al., 2007). These trout lines thus provide an excellent model of coping style and the concomitant relationship between heritable stress responses and behavioural phenotype which is, furthermore, reflected in natural populations (Cockrem, 2007; Koolhaas et al., 1999).

Ultimately, many of these heritable differences in behaviour manifest as differences in gene expression: a microarray analysis comparing the expression of 20,000 genes in an outbred population of *O. mykiss* highlighted ~1,000 genes which were

differentially expressed in the brain of fish showing either consistently bold or shy responses to novelty (LU Sneddon, unpublished data). Therefore differential gene regulation between bold and shy fish indicate that bold fish have either a different gene expression profile or more profoundly regulate relevant genes, and may also account for divergence of behaviour or stress physiology in these animals. If these genes identified by Sneddon et al. (unpublished data) play a role in defining bold and shy phenotypes, they might be expected to show a different pattern of expression between HR and LR fish. No previous studies have specifically examined bold/shy behaviour in detail within these lines or explored the possibility that if variation in boldness occurs between these lines of selected fish it may be linked to discrete individual differences in brain gene expression. These lines thus offer a unique model of the putative link between behavioural polymorphism and physiological stress responsiveness and, for the first time, to correlate these aspects of animal personality and coping style by quantifying the expression of a suite of candidate genes.

The aim of this study was to determine the extent to which divergent high or low neuroendocrine responses to stress correlate with differences in individual boldness. We hypothesise (1) that LR individuals would approach a novel object more quickly and exhibit a lower stress response than HR individuals and (2) that this divergence in behavioural and endocrine responses would be associated with clear differences in the expression of genes associated with boldness and/or the stress response.

2.3 Materials and Methods

2.3.1 Experimental fish

The following experiment was conducted humanely under Home Office, UK, guidelines according to the Animal (Scientific Procedures) Act 1986, and following local ethical approval. Rainbow trout, *Oncorhynchus mykiss* Walbaum, from inbred lines selected for high (HR) or low (LR) cortisol responsiveness to a standardised stressor (Pottinger and Carrick, 1999) were transferred from CEH Windermere to Liverpool where each line was held separately (~140 fish per tank) in two stock tanks (2 x 2 x 0.5 m) in a semi-recirculating system. Tanks were supplied with filtered aerated freshwater and maintained at 13 (range ± 2°C) on an ambient 14:10 h light:dark regime. Half of the tank had an opaque overhead cover for shelter. Fish

were inspected twice daily and fed commercial pellets (Skretting, UK) at 1 % body mass per day. After a period of at least 4 months to allow fish to acclimate, trout (HR: n = 44, 343.0 ± 14.7 g; LR: n = 33, 356.5 ± 11.0 g) were selected at random from the stock tanks and placed into individual glass tanks (90 x 50 x 45 cm) which were screened from visual disturbance. All tanks were supplied with a constant flow of UV-filtered freshwater in a semi-closed system maintained at 10 ± 1°C with aeration. The trout were left to acclimate for a minimum of one week and fed daily. Experiments were conducted on fish that had resumed feeding after this period.

2.3.2 Behaviour

A purpose-built low-light video camera was situated in front of the tank and a second camera placed to the side of the tank. Measuring rulers (0.5 cm intervals) were arranged horizontally and vertically along the front of the tank to measure proximity of the fish to the novel object. The fish were allowed 10 minutes to acclimatise to the potential disturbance arising from setting up the cameras. Behaviour of the fish without disturbance was then recorded for 10 minutes, before a novel object was added. The novel object test is a standard paradigm to differentiate between bold and shy individuals (Wilson et al., 1993). The novel object was placed as near to the centre of the tank as possible, and the behaviour of the fish was recorded for a further 10 minutes after which the object was carefully removed. This test was repeated a week later to assess the level of consistency of behaviour displayed by the experimental individuals. Novel objects were varied between trials to ensure the fish did not become habituated to a familiar shape, and consisted of an orange frustum-shaped nonreactive rubber bung (7.05 cm mean diameter, 4.9 cm height) and a bipyramidal Duplo$^{(R)}$ construct (height 13.5 cm, and maximum widths 7.6 x 6.3 cm) of black, red and blue plastic bricks.

Scoring of the behaviour was accomplished using custom designed behavioural analysis software. Nine behaviours were initially scored based on the activity levels of the subject and its proximity to the novel object (see Frost et al., 2007). Principal components analysis (Minitab ver.15.1) was subsequently used to identify the key behaviours that differentiated bold fish from shy. Latency to approach within 5 cm (s) of the object was strongly represented in the first principle component (eigenvalue = 3.53, loading for 5 cm latency = -0.41) and could be solely used to

differentiate between bold and shy groups. This measure has previously been used to identify boldness in fish (Coleman and Wilson, 1998; Frost et al., 2007). Loadings for six of the behaviours were well represented in the first principal component, and two of these, frequency of entering 10 cm zone (min^{-1}) centred on the object (loading = 0.459) and duration (s) spent passive (loading = -0.381), were selected for further analysis. Passive behaviour was defined to exclude swimming (movement of the fish generated by propulsion using the fins, of no less than approximately one body length) but include drifting, fish pivoting on their own axis, any minor movements made to maintain position, and resting on the bottom of the tank.

2.3.3 Hormone analysis and quantification of gene expression

Subsequent to, and on the same day as, the final behavioural trial, approximately half of the fish (n = 34) were netted and exposed to air for 60 s to induce an acute physiological stress response before being placed back into their tank (Pickering and Pottinger, 1989). Fifteen minutes after emersion, the trout were netted again before being killed humanely by concussion. To obtain unstressed plasma cortisol concentrations, fish were killed by concussion without this treatment. Individuals were killed at the same time each day to ensure that interpretation of differences in hormone levels was not compromised by diel fluctuations in plasma cortisol (Pickering and Pottinger, 1983). Immediately after euthanasia, a 2 ml blood sample was taken from the caudal vessels using sterile 25 g needles and heparinised 2 ml syringes, the supernatant plasma was aspirated, divided into aliquots and frozen at -20°C. Plasma cortisol levels were determined by radioimmunoassay (Pottinger and Carrick, 2001b).

Immediately following blood sampling, the whole brain was removed and stored at -80°C until RNA extraction, and fish were sexed. Total RNA was extracted from trout brain using TRIzol® (Invitrogen Life Science, UK), with RNA eluted into 50 µl RNase-free water. RNA concentrations were determined by optical density at 260 nm using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK) system and the quality of the samples assessed by 2 % agarose gel electrophoresis. For each sample, approximately 1 µg of mRNA was reverse-transcribed into first-strand cDNA using random hexamers and SuperScript™ III reverse transcriptase (Invitrogen Life Science, UK), following the manufacturer's protocol.

For RT-PCR, ~0.15 μg of the resulting cDNA was amplified in a 10 μl PCR (using 5 μl Fast SYBR Green, Invitrogen Life Science, UK) primed with 2 pmol each primer (Table 2.1). Eight pairs of primers were developed using Primer Express® 3.0 software against *O. mykiss* sequences for genes functionally associated with boldness or the stress response (Table 2.2). Six of these genes, ependymin, GABA$_A$, calmodulin, MHCI, Hbα4, and a lipocalin, retinol binding protein, were differentially regulated between bold and shy rainbow trout in a previous study (LU Sneddon, unpublished data); the remaining two, proopiomelanocortin and arginine vasotocin, were chosen based on the importance of their roles in physiological stress responses and related behaviour (Goodson and Bass, 2001; Winberg and LePage, 1998). Thermal cycling conditions, using a 7500 Fast Real-Time PCR System (Applied Biosystems), were: 10 min at 95°C, followed by 40X [95°C 3 s, 60°C 30s] and then [95°C for 15 s, 60°C for 60 s, 95°C for 15 s and 60°C for 15 s], which allowed the construction of a melting curve to assess the specificity of the product. Gene expression levels were normalised against glyceraldehydes-3-phosphate dehydrogenase (GAPDH), a housekeeping gene often used in relative gene expression studies across a variety of animal and fish species (e.g. Bernier et al., 2008; Reilly et al., 2008).

2.3.4 Data Analysis

None of the data were normally distributed (Anderson-Darling; Minitab, ver.15.1) and thus non-parametric tests were applied. A Wilcoxon Signed Rank Test was used to analyse the difference between behavioural scores of the first and second trial to test for consistency in latency to approach within 5 cm of the novel object (Minitab, ver.15.1). Subsequently, data were separated for trout showing consistently bold (approach to 5 cm of the object within 180s in both trials; $n = 28$) or shy (do not approach to 5 cm within 300 s in both trials; $n = 13$) behaviour. Scores for each of the behaviours were then averaged over the two trials and compared between bold and shy groups using Mann-Whitney U-tests (R, ver.2.7.0), including sequential Bonferroni treatment (Rice, 1989) for multiple tests.

Table 2.1: Primer sequences for RT-PCR for eight genes implicated in behavioural responses, and for a reference gene (*), including accession number (where primers were generated from a single sequence), and amplicon size and melting temperature, T_m.

Gene Accession No.	Forward (5' – 3')	Reverse (5' – 3')	Size (bp)	T_m (°C)
Ependymin NM_001124693	CTC ATG CTC ACG CTC TGG AA	CCA AAA ACA GCT CAA CCT GAT G	60	83
GABA_A BT073523	CTC ATC CGA AAG CGA ATC CA	CAC ACT CTC GTC ACT GTA GG	156	81
Calmodulin	CCG GGA GGC TGA TAT CGA T	CGT CAT CAT CTG CAC AAA TTC TTC	64	81
MHC1	AGT CCC TCC CTC TGT GTT TCT G	TCG CGT GGC AGG TCA CT	62	62
POMC NM_001124718	AGC GCT ATG GAG GGT TCA TG	CAA CGT GAG CAG TGG TTT CTG	62	82

Table 2.1 continued

Gene Accession No.	Forward (5' – 3')	Reverse (5' – 3')	Size (bp)	T_m (°C)
Hbα4 BT074353	GAA GAA GCG CGG CAT CAC	TCG TCC ATG TGG CCA ACA	60	81
AVT DQ291141	ACC CAG CGG TCC TAT ATT ATG ATC	GGC ATG CTG AGG ACC AGA CT	62	81
RBP NM_001124278	GGA CAA TGT CGT CGC TCA GTT	CGT GGG CAG TTG CAG TCA	62	80
GAPDH* AF027130	TGT TGT GTC TTC TGA CTT CAT TGG	CCA GCG CCA GCA TCA AA	60	81

32

Table 2.2: Genes (including abbreviations and known major functions) used in this study and their primer sequences. Italicised genes showed differential expression between bold and shy rainbow trout, *Oncorhynchus mykiss*, in a previous microarray study (LU Sneddon, unpublished data). Primers were developed using Primer Express® 3.0 software, and were diluted to a working concentration of 10 pmol μl^{-1}.

Gene	Abbr.	Functions
Ependymin	Epd	Memory/learning[1]; Cold tolerance[2]; Regeneration[3]
γ-Aminobutyric acid A	GABA$_A$	Anxiety[4]; Aggression[5]; Memory[4]
Calmodulin	CaM	Calcium binding (Memory[6]; Nerve growth[6]; Immune system[7])
Major histocompatability complex Class I	MHC I	Immune system[8]; Kin recognition[8]
Haemoglobin α4 subunit	Hbα4	Oxygen transport
(Arginine) vasotocin	AVT	ACTH secretion[9]; Modulation of social and non-social behaviour[9]
Proopiomelanocortin	POMC	Stress response[10]
Retinol binding protein	RBP	Vitamin A transport[11]; Stress/Immune response[12]

[1]Shashoua (1991), [2]Tang et al. (1999), [3]Suárez-Castillo et al. (2004), [4]Kalueff and Nutt (1997), [5]Miczek et al. (2003), [6]Stevens (1983), [7]Racioppi and Means (2008), [8]Götze (1977), [9]Goodson and Bass (2001), [10]Winberg and LePage (1998), [11]Goodman (1980), [12]Flower (1996).

Plasma cortisol concentrations for stressed and unstressed trout were compared between the two stress lines (unstressed: HR $n = 13$, LR $n = 23$; stressed: HR $n = 27$, LR = 7), between consistently bold and shy trout (unstressed: bold $n = 12$, shy $n = 5$; stressed: bold $n = 14$, shy $n = 7$) and between sexes (female $n = 17$, male $n = 15$) using Mann-Whitney U Tests (R, ver.2.7.0). For RT-PCR, cycle threshold (Ct; the first cycle number at which fluorescence is significantly greater than background levels) and efficiency values for each gene were exported into REST (ver.2.0.7; Pfaffl et al., 2002) whereby the relative expression of each gene between bold and

shy fish or between fish from each of the two stress lines, normalised to a reference gene (GAPDH), was calculated. Statistical analysis was subsequently accomplished through REST's bootstrap randomisation procedure.

2.4 Results

In unstressed rainbow trout, (Fig. 2.1A) median plasma cortisol concentrations were significantly and almost threefold greater in LR fish compared with the HR line (3.16 and 1.34 ng ml^{-1} respectively; $W = 47.0$, $p < 0.01$, $n_1 n_2 = 23,13$), with no significant difference between sexes ($W = 89.0$, $p = 0.15$, $n_1 n_2 = 17,15$). By contrast, after exposure to a stressor, HR trout exhibited over double the plasma cortisol concentrations than did LR fish (67.42 ng ml^{-1} and 27.14 ng ml^{-1} respectively; $W = 158.0$, $p < 0.01$, $n_1 n_2 = 27,7$; Fig. 2.1B), and while blood-cortisol concentrations were almost twice as high in female trout (73.53 ng ml^{-1}) than in males (46.36 ng ml^{-1}), the response was highly variable so insignificant ($W = 177.0$, $p = 0.06$, $n_1 n_2 = 17,15$).

Individual trout were consistent in their latency to approach within 5 cm of a novel object over two trials ($W = 913.0$, $p = 0.113$, $n = 77$), thus confirming the utility of this measure. Rather than being associated predominantly with one or other line, both bold and shy fish were identified within each line. Moreover, there was a tendency for fish to be bold rather than shy in both lines (Fig. 2.2); although there were proportionately more shy fish in the HR line compared to the LR line (15:9 bold and shy compared to 13:4 bold and shy respectively), this difference was not significant ($\chi^2_1 = 0.891$, $p = 0.344$). Furthermore, although plasma cortisol concentrations profoundly differed between the two lines, there was no significant difference observed in cortisol concentration between bold and shy fish, regardless of whether they were unstressed ($W = 37.0$, $p = 0.51$, $n_1 n_2 = 12,5$; Fig. 2.1A) or stressed ($W = 89.0$, $p = 0.15$, $n_1 n_2 = 17,15$; Fig. 21B). As such, data for HR and LR fish were pooled together for further behavioural comparisons.
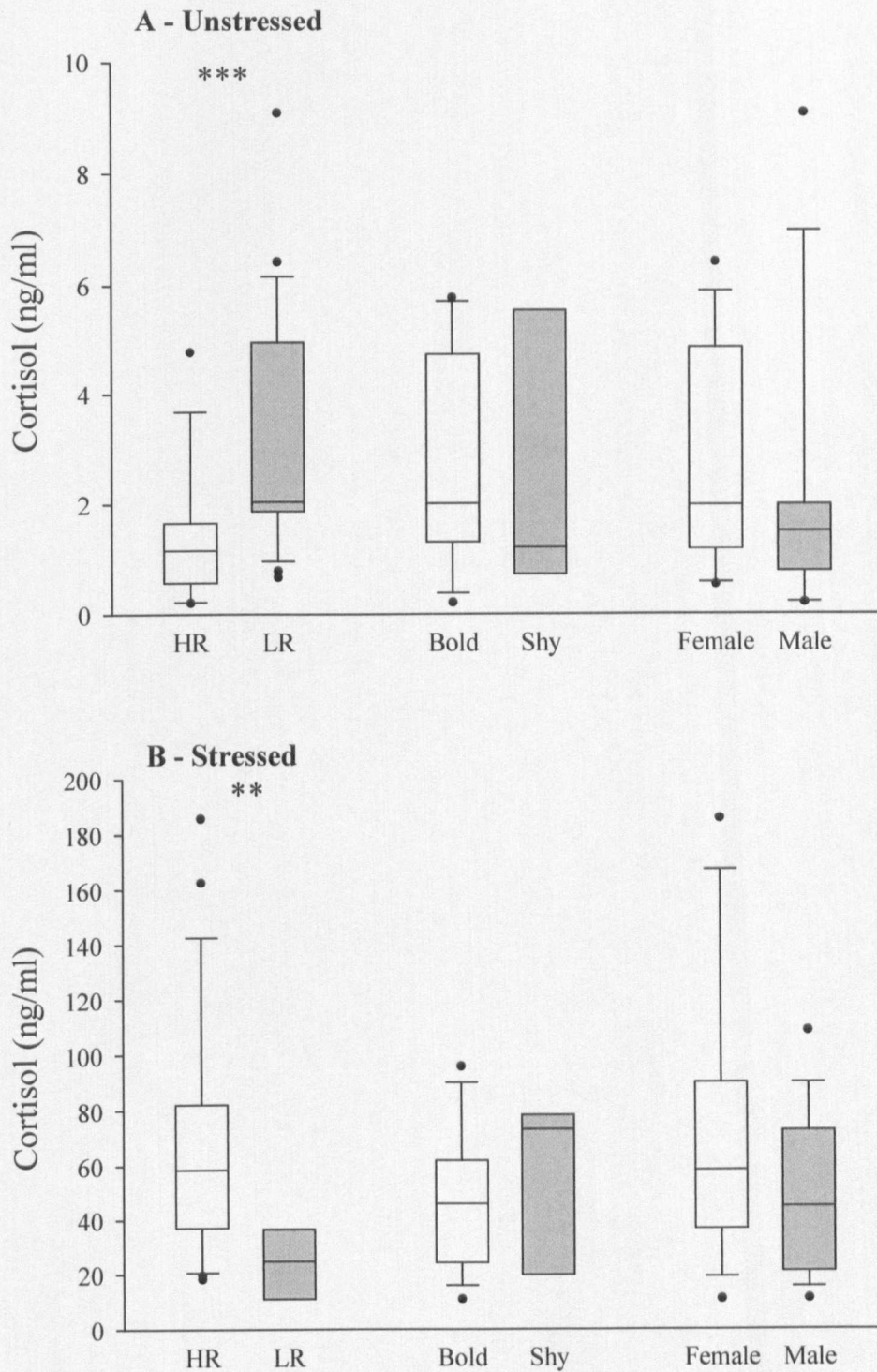
Figure 2.1: Median plasma cortisol (ng ml^{-1}; ± 90th and 10th percentiles; dots indicate values >1.5x interquartile range) in unstressed (A; $n = 36$) and stressed (B; $n = 34$) *Oncorhynchus mykiss*. In each case, comparisons were made between high (HR) and low (LR) stress responsive lines, between individuals determined bold and shy by a novel object test, and by sex. Asterisks denote significant difference between groups (Mann-Whitney test): **, $p < 0.01$; ***, $p < 0.001$.
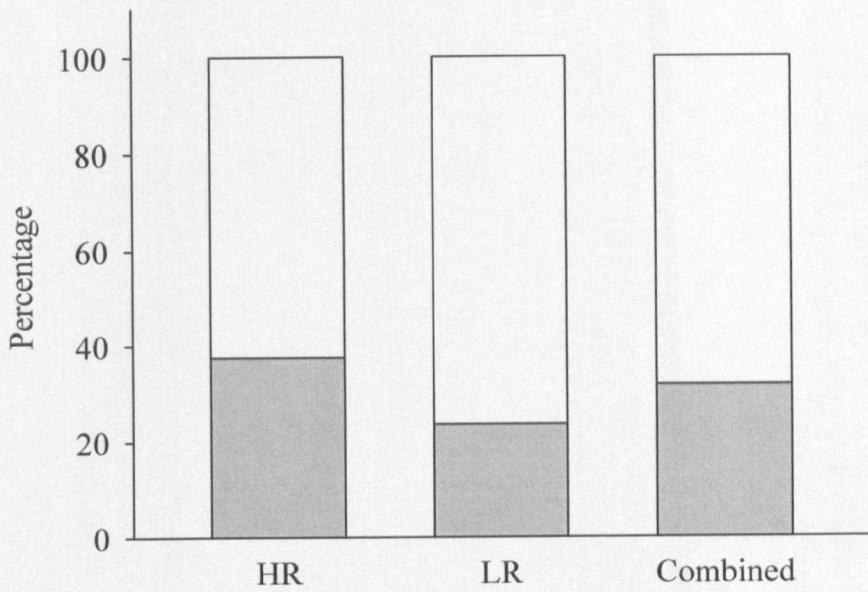
Figure 2.2: Percentage of rainbow trout, *Oncorhynchus mykiss*, showing consistently bold (white) or shy (grey) behaviour in lines bred for high (HR; $n = 24$) and low (LR; $n = 17$) cortisol response to stress, and in both groups combined.
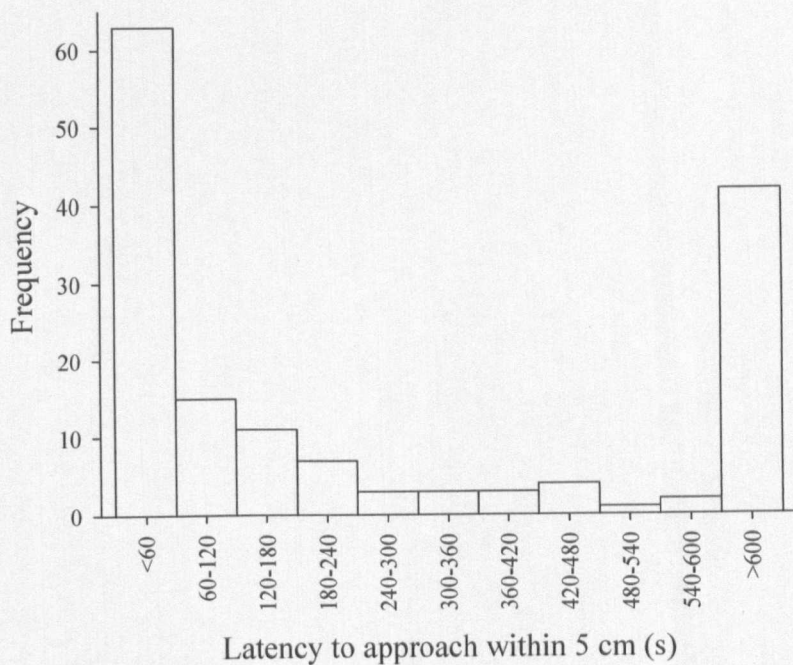
Figure 2.3: Frequency of individual trials in which individual rainbow trout, *Oncorhynchus mykiss*, approached within 5 cm of a novel object within a certain period of time ($n = 154$).

Consistent with other studies, boldness showed a bimodal (i.e. u-shaped) distribution and tended towards extremes in individual trials (Fig. 2.3), with fish acting either extremely boldly (approaching 5 cm of the object within 60 s; $n = 63$) or extremely shyly (not approach within 5 cm during the trial; $n = 42$). Consistently bold fish spent less time overall being passive ($W = 477.0$, $p < 0.01$, $n_1 n_2 = 28,13$; Fig. 2.4A) than did shy trout, and also entered the 10 cm zone about the object approximately ten times more frequently ($W = 726.5$, $p < 0.01$, $n_1 n_2 = 28,13$; Fig. 2.4B), thus providing evidence of a behavioural divergence in these fish.

Table 2.3: Relative expression (normalised to a control gene, GAPDH; RE) and p values for the comparisons of expression of eight genes, selected for implicated roles in boldness, between bold and shy or between high (HR) and low (LR) stress responsive rainbow trout, *Oncorhynchus mykiss*. Asterisks denote significant difference between the groups (REST, in Pfaffl et al., 2002): *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

	Boldness		Stress Line	
	RE	*p*	**RE**	*p*
Epd	0.82	0.52	2.63	***
MHC I	0.69	0.46	5.92	***
CaM	0.75	0.31	2.09	**
GABA$_A$	1.02	0.96	1.93	**
POMC	1.03	0.98	0.76	0.70
Hbα4	0.94	0.88	0.99	0.98
AVT	0.90	0.72	1.89	*
RBP	0.80	0.42	2.01	**

A - Duration of Passive Behaviour

B - 10 cm Approach Frequency
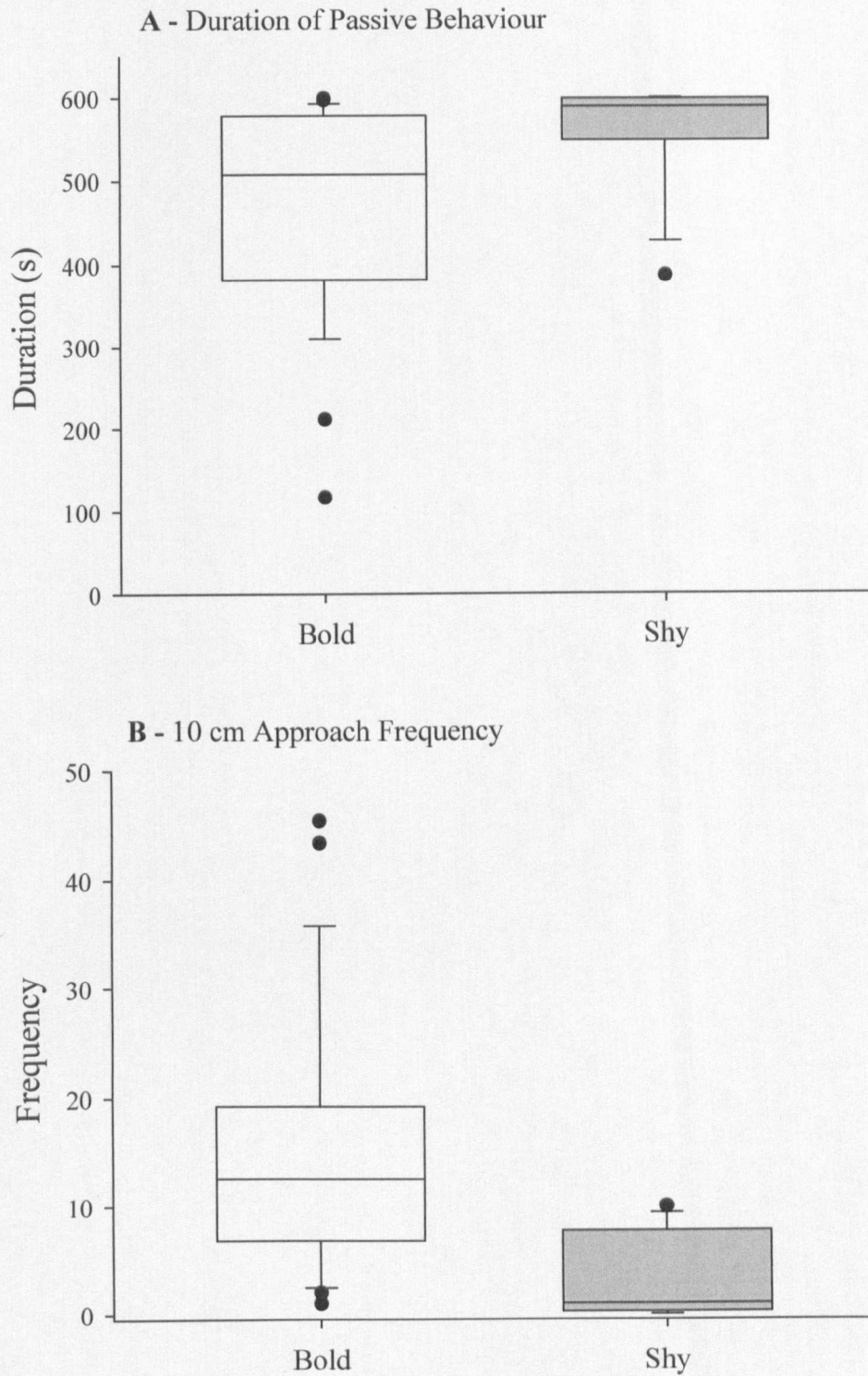
Figure 2.4: Median ($\pm$ 90th and 10th percentiles; dots indicate values >1.5x interquartile range) duration of passive behaviour (A) and frequency of approaching to within 10 cm of a novel object (B) for bold (white; $n = 28$) and shy (grey; $n = 13$) rainbow trout, *Oncorhynchus mykiss*.

The genetic dissimilarity between the stress lines was reflected in the candidate gene expression between them in six of the candidate genes; ependymin, calmodulin, MHC I, $GABA_A$, vasotocin and RBP were significantly upregulated in the brains of LR fish compared with HR fish (Table 2.3; Fig. 2.5B). Average fold change varied from an upregulation factor of 1.89 in AVT up to 5.92 in MHCI. In contrast, expression of both POMC and $Hb\alpha4$ were almost identical between the lines. However, bold and shy fish did not significantly differ in the expression levels of any of these genes, with the expression of most genes marked by large variance due to pooling of samples within the stress lines (Fig. 2.5A).

2.5 Discussion

Boldness is a complex behavioural trait that is associated with coping style (Koolhaas et al., 1999), and may thus be assumed to correlate with the magnitude of the physiological stress response. In this study, bold and shy rainbow trout were successfully identified by way of their behavioural response to novelty; this is the first characterisation of both bold and shy phenotypes within these lines. In addition, divergent plasma cortisol responses to a stressor were evident between the HR and LR lines, consistent with earlier findings (summarised in Øverli et al., 2005). However, no significant relationship between boldness and stress responsiveness was found within each line. Although a slightly larger proportion of LR trout exhibited a bold phenotype than HR trout this was not significant and no associated differences were observed in post-stress plasma cortisol levels between bold and shy individuals independent of selection line. Physiological divergence between the lines was correlated with differences in regulation of six candidate genes in the brain. However, bold and shy fish within each line did not exhibit the dissimilarity in gene regulation.

2.5.1 Behaviour

The results demonstrate that boldness remains consistent in the trout stress lines within a constant environment. Furthermore, the existence of a behavioural syndrome or personality within these fish was evident: boldness was significantly linked with activity levels, suggestive of risk-taking and risk-averse strategies in bold and shy fish respectively (Sneddon, 2003). Bold fish spend more time in the

A - Bold vs. Shy

B - HR vs. LR
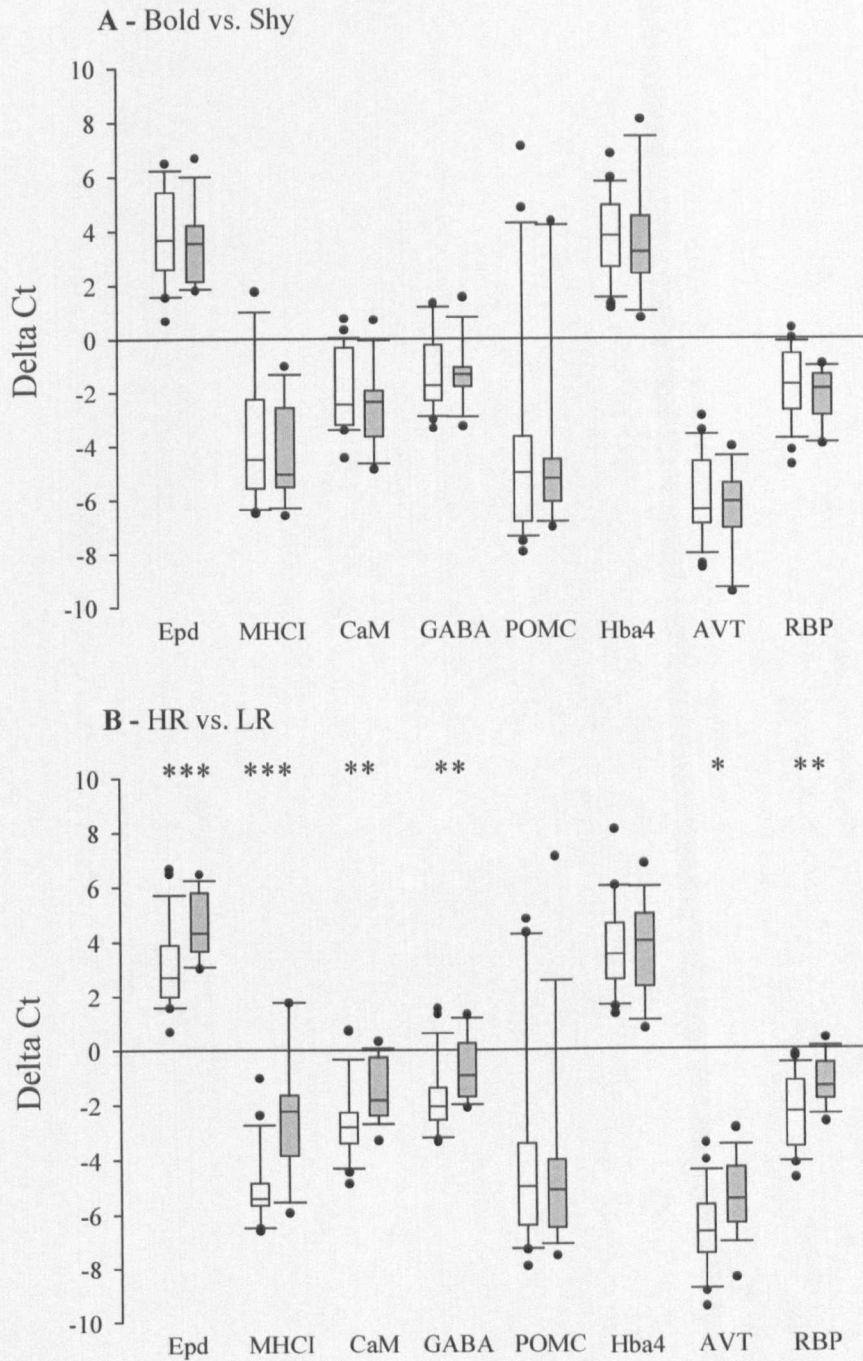
Figure 2.5: Median relative expression (Ct_{target} - $Ct_{reference}$; ± 90th and 10th percentiles; dots indicate values >1.5x interquartile range) of eight candidate genes compared between (A) bold ($n = 28$; white) and shy ($n = 13$; grey), and (B) high (HR; white; $n = 22$-25) and low (LR; grey; $n = 17$) stress responding rainbow trout, *Oncorhynchus mykiss*. Asterisks denote significant difference between the groups (REST, in Pfaffl et al., 2002): *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

open, even in the presence of potential threats, whilst shy fish, unless accompanied by bold conspecifics, are found outside of cover less often (Magnhagen and Staffan, 2005). Bold individuals in this study were characterised by making more use of the available tank space and being less inclined to actively avoid the object, although assumptions as to whether individuals were investigating the object cannot be made. Shy fish, on the other hand, were more reluctant to approach the object, and in some cases their behaviour could be compared to that of the withdrawal and immobility exhibited by the reactive coping style of rodents when confronted by a stressor (Koolhaas et al., 1999). In the present study individuals tended to exhibit one behaviour type or the other, with the distribution of shyness/boldness approximating bimodality. The strongly bimodal response to novel objects and the frequency of bold and shy fish within line and as a whole were similar to those observed by Frost et al. (2007) in an outbred population of rainbow trout. Boldness thus apears to be bimodally distributed in this species, a response seemingly maintained even in lines selected for divergent responsesiveness to a stressor. Other species may exhibit different distributions, such as a normal distribution with relatively fewer bold and shy compared to intermediate fish in pumpkinseed sunfish (Wilson et al., 1993). Thus bold/shy distributions may reflect interspecific or between-population differences in intrinsic factors or extrinsic pressures that may drive variation in personality. Even rearing conditions can cause a prevalence of certain behavioural types within a population of salmonid fish (Sundström et al., 2004).

2.5.2 Coping Styles and Gene Expression

A clear finding here was the consistent divergence in the HPI reactivity to stress, reflected in an equally strong divergence in the genes involved in the stress axis, further demonstrating the strong genetic basis that underpins the stress response in rainbow trout (Pottinger and Carrick, 1999, 2001b) and possibly other vertebrates (Yao and Denver, 2007). Further work should focus on determining whether these responses are consistent throughout the entire pathway or whether genetic regulation occurs only at key points of the response. In unstressed fish plasma cortisol concentrations were higher in LR fish than in HR fish, the reverse of an earlier observation in these lines (Pottinger and Carrick, 2001a). This would concur with the idea that these phenotypes differ when facing a novel challenge such as a threat to

homeostasis with resting hormone concentrations not indicative of stress coping style.

Differences in whole-brain gene expression between the stress lines represent the first evidence that a distinct phenotypic divergence between the lines, particularly of the stress responsiveness, is reflected by a broader suite of correlated molecular responses. This may indicate divergence in the genetic control of the stress response, and each of the candidate genes tested has a functional role linked with boldness or stress physiology. Immune function can be compromised by chronic stress possibly explaining why MHC, CaM and RBP were each upregulated in LR fish relative to HR fish, since the corresponding proteins are associated with the immune system or response. The Ca^{2+}/CaM complex directly or indirectly controls a number of mechanisms and enzymes involved in the immune response, including aspects of the MHC and the serine-threonine kinases CaMK I, II and IV (Racioppi and Means, 2008). RBP meanwhile has been implicated in inflammatory processes associated with immune responses (Flower, 1996). Low stress-responding animals are often characterised as having improved health over those with a high response, and a major issue associated with sustained elevation of cortisol is a reduction in immunity and increased susceptibility to pathogens (Wendelaar Bonga, 1997). Some aspect of divergent immunological parameters between low and high stress responders thus appears to be controlled at the molecular level, which may itself be influenced by circulating steroid concentration, in addition to direct causal relationships between stress hormones and immune responses.

Both GABA$_A$ and AVT were upregulated in LR fish, and changes in expression of both genes have been related to aggressive behaviour (Backström and Winberg, 2009; Miczek et al., 2003). Aggression is a defining characteristic of stress coping styles and of the stress lines: LR/proactive individuals are more aggressive, and win more dyadic contests against HR/reactive conspecifics (Pottinger and Carrick, 2001b). However, high levels of AVT tend to inhibit aggression in territorial teleosts such as the rainbow trout (Backström and Winberg, 2009). High expression of AVT may not reflect actual active protein levels due to mechanisms such as post-translational modification, and therefore future studies should target protein expression. Backström and Winberg (2009) suggest that the aggressive output

influenced by AVT could be mediated by other systems, in particular the brain serotonergic system, and thus studies incorporating serotonergic activity with AVT concentration or expression could more accurately identify determinants of aggression.

Expression of POMC may not differ between subjects with different stress-coping abilities (Centeno et al., 2007), but rather physiological variation in the HPI axis may occur downstream during post-translational modification, and this may indeed be the case for the HR and LR trout lines. Hormonal activity is dependent upon the relative concentrations of hormones and their receptors, which are usually in excess of that required for any individual response. An increase in relative receptor concentration results in increased tissue sensitivity to the respective ligand. For example, the rainbow trout stress lines differ in their expression of forebrain mRNA coding for the corticotrophic releasing factor receptors CRFR1 and CRFR2 (Backström et al., 2011). In response to a confinement stress high responding trout exhibited greater CRFR1, to which CRF binds to stimulate the secretion of ACTH and, consequently, cortisol. In contrast, these HR trout showed lower CRFR2 expression; in mammalian systems, urocortin rather than CRF binds to CRFR2 thereby reducing stress reactivity (Charmandari et al., 2005). Expression of the mRNA for the genes for these receptors therefore appears consistent with stress physiology in the LR and HR trout lines. Downstream of the CRF system, some data suggest concentrations of adrenocorticotrophic hormone (ACTH) in HR and LR fish did not differ significantly during stress; instead, the sensitivity of the interrenal to ACTH differed between the lines (Pottinger and Carrick, 2001a). Variation in tissue sensitivity to ACTH appears to be an important aspect of the stress response (e.g. Carsia et al., 1988; Gómez et al., 1996), and some of this may be derived from genetic polymorphisms (Charmandari et al., 2005). For example, a single nucleotide polymorphism in the ACTH receptor promoter in humans results in a reduced cortisol response to an injection of CRF (Slawik et al., 2004). Additionally, receptor concentration can be controlled by the activity of their ligands: high concentrations of ligand nay result in upregulation of receptor concentration and therefore greater tissue sensitivity (e.g. estradiol; Ing & Tornesi, 1997). In contrast, other receptors may be downregulated at high ligand concentration. For instance, whilst Backström et al. (2011) found no difference in AVT receptor mRNA expression, there did

appear to be a difference in the speed of stress-induced downregulation of this gene between stress lines. Divergent stress responsiveness in teleosts may therefore, at least in part, be due to differences in tissue sensitivity to hormones. However, the extent to which factors other than hormone concentration causes divergence in overall stress responsiveness in teleosts still remains unclear (Pottinger & Carrick, 2001a) and provides an opportunity for further research.

The absence of a well-defined link between post-stress plasma cortisol levels and boldness within the lines was surprising given previously observed correlations between the magnitude of the stress response and behaviour (Koolhaas et al., 1999; Øverli et al., 2005). Both boldness and shyness were represented within each coping style although it must be noted that in an earlier study (Schjolden et al., 2005) we were unable to demonstrate differences between the lines in locomotory activity in the presence of a novel object: direct correlations between stress responsiveness and behaviour or behavioural type thus are not always observed. One reason may be that boldness is context-specific: individual behaviour varies dependent upon the situation (e.g. in familiar compared to unfamiliar environments; Schjolden et al., 2005) and thus would potentially confer adaptive advantages particularly in an inconsistent environment (Bell, 2007; Coleman and Wilson, 1998; Wilson and Stevens, 2005). Such variation may be elicited by the type or severity of the stressor or to familiarity with the test environment (Brelin et al., 2008; Misslin and Ropartz, 1981; Schjolden et al., 2005) and contrasting behavioural responses observed between studies may arise from variation in methodological approach to characterising boldness. Furthermore, Schjolden et al. (2005) could not find consistent differences in behavioural responses between HR and LR rainbow trout across several tests including the response of the subjects to a novel object: thus whilst aggression, a defining component of coping styles and a putative element of boldness, may strongly and consistently correlate with HPI axis reactivity the same is not necessarily true of responses to novelty. It may thus be more important at this stage to measure individual responses across a range of contexts and correlate with physiological parameters.

Alternatively, the existence of bold and shy phenotypes within each stress line instead of correlating with stress responsiveness, according to coping style theory

(Koolhaas et al., 1999), may suggest that divergent personality traits persist within a population or species irrespective of other factors. Experience, brought about by environmental or social influences, can shape an individual's behavioural strategy (Brown et al., 2007; Frost et al., 2007). Moreover, behavioural variation can occur within a group regardless of genetic background, and when environmental conditions are identical for each individual (Metcalfe et al., 1989).

With this in mind, it is not surprising that recent work has highlighted the complex nature behind any genetic control of personalities (Korsten et al., 2010). Our data reinforce this, since, despite previous studies that identified different gene expression profiles between rainbow trout with different behaviours (e.g. dominance, Sneddon et al., 2005; boldness, L.U. Sneddon, unpublished data), no such divergence between bold and shy fish was uncovered in the present study. Gene expression may vary between discrete regions of the brain (Feldker et al., 2003), and thus a single measurement encompassing all brain regions could obscure more fine-scale differences in expression. Variation in the expression of genes at different brain loci have been observed in teleosts, including GABA (Bernier et al., 1999; Larson et al., 2006). Moreover, differences in territoriality have been directly related to variation in expression of AVT across the brain of cichlids (Greenwood et al., 2008). Thus, whilst no difference in expression of the studied genes was found across the entire brain, that is not to say that bold and shy individuals express these genes in different localised areas of the brain: whilst differential expression of these genes between the stress lines was profound, variation amongst bold and shy groups may be more subtle. It is of course possible that within each line the genetic variation is low, or that different genes may be involved in the expression of bold/shy behaviour. However, the clear divergence in whole-brain expression of some of the examined genes in a previous study (LU Sneddon, unpublished data) suggests the latter not to be the case, but does emphasise the complexity of bold and shy personalities in rainbow trout. Given that the expression of boldness was entirely independent of selection line, it is likely that the genetic control of boldness is unrelated to the controlling divergent elements of the selected stress response.

2.5.3 Conclusions and Implications

The results of this study are consistent with earlier observations (Ruiz-Gomez et al., 2008; Schjolden et al., 2005) that suggest that the putative relationship between stress responsiveness and behaviour in the HR and LR lines of rainbow trout is complex. Whilst stress responsiveness is a hereditary trait (Pottinger and Carrick, 1999, 2001b) and was linked to the differential expression of six genes with functions in relevant behaviour and physiology between the two stress lines, the same was not true of our measures of boldness in response to novelty within each line. This suggests that the adoption of these contrasting behavioural strategies may not be explained entirely by genetic background or stress coping style and may instead be influenced by external factors. Experience and environmental influences may cause quite distinct changes in behavioural responses throughout an animal's life history (Frost et al., 2007; Ruiz-Gomez et al., 2008), evidently resulting in behavioural polymorphism even within coping styles, and it is important for future studies to take account of how experience and external factors shapes boldness. This may explain why variation in behavioural phenotypes persist in natural populations to ensure a proportion of individuals can adapt to and survive any perturbations.

3 Gene expression within the hypothalamo-pituitary-interrenal axis in two lines of rainbow trout, *Oncorhynchus mykiss*, selected for a divergent stress response

3.1 Abstract

Activation of physiological stress axes provides the physiological and behavioural stimulation to ameliorate or escape potential stressors. Numerous studies have identified consistent relationships between the magnitude of the neuroendocrine stress response and behaviour. However, these correlations are not always observed, and this may be due to individual plasticity in behaviour or physiology. Thus, it is critical to understand the molecular pathways within the stress response to identify the mechanisms of behavioural and physiological control during stress. Here the expression of eight candidate genes with roles throughout the teleostean hypothalamo-pituitary-interrenal (HPI) axis was determined in the brains of two lines of rainbow trout, *Oncorhynchus mykiss*, selectively bred for their divergent physiological responses to stress. Seven of these genes were significantly upregulated in the low responding (LR) line compared to the high responding (HR) line. Uniform upregulation of these genes does not translate to equivalent post-stress levels of their associated end-products. Thus, differences in the magnitude of HPI axis activation between lines may be controlled at the level of gene expression whilst finer scale regulation occurs via physiological feedback mechanisms. These data are the first to characterise the response to stress at the level of gene expression and clearly demonstrate molecular differentiation between animals of high and low physiological stress responding ability.

3.2 Introduction

For an organism in a challenging environment, the neuroendocrine stress response provides an adaptive solution by driving physiological and, subsequently, behavioural responses to alleviate the effects of stress (Wendelaar Bonga, 1997). However, chronic or repeated elevations of stress hormones can be detrimental to animal health (Charmandari et al., 2005). This is particularly important in intensive animal-rearing environments, such as in aquaculture where fish are necessarily and frequently exposed to environmental stressors (Barton, 1997). Chronic or repeated activation of the stress axis over an extended period of time can result in growth retardation (Pickering, 1993b) and reduce reproductive capacity (Campbell et al., 1992) and disease resistance (Pickering and Pottinger, 1989), and the associated reduction in flesh quality and yield (Pottinger, 2001) is of particular importance to the aquaculture industry. It is thus vital to gain a full understanding of stress responses with the aim of improving methods of animal welfare in stressful environments; one such method is through modification of stress responses through selective breeding programs (Pottinger, 2000; Pottinger and Pickering, 1997).

The stress response is coordinated through two key neuroendocrine axes. The sympathetic-chromaffin (adrenergic) axis utilises the catecholamines adrenaline and noradrenaline as hormonal end products. In contrast, the hypothalamo-pituitary-interrenal (HPI, in fish; Fig. 1.1) or -adrenal (HPA, in other vertebrates) axis is characterised by the release of corticosteroids (Wendelaar Bonga, 1997). The final product of this axis depends on the taxon, with many vertebrates utilising corticosterone but teleost fish use cortisol. The importance of this hormone in fish is evident considering the role it plays in hydromineral balance, such as that necessary in salinity acclimation, in addition to metabolic activity (Shaw et al., 2007). Due to the ease with which it is measured and because of its involvement in many of the deleterious effects associated with stress, post-stress plasma cortisol levels provide a strong candidate for selective breeding programs in fish (Pottinger and Carrick, 1999). The HPI response is a heritable trait in rainbow trout, *Oncorhynchus mykiss*, where the progeny of low stress-responding individuals also have a reduced cortisol response to the same stressor (Pottinger and Carrick, 1999, 2001b). Conversely, offspring from high stress-responding parents tend to characteristically display higher circulating cortisol levels after stress. The heritability of this trait is moderate

to high (h^2 = 0.41 - 0.73, Fevolden et al., 1999; Pottinger and Carrick, 1999), and the divergent HPI response was still evident in the F4 generation (Thomson et al., 2011).

Consistent correlations between the magnitude of the stress response and a suite of behaviours, termed stress coping styles, have been frequently identified in the laboratory (Koolhaas et al., 1999). Contemporary studies, however, have highlighted that there is also variation in behavioural responses within coping styles (Koolhaas et al., 2010); some of this behavioural variation may arise from the modifying effects of experience (Brown et al., 2007; Frost et al., 2007) and can conflict with current coping style theory (Ruiz-Gomez et al., 2008). However, considering the effects of hormones on behaviour it remains likely that different behavioural responses to stress, and personalities associated with stress responsiveness, are determined by variation in neuroendocrine activity. Given that both behaviour (e.g. Frost et al., 2007) and physiology (e.g. Auperin and Geslin, 2008) are to some extent plastic traits, and that coping style itself is not entirely rigid within an individual (Koolhaas et al., 1999), it is important now to study the molecular mechanisms shaping these traits to further our understanding of their operation and adaptive significance in natural populations.

Whilst the neuroendocrine pathways of the stress response axes are well documented, to date few studies have explored the correlated gene expression behind the control of the stress response in teleost fish. In a previous study, six candidate genes were differentially expressed between the two rainbow trout stress lines (Thomson et al., 2011). Here, we further characterise gene expression differences between the lines by investigating the expression patterns of a further eight candidate genes within the stress axis to examine in more detail the mechanism(s) underlying the observed differences in the cortisol response of HR and LR rainbow trout to stress. This information is relevant to understanding differences in stress responsiveness within populations. The genes selected for this study were chosen on the basis of their role in the HPI axis of the teleost stress response (Table 3.1). Included within this definition are: hormones involved in the neuroendocrine cascade resulting finally in the secretion of cortisol; receptors for these hormones; enzymes responsible for the inhibition or stimulation of hormone release or the processes responsible for hormone release; and neurotransmitters that modulate processes

within the stress axis. The HPI axis is also regulated by and interacts with various other pathways; for example, the serotonin pathway and HPI axis modulate each other through feedback mechanisms (Konakchieva et al., 1997; Winberg et al., 1997), and as such two measures of this pathway – the receptor Mel_{1A} and the enzyme AANAT – were thus included in this study.

3.3 Methodology

Experiments were conducted at the University of Liverpool under Home Office (UK) licensing and after local ethics approval. Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from CEH Windermere, and represented the fourth (F4) generation of lines selected for their low (LR, $n = 6$; 367.46 ± 31.39 g) and high (HR, $n = 6$; 266.11 ± 31.66 g) cortisol response to a confinement stress (Pottinger and Carrick, 1999). Lines were held separately in two stock tanks (2 x 2 x 0.5 m) with circulating UV-filtered fresh water at $13 \pm 2°C$ on an ambient 14:10 light:dark cycle, and allowed a period of at least 4 months to acclimate. These fish were transferred to individual glass tanks (90 x 50 x 45 cm), screened from visual disturbance in a semi-closed system with flowing aerated freshwater maintained at $10 \pm 1°C$ under a 14:10 h light:dark regime. Fish were fed 1% body weight per day with pellets (Skretting, UK). Fish were netted and killed by concussion and, to minimise any variation introduced by diel fluctuations in circulating cortisol levels (Pickering and Pottinger, 1983), this procedure was performed at the same time each day. Immediately after euthanasia a blood sample was extracted into a heparinised 2 ml syringe using sterile 25 G needles. The blood was centrifuged, and the supernatant plasma aspirated and stored at -20°C. Plasma cortisol levels were determined by radioimmunoassay (Pottinger and Carrick, 2001b). Following blood sampling, brains were removed and immediately frozen on dry ice prior to storage at -80°C until RNA extraction.

Total RNA was extracted from trout brain using TRIzol® (Invitrogen Life Science, UK), with DNase-treated RNA eluted into 50 µl RNase-free water. RNA concentrations were determined by optical density at 260 nm using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK) system and the quality of the samples assessed by 2% agarose gel electrophoresis. For each sample, approximately

Table 3.1: Names and abbreviations for genes used in this study (in alphabetical order), including a reference gene (*).

Gene and Abbreviation		Function
Arylalkylamine N-acetyltransferase	AANAT	An enzyme involved in the synthesis of melatonin from tryptophan in the pineal gland – AANAT catalyses the acetylation of serotonin (5-hydroxytryptamine) into N-acetylserotonin, the immediate precursor to melatonin. Melatonin has been linked with HPA/I axis regulation by attenuation of CRF release and interfering with declines in ACTH activity under chronic stress[1].
Calcineurin	CaN	A phosphatase involved in negative feedback of adrenocorticotrophic hormone (ACTH) secretion by mediating the Ca^{2+}-influenced inhibition of CRF action[2,3].
Corticotrophin Releasing Factor	CRF	Neurotransmitter hormone; released from the hypothalamus into the anterior lobe of the pituitary where it binds to CRF Type I receptors to stimulate the secretion of adrenocorticotrophic hormone, thereby initiating the HPI axis response [4].
Glucocorticoid Receptor Type II	GRII	Steroid receptor of cortisol (and other glucocorticoid ligands). Responsible for mediating the negative feedback of the HPI axis (inhibition of ACTH and CRF secretion), particularly under stressful conditions[5]. Also implicated in hydromineral acclimation[6].
Melatonin Receptor 1a	Mel$_{1A}$	Receptor for melatonin, the final metabolite of the serotonin-melatonin pathway. Melatonin has been linked with HPA/I axis regulation by attenuation of CRF release and interfering with declines in ACTH activity under chronic stress[1].

Table 3.1 continued

Gene and Abbreviation			Function
Melanocortin 2 Receptor		MC2R	Melanocortin receptor specific to adrenocorticotrophic hormone. Binding of ACTH stimulates the synthesis and secretion of cortisol in the interrenal[7].
Mineralocorticoid Receptor		MR	Steroid receptor; binds glucocorticoids (e.g. cortisol) and mineralocorticoids (e.g. deoxycorticosterone). Linked to stress regulation through feedback[5,8]; otherwise, as yet undefined role in teleost stress physiology.
Urotensin I		UI	Teleost orthologue of urocortin, and a member of the corticotrophin releasing factor family of neuropeptides. UI stimulates ACTH secretion in the teleostean pituitary, thereby initiating the HPI axis[9].
*Glyceraldehyde-3-phosphate dehydrogenase**		*GAPDH*	*Reference gene[10]*

[1]Konakchieva et al. (1997); [2]Antoni et al. (1994); [3]Shipston et al. (1994); [4]Chrousos and Gold (1992); [5]Charmandari et al. (2005); [6]Shaw et al. (2007); [7]Mountjoy (2010); [8]De Kloet et al. (1998); [9]Fryer et al. (1983); [10]Reilly et al. (2008).

1 µg of mRNA was reverse-transcribed into first-strand cDNA using random hexamers and SuperScript™ III reverse transcriptase (Invitrogen Life Science, UK), following the manufacturer's protocol. For RT-PCR, ~0.15 µg of the cDNA was amplified in a 10 µl PCR (using 5 µl Fast SYBR Green, Invitrogen Life Science, UK) primed with 2 pmol each primer. Pairs of primers for these genes were developed using Primer Express® 3.0 software against *O. mykiss* sequences (Table 3.2). Thermal cycling conditions, using a 7500 Fast Real-Time PCR System (Applied Biosystems), were: 10 min at 95 °C, followed by 40× [95 °C 3 s, 60 °C 30 s] and then [95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s and 60 °C for 15 s], which allowed the construction of a melting curve to assess the specificity of the product.

None of the data were normally distributed (Anderson-Darling; Minitab, ver. 15.1) and thus were analysed with non-parametric techniques. Cortisol values were compared between HR and LR fish using a Mann-Whitney U Test (R, ver. 2.7.0). Ct values for each gene, relative to the control gene and accounting for efficiency, were compared between HR and LR lines using REST© software (ver. 2.0.13; Pfaffl et al., 2002), which accomplishes statistical analysis using bootstrap randomisation techniques. ΔCt values (target – reference) for each gene were compared to plasma cortisol concentration using Spearman rank correlation coefficient (R, ver. 2.7.0).

3.4 Results

The LR individuals exhibited a slightly greater plasma cortisol concentration compared to the HR fish which was not significant ($W = 51.0$, $p = 0.07$, $n_1 n_2 = 6,6$; Fig. 3.1). These samples represent an unstressed or mildly stressed state and thus cortisol values were not expected to differ. Fish from these two stress lines did show the expected divergence in stress response after they were exposed to an acute stressor (Thomson et al., 2011).

RNA expression of seven of the investigated genes – CRF, UI, MC2R, CaN, Mel$_{1a}$, MR and GRII – was significantly upregulated in the LR fish compared to HR trout (Fig. 3.2). Only AANAT was not significantly differentially expressed between the two stress lines. These differences represented a mean fold change of between 2.46 in GRII up to 5.29 in MC2R. AANAT also exhibited a mean fold change of approximately 3.0, but this was associated with a large variance. Additionally, increased expression of AANAT, CRF, UI and CaN was significantly correlated with

Table 3.2: Primer sequences for each gene used in the study and a reference gene (*), with accession numbers, and product size (number of base pairs) and melting temperature (T_m, °C). Where more than one accession number is present, primer sequences were developed from aligned mRNA sequences. All primers were developed using Primer Express® 3.0 software except GRII and MR.

Gene	Accession No.	Forward (5'-3')	Reverse (5'-3')	Size (bp)	T_m (°C)
AANAT	AF033500	TCT CCT TGG GCT GGT TTG AA	AGC CTG AGC CGA TGA TGA AC	57	84
CaN	NM_001160568	TTC GAC ACC GAT GGA AAC G	CTG TGA GAC TCC CTC GAT GAA TT	60	81
CRF	AF296672	GTG GTT CTG CTC ATT GCT TTC TT	CGC CAG GGC TCT CGA TAG	61	82
GRII	AY495372	CCA GCA CCT CTA TGA CAA CC	AAG CCA GAT TCC TTC TCC	64	81
MC2R	NM_001124680	CGC TAC GTC ACC ATC TTC CA	CGC CTC GTG GTC ATG ATG T	56	84
Mel1a	NM_001124262	AAG GCT GGA ACC TGG GAT ATG T	GCT GAC GCC CAT GAG AAA A	59	82
MR	AY495584; AY495585	CAA CGA TGG CAG CTA CTA CC	GTC CGC CTG AAT TAA CAC C	80	81
UI	NM_001124343	CCG TCT GTC CAG CAC TAT GAA G	TGA GGA GAA CAG TGG CAA GGA	62	83
GAPDH*	AF027130	TGT TGT GTC TTC TGA CTT CAT TGG	CCA GCG CCA GCA TCA AA	60	81
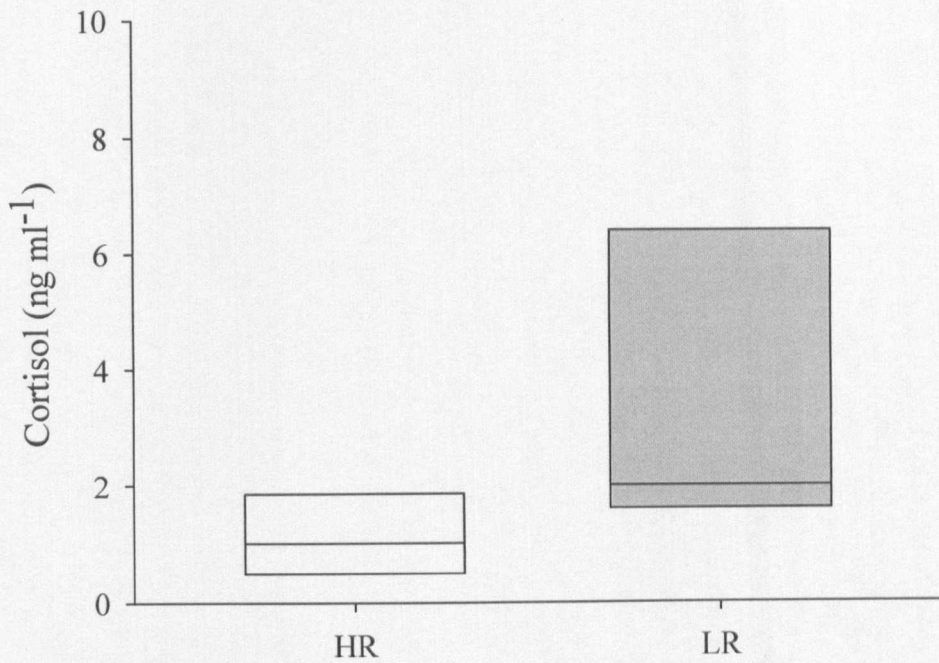
Figure 3.1: Median plasma cortisol (ng ml^{-1}; with 25th and 75th percentiles) in unstressed high (HR; $n = 6$) and low (LR; $n = 6$) stress responding lines of rainbow trout, *Oncorhynchus mykiss*.

Table 3.3: Spearman's rho (ρ) and statistical significance for the expression of eight candidate genes (ΔCt) and plasma cortisol concentration (ng ml^{-1}) in the rainbow trout, *Oncorhynchus mykiss* ($n = 12$ except for MC2R, $n = 11$).

Gene	ρ	p
CRF	-0.65	0.026
UI	-0.59	0.046
MC2R	-0.15	0.635
CaN	-0.72	0.011
Mel$_{1a}$	-0.47	0.128
AANAT	-0.77	0.005
MR	-0.57	0.055
GRII	-0.52	0.089
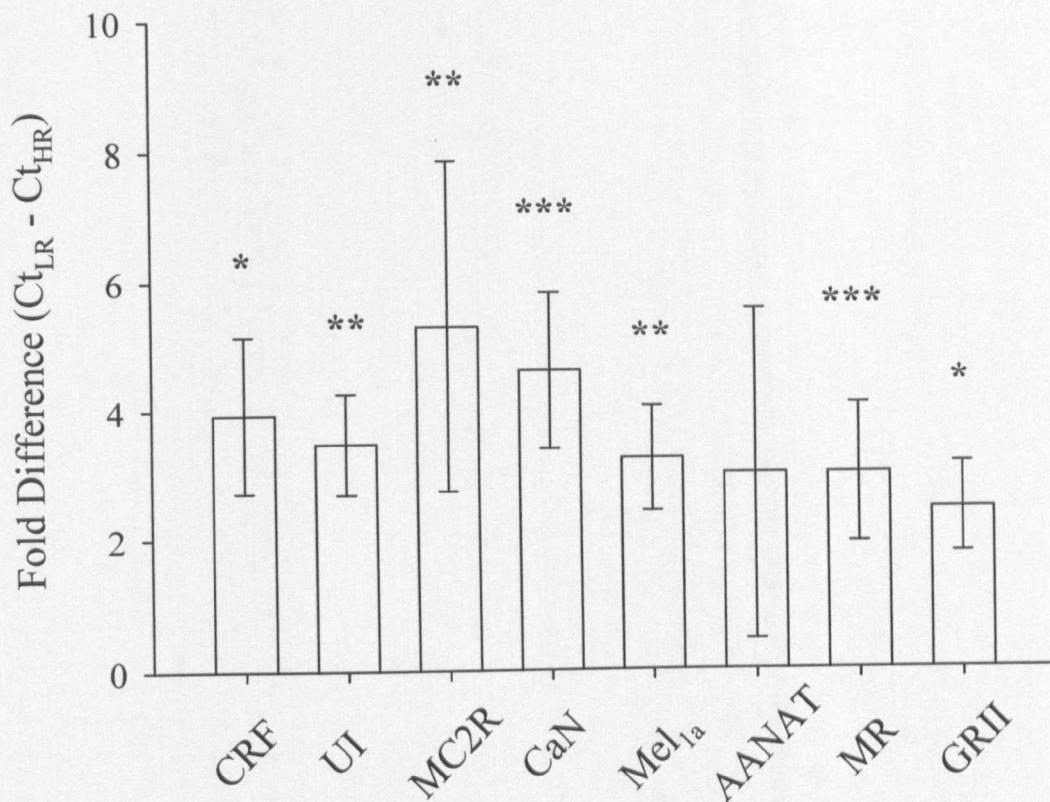
Figure 3.2: Fold difference (± SD) in the expression of eight genes between the brains of high ($n = 6$ except MC2R, $n = 5$) compared with low ($n = 6$) stress responding lines of rainbow trout, *Oncorhynchus mykiss*. Fold difference was calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔCt was calculated as ($Ct_{LR} - Ct_{HR}$). Probability values indicate differences in expression between LR and HR fish (obtained using Rest© analysis software, ver. 2.0.13): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

increased cortisol concentration (Table 3.3; Fig. 3.3); more negative Ct values indicate a greater copy number of transcript.

3.5 Discussion

The magnitude of the neuroendocrine response to a stressor is a heritable trait in rainbow trout, and thus selective breeding can be used to produce lines of animals exhibiting divergent stress responsiveness (Pottinger, 2000). Whilst the physiological

A - CRF

B - UI

C - MC2R

D - CaN

E - Mel$_{1A}$

F - AANAT

Cortisol (ng ml^{-1})

ΔCt
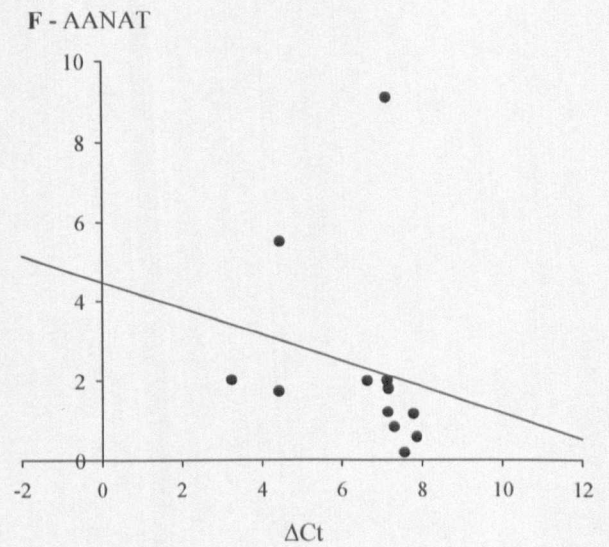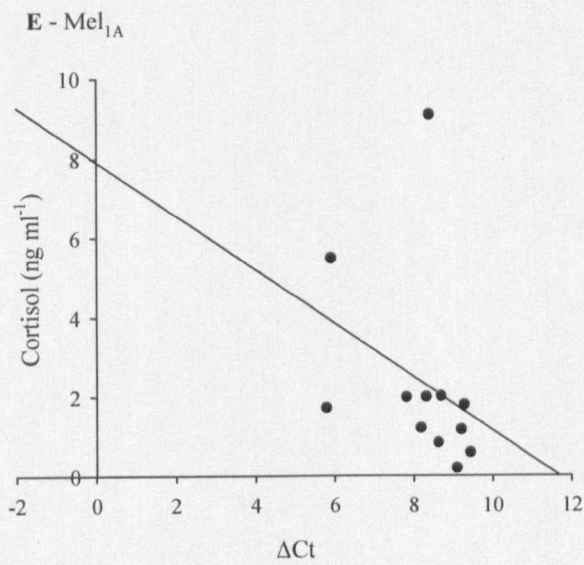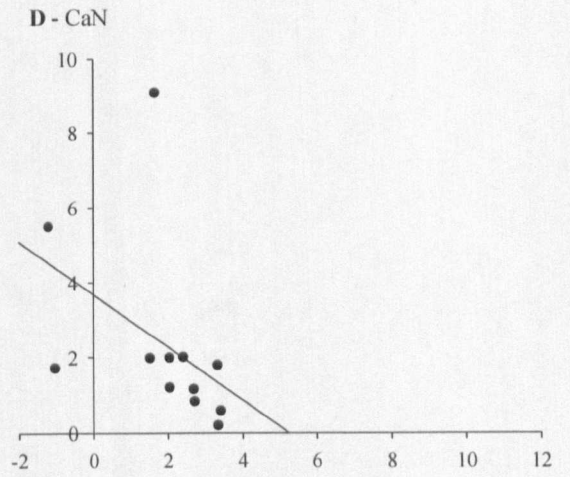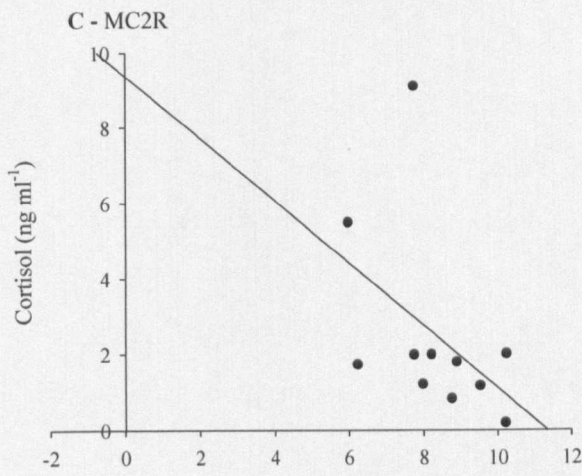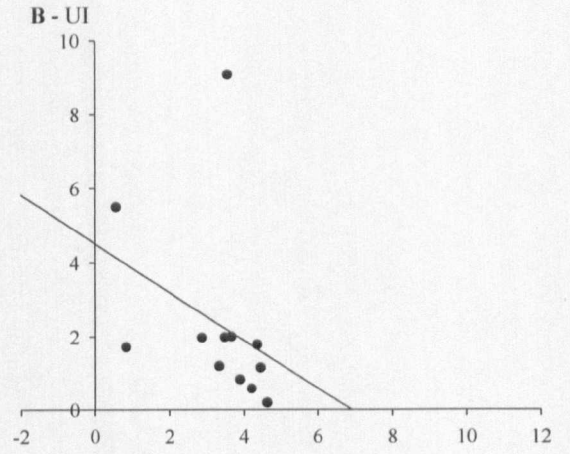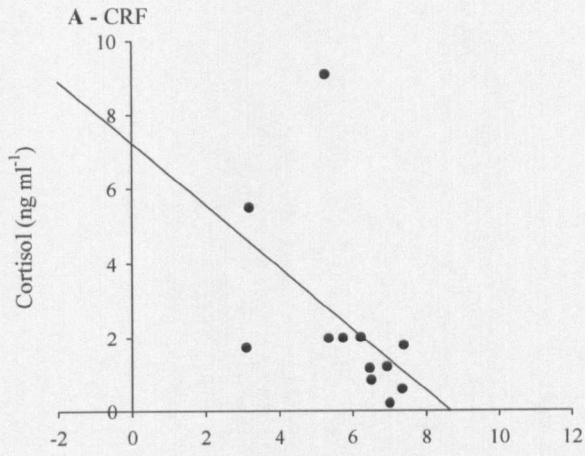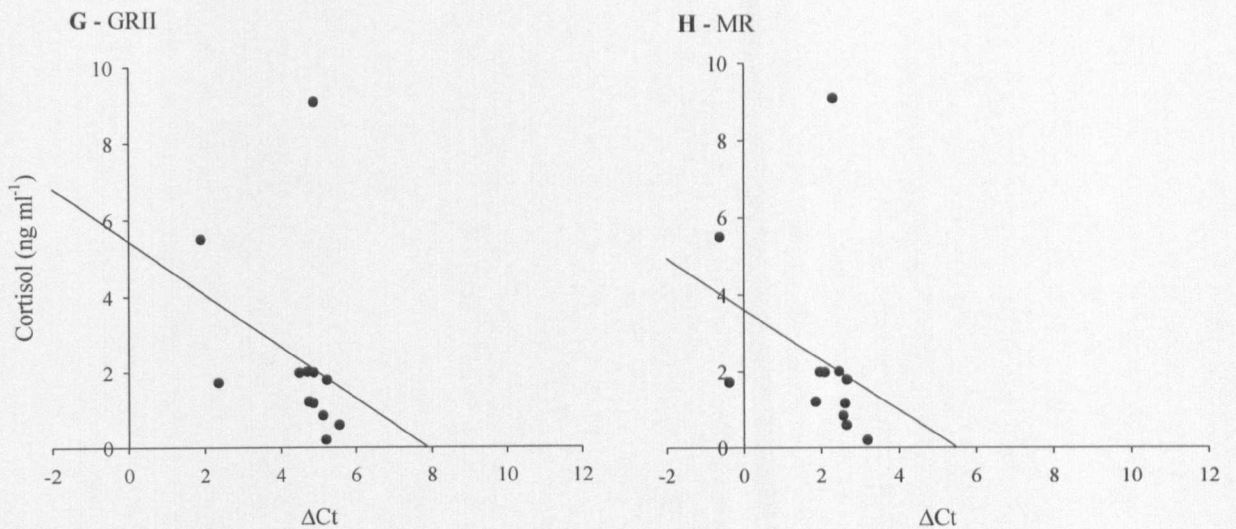
Figure 3.3: Correlations between plasma cortisol concentration (ng ml^{-1}) and the expression (ΔCt) of eight genes in the brains of rainbow trout, *Oncorhynchus mykiss* (n = 12 except for MC2R, n = 11): (A) corticotrophic releasing factor; (B) urotensin I; (C) melanocortin 2 receptor; (D) calcineurin; (E) Melatonin receptor 1A; (F) aralkylamine-N-acetyltransferase; (G) glucocorticoid receptor II; (H) mineralocorticoid receptor.

mechanisms behind these differences have been thoroughly explored (e.g. Sumpter, 1997; Charmandari et al., 2005), the genetic basis of variation in the neuroendocrine stress response has received less attention, particularly in fish. Here we have shown that there are clear differences in the expression of genes throughout a major neuroendocrine stress axis in rainbow trout, with seven of eight investigated genes upregulated in fish bred for a low cortisol response to a confinement stressor compared with those showing a high response. Furthermore, four of these genes were correlated with plasma cortisol concentration.

Eight genes were selected based on the roles they play in the hypothalamo-pituitary-interrenal stress axis of the rainbow trout; these complement a further six genes analysed in a previous study, four of which are involved in the HPI axis (Thomson et al., 2011). The genes considered in the present study (Table 3.1) represent a diverse range of functions, including peptide hormones (CRF, UI), hormone receptors (MR,

GRII, Mel_{1a}, MC2R), and enzymes (CaN, AANAT). Whilst each is specifically involved in the stress axis (or, in the case of AANAT and Mel1a, utilised as part of a separate molecular pathway which interacts with the HPI axis; Dinan, 1996; Kreke and Dietrich, 2008), the products of these genes also have functional significance in individual aspects of stress-related physiology or behaviour. However, the functional roles of these genes and their products depends upon the tissue in which they are expressed; thus, whilst these genes are linked with the HPI axis, expression levels within the brain, as measured here, cannot necessarily be directly linked with HPI activity *per se*.

CRF and UI both belong to the corticotrophin releasing factor family, and principally activate the HPI axis by stimulating ACTH secretion from the anterior pituitary (Lovejoy and Balment, 1999). Furthermore, UI may act synergistically with ACTH to stimulate cortisol secretion directly at the interrenal. It is therefore surprising that both these genes were expressed at a higher level in LR fish than in HR individuals since increased expression of both UI and CRF might be expected to result in increased HPI activity and, therefore, higher cortisol secretion, which is at odds with post-stress levels of cortisol observed in these fish (Pottinger and Carrick, 1999; Thomson et al., 2011). Backström et al. (2011) found the reverse relationship in CRF expression, but this may be explained by their samples being taken during the stress response. Similarly, increased expression of receptors would indicate heightened sensitivity of target tissue to stimulation; therefore, if functionally active receptor concentration was directly linked with gene expression then the greater expression of MC2R, to which ACTH binds to stimulate cortisol secretion (Metz et al., 2005), would also lead to an increased yield of cortisol. Alternatively, the HPI axis is self-regulated by feedback from its own products; increased receptor concentrations, and therefore tissue sensitivity, would result in more rapid induction of negative feedback on the system, thus reducing overall cortisol yield. CaN may also assist in the modulation of the HPI cascade through negative feedback by inhibiting the secretion of ACTH (Antoni, 1996; Shipston et al., 1994), and is itself modulated by calmodulin which is also upregulated in LR fish (Thomson et al., 2011).

Additionally, it seems that many of the receptors (e.g. MR, GRII for glucocorticoids, and Mel_{1A} for melatonin) are also upregulated in LR trout; previously MR, but not

the glucocorticoid receptors, had been shown to be differentially expressed between the lines (Johansen et al., 2011). If upregulation of these genes translates into greater concentrations of the receptors then it would suggest that the target tissue in low responding animals has increased sensitivity to the stress hormones. Thus, assuming expression in the brain is equivalent to that in other target tissue, LR fish, which have reduced glucocorticoid output from the HPI axis, may still produce an equivalent secondary and tertiary stress response to that observed in HR trout. Similarly Mel$_{1A}$ was upregulated in LR fish and could indicate a heightened regulatory role of melatonin on the HPI axis in low-responding fish. These receptor-coding genes were not, however, correlated with plasma cortisol concentration, suggesting that receptors may be regulated by cortisol only at high levels encountered during stress (e.g. Johansen et al., 2011).

In contrast AANAT, an enzyme involved in melatonin synthesis, did not differ in expression between the two stress lines in this study, although expression of the gene was correlated with cortisol concentration. Both melatonin and its precursor, serotonin, have important roles in the stress axis in terms of regulation at the hypothalamic and pituitary levels (Dinan, 1996; Konakchieva et al., 1997): melatonin may be involved in circadian regulation of the axis (Arendt, 1988; Konakchieva et al., 1997), but serotonin also features heavily in the control of aggressive behaviour, a defining feature of coping styles (Koolhaas et al., 1999; Summers and Winberg, 2006). Since AANAT expression was highly correlated to HPI activity, the high variance may be explained by the involvement of this gene in alternative processes that may not have been controlled in this study.

The HPI response is not a simple cascade producing a single, final product, but is instead an extremely complex web of interactions where its own products inhibit or promote further secretion (Wendelaar Bonga, 1997). Other molecular pathways, such as the serotonin-melatonin pathway, also interact with the axis to the same effect (Dinan, 1996). As a result appropriate levels of cortisol are maintained within the system, but this level of complexity makes it difficult to identify how alterations in individual aspects of the system are translated into variation in circulating cortisol after a stressful event. In this case the differentially-expressed genes were upregulated in the brains of LR trout. Furthermore, four of these genes – CRF, UI,

CaN and AANAT – were directly correlated with plasma cortisol concentration. Without additional information regarding post-translational modification of these genes it is unclear how gene expression translates into concentration of these proteins and molecules, and whether or not such modification also varies between the two lines. There are multiple potential processing events downstream of mRNA synthesis, including translocation throughout the cell, cleavage of specific parts of the sequence (e.g. for activation of the molecule), or bonding with other groups such as through glycosylation or phosphorylation (Feder & Walser, 2005; Gygi et al., 1999); seemingly, though, there has been little work to elucidate the importance of these processes in the stress response *per se*. Proteolytic cleavage is one critical aspect of the stress response as it is the process by which various stress proteins, including the melanocyte stimulating hormones and ACTH amongst others, are derived from the precursor molecule POMC (Rotllant, 2000). ACTH is a pivotal molecule in the HPI axis and appears to be differentially produced by animals with low and high HPI responses to stress (Balm & Pottinger, 1995). Furthermore, mRNA abundance does not provide details on rates of protein synthesis and degradation, and it is therefore difficult to obtain an accurate view of protein activity by using relative mRNA concentration alone (Feder & Walser, 2005). This may, in part, be a result of independent actions of factors controlling gene expression compared with those controlling translation, which may themselves vary dependent on current intracellular protein concentrations (Buckley et al., 2006). Furthermore, the site of action of the products of some of these genes, when involved in the HPI axis and the control of the stress response, occur outside of the brain or in unrelated tissues (e.g. interrenal binding of ACTH to MC2R). Thus, measurements of the expression of these genes and their activity within brain tissue does not necessarily reflect their relative importance in the axis itself, but may indicate divergence in stress-related functionality between the two stress lines. It is therefore imperative that future work not only identifies those post-translational processes relevant to the stress response but also should seek to determine differences in these processes between animals exhibiting high and low physiological responses to stress.

Another possibility is that, rather than these genes controlling the regulation of cortisol secretion, behavioural responses to stress may be controlled by the action of cortisol on gene expression, and thus divergent cortisol production between high and

low stress-responding animals may result in differential gene expression. It is important to note that expression of mRNA does not necessarily directly relate to protein concentration (Bustin, 2002; Gygi et al., 1999), perhaps indicative of variation in the timing of gene expression and protein synthesis (Buckley et al., 2006), although strong links between the two have previously been observed (Buckley et al., 2006). The disparity between gene expression and concentration of their products may thus be a result of variation in post-transcriptional translation of the genes or the continued feedback control within the stress axis, or indeed a combination of the two. Furthermore, in this study, we observed that in an unstressed state increased expression of CRF, UI, CaN and AANAT was correlated with increased plasma cortisol, whereas the reverse is likely in stressed fish (Thomson et al., 2011). Gene expression is thus intimately linked with each line, but coordination between genetic and physiological mechanisms may differ at different stages of the stress axis. This could explain different patterns of gene expression observed at different time points, or even brain regions (Backström et al., 2011; Johansen et al., 2011), and may indicate a facet of HPI axis control that remains unexplored. It is, therefore, critical for further studies to determine a direct functional relationship between gene expression and protein activity to establish how they are related throughout the HPI axis both during the stress response and when it is inactive.

These data represent a fundamental difference in the operation of the HPI axis in rainbow trout. Few studies have investigated the expression of stress-related genes in relation to hereditary divergence in cortisol reactivity to stress (e.g. Backström et al., 2011; Johansen et al., 2011) but to our knowledge this is the first to demonstrate this divergence throughout many levels of the HPI axis. Whilst stress responses are heritable, the development of the HPI axis can be affected throughout an organism's life (Charmandari et al., 2005; Wendelaar Bonga, 1997), particularly through early ontogeny where, for example, prenatal exposure to stress hormones can define neuroendocrine responses in animals at later stages of development (Auperin and Geslin, 2008). Pursuing a similar characterisation of the molecular differences between high and low responders after such a shift in neuroendocrine response would provide important data regarding the molecular control of the stress response.

4 Social transmission of bold and shy behaviour in the rainbow trout, *Oncorhynchus mykiss*

4.1 Abstract

Personality, as measured by the degree of boldness in animals, has a major impact on decision-making and influences individual reactions to environmental challenges. Individual variation within populations leads to bold or shy behavioural strategies and in certain contexts may have implications for overall fitness and survival. Recently boldness has been determined to be plastic and is shaped by both hereditary background and experience. Studies have postulated that bold or shy behavioural traits can be transmitted between individuals. Here we investigated social learning in rainbow trout, *Oncorhynchus mykiss*, specifically how individual boldness may be modified by learning from bold and shy conspecifics. Individual focal fish were assessed for boldness using a standard novel object test before being placed into a group of completely bold or completely shy trout. The focal fish were allowed a week within the group, during which they were exposed to various environmental cues to stimulate behavioural transmission, before they were reassessed for boldness. Bold trout were more behaviourally labile and became shyer regardless of the group they were placed in. In contrast shy trout generally remained shy, the exception being that both bold and shy focal fish reduced the number of swims they made after placement in the shy group. Stress-induced plasma cortisol levels were significantly different between initially bold and shy trout, regardless of treatment, and resembled the expected responses from proactive and reactive physiological coping styles respectively. Trout may therefore be able to make behavioural adaptations to their environment based upon the responses of others, but the extent to which individuals can do this may depend on their previous level of boldness.

4.2 Introduction

Consistent intraspecific variation in animal behaviour is thought to be critical for how populations or species respond and adapt to environmental or exogenous variation (Slater, 1981). Often, different behavioural traits are linked to form an animal personality (Gosling, 2001), where these traits are considered to be consistently correlated across contexts (Bell and Stamps, 2004; Sih et al., 2003). One such personality factor is boldness, which defines the amount of risk an animal is prepared to take in situations of novelty, and has been linked with aggressiveness and levels of activity and exploration (Lima and Dill, 1990; Sneddon, 2003; van Oers et al., 2005b).

Both bold and shy behaviour can improve fitness dependent upon the prevailing environment and conditions (Dingemanse et al., 2004), potentially resulting in population compositions skewed towards one end of the bold-shy continuum (e.g. Bell, 2005; Brown et al., 2005b). However, in social organisms the overall behaviour of a group may be more important in determining fitness, and is generally influenced by the interactions of individual animals within that group (Michelena et al., 2011). As such, the benefits and costs of alternative behavioural strategies, such as boldness or shyness, may be shared or moderated throughout the group (Magnhagen and Bunnefeld, 2009; Sih and Watters, 2005).

Personality has repeatedly been linked with physiological responses to stressors (e.g. Koolhaas et al., 1999) where individuals are described according to their coping style: proactive animals respond to stress with low hypothalamo-pituitary-interrenal (HPI) axis activity, and tend to exhibit bold behaviour. Conversely, those with a high HPI response are characterised by inactivity and shyness, and are described as reactive. Additionally, proactive and reactive animals have different propensities towards behavioural change, with proactive animals generally being more likely to form routines and having more rigid personalities than the more behaviourally labile reactive animals (Cockrem, 2007; Koolhaas et al., 1999; Ruiz-Gomez et al., 2011). These adaptive individual differences are a heritable trait and are likely to provide solutions to alternative environmental pressures (van Oers et al., 2005a). However, some recent data suggest that proactive and reactive profiles are not in themselves rigid, but that the link between physiological stress reactivity and social behaviour

can be decoupled under certain situations (Ruiz-Gomez et al., 2008). If behavioural traits such as boldness are strongly linked with stress physiology in laboratory conditions yet behaviour can be labile, then this raises the question of whether physiological responses to stimuli exhibit a similar plasticity, and whether measured stress responses reflect current behavioural phenotypes or those of an earlier life stage. It is therefore important to further consider how, and to what extent, changes in personality modify stress responsiveness.

Different aspects of behaviour are, in part, hereditary, but whilst the behaviour of early life stages of animals may be strongly influenced by genetic background, personality can be influenced by the environment (Robinson, 2004). Boldness, in particular, may be maintained across generations (Bell and Sih, 2007), but interactions with exogenous stimuli often lead to changes in behaviour along the bold-shy continuum (Bell and Stamps, 2004; Cockrem, 2007; Frost et al., 2007). This behavioural plasticity may be manifest as rapid, reversible changes such as a switch to reduced activity in the presence of a predator. Alternatively, learned or experiential variations in behavioural strategy may be drawn from a pre-existing repertoire, and are often regarded as irreversible (Mery and Burns, 2010). Learning can, however, be a slow process which is often associated with the cost of suboptimal behaviour performed in a novel situation (Mery and Burns, 2010). Learning from other animals can facilitate the process and thus bypass the need for trial-and-error mechanisms. Amongst social animals the transmission of behaviour is accomplished through observation or interaction with a demonstrator (Heyes, 1994). Since social animals spend the majority of their time amongst conspecifics, behaviour would be shared across the group (Galef, 1995), potentially resulting in behavioural changes in individual members (Magnhagen and Bunnefeld, 2009). However, it remains unclear whether these changes are temporary adaptations to group living or learned behavioural strategies which are maintained and utilised outside of a group context.

Whilst recent studies have focussed on individual behavioural characteristics few have attempted to determine how personality affects behavioural composition within a group or to assess how bold and shy personalities, believed to vary in terms of their propensity towards plasticity, may be modulated by the composition of a social

population (e.g. Magnhagen, 2007). Likewise, the link between behavioural plasticity and that of physiological changes linked with behaviour remains largely unexplored.

The main aim of this study was therefore to determine to what extent individual levels of boldness in rainbow trout, *Oncorhynchus mykiss*, can be influenced by social composition. Specifically, we examined whether 1) individual trout will assume the behavioural characteristics of a population with which they live, and; 2) whether behavioural plasticity is reflected in plasticity in the physiological stress response, measured as post-stress plasma cortisol concentrations, in these fish.

4.3 Methodology

The following experiment was conducted humanely under Home Office, UK, guidelines according to the Animal (Scientific Procedures) Act 1986, and following local ethical approval. Rainbow trout, *Oncorhynchus mykiss*, obtained from a commercial supplier were placed directly into stock tanks (2 x 2 x 0.5 m) where they were maintained on a semi-recirculating system on a 14:10 h light:dark cycle at $13 \pm 1°C$, and fed 1% body weight per day on commercial trout feed (Skretting, UK). Half of the tank had an opaque cover for shelter. For experimental procedures, fish ($n = 34$, 35.64 ± 3.09 g) were caught at random and transferred to individual glass aquaria (90 x 50 x 45 cm) which were screened from visual disturbance. All tanks were supplied with filtered freshwater and maintained at $10 \pm 1°C$ with constant aeration, and feed provided at 1% body weight per day at the same time each day. Fish were allowed to acclimatise for at least one week, or until the resumption of feeding, before being evaluated for boldness.

4.3.1 Boldness trials

In all cases, boldness was assessed using novel object tests, which have previously been used to distinguish bold and shy behaviour in rainbow trout and other teleosts (e.g. Frost et al., 2007; Thomson et al., 2011). A custom-built low-light video camera was positioned in front of the tank, and measuring rulers placed along the tank to accurately assess the position of the fish and object. Fish were allowed 10 minutes to recover from the initial disturbance before recording began. After 10 minutes the novel object was placed into the centre of the tank, and behaviour of the fish was

recorded for a further 10 minutes. Different novel objects were used throughout to avoid fish becoming habituated to a familiar shape, and included an orange frustum-shaped bung (7.1 cm mean diameter, 4.9 cm height), a blue box weighted with gravel (7.5 x 5.3 x 3.8 cm), or Lego Duplo™ constructs of various shapes, sizes and colours. Boldness was scored using custom-designed behavioural-analysis software according to three factors: latency to approach to within 5 cm of a novel object, duration of time that individuals were passive, and the frequency of initiating passive behaviour. Passive behaviour included the subject resting at the base of the tank, pivoting on its own axis, and drifting across the tank, but excludes swimming under its own propulsion more than one body length.

4.3.2 Population tanks

Separate populations of either nine bold or nine shy trout were set up in large tanks (~149 x 92 x 37 cm) in identical conditions to the stock tanks; each tank contained two populations of the same behavioural type that were separated by a divider and thus unable to interact, although they did share the same flow-through of water. Populations were established over a one-month period, with the same fish utilised throughout the experiment (to minimise total numbers of animals used in accordance with Home Office guidelines), although any losses were replaced with individuals recently assessed for boldness.

4.3.3 Focal Subjects

The day following transfer from stock tanks to test tanks, focal trout were netted and anaesthetised (benzocaine (Sigma-Aldrich Co., UK) at 0.033 g l⁻¹) and tagged using yellow VIE (visible implant elastomer, Northwest Marine Technology inc.) directly behind the eye. Each fish was weighed to 0.01g, returned to its individual aquarium and allowed to acclimatise for one week or until resumption of feeding. Focal fish were then given a novel object test to assess initial boldness before being placed into one of the population tanks.

Over a period of 1 week each of the following cues were used, in random order, to reinforce or enhance interactions and facilitate possible social learning between the focal fish and other conspecifics within their populations:

1) Novel object exposure - a novel object was placed centrally into the tank and left for 15 minutes before being retrieved with a net.

2) Simulated predator attack – food pellets were presented to the group and when one fish had attempted to feed, predator threat was simulated using a model heron's (*Ardea cinerea*) head that was thrust towards the group of fish twice in succession (see e.g. Johnsson et al., 2001b; Jönsson et al., 1996).

3) Net chase - the group was chased with a small hand-net for 2 minutes, with the net moved around the tank to ensure that each fish was chased.

After one week, the focal fish was identified by its VIE tag, carefully retrieved and replaced in an individual tank where it was allowed 24 hours to recover, after which point it was retested for boldness using a second, unfamiliar novel object.

On the same day as the final behavioural trial, focal fish were given an emersion stress by being netted and lifted from the water for 60 s to induce a physiological stress response (Pickering and Pottinger, 1989). The fish were then replaced and left for 15 min to allow a maximal cortisol response to the stressor develop. At this point the fish was netted again and killed humanely by concussion. Fish were killed at the same time each day to ensure that hormonal levels were not compromised by diel fluctuations (Pickering and Pottinger, 1983). Immediately after they were killed the fish were weighed before a blood sample was taken from the caudal vessels into a 2 ml heparinised syringe using sterile 25G needles. The blood was centrifuged at 3 500 RPM for 5 min at 4°C, and the supernatant plasma aspirated and frozen at -20°C until further analysis. Cortisol concentrations were subsequently determined using a radioimmunoassay procedure (Pottinger and Carrick, 2001b).

4.3.4 Data Analysis

Change in three behavioural parameters (latency to approach within 5 cm of the novel object (s); duration of passive behaviour (s); frequency of passive behaviour, specific growth rate (G), and post-stress plasma cortisol levels (ng ml^{-1}) were compared using a general linear model, using initial boldness and test population as parameters. Specific growth rate was calculated according to Höjesjö et al. (1999) as:

$G = 100 \times [log(w_f) - log(w_i)] \times t^{-1}$, where w_f = final weight (g), w_i = initial weight (g) and t = time in days. *Post-hoc* analyses were accomplished using a

Bonferroni adjustment. Data met the assumptions of a GLM. To determine if there was a significant change in median boldness, comparisons of change in latency to approach the object to within 5 cm for each treatment were compared using Wilcoxon signed rank tests. All analyses were performed using R (ver.2.9.0).

4.4 Results

Overall, two populations each of bold and shy fish were established, and 34 focal fish were tested (in bold populations: 5 bold and 11 shy; in shy populations: 8 bold and 10 shy). Boldness changed principally amongst bold trout: both latency to approach within 5 cm of the object ($F_{1,30}$ = 36.14, p < 0.001) and duration of passive behaviour ($F_{1,30}$ = 5.43, p = 0.027) significantly differed between initially bold and shy individuals, but the population they were placed in did not impact on these behaviours (Figs 4.1A, 4.1B). Bold trout generally became shyer, taking over twelve times longer to approach the object and spending 30% more time being passive; in contrast, shy fish exhibited approximately 10% increase in latency, which was not significant (Table 4.1), and no change in their levels of activity. Both bold and shy trout placed into a shy population almost halved their frequency of passive behaviour, accounting for a significant reduction ($F_{1,30}$ = 4.20, p = 0.049; Fig. 4.1C) compared to those placed in a bold population which did not change this behaviour.

Table 4.1: Wilcoxon signed rank test values for median change in latency to approach within 5 cm of a novel object of rainbow trout, *Oncorhynchus mykiss*, which differ in initial boldness and placed into either a bold or shy behavioural population.

Treatment	n	V	p
Bold in bold population	5	15	0.06
Bold in shy population	8	36	<0.01
Shy in bold population	11	10	0.10
Shy in shy population	10	6	0.18

Most focal individuals generally lost a small amount of weight throughout the test period (Fig. 4.2), with only shy trout placed into a shy population showing a mean increase in specific growth rate. However, growth rate between trials was not significantly different either between bold and shy rainbow trout ($F_{1,29} = 0.40$, $p = 0.53$) or between populations ($F_{1,29} = 2.19, p = 0.15$).

All of the trout in the study exhibited raised cortisol levels indicative of a physiological response to the emersion stress. Similar to aspects of the behaviour, post-stress plasma cortisol levels (ng ml^{-1}) significantly differed dependent on initial boldness: irrespective of the population they were placed into, shy fish showed an almost threefold greater physiological response to stress than bold fish ($F_{1,30} = 11.31$, $p = 0.002$; Fig. 4.3).

4.5 Discussion

Social transmission of behaviour between individuals is an important method of acquiring novel behavioural responses to environmental stimuli (Heyes, 1993). In this study, when held in a group of either bold or shy fish, bold rainbow trout were behaviourally labile, capable of altering their phenotype towards a shy personality regardless of the behavioural profile of the group. In contrast, shy fish did not significantly alter their behaviour when placed in a bold or shy group, generally remaining shy throughout. Initially bold and shy fish also had distinct physiological stress-response profiles which were not affected by individual treatments. Additionally, none of the treatments resulted in differential weight change in trout. Thus, behavioural change did not affect the physiological profile of these fish.

4.5.1 Behaviour

Individual animals may copy others if the behavioural outcomes are beneficial (Brown and Laland, 2002), and here bold fish placed into a shy population shifted their behavioural phenotype towards that presented by the group, becoming more neophobic and less active. Shy fish are generally less active and exploratory than bold animals, particularly when reacting to unfamiliar stimuli (Frost et al., 2007; Wilson et al., 1993). Bold focal fish, observing and experiencing the shy behavioural responses during exposure to the three threatening cues, appear to learn or adopt shy characteristics within the seven day period. Previous data suggests that the adoption
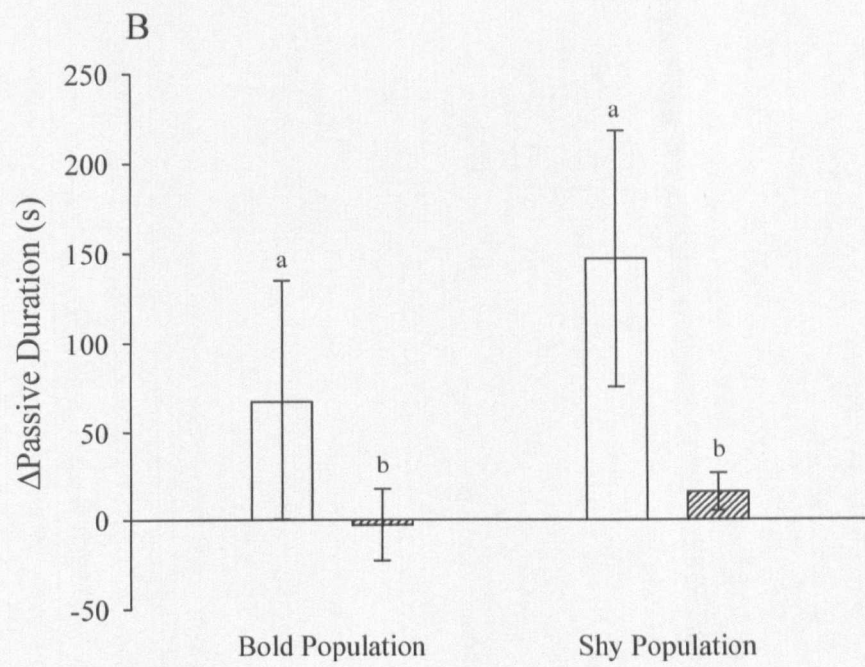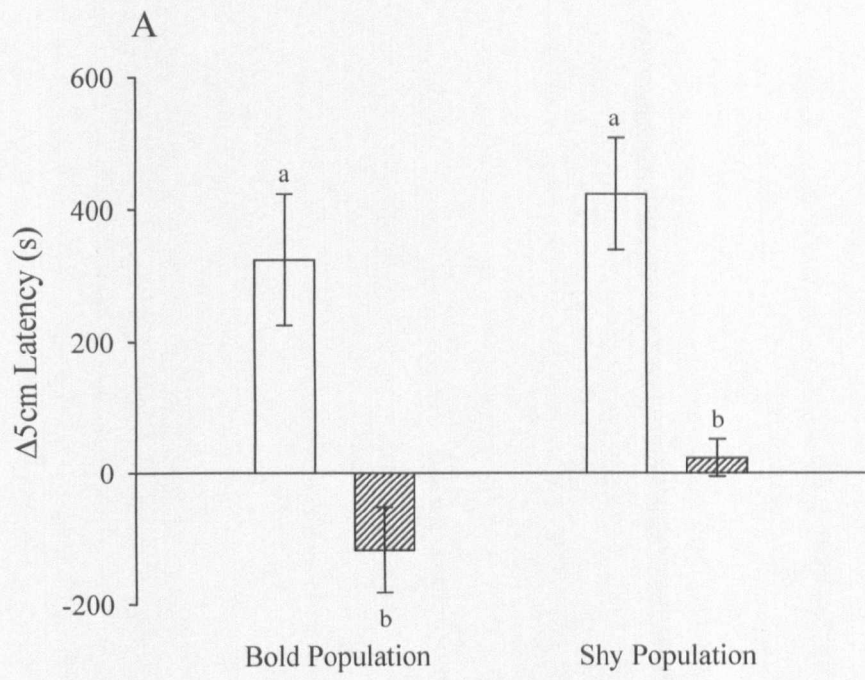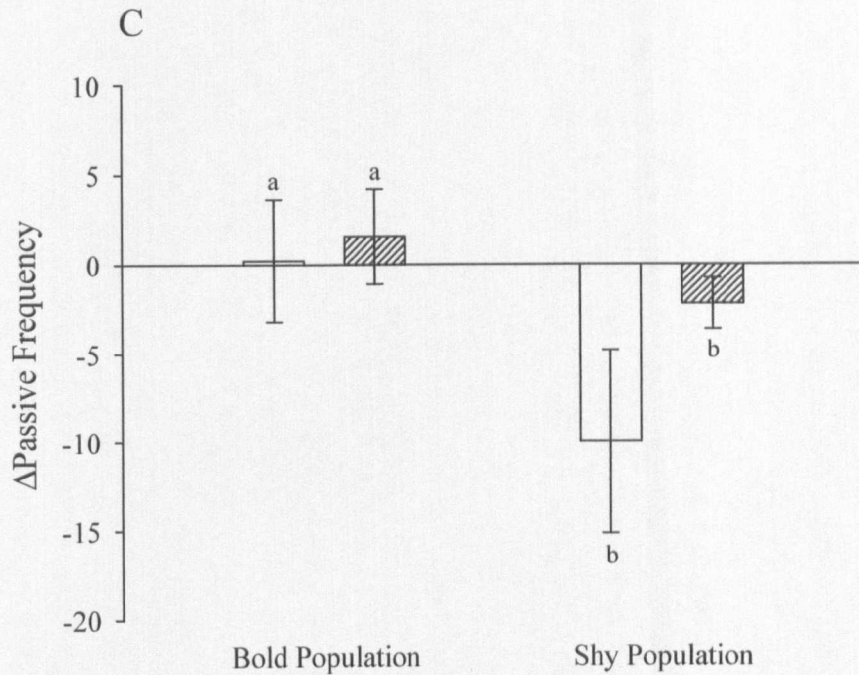
Figure 4.1: Mean (±SE) change in A) latency to approach within 5cm of a novel object, B) duration of passive behaviour, C) passive frequency in bold (white bars) and shy (hatched bars) rainbow trout, *Oncorhynchus mykiss*. Individual trout were placed into a group of either nine bold or nine shy conspecifics for one week between novel-object trials. Significance values represent differences between treatments: groups which do not share a common lower case letter were significantly different ($p < 0.05$). $n = 5,8$ for bold trout and $n = 11,10$ for shy trout in a bold and shy population respectively.

of social tactics through interaction with conspecifics tends to occur between a period of 3 and 7 days (Frost et al., 2006; LePage et al., 2005; Winberg et al., 2001). Adopting the general behavioural profile of a group facilitates group cohesion and, therefore, the sharing of useful public information (Krause and Ruxton, 2002; Magnhagen and Bunnefeld, 2009) and is therefore of great benefit to the individual. Furthermore, behaving differently to the group is an obvious extension to the oddity effect (Krause and Ruxton, 2002): it would be maladaptive to stand out from other animals, particularly here where there is increased predator threat. In contrast, without experience of alternative strategies or the necessary inclination to adopt them, the shy focal fish maintained within a shy population did not significantly alter their behavioural characteristics.

72
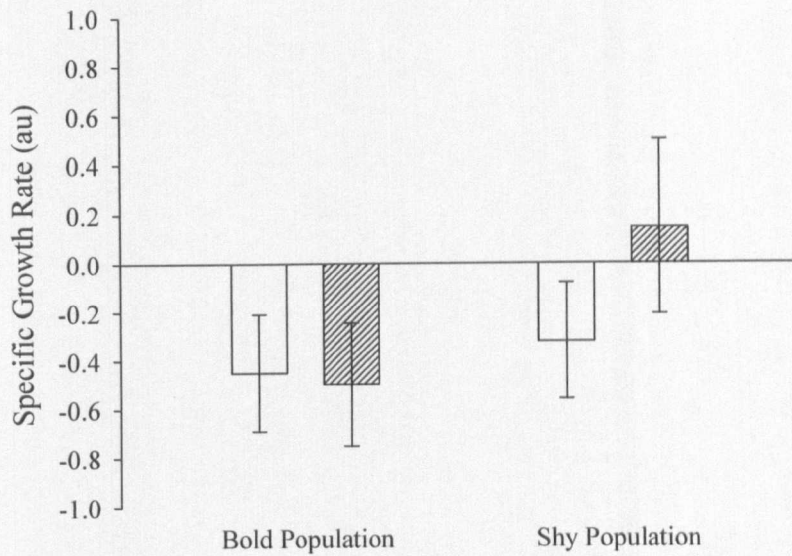
Figure 4.2: Mean (±SE) specific growth rate (arbitrary units) of bold (white) and shy (hatched) rainbow trout, *Oncorhynchus mykiss*, placed for one week into a population of nine bold (*n* = 16 bold, shy) or nine shy (*n* = 18 bold, shy) trout.

A shift towards shyness when placed in a bold group likely reflects alternative social dynamics in the rainbow trout. One reason may be that the focal fish were subjected to territorial aggression: salmonids, including rainbow trout, form dominance hierarchies through agonistic interactions (Sneddon et al., 2005; Winberg and LePage, 1998). These hierarchies are relatively stable but, particularly amongst bold animals, the introduction of a new fish may result in heightened aggression and tension within the group aimed towards the intruder (Höjesjö et al., 1998; Johnsson, 1997). The focal fish may therefore need to alter its behavioural strategy to accommodate itself within the population, perhaps taking on more submissive and less active behaviours which then persist when that animal is placed into isolation (Blanchard et al., 1993; Coussi-Korbel and Fragaszy, 1995; Øverli et al., 1998). Animals may also reduce their levels of aggression if subject to high levels of aggression from more dominant fish (Øverli et al., 2004); bold animals are often more aggressive (Sih et al., 2004a) and, if true of these trout, this may therefore also help to explain the reduction in boldness observed in the focal fish. Shy fish, in
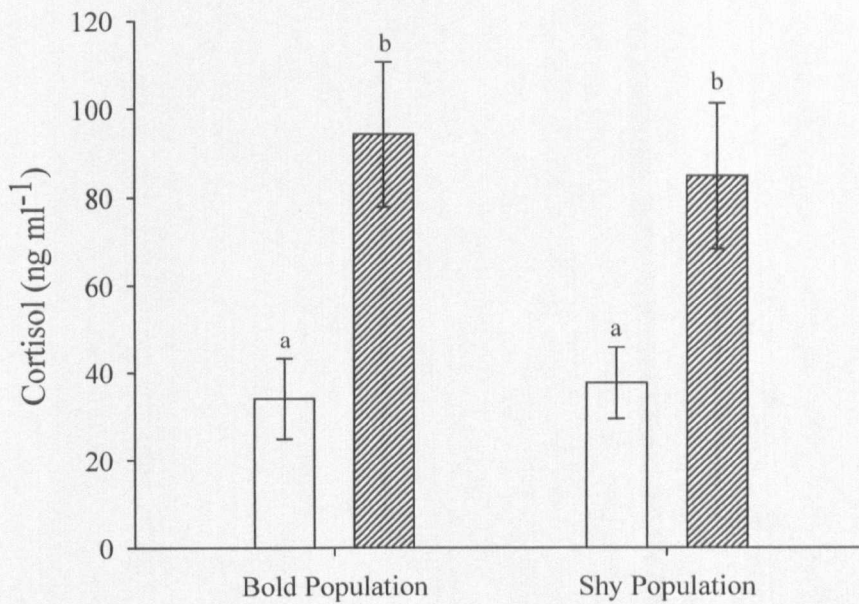
Figure 4.3: Mean (±SE) plasma cortisol concentrations in bold (white bars) and shy (hatched bars) rainbow trout, *Oncorhynchus mykiss*, 15 min after a 60 s emersion stress. Individual trout were placed into a group of either nine bold or nine shy conspecifics for one week before sampling. Groups which do not share a common lower case letter were significantly different ($p < 0.05$). $n = 5,8$ for bold trout and $n = 11,10$ for shy trout in a bold and shy population respectively.

contrast, are generally already submissive (Bell, 2005; Huntingford, 1976; Sundström et al., 2004) and would potentially require less behavioural adaptation to fit into the group. However, the relationship between aggression and boldness can be complex, for example depending upon exogenous or environmental influences, and under such conditions it is possible for the two to become decoupled (Bell and Sih, 2007). As such, it is important to consider rank within a hierarchy relative to boldness, and future work should focus on the dynamics of behavioural change within the group in addition to overall change after removal from it. Furthermore, the effects of social dominance may not be as strong in species with different social strategies.

An alternative explanation for the change in behaviour of bold but not shy trout may be due to fundamental differences in learning ability or motivation between the two

personalities. Bold rainbow trout tend to learn more quickly and retain memories for longer (Sneddon, 2003); as such, here bold trout may have been able to adjust their behaviour within a short time span, whereas the shy trout were unable or unwilling to do so in the seven day period with which they spent the group. Furthermore, bold and shy fish may differ in their propensity for social learning or for learning by experience (Magnhagen and Staffan, 2003); this aspect of learning remains to be fully explored. Additionally, the behaviour of social animals within a group may differ to that when in isolation: shoaling perch, for example, tend to become shyer after having been removed from their group, and this may reflect an increase in perceived risk when kept alone (Magnhagen and Bunnefeld, 2009). The increased shyness observed here in focal individuals after one week spent in a small group may be explained by a similar mechanism. However, all fish were originally taken from stock tanks, and this theory cannot therefore explain the wide variation in boldness between individual fish in the first novel object trial.

In addition to the potential decoupling of the aggression-boldness syndrome, a further possible dissociation of behaviour within these fish was observed with frequency of passive behaviour which, in contrast to change in neophobia and activity levels, was reduced in both bold and shy fish when they had been placed into a shy population. This may reflect that within the shy population, where aggression is thought to be lower (Sih et al., 2004a), there may be a less competitive environment (Ward et al., 2004). Within a bold, and potentially aggressive, population individuals may need to avoid agonistic interactions and compete for scarcer food pellets, resulting in skittish or flighty behaviour, which may not be true of the shy population.

An additional complication is that behavioural change in these animals may have been due directly to the environmental cues rather than learning from the group. Novel object presentations generally do not cause a shift in behaviour, at least in rainbow trout (Frost et al., 2007; Thomson et al., 2011), whilst net chasing may cause a shift towards boldness (Brown et al., 2007) although this was not observed here. In general animals will reduce their activity levels when predators are present (Johnsson et al., 2001b; Lima and Dill, 1990), and simulated predator attacks may cause a similar response (Jönsson et al., 1996). Adaptive responses to a threat would be to

seek cover and remain inactive, thereby avoiding danger. However, the fish used in the present study originated from a farm environment where natural predator threat is low, and thus effective anti-predator responses may not be inherent in these fish (Álvarez and Nicieza, 2003; Malavasi et al., 2004; although cf. Scheurer et al., 2007). Furthermore, the effect of simulated predator attacks on overall boldness is unclear as, although activity is often reduced, in some instances activity and exploration may increase under higher levels of threat (Archard and Braithwaite, 2011). Therefore, it seems unlikely that behaviour would be skewed one way or another as a result of the environmental treatments alone. In contrast, we expect environmental challenges (as simulated here by each cue) to reinforce learning from conspecifics (Smith, 1997) and for the focal fish to obtain information on the perceived riskiness of the environment. A further possibility is that the population fish became habituated to the environmental cues since these populations contained the same individuals throughout the study. As such their own responses to cues may change as they habituate to the stimuli (Barton et al., 1987; Magurran and Girling, 1986). Here we varied the order and timing of presentation and used differently shaped and coloured objects, a process which can mitigate any effects of habituation (Frost et al., 2007; Sneddon et al., 2003).

Weight and size are important indicators of overall health but change in these parameters are also useful indicators of feeding rate and/or gastrointestinal health, both markers of stress (Olsen et al., 2005; Wendelaar Bonga, 1997) and proxies for food acquisition in hierarchies (Sneddon et al., 2006). That neither the behaviour of the population nor the initial boldness of the focal fish appeared to influence specific growth rate in the focal fish could be due to a variety of factors. In social groups, dominant fish typically have greatest access to food (Sneddon et al., 2006; Ward et al., 2006). However, here the focal fish may be treated as an intruder and thus, at least initially, take on a subordinate role and have less access to the pellets than expected from their behavioural profile. Furthermore, if focal fish were under stress due, for instance, to exclusion from the group or moving between tanks, then this may affect their feeding habits (Bernier and Peter, 2001; Øverli et al., 2006); however, where possible attempts were made to mitigate these potential effects. Nonetheless, test populations had longer to acclimate to the group environment compared to the focal fish that were transferred from individual tanks into unfamiliar

environments with an already-established population. Given only a week within the population, these focal fish may not have fully recovered and may yet have been ready to feed to satiation.

4.5.2 Physiology

The post stress cortisol levels clearly indicated a distinct and significant difference between originally bold and shy focal fish in their physiological responses to emersion stress, with the pattern being similar to that observed in proactive and reactive coping styles respectively (Koolhaas et al., 1999; Øverli et al., 2007). Despite bold fish becoming shyer and shy fish remaining shy, physiological differences in stress responsiveness were still apparent and concordant with values expected for originally bold and shy animals. In a previous study, a switch in behavioural phenotype between high (HR) and low (LR) stress-responding lines of rainbow trout was not linked with a concurrent switch in stress response profile (Ruiz-Gomez et al., 2008); behavioural responses to challenges may thus be more labile than physiological responses, suggesting that while boldness and stress responsiveness may be linked in terms of coping style they can be decoupled by experience or other influences on behaviour, whilst physiological responses remain static. However, clear and consistent differences in the physiological stress responsiveness of bold and shy trout are not always apparent (e.g. in the HR and LR lines of trout: Schjolden et al., 2005; Thomson et al., 2011); indeed, a lack of consistent correlation may point to an artefact of the selection process which has decoupled certain behavioural and physiological traits, but the correlation between boldness and stress physiology may also be highly context specific (Øverli et al., 2007). As a result the mechanisms of coping style theory in fish are relatively unclear (Koolhaas et al., 2010), and future work needs to elucidate the connection.

4.5.3 Conclusions and Implications

The results of this study indicate behavioural lability in rainbow trout, with bolder animals generally showing greater propensity to alter their behaviour than shy individuals. Behavioural plasticity was, seemingly, independent of the overall behavioural profile of the population with which focal fish were grouped, and thus it remains unclear what the primary drivers of behavioural change may be between members of a group. Future work should, therefore, focus on the dynamics of

behavioural plasticity or social learning whilst individuals are within the group rather than the before-and-after effect. Initially bold trout also had lower physiological responses to stress; stress profiles in bold and shy fish in this study thus conformed to current coping style theory, whereas behavioural plasticity in these animals proved contrary, as shy animals are generally assumed to be more flexible in their behavioural responses to stimuli, potentially due to divergence in learning and memory capabilities between personalities (Koolhaas et al., 1999; Moreira et al., 2004; Ruiz-Gomez et al., 2011; Sneddon, 2003). Whilst this may reflect the treatments themselves engendering shyer behaviour in the focal fish, there remains inconsistent evidence for the existence of coping styles in fish (Koolhaas et al., 2010), and thus future work needs to focus on the relationship between stress physiology and behaviour.

5 Plasticity of boldness in rainbow trout, *Oncorhynchus mykiss*: does hunger and predation influence risk-taking behaviour?

5.1 Abstract

Boldness, a measure of an individual's propensity for taking risks, is an important determinant of fitness but is not necessarily a fixed trait. Dependent upon an individual's state, and given certain contexts or challenges, individuals may be able to alter their inclination to be bold or shy in response. Furthermore, the degree to which individuals can modulate their behaviour has been linked with physiological responses to stress. Here we attempted to determine whether bold and shy rainbow trout, *Oncorhynchus mykiss*, can exhibit behavioural plasticity in response to changes in state (nutritional availability) and context (predation threat). Individual trout were initially assessed for boldness using a standard novel object paradigm; subsequently, fish experienced, each day for one week, either high (unpredictable), low (predictable) or no simulated predator threat in combination with a high (2% body weight) or low (0.15% bw) food ration, before being reassessed for boldness. Bold trout were generally more plastic, altering levels of neophobia and activity relevant to the challenge, whereas shy trout were more fixed and remained shy. Increased predation risk generally resulted in increased plasma cortisol and an increase in the expression of three candidate genes with roles linked to boldness and in the hypothalamo-pituitary-interrenal stress axis. The results suggest a divergence in the ability of bold and shy trout to alter their behavioural profiles in response to internal and exogenous factors, and has important implications for our understanding of the maintenance of different behavioural phenotypes in natural populations.

5.2 Introduction

Any number of potential behaviours may be available to individual animals in response to an environmental challenge. However, only few of these, gained through experience or through genetic predisposition, will be adaptive in the context of any particular challenge. Correlated groups of such behavioural responses are termed animal personalities, and they are often considered consistent across contexts (Gosling, 2001). One important axis of behavioural variation is that of boldness, a measure of individual responses to novelty: bold animals are generally more active, more likely to explore novel objects or environments and spend more time in the open compared to shy conspecifics (Sih et al., 2004a; Sneddon, 2003; van Oers et al., 2005b), and this variation exists along a continuum from bold to shy. Boldness, also regarded as a willingness to take risks, is often correlated across other behavioural axes (e.g. increased boldness with higher aggression and activity; Adriaenssens and Johnsson, 2011; Bell, 2005).

Behaviour may have a heritable component (Giles and Huntingford, 1984; van Oers et al., 2004), and though .various studies have identified candidate genes implicated in behaviour (Greenwood et al., 2008; Ingram et al., 2005; Sneddon et al., 2011), it can also be shaped by experience throughout ontogeny and can vary according to local conditions (Dall et al., 2004; Slater, 1981). In particular, bold and shy animals are known to be able to alter their behaviour along the bold-shy axis, and these shifts may be a result of extrinsic (environmental; e.g. Chapman et al., 2010; Frost et al., 2007) or intrinsic (age, size etc.; Bell and Stamps, 2004; Brown and Braithwaite, 2004) factors. Boldness influences decisions and determines fitness dependent upon prevailing conditions (Frost et al., 2007; Lima and Dill, 1990), such that the suitability of bold or shy behaviour would differ according to the environment. The ability to alter behaviour regardless of phenotypic or genotypic propensity therefore has important ecological implications, and a plastic behavioural profile would be considered adaptive particularly in a fluctuating environment (Dall et al., 2004). The present study tests this by determining the degree to which bold or shy behaviour changes in the context of extrinsic (risk, measured as predation threat) and intrinsic (nutritional status) factors.

Predation threat is a significant environmental pressure, where the wrong behavioural choice will have profound consequences for the individual. High threat may encourage taking risks in order to forage (Brown et al., 2005b) or, alternatively, may engender a shy approach to limit exposure through activity and exploration (Archard and Braithwaite, 2011; Brydges et al., 2008). Whilst individuals may adjust their behavioural profile to correspond with prevailing predator threat, resulting in populations exhibiting a trend towards a particular profile (Brown et al., 2007; Magnhagen and Borcherding, 2008), there does not appear to be one single correct behavioural response to variations in predation pressure; instead, the relationship appears to be confounded by additional factors, such as habitat stability (Brydges et al., 2008), age (Magnhagen and Borcherding, 2008), size (Werner et al., 1983), and food availability (Borcherding and Magnhagen, 2008). Predation risk, however, does impact upon the expression of boldness (Bell and Sih, 2007) yet little is known regarding how hunger levels may modulate changes in the expression of bold or shy behaviours in this context.

Nutritional status is an important determinant of activity levels, since hungry animals will both have lower energy reserves yet also need to increase activity levels to forage (Borcherding and Magnhagen, 2008; Vehanen, 2003). Like predator threat, foraging profitability varies spatiotemporally. Animals therefore need to adjust foraging rates and activity dependent on both profitability (Croy and Hughes, 1991) and prevailing predation risk (Metcalfe et al., 1987; Vehanen, 2003; Werner et al., 1983). When resources are scarce, and predators are present, individuals must accept the higher risk associated with increasing activity and foraging (Borcherding and Magnhagen, 2008; Lima and Bednekoff, 1999). The extent to which individuals exhibit antipredator behaviour will therefore be a function of current predator threat, satiety and propensity towards risk-taking (Lima and Dill, 1990).

Threat necessarily induces a stress response in animals, providing a physiological and, subsequently, behavioural reaction to facilitate escape or avoidance of the stressor e.g. a predator. In fish this is accomplished through activation of, primarily, the hypothalamo-pituitary-interrenal (HPI) axis, resulting in the release of cortisol (Wendelaar Bonga, 1997). The control of feed intake is likewise controlled in the hypothalamus by a complex series of interconnecting pathways regulating orexigenic

and anorexigenic signals (Kalra et al., 1999), and appetite is regulated by hormones and neurotransmitters shared with the HPI axis e.g. γ-aminobutyric acid (GABA; Pu et al., 1999) and corticotrophin releasing factor (CRF; Bernier and Craig, 2005). Appetite and feeding rates are known to be reduced in stressed animals (Beitinger, 1990; Wendelaar Bonga, 1997). Thus, whilst behavioural changes would result in lower feeding rates in the presence of a predator, stress associated with that risk may evoke appetite reduction through stimulation of the physiological response (Scheuerlein et al., 2001).

Physiological stress responses can diverge within a population, and are linked with boldness such that individuals with a low cortisol response to stress tend to be bolder and more aggressive than high-responding conspecifics (Koolhaas et al., 1999; Øverli et al., 2002b; Pottinger and Carrick, 2001b). In rainbow trout, these links between behaviour and physiology, termed stress coping styles, may only be apparent during activation of the HPI axis (Øverli et al., 2005). Divergence in the stress response and in behaviour are both hereditary traits (Øverli et al., 2007; Pottinger and Carrick, 1999), and previous studies using lines of trout bred for divergent stress responses revealed significantly different patterns of gene expression between low- and high-responding fish (Backström et al., 2011; Thomson et al., 2011).

Bold and shy animals clearly respond to threat differently, and each will prioritise antipredator behaviour or feeding to different degrees. However, whilst boldness is a heritable trait (e.g. Bell, 2005; Brown et al., 2007) it is also plastic and can be shaped by experience. The behavioural strategies of bold and shy fish are well studied under risky and food-deprived regimes, though it remains unclear what effect sustained predation pressure, and the associated activation of the stress response, has on boldness. Furthermore, previous studies have highlighted the importance of individual genes in shaping behaviour, and there is an emerging literature on the expression of candidate genes related to boldness in particular. It is therefore imperative to our understanding of the molecular control of behaviour to identify how gene expression correlates with changes in behaviour brought about through experience.

The aim of this study was to determine how behavioural decisions in bold and shy rainbow trout, *Oncorhynchus mykiss*, may be influenced by exposure to varying levels of predator threat and metabolic demand. Circulating plasma cortisol levels were assessed to determine variation in stress levels in these fish, and behavioural and physiological differences were related to the expression of genes known to be involved in processes of behaviour, the stress response and appetite regulation. Specifically, we hypothesised that (1) individuals would vary their behaviour according to prevailing risk, where satiated fish would reduce risk-taking activity whilst food-deprived animals would take more chances; (2) fish under higher predation and/or restricted dietary regimes would experience elevated activation of the stress response, and alterations in the expression of three candidate genes implicated in feeding- and boldness-related behavioural processes.

5.3 Methodology

5.3.1 Test Animals

The following experiment was conducted under Home Office, UK, guidelines according to the Animal (Scientific Procedures) Act 1986, and following local ethics approval. Rainbow trout, *Oncorhynchus mykiss*, obtained from a commercial supplier were placed directly into stock tanks (2 x 2 x 0.5 m) where they were maintained in a semi-recirculating system on a 14:10 h light:dark cycle at 13 ± 1°C, and fed 1% body weight per day on commercial trout feed (Skretting, UK). Half of the tank had an opaque cover for shelter. For experimental procedures, fish ($n = 75$, 93.48 ± 3.94 g) were caught at random and transferred to individual glass aquaria (90 x 50 x 45 cm) which were screened from visual disturbance. All tanks were provided with filtered freshwater and maintained at 10 ± 1°C with constant aeration provided, and feed provided at 1% body weight per day at the same time each day. On the day following transfer to experimental aquaria, trout were netted, anaesthetised (0.033 g l^{-1}, benzocaine (Sigma-Aldrich Co., UK) in a 25 L bucket of aerated water), and weighed. The fish were returned to individual aquaria and allowed to acclimatise for at least one week or until the resumption of feeding.

5.3.2 Novel Object Tests

Boldness was assessed using standard novel object tests, which have previously been used to distinguish bold and shy behaviour in trout and other teleosts (e.g. Frost et

al., 2007; Thomson et al., 2011). Custom-built low-light cameras were positioned in front and to the side of the tank, and measuring rulers (0.5 cm) placed horizontally and vertically to accurately quantify the location of the subject relative to the novel object. The fish were allowed ten minutes to recover from any disturbance arising from setting up the equipment before recording began. After 1 min the novel object was dropped centrally into the tank, and the subsequent behaviour of the trout was recorded for a further ten minutes after which the object was removed. The novel objects used comprised an orange frustum-shaped bung (7.1 cm mean diameter, 4.9 cm height) and a blue-coloured transparent box weighted with gravel (7.5 x 5.3 x 3.8 cm).

Scoring of the behaviour was accomplished using custom-designed behavioural-analysis software. Consistent with a previous study (Thomson et al., 2011), we focussed on two key behavioural responses: 1) latency to approach to within 5cm of the novel object (s): those fish which approached within 180 s were considered bold ($n = 35$), those which did not approach within 300 s as shy ($n = 36$), and the remainder were classed as intermediate ($n = 4$) and discarded from further analysis; and, 2) the duration of passive behaviour (s). Passive behaviour included the subject resting at the base of the tank, pivoting on its own axis, and drifting across the tank, but excluded swimming greater than one body length under its own propulsion.

5.3.3 Predation Risk and Diet Manipulations

Each day subsequent to the first behavioural test fish were subjected to one of three treatments varying the level of perceived predation risk and imposing one of two levels of feed provision (Table 5.1). Predation risk was simulated by using a plastic heron's head (*Ardea cinerea*) mounted on a pole to simulate a predator attack. Attacks were made from behind a screen to prevent human association, and consisted of three swift strikes into the water followed by immediate removal of the model. Simulated attacks such as these have previously been successfully used to stimulate fright responses in various teleost species, including rainbow trout (Johnsson et al., 2001b; Jönsson et al., 1996). Simulated attacks coincided with the injection of 20 ml trout alarm pheromone into the water to provide a chemical stimulus of risk. Alarm substance extracted from conspecific skin has previously been demonstrated to increase antipredator behaviour in this species (Brown and Smith, 1998), and was

prepared as per this study with minor modifications. Skin was dissected from sacrificed non-experimental rainbow trout and washed with distilled water, then placed into individual 50 ml Falcon tubes with 6.25 ml sterile distilled water per 1 g skin. The contents were homogenised and then centrifuged at 4°C for 20 min at 8 000 RPM. The supernatant was pipetted into aliquots in fresh Falcon tubes and frozen at -20°C. On the day of experimentation, a tube was thawed and combined with 7 ml distilled water per 1 ml pheromone, with a final volume of 20ml solution used for each tank. Fish experiencing no risk were fed at the same time each day without a simulated predator attack. Low risk fish were subjected to a simulated attack each day at the same time, one hour after feeding, to create a predictable threat. High risk trout were exposed to an unpredictable environment where the timing of the attack varied each day, and was determined using a random number generator.

To provide different levels of hunger in the subjects, fish were fed one of two quantities of feed. Those on a high diet were fed 2% ± 0.01g body weight per day, whilst to induce fasting those fed on a low feed routine were only provided with 0.15% ± 0.01g body weight per day. The manufacturer's recommendations for this food are 1 - 1.5% body weight; therefore, these represent divergent feeding regimes. Short-term food deprivation occurs in the wild due to limited food availability or low temperatures, and therefore fasting is a non-stressful natural process in rainbow trout causing a reduction in weight and condition factor (Pottinger et al., 2003); upon resumption of feeding, individuals quickly return to their former health status with no further ill-effects. Furthermore, the subjects in this study did not lose a significant amount of weight in the trial period and a low sample size was adopted. Fish were fed at the same time each day regardless of threat or diet regime.

These conditions were applied each day for seven days, at which point a second and final novel-object test took place to assess any change in behaviour after this time.

5.3.4 Hormone Analysis and Quantification of Gene Expression

Approximately 2 hours after the final behavioural assessment, fish were netted and killed by concussion. Fish were killed at the same time each day to ensure interpretation was not compromised by diel fluctuations in levels of plasma cortisol

85

(Pickering and Pottinger, 1983). The fish were immediately weighed, and a 2 ml blood sample taken from the caudal vessels into a heparinised 2 ml syringe using sterile 25 g needles. The blood was centrifuged at 3 500 RPM for 5 min at 4°C, the supernatant plasma was aspirated, divided into aliquots and frozen at -20°C until further analysis.

Table 5.1: Treatment combinations and sample size for individual rainbow trout, *Oncorhynchus mykiss*, used in this study; each trout was exposed to one level of threat alongside one diet regime throughout the seven-day test period.

Threat regime	Diet regime	n	
		Bold	Shy
None	Low (0.15% bw)	7	8
	High (2% bw)	8	8
Low (Predictable)	Low (0.15% bw)	5	5
	High (2% bw)	5	5
High (Unpredictable)	Low (0.15% bw)	5	5
	High (2% bw)	5	5

Immediately following sampling, the whole brain was removed and stored at -80°C until RNA extraction. Total RNA was extracted using TRIzol® (Invitrogen Life Science, UK), with DNase-treated RNA eluted into 50 µl RNase-free water. RNA concentrations were determined by optical density at 260 nm using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK) and the quality of the samples assessed by 2% agarose gel electrophoresis. For each sample, approximately 1 µg of mRNA was reverse-transcribed into first strand cDNA using random hexamers and SuperScript™ III reverse transcriptase (Invitrogen Life Science, UK), following the manufacturer's protocol.

For RT-PCR, ~0.15 µg of the resulting cDNA was amplified in a 10 µl PCR (using 5 µl Fast SYBR Green, Invitrogen Life Science, UK) primed with 2 pmol of each primer: primer pairs were developed using Primer Express® 3.0 software against *O. mykiss* sequences (Table 5.2). Thermal cycling conditions, using a 7500 Fast Real-Time PCR System (Applied Biosystems), were: 10 min at 95°C, followed by 40X [95°C 3 s, 60°C 30 s] and then [95°C for 15 s, 60°C for 6 s, 95°C for 15 s and 60°C for 15 s], which allowed the construction of a melting curve to assess the specificity of the product. Relative quantitation of product was determined using ΔCt (Ct_{target} − $Ct_{reference}$) normalised against GAPDH, a housekeeping gene previously utilised in relative gene expression studies (e.g. Bernier et al., 2008; Reilly et al., 2008).

5.3.5 Data Analysis

Differences between bold and shy trout (mean initial 5 cm latency (s) ± SE = 34.20 ± 6.56 s and 578.67 ± 9.03 s respectively) in initial passive duration and in the latency to approach the object after the second trial were determined using a Mann-Whitney U Test. Subsequently, change in boldness across the two trials was assessed for initially bold and shy fish using a Wilcoxon signed rank test on change in latency to approach the object. Response variables (change in behaviour between the trials; specific growth rate (%); plasma cortisol (ng µl⁻¹); ΔCt of target genes) were analysed with a factorial analysis using a general linear model; non-significant terms were systematically removed, and AIC values compared, to obtain the minimum acceptable model. With the exception of cortisol, all response variables fit the assumptions of GLM; cortisol data were log_{10}-transformed for analysis. Specific growth rate was calculated as: $G = 100 \times [log(w_f) - log(w_i)] \times t^{-1}$, where w_f = final weight (g), w_i = initial weight (g) and t = time in days, as per Höjesjö et al. (1999).

Plasma cortisol concentration data included three points with large residuals: one shy individual (high risk) had a cortisol concentration in excess of 100 ng ml⁻¹, typical of the response to a moderate stressor in this species (Øverli et al., 2002b; Pottinger and Carrick, 1999) and far in excess of those exhibited by the remainder of the group. One bold fish in each of the no threat and low threat group exhibited cortisol concentrations of ~20 ng ml⁻¹ which, whilst high, has previously been observed in

87

unstressed trout (Øverli et al., 2002b). However, it is unlikely these fish exhibited the same physiological profile as the remainder of their groups, which exhibited cortisol concentrations <4 ng ml^{-1} which are more typical of an unstressed state (Balm and Pottinger, 1995; Thomson et al., 2011). On these bases, and verified through Grubbs' Tests, these data were assumed to be outliers and analyses are thus presented with these data excluded. All analyses were performed in R (ver. 2.7.0).

5.4 Results

5.4.1 Behaviour

Initially, bold fish were significantly less passive than shy trout (median passive duration = 476.84 and 572.95 s respectively; $W = 151.0$, $p < 0.001$, $n_1 n_2 = 33, 35$). Analysis of the behavioural parameters indicated that whilst some behaviour changed after a period of simulated predator attacks others did not, suggesting that these behaviours were, to some degree, free to vary independently. Change in the latency to approach within 5cm of the novel object varied dependent on initial boldness ($F_{1,66} = 26.04$, $p < 0.001$; Fig. 5.1): bold trout in general became significantly shyer, reflected in an ($W_{33} = 462.0$, $p < 0.001$), whereas shy fish did not alter their latency to approach the object ($W_9 = 8.0$, $p = 0.097$). Despite the increase in latency, bold fish did not become as neophobic as shy fish (median 5cm latency after treatments = 137.9 and 600.0 s for bold and shy fish respectively; $W = 293$, $p < 0.001$, $n_1 n_2 = 33, 35$).

The duration of passive behaviour varied according to a three-way interaction of all parameters ($F_{2,56} = 4.95$, $p = 0.010$; Fig. 5.2), where activity levels varied more in bold fish than shy: under no threat, bold fish became more passive on a low diet but slightly more active on a high diet, whereas under low threat, this pattern was reversed. Similarly, under high threat bold fish became more passive on a high diet. Shy fish were marked by almost no change in any treatment except high threat on a high diet, where in contrast to bold trout they appeared to become slightly more active.

Unsurprisingly, those fish on a higher diet had an approximately five-fold greater specific growth rate than those on a low diet ($F_{1,58} = 74.47$, $p < 0.001$; fig. 5.3). There was no other significant difference between the two groups.

Table 5.2: Accession numbers, sequences, product size (number of base pairs) and melting temperature (T_m, °C) for each gene used in this study and for a reference gene (*). All primers were developed using Primer Express® 3.0 software.

Gene	Accession	Forward	Reverse	Size (bp)	T_m (°C)
Epd	NM_001124693	CTC ATG CTC ACG CTC TGG AA	CCA AAA ACA GCT CAA CCT GAT G	60	83
GABA$_A$	BT073523	CTC ATC CGA AAG CGA ATC CA	CAC ACT CTC GTC ACT GTA GG	156	81
CRF	AF296672	GTG GTT CTG CTC ATT GCT TTC TT	CGC CAG GGC TCT CGA TAG	61	82
GAPDH*	AF027130	TGT TGT GTC TTC TGA CTT CAT TGG	CCA GCG CCA GCA TCA AA	60	81

89

5.4.2 Physiology

After removal of outliers, no significant trend was observed between $\log_{10}$-transformed plasma cortisol and any treatment. ($F_{2,54}$ = 2.82, p = 0.068): the greatest plasma cortisol was recorded from shy fish under a low-threat regime, whilst all fish in the high risk group generally showed higher concentrations than those under low risk (Fig. 5.4).

5.4.3 Gene Expression

Relative expression of all three genes varied according to the levels of threat experienced by individual trout. Expression of CRF was significantly (approximately 5-fold) in fish under high threat than those under no threat ($F_{2,50}$ = 9.71, p < 0.001; Fig. 5.5). Similarly, ependymin expression was significantly (approximately 3-fold) higher in those experiencing low or high threat compared with those on a no threat regime ($F_{2,48}$ = 9.37, p < 0.001). A possible interaction between threat and boldness may also be evident in driving the expression of ependymin ($F_{2,48}$ = 2.93, p = 0.063). The expression of $GABA_A$ also increased with increasing threat, but a significant interaction term indicated that boldness influenced the levels of expression of this gene (*Threat*Boldness*: $F_{2,48}$ = 3.29, p = 0.046): shy fish under high threat had the highest expression followed by bold under both high and low risk and shy under low risk. Fish under the no risk regime had the lowest expression, almost five times lower than shy fish under high threat (Fig. 5.6).

5.5 Discussion

Animals may exhibit a divergence in their behavioural plasticity, which has important implications for individual fitness: those with rigid behavioural profiles are better adapted for stable environments, but plasticity may be more adaptive where there are new or fluctuating environmental challenges (Dall et al., 2004). Here we demonstrate that, at least in some contexts, personality may be one determinant of the extent to which individuals can alter their behavioural profile in response to such challenges. Bold fish were behaviourally labile and performed an alternative strategy dependent upon the context. In contrast, shy fish generally remained shy regardless of the potential threat or nutritional status suggesting that the shy personality is relatively inflexible in these contexts. Risk itself appeared to induce physiological and molecular changes in these fish regardless of food availability or, to some extent,

the level of boldness; thus, bold and shy behavioural profiles may arise from individual differences in the ability to respond to both external and internal cues.

5.5.1 Behaviour

Animals should be able to allocate foraging behaviour to times when predation threat is low (the risk allocation hypothesis; Lima and Bednekoff, 1999; Metcalfe et al., 1987; Vehanen, 2003), but when threat is unpredictable animals may have to continue behaving cautiously (Ferrari et al., 2008). In this study, bold fish altered their propensity for taking risks and their levels of activity dependent on the environmental regime, in general reducing activity (increasing passivity) when under threat, a general response in animals to reduce the chances of being detected by a potential predator (e.g. Anholt and Werner, 1995; López et al., 2005; Zimmer et al., 2011). The exception was when threat was predictable and food availability was low; food-deprived animals usually increase foraging effort and activity to increase the chances of acquiring food (Vehanen, 2003), and bold animals in particular will have an advantage in terms of their propensity to take risks (Brown et al., 2005b). These fish may, therefore, have been behaving according to the risk allocation hypothesis, perhaps increasing activity between predictable attacks to maximise foraging. Likewise, bold fish under no threat and on a low diet increased foraging activity, whilst those with plenty of feed may have decreased their activity to conserve energy if habituated to being fed at a certain time (Chen and Tabata, 2002).

Current literature describes a dichotomy in behavioural responses to high predation threat, with some animals showing predominantly bold behaviour in areas of high predation (e.g. Brown et al., 2005b; Magnhagen and Borcherding, 2008) and others showing a predominantly shy personality under the same conditions (e.g. Brydges et al., 2008). This may reflect interspecific variation in antipredator strategies, but is likely to also indicate manifold other differences in the environments and challenges these animals experience: both bold and shy responses may be adaptive dependent upon the context (Coleman and Wilson, 1998). Shy fish in this study did not alter their behaviour, but the presented data cannot discriminate between a lack of behavioural plasticity in shy fish or whether being shy is simply the more appropriate behavioural response to these challenges and contexts.
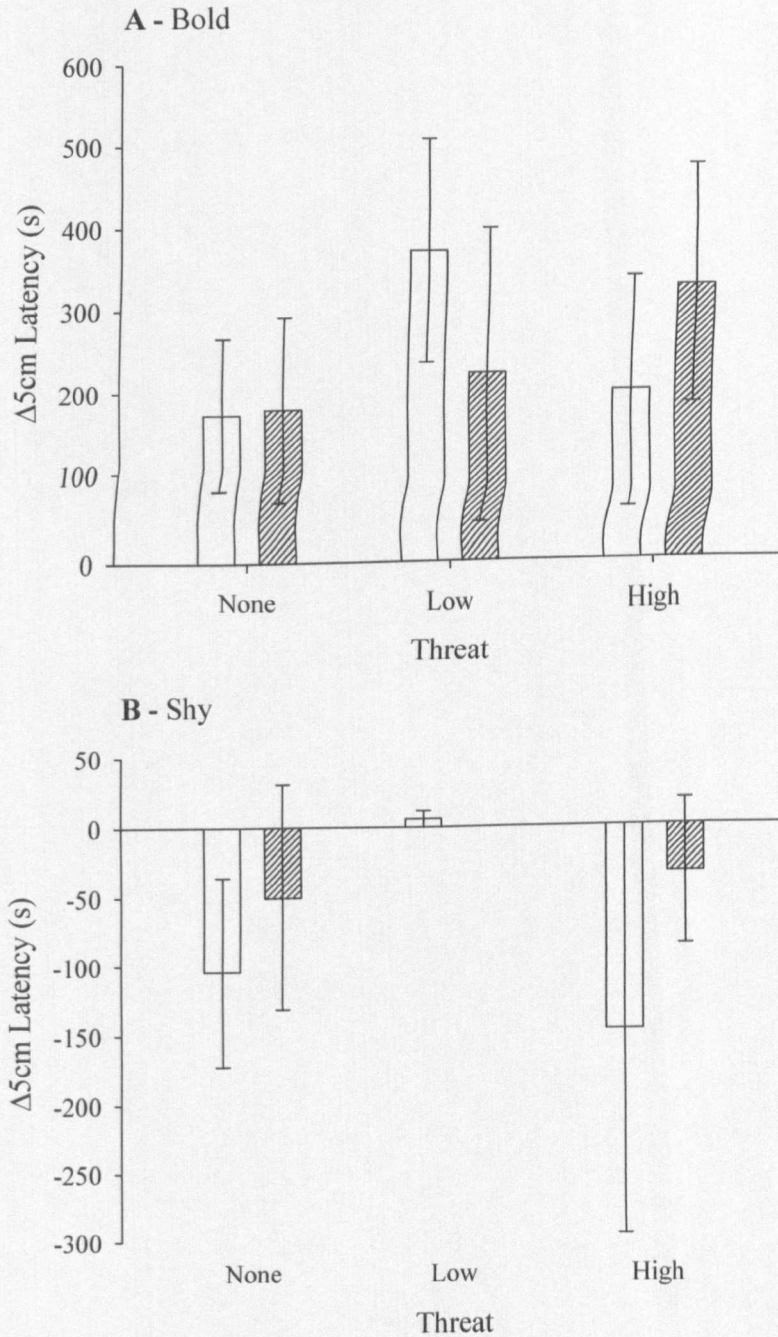
A - Bold

B - Shy

Figure 5.1: Mean (± SE) change in latency between trials (s) for (A) bold and (B) shy rainbow trout, *Oncorhynchus mykiss*, to approach within 5 cm of a novel object. Individual trout experienced no, low or high predator threat and had either low (white) or high (hatched) feed availability. In each treatment $n = 5$ except for no threat-high diet (bold and shy $n = 8$) and no threat-low diet (bold and shy $n = 7$ and 8 respectively). Change in latency was significantly different between initially bold and shy trout ($p < 0.001$).
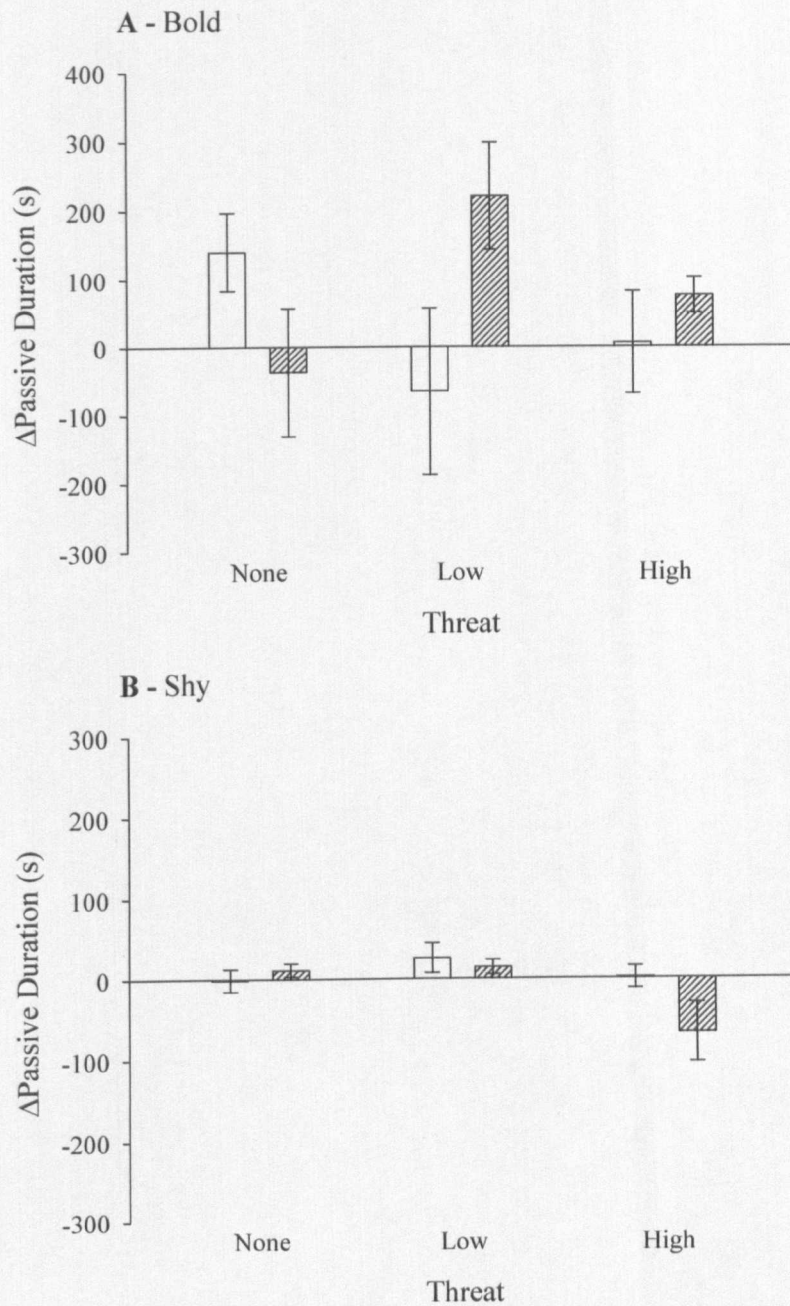
A - Bold

B - Shy

Figure 5.2: Mean (± SE) change in duration of passive behaviour between trials (s) for (A) bold and (B) shy rainbow trout, *Oncorhynchus mykiss* under a no, low or high threat regime and low (white) or high (hatched) food availability. In each treatment $n = 5$ except for no threat-high diet (bold and shy $n = 8$) and no threat-low diet (bold and shy $n = 7$ and 8 respectively).
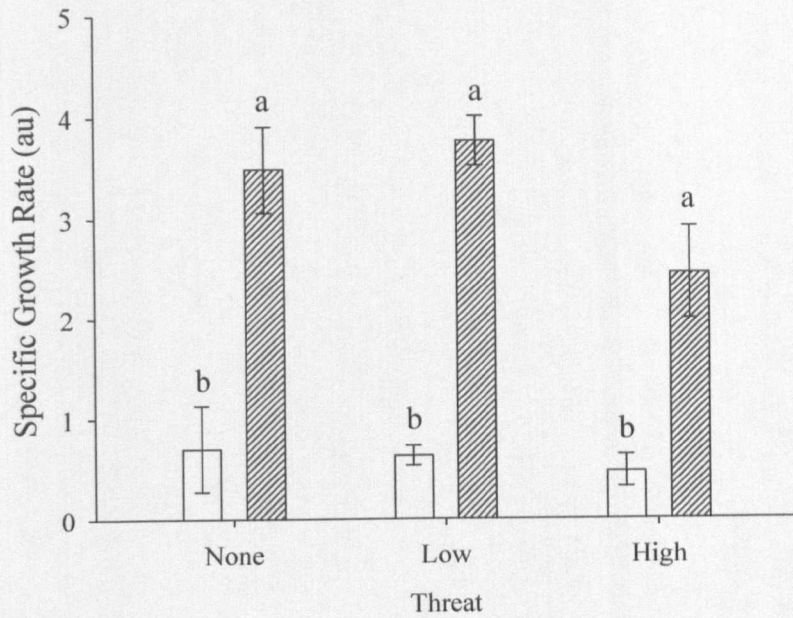
Figure 5.3: Mean (± SE) specific growth rate (arbitrary units) for rainbow trout, *Oncorhynchus mykiss*, under no, low or high predation threat and low (white) or high (hatched) feed availability. *n* (no, low, high threat) for low diet = 12, 10, 10 and for high diet = 13, 9, 10. Lower case letters indicate significant differences ($p < 0.05$).
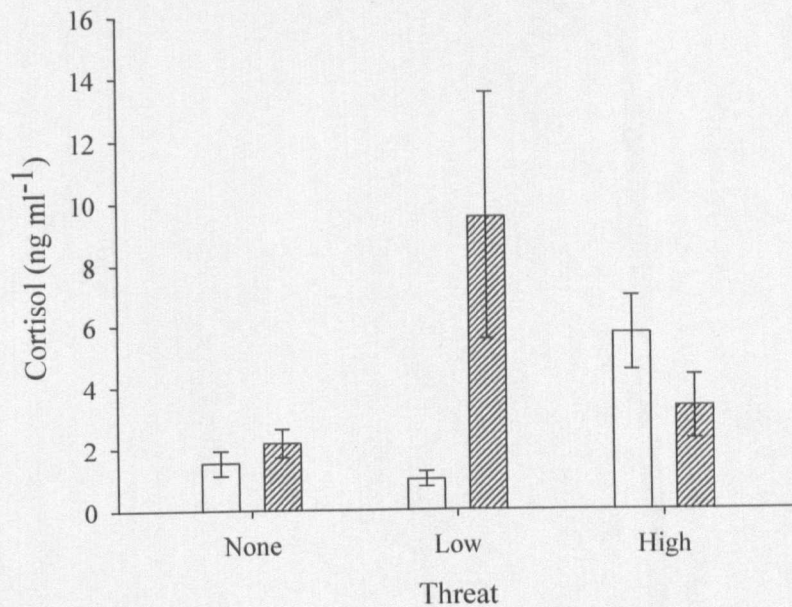
Figure 5.4: Mean (±SE) plasma cortisol (ng ml^{-1}) in bold (white) and shy (hatched) rainbow trout, *Oncorhynchus mykiss*, exposed to no, low or high levels of predation threat. *n* (no, low, high threat) for bold trout = 10, 9, 10 and for shy trout = 14, 10, 10.
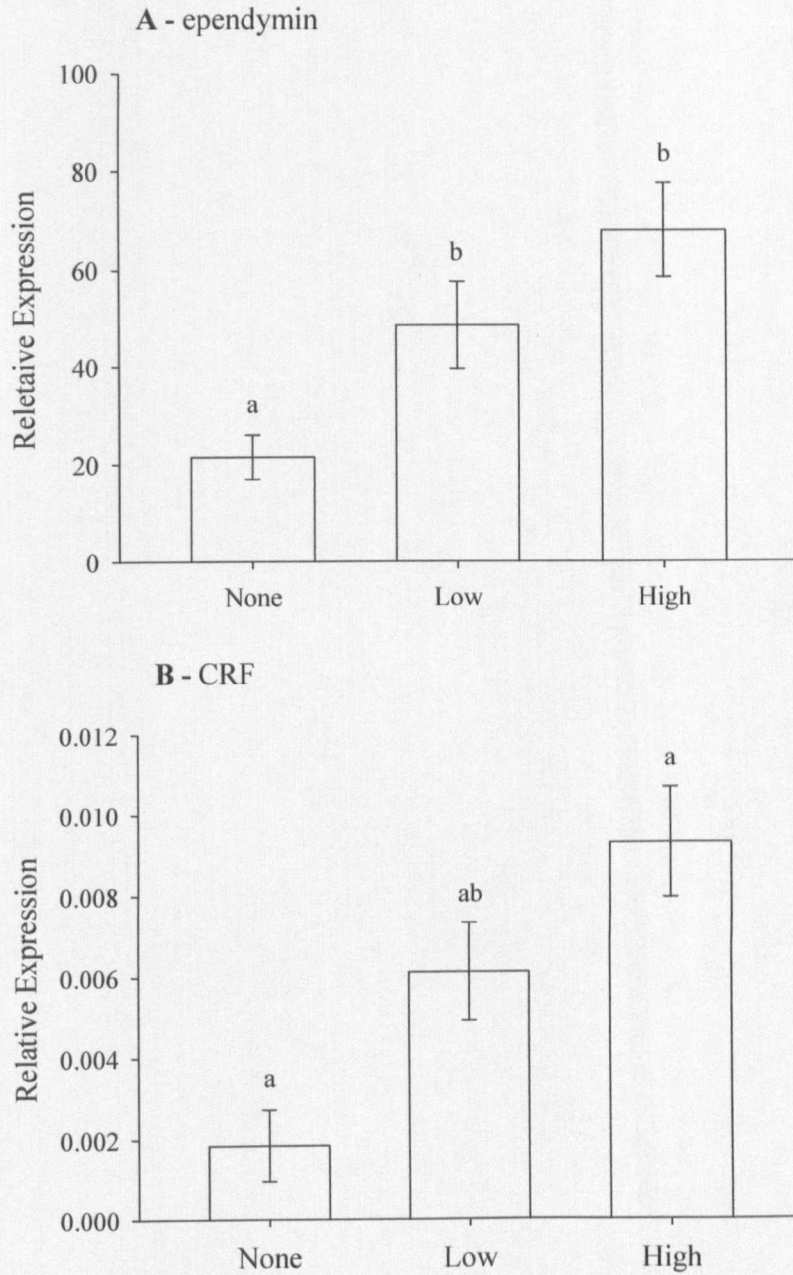
A - ependymin

B - CRF

Figure 5.5: Mean (± SE) relative expression, $\left(e_{Ref}{}^{Ct_{Ref}}\right)\!/\!\left(e_{Tgt}{}^{Ct_{Tgt}}\right)$ (where e = reaction efficiency and Ct = cycle threshold of reference and target genes) of (A) ependymin and (B) CRF (corticotrophin releasing factor) in the brains of rainbow trout under no, low and high predation threat. n = 19 for each treatment. Means that do not share a common lower case letter are significantly different (Tukey HSD, $p < 0.05$).
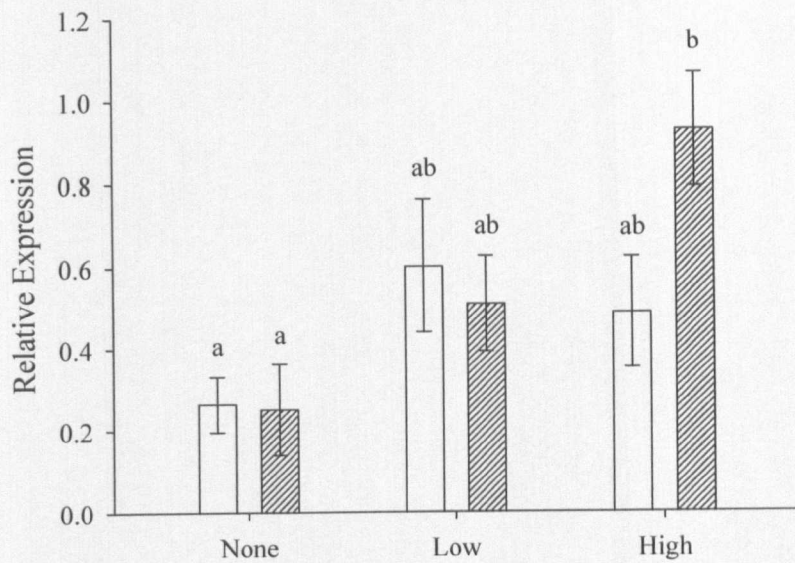
Figure 5.6: Mean (± SE) relative expression, $\left(e_{Ref}^{Ct_{Ref}}\right)\big/\left(e_{Tgt}^{Ct_{Tgt}}\right)$ (where e = reaction efficiency and Ct = cycle threshold of reference and target genes) of GABA$_A$ (γ-aminobutyric acid A) in the brains of bold (white) and shy (hatched) rainbow trout, *Oncorhynchus mykiss*, under no, low or high predation threat. $n = 10$ for bold and for shy trout at each level of threat (except no threat-shy, where $n = 9$). Means that do not share a common lower case letter are significantly different (Tukey HSD, $p < 0.05$).

In general, animals avoid being in the open in the presence of a predator (Metcalfe et al., 1987; Smith, 1997; Werner et al., 1983) even if it results in a reduction in feed intake. Rainbow trout appear to favour the more cautious response to risk, with initially shy fish generally not risking increasing activity to forage. Each group, however, gained weight throughout the treatment period, indicating that the energetic constraint may not have been enough to induce a change to a behavioural profile favouring foraging even when risk is high. The volume of feed provided in the low diet regime was similar to that used in other studies (e.g. Höjesjö et al., 1999) wherein a negative impact on body condition was observed, which may suggest differences in the quality of feed. As such, future studies may need to more accurately profile nutrient availability in feed rather than estimate supply of feed by weight (e.g. in Borcherding and Magnhagen, 2008). In addition to mortality, the

threat of predatory attack *per se* may cause a variety of non-lethal effects in potential prey which are still detrimental to heath, such as reduced feeding and growth (and, therefore, increased stress; Lima, 1998). Indeed, whilst growth rates were positive, these data suggested a possible lower rate of growth in the high risk group compared to low and no threat. The model of predation risk presented to fish in this study does elicit behavioural and physiological antipredator responses (Brown and Smith, 1998; Jönsson et al., 1996), and, therefore, it is likely that a sustained period of high threat would result in a more conspicuous deterioration in weight gain relative to that seen at lower threat levels.

One important difference observed in these fish was the ostensibly enhanced behavioural plasticity observed in bold fish compared to shy. Whilst behavioural plasticity provides adaptive flexibility, particularly in a fluctuating environment, it can also be costly (DeWitt et al., 1998) and, within populations or groups, animals may therefore exhibit variation in their propensity for behavioural change. Shy animals may be able to place less investment into these costs as some, such as the acquisition of environmental information, necessitate risky behaviour (DeWitt et al., 1998), resulting in shy individuals generally less phenotypically plastic than bold animals. Bold fish here altered their responses dependent on a potential trade-off between internal energy status and risk, whereas shy fish were either limited in their plasticity or, alternatively, they exhibited levels of activity suitable to the combination of state and context they experienced. However, exceptions may occur as a function of an individual's state, whereby, for example, the potential cost of exposure to threat may be mitigated by a need to forage driven by extremely low nutritional state (Dall et al., 2004). As such, when energetic levels fall below a certain threshold determined by the level of threat, an otherwise shy animal may begin to forage (Dall et al., 2004; Höjesjö et al., 1999). Considering all animals in this study gained weight, it is plausible that this threshold may never have been reached and the requirement of foraging regardless of danger was never achieved. It was also likely, however, that shy fish simply have a lower threshold than bold; since bold animals are, by definition, less averse to taking risks they may be prepared to forage at slightly healthier energetic status than shy individuals.

5.5.2 Physiology

In contrast to the behavioural responses of these fish, the molecular data suggest that both bold and shy fish were experiencing similar physiological and genetic responses to the stimuli. Cortisol levels were marginally higher in both bold and shy fish under high predation threat compared to none: in general, fish in or from a riskier environment tend to show heightened stress indicators (Brown et al., 2005a; Woodley and Peterson, 2003), though in this case the cortisol increase was not as profound as what would be expected after an acute and substantial stress (cf. confinement: Øverli et al., 2002b; Pottinger and Carrick, 1999; emersion: Sloman et al., 2001; Thomson et al., 2011). Rather than being an acute response to the behavioural test, these values may therefore reflect baseline HPI activity which has been modified by exposure to the treatment regimes. The functional significance of variation in unstressed fish remains relatively unknown, yet here there was a clear, if not significant in this case, increase in plasma cortisol concentrations in shy fish under a low threat compared to other treatments. The predictability of the once-daily simulated attacks may therefore be more stressful to shy trout than bold, manifesting in these fish as sustained or periodically elevated circulating cortisol. Future work should address this potential difference in the physiology of bold and shy fish in response to the predictability of a stressor.

5.5.3 Gene Expression

Contrary to expectations, gene expression was not correlated with diet, despite both CRF and $GABA_A$ being involved in neural pathways controlling appetite and feed intake (Bernier and Craig, 2005; Pu et al., 1999). Since these genes are both involved in stress reactivity (CRF, Chrousos and Gold, 1992; GABA, Makara and Stark, 1974), and $GABA_A$ in the expression of fear (Caldji et al., 2000) it is likely that upregulation of these genes in individuals under higher risk represents a molecular response to predation risk. However, the chronic stress associated with predation risk can result in reduced condition (Barton and Iwama, 1991; Barton et al., 1987) and, potentially, reduced foraging without a concomitant loss of appetite (Metcalfe et al., 1987); these factors would indeed suggest an increase in appetite despite the reduction in feed intake. Upregulation of CRF and $GABA_A$ in those fish under higher predation risk may, therefore, represent simultaneous activation of both physiological stress response and appetitive pathways.

Ependymin is linked with a variety of processes linked with environmental interaction, some of which are related to behaviour and to stress (Shashoua, 1991; Tang et al., 1999). Ependymin has previously been associated with memory formation (Shashoua, 1991), but it remains unclear whether variation in the expression of this gene can be linked with the anticipation of attacks in this study. Upregulation of ependymin has also, however, been linked with a reduction in competitive ability (Aubin-Horth et al., 2005; Sneddon et al., 2011), and may therefore be related to the trend towards shyness and inactivity observed in the trout in the present study.

Fish originating from aquaculture environments, such as those used in this study, may have impaired antipredator responses (Álvarez and Nicieza, 2003; Malavasi et al., 2004). Although there is a genetic component to these responses (Bell, 2005), much of an individual's response to predator threat derives from experience and environmental influences (Kelley and Magurran, 2003), and a lower survivability of fish without this experience may be due to inherent differences in both their physiology and their behaviour (Brown et al., 2005a; Johnsson et al., 2001a). Thus the responses to threat observed in this study may not reflect the magnitude of the response in animals from populations which encounter predator threat on a more frequent basis. However, alarm pheromone does elicit innate anti-predator responses such as increased refuge use in farmed rainbow trout (Ashley et al., 2009). Future studies should use wild caught individuals to explore whether such differences do occur. Furthermore, these results indicate that our preconceptions of predictability and unpredictability in the context of threat being 'low' and 'high' respectively may not be entirely accurate. These labels were based on hypotheses that predictable attacks would be easier for a fish to cope with; however, whilst bold fish seemed to be able to deal with a predictable threat (which, if they are routine-forming as predicted by coping style theory, would make sense; Koolhaas et al., 1999), shy fish were found to be under considerable stress in this situation. In contrast, expression of the three candidate genes increased linearly from no to low to high threat. Overall, the hypothesis that unpredictable threat is 'higher' risk than predictable may be true but highlights that individual reactions to these contexts are extremely divergent.

5.5.4 Conclusions and Implications

In the present study we demonstrated clear differences in the behavioural responses of bold and shy rainbow trout to variations in nutritional state and predation threat, where bold fish exhibited greater behavioural plasticity than shy fish. In contrast, aside from slight differences between bold and shy fish in plasma cortisol concentrations, molecular trends were dominated by responses to predation threat rather than initial boldness. These data therefore highlight the divergence of phenotypic plasticity within a species whilst providing information on the roles of physiology and gene expression in response to these contexts. Both bold (e.g. Frost et al., 2007; Ruiz-Gomez et al., 2008; Chapter 3) and shy (particularly in the context of coping style theory, e.g. Benus et al., 1991; Koolhaas et al., 1999) animals can exhibit phenotypic plasticity, and thus a propensity for behavioural change. State and context-dependent effects, such as prevailing predation pressure, may generate evolutionary constraints particular to one species or population. Future work therefore needs to focus on how animals of differing personality respond to multiple challenges and apply this to complex environments to elucidate the functional significance of variation in both behaviour and its plasticity in nature, and continue to attempt to determine how this is related to key physiological and genetic mechanisms which may drive these differences.

6 Behavioural plasticity of bold and shy rainbow trout, *Oncorhynchus mykiss*, exposed to a predation threat and two environmental stressors, hypoxia and increased temperature

6.1 Abstract

Animals exhibit wide spectra of individual behavioural traits which may be correlated through time or across contexts, and are termed personalities. One important personality measure is boldness, defined as a response towards novelty or risk, and bold and shy behavioural profiles are linked with physiological stress responsiveness. Individual differences in boldness may be modulated through state and context-dependent conditions; here the behavioural and physiological responses of bold and shy rainbow trout, *Oncorhynchus mykiss*, towards a variety of biotic (predation risk) and abiotic (temperature increase and hypoxia) environmental challenges were examined to determine whether risk or internal state altered the degree of boldness. Trout were tested for boldness using a standard novel object paradigm: bold fish approached to within 5 cm of the object within 180 s whilst shy trout did not approach within 250 s. For the following 14 days fish were exposed to either no, low (predictable) or high (unpredictable) risk via exposure to a model predator and trout alarm substance. For the final 7 days of this study period, test subjects were also exposed either to an increase in temperature (from 11 to 19°C), or a reduction in water oxygen content (from 100% to 50% O_2 saturation). In response bold fish generally became shyer, particularly under unpredictable threat, whilst shy fish became somewhat bolder except when facing a predictable threat. Risk and, for bold fish, higher temperature resulted in increase stress axis activation. These responses were linked with behavioural and physiological acclimation to environmental challenges, and have important implications with environmental extremes more frequent due to anthropogenic activity and climate change.

6.2 Introduction

Individual animals exhibit consistent differences in behavioural traits, and considered together these are termed animal personalities (Dall et al., 2004; Gosling, 2001). One important aspect of personality across a variety of contexts in animals is boldness, which describes an individual's response to novelty or fear: bold animals tend to be more aggressive, more active and more likely to explore new environments or objects than shy conspecifics (Sih et al., 2004a; Sneddon, 2003; van Oers et al., 2005b). Rather than existing as two discrete states of bold and shy, boldness exists as a continuum along which individuals are able to vary their behaviour (Brown et al., 2005b; Frost et al., 2007; Wilson et al., 1993; Chapters 4 and 5). Therefore, whilst the response of a bold or shy individual to a stimulus is important, the effect of environmental influences and experience on individual behaviour is also of interest since it may be adaptive for animals to alter their behavioural strategy to meet current environmental demands (Chapman et al., 2010; Dall et al., 2004).

Animal personalities are associated with natural variation in how individuals respond to exogenous challenges (Carere and Eens, 2005; Dall et al., 2004; Frost et al., 2007; Lima and Dill, 1990). Much of this variation derives from inherited characteristics (e.g. Brown et al., 2007; Giles and Huntingford, 1984; van Oers et al., 2004) though experience and learning also play key roles in shaping individual responses (Brown et al., 2007; Mery and Burns, 2010). Animals may therefore adapt to the conditions of their local environment, thereby increasing fitness. However, personality traits such as being bold or shy may affect the degree to which individuals can change their behaviour, or their propensity for doing so (Benus et al., 1991; Carere et al., 2005; Koolhaas et al., 1999; Sinn et al., 2008; Chapters 4, 5). Furthermore, continuously responding to a variable environment and modifying behaviour to suit can be costly (Dall et al., 2004; DeWitt et al., 1998). Being able to alter behaviour therefore relies on the physiological capacity to do so, and this may compromised by other variables including individual health or state (Dall et al., 2004). Intraspecific variation in boldness, and thus phenotypic plasticity, therefore has implications for the ability of individuals or populations to survive both in fluctuating and in stable environments. Therefore, the present study will explore factors that drive plasticity in boldness to determine the impact of physiological state and context on behavioural decisions.

Fish are naturally exposed to environmental fluctuations such as variation in water quality, temperature and oxygen concentration. Different species occupy particular habitats or niches according to their tolerance limits, but spatiotemporal variations are expected to select for certain genotypes within a species. For example, aquatic habitats often experience seasonal declines in oxygen content (hypoxia; e.g. Rabalais et al., 1994) and variations in temperature (e.g. Matthews and Berg, 1997), and individuals must adapt to these stressors. When exposed to hypoxia, fish can acclimate firstly through short-term behavioural changes: a general reduction in activity to conserve energy (Herbert and Steffensen, 2005), combined with increased aquatic surface respiration (ASR) whereby the fish will ventilate near the relatively oxygen-rich air-water interface (Shingles et al., 2005; Timmerman and Chapman, 2004). Where hypoxia is chronic, fish will secondarily undergo physiological acclimation, resulting in increased red blood cell counts and haemoglobin concentration, and tissue-specific alterations to enzymatic activity to rebalance energy demands (Lai et al., 2006; Martínez et al., 2006; Timmerman and Chapman, 2004). These changes are in general specific to individual species and even to particular oxygen concentrations (Herbert and Steffensen, 2005). However, fish performance is often decreased and escape responses from predators are therefore compromised (Lefrancois et al., 2005; Timmerman and Chapman, 2004); ASR, in particular, can lead to increased susceptibility to attack from predators (Shingles et al., 2005). Similarly, increases in ambient temperature may provide some benefit (Barron et al., 1987), but tolerance to environmental stress is rapidly diminished when such stressors are experienced in combination, often synergistically causing rapidly elevated levels of mortality (Matthews and Berg, 1997; Shimps et al., 2005).

Anthropogenically-induced elevated temperature and reduced water oxygen content are an increasing problem (Timmerman and Chapman, 2004). Increasing global temperatures and frequency of eutrophication in susceptible areas may cause significant physiological barriers to many species or populations (e.g. Herbert and Steffensen, 2005; Jensen et al., 1993; Johannessen and Dahl, 1996; Landman et al., 2005), particularly those which are living on the edge of their physiological tolerance (e.g. Herbert and Steffensen, 2006; Matthews and Berg, 1997). Even towards these limits energy allocation is diverted from some processes e.g. growth and reproduction, in order to fuel other biochemical processes necessary for survival in

harsher conditions (Roessig et al., 2004). Such conditions may therefore affect individual state, thereby limiting the available behavioural response to further challenges, such as predation threat (Dall et al., 2004). Understanding how individuals respond to a range of environmental challenges will thus inform models on how populations and species may adapt to a rapidly changing climate. Thus the impact of hypoxia and increased temperature will be examined by determining how bold and shy individuals alter their propensity to take risks in the face of a novel challenge.

Predators provide a consistent threat throughout the lives of most animals, and many activities, such as foraging (Huntingford et al., 1988; Lima and Bednekoff, 1999), reproduction (Scheuerlein et al., 2001), habitat choice (Bardonnet and Heland, 1994; Gristina et al., 2011) and so on, are balanced to maximise the benefits accrued whilst simultaneously minimising exposure to potential predators. Bold and shy animals exhibit fundamentally different antipredator strategies, since by nature shy animals are reclusive and tend towards shelter when under threat, whilst bold animals may be more likely to spend time in the open even when dangerous to do so (Sih et al., 2004a; Wilson et al., 1993). However, when under stress and/or homeostatic threat, the ability to avoid a predator may be compromised. This may be due to an inability to mobilise the physiological system to enact an appropriate escape response since it is devoted to utilising less efficient energy sources (Lefrancois et al., 2005) or simply because the environmental challenge leaves the individual in a vulnerable or weakened state (Shingles et al., 2005). Therefore, bold and shy individuals will be exposed to varying risk to determine whether this influences behavioural decisions in the face of hypoxia or increased temperature.

Responses to stress often involve activation of physiological stress axes, which prepare the body by mobilising and reallocating energy dependent upon the stressor (Wendelaar Bonga, 1997). One such axis, the hypothalamo-pituitary-interrenal/adrenal (HPI/A) axis, results in the release of pituitary cortisol and is considered the dominant axis in teleost fish. Physiological responses often correlate with animal personality, and divergence in HPI or HPA activity is linked with boldness, whereby bold animals tend to produce lower concentrations of cortisol in response to a stressor than shy conspecifics (Koolhaas et al., 1999). These correlated

differences are termed coping styles, and rather than 'bold' or 'shy' are referred to as proactive and reactive respectively (Koolhaas et al., 1999). However, whilst numerous studies have identified coping styles in rainbow trout and other salmonids (e.g. Brelin et al., 2008; Øverli et al., 2007), the relationship between behaviour and physiology is not always clear, particularly in the rainbow trout (Koolhaas et al., 2010), and may reflect some degree of behavioural plasticity or even decoupling between correlated behaviours (Ruiz-Gomez et al., 2008; Schjolden et al., 2005; Thomson et al., 2011). There is no doubt that physiological responses are connected to behaviour, and that the distribution of bold and shy behaviours within a population is linked to HPI axis reactivity, but further work is necessary to fully understand how divergent stress responsiveness is maintained notwithstanding behavioural plasticity. Cortisol concentrations will therefore be determined to correlate these with behavioural changes within the contexts of risk and environmental variation.

Natural environments provide multiple challenges, often simultaneously, and individuals need to be able to deal with each stress appropriately. Many studies focus on one or two potential stressors, and whilst this provides important and useful information regarding individual responses they do not necessarily reflect the natural scenarios in which marine and aquatic organisms may often find themselves. It is, therefore, imperative for studies to focus on such stressors in combination to gain a greater understanding of how organisms not only survive but adapt to a range of natural pressures. The overall aim of this study was therefore to 1) characterise the behavioural changes in bold and shy rainbow trout, *Oncorhynchus mykiss*, exposed to combinations of three environmental stressors: periodic risk, reduced water oxygen content and increased temperature; and to 2) link boldness, and its plasticity, to the neuroendocrine response to stress. This approach will investigate whether these divergent phenotypes will alter their behaviour in response to a risky environment, and also how whether these changes may be mediated or augmented by state by imposing changes in metabolism through hypoxia or increased temperature.

6.3 Methodology

Rainbow trout, *Oncorhynchus mykiss* (weight ± SE = 97.98 ± 3.21 g) , were obtained from a commercial supplier and placed directly into stock tanks (2 x 2 x 0.5m) where they were maintained on a semi-recirculating system at 12 ± 1°C, with constant

aeration and on a 14:10 h light/dark cycle. Fish were fed *ad libitum* on commercial trout feed (Skretting, UK). For experiments, fish were transferred into individual glass aquaria (90 x 50 x 45 cm) which were identically equipped, screened from visual disturbance and provided with constant filtered freshwater and aeration. Light, temperature and feeding regimes were identical to the stock tanks, and fish were allowed seven days to acclimatise. Fish were assessed for boldness before entering a 14-day test period during which they were exposed to individual procedures, varying levels of risk, temperature or oxygen concentration (Table 6.1). To avoid imposing two stressors simultaneously application of treatments was staggered: simulated predation risk, if applied to the individual, was performed through days 1-14, whilst any changes to temperature or water oxygen content took place through days 8-14. After the test period, individuals were retested for boldness.

6.3.1 Bold Shy Assessments

Boldness in all cases was assessed using a standard novel object paradigm, which has previously been used to distinguish bold and shy behaviour in rainbow trout and other animals (Bergman and Kitchen, 2009; Frost et al., 2007; Wilson et al., 1993). Novel objects consisted of non-reactive plastic Lego Duplo™ constructs of various sizes, colours, and dimensions to a maximum of 10 x 7 x 4 cm as per Sneddon et al. (2003). Low-light cameras, built in-house, were set up to the front and sides of the tank, and measuring rulers placed along the tank edges to accurately measure the proximity of the fish to the novel object. Fish were allowed 10 min to recover from the disturbance of setting up the equipment, after which a novel object was dropped approximately 10 cm in front of the subject and their behaviour recorded for a further 10 min.

The principle measure of boldness in these fish, consistent with other studies (Frost et al., 2007; Thomson et al., 2011; Chapters 4, 5), was latency to approach to within 5cm of the novel object. Fish that approached within 180 s were considered bold, subjects which did not approach within 250 s as shy, and the remainder were classed as intermediate and were discarded from further analysis (~1.4% of fish). The duration of active behaviour, defined as the time spent swimming or moving more than one body length under the power of the subject's own propulsion, was also recorded. Fish were anaesthetised (benzocaine (Sigma-Aldrich Co., UK) at 0.033g

l^{-1}) in a 25 L bucket and the weight (to 0.01g) of each individual was recorded prior to the initial trial and after the second novel object test. Subjects were retested for boldness after a 14-day test period subsequent to the first novel object trial; the second trial used a different object to prevent habituation to a familiar shape (Sneddon et al., 2003). A control group of trout ($n = 5$ bold and 7 shy) were kept at standard conditions and received no treatments during this period.

Table 6.1: Treatment combinations and numbers of bold and shy rainbow trout, *Oncorhynchus mykiss*, used in the study. Risk treatments occurred over days 1-14 of the test period, whereas variation in oxygen and temperature occurred over days 8-14 of this period. Control treatments were defined as no risk, 100% dissolved oxygen, and 12°C temperature. Samples obtained by JST indicated by * (n=1) and ** (n=2).

Risk	pO$_2$ (%)	Temperature (°C)	n Bold	Shy
None	100	11	5*	7
	50	11	9	6
	100	19	7	8
Low (Predictable)	100	11	5	7
	50	11	5	7
	100	19	7	10
High (Unpredictable)	100	11	5**	7
	50	11	13	5
	100	19	14	13

6.3.2 Predation Risk

Following initial assessment, fish were exposed either to a low (predictable) or high (unpredictable) threat regime. Low risk involved a consistent feeding regime and simulated predator attacks each performed at the same time daily, am and pm, throughout the 14-day test period. The high risk regime consisted of an inconsistent

time of feeding and an unpredictable number of attacks performed randomly each day. Predator threat was simulated by injection of 15 ml alarm pheromone into the tank followed immediately by three swift strikes into the water using a model heron (*Ardea cinerea*) head. The use of model predators generates fright responses in teleosts, including rainbow trout (Johnsson et al., 2001b; Jönsson et al., 1996). Alarm pheromone likewise elicits an antipredator response in fish (Brown and Smith, 1998), and was prepared from the skin of other rainbow trout as per Brown and Smith (1998) with some minor modifications: skin was removed from both sides of the fish and washed with distilled water, and then homogenised in 25ml distilled water. The skin was centrifuged at 8 500 RPM for 15 min, and the supernatant subsequently removed and stored at -20°C. Before the day of experimentation the samples were defrosted overnight at 4°C. The preparation was then made up to 200ml with distilled water. Simulated attacks were performed from behind a dark screen to prevent human association. After the test period individuals were reassessed for boldness before being killed by concussion.

6.3.3 Abiotic Variation

Following the initial novel object test subjects were allowed 7 days under experimental (no, low or high risk) conditions. On day 8, trout were exposed either to hypoxia or to an increase in temperature to 19°C over 16 h (control individuals were exposed to no further treatment) for the remaining 7 days of the test period. Hypoxia was achieved by bubbling nitrogen into the tank water, monitored by a temperature-compensated probe connected to an oxygen controller (Eutech, Cole Parmer, USA) linked to a solenoid which reduced the oxygen concentration to $50 \pm 1\%$ normoxia. To prevent oxygen diffusion at the surface floating lids were constructed from polystyrene (89.8 x 49.8 cm) and covered with black plastic such that they minimised the air-water interface. Each lid had a section (17 x 28 cm) which could be removed to allow feeding and predator attacks. Temperature increases from 12 to 19°C were achieved using individual internal thermostatic heaters (Tetratec, UK). Following this period, individuals were retested for boldness before being killed by concussion.

6.3.4 Hormone Analysis and Quantification of Gene Expression

All fish were killed at approximately the same time each day (1 pm ± 1h) to ensure interpretation was not compromised by diel variations in plasma cortisol

concentrations (Pickering and Pottinger, 1983). A blood sample was immediately taken into heparinised 2 ml syringes using sterile 25 g needles. The blood was centrifuged at 3 500 RPM and 4°C for 5 minutes, the supernatant plasma aspirated and frozen at -20°C until further analysis. Cortisol concentrations were subsequently determined using a radioimmunoassay procedure (Pottinger and Carrick, 2001b).

With the exception of three data (one bold control and two bold samples from the high risk group at 11°C and normoxia) these data were contributed by Ashley Frost (n = 62), Charlotte Smith (n = 12), Hannah Burton (n = 12) and Ben Davis (n = 15) under the supervision of LS, and by LS (n = 35).

6.3.5 Data Analysis

Change in behavioural measures (latency to approach within 5cm of the novel object, and duration of active behaviour), specific growth rate (G), and plasma cortisol concentration (ng ml^{-1}) were each assessed using GLM (R, ver. 2.9.0). Specific growth rate was calculated as: $G = 100 \times [log(w_f) - log(w_i)] \times t^{-1}$ where w_f = final weight (g), w_i = initial weight and t = time as days in study (Höjesjö et al., 1999). Initial models contained all main terms, all two-way interactions except [oxygen-content * temperature], and included two three-way interaction terms: [risk * oxygen-content * boldness] and [risk * temperature * boldness]. Models were reduced to minimum adequate models: selection of terms in the models was based on minimising Akaike's Information Criterion (AIC) using the stepAIC function (MASS package; Venables and Ripley, 2002), with the final model selected by choosing the model with the lowest AIC. All data fit the assumptions of GLM with the exception of cortisol concentrations, which were therefore log_{10}-transformed for statistical analysis. A correlation between log_{10}-transformed cortisol and G was tested for using a Spearman rank correlation coefficient.

6.4 Results

Although boldness is considered constant in a consistent environment (Frost et al., 2007; Thomson et al., 2011), environmental challenges, such as those presented here, can elicit a change in behaviour; responses, in terms of latency to approach within 5cm of the novel object, were complex and varied according to a three way interaction between risk, temperature and initial boldness ($F_{2,122}$ = 3.77, p = 0.026).

In general, bold fish increased their latency to approach the object, becoming more neophobic, represented as a linear increase in latency with increasing threat for fish kept at a low temperature, or as a single peak response at the highest threat for those at high temperature, a ~360% increase in change from no to high risk (Fig. 6.1A). In contrast shy fish, regardless of temperature, remained shyest on a low threat regime, became bolder when under no threat, and decreased approach latency the most, by on average almost 400s, under high threat and low temperature (Fig. 6.1B). Boldness also varied according to a main effect of oxygen ($F_{1,122}$ = 4.21, p = 0.042), whereby fish which spent time in normoxic conditions generally became bolder and reduced their latency to approach a novel object, whilst those which underwent hypoxia increased their latency (mean ± SE change in latency: -17.8 ± 18.9 and 41.9 ± 47.6s for normoxic and hypoxic fish respectively), but this wasn't related to any other treatments (Fig. 6.2). A main effect of initial boldness similarly influenced change in approach latency (mean ± SE change in latency: 129.9 ± 23.8 and -127.1 ± 23.9 s for bold and shy fish respectively; $F_{1,122}$ = 78.44, p < 0.001).

In contrast to change in latency, change in levels of activity varied only according to initial boldness ($F_{1,137}$ = 7.97, p = 0.005; Fig. 6.3), with no significant interactions. Bold trout reduced their activity levels (mean ± SE), regardless of environmental challenge, by some 29.6 ± 16.5s (-12.81 %), whereas shy fish increased their activity to roughly the same degree: by 29.4 ± 15.7s (17.98 %).

In general, trout lost weight (mean body mass ± SE = -11.29 ± 1.08 %) throughout the experiments. This was evident in negative specific growth rates, and was due to interactions between risk and oxygen ($F_{2,125}$ = 13.89, p < 0.001) and risk and initial boldness ($F_{2,123}$ = 3.91, p = 0.023). In each case growth rate was highest in the group experiencing no risk and lowest in the low risk groups, on average a 3.5-fold decrease in G; growth rate decreased dramatically to a minimum in fish where low risk was combined with hypoxia, a 6-fold difference compared to no threat exposure (Fig. 6.4). Additionally, all main effects were significant except for initial boldness.

A - Bold

B - Shy

Figure 6.1: Mean (±SE) change in the latency (s) for A) bold and B) shy rainbow trout, *Oncorhynchus mykiss*, to approach within 5cm of a novel object after a two-week experiment period. During this period, individual trout experienced either no, low (predictable) or high (unpredictable) predator threat (1st and 2nd week) in combination with being kept at low (11°C; white) or high (19°C; hatched) temperature (2nd week). Bars which do not share a common lowercase letter were significantly different ($p < 005$, Tukey HSD), and these are continuous between figures A and B.
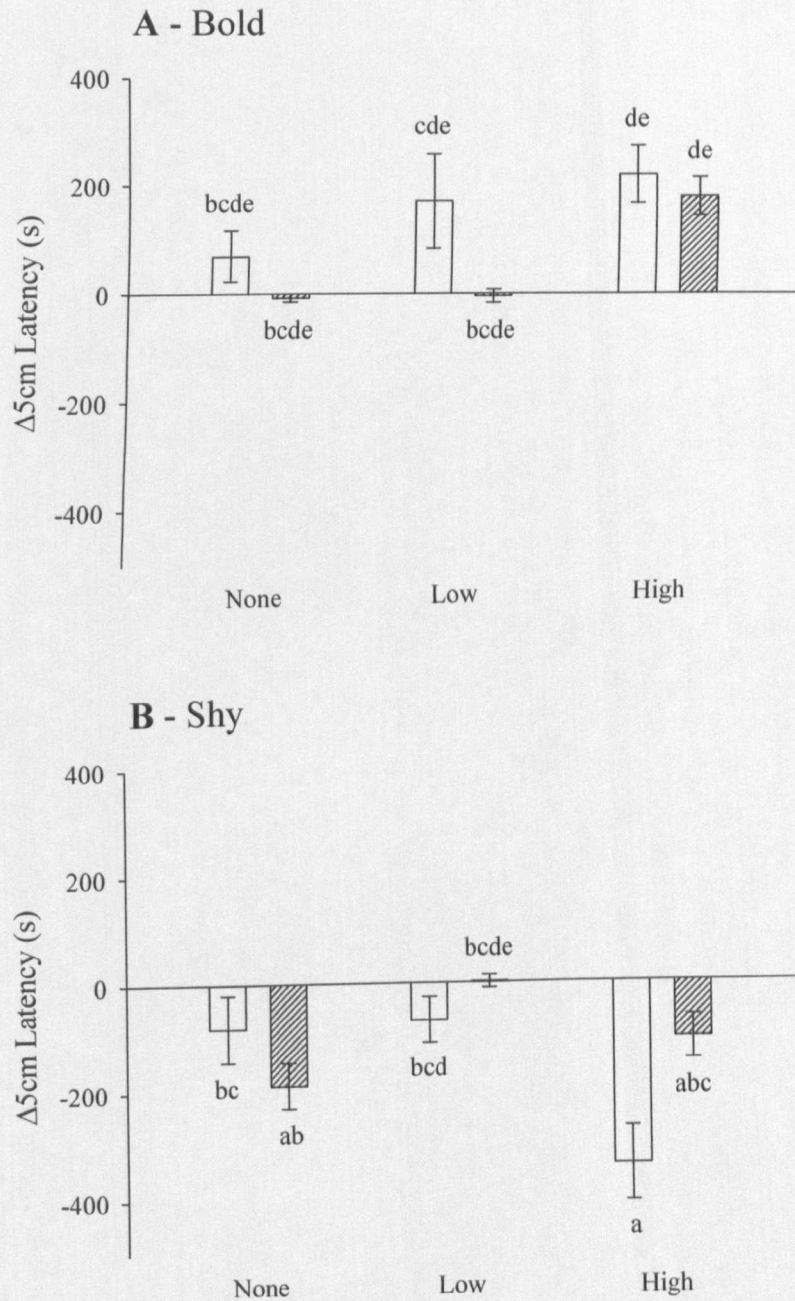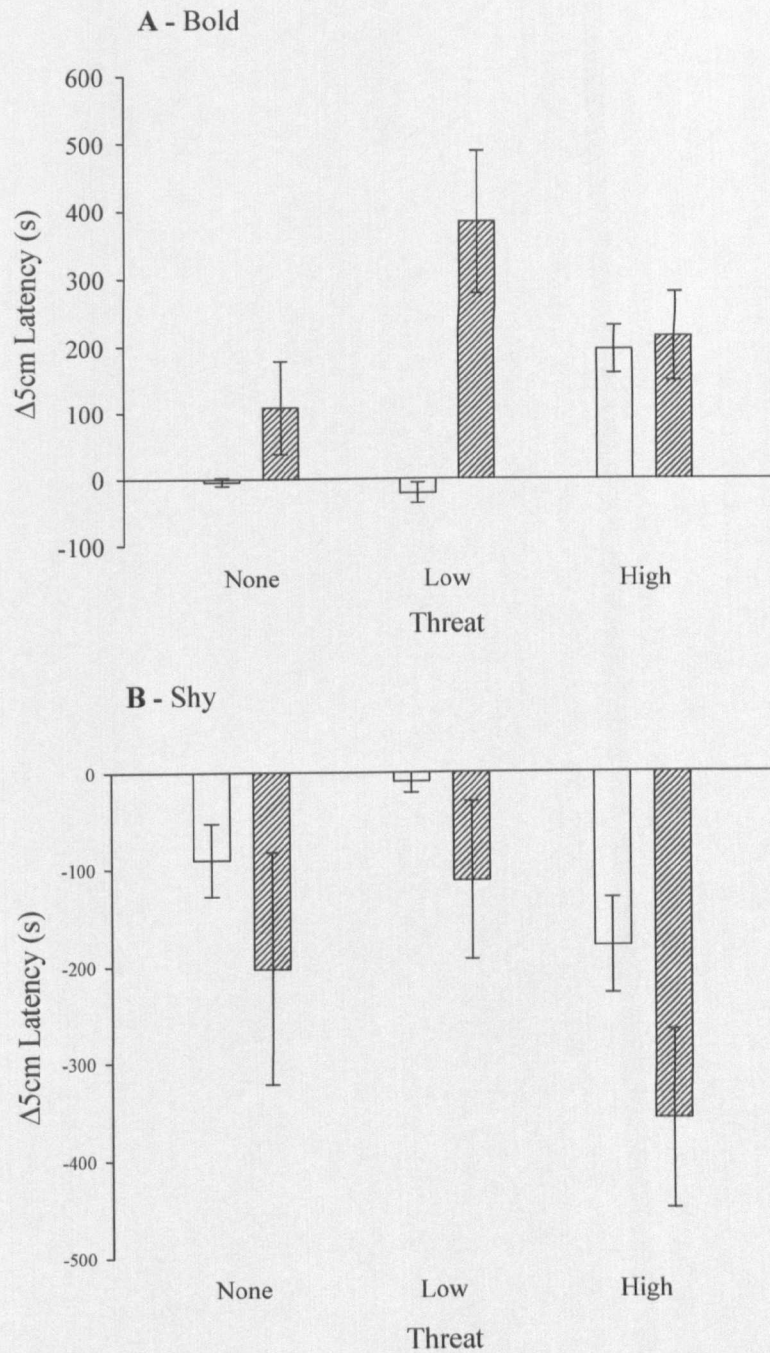
A - Bold

B - Shy

Figure 6.2: Mean (±SE) change in the latency (s) for A) bold and B) shy rainbow trout, *Oncorhynchus mykiss*, to approach within 5cm of a novel object after a two-week experiment period. During this period, individual trout experienced either no, low (predictable) or high (unpredictable) predator threat (1st and 2nd week) in combination with being kept at normoxia (dissolved O_2 = 100%; white bars) or hypoxia (dissolved O_2 = 50; hatched bars) during the 2nd week.
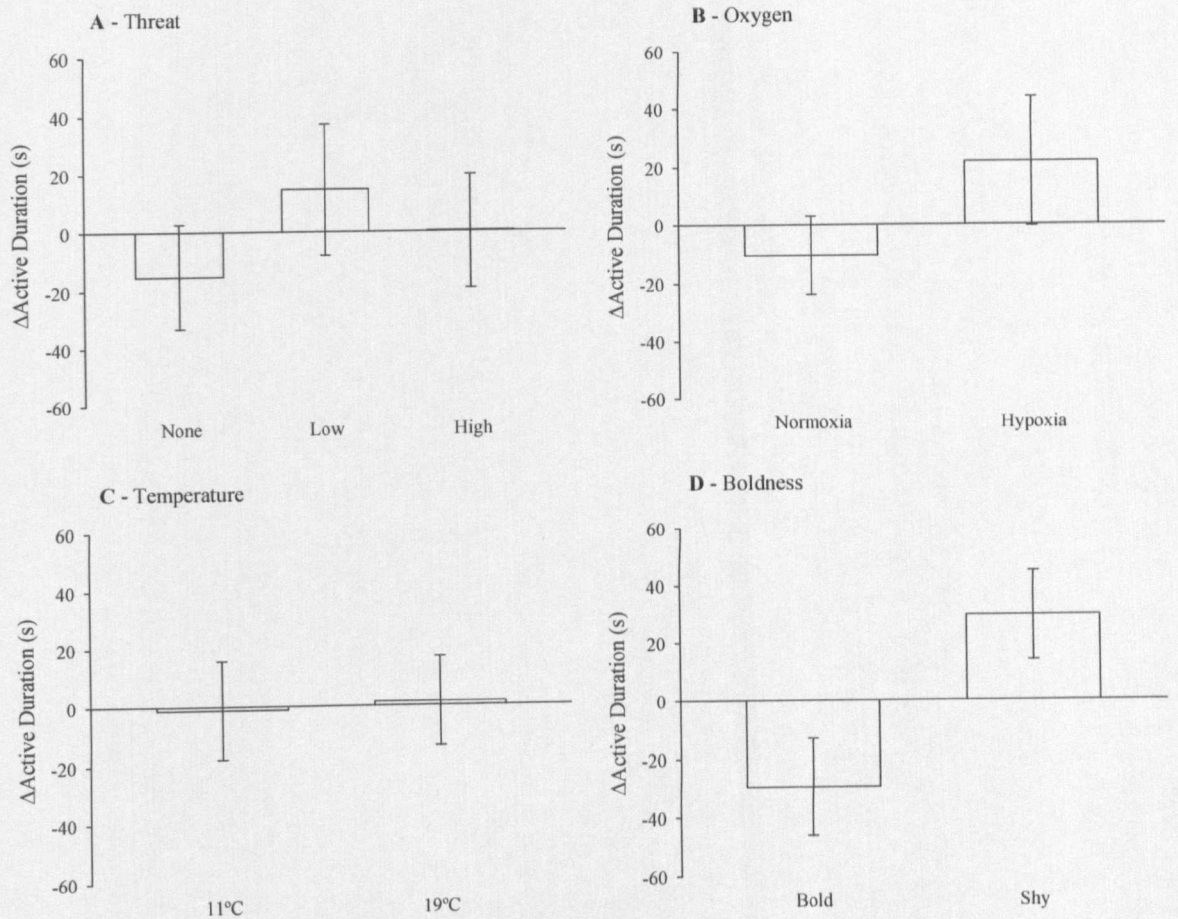
Figure 6.3: Mean (±SE) change in the duration of active behaviour (s) between two novel object trials for rainbow trout, *Oncorhynchus mykiss*, exposed to (A) no ($n = 42$), low (predictable, $n = 41$) or high (unpredictable, $n = 57$) predation threat; (B) normoxia (dissolved O_2 = 100%; $n = 95$) or hypoxia (dissolved O_2 = 50%, $n = 45$); (C) low (11° C, $n = 81$) or high (19°C, $n = 59$) temperature; or (D) classified as bold ($n = 70$) or shy ($n = 70$).

A - Behaviour

B - Oxygenation

C - Temperature
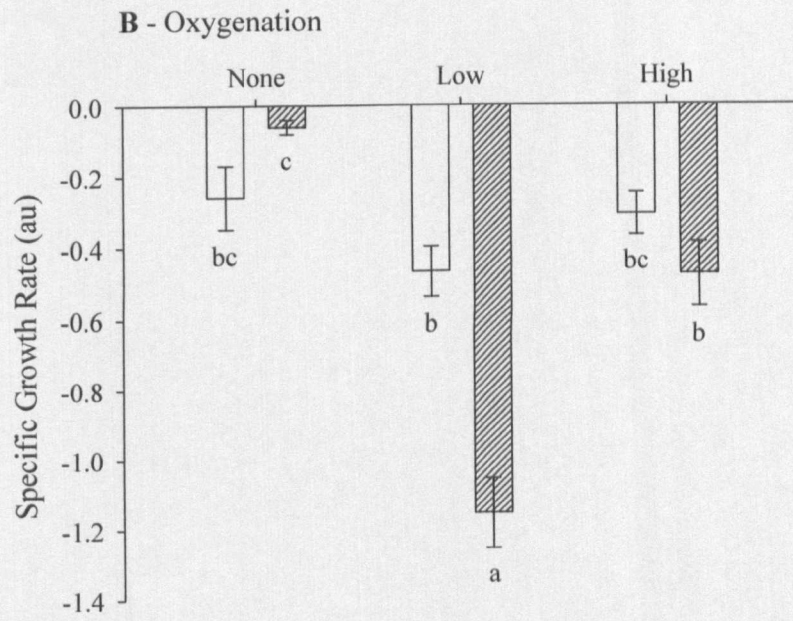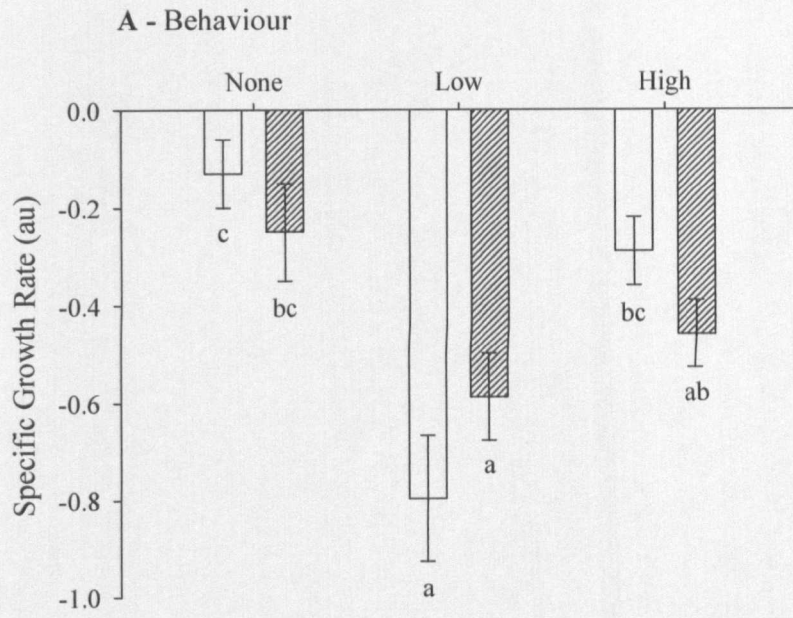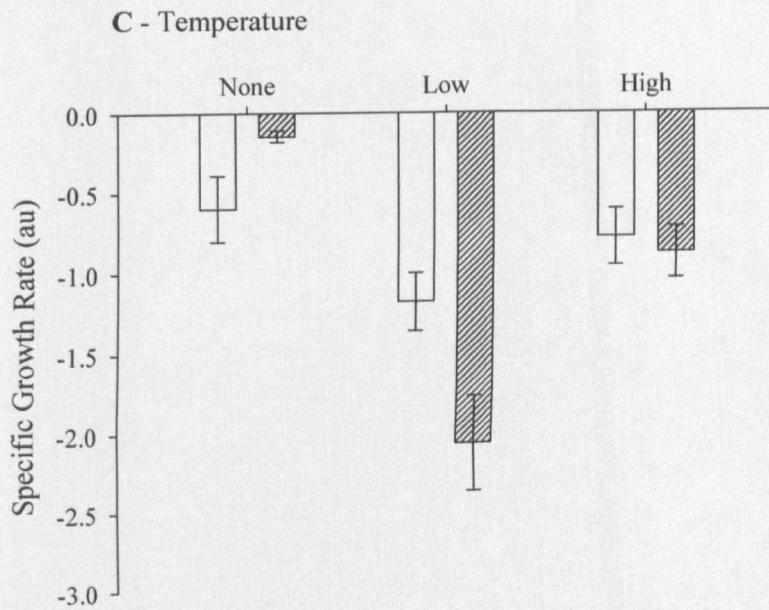
Figure 6.4: Mean (±SE) specific growth rate for rainbow trout, *Oncorhynchus mykiss*, at no, low and high predation risk and either (A) initially bold behaviour (white bars, $n = 21, 17, 32$) or shy behaviour (hatched bars, $n = 21, 24, 25$) towards a novel object; (B) kept under normoxic (white bars, dissolved oxygen = 100%, $n = 27, 29, 39$) or hypoxic (hatched bars, dissolved oxygen = 50%, $n = 15, 12, 18$) conditions; or, (C) kept under low (11°C; $n = 27, 24, 30$) or high (19°C; $n = 15, 17, 27$) temperature. Where present, bars which do not share a lower case letter were significantly different ($p < 0.05$, Tukey HSD).

Cortisol levels were generally high throughout the experiments, but differed dependent on treatment. Thus, log_{10}-transformed cortisol concentrations varied according to an interaction between risk and temperature ($F_{2,131} = 4.77$, $p = 0.010$), where concentrations were lowest at no threat but peaked at six times the cortisol concentration under a combination of low threat and high temperature (Fig. 6.5A). Cortisol also varied as an interaction between temperature and initial boldness ($F_{1,131} = 5.17$, $p = 0.025$), where it appears cortisol was relatively consistent across temperature for shy fish but increased with increasing temperature from ~40 to ~70 ng ml^{-1} for bold trout (Fig. 6.5B), but was not linked with oxygen availability (Fig. 6.6). Cortisol was additionally linked with risk as a main effect, where log_{10}-transformed cortisol was greatest in those fish under low threat and lowest under no

A - Predation threat vs. Temperature
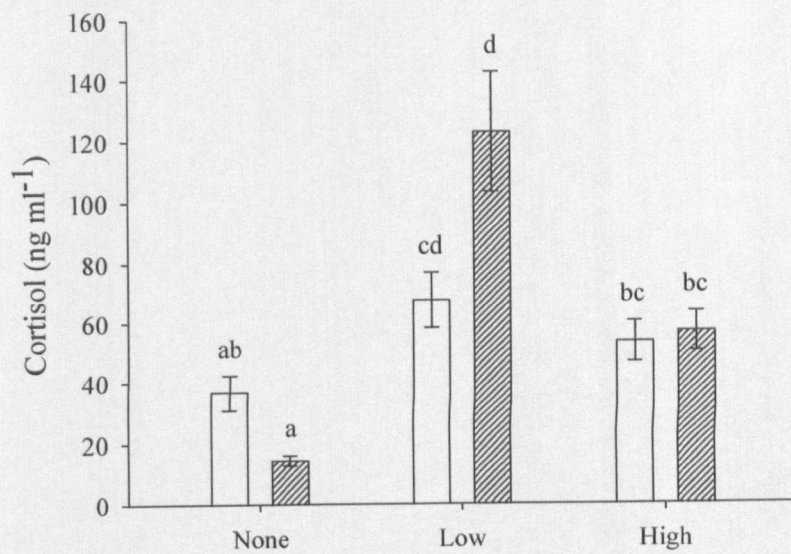
B - Temperature vs. boldness
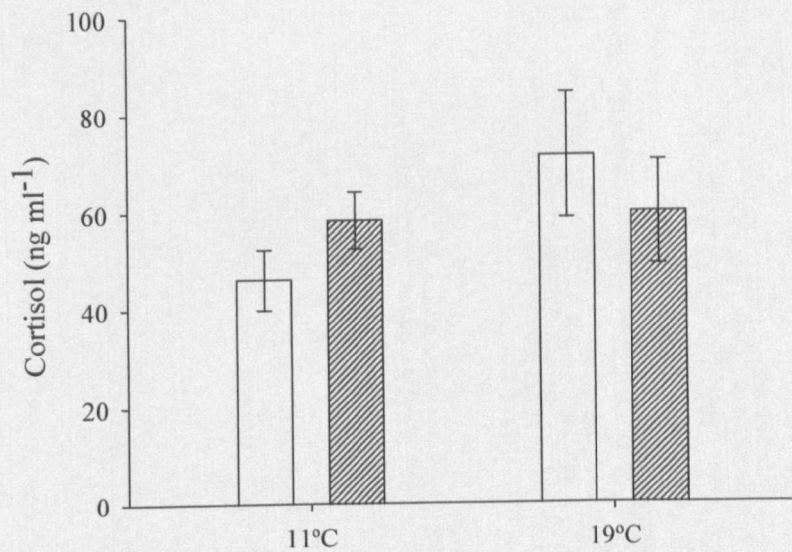
Figure 6.5: Mean (±SE) plasma cortisol concentrations (ng ml^{-1}) for rainbow trout, *Oncorhynchus mykiss*, after a two week treatment period. Trout were exposed either to A) no, low (predictable) or high (unpredictable) predation threat and low (11°C, white bars, $n = 27, 23, 30$) or high (19°C, hatched bars, $n = 15, 17, 27$) temperature; or, B) low and high temperature but separated by initially behaving boldly (white bars, $n = 41, 28$) or shyly (hatched bars, $n = 39, 31$). Bars which do not share a common lower case letter are significantly different ($p < 0.05$, Tukey HSD); letters are not continuous across graphs.
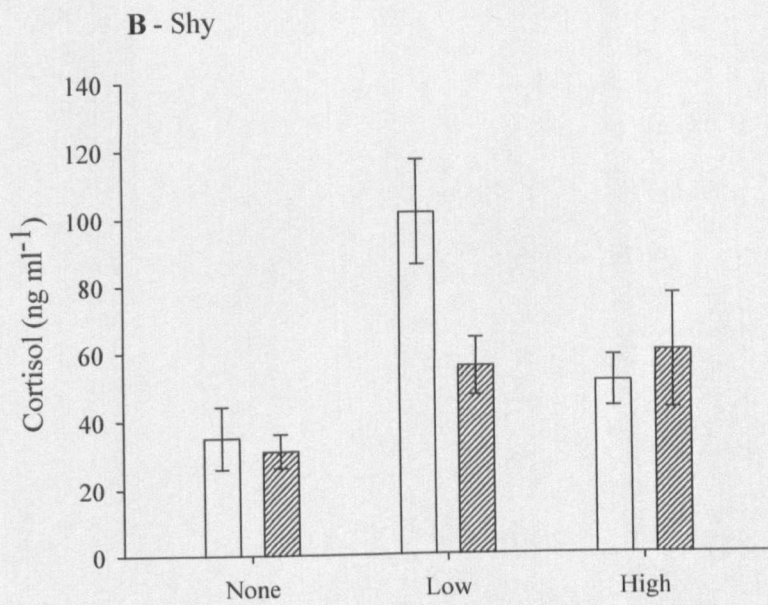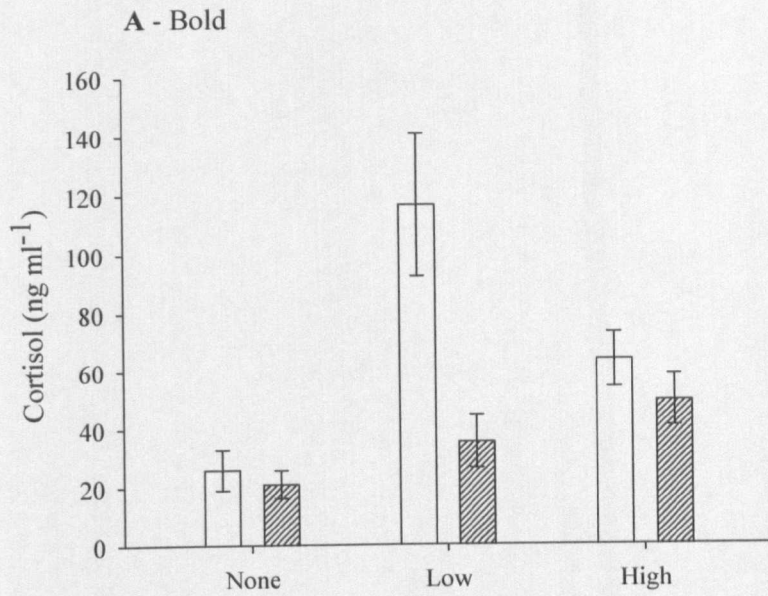
A - Bold

B - Shy

Figure 6.6: Mean (±SE) plasma cortisol concentrations (ng ml^{-1}) for (A) bold and (B) shy rainbow trout, *Oncorhynchus mykiss*, after a two week treatment period. Fish were exposed to no, low (predictable) and high (unpredictable) predation risk during the two weeks, and either kept at normoxia (dissolved O_2 = 100%, white bars) or hypoxia (dissolved O_2 = 50%, hatched bars) for the final 7 days of the trial period.

threat ($F_{2,131}$ = 23.04, $p < 0.001$; mean cortisol ± SE = 28.68 ± 4.05, 91.28 ± 10.70, 55.59 ± 4.77 ng ml^{-1} for no, low and high risk respectively). Across all fish, cortisol levels were negatively correlated with SGR (ρ = -0.363, $p < 0.001$).

6.5 Discussion

Behavioural plasticity is an important attribute which may provide an adaptive advantage, particularly when environmental challenges are inconsistent and change rapidly (Dall et al., 2004). However, not all individuals within a population display plasticity, possibly due to the costs associated with tracking environmental information and subsequently changing physiology and behaviour to suit conditions (DeWitt et al., 1998). In this study, bold and shy rainbow trout differed in their behavioural responses to some, but not all, combinations of environmental stressors – 50% hypoxia did not appear to elicit a behavioural change, whereas predation risk and increased temperature caused shifts in bold and shy fish. These changes in behaviour were associated with overall reductions in the weight of individuals, particularly when under environmental stress, and with concurrent differences in the physiological response to a stressor.

6.5.1 Behaviour

The changes in neophobia observed here suggest fundamental differences in how bold and shy individuals are able to respond to multiple stressors: in this case, increased risk within the context of temperature variation. Animals must weigh the potential benefits of any action that might expose it to increased threat (Lima and Bednekoff, 1999): though they often prefer to hide and reduce activities when risk is high (Carvalho and Del-Claro, 2004; Huntingford et al., 1988), animals may choose to alter their behaviour, with this decision mediated by energetic state (Anholt and Werner, 1995; Dall et al., 2004; Höjesjö et al., 1999; Lima and Bednekoff, 1999; Vehanen, 2003). Shy fish appear to find predictable threat most stressful and generally remain the shyest under these conditions (Chapter 5). Unexpectedly, an unpredictable risk caused a shift in the behaviour of shy trout towards boldness. In contrast, at high risk bold fish became shyer. When risk is predictable it makes sense to partition time into periods of antipredator activity when risk is high and to resume normal activity when risk is low. However, an unpredictable regime may engender a

more intermediate approach: without foreknowledge of when a predator will strike, it may be maladaptive to act too boldly, thereby risking being attacked, or to act too shyly and miss out on foraging opportunities. This change was more distinct at low temperature in shy fish: an increase in temperature still imposes a greater metabolic demand on the animal (Evans, 1990) which, combined with the cost of behavioural plasticity (DeWitt et al., 1998), may inhibit such a dramatic change in the behaviour of shy fish. Thus, internal physiological state may constrain adaptive decisions

The combinations of factors driving behavioural change in bold fish differ to those of shy trout, and thus require alternative explanatory mechanisms. Boldness may be considered adaptive when risk is high yet nutritional status low, since foraging when under predation threat necessitates risk-taking behaviour (Brown et al., 2005b). The weight loss in these fish would indicate that the requisite nutrition was not being obtained, either through stress-related reduction in foraging and assimilation (Beitinger, 1990; Bernier and Peter, 2001) or insufficient food supply, and thus fish would be expected to behave more boldly. In this instance, however, it is possible that bold trout opted for an energy-saving strategy rather than increasing foraging effort. Strikingly, almost no change in behaviour was observed at all at higher temperatures when under no and low threat. This may again be due to the energetic costs associated both with plasticity (DeWitt et al., 1998) and increased temperature (Evans, 1990), and would suggest that for bold fish it is an unpredictable risk that provides the necessary impetus to alter their behavioural strategy.

Hypoxia appeared to play a role in overall behavioural change in these trout. In response to reduced dissolved O_2, fish will initially increase activity as an escape response, in addition to performing ASR to utilise the most oxygen-rich water available. Subsequently, fish may reduce activity and, therefore, oxygen expenditure. Here, animals under hypoxia reduced their latency to approach the object, which may reflect the heightened energetic demand resulting in greater caution towards novelty. Indeed, hypoxia may promote hiding behaviour as a response to potential threat since it has a low energetic cost (Kramer, 1987). In extremely low oxygen saturation, cessation of activity is critical for survival but not observed in all animals (van Raaij et al., 1996; Vianen et al., 2001). This divergence in behavioural change has been attributed to variation in coping styles (Höglund et al., 2008; Laursen et al., 2011),

and thus should be linked with bold and shy traits but this was not observed here. Such variation may only be apparent at extremely low dissolved O_2 (e.g. <35%, Höglund et al., 2008), seemingly at the critical point of oxygen saturation for survival (Vianen et al., 2001). Rainbow trout are considered highly sensitive to hypoxia (e.g. Dean and Richardson, 1999; Landman et al., 2005; Vianen et al., 2001), yet there remains relatively little published data on behavioural or physiological responses within an intermediate range (35-65% dissolved O_2). Behavioural differentiation may occur between 40 and 60% oxygen saturation in some salmonids (Brelin et al., 2005), depending on the type of behaviour recorded, but there does not yet seem to be a consensus for rainbow trout. Furthermore, the point of behavioural divergence may depend upon the interplay of hypoxia with additional factors, such as temperature or internal state (Schurmann et al., 1991; Shimps et al., 2005). Multiple studies indicate that for rainbow trout, at the point of behavioural divergence due to hypoxia, an incorrect behavioural decision is fatal (van Raaij et al., 1996; Vianen et al., 2001), and it may, therefore, not be possible to run long-term behavioural experiments under these conditions in this species.

Both bold and shy fish altered their levels of activity but this was not in response to differences in predation threat, dissolved oxygen or temperature. This was surprising since higher temperature leads to a higher metabolic rate, often coinciding with an increase in activity (Evans, 1990; Peterson and Anderson, 1969) whilst, aside from an initial escape response, hypoxia leads to reduced activity to minimise energetic and oxygen expenditure (Herbert and Steffensen, 2005; Lefrancois et al., 2005). Levels of activity are, however, often associated with boldness (Sneddon, 2003; Thomson et al., 2011; Wilson and Godin, 2009), and indeed the change in activity observed in these fish mirrored their behavioural responses towards a novel object i.e. in general, shy fish became bolder and more active whilst bold trout showed the reverse response. Since these changes were not related to the treatments, it is possible that the fish were altering their levels of activity relative to an unmeasured factor, or even commensurate with their nutritional status, balancing energetic input and output and habituating to the prevailing conditions. Future studies should explore trade-offs in energy expenditure and intraspecific variation in the context of environmental variation in more detail.

Attention must be drawn to the variation in water oxygen content with changing temperature. Although in the present study changes to temperature and dissolved oxygen content were not made in combination, higher temperatures result in reduced water oxygen content; additionally, the metabolic rate (and therefore the oxygen requirement) of poikilotherms such as fish increases with increasing temperature (Solbé, 1988). In the present study there was an approximately 18% decrease in oxygen content from just over 11 mg l^{-1} at 11°C to ~9 mg l^{-1} at 19°C. Thus, even though the water was fully saturated at these temperatures there was still a sizeable reduction in oxygen available to the fish with increased temperature. However, even at the higher temperature the oxygen availability is considerably higher than the hypoxic treatment (50% saturation at 11°C was equivalent to approximately 5.5 mg l^{-1} dissolved oxygen) and is in excess of the recommended optimum oxygen concentration for rainbow trout ($\geq$9 mg l^{-1} at temperatures over 15°C; Raleigh et al., 1984). Absolute minima for rainbow trout are suggested to be 6 mg l^{-1} (Solbé, 1988), only achieved in the present study in the hypoxic treatment. It therefore seems likely that the hypoxic treatment represented a distinct stressor to these fish, but that the decrease in oxygen availability for fish kept at the higher temperature, particularly over the time scale of the change (16 h), would not be enough to invoke a stress response.

Coping style theory predicts that reactive animals, analogous to shy, have more flexible behaviour than bolder conspecifics (Koolhaas et al., 1999), yet recent evidence suggests that this is not always the case (Frost et al., 2007; Ruiz-Gomez et al., 2008). Indeed both bold and shy rainbow trout can alter their behaviour dependent upon recent experience (Frost et al., 2007) and it seems that here a similar process took place. Some of these changes may represent the accommodation of a more intermediate personality under the combinations of challenges presented, which may be more adaptive in the presented contexts than being completely bold or shy. Rather than defining the ability of individuals to alter behaviour, animals of different coping style may simply respond to different stimuli, or to the same stimuli in different ways. Despite some interesting and informative studies identifying key behavioural and physiological characteristics of coping styles in rainbow trout (Øverli et al., 2007 and references therein) there remains a great deal of variability in these responses (Koolhaas et al., 2010). Future studies, therefore, need to determine

the causative factors of this variation across a variety of states and contexts and relate these to natural ecology.

Many of the animals in this study showed negative growth rates indicating that they were not obtaining sufficient nutrition from the food they received. This may have been partly due to the quantity or quality of feed provided, but here even those fish receiving no treatment may have been slightly stressed, and this can often reduce rates of food attack and ingestion, and cause the assimilation of ingested food to be less efficient compared to unstressed animals (Beitinger, 1990; Bernier and Peter, 2001; Wendelaar Bonga, 1997). Both bold and shy fish exposed to threat in this study, particularly a predictable threat, in general had lower growth rates, and this is likely as a result of reduced foraging, both as one aspect of an individual's antipredator strategy and as a result of stress (Lima, 1998; Metcalfe et al., 1987). Hypoxia can cause reduced growth rates by inhibition of feeding, since feeding and digestion imposes a large oxygen cost, particularly in salmonids (Bernier and Craig, 2005; Brett and Groves, 1979; Chabot and Dutil, 1999). However, the greatest effect of hypoxia on growth rate was evident in fish experiencing predictable predator attacks, suggesting that in combination these treatments caused a significant reduction in feed intake and/or assimilation efficiency.

6.5.2 Physiology

Baseline cortisol levels in these fish were consistent with those observed in some previous studies (Øverli et al., 2002b; Pottinger and Carrick, 1999). The predictable threat appeared to be the most stressful for these fish, particularly at 19°C compared with 11°. High ambient temperatures are associated with more rapid and pronounced stress responses in fish (Pottinger and Carrick, 2000; Sumpter et al., 1985), and previous data suggests that the predictability of the low risk situation may be more stressful than unpredictable attacks (Chapter 5). Such an elevation in HPI activity when more than one stress is applied may indicate activation of the physiological response in multiple cell-types, each attuned to particular stressors, and, therefore, a synergistic effect on increases in cortisol secretion (Sumpter et al., 1985). The effects of temperature were specifically dependent on initial boldness, where HPI activation in shy fish was generally immutable in the context of temperature but bold fish were more sensitive to change. This may suggest different thresholds for temperature

tolerance between bold and shy individuals which, for bold trout, may lie somewhere between 11 and 19°C. If such a threshold exists, then for shy fish it may lie outside of this range and, therefore, closer to the limits of thermal tolerance in this species. Alternatively, the high levels of cortisol still represent activation of the HPI axis in these fish, and the apparently stable cortisol level in shy trout may indicate a more prominent role of these other stressors in comparison to temperature.

The fish in this study did not, however, experience an acute stressor such as those used in previous studies to characterise stress responses (e.g. Øverli et al., 2002b; Pottinger and Moran, 1993; Thomson et al., 2011) and thus the measured cortisol levels may represent chronic stress from daily and repeated exposures. In contrast, in a previous study lower cortisol concentrations were found in the plasma of trout in otherwise similar conditions (Chapter 5), suggestive of an intrinsic difference in the physiological condition of the fish. This may be due to differences in the husbandry of these fish, but more likely may represent differences in protocol wherein the fish in the present study were sampled immediately after the second novel object test rather than later in the day. Therefore, future studies should perhaps standardise their final stress test since it is known that the divergence in cortisol release between coping styles is only evident after an acute stress test (Øverli et al., 2005).

6.5.3 Conclusions and Implications

Whilst individual behavioural and physiological responses to environmental stimuli are well-characterised, there remains a lack of information as to how individuals respond to a variety of such challenges, and how intraspecific variation in these responses may be driven by individual differences in personality. Here behavioural responses to a combination of threats were extremely complicated when personality is considered, and previous theory on responses to single stressors may not always be applicable when other threats to homeostasis are present. Responses to risk are important to assess since incorrect decisions can be immediately fatal, and here fish adjusted their responses in accordance with the environmental context. Furthermore we demonstrated that energetic and physiological state can be adversely affected by not only risk but also by environmental stressors, and this in turn has implications for survivability and fitness in a challenging environment. Understanding intraspecific variation is critical since natural environments frequently impose abiotic challenges

on their inhabitants, and these may become more common or extreme due to climate change and other anthropogenic influences; a fuller understanding of these interactions can therefore inform models of individual and population fitness in increasingly stressful habitats.

7 Discussion

7.1 Overview

Boldness is an important personality measure in animals, describing individual responses to novelty or risk and, therefore, a key determinant of survivability dependent upon particular environmental challenges. Boldness is also linked with physiological stress responsiveness such that bold and aggressive animals often have a distinct neuroendocrine profile with reduced HPI axis reactivity compared to shy animals. Previous studies have suggested that the degree of boldness is a fixed trait. Here I show that boldness is a consistent trait when no environmental challenges or stressors are presented to individuals (Chapter 2). However, variations in context (biotic, such as threat or social status; or abiotic, such as temperature and dissolved oxygen content) as well as state (e.g. energetic and nutritional status) can modify individual levels of neophobia and activity (Chapters 4, 5, 6). These variations appeared to be proportional, with appropriate directionality, to the stimulus, and are therefore likely aimed at improving individual fitness relative to the context. No correlation was observed between boldness and physiological response to stress between lines selected for a divergent stress response as would have been expected according to coping style theory (Chapter 2), although this divergence was apparent in outbred trout. Furthermore, bold and shy fish did exhibit divergent stress responses to environmental challenges, with shy fish in particular finding a predictable risk highly stressful (Chapters 4, 5, 6). Stress responsiveness was highly correlated with the expression of behaviour- and stress-related candidate genes (Chapters 2, 3), and whilst boldness was not linked with gene expression within the stress lines, gene expression did differ in fish dependent upon environmental stress (Chapter 5).

7.2 Boldness

Trout were consistent in several measures of their behavioural response towards novelty across two trials separated by a week when kept in constant conditions, thereby demonstrating individual personalities in these animals. Subsequent exposure to environmental stimuli and social learning opportunities caused these trout to modify their behaviour in response. Bold fish displayed significantly greater behavioural plasticity than shy fish; bold trout generally became shyer when placed into a social group, and also increased neophobia and altered activity levels in

response to environmental stressors. In contrast shy fish displayed only limited flexibility, remaining shy in response to most stimuli but becoming slightly bolder after a two-week exposure to a high (unpredictable) predation threat regime.

These data contrast with coping style theory, which predicts that bold, proactive animals are generally rigid in behaviour, whereas shy, reactive animals are more flexible in the way they respond to the environment (Koolhaas et al., 1999; Sih et al., 2004a). In contrast, bold trout have demonstrated greater behavioural plasticity in response to intraspecific aggression (Frost et al., 2007). Similarly, LR trout modified their activity levels in an open field test to a greater extent than HR trout (Schjolden et al., 2005). Altogether this would suggest that other factors play a role in determining coping styles in these fish. For instance, animals may react differently depending on their familiarity with the environment (Schjolden et al., 2005; Sluyter et al., 1996), and differences in the speed at which bold and shy individuals can learn has important ramifications for how quickly they may adjust their behaviour towards any stimulus, particularly if repeated or chronic (Sneddon, 2003). A rigid behavioural profile is maladaptive in a changing environment and it makes sense for animals to be able to alter behavioural responses regardless of physiology. Indeed, the physiological response provides the necessary mechanisms and energy for behaviour, and whilst divergent sympathetic and HPI stress profiles may favour any particular behavioural strategy, the freed resources may still be put to use in alternative behaviour. Whilst physiology and behaviour are inherently linked, how boldness correlates with the neuroendocrine response to stress needs to be more fully explored, particularly during early life stages since this provides minimal opportunity for environmental and ontogenetic influences on behaviour.

Appropriate behavioural responses often depend on both context (i.e a functional behaviour such as responding to a predator attack, foraging, or competition with a conspecific; Sih et al., 2004b) and on state (current external or internal charactistics of an individual, such as nutritional status or recent experience; Dall et al., 2004). Context dependency of behaviour is important since responding the same way to every challenge is likely to be maladaptive. Similarly, different states may necessitate behaving differently: foraging, for instance, becomes increasingly important as nutritional state decreases, but at the same time state can limit the

available behavioural repertoire (Dall et al., 2004). In this thesis, the behavioural responses of fish to variations in threat were mediated by their energetic state: bold fish would reduce activity and become shyer if they were exposed to threat but had sufficient resources (high food availability). However, if food availability was reduced, these fish became more active, presumably as a need to forage, even when risk was higher. Animals, therefore, must make cost-benefit analyses of context versus state to determine the most appropriate behavioural response to their scenario. In this manner, differences between individuals can be generated since, even exposed to identical contexts, animals will behave according to their state. If bold and shy personalities exhibit different levels of behavioural plasticity then this may further limit the extent to which animals can respond to the environment. The available evidence seems to suggest that both shy and bold fish may exhibit more or less plasticity dependent, conversely, on context (Schjolden et al., 2005). Thus the bold-shy continuum provides a wide array of potential behavioural responses to environmental challenges, and the extent to which animals can draw upon this repertoire may itself depend upon context and state.

Behavioural change was not only linked with adaptive changes towards environmental stressors but also with social interaction. Learning from conspecifics is an integral method of gaining valuable experience about the environment, particularly amongst social animals, since it negates the need for the energetically- and temporally-costly processes of trial and error (Galef and Laland, 2005). Since, by definition, social learning requires social interactions between individuals for behaviour to be transmitted, it may be favoured by shyer animals which are naturally less aggressive, and therefore, within a shy population, knowledge about the environment and the appropriate responses to it may be shared. However, bold animals tend to be more aggressive, and boldness has, therefore, been linked with dominance (Brick and Jakobsson, 2001; Dingemanse and de Goede, 2004; Sundström et al., 2004). Frost et al. (2007) demonstrated that intraspecific aggression could cause shifts in behaviour of bold and shy animals depending on whether they won or lost a dyadic encounter, or even if they observed others doing so. Being in the presence of a dominant whilst simultaneously being a newcomer to a group may be stressful and inhibit the transfer of behaviour (Höjesjö et al., 1998; Sneddon et al., 2005; Winberg and LePage, 1998); rather, such individuals are likely to exhibit

subordinate behaviour. Thus even an originally bold individual may become shy and reclusive when subordinate to a relatively larger or stronger fish. Behavioural change can therefore be caused by interactions with conspecifics, but the mechanisms of change are dependent upon the social environment. Becoming bolder or shyer when in a group may depend as much on learning as on finding one's place in the hierarchy, and is likely also a result of contextual influence on the individual and group as a whole.

7.3 Coping Styles

Contrary to expectations, in lines bred for divergent stress responsiveness no correlations between boldness and HPI activity were found. Coping style theory predicts that aggressive and bold behaviour is correlated with a low HPI response to stress, whereas shy behaviour is linked with higher cortisol responses (Koolhaas et al., 1999). Rather, within each line I found both bold and shy rainbow trout, suggesting either that the HR and LR lines of trout were not equivalent to shy and bold respectively or that, whilst a genetic link between boldness and stress responsiveness existed, this was no longer evident due to decoupling of these traits. Coping styles appear prevalent across many animal groups, including teleosts, but the evidence is less clear in rainbow trout (Koolhaas et al., 2010). Indeed, decoupling has previously been demonstrated in these lines after a significant stress, and although coping styles were not restored in these trout they were evident in the subsequent generation (Ruiz-Gomez et al., 2008). This evidence would suggest that boldness and shyness are equivalent to proactive and reactive coping styles and that these responses are hereditary traits. However, whilst physiology may be quite rigid, personality can display plasticity as a result of environmental factors (experience, learning and, potentially, state).

Coping styles should, according to theory, provide a package of behavioural and physiological responses that, taken together, allow animals to respond appropriately to any challenge that could damage health (Koolhaas et al., 1999). The net result of these behavioural and physiological interactions are the archetypal proactive and reactive strategies. It is, however, unlikely that reactive and proactive strategies are adaptive across all contexts and states, but that environmental pressure can generate these extreme phenotypes in particular species and in particular scenarios (Koolhaas

et al., 1999). One clue as to how selection works on both boldness and coping styles can be obtained by looking at population distribution patterns. Rainbow trout appear to generally show either extremely shy or bold behaviour (Frost et al., 2007; Chapter 2), whilst pumpkinseed sunfish *Lepomis gibbosus*, for instance, show a normal distribution with relatively more intermediate personalities represented (Wilson et al., 1993). Therefore it seems likely that proactive and reactive strategies represent extremes, but in between these strategies there may exist a large amount of scope for behavioural or physiological variation and plasticity. Post-stress cortisol levels tends to remain extremely consistent across studies whereas behaviour appears more malleable, and therefore decoupling of these traits may more likely be due to behavioural adaptation rather than physiological change. Nevertheless, coping styles could, therefore, provide useful models for understanding adaptive responses to environmental stress but exclude, in some cases, a significant proportion of information. Conversely, including personality within physiological and behavioural analysis provides us with a means to explain the high individual variation often seen in studies with ill-defined groups, and future studies should explore intraspecific variation as a factor in explaining any divergent responses (e.g. MacKenzie et al., 2009).

Furthermore, whilst boldness has been linked to stress responsiveness, the correlation between behaviour and physiological stress responsiveness may only be apparent during activation of the HPI axis (Øverli et al., 2002a; Øverli et al., 2007), and thus if novel objects are not a sufficiently stressful stimulus then coping style will not be apparent. This may have confounded some reports on boldness in trout which were unable to find significant links with stress physiology in all cases (e.g. Schjolden et al., 2005), but ultimately highlights an inherent problem in the analysis of stress coping styles. Since measures of boldness are, by definition, responses to novelty, and if these responses are not correlated with stress physiology, then coping styles really cannot be related to boldness. Much stronger correlations are observed with aggression (Pottinger and Carrick, 2001b) which may generally be a stronger tool for development of coping style theory.

One important feature of the rainbow trout stress lines was the strong divergence in the expression in the brain of several candidate genes implicated in roles in boldness-

related behaviour and in the stress response. These data were corroborated by a second study (Chapter 3) in which I demonstrated this trend in genes representative of the entire HPI axis and in ancillary metabolic pathways. With the exception of two genes (POMC and Hbα4), all of the genes investigated were significantly upregulated in low stress responding trout compared to high responding fish. Additionally, five of these were directly correlated with plasma cortisol concentration. This demonstrates the strength of genetic mechanisms involved in physiological responses to stress, though it is unclear at this stage whether control of responses is through the actions of physiology on expression or vice-versa. Physiological responses are extremely rapid (a matter of seconds for the sympathetic response, and slightly longer for the HPI axis), and therefore in all likelihood regulation of the response occurs first with hormone secretion and latterly with up- or down-regulation of the relevant gene network to modulate the response.

7.4 Wider Implications

Personality and boldness, and the physiological and genetic mechanisms associated with them, are receiving increasing amounts of attention due to the importance of behavioural responses to environmental stress associated both with animal care and with climatic change. However, until recently few studies had attempted to understand how animals respond to complex variations in their environment, and how individual variation in personality traits provides adaptive responses to these challenges. Here I have shown that rainbow trout are able to modulate their behaviour in response to combinations of such challenges. One would therefore expect the fitness of bold and shy behavioural strategies to differ on a context-dependent basis: bold animals may thrive where individuals must be competitive for few resources, whereas shy animals may do better where being aggressive is likely to be maladaptive, but the fitness consequences of these phenotypes will be modulated by differences in behavioural flexibility, particularly in a changing environment. Contrary to coping style theory, I have demonstrated that this behavioural plasticity is much more prevalent in bold trout than shy, although this may depend upon context and state. Whilst I could not demonstrate a correlation between boldness and stress physiology using two lines of trout selected for stress responsiveness, such a link was evident in outbred fish and I have shown that trout of different personality also show divergent HPI reactivity dependent upon context.

Despite the importance of genes in driving behaviour and the hereditary aspects associated with these traits, few studies have investigated the role of individual genes in generating personality or in the control of the stress response. Here I demonstrated that behavioural responses to environmental stimuli are linked with differences in the expression of three candidate genes, at least one of which, $GABA_A$, covaries with the boldness of the individual. Although it is impossible to determine whether expression of these genes regulates behaviour or vice-versa, this work adds novel information to a growing field of genetic studies which have already linked differences in the expression of individual genes to variation in behaviours such as foraging (Ben-Shahar et al., 2002; Ingram et al., 2005). Furthermore, whilst physiological stress responsiveness and the role of the HPI axis in teleostean responses are well studied, few have attempted to correlate HPI activity with the expression of stress-related genes. In this thesis I have characterised the expression of a broad range of such genes, linking them directly to divergence in the stress response. This is also the first study, to my knowledge, to characterise gene expression throughout the entire stress axis. This information can inform future studies investigating the molecular mechanisms controlling, or controlled by, neuroendocrine stress responsiveness. Of particular importance may be determining the chromosomal location of the genes of interest relative to those of other related traits and the potential genetic knock-on effects of selection.

7.5 Limitations and Future Work

Boldness is an important personality parameter, but its measurement varies across studies, even within context. Here I used a novel object test to determine bold and shy animals, and this is a frequently used method in fish (Bergman and Kitchen, 2009; Frost et al., 2007; Wilson et al., 1993). Alternative methods include how quickly individuals eat a novel food type (Frost et al., 2007; Magnhagen and Staffan, 2003; Sneddon, 2003), and the latency to exit a shelter into a novel environment (Brown and Braithwaite, 2004; Schjolden et al., 2005; Verbeek et al., 1994). Whilst each reflects on a response to novelty, it could be argued that they target different cognitive mechanisms. For example, interacting with a potential food item is very different to encountering an object which may prove dangerous (Frost et al., 2007), whilst interacting with novelty within a familiar environment also contrasts with exposure to an unfamiliar setting; indeed, Schjolden et al. (2005) observed quite

different responses to a novel object and a novel environment in the same fish. Furthermore, boldness is linked with a variety of other behaviours, such as aggression and activity; whilst they are often correlated, there is still debate over whether all of these behaviours can be encompassed within a single personality (i.e. boldness) or whether they each represent different personality continua. Relating behavioural findings to previous work therefore entails careful assessment of the cognitive processes actually being tested, and this also bears some influence on the conclusions drawn from studies on boldness.

Genes have been well mapped in rainbow trout, since salmonids are an important model species in genetic and genomic studies due both to a genome duplication event in their evolutionary history as well as their importance as an aquaculture and fishery product (Guyomard et al., 2006). However, mapping efforts are not complete and therefore there remain some genes which have not been characterised, and their sequences as yet undetermined. Rainbow trout-specific sequences of some genes influential in boldness or behaviour or in the HPI axis have not yet been determined and knowledge of their expression may prove valuable in understanding intraspecific variation in the traits of interest. For example, the *DRD4* gene controls novelty-seeking behaviour and is implicated in exploratory behaviour; whilst in itself *DRD4* provides a useful tool for understanding boldness, polymorphisms in this gene in particular have the potential to further our understanding of the evolutionary development of personality across taxa (Bailey et al., 2007; Benjamin et al., 1996; Fidler et al., 2007). Whilst coverage of the HPI axis itself was thorough, analysis of a wider range of targeted candidate genes could provide a better understanding of the processes related both to boldness and the stress response.

Additionally, whilst relative expression of genes may differ between brains of different animals, expression may also vary across discrete regions or cell types of the brain (Feldker et al., 2003). This may have implications for some genes which have numerous roles dependent on where, or in what, tissue they are expressed. It was impossible to determine differences on such a fine scale in this study but microdissection techniques could be used to accomplish this. Furthermore, whilst the expression of candidate genes can provide useful information regarding the mechanisms of behavioural and physiological control, and how individual

differences may evolve across generations, in between gene expression and higher mechanisms there remains a gap in knowledge. For instance, processes involved in protein synthesis from their genes, such as post-translational modification, have not been characterised in many cases, and thus there should be care in drawing conclusions from gene expression alone. Future work needs to address this gap in our knowledge, but will provide insight into how behavioural polymorphisms may be maintained within natural populations. Such studies can also help improve our knowledge of how behavioural polymorphisms are maintained and expressed within natural populations.

7.6 Conclusions

Consistent bold and shy responses to novelty were observed in rainbow trout, and these were linked with divergence in levels of activity when conditions remained constant. However, bold and shy behaviour was evident within two lines selected for low and high neuroendocrine stress responses, and therefore not consistent within respective line as expected. Whilst boldness could not be related to the expression of candidate genes, chosen on the basis of their roles in behaviour and physiology, expression was significantly different between the two stress lines and may be linked to stress. Throughout the HPI axis, and amongst the genes tested, LR trout tend to show uniform upregulation of these genes compared with shy trout and may explain divergent stress responsiveness.

Trout altered their level of boldness based on the social context. In general, bold fish became shyer whilst shy fish remained shy after exposure to environmental challenges, regardless of whether placed into a bold or shy population. This may suggest that a bold, dominant group causes submission in intruder fish, whereas new members of a shy group take on shy characteristics. In these outbred fish divergent stress responses between originally bold and shy fish could be observed; cortisol levels were as expected from coping style theory.

Boldness and activity levels also changed in fish exposed to a variety of biotic and abiotic stressors. Bold animals tended to alter their behaviour according to interactions between context and state, thereby producing behaviour more appropriate to individual scenarios. In contrast, shy fish generally did not alter their

behaviour, suggesting either that shyness and inactivity were adaptive responses to these situations, or that shy trout were not capable of exhibiting behavioural plasticity. Together, these findings provide new evidence for dynamic behavioural responses primarily in bold individuals that are dependent upon context and physiological state and may be relevant to theoretical models on the ecology and evolution of animal personalities.

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