Genomic analysis of stress, aggression and boldness in rainbow trout

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Abstract

Behavioural phenotypes are under many influences from external and internal environments. The extent to which genetics affects behaviour has been a focus of recent study. Gradually, evidence demonstrating the influences of genes upon behaviour has mounted in a wide range of studies from family resemblance to gene knockouts to microarrays. A major challenge in behavioural ecology is to investigate the genomic architecture of behaviour, including the numbers, locations and effects of genes associated with behaviour, with the aim of understanding how complex behaviours are influenced by genetic variation. A recent transition in the study of animal behaviour has been in the interest of studying those behaviours that are correlated. When behaviours are correlated, there may be underlying genetic correlations that act as a constraint from performing the most optimal behaviour for the situation. Three traits that are often associated are aggression, boldness and stress responsiveness and it is useful to study these together and separately in order to establish whether genetic correlations exist. Stress, and the related behaviours aggression and boldness, have a significant bearing on the welfare of rainbow trout in aquaculture and are important when considering breeding programmes and rearing conditions for fish farming. These studies aimed to assess the genomic complexity of the evolutionarily important traits, stress responsiveness, aggression and boldness, including the genomic links between behaviours, so as to provide empirical evidence for underlying mechanisms of behavioural syndromes. In addition, this study aimed to identify candidate genes associated with stress and aggression using novel genomic techniques. A combination of genomic approaches was used to understand genomic components of animal behaviour, under the wider context of a behavioural syndrome, where behaviours are correlated across context. The association between heterozygosity and stress responsiveness and the related behaviours was tested to determine genomic influences on behaviour. The number and genomic locations of regions associated with stress responsiveness were determined to understand the level of complexity associated with stress. Using next generation sequencing, the transcriptomes of aggressive and less aggressive fish were characterised, with the aim of identifying the numbers of genes associated with aggressiveness. Crucially, novel candidates for the study of individual and correlated behaviours were identified. Moreover, candidate genes were studied in relation to both stress responsiveness and aggressive behaviour. The results showed that genetic diversity was linked with aggression but not stress responsiveness or boldness. Moreover, genome-wide heterozygosity, rather than heterozygosity at single loci, appeared to be associated with aggressiveness. Similarly, genome regions potentially associated with stress responsiveness were located across the genome. Genomic control of behaviour was

complex, where many genes were associated with aggressive behaviour and these effects may interact with the effects of other genes. Furthermore, I showed that novel applications of techniques can yield novel candidates for behaviour, where I identified genome regions that are potentially associated with stress responsiveness and candidate genes associated with aggression using a transcriptome, including unidentified sequences. These results highlighted the complex mechanisms that regulate apparently correlated behaviours. To further the study of behavioural genomics, the impact of environmental conditions and previous experience to investigate non-genomic effects should be considered. Moreover, there may be regulatory systems and pathways that interact both at the genetic and environmental level, which may be studied with the use of next generational genomic tools. These findings may be relevant for better understanding the effects of stress and the related behaviours aggression and boldness in rainbow trout in aquaculture. By determining novel candidates for these traits, it may be possible to use these as biomarkers for the early detection of negative effects or to aid marker-assisted breeding programs.

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Declaration of originality

I declare that all the work presented in this thesis is my own original research with the following acknowledgements:

Chapter 2: The rainbow trout samples used to assess boldness and stress responsiveness and the associated behavioural data were collected by Jack Thomson and are also presented in Thomson *et al.* (2011).

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Abbreviations

ACTH	Adrenocorticotrophic hormone
AVP	Arginine vasopressin
AVT	Arginine vasotocin
CRH	Corticotrophic releasing hormone
EPD	Ependymin
EST	Expressed sequence tag
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GR	Glucocorticoid receptor
ID	Identity disequilibrium
LD	Linkage disequilibrium
HFC	Heterozygosity-fitness correlation
HHC	Heterozygosity-heterozygosity correlation
HPA	Hypothalamic-pituitary-adrenal axis
HPI	Hypothalamic-pituitary-interrenal axis
HR	High responding line
LR	Low responding line
MR	Mineralocorticoid receptor
$P450_{SCC}$	Cytochrome P450 side-chain cleavage enzyme
PHt	Proportion of heterozygous loci
QTL	Quantitative trait loci
StAR	Steroidogenic acute regulatory protein
US	Unselected line
V1a	Vasotocin receptor 1a
VACM-1	Vasopressin-activated calcium-mobilizing receptor
GO	Gene Ontology

Contents

Chapter 1 General Introduction

Behavioural phenotypes are under many influences from the external and internal environments. Animals can alter their behaviour depending on previous experiences (Bendesky & Bargmann 2011). Moreover, behaviour shows plasticity in the face of differing environmental cues (Boake *et al.* 2002), such as a change in risk-taking behaviour in the presence and absence of a predator in rainbow trout, *Oncorhynchus mykiss* (Vilhunen *et al.* 2008; Thomson *et al.* 2012). Until recently, these non-genetic influences on behaviour led to a belief that the genetic component of behaviour was minimal, as described by the "phenotypic gambit" (Boake *et al.* 2002). Gradually, evidence demonstrating the influences of genes upon behaviour has mounted in a wide range of studies from family resemblance to gene knockouts to microarrays (Section *1.1*). Now, the major challenge in behavioural ecology is to investigate the genomic architecture of behaviour, including the numbers, locations and effects of all genes associated with behaviour, with the aim of understanding how complex phenotypes are influenced by genetic variation intraspecifically.

1.1 Genomics of behaviour

Despite strong environmental and experiential influences, the study of the genomics of behaviour is vital to understand the underlying propensities for a suite of behaviours that individuals possess due the large numbers of genes and networks associated with behaviour. A first step in demonstrating genetic components in behaviour is to investigate heritability. For example, aggression in great tits, *Parus major*, was heritable (h^2=0.247) (Drent *et al.* 2003), as were the correlated behaviours boldness and aggression in three-spined sticklebacks, *Gasterosteus aculeatus*, ($0.011<h^2<0.837$) (Bell 2005). Whilst determining heritability is useful to reveal a genetic component, it cannot determine the effects of specific genes or pathways involved in the underlying mechanisms. Therefore the actions of particular genes in the expression of behaviour have been demonstrated using gene knockouts. For example, in *Drosophila melanogaster,* learning has been linked with the *dunce* and *rutabaga* genes (Anholt & Mackay 2004). In mice, *Mus musculus*, a mutation causing an absence of the arginine vasopressin receptor 1b (AVPr1b) reduces aggressive behaviour (Stevenson & Caldwell 2012). Studying single knockout or antisense genes, can give useful information about behaviour, although a single gene rarely controls a specific behaviour entirely. For example, the absence of a functional AVPr1b does not cause an absence of aggressive behaviour, only reduces the number of attacks towards a conspecific, implicating many genes in complex traits. The combined results from many candidate gene studies have made it apparent that behaviour is under the influence of more than one gene

(Greenspan 1997). Indeed, mammalian studies have shown that behaviour is the result of interactions among gene products in networks (Kinnally *et al.* 2010). For example in Rhesus macaques, *Macaca mulatta*, serotonin modulates response to an intruder, which is in turn modulated by monoamine oxidase A (Kinnally *et al.* 2010). Interactions among genes may occur among genotypes, where behaviour is influenced by heterozygous effects, such as dominance, or additive effects of alleles. Moreover, more dynamic gene interactions may occur between transcripts (Mackay 2001). The challenge for the study of behavioural genetics is to identify this genomic complexity to explain phenotypic variation.

The expression of almost all behaviours is often the result of the effects of many genes. It is thus important to study the genomic architecture of behaviour, including the number and location of loci, dominance effects of multiple alleles at a locus, additive effects of alleles (Vallejo *et al.* 2009), pleiotropy (where a gene has effects on many traits) or epistasis (where the effect of a gene is modulated by at least one other gene). Many types of study can be used to determine the numbers and locations of genes in complex traits. For example, assessing the level of variation in genome-wide heterozygosity may indicate local effects of single genes or general effects due to inbreeding. Heterozygosity is often related to fitness traits due to a reduction in the expression of deleterious recessive alleles (dominance) or heterozygote advantage (overdominance) (Slate *et al.* 2004). When fitness-related traits are correlated with heterozygosity, this relationship may be caused by two effects when neutral markers are used (Hansson & Westerberg 2002). The first is the local effect hypothesis, where neutral loci are in linkage disequilibrium (LD) with one or more fitness genes. The second is the general effect hypothesis, where the level of heterozygosity across a large set of neutral markers is generally correlated across loci within an individual's genome (ID), where the heterozygosity at neutral markers is thought to represent genome-wide heterozygosity due to inbreeding (Weir & Cockerham 1973; Szulkin *et al.* 2010) (for more details, see Chapter 2). For example, Tiira *et al.* (2003) showed that aggressive behaviour in landlocked salmon, *Salmo salar,* was affected by genome-wide heterozygosity (estimated at 11 neutral markers), indicating that the effects of heterozygosity are associated with aggressive behaviour. However, single locus effects were detected in dominant brown trout, *Salmo trutta,* where two genomic regions showed a particular genetic influence (Tiira *et al.* 2006). Moreover, this approach may indicate how genotypic effects influence behaviour. Thus, these effects may affect a phenotype associated with heterozygosity.

Another method for identifying numbers and locations of genes is to use quantitative trait loci (QTL) mapping, which allow one to find genotypic information about the physical chromosomal location of genes involved in a particular phenotype (Solberg *et al.* 2006;

Drew *et al.* 2007) and this may increase understanding of the networks involved in behavioural control. QTLs are a set of loci that attempt to explain genetic control of a quantitative trait that is under complex control, such as behaviour. Identifying QTLs typically involves breeding animals that exhibit a phenotypic trait. By back-crossing these individuals the location of polymorphic neutral markers may be statistically mapped (Jones *et al.* 1997). For example, the control of defensive behaviour in the honeybee, *Apis melifera*, was found to be associated with 13 genomic regions (Lobo *et al.* 2003) using this method. However, this approach may be time-consuming due to the extensive breeding programs involved. Genome scans use neutral molecular markers to detect genetic differentiation among populations to identify genome regions under natural selection, which can be used to infer adaptation to various situations (Nielsen 2005; Oleksyk *et al.* 2010). This may offer an alternative to QTL mapping for identifying candidate genes. By detecting genetic differentiation among groups or populations of animals, it may be possible to identify regions of the genome as candidates for a particular trait (Chapter 3). For example, approximately 40 physiologically important genes were under selection between groups of three-spined stickleback, *Gasterosteus aculeatus,* adapted to differing environmental salinity (Shimada *et al.* 2011). Selection for different types of behaviour among populations thus presents an opportunity to identify regions associated with the genetic basis of behavioural traits – a method of identifying novel candidate genes (Kayser *et al.* 2003; Kane & Rieseberg 2007).

Heterozygosity and genome scans are highly useful for characterising numbers and locations of genes with a role in phenotypic traits, where this can be applied to any fitness-related trait such as aggressive behaviour. Thus novel candidates and genotypic effects associated with phenotypic traits may be identified. These approaches may be built upon by using techniques that are able to characterise the full complement of genes with behavioural function and capture of the networks of gene interactions that regulate expression of behaviour. The use of microarrays can vastly increase the number of genes studied for a particular behaviour. By assessing gene expression involved in a behaviour, and where the array contains the appropriate genes, new interactions can be identified. For example, microarrays were used to identify candidate genes for studying alcohol-induced behaviour in mice (Rulten *et al.* 2006). Two particular genes, among a number of others, were found to be down-regulated in mice carrying out "ethanol-related behaviours". These two genes can be used in further studies to explore genes that affect alcohol-induced behaviour. In other studies, microarrays have identified 266 genes associated with aggression in *Drosophila* (Edwards *et al.* 2009a). Moreover 1165 genes differed between fish of different social status (Sneddon *et al.* 2005). This shows the sheer number of genes with a behavioural role, which

may show pleiotropic or epistatic effects. Microarrays have cDNA probes or oligonucleotides fixed to a slide to which cDNA from samples bind, which can be visualised by fluorescence (Hoheisel 1997). This means that only known sequences can be probed, making it difficult to study gene expression of non-model organisms using microarrays. Next generation sequencing technology (NGS), such as the GS FLX 454 sequencer, makes it possible to sequence all transcripts in a sample irrespective of whether sequences are known or not (Wicker *et al.* 2006) and this could allow a more complete view of the genetic control of behaviour.

Next generation sequencing allows the *de novo* identification of novel candidate sequences without *a priori* knowledge of function (Wilhelm & Landry 2009). This has led to enterprising studies that discover candidate genes associated with complex phenotypic traits, such as the ecophysiological response to salinity (Lowe *et al.* 2011), birdsong (Lovell *et al.* 2008) and dominance (Sneddon *et al.* 2011). In addition, because NGS can sequence all the mRNA from a sample, not only can known genes be associated with a novel phenotype, but previously unknown genes can be sequenced, which is particularly important for non-model organisms (Hudson 2008) and has been used to generate sequence data for many non-model organisms. Moreover, the sequencing of transcriptomes may allow the identification of many genes with interacting effects in a phenotype and allow mapping of networks of genes and their interactions by using changes in Gene Ontology – a system of categorising genes according to their function (Liinamo *et al.* 2007). NGS could be a powerful tool with which to tackle questions in behavioural ecology. However, examples of the technology's use in this way are rare and limited to invertebrates (Toth *et al.* 2007). Genomic study of behaviour is important to reveal the complex associations between genes and behaviour.

1.2 Animal behaviour

A recent transition in the study of animal behaviour has been in the interest of studying those behaviours that are correlated. When behaviours are correlated, there may be underlying genetic correlations that act as a constraint from performing the most optimal behaviour for the situation (Sih *et al.* 2004a). The interest in understanding the genetic basis of this complex association of behaviours is consequentially increasing. The behavioural and physiological correlation among behaviours has been termed temperament (Réale *et al.* 2007), personality (Wolf *et al.* 2007) and behavioural syndromes (Sih *et al.* 2004a; Sih *et al.* 2004b). Behavioural syndromes are defined as a suite of behaviours that are correlated across context (Sih *et al.* 2004a; Sih *et al.* 2004b) and describe individual variation of consistent behavioural types within a population or species. Behavioural syndromes have been shown in a diverse range of taxa, including mammals, birds, fish and squid

(Huntingford 1976; Drent *et al.* 2003; Sneddon 2003; Sinn *et al.* 2006; Beausoleil *et al.* 2008; Evans *et al.* 2010). Specifically, in sticklebacks, *Gasterosteus aculeatus*, that are aggressive in the context of intraspecific contests over territories also show risk-taking behaviour in the presence of a predator (Huntingford 1976). Whilst the level of aggression varies between contexts, some individuals are consistently more aggressive than others and this suggests a lack of behavioural plasticity (Conrad *et al.* 2011).

A specific example of behavioural syndromes is stress coping styles (Koolhaas *et al.* 1999; Koolhaas *et al.* 2007), which describes how an individual copes with an external stressor. The coping styles are usually distributed bimodally and the two extremes are termed proactive and reactive. These names describe the behavioural types that encompass the suite of correlated behavioural phenotypes, including aggressiveness, stress coping and routine formation. In rats, stress coping styles were determined by their reaction to a shock prod. Aggressive rats exhibited proactive burying of the prod and less-aggressive exhibited reactive avoidance of the prod (Benus *et al.* 1990). Further study also showed that aggressive males formed routines more easily (Koolhaas *et al.* 1999). There is evidence for coping styles in many vertebrates (Koolhaas *et al.* 2007), including in salmonids (Øverli *et al.* 2004; Øverli 2007). For example, Øverli *et al.* (2002b) showed that rainbow trout, *Oncorhynchus mykiss*, that resumed feeding sooner showed less activity in response to a stressor. Moreover, lines of rainbow trout bred for divergent plasma cortisol responses to stress (Pottinger *et al.* 1992; Pottinger & Carrick 1999) show behavioural differences in levels of aggression (Pottinger & Carrick 2001; Øverli *et al.* 2002b). Three traits that are often associated are aggression, boldness and stress responsiveness and it is useful to study these both together and separately in order to establish whether genetic correlations exist.

1.2.1 The stress response

The stress response is a set of physiological and behavioural responses that enable an individual to cope with abiotic or biotic change that challenge homeostasis, such as the introduction of a predator, or a sudden change in abiotic environment. In vertebrates, stress can be categorised into different levels of response, from alarm and resistance, in which the sympathetic nervous system and other stress-regulating systems are invoked, to adaptation and exhaustion, where homeostasis is threatened (as reviewed in Ellis *et al.* (2012) and Johnson *et al.* (1992)). The acute stress response can be adaptive by allowing an animal to maintain homeostasis through physiological and behavioural change, which is also termed allostasis (McEwen & Stellar 1993). However, if a stressor is prolonged or repeated, stress becomes chronic and the chronic response can cause deleterious effects that impact upon an individual's fitness: the physiological response may fail to retain homeostatic balance,

termed allostatic load. For example, chronic stress can cause impaired reproduction (Campbell *et al.* 1992), growth (Pickering 1990; Pottinger 2006) or immunocompetency (de Kloet *et al.* 2005). These effects are seen in wild populations where chronic stress may impact upon individual fitness due to environmental challenges. Similarly, deleterious effects are also common in domesticated animals, where stressors may arise from husbandry processes due to, for example, crowding or handling (Ellis *et al.* 2012) and this may impact upon welfare or economic return. For example, the impact of stress upon reproduction, growth and immunocompetency may affect number or quality of the animals or may leave livestock more susceptible to disease.

The neuroendocrine stress response is primarily controlled by the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar-Bonga 1997) (hypothalamic-pituitary-adrenal, HPA, in mammals), which regulates hydromineral balance and energy metabolism, along with the hypothalamic-sympathetic-chromaffin axis (HSC), which, via catecholamines, has a role in oxygen transport. Once a stressor is perceived, the HPI/HPA commences with the hypothalamus releasing corticotrophin-releasing hormone (CRH), which increases serotonin, dopamine (Øverli *et al.* 2005) and arginine vasotocin (AVT) (Gilchriest *et al.* 2001) in the brain. These substances stimulate the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary (Mommsen *et al.* 1999) into the blood stream where ACTH stimulates the interrenal cells (adrenal gland in mammals) to release glucocorticoids into the bloodstream. Elevated levels of glucocorticoids, specifically cortisol in teleost fish and corticosterone in mammals and birds, act on the liver to facilitate the release of glucose, via gluconeogenesis, into the bloodstream. Glucocorticoids also regulate their own release by negative feedback (Wendelaar-Bonga 1997).

An understanding of the genetic basis of the stress response is rapidly advancing and many studies investigate the action of a single candidate gene, such as vasotocin (Gilchriest *et al.* 2000), serotonin transporters (Kinnally *et al.* 2010) and carbonic anhydrase in salinity stress (Pongsomboon *et al.* 2009). Studies often test stress responsiveness by investigating gene expression of a small subset of genes associated with ecologically relevant stressors (Chapter 6). For example, proopiomelanocortin (POMC) was upregulated in channel catfish, *Ictalurus punctatus*, after low water stress (Karsi *et al.* 2005), steroidogenic acute regulatory protein (StAR) showed no change after high water flow in white sturgeon, *Acipenser transmontanus* (Kusakabe *et al.* 2009) and five genes were investigated in piglets (*Sus scrofa*) after social isolation (Kanitz *et al.* 2009). Other studies focus on QTLs associated with stress in order to identify genetic loci associated with stress (Ahmadiyeh *et al.* 2005; Solberg *et al.* 2006; Drew *et al.* 2007). More recently, microarrays have allowed gene

expression profiling on a large scale that is associated with a range of stressors, for example, cold stress in carp, *Cyprinus carpio* (Gracey *et al.* 2004) and handling stress in rainbow trout (Krasnov *et al.* 2005).

1.2.2 Aggressive behaviour

Animal interactions can result in mutually beneficial outcomes, which is termed cooperation, or in one individual receiving benefit at the cost of another, termed conflict. Where resources are limited, conflict arises to decide which individual obtains resources, such as food. Conflict over resources is manifest in avoidance, manipulation and physical coercion, the latter is also called aggression. Aggression differs from other forms of conflict due to deliberate infliction of injury via physical threat or attack, whilst other forms of conflict result in gaining resources and removal of a competitor without intent of injury. Aggression encompasses both physical display and the internal physiological and emotional states that accompany attack, which determine intent (reviewed in Huntingford & Turner (1987)) and is used by conspecifics to engage in physical conflict. Aggression can be adaptive by increasing fitness. For example, the protection of offspring leads to reproductive success, whereas obtaining a territory may mean increased survival (through access to food) or increased reproduction (through access to mates) (Maxson & Canastar 2005). However, aggression may also be maladaptive by increasing of injury or death, which impairs reproductive potential and survival. Moreover, individuals with a tendency for aggressive behaviour may also direct their aggression towards offspring, diminishing their reproductive success, or towards predators, increasing their risk of death. Populations show variation in aggressive behaviour, but both aggressive and non-aggressive behaviours persist in populations, meaning both behaviours are effective survival strategies.

The physiological control of aggression in vertebrates is well-documented and exhibits a range of biochemical changes, including those that show similarities with the physiology of the stress response. In the vertebrate brain, neurotransmitters that have general effects on behaviour, such as noradrenaline, dopamine and acetylcholine cause increased aggression, whilst serotonin inhibits (Huntingford & Turner 1987). More specifically, hormones that are involved in stress affect aggressive behaviour. Since agonistic encounters are stressful for an individual, it follows that similar endocrine responses are seen. Indeed, genes associated with the HPI axis are implicated. POMC is a precursor to ACTH, α-melanocyte stimulating hormone (α-MSH) and β-endorphin, all of which are implicated in stress-responsiveness (Wendelaar-Bonga 1997) and aggression (Höglund *et al.* 2000). ACTH (Kruk 1991; Veenema *et al.* 2003), glucocorticoids (Gammie *et al.* 2007; Øverli 2007) and their receptors

(Schjolden *et al.* 2009) are seen to increase in response to an encounter. Moreover, there are differing responses between dominant and subordinate animals, where subordinates usually exhibit elevated glucocorticoid levels for longer (Sloman *et al.* 2001). Whilst the physiological control of aggression is quite well-known, it is also complex and depends upon both the previous experiences of the animal, motivation to engage in aggression, resource value and upon environmental conditions. Indeed, these factors affect the decision-making process of an animal during an aggressive interaction (Tinbergen 1951).

As an evolutionarily and economically important behaviour, there is much interest in understanding the genetic mechanisms underlying this complex phenotype. Indeed, phenotypic differences in behaviour have been attributed to differences in candidate genes. For example, different genotypes for MHC Ia locus in rainbow trout, *Oncorhynchus mykiss*, confer differences in aggressive behaviour, where aggressive and less aggressive are homozygous for different alleles and intermediate behavioural types are heterozygous (Azuma *et al.* 2005). Moreover, the relationship between genotype and gene transcripts has been studied for candidate genes. For example, reduced transcript expression of the candidate gene tryptophan hydroxylase 2, which is involved in the synthesis of serotonin, was related to a gene mutant for this gene in mice, *Mus musculus* (Kulikov *et al.* 2005). Indeed, gene expression of single candidate genes which are related to aggressive behaviour have been well-studied in many taxa, including *Drosophila* (Simon & Krantz 2007), domestic dogs, *Canis lupus familiaris* (Van Den Berg *et al.* 2008) and other mammals (Popova 2008). Particularly well-studied are the gene expression neurotransmitters, including serotonin (Mosienko *et al.* 2012), its transporters (Naumenko *et al.* 2009) and metabolites (Craig 1994; Hashizume *et al.* 2003), vasopressin/vasotocin and its receptors (Gilchriest *et al.* 2001; Liu *et al.* 2001; Albers *et al.* 2006; Caldwell *et al.* 2008).

Indeed, many candidate genes are implicated in aggressive behaviour, such as the elevation of POMC in subordinate rainbow trout (Winberg & Lepage 1998). However, it is becoming more evident that complex phenotypes are under the control of many genes (Robinson 2004; Robin *et al.* 2007). Edwards *et al.* (2009b) showed that single nucleotide polymorphisms (SNPs) in many genes mediated variation in aggressive behaviour and that these genes were differentially expressed in aggressive and less aggressive flies. More recently it has been possible to study the changes in many genes (Pavlidis *et al.* 2011) or a number of tissues (Jeffrey *et al.* 2012). Advances in genetic techniques have allowed the study of transcriptomes in aggressive behaviour in invertebrates (Alaux *et al.* 2009; Edwards *et al.* 2009a) and this has only recently been extended to vertebrates (Gammie *et al.* 2007; Renn *et al.* 2008). For example, over one thousand genes were differentially expressed in dominant,

subdominant and subordinate rainbow trout (Sneddon *et al.* 2011), which shows a complex genetic association with aggressive behaviour.

1.2.3 Bold-shy continuum

Boldness is a trait often studied in behavioural syndromes/temperament and is a trait related with aggressive behaviour (Wilson *et al.* 1993). Boldness was often used to describe any risk-taking behaviour, including reactions to novelty (Wilson *et al.* 1993; Sneddon 2003) and this is the definition used here, although more recently, boldness has been defined to encompass risk-taking behaviour only, and exploration to deal with novelty (Réale *et al.* 2007; Conrad *et al.* 2011). The fitness consequences of boldness are unclear, since some examples indicate that bold behaviour is advantageous. For example, female Trinidadian guppies, *Poecilia reticulata*, chose bold male guppies as mates irrespective of their colour (Godin & Dugatkin 1996). However, other examples indicate boldness confers a disadvantage. For example, bold female mosquitofish, *Gambusia holbrooki*, were smaller and had lower fecundity than shy mosquitofish (Wilson *et al.* 2010). These contrasting examples along with others that show trade-offs, such as Agamas, *Agama planiceps*, that feed more but experience higher injury from predators (Carter *et al.* 2010) are consistent with boldness being an optimal strategy or showing frequency-dependent fitness (Wilson *et al.* 1994; Bell 2009). Indeed the ecology of boldness may be species- or population-specific and it may show fitness advantages in particular contexts. Alternatively, boldness may be physiologically constrained and thus be exhibited due to behavioural syndromes, although evidence for this is weak (Decker & Griffen 2012; Garamszegi *et al.* 2012). Indeed, boldness shows consistency with stress coping styles, since bold rainbow trout, *O. mykiss*, take more risks and learn a conditioned response quicker (Sneddon 2003).

Risk-taking behaviour is often specific to a predation context, but can also include aggression towards a conspecific (Bell 2009). Boldness, in the broader sense, may produce benefits for an individual. For example, boldness in the presence of a predator may seem maladaptive, but may allow protection of offspring. Furthermore, the relationship between boldness and other behaviours may place physiological constraints upon the degree of boldness. For example, Huntingford (1976), found that bold behaviour towards predators was correlated with bold behaviour towards conspecifics and she suggested this may be due to similar effects of intruder stimuli, regardless of species. Moreover, boldness is often related to other fitness enhancing behaviours such as dominance (Sundström *et al.* 2004), learning (Sneddon 2003) and mate attraction (Dugatkin & Wilson 1993). Whilst risk-taking may affect survival when related to predator avoidance, novelty inspection may enable an individual to find new habitats or food sources (Budaev 1997; Réale *et al.* 2007). Indeed,

obtaining food may benefit an individual despite presence of predators (Wilson *et al.* 1994), particularly if the individual experiences reduced food availability (Thomson *et al.* 2012). Relatively few studies linking boldness with physiological parameters have been conducted although evidence suggests the physiology of boldness differs from the physiology of other behaviours, for example, in collared flycatchers, *Ficedula albicollis*, cortisol was not correlated between boldness and stress coping style (Garamszegi *et al.* 2012). Similarly, plasma cortisol, an indicator of stress responsiveness, was not linked to boldness (Thomson *et al.* 2011).

Genetic and genomic studies associated with boldness are not numerous but they do indicate some level of genetic control. Many vertebrates exhibit a bold-shy continuum and this is often heritable. For example, poeciliid fish, *Brachyraphis episcopi*, exhibit family resemblance in boldness, (Brown *et al.* 2007). Furthermore, three-spined sticklebacks, *Gasterosteus aculeatus* show population differences in heritability of boldness ($0.011<h^2<0.837$) (Bell 2005; Dingemanse *et al.* 2009). In addition, bighorn sheep, *Ovis Canadensis* show weak heritability ($h^2=0.21$) (Réale *et al.* 2000), as do domestic dogs, *Canis familiaris* show similar levels ($h^2=0.27$) (Strandberg *et al.* 2005). These differences show an often weak effect of heritability upon boldness, indicating complex influencing factors. For example, in brown trout, *Salmo trutta*, genetic diversity only relates to bold behaviour when it is assessed in the presence of a predator (Vilhunen *et al.* 2008). This context-dependent association with genetics is apparent in great tits, *Parus major*, where birds that possess different SNPs for the dopamine receptor gene DRD4 also exhibit differences in exploratory behaviour, where the effect of the gene is weak ($0.045<r^2<0.058$) and these differences are seen in some populations but not others (Korsten *et al.* 2010). Behavioural differences are also seen in rainbow trout that possess different combinations of two MHC alleles (Azuma *et al.* 2005). Few studies have assessed gene expression associated with boldness and the results appear to support context-dependence genetic associations (Thomson *et al.* 2012). Current knowledge in the complex traits aggression, boldness and stress responsiveness, shows correlations of behaviours across contexts, along with complex physiological control. Moreover, genetic studies are beginning to implicate complex pathways and novel candidates with a role in behaviour. By using multiple genomic approaches, the complexities underlying these behaviours and the correlations between them, may be revealed.

1.3 The rainbow trout model

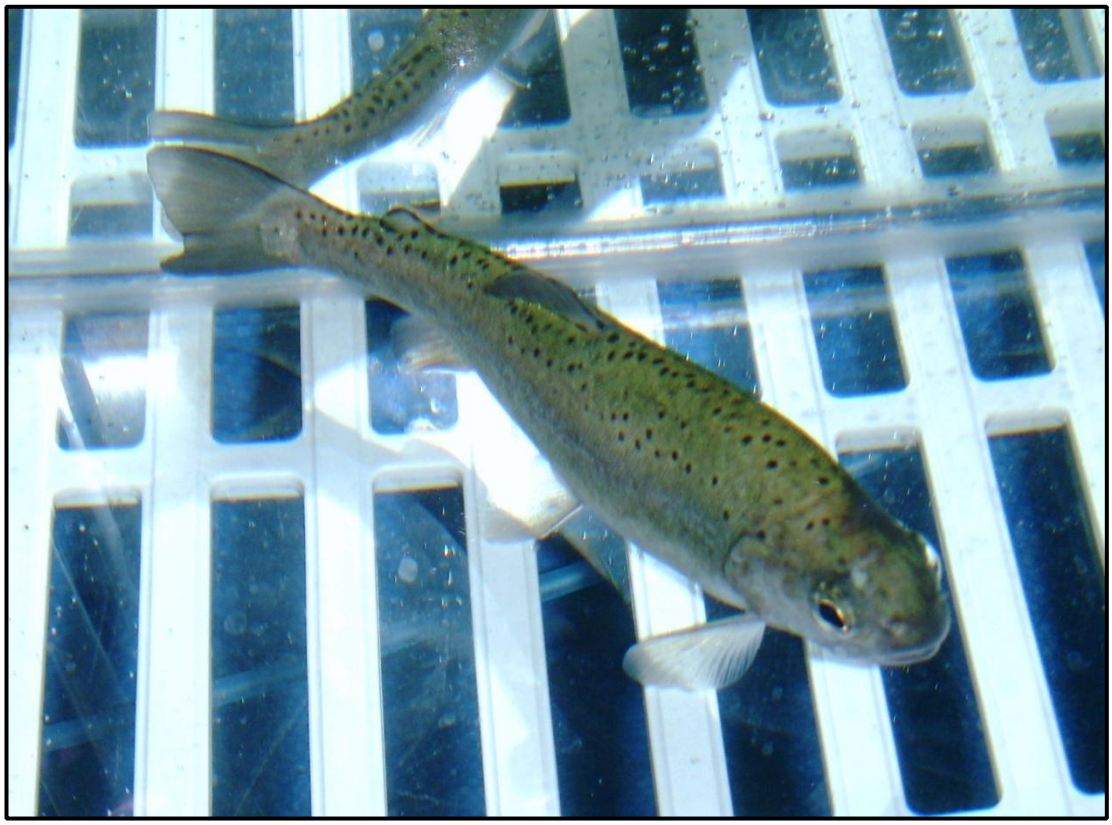

Figure 1.1. Rainbow trout (*Oncorhynchus mykiss*) in a tank in the Liverpool aquarium.

Rainbow trout (*Oncorhynchus mykiss;* Figure 1.1) from the family Salmonidae are related to trout, salmon, particularly of the *Salmo* and *Oncorhynchus* genera, chars, freshwater whitefishes and graylings. *Oncorhynchus* species are native to north America, China and Siberia, where *O. mykiss* occupies freshwater rivers and lakes north of California on the east of the Pacific (Crisp 2000). In addition, some rainbow trout (referred to as steelhead trout) are anadromous and migrate to sea; however this thesis is concerned with those that remain in fresh water during their entire lives. In the 19th century, Victorian anglers transported the American rainbow trout to Europe, including the UK (Sutterby *et al.* 1990). This was successful in rainbow trout, as opposed to other *Oncorhynchus* species due to easy propagation and fast growth. Both in their natural and introduced environments, rainbow trout, along with other salmonids, represent an important economical asset. Popularity for sport-fishing and for the food industry has driven the creation of trout farms (Crisp 2000; Pounder Pers. Comms.). Whilst aquaculture provides a managed system of renewable fish stocks, procedures during the farming of trout can increase stress (introduced below). It is

thus important to understand the underlying causes of stress responsiveness and related behaviours.

Rainbow trout, *Oncorhynchus mykiss,* are a popular farmed species and, as such, are an important aquaculture model for salmonids. Trout in aquaculture are well-studied for aggression, boldness and stress responsiveness for three key reasons. First, animals face stressors in the form of transport, netting and human handling (Zuberi *et al.* 2011). Second, stressors may be in the form of social stress due to crowding from high stocking densities. Third, animals may encounter different types of novel objects, environments or situations. Rainbow trout are highly tractable for studies in aggression due to territorial behaviour and readily engage in contests. Moreover, they show a profound divergence in bold behaviour and have been studied in relation to behavioural syndromes or stress coping styles. Importantly, lines have been selected for divergent responses to stress, which allow easy study of stress responses and related traits. By studying the genomic relationship with these related behaviours, it may be possible to identify genomic complexity associated with economically important behaviours for hatchery trout. Moreover, it may be possible to draw conclusions for natural populations, where these traits are evolutionarily important for survival.

Stress is a major problem in aquaculture due to the adverse effects (Section *1.2.1*) of prolonged or repeated stress that can be caused by routine procedures. To investigate stress response in farmed fish, two lines of rainbow trout were selectively bred for divergent plasma cortisol response to stress (Pottinger *et al.* 1992; Pottinger & Carrick 1999). Fish that had the highest cortisol response to a standard stressor were bred with each other to form the high responding (HR) line and fish that had the lowest cortisol response formed the low responding (LR) line (Pottinger & Carrick 1999). Briefly, 250 rainbow trout (Stirling strain) were subjected to confinement stress in 50L water for 3h with 6-7 other fish. Plasma cortisol was measured to determine the strength of the response. The four highest responding (HR) and four lowest responding (LR) from each of 6 stock tanks were used to breed 24 HR and 24 LR groups of the next generation. 15 HR families and 14 LR families were used to breed subsequent generations in a similar way, where the highest and lowest responding individuals were bred. F4, the generation used in the current study, was bred from any F3 HR fish, as opposed to only the highest responding to derive the new HR group and any F3 LR fish to derive the new LR group. The cortisol response to stress was found to be heritable during this breeding program ($0.41<h^2<0.73$).

The HR and LR lines show many behavioural and physiological differences and these have been equated to differences in stress coping style (Øverli *et al.* 2005; Øverli 2007). LR fish are more often dominant than HR (Pottinger & Carrick 2001), which links to plasma cortisol levels, since in rainbow trout hierarchies, the dominant fish is the least stressed, whereas the subdominant is the most stressed (Sloman & Armstrong 2002), as measured by plasma cortisol concentrations. Furthermore, LR fish resume feeding after stress-induced anorexia sooner than HR fish and show a lower amount of activity in the presence of an intruder (Øverli *et al.* 2002b) and retain a conditioned response longer than do HR fish (Moreira *et al.* 2004). There were unexpectedly no differences in boldness between lines (Thomson *et al.* 2011), but they differ in behaviour when challenged in novel environments as opposed to in home tanks (Schjolden *et al.* 2005). This indicates a level of plasticity in boldness, which may be associated with transcriptional gene effects

The cortisol stress response is a heritable trait in rainbow trout (Fevolden *et al.* 1999; Pottinger & Carrick 1999) and is well-studied in terms of gene expression: the expression of single genes or subsets of genes show clear evidence of transcriptional changes of key genes in response to many types of stress. For example, mineralocorticoid receptor in interrenal tissue is upregulated in response to simulated stress by ACTH exposure (Aluru & Vijayan 2008). Thomson *et al*. (2011) show differences in relative expression of a number of genes between HR and LR trout. Microarrays enable the assessment of transcriptional changes of many genes and this has been employed to investigate various stressors in trout. For example, the transcriptome response in the brain, kidney (Krasnov *et al.* 2005) and liver (Wiseman *et al.* 2007) to handling stress; the response of seven tissue types after low water stress (Momoda *et al.* 2007); the response of the heart to changing temperatures (Vornanen *et al.* 2005). In addition, multiple genes, or quantitative trait loci (QTLs), have been located to regions of the trout genome that are associated with the cortisol response to stress, both in the rainbow trout lines (Quillet *et al.* 2010) and in hatchery trout (Drew *et al.* 2007).

Although the physiological mechanisms and genetics of aggression in mammals are well-documented, in rainbow trout, genetics and transcriptional changes of aggression are less well-studied and these are mostly limited to the expression of a few genes (Winberg & Lepage 1998; Gilchriest *et al.* 2001; Jeffrey *et al.* 2012) and a microarray of trout from a stable hierarchy (Sneddon *et al.* 2011). Similarly, few studies have attempted to identify the genetic basis of boldness in rainbow trout, but results show that different MHC Ia alleles are related to differences in behaviour (Azuma *et al.* 2005). Moreover, whilst no differences in the expression of candidate genes were seen in rainbow trout tested for boldness by presentation of a novel object (Thomson *et al.* 2011), differences in gene expression were

seen in trout exposed to differing levels of predation threat (Thomson *et al.* 2012). As such, the genetic components of boldness are unclear.

Though the rainbow trout genome has not yet been sequenced, due to difficulties arising from genome duplication, behavioural genetics of the rainbow trout is facilitated by the availability of a number of genomic tools. These include microsatellite linkage maps (Young *et al.* 1998; Sakamoto *et al.* 2000; Nichols *et al.* 2003; Guyomard *et al.* 2006; Rexroad *et al.* 2008). The most recent linkage map (Rexroad *et al.* 2008) comprises 1124 microsatellites and has comparative homology with zebrafish, *Danio rerio*, and a number of other teleost fish. A physical BAC map (Palti *et al.* 2009) allows fine scale mapping and identification of candidate gene positions, although the positions of markers are still being added. Moreover many of the contigs in this map require sequencing to allow further integration with the microsatellite maps. There are also a number of expressed sequence tags (ESTs) present in the SalmonDB database (Di Génova *et al.* 2011), which incorporates EST sequences from *Salmo salar, Oncorhynchus mykiss*, along with the whole genome sequences of *Danio rerio, Gasterosteus aculeatus, Tetraodon nigroviridis, Oryzias latipes* and *Takifugu rubripes*. Transcriptomes can give useful sequence information for non-model organisms, particularly those with large genomes and next generation sequencing technology allows this (Salem *et al.* 2010). Despite the status of rainbow trout as a non-model organism, important evolutionary and ecological questions can be answered using these tools. Molecular markers, for example can be used to determine differences between individuals or populations. Moreover, next generation sequencing can be used to identify differences in *de novo* gene expression among individuals. These tools can thus be used to determine genomic complexity associated with phenotypic traits, such as behaviour.

1.4 Aims

Stress and the related behaviours aggression and boldness have a significant bearing on the welfare of rainbow trout in aquaculture and are important when considering breeding programmes and rearing conditions for fish farming. In natural populations, it is clear that behaviour has evolutionary fitness implications. It is thus important to discover genomic factors that affect behaviour. Complex phenotypic traits are often under the control of many genes and a major undertaking is to understand the genomic architecture affecting complex traits. The challenge for the study of behaviour is to identify the numbers and genomic locations of genes involved in behavioural traits and to recognise single genes as components of pathways and networks, rather than acting solely. Moreover, by studying related behavioural and physiological traits, it may be possible to understand genetic mechanisms that underlie correlations. This thesis will describe some of the complex genomic

architecture of stress responsiveness and related behaviours. Genomic relationships with correlated behaviours will be characterised, the locations of genome regions and the numbers of genes associated with behavioural traits will be determined. Crucially, novel candidates for the study of individual and correlated behaviours will be identified. Moreover, candidates will be studied in relation to specific behaviours. Consequentially, it may be possible to comprehend the complex nature of the genomic factors underlying correlations between the evolutionarily and economically important traits: stress responsiveness and the related behaviours, aggression and boldness.

1.5 Chapter outlines

Chapter 2: Heterozygosity of three behaviours

It is unclear whether the relationship between genome-wide heterozygosity, which is often correlated with fitness traits, and the evolutionary and economically important behavioural traits stress responsiveness, aggression and boldness exists. Moreover, little work has been conducted to examine genetic links between correlated behaviour. By assessing genome-wide genetic variation, aggression, boldness and stress responsiveness, I can determine genomic influences on behaviours and show whether there are similarities in the patterns of heterozygosity among behaviours. Under the hypothesis that aggression is related to fitness, it would be expected that more aggressive individuals will be more heterozygous and as boldness and stress responsiveness relates to aggressiveness, it is also expected that LR and bolder individuals will be more diverse.

Chapter 3: Genome scan of selective breeding for stress responsiveness

The number and location of genome regions associated with stress responsiveness is unknown. I will assess genetic differentiation between lines of trout selectively bred for divergent responses to stress to identify neutral markers that diverge between the lines, which may be indicators of selection. Knowledge of the locations of the genetic markers provides an opportunity to identify (and therefore enumerate) candidate regions that are associated with the selective breeding program for stress. Since the stress response is under complex control, I expect genetic differences between the lines to emerge at widespread locations.

Chapter 4: *De novo* sequencing of aggressive behaviour

The known candidate genes for aggressive behaviour are likely to explain a small proportion of genetic variance and the full complement of genes involved in this complex behaviour has not been sequenced *de novo.* By sequencing the transcriptome of aggressive behaviour, it may be possible to identify the number and identity of differentially expressed genes

between behavioural types. Moreover, novel candidates may be identified using this approach. Since there are complex external cues associated with social behaviour, such as those specific to an opponent, I expect many genes to be differentially expressed between aggressive and less-aggressive trout.

Chapter 5: Changes in transcripts after aggressive interactions

Little is known about the transcriptional changes that occur during recovery from social stress or aggressive interactions. By tracking changes in genes in the brain, it may be possible to understand how winners and losers differentially modulate their responses during recovery from social stress and to determine the role of candidate genes in aggressive interactions. I expect winners and losers to show differential expression in four candidate genes, due to known differences in physiology between dominant and subordinate trout. In addition, I expect that initial changes will be followed by a recovery to control levels.

Chapter 6: Transcript changes in response to an acute stressor

The transcriptional changes of key candidate genes in an axis in multiple tissues in response to an acute stressor have not been studied. The main aim of this chapter is to quantify transcriptional changes of key genes in the HPI axis in the 24 hours following a standard acute stressor, in the brain, head kidney and liver. I expect a distinct change in transcription immediately following the stressor with a subsequent recovery to control levels.

Chapter 2 Is genome-wide heterozygosity related to the evolutionarily important traits aggression, boldness and stress responsiveness?

2.1 Introduction

Behavioural traits form complex phenotypes whose expression represents the response to a variety of influences, including genotype, external environment, emotional state and previous experience (Boake *et al.* 2002; Bendesky & Bargmann 2011). A long-standing and major challenge in evolutionary biology is to understand the associations between genotype and phenotypes, with a recent emphasis on determining the role of genes upon apparently complex behaviours. Currently, many studies focus on the effect that polymorphisms in specific candidate genes have upon behaviour, such as variation in the dopamine receptor D4 (DRD4), which is associated with novelty seeking in great tits, *Parus major* (Fidler *et al.* 2007). Another example is differences in the major histocompatibility complex (MHC) class Ia among rainbow trout, *Oncorhynchus mykiss*, that exhibit correlated behaviour: trout that were homozygous for one genotype were bold and aggressive whereas trout with an alternate homozygous genotype were shy and passive and heterozygous trout had intermediate levels of boldness (Azuma *et al.* 2005). However, there are several problems with this approach. First, the expression of complex phenotypic traits is only rarely determined by the action of one or few specific genes, and many studies have highlighted a wider role of the effect of variation in the amount of genetic diversity (heterozygosity) among individuals in determining the expression of certain behaviours. Second, such genetic correlates of behaviour should examine their contribution to correlated behavioural traits. Certain behavioural traits confer fitness upon an individual. For example, aggression increases an animal's competitive ability and thus allows it to obtain mates (Meagher *et al.* 2000). Moreover, heterozygosity is related to many fitness traits (Kempenaers 2007). By examining the variation in behavioural traits alongside the variation in heterozygosity, it may be possible to determine how genome-wide heterozygosity affects the expression of behaviour.

2.1.1 Heterozygosity-fitness-correlations

Genome-wide heterozygosity is a robust representation of genetic diversity, measured as a proportion of heterozygous loci within an individual. Heterozygosity is often related to fitness traits due a reduction in the expression of deleterious recessive alleles (dominance) or heterozygote advantage (overdominance) (Slate *et al.* 2004). When a fitness-related trait is associated with heterozygosity, it is termed a heterozygosity-fitness-correlation (HFC) (David 1998; Chapman *et al.* 2009). There are many examples of HFCs, such as survival (Coulson *et al.* 1998; Silva *et al.* 2009), reproductive success (Olano-Marin *et al.* 2011;

Wetzel *et al.* 2012), disease resistance (Acevedo-Whitehouse *et al.* 2005; Rijks *et al.* 2008) and growth rate (Pogson & Fevolden 1998; Bierne *et al.* 2000). In addition, many morphological traits that could have some impact upon fitness are correlated with heterozygosity, for example, antler size in Iberian red deer, *Cervus elaphus hispanicus* (Pérez-González *et al.* 2010). Crucially, the expression of a number of important behavioural traits, such as aggression (Charpentier *et al.* 2008) and territoriality (Lieutenant-Gosselin & Bernatchez 2006), is associated with heterozygosity. Whilst the traits studied in this chapter are not fitness traits *per se*, the term heterozygosity-fitness-correlation will be applied in a general sense in absence of a more appropriate term.

For putative neutral genetic markers, such as microsatellites, there are two hypotheses for the existence of any HFC (Hansson & Westerberg 2002). The first hypothesis is the local effect hypothesis, where neutral loci are in linkage disequilibrium (LD) with one or more fitness genes. For example, single locus effects of three neutral markers, thus LD, were detected in relation to survival to adult age in great reed warblers, *Acrocephalus arundinaceus* (Hansson *et al.* 2004). In addition, survivors tended to possess more diverse major histocompatibility complex (MHC) regions. However, LD is unlikely to be the sole cause of HFCs because linkage between two loci within a genome will also generate heterozygote excess and, thus, identity disequilibrium (ID) (Szulkin *et al.* 2010). The second hypothesis is the general effect hypothesis, where the level of heterozygosity across a large set of neutral markers is generally correlated across loci within an individual's genome (ID) (Weir & Cockerham 1973; David 1998). This correlated heterozygosity can be measured by the level of heterozygote excess compared with expectations under random mating (Weir & Cockerham 1973; David 1998; Szulkin *et al.* 2010). When inbreeding occurs, there is a non-random association of genotypes across the genome. Therefore, the heterozygosity at neutral markers is thought to represent genome-wide heterozygosity due to inbreeding (Weir & Cockerham 1973; Szulkin *et al.* 2010) and thus is expected to be more common in small populations (Slate *et al.* 2004). The majority of HFCs have a weak effect size (i.e. $r^2<0.05$) (David 1998; Chapman *et al.* 2009), which is thought to be due to insufficient numbers of genetic markers used to accurately characterise genome-wide heterozygosity reflecting inbreeding (Balloux *et al.* 2004; Slate *et al.* 2004; Grueber *et al.* 2008). Thus, many studies invoke local effects as the cause of HFC (Hansson *et al.* 2004; Lieutenant-Gosselin & Bernatchez 2006; Tiira *et al.* 2006; Charpentier *et al.* 2008). Whereas, both linkage and identity disequilibria may explain HFC (Grueber *et al.* 2008; Szulkin *et al.* 2010).

2.1.2 Behavioural traits

Aggressive behaviour is likely to impact upon fitness since it allows an individual to gain dominance and territoriality, and consequentially access to limited resources such as mates and food. Indeed, the evidence for fitness benefits associated with aggression is clear in many species: aggressive behaviour has been related to male mating success in the common loon, *Gavia immer* (Mager III *et al.* 2008), dominance has been related to reproductive success in meerkats, *Suricata suricatta* (Hodge *et al.* 2008), and male territoriality has been related to female reproductive success in the African lion, *Panthera leo* (Mosser & Packer 2009). Also, reproductive success is related to aggressiveness and dominance in salmonids, for example Chinook salmon, *Oncorhynchus tshawytscha,* and rainbow trout (Gallardo & Neira 2005; Tatara *et al.* 2008; Schroder *et al.* 2010). As a fitness-related trait, the expression of aggression has been quantified in relation to differences in heterozygosity, with significant associations reported in primates (Charpentier *et al.* 2005; Charpentier *et al.* 2008), birds (Seddon *et al.* 2004; Ryder *et al.* 2010) and salmonids (Tiira *et al.* 2003; Tiira *et al.* 2006). In landlocked salmon, *Salmo salar,* aggressiveness was associated with higher genetic diversity, where this link was thought to be due to inbreeding (Tiira *et al.* 2003). Similarly, in brown trout, *Salmo trutta*, dominance, a trait linked with aggression was associated with higher levels of heterozygosity, however here this association was apparently due to local effects (Tiira *et al.* 2006). However, both these studies use a small number of markers to estimate genome-wide heterozygosity and may therefore lack the statistical power to detect LD. Together, these studies demonstrate a clear link between the level of heterozygosity and the expression of aggressive behaviour in salmonid fishes, even if the underlying cause (*i.e.* local versus single locus effect) is unclear. By following a similar framework, the link between the fitness-related traits aggression and heterozygosity may be shown in rainbow trout.

If the association between aggression and heterozygosity is a general salmonids phenomenon, then this may be used as a standpoint from which to study other phenotypic traits. Aggression has been correlated with other behaviours, such as boldness and stress responsiveness. Correlated behaviours within an individual are termed behavioural syndromes which are suites of correlated behaviours that are consistent across context and show consistent physiological responsiveness (Sih *et al.* 2004a; Sih *et al.* 2004b) Section *1.2*). A keystone study in demonstrating behavioural syndromes in fishes showed correlations between aggression and boldness in three-spined sticklebacks, *Gasterosteus aculeatus* (Huntingford 1976) and this has been replicated in many fishes since, including the guppy, *Poecilia reticulata,* (Budaev 1997), European grayling, *Thymallus thymallus* (Salonen & Peuhkuri 2006) and brown trout, *Salmo trutta* (Sundström *et al.* 2004). In

addition aggression and boldness have been correlated with stress responsiveness in stress coping styles. Stress coping styles are characterised in many vertebrates including mammals and fish and are important topics in the fields of behavioural ecology and physiology (Koolhaas *et al.* 1999; Koolhaas *et al.* 2007). For example, Verbeek *et al.* (1996) showed that great tits, *Parus major*, selected for divergent stress coping style also exhibited divergence in boldness and in aggressiveness. Stress coping style has been demonstrated in rainbow trout, where different lines bred for high and low stress responsiveness exhibit differences in aggressive behaviour (Pottinger & Carrick 2001; Øverli *et al.* 2002b). The complex nature of the correlation between behavioural traits means that the genetic basis of behavioural syndromes is relatively understudied. Moreover, the behavioural trait boldness and the physiological trait cortisol release during stress are, individually, complex traits with potentially complex underlying genetic mechanisms, which warrant further investigation.

A key component of behavioural syndromes is risk-taking or exploration of novel objects or environments, termed boldness (Huntingford 1976; Fraser *et al.* 2001; Sneddon 2003; Eriksson *et al.* 2010). Fitness advantages of boldness are unclear and may be dependent upon context. Sometimes, boldness is advantageous, for example, female Trinidadian guppies, *Poecilia reticulata*, mated with bold male guppies after observing the risk-taking behaviour of potential mates and this was irrespective of male colour (Godin & Dugatkin 1996). However, other examples indicate boldness confers a disadvantage: bold female mosquitofish, *Gambusia holbrooki*, were smaller and had lower fecundity than shy mosquitofish (Wilson *et al.* 2010). Therefore, it is likely that, whilst boldness confers advantages in some contexts, in others, individuals that retain this trait have reduced fitness. This context-dependency is seen in Panamanian bishops, *Brachyrhaphis episcopi*, where fish are bolder in the presence of a predator but not in populations without predation (Brown *et al.* 2005).

The genetic basis of boldness has not received much attention but boldness is heritable in the great tit, *Parus major* (Drent *et al.* 2003), and in *Brachyrhaphis episcopi* (Brown *et al.* 2007). The values of heritability are varied among species and populations but remain low to moderate $h^2<0.5$. Furthermore, a genetic component of boldness is evident in the gene DRD4, which was associated with exploratory behaviour in great tits, but only in certain populations (Korsten *et al.* 2010). These studies suggest the contribution of a genetic component, but also that boldness may be under many influences. The apparently plastic nature of boldness, or at least different underlying mechanism among taxa and contexts, makes it difficult to assess genetic influences upon this behavioural trait. Nonetheless, by assessing the relationship between genetic variation and the variation in bold behaviour, the

link between heterozygosity and boldness may be elucidated. Furthermore, since aggression is correlated with both boldness and heterozygosity in salmonids, comparing the respective associations of heterozygosity with aggression and boldness may continue the understanding of how genetic links affect behavioural links in rainbow trout.

The cortisol response to stress is a well-studied trait with implications for fitness, having both evolutionary and economical importance. Chronic stress can cause deleterious effects on growth processes (Pickering *et al.* 1991), the reproductive system (Campbell *et al.* 1992) and immunocompetence (Pickering & Pottinger 1989) and this can impact directly upon the reproduction and survival of animals. Lines of rainbow trout were bred for high (HR) and low (LR) plasma cortisol levels in response to a confinement stressor (for details see Section *1.3*) and this response was heritable. Moreover, these lines diverged in aggressive behaviour with LR being more aggressive than HR (Øverli *et al.* 2002; Pottinger & Carrick 2001). That it is possible to generate lines of trout based upon their cortisol responses to stress demonstrates a genetic basis to the stress response. Evidence of consistent differences in aggressive behaviour implies some constraint on coping style, which may be genetic. Moreover, Thomson *et al.* (2011) showed differential gene expression between HR and LR in response to an acute stressor, implicating a small number of candidate genes in the stress response. However, whether the stress response and the link with behaviour is due to a few candidate genes is not known, which would be evident as local effects in a HFC, or due to genome-wide levels of heterozygosity, which would be generated by inbreeding. Therefore, these inbred lines present an opportunity to study the genetic basis of stress coping styles.

Currently, how heterozygosity influences a suite of correlated behaviours, both individually and with the context of an overall behavioural syndrome is unclear. Aggressive behaviour is a well-studied fitness-related trait in terms of its association with heterozygosity, where more aggressive individuals show higher genetic diversity. However, the genetic architecture, for example the number of genes implicated in a trait, associated with stress responsiveness and boldness are less well-characterised. Moreover, the genetic basis underlying the correlation of behaviours is not well-understood.

The aims of the present study is to explore the effect of heterozygosity in three complex phenotypic traits, aggression, boldness and stress responsiveness, to improve our understanding of the genetic basis of complex traits. The HFC approach allows general inbreeding effects to be partitioned from local effects and thus indicate a level of genomic complexity associated with individual behaviours, which can also be expanded upon in future study (Chapter 3). I expect that aggressive individuals will be more heterozygous than

less aggressive individuals due to previous work on many animals and in particular salmonids. Moreover, I hypothesise that because low stress responsiveness and boldness are often correlated with aggression in salmonids, I also expect that individuals showing these fitness-related traits to be heterozygous.

2.2 Methods

2.2.1 Rainbow trout husbandry

All experiments were conducted in a humane manner according to Home Office UK licensing and after local ethics approval. Rainbow trout, *Oncorhynchus mykiss*, were tested for two behaviours: aggression and boldness. To do this, two groups of fish were used. For aggression, I used a commercially obtained hatchery strain and for boldness I used lines of trout with divergent responses to stress. The fish tested for aggression comprised two groups of rainbow trout that were transported from a commercial supplier to the aquaria at Liverpool on 11/08/2010 (n=100, approximate weight=90 g) and 25/10/2010 (n=70, approximate weight=90 g). Rainbow trout interactions are influenced by previous experience of winning or losing and, in order to reduce effects of previous experience upon aggressive behaviour, the trout were held in two separate stock tanks (2x2x0.5 m; 2,000L). The holding tanks were supplied with aerated recirculating freshwater (semi-closed system) at 13±2°C, with a 14:10 hr light:dark regime, similar to ambient. Trout were fed commercial pellets (Skretting, UK) at a rate of 1% body weight daily according to manufacturer's recommendations. Fifteen pairs of fish, one from each tank, were caught at random using a hand net, anaesthetised in 33.3 μgml^{-1} benzocaine (Sigma, UK) in a 25 L bucket to minimise stress during weighing; fish were weighed to the nearest 0.01 g and transferred to experimental tanks (90x45x50 cm; 200 L) at 11±1°C, which were arranged in three semi-closed recirculating systems (n=15 tanks) with aerated freshwater and were screened from visual disturbance. Each tank contained two fish of equal weight (±10%), so as not to influence the outcome of the interaction as relatively larger fish tend to be dominant (van Leeuwen *et al.* 2011), one fish in each of two compartments, halved by an opaque divider. Fish were fed daily in each compartment at the same rate as in the stock tanks.

The inbred lines selected for high (HR) or low (LR) cortisol responsiveness to a standardised stressor (Section *1.3*; Pottinger & Carrick, 1999) were used to assess the associations between heterozygosity with both boldness and stress responsiveness. These rainbow trout were transferred from CEH Windermere to Liverpool where each line was held separately (~140 fish per tank) as described above and in Thomson *et al.* (2011). Rainbow 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 (90×45×50 cm; 200 L), as above. Experiments were conducted on fish that had resumed feeding after this period.

2.2.2 Behavioural tests

Once fish resumed feeding and after a period of at least 7 days (reduced feeding is a standard indicator of stress (Carr 2002)), the tests for aggressive behaviour began. All interactions took place between 12:00 and 14:00 to minimise any effects of diurnal variation in physiology. At least 15 min prior to each experiment low light level cameras that were linked to a remote monitoring system were installed at the front of the tanks to allow fish to minimise disturbance immediately preceding the behavioural trial. Each experiment began by removing the divider (using a pulley system to prevent fish being aware of the operator), after which the pairs were allowed to interact for a maximum of 15 min. An opaque tube (160x90x90 mm) was supplied as a refuge for each fish, should it choose to remain out of sight of its opponent, such that any interactions were voluntarily performed by the fish and the stress of the encounter was minimised. The videos of the interactions were assessed blind to determine the amount of aggression that each fish performed. The total number of aggressive acts were determined by measuring the following three types of aggressive behaviour: (1) displacement, where one fish moves slowly towards its opponent and the opponent retreats in response; (2) chase, where one fish moves rapidly towards its opponent resulting in either contact between the two fish or the opponent retreating; (3) circling, where both fish circle each other. An aggressiveness score for each fish was calculated by subtracting the total number of aggressive actions of its opponent from the total number of acts of the fish. A fish's aggressiveness is dependent upon the degree of aggressiveness exhibited by its opponent (Haller *et al.* 1996; Neat *et al.* 1998; Dugatkin & Druen 2004) (Appendix 1, Section *A1.1*) and subtraction of one fish's score from its opponent's score accounts for this. Thus, fish with the highest score were deemed to be "aggressive" (n=15) and those with the lowest were "less aggressive" (n=15). Dividers were replaced and each trout humanely killed at the end of the interaction period using concussion and exsanguination. Fish were weighed and sexed, and muscle tissue was collected onto dry ice and frozen at -80°C.

For the boldness test, rainbow trout from the selected lines were treated as described previously (Thomson *et al.* 2011). Briefly, trout were caught at random (HR: n=44, LR: n=33) from their stock tanks and placed individually into an experimental tank for 7 days to allow acclimation, where its behaviour was observed on day 8 for 10 mins, before a novel object was added. This is a standard fear test to measure neophobia and the propensity of fish to take risks during a novel situation. The behavioural responses to the novel object

were measured as the latency to approach within 5 cm and 10 cm, frequency of entering within 5 cm and 10 cm and time spent within 5 cm and 10 cm zones of the novel object as well as latency, frequency and duration of time spent outside of these zones (passive). The trial was repeated one week later to ensure consistency of response using a different object to prevent habituation. Fish that behaved consistently i.e. bold or shy over both trials were used for genotyping. Surprisingly, both HR and LR lines had bold (HR: n=15, LR: n=13), intermediate (HR: n=20, LR: n=16) and shy (HR: n=9, LR: n=4) individuals (Thomson *et al.* 2011), therefore, both line and boldness were investigated. Following the second trial, fish were killed humanely by concussion and exsanguination and muscle tissue sampled and immediately frozen at -80°C.

2.2.3 Genotyping

I assessed the association between genome-wide heterozygosity with aggression (hatchery strain), boldness and stress responsiveness (stress lines). To do this, I genotyped the stress lines at 82 microsatellite markers, corresponding to approximately three loci from each of the published rainbow trout linkage groups (Guyomard *et al.* 2006; Rexroad *et al.* 2008). The number of loci was reduced to 24 to assess aggression in the hatchery strains for logistical reasons (all markers in Appendix 1, Table A1). For PCR, genomic DNA from 44 LR and 33 HR rainbow trout (*Oncorhynchus mykiss*), as well as from 15 aggressive and 15 less aggressive fish was extracted from muscle tissue using a high salt method (Aljanabi & Martinez 1997). Microsatellite alleles were amplified in a 10 µl PCR on a Dyad DNA Engine (MJ Research Inc.). A tailed primer method was used to label PCR products (see Schuelke 2000), whereby the PCR reaction contained forward primers that are synthesised to include a 5' (or tail) M13 sequence (AGCGGATAACAATTTCACACAG). The reaction also contained the M13 sequence, which was labelled with either 6-FAM, NED, PET or VIC fluorophores (Applied Biosystems). Best results were achieved using two rounds of PCR. The PCRs consisted of 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 5-50 ng template DNA, 10 µg BSA and 0.25 U *Taq* polymerase (ABgene). The first round of PCR contained 1 pmol of tailed-forward and reverse primer and PCR conditions were 95°C for 5 min, 30X [95°C 30 s, 57°C 45 s, 72°C 45 s], 72°C 30 min (all loci amplified at the same annealing temperature). The second round of PCR contained 2 pmol reverse primer and 2 pmol of labelled M13 primer; thermal cycling conditions were 95°C for 5 min, 10X [95°C 30 s, 50°C 45 s, 72°C 45 s], 72°C 30 min. PCR products were pooled with a 500 bp (LIZ) size standard (Applied Biosystems), separated by capillary electrophoresis on an ABI3130*xl* and sized using GENEMAPPER v.4.0 software (Applied Biosystems).

2.2.4 Data analyses

Loci were tested for departure from Hardy-Weinberg using Genepop on the web *v*.4.0.10 (Raymond & Rousset 1995; Rousset 2008). Multilocus heterozygosity, generally one of the most robust measures of heterozygosity (Chapman *et al.* 2009; Szulkin *et al.* 2010), is a count of the number of heterozygous loci and was standardised by the total number of loci used for genotyping to generate a proportion of heterozygous loci (PHt). PHt was calculated using GENHET (Coulon 2010) separately for the hatchery strain and the stress lines. It is also possible to calculate other measures of heterozygosity, observed heterozygosity, expected heterozygosity, internal relatedness and homozygosity by locus, using GENHET. These measures were significantly correlated (all r^2>(-)0.9, all P<0.0001; Table 2.1). Since the level of aggression is dependent on the opponent, variation in levels of heterozygosity of aggressive individuals and less aggressive individuals was compared between two groups in concordance with previous methods used to assess heterozygosity amongst aggression types in salmonids (Tiira *et al.* 2006). In addition, sex was included as a covariate. Since many measures were used to assess boldness, a principal component analysis, based on a correlation matrix, of the second boldness trial was conducted to identify which measures of boldness best describe variation in the data. A HFC was assessed by correlation between PHt and PC1, which was used to characterise boldness (Thomson *et al.* 2011) and with PC2. As with aggression, the difference in heterozygosity was assessed between HR and LR rainbow trout. Since there were two stocks of rainbow trout used for the aggression trial, I tested whether there was a difference in heterozygosity between the two stocks and whether there was a bias in number of winners originating from each tank. In addition, since it was not possible to determine the sex of the fish prior to the experiment, I tested for an effect of sex upon winning. Finally, since it was not always possible to obtain fish of equal weight, I assessed whether there was an effect of the difference in weight upon the difference in aggressive acts.

Table 2.1. Correlation coefficients for the correlations between the proportion of heterozygous loci (PHt) and four other measures of heterozygosity: observed heterozygosity (H_o), expected heterozygosity (H_e), internal relatedness (IR) and homozygosity by loci (HL).

Measure of heterozygosity	R^2	**P-value**
H_o	0.996	***
H_e	0.999	***
IR	-0.928	***
HL	-0.969	***

To assess the underlying mechanisms of a HFC, it is necessary to test for local effects (LD) or general effects (ID). ID can be quantified by heterozygosity-heterozygosity correlations (HHCs), which assess whether heterozygosity at loci is correlated within individuals and this is central to inbreeding effects (Balloux *et al.* 2004). HHCs work by arbitrarily dividing the panel of marker loci into two sets and then assessing the correlation in multilocus heterozygosity between the two sets. This is repeated multiple times where the assignment of loci to the two groups is random. Rhh (Alho *et al.* 2010) was used to calculate the mean HHC using PHt from the outbred trout used to measure aggression and separately for the inbred lines. The validity of HHCs has been questioned recently because of a lack of independence of the resulting correlation coefficients (Szulkin *et al.* 2010). Whilst HHCs provide a sufficient estimate of inbreeding, a more appropriate parameter to capture ID is by the parameter g_2. This is defined as the excess of double heterozygotes at two loci compared with expectation under random association (with g_2=0 indicating a lack of ID) (David *et al.* 2007). By testing for ID, whether the genotyped markers represent the underlying inbreeding coefficient in the population used can be indicated. I measured g_2 using RMES software (David *et al.* 2007) for both the hatchery strain used to measure aggression and for the inbred lines used to measure boldness and which characterise stress responsiveness. An additional specific test, which assesses the effects of single loci upon the phenotype was carried out using GEPHAST (Amos & Acevedo-Whitehouse 2009). In this test loci are sorted according to a maximum phenotype-genotype association. This is repeated randomly to detect a strong association of a single locus with phenotype; this procedure was done using 1,000 randomisations and corrected for multiple testing using a sequential Bonferroni test (Rice 1989) to maintain a type-I error rate of 0.05. However, the trout used to assess the association between boldness and stress responsiveness and heterozygosity were not tested for single locus effects on phenotype, due to finding no initial phenotypic relationship with heterozygosity.

2.3 Results

2.3.1 Behaviour

Aggressive individuals performed about 45% more aggressive actions against their opponent (n=15, 49.3 ± 9.51 acts) than did their less aggressive counterparts (n=15, 33.6 ± 8.18 acts; Figure 2.1), with no apparent effect of stock origin upon aggressive behaviour (χ^2=0, df=1, P=1.00; Appendix 1, Table A3). The categorical assignment of aggression means that in a pairwise contest, the aggressive individual always performed significantly more aggressive acts than the less aggressive (paired t test: t=6.11, df=14, $P<1x10^{-4}$). To assess the effect of sex upon the level of aggression, a linear mixed effects model was fit to the data in R (R

Development Core Team 2009) with aggressiveness and sex as fixed effects and the pairing of individuals as a random effect. This showed there to be no significant difference in aggression between males and females (F_{12}=0.687, P=0.505; Appendix 1, Figure A1). Additionally, differences in weight were minimised to 8.9±6.3% difference, as body size can affect the outcome of interactions. These minor differences in weight did not affect the number of aggressive acts, thus the outcome of the interaction (paired t-test: t=-0.67, df=31, p=0.509; Appendix 1, Figure A2).

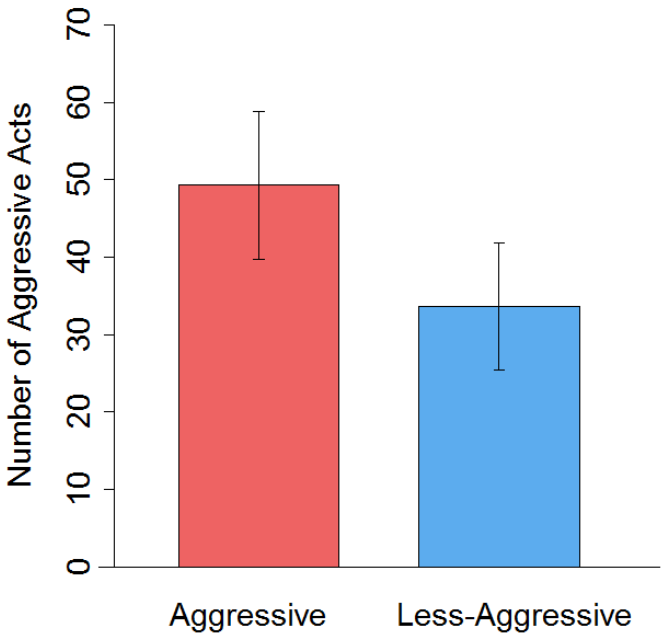

Figure 2.1. The total number of aggressive acts, quantified as displacements, chases and circles, carried out by aggressive (red; n=15) and less aggressive (blue; n=15) rainbow trout. Error bars show standard error of the mean.

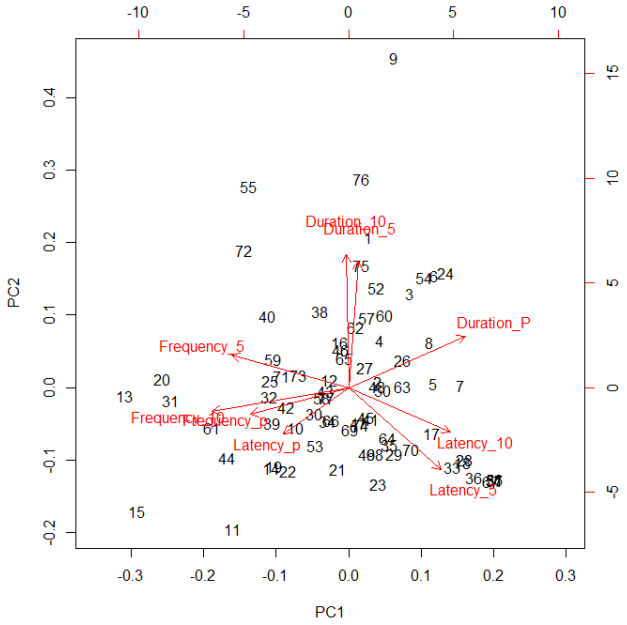

Figure 2.2 Principal component analysis of nine measures of boldness (latency to enter within 5 cm, 10 cm and passive latency, frequency of entering within 5 cm, 10 cm and passive frequency, duration of time spent within 5 cm, 10 cm and passive duration spent near a novel object) shows the loadings of PC1 and PC2 of these nine measures.

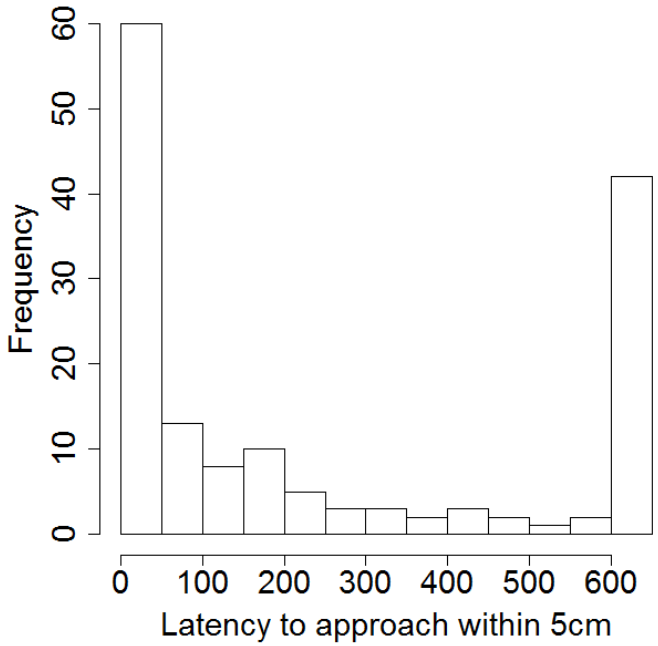

Figure 2.3. The distribution of boldness amongst bold and shy rainbow trout, characterised by the latency to approach within 5cm of a novel object across two tests. Data from Thomson *et al.* (2011).

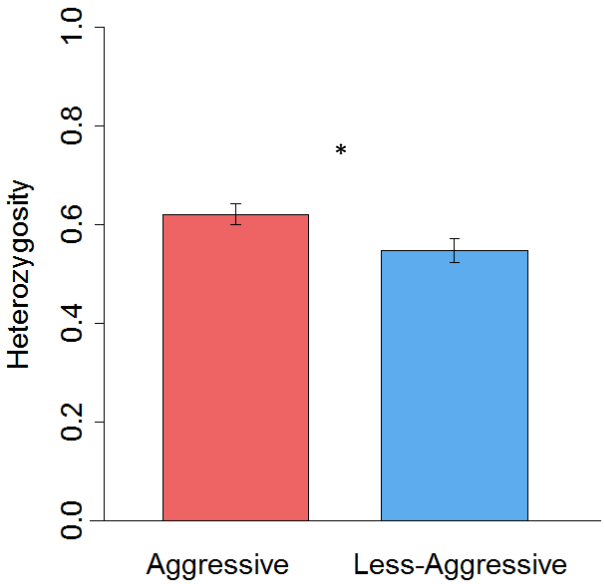

Figure 2.4. The difference in heterozygosity, measured as the proportion of heterozygous loci of the total number of loci, between aggressive (red; n=15) and less aggressive (blue; n=15) rainbow trout. Error bars show the standard error of the mean. * indicates P<0.05.

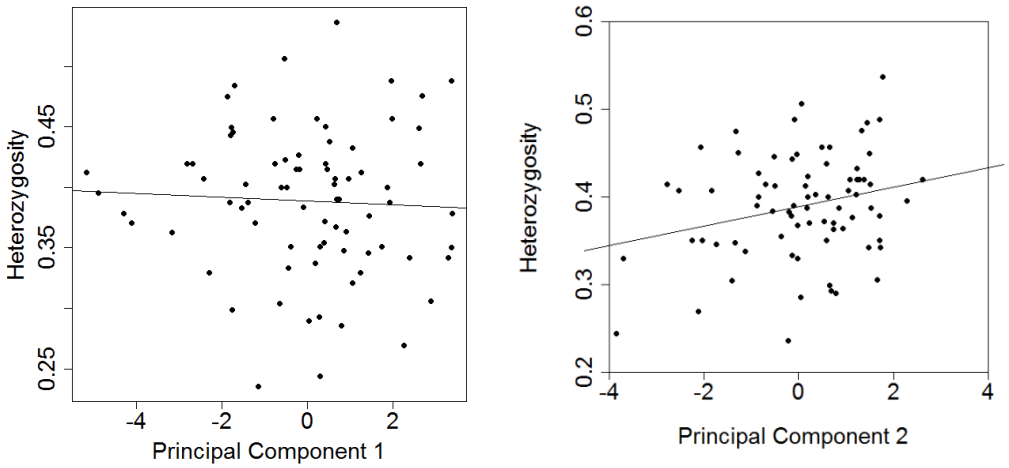

Figure 2.5. The relationship between a. principal component 1, which describes 40% of variance of boldness and is described by latency to approach within 5 and 10 cm of a novel object performed by bold and shy rainbow trout (n=77), and b. principal component 2, which describes 25% of variance of boldness and is described by the duration of time spent within 5 and 10 cm of a novel object and heterozygosity, measured as the proportion of heterozygous loci of the total number of loci.

Table 2.2. Principal component loadings (derived using a correlation matrix) for nine measures of boldness in lines of rainbow trout selected for divergent cortisol responses to stress.

Boldness Measure	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Duration 10 cm	-0.00778	0.597737	-0.20619	0.143173	-0.19094	0.359671	-0.42648	0.479189	-0.04806
Duration 5 cm	0.03407	0.568579	-0.25121	0.263452	-0.3112	-0.32135	0.372966	-0.45	-0.0364
Passive Duration	0.416508	0.232012	0.290897	-0.05065	0.296565	-0.53033	0.06703	0.393837	-0.39586
Frequency 10 cm	-0.48584	-0.10766	-0.06556	0.255699	0.115309	0.193174	0.201395	0.024897	-0.7687
Frequency 5 cm	-0.41719	0.146503	0.07409	0.439738	0.508449	-0.13919	0.193677	0.238841	0.482886
Passive Frequency	-0.35007	-0.12026	0.489506	0.2379	-0.54241	-0.39095	-0.3409	0.058626	0.008459
Latency 10 cm	0.362093	-0.2001	-0.15365	0.626207	0.247577	-0.03836	-0.49156	-0.3169	-0.09098
Latency 5 cm	0.3281	-0.36715	-0.1643	0.401726	-0.38905	0.082916	0.441632	0.456873	0.084671
Passive Latency	-0.23216	-0.20796	-0.71397	-0.19663	-0.00799	-0.51792	-0.20871	0.206173	-0.00802
Standard deviation	1.9079	1.5143	1.0774	0.90088	0.68427	0.50305	0.41179	0.383	0.238
Proportion of Variance	0.4045	0.2548	0.129	0.09018	0.05202	0.02812	0.01884	0.0163	0.0063
Cumulative Proportion	0.4045	0.6593	0.7883	0.87843	0.93045	0.95857	0.97741	0.9937	1

The selected HR and LR lines have consistently displayed a divergent cortisol stress response through four generations (F1-F3: Øverli *et al.* 2005; F4: Thomson *et al.* 2011). Boldness was tested in these lines: the results for the boldness assessment are described in full in Thomson *et al.* (2011) but are briefly recapitulated here. Principal component analysis (Table 2.2) of the final bold-shy assessment demonstrated that PC1 described 40.5% of the variation and PC2 described a further 25.5%. The major contributor to PC1 was latency to approach within 5cm and 10 cm, whereas PC2 described duration of time spent within 5 cm and 10 cm of the novel object (Figure 2.2). The distribution of time to approach within 5 cm of the novel object was bimodal (Figure 2.3), where individuals tended towards either the bold (quick to approach) or shy (slow to approach) extreme. Of the 77 individuals examined, 41 were consistently bold or shy over two trials, where 28 were bold and 13 were shy. The lines showed similar numbers of bold (HR: n=15, LR: n=13), intermediate (HR: n=20, LR: n=16) and shy (HR: n=9, LR: n=4) individuals, but differed in proportion, where LR had a slightly higher proportion of bold fish (Appendix 1, Figure A3). Additionally, the trout from the two lines showed no difference in weight (two sample t-test: t=-0.735, df=73.9, P=0.465).

2.3.2 Heterozygosity-behaviour-correlations

The fish used for examining aggression had a mean number of alleles of 5.8, ranging from two to twelve (Appendix 1, Table A4). Two loci differed significantly from Hardy-Weinberg equilibrium: OMM1780 and OMM1374 (Appendix 1, Table A4). There were no differences in PHt between the two stocks (t test: t=0.664, df=25.1, P=0.513; Appendix 1, Figure A4). In the stress lines, which were characterised for boldness, the mean number of alleles was 3.8, ranging from two to nine (Appendix 1, Table A5). Five monomorphic loci were excluded (OmyRGT40TUF; OMM1762; OMM1116; Omy1136INRA and OMM1797) and seventeen loci (Appendix 1, Table A7) were found to differ from Hardy-Weinberg equilibrium (P<0.01) after sequential Bonferroni correction (Rice 1989).

Table 2.3. The differences in five measures of heterozygosity between aggressive (n=15) and less aggressive (n=15) rainbow trout: proportion of heterozygous loci (PHt) and observed heterozygosity (H_o), expected heterozygosity (H_e), internal relatedness (IR) and homozygosity by loci (HL)

Measure of heterozygosity	**Mean difference**	**Paired *t*-value**	**Degrees of Freedom**	***P*-value**
PHt	0.0672	2.53	14	0.0241
H_o	0.113	2.31	14	0.0366
H_e	0.113	2.47	14	0.0268
IR	-0.0720	-1.49	14	0.159
HL	-0.0665	-1.98	14	0.0682

Table 2.4. The correlation coefficients for the relationship between five measures of heterozygosity and rainbow trout tested for PC1 (boldness towards a novel object; n=77): proportion of heterozygous loci (PHt) and observed heterozygosity (H_o), expected heterozygosity (H_e), internal relatedness (IR) and homozygosity by loci (HL)

Measure of heterozygosity	R^2	***P*-value**
PHt	-0.0506	0.6623
H_o	-0.0565	0.6254
H_e	-0.0568	0.6237
IR	0.0588	0.6114
HL	0.0398	0.7314

Table 2.5 The correlation coefficients for the relationship between five measures of heterozygosity and rainbow trout tested for PC2 (n=77): proportion of heterozygous loci (PHt) and observed heterozygosity (H_o), expected heterozygosity (H_e), internal relatedness (IR) and homozygosity by loci (HL)

Measure of Heterozygosity	R^2	***P*-value**
PHt	0.28	0.013
H_o	0.27	0.016
H_e	0.28	0.013
IR	-0.28	0.014
HL	-0.3	0.008

Table 2.6. The differences in five measures of heterozygosity between rainbow trout with a high (HR; n=44) and low (LR; n=33) cortisol response to confinement stressor: proportion of heterozygous loci (PHt) and observed heterozygosity (H_o), expected heterozygosity (H_e), internal relatedness (IR) and homozygosity by loci (HL)

Measure of heterozygosity	**Line**	**Mean Value**	***t*-value**	**Degrees of Freedom**	***P*-value**
PHt	HR	0.378	-1.85	73.3	0.069
	LR	0.403			
H_o	HR	0.972	-1.86	72.7	0.067
	LR	1.03			
H_e	HR	0.802	-1.81	73.1	0.074
	LR	0.854			
IR	HR	0.170	-0.52	74.0	0.603
	LR	0.184			
HL	HR	0.540	0.58	74.3	0.564
	LR	0.532			

Table 2.7 Output of 2-way ANOVA where the difference in heterozygosity was tested between winning and sex and the interaction between winning and sex of rainbow trout.

	DF	**SS**	**MS**	**F value**	***P*-value**
Winning	1	0.04104	0.04104	5.136	0.032
Sex	1	0.0026	0.0026	0.325	0.573
Winning:sex	1	0.01342	0.01342	1.679	0.206
Residuals	26	0.20773	0.00799		

Consistent with expectations due to studies in other salmonids, aggressive rainbow trout had significantly higher levels of heterozygosity than less aggressive fish (PHt aggressive and less aggressive=0.623±0.025 and 0.556±0.024 respectively) (Figure 2.4; paired t test: t_{14}=2.53, P=0.0126; data for PHt, see Table 2.3 for other measures of heterozygosity). Additionally, where sex was included as a covariate, there was an effect of aggression on heterozygosity, but not sex nor the interaction between sex and aggression (Table 2.7). Contrary to expectations, boldness in the lines, measured as PC1, was not correlated with PHt (Figure 2.5a; Pearson's correlation: r^2=-0.05, n=77, P=0.662; data for PHt, see Table 2.4 for other measures of heterozygosity). However, PC2 was correlated with PHt (Figure 2.5b; Pearson's correlation: r^2=-0.228, n=77, P=0.046; data for PHt, see Table 2.5 for other measures of heterozygosity). Moreover, there was no significant difference in heterozygosity between the HR and LR lines, which differ in their cortisol responses to stress

(two-sample t test: t=-1.85, df=73, P=0.069; data for PHt, see Table 2.6 for other measures of heterozygosity), although LR showed a nonsignificant tendency to have higher heterozygosity (Figure 2.6).

2.3.3 Causes of HFCs

The hatchery strain used to measure aggressive behaviour showed no evidence for significant ID, with the heterozygosity-heterozygosity correlations (HHCs) indicating a low inbreeding signal (r=-0.056, 95% confidence interval: -0.304–0.239) and g_2 was low and non-significant (g_2=0.00457, P=0.283). Furthermore, no evidence for single locus effects on the aggressive phenotype was found, whereby no locus affected the aggression-heterozygosity association more than any other (Appendix 1, Table A6). In contrast, the stress lines showed a presence of significant ID using both HHC (r=0.232, 95% confidence interval: -0.0517–0.471) and g_2 (g_2=0.181, P<0.0001); thus the pattern of heterozygosity at one or few of these loci in these lines is representative of the general genome-wide variation in diversity that occurs due to inbreeding.

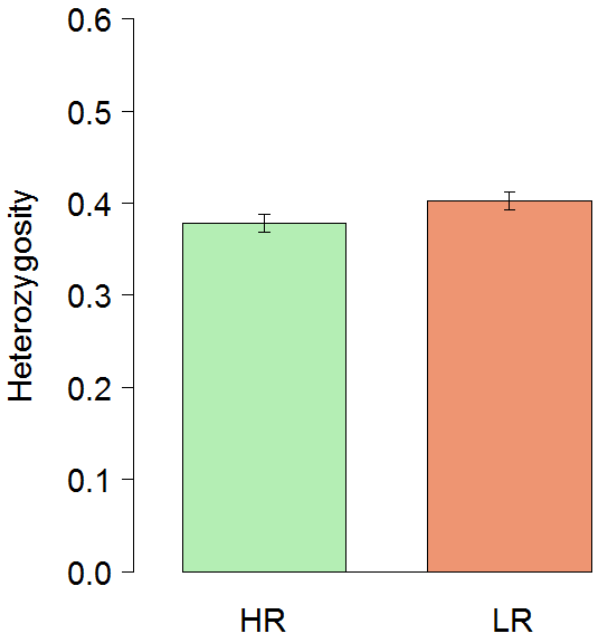

Figure 2.6. Heterozygosity of high responding (HR; green; n=44) and low responding (LR; orange, n=33) rainbow trout bred for their divergent cortisol responses to a confinement stressor. Error bars show the standard error of the mean.

2.4 Discussion

Few studies have quantified the wider role that differences in heterozygosity *per se* drive variation in behavioural traits. Fewer still have assessed whether such putative inbreeding effects can be extended to a suite of correlated behaviours that define a behavioural syndrome. This information is valuable for understanding how behavioural syndromes arise (Sih *et al.* 2004a; Bell 2009; Conrad *et al.* 2011). My data indicate that aggressive rainbow trout have higher heterozygosity than less aggressive individuals, consistent with studies on a range of salmonids and other taxa, but that heterozygosity has no relationship with the level of boldness or stress responsiveness. These results suggest that aggressive behaviour has a substantial genetic component driven by variation in genome-wide diversity. There is conflicting evidence for single loci or genome-wide heterozygosity to drive associations between genotype and phenotype in salmonids, likely arising from the use of a small number of markers to reflect heterozygosity across the genome. The use of a large set of markers maximises the ability to distinguish these effects. Whilst boldness and stress responsiveness have a genetic component (Section *2.1.2*), the results suggest that the association between genotype and phenotype for these traits may be more complex. Indeed, many factors affect these traits, including environmental context or individual experience, meaning that flexibility, rather than fixed genotypes may be required. Moreover, these behaviours showed distinct relationships with heterozygosity, suggesting that heterozygosity does not contribute to correlations in behaviour, therefore that mechanisms for behavioural syndromes are complex.

2.4.1 The relationship between heterozygosity and aggression

That more aggressive rainbow trout are more heterozygous than relatively less aggressive individuals is in line with previous studies on salmonids. Specifically, landlocked salmon, *Salmo salar*, with higher heterozygosity were more aggressive (Tiira *et al.* 2003) and dominant brown trout, *Salmo trutta*, were more heterozygous than subordinates (Tiira *et al.* 2006). As putative neutral markers, HFCs can be detected due to two reasons: the general effect, creating genome-wide heterozygosity and the local effect of single loci (Hansson & Westerberg 2002; Slate *et al.* 2004; Szulkin *et al.* 2010). The HFCs in *S. salar* and *S. trutta* were attributed to general and local effects respectively. However, neither local nor general effects were detected in these data, but it is likely that both general effects due to inbreeding and single loci affect aggressive behaviour in trout. Heterozygosity at single loci affects behavioural traits, for example, differences in heterozygosity at the MHCIa locus affected the level of aggression and boldness in rainbow trout (Azuma *et al.* 2005). Szulkin *et al.* (2010) argue that local effects are unlikely to be the cause of most HFCs because the effects are usually small and difficult to detect; moreover, the lack of evidence for LD is not

surprising, given that markers were selected to cover widespread locations across chromosomes.

While computer simulations indicate that the use of a small panel of loci (<100) can reduce statistical power to detect the pattern of genome-wide heterozygosity (Balloux *et al.* 2004), other studies have identified ID using few loci (Olano-Marin *et al.* 2011). Szulkin *et al.* (2010) suggest that most natural populations depart from the conditions that would drive an association between heterozygosity and inbreeding, particularly small population size due to a recent bottleneck (Balloux *et al.* 2004; Grueber *et al.* 2008; Slate *et al.* 2004). Thus, the use of hatchery fish, which are characterised by small effective population size, and therefore a high rate of inbreeding due to selection for fast growth and/or large body size, increases the opportunity of finding HFC. Furthermore, the non-significant values of g_2 reported here do not contradict an association between heterozygosity and aggression, since fitness-related traits are often under the control of more loci than can be genotyped. Thus inbreeding effects may not be represented by the genotyped loci whilst being detected by association with the phenotype (Szulkin *et al.* 2010). To further investigate the causes of HFC in rainbow trout, additional genetic markers could be used to increase the chances of finding a correlation between inbreeding and heterozygosity, although an increase in the number of markers does not always increase the chance of finding this effect (Olano-Marin *et al.* 2011). Nonetheless, these findings suggest that there is a general genetic influence upon aggressive behaviour through genome-wide heterozygosity, whereby heterozygotes are more aggressive. Moreover, complex genotypes may relate to many genes constituting many pathways. To further characterise genomic effects of genes upon aggressive behaviour, the transcriptome profile of aggressive trout should be studied further (Chapter 4).

Work on salmonids tends to be focussed on welfare within hatchery populations but these data have wider implications in natural populations as aggression confers greater competitive ability, and potentially greater fitness, in other species. For example, male wild house mice, *Mus domesticus,* with low heterozygosity had poor competitive ability for mates and thus sired fewer offspring (Meagher *et al.* 2000). Likewise, group heterozygosity explained territory size and reproductive success in a co-operatively-breeding bird, *Monias benschi* (Seddon *et al.* 2004). Seddon *et al*. (2004) suggest that the effect observed in *M. benschi* was a consequence of a correlation between heterozygosity and the level of disease resistance and energy metabolism that ultimately determined the group's ability to be aggressive and maintain a larger territory. Indeed, heterozygosity is related to many indicators of fitness, including survival, reproductive success, immune function and, crucially, competitive ability (reviewed in Kempenaers (2007)). This suggests that

heterozygosity may indicate general fitness either through inbreeding or because of an association with a specific fitness locus (see Chapter 3). That the current study shows there is a difference in heterozygosity between the two behavioural types of trout shows that these fish may possess a genotype that underlies the propensity to win fights via aggressive behaviour. The fitness consequences of this in wild salmonids have not been studied but by analogy with other studies more heterozygous individuals are expected to be generally "fitter" than homozygotes through dominance and territorial behaviour that provides greater access to limited resources such as food, breeding territories and mates.

2.4.2 Stress, boldness and heterozygosity

In contrast with aggression, rainbow trout certain aspects of boldness were not associated with heterozygosity, whereas others were. There are a number of possible reasons for this, such as boldness being determined by specific alleles at one or more genes (*i.e.* an undetected local effect) or even a wider interaction between genes and the environment, with the outcome that differences in genome-wide heterozygosity *per se* have no apparent effect upon boldness. Indeed, it is apparent that many factors, including environment, life-history and previous experience, influence boldness (Section *2.1.2*). (Frost *et al.* 2007)(Álvarez & Bell 2007)(2011)Despite this variety of influencing factors other studies have indicated a clear genetic basis to the expression of boldness, where boldness is heritable in some species of fish (Sundström *et al.* 2004; Brown *et al.* 2007; Dingemanse *et al.* 2009). (Fidler *et al.* 2007)However, consistent with my data is the apparent complexity of genetic components, with boldness not heritable in all fish species (Riesch *et al.* 2009)(Korsten *et al.* 2010; Tschirren & Bensch 2010). The results from the present study suggest that association of genotype and boldness is likely to be context-dependent. Indeed, theoretical work has shown that boldness may be context-dependent, for example upon frequency of behavioural type and upon population size and density, where boldness is favoured in large populations and shyness in small populations, but both at low frequency (Ji *et al.* 2009). Moreover, the results suggest that certain aspects of boldness may be more closely linked with heterozygosity than others. Genetic diversity appears to drive bold behaviour in the presence of predators in brown trout, *Salmo trutta,* (Vilhunen *et al.* 2008), where bolder trout were more heterozygous, in populations with the presence of predation threat. This indicates a genetic basis to boldness, which is dependent upon context, where boldness is adaptive in a predation context due to a need for survival. This reliance upon environmental conditions (e.g. predation risk) and upon individual learning means that the genetic component of boldness may be weak since many studies have shown boldness is plastic. Further work could be conducted to determine whether heterozygosity reflects flexible, rather than fixed behaviour. Despite maximising the chance of detecting HFC through the use of a large panel

of markers and an inbred population, there were inconclusive results pertaining to the association between boldness and heterozygosity, likely due to complex determining factors of boldness.

Stress responsiveness showed no association with heterozygosity and this may be for different reasons than boldness. The cortisol response to stress is well-characterised in a broad range of taxa, including axes that result in, and regulate, its production. Moreover, many candidate genes are implicated in the pathways that cause cortisol production. For example, 314 genes were differentially expressed after an acute stressor in rainbow trout (Cairns *et al.* 2008). In addition, the stress lines diverged in the expression of six genes associated with behavioural traits (Thomson *et al.* 2011), where the expression in these genes was correlated. This may indicate that complex pathways may be controlled by one or a few genes that initiate the pathway. Whilst genetic effects in transcription do indicate genetic mechanisms, it does not necessarily reflect how stress responsiveness should be related to heterozygosity. Moreover, the numerous amounts of genes that are implicated in regulation of stress responsiveness may be restricted to a number of specific pathways, which may be under the control of a few genes.

2.4.3 Genetic background of behavioural syndromes

Behavioural syndromes are a suite of correlated behaviours and physiology across multiple contexts and exist in a number of species (Koolhaas *et al.* 1999; Sih *et al.* 2004a; Sih *et al.* 2004b). Studies that determine genetic bases for behavioural syndromes are lacking (reviewed in Bell 2009; van Oers *et al.* 2005). Since aggression, boldness and stress responsiveness are correlated in rainbow trout, and aggression is associated with heterozygosity, it may be expected that correlated behavioural traits should show similar relationships with heterozygosity if they are associated with inbreeding. A key finding from the current study is the difference in pattern of genetic diversity between aggression, boldness and stress responsiveness, which suggests that these behaviours have differing genetic mechanisms and thus an absence of general genetic basis (in this case variation in genome-wide heterozygosity) for a behavioural syndrome.

The rainbow trout stress lines show consistent and clear divergence in behavioural type for aggression (Øverli *et al.* 2002; Pottinger & Carrick 2001), with LR are more aggressive. Whilst aggression is often correlated with boldness (Huntingford 1976; Johnson & Sih 2005; Wolf *et al.* 2007), Bell (2005) found weak genetic correlations for boldness and aggression in low-predator populations of stickleback, *Gasterosteus aculeatus*. Similarly, in the current

study, the relationship between heterozygosity of aggression, stress and boldness differ, suggesting the association is attributable to mechanisms outside of those due to genome-wide heterozygosity and thus not consistent across time and context. For example, if a small number of genes are shared between behaviours constituting a behavioural syndrome, this would be difficult to detect using HFC.

An alternative explanation for the lack of similarity in the associations between the three behaviours and genome-wide heterozygosity may be that the behavioural syndrome is decoupled in this species. In other fishes, a lack of behavioural correlation has been shown for aggression and boldness, for examples in the convict cichlid, *Cichlasoma nigrofasciatum* (Budaev *et al.* 1999), and in golden shiners, *Notemigonus crysoleucas* (Leblond & Reebs 2006). Moreover, boldness was weakly associated with stress responsiveness in the stress lines (Thomson *et al.* 2011). Whilst this may explain differences in HFCs for boldness and stress, this does not explain why stress and aggression do not show similar patterns, since the stress lines show differences in aggressive behaviour. Thus, rather than a lack of behavioural syndrome, it may be that specific contexts are required to be able to detect behavioural syndromes. There are many examples where boldness only exhibits consistency or correlations with other traits in the presence of predators. One such example is in sticklebacks, *G. aculeatus* from low-predator, small ponds exhibit a lack of behavioural syndrome, whereas sticklebacks from large ponds with high predation demonstrate an association between aggressiveness, activity and exploration (Dingemanse *et al.* 2007). This lack of consistency in correlated behaviours may be driven by the environmental dependence of boldness: Álvarez & Bell (2007) showed that boldness in *G. aculeatus* was affected by environment type, which may suggest a decoupling of boldness from behavioural syndromes. It was suggested that a lack of selection pressure upon a behavioural syndrome was the mechanism responsible for reducing the association in behaviours in low-predator environments. Since the rainbow trout used in this study originated from hatcheries where predation is minimal, it is possible that the same mechanism to dissociate the behaviours is present whereby a lack of predators relaxes selection pressure for boldness. These results suggest the general genetic basis for the behavioural correlation between aggression, stress and boldness, at least at the species level, is lacking.

2.4.4 Conclusions

This study investigated the link between heterozygosity and behaviours that form a behavioural syndrome, aggression and boldness, along with a complex physiological trait that is often correlated with these behaviours, stress responsiveness. I confirmed a general phenomenon associating aggression with heterozygosity in salmonids. Since aggressiveness

is related to both boldness and stress responsiveness, it was expected that these two complex traits would show a similar association with heterozygosity to aggression. However, I did not find an association with heterozygosity for stress responsiveness, but found conflicting results for boldness. This inconsistency of association in boldness is possibly due to context-dependence, where both environment and experience affect the expression of boldness. These factors may act solely upon boldness or in concert with the effects of single genes, although these effects may be weak and inconsistent. In stress responsiveness, on the other hand, the lack of association with heterozygosity may be due to an undetected local effect of a small number of genes, whereby a small set of genes controls a complex pathway. Whilst these results may have important implications for understanding behavioural syndromes in natural populations, they may also help understand how fitness-related behavioural traits affect fitness in the wild. To elucidate HFCs more specifically, studies should be conducted in different contexts (*e.g.* predation risk, competition), particularly given the plastic nature of boldness. This study is the first to assess how heterozygosity affects the genetic link among correlated behavioural and physiological traits, by demonstrating that behaviours that are often related to one another do not necessarily show a simple genetic association.

Chapter 3 The genomic consequences of selective breeding for divergent responses to stress.

3.1 Introduction

Response to selection can leave a profound footprint on genome architecture including the distribution and diversity of genes (Beaumont & Nichols 1996; Schlötterer 2002b; Nielsen 2005). The modes of selection underlying mechanisms of adaptation can be better understood by identifying the number and locations of distinct regions of the genome affected by selection. Genomic patterns in genetic variation may be present as a result of genetic drift, where mutations may increase in frequency due to random factors (Kimura 1968). However, certain instances of genetic variation are distinctive from this "neutral" variation due to drift. Notably, positive selection can be characterised in two ways: a reduction in genetic diversity or an increase in genetic differentiation amongst populations at a specific locus, due to an advantageous allele increasing in frequency. Balancing selection is distinguished from this by showing high levels of heterozygosity, due to for example frequency-dependent selection, which maintains polymorphism at a locus (Nielsen 2005; Oleksyk *et al.* 2010). The utility of detecting selection comes when performing a genome scan using a large number of genetic markers, distributed across known locations on the genome, from which neutral variation can be determined. From this it is possible to detect outliers, which show extreme levels of differentiation or diversity and which may, therefore, be under selection. Thus it may be possible to identify functionally important genomic regions associated with a complex phenotypic trait.

3.1.1 Genome scans for selection

Genome scans have been used to highlight the genetic basis of population or species divergence. Within a wide range of species, identifying markers that show genetic differentiation has identified genome regions under natural selection, which can be used to infer adaptation to various situations. For example, genome regions have been associated with adaptation to altitude in the common frog, *Rana temporaria* (Bonin *et al.* 2006), with temperature in white spruce, *Picea glauca* (Namroud *et al.* 2008) and to new habitat in the gilthead sea bream, *Sparus aurata* (Coscia *et al.* 2012). Moreover, genome scans can be applied to different species in order to determine the causes of speciation: allopatric speciation can be distinguished from sympatric speciation by differential signatures of divergence. Indeed, this approach has been used to show sympatric speciation (identifiable by low numbers of divergent loci) between two species of palm (Arecaceae) on a Pacific island (Savolainen *et al.* 2006). An important application of genome scans is to be able to identify regions of the genome that may be associated with distinct phenotypic traits. For

example, approximately 40 physiologically important genes were under selection between groups of three-spined stickleback, *Gasterosteus aculeatus*, adapted to differing environmental salinity (Shimada *et al.* 2011). By identifying the number and genomic location of outliers associated with a phenotypic trait, it may be possible to understand the genetic architecture underlying complex traits. Moreover, these genomic regions can be putative candidates for phenotypic traits.

In genome scans, outlier loci indicate the action of selection either because they reside within a functional gene or because they are situated in a "neutral" region of the genome that is linked (i.e. they "hitchhike" (Maynard Smith & Haigh 1974) with a gene under selection) (Schlötterer 2003). Crucially, a genome scan can be used to detect the action of selection without reference to either a full genome sequence or pedigree data and this technique is widely employed in natural populations where classic breeding and mapping approaches used to identify quantitative trait loci (QTLs) are impractical (but see Rogers & Bernatchez, 2007). However, there can be a number of disadvantages to using genome scans, with certain demographic features, such as bottlenecks in particular, capable of generating a similar genomic signature to the action of selection (Wall *et al.* 2002). As the recent demography of many natural populations is often unknown, it can be difficult to determine the action of selection unambiguously (Teshima *et al.* 2006; Kane & Rieseberg 2007). Indeed, recent studies have criticised the efficacy of genome scans, either through a lack of sensitivity or because a high rate of false positive loci are identified (Hermisson 2009). Recent improvements to the underlying statistical assumptions have addressed many concerns about the efficacy of genome scans to detect selection, particularly when populations have hierarchical structure or have experienced a bottleneck (Foll & Gaggiotti 2008; Excoffier *et al.* 2009). However there are still uncertainties about the rate of false positives; thus a typical approach is simply to "apply several statistical tests" to a large number of markers to identify consistent outlier loci.

3.1.2 Genome scans for candidate genes

Even when many loci are identified it is still problematic to relate such candidate loci to a specific phenotypic trait as there is often a lack of *a priori* information about the underlying selective process (Chapman *et al.* 2008; Nielsen *et al.* 2009) or even the phenotypic trait under selection (Li & Merila 2010). For example, selection may be identified among populations whose habitat differs in altitude, but selection may be acting on genes associated with coping with temperature. Thus, it is not always clear upon which trait natural selection has acted. This makes it difficult to decide which outlier loci should be studied further to characterise a specific trait. Few studies perform genome scans based on strong selection for

a known trait. However, some studies identify outliers using a correlative approach, which attempts to link outlier loci with environmental variables (Bonin *et al.* 2009; Manel *et al.* 2009; Nunes *et al.* 2011). Other studies, particularly those that use SNPs to identify selection, use sequence data to annotate and assign function to selected loci (Kane & Rieseberg 2007). These studies use natural populations and, therefore, the reasons behind selective differences between the populations may be less clear than lines of animals selected for a known trait. Where there is information about a specific selective mechanism behind divergence, as with artificial selection, the function of regions of selection can be inferred. Moreover, assigning regions of selection to a phenotypic trait may be achieved with the use of markers associated with a functional gene.

It is also desirable to locate genetic markers associated with selection for a phenotypic trait. Identifying the number and location of candidate loci for complex phenotypic traits is traditionally achieved by selective breeding and mapping quantitative trait loci (QTL) (Allen Orr 2001). An advantage of QTL mapping is that underlying genetic architecture of a trait can be learnt due to identifying locations of regions associated with the trait in question. However, this is sometimes impractical due to the extensive breeding programs required to achieve differences in phenotype and back-crossing these families, particularly when the unit of generation time is years. The use of genome scans allows the identification of the number of markers associated with selection for a trait, but may lack information about the location of these markers. By choosing markers from known locations, such as when using a linkage map, the identity of candidate genomic regions associated with a phenotypic trait can be found (Bonin *et al.* 2009). Moreover, by comparing regions identified using a genome scan with those identified by QTL mapping, the benefits of both approaches may be captured (Rogers & Bernatchez 2005). This potentially powerful approach not only allows the future allocation of function to specific genomic regions, but it can also provide information about the cause of divergence. Thus, it is useful to examine populations that have (1) undergone selection for a known trait and (2) map outlier loci to genomic locations to verify the efficacy of the genome scan approach against QTL.

Genome scans are useful for linking genomic regions with complex (i.e. involving many genes) traits (Casto & Feldman 2011). Where traits are complex, individuals that have been artificially selected for a single aspect of the target trait represent an efficient way of identifying the number of genomic locations associated with that trait, and thus the potential level of genomic complexity, while reducing confounding effects of other factors that differ between populations or individuals. The cortisol response to stress is a complex phenotypic trait with an important role in the fitness and health of both natural populations and domestic

animals, having both evolutionary and economic importance. When prolonged or repeatedly activated, the stress response can cause deleterious effects on growth processes, (Pickering *et al.* 1991), the reproductive system (Campbell *et al.* 1992) and immunocompetence (Pickering & Pottinger 1989). This can impact directly upon the reproduction and survival of not only wild animals but also of domestic animals, with respect to stock improvement and welfare and this is a major driver to understanding both general and specific underlying genetic mechanisms controlling this adaptive trait. The stress response is a complex physiological and behavioural response that helps an animal to cope with, or escape from, an external threat or challenge. Although the stress response comprises a complex neuroendocrine pathway (Wendelaar-Bonga 1997), the magnitude of the response can be consistently expressed as the extent to which plasma levels of the major species-specific corticosteroid hormone are elevated as has been shown within individuals in a number of taxa, including humans, *Homo sapiens* (Steptoe *et al.* 2009), rats and poultry (see Pottinger (2000)) and fish (e.g. common carp, *Cyprinus carpio L.*, (Tanck *et al.* 2001) and rainbow trout, *Oncorhynchus mykiss* (Fevolden *et al.* 1999; Pottinger & Carrick 1999)).

3.1.3 Is the genetic influence on stress responsiveness complex?

Rainbow trout, *O. mykiss*, kept in aquaculture experience many stressors due to intrinsic farming procedures such as handling or transport and, as such, may experience chronic stress. Plasma cortisol concentrations are consistently elevated in response to stress in rainbow trout (Pickering & Pottinger 1989) and, as such, cortisol can be used as an indicator of the general physiological stress response. The stress response is heritable ($0.41<h^2<0.73$) in rainbow trout and this has been applied to selectively breed stress lines for divergent (high and low) cortisol responses to a confinement stressor (Section *1.3)* (Pottinger *et al.* 1992). These selected lines have been behaviourally and physiologically characterised (Pottinger & Carrick 2001; Øverli *et al.* 2005; Schjolden *et al.* 2005) and detailed knowledge of the genetic architecture associated with the stress response is growing. Many studies focus on the expression of candidate genes; microarrays show a large number of genes conferring pathways involved in stress responsiveness (Section *1.3*). These studies highlight the need to identify the number and location of genes, in order that specific mechanisms, such as epistasis or pleiotropy can be investigated. In Chapter 2, I show that genome-wide heterozygosity is not related with stress responsiveness in these lines, thus requiring further investigation into genetic architecture in order to identify novel candidates associated with this trait. QTL studies may be drawn upon to enable a comparison of the genome scan approach with the QTL mapping approach. Two such studies have identified QTL in rainbow trout, *O. mykiss* (Drew *et al.* 2007; Quillet *et al.* 2010). The cortisol response to handling stress yielded two QTLs (Drew *et al.* 2007) and a more recent study (Quillet *et al.*

2010) identified twelve QTLs in the selectively bred lines of rainbow trout which were associated with the response to a confinement stressor. These studies may help verify that candidate loci are associated with the trait that was selected. Moreover, by using lines of rainbow trout that have undergone artificial selection for a known trait, we can infer that selected regions are associated with the selected trait. However, to confirm this inference it is necessary to associate candidate genes that have been previously related to the stress response, with markers under selection from the current study. This can be achieved by using markers associated with genes linked with the stress response, thus indicating that searching for signatures of selection is a valid approach to identifying candidate loci for complex phenotypic traits.

The broad aim of this study was to use a genome scan to detect the number and locations of distinct regions of the rainbow trout genome associated with a complex trait, the plasma cortisol response to stress, with a view to ascertaining genomic complexity underlying the trait. By using a model with known demographic history, the instance of false positives may be minimised and allow regions showing evidence for selection to be put forward for further study. By confirming regions of selection, the confidence in the genome scan may be enhanced. Using animals that have demonstrated a specific heritable response to selection for differing cortisol response to confinement stress (Pottinger & Carrick 1999), regions under selection may be assigned function more easily. Using functional markers associated with stress responsiveness and previously identified QTL, the genome scan may yield candidate loci associated with selective breeding for the stress response. Furthermore, a large number of markers at known genomic locations (Rexroad *et al.* 2008), may allow the genomic complexity of stress responsiveness to be determined.

3.2 Methods

3.2.1 Rainbow trout husbandry

All experiments were conducted in a humane manner according to the UK Animals (Scientific Procedures Act 1986) and after local ethics approval. Two lines of rainbow trout (*Oncorhynchus mykiss*) that were selected for a high cortisol response (HR) and a low cortisol response (LR) to a confinement stressor over four generations (Section *1.3*) (Pottinger & Carrick 1999, 2001) were transported to the aquarium at Liverpool University (HR: n=44, body mass=343.00±14.68 g; LR: n=33, body mass=356.47±10.97 g). A third group of trout, unselected for cortisol responsiveness (US), was obtained from a commercial supplier (n=16; body mass=103.65±5.72 g). The trout were held in three separate 2000 L holding tanks (2x2x1 m) supplied with aerated recirculating freshwater (semi-closed system)

at 14±2°C, with a light:dark regime of 14:10 hours and were fed commercial pellets (Skretting, UK) at 1% body weight daily.

3.2.2 Genotyping

104 microsatellite loci were selected from the rainbow trout linkage map (Guyomard *et al.* 2006; Rexroad *et al.* 2008), with approximately three microsatellites from each of the 30 linkage groups (Appendix 1, Table A1). One locus, OMM5308 (linkage group RT11; or chromosome Omy27), was linked with a homologue of vasopressin-activated calcium-mobilizing receptor (VACM-1) (Coulibaly *et al.* 2005), which is implicated in the stress response (Coulibaly *et al.* 2005). Five markers associated with ESTs were also used (Väsemagi *et al.* 2005): CA048687, CA042613, CA054538, CA058580, CA059136 (for known functions see Appendix 1, Table A1). In addition, microsatellites that were associated with genes linked to aggressive behaviour were used, since this is known to share physiological control with the stress response (Sloman *et al.* 2001). To obtain sequences containing these microsatellites, 454 read sequences from differentially expressed reads between aggressive and less-aggressive trout (Chapter 4) were mined for repeat motifs using QDD2 (Meglécz *et al.* 2010). These read sequences were aligned against non-redundant (nr) database (*blast.ncbi.nlm.nih.gov*) using BLAST (Altschul *et al.* 1990) to determine the identity of five genes associated with aggression: NADH dehydrogenase, proopiomelanocortin (POMC), calmodulin, lipoprotein receptor and glucose-6-phosphate dehydrogenase. Primers (for primer sequences: Appendix 1, Table A2) for the sequences containing microsatellites were identified using Primer 3, v 0.4.0 (Rozen & Skaletsky 2000).

For PCR, genomic DNA from 33 LR, 44 HR and 16 unselected rainbow trout was extracted from muscle tissue using a high salt method (Aljanabi & Martinez 1997). Microsatellite alleles were amplified in a 10 µl PCR on a Dyad DNA Engine (MJ Research Inc.). A tailed primer method, whereby forward primers are synthesised with a 5' (or tail) sequence, M13, AGCGGATAACAATTTCACACAG, labelled with either 6-FAM, NED, PET or VIC fluorophores (Applied Biosystems), was used to label PCR products (see Schuelke (2000)). Best results were achieved using two rounds of PCR. The PCRs consisted of 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 5-50 ng template DNA, 10 µg BSA and 0.25 U *Taq* polymerase (ABgene). The first round of PCR contained 1 pmol of forward and reverse primer and PCR conditions were 95°C for 5 min, 30X [95°C 30 s, 57°C 45 s, 72°C 45 s], 72°C 30 min (all loci amplified at the same annealing temperature). The second round of PCR contained 2 pmol reverse primer and 2 pmol of a primer labelled with one of four fluorophores; thermal cycling conditions were 95°C for 5 min, 10X [95°C 30 s, 50°C 45 s, 72°C 45 s], 72°C 30 min. PCR products

were pooled with a 500 bp (LIZ) size standard (Applied Biosystems), separated by capillary electrophoresis on an ABI3130*xl* and sized using GENEMAPPER v.4.0 software (Applied Biosystems).

3.2.3 Statistical analyses

Basic measures of genetic diversity: expected heterozygosity (H_e), allele frequencies and numbers of alleles (N_a) were calculated for successfully amplified loci using MSANALYZER v.4.05 (Appendix 1, Table A7) (Dieringer & Schlötterer 2003). Genetic differences among HR, LR and US (F_{ST}) were calculated across all loci using GenePop *v*.4.0.10 (Raymond & Rousset 1995; Rousset 2008) and tested for global differentiation using Arlequin *v*3.5.1.3 (Excoffier *et al.* 2005). Loci that were monomorphic in all three lines were discarded. Loci were examined for departure from Hardy-Weinberg equilibrium and null alleles using GenePop *v*.4.0.10 (Raymond & Rousset 1995; Rousset 2008) and with a sequential Bonferroni correction for multiple testing (Rice 1989). Loci that were not in Hardy-Weinberg equilibrium (Appendix 1, Table A7) were retained because these populations were artificially maintained and were not random mating. Thus it is expected that some markers will deviate from Hardy-Weinberg assumptions due to selection or inbreeding. Markers with evidence for null alleles were excluded from further analysis (Appendix 1, Table A7). Since genetic divergence can be due to demographic history instead of selection, all lines were examined for evidence of a recent bottleneck, by testing for heterozygosity excess using a two-phased model (TPM) of mutation, incorporating the Wilcoxon test using Bottleneck, v.1.2.02 (Cornuet & Luikart 1996).

Evidence for positive selection was examined using two types of statistical tests to detect outlier loci (Storz 2005); (1) increased genetic differentiation, using F_{ST}, and (2) reduced genetic diversity, using lnRΘ. Generally, these methods differ in their sensitivity to population demography, where the methods based on variation in genetic differentiation are more sensitive to demographic conditions than methods based on variation in genetic diversity (Schlötterer 2002a). To detect divergent patterns of genetic differentiation at specific loci among lines three statistical methods based on F_{ST} were used. The first approach, Fdist2 (Beaumont & Nichols 1996), which was implemented in the software LOSITAN (Antao *et al.* 2008) was the first test to compare F_{ST} with heterozygosity (H_e), rather than with allele frequencies, enabling divergence to be detected using dominant markers. This method uses simulations to generate an expected distribution of F_{ST} and heterozygosity based on the empirical average genetic differentiation among samples (over all samples and loci). Outliers are then derived from this neutral distribution based on 95% confidence intervals. 1,000,000 simulations and a false discovery rate of 10% were used to

identify outlier loci. The second method, implemented in BayesFst v.OCT06 (Beaumont & Balding 2004), builds on the original approach by accounting for population-specific effects by allowing F_{ST} to differ among populations. Finally, the third method, by Foll and Gaggiotti (2008), BayeScan v.1.0, is another Bayesian method, which directly tests whether a locus is subject to selection by estimating the posterior probability that a locus favours one of two models; the first includes and the second excludes selection. The loci were tested using parameters that represent the rainbow trout genotypes: 84 loci, 3 populations and the observed number of alleles at each locus, over 1000 simulations.

The second type of statistical test detects selection by identifying reduced diversity, instead of F_{ST}: lnRV (Schlötterer 2002a) measures the ratio of variance in microsatellite repeat number between pairs of populations and lnRH (Kauer *et al.* 2003) measures the ratio in gene diversity (*i.e.* expected heterozygosity H_e) between pairs of populations. Both statistics are suited to detecting recent and extreme selection (Schlötterer 2003) and are less sensitive to demographic events, such as bottlenecks, than the F_{ST}-based statistics (Schlötterer 2002b). For both lnRV and lnRH, the stress lines and hatchery strain were compared in a pairwise manner, due to the nature of the test. Using a Gaussian distribution density function (see Schlötterer (2002a) and Kauer (2003)), standardised *p* values (using an overall mean of 0 and standard deviation of 1) were assigned to the lnRV and lnRH value of each locus. In addition, the two statistics were combined, which reduces the number of false positive outlier loci by approximately three-fold (Schlötterer & Dieringer 2005). Loci with strong evidence for selection were those that were identified with the lowest p value threshold in any given test.

3.2.4 Verification of outliers

To verify three regions showing signatures of selection, a further eight markers in these regions were tested for evidence of selection. Following the initial genome scan, three genomic regions impacted by differing signatures of selection were chosen (see Section *3.3.3*): the first contained the marker OMM5261 (chromosome 27, linkage group 11), the second region contained OmyRGT40TUF (chromosome 13, linkage group 2) and the third contained OMM1762 (chromosome 28, linkage group 13). The eight markers were chosen based on their proximity to the three regions showing evidence for selection, according to the microsatellite linkage map (Rexroad *et al.* 2008): three loci were selected around the locus OmyRGT40TUF (OMM1002, OMM1671, OMM1037), three loci around OMM5261 (OMM5178, OMM1310, OMM5332) and two around OMM1762 (OMM3022, OMM1388) (Figure 3.3). The above analysis was repeated with the original 84 successfully amplified loci plus the seven successfully amplified loci of the eight extra loci. It was expected that,

by virtue of linkage, these loci would also be identified as outliers. Additionally, selected regions were compared with QTLs previously identified to be associated with the stress response in rainbow trout (Drew *et al.* 2007; Quillet *et al.* 2010).

3.3 Results

Ninety-one of the original 104 microsatellites were successfully amplified, including four EST-associated markers and three markers associated with aggressive behaviour. Four markers were monomorphic and thus excluded. These were OMM1797, Omy1136INRA, OMM1333, the EST-associated marker CA048687. A further three loci were removed due to significant null alleles, leaving a final tally of 84 loci distributed throughout the rainbow trout genome, including three EST-associated markers: CA054538, CA059136 and CA058580 and one aggression-related marker: NADH dehydrogenase. Although the HR and LR groups had undergone an intense regime of selective breeding over four generations (Pottinger & Carrick 1999) they retained a reasonable level of polymorphism, with numbers of alleles ranging from one to nine in the HR line and from one to seven in the LR line compared with between one to eleven in the hatchery strain. The HR line possessed six monomorphic loci and the LR line possessed seven, where five of these loci were similarly monomorphic in both lines. There were 26 loci in the HR line, 19 in LR and 15 in US that were not in Hardy-Weinberg equilibrium (Appendix 1, Table A7) and these were retained.

Values of gene diversity (H_e) varied between 0.030 and 0.728 for the HR line, from 0.031 to 0.781 in the LR line, whereas, H_e in the unselected group ranged from 0.063 to 0.913. Thus, the level of genetic variability did not differ significantly between the two lines (Mann Whitney: W=3854, n=91, P=0.421), although the unselected trout retained significantly more genetic variation (Kruskal-Wallis: H=73.8, n=240, P<0.001) than either of the two stress lines, with a median H_e of 0.654, compared with 0.403 for HR and 0.442 for LR. Moreover, none of the groups of trout showed evidence for having been through a bottleneck when testing for heterozygosity excess from the two-phased model of mutation (HR: P=0.408; LR: P=0.134; US: P=0.139). Additionally, the three populations showed no evidence for global differentiation (P>0.05), where HR and US were more differentiated (F_{ST}=0.288) than HR and LR (F_{ST}=0.266) or LR and US (F_{ST}=0.252).

Table 3.1. Microsatellite markers were tested for signatures of selection in rainbow trout selected for high (HR) and low (LR) cortisol response to stress in comparison with an unselected (US) hatchery strain. Markers were tested with four tests: Fdist2, BayesFst, BayeScan and lnRθ. Loci identified by lnRV, lnRH and lnRθ are represented by the pair in which the locus was identified. * indicates significance at $P<0.05$, ** $P<0.01$ and *** $P<0.001$. The verification loci are extra markers investigated around regions showing evidence for selection. Loci under balancing selection as opposed to positive selection are denoted with b.

Locus	Fdist 2	Bayes Fst	Baye Scan	lnRV			lnRH			lnRθ		
				HR LR	HR US	LR US	HR LR	HR US	LR US	HR LR	HR US	LR US
OMM3006						*						
OMM5320	**											
OmyRGT40 TUF					*	*		*	*		*	*
OMM1151					*	*		*			*	
OmyRGT17 TUF			* b									
OMM5254					*			*			*	
OMM1710								*	*			
OMM5261				*		*	*		*	*		*
OMM1154							*		*			
OMM1762	**							*	*			
OMM1374								*				
OmyRGT12 TUF	**											
OMM1384				*								
OMM1134				*				*			*	
OMM1824								*				
OMM5133				*					*			
Omy1501 INRA	**											
OMM1322									*			
OMM1690		* b	*** b						*			
Omy1259 INRA					*	*		*	*		*	*
OMM1767		*	*		*	*						
Omy1398 INRA						*		*				
OMM1505	**		*									
Omy1308 INRA	**											
CA058580					*	*		*	*		*	*
CA054538					*	*		*	*		*	*
Verification loci												
OMM5178							*		*			
OMM1310	**	*			*	*	*		*			*
OMM1388		*										

3.3.1 Genome-wide selection

Overall, seventeen microsatellite loci (20% of the loci analysed) showed evidence for response to selection in at least one of the four different statistical tests (Table 3.1), with fifteen loci responding to divergent selection and two loci to balancing selection. The outlier tests varied in the number and identity of outlier loci they identified as indicative of the stress response. The three tests for genetic differentiation amongst populations identified nine outliers overall. Fdist2 identified six outlier loci, (Table 3.1; Figure 3.1a), BayesFst yielded four, including one under balancing selection (Table 3.1; Figure 3.1b) and BayesScan identified four, including two under balancing selection (Table 3.1; Figure 3.1c). Three of these loci were consistently identified using two tests: BayesFst and BayeScan both identified one locus, OMM1767, to be under positive selection and one, OMM1690, to be under balancing selection. In addition, both Fdist2 and BayeScan identified OMM1505 as being under positive selection. The tests for reduced diversity, lnRH and lnRV, yielded twenty-two outlier loci when considered separately, but this value was reduced to just eight loci when the tests were considered in combination, as recommended by Schlötterer & Dieringer (2005) (Figure 3.2). In the direct comparison between lines one of these eight outliers, OMM5261, showed reduced diversity in LR compared with both HR and the hatchery strain. Three regions showing evidence for selection were chosen for further investigation into signatures of genetic variation. The first contained the marker OMM5261, which showed reduced diversity in LR compared with the other two groups. Moreover, this region contained a second marker OMM1154, which showed reduced heterozygosity in LR. The second region contained OmyRGT40TUF, which showed reduced diversity in both lines compared with the hatchery group. The third contained OMM1762, which showed genetic differentiation among the three groups as well as reduced diversity in the lines compared with the hatchery group.

3.3.2 A link with function

Two EST-associated markers, CA058580 (unknown function) and CA054538 (associated with cyclin e) showed reduced diversity in both HR and LR in their respective comparisons with US. The other functional loci that were investigated for signatures of selection (one of the three EST-associated and one aggression-related marker) were not detected as outliers. Moreover, the locus OMM5308, a microsatellite linked with a homologue of vasopressin-activated calcium-mobilizing receptor (VACM-1), did not show evidence for selection. In addition five microsatellite loci shared linkage with QTL found using mapping for the cortisol response to stress (Drew *et al.* 2007; Quillet *et al.* 2010). This proportion (29%) is equivalent to previously found proportions (30%) of QTL matching loci identified with a genome scan (Rogers & Bernatchez 2005).

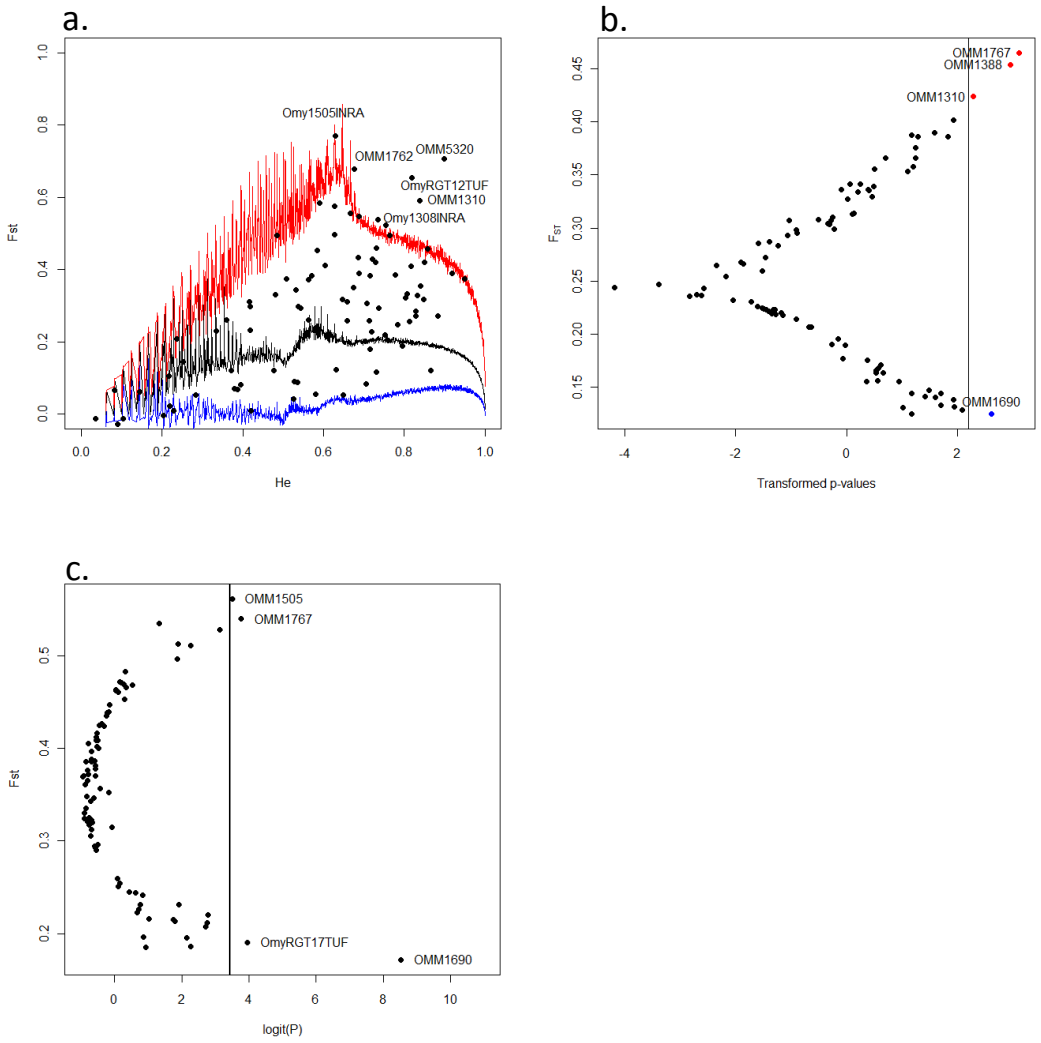

Figure 3.1. F_{ST} of the three populations (high responding – HR, low responding – LR and unselected – US), of *O. mykiss* against **a.** heterozygosity (H_e) using Fdist2, where the lines show 0.05 (blue), 0.5 (black) and 0.95 (red) quantiles. Loci above the red line show evidence for directional selection. **b.** F_{ST} against posterior probabilities, using BayesFst, where the line shows the point at which loci become outliers. Red points show loci subject to directional selection whereas blue points are subject to balancing selection. **c.** F_{ST} plotted against the posterior probability that a locus is subject to selection, using BayeScan, where the line shows the point at which loci become outliers and loci to the right of this line are outliers.

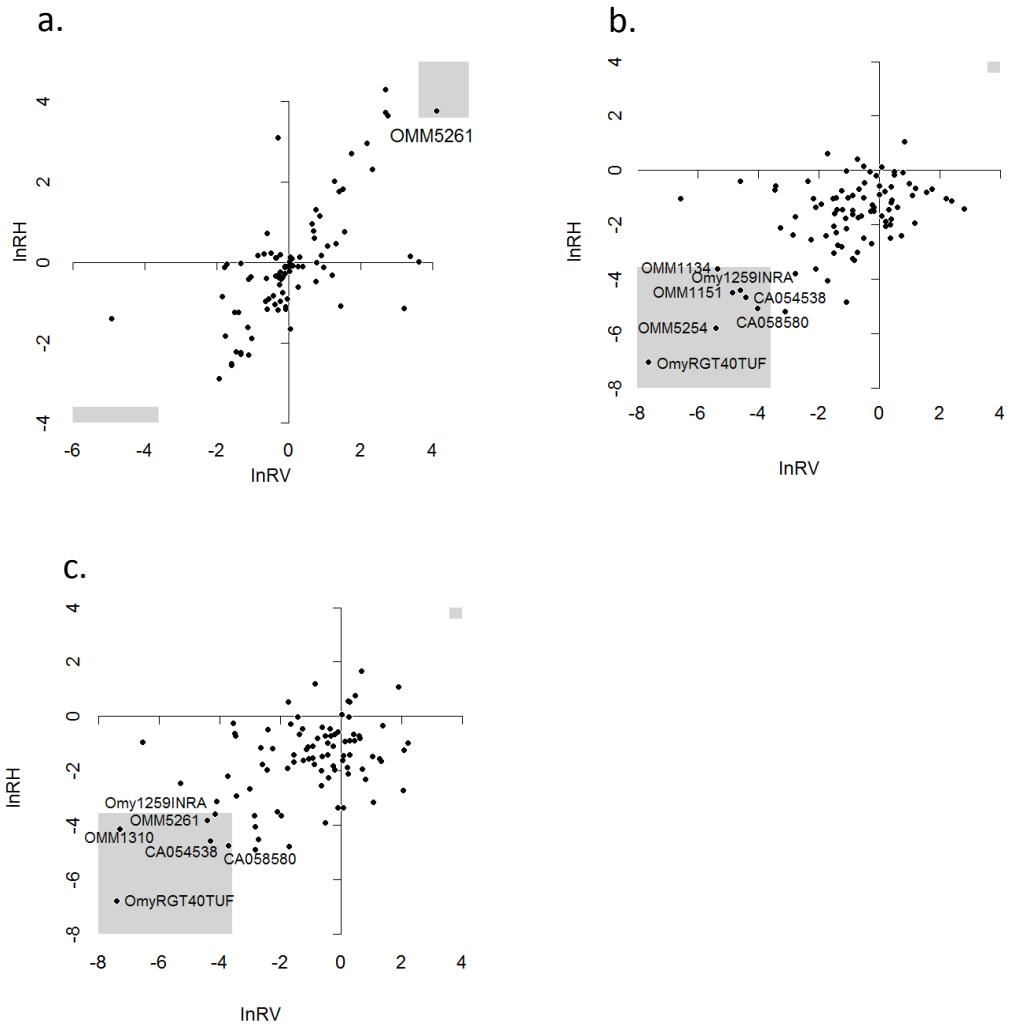

Figure 3.2. LnRV plotted against lnRH. Grey boxes represent a significant reduction in variability at p=0.95. for a. the high responding (HR) and low responding (LR) b. the HR line and unselected (US) group c. the LR and US groups.

Table 3.2. Loci identified in QTL studies (Drew et al 2007 and Quillet et al 2010), shown with their p values. These correspond with loci found in the current study, as shown by the tests with which they were identified. Pvalues are indicated by '.' $P<0.1$, '*' $P<0.05$, '**' $P<0.01$, '***' $P<0.001$

Locus	**Pairs identified with lnRΘ**	**Fdist2**	**Linkage Group**	**Significance in Quillet *et al.* (2010)**	**Significance in Drew *et al.* (2007)**
OMM1151	1		6	***	N/A
OMM1134	1		20	.	N/A
Omy1501INRA		**	23	.	**
Omy1308INRA		**	31	*	**
CA058580	2		6	***	N/A

The two tests for QTL find roughly equivalentnumbers – the genome scan identified 17 and the QTL mapping approach used by Quillet *et al.* (2010) identified 12, although Drew *et al.* (Drew *et al.* 2007) found only 3. Outliers that matched QTL generally showed weak evidence for selection, that is, show evidence from only one test. Omy1308INRA and Omy1501INRA were identified with Fdist2; OMM1134 and OMM1151 showed reduced diversity in HR compared with US. One marker that corresponded with QTL and that showed evidence for reduced diversity in the two comparisons involving the hatchery strain was CA058580 (Table 3.2).

3.3.3 Verification of outliers

Seven of the eight markers from regions with evidence for selection were successfully amplified by PCR to provide consistent and interpretable genotypes. Of the seven loci chosen around those regions with evidence for selection, two were found to be under selection (Table 3.1). In the region around OMM5261, OMM1310 showed evidence for selection in two F_{ST}-based tests, providing further evidence that this region is under selection. Moreover, this locus showed reduced diversity in LR compared with US, which is consistent with both OMM5261 and OMM1154 found in this region. However, OMM5178, which was the furthest physical distance away from the selected region only showed evidence for reduced heterozygosity and thus did not survive the combination of lnRH and lnRV. OMM5332 was not significant in any test. In the second region (around OmyRGT40TUF), no markers showed evidence for selection. The third region was around OMM1762, where OMM1388, but not OMM3022 showed evidence for selection using BayesFst.

These results revealed a widespread distribution of markers across the genome with seventeen of the putative outlier loci being found on 14 of 29 chromosomes (Figure 3.3). Linkage group RT11 (or chromosome 27) had the largest amount of markers under selection upon it, whereas nine linkage groups showed evidence at only one marker. Five of these markers were associated with QTL and were located across four linkage groups. Moreover, two regions showed consistent selection with at least two markers. Selection, in this group of selectively bred rainbow trout, is not only located genome-wide, but was confirmed in specific, localised genomic areas.

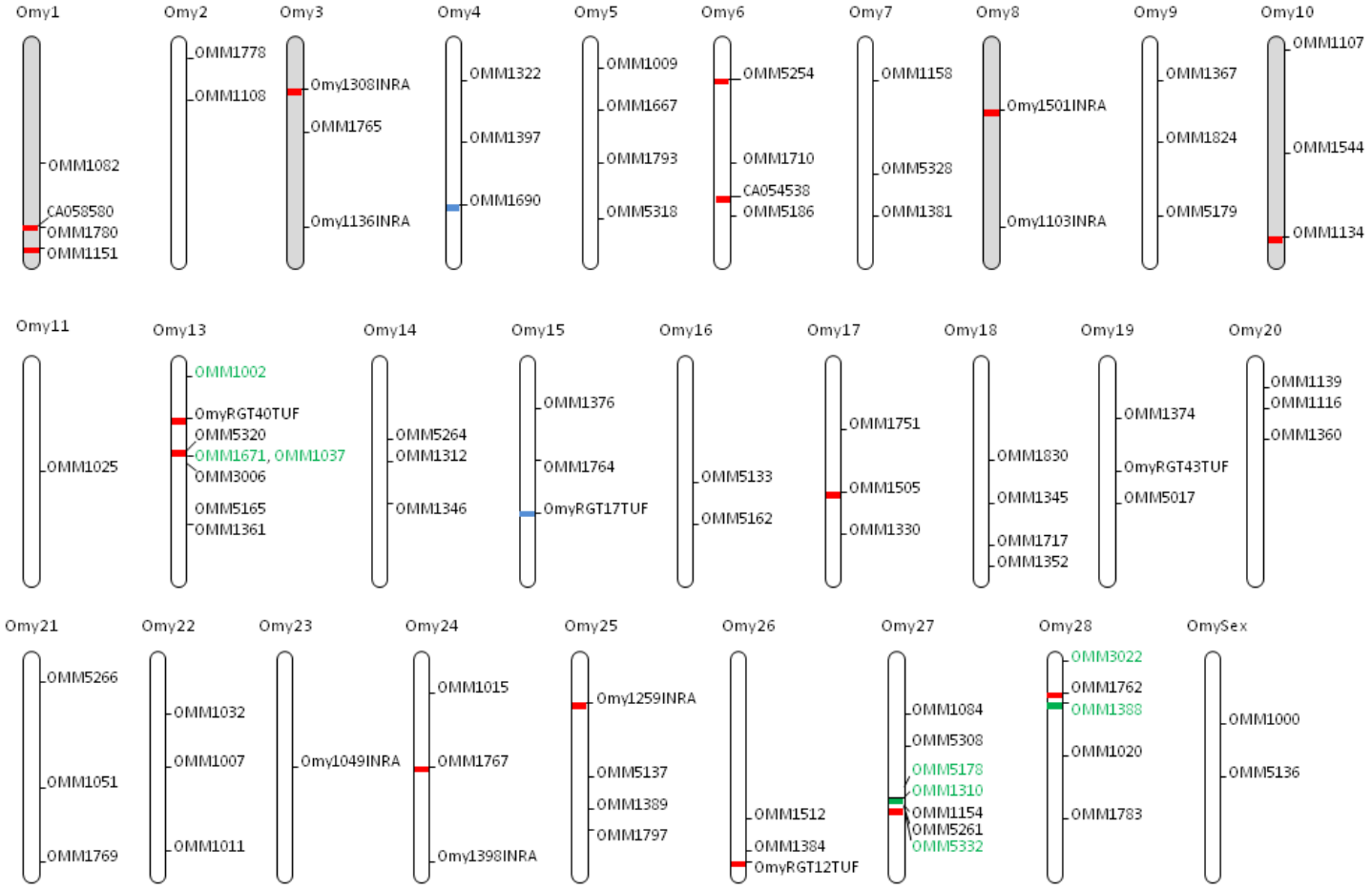

Figure 3.3. The position of all loci on the linkage groups of rainbow trout, numbered as chromosomes as in Rexroad *et al.* (2008). Red lines indicate loci subject to directional selection and blue lines indicate loci subject to balancing selection. Locus names in green are loci that were chosen to test regions that had been found to be under selection. Chromosomes filled in grey are those where QTLs from previous studies are found (Drew *et al.* 2007; Quillet *et al.* 2010).

3.4 Discussion

The cortisol response to stress is an important phenotypic trait in rainbow trout, since intrinsic procedures in aquaculture can cause chronic stress, which can have negative consequences for growth, reproduction and immune function (see Section *1.2.1*). The genomic architecture for the stress response is largely unknown (but see Chapter 2) but identifying genomic effects and novel candidates may be beneficial for marker-assisted breeding programs. Using a genome scan to identify and locate the number of candidate genomic regions that underlie variation within a particular trait is often difficult for several reasons, including the range of potential additional mechanisms that may drive divergence (particularly in natural populations), difficulties in identifying false positive outlier loci and little/no information about the physical position of markers throughout the genome. Here, two lines of rainbow trout with a known selective regime were used to identify 17 candidate loci on 14 chromosomes that show signatures of selection, including 15 responding to directional selection and two to balancing selection. Using information about the population history and demography, these tests could reliably show regions responding to selection. Two of the selected regions were further validated by use of additional loci, thus increasing confidence in the ability of this genome scan to accurately identify regions under selection. A major challenge when using genome scans for non-model organisms is to assign function to selected regions. By using rainbow trout artificially selected for a known trait, the cortisol response to stress, the function of selected regions may be inferred. Moreover, regions under selection were compared with regions associated with stress responsiveness in a QTL mapping study and five loci that corresponded between both were found. However, markers associated with physiological or behavioural function did not show evidence for selection. I thus identify potential candidate regions for further investigation with regards to their association with stress responsiveness and showed that novel candidates associated with a phenotype are important to study. Finally, having identified markers responding to selection from across the genome, I can infer that the genomic architecture of the phenotypic trait, cortisol response to stress, is complex.

3.4.1 Success of the genome scan

The utility of genome scans for identifying regions responding to adaptation is in contention (Section *3.1.1*). This is because tests for adaptation may produce false positives due to population structure or demographic features affecting genome regions in similar ways to selection (Storz 2005). For example, both positive selection and bottlenecks may produce reduced diversity at some loci. Where the number of loci used to detect selection is small, this may be a problem: bottlenecks produce genome-wide reduced diversity, whereas selection produces reduced diversity against a background of neutral variation. It is thus

crucial to use a large number of markers to detect selection. However, a large number of markers may increase the number of false positives, where 0.4-24.5% of markers are expected to be detected by chance, which is dependent upon the number of markers and populations (Nosil *et al.* 2009). In this chapter, I identified 20% of markers, which is in keeping with the expected amount, particularly since small numbers of populations and inclusion of functional markers increase the number of outliers. The different statistical methods of detecting loci under selection are sensitive to population-specific demography. It is thus important to know the particular structure and history of the populations being studied. When attempting to associate regions under selection with a phenotypic trait, it is imperative to be able to trust that the genome scan has performed well by minimising false positives and confirming regions of particular interest.

In this study two types of statistical test were used to identify signatures of selection. The first, based on the measure of genetic differentiation, F_{ST}, evaluates the within-population diversity with the between-population diversity of loci, where selected loci show reduced diversity within populations and increased diversity between populations (Storz 2005). The three F_{ST}-based tests identified similar numbers of markers differentiated among populations. However, only three were consistent in two of these tests and none were consistent across all three tests. Thus, it is important to consider the utility of each test for reducing false positives due to population history in order to determine whether the markers represent those under selection. For example, BayeScan and BayesFst account for locus- and population-specific effects of F_{ST} and are thus believed to be appropriate for most types of data as they are able to consider population structures, such as bottlenecks or complex hierarchical structure by accounting for population differences in F_{ST} (Beaumont & Balding 2004; Foll & Gaggiotti 2008; Excoffier *et al.* 2009). Therefore, these tests should produce the most reliable data across a range of scenarios, including analysis of a few populations (Nielsen *et al.* 2009; Hansen *et al.* 2010). However, in the present study, even Fdist2 should perform well. The drawback of this method is that demography is not taken into account. By showing that the stress lines had not experienced a bottleneck event, it was possible to eliminate at least one other source of reduced diversity and thus attribute more confidently the divergence found with Fdist2 to selection.

The second type of test identifies markers that show reduced diversity, either using heterozygosity (lnRH) or variance in microsatellite repeat number (lnRV). In contrast with the F_{ST}-based tests, lnRH and lnRV are suited to comparing pairs of populations and detecting recent, as opposed to ancient selection (Schlötterer 2003). Considering that the stress lines underwent intensive selection in the last four generations, this reduced diversity

test may be the most appropriate. Moreover, by examining pairs of populations, the population showing reduced diversity at a locus can be shown. For example, the locus OMM5261 and the locus confirming selection in this region, OMM1310, showed reduced diversity in the LR group as characterised by the selection regime in the current study (discussed further in Section *3.4.2*).

In addition to assessing the performance of the tests, thus increasing confidence that the regions were under selection, three regions showing signatures of divergence were investigated in an attempt to confirm that selection was occurring at these regions. Two of these three regions showed evidence with one extra marker. None of these regions were chosen based upon a strong signature of selection. That two of these regions were verified with extra testing shows that even the markers identified with weaker evidence warrant further investigation. Moreover, confirmation in these regions potentially indicates selective sweeps, where reduced diversity is found in the surrounding sequence of selected regions (Butlin 2010).

3.4.2 Candidate regions

Having assessed the suitability of this genome scan to accurately detect evidence for selection, the task is to now to determine whether these regions are associated with the selective breeding program and thus identify candidate genes associated with the trait. In this study, 17 loci that had responded to selection for the complex trait cortisol response to confinement stress were identified. Few studies perform genome scans based on strong selection for a known trait and those that do use natural populations (Bonin *et al.* 2009; Manel *et al.* 2009; Nunes *et al.* 2011). Therefore, the reasons behind selective differences between the populations may be less clear than lines of animals selected for a known trait. By using rainbow trout selected for the cortisol response to stress more confidence can be placed in the phenotypic relationship these markers have.

Furthermore, five regions under selection were from the same linkage groups as loci identified using QTL mapping for stress responsiveness from previous studies. That five of the outlier loci identified here corresponded with five (of twelve) QTLs is equivalent to the rate of correspondence in a previous study that integrated QTL data and a genome scan, with approximately 30% of directionally selected loci corresponding with QTLs (Rogers & Bernatchez 2005). I was thus able to confirm these five regions of selection. Drew *et al.* (2007) used amplified fragment length polymorphisms (AFLPs) to identify three linkage groups (RT23, RT27 and RT31) associated with the cortisol response to stress and body weight, which could indicate a high rate of false positive loci identified using the genome

scan. However, in a more recent study, using the same lines of HR and LR trout used here, twelve QTLs for the cortisol response to stress were more widespread across the genome, being found on twelve chromosomes (Quillet *et al.* 2010). Unfortunately, only loose associations between QTLs and regions under selection from the current study can be made, since AFLP QTLs cannot easily be located on a microsatellite linkage map and specific locations of QTLs other than on which chromosome the QTL lies have not yet been published. Still, this comparison has shown that both genome scans and QTL mapping are effective at discovering QTL and both studies are competent at identifying a number of candidates for further study. One particular candidate shown in this comparison is the EST-associated marker CA058580.

Moreover, two regions that were verified using extra markers form potential stress response-related candidates for further study. These two regions were chosen based upon differences in signatures of selection: one – OMM5261 – showed reduced diversity in one population (LR) compared with the other two populations; the second – OMM1762 – showed reduced diversity and increased genetic differentiation. This suggests that the selective breeding program produced different genetic responses. That OMM5261 and the confirming marker, OMM1310, both showed reduced diversity in the LR line suggests that this region may contain a gene specific to producing a low cortisol response to stress. Sequencing these regions may elucidate function.

In addition to identifying novel candidates in the form of selected genome regions, five functionally-related markers were investigated: three EST-associated markers and one aggression-related marker. In addition, fish were genotyped at markers linked to a candidate stress response gene, VACM-1. Two EST markers showed reduced diversity in both lines compared with the US strain, which suggests that these could be novel candidates for stress responsiveness in rainbow trout. However, the third EST marker was linked with a glycoprotein, which is generally implicated in many functions, such as immune response and, crucially, stress responsiveness. This marker was not under selection. Moreover, the locus OMM5308 (found within the VACM-1 sequence) was not under selection. This suggests that the selection identified at OMM5261, which is located on the same linkage group as OMM5308 – RT11 (Omy27) – is not associated with VACM-1. However, candidate genes for a trait may not be selected. Whilst important to the trait, its differential expression may be due to other factors. For example, melanism in the peppered moth, *Biston betularia*, is known to be associated with 16 candidate genes. A genome scan incorporating these genes failed to detect selection at these loci (van't Hof & Saccheri 2010). This suggests that other genes, such as transcription factors or other regulatory mechanisms

may be the subject of selection. This may mean that studying typical candidate genes associated with phenotypic variation may be less meaningful than studying pathways associated with a trait (Chapter 4). Generally, though, this approach has shown the utility of using both functionally-related markers and neutral markers.

3.4.3 Complexity of the stress response

Complex phenotypic traits typically involve many genes that can be widely distributed across the genome. The 17 outlier loci were distributed across 14 (of the 29) rainbow trout chromosomes and show a widespread distribution of loci associated with the selection programme in these lines. The detection of many regions suggests that the stress response is under polygenic control, as has been demonstrated previously for other complex traits (Zimmerman *et al.* 2004; Haidle *et al.* 2008). Selection for a trait, particularly when controlled by many genes, can cause a correlated response in other traits due to, for example, linkage and pleiotropy (Casto & Feldman 2011). These genomic effects remain to be assessed in rainbow trout. Nonetheless, substantial divergence between these lines of trout were detected and these regions were validated with additional loci.

The detection of balancing selection at two regions of the genome may be useful for understanding the effects of artificial selection for stress responsiveness on genetic variation. Balancing selection maintains genetic polymorphism of alleles at a locus (Oleksyk *et al.* 2010). The two regions under balancing selection in the present study may represent genes that are vital to the stress response phenotype, regardless of any divergence in strength of response. An obvious example would be that all rainbow trout require the ability to produce cortisol; therefore genes associated with producing functional cortisol are required by all individuals and would thus be present in all trout. However, balancing selection often represents frequency-dependent selection, where the maintenance of genetic polymorphism is required for variable environmental factors. For example, *Drosophila melanogaster*, possessing one of two alleles for the foraging gene *for,* that are raised in low nutrient conditions show frequency-dependent fitness, where both alleles confer fitness at low frequencies in the population. This is not seen in high nutrient conditions (Fitzpatrick *et al.* 2007). This kind of frequency-dependent selection may be occurring at genes associated with selection for stress responsiveness. Moreover, since selection for divergent stress responsiveness has caused divergence for aggressive behaviour and growth rate (Pottinger & Carrick 2001; Øverli *et al.* 2002b; Pottinger 2006), further study of these regions may aid understanding of pleiotropic effects of genes in behavioural syndromes where different behaviours correlate to produce a specific phenotype.

3.4.4 Conclusions

A genome scan approach was used to quantify the number of specific genomic regions directly associated with the stress response and identified 17 candidate loci, distributed across 14 chromosomes, 19 of which were identified as being under directional selection and two under balancing selection. Five of these loci were associated with QTLs for the cortisol response to stress (Drew *et al.* 2007; Quillet *et al.* 2010) and a further two regions were confirmed as having evidence for selection by investigating the regions with extra microsatellites. These regions may be associated with genes with a low phenotypic effect (Wen *et al.* 2009) or genes with pleiotropic effects (Barendse *et al.* 2009), but further study is needed to confirm or refute these possibilities, which is more difficult given the absence of a sequenced genome. However, even without functional information, the loci identified in this study provide a basis with which to understand the genetic consequences of selective breeding for the cortisol response to stress. Moreover, these markers could be used for future study of selection in these genomic regions in wild rainbow trout populations. In addition, using known lines of rainbow trout with selection for a known trait, these results show that, despite drawbacks associated with genome scans, they can successfully detect selection of a complex trait.

Chapter 4 Behavioural genomics: *de novo* transcriptome sequencing of aggressive behaviour

4.1 Introduction

Aggression is an important behaviour that can increase individual fitness, for example by facilitating parental protection of offspring, acquisition of mates, territories or dominance status (Huntingford & Turner 1987). In farmed animals, aggression may have economic consequences, since repeated agonistic encounters may elevate stress in subordinates and, therefore, may increase concerns for animal welfare by reducing growth or impairing immune and/or reproductive function (Pickering 1990; Campbell *et al.* 1992; de Kloet *et al.* 2005). As an evolutionarily and economically important behaviour, there is much interest in understanding the genetic architecture (*e.g.* the number or effect of genes) and thus the underlying genetic mechanisms associated with this complex phenotype.

Indeed, characterising transcriptomes has shown that aggressive behaviour was associated with the differential expression of many (>100) genes in diverse taxa such as honey bees, *Apis mellifera*, (Alaux *et al.* 2009), in hens, *Gallus gallus* (Buitenhuis *et al.* 2009) and in rainbow trout, *Oncorhynchus mykiss* (Sneddon *et al.* 2011). In addition, there may be as yet unidentified genes that have a role in the expression of complex behaviours. By associating transcriptome profiles with aggression, it is possible to determine functional pathways, along with the number of expressed genes, which may incorporate novel candidates, implicated in aggressive behaviour.

4.1.1 Genetics of aggression

Aggressive interactions in salmonids, determine those individuals that become dominant. In wild fish, this influences which individuals gain territories and the associated resources, which can increase individual fitness (Huntingford & Turner 1987). Similarly, in aquaculture, rainbow trout form dominance hierarchies, where dominant fish monopolise food. In both cases, once dominance is established, dominant individuals show distinct behavioural and physiological differences relative to subordinates (Sloman & Armstrong 2002). For example, subordinate Arctic char, *Salvelinus alpinus*, show a reduced number of aggressive attacks once a hierarchy was established. Moreover, subordinate *S. alpinus* showed higher hypothalamic serotonin metabolite: serotonin ([5-HIAA]/[5-HT]), ratios. Aggression is a trait behaviourally and physiologically correlated with boldness and stress responsiveness in a behavioural syndrome (Section *2.1.2*). Physiological correlations with stress responsiveness include plasma cortisol levels, where subordinate rainbow trout and brown trout, *Salmo trutta,* exhibit elevated plasma cortisol levels (Sloman *et al.* 2001). As

such, aggressiveness also shows similarities in other HPI components, for example proopiomelanocortin (POMC) is upregulated in subordinate rainbow trout (see Sections *1.2.1* and *1.2.2*). Differences in physiology between dominant and subordinate fish indicate that the ability to gain dominance, thus the associated benefits, through aggressive behaviour may be under a genetic influence. Indeed, understanding this link between behavioural phenotype and the underlying genetic mechanisms is the focus of many studies in behavioural ecology.

Phenotypic differences in aggressive behaviour have been attributed to genotypic differences in candidate genes as well as putative levels of inbreeding as measured by genome-wide heterozygosity (see Chapter 2). For example, aggressive and less aggressive rainbow trout, *Oncorhynchus mykiss*, are homozygous for different alleles of the major histocompatibility complex Ia (MHC Ia) and intermediate behavioural types are heterozygous (Azuma *et al.* 2005). Since genotypic differences in genome-wide heterozygosity indicate a genetic component of aggressive behaviour, this may mean that there are complex transcriptional changes that occur during aggressive behaviour (Chapter 4). Indeed, the relationship between genotype at important behaviour-related candidate genes and the transcript expression of these genes is evident in a range of animals. For example, reduced transcript expression of the candidate gene for the enzyme tryptophan hydroxylase 2 (*Tph2*), which is involved in the synthesis of serotonin, was related to a gene mutant for in *Tph2* in mice, *Mus musculus* (Kulikov *et al.* 2005). Identifying a specific transcriptional difference that is linked with a difference in aggressive behaviour can aid the understanding of how aggressive behaviour is generated. However, complex phenotypes often show complex associations with many genes (Robinson 2004; Robin *et al.* 2007). Indeed, Edwards *et al.* (2009b) showed that nine genes were differentially expressed between aggressive and less aggressive *Drosophila melanogaster*. These studies have made it clear that many genes are involved in aggressive behaviour, thus interest has grown in the association of hundreds of genes in expression profiling.

4.1.2 Transcriptome profiling

Determining transcriptome profiles may allow the identification of gene pathways and novel candidate genes associated with complex phenotypes, such as aggressive behaviour. Recently, the use of microarrays in behavioural ecology has shown its utility by estimating the huge number of genes and associated pathways, which are differentially expressed in aggression (Anholt & Mackay 2004; Bell & Aubin-Horth 2010) and also identifying novel candidate genes. For example, Renn *et al.* (2008) identified 171 genes differentially expressed between dominant and subordinate African cichlids, *Astatotilapia burtoni.*

Moreover, the number of genes expressed in the honey bee, *Apis mellifera,* was associated with the level of involvement in colony defence and thus with aggressive behaviour (Alaux *et al.* 2009). In addition, the identity of differentially expressed genes – many were involved in regulation of aggression – indicates that typical candidate genes, such as that which codes for the neurotransmitter serotonin, may lack the ability to describe phenotypic variation in this trait. Therefore, novel candidates may need to be explored in order to discover detailed mechanisms involved in expressing behaviour. Indeed, a microarray, to profile transcriptomes of dominant and subordinate rainbow trout, *Oncorhynchus mykiss,* identified the novel candidate for dominance, ependymin, which was previously overlooked (Sneddon *et al.* 2005). Whilst microarrays have allowed the investigation of thousands of genes, they are limited by the need to use gene probes that are known *a priori*. Therefore, completely novel candidate genes cannot be identified in this way.

Advances in high through-put sequencing, such as the 454 GS FLX (Roche), have allowed sequencing of huge amounts of DNA by sequencing many DNA segments in parallel. Such next generation sequencing (NGS) technologies allow *de novo* sequencing of transcriptomes. Not only can NGS technologies match the ability of microarrays to identify differentially expressed genes, but it can also surpass this ability by revealing previously unsequenced gene transcripts which are associated with behaviour (Hudson 2008). Moreover, these transcripts can be associated with underlying mechanisms of phenotypic traits (Wilhelm & Landry 2009). Indeed, NGS has led to the discovery of candidate genes of phenotypic traits, such as those in response to salinity in the dinoflagellate *Oxyrrhis marina* (Lowe *et al.* 2011) and in birdsong in zebra finches, *Taeniopygia gutatta* (Lovell *et al.* 2008). In addition, the ability of NGS technologies to rapidly sequence novel gene transcripts means that they are a crucial tool for studying ecological and evolutionary questions in non-model organisms (Hudson 2008). Moreover, NGS can be used to generate genomic resources in the form of transcript sequences, where a genome sequence does not exist. For example, expressed sequence tag (EST) libraries were generated using NGS in relation to reproductive status in the paper wasp, *Polistes metricus* (Toth *et al.* 2007) and in the liver of rainbow trout, *Oncorhynchus mykiss* (Salem *et al.* 2008). By applying NGS to behavioural ecology questions, it may be possible to determine the numbers and identities of genes associated with complex behaviour. Moreover, these sequences will contribute to EST databases of non-model organisms.

In the rainbow trout, aggression is an important trait for both wild and domestic populations. Recent studies have shown evidence for a relationship between genes and aggressive behaviour: for example, the candidate genes MHC Ia (Azuma *et al.* 2005) and ependymin

show a relationship with aggression (Sneddon *et al.* 2011); differential expression of over a thousand genes characterised in a transcriptome were associated with dominance status (Sneddon *et al.* 2011); and genome-wide heterozygosity was higher in aggressive trout than in less aggressive trout (Chapter 2). This work suggests that there is a clear, but complex genetic component of aggressive behaviour that is best quantified using next generation sequencing to make an unbiased assessment of the differentially expressed genes involved with the expression of this behaviour: a feat not possible with microarrays because gene probes for microarrays must be known. Moreover, rainbow trout genomic resources are increasing but limited: these exist in the form of ESTs (Govoroun *et al.* 2006), a microsatellite linkage map (Rexroad *et al.* 2008), an in-progress BAC map (Palti *et al.* 2009) and a transcriptome characterisation of the liver (Salem *et al.* 2010). However, sequencing of the whole genome, despite the advances in NGS that offer rapid and large amounts of sequence data to be obtained, is not yet complete and novel genomic resources are therefore welcome. The present study will use NGS to sequence the transcriptome, and thus differentially expressed transcripts, of aggressive and less aggressive rainbow trout. Moreover, these differentially expressed genes, including the identification of previously uncharacterised genes, may present novel candidates for further study in behavioural genetics. Finally, these findings will contribute to genomic resources of the rainbow trout. I hypothesise that genes previously known to have a role in aggression will be differentially expressed between individuals with different rates of aggression. This is the first study to use NGS to address a behavioural question in vertebrates and thus assign *de novo* transcriptome profiles to a complex phenotypic trait.

4.2 Methods

4.2.1 Rainbow trout husbandry

Rainbow trout, *Oncorhynchus mykiss*, used in this chapter were those assessed for their aggressive behaviour in chapter 2. All experiments were conducted in a humane manner according to Home Office UK licensing and after local ethics approval. Rainbow trout were transported from a commercial supplier to the aquaria at Liverpool on 11/08/2010 (n=100, approximate weight=90 g) and 25/10/2010 (n=70, approximate weight=90 g). The trout were kept in two separate holding tanks (2x2x0.5 m; 2000 L) supplied with aerated recirculating freshwater (semi-closed system) at 13°C±2°C, with a 14:10 hr light:dark regime, similar to ambient. Trout were fed commercial pellets (Skretting, UK) at a rate of 1% body weight daily according to manufacturer's recommendations. Fifteen pairs of fish, one from each tank, were caught at random using a hand net, anaesthetised in 33.3 μgml^{-1} benzocaine (Sigma, UK) in a 25L bucket to minimise stress during weighing; fish were weighed to 0.01 g and transferred to experimental tanks (90x45x30cm; 200L) at 11±1°C,

which were arranged in three semi-closed recirculating systems (*n*=15 tanks) with aerated freshwater and were screened from visual disturbance. Each tank contained two fish of equal weight (±10%), so as not to influence the outcome of the interaction as relatively larger fish tend to be dominant (Huntingford *et al.* 1990; van Leeuwen *et al.* 2011). One fish was placed in each of two compartments, halved by an opaque divider. Fish were fed daily in each compartment at the same rate as in the stock tanks.

4.2.2 Behavioural tests

Once fish resumed feeding for at least 7 days (reduced feeding is a standard indicator of stress (Carr 2002)), the tests for aggressive behaviour began. All interactions were conducted between 12:00 and 14:00 to minimise any effects of diurnal variation in physiology. At least 15 min prior to each experiment low light level cameras that were linked to a remote monitoring system were installed at the front of the tanks to allow fish to minimise disturbance immediately preceding the behavioural trial. Each experiment began by removing the divider (using a pulley system to prevent fish being aware of the operator), after which the pairs were allowed to interact for a maximum of 15 min. An opaque tube (160x90x90 mm) was supplied as a refuge for each fish, so that they could opt to remain out of sight of their opponents and thus any interactions were conducted voluntarily such that the stress of these encounters were minimised. The videos of the interactions were assessed blind to determine the amount of aggression that each fish performed. The total number of aggressive acts were determined by measuring the following three types of aggressive behaviour: (1) displacement, where one fish moves slowly towards its opponent and the opponent retreats in response; (2) chase, where one fish moves rapidly towards its opponent resulting in either contact between the two fish or the opponent retreating; (3) circling, where both fish circle each other. Aggressiveness is considered dependent upon the degree of aggressiveness exhibited by an opponent (Haller *et al.* 1996; Neat *et al.* 1998; Dugatkin & Druen 2004); Appendix 1, Section A1.1). To account for this, an aggressiveness score for each fish was calculated by subtracting the total number of aggressive actions of its opponent from the total number of acts of the fish. Fish with the highest aggressive score were defined as “aggressive” (*n*=15) whereas those with the lowest aggressive score were “less aggressive” (*n*=15). Dividers were replaced and each trout humanely killed at the end of the interaction period using concussion and exsanguination. Fish were weighed, sexed and the brain (including the pituitary) collected and frozen at -80°C. The brains of the five most aggressive and the five least aggressive were used as material to generate a transcriptome.

4.2.3 cDNA Library preparation

RNA was extracted from brain tissue using Trizol™ following the manufacturer's instructions (Invitrogen, UK) and homogenised using a TissueLyser (Qiagen, UK) for 2 minutes at 30Hz. RNA was purified using Ambion® PureLink™ RNA Mini Kit (Invitrogen, UK). For first strand synthesis, a 20µl reaction mix comprising 2.7µg of starting RNA, 1pmol each of 3' SMART^{TM} CDS Primer II A (5'-AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$VN-3') and SMART^{TM} II A Oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3') (Clontech), 2mM of each dNTP and template RNA. This was incubated at 65°C for 5 min before being placed on ice. 4µl of 5X First strand buffer (Invitrogen) and 0.1 mM DTT (Invitrogen) were added to each well. This was incubated at 42°C for 2 min and 400 units of Superscript II RT (Invitrogen) was added. This was incubated at 42°C for 50 min and at 70°C for 15 min. For PCR a reaction mix comprising 200 µM dNTPs, 0.5 µM 5' PCR II A (5'-AAGCAGTGGTATCAACGCAGAGT-3'), 0.02 u µl^{-1} Advantage DNA Polymerase and 1 µl first strand cDNA. PCR conditions were 98^{o}C for 30 s, 16X [98^{o}C 10 s, 72^{o}C 6 min], 72^{o}C 10 min. The reaction was purified using Agencourt® Ampure® beads (Beckman Coulter, New England BioLabs).

Individual samples were pooled into aggressive and less aggressive behavioural treatments and each pool of cDNA was quantified using a Qubit® Fluorometer (Life Technologies, UK). Approximately 2.5 µg of cDNA for each pool was submitted for library construction and sequencing, with the cDNA samples multiplex identified (MID-tagged) and then pooled for sequencing using half GS454flx Titanium (Roche) sequencing run at the Centre for Genomic Research, University of Liverpool, UK (www.liv.ac.uk/genomic-research/).

4.2.4 Differential gene expression

The analysis for differential gene expression required the two samples to be assembled together to obtain contigs that were contributed to by both samples. In this way, the number of reads from each sample that contributed to a contig may be obtained. To identify genes that were differentially expressed between aggressive and less aggressive rainbow trout, the reads generated from 454 sequencing were assembled using CAP3 (Huang & Madan 1999), with parameters altered for an increase in stringency, such that quality value cutoff was 15, depth of good coverage was 4, match score factor was 3, mismatch score factor was -6, gap penalty factor was 8, overlap length cutoff was 50 and overlap identity was 95%. Sequencing reads with a quality score lower than 15 and which were shorter than 100bp were removed prior to assembly. In addition, these reads had SMART adaptor and MID-tag

sequences removed prior to assembly. Contigs that existed in only one sample were discarded to reduce the instance of false positives in differential gene expression analysis.

The overall read abundance was normalised (read number) for variation in read length by dividing the counts by the length of the contig. This accounts for higher read counts in longer transcripts (Oshlack *et al.* 2010). The program IDEG6 (Romualdi *et al.* 2003) was used to obtain differential gene expression by using the read number of each sample to calculate a measure of gene expression. The level of gene expression for each contig was compared between aggressive and less aggressive rainbow trout. To allow this comparison, the read number of each sample was normalised for library size by adjusting for the total read number in the respective libraries. This accounts for greater numbers of reads in each gene if the library is sequenced to a greater depth (Oshlack *et al.* 2010). The normalised read numbers assessed for differential expression using Audic and Claverie, Fisher exact and Chi-squared tests, with a Bonferroni correction applied to maintain a type 1 error rate of $p<0.05$. Because of this nature in which gene expression is calculated, IDEG6 only identifies contigs that are upregulated in one sample compared to the other, but it cannot calculate downregulation.

Annotation of the differentially expressed contigs was carried out in two ways. First, the combined assembly was submitted to BLAST the translated contig sequences against the zebrafish Uniprot database (accessed in October 2009) with an e-value cut-off of $1e^{-5}$ using blastall function of BLAST (Altschul *et al.* 1990). Second, translated contig sequences were submitted against the NCBI non-redundant (nr) protein database (accessed in May 2012) using the BLAST step in Blast2GO® (Conesa *et al.* 2005) with default settings. The BLAST results of the differentially expressed genes were inspected to ensure that each contig was associated with only a single gene (i.e. to correct for partial contig and transcript variants) and where two or more contigs aligned with the same gene, the read data were combined to enable more accurate representation of gene expression. Sequences that were not identified in these two ways were individually submitted against the NCBI non-redundant (nr) protein database (accessed in December 2012) using the blastx function. In addition, these sequences were submitted against the NCBI non-redundant (nr/nt) nucleotide collection (accessed in December 2012) using blastn.

4.2.5 Gene Ontology of aggression

To obtain the function of genes expressed in aggressive and less aggressive trout, I identified Gene Ontology (GO) terms (Liinamo *et al.* 2007) for the differentially expressed genes and conducted an enrichment analysis of the sequences from separately assembled samples.

Blast2GO® runs three steps to obtain annotated sequences. The first is alignment using BLAST to assign translated contig sequences to proteins; the second is mapping, which associates BLAST hits, *i.e.* gene names, with GO terms; and the third is annotation, to assign the most specific GO term to the given sequence. To identify GO terms of the differentially expressed genes, the sequences were aligned, mapped and annotated in Blast2GO®. Gene Ontology terms comprise three vocabularies of terms describing the characterisation and annotation of gene products: biological processes, cellular components and molecular functions. These are structured in many levels to identify gene function from broad to narrow sense.

Whereas the analysis for differential gene expression required the two samples to be assembled together, the enrichment analysis of differential GO terms, implemented in Blast2GO® required the samples to be assembled separately, since the test counts the number of contigs assigned to GO terms from each sample and then compares GO terms between samples. To obtain separately assembled samples, the sequence reads from each sample were quality trimmed to remove low quality sequences and had SMART primers and MID tags removed before being assembled separately using Newbler (454 Life Sciences, www.454.com) by the CGR (www.liv.ac.uk/genomic-research/) University of Liverpool, UK. The assemblies were combined into a single FASTA file and uploaded into Blast2GO®.

Following alignment, mapping and annotation in Blast2GO®, the enrichment analysis using GOSSIP (Blüthgen *et al.* 2005) was applied to the data. GOSSIP is a function to identify GOs that are over- or under-represented in one sample in comparison with another. To implement this, Blast2GO® was given a list of contig names from the aggressive sample, which was used as the reference sample and a list of contig names from the less aggressive sample, which was used as the test sample. Using Fisher's exact test, Gossip then identified GO terms, and thus functions, which are under- or over-represented between aggressive and less aggressive trout to a false discovery rate of 0.05.

4.3 Results

4.3.1 Behaviour

Aggressive individuals performed about 45% more aggressive actions against their opponent (n=15, 49.3±9.51 acts) than did their less aggressive counterparts (n=15, 33.6±8.18 acts; Figure 2.1), with no apparent effect of stock origin upon aggressive behaviour (χ^2=0, df=1, p=1.00; Appendix 1, Table A3).

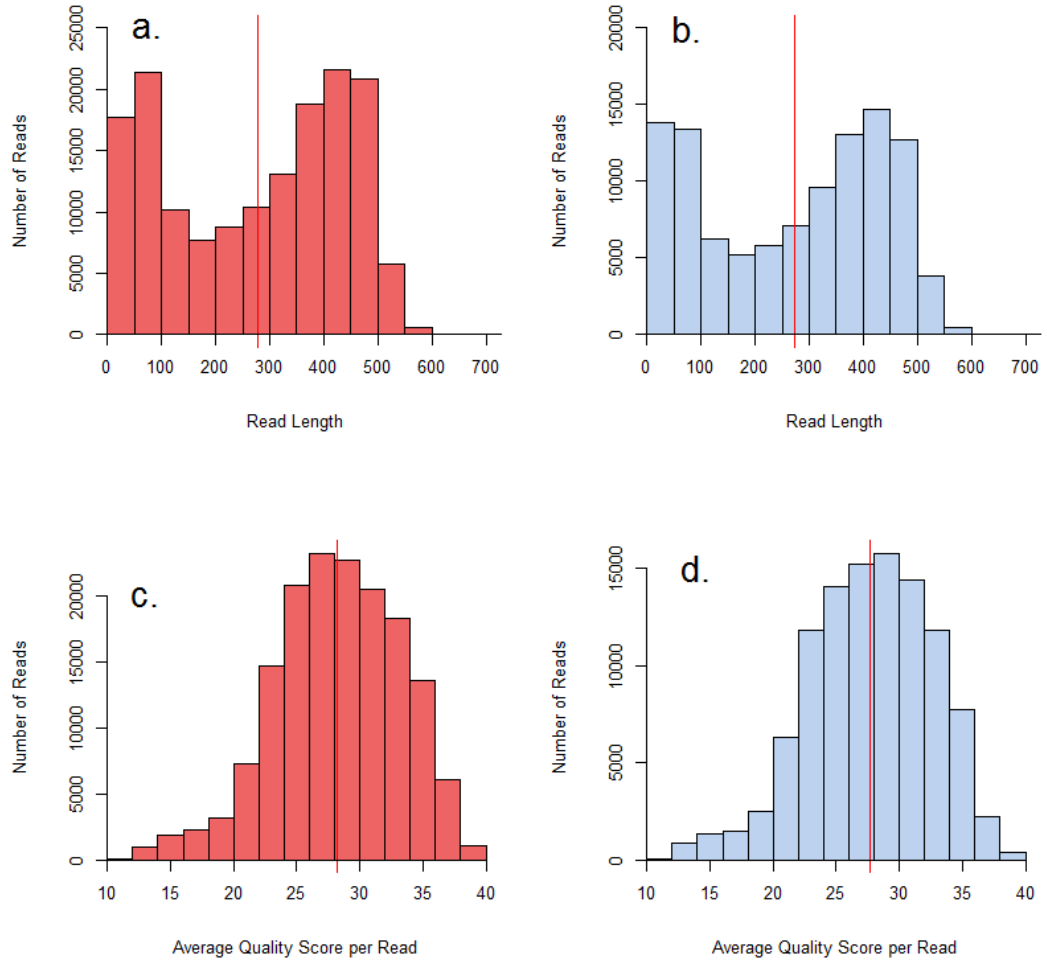

Figure 4.1. Pyrosequencing read information of aggressive (red) and less aggressive (blue) *Oncorhynchus mykiss*, showing the distributions of read lengths (a. and b.), where red lines indicate median read lengths. The average quality score of reads is shown for aggressive (c.) and subordinate (d.). Red lines indicate mean quality score per read, which is 29.41 for aggressive and 28.96 for less aggressive.

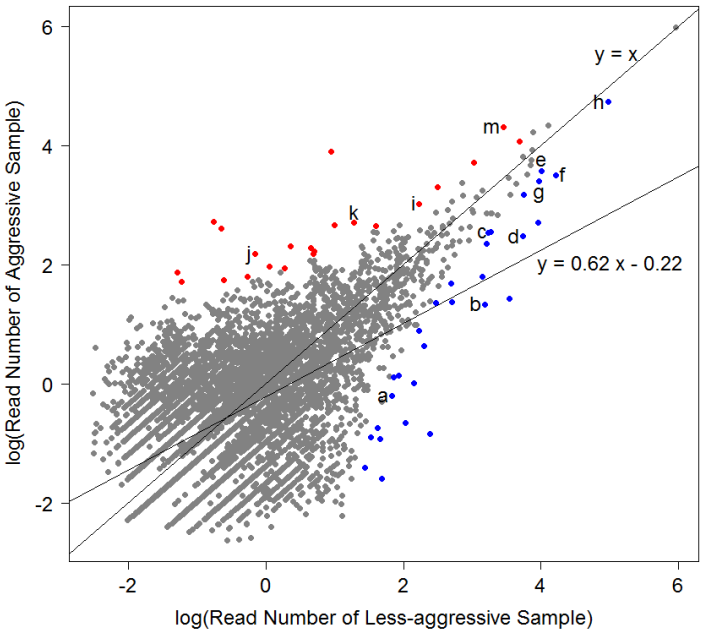

Figure 4.2. Expression of contigs of aggressive and less aggressive *Oncorhynchus mykiss* as measured by the normalised number of reads that each sample contributed to a contig. Contigs that were significantly differentially expressed after sequential Bonferroni correction ($P<0.05$) are shown in red (upregulated in aggressive individuals) or blue (upregulated in less aggressive individuals). Letters indicate contigs that have been nominally identified by BLAST (see Table 1 for identities). Regression lines show y=x (*i.e.* equal expression) and y=0.62x-0.22, which is the empirical relationship between genes expressed in aggressive and less aggressive trout.

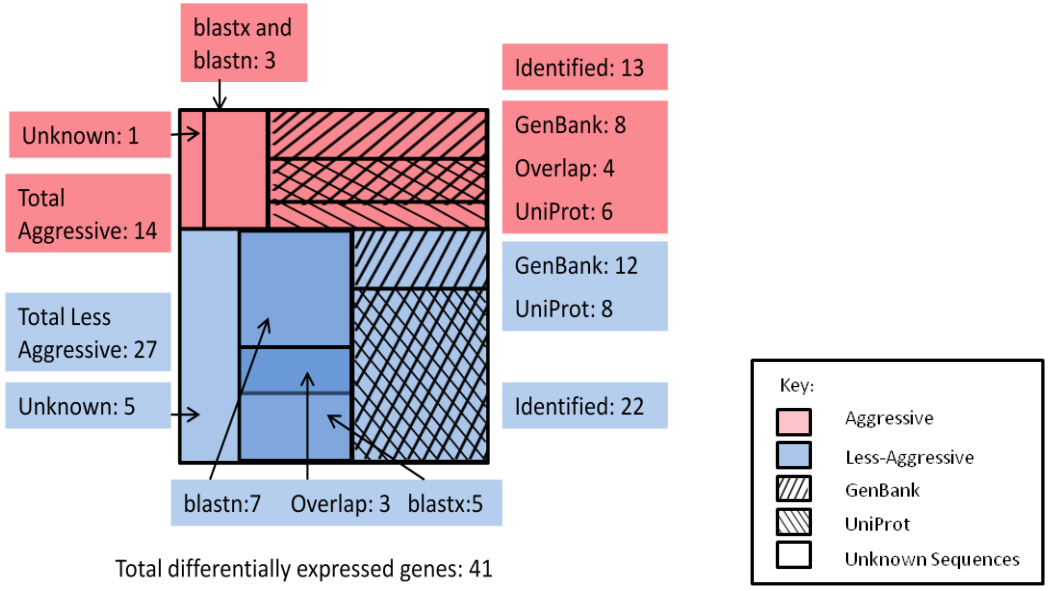

Figure 4.3. Diagrammatic representation of the 41 differentially expressed genes in aggressive and less aggressive *Oncorhynchus mykiss*. Each unit area represents one gene. The categories show all the sequences that were unknown and identified from both the non-redundant GenBank (right hatched) and *Danio rerio* UniProt (left hatched) databases and sequences that were unidentified (no hatching). Also shown are the sequences identified by individual blastn and blastx.

The categorical assignment of aggression means that, in a pairwise contest, the aggressive individual always performed significantly more aggressive acts than the less aggressive (paired t test: t=6.11, df=14, $p<1x10^{-4}$). To assess the effect of sex upon the level of aggression, a linear mixed effects model was fit to the data in R (R Development Core Team 2009) with aggressiveness and sex as fixed effects and the pairing of individuals as a random effect, which showed that there was no difference in aggression between males and females (F_{12}=0.687, p=0.505; Appendix 1, Figure A1). Additionally, differences in weight were minimised to 8.9±6.3% difference, as body size can affect the outcome of interactions. These minor differences in weight did not affect the number of aggressive acts, thus the outcome of the interaction (paired t-test: t=-0.67, df=31, p=0.509; Appendix 1, Figure A2).

4.3.2 Gene expression and candidate genes

The 454 sequencing run yielded a total 262,805 reads (and some 72,527,691 base pairs), of which 156,902 (43,478,246 bp) were from aggressive trout and 105,903 (29,049,445 bp) were from less aggressive trout. Both samples had a high proportion of short (*i.e.* less than 200 bp) reads with average quality scores of 29.41 and 28.96 respectively (Figure 4.1). After trimming to remove SMART primers, MID tag sequence and to remove the short and low-quality reads, there were 226,716 reads remaining. The CAP3 assembly generated 63,588 singletons and 20,070 contigs of which 8,434 were retained after removal of the contigs that had zero reads from one of each sample. Mean contig length was 599 bp with a median read number of 5 (ranging from 2-4,490 reads).

Forty-one contigs were significantly ($p<1x10^{-5}$) differentially expressed between aggressive and less aggressive fish (Figure 4.2), as identified with all 3 tests (*i.e.* Audic and Claverie, Fisher exact and Chi-squared tests) implemented by IDEG6. Fourteen of these contigs were upregulated (1.47<88.0 fold-difference) in the aggressive trout, whereas twenty-seven contigs were upregulated (1.28<27.0 fold-difference) in the less aggressive trout. In the aggressive trout ten contigs (2.19<88.0 fold-difference) were identified by BLAST against either the zebrafish protein or the nr protein database and in the less aggressive trout twelve (1.28<26.8 fold-difference) were identified by BLAST (Table 4.1). The two databases produced BLAST hits of similar proportions: the Genbank nr database identified eight genes in aggressive and twelve upregulated in less aggressive trout (Figure 4.3). Similarly, using the *Danio rerio* UniProt database six genes upregulated in aggressive and eight in less aggressive trout were identified (Table 4.1).

Table 4.1. BLAST matches of differentially expressed genes in aggressive and less aggressive rainbow trout against two databases: non-redundant protein sequences (nr) and UniProt *Danio rerio* database. Presented are accession number, contig ID and gene ontology number and term of differentially expressed genes identified using *Danio rerio* Uniprot database and Genbank nonredundant database of aggressive and less aggressive *Oncorhynchus mykiss.*

Sample	Gene name	Accession number	GO number for nr genes	GO term for nr genes	Letter on Graph	Aggressive normalised read	Less Aggressive normalised	Fold Difference	Database	Contig ID(s)
Aggressive upregulated	Hemoglobin subunit alpha-1	NP_001118023	GO:0019825	Oxygen binding	i, m	94.7	40.7	2.33	nr, UniProt	13590, 4845
	rRNA intron-encoded endonuclease	BAD18907				41.2	20.4	2.02	nr	16600
	Glutathione peroxidase 1	NP_001117997	GO:0006979	Response to oxidative stress	k	15.1	3.7	4.08	nr, UniProt	18861
	Proopiomelanocortin a precursor	NP_001118190			j	13.6	0.5	27.2	nr	15748
	Proopiomelanocortin b precursor	NP_001118191	GO:0005179	Hormone activity		8.8	0.1	88.0	nr, UniProt	12723
	Olfactory receptor family c subfamily 2 member 2	AEB77813				6.4	0.2	32.0	nr	12527
	Neuromedin s	ACJ64069				5.5	0.2	27.5	nr	5108
	PREDICTED: hypothetical protein LOC793455	XP_001333219.1				49.7	2.7	18.4	UniProt	8433
	PREDICTED: hypothetical protein LOC335618	XP_001920014.1				15.3	0.5	30.6	UniProt	8068

Sample	Gene name	Accession number	GO number for nr genes	GO term for nr genes	Letter on Graph	Aggressive normalised read	Less Aggressive normalised	Fold Difference	Database	Contig ID(s)
Less Aggressive upregulated	NADH dehydrogenase subunit 4	ABB78034	GO:0070469	Respiratory chain	b, g	34.1	76.9	2.26	nr, UniProt	6905, 1672
	Nadh dehydrogenase subunit 2	ABB78065	GO:0070469	Respiratory chain	h	113.4	145.6	1.28	nr, UniProt	19434
	Fatty acid-binding heart	ACI66303	GO:0008289	Lipid binding	c	12.9	26.3	2.04	nr, UniProt	2679
	Fatty acid-binding brain	ACI66833	GO:0008289	Lipid binding	f	33.3	67.9	2.04	nr, UniProt	8480
	Cytosolic non-specific dipeptidase	ACI34318	GO:0016805	Dipeptidase activity	d	11.1	41.9	3.77	nr, UniProt	5566
	60s ribosomal protein l7	ACH70988	GO:0030528	Transcription regulator activity	e	35.9	55	1.53	nr, UniProt	15885
	Transposase [Salmo salar]	ABV31710				0.2	4.1	20.5	nr	4344
	Serologically defined colon cancer antigen 1 homolog	NP_001167106				0.6	7.5	12.5	nr	529
	Predicted protein [Nematostella vectensis]	XP_001624684				0.4	10.7	26.8	nr	809
	Overexpressed breast tumor protein homolog	ACI68525				0.4	4.6	11.5	nr	18524
	Neurofilament medium polypeptide	NP_001158883	GO:0005198	Structural molecule	a	0.7	6.3	9.00	nr, UniProt	3709

Table 4.2. Sequences that remained unidentified using BLAST2GO and blastall were submitted individually to BLAST using both protein and nucleotide databases. Relative expression, fold difference, gene identity and accession numbers are shown for sequences that were differentially expressed between aggressive and less aggressive *Oncorhynchus mykiss*.

Sample	Contig Number	Aggressive normalised read number	Less Aggressive normalised read number	Fold Difference	blastx top hit	blastx accession number	blastn top hit	blastn accession number
Aggressive upregulated	Contig15933	10.1	1.5	6.73	UPF0527 membrane protein [*Salmo salar*]	ACI68813.1	*Salmo salar* clone ssal-rgf-525-176 unknown large open reading frame mRNA, novel cds	BT072433.1
	Contig16293	6.1	0.7	8.71	NADH dehydrogenase 1 alpha subcomplex subunit 1 [*Salmo salar*]	ACI66222.1	*Oncorhynchus mykiss* NADH dehydrogenase 1 alpha subcomplex subunit 1 (ndua1), mRNA complete cds	NM_001160667.1
	Contig2115	7.2	0.1	72.00	PREDICTED: similar to predicted protein [*Hydra magnipapillata*]	XP_002161911.1	*Salmo salar* clone BAC CHORI214-114L13 von Willebrand factor A domain containing 5A (VWA5A) gene, complete cds	FJ969488.1
Less Aggressive upregulated	Contig19318	15.1	52.1	3.45	Cytosolic non-specific dipeptidase [*Salmo salar*]	ACI34318.1	*Salmo salar* clone ssal-rgf-540-171 Cytosolic non-specific dipeptidase putative mRNA, complete cds	BT046056.1
	Contig6671	23.9	42.4	1.77			Zebrafish DNA sequence from clone DKEY-69N2 in linkage group 11, complete sequence	CR855274.12
	Contig5244	4.2	34.6	8.24	Cytosolic non-specific dipeptidase [*Salmo salar*]	ACI34318.1	*Salmo salar* clone ssal-rgf-540-171 Cytosolic non-specific dipeptidase putative mRNA, complete cds	BT046056.1

	Contig Number	Aggressive normalised read number	Less Aggressive normalised read number	Fold Difference	blastx top hit	blastx accession number	blastn top hit	blastn accession number
Less Aggressive upregulated	Contig3866	12.7	25.6	2.02			*Salmo salar* clone ssal-rgf-512-275 Probable glutamate receptor precursor putative mRNA, complete cds	BT059568.1
	Contig13575	6.1	23.4	3.84	Cytosolic non-specific dipeptidase [*Salmo salar*]	ACI34318.1	*Salmo salar* clone ssal-rgf-540-171 Cytosolic non-specific dipeptidase putative mRNA, complete cds	BT046056.1
	Contig285	3.9	11.7	3	PREDICTED: protein ETHE1, mitochondrial-like [*Equus caballus*]	XP_001916928.1		
	Contig2338	1.1	6.8	6.18			*Salmo salar* clone ssal-rgf-502-146 Vesicle-associated membrane protein-associated protein A putative mRNA, complete cds	BT059404.1
	Contig8559	1.1	6.3	5.73	PREDICTED: PEX5-related protein-like [*Oreochromis niloticus*]	XP_003440010.1	Salmo salar IgH locus A genomic sequence	GU129139.1
	Contig5703	0.4	5.4	13.5			*Salmo salar* DNA damage-binding protein 1 (ddb1), mRNA complete cds	NM_001165380.1
	Contig2044	0.6	5.1	8.50	ribosome small subunit-dependent GTPase A [*Prevotella tannerae* ATCC 51259]	ZP_05735056.1		

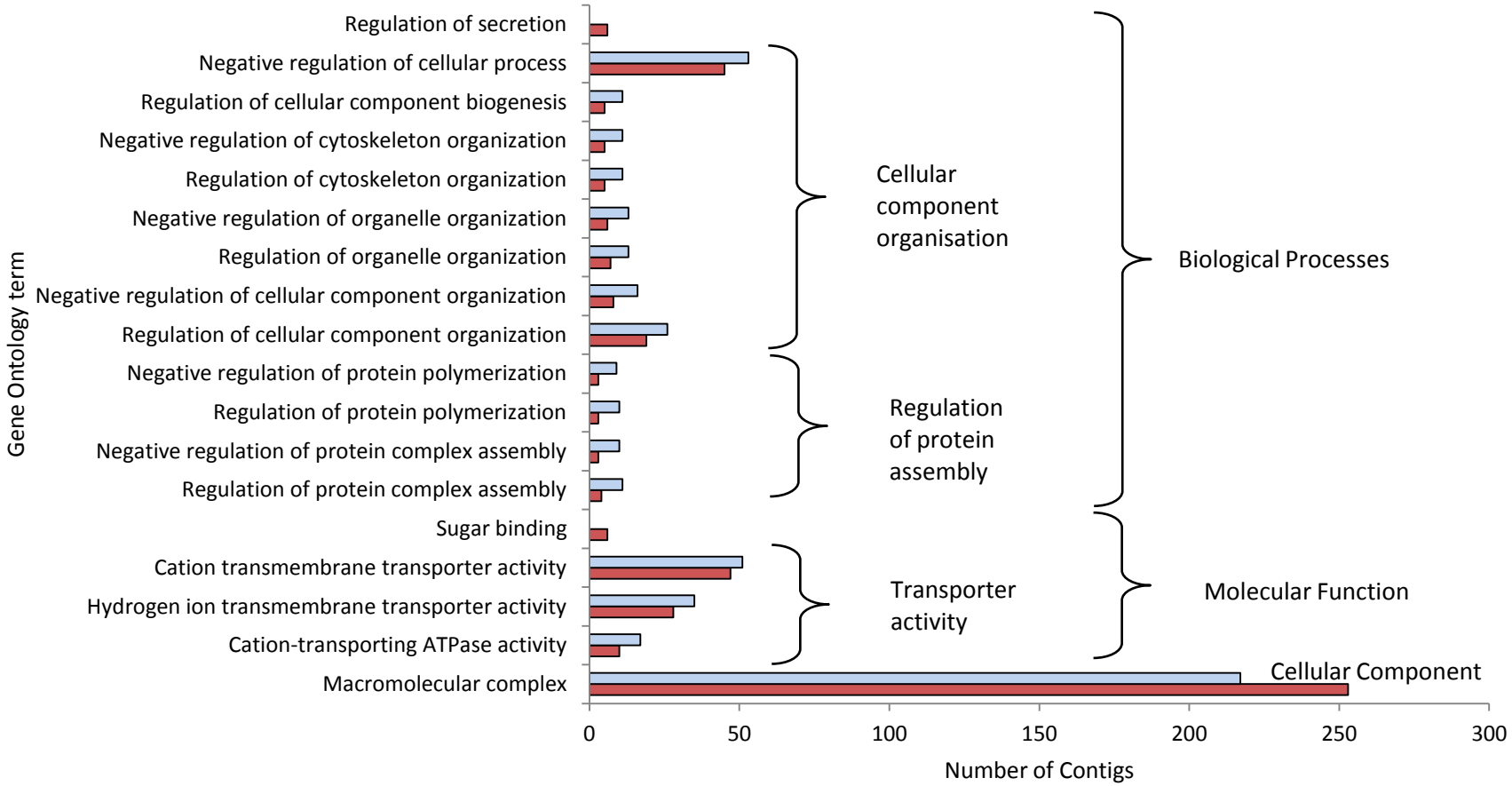

Figure 4.4. Gene Ontology terms that are under- or over-represented between the transcriptomes of aggressive (red) and less aggressive (blue) *Oncorhynchus mykiss*, as identified using GOSSIP implemented in Blast2GO®. Also shown are higher level GO terms.

There were two instances where two contigs matched the same BLAST hit: haemoglobin subunit alpha 1, which was upregulated in the aggressive trout (contig 13590: 2.36 fold-difference; contig 4845: 2.19 fold-difference) and NADH dehydrogenase subunit 4, which was upregulated in the less aggressive trout (contig 19434: 1.28 fold- difference; contig 1672: 1.75 fold-difference). In addition, thirteen contigs were submitted to BLAST individually and identified using either blastx or blastn (Appendix 1; Table A8). Three were upregulated in the aggressive sample, where these were identified using both blastx and blastn. A further ten were upregulated in the less aggressive sample, where three were identified using blastx and blastn, two with blastx and four with blastn (Table 4.2). Notably, two proopiomelanocortin (POMC) precursors – POMC is a precursor to ACTH, involved in stress responses – were upregulated in aggressive trout (POMCa: 27.2 fold-difference; POMCb: 88 fold-difference). Furthermore, glutathione peroxidase – a protein involved in oxidative stress – was also upregulated in aggressive fish. Predominantly genes associated with respiration were found in less aggressive trout (Table 4.1). In addition, there were six unidentified contigs that were differentially expressed (Appendix 1; Table A9): one in the aggressive trout and five in the less aggressive group.

4.3.3 Gene Ontology of aggression

The Newbler assembly for the separate samples generated 4,069 contigs (1,934,879 bp) and 51,935 singletons in the aggressive sample, with a mean length of 475 bp (1<2230 bp). There were 1,572 large contigs (≥500 bp), with mean size 475 bp. In the sample from less aggressive fish, there were 2,502 (1,151,736 bp) contigs and 35,493 singletons, with mean contig length of 460 bp (1<2340 bp). There were 884 large reads, with average size 731 bp.

Gene Ontology terms comprise three vocabularies of terms describing the characterisation and annotation of gene products: biological processes, cellular components and molecular functions. BLAST2GO® annotates sequences by assigning the most specific GO term identified from the BLAST hit to the query sequence. Enrichment analysis of the annotated contig sequences using GOSSIP, revealed 18 GO terms that were differentially expressed between aggressive treatments, where three were over-represented in the aggressive and 15 over-represented in the less aggressive rainbow trout. These comprised one cellular component term, four molecular function terms and thirteen biological process terms (Figure 4.4). Macromolecular complex is the only cellular component term and includes cellular components that form complexes with proteins or DNA and is over-represented in less aggressive trout. One molecular function GO term, sugar binding activity, associated with immune function, was over-represented in aggressive trout. In contrast, three molecular function GO terms were over-represented in the less aggressive sample and are terms

associated with transporter activity, which is the movement of substances into, out of or between cells. This is particularly notable for the energy transfer implications of ATPase activity. One biological process term was over-represented in the aggressive fish, regulation of secretion. Twelve were over-represented in the less aggressive sample and these were all cellular component organisation or protein assembly terms.

4.4 Discussion

Aggression, as a complex phenotype, has been the focus of many genetic and genomic studies. Using next generation sequencing it has been possible to study the transcriptome involved in aggressive behaviour rather than just one or a few genes and which was unbiased by *a priori* EST data as occurs with microarrays. By sequencing the transcriptomes of aggressive and less aggressive rainbow trout, 41 differentially expressed genes were identified, six of which were previously uncharacterised. I have confirmed previous candidates for aggressive behaviour (POMC) and identified novel candidates for aggression (neuromedin S) which may show pleiotropic effects in stress responsiveness. Moreover, through GO-analysis a wider role of metabolism-related genes are implicated in differential energy use between aggressive and less aggressive trout. The genes differentially expressed in this study may be used to further study aggression and related behaviour (Chapter 3). Furthermore, more than 8,000 contigs have been sequenced and assembled which can be used for future genomic studies of the rainbow trout so this contributes an important resource to the genetic databases concerning this non-model species. This study is one of the first to use NGS to sequence *de novo* the transcriptome of an important behavioural phenotype in a vertebrate.

4.4.1 Old and new candidates for aggressive behaviour

A small number of differentially expressed transcripts could not be identified but these could act as potential new targets for the study of aggression when the trout genome is sequenced. Where gene sequences were identified, the well-known candidate gene products for aggressive behaviour, such as MHC Ia or serotonin (5-HT) and associated enzymes (*e.g. Tph2*) and metabolites (*e.g.* 5-HIAA) were not represented. However, transcripts associated with aggressive behaviour in the present study were associated with increased HPI function, an axis involved in stress responsiveness and aggressive behaviour (Pottinger & Carrick 2001; Sloman *et al.* 2001; Jeffrey *et al.* 2012). POMC is a precursor to adrenocorticotrophic hormone (ACTH), which leads to the release of cortisol, the principal hormone secreted during stress in many animals including trout (Sloman *et al.* 2001). In the present study, the two hormone precursors POMCa and POMCb, implicated in subordinate behaviour in rainbow trout (Winberg & Lepage 1998), were upregulated 27.2 and 88-fold, respectively, in

aggressive trout. Subordinates normally show a larger plasma cortisol response to stress (Øverli *et al.* 1999; Pottinger & Carrick 2001; Øverli *et al.* 2004) and an increased level of POMC (Winberg & Lepage 1998; Renn *et al.* 2008). Therefore, this upregulation of POMC in aggressive trout may be an unusual finding. However, POMC mRNA expression was not different between rainbow trout selectively bred for high and low cortisol responses to stress in response to an emersion stressor (Thomson *et al.* 2011). The upregulation of POMC in aggressive fish in the present study contrasts with previous findings and this may indicate that aggressive behaviour has a distinct transcriptional profile compared with dominance. However, the present study did not investigate the relationship between aggression and dominance, but this could be studied further.

In addition, the upregulation of POMC in aggressive rainbow trout suggests pleiotropic effects, due to its role as a precursor. POMC is a precursor to ACTH in addition to two other hormones, α-melanocyte stimulating hormone (α-MSH) and β-endorphin. α-MSH is involved in skin darkening in salmonids, where subordinate Arctic char, *Salvelinus alpinus,* show higher levels of α-MSH and skin darkening (Höglund *et al.* 2000). In contrast, low stress-responding rainbow trout showed higher levels of α-MSH (Kittilsen *et al.* 2009). Moreover, β-endorphin is upregulated in response to stress, though this has analgesic effects (Sumpter *et al.* 1985). These contrasting effects, along with the results from the present study, indicate that POMC has many functions. Taken further, this may indicate a flexible role of POMC during aggressive behaviour. Moreover, there may be differences in the expression of POMC at the transcriptional level compared with the biologically active protein. Nonetheless, this transcriptome study confirms a role for POMC in aggression, where this role contrasts with previous findings.

In addition to confirming an existing aggression candidate, this study identified a gene not previously associated with aggression. The current study showed a 27.5-fold upregulation of neuromedin S in aggressive rainbow trout. This gene has been implicated in feeding behaviour, which relates to aggression, but this is the first time it has been directly associated with aggressive behaviour and thus constitutes a novel candidate. For example, injection of neuromedin S in rats, *Rattus norvegicus,* caused increased mRNA levels of POMC and corticotrophin releasing hormone (CRH) and, subsequently, the suppression of feeding behaviour (Ida *et al.* 2005; Miyazato *et al.* 2008). Moreover, a similar suppression of feeding behaviour in response to neuromedin S was seen in the domestic chicken, *Gallus gallus* (Tachibana *et al.* 2010), and in the Japanese quail, *Coturnix coturnix japonica* (Shousha *et al.* 2006). Since aggression is used to monopolise food in rainbow trout, it is not surprising that this gene with a role in feeding behaviour is upregulated in aggressive fish.

The co-upregulation of neuromedin S and POMC implies pleiotropic effects, via a role in feeding and aggression. Furthermore, these genes may exhibit epistatic effects, where neuromedin S may regulate the expression of POMC. This is the first time neuromedin S has been implicated in aggressive behaviour and thus highlights the need to study pleiotropy and epistasis of candidate genes.

In addition, genes associated with oxidative stress were upregulated in aggressive trout, for example, Haemoglobin subunits and glutathione peroxidase. These genes are associated with antioxidant function: haemoglobin, when present in neurons rather than blood, maintains oxygen homeostasis and controls oxidative phosphorylation (Biagioli *et al.* 2009). Glutathione peroxidase is involved in the reduction of reactive oxygen species in the cell, such as H_2O_2, and is upregulated in salmonids (*O. mykiss* and *S. salar*) in response to environmental pollutants (Li *et al.* 2010a; Li *et al.* 2010b). Moreover, glutathione peroxidase responds to a dose of corticosterone in rat hippocampal slices *in vitro*, suggesting that its role in aggressive behaviour may be due to social stress (Sood *et al.* 2011). These genes indicate that responses to toxic stress elicit a similar response to social stress, perhaps through pleiotropy. Overall, these genes represent novel candidates, implicated in aggressive behaviour for further study.

4.4.2 Genes in less aggressive trout

Despite there being a larger number of upregulated genes in less aggressive fish, these genes represented a smaller range of functions than in aggressive fish and were mostly covered by a small number of GO terms. These were associated with increased respiration, protein assembly and lipid-binding and are consistent with increased metabolism, including the production of proteins or hormones, and with a fight or flight response (Haller 1995). These types of gene function are consistent with those upregulated in subdominant rainbow trout using a microarray (Sneddon *et al.* 2011). The differences in genes indicate that aggressive fish invest less in energy metabolism during an interaction, whilst less aggressive fish invest resources into energy release. This difference is consistent with previous findings, where Siamese fighting fish, *Betta splendens*, that lose dyadic contests incur a higher energetic cost, by showing a greater increase of free glucose and reduction of stored glycogen in muscle (Haller 1991). Similarly, male cichlids, *Tilapia zillii*, that lose contests have higher levels of lactate (Neat *et al.* 1998). These differences in gene identity between aggressive and less aggressive may indicate genes that confer an underlying propensity for competitive ability and thus ability for winning an interaction.

4.4.3 Gene Ontology of aggression

Aggressive and less aggressive trout differed in expression of genes which showed differing function or Gene Ontology (GO) (Liinamo *et al.* 2007), suggesting that the two behavioural types employ differing biological function during an aggressive encounter. Aggressive fish showed over-representation of secretion and regulation of sugar binding, whereas less aggressive individuals showed over-representation in the categories of cation transport and protein assembly. These GO terms in aggressive fish correspond with the GO terms identified for the differentially expressed genes, as identified using IDEG6. Secretion is associated with hormone activity and sugar-binding may be involved in signalling or glucose metabolism. The GO terms in less aggressive trout identified from the differentially expressed genes had a role in respiration, which is consistent with the wider enrichment analysis. However, also identified was lipid-binding, implicating cell signalling in less aggressive trout. Overall, these terms are similar to GO terms found to differ between dominant and subordinate male African cichlids, *Astatotilapia burtoni* (Renn *et al.* 2008), where hormone signalling was upregulated in dominant fish and regulation of cation transport was upregulated in subordinates. In addition, dominant *A. burtoni* also showed upregulation for cytoskeleton/structural molecules and iron ion binding, not shown in the present results. Nonetheless, this GO characterisation of aggression in rainbow trout shows fundamental differences in the transcript biological functioning in the brains of aggressive and less aggressive trout. Moreover, this indicates differences in the allocation of resources towards hormone secretion and antioxidation in more aggressive fish compared with structural and energy-releasing functions in the less aggressive trout.

4.4.4 Implications for behavioural syndromes

Aggression is a trait behaviourally and physiologically correlated with boldness and stress responsiveness in a behavioural syndrome (Section *2.1.2*). Moreover, it is intrinsically related with dominance, since aggressive individuals tend to gain dominance. Whilst genome-wide heterozygosity was related to aggressive behaviour (Chapter 2), this was not the case for boldness or stress responsiveness, indicating that there may be genomic differences in these traits. However, the present study indicates potential pleiotropic effects of transcripts that may describe an underlying genetic link between these correlated behaviours. For example, neuromedin S and POMC are both molecules involved in the HPI axis and, as such, are implicated in stress responsiveness. POMC is implicated in many functions – it is known to be involved in both aggression (Winberg & Lepage 1998) and stress responsiveness (Gilchriest *et al.* 2000) – due to its role as a precursor to ACTH, α-MSH and β-endorphin. Similarly, neuromedin S is implicated in the suppression of feeding, a behaviour that is related to both stress and aggression. These transcripts indicate that

pleiotropic effects of genes may be involved in the correlation of behaviours. By identifying novel functions of genes, it may be possible to obtain insights into the apparent decoupling of behavioural syndromes implied by the differences in genome-wide heterozygosity among aggression boldness and stress (Chapter 2). Moreover, expression of transcripts may be a specific response to environmental cues and future work could be conducted to inform the mechanisms underlying the flexible and context-dependent nature of both individual behaviours and behavioural syndromes. Future studies should explore possible epistatic, epigenetic and/or pleiotropic effects on gene expression linked to aggressive behaviour, boldness and stress coping style of these novel candidate genes.

Differential expression of genes involved in energy metabolism between aggressive and less aggressive rainbow trout may be related to the differences in genome-wide heterozygosity in these trout (Chapter 2). Low heterozygosity, seen in less aggressive trout may drive less efficient use of resources. Indeed, inbreeding depression in crickets, *Gryllodes sigillatus*, was associated with high energy metabolism (Ketola & Kotiaho 2009). Less aggressive or subordinate individuals generally show higher energy metabolism during an interaction, characterised by increased anaerobic respiration, increased glucose release and reduced glycogen stores (Haller 1991, 1995; Haller *et al.* 1996; Neat *et al.* 1998). This is consistent with the genes expressed in the present study, where less aggressive fish show upregulation of genes associated with increased respiration. This may also indicate that aggressive animals possess a greater ability to prepare metabolically for an interaction (Haller 1995; Haller *et al.* 1996) by, for example, having greater glycogen reserves. Indeed, aggression is correlated with higher resting metabolic rates which may correspond to a greater release of energy for subordinates during aggressive behaviour (Biro & Stamps 2010). Moreover, these differences in metabolic rate extend to correlated phenotypes, such as fast growth and boldness (Biro & Stamps 2010). The genes involved in metabolism in this study indicate an important genetic link among these correlated behaviours, which could extend to some of the physiological parameters involved in behavioural syndromes. However, further study of muscle and liver tissue would be needed to elucidate the function of energy metabolism in these fish. These results highlight the importance of identifying pathways associated with even well-studied behaviours, thus identifying novel candidates, which may have pleiotropic effects upon other phenotypic responses.

4.4.6 Genomic resources

This study has also highlighted the importance of using NGS for *de novo* transcriptome sequencing for non-model organisms. I have employed the use of NGS to identify novel gene transcripts in the non-model organism, the rainbow trout. Critically, for non-model

organisms, it is important to provide new sequence data and the utility of this application has been shown previously in *Oxyrrhis marina* (Lowe *et al.* 2011) paper wasps, *Polistes metricus* (Toth *et al.* 2007) and zebra finches, *Taeniopygia gutatta* (Lovell *et al.* 2008). By associating transcripts with a complex phenotypic trait, novel gene transcripts may be provided with function and may contribute to the growing EST libraries for rainbow trout (Salem *et al.* 2008).

4.4.5 Conclusions

This study used an original combination of behavioural and quantitative next generational sequencing approaches to study the genomic basis of aggressive behaviour of the non-model organism rainbow trout, *Oncorhynchus mykiss*. I found 51 genes that were differentially expressed between aggressive and less aggressive trout, of which 29 were unidentified and, as such, are potential novel candidates for aggressive behaviour in the future. In addition, this study confirmed a role for POMC in aggressive behaviour as well as genes associated with energy metabolism. Moreover, I identified neuromedin S, which may have pleiotropic effects on aggression and feeding behaviour. This study also showed that genes associated with energy metabolism were highlighted in less aggressive trout. This, along with many of the genes identified here, may be used as candidates for further study into the genomic architecture of aggression and related behaviour (Chapter 3). Not only is this generation of ESTs important for providing novel candidates, but is also important for adding to genomic resources for non-model organisms. This is one of the first studies to demonstrate that next generation sequencing can be used as a tool for behavioural ecology of non-model organisms as previously postulated (Bell 2009). I have generated novel candidates associated with aggressive behaviour and a set of sequences which can be used as a genomic resource for rainbow trout for further study in behavioural ecology and other disciplines.

Chapter 5 How do genes involved in the hypothalamic-pituitary-interrenal axis relate to winning and losing in rainbow trout?

5.1 Introduction

Aggressive behaviour in rainbow trout, *Oncorhynchus mykiss,* is important for both farmed fish and for natural populations. In farmed animals, aggression due to repeated agonistic encounters may elevate stress in subordinates and, therefore, may increase concerns for animal welfare by reducing growth or impairing immune and/or reproductive function (Pickering 1990; Campbell *et al.* 1992; de Kloet *et al.* 2005). Whereas, in wild fish, aggression may affect individual fitness, for example by facilitatingacquisition of mates, territories or dominance status (Huntingford & Turner 1987). As an evolutionarily and economically important behaviour, there is much interest in understanding the molecular mechanisms underlying winning a single social encounter.

Aggressive behaviour is physiologically correlated with stress responsiveness, whereby aggressive rainbow trout, *Oncorhynchus mykiss,* exhibit lower cortisol responses to stress (Pottinger & Pickering 1992; Pottinger & Carrick 2001; Øverli *et al.* 2004). The HPI axis is key in initiating and modulating the response to social stress and is a hormonal cascade that results in the release of cortisol into the bloodstream (Wendelaar-Bonga 1997). Aggressiveness has been linked with cortisol production (Gilmour *et al.* 2005), for example, aggression negatively correlates with cortisol in threespine sticklebacks, *Gasterosteus aculeatus* (Aubin-Horth *et al.* 2012); moreover, *O. mykiss* given doses of cortisol in their food exhibited reduced levels of aggression (Øverli *et al.* 2002a) and subordinate *O. mykiss* and brown trout, *Salmo trutta*, had relatively higher levels of cortisol than dominant individuals (Sloman *et al.* 2001). In addition, HPI genes, including glucocorticoid receptors (GR), have been associated with social status in *O. mykiss,* where GR was upregulated in subordinates (Jeffrey *et al.* 2012). By quantifying the changes in expression in genes from the HPI axis, it may be possible to identify the relationship between gene expression and social stress in individuals that undergo a positive or negative social encounter.

Transcript profiling of brain genes demonstrated that a number of genes were differentially expressed between aggressive and less aggressive trout (Chapter 4) and between subdominant and dominant trout (Sneddon *et al.* 2011). However, few studies have investigated the changes in genes after one initial social encounter (Tang *et al.* 1999; Gilchriest *et al.* 2000; Wiseman *et al.* 2007; Yada *et al.* 2007; Lema 2010), which has important implications for understanding the primary manifestations of stress following an interaction in a territorial species such as rainbow trout. This fish is particularly important in

aquaculture where repeated aggressive interactions may be unavoidable in captivity but the deleterious impact of being subordinate results in reduced growth fecundity and economic return. Changes in specific mRNA transcript abundance can be useful indicators of the initial mechanisms behind aggressive behaviour in a social interaction and indicate how individuals cope with social stress. By studying gene expression in rainbow trout brains after a single social encounter and, because aggressive encounters are used to establish dominance, it may be possible to discover how specific genes are involved in early stages of dominance.

Four key genes of interest, three of which are genes associated with the HPI axis, and are thus implicated in both dominance and stress, will be investigated following an initial aggressive encounter. Specifically, these are vasotocin receptor 1a (V1a), mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). The fourth gene, ependymin (EPD), is also linked with stress, dominance and memory formation (Shashoua & Moore 1978; Tang *et al.* 1999; Sneddon *et al.* 2011). Arginine vasotocin (AVT) is a neurohormone associated with stress responses in *O. mykiss* (Gilchriest *et al.* 2001) and with memory and learning, and social behaviour in a variety of taxa (de Kloet 2010). Subordinate mammals are often reported to show low levels of arginine vasopressin (AVP), the mammalian homologue of AVT (Marler *et al.* 1999; Almeida *et al.* 2012) and experimental administration of AVT reduces aggression in trout (Backström & Winberg 2009). However, this effect is dependent on territoriality (Goodson & Bass 2001), where non-territorial species show the reverse effect. Binding of AVT/AVP to V1a alters behaviour, where V1a binding was associated with decreased aggression in Long-Evans hooded rats (Askew *et al.* 2006) but was associated with increased aggression in Syrian hamsters, *Mesocricetus auratus,* thus indicating conflicting evidence of role (Cooper *et al.* 2005; Albers *et al.* 2006). Few studies have investigated mRNA changes in V1a at the transcript level after an acutely aggressive interaction and thus the present study may provide valuable new information regarding the role it plays.

Ependymin (EPD) is a glycoprotein secreted in the meninx into the cerebrospinal fluid and has been implicated in neurogenesis (Castillo *et al.* 2008), learning (Rother *et al.* 1995), stress (Tang *et al.* 1999); Chapter stress 6) and dominance (Sneddon *et al.* 2011). The latter study revealed complex effects where EPD was upregulated in subdominant *O. mykiss* compared with dominants. Moreover, experimental blockade of this protein in zebrafish, *Danio rerio,* resulted in increased aggressive behaviour from the subdominant and reduced aggression in dominants. The role of ependymin in behaviour is relatively unstudied and, as such, further evidence for its role in aggression is required.

The mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) are binding sites for cortisol and, as such, have a role in tonic inhibition of the HPI axis (Reul *et al.* 2000) and negative feedback (de Kloet 2004) in response to a stressor. Both MR and GR have also been directly related to dominance, where subordinate rainbow trout showed downregulation of GR (Jeffrey *et al.* 2012) and where dominant *D. rerio* showed upregulation of MR but no change in GR in comparison with control fish (Pavlidis *et al.* 2011). Indeed, this balance between MR and GR is implicated in the capacity to cope with, and recover from, a social encounter or stressor (de Kloet & Derijk 2004).

The main aim of the current study was to quantify changes in gene expression of these four key genes in the brain, namely V1a, EPD, MR and GR, which are associated with regulation of stress response and/or aggressive behaviour and memory. Fish that are winners in a single pair-wise interaction were compared with losers at time points up to 24h following a social interaction. This will enable understanding of how winners and losers differentially modulate their responses during recovery from social stress and to determine what role the candidate genes have in aggressive interactions.

5.2 Methods

5.2.1 Husbandry

All experiments were conducted in a humane manner according to Home Office UK licensing and after local ethics approval. Rainbow trout, *Oncorhynchus mykiss*, were transported from a commercial supplier (n=129; mean weight=117.68±2.55 g), to the Liverpool aquarium. The trout were kept in two separate holding tanks (2x2x0.5 m; 2000 L) supplied with aerated recirculating freshwater (semi-closed system) at 13°C±2°C, with a 14:10 hr light:dark regime, similar to ambient. Trout were fed commercial pellets (Skretting, UK) at a rate of 1% body weight daily according to manufacturer's recommendations. Fish were caught at random (n=102) using a hand net, anaesthetised in 33.3 $\mu g ml^{-1}$ benzocaine (Sigma, UK) in a 25L bucket to minimise stress during weighing; fish were weighed to 0.01g and transferred to experimental tanks (90x45x30cm; 200L) at 10±1°C, which were arranged in three semi-closed recirculating systems (n=15 tanks) with aerated freshwater and were screened from visual disturbance. Each tank contained two fish of equal weight (±10%), so as not to influence the outcome of the interaction as relatively larger fish tend to be dominant (Huntingford *et al.* 1990; van Leeuwen *et al.* 2011), one fish in each of two compartments, halved by an opaque divider. Fish were fed daily in each compartment at the same rate as in the stock tanks.

5.2.2 Behavioural tests

Once fish resumed feeding (reduced feeding is a standard indicator of stress (Carr 2002)), the tests for aggressive behaviour began. All interactions were conducted between 12:00 and 14:00 to minimise any effects of diurnal variation in physiology. At least 15 min prior to each experiment low light level cameras that were linked to a remote monitoring system were installed at the front of the tanks to allow fish to minimise disturbance immediately preceding the behavioural trial. Each experiment began by removing the divider (using a pulley system to prevent fish being aware of the operator), after which the pairs were allowed to interact for a maximum of 15 min. An opaque tube (160x90x90 mm) was supplied as a refuge for each fish, so that they could opt to remain out of sight of their opponents and thus any interactions were conducted voluntarily such that the stress of these encounters were minimised.

Videos were assessed to determine the winners and losers in a pair by observing the outcome of an interaction. An interaction began when one fish exhibited one of three aggressive acts: (1) a displacement, where one fish moves slowly towards its opponent, in response to which the opponent retreats; (2) a chase, where one fish moves rapidly towards its opponent resulting in either contact between the two fish or the opponent rapidly retreating; (3) circles, where both fish circle around each other. The latency to begin an interaction by performing one of these behaviours was recorded to assess the variability in the distribution of aggression. Fish in experiments where they did not interact within 10 minutes were excluded from analysis. If fish continued aggressive acts, the interaction was defined as either settled or escalated. An interaction was settled when one individual was assigned as the winner due to its consistent chasing of a submissive loser which in turn consistently retreated until the end of the fifteen minute period. An escalated interaction was defined as one in which neither fish was observed to consistently chase or retreat; since fish could not be assigned as winner or loser, these experimental animals were excluded from analysis. Control fish that did not have an opponent present upon removal of the divider were also sampled (n=6). At different time points – immediately, 2 hours, 8 hours and 24 hours – after the fifteen minute interaction or control period, the divider was replaced. Fish were caught with a hand net and were humanely killed by concussion and exsanguination. The brains of winners and losers were dissected out and frozen at -80°C prior to RNA extraction.

Table 5.1. Sequences of target specific primers, as designed using Primer3 (Rozen & Skaletsky 2000) on Primer-BLAST, ncbi, showing the accession number from which the primers were designed and the resulting melting temperature (T_m°C).

Gene	Accession	Primer sequence	T_m°C
EPD	NM_001124693	**5'-3'**CTCATGCTCACGCTCTGGAA	80.3
		3'-5'CCAAAAACAGCTCAACCTGATG	
V1a	DQ291141	**5'-3'**CAGCCCACCCAGCGGTCCTA	79.9
		3'-5'TACGCCTTTACGCCCCACGGTT	
MR	NM_001124483	**5'-3'**CCAGCAACACCGCCACTTGACA	77.4
		3'-5'TGTTGGCCGAAGCCGCCAAAGT	
GR	AY495372	**5'-3'**CGTGTCCTGCTACGATTCGCAAGG	81.6
		3'-5'AGGAACAAGGCGCGATGGTGGT	
GAPDH	AF027130	**5'-3'**TGTTGTGTCTTCTGACTTCATTGG	76.7
		3'-5'CCAGCGCCAGCATCAAA	

5.2.3 Gene expression

RNA was extracted from the brain tissue using Trizol™, following the manufacturer's instructions (Invitrogen, UK) and homogenised using a TissueLyser (Qiagen, UK) for 2 minutes at 30Hz. RNA was purified using Ambion® PureLink™ RNA Mini Kit (Invitrogen, UK) and cDNA was generated with 1µg RNA and SuperScript™ III Reverse Transcriptase (Invitrogen). qPCR was carried out on ABI 7500 Fast Real-Time PCR System using 7.5ng cDNA in a 10µl reaction, 2pmol target-specific primers (TSPs), *Power*SYBR® Green PCR Master Mix (Applied Biosystems). TSPs (Table 5.1) were the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (de Santis *et al.* 2011) vasotocin receptor 1a (V1a), ependymin (EPD), mineralocorticoid receptor 1 (MR) and glucocorticoid receptor (GR). Cycle threshold (CT) values for each sample for each gene were generated using ABI 7500 Fast software v.2.0.5, which were converted to relative expression ratios according to the formula: $Ratio=2^{-[\Delta CT]}$ (Pfaffl 2001) where ΔCT is the difference in CT between the gene of interest and the reference gene GAPDH.

5.2.4 Data analysis

All data analysis was conducted in R v2.9.0 (R Development Core Team 2009). Log_e transformed gene expression ratios were normally distributed (Shapiro-Wilk tests: $P>0.05$). Two-way ANOVAs were used to analyse differences in gene expression between time points and between winner and losers followed by post-hoc Tukey HSD tests. A polynomial linear regression with time as a quadratic term and with winning as a second explanatory variable was used to analyse the pattern of gene expression over time. The control group was not included in the linear models, since the model describes the respective shapes of the curves of winners and losers and control fish do not fit into either category.

5.3 Results

Forty-eight pairs of rainbow trout, *Oncorhynchus mykiss*, were allowed to interact to determine a winner and loser of each pair. Sixteen pairs that failed to interact within 10 minutes and 9 pairs that did not resolve the interaction were excluded, leaving 23 winners and 23 losers. The distribution of aggressive behaviour, as measured by the latency for a pair to begin an interaction, for these 23 pairs was bimodal, with most pairs initiating interactions within 200 s and after 400 s and only few pairs initiating between this (Figure 5.1).

When genes were correlated regardless of winning or time after social encounter, the expression of the four genes showed a high degree of similarity and were all significantly correlated (all Pearson's correlations: r^2>0.45, P<0.001; Table 5.2), where EPD and GR were more correlated with one another than with V1a or MR, which were also highly correlated (Figure 5.2). In general, candidate gene expression varied among time points and between winners and losers, with the specific effects depending upon the particular gene (Figure 5.1). Even though V1a expression was correlated with MR and EPD with GR, these genes showed distinct effects of treatment and distinct patterns. V1a was the only gene to show an effect of time since the interaction plus winning, whereas MR and EPD showed only an effect of time and GR did not show any significant effects, but did exhibit a trend for an effect of time.

Specifically, expression of V1a changed significantly in both winners and losers following the conclusion of the fight ($F_{3,43}$=3.90, P=0.015: Figure 5.3a). V1a was upregulated immediately after the aggressive interaction in both winners and losers when compared with the control group (Tukey test: t=2.80, P=0.008; Figure 5.4a). In winners V1a expression remained upregulated at 2 hours after the aggressive interaction (Tukey test: t=2.45, P=0.019; Figure 5.3a) whereas V1a expression in losers returned to control levels. The subsequent time points showed no difference compared with controls. V1a also exhibited a near-significant trend for a quadratic pattern in gene expression, with winning or losing showing a trend for a difference in pattern (linear model: $F_{1,40}$=3.81, P=0.058: Figure 5.3a), where winners showed an increase in expression until 2 hours after the interaction, which, at 24 hours was reduced to control levels. Losers showed the inverse pattern, with an initial increase followed by a return to control levels with a gradual increase until 24 hours.

In contrast, although MR expression changed significantly with time ($F_{4,43}$=3.59, P=0.013), it was not affected by winning or losing ($F_{3,43}$=2.59, P=0.065; Figure 5.3b). There were,

however, differences between most time points and the control, with expression upregulated immediately after conclusion of the fight (Tukey test: t=3.34, P=0.002), at 2 hours (Tukey test: t=2.25, P=0.029) and at 24 hours (Tukey test: t=2.87, P=0.006) after the interaction (Figure 5.4b). These results were not reflected in a linear model that attempted to describe the shape of gene expression over time ($F_{5,40}$=0.352, P=0.878). Similarly, there were no significant changes in EPD expression with time and no differences were evident between winners and losers ($F_{3,43}$=2.18, P=0.134: Figure 5.3c). The linear model did not show a quadratic pattern ($F_{5,40}$=1.24, P=0.309). The expression of GR was not different between time points or between winners and losers ($F_{3,43}$=1.49, P=0.104: Figure 5.3d). However, GR showed a non-significant quadratic trend of relative expression, regardless of winning, with a general downregulation in expression until 8 hours when expression returned to control (Linear model: $F_{1,40}$=3.79, P=0.0587: Figure 5.4d).

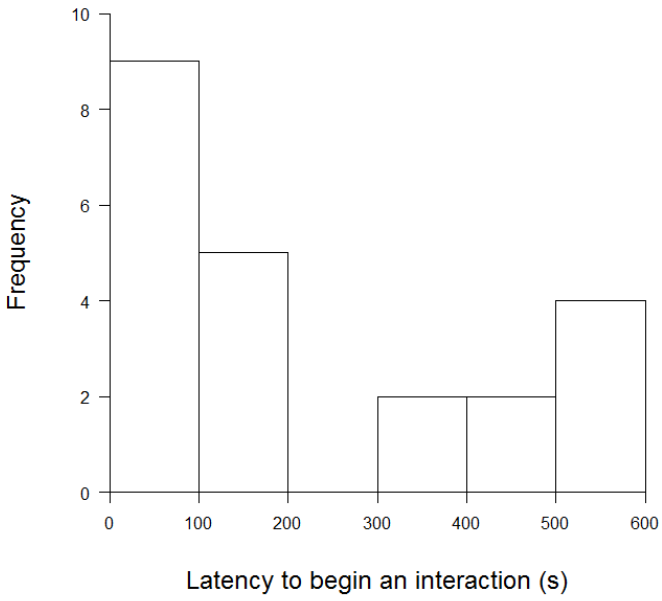

Figure 5.1. The distribution of the latency in seconds before an interaction was initiated.

Table 5.2. Correlation coefficients and bonferroni-adjusted p values of each gene: V1a, EPD, MR and GR correlated with every other gene.

Gene 1	Gene 2	Coefficient	P value
EPD	V1a	0.727	1.84E-11
EPD	MR	0.452	4.16E-09
EPD	GR	0.791	4.10E-09
V1a	MR	0.730	3.10E-09
V1a	GR	0.727	4.13E-07
MR	GR	0.648	7.72E-04

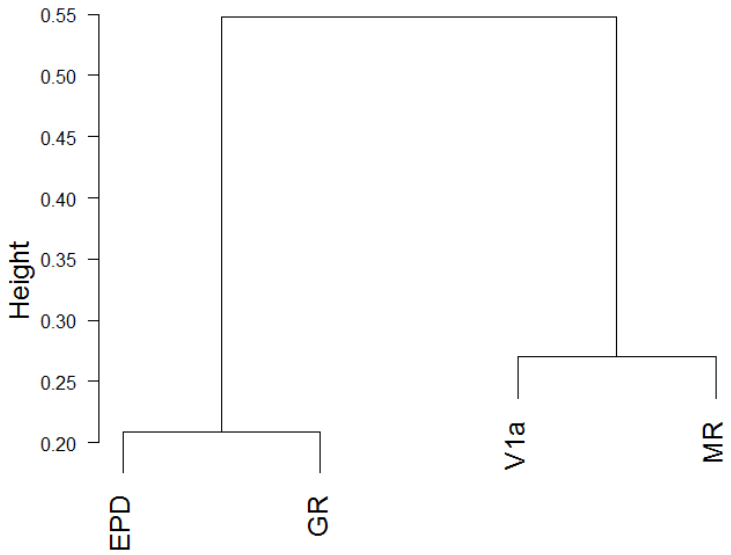

Figure 5.2. Dendrogram of the relationships between gene expression of each target gene with every other, based on correlation coefficients (Table 2.)

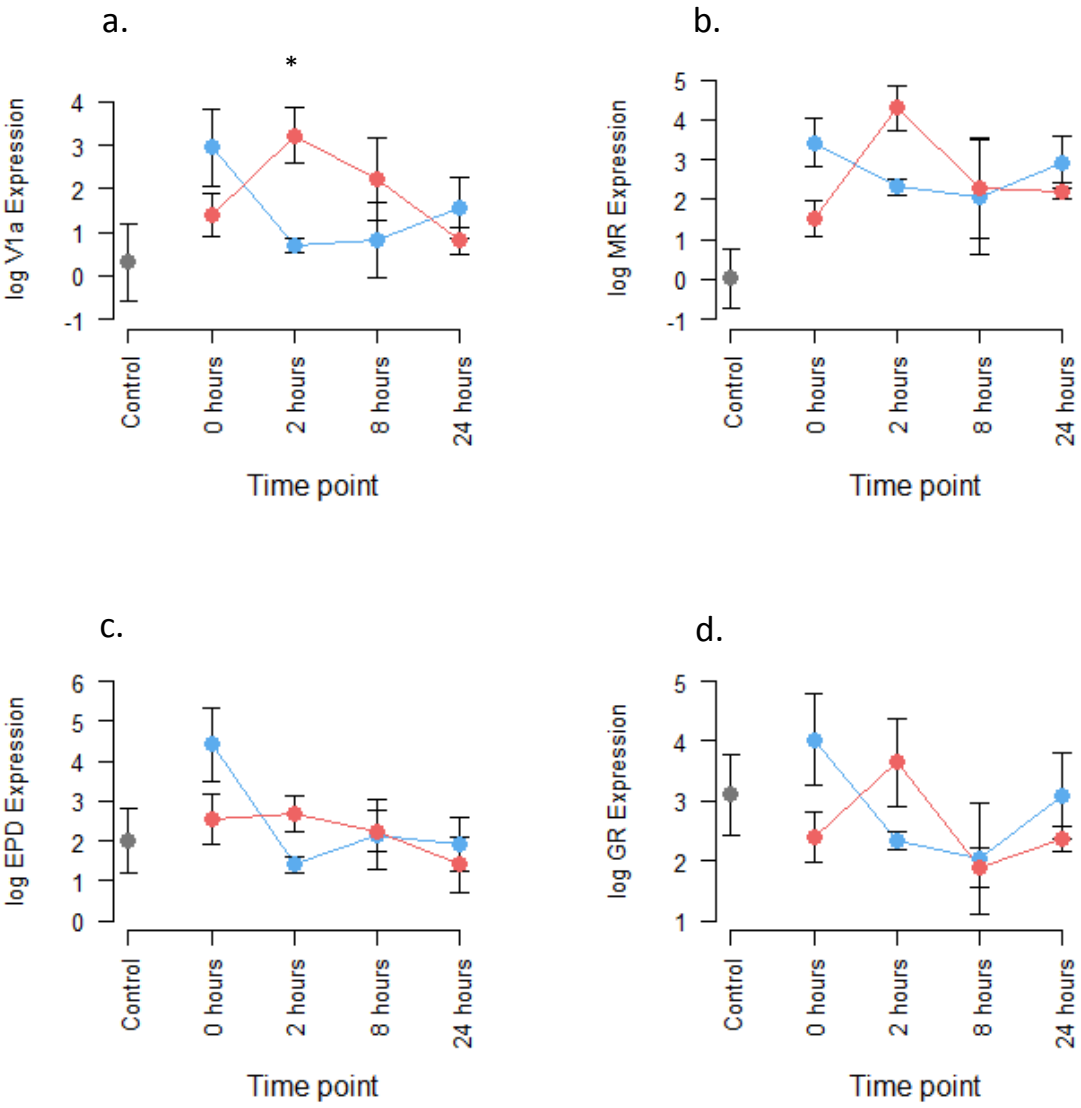

Figure 5.3. Relative gene expression of a. V1a, b. MR, c. EPD and d. GR of control (grey; *n*=6), loser (blue) and winner (red) rainbow trout after 0hrs (winners: *n*=6; losers: *n*=6), 2hrs (winners: *n*=6; losers: *n*=6), 8hrs (winners: *n*=5; losers: *n*=5) and 24hrs (winners: *n*=6; losers: *n*=6) following a fifteen minute interaction. * indicates a significant difference at *P*=0.05 between winners and losers. *P*-values are shown in Table 5.3. Where interaction terms (*i.e.* Time:Winning) are not significant, these are shown in Figure 5.4.

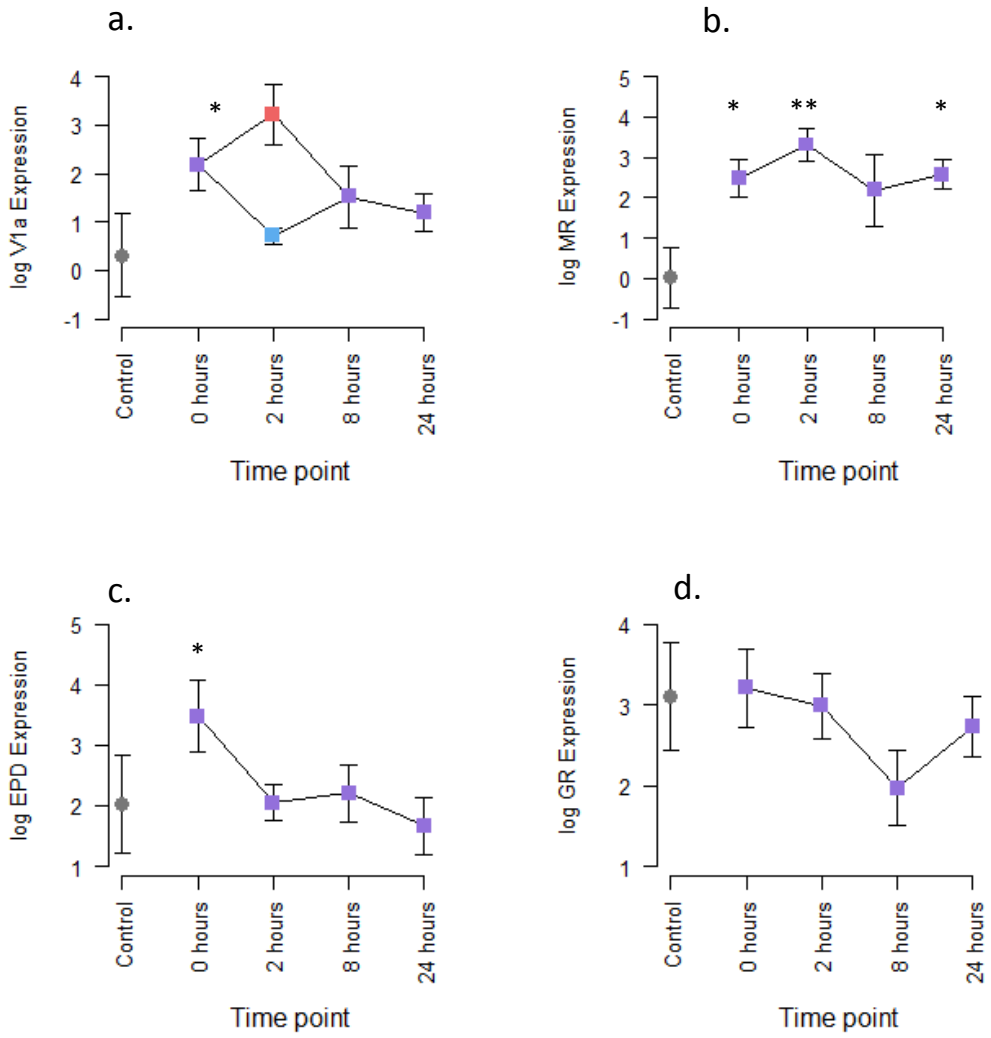

Figure 5.4. Relative gene expression ratios of a. V1a b. MR, c. GR and d. EPD in rainbow trout 0hrs (*n*=12), 2hrs (*n*=12), 8hrs (*n*=10) and 24hrs (*n*=12) after a fifteen minute interaction with winners and losers pooled (purple) and control (grey,*n*=6). * indicates a significant difference at *P*=0.05 and ** indicates *P*=0.01 between gene expression of pooled winners and losers at a time point and control. V1a expression at 2hrs was kept separate due to a significant difference between winners and losers.

Table 5.3 Output of ANOVA showing differences in gene expression of V1a, MR, EPD and GR between winners, losers and control rainbow trout after 0hrs, 2hrs, 8hrs and 24hrs following a fifteen minute interaction.

		DF	**SS**	**MS**	**F-value**	***P*-value**
V1a	Time point	4	17.637	4.409	1.6456	0.18029
	Winning	1	1.481	1.481	0.5527	0.46128
	Time:Winning	3	31.384	10.461	3.9043	0.01493
	Residuals	43	115.215	2.679		
MR	Time point	4	44.563	11.141	3.5937	0.01295
	Winning	1	0.163	0.163	0.0527	0.81958
	Time:Winning	3	24.129	8.043	2.5944	0.06477
	Residuals	43	133.303	3.1		
EPD	Time point	4	22.82	5.705	2.1808	0.0872
	Winning	1	0.864	0.864	0.3302	0.5685
	Time:Winning	3	15.376	5.125	1.9592	0.1344
	Residuals	43	112.486	2.616		
GR	Time point	4	10.019	2.505	1.215	0.3184
	Winning	1	1.043	1.043	0.5059	0.4808
	Time:Winning	3	13.52	4.507	2.1862	0.1035
	Residuals	43	88.645	2.062		

5.4 Discussion

Aggression, and the consequent stress response, is modulated by the HPI axis in rainbow trout *Oncorhynchus mykiss*. Most studies have shown subordinates are less aggressive and have elevated cortisol in response to their lower social status compared with dominants (Sloman *et al.* 2001; Øverli *et al.* 2004). This has detrimental effects upon immunity and growth if this becomes a chronic situation as seen in rainbow trout held in groups for six weeks (Pottinger & Pickering 1992). Key genes that modulate the stress response, aggressive behaviour and learning are important in understanding the mechanisms behind social interactions. Although the pattern of gene expression following a social interaction has not been well-studied (but see: (Tang *et al.* 1999; Gilchriest *et al.* 2000; Wiseman *et al.* 2007; Yada *et al.* 2007; Lema 2010), it was predicted that winners and losers should show differential expression of genes related to both stress and aggression: genes with functions associated with the stress axis are likely to be elevated in losers whereas genes associated with aggressive behaviour, such as EPD, is likely to be enhanced in winners as found in previous studies (Sneddon *et al.* 2011). There were, however, limited differences in gene expression between winners and losers, contrary to expectations, with only one gene (V1a) showing significant differences. However, three of the studied genes were upregulated in both winners and losers in response to a social interaction. While there was a general correlation between changes in the expression of these genes, this study highlights important variation in the actions of genes with seemingly similar functions and thus the need to quantify many genes across an axis.

5.4.1 Vasotocin receptor 1a

The AVT receptor V1a plays an important role in the control of dominance, stress and memory. In the present study, this gene was upregulated in both winners and losers immediately after an interaction but only in winning fish 2h after the trial. Whilst some studies show that V1a binding is elevated in subordinate Long-Evans hooded rats (Askew *et al.* 2006), others have recorded the reverse (Cooper *et al.* 2005; Albers *et al.* 2006). For example, socially experienced Syrian hamsters, *Mesocricetus auratus*, show greater amounts of V1a binding. Since the actions of V1a have not been related to gene expression levels, the upregulation of mRNA may not be related to binding activity. Nonetheless, these differences may be dependent upon previous experience or may be species-specific. Since V1a is implicated in social recognition (Wacker & Ludwig 2012), the difference between winners and losers in V1a expression at 2h could be indicative of differential social memory employed by dominants and subordinates. Forming a memory of the interaction and recognising a dominant may be more important to a stressed subordinate to avoid future confrontations and additional stress.

Furthermore, the difference in pattern could indicate that V1a is implicated in the differential recovery from social stress. Perhaps unusually, V1a expression over time in the dominant group is consistent with the pattern of gene expression over time seen after recovery from an acute stressor (Chapter 6), whereas subordinates showed the reverse pattern. Since aggressiveness was negatively correlated with cortisol concentrations in rainbow trout, where subordinate trout were more stressed (Øverli *et al.* 2004), this result may not be expected. However, the current result of an upregulation in V1a in the winners may be related to elevated expression of the AVT gene, which is upregulated in response to stress in rainbow trout (Gilchriest *et al.* 2001). AVT/AVP levels are modulated by other factors, specifically by serotonin (Backström & Winberg 2009), testosterone (Rodríguez & Specker 1991; Marler *et al.* 1999) and corticotrophic releasing hormone (Mikhailova *et al.* 2007). However, it is unknown how these factors interact to create responses to different types of stress or aggressive behaviour in different systems of territoriality so may be of interest for future studies.

5.4.2 Ependymin

EPD is a gene upregulated in dominant rainbow trout (Sneddon *et al.* 2011), during cold stress in zebrafish, *Danio rerio*, and common carp, *Cyprinus carpio* (Tang *et al.* 1999) and with memory formation in goldfish, *Carassius auratus* (Shashoua & Moore 1978). The current study identified that EPD was upregulated in rainbow trout following an aggressive social interaction but that there was no difference in expression levels between winners and losers. The increase in EPD expression could be due to the single aggressive interaction inducing a stress response, since EPD is implicated in stress responsiveness of rainbow trout selected for divergent cortisol response to stress (Thomson *et al.* 2011). The lack of difference between winners and losers contrasts with previous findings showing distinct differences in EPD expression between dominant, subdominant and subordinate rainbow trout after 15 minute interactions repeated twice a day for seven days (Sneddon *et al.* 2011) and also with the results of V1a expression as detailed above. Since EPD is associated with memory formation and learning (Shashoua & Moore 1978), upregulation immediately after the interaction indicates that both winners and losers may form memories during the interaction but this remains to be tested.

5.4.3 Mineralocorticoid and glucocorticoid receptors

The mineralocorticoid receptor is implicated in tonic inhibition of the stress response (Reul *et al.* 2000) and the upregulation of MR following an aggressive encounter shown here is consistent with this. A lack of difference between winners and losers suggests that both

respond similarly to a single social interaction. Indeed, there is evidence to indicate that the role of MR in social encounters is to allow the animal to adapt to social stress (van der Staay *et al.* 2008). Since MRs are implicated in the maintenance of homeostasis and the tonic inhibition of the HPI axis (Reul *et al.* 2000), an upregulation of this receptor indicates suppression of the HPI axis. This may be associated with attenuation of the stress response and, therefore, adaptation to a social encounter. In addition, MR gene expression is elevated after the social encounter and does not return to control levels within the timeframe studied here. Since MR elevation indicates an increased effort to retain homeostasis, the results here indicate that this increased effort continues for at least 24 hours. This is consistent with previous work in pigs, *Sus scrofa* (van der Staay *et al.* 2008), but requires further investigation in rainbow trout.

The glucocorticoid receptor (GR) is associated with mediating negative feedback after stress (de Kloet 2004) and with dominance in fish (Jeffrey *et al.* 2012). The present study showed no differences in GR expression between time points nor between winners and losers. These results, along with those of elevated MR expression are consistent with previous findings indicating dominance and a stress response. In studies comparing hippocampal GR, repeated social defeat did not affect GR expression in pigs, *Sus scrofa* (van der Staay *et al.* 2008), nor was it different between dominant and subordinate zebrafish, *Danio rerio* (Pavlidis *et al.* 2011). This suggests that whilst no differences in gene expression were found, that this is a normal response to social stress. Moreover, studies in mammals show that a rise in MR but not GR, as shown here is consistent with a stress response. After acute stress in rats, forced swimming produced a rise in hippocampal MR but not GR (Gesing *et al.* 2001). These results show that whilst GR expression did not differ between winners and losers, the contrasting upregulation of MR indicates that maintaining homeostasis of the HPI axis is important after a social encounter, rather than eliciting a measurable stress response and recovery from the interaction.

5.4.4 Conclusions

This study was designed to quantify the expression of four candidate genes associated with stress and learning in the period immediately following a social encounter as well as in winners and losers. The results showed that, whilst all four genes quantified were correlated with one another, the expression pattern of each gene differed following the social encounter. This highlights the importance of quantifying many genes across an axis, since the expression of single genes may not be correlated with a complicated behaviour. Only one gene, V1a, showed differential expression between winners and losers whereas EPD, GR and MR did not, possibly due to the fact that the encounter was stressful for both winners and

losers. EPD and GR were not differentially expressed over time thus they did not appear to be involved in recovery from the interaction. The expression of MR indicated that homeostasis of the HPI was maintained in the face of a potentially challenging social encounter, whilst EPD expression indicated a mild stress response. Moreover, since the genes that were upregulated are known to be involved in stress responses, this supports other data showing that social encounters, even when short and unrepeated are acutely stressful for rainbow trout. Repeated interactions are likely to have a more profound impact upon the health and welfare of this important aquaculture species. Future studies should investigate a greater range of genes and explore other potential pathways such as the adrenergic or serotonergic pathways.

Chapter 6 How do genes involved in the hypothalamic-pituitary-interrenal axis relate the response to an acute stressor?

6.1 Introduction

Organisms react to stressors, stimuli that perturb the external or internal environment, via the stress response, which allows the organism to potentially avoid or overcome the disturbance to homeostasis (Section *1.2.1;*(Johnson *et al.* 1992; Wendelaar-Bonga 1997). Homeostasis is maintained through changes in physiological and behavioural mechanisms through allostasis (McEwen & Stellar 1993; Romero *et al.* 2009). The magnitude of the stress response is proportional to the severity of the stressor, ranging from a mild disturbance to homeostasis (acute) to a repeated or prolonged stimulus (chronic). Aquaculture imposes a range of stressors on fish, including for example, handling during capture and transport between tanks or sites (Barton & Schreck 1987) and stressors can impact upon fish welfare (Veissier & Boissy 2007), particularly if the stimulus is chronic. When stress is chronic, an animal alters its physiology and behaviour due to allostatic load, where energy demands exceed energy gains (Romero *et al.* 2009). This is not only relevant to animal welfare, but stress can adversely affect organismal fitness, since prolonged activation of the stress axis may deleteriously affect several traits, such as growth, reproductive capability and immunity (Pickering & Pottinger 1989; Campbell *et al.* 1992). Acute stressors allow necessary coping and recovery via activation of the hypothalamic-pituitary-interrenal axis (HPI; HP-adrenal (HPA) in mammals) and the sympathetic nervous system (Wendelaar-Bonga 1997). Many studies have quantified the changes in the expression of key HPI genes in response to acute stressors (Geslin & Auperin 2004; Wiseman *et al.* 2007; Kusakabe *et al.* 2009; Alderman *et al.* 2012) since animals often encounter short-term stress. However, these studies commonly focus on a single gene, protein or tissue, such as the liver. How expression of key HPI axis genes and the suite of correlated responses across the axis are regulated after an acute stressor and how this links to cortisol production (end product of HPI) has not been well studied, particularly with regards to recovery. Furthermore, how gene regulation is correlated within and between key tissues such as the brain, liver and head kidney has not been fully investigated.

6.1.1 Stress physiology

The vertebrate stress response is neuroendocrine in nature and the hormonal response is well-studied. However, the role of gene transcripts is less understood, yet these underlie the production of hormones. The hypothalamic-pituitary-interrenal (HPI/HPA) axis (Wendelaar-Bonga 1997) is initiated in higher centres of the brain resulting in the release of hypothalamic corticotrophin-releasing hormone (CRH), which in turn stimulates the release

of adrenocorticotrophic hormone (ACTH) from the pituitary (Pierson *et al.* 1996). Arginine vasotocin (AVT) in teleosts co-localises with CRH in the brains of teleosts (Yulis & Lederis 1987) and possesses similar ACTH-stimulating actions (Fryer & Leung 1982). Whilst the additive effect of AVT and CRH has not been studied in teleosts, the combined action of AVT and CRH stimulates a greater than additive amount of corticosterone in chickens, *Gallus gallus domesticus,* (Mikhailova *et al.* 2007). Following stimulation by ACTH the interrenal cells release corticosteroids into the bloodstream. Elevated levels of corticosteroids, specifically cortisol in teleosts, act on the gills, intestine and liver and facilitate the maintenance of hydromineral balance and redirection of energy metabolism (Wendelaar-Bonga 1997). Cortisol also has a role in negative feedback within the stress axis, acting at the pituitary and hypothalamus to suppress release of ACTH and CRH. These physiological changes are also thought to alter the genomic molecular response to stress, in particular the transcriptional changes of key HPI genes, which alters during the time-frame of an acute stressor (Morsink *et al.* 2006; Bury & Sturm 2007). It is suggested that these transcriptional changes have an important role in the mediation of negative feedback of the cortisol response to stress and this could have implications for understanding the mechanisms behind recovery from an acute stressor.

6.1.2 Genes in the hypothalamic-pituitary-interrenal axis

Whilst the stress-induced changes in critical HPI components are relatively well-understood, knowledge of transcriptional changes is limited, particularly with regard to recovery from stress. The HPI axis comprises the hypothalamus in the brain, the pituitary, and interrenal cells in the head kidney. Cortisol, the primary end product of the HPI axis acts at many sites throughout the body to initiate adaptive and stress-coping mechanisms. A number of important genes from these tissues have critical roles in initiating and mediating the stress response: for brain, vasotocin receptor 1a (V1a), mineralocorticoid receptor 1 (MR), corticotrophin releasing hormone (CRH) and ependymin (EPD); for kidney, steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage enzyme ($P450_{SCC}$) and glucocorticoid receptor (GR); for liver, MR and GR. The roles in regulating the stress response and responses of these genes to acute and chronic stressors is introduced briefly (Table 6.1): two key hormones are involved in the stimulation of ACTH release, namely AVT, whose effects are mediated by the vasotocin receptor V1a, and CRH (Yulis & Lederis 1987). In addition to changes in protein levels, the mRNA coding for both AVT and V1a increases in the hypothalamus after acute stress (Lema 2010; Almeida *et al.* 2012), which indicates an early role of transcription in the mediation of the stress response.

Table 6.1. Previous work on mRNA expression of the genes used in this study in response to various acute or chronic stressors. ↑ indicates upregulation after stress, ↓ indicates downregulation and – indicates no change.

Gene	Acute	Chronic	Species	Reference
V1a	↑		*Cyprinodon nevadensis amargosae*	Lema 2010
EPD	↑		*Cyprinus carpio; Danio rerio*	Tang *et al.* 1999
CRH	↑		*Rattus norvegicus*	Hsu *et al.* 2001
	↑		*Mus musculus*	Weninger *et al.* 1999
	↓	↑	*Gallus gallus domesticus*	Wang & Xu 2008
$P450_{SCC}$	–	↑	*Oncorhynchus mykiss*	Geslin & Auperin 2003
StAR	–	↑	*Oncorhynchus mykiss*	Geslin & Auperin 2003
	–		*Acipenser transmontanus*	Kusakabe *et al.* 2009
MR	↓		*Rattus norvegicus*	Olsson *et al.* 1997
	↑		*Sus scrofa*	Kanitz *et al.* 2006
GR	↓		*Oncorhynchus mykiss*	Yada *et al.* 2007
	↑		*Oncorhynchus mykiss*	Alderman *et al.* 2012
	↑		*Oncorhynchus mykiss*	Wiseman *et al.* 2007

Since CRH shares ACTH-releasing function with AVT, it follows that CRH mRNA is similarly upregulated after acute stress (Weninger *et al.* 1999; Hsu *et al.* 2001). However, the role of transcriptional changes of CRH mRNA in response to acute stress in the fish brain is unclear, since, in common carp, *Cyprinus carpio,* CRH mRNA was downregulated in response to acute stress (Mazon *et al.* 2006) but, in rainbow trout, *Oncorhynchus mykiss,* it was upregulated (Doyon *et al.* 2005). Additionally, changes in CRH may have more wide-reaching effects than HPI axis control of stress, as it has behaviour-mediating effects in regions of the brain other than the hypothalamus and pituitary (Heinrichs *et al.* 1995).

Another protein that is also involved in behaviour is ependymin (EPD). EPD is not an HPI axis glycoprotein but it is located in the meninges of teleost fish (Königstorfer *et al.* 1990). It was first characterised for its function in neurogenesis but is also involved in stress responses. In zebrafish, *Danio rerio,* and carp, *Cyprinus carpio,* EPD levels were elevated after 2 hours and peaked at 10 hours in response to cold-induced stress (Tang *et al.* 1999). Moreover, this protein has been implicated in memory and learning in goldfish, *Carassius auratus* (Rother *et al.* 1995), and, more recently, in dominance status in rainbow trout, *Oncorhynchus mykiss* (Sneddon *et al.* 2011). Relatively little is known about the role of EPD in the stress response, particularly the transcriptional changes and interactions with other genes thus more information on the role it may play is required.

Two key steps in the ACTH-stimulated production of cortisol by the interrenal are controlled by cytochrome P450 cholesterol side chain cleavage protein ($P450_{SCC}$) and steroidogenic acute regulatory protein (StAR). $P450_{SCC}$ is an inner mitochondrial membrane protein that converts cholesterol into pregnenolone (Payne & Hales 2004), which is a cortisol precursor, whereas StAR transports cholesterol from the outer to the inner membrane, which is the site of action of $P450_{SCC}$. These two proteins have clear roles in the response to stress and this has been demonstrated in Atlantic cod, *Gadus morhua*, by a difference in gene expression between strains bred for a high and low cortisol response to stress (Hori *et al.* 2012). Whilst a change in $P450_{SCC}$ mRNA expression has been implicated in acclimation to chronic stress, it shows no change in response to acute stress (Geslin & Auperin 2004) and, similarly, StAR mRNA expression also shows no change in response to acute stress in rainbow trout (Kusakabe *et al.* 2009).

Mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) have wide-ranging roles in energy metabolism and hydromineral balance and are found in many tissues, particularly the liver where they have a role in glucose and glycogen metabolism and the brain and kidney to regulate cortisol production. In the mammalian brain, MR is bound by corticosterone (Reul *et al.* 2000) resulting in a role of tonic inhibition of the HPA axis (de Kloet & Reul 1987), thus reduction of MR induces an elevated response to stress. This decrease in MR, as has been shown to occur after an acute stressor (Olsson *et al.* 1997; Dickens *et al.* 2009; Kanitz *et al.* 2009) also has a role in negative feedback and reduces cortisol production. Similarly, in teleosts, MRs bind cortisol, suggesting a similar function in tonic inhibition of the HPI (Sturm *et al.* 2005). Whilst MRs are occupied by glucocorticoids at a high level under basal conditions, GRs are occupied at a lower level, due to differences in affinity and specificity for binding glucocorticoids. This indicates a function in adaptation to stressors, rather than tonic inhibition for GRs (Reul *et al.* 1987). Indeed, GRs are implicated in adaptation through transcriptional regulation (Morsink *et al.* 2006) and negative feedback. GR mRNA levels are often downregulated in response to stress (Yada *et al.* 2007; Alderman *et al.* 2012) reflecting down-regulation of functional binding sites by cortisol (Pottinger 1990) but have also shown an increase in relation to a decrease in protein levels (Wiseman *et al.* 2007).

Stress is a physiologically complex trait in teleost fish, mediated by the HPI axis and involving many genes and multiple tissues, which are rarely studied in concert. Furthermore, recovery from stress has only been investigated in distinct tissues, for example microarrays of liver tissue (Momoda *et al.* 2007; Wiseman *et al.* 2007). However, the transcriptional changes of genes across an axis in multiple tissues has not been investigated.

By choosing genes known to be involved in the initiation and mediation of the stress response and, with an idea of the changes these genes have previously made in response to acute stress, transcriptional changes can be quantified. The main aim of this study is to quantify transcriptional changes of key genes in the HPI axis in the 24 hours following a standard acute stressor, namely V1a, EPD, CRH and MR in the brain, StAR, $P450_{SCC}$ and GR in the head kidney and GR and MR in the liver. Not only does this allow the investigation of the initiation of a response to an acute stressor, but it also indicates the role of these genes in regulation and attenuation of the stress response.

6.2 Methods

6.2.1 Husbandry

All experiments were conducted in a humane manner according to Home Office UK licensing and after local ethics approval. Rainbow trout (*Oncorhynchus mykiss*) were collected from a commercial supplier and transported to a stock tank (2x2x0.5 m; 2,000 L) in the Liverpool aquarium (*n*=100, approx weight=300 g). The fish were kept at 13±2°C and a light:dark regime of 14:10 hours, similar to ambient, for two weeks. The fish were fed commercial trout pellets (Skretting, UK) at a rate of 1% body weight daily according to manufacturer's recommendations. Fish (*n*=26, mean weight=328.98±13.29 g) were caught at random using a hand net and transferred to experimental tanks (90x45x30 cm; 200 L) at 10±1°C, which were arranged in three semi-recirculating systems with aerated freshwater and screened from visual disturbance. Fish were allowed to settle until all fish in a system had resumed feeding for two consecutive days (reduced feeding is a standard indicator of stress (Carr 2002)). Between 12:00 and 14:00, minimising diurnal variation in physiology, fish were subject to an acute stressor. Fish were caught using a hand net and were emersed from water for one minute. Fish were either killed by concussion and exsanguination immediately or returned to the water for 2 hours, 8 hours or 24 hours and subsequently killed for samples. Control fish that did not undergo the acute stressor were killed as above and sampled. A blood sample was taken using a 25g sterile needle and 1ml heparinised syringe via caudal venipuncture and blood plasma was collected by centrifugation (5 min at 3500rpm), stored at -20°C and was analysed blind for cortisol concentrations by a validated radioimmunoassay at CEH Lancaster. The whole brain, the head kidney and approximately 500mg liver were removed and stored at -80°C.

Table 6.2. Sequences of target specific primers, as designed using Primer3 (Rozen & Skaletsky 2000) on Primer-BLAST, ncbi, showing the accession number from which the primers were designed and the resulting melting temperature (T_m°C).

Gene	**Accession**	**Forward (5'-3') primer sequence**	**Reverse (3'-5') primer sequence**	**T_m°C**
CRH	NM_001124286	GTGGTTCTGCTCATTGCTTTCTT	CGCCAGGGCTCTCGATAG	78.1
EPD	NM_001124693	CTCATGCTCACGCTCTGGAA	CCAAAAACAGCTCAACCTGATG	80.3
V1a	DQ291141	CAGCCCACCCAGCGGTCCTA	TACGCCTTTACGCCCCACGGTT	79.9
MR	NM_001124483	CCAGCAACACCGCCACTTGACA	TGTTGGCCGAAGCCGCCAAAGT	77.4
$P450_{SCC}$	S57305	CCAGTGCCCGCCACAACTCC	GATGGCTGGCATCTCCGGCT	83.7
StAR	AB047032	GCTCAGCTCGCGGATCGAGG	GCGCTTGGCACAGCGAACAC	85.3
GR	AY495372	CGTGTCCTGCTACGATTCGCAAGG	AGGAACAAGGCGCGATGGTGGT	81.6
GAPDH	AF027130	TGTTGTGTCTTCTGACTTCATTGG	CCAGCGCCAGCATCAAA	76.7

6.2.2 Gene expression

RNA was extracted from the total brain, head kidney and 100mg of liver for each time point using Trizol™, following the manufacturer's instructions (Invitrogen, UK) after the tissue samples were homogenised using a TissueLyser (Qiagen, UK) for 2 minutes at 30Hz. Total RNA was purified to remove contaminants using Ambion® PureLink™ RNA Mini Kit (Invitrogen, UK) and cDNA was generated with 1µg RNA and SuperScript™ III Reverse Transcriptase according to the manufacturer's protocol (Invitrogen, UK). Real time quantitative PCR (RT-qPCR) was carried out on ABI 7500 Fast Real-Time PCR System using 3µl cDNA, 2pmol target-specific primers (TSPs), *Power* SYBR® Green PCR Master Mix (Applied Biosystems) to a final reaction volume of 10µl. TSPs were developed using Primer3 (Rozen & Skaletsky 2000) on primer-BLAST, ncbi and were as follows: the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (de Santis *et al.* 2011) for all tissues; for brain, vasotocin receptor 1a (V1a), mineralocorticoid receptor 1 (MR), corticotrophin releasing hormone (CRH) and ependymin (EPD). For kidney TSPs were steroidogenic acute regulatory protein (StAR), cytochrome P450 ($P450_{SCC}$) and glucocorticoid receptor (GR). For liver, TSPs were MR and GR (Table 6.2). CT values for each sample/gene combination were generated using ABI 7500 Fast software v2.0.5, which were converted to relative expression ratios according to the formula: Ratio = $2^{-[\Delta CT]}$ (Pfaffl 2001), where ΔCT is the difference in CT between the gene of interest and the reference gene GAPDH.

6.2.3 Data analysis

All data analysis was conducted in R v.2.9.0 (R Development Core Team 2009). Log transformed gene expression ratios and cortisol concentrations were normally distributed (Shapiro-Wilk normality tests: $P>0.05$). For each gene in each tissue and also for plasma cortisol concentrations, a one-way ANOVA was used to assess differences in gene expression between specific time points. A polynomial linear regression with either a quadratic or cubic term was used to analyse whether gene expression changes non-linearly over time and these were assessed using AIC values. Multiple regression with cortisol as the explanatory variable and expression of all genes from all tissues as response variables was used to assess the relationship between gene expression and cortisol concentrations.

6.3 Results

The results from this study show tissue-specific trends in gene expression, whereby brain transcripts showed similar patterns to kidney transcripts. Within each tissue, there were no categorical differences in gene expression in stressed fish at any time point in comparison with unstressed control fish.

Table 6.3. Correlation coefficients and bonferroni-adjusted p values of each gene correlated with every other gene.

Tissue of Gene 1 and Gene 2	Gene 1	Gene 2	Correlation Coefficient	Adjusted p value	Significance Level
Brain-Brain	V1a	CRH	0.894	0.000	***
	V1a	Brain MR	0.812	0.000	***
	V1a	EPD	0.753	0.001	**
	Brain MR	CRH	0.926	0.000	***
	Brain MR	EPD	0.723	0.001	**
	CRH	EPD	0.717	0.002	**
Kidney-Kidney	StAR	$P450_{SCC}$	0.869	0.000	***
	StAR	Kidney GR	0.795	0.000	***
	$P450_{SCC}$	Kidney GR	0.827	0.000	***
Liver-Liver	Liver GR	Liver MR	0.977	0.000	***
Brain-Kidney	V1a	StAR	0.658	0.009	**
	V1a	$P450_{SCC}$	0.619	0.021	*
	V1a	Kidney GR	0.685	0.004	**
	Brain MR	StAR	0.828	0.000	***
	Brain MR	$P450_{SCC}$	0.861	0.000	***
	Brain MR	Kidney GR	0.810	0.000	***
	CRH	StAR	0.713	0.002	**
	CRH	$P450_{SCC}$	0.779	0.000	***
	CRH	Kidney GR	0.718	0.002	**
	EPD	StAR	0.752	0.001	**
	EPD	$P450_{SCC}$	0.581	0.047	*
	EPD	Kidney GR	0.532	0.112	
Brain-Liver	V1a	Liver GR	0.146	0.993	
	V1a	Liver MR	0.103	0.631	
	Brain MR	Liver GR	0.410	0.516	
	Brain MR	Liver MR	0.369	0.550	
	CRH	Liver GR	0.333	0.897	
	CRH	Liver MR	0.297	0.953	
	EPD	Liver GR	0.252	0.942	
	EPD	Liver MR	0.177	1.000	
Kidney-Liver	StAR	Liver GR	0.455	0.356	
	StAR	Liver MR	0.365	0.796	
	$P450_{SCC}$	Liver GR	0.422	0.520	
	$P450_{SCC}$	Liver MR	0.329	0.813	
	Kidney GR	Liver GR	0.344	0.901	
	Kidney GR	Liver MR	0.265	1.000	

Table 6.4. AIC values of linear models with time as linear, quadratic and cubic terms. A low AIC indicates a better fitting model.

Model	**AIC**
VR1a~time	66.2
VR1a~time+time^2	68.2
VR1a~time+time^2+time^3	64.1
EPD~time	65.0
EPD~time+time^2	66.4
EPD~time+time^2+time^3	63.7

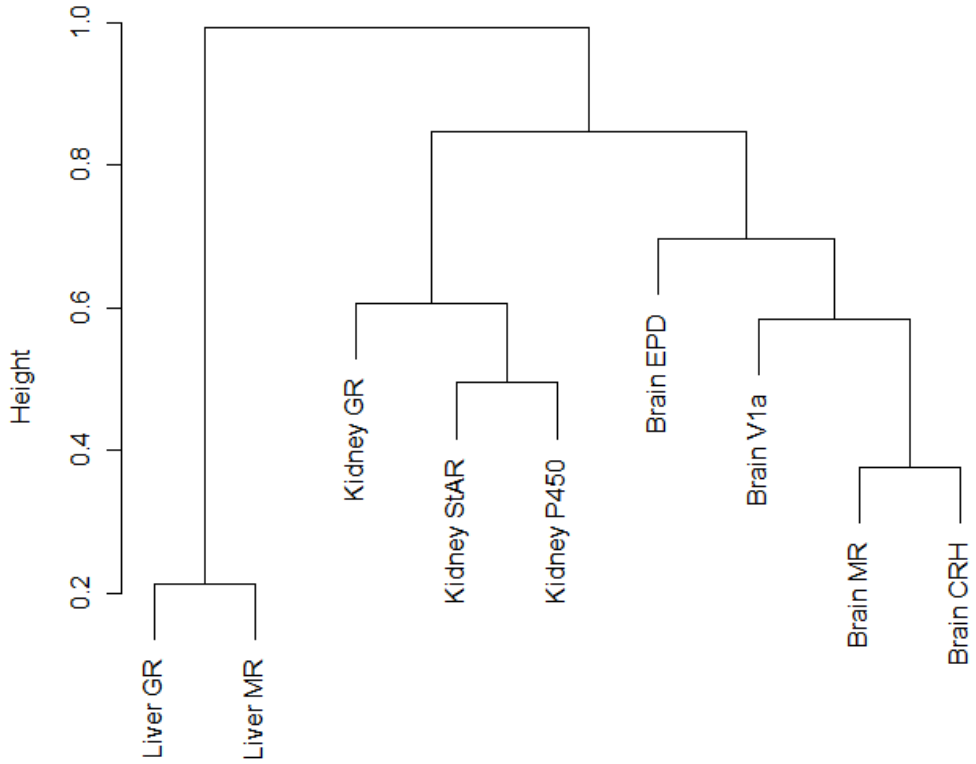

Figure 6.1. Dendrogram of the relationships between gene expression of each target gene from each tissue with every other, based on correlation coefficients (Table 2.)

Moreover, there were no categorical differences in gene expression among time points, where the pattern of gene expression was not correlated with cortisol concentrations. However, two genes in the brain, V1a and EPD showed a nonlinear pattern of gene expression over time, where there was an elevation in relative transcripts at 2 hours followed by a drop at 8 hours with a subsequent rise at 24 hours.

6.3.1 Cortisol response to an acute stressor

Similar to expected pattern with stress response, log plasma cortisol followed a quadratic pattern (Linear model: $F_{1,\ 23}$=5.6484, P<0.05), with a peak in concentration at 2 hours (20.8±15.2 ngml^{-1}) that returned to control levels (1.76±0.55 ngml^{-1}) after 24 hours (Figure 6.5 shows log data, for actual values see Appendix 1, Figure A5). Furthermore, plasma cortisol levels varied with time and were significantly elevated in comparison with control levels (ANOVA: $F_{4,21}$=3.73, P=0.019), specifically, at 2h (20.8±15.2 ngml^{-1}; Tukey test: t=3.21, P=0.004) and 8h (8.2±4.3 ngml^{-1}; Tukey test: t=2.35, P=0.029) but did not differ between control fish (1.1±0.5ngml^{-1}) and fish that were sampled immediately after an acute stressor (1.8±0.6ngml^{-1}). The temporal variation in plasma cortisol response was independent to those patterns exhibited in all genes in all tissues studied here (multiple regression: $F_{9,14}$=0.584, P=0.790).

6.3.2 Gene expression

Gene expression following the stressor showed tissue-specific, rather than more general, trends, with the correlation in standardised gene expression greater between genes within a tissue than between genes from different tissues (Figure 6.1); for example, all pair-wise correlations of standardised gene expression within the brain were significant (all Pearson's correlations: r^2>0.7, P<0.001: Table 6.3) but there were no significant correlations between any pairs of genes from the brain and liver (all Pearson's correlations: r^2<0.5, P>0.5: Table 6.3). Similarly, there was greater correlation between genes within the kidney (all Pearson's correlations: r^2>0.8, P<0.0001: Table 6.2) than between genes expressed in the kidney and from the brain (all Pearson's correlations: r^2>0.5, P<0.05: Table 6.3) and with those from the liver (all Pearson's correlations:r^2<0.5, P>0.3: Table 6.3) and the two genes from the liver were the most highly correlated (Pearson's correlation: r^2=0.98, P<0.0001: Table 6.3).

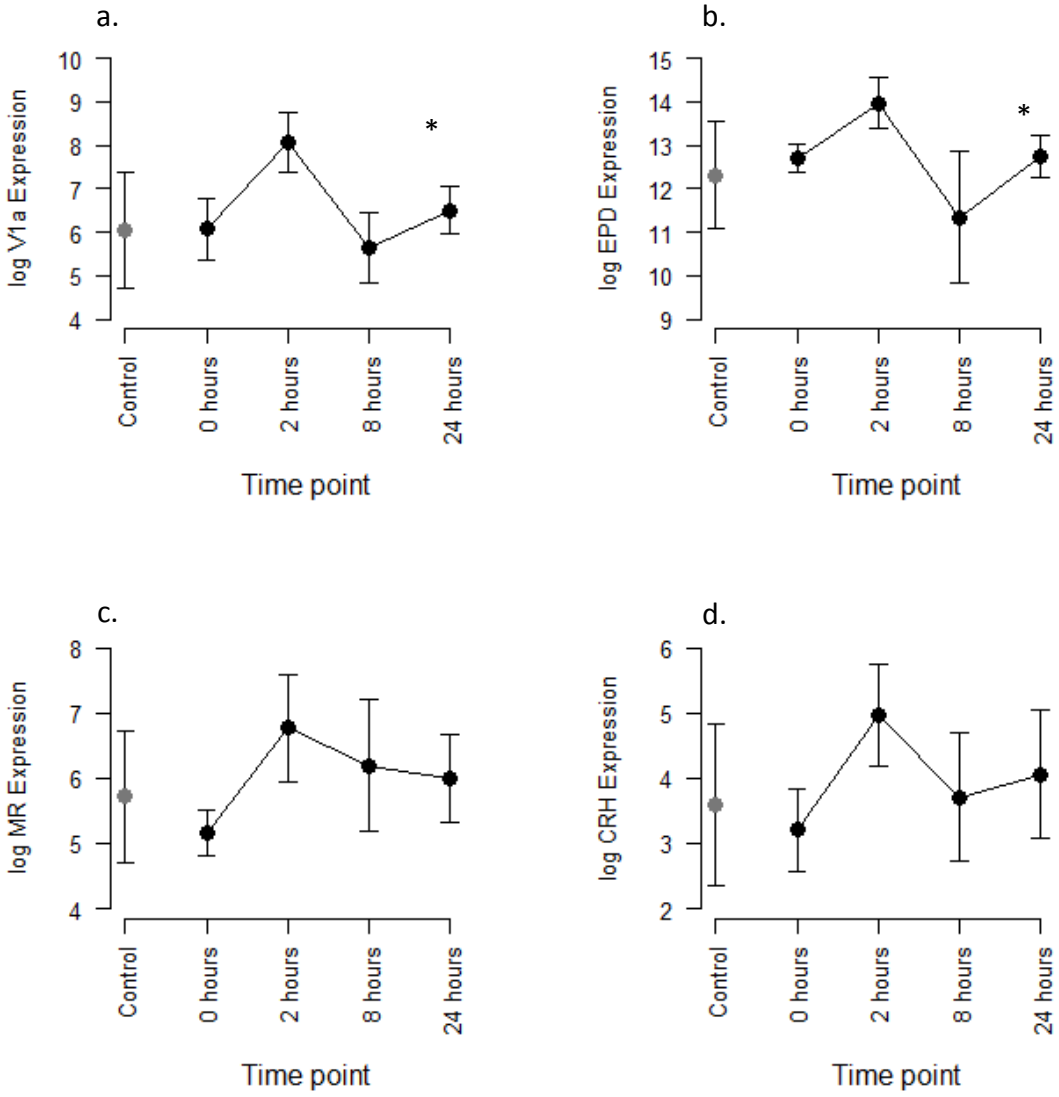

Figure 6.2. Gene expression of a. V1a, b. EPD, c. MR and d. CRH in rainbow trout brain after no stress (control, *n*=5), immediately (*n*=6), 2 hours (*n*=5), 8 hours (*n*=5) and 24 hours (*n*=5) after an acute one minute emersion stressor. * indicates a significant non-linear pattern of gene expression over time at $P<0.05$.

Table 6.5 Output of ANOVA showing differences in gene expression of V1a, EPD, MR and CRH in the brains of rainbow trout treated with an acute emersion stress after 0hrs, 2hrs, 8hrs and 24hrs.

		DF	**SS**	**MS**	**F-value**	***P*-value**
V1a	Time	4	3.276	0.819	1.374	0.280
	Residuals	19	11.325	0.596		
EPD	Time	4	3.031	0.758	1.262	0.319
	Residuals	19	11.407	0.600		
MR	Time	4	1.430	0.357	0.706	0.598
	Residuals	19	9.621	0.506		
CRH	Time	4	1.734	0.433	0.595	0.671
	Residuals	19	13.845	0.729		

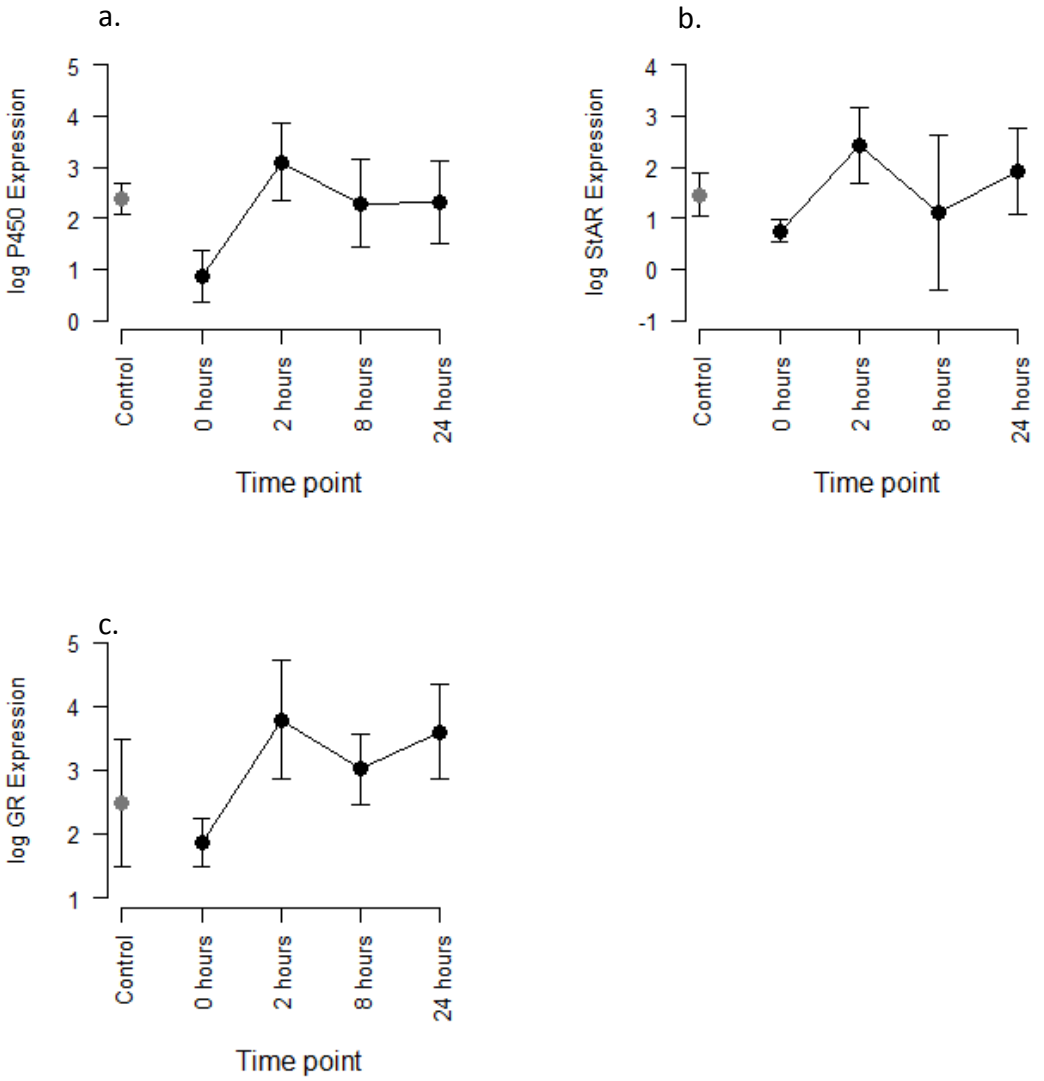

Figure 6.3. Gene expression of a. $P450_{SCC}$, b. StAR and c. GR in rainbow trout head kidney after no stress (control, *n*=5), immediately (*n*=6), 2 hours (*n*=5), 8 hours (*n*=5) and 24 hours (*n*=5) after an acute one minute emersion stressor.

Table 6.6 Output of ANOVA showing differences in gene expression of $P450_{SCC,}$ StAR and GR in the kidneys of rainbow trout treated with an acute emersion stress after 0hrs, 2hrs, 8hrs and 24hrs.

		DF	**SS**	**MS**	**F-value**	***P*-value**
$P450_{SCC}$	Time	4	2.779	0.695	1.690	0.194
	Residuals	19	7.812	0.411		
StAR	Time	4	1.699	0.425	0.763	0.562
	Residuals	19	10.581	0.557		
GR	Time	4	2.561	0.640	1.337	0.293
	Residuals	19	9.096	0.479		

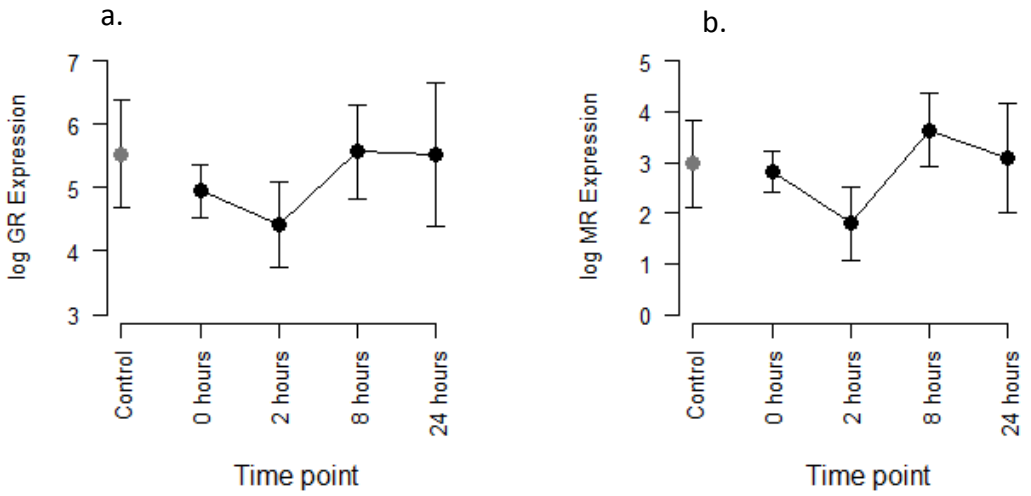

Figure 6.4. Gene expression of a. GR and b. MR in rainbow trout liver after no stress (control, *n*=5), immediately (*n*=6), 2 hours (*n*=5), 8 hours (*n*=5) and 24 hours (*n*=5) after an acute one minute emersion stressor.

Table 6.7 Output of ANOVA showing differences in gene expression of GR and MR in the livers of rainbow trout treated with an acute emersion stress after 0hrs, 2hrs, 8hrs and 24hrs.

		DF	**SS**	**MS**	**F-value**	***P*-value**
GR	Time	4	0.935	0.234	0.424	0.789
	Residuals	19	10.475	0.551		
MR	Time	4	1.580	0.395	0.737	0.578
	Residuals	19	10.190	0.536		

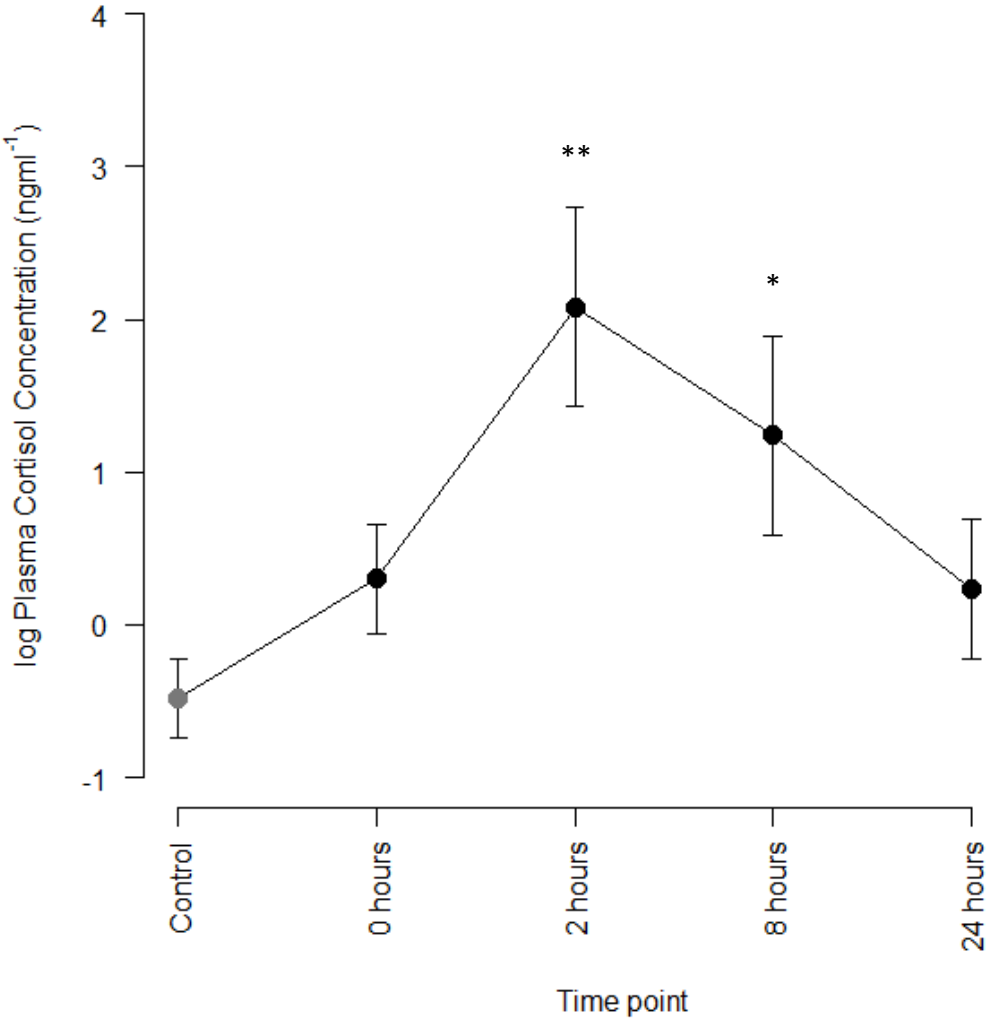

Figure 6.5. log plasma cortisol concentrations ($ngml^{-1}$) after no stress (control, *n*=5), immediately (*n*=6), 2 hours (*n*=5), 8 hours (*n*=5) and 24 hours (*n*=5) after an acute one minute emersion stressor. * indicates a significant difference between a time point and control at *P*=0.05 and ** at *P*=0.01.

Despite the correlated response of gene expression within tissues, the significance of these patterns was gene-specific. There were no significant linear variations among time points for any gene (Table 6.5) but the mRNA levels of V1a and EPD followed non-linear patterns of expression over time: expression of V1a and EPD followed a cubic pattern rather than simple linear variation (Table 6.4), whereby gene expression was upregulated after 2 hours, reduced after 8 hours and increased slightly at 24 hours (V1a: $F_{1,22}$=5.75, P=0.0254; EPD: $F_{1,22}$=4.37, P=0.0484: Figure 6.2a and 6.2b). Conversely, MR (Figure 6.2c) and CRH (Figure 6.2d) followed neither a linear nor a polynomial pattern of gene expression (MR: $F_{3,22}$=1.12, P=0.364; CRH: $F_{3,22}$=0.845, P=0.484), but, interestingly, these genes were the most closely correlated genes in the brain (Pearson's correlation: r^2=0.93, P<0.0001; Table 6.3). In contrast, no gene in the kidney (Figure 6.3) showed any polynomial pattern over time ($P450_{SCC}$: $F_{1,21}$=0.955, P=0.433; StAR: $F_{1,21}$=0.005, P=0.945; GR: $F_{1,21}$=0.945, P=0.405) and similar results were shown in the two genes in the liver (MR: $F_{2,23}$=0.225, P=0.800; GR: $F_{2,23}$=0.380, P=0.688; Figure 6.4). Moreover, there were no differences in timepoints in any of the genes in the kidney (Table 6.6) or in the liver (Table 6.7).

6.4 Discussion

The stress response is a complex trait partly controlled by the HPI axis (Wendelaar-Bonga 1997) and mediated by transcriptional changes of key HPI and associated genes (Olsson *et al.* 1997; Jezova *et al.* 1999; de Kloet 2004; Lema 2010). However, few studies have examined a suite of correlated responses and temporal variation (Lema 2010; Jeffrey *et al.* 2012). This study aimed to quantify changes over time in genes involved in promoting the stress response in the brain and kidney together with genes associated with negative feedback of the stress response in the liver and how these related to a standard indicator of the stress response: plasma cortisol concentrations. Since the brain and kidney are involved in initiating a stress response, it was expected that genes in these tissues would be more closely related than with genes in the liver, which is involved in energy metabolism and negative feedback. As expected, the expression of genes in the brain and kidney were more closely related to one another than with genes from the liver, suggesting genetic changes in the liver are distinct from those in the brain and kidney. Furthermore, whilst the plasma cortisol concentrations observed here are consistent with responses to acute stress, as has been shown previously in the rainbow trout (Pickering & Pottinger 1989; Pickering *et al.* 1991), expression of these genes was independent of levels of plasma cortisol, showing that the transcriptional changes of these genes appear not to have a direct effect on the control of cortisol release and it may be that protein changes are required for this. Most of the genes studied here did not change in expression which is inconsistent with a stress response. However, two genes, V1a and EPD exhibited similar results to those previously found in

other studies. By contrast, the gene expression results reported here are inconsistent with gene expression usually associated with an acute stress response, which usually invokes changes in CRH (Hsu *et al.* 2001; Liu *et al.* 2001; Wang & Xu 2008) and the corticosteroid receptors MR and GR (Poletto *et al.* 2006; Yada *et al.* 2007; Jeffrey *et al.* 2012) along with no change in interrenal genes (Geslin & Auperin 2004; Kusakabe *et al.* 2009). Changes in the abundance of brain transcripts of V1a and EPD are consistent with a stress response, but the evidence for this response weakens further down the HPI axis. Additionally, changes are undetectable for some genes at the time points used in this study but may have occurred prior to the first time point. These results are consistent with previous findings that the response to an acute stressor is mediated quickly by the brain and kidney, but that transcript levels may be less important than protein levels.

6.4.1 Relationship between cortisol and gene expression

The elevation of plasma cortisol levels is a well-documented physiological response to acute and chronic stressors (Pickering & Pottinger 1989; Pickering *et al.* 1991). The present results show an initial rise to 20.8±15.2 $ngml^{-1}$, followed by return to control levels within 24 hours. This is in accordance with previous studies, which showed an elevation of plasma cortisol to 40-80 $ngml^{-1}$ 30 mins after a 30 second emersion stressor, where this was reduced to <20 $ngml^{-1}$ at 2 hours post stress (Pickering & Pottinger 1989). These findings confirm that the rainbow trout in the present study were exhibiting a physiological change in cortisol concentrations in response to the emersion stressor.

The present study also sought to quantify the correlation between cortisol and the expression of selected genes within the stress axis but found that the cortisol response was not correlated with gene expression. Previous findings show that circulating cortisol concentration is positively correlated with $P450_{SCC}$ and StAR mRNA expression up to 5 days after ACTH treatment in Chinook salmon, *Oncorhynchus tshawytscha* (McQuillan *et al.* 2011), where ACTH stimulates the release of cortisol. Moreover, Thomson *et al*. (unpub.) showed that trout selected for high (HR) and low (LR) cortisol responses to stress exhibited differential gene expression of HPI-related genes, including CRH and MR. These findings show that transcriptional changes of genes coding for proteins in the HPI axis may be less important than the protein levels themselves. Indeed, Akinbami *et al.* (1999) showed that three hours of immobilisation stress in rats, *Rattus norvegicus*, caused a reduction in $P450_{SCC}$ protein levels, but no change in mRNA levels. This may suggest that an important step in controlling or regulating stress responses may be in translation.

Whilst there was no correlation between gene expression and cortisol levels, the present results show that genes were more highly correlated within tissues than between tissues. Genes in the brain were more highly correlated with those in the kidney than the liver and, likewise, the relationship of genes in the kidney was stronger with the brain than with the liver. Correlated gene expression in a tissue may be due to the specific role of a tissue in stress responsiveness and therefore the specific roles of the genes. For example, the genes measured in the liver were MR and GR, which are associated with mediation of negative feedback and with tonic inhibition of the stress response (Reul *et al.* 2000; de Kloet 2004). However, the expression of MR and GR was also investigated in the brain and kidney, respectively. In neither case was gene expression different to unstressed control fish at any time point. This may mean that the HPI axis ceases to produce cortisol before the stress response reaches the liver, or that the relationship between transcript and protein has an important role in mediating the cortisol stress response. Future studies should explore tissue specific expression in more detail.

6.4.2 Genes in the brain

Whilst no categorical difference in gene expression was shown in the current study, a general relationship between gene expression of V1a and time was shown, where V1a mRNA was upregulated after 2 hours and decreased at 8 hours. The gene encoding the AVT receptor, V1a, was similarly upregulated 5 and 20 hours after osmotic challenge in pupfish, *Cyprinodon nevadensis amargosae* (Lema 2010). This may confirm findings in the present study; however, genetic changes may be different between specific stressors. This initial upregulation is consistent with previous results where AVT, the neurohormone that binds V1a is upregulated in rainbow trout 2 h after stress (Gilchriest *et al.* 2000). However, this study shows a continuation of upregulation in AVT even after 26 hours, indicating that AVT and its receptor have distinct roles in the response to acute stress, although future studies are need to quantify this relationship. Perhaps unusually, V1a expression over time in this response to an acute stressor is consistent with the pattern of gene expression over time seen after recovery from an aggressive interaction in dominant trout (Chapter 5). This may suggest pleiotropic effects of V1a in both stress and aggression.

Similarly, acute stress was expected to upregulate EPD, since this has been implicated in stressed zebrafish, *Danio rerio,* and carp, *Cyprinus carpio* (Tang *et al.* 1999), and in stressed rainbow trout bred for divergent responses to stress (Thomson *et al.* 2011). The results of EPD upregulation is consistent with an acute response to stress. Since this is the first time that temporal EPD mRNA changes in response to an acute stressor have been studied, there is a lack of evidence with which to compare the decrease in EPD at 8 hours following stress.

Tang *et al.* (1999) showed a 2 fold-change of EPD mRNA during a 24 hour response to cold stress, but did not investigate the recovery period. Therefore, these results are the first to show that EPD rises 2 hours and falls 8 hours after acute stress suggesting a role in acute stress responsiveness and recovery.

The gene coding for CRH is normally upregulated after acute stress (Hsu *et al.* 2001; Liu *et al.* 2001; Wang & Xu 2008). Since chronic stress elicits no change in CRH protein levels (Yulis & Lederis 1987), the lack of change in CRH gene expression shown in the current study may be evident of chronic stress in these trout. Perhaps, for example, due to prolonged stress caused by their hatchery background that exposes them to repeated handling. Such effects were minimised during the experiments as the fish were allowed time to settle from both transport to the aquarium and transfer from holding tank to experimental tank. As such, it is more likely that the experimental stress response was too mild to elicit an observable response from the CRH-ACTH axis. In addition, the lack of change in CRH is contrary to expectations because of the similar actions of CRH and AVT: they both act to stimulate release of ACTH (Mikhailova *et al.* 2007). However, this effect was different for the two genes in goldfish, *Carassius auratus*, whereby CRH had twice the ACTH-stimulating capacity of AVT (Fryer 1989). This could also be the case for rainbow trout, so that less CRH than AVT is required to elicit a response to an acute stressor. In addition, CRH and arginine vasotocin (AVT) are the hormones that stimulate ACTH production, whilst their receptors mediate this action. Therefore, the results here indicate that by 2h, the stress response is not being stimulated by CRH, but that it is being mediated by V1a. Moreover, these results indicate that transcriptional changes in V1a negate the need for similar changes in CRH.

The final gene investigated in the brain was MR, which is involved in tonic inhibition of the HPI axis. MR expression changes differ in relation to the type and strength of the stressor. Often, MR is downregulated after acute stress (Olsson *et al.* 1997; Poletto *et al.* 2006; Kanitz *et al.* 2009), which demonstrates association with the elevation of the cortisol response. However, other times, as here, gene expression does not change and this has been attributed to possible post-transcriptional regulations, evident from differences between heteronuclear RNA (hnRNA) and mRNA (Herman & Watson 1995; Paskitti *et al.* 2000). It is therefore possible that post-translational regulation maintains MR mRNA levels at 2 hours after stress, the peak cortisol concentration in the present study. This may indicate that the cortisol response to stress may be regulated within two hours of the stressor. Alternatively, MR mRNA levels may not change due to the system functioning to maintain homeostasis in response to emersion stress.

6.4.3 Genes in the kidney and liver

Given that not all of the genes in the brain showed significant changes in expression and that the genes from the brain and genes from the head kidney are not strongly correlated, the results that neither StAR nor $P450_{SCC}$ differed in mRNA expression in response to acute stress may be expected. These findings are also consistent with previous findings (Geslin & Auperin 2004). However, StAR and $P450_{SCC}$ are known to exhibit downregulation in response to chemical stress in rainbow trout (Aluru *et al.* 2005), thus changes in expression of these two genes is dependent upon the strength of the stressor. Since changes in mRNA expression of StAR and $P450_{SCC}$ are associated with production of cortisol, the lack of change here suggests rapid mediation of the cortisol response, by prevention of translation of these proteins. Indeed, in rats, mRNA expression and in protein levels were different (Akinbami *et al.* 1999), meaning it is possible that transcriptional changes are not representative of protein levels.

The liver is an important target for cortisol actions and MR and GR are involved in negative feedback of the cortisol response to stress as well as mediating effects of corticosteroids on target tissues. No change in either of these receptors was found in the liver or for GR in the kidney. These results, along with previous findings that GR mRNA expression is elevated 1h post-stressor (Wiseman *et al.* 2007), further corroborate the implication that the cortisol response to stress seen 2 hour after emersion stress was regulated rapidly through homeostasis. Whereas MR controls tonic inhibition of the HPI, GR is involved in negative feedback (Sathiyaa & Vijayan 2003), where an increase in cortisol elevates mRNA and lowers GR protein. No change in these receptors indicates that tonic inhibition of the HPI is retained and, therefore, that negative feedback from GR is not necessary.

6.4.4 Conclusions

Stress is a major problem in aquaculture due to repeated (handling or transport) or prolonged (overcrowding) stressors. The cortisol response to stress is a well-studied trait in rainbow trout and its release is initiated and regulated by the HPI axis. However, the changes in gene transcripts along the HPI axis over time have not been characterised before. This study aimed to quantify changes in candidate gene transcripts across the HPI axis in three key tissues, with the aim of detecting changes in stress response during recovery after an acute stressor. Gene expression was correlated along the HPI axis within tissues, but not with plasma cortisol concentrations. Additionally, weak or no temporal variation was found in gene expression, despite a normal cortisol response. These results imply that the release of cortisol in response to an acute stressor is mediated within a short time-frame. Alternatively,

changes in gene transcription may be less important than protein levels in generating an elevated cortisol response. However, further work should be carried out to quantify protein changes compared with transcriptomic changes to elucidate the speed of mediation of the stress response. There was a correlated response of gene expression at different points in the HPI axis and therefore, these genes were upregulated in concert to initiate and attenuate the cortisol response to stress. This study highlights the importance of time course studies, both at the level of the transcript and the protein to understand how the stress response is mediated over time. Future studies should explore other pathways in multiple key tissues across a range of stressors to provide a comprehensive overview of stress.

Chapter 7 General Discussion

Behavioural ecologists have recently highlighted the genetic influences upon behaviour (McGuffin *et al.* 2001; Boake *et al.* 2002; Dick & Rose 2002; Bell 2009). Research in this field has mostly been limited to studies of single candidate genes or quantitative trait loci (QTLs) (Reif & Lesch 2003). However, candidate genes explain only a small amount of genetic variation associated with behaviour, whereas many genes, potentially with small, interacting or regulatory effects may be the underlying cause of behavioural differences (McGuffin *et al.* 2001; Boake *et al.* 2002; Dick & Rose 2002). Contemporary technologies make it possible to detect the level of genomic complexity involved in complex behavioural traits. I used a combination of genomic approaches to understand genomic components of animal behaviour, under the wider context of a behavioural syndrome. Aggression in rainbow trout does show a relationship with heterozygosity, but neither boldness nor stress responsiveness yielded significant results. Moreover, aggressive behaviour was associated with differences in global transcript expression. These results highlighted the complex mechanisms that regulate apparently correlated behaviours. In rainbow trout selected for divergent stress responsiveness, neutral markers showing signatures of selection were widespread across the genome, indicating genome-wide distribution of genes associated with stress responsiveness. Furthermore, small changes in candidate gene expression occurred after acute emersion and aggressive interactions. These findings are relevant to understanding the genomic architecture, which describes the number and chromosomal locations of genes, associated with complex behaviour, potential epistatic effects of many genes and pleiotropic effects of a few genes (Mackay 2009). Moreover, I have demonstrated the utility of genomics in behavioural ecology to identify novel candidates for further study. In addition, these results have wider implications for behavioural syndromes, applications for aquaculture and fitness effects of important behaviours.

7.1 Genomics to identify behavioural candidate genes

Novel targets for stress and aggression in the form of genome regions (Chapter 3) and transcript sequences (Chapter 4) have been determined here using the rainbow trout. In Chapter 3 I used a genome scan to identify regions under selection, which are potentially associated with stress. These regions were spread across the genome and a small number corresponded with previously identified QTLs for the cortisol responses to stress (Drew *et al.* 2007; Quillet *et al.* 2010). With the absence of a sequenced genome, these regions require validation through sequencing and annotation. Indeed, in model organisms, such as humans, *Homo sapiens*, and mosquitoes, *Anopheles gambiae*, it is simpler to allocate function to genomic regions that show evidence for selection. For example, the distances

between microsatellites and genes can be obtained (Storz *et al.* 2004). Furthermore, it is possible to scan the genome for polymorphisms (*i.e.* SNPs) that differ among populations and also to identify the function of a wider suite of genes under selection using Gene Ontology (The Gene Ontology Consortium 2000) as completed recently to understand human evolution (Tang *et al.* 2007). A further approach used in model organisms was to apply Diversity Arrays Technology (DArT markers) to identify genes under selection and then identify particular SNPs that are under selection (Bonin *et al.* 2009). However, the use of microsatellites in rainbow trout allows the location of selected regions to be identified and is the initial step in identifying functional information, which can be studied further via sequencing and gene ontology information. Nonetheless, this study represents the first time selection has been specifically studied for the purpose of identifying candidate genes for behaviour.

Using next generation sequencing (NGS), key genes associated with aggressive behaviour were identified (Chapter 4). The alternative approach for characterising transcriptomes associated with phenotypes is to use microarrays (Section *4.1.2*). However, microarrays require *a priori* knowledge of the gene probes to be assessed, which excludes any unsequenced genes, whereas NGS allows *de novo* sequencing of the full complement of genes associated with a phenotype. Thus, this approach is essential for the study of evolutionary and ecological questions in non-model organisms. By applying NGS for the study of aggressive behaviour, it is possible to identify novel candidate genes, which may not have been previously implicated in this function. Indeed, in Chapter 4, not only were previous candidate genes for aggressive behaviour confirmed (proopiomelanocortin; POMC), but novel candidates previously not associated with aggression were identified (neuromedin S). POMC is known to be involved in stress responsiveness and other behaviours, which may indicate a range of pleiotropic effects. As well as identifying expected genes, the study aimed to identify novel candidates, of which there were two types: the first were identifiable genes, but which are not commonly considered in behavioural studies, possibly due to small, previously undetected effects on behaviour. The second are those which are novel sequences that did not align with currently identified sequences and should therefore be studied further. By identifying novel candidates for aggression and stress responsiveness, the mechanisms involved in the control of these behaviours may be better-known. For example, by identifying genes involved in oxygen metabolism or hormone secretion (Chapter 4), the pathways involved in the control of different behavioural types may be understood. Overall, the novel candidates for stress responsiveness and aggressive behaviour, in the form of genomic regions and differentially expressed genes,

implicate many genes, and therefore pathways may be more important in the expression of behaviour than are single genes.

7.2 Genomic control of behaviour is complex

The genomic architecture of behaviour is typically complex, encompassing the action of genetic variation in DNA sequence, through to the factors that regulate gene expression. Furthermore, behaviours are typically influenced by many interactions between gene networks and internal and external environmental stimuli (Mackay 2009). The study of behavioural genetics has made a transition towards disseminating the genomic architecture involved in behaviour since it is known that genes show interactions, such as regulatory or modulatory effects (Boake *et al.* 2002; Reif & Lesch 2003; Mackay 2009). The results from Chapters 2-6 demonstrated many genes were involved in the control of behaviour (Chapter 4), which are potentially distributed across the genome (Chapter 3), indicating complex genomic control. Moreover, there was evidence of pleiotropic effects of particular genes. In addition, potential epistatic effects may explain small changes in genes involved in the hypothalamic-pituitary-interrenal (HPI) axis (Chapters 5 & 6). Differences in genetic diversity among stress responsiveness, aggression and boldness were also found (Chapter 2) suggesting that trout may have a genetic propensity to win fights but not to be bold. Overall, these results may contribute to the knowledge of the complex genomic association with individual behaviour as well as genomic effects involved with behavioural syndromes.

Pleiotropy, where a gene may have effects in many phenotypes, may be implicated when the expression of particular genes in one behaviour are the same genes known to be associated in other behaviours or phenotypes (Anholt & Mackay 2004; Barendse *et al.* 2009; Edwards *et al.* 2009b). For example, in Chapter 4, neuromedin S was identified as a novel candidate gene associated with aggression, whereas it was previously implicated in feeding behaviour (Ida *et al.* 2005; Miyazato *et al.* 2008). Similarly, proopiomelanocortin (POMC) was upregulated in aggressive trout. POMC is a precursor to adrenocorticotrophic hormone (ACTH), which leads to the release of cortisol, the principal hormone secreted during stress in many animals including trout (Sloman *et al.* 2001). As such, POMC is a crucial part of the HPI axis, which is involved in the control of stress responsiveness and aggression. Moreover, pleiotropy may be evident in Chapters 5 and 6, where similar genes were studied in the brains of fish that had experienced aggression and stress. Whilst there were some weak changes in HPI genes, some of these changes were similar between aggressive fish and those that had received an acute stressor. The vasotocin receptor V1a showed similar expression patterns in aggressive fish as in stressed fish. Not only does this support pleiotropy due to similarities in gene expression in the brain of these two behaviours, but

also due to the role of vasotocin and its receptors in learning and memory (de Kloet 2010). However, other genes, mineralocorticoid (MR) and ependymin (EPD) did not show similar patterns between stress and aggression tests, which indicate that only particular genes potentially exhibit pleiotropic effects. These Chapters highlight, that while the study of individual candidate genes is important, the study of networks of interacting genes may be more informative when studying complex behavioural traits.

Using transcriptomes may be more informative than single candidate genes to determine the number of genes involved in a complex trait. In Chapter 4 many genes were correlated with aggressiveness. Moreover, transcriptomic data provides initial networks from which gene interactions may be studied and enable a move towards exploring networks of interacting genes, which may have a small effect upon phenotype and that combine to produce a specific behavioural outcome (Anholt & Mackay 2004). Using this transcriptome data, it may be possible to map epistatic effects of genes, where the expression of certain genes or gene products regulates the expression of other genes (Reif & Lesch 2003). In Chapter 5, some genes may be under epistatic control of other factors. For example, arginine vasotocin (AVT), the agonist of V1a suppressed aggression in rainbow trout, where this may be regulated by serotonin (5-HT), which suppresses AVT (Backström & Winberg 2009). Thus a lack of gene expression changes, in genes expected to be involved in stress and aggression, may be due to the control of genes not studied in these chapters.

Of course, behaviour is not solely under the control of genes and it is well-known that genes are both inherited and environmentally responsive for most phenotypes, but this is a relatively new concept in behavioural ecology (McGuffin *et al.* 2001; Dick & Rose 2002; Bell 2009). Gene transcripts can have a biological role in causing behavioural phenotypes, as they link phenotypic variation with gene expression (Boake *et al.* 2002). However, the transcript differences seen in Chapters 4-6 may be expressed because of the specific previous experiences or environmental effects influencing a response in gene transcripts of individual fish. Indeed, behaviour is known to alter depending upon environment, for example, boldness in rainbow trout was dependent upon food availability and predation threat (Thomson *et al.* 2012). Furthermore, rainbow trout alter their boldness based upon previous experience or observation (Frost *et al.* 2007). Similarly, a winning experience altered the type of aggressive behaviour used to initiate fights in the cyprinid fish *Rivulus marmoratus* (Hsu & Wolf 2001). Gene-environment interactions also affect behaviour, for example, three transcriptional profiles of aggression in *Drosophila melanogaster* differed between highly and less aggressive flies but showed little overlap (Bendesky & Bargmann 2011). It is often the case that behaviour is affected by gene-gene interactions as well as gene-

environment interactions. For example the interactions of serotonin with monoamine oxidase a and rearing environment affected behavioural responses to a human intruder in rhesus macaques, *Macaca mulatta* (Kinnally *et al.* 2010). These studies suggest that transcript profiles from this thesis should be considered within the context of the experimental environment in mind. Furthermore, by emulating natural environments or by controlling previous experiences during the life of a fish, transcriptome studies questions about the context-dependent nature of behaviour may be approached.

7.3 Genomics of behavioural syndromes

Behavioural syndromes are behavioural and physiological correlations that are consistent across context (Sih *et al.* 2004a; Sih *et al.* 2004b; Conrad *et al.* 2011). However, it is unknown whether the behavioural and physiological correlations have a genetic component, but it is expected that genes play a substantial role (Koolhaas *et al.* 1999; Bell 2009; St-Cyr & Aubin-Horth 2009; Prunet *et al.* 2012). The present study's findings indicated a decoupling of genomics and behaviour. Firstly, there is no relationship with genetic diversity between behaviours that usually constitute behavioural syndromes (Chapter 2), whereby aggression was affected by heterozygosity, but boldness and stress responsiveness were not. Second, there were differences in patterns amongst MR and EPD transcript expression between behavioural tests (Chapters 5 & 6), indicating that pleiotropic effects of these genes are not responsible for behavioural syndromes. However, behavioural syndromes were not explicitly studied in these fish; therefore, the effects of these genes may be linked between stress and aggression. Indeed, previous studies implicate both MR and EPD in stress and aggression (Sections *5.4.3* & *6.4.3*). Nonetheless, there is evidence to suggest that there is a decoupling of behavioural traits in a behavioural syndrome (Vaz-Serrano *et al.* 2011), but the genomic effects upon behavioural syndromes are still unclear and should thus be studied in further detail. By using next generation sequencing, the pathways of interacting genes may be studied to identify the genetic links in behavioural syndromes and provide new avenues for future research.

Of course, in addition to genomic effects upon behavioural syndromes, other influences, such as environment, may influence the correlation of behaviour. In the aquaculture environment, there are obvious differences compared with natural environments, notably, the absence of predators and presence of many conspecifics. These differences in the environment may place different selection pressures, as suggested by Conrad & Sih (2009), whereby reduced threat of predation may relax selection pressure for bold behaviour. Indeed, the results in Chapter 2, where boldness, measured in the absence of predators, does not exhibit a heterozygosity-fitness correlation (HFC) shows similarities with the results in

Vilhunen *et al.* (2008). In this study, boldness measured in the absence of predators was not associated with heterozygosity but boldness measured in the presence of predators was. This may show that the ecology of the hatchery environment alters the coupling of traits, either due to changes in genetic diversity or behavioural plasticity. Comparing genomics of aggression with that of boldness and stress responsiveness between hatchery and natural populations, may indicate how environment affects genomic correlations in rainbow trout.

7.4 Wider implications

Studying the genomics of behaviour in farmed fish may have important applications for breeding programs and welfare. For example, by investigating selection lines (Chapter 3) it may be possible to identify genetic markers with which to aid marker-assisted selection programs (Yeo *et al.* 2000; Avila *et al.* 2005). Furthermore, novel candidates identified using gene transcription of stress responsiveness and aggressive behaviour may act as biomarkers to detect the effects of stress and aggression produced by aquaculture procedures, such as overcrowding.

Heterozygosity is correlated with many fitness-related traits, such as survival (Coulson *et al.* 1998; Silva *et al.* 2009), reproductive success (Olano-Marin *et al.* 2011; Wetzel *et al.* 2012), disease resistance (Acevedo-Whitehouse *et al.* 2005; Rijks *et al.* 2008) and growth rate (Pogson & Fevolden 1998; Bierne *et al.* 2000). Moreover, the expression of a number of important behavioural traits, such as aggression (Charpentier *et al.* 2008) and territoriality (Lieutenant-Gosselin & Bernatchez 2006), is associated with heterozygosity. Heterozygosity is often related to fitness traits due a reduction in the expression of deleterious recessive alleles (dominance) or heterozygote advantage (overdominance) (Slate *et al.* 2004). When fitness-related traits are correlated with heterozygosity, this relationship may be caused by two effects when neutral markers are used (Hansson & Westerberg 2002). The first is the local effect hypothesis, where neutral loci are in linkage disequilibrium (LD) with one or more fitness genes. The second is the general effect hypothesis, where the level of heterozygosity across a large set of neutral markers is generally correlated across loci within an individual's genome (ID), where the heterozygosity at neutral markers is thought to represent genome-wide heterozygosity due to inbreeding (Weir & Cockerham 1973; Szulkin *et al.* 2010). When this occurs, individuals may show fitness across many phenotypic traits. Heterozygosity at many loci can affect immunocompetence, growth and survival (reviewed in (Kempenaers 2007)) and these traits may enable an individual to increase its competitive ability. This may allow an individual to increase its fitness in terms of survival and reproductive success through gaining food or mates.

In Chapter 2, aggressive rainbow trout were more heterozygous than less aggressive trout, which was most likely due to general effects. This may indicate that individuals exhibit a number of fitness-enhancing traits. In Chapter 4, the transcriptomes of a pool of five aggressive individuals from Chapter 2 were characterised in comparison with less aggressive individuals. Aggressive and less aggressive individuals expressed expressed different genes associated with energy metabolism: aggressive trout expressed genes with antioxidant effects and less aggressive trout expressed genes associated with increased respiration. Whilst the relationship between heterozygosity and gene expression was not explicitly investigated here, there is a possibility that the differential expression of genes may be associated with genome-wide heterozygosity. This may be due to a high number of loci with dominant or overdominant effects, whereby beneficial alleles are expressed more in heterozygous individuals. Individuals that are more efficient at storing or mobilising energy may be better able to compete and thus win territories, food or mates. These results may reveal some of the mechanisms behind the fitness-related trait aggression. However, genes that are differentially expressed between aggressive and less aggressive trout should be genotyped at the corresponding loci to determine heterozygosity and thus discover whether heterozygosity influences the expression of aggressive behaviour.

7.5 Conclusions

These studies aimed to assess the genomic complexity of the evolutionarily important behavioural traits, stress responsiveness, aggression and boldness, including the genomic links between behaviours, so as to provide empirical evidence for underlying mechanisms of behavioural syndromes. In addition, this study aimed to identify candidate genes associated with stress and aggression using novel genomic techniques. The results showed that genetic diversity was linked with aggression but not stress responsiveness or boldness (Chapter 2). Moreover, genome-wide heterozygosity, rather than heterozygosity at single loci, appeared to be associated with aggressiveness. Similarly, genome regions potentially associated with stress responsiveness were located across the genome (Chapter 3). I also showed that genomic control of behaviour was complex, where many genes were associated with aggressive behaviour and these may have pleiotropic or epistatic effects (Chapter 4). Pleiotropic effects may be present in some genes, (*e.g.* V1a: Chapters 5 & 6; or POMC: Chapter 4), but not others, (*e.g.* EPD: Chapters 5 & 6). Moreover, I showed that novel applications of techniques can yield novel candidates for behavioural investigations, where I identified genome regions that are potentially associated with stress responsiveness (Chapter 3) and candidate genes associated with aggression using a transcriptome, including unidentified sequences (Chapter 4). These results demonstrated that behavioural ecology can shift from the study or one or a few candidate genes and towards a network view of

genomics, where many genes and their interactions control complex behaviours. Moreover, this idea of multiple genes can be applied to single behavioural traits and to behavioural syndromes, where pleiotropy may be restricted to the effects a few genes, which are regulated by the interactions of many genes. To further the study of behavioural genomics, the impact of environmental conditions and previous experience to investigate the non-genomic control should be considered. Epigenetic or maternal effects may influence intraspecific behaviour and studies are beginning to explore indirect genetic effects. Moreover, there may be regulatory systems and pathways that interact both at the genetic and environmental level, which may be studied with the use of next generational genomic tools.

Appendix 1

A1.1 Opponent-dependent levels of aggression – Preliminary study

In Chapters 3, 4 & 5, I studied the genetic differences in aggressive and less-aggressive groups of rainbow trout. Originally, I wanted to base the study upon aggressive and less-aggressive individuals that became winners by a greater or lesser margin when encountering opponents. However, it was difficult to know whether the margin was due to the fish being a better fighter or whether it was due to an effect of the opponent. To test this, I used twelve rainbow trout that encountered three different opponents that were 50% of the size of the test fish on three separate occasions. Small fish were used to ensure that the test fish always won. I measured the latency to begin an interaction and the frequency of aggressive acts, as outlined in chapter 1. I tested for a difference in aggressiveness among individuals. I found no differences in number of aggressive acts carried out by each individual (Figure A1.1: K_{11}=15.7, p=0.153) or in the latency to begin an interaction (Figure A1.2: K_{11}=12.0, p=0.366). Therefore, this was not deemed to be an effective or consistent way to measure inter-individual levels of aggression. This also seems to indicate that, while a large fish is likely to win, opponents affect the amount of aggression an individual performs. Thus an approach to measure aggression that accounts for opponent effects was used in chapter 1.

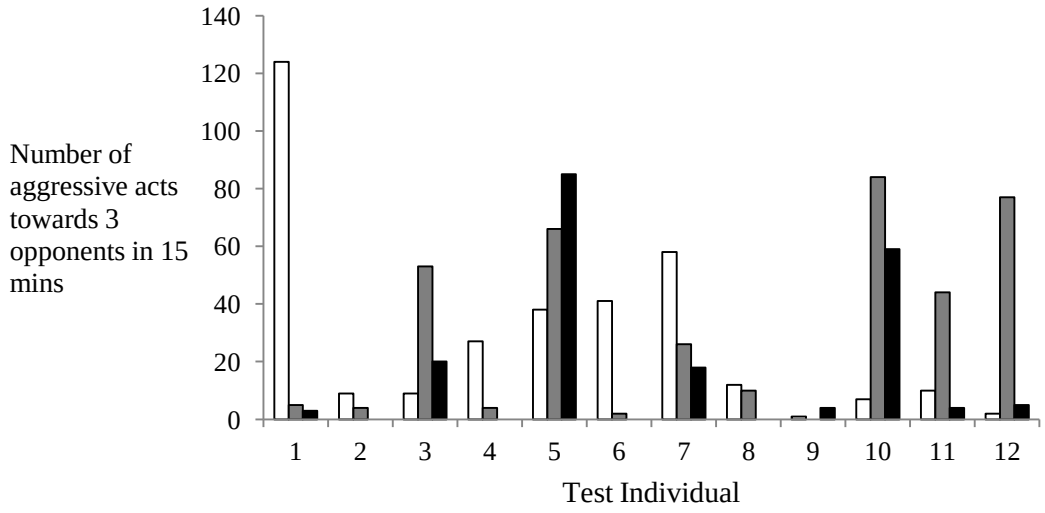

Figure A1.1. The number of aggressive acts carried out by large fish that interacted with three smaller (50%) fish on three separate occasions (white bars are against opponent 1, grey bars are opponent 2 and black bars are opponent 3).

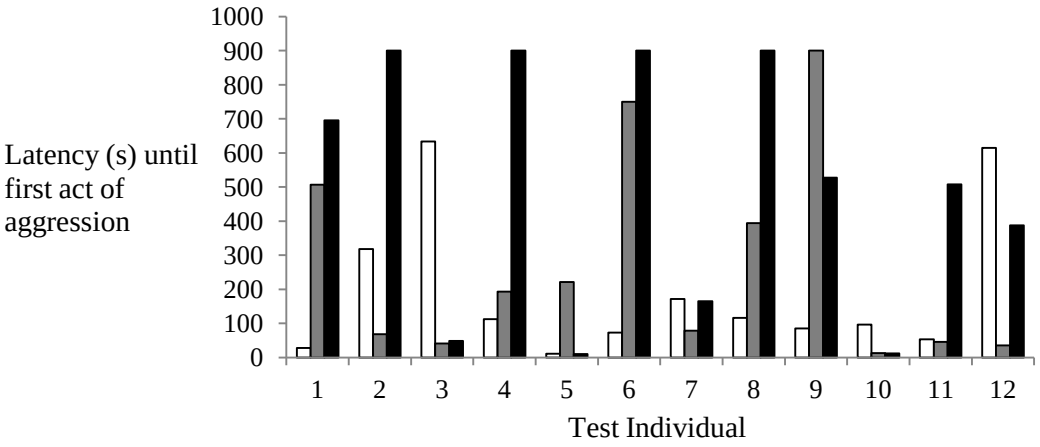

Figure A1.2. The latency in seconds (to a maximum of 900s) until the large fish began to attack one of three smaller (50%) opponents. White bars are against opponent 1, grey bars are opponent 2 and black bars are opponent 3.

Chapters 2 & 3

Table A1. Primer sequences, linkage group, GenBank accession number and reference for all successfully amplified microsatellite markers used to characterise genetic variation between lines of rainbow trout selected for divergent cortisol responses to stress or aggressive behaviour in a hatchery strain of rainbow trout (denoted by L/S).

Marker	Forward Primer	Reverse Primer	Linkage Group	GenBank Accession	Reference	Line (L)/ Strain (S)	Chapter 2 or 3
OMM1665	CGTGTCACAAACCGTTACC	CCTCCAATGTTCCAACTCA	RT1	BV212292	Coulibaly et al 2005	L	3
OMM5136	CCAGCACTTTCTGTCTCATA	CTCCACAGGCCTTATTACTT	RT1	BV211865	Coulibaly et al 2005	L	2 & 3
OMM1000	GACCACCAGCTCTTTCAATTA	GCAAACTGGGTTTTAGATCAG	RT1	AF346664	Rexroad et al 2002	L & S	2 & 3
OMM3006	CCCCTGTTTATTACAGTGGATGAGA	GAGATTGTACTGGTCTACTTATTCTCCGTC	RT2A	G73806	Rexroad and Philips	L	2 & 3
OMM5165	TCAGAGAAGGGATGTCGG	TCTGCTGAACCAGTGCAT	RT2A	BV211893	Coulibaly et al 2005	L	2 & 3
OMM1361	TCCCTCCTGCTGGTTTACACT	ACGTCAGCCTGTTGGTCATTC	RT2A	BV005153	Palti et al 2002	L	2 & 3
OMM5319	TGCACTCTCCTTTGATGTC	AGACAAAGCCTTGAACGAG	RT2B	BV212027	Coulibaly et al 2005	L	2 & 3
OmyRGT40TUF	GCAGATAAGGCACCAACCAT	TATGCTTAGAGCCCCCTGTG	RT2B	AB087608	Sakamoto et al 2000	L	2 & 3
OMM5320	CGCTTATGTCGCTTATGC	GGAGACAGGCTTAGGGAC	RT2B	BV212028	Coulibaly et al 2005	L	2 & 3
OMM5264	AAGCATCATTGCCGTGAC	TCTCTTCGCAGGGATTCT	RT3	BV211979	Coulibaly et al 2005	L & S	2 & 3
OMM1312	AGGTCCAGACAGCAATCC	GGTGAGTTTAGCGAGGTA	RT3	G73552	Palti et al 2002	L	2 & 3
OMM1346	CTCATGGGAATGTTAAGGATGATT	AGCCTTTTAAACCATTGACCTCT	RT3	G73577	Palti et al 2002	L	2 & 3
OMM5137	TGTTCGTGCTGGAGTACC	GGAAGTCTGCAGGCTAGG	RT4	BV211866	Coulibaly et al 2005	L	2 & 3
OMM1032	GCGAGGAAGAGAAAGTAGTAG	CCCATCTTCTCTCTGATTATG	RT5	AF352767	Rexroad et al 2002b	L	2 & 3
OMM1007	CATAGTTTTCCTGGTTCAC	CCCTTAACTGACGCTATT	RT5	AF346669	Rexroad et al 2002	L	2 & 3
OMM1011	CAAGGATTCGGGACAT	CACCCCTAAAGTAGAGCA	RT5	AF346672	Rexroad et al 2002	L & S	2 & 3
OMM1082	CAAGAGCACTAACGACCATGT	CGCAAGCAAGCTAACACA	RT6	AF352753	Rexroad et al 2002b	L	2 & 3
OMM1780	ATTCACAAGGCTCTGATCCC	GCAGACAATTTCCTCTACCG	RT6	BV212247	Coulibaly et al 2005	L & S	2 & 3
OMM1151	GTCTCATCAGCCATGCGACTCAAA	TGCCAGGGAATACAGTCTGTAATGACC	RT6	AY039633	Rexroad et al 2002a	L	2 & 3
OMM1376	GCTGTTGTGGGATTAGAACTG	AACCGGTCTATCAAGTTAGGC	RT7	BV078062	Rodriguez et al 2003	L	2 & 3
OmyRGT17TUF	GGTCAGTGGCCATTCAGATT	ACCAGCTCCTCCCTTGTTCT	RT7	AB087594	Sakamoto et al 2000	L	2 & 3
OMM1764	GCTACCCGCTACCCTAACAT	GCCCACCTACAGAGATTGTC	RT7	BV212233	Coulibaly et al 2005	L	2 & 3
OMM1009	ACTGGAATCCAATAACAACCC	CGGAGGTTTGATGAGTCATT	RT8	AF346671	Rexroad et al 2002	L	2 & 3

Marker	Forward Primer	Reverse Primer	Linkage Group	GenBank Accession	Reference	Line (L)/ Strain (S)	Chapter 2 or 3
OMM1667	CTTACCCAAACAGTCGCAATTC	TCATTCTGAGCGGACTAACACA	RT8	BV212294	Coulibaly et al 2005	L	2 & 3
OMM1793	CTGTAACTGTGAGCAAGCAAAC	GAGACTGCTGGGAGAGGTATCT	RT8	BV212254	Coulibaly et al 2005	L & S	2 & 3
OMM5318	TCCTCCATCTCCACTAAC	GGGTCAGCATTTCTAATC	RT8	BV212026	Coulibaly et al 2005	L	2 & 3
OMM5254	CTGTCTGGGTTGCGTTTGCTA	CCTGTGGCACACAATGGATTC	RT10	BV211969	Coulibaly et al 2005	L & S	2 & 3
OMM1710	GGCAGACATTTGCTTCAGTC	GGTTCTGGGTAATGGTTGTG	RT10	BV212191	Coulibaly et al 2005	L	2 & 3
OMM5186	TGACCACAGCAGACATATAGCCAG	GCTTGTGTTGGGTTTGATATAGGG	RT10	BV212286	Coulibaly et al 2005	L	2 & 3
OMM5261	AGGTCCTGCGACTGTTCTA	CAAAGACCTTGTGCTCAAC	RT11	BV211976	Coulibaly et al 2005	L	2 & 3
OMM1154	GGCTGTGCATTAATTCCAG	TGTGAGAGAAGGAGAAGCTGA	RT11	AY039636	Rexroad et al 2002a	L	2 & 3
OMM1084	CGAGACAAGCAGCCAGATAGAG	CACTGACTGTCTGTCTTGGCTATC	RT11	AF352754	Rexroad et al 2002b	L	3
OMM1127	GAGCTCATCTGATCGTGAC	GGAGAGATGAGAGGATGTATG	RT12	AF375029	Rexroad et al 2001	L	3
OMM1158	CCCATGACGCTGTAAACC	ATGTGTGCTTAAATCTCACCC	RT12	AY039640	Rexroad et al 2002a	L	2 & 3
OMM5328	AATGAAACCCAAGCCTAGC	GGGTCTTTCCAGATGCACT	RT12	BV212036	Coulibaly et al 2005	L	2 & 3
OMM1381	TGTTCTTGGTCCGCATTGA	GTCCTTTTCGTCGTTGTTGTG	RT12	BV078067	Rodriguez et al 2003	L & S	2 & 3
OMM1762	CCTCTAATTTCACTCGGATG	CCTTTGTCTGTCTTAAACCC	RT13	AF469960	Coulibaly et al 2005	L	2 & 3
OMM1020	CCTGTGAGTGTTAATTCGACCTGT	GGTCTTACCTCAACATCGGTGA	RT13	AF346679	Rexroad et al 2002	L	2 & 3
OMM1783	GAGAACTGAACGGGTTGA	TGACAGCCGACCTAAATC	RT13	BV212249	Coulibaly et al 2005	L	2 & 3
OMM1374	CGTGTATTTGGGTCATTATGC	ATTCTGTTATCTGCGTTTGG	RT14	BV005163	Palti et al 2002	L & S	2 & 3
OmyRGT43TUF	TTACTGTGCATCCTACAGGCC	CATTGCTCATTCATCCCTGA	RT14	AB087610	Sakamoto et al 2000	L	2 & 3
OMM5017	TTGAGCCAAACATGCCTC	CACAGCATCTAGACAGTTCCC	RT14	CO805122	Rexroad unpub	L	2 & 3
OMM5266	GAAGTGTGCTGCATGTCG	CAAGGTCCCCGTGAAATA	RT15	BV211981	Coulibaly et al 2005	L	2 & 3
OMM1051	CCTACAGTAGGGATTAACAGC	CATGCCCACACATTACTAC	RT15	AF346695	Rexroad et al 2002	L	2 & 3
OMM1769	GCCAGGATCTCTGACTCGC	ACACAGTGCCAAGGTCGTG	RT15	BV212238	Coulibaly et al 2005	L & S	2 & 3
OMM1352	GTGTTACGGTTGACATACCTG	AGATTTTCCCTGGTTGCT	RT16	BV005145	Rexroad 2002 unpub	L	2 & 3
OMM1830	TGGACATCTTGAAGCACA	CCATAAGAGCGGCAGTTC	RT16	BV212277	Coulibaly et al 2005	L	2 & 3
OMM1345	CCCTGGATTCTCCTGTTAG	ACATAGACACAGCACTCATGG	RT16	G73576	Palti et al 2002	L & S	2 & 3

Marker	Forward Primer	Reverse Primer	Linkage Group	GenBank Accession	Reference	Line (L)/ Strain (S)	Chapter 2 or 3
OMM1717	CTCCTCTCTGGCTTCATTT	GGAGAGGAGGAGAGTGATG	RT16	BV212198	Coulibaly et al 2005	L	2 & 3
OMM1139	AAGACAGAAGGAAAGCGAGAG	AGGGGATACAGCATTGTTCA	RT17	AF375036	Rexroad et al 2001	L	2 & 3
OMM1116	GACAAAGACAGAGAGGGACGA	AGCACCAAGATCGAAACTCC	RT17	AF352771	Rexroad et al 2002b	L & S	2 & 3
OMM1360	TATGGTGGGAGGTGTCATTGT	GGGGAAAGGTGTGAGTTATAC	RT17	BV005152	Palti unpub	L	2 & 3
OMM1384	TCGTCTATCCCTTCGTGTGAG	AAAGAGAGGAGAGACGGCAGA	RT18	BV078070	Rodriguez et al 2003	L	2 & 3
OMM1512	CAAATCAGCCCAGGTTACAG	GATTACCTGCAGGTGTTTCC	RT18	BV212048	Coulibaly et al 2005	L	2 & 3
OmyRGT12TUF	TGAAGACGTTGTGGCTCCTA	CAAAGCACCTGGCCTGTAAT	RT18	AB087591	Sakamoto et al 2000	L & S	2 & 3
Omi127TUF	GGGAACATTCCCACACCTTA	CAGGGCTACAGGGTAAGTGG	RT19	AB105850	Hara et al unpub	L	3
OMM1025	CGCCATTGTAGTCTCGTC	AGTCCGCTATGTTGTTATGTC	RT19	AF346682	Rexroad et al 2002	L	2 & 3
OMM1333	TTAACTCTTCCATACCCTC	ATGTCTGGCTGACAATACAAC	RT19	G73567	Palti et al 2002	L	3
OMM1107	ACCTTATCCTGTTGCTGCTAC	ATTGCCAGAGGAAACGTC	RT20	AF375022	Rexroad et al 2001	L	2 & 3
OMM1544	AGAGGCCACCACGTTAGA	GAGGAGGCAGCTATGTG	RT20	BV212073	Coulibaly et al 2005	L & S	2 & 3
OMM1134	GAAGTTCATCTCCAGGTCAAACTG	TGCGTAGGTTGATGAATCCTC	RT20	AY039628	Rexroad et al 2002a	L	2 & 3
OMM1367	GCATCAGGCTTTGGGTAACTG	GGTGCACAAGAAGACGCTGAA	RT21	BV005161	Palti et al unpub	L	2 & 3
OMM1824	GAGGACATTGCAGCAATAAGG	TATGGTTACAGCGACGCAACA	RT21	BV212272	Rexroad et al unpub	L	2 & 3
OMM5179	CCCTGTCACATGGATGCT	GATTTGGCAACCGAACAC	RT21	BV211905	Coulibaly et al 2005	L & S	2 & 3
OMM5162	GATACTGTGCAGATTCCGAATG	GCGCTTCAATGTTACGATTACC	RT22	BV211890	Coulibaly et al 2005	L	2 & 3
OMM5133	TGAATAGCATGGCACACTC	CACCATAGGAAATTGACCC	RT22	BV211864	Coulibaly et al 2005	L & S	2 & 3
Omy1158INRA	CCACACAATCACCGTTGC	TGATGGGTGCTATTGACTCG	RT22	BV681443	Guyomard et al 2006	L	3
OMM1538	CAATGTCTACCTTCCGCAAA	AGAGGGGATGGCAGATAGAT	RT23	BV212067	Coulibaly et al 2005	L	3
Omy1501INRA	AAGAGGTGGAAAGAGGAGAGG	TTCACGGCTCAAAGTCTAGG	RT23	BV681375	Guyomard et al 2006	L & S	2 & 3
OMM1719	CGCTACCAACGTGTTAATGT	TTTCTCACACAGTCTCTTGC	RT23	BV212200	Coulibaly et al 2005	L	3
Omy1103INRA	TCAAACTCGGTCCTGTCC	CTGCTCAGTTTACAACAAATGC	RT23	BV681351	Guyomard et al 2006	L	2 & 3
OMM1322	GCGCTCCTTTCATCTCTGATACAG	GGTGAATACTTTCGCAAGCC	RT24	G73560	Palti et al 2002	L	2 & 3
OMM1397	CCGGCTCCACATTGATTAT	TCTTATCCTCTGGCTACAGCA	RT24	BV078083	Palti et al 2003	L	2 & 3

Marker	Forward Primer	Reverse Primer	Linkage Group	GenBank Accession	Reference	Line (L)/ Strain (S)	Chapter 2 or 3
OMM1690	CTCCAGACCTCTCCTAAGC	GGAGAGAGGGATACGAATG	RT24	BV212176	Coulibaly et al 2005	L & S	2 & 3
OMM1389	ACGGCTCCAGTGAAGATTATC	AACTGGTTGTATCGCCTGA	RT25	BV078075	Rodriguez et al 2003	L	2 & 3
OMM1797	AGGATGTAGCGGGTACGG	TCCACCCTCAACGCTTCT	RT25	BV212257	Coulibaly et al 2005	L	2 & 3
Omy1259INRA	ACTGGGAAATGCACAGACC	GATGAACGCCACTGTAGTATGG	RT25	BV681366	Guyomard et al 2006	L	2 & 3
Omy1398INRA	CCCAGAGGTCAAAGATGTGG	TGGCAGTAGAGAATGACAGTGG	RT26	BV681586	Guyomard et al 2006	L	2 & 3
OMM1767	GGGTTCTACCAGTTCTCTTAAC	TTACCTGTCTGCACTAGCTCTC	RT26	BV212236	Coulibaly et al 2005	L	2 & 3
OMM1015	GACAAATTCACCCTCTTATG	CATGAGAACTGTTGCCA	RT26	AF346675	Rexroad et al 2002	L	2 & 3
Omy1178INRA	GGAAATCTGGAGTTGCTTCC	TCCAATCGTTTCTGTTCC	RT27	BV686459	Guyomard et al 2006	L	3
Omy1423INRA	GCTCGTCAGACAGCTTTGC	AGGTCCTGGACATTACTGC	RT27	BV681518	Guyomard et al 2006	L	3
OMM1108	CACAGGTGAGAACATGCCGCTAAT	AGAGCGGGAGCAAATGTGACAGATAGA	RT27	AF352769	Rexroad et al 2002b	L	2 & 3
OMM1778	GTGTTCCTGGTTCCCATAG	GTTGGGTGGACCTCATAAC	RT27	BV212246	Rexroad et al 2001	L & S	2 & 3
OMM1751	GGCCCTGTCTGCAATACC	GGCCCTGTCTGCAATACC	RT29	BV212225	Coulibaly et al 2005	L	2 & 3
OMM1330	CCAGGAAAATAATTGAC	TGGAGGATGTCTATTAGTGTA	RT29	G73565	Palti et al 2002	L	2 & 3
OMM1505	TCTATGGGGCATTAAGTG	CTTCCCGGAAGAGTAGAG	RT29	BV212041	Coulibaly et al 2005	L & S	2 & 3
OMM1395	CAAAACAGGGAGATACAG	CCTTTACTGGGACTATTC	RT30	BV078081	Rodriguez et al 2003	L	3
Omy1049INRA	ATGGAATGAACTGGGTCTGC	AATGTGTATTGACCAGCAAGG	RT30	BV681394	Guyomard et al 2006	L & S	2 & 3
OMM1723	GGGTGTTTATGTAAAGGG	CGTTCATGGCTAGTATCC	RT30	BV212204	Coulibaly et al 2005	L	3
Omy1308INRA	CTATGTTGATAGGGACAAAGG	TGAAACATCCAACAGGTAGC	RT31	BV681585	Guyomard et al 2006	L	2 & 3
OMM1765	CAGGAGCCAAACGACAAGGG	TGACTGGCCTTTCGCGTGAG	RT31	BV212234	Coulibaly et al 2005	L & S	2 & 3
Omy1136INRA	TTCCCAGCAAAGGATAAGG	GGTGGGTAAGAGAGCTTAGAGG	RT31	BV681527	Guyomard et al 2006	L	2 & 3
OMM5308	AGGAGGGAGGAGTGGTTG	AGGGGCACCACAGTCTGA	Vasopressin	BV212017	Coulibaly et al 2005	L	2 & 3
CA048687	CAGAGACAGAGGGTCAGCCTA	CCCATCATCGTAGTCCACA	Blood group associated glycoprotein	CA048687	Rise et al 2004	L	3
CA042613	GCCAAGTGTCTTCCTGTGAAA	CAGTCCACCTCGGAAAATC	EST RT16	CA042613	Rise et al 2004	L	3

Marker	Forward Primer	Reverse Primer	Linkage Group	GenBank Accession	Reference	Line (L)/ Strain (S)	Chapter 2 or 3
CA054538	AGCTACTGGTCCCCAAACCT	AAGGTGGACTTGGCTTGATG	Cyclin E RT10	CA054538	Rise et al 2004	L	3
CA058580	ATAACATGCAAGCGGTTTCC	GCTGGAAGTGTTGAGTTGC	EST RT6	CA058580	Rise et al 2004	L	3
CA059136	AGGGTAGTGAGAAAGCAGCAA	AACTGGCTGGCCATAGG	ESTRT22	CA059136	Rise et al 2004	L	3

Table A2. Primer sequences, location, accession number and reference for the eight microsatellite markers used to verify regions of the genome with evidence for selection in Chapter 3. Also presented are the primer sequences for genes associated with behaviour identified from read sequences generated in Chapter 4.

Marker	Forward Primer	Reverse Primer	Linkage Group	GenBank Accession	Reference	Line (L)/ Strain (S)	Chapter 2 or 3
OMM3022	CAGTGCGCTGTCAGGTTGTA	GCTGTGGTTCTGGCCATTAG	RT11	BV718483	Rexroad et al unpub	L & S	2 & 3
OMM1671	CTGACACTGCGGCATGTG	GGTCCCTGCCCTTGATCT	RT2B	BV212163	Coulibaly et al 2005	L	2 & 3
OMM1002	ATTCAGAATGCCAGATCGTC	CATCAGTATCATCATCATCGC	RT13	AF346666	Rexroad et al 2002	L	2 & 3
OMM5178	ATAAAGATGACCCCTATCCC	GTGGTATCTGCCTTGGACTC	RT11	BV211904	Coulibaly et al 2005	L & S	2 & 3
OMM5332	CTGCTACCATGTTGTGTTTC	CGTGCAATCAATCTGTAAGG	RT11	BV212039	Coulibaly et al 2005	L	3
OMM1037	GCGACTGGATTTAATACTGC	TCCTCTGACTGCCATTACATC	RT2B	AF346687	Rexroad et al 2002	L & S	2 & 3
OMM1388	ACTGTCGACGTGCTAACT	TCCTCTCTGTGCTGAATC	RT13	BV078074	Rodriguez et al 2003	L	2 & 3
OMM1310	CGCGTGACAGTGAAAAGTAATAGC	TTATCATTCCCTACCAATCGATCC	RT11	G73550	Palti et al 2002	L	2 & 3
POMC	AGAGCCCAGATCGGCTGCCC	TGGGTTTGCCCCAGCGGAAG	NA			L	**3**
nadh_dehy	TCTGGATGTGGTACTTGCCC	ACGCTCCTACTGTCCACGAG	NA			L	3
calmod	AGCTCACAGACGAGGAGGTG	TGATCTTAGGAGGACAAGCAATG	NA			L	3
lipo_rec	TTCCAAATGGACATTGACCC	TCCATTCCTCCTGACAGAGC	NA			L	3
G6P	TTCAGGGTTGATGTTGAGGG	GAACATTGGCTTCCAGATCG	NA			L	3

Table A3. The numbers of Aggressive and Less Aggressive rainbow trout that originated from Stocks 1 and 2, which were transported to the Liverpool aquarium two months apart. These fish were used in Chapters 2 & 4.

		Aggression category	
		Less Aggressive	Aggressive
Stock Tank	Stock 1	8	7
	Stock 2	7	8

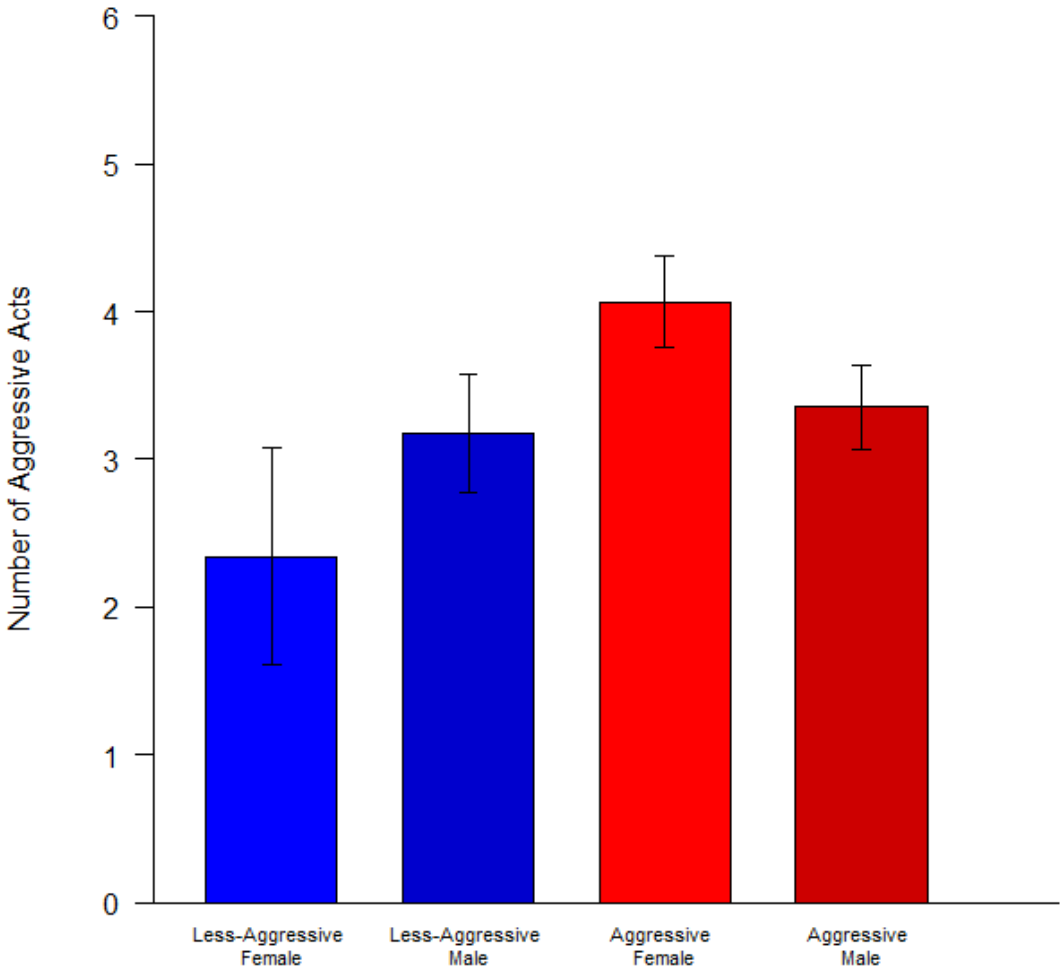

Figure A1. The number of aggressive acts performed by less aggressive females (n=6; blue) less aggressive males (n=9; dark blue) and aggressive females (n=5; red) and aggressive males (n=10; dark red). Error bars show standard error of the mean.

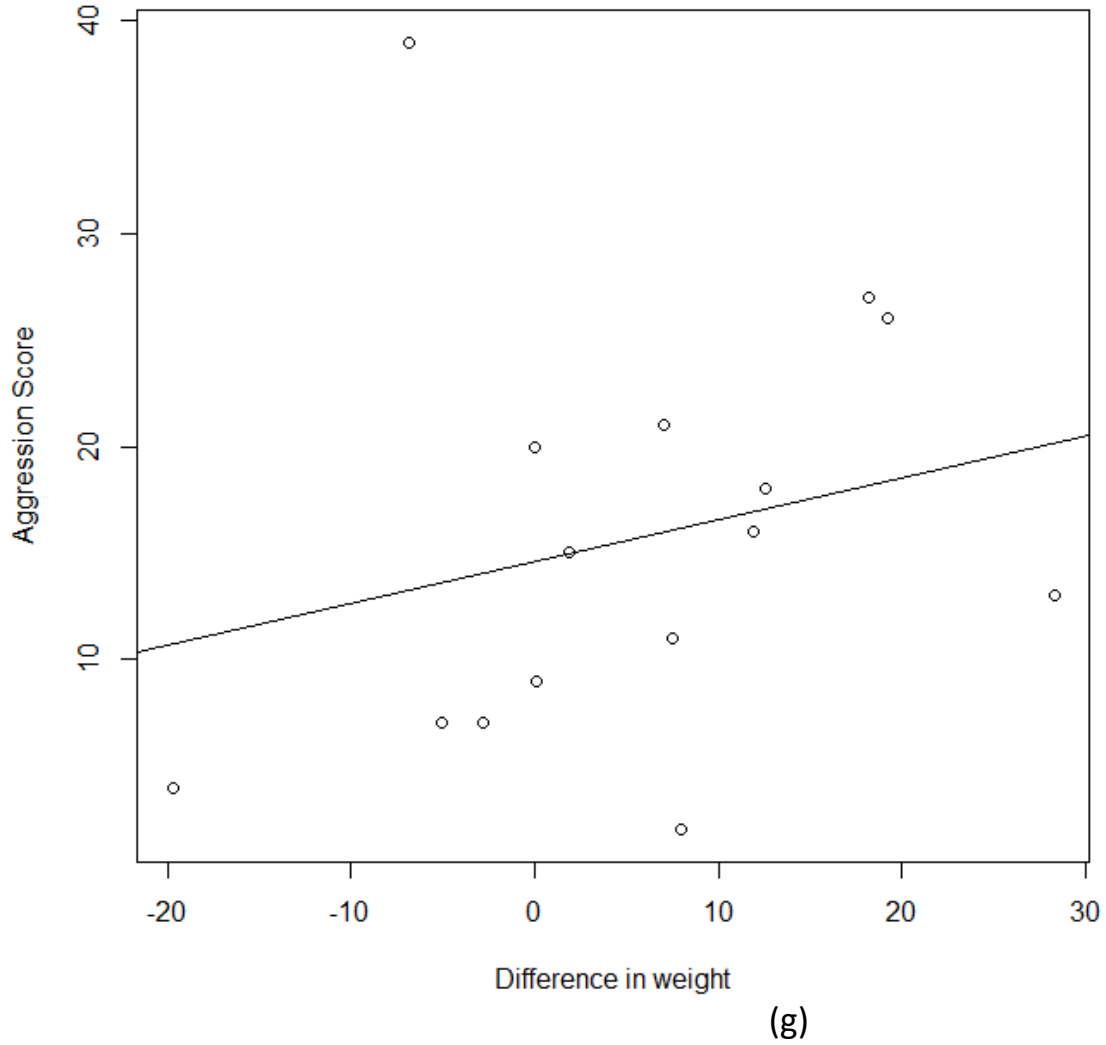

Figure A2. The relationship between the difference in weight (g) between two fish in a pair and the positive aggression score of that pair (n=15 pairs).

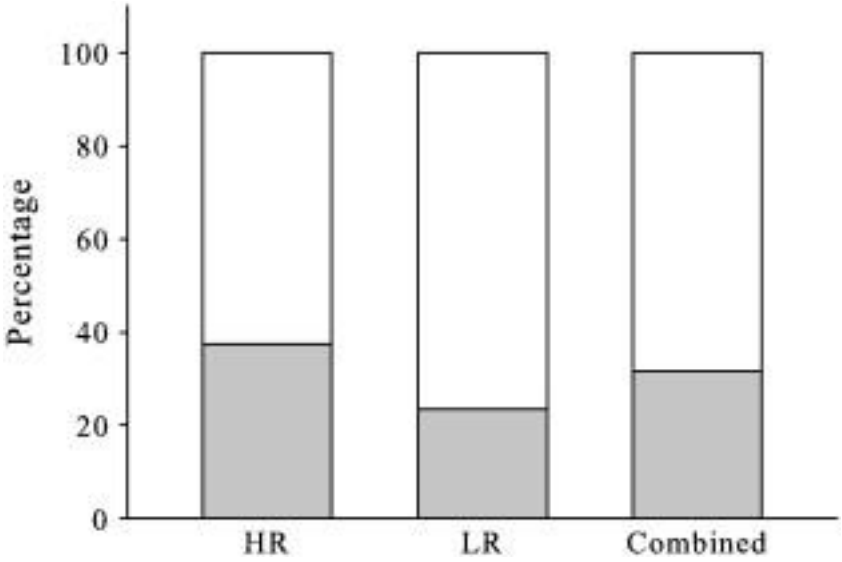

Figure A3. Percentage of rainbow trout, *Oncorhynchus mykiss*, showing consistently bold (white) or shy (grey) behaviour in lines bred for high (HR; n=24) and low (LR; n=17) cortisol response to stress, and in both groups combined. Reproduced from Thomson *et al.* (2011) with permission from Elsevier.

Table A4. Markers used to assess an association of heterozygosity with aggressive behaviour. Presented are the markers that deviate from HWE as well as allele number and average allele number.

Locus	W&C stat	P-value	Significant after sequential Bonferroni	Max number of alleles for locus	Number alleles aggressive	Number alleles less aggressive
OMM1000	-0.0787	1		4	6.33	5.81
OMM1011	-0.0769	1		3	3.005	2.78
OMM1037	0.1447	0.1251		8	6.355	5.26
OMM1116	0.2857	0.2582		4	3.8	4.25
OMM1345	0.3182	0.0354		4	3.295	5.715
OMM1374	0.5109	0.0077		8	3.69	3.775
OMM1381	0.2971	0.0591		7	5.685	6.035
OMM1505	0.1	0.1201		4	2.53	2.96
OMM1544	0.413	0.0968		7	3.26	3.95
OMM1690	-0.1269	0.2713		12	1.905	2.595
OMM1765	-0.0769	1		2	5.905	3.895
OMM1769	0.2518	0.3206		5	4.615	5.29
OMM1778	-0.213	1		5	2.63	3.75
OMM1780	0.3788	0.0014	*	10	3.44	3.865
OMM1793	0.0714	0.7471		4	3.445	3.425
OMM3022	0.3931	0.1391		5	2	2
OMM5133	0.4416	0.0002	*	8	2.775	1.875
OMM5178	-0.3391	0.7488		5	4.46	5.765
OMM5179	0.1333	0.2222		6	5.645	4.795
OMM5254	-0.0667	0.2934		8	2.985	2.75
OMM5264	-0.211	0.6182		5	7.58	7.31
Omy1049INRA	-0.0512	0.6053		7	4.51	5.3
Omy1501INRA	0.027	0.1013		3	4.26	2.96
OmyRGT12TUF	0.1284	0.2124		4	4.445	4.645

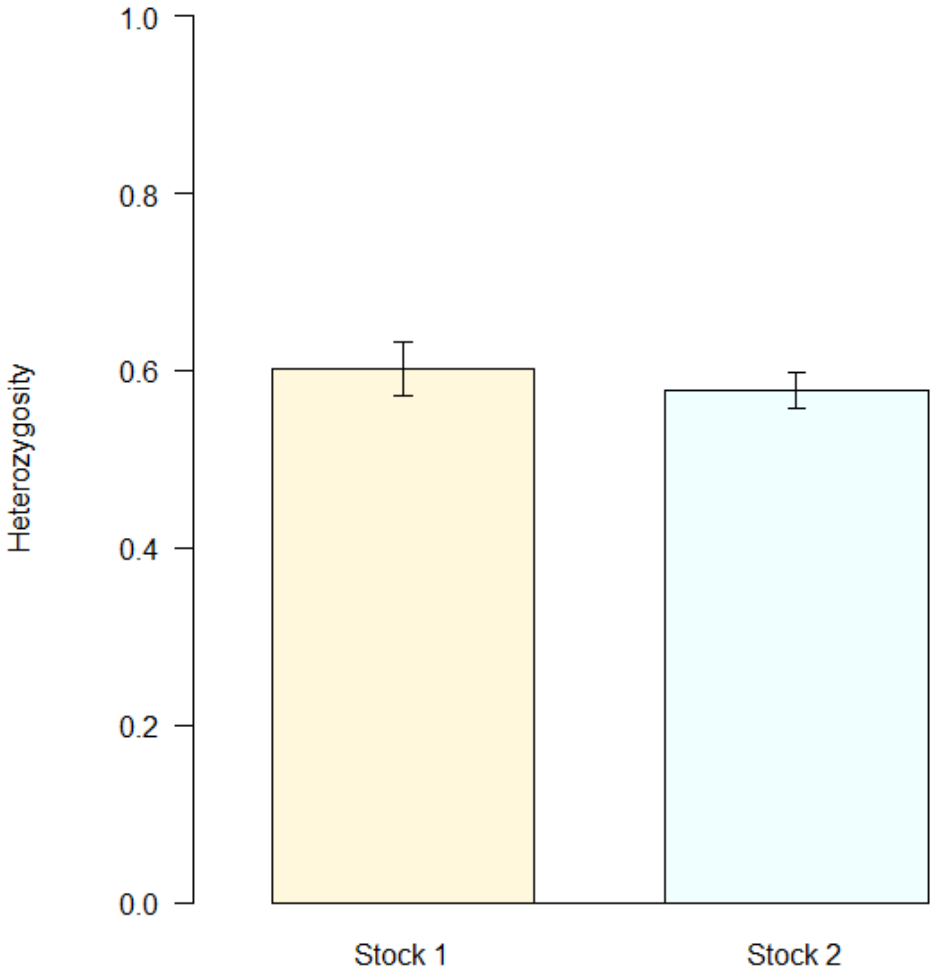

Figure A4. The level of heterozygosity of the rainbow trout tested for aggression. The rainbow trout used in these studies were kept in separate stock tanks and this graph shows the average heterozygosity in stock 1 (n=15; cream) and stock 2 (n=15; pale blue). Error bars show standard error of the mean.

Table A5. Microsatellite markers deviating from HWE and the average number of alleles at that locus for markers used to determine heterozygosity for boldness in the selected lines of rainbow trout in Chapter 2. * indicates p<0.05.

Locus	W&C stat	P-value	Significant after sequential Bonferroni	Max number of alleles for locus	Number of alleles HR	Number of alleles LR
OMM1000	NA	1		4	2.275	1.56
OMM1007	-0.059	0.6758		4	3.515	3
OMM1009	0.2967	0.0051		3	3	2.865
OMM1011	1	0.0008		3	3	3
OMM1015	-0.2245	0.0172		5	3.215	3.57
OMM1020	-0.2605	0.1859		3	2.9	3
OMM1025	0.4554	0.0001	*	5	3.885	3.475
OMM1032	-0.0261	0.2262		3	2.9	3
OMM1051	0.0204	0.0803		1	1	1
OMM1082	0.2887	0.0084		3	1	2.23
OMM1107	0.2998	0.0103		4	3.465	3.47
OMM1108	0.479	0.0092		2	2	1
OMM1116	NA	1		3	3	3
OMM1134	-0.0213	1		7	4	4.545
OMM1139	0.0323	1		6	5.41	4.73
OMM1151	0.0502	0.3205		3	2.47	2.8
OMM1154	NA	1		4	3.735	3.475
OMM1158	0.1398	0.6004		5	3	3.71
OMM1312	-0.0584	0.0006	*	2	2	2
OMM1322	0.6503	0	*	3	2.4	2
OMM1330	-0.1125	0.0014		6	4.49	4.535
OMM1345	-0.2611	0.3429		8	5.74	4.515
OMM1346	-0.0508	1		4	4	3.38
OMM1352	0.7935	0.0005	*	4	2.835	3.525
OMM1360	0.0805	0.0024		4	3.775	3.72
OMM1361	-0.2813	0.5252		4	2.925	3.445
OMM1367	0.4007	0.0003	*	9	7.175	4.46
OMM1374	0.0254	1		3	2.725	2.995
OMM1376	1	0.016		3	2	2.91
OMM1381	0.1987	0.002		2	2	2
OMM1384	0.3536	0	*	5	3.94	4.345
OMM1389	-0.2226	0.2816		3	3	3
OMM1397	0.1367	0.0406		4	3	2
OMM1505	-0.1095	0.8469		3	3	2
OMM1512	-0.2378	0	*	1	1	1
OMM1544	-0.1408	0.7438		7	5.215	3.445
OMM1667	0.2765	0.0084		2	2	2
OMM1690	0.2429	0.0007	*	5	3.88	3.51

Locus	W&C stat	P-value	Significant after sequential Bonferroni	Max number of alleles for locus	Number of alleles HR	Number of alleles LR
OMM1710	-0.2126	0.2699		3	2	2.33
OMM1717	-0.0057	0.0162		5	3.415	3.535
OMM1751	-0.003	0.7109		3	2	2.515
OMM1762	NA	1		6	5.07	5.405
OMM1764	-0.0479	0.5222		3	2.95	3
OMM1765	0.0712	0.033		3	3	2
OMM1767	-0.0502	1		6	4.49	2.51
OMM1769	-0.2087	0.2833		2	2	2
OMM1778	-0.5032	0	*	4	3.335	3
OMM1780	0.0971	0.0001	*	4	3.775	2.87
OMM1783	-0.1013	0.7235		3	3	2
OMM1793	0.1268	0.499		3	3	2.5
OMM1797	NA	1		4	3.905	4
OMM1824	-0.0708	0.9346		1	1	1
OMM1830	0.2152	0.2725		2	2	2
OMM3006	0.6596	0.0463		3	1.515	2
OMM5017	-0.5526	0	*	4	3.54	3
OMM5133	0.2163	0.0105		2	2	2
OMM5136	0.225	0.0931		6	4.535	4.435
OMM5137	0.8348	0	*	9	5.31	4.9
OMM5162	0.8242	0	*	6	4.255	4.005
OMM5165	-0.2658	0.0008		2	2	2
OMM5179	-0.0753	0.8004		6	3.205	3.475
OMM5186	1	0.0008		2	2	2
OMM5254	0.0229	1		2	2	2
OMM5261	NA	1		4	3.535	2.705
OMM5264	-0.0271	1		2	2	2
OMM5266	-0.234	0.262		3	3	3
OMM5308	-0.0097	0.8579		5	4	3.64
OMM5318	0.2363	0.0069		4	3	3.24
OMM5319	0.0043	0.4213		3	2	2.525
OMM5320	0.0549	1		2	1.985	2
OMM5328	-0.0187	0.8518		3	2.77	3
Omy1049INRA	-0.0294	0.1518		1	1	1
Omy1103INRA	-0.6	0.0005	*	3	2	3
Omy1136INRA	NA	1		2	2	2
Omy1259INRA	0.5799	0.0016		2	2	2
Omy1308INRA	1	0.0001	*	4	3.75	3.54
Omy1398INRA	1	0.0175		2	1.9	2
Omy1501INRA	0.3561	0.0001	*	2	2	1
OmyRGT12TUF	0.3384	0.0871		3	3	3
OmyRGT17TUF	0.0147	0	*	2	2	2
OmyRGT40TUF	NA	1		2	2	2
OmyRGT43TUF	0.0349	0.0839		1	1	1

Table A6. The results of testing for single locus effects of heterozygosity upon aggressive behaviour in rainbow trout. No locus showed significant effects after sequential Bonferroni correction.

Locus	***P* value**	**Randomisations**	**Bonferroni *P* value**
OMM1778	0.014	2000	0.00208
OMM1544	0.045	1000	0.00217
OMM1037	0.085	1000	0.00227
OMM5133	0.099	1000	0.00238
OMM3022	0.106	1000	0.0025
OMM1000	0.142	1000	0.00263
OMM5178	0.150	1000	0.00278
OMM1345	0.181	1000	0.00294
OMM1011	0.258	1000	0.00313
OMM1374	0.484	1000	0.00333
Omy1049INRA	0.489	1000	0.00357
OMM5264	0.501	1000	0.00385
OMM1769	0.515	1000	0.00417
OMM1116	0.577	1000	0.00455
OMM1690	0.617	1000	0.005
OmyRGT12TUF	0.635	1000	0.00556
OMM5179	0.699	1000	0.00625
OMM1381	0.736	1000	0.00714
OMM1780	0.764	1000	0.00833
OMM1793	0.772	1000	0.010
OMM5254	0.819	1000	0.0125
Omy1501INRA	0.827	1000	0.0167
OMM1505	0.893	1000	0.025
OMM1765	0.898	1000	0.050

Table A7. Microsatellite markers were used to detect association with heterozygosity (Chapter 2) and signatures of divergence (Chapter 3) in rainbow trout that were selectively bred for high (HR) and low (LR) cortisol responses to stress. Presented are the linkage group, the number of loci that amplified, the number of loci that were monomorphic in each line, the number of alleles significant for null alleles. Also shown for loci used in the genome scan are loci that deviate from Hardy-Weinberg equilibrium (HWE), expected heterozygosity H_e and the number of alleles per stress line (HR and LR) and hatchery strain (US).

Locus name	Linkage group	Did not amplify	Mono-morphic Loci	Null allele	Genome scan markers	Loci deviating from HWE			H_e			Number of alleles		
						HR	LR	US	HR	LR	US	HR	LR	US
CA048687	NA		x											
NADH	NA				x				0.339	0.417	0.315	2	2	2
Calmod	NA	x												
G6P	NA	x												
Lipo	NA	x												
POMC	NA	x												
OMM1000	RT1				x				0.068	0.033	0.123	3	2	3
OMM1665	RT1			x										
OMM5136	RT1				x			x	0.400	0.119	0.692	2	3	4
OMM1361	RT2A				x				0.649	0.445	0.756	3	5	5
OMM3006	RT2A				x				0.506	0.089	0.579	2	2	3
OMM5165	RT2A				x	x			0.619	0.762	0.899	6	6	11
OMM5319	RT2B				x				0.525	0.549	0.736	4	3	4
OMM5320	RT2B				x	x			0.203	0.494	0.575	3	2	5
OmyRGT40TUF	RT2B				x				0.023	0.029	0.867	2	2	9

Locus name	Linkage group	Did not amplify	Mono-morphic Loci	Null allele	Genome scan markers	Loci deviating from HWE			H_e			Number of alleles		
						HR	LR	US	HR	LR	US	HR	LR	US
OMM1312	RT3				x	x	x	x	0.542	0.666	0.902	5	7	11
OMM1346	RT3				x				0.427	0.480	0.466	2	2	2
OMM5264	RT3				x				0.473	0.335	0.607	6	3	4
OMM5137	RT4				x		x		0.368	0.367	0.494	5	4	3
OMM1007	RT5				x				0.465	0.626	0.750	3	3	7
OMM1011	RT5				x				0.458	0.129	0.262	2	2	3
OMM1032	RT5				x				0.439	0.659	0.722	4	4	8
CA058580	RT6				x				0.023	0.031	0.659	2	2	3
OMM1082	RT6				x				0.568	0.526	0.802	3	4	6
OMM1151	RT6				x	x			0.176	0.583	0.849	4	4	11
OMM1780	RT6				x	x	x		0.216	0.515	0.736	3	3	8
OMM1376	RT7				x				0.071	0.061	0.063	3	2	2
OMM1764	RT7				x				0.469	0.537	0.711	2	3	4
OmyRGT17TUF	RT7				x		x		0.521	0.710	0.863	5	5	9
OMM1009	RT8				x	x			0.563	0.578	0.677	4	3	6
OMM1667	RT8				x				0.503	0.544	0.760	4	4	4
OMM1793	RT8				x				0.294	0.500	0.548	3	2	5
OMM5318	RT8				x	x		x	0.329	0.396	0.770	3	3	8
CA054538	RT10				x				0.024	0.027	0.607	2	2	4
OMM1710	RT10				x				0.103	0.446	0.742	2	2	7
OMM5186	RT10				x	x	x		0.210	0.117	0.387	3	2	2
OMM5254	RT10				x				0.072	0.493	0.865	2	2	8

Locus name	Linkage group	Did not amplify	Mono-morphic Loci	Null allele	Genome scan markers	Loci deviating from HWE			H_e			Number of alleles		
						HR	LR	US	HR	LR	US	HR	LR	US
OMM1084	RT11	x												
OMM1154	RT11				x			x	0.452	0.029	0.613	2	2	3
OMM5261	RT11				x				0.504	0.033	0.513	2	2	4
OMM5308	RT11				x				0.542	0.599	0.690	4	3	4
OMM1127	RT12	x												
OMM1158	RT12				x			x	0.585	0.599	0.801	3	3	7
OMM1381	RT12				x			x	0.663	0.565	0.790	5	5	8
OMM5328	RT12				x				0.606	0.522	0.609	3	3	4
OMM1020	RT13				x	x		x	0.545	0.526	0.766	3	3	4
OMM1762	RT13				x				0.024	0.032	0.683	2	2	5
OMM1783	RT13				x				0.377	0.496	0.387	4	2	2
OMM1374	RT14				x				0.207	0.364	0.885	2	2	9
OMM5017	RT14				x	x	x		0.656	0.642	0.837	4	4	7
OmyRGT43TUF	RT14				x				0.599	0.604	0.793	7	7	7
OMM1051	RT15				x				0.464	0.541	0.813	3	3	8
OMM1769	RT15				x			x	0.495	0.474	0.375	2	2	4
OMM5266	RT15				x				0.414	0.486	0.605	2	2	5
CA042613	RT16	x												
OMM1345	RT16				x				0.240	0.394	0.681	3	3	6
OMM1352	RT16				x		x		0.652	0.147	0.745	3	3	5
OMM1717	RT16				x				0.085	0.328	0.466	3	3	2
OMM1830	RT16				x				0.506	0.479	0.609	2	2	4

Locus name	Linkage group	Did not amplify	Mono-morphic Loci	Null allele	Genome scan markers	Loci deviating from HWE			H_e			Number of alleles		
						HR	LR	US	HR	LR	US	HR	LR	US
OMM1116	RT17				x				0.026	0.029	0.063	2	2	2
OMM1139	RT17				x				0.103	0.502	0.359	2	2	3
OMM1360	RT17				x			x	0.599	0.423	0.869	4	4	8
OMM1384	RT18				x	x	x	x	0.582	0.580	0.829	7	5	8
OMM1512	RT18				x		x	x	0.506	0.567	0.712	3	4	4
OmyRGT12TUF	RT18				x	x		x	0.173	0.326	0.542	2	2	3
Omi127TUF	RT19	x												
OMM1025	RT19				x	x	x		0.485	0.652	0.692	3	3	5
OMM1333	RT19		x											
OMM1107	RT20				x				0.515	0.491	0.699	3	3	3
OMM1134	RT20				x				0.024	0.087	0.411	2	3	3
OMM1544	RT20				x	x			0.663	0.634	0.745	4	4	6
OMM1367	RT21				x	x	x		0.670	0.256	0.796	4	4	6
OMM1824	RT21				x			x	0.219	0.562	0.815	4	4	6
OMM5179	RT21				x				0.449	0.646	0.763	3	3	7
CA059136	RT22				x	x	x		0.136	0.512	0.284	2	2	3
OMM5133	RT22				x	x		x	0.605	0.580	0.878	4	4	7
OMM5162	RT22				x	x	x		0.169	0.172	0.546	2	3	3
Omy1158INRA	RT22			x										
OMM1538	RT23	x												
OMM1719	RT23	x												
Omy1103INRA	RT23				x		x		0.366	0.473	0.476	2	2	6

Locus name	Linkage group	Did not amplify	Mono-morphic Loci	Null allele	Genome scan markers	Loci deviating from HWE			H_e			Number of alleles		
						HR	LR	US	HR	LR	US	HR	LR	US
Omy1501INRA	RT23				x	x	x		0.248	0.477	0.186	4	3	2
OMM1322	RT24				x	x	x	x	0.480	0.173	0.810	4	3	9
OMM1397	RT24				x				0.547	0.526	0.708	3	4	4
OMM1690	RT24				x	x	x		0.596	0.522	0.913	9	6	12
OMM1389	RT25				x				0.550	0.569	0.770	4	4	6
OMM1797	RT25		x											
Omy1259INRA	RT25				x				0.283	0.437	0.887	2	2	8
OMM1015	RT26				x				0.532	0.553	0.708	4	5	4
OMM1767	RT26				x				0.473	0.491	0.655	2	2	5
Omy1398INRA	RT26				x				0.023	0.067	0.398	2	2	3
OMM1108	RT27				x				0.266	0.484	0.685	2	2	5
OMM1778	RT27				x	x	x		0.655	0.668	0.722	4	5	6
Omy1178INRA	RT27			x										
Omy1423INRA	RT27	x												
OMM1330	RT29				x	x			0.716	0.729	0.802	6	5	5
OMM1505	RT29				x				0.108	0.519	0.312	2	3	2
OMM1751	RT29				x				0.427	0.635	0.758	3	3	8
OMM1395	RT30	x												
OMM1723	RT30	x												
Omy1049INRA	RT30				x	x			0.593	0.668	0.760	4	4	5
OMM1765	RT31				x	x			0.148	0.538	0.660	2	3	3

Locus name	Linkage group	Did not amplify	Mono-morphic Loci	Null allele	Genome scan markers	Loci deviating from HWE			H_e			Number of alleles		
						HR	LR	US	HR	LR	US	HR	LR	US
Omy1136INRA	RT31		x											
Omy1308INRA	RT31				x	x	x		0.173	0.170	0.500	2	2	4

Chapter 4

Table A8. Contigs that were differentially expressed between aggressive and less aggressive rainbow trout and which were identified using blastx and blastn functions of BLAST.

Contig Number	Less Aggressive normalised read number	Aggressive normalised read number	Fold Difference	Contig Sequence
Contig285	11.7	3.9	3	ACCACGTCTGGAACCAACACGACCCTGAACTTTTAGTCTTTACTTATTAACCTAAGTCCT TAAGACTGCGAAATTAGCTCTTCAAATGGCTACCCGGACTACATGCATTGACTATGTACA CACAGGACATTCAGTAACTTACACTAACAACACACACCAAATACGCTACTGCCAATGTAC TGTCTATCATCTATACTAAGCACGCACACACACACACGGGACTCTCAGACATTCAGTACT TACACTGACAACACACGCCCACACACGAAATGCACTGCTGCCAATGTACTGTCTTATCTA TCCAAGGCACACAGGGGATA
Contig2044	5.1	0.6	8.50	TGCAGTTGTTTTTTATTTCTCATGAATTAACCTACAACTCCAGCACCTGACAAAAAGAAC CTGGATGTGCGTATGCTCTTCAGCTTTTGGACAATGTCCTACATTCACCCACAATTCATT TACAATATGTACACGTCATGATTAACAAAACACAGCACTATTGTGAAGGAAAAAAAGACG CATTGGCAT
Contig2115	0.1	7.2	72.00	AGGGGAACGGATGATCTCCACGTGTGTGGTTCCCACCGTGAAGCATGGAGGTGGTGGTGT GATGGTGCTTTGCTCGTGACACTGATTTATTTAGAATTCAAGGCACACAGGGGATA

Contig Number	Less Aggressive normalised read number	Aggressive normalised read number	Fold Difference	Contig Sequence
Contig2338	6.8	1.1	6.18	ACGGGGCACTGGGTTTGTGACCTTGGTTGAAATCGGAAGCAAAAAAATAAATAAAAAAAT GAGCTCTCTCTCTCTGTGGTTTTAATAAGAGTTTGAATGTTCAGTGTCTTGTCCTGTTAT TTTAATAATTTTAAGAAAATGGGATGAAGAAATAGCCTTCCAAACAAAAGACTCTGCTAC ATGTTGCCTAAACATTCTCATTTTCACTCAATTCTTCTAATCTTGGTTTTTCTTGAGGTA TGTGAAAAGATACAGTATGCTGGGAATATAGGTGTCTCCTACTTTTCCTTTTCCCTACTG ACATAAACACACAATTATAAAAAATTGAATTTTTATTATTATTCTTTCTAGTAATTTTTT TTTTTCCTTTTTAAAACAAATTACTATATCCCTTCGTTGAGGCAAAAATAATCTACGTTC TTGTCAGTCCTGTCCCATGGTATTTAACTATTGATGAAAAATAAAATAAAATAACTTTAA
Contig3866	25.6	12.7	2.02	AACGGGGATGTCAGAGAAGAGCAAAGCGTAAAGGATACGTTTCACTGGCTGGTTCCTAAC AACATCAGAGTTGGATATGCAAATAAGACCGTGTAAATCCGTTCCTGTCCTTGAGAGCAG CAGTGTCTTCCCTATCATAGTGTATCTACAGCAATATGTTCTGTTTCATAACGTACCACA CAGCTACCAAGTTCCCCACGTGTAAATACAATGGCTTGTTAGAGTAGATTCTACTATAGA TTCCACTCTGAAAAGGTATTATAGAAAACAGCAACAATAGCATTTCCTGTCCTCAGACGA CATGGTCCATAGCAGAAATGAACTAACAGTGATGAAATATTAAGTTGGATACAGGCTCTT CAATGAACCTCCAGATGAATGTGTAAGTGTATGTTATTGTAGGCCTATTTGTTGTTGATA TGAACAGGTTGTATTTTTTGTTACTGCCAAAATAATGTTGTACTAAATCACATCTGATTG GCTTTTAAGTTTGTTTCTATTAACTGTTCTGTGTAACGTTTAACTGTCTACCAAATAAAT ACCTAGACACAAAAAAAAANAAAAAAAAAAAAAGT

Contig Number	Less Aggressive normalised read number	Aggressive normalised read number	Fold Difference	Contig Sequence
Contig5244	34.6	4.2	8.24	ACACGGGGACCAAGTTGTTCATCGCGTACCTCCATGAAGTGGCCCATCTCAAGAAGGACT GAGAGCACCTCTTTTCATCACCACCGTTATAAGACAGTTCTATATTGTTTAGCTTGTATG AGATCCCATCAGTGGAGTCTGGCTTTGTTTAGCCTTTTTCCTGACATTAACTGGTAACTT TTCTTGTTTCTTTCCCTCTAAGCAAAACATTCCAGAAACCCTGTGAGTTCTTGGCATCTG ATATAGCCTCAACACACTGCCTCTCTCTCGCGCTCTCTCTCTCTCTTTCTGAATCTTTCT TCCCCCCTCACGTTCCTCCTTTCTCTCTCTCAATGTCTCTCTCTCTCATTCTCTCTCATT CACATACAAATGTCTTACTTCGTATAGAGTAAAATAAAACTAGGTTTGTACGACACTAAA
Contig5703	5.4	0.4	13.5	ACGGGGAGAGATGACTTGTGGATTTGACAGGGATCCACTAAAGTAGGAATGGTTGAAGTC TGGAGAGAACATGAGTAAAAGAGAATGAAGGAATGGGTGGAAAATGATACATTAGCTGTT TTGTCTGCCGTTTCTCCAAATAAAGGTTGCTTTAGTACTGTAGAAAAAAAAAAAAAAAAA
Contig6671	42.4	23.9	1.77	ACGCGGGGATGTAATGAAAGAATGTTTTTGTTTGTTACTTGCCAGTTATTCACTAAAGAG GGGGAACTGCAAACGCGGGGAGGCAGACCATTTAACACTTTAAACTAAATGTTGAAACAT TACATTACAACAACAAAAATGAAGAAAAGATCAACATGGAATGAAGGAATACATTCTTCC TTAATTAAAGAAAAAAAAAAAATACCCTTGATTGAGAGTCTTGAGAGCTGCCTGATGATTC TGGTTATGATGTTACCCGTTTATTCCGGCTATGAAATCTCTTTTTTTCTTGCTCTCTATC TCTCTGCTATTTCTACCAGTGCATTTTGTTACTCCGAACTGAACTCTTCCTAAGGAACCT GAATAAAACCACAGAGAGAATGACCGCGGGGAGGCAGACCATTTAACACTTTAAACTAA ATGTTGAAACATTACATTACAACAACAAAAATGAAGAAAAGATCAACATGGA ATGAAGGAATACATTCTTCCTTAATTAAAGAAAAAAAAAAAATACCCTTGATTGAGAGTC TTGAGAGCTGCCTGATGATTCTGGTTATGATGTTACCCGTTTATTCCGGCTATGAAATCT CTTTTTTTCTTGCTCTCTATCTCTCTGCTATTTCTACCAGTGCATTTTGTTACTCCGAAC TGAACTCTTCCTAAGGAACCTGAATAAAACCACAGAGAGAATGACAAAAAAAAAAAAAAA

Contig Number	Less Aggressive normalised read number	Aggressive normalised read number	Fold Difference	Contig Sequence
Contig8559	6.3	1.1	5.73	GATGCTCTCAATTGTGCATCTCATGAAGCTGGTTGAGAGAATGCCAAAGGTGTGCAAAGC TGTCATCAAGGCAAAGGATGGCTACTTTGAAGAATCTCGCCTTCTGGATGATAGCGGGAT GAACAGGCAGTGGCTCGGGTGGTTGTTGTTCTTGATGATCTTTTTGGCTTCCTGTGTCAT TGGGTGCTGTAGGTGTCCTGGAGGGCAGGTAGTTTTCCCCCGGTGTACGTCTTGCGTTTG TAGTA
Contig13575	23.4	6.1	3.84	AAAACGGGGACCAAGTTGTTCATCGCGTACCTCCATGAAGTGGCCCATCTCAAGAAGGAC TGAGAGCACCTCTTTTCATCACCACCGTTATAAGACAGTTCTATATTGTTTAGCTTGTAT GAGATCCCATCAGTGGAGTCTGGCTTTGTTTAGCCTTTTTCCTGACATTAACTGGTAACT TTTCTTGTTTCTTTCCCTCTAAGCAAAACATTCCAGAAACCCTGTGAGTTCTTGGCATCT GATATAGCCTCAACACTCTGCCTCTCTCTCTCGCTCTCTCTCTCTCTCTTCTGTATCTTC
Contig15933	1.5	10.1	6.73	ACGCGGGCAGATAGACAGATTTTATTTTACCTTGTCAGGTCGGGGATTCGATGTAGCAAC CTTTCTGTTACTGGCCCAACGAGCTAACCACTAACCTGCCGCCCCAAAATCAAGGCACAC AGGGGATAATGTACGCACACTGTCTGATGTAAATAAATAAACACTGAGAGAAATGTCAAA TGGTCACATCATGCTGCCACTAGATGGACCCACTATTCAAATGTTCAAATGGCGATGTAA GCAGTACTTTAAACATATCTGTTTGATCAAGGTAGCGACTATGTTTACAAGCTTGTTGAA ACGCATCTATAAATGCTGTATTACGTGTCTATGTAAGTGTTATAAAGAGCTTTATAAGTG TCTATAATGCCACTGTTCCAAACATTTCAACCTACTATGTTTGTCTAAGAGTGTGAATAT ATCT

Contig Number	Less Aggressive normalised read number	Aggressive normalised read number	Fold Difference	Contig Sequence
Contig16293	0.7	6.1	8.71	GTCTGATGGAGAGAGACAGACGTGTGTCAGGAGTGGATTTGTATTACAAATCGAAGGGAC TTGAGAATATCCACTGATCCCAGATCTCACCTATGGAATGGATTTCTGTCAAGAATTCGT TGTACATTAAAAGTTATGTATTGTCTGAATTTAAAAAAAAAAAAAAAAAAAAAAAAAA
Contig19318	52.1	15.1	3.45	AGAGGGGACCAAGTTGTTCATCGCGTACCTCCATGAAGTGGCCCATCTCAAGAAGGACTG AGAGCACCTCTTTTCATCACCACCGTTATAAGACAGTTCTATATTGTTTAGCTTGTATGA GATCCCATCAGTGGAGTCTGGCTTTGTTTAGCCTTTTTCCTGACATTAACTGGTAACTTT TCTTGTTTCTTTCCCTCTAAGCAAAACATTCCAGAAACCCTGTGAGTTCTTGGCATCTGA TATAGCCTCAACACTCTGCCTCTCTCTCTCTCTCTCTCTTTCTGTATTCTTTCTTCCTCC TCTACTTTCCTCCTATTCTCTCTCATGTCTCTCTCTCTCTATTTCTCTCCCCGTTCACAC ACAAATATCTACATAAAAACAGACAAAATAATCGTTGGTTTCGACGACAAAAAAACTAAA

Table A9. The *de novo* transcriptomes of aggressive and less aggressive rainbow trout, *Oncorhynchus mykiss*, were analysed for differentially expressed transcripts. The unidentified sequences that were differentially expressed are shown here. The normalised read number and fold differences are shown.

Contig Number	Less Aggressive normalised read number	Aggressive normalised read number	Fold Difference	Contig Sequence
Contig8207	4.9	14.2	2.90	AACCCGGGGCCACGCACGGACAGAATACACACGCTGCCTCGGACACAAACAGACATGCTT GCCCATACGCAGTCACACGCACATACACTCACACACGCACACACAACTATTTCAGATGGG TGAAATGGTGCCAAATAACCTGAGTGAAATGCTAGCTGTACGGCTTTGACAACTTCAGGA ATATTTCACTCGTCTAAACAAATATTTATAAAACTAAGAACCAAAGACCGTACGCATTGA CTACATCTTTGAAGCATGGATGTATCTGCAGTTACTACCATGTAGTTTAAAATGCCCTTG GGGAATCTGTGTTTTTGAGGTACTTTGTTTATAAAAAAAATATAAAATTATATCCTTGTT TTTTGTATAAAGC
Contig212	8.8	0.9	9.78	ACGCGGGCCCTTTACTGAGTCGACATACTGTATTAAGTAGAGGACATGTTGGCTCTTCTG ATTTCTACTCCTGTAAAAGCATTTTATCCATTTGTTGGATTGCTTGTGAATTAATGTATT AATTTATTAAGTGTTTTTCAGTTGAAAAAGAAGTGTCAAAACAAACAAACAAAAAAAAAA AGC
Contig1022	24.8	10.5	2.36	CCGTAACGCGGGGTTCTGTCCCCGCAAGGCTGTTTGCCGTTTTTACGAGCTTCACGTATG CAACATTGTGCCAGTAAAATTGAAACTTCATCAGCGCCAGCTGGCCTCCCCACCGCTAGC TGGCTAGCGAACTAATGGAAATTACTTTAGTATAACTGGGCTTCAATCTTCCCACACTTG CCCTGATGCCAGACTGACTATGCAGTAGAAATGCGGCTCTTGCAGATAATCAAGGTACCA GTTTAACCCACAGACTGAAATTAACAGGCTATGATGGCTCCCCTGAGAAAAGACTGCAGG TCACTGTCGGTCGGACCAAAGGGGAGCAGCAACAAAACAACTCACCGGTCCTTCCCTTCA CTCCTTCCTGGGAGAAACAAAACACTGTCATCATAAAGAAAAAAAAAAAAAAAAAAAAAA AAAAAAACTTACT
Contig	**Less**	**Aggressive**	**Fold**	**Contig Sequence**

Number	Aggressive normalised read number	normalised read number	Difference	
Contig5380	5.4	0.2	27.0	GACGTACAACAATAGCTTGTGACACACAGCTTAATAAGGGTTTGTTGGCAACAGTGAAGC GGACATTCACTCTAGATAGAAAATAGCATTGATAGCCTACAATAGATTAACATAGACATT CACTCTAGATAGAAAATAGCATTGATAGCCTACAATAGATTAACATAGACATTCACTCTA GATAGAAAATAACATTGATAGCCTACAATAGATTAACATAGAAATTCATTCTAGATAGAA AATAGCATTGATAGCCTACAATAGATGAACATAGACATTCATAAGTGATAGAAAATAGCA CTGATAGCCTACAATAGATGAACATAGACATTCATAAGTGCATCTGTTACTTTTTAAGAA GAGACATTTTAGATACAGTTACAGAGCTTTTTGCCTTCTTGCCTGTGACAGACTACACAC TGAATAACTGGTCACAAGTCTCAGGAAACTGT
Contig6039	14.6	5.3	2.75	ACAGAGTAACGGGGGAGGAGAAGGCTACGGAGGAGAAGGCTACGGAGGAGGAGAAGGCTA CGGAGGAGGAGAAGGCTACAGCGGAGGAGGAGAAGGCTACGGAGGAGGAGAAGGCTACGG AGGAGGAGAAGGCTACGGAGGAGGAGAAGGCTACAGCGGAGGAGAAGGCTACAGAGGAGG AGAAGGCTACAGAGGAGGAGAAGGCTACAGAGGAGGAGAAGGCTACAGAGGAGGAGAAGG CTACAGCGGAGGAGAAGACAACAAAGTGAATAAGAGAACCAGATCCTCACCGTGTTGCCC AACAAAACCTCCCTTTAGTGGAACTTTGATCCCATAACCACTAACCAAATACCACTTGTA CTTCCATGCACTTCAAACCATGTCTTACATACTCATGACACATTCTAGGTGCTGTATTGT TAAAAAATGCTAAAGTTTTTACCATGAACTGTGTACTTTTGCCGGTTAATTGATAACAGT TCAAGGTATCTTAAATAACAAAATATGACTAGTTCAAACAAAGACATTTAAATAAAACAC AATACAACCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA ANAAAAAANAAAAANAAAANAAANAAANAAN

Contig Number	Less Aggressive normalised read number	Aggressive normalised read number	Fold Difference	Contig Sequence
Contig13776	9.3	2.4	3.88	ACGTCGGGTAATGTATACCTAACAATTGGAATAACGATTGAAACCCCATTGGATTTTTTT CTTTACGTTTATTTTTTGTTTATCATCTGTGTGTTGAAAAAAAAATCATTTAAAAAAACG TTTCTCGGTGATCTGCTATTGAAGTTCCTGATGCATGTAGGGGTGCACTACATCTGAGAA TTGTTGTGGAGTTGTAAGGTTTTATGTGATGCTGTTTTGTTTTTATAGAGGACTAGAATA GGATTCTGAGTAGGATATAAAACGATGTATTGTCAATTATGTTGCTCACACCAAAAAAGG GAAAATGTCATGTACAATAAAGAACCACATTTACAAGTGGCCTGTTGACTGACTAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAANAAAAAAAANAAAAAATAAAAANAAAATAAAA

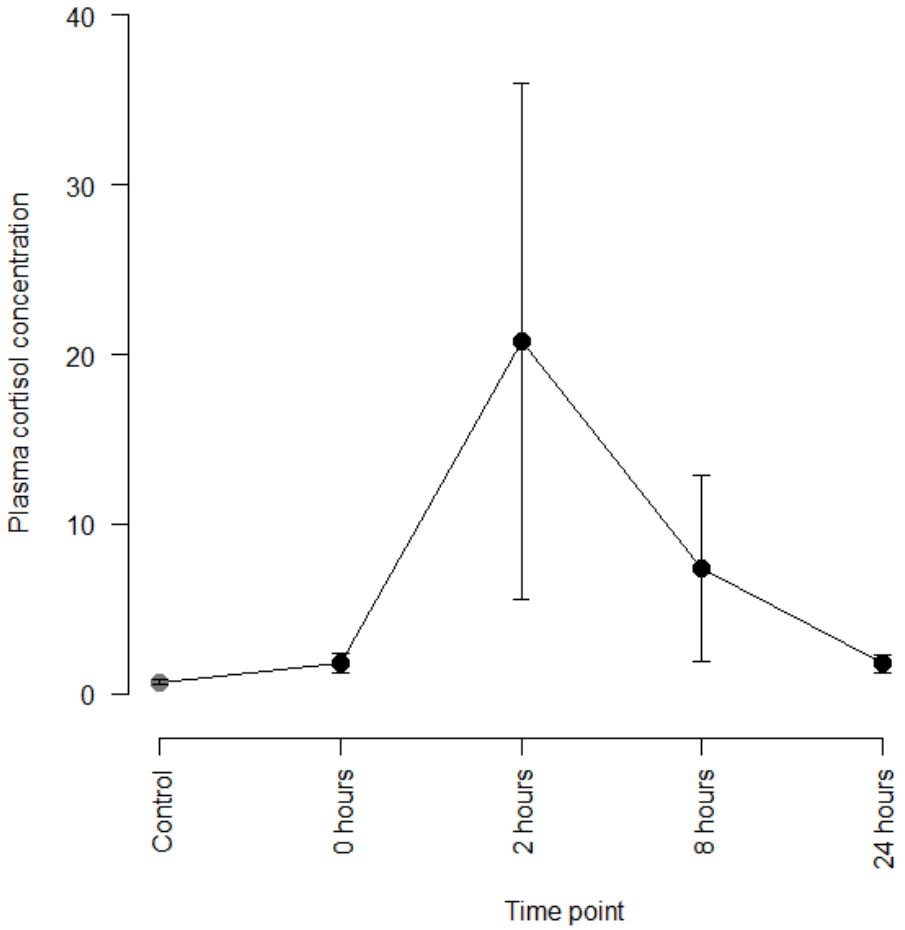

Figure A5. Absolute values of plasma cortisol concentrations ($ngml^{-1}$) after no stress (control, n=5), immediately (n=6), 2 hours (n=5), 8 hours (n=5) and 24 hours (n=5) after an acute one minute emersion stressor.

References

Acevedo-Whitehouse K, Vicente J, Gortazar C, *et al.* (2005) Genetic resistance to bovine tuberculosis in the Iberian wild boar. *Molecular Ecology* **14**, 3209-3217.

Ahmadiyeh N, Churchill G, Solberg L, *et al.* (2005) Lineage is an epigenetic modifier of QTL influencing behavioral coping with stress. *Behavior Genetics* **35**, 189-198.

Akinbami MA, Philip GH, Sridaran R, Mahesh VB, Mann DR (1999) Expression of mRNA and proteins for testicular steroidogenic enzymes and brain and pituitary mRNA for glutamate receptors in rats exposed to immobilization stress. *The Journal of Steroid Biochemistry and Molecular Biology* **70**, 143-149.

Alaux C, Sinha S, Hasadsri L, *et al.* (2009) Honey bee aggression supports a link between gene regulation and behavioral evolution. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 15400-15405.

Albers HE, Dean A, Karom MC, Smith D, Huhman KL (2006) Role of V1a vasopressin receptors in the control of aggression in Syrian hamsters. *Brain Research* **1073-1074**, 425-430.

Alderman SL, McGuire A, Bernier NJ, Vijayan MM (2012) Central and peripheral glucocorticoid receptors are involved in the plasma cortisol response to an acute stressor in rainbow trout. *General and Comparative Endocrinology* **176**, 79-85.

Alho JS, Välimäki K, Merilä J (2010) Rhh: An R extension for estimating multilocus heterozygosity and heterozygosity-heterozygosity correlation. *Molecular Ecology Resources* **10**, 720-722.

Aljanabi S, Martinez I (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR- based techniques. *Nucleic Acids Research* **25**, 4692-4693.

Allen Orr H (2001) The genetics of species differences. *Trends in Ecology & Evolution* **16**, 343-350.

Almeida O, Gozdowska M, Kulczykowska E, Oliveira RF (2012) Brain levels of arginine-vasotocin and isotocin in dominant and subordinate males of a cichlid fish. *Hormones and Behavior* **61**, 212-217.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Aluru N, Renaud R, Leatherland JF, Vijayan MM (2005) Ah receptor-mediated impairment of interrenal steroidogenesis involves StAR protein and P450scc gene attenuation in rainbow trout. *Toxicological Sciences* **84**, 260-269.

Aluru N, Vijayan MM (2008) Molecular characterization, tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout. *Endocrinology* **149**, 4577-4588.

Álvarez D, Bell AM (2007) Sticklebacks from streams are more bold than sticklebacks from ponds. *Behavioural Processes* **76**, 215-217.

Amos W, Acevedo-Whitehouse K (2009) A new test for genotype-fitness associations reveals a single microsatellite allele that strongly predicts the nature of tuberculosis infections in wild boar. *Molecular Ecology Resources* **9**, 1102-1111.

Anholt RRH, Mackay TFC (2004) Quantitative genetic analyses of complex behaviours in *Drosophila*. *Nature Reviews Genetics* **5**, 838-849.

Antao T, Lopes A, Lopes R, Beja-Pereira A, Luikart G (2008) LOSITAN: A workbench to detect molecular adaptation based on a Fst-outlier method. *BMC Bioinformatics* **9**, 323.

Askew A, Gonzalez FA, Stahl JM, Karom MC (2006) Food competition and social experience effects on V1a receptor binding in the forebrain of male Long-Evans hooded rats. *Hormones and Behavior* **49**, 328-336.

Aubin-Horth N, Deschênes M, Cloutier S (2012) Natural variation in the molecular stress network correlates with a behavioural syndrome. *Hormones and Behavior* **61**, 140-146.

Avila CM, Sillero JC, Nadal S, *et al.* (2005) QTL detection for agronomic traits in Faba bean (*Vicia faba* L.). *Agriculturae Conspectus Scientificus* **70**, 65-73.
Azuma T, Dijkstra JM, Kiryu I, *et al.* (2005) Growth and behavioral traits in Donaldson rainbow trout (*Oncorhynchus mykiss*) cosegregate with classical major histocompatibility complex (MHC) class I genotype. *Behavior Genetics* **35**, 463-478.
Backström T, Winberg S (2009) Arginine-vasotocin influence on aggressive behavior and dominance in rainbow trout. *Physiology & Behavior* **96**, 470-475.
Balloux F, Amos W, Coulson T (2004) Does heterozygosity estimate inbreeding in real populations? *Molecular Ecology* **13**, 3021-3031.
Barendse W, Harrison B, Bunch R, Thomas M, Turner L (2009) Genome wide signatures of positive selection: The comparison of independent samples and the identification of regions associated to traits. *BMC Genomics* **10**, 178.
Barton BA, Schreck CB (1987) Influence of acclimation temperature on interrenal and carbohydrate stress responses in juvenile chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture* **62**, 299-310.
Beaumont MA, Balding DJ (2004) Identifying adaptive genetic divergence among populations from genome scans. *Molecular Ecology* **13**, 969-980.
Beaumont MA, Nichols RA (1996) Evaluating loci for use in the genetic analysis of population structure. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **263**, 1619-1626.
Beausoleil NJ, Blache D, Stafford KJ, Mellor DJ, Noble ADL (2008) Exploring the basis of divergent selection for 'temperament' in domestic sheep. *Applied Animal Behaviour Science* **109**, 261-274.
Bell AM (2005) Behavioural differences between individuals and two populations of stickleback (*Gasterosteus aculeatus*). *Journal of Evolutionary Biology* **18**, 464-473.
Bell AM (2009) Approaching the genomics of risk-taking behavior. *Advances in genetics* **68**, 83-104.
Bell AM, Aubin-Horth N (2010) What can whole genome expression data tell us about the ecology and evolution of personality? *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **365**, 4001-4012.
Bendesky A, Bargmann CI (2011) Genetic contributions to behavioural diversity at the gene–environment interface. *Nature Reviews Genetics* **12**, 809-820.
Benus RF, Den Daas S, Koolhaas JM, Van Oortmerssen GA (1990) Routine formation and flexibility in social and non-social behaviour of aggressive and non-aggressive male mice. *Behaviour* **112**, 176-193.
Biagioli M, Pinto M, Cesselli D, *et al.* (2009) Unexpected expression of alpha- and beta-globin in mesencephalic dopaminergic neurons and glial cells. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 15454-15459.
Bierne N, Beuzart I, Vonau V, Bonhomme F, Bédier E (2000) Microsatellite-associated heterosis in hatchery-propagated stocks of the shrimp *Penaeus stylirostris*. *Aquaculture* **184**, 203-219.
Biro PA, Stamps JA (2010) Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? *Trends in Ecology & Evolution* **25**, 653-659.
Blüthgen N, Brand K, Cajavec B, *et al.* (2005) Biological profiling of gene groups utilizing Gene Ontology. *Genome Informatics* **16**, 106-115.
Boake Christine RB, Arnold Stevan J, Breden F, *et al.* (2002) Genetic tools for studying adaptation and the evolution of behavior. *The American Naturalist* **160**, S143-S159.
Bonin A, Paris M, Tetreau G, David J-P, Despres L (2009) Candidate genes revealed by a genome scan for mosquito resistance to a bacterial insecticide: sequence and gene expression variations. *BMC Genomics* **10**, 551.
Bonin A, Taberlet P, Miaud C, Pompanon F (2006) Explorative genome scan to detect candidate loci for adaptation along a gradient of altitude in the common frog (*Rana temporaria*). *molecular biology and evolution* **23**, 773-783.

Brown C, Burgess F, Braithwaite VA (2007) Heritable and experiential effects on boldness in a tropical poeciliid. *Behavioral Ecology and Sociobiology* **62**, 237-243.

Brown C, Jones F, Braithwaite V (2005) In situ examination of boldness-shyness traits in the tropical poeciliid, *Brachyraphis episcopi*. *Animal Behaviour* **70**, 1003-1009.

Budaev SV (1997) 'Personality' in the guppy (*Poecilia reticulata*): A correlational study of exploratory behavior and social tendency. *Journal of Comparative Psychology* **111**, 399-411.

Budaev SV, Zworykin DD, Mochek AD (1999) Individual differences in parental care and behaviour profile in the convict cichlid: A correlation study. *Animal Behaviour* **58**, 195-202.

Buitenhuis B, Hedegaard J, Janss L, Sørensen P (2009) Differentially expressed genes for aggressive pecking behaviour in laying hens. *BMC Genomics* **10**, 544.

Bury NR, Sturm A (2007) Evolution of the corticosteroid receptor signalling pathway in fish. *General and Comparative Endocrinology* **153**, 47-56.

Butlin R (2010) Population genomics and speciation. *Genetica* **138**, 409-418.

Cairns MT, Johnson MC, Talbot AT, *et al.* (2008) A cDNA microarray assessment of gene expression in the liver of rainbow trout (*Oncorhynchus mykiss*) in response to a handling and confinement stressor. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics* **3**, 51-66.

Caldwell HK, Lee H-J, Macbeth AH, Young Iii WS (2008) Vasopressin: Behavioral roles of an "original" neuropeptide. *Progress in Neurobiology* **84**, 1-24.

Campbell PM, Pottinger TG, Sumpter JP (1992) Stress reduces the quality of gametes produced by rainbow trout. *Biology of Reproduction* **47**, 1140-1150.

Carr JA (2002) Stress, neuropeptides, and feeding behavior: A comparative perspective. *Integrative and Comparative Biology* **42**, 582-590.

Carter AJ, Goldizen AW, Tromp SA (2010) Agamas exhibit behavioral syndromes: Bolder males bask and feed more but may suffer higher predation. *Behavioral Ecology* **21**, 655-661.

Castillo J, Castellana B, Acerete L, *et al.* (2008) Stress-induced regulation of steroidogenic acute regulatory protein expression in head kidney of Gilthead seabream (*Sparus aurata*). *Journal of Endocrinology* **196**, 313-322.

Casto AM, Feldman MW (2011) Genome-wide association study SNPs in the human genome diversity project populations: Does selection affect unlinked SNPs with shared trait associations? *PLoS genetics* **7**, e1001266.

Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC (2009) A quantitative review of heterozygosity fitness correlations in animal populations. *Molecular Ecology* **18**, 2746-2765.

Chapman MA, Pashley CH, Wenzler J, *et al.* (2008) A genomic scan for selection reveals candidates for genes involved in the evolution of cultivated sunflower (*Helianthus annuus*). *Plant Cell* **20**, 2931-2945.

Charpentier M, Setchell JM, Prugnolle F, *et al.* (2005) Genetic diversity and reproductive success in mandrills (*Mandrillus sphinx*). *Proceedings of the National Academy of Sciences of the United States of America* **102**, 16723-16728.

Charpentier MJE, Prugnolle F, Gimenez O, Widdig A (2008) Genetic heterozygosity and sociality in a primate species. *Behavior Genetics* **38**, 151-158.

Conesa A, Götz S, García-Gómez JM, *et al.* (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674-3676.

Conrad JL, Sih A (2009) Behavioural type in newly emerged steelhead *Oncorhynchus mykiss* does not predict growth rate in a conventional hatchery rearing environment. *Journal of Fish Biology* **75**, 1410-1426.

Conrad JL, Weinersmith KL, Brodin T, Saltz JB, Sih A (2011) Behavioural syndromes in fishes: A review with implications for ecology and fisheries management. *Journal of Fish Biology* **78**, 395-435.

Cooper Ma, Karom M, Huhman KL, Albers HE (2005) Repeated agonistic encounters in hamsters modulate AVP V1a receptor binding. *Hormones and Behavior* **48**, 545-551.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**, 2001-2014.

Coscia I, Vogiatzi E, Kotoulas G, Tsigenopoulos CS, Mariani S (2012) Exploring neutral and adaptive processes in expanding populations of gilthead sea bream, *Sparus aurata* L., in the North-East Atlantic. *Heredity* **108**, 537-546.

Coulibaly I, Gharbi K, Danzmann RG, Yao J, Rexroad III CE (2005) Characterization and comparison of microsatellites derived from repeat-enriched libraries and expressed sequence tags. *Animal Genetics* **36**, 309-315.

Coulon A (2010) GENHET: an easy-to-use R function to estimate individual heterozygosity. *Molecular Ecology Resources* **10**, 167-169.

Craig I (1994) Psychiatric Genetics: Misbehaving monoamine oxidase gene. *Current Biology* **4**, 175-177.

Crisp DT (2000) *Trout and salmon: ecology, conservation and rehabilitation*, 1st edn. Fishing News Books, Oxford.

David P (1998) Heterozygosity-fitness correlations: new perspectives on old problems. *Heredity* **80**, 531-537.

David P, Pujol B, Viard F, Castella V, Goudet J (2007) Reliable selfing rate estimates from imperfect population genetic data. *Molecular Ecology* **16**, 2474-2487.

de Kloet ER (2004) Hormones and the stressed brain. *Annals of the New York Academy of Sciences* **1018**, 1-15.

de Kloet ER (2010) From vasotocin to stress and cognition. *European Journal of Pharmacology* **626**, 18-26.

de Kloet ER, Derijk R (2004) Signaling pathways in brain involved in predisposition and pathogenesis of stress-related disease: Genetic and kinetic factors affecting the MR/GR balance, pp. 14-34.

de Kloet ER, Joels M, Holsboer F (2005) Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* **6**, 463-475.

de Kloet ER, Reul JMHM (1987) Feedback action and tonic influence of corticosteroids on brain function: A concept arising from the heterogeneity of brain receptor systems. *Psychoneuroendocrinology* **12**, 83-105.

de Santis C, Smith-Keune C, Jerry DR (2011) Normalizing RT-qPCR Data: Are we getting the right answers? An appraisal of normalization approaches and internal reference genes from a case study in the finfish *Lates calcarifer*. *Marine Biotechnology* **13**, 170-180.

Decker R, Griffen B (2012) Correlating context-specific boldness and physiological condition of female sand fiddler crabs (*Uca pugilator*). *Journal of Ethology* **30**, 403-412.

Di Génova A, Aravena A, Zapata L, *et al.* (2011) SalmonDB: A bioinformatics resource for *Salmo salar* and *Oncorhynchus mykiss*. *Database* **2011**.

Dick DM, Rose RJ (2002) Behavior Genetics: What's New? What's Next? *Current Directions in Psychological Science* **11**, 70-74.

Dickens M, Romero LM, Cyr NE, Dunn IC, Meddle SL (2009) Chronic stress alters glucocorticoid receptor and mineralocorticoid receptor mRNA expression in the European starling (*Sturnus vulgaris*) brain. *Journal of Neuroendocrinology* **21**, 832-840.

Dieringer D, Schlötterer C (2003) Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *molecular ecology notes* **3**, 167-169.

Dingemanse NJ, Van der Plas F, Wright J, *et al.* (2009) Individual experience and evolutionary history of predation affect expression of heritable variation in fish personality and morphology. *Proceedings of the Royal Society B: Biological Sciences* **276**, 1285-1293.

Dingemanse NJ, Wright J, Kazem AJN, *et al.* (2007) Behavioural syndromes differ predictably between 12 populations of three-spined stickleback. *Journal of Animal Ecology* **76**, 1128-1138.

Doyon C, Trudeau VL, Moon TW (2005) Stress elevates corticotropin-releasing factor (CRF) and CRF-binding protein mRNA levels in rainbow trout (*Oncorhynchus mykiss*). *Journal of Endocrinology* **186**, 123-130.

Drent PJ, Van Oers K, Van Noordwijk AJ (2003) Realized heritability of personalities in the great tit (*Parus major*). *Proceedings of the Royal Society B: Biological Sciences* **270**, 45-51.

Drew RE, Schwabl H, Wheeler PA, Thorgaard GH (2007) Detection of QTL influencing cortisol levels in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **272**, 183-194.

Dugatkin LA, Druen M (2004) The social implications of winner and loser effects. *Proceedings of the Royal Society B: Biological Sciences* **271**, S488-S489.

Dugatkin LA, Wilson DS (1993) Fish behaviour, partner choice experiments and cognitive ethology. *Reviews in Fish Biology and Fisheries* **3**, 368-372.

Edwards AC, Ayroles JF, Stone EA, *et al.* (2009a) A transcriptional network associated with natural variation in *Drosophila* aggressive behavior. *Genome Biology* **10**.

Edwards AC, Zwarts L, Yamamoto A, Callaerts P, Mackay TFC (2009b) Mutations in many genes affect aggressive behavior in *Drosophila melanogaster*. *BMC Biology* **7**.

Ellis T, Yildiz HY, López-Olmeda J, *et al.* (2012) Cortisol and finfish welfare. *Fish Physiology and Biochemistry* **38**, 163-188.

Eriksson AC, Booth DJ, Biro PA (2010) 'Personality' in two species of temperate damselfish. *Marine Ecology Progress Series* **420**, 273-276.

Evans J, Boudreau K, Hyman J (2010) Behavioural syndromes in urban and rural populations of song sparrows. *Ethology* **116**, 588-595.

Excoffier L, Estoup A, Cornuet J-M (2005) Bayesian Analysis of an Admixture Model With Mutations and Arbitrarily Linked Markers. *Genetics* **169**, 1727-1738.

Excoffier L, Hofer T, Foll M (2009) Detecting loci under selection in a hierarchically structured population. *Heredity* **2009**, 285-298.

Fevolden SE, Roed KH, Fjalestad KT, Stien J (1999) Poststress levels of lysozyme and cortisol in adult rainbow trout: heritabilities and genetic correlations. *Journal of Fish Biology* **54**, 900-910.

Fidler AE, van Oers K, Drent PJ, *et al.* (2007) Drd4 gene polymorphisms are associated with personality variation in a passerine bird. *Proceedings of the Royal Society B: Biological Sciences* **274**, 1685-1691.

Fitzpatrick MJ, Feder E, Rowe L, Sokolowski MB (2007) Maintaining a behaviour polymorphism by frequency-dependent selection on a single gene. *Nature* **447**, 210-212.

Foll M, Gaggiotti O (2008) A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: A bayesian perspective. *Genetics* **180**, 977-993.

Fraser DF, Gilliam JF, Daley MJ, Le AN, Skalski GT (2001) Explaining leptokurtic movement distributions: Intrapopulation variation in boldness and exploration. *The American Naturalist* **158**, 124-135.

Frost AJ, Winrow-Giffen A, Ashley PJ, Sneddon LU (2007) Plasticity in animal personality traits: does prior experience alter the degree of boldness?

Fryer JN (1989) Neuropeptides regulating the activity of goldfish corticotropes and melanotropes. *Fish Physiology and Biochemistry* **7**, 21-27.

Fryer JN, Leung E (1982) Neurohypophysial hormonal control of cortisol secretion in the teleost *Carassius auratus*. *General and Comparative Endocrinology* **48**, 425-431.

Gallardo JA, Neira R (2005) Environmental dependence of inbreeding depression in cultured Coho salmon (*Oncorhynchus kisutch*): Aggressiveness, dominance and intraspecific competition. *Heredity* **95**, 449-456.

Gammie SC, Auger AP, Jessen HM, *et al.* (2007) Altered gene expression in mice selected for high maternal aggression. *Genes, Brain and Behavior* **6**, 432-443.

Garamszegi LZ, Rosivall B, Rettenbacher S, *et al.* (2012) Corticosterone, avoidance of novelty, risk-taking and aggression in a wild bird: No evidence for pleiotropic effects. *Ethology* **118**, 621-635.

Gesing A, Bilang-Bleuel A, Droste SK, *et al.* (2001) Psychological stress increases hippocampal mineralocorticoid receptor levels: Involvement of corticotropin-releasing hormone. *Journal of Neuroscience* **21**, 4822-4829.

Geslin M, Auperin B (2004) Relationship between changes in mRNAs of the genes encoding steroidogenic acute regulatory protein and P450 cholesterol side chain cleavage in head kidney and plasma levels of cortisol in response to different kinds of acute stress in the rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **135**, 70-80.

Gilchriest BJ, Tipping DJ, Hake L, Levy A, Baker BI (2001) Differences in arginine vasotocin gene transcripts and cortisol secretion in trout with high or low endogenous melanin-concentrating hormone secretion. *Journal of Neuroendocrinology* **13**, 407-411.

Gilchriest BJ, Tipping DR, Levy A, Baker BI (2000) The effects of acute and chronic stresses on vasotocin gene transcripts in the brain of the rainbow trout (*Oncorhynchus mykiss*). *Journal of Neuroendocrinology* **12**, 795-801.

Gilmour KM, DiBattista JD, Thomas JB (2005) Physiological causes and consequences of social status in salmonid fish. *Integrative and Comparative Biology* **45**, 263-273.

Godin JG, Dugatkin LA (1996) Female mating preference for bold males in the guppy, *Poecilia reticulata*. *Proceedings of the National Academy of Sciences* **93**, 10262-10267.

Goodson JL, Bass AH (2001) Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates. *Brain Research Reviews* **35**, 246-265.

Govoroun M, Le Gac F, Guiguen Y (2006) Generation of a large scale repertoire of Expressed Sequence Tags (ESTs) from normalised rainbow trout cDNA libraries. *BMC Genomics* **7**, 196.

Gracey AY, Fraser EJ, Li W, *et al.* (2004) Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 16970-16975.

Greenspan RJ (1997) A kinder, gentler genetic analysis of behavior: dissection gives way to modulation. *Current Opinion in Neurobiology* **7**, 805-811.

Grueber CE, Wallis GP, Jamieson IG (2008) Heterozygosity-fitness correlations and their relevance to studies on inbreeding depression in threatened species. *Molecular Ecology* **17**, 3978-3984.

Guyomard R, Mauger S, Tabet-Canale K, *et al.* (2006) A type I and type II microsatellite linkage map of rainbow trout (*Oncorhynchus mykiss*) with presumptive coverage of all chromosome arms. *BMC Genomics* **7**, 302.

Haidle L, Janssen J, Gharbi K, *et al.* (2008) Determination of quantitative trait loci (QTL) for early maturation in rainbow trout (*Oncorhynchus mykiss*). *Marine Biotechnology* **10**, 579-592.

Haller J (1991) Muscle metabolic changes during the first six hours of cohabitation in paris of male *Betta splendens*. *Physiology & Behavior* **49**, 1301-1303.

Haller J (1995) Biochemical background for an analysis of cost-benefit interrelations in aggression. *Neuroscience and Biobehavioral Reviews* **19**, 599-604.

Haller J, Miklósi A, Csányi V, Makara GB (1996) Behavioral tactics control the energy costs of aggression: The example of *Macropodus opercularis*. *Aggressive Behavior* **22**, 437-446.

Hansen MM, Meier K, Mensberg K-LD (2010) Identifying footprints of selection in stocked brown trout populations: A spatio-temporal approach. *Molecular Ecology* **19**, 1787-1800.

Hansson B, Westerberg L (2002) On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology* **11**, 2467-2474.

Hansson B, Westerdahl H, Hasselquist D, A°Kesson M, Bensch S (2004) Does linkage disequilibrium generate heterozygosity-fitness correlations in great reed warblers? *Evolution* **58**, 870-879.

Hashizume C, Suzuki M, Masuda K, *et al.* (2003) Molecular cloning of canine monoamine oxidase subtypes A (MAOA) and B (MAOB) cDNAs and their expression in the brain. *The Journal of Veterinary Medical Science* **65**, 893-898.

Heinrichs SC, Menzaghi F, Pich EM, Britton KT, Koob GF (1995) The role of CRF in behavioral aspects of stress, pp. 92-104. Annals New York Academy of Sciences.

Herman JP, Watson SJ (1995) Stress regulation of mineralocorticoid receptor heteronuclear RNA in rat hippocampus. *Brain Research* **677**, 243-249.

Hermisson J (2009) Who believes in whole genome-scans for selection? *Heredity* **103**, 283-284.

Hodge SJ, Manica A, Flower TP, Clutton-Brock TH (2008) Determinants of reproductive success in dominant female meerkats. *Journal of Animal Ecology* **77**, 92-102.

Höglund E, Balm PHM, Winberg S (2000) Skin darkening, a potential social signal in subordinate Arctic charr (*Salvelinus alpinus*): The regulatory role of brain monoamines and pro-opiomelanocortin-derived peptides. *Journal of Experimental Biology* **203**, 1711-1721.

Hoheisel JD (1997) Oligomer-chip technology. *Trends in Biotechnology* **15**, 465-469.

Hori TS, Rise ML, Johnson SC, Afonso LOB, Gamperl AK (2012) The mRNA expression of cortisol axis related genes differs in Atlantic cod (*Gadus morhua*) categorized as high or low responders. *General and Comparative Endocrinology* **175**, 311-320.

Hsu DT, Lombardo KA, Herringa RJ, *et al.* (2001) Corticotropin-releasing hormone messenger RNA distribution and stress-induced activation in the thalamus. *Neuroscience* **105**, 911-921.

Hsu Y, Wolf LL (2001) The winner and loser effect: what fighting behaviours are influenced? *Animal Behaviour* **61**, 777-786.

Huang X, Madan A (1999) CAP3: A DNA sequence assembly program. *Genome Research* **9**, 868-877.

Hudson ME (2008) Sequencing breakthroughs for genomic ecology and evolutionary biology. *Molecular Ecology Resources* **8**, 3-17.

Huntingford F, Turner A (1987) *Animal Conflict*, 1 edn. Chapman and Hall Ltd., London New York.

Huntingford FA (1976) The relationship between anti-predator behaviour and aggression among conspecifics in the three-spined stickleback, *Gasterosteus aculeatus*. *Animal Behaviour* **24**, 245-260.

Huntingford FA, Metcalfe NB, Thorpe JE, Graham WD, Adams CE (1990) Social dominance and body size in Atlantic salmon parr, Salmo salar L. *Journal of Fish Biology* **36**, 877-881.

Ida T, Mori K, Miyazato M, *et al.* (2005) Neuromedin S is a novel anorexigenic hormone. *Endocrinology* **146**, 4217-4223.

Jeffrey JD, Esbaugh AJ, Vijayan MM, Gilmour KM (2012) Modulation of hypothalamic-pituitary-interrenal axis function by social status in rainbow trout. *General and Comparative Endocrinology* **176**, 201-210.

Jezova D, Ochedalski T, Glickman M, Kiss A, Aguilera G (1999) Central corticotropin-releasing hormone receptors modulate hypothalamic- pituitary-adrenocortical and sympathoadrenal activity during stress. *Neuroscience* **94**, 797-802.

Ji T, Zhang B, Sun Y, Tao Y (2009) Evolutionary dynamics of fearfulness and boldness. *Journal of Theoretical Biology* **256**, 637-643.

Johnson EO, Kamilaris TC, Chrousos GP, Gold PW (1992) Mechanisms of stress: A dynamic overview of hormonal and behavioral homeostasis. *Neuroscience & Biobehavioral Reviews* **16**, 115-130.

Jones N, Ougham H, Thomas H (1997) Markers and mapping: we are all geneticists now. *New Phytologist* **137**, 165-177.

Kane NC, Rieseberg LH (2007) Selective sweeps reveal candidate genes for adaptation to drought and salt tolerance in common sunflower, *Helianthus annuus*. *Genetics* **175**, 1823-1834.

Kanitz E, Puppe B, Tuchscherer M, *et al.* (2009) A single exposure to social isolation in domestic piglets activates behavioural arousal, neuroendocrine stress hormones, and stress-related gene expression in the brain. *Physiology and Behavior* **98**, 176-185.

Karsi A, Waldbieser GC, Small BC, Wolters WR (2005) Genomic structure of the proopiomelanocortin gene and expression during acute low-water stress in channel catfish. *General and Comparative Endocrinology* **143**, 104-112.

Kauer MO, Dieringer D, Schlotterer C (2003) A microsatellite variability screen for positive selection associated with the "Out of Africa" habitat expansion of *Drosophila melanogaster*. *Genetics* **165**, 1137-1148.

Kayser M, Brauer S, Stoneking M (2003) A genome scan to detect candidate regions influenced by local natural selection in human populations. *molecular biology and evolution* **20**, 893-900.

Kempenaers B (2007) Mate choice and genetic quality: A review of the heterozygosity theory. *Advances in the study of behaviour* **37**, 189-278.

Ketola T, Kotiaho JS (2009) Inbreeding, energy use and condition. *Journal of Evolutionary Biology* **22**, 770-781.

Kimura M (1968) Evolutionary rate at the molecular level. *Nature* **217**, 624-626.

Kinnally EL, Tarara ER, Mason WA, *et al.* (2010) Serotonin transporter expression is predicted by early life stress and is associated with disinhibited behavior in infant rhesus macaques. *Genes, Brain and Behavior* **9**, 45-52.

Kittilsen S, Schjolden J, Beitnes-Johansen I, *et al.* (2009) Melanin-based skin spots reflect stress responsiveness in salmonid fish. *Hormones and Behavior* **56**, 292-298.

Königstorfer A, Sterrer S, Hoffmann W (1990) Ependymins are expressed in the meninx of goldfish brain. *Cell and Tissue Research* **261**, 59-64.

Koolhaas JM, de Boer SF, Buwalda B, van Reenen K (2007) Individual variation in coping with stress: A multidimensional approach of ultimate and proximate mechanisms. *Brain, Behavior and Evolution* **70**, 218-226.

Koolhaas JM, Korte SM, De Boer SF, *et al.* (1999) Coping styles in animals: current status in behavior and stress-physiology. *Neuroscience & Biobehavioral Reviews* **23**, 925-935.

Korsten P, Mueller JC, HermannstÄDter C, *et al.* (2010) Association between DRD4 gene polymorphism and personality variation in great tits: a test across four wild populations. *Molecular Ecology* **19**, 832-843.

Krasnov A, Koskinen H, Pehkonen P, *et al.* (2005) Gene expression in the brain and kidney of rainbow trout in response to handling stress. *BMC Genomics* **6**.

Kruk M (1991) Ethology and pharmacology of hypothalamic aggression in the rat. *Neuroscience and Biobehavioral Reviews* **Vol. 15**, pp. 527-538.

Kulikov AV, Osipova DV, Naumenko VS, Popova NK (2005) Association between Tph2 gene polymorphism, brain tryptophan hydroxylase activity and aggressiveness in mouse strains. *Genes, Brain and Behavior* **4**, 482-485.

Kusakabe M, Zuccarelli MD, Nakamura I, Young G (2009) Steroidogenic acute regulatory protein in white sturgeon (*Acipenser transmontanus*): cDNA cloning, sites of expression and transcript abundance in corticosteroidogenic tissue after an acute stressor. *General and Comparative Endocrinology* **162**, 233-240.

Leblond C, Reebs SG (2006) Individual leadership and boldness in shoals of golden shiners (*Notemigonus crysoleucas*). *Behaviour* **143**, 1263-1280.

Lema SC (2010) Identification of multiple vasotocin receptor cDNAs in teleost fish: Sequences, phylogenetic analysis, sites of expression, and regulation in the hypothalamus and gill in response to hyperosmotic challenge. *Molecular and Cellular Endocrinology* **321**, 215-230.

Li M-H, Merila J (2010) Sex-specific population structure, natural selection, and linkage disequilibrium in a wild bird population as revealed by genome-wide microsatellite analyses. *BMC Evolutionary Biology* **10**, 66.

Li ZH, Li P, Randak T (2010a) Effect of a human pharmaceutical carbamazepine on antioxidant responses in brain of a model teleost in vitro: An efficient approach to biomonitoring. *Journal of Applied Toxicology* **30**, 644-648.

Li ZH, Zlabek V, Grabic R, *et al.* (2010b) Effects of exposure to sublethal propiconazole on the antioxidant defense system and Na+-K+-ATPase activity in brain of rainbow trout, *Oncorhynchus mykiss*. *Aquatic Toxicology* **98**, 297-303.

Lieutenant-Gosselin M, Bernatchez L (2006) Local Heterozygosity-fitness correlations with global positive effects on fitness in Threespine Stickleback. *Evolution* **60**, 1658-1668.

Liinamo A-E, van den Berg L, Leegwater PAJ, *et al.* (2007) Genetic variation in aggression-related traits in Golden Retriever dogs. *Applied Animal Behaviour Science* **104**, 95-106.

Liu Y, Curtis JT, Fowler CD, *et al.* (2001) Differential expression of vasopressin, oxytocin and corticotrophin-releasing hormone messenger RNA in the paraventricular nucleus of the prairie vole brain following stress. *Journal of Neuroendocrinology* **13**, 1059-1065.

Lobo NF, Ton LQ, Hill CA, *et al.* (2003) Genomic analysis in the sting-2 quantitative trait locus for defensive behavior in the honey bee, *Apis mellifera*. *Genome Research* **13**, 2588-2593.

Lovell PV, Clayton DF, Replogle KL, Mello CV (2008) Birdsong "transcriptomics": Neurochemical specializations of the oscine song system. *PloS one* **3**.

Lowe CD, Mello LV, Samatar N, *et al.* (2011) The transcriptome of the novel dinoflagellate *Oxyrrhis marina* (Alveolata: Dinophyceae): response to salinity examined by 454 sequencing. *BMC Genomics* **12**, 519.

Mackay T (2009) The genetic architecture of complex behaviors: lessons from *Drosophila*. *Genetica* **136**, 295-302.

Mackay TFC (2001) The genetic architecture of quantitative traits. *Annual Review of Genetics* **35**, 303.

Mager III JN, Walcott C, Piper WH (2008) Nest platforms increase aggressive behavior in common loons. *Naturwissenschaften* **95**, 141-147.

Manel S, Conord C, Despres L (2009) Genome scan to assess the respective role of host-plant and environmental constraints on the adaptation of a widespread insect. *BMC Evolutionary Biology* **9**, 288.

Marler Ca, Boyd SK, Wilczynski W (1999) Forebrain arginine vasotocin correlates of alternative mating strategies in cricket frogs. *Hormones and Behavior* **36**, 53-61.

Maxson SC, Canastar A (2005) Genetic aspects of aggressions in nonhuman animals. In: *Biology of Aggression* (ed. Nelson R). Oxford University Press, Incorporated, Cary, NC, USA.

Maynard Smith J, Haigh J (1974) The hitch-hiking effect of a favourable gene. *Genetics Research* **23**, 23-35.

Mazon AF, Verburg-van Kemenade BML, Flik G, Huising MO (2006) Corticotropin-releasing hormone-receptor 1 (CRH-R1) and CRH-binding protein (CRH-BP) are expressed in the gills and skin of common carp *Cyprinus carpio* L. and respond to acute stress and infection. *Journal of Experimental Biology* **209**, 510-517.

McEwen BS, Stellar E (1993) Stress and the individual: Mechanisms leading to disease. *Archives of Internal Medicine* **153**, 2093-2101.

McGuffin P, Riley B, Plomin R (2001) Toward behavioral genomics. *Science* **291**, 1232-1249.

McQuillan HJ, Kusakabe M, Young G (2011) Effects of chronic manipulation of adrenocorticotropic hormone levels in Chinook salmon on expression of interrenal steroidogenic acute regulatory protein and steroidogenic enzymes. *General and Comparative Endocrinology* **174**, 156-165.

Meagher S, Penn DJ, Potts WK (2000) Male-male competition magnifies inbreeding depression in wild house mice. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 3324-3329.

Meglécz E, Costedoat C, Dubut V, *et al.* (2010) QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* **26**, 403-404.

Mikhailova MV, Mayeux PR, Jurkevich A, *et al.* (2007) Heterooligomerization between vasotocin and Corticotropin-Releasing Hormone (CRH) receptors augments CRH-stimulated 3′,5′-cyclic adenosine monophosphate production. *Molecular Endocrinology* **21**, 2178-2188.

Miyazato M, Mori K, Ida T, *et al.* (2008) Identification and functional analysis of a novel ligand for G protein-coupled receptor, Neuromedin S. *Regulatory Peptides* **145**, 37-41.

Mommsen TP, Vijayan MM, Moon TW (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* **9**, 211-268.

Momoda TS, Schwindt AR, Feist GW, *et al.* (2007) Gene expression in the liver of rainbow trout, *Oncorhynchus mykiss*, during the stress response. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* **2**, 303-315.

Moreira PSA, Pulman KGT, Pottinger TG (2004) Extinction of a conditioned response in rainbow trout selected for high or low responsiveness to stress. *Hormones and Behavior* **46**, 450-457.

Morsink MC, Steenbergen PJ, Vos JB, *et al.* (2006) Acute Activation of Hippocampal Glucocorticoid Receptors Results in Different Waves of Gene Expression Throughout Time. *Journal of Neuroendocrinology* **18**, 239-252.

Mosienko V, Bert B, Beis D, *et al.* (2012) Exaggerated aggression and decreased anxiety in mice deficient in brain serotonin. *Translational Psychiatry* **2**, e122.

Mosser A, Packer C (2009) Group territoriality and the benefits of sociality in the African lion, *Panthera leo*. *Animal Behaviour* **78**, 359-370.

Namroud M-C, Beaulieu J, Juge N, Laroche J, Bousquet J (2008) Scanning the genome for gene single nucleotide polymorphisms involved in adaptive population differentiation in white spruce. *Molecular Ecology* **17**, 3599-3613.

Naumenko VS, Kozhemjakina RV, Plyusnina IZ, Popova NK (2009) Expression of serotonin transporter gene and startle response in rats with genetically determined fear-induced aggression. *Bulletin of Experimental Biology and Medicine* **147**, 81-83.

Neat FC, Taylor AC, Huntingford FA (1998) Proximate costs of fighting in male cichlid fish: the role of injuries and energy metabolism. *Animal Behaviour* **55**, 875-882.

Nichols KM, Young WP, Danzmann RG, *et al.* (2003) A consolidated linkage map for rainbow trout (*Oncorhynchus mykiss*). *Animal Genetics* **34**, 102-115.

Nielsen R (2005) Molecular signatures of natural selection. *Annual Review of Genetics* **39**, 197-218.

Nielsen R, Hubisz MJ, Hellmann I, *et al.* (2009) Darwinian and demographic forces affecting human protein coding genes. *Genome Research* **19**, 838-849.

Nosil P, Funk DJ, Ortiz-Barrientos D (2009) Divergent selection and heterogeneous genomic divergence. *Molecular Ecology* **18**, 375-402.

Nunes VL, Beaumont Ma, Butlin RK, Paulo OS (2011) Multiple approaches to detect outliers in a genome scan for selection in ocellated lizards (*Lacerta lepida*) along an environmental gradient. *Molecular Ecology* **20**, 193-205.

Olano-Marin J, Mueller JC, Kempenaers B (2011) Correlations between heterozygosity and reproductive success in the blue tit (*Cyanistes caeruleus*): An analysis of inbreeding and single locus effects. *Evolution* **65**, 3175-3194.

Oleksyk TK, Smith MW, O'Brien SJ (2010) Genome-wide scans for footprints of natural selection. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **365**, 185-205.

Olsson T, Håkansson Å, Seckl JR (1997) Ketanserin selectively blocks acute stress-induced changes in NGFI-A and mineralocorticoid receptor gene expression in hippocampal neurons. *Neuroscience* **76**, 441-448.

Oshlack A, Robinson M, Young M (2010) From RNA-seq reads to differential expression results. *Genome Biology* **11**, 220.

Øverli Ø (2007) Evolutionary background for stress-coping styles: Relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates. *Neuroscience and Biobehavioral Reviews* **31**, 396-412.

Øverli Ø, Harris CA, Winberg S (1999) Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships on brain monoamines and cortisol in rainbow trout. *Brain, Behavior and Evolution* **54**, 263-275.

Øverli Ø, Korzan WJ, Höglund E, *et al.* (2004) Stress coping style predicts aggression and social dominance in rainbow trout. *Hormones and Behavior* **45**, 235-241.

Øverli Ø, Kotzian S, Winberg S (2002a) Effects of Cortisol on Aggression and Locomotor Activity in Rainbow Trout. *Hormones and Behavior* **42**, 53-61.

Øverli Ø, Pottinger TG, Carrick TR, Overli E, Winberg S (2002b) Differences in behaviour between rainbow trout selected for high- and low-stress responsiveness. *The Journal of Experimental Biology* **205**, 391-395.

Øverli Ø, Winberg S, Pottinger TG (2005) Behavioral and neuroendocrine correlates of selection for stress responsiveness in rainbow trout—a review. *Integrative and Comparative Biology* **45**, 463-474.

Palti Y, Luo M-C, Hu Y, *et al.* (2009) A first generation BAC-based physical map of the rainbow trout genome. *BMC Genomics* **10**, 462.

Paskitti ME, McCreary BJ, Herman JP (2000) Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: Time-course analysis. *Molecular Brain Research* **80**, 142-152.

Pavlidis M, Sundvik M, Chen Y-C, Panula P (2011) Adaptive changes in zebrafish brain in dominant–subordinate behavioral context. *Behavioural Brain Research* **225**, 529-537.

Payne AH, Hales DB (2004) Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine reviews* **25**, 947-970.

Pérez-González J, Carranza J, Torres-Porras J, Fernández-García JL (2010) Low heterozygosity at microsatellite markers in Iberian red deer with small antlers. *Journal of Heredity* **101**, 553-561.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* **29**, e45.

Pickering AD (1990) Stress and the suppression of somatic growth in teleost fish. *Progress in clinical and biological research* **342**, 473-479.

Pickering AD, Pottinger TG (1989) Stress responses and disease resistance in salmonid fish - effects of chronic elevation of plasma-cortisol. *Fish Physiology and Biochemistry* **7**, 253-258.

Pickering AD, Pottinger TG, Sumpter JP, Carragher JF, Le Bail PY (1991) Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* **83**, 86-93.

Pierson PM, Guibbolini ME, Lahlou B (1996) A V1-type receptor for mediating the neurohypophysial hormone-induced ACTH release in trout pituitary. *Journal of Endocrinology* **149**, 109-115.

Pogson GH, Fevolden SE (1998) DNA heterozygosity and growth rate in the Atlantic cod *Gadus morhua* (L). *Evolution* **52**, 915-920.

Poletto R, Steibel JP, Siegford JM, Zanella AJ (2006) Effects of early weaning and social isolation on the expression of glucocorticoid and mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase 1 and 2 mRNAs in the frontal cortex and hippocampus of piglets. *Brain Research* **1067**, 36-42.

Pongsomboon S, Udomlertpreecha S, Amparyup P, Wuthisuthimethavee S, Tassanakajon A (2009) Gene expression and activity of carbonic anhydrase in salinity stressed

Penaeus monodon. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **152**, 225-233.
Popova NK (2008) From gene to aggressive behavior: The role of brain serotonin. *Neuroscience and Behavioral Physiology* **38**, 471-475.
Pottinger TG (1990) The effect of stress and exogenous cortisol on receptor-like binding of cortisol in the liver of rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* **78**, 194-203.
Pottinger TG (2000) Genetic selection to reduce stress in animals. In: *The Biology of animal stress: Basic principles and implications for animal welfare* (eds. Moberg GP, Mench JA). CAB International, New York. .
Pottinger TG (2006) Context dependent differences in growth of two rainbow trout (*Oncorhynchus mykiss*) lines selected for divergent stress responsiveness. *Aquaculture* **256**, 140-147.
Pottinger TG, Carrick TR (1999) Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. *General and Comparative Endocrinology* **116**, 122-132.
Pottinger TG, Carrick TR (2001) Stress responsiveness affects dominant-subordinate relationships in rainbow trout. *Hormones and Behavior* **40**, 419-427.
Pottinger TG, Pickering AD (1992) The influence of social interaction on the acclimation of rainbow trout, *Oncorhynchus mykiss* (Walbaum) to chronic stress. *Journal of Fish Biology* **41**, 435-447.
Pottinger TG, Pickering AD, Hurley MA (1992) Consistency in the stress response of individuals of two strains of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **103**, 275-289.
Pounder K (Pers. Comms.).
Prunet P, Øverli O, Douxfils J, *et al.* (2012) Fish welfare and genomics. *Fish Physiology and Biochemistry* **38**, 43-60.
Quillet E, Krieg F, Dechamp N, *et al.* (2010) Mapping QTLs affecting cortisol response to confinement stress in rainbow trout. In: *9th World Congress on Genetics applied to Livestock Production (WCGALP)*.
R Development Core Team (2009) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Raymond M, Rousset F (1995) GENEPOP (Version-1.2) - Population-genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**, 248-249.
Réale D, Gallant BY, Leblanc M, Festa-Bianchet M (2000) Consistency of temperament in bighorn ewes and correlates with behaviour and life history. *Animal Behaviour* **60**, 589-597.
Réale D, Reader SM, Sol D, McDougall PT, Dingemanse NJ (2007) Integrating animal temperament within ecology and evolution. *Biological Reviews* **82**, 291-318.
Reif A, Lesch K-P (2003) Toward a molecular architecture of personality. *Behavioural Brain Research* **139**, 1-20.
Renn SCP, Aubin-Horth N, Hofmann HA (2008) Fish and chips: functional genomics of social plasticity in an African cichlid fish. *Journal of Experimental Biology* **211**, 3041-3056.
Reul J, Gesing A, Droste S, *et al.* (2000) The brain mineralocorticoid receptor: greedy for ligand, mysterious in function. *European Journal of Pharmacology* **405**, 235-249.
Reul JMHM, Van den Bosch FR, De Kloet ER (1987) Differential response of type I and type II corticosteroid receptors to changes in plasma steroid level and circadian rhythmicity. *Neuroendocrinology* **45**, 407-412.
Rexroad C, Palti Y, Gahr S, Vallejo R (2008) A second generation genetic map for rainbow trout (*Oncorhynchus mykiss*). *BMC Genetics* **9**, 74.
Rice WR (1989) Analyzing tables of statistical tests. *Evolution* **43**, 223-225.
Riesch R, Duwe V, Herrmann N, *et al.* (2009) Variation along the shy-bold continuum in extremophile fishes (*Poecilia mexicana, Poecilia sulphuraria*). *Behavioral Ecology and Sociobiology* **63**, 1515-1526.

Rijks JM, Hoffman JI, Kuiken T, Osterhaus ADME, Amos W (2008) Heterozygosity and lungworm burden in harbour seals (*Phoca vitulina*). *Heredity* **100**, 587-593.
Robin C, Daborn PJ, Hoffmann AA (2007) Fighting fly genes. *Trends in Genetics* **23**, 51-54.
Robinson GE (2004) Genomics: beyond nature and nurture. *Science* **304**, 397-399.
Rodríguez M, Specker JL (1991) In vitro effects of arginine vasotocin on testosterone production by testes of rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **83**, 249-257.
Rogers SM, Bernatchez L (2005) Integrating QTL mapping and genome scans towards the characterization of candidate loci under parallel selection in the lake whitefish (*Coregonus clupeaformis*). *Molecular Ecology* **14**, 351-361.
Romero LM, Dickens MJ, Cyr NE (2009) The reactive scope model — A new model integrating homeostasis, allostasis, and stress. *Hormones and Behavior* **55**, 375-389.
Romualdi C, Bortoluzzi S, d'Alessi F, Danieli GA (2003) IDEG6: a web tool for detection of differentially expressed genes in multiple tag sampling experiments. *Physiological Genomics* **12**, 159-162.
Rother S, Schmidt R, Brysch W, Schlingensiepen K-H (1995) Learning-induced expression of meningeal ependymin mRNA and demonstration of ependymin in neurons and glial cells. *Journal of Neurochemistry* **65**, 1456-1464.
Rousset F (2008) GENEPOP ' 007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* **8**, 103-106.
Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. (ed. Krawetz S MS), pp. 365-386. Humana Press, Totowa, NJ
Rulten SL, Ripley TL, Hunt CL, Stephens DN, Mayne LV (2006) Sp1 and NFκB pathways are regulated in brain in response to acute and chronic ethanol. *Genes, Brain & Behavior* **5**, 257-273.
Ryder TB, Tori WP, Blake JG, Loiselle BA, Parker PG (2010) Mate choice for genetic quality: A test of the heterozygosity and compatibility hypotheses in a lek-breeding bird. *Behavioral Ecology* **21**, 203-210.
Sakamoto T, Danzmann R, Gharbi K, *et al.* (2000) A microsatellite linkage map of rainbow trout (*Oncorhynchus mykiss*) characterized by large sex-specific differences in recombination rates. *Genetics* **155**, 1331 - 1345.
Salem M, Kenney PB, Rexroad CE, Yao J (2008) Development of a 37 k high-density oligonucleotide microarray: a new tool for functional genome research in rainbow trout. *Journal of Fish Biology* **72**, 2187-2206.
Salem M, Rexroad CE, Wang J, Thorgaard GH, Yao J (2010) Characterization of the rainbow trout transcriptome using Sanger and 454-pyrosequencing approaches. *BMC Genomics* **11**, 564.
Salonen A, Peuhkuri N (2006) The effect of captive breeding on aggressive behaviour of European grayling, *Thymallus thymallus*, in different contexts. *Animal Behaviour* **72**, 819-825.
Sathiyaa R, Vijayan MM (2003) Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. *American Journal of Physiology - Cell Physiology* **284**, C1508-C1515.
Savolainen V, Anstett MC, Lexer C, *et al.* (2006) Sympatric speciation in palms on an oceanic island. *Nature* **441**, 210-213.
Schjolden J, Backström T, Pulman KGT, Pottinger TG, Winberg S (2005) Divergence in behavioural responses to stress in two strains of rainbow trout (*Oncorhynchus mykiss*) with contrasting stress responsiveness. *Hormones and Behavior* **48**, 537-544.
Schjolden J, Basic D, Winberg S (2009) Aggression in rainbow trout is inhibited by both MR and GR antagonists. *Physiology & Behavior* **98**, 625-630.
Schlötterer C (2002a) A microsatellite-based multilocus screen for the identification of local selective sweeps. *Genetics* **160**, 753-763.

Schlötterer C (2002b) Towards a molecular characterization of adaptation in local populations. *Current Opinion in Genetics & Development* **12**, 683-687.
Schlötterer C (2003) Hitchhiking mapping - functional genomics from the population genetics perspective. *Trends in Genetics* **19**, 32-38.
Schlötterer C, Dieringer D (2005) A novel test statistic for the identification of local selective sweeps based on microsatellite gene diversity. In: *Selective Sweep* (ed. Nurminsky D), pp. 55-64.
Schroder SL, Knudsen CM, Pearsons TN, *et al.* (2010) Behavior and breeding success of wild and first-generation hatchery male spring chinook salmon spawning in an artificial stream. *Transactions of the American Fisheries Society* **139**, 989-1003.
Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* **18**, 233-234.
Seddon N, Amos W, Mulder RA, Tobias JA (2004) Male heterozygosity predicts territory size, song structure and reproductive success in a cooperatively breeding bird. *Proceedings of the Royal Society B: Biological Sciences* **271**, 1823-1829.
Shashoua VE, Moore ME (1978) Effect of antisera to β and γ goldfish brain proteins on the retention of a newly acquired behavior. *Brain Research* **148**, 441-449.
Shimada Y, Shikano T, Merilä J (2011) A high incidence of selection on physiologically important genes in the three-spined stickleback, *Gasterosteus aculeatus*. *molecular biology and evolution* **28**, 181-193.
Shousha S, Nakahara K, Sato M, *et al.* (2006) Effect of neuromedin S on feeding regulation in the Japanese quail. *Neuroscience Letters* **391**, 87-90.
Sih A, Bell A, Johnson JC (2004a) Behavioral syndromes: An ecological and evolutionary overview. *Trends in Ecology and Evolution* **19**, 372-378.
Sih A, Bell AM, Johnson JC, Ziemba RE (2004b) Behavioral syndromes: An integrative overview. *Quarterly Review of Biology* **79**, 241-277.
Simon AF, Krantz DE (2007) Road rage and fruit flies. *Nature Genetics* **39**, 581-582.
Sinn DL, Apiolaza LA, Moltschaniwskyj NA (2006) Heritability and fitness-related consequences of squid personality traits. *Journal of Evolutionary Biology* **19**, 1437-1447.
Slate J, David P, Dodds KG, *et al.* (2004) Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity* **93**, 255-265.
Sloman KA, Armstrong JD (2002) Physiological effects of dominance hierarchies: Laboratory artefacts or natural phenomena? *Journal of Fish Biology* **61**, 1-23.
Sloman KA, Metcalfe NB, Taylor AC, Gilmour KM (2001) Plasma cortisol concentrations before and after social stress in rainbow trout and brown trout. *Physiological and Biochemical Zoology* **74**, 383-389.
Sneddon LU (2003) The bold and the shy: individual differences in rainbow trout. *Journal of Fish Biology* **62**, 971-975.
Sneddon LU, Margareto J, Cossins AR (2005) The use of transcriptomics to address questions in behaviour. *Physiological and Biochemical Zoology* **78**, 695-705.
Sneddon LU, Schmidt R, Fang Y, Cossins AR (2011) Molecular correlates of social dominance: A novel role for ependymin in aggression. *PloS one* **6**, e18181.
Solberg LC, Baum AE, Ahmadiyeh N, *et al.* (2006) Genetic analysis of the stress-responsive adrenocortical axis. *Physiol. Genomics* **27**, 362-369.
Sood PK, Nahar U, Nehru B (2011) Curcumin attenuates aluminum-induced oxidative stress and mitochondrial dysfunction in rat brain. *Neurotoxicity Research* **20**, 351-361.
St-Cyr S, Aubin-Horth N (2009) Integrative and genomics approaches to uncover the mechanistic bases of fish behavior and its diversity. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **152**, 9-21.
Steptoe A, van Jaarsveld CHM, Semmler C, Plomin R, Wardle J (2009) Heritability of daytime cortisol levels and cortisol reactivity in children. *Psychoneuroendocrinology* **34**, 273-280.

Stevenson EL, Caldwell HK (2012) The vasopressin 1b receptor and the neural regulation of social behavior. *Hormones and Behavior* **61**, 277-282.

Storz J, F., Payseur BA, Nachman MW (2004) Genome scans of DNA variability in humans reveal evidence for selective sweeps outside of Africa. *molecular biology and evolution* **21**, 1800-1811.

Storz JF (2005) Using genome scans of DNA polymorphism to infer adaptive population divergence. *Molecular Ecology* **14**, 671-688.

Strandberg E, Jacobsson J, Saetre P (2005) Direct genetic, maternal and litter effects on behaviour in German shepherd dogs in Sweden. *Livestock Production Science* **93**, 33-42.

Sturm A, Bury N, Dengreville L, *et al.* (2005) 11-Deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. *Endocrinology* **146**, 47-55.

Sumpter JP, Pickering AD, Pottinger TG (1985) Stress-induced elevation of plasma α-MSH and endorphin in brown trout, *Salmo trutta* L. *General and Comparative Endocrinology* **59**, 257-265.

Sundström LF, Petersson E, Höjesjö J, Johnsson JI, Järvi T (2004) Hatchery selection promotes boldness in newly hatched brown trout (*Salmo trutta*): Implications for dominance. *Behavioral Ecology* **15**, 192-198.

Sutterby R, Greenhaulgh M, Farrell S (1990) *The wild trout: The natural history of an endangered fish*, 1st edn. Philip's, Exeter.

Szulkin M, Bierne N, David P (2010) Heterozygosity-fitness correlations: A time for reappraisal. *Evolution* **64**, 1202-1217.

Tachibana T, Matsuda K, Khan MSI, Ueda H, Cline MA (2010) Feeding and drinking response following central administration of neuromedin S in chicks. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology* **157**, 63-67.

Tanck MWT, Vermeulen KJ, Bovenhuis H, Komen H (2001) Heredity of stress-related cortisol response in androgenetic common carp (*Cyprinus carpio L.*). *Aquaculture* **199**, 283-294.

Tang K, Thornton KR, Stoneking M (2007) A new approach for using genome scans to detect recent positive selection in the human genome. *PLoS Biol* **5**, e171.

Tang SJ, Sun KH, Sun GH, *et al.* (1999) Cold-induced ependymin expression in zebrafish and carp brain: implications for cold acclimation. *FEBS Letters* **459**, 95-99.

Tatara CP, Riley SC, Scheurer JA (2008) Environmental enrichment in steelhead (*Oncorhynchus mykiss*) hatcheries: field evaluation of aggression, foraging, and territoriality in natural and hatchery fry. *Canadian Journal of Fisheries and Aquatic Sciences* **65**, 744-753.

Teshima KM, Coop G, Przeworski M (2006) How reliable are empirical genomic scans for selective sweeps? *Genome Research* **16**, 702-712.

The Gene Ontology Consortium (2000) Gene Ontology: tool for the unification biology. *Nature Genetics* **25**, 25-29.

Thomson JS, Watts PC, Pottinger TG, Sneddon LU (2011) Physiological and genetic correlates of boldness: Characterising the mechanisms of behavioural variation in rainbow trout, *Oncorhynchus mykiss*. *Hormones and Behavior* **59**, 67-74.

Thomson JS, Watts PC, Pottinger TG, Sneddon LU (2012) Plasticity of boldness in rainbow trout, *Oncorhynchus mykiss*: do hunger and predation influence risk-taking behaviour? *Hormones and Behavior* **61**, 750-757.

Tiira K, Laurila A, Enberg K, *et al.* (2006) Do dominants have higher heterozygosity? Social status and genetic variation in brown trout, *Salmo trutta*. *Behavioral Ecology and Sociobiology* **59**, 657-665.

Tiira K, Laurila A, Peuhkuri N, *et al.* (2003) Aggressiveness is associated with genetic diversity in landlocked salmon (*Salmo salar*). *Molecular Ecology* **12**, 2399-2407.

Tinbergen N (1951) *The study of instinct* Oxford University Press, New York.

Toth AL, Varala K, Newman TC, *et al.* (2007) Wasp gene expression supports an evolutionary link between maternal behavior and eusociality. *Science* **318**, 441-444.

Tschirren B, Bensch S (2010) Genetics of personalities: no simple answers for complex traits. *Molecular Ecology* **19**, 624-626.
Vallejo RL, Rexroad CE, III, Silverstein JT, Janss LLG, Weber GM (2009) Evidence of major genes affecting stress response in rainbow trout using Bayesian methods of complex segregation analysis. *J. Anim Sci.* **87**, 3490-3505.
van't Hof AE, Saccheri IJ (2010) Industrial melanism in the peppered moth is not associated with genetic variation in canonical melanisation gene candidates. *PloS one* **5**, e10889.
Van Den Berg L, Vos-Loohuis M, Schilder MBH, *et al.* (2008) Evaluation of the serotonergic genes htr1A, htr1B, htr2A, and slc6A4 in aggressive behavior of Golden Retriever dogs. *Behavior Genetics* **38**, 55-66.
van der Staay FJ, de Groot J, Schuurman T, Korte SM (2008) Repeated social defeat in female pigs does not induce neuroendocrine symptoms of depression, but behavioral adaptation. *Physiology & Behavior* **93**, 453-460.
van Leeuwen TE, Rosenfeld JS, Richards JG (2011) Failure of physiological metrics to predict dominance in juvenile pacific salmon (Oncorhynchus spp.): Habitat effects on the allometry of growth in dominance hierarchies. *Canadian Journal of Fisheries and Aquatic Sciences* **68**, 1811-1818.
Väsemagi A, Nilsson J, Primmer CR (2005) Seventy-five EST-linked Atlantic salmon microsatellite markers and their cross-amplification in five salmonid species. *molecular ecology notes* **5**, 282-288.
Vaz-Serrano J, Ruiz-Gomez ML, Gjøen HM, *et al.* (2011) Consistent boldness behaviour in early emerging fry of domesticated Atlantic salmon (*Salmo salar*): decoupling of behavioural and physiological traits of the proactive stress coping style. *Physiology and Behavior* **103**, 359-364.
Veenema AH, Meijer OC, de Kloet ER, Koolhaas JM, Bohus BG (2003) Differences in basal and stress-induced HPA regulation of wild house mice selected for high and low aggression. *Hormones and Behavior* **43**, 197-204.
Veissier I, Boissy A (2007) Stress and welfare: Two complementary concepts that are intrinsically related to the animal's point of view. *Physiology & Behavior* **92**, 429-433.
Verbeek MEM, Boon A, Drent PJ (1996) Exploration, aggressive behaviour and dominance in pair-wise confrontations of juvenile male great tits. *Behaviour* **133**, 945-963.
Vilhunen S, Tiira K, Laurila A, Hirvonen H (2008) The bold and the variable: Fish with high heterozygosity act recklessly in the vicinity of predators. *Ethology* **114**, 7-15.
Vornanen M, Hassinen M, Koskinen H, Krasnov A (2005) Steady-state effects of temperature acclimation on the transcriptome of the rainbow trout heart. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **289**, R1177-1184.
Wacker DW, Ludwig M (2012) Vasopressin, oxytocin, and social odor recognition. *Hormones and Behavior* **61**, 259-265.
Wall JD, Andolfatto P, Przeworski M (2002) Testing models of selection and demography in *Drosophila simulans*. *Genetics* **162**, 203-216.
Wang JW, Xu SW (2008) Effects of cold stress on the messenger ribonucleic acid levels of corticotrophin-releasing hormone and thyrotropin-releasing hormone in hypothalami of broilers. *Poultry Science* **87**, 973-978.
Weir BS, Cockerham CC (1973) Mixed self and random mating at two loci. *Genetical Research* **21**, 247-262.
Wen Y, Liu Y, Xu Y, *et al.* (2009) Loss-of-function mutations of an inhibitory upstream ORF in the human hairless transcript cause Marie Unna hereditary hypotrichosis. *Nature Genetics* **41**, 228-233.
Wendelaar-Bonga SE (1997) The stress response in fish. *Physiological Reviews* **77**, 591-625.
Weninger SC, Dunn AJ, Muglia LJ, *et al.* (1999) Stress-induced behaviors require the corticotropin-releasing hormone (CRH) receptor, but not CRH. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 8283-8288.

Wetzel DP, Stewart IRK, Westneat DF (2012) Heterozygosity predicts clutch and egg size but not plasticity in a house sparrow population with no evidence of inbreeding. *Molecular Ecology* **21**, 406-420.

Wicker T, Schlagenhauf E, Graner A, *et al.* (2006) 454 sequencing put to the test using the complex genome of barley. *BMC Genomics* **7**, 275.

Wilhelm BT, Landry JR (2009) RNA-Seq-quantitative measurement of expression through massively parallel RNA-sequencing. *Methods* **48**, 249-257.

Wilson ADM, Godin JGJ, Ward AJW (2010) Boldness and reproductive fitness correlates in the eastern mosquitofish, *Gambusia holbrooki*. *Ethology* **116**, 96-104.

Wilson DS, Clark AB, Coleman K, Dearstyne T (1994) Shyness and boldness in humans and other animals. *Trends in Ecology & Evolution* **9**, 442-446.

Wilson DS, Coleman K, Clark AB, Biederman L (1993) Shy-bold continuum in pumpkinseed sunfish (*Lepomis gibbosus*): An ecological study of a psychological trait. *Journal of Comparative Psychology* **107**, 250-260.

Winberg S, Lepage O (1998) Elevation of brain 5-HT activity, POMC expression, and plasma cortisol in socially subordinate rainbow trout. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **274**, R645-R654.

Wiseman S, Osachoff H, Bassett E, *et al.* (2007) Gene expression pattern in the liver during recovery from an acute stressor in rainbow trout. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics* **2**, 234-244.

Wolf M, Van Doorn GS, Leimar O, Weissing FJ (2007) Life-history trade-offs favour the evolution of animal personalities. *Nature* **447**, 581-584.

Yada T, Azuma T, Hyodo S, *et al.* (2007) Differential expression of corticosteroid receptor genes in rainbow trout (*Oncorhynchus mykiss*) immune system in response to acute stress. *Canadian Journal of Fisheries and Aquatic Sciences* **64**, 1382-1389.

Yeo JS, Kim JW, Chang TK, Park YA, Nam DH (2000) Utilization of DNA marker-assisted selection in korean native animals. *Biotechnology and Bioprocess Engineering* **5**, 71-78.

Young W, Wheeler P, Coryell V, Keim P, Thorgaard G (1998) A detailed linkage map of rainbow trout produced using doubled haploids. *Genetics* **148**, 839 - 850.

Yulis CR, Lederis K (1987) Co-localization of the immunoreactivities of corticotropin-releasing factor and arginine vasotocin in the brain and pituitary system of the teleost *Catostomus commersoni*. *Cell and Tissue Research* **247**, 267-273.

Zimmerman A, Evenhuis J, Thorgaard G, Ristow S (2004) A single major chromosomal region controls natural killer cell-like activity in rainbow trout. *Immunogenetics* **55**, 825 - 835.

Zuberi A, Ali S, Brown C (2011) A non-invasive assay for monitoring stress responses: A comparison between wild and captive-reared rainbowfish (*Melanoteania duboulayi*). *Aquaculture* **321**, 267-272.