GENETIC ALALYSIS OF CORTISOL RESPONSE IN A WILD X DOMESTIC RAINBOW TROUT CROSS

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GENETIC ALALYSIS OF CORTISOL

RESPONSE IN A WILD X DOMESTIC

RAINBOW TROUT CROSS

Abstract

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Stressors in aquaculture can interfere with efficient fish production. Chronic stress decreases immune response, inhibits growth and negatively affects reproduction through various physiological pathways. It has been shown that stress response can be under strong genetic control, indicating the possibility for vast improvements in aquaculture broodstocks through breeding programs. Doubled haploids were created in a cross of our highly domestic Arlee (AR) and wild Whale Rock (WR) clonal lines. After 6 weeks on exogenous feed, seventy six doubled haploid progeny were individually exposed to a handling stressor and whole body cortisol levels were measured at 30 min. after termination of the stressor. Fish showing high cortisol response exhibited decreased body length (r^2=0.131, p=0.001) and mass (r^2=0.099, p=0.005). A total of 453 polymorphic AFLP markers were used to create a genetic linkage map revealing 32 rainbow trout linkage groups at a marker density of one marker every 8.0cM. Composite interval mapping revealed 5 significant QTL, two for whole body cortisol levels, two for body length, and one for body mass. QTL for increased cortisol and short body length associated with the WR allele mapped to the same location on linkage group WA-XIV. A second cortisol QTL identified on the rainbow trout sex chromosome (WA-I) mapped

in close association to the gene coding for carbonyl reductase, the analogue of a 20beta-hydroxysteroid dehydrogenase in rainbow trout, which is known to contribute to cortisol clearance. The identification of these regions influencing cortisol response may lead to mapping of stress-related genes, or marker assisted selection leading to a reduction in stress response and its associated negative effects.

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CHAPTER 1

GENERAL INTRODUCTION

Animals have developed a number of mechanisms to maintain homeostasis and defend against unfavorable environmental conditions. In natural environments, response to acute stressors is advantageous; however chronic stress can have detrimental effects. The effects and metabolic regulation of stress have been the subject of study in many domestic species as the transition from natural environments to those of intensive animal culture often results in a chronically-elicited stress response. Long term captive rearing exposes populations to natural and artificial selection that results in physiological, behavioral and genetic changes (Price, 1999). These changes often include reduction in stress response, which has effects on important survival, growth and reproductive processes (Wendelaar Bonga, 1997). The study of stress physiology and its genetic control may lead to better understanding of the domestication process and, through marker-assisted breeding, production of fish better suited for captive environments. Conversely, fish intended for release or supplementation of wild populations may benefit by retaining natural levels of stress responsiveness. Understanding the genetics of stress response may lead to accelerated domestication for aquaculture, and domestication prevention for conservation as well as an overall understanding of the genetic control of stress.

Stress response has three main levels: perception of a stressor, response, and the effects of the response (Moberg, 1985). The perception of stress is recognition of anything chemical, physical or perceived that elicits a stress response.

In the wild, stressors can be invasion of the territory by a conspecific, competition for food, or predators. The stressors in aquaculture setting include constant crowding, handling, exposure to humans, poor water quality, and confinement.

The response itself has been further broken down into primary and secondary responses, which in turn have effects on the whole organism. Like many other terrestrial vertebrates the physiological response to a stressor in fish involves two different pathways. The first is the rapid release and effects of catecholamines by the sympathetic pathways, and the second is the release and effects of corticosteroids including cortisol. Rather than having an adrenal medulla, teleost fish possess chromaffin cells located in the head kidney. When a stressor is perceived by the brain hypothalamus, sympathetic nerve fibers stimulate the chromaffin cells to produce and release catecholamines including epinephrine and norepinephrine (Wendelaar Bonga, 1997). These catecholamines are identified with the fight or flight response in vertebrates, which includes increases in ventilation and oxygen uptake, blood oxygen transport, and blood glucose levels (Wendelaar Bonga, 1997). Heart rate in rainbow trout is controlled by adrenergic and cholinergic stimuli. Therefore increased circulation of epinephrine produces increased heart rate (Wendelaar Bonga, 1997). It has also been shown that epinephrine increased Na^+/H^+ exchange and decreases Cl^-/HCO_3^- exchange across the membrane of erythrocytes, thus causing changes in the pH and hemoglobin affinity for oxygen. Both epinephrine and norepinephrine lead to increased blood glucose. It is thought that they stimulate glycogen phosphorylase which induces glycogenolysis in the liver, in turn causing increased blood glucose levels (Wendelaar Bonga, 1997). Although no

conclusive evidence has been found, it has been suggested that catecholamines also play a role in increased blood free fatty acid levels.

Corticosteroids, the second highly influential stress hormones are regulated via the Hypothalamic-pituitary-interrenal axis. When a stressor is perceived the hypothalamus releases a complex mixture of hormones and neurotransmitters into the blood including but not limited to corticotrophin releasing hormone (CRH), dopamine, neuropeptide Y, and arginine vasotocin and isotocin, which all have a stimulatory effect on the pituitary gland. Once stimulated, the pituitary gland releases adrenocorticotropic hormone (ACTH), melanophore-stimulating hormone (MSH) and β-endorphin (β-END) into the blood. These then stimulate the interrenal cells to produce cortisol. Once in circulation, cortisol acts as a negative feedback directly on the interrenal cells, and also on the pituitary gland and hypothalamus (Wendelaar Bonga, 1997).

Elevated blood levels of corticosteroids then cause secondary responses. Components of the secondary response include alterations of metabolic, osmoregulatory and hematological functions and changes in growth. As previously mentioned a change occurs from energy storage (anabolic state) to energy utilization (catabolic state). Elevated cortisol levels have been shown to increase levels of all key enzymes controlling gluconeogenesis (6-phosphatase, fructose1,6-bisphosphates and PEPCK) (Mommsen *et al.*, 1999). In the liver, these enzymes promote gluconeogenesis which results in increased blood glucose levels.

Cortisol also has osmoregulatory effects as it is influential in adaptation from freshwater to salt water in salmonids. This is thought to occur by expression of the α-

subunit mRNA of Na+/K+-ATPase, causing increased Na+/K+-ATPase function in the gills (Mommsen *et al.*, 1999).

During times of stress, energy is diverted from growth and reproduction and made available for processes leading to restoration of homeostasis. The effect of chronically elicited stress on rainbow trout has been the topic of considerable study (Barton, Iwama, 1991; Jentoft *et al.*, 2005; Pickering, 1992; Van Weerd, Komen, 1998; Weil *et al.*, 2001). Decreased growth has been associate with both increased longevity of (Weil *et al.*, 2001), and peak (Jentoft *et al.*, 2005) cortisol levels. Likewise, increased cortisol levels are commonly associated with decreased reproductive success (Barton, Iwama, 1991) and offspring quality(Schreck *et al.*, 2001). Among aquaculture species elevated stress levels have also been shown to repress immune function (Davis *et al.*, 2003; Maule *et al.*, 1989) and increased disease and mortality (Pickering, Pottinger, 1989).

Many factors involved with stress response are under genetic control (Barton, 2002), and have a significant heritable component (Fevolden *et al.*, 1999; Pottinger, Carrick, 1999; Pottinger *et al.*, 1992). Quantitative trait locus (QTL) analysis is a powerful way of detecting genomic regions influencing heritable traits. Complex traits such as stress response are under the control of many different genetic loci. QTL analysis provides a way to identify the number, and effect of loci controlling various traits. In some instances, QTL detection can lead to identification of specific genes controlling trait variation (Oliver *et al.*, 2005; Van Laere *et al.*, 2003) however this process is complex (Glazier *et al.*, 2002). Alternatively, identification of specific nucleotides can be associated with trait variances can be identified (Grisart *et al.*, 2004; Ron, Weller, 2007) and applied to marker-assisted selection.

Behavioral and phenotypic variations between wild and domestic salmonids populations have been identified. Captive-bred populations have been shown to exhibit reduced predator avoidance (Johnsson *et al.*, 1996; Yamamoto, Reinhardt, 2003), increased aggression and dominance (Metcalfe *et al.*, 2003) and less time under cover (Fleming, Einum, 1997) than their wild counterparts. Domesticated salmonids also tend to show reduced stress responsiveness to handling and crowding (Mazur, Iwama, 1993; Shrimpton *et al.*, 1994; Woodward, Strange, 1987).

The doubled haploid design is more powerful for detecting QTL than a backcross design (Carbonell *et al.*, 1993). Clonal rainbow trout produced by androgenesis and gynogenesis provide the foundation for producing doubled haploids form QTL analysis (Parsons, Thorgaard, 1984). As described above, numerous behavioral and physiological differences have been seen between wild and domestic salmonids. In this study, QTL analysis is applied to a doubled haploid population derived from wild (WR) x domestic (AR) hybrid rainbow trout. QTL identified for post stress cortisol levels will be evaluated for co-localization with body length and body mass. This will allow testing for genetic linkage between stress response and growth.

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CHAPTER TWO

GENETIC ANALYSIS OF CORTISOL RESPONSIVENESS IN A WILD X DOMESTIC RAINBOW TROUT CROSS

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Abstract

Stressors in aquaculture can interfere with efficient fish production. Chronic stress decreases immune responses, inhibits growth and negatively affects reproduction through various physiological pathways. It has been shown that the hormonal stress response can be under strong genetic control, indicating the possibility for vast improvements in aquaculture broodstocks through breeding programs. Doubled haploids were created in a cross of our wild Whale Rock (WR) and highly domesticated Arlee (AR) clonal lines. After 6 weeks on commercial starter feed, seventy six doubled haploid progeny were individually exposed to handling stress and whole body cortisol levels were measured at 30 min. post onset of the stressor. Fish showing high cortisol response exhibited decreased body length (r^2=0.131, p=0.001) and mass (r^2=0.099, p=0.005). A total of 453 polymorphic AFLP markers were used to create a genetic linkage map revealing 32 rainbow trout linkage groups at a marker density of one marker every 8.0cM. Composite interval mapping revealed 5 significant quantitative trait loci (QTL), two for whole body cortisol concentrations, two for body length, and one for body mass. QTL for increased body cortisol and short body length associated with the WR allele mapped to the same location on linkage group WA-XIV. A second cortisol QTL identified on the rainbow trout sex chromosome (WA-I) mapped in close association to the gene coding for carbonyl reductase, and enzyme analogous to 20beta-hydroxysteroid dehydrogenase in rainbow trout, which is known to contribute to cortisol clearance in the liver. The identification of these regions influencing cortisol body concentrations may lead to mapping of stress-related genes, or marker assisted selection leading to a reduction in hormonal stress responsiveness and its associated negative effects.

1. Introduction:

In aquaculture, chronic stress plays a significant role in animal health and overall yield (Davis, 2006). Minimizing stress through various methods can potentially be extremely beneficial to the efficiency of fish production as it has been with many domesticated species. Environments differ dramatically between native and farm environments for example with regard to density, competition for food, and disease exposure. The process of domestication produces animals that are best adapted for survival in human-controlled environments.

The endocrine stress response in fish is controlled through the Hypothalamic-pituitary-interrenal (HPI) axis. Following perception of a stressor the hypothalamus releases corticotropin releasing hormone (CRH) which stimulates the release of melanophore-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) from the pituitary gland (reviewed by Wendelaar Bonga, 1997). ATCH and α-MSH stimulate the interrenal cells in the head kidney to synthesize and release cortisol into the bloodstream to act on a number of osmoregulatory, hematological and metabolic functions that serve to restore the fish to a pre-stress homeostatic state (Wendelaar Bonga, 1997). This is primarily achieved by diverting energy investment from growth and reproduction to activities such as locomotion, respiration, and tissue repair (Wendelaar Bonga, 1997). During short term acute stress, diversion of energy from growth and reproduction can be beneficial to fish survival. Chronic stress, on the other hand, such as those experienced in aquaculture settings can prolong cortisol secretion resulting in negative effects on fish health. Because of its consistent post stress presence

in the bloodstream cortisol is generally considered the a good indicator of the stress response due to its (Barton, 2002).

In an aquaculture setting, fish experience chronic stressors including crowding, handling and human interaction (Davis *et al.*, 1993), exposure to poor water quality and toxins (Schlenk *et al.*, 1999), aggression from conspecifics (Mommsen *et al.*, 1999) and competition for food. Although there are conflicting data about the relationship of stress and various measures of fish performance (Davis, 2006), studies reveal a negative correlation between cortisol levels and growth (Jentoft *et al.*, 2005; Weil *et al.*, 2001), immune responses and disease resistance (Davis *et al.*, 2003; Maule *et al.*, 1989) and reproduction (Schreck *et al.*, 2001).

The process of domestication has produced animals that are better suited for rearing and breeding in captive environments. Among the most profound changes in wild versus domesticated animals are an increased threshold for behavioral and physiological responses to environmental challenges (Price, 1999). One of the major physiological changes from wild to domestic animals is a reduction in the endocrine stress response. Reduced stress response of domesticated vs. wild animals has been seen in a number of species including salmonids fishes(Mazur, Iwama, 1993; Shrimpton *et al.*, 1994), mice (Treiman, Levine, 1969) and guinea pigs (Kunzl, Sachser, 1999). In these and other species, genetics manipulation has been associated with the reduction of stress response. Observed variations in stress response have been shown to have a genetic component in many species including rainbow trout (Drew *et al.*, 2007), pigs (Desautes *et al.*, 2002), cattle (Gauly *et al.*, 2002) and chickens (Buitenhuis *et al.*, 2003). Variations in chicken

egg corticosterone concentrations have also been shown to influence various behaviors in offspring, indicating a non-genetic effect of corticosterone (Janczak *et al.*, 2007).

In rainbow trout, variation in stress responsiveness has been the subject of much study by several research groups. In general it has been shown that stress responsiveness has a heritable component (Fevolden *et al.*, 1999; Pottinger, Carrick, 1999) and can be altered by selective breeding (Pottinger, Carrick, 1999). This lays the foundation for potential improvements in broodstock through advanced selective breeding procedures supplemented by genetic understanding of the endocrine system. Marker-assisted selection has the potential to increase efficiency of fish production by allowing selection based entirely upon genotype rather than waiting for grow out and measurement of phenotypic characteristics such as cortisol body concentrations. Genetic understanding of the stress responsiveness may also be beneficial for raising fish intended for release into the wild. In this case, retention of an appropriate stress response may increase chances of survival in the natural environment thereby increasing the impacts of hatchery conservation efforts.

Our lab has shown considerable differences in body cortisol stress responsiveness among several clonal rainbow trout lines (Drew *et al.*, 2007). Although direct correlation between domestication and a reduced stress response was not found, these lines offer promising study system for identifying genetic loci affecting the stress response and other quantitative traits. The clonal lines represent genetically identical individuals created through two generations of either gynogenesis or androgenesis. The clonal lines encompass a broad geographic distribution, large variation in domestication, and variation in behavioral characteristics (Lucas *et al.*, 2004), development rate (Nichols *et*

al., 2007; Robison *et al.*, 1999) stress response (Drew *et al.*, 2007), disease resistance (Nichols *et al.*, 2003a) and various meristic traits (Nichols *et al.*, 2004; Zimmerman *et al.*, 2005). QTL analysis has revealed loci influencing development rate (Robison *et al.*, 2001), meristic traits (Nichols *et al.*, 2004), immune function (Zimmerman *et al.*, 2004) and the stress response (Drew *et al.*, 2007).

In this paper we employ methods previously utilized to quantify the interrenal stress response differences in a doubled haploid cross. The wild Whale Rock (WR) female clonal line from central California was crossed with the highly domesticated Arlee line to produce hybrids. The hybrids were then used to produce doubled haploids by androgenesis. The domestic AR line has previously been shown to have a reduced stress response (Drew *et al.*, 2007), therefore our hypothesis is that the WR x AR doubled haploids will show large variations in individual stress responses that will reveal QTL influencing the trait. Comparisons of these QTL can then be made with those found to influence the stress response in the OSUxAR cross (Drew *et al.*, 2007).

2. MATERIALS AND METHODS

2.1 Parental lines

Two clonal rainbow trout lines were used to create doubled haploid individuals. In 2000, the milt of wild rainbow trout (landlocked steelhead) from the Whale Rock Reservoir in central California was collected and used to create an androgenetic population. From the resulting offspring a female was selected and eggs were fertilized with UV-irradiated sperm and diploidy was restored by heat shock treatment to create a homozygous XX female line. In order to maintain wild characteristics and try to prevent domestication, the WR line has been kept in hatchery environment that imitates a natural

stream environment and had minimal human interaction. It is predicted that these fish maintain much of their natural physiological and behavioral adaptations as clones were produced from gametes of wild fish. The Arlee (AR) is a clonal (YY) line derived from the Arlee Hatchery in Montana. Initial propagation of this line is thought to have originated from wild rainbow trout populations of the McCloud River (California) over 100 years ago. Studies done previously with the AR clonal line revealed comparatively low cortisol levels in response to stressors and other behavioral characteristics observed in highly domesticated fishes (Drew *et al.*, 2007; Lucas *et al.*, 2004).

2.2 Mapping population

Clonal WR and AR individuals described above were spawned to create hybrid offspring. Upon sexual maturity, a hybrid male was used to produce doubled haploids by the process of androgenesis (Parsons, Thorgaard, 1984). Eggs were obtained from a single outbred hatchery female (Trout Lodge, Inc., Summer, WA) and divided into three groups. Each group was then exposed to γ-radiation to destroy maternal DNA and then fertilized with sperm from a WR x AR hybrid male. Heat shock treatment was used to block cell cleavage and restore the gametes to diploidy (Young *et al.*, 1998). This process produced doubled haploids, homozygous at all loci. Incubation of fertilized eggs occurred in a 10°C cold room using a Heath-Style incubator. Fish were disturbed as little as possible during development to minimize disease contamination and developmental disruptions. Upon hatch and absorption of the yolk sac, fish were transferred to a single 19L tank (30-cm diameter) in an indoor recirculation style hatchery and given commercial starter feed.

2.3 Stress treatment

Stress treatments took place 6 weeks after the fish were transferred to commercial feed and occurred on 6 consecutive days between July 12-17 2006. Treatment was done in a 10°C cold room fitted with 15 individual compartments. Compartments were visually isolated from one another and the experimenter by dividing walls and dark curtains to minimize stress response induced by unwanted visual disturbances. Compartments contained a plastic aquarium (26.5cm L x 16.0cm W x 17cm H) painted white on three sides to further minimize visual stimulation, an air supply and an incandescent light source. Each tank was lined with a clear plastic bag that was removed after each test to minimize carryover of anesthetics or other chemicals from one trial to the next. The day before the scheduled testing, between 1400 and 1530 hours, fish were transferred from the hatchery tank to the cold room. Single fish were randomly assigned to a tank and allowed to acclimate overnight with the same photoperiod as experienced in the hatchery. Stress treatments took place the following morning between 900 and 1200 hours. The time of the stress event was randomly assigned to each compartment. At the assigned time the dividing curtain was lifted and the fish was caught in a small mesh net, held above the water for 30 seconds and returned to the water. Previous studies revealed that body cortisol levels in response to this stressor was greatest after 30 minutes; therefore all fish were given 30 minutes to recover before collection (Drew *et al.*, 2007).

To minimize further induction of stress, anesthesia was delivered remotely via thin tubes from behind the dividing curtain, a method shown to have minimal effect on cortisol levels (Gerwick *et al.*, 1999). Twenty five ml of tricaine methanosulfate (MS222) in a concentration of 44mg/ml was injected into the aquarium for a final concentration of 200mg/L, the manufactures suggested concentration for rapid

anesthesia. After injection of MS222, the line was immediately flushed with 30mL of water to ensure all the anesthetic was flushed into the tank. Once the fish stopped all movement (usually within 1-2 min) they were netted, measured for length, flash frozen in liquid nitrogen and then stored at -20°C until cortisol extraction. The water was then dumped, the plastic bag from each aquarium was removed and replaced with a fresh one. Air stones were rinsed clean of anesthesia and tanks were refilled with de-chlorinated water for the next stress treatment.

2.4 Cortisol extraction and assay

The procedure for cortisol extraction was modified by Drew et al. (2007) from that described by Hiroi (Hiroi *et al.*, 1997). Before cortisol extraction, wet body mass was measured and the caudal and dorsal fins were removed and stored for DNA extraction. Fish were then diced, placed in glass culture tubes (12 x 75mm) and homogenized in 5 volumes (w/v) PBS. Homogenization was done using a Tissue-Tearor homogenizer (Biospec Products, Bartlesville, OK) on the highest setting for approximately 1 minute.

Samples were vortexed for 30 sec and 300ul of the homogenate was transferred to a 16 x 150 mm glass tube. Steroids were extracted twice with 3mL of diethyl ether and combined extracts were then evaporated in a 37°C water bath until dry. Lipids were removed from the samples with 300ul of tetrachloromethane. Samples were vortexed for 4 min and 500ul of assay buffer was added before vortexing another 2 min. Samples were then centrifuged at 4°C and 3000rpm for 10 min and transferred to a clean glass test tube. A 1:10 dilution was made and cortisol concentration was assayed with the Cortisol Enzyme Immunoassay kit (Cayman Chemical Co., Ann Arbor, MI) according to the provided protocol. Each sample was assayed in triplicate to control for inter-assay

variability. For control, 100pg/ml cortisol was added to selected samples and extraction efficiency was calculated to be 92.6%.

2.5 Genotyping

The fin clips removed before homogenization provided tissue for DNA extractions. Extraction of genomic DNA was done according to manufacturer's protocol for Purgene Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN). Amplified fragment length polymorphism (AFLP) method was used for genotyping of doubled haploid individuals (Vos *et al.*, 1995). The protocol summarized here was adapted by Robison et al for linkage mapping (Robison *et al.*, 2001). A total of 500ug of DNA was digested with EcoR1 and MSE1 restriction enzymes and adapters were ligated onto the produced sticky ends. Primers recognizing the adapters, restriction site and one specified nucleotide were used for selective +1 amplification. Selective +3 amplifications were then done with a Cy-5 labeled primer recognizing EcoR1, and an unlabeled MSE1 primer. The PCR product was run on a 6% acrylamide gel and scanned with a Typhoon 9400 imager (Amerisham Biosciences, Piscataway, NJ). Polymorphic markers were named beginning with "E" for Eco followed by the 3 nucleotide recognition sequence of the +3 primer for Eco first then MSE. The number following the primer sequence represents size from smallest (01) to largest. Markers were also designated "w" or "a" indicating WR or AR markers. Markers matched to previously published maps (Nichols *et al.*, 2003b) were given the same name as previously published. Microsatellite markers (OMM 1026, OMM 1046, OMM1048 and OMM1131) were used to confirm linkage group correspondence between maps. In total, 53 AFLP and 4 microsatellite primer combinations provided marker data for mapping.

2.6 Linkage analysis

Linkage analysis was done using MAPMAKER/EXP version 3.0b (Dr. Scott Tingey, Dupont Experimental Station, Wilmington, DE, U.S.A). Markers that were scored for less than 70% of individuals were removed from the analysis and remaining markers were tested for linkage disequilibrium using χ^2 analysis with Yates correction for continuity. The genotypes of markers showing deviation from Mendelian segregation were checked for errors and if found to be unreliable were removed from further analysis. Markers remaining in the analysis showing greater than expected WR genotypes were labeled with ^ and those showing greater than expected AR genotypes were labeled with * at the end of the marker name. Markers having identical genotypes for all individuals were identified as clusters and the marker with the largest number of individuals genotyped was selected to represent the cluster. Markers were initially linked using Mapmaker's "group" command with a LOD of 3 and maximum distance of 37.2cm (Kosambi Map function). Large groups were further divided by increasing the LOD score to 5. Mapmaker's "compare" command was then used to determine initial marker order for 6 to 7 markers in each linkage group and subsequent markers were added using the "try" command. Genotypes identified as having high probability of error by Mapmaker's error detection were double checked. If the marker had been miss-scored, the appropriate corrections were made. Linkage group marker order was then tested using the "ripple" command to determine final order.

2.7 QTL Analysis

Composite interval mapping (CIM) was used to detect significance and position of QTL throughout the genome at intervals of 2.0cM (Basten *et al.*, 1994; Zeng, 1994).

Analysis was done with Windows QTL Cartographer Version 2.5 (Wang *et al.*, 2005). Background markers were added through stepwise linear regression with forward addition and backward elimination using model 6. To reduce over-parameterization of the model, the type I error rate was set at 0.05 for inclusion and 0.10 for elimination. The model retained the 5 most significant background markers outside a 10cM window. QTL significance thresholds for each trait were calculated by the permutation method with 1000 replications at the 5% significance level (Churchill, Doerge, 1994; Doerge, Churchill, 1996). Composite interval mapping was used to test for QTL associated with cortisol levels, body weight and body length. QTL were considered significant if they reached the calculated threshold with a significance level of 0.05.

3. Results

3.1 QTL analysis in doubled haploids:

Pearson correlation coefficient was positive for body mass and body length (0.943) and negative for cortisol and body length (-0.362) and mass (-0.312). Linear regression analysis also detected a negative relationship between cortisol levels and body length (r^2=0.131, F=11.3, p=0.001) and cortisol and body mass (r^2=0.099, F=8.29, p=0.005) (fig 3a,b).

Individual cortisol responses among doubled haploids varied greatly ranging from 4.5 to 94.4 ng/g with an average of 23.0ng/g (Fig 1). Body length and mass also showed significant variation. A ten fold difference was seen in body mass with an average of 0.45g and a minimum and maximum of .13 and 1.3g respectively. Overall body length ranged from 25 to 47mm with a mean of 33.4mm.

The 53 AFLP and 4 microsatellite primer sets produced 512 markers polymorphic between the parental lines. Of those, 457 were used to create a genetic linkage map. The remaining 54 markers were either removed from the analysis due to deviations from expected Mendelian segregation, inconsistencies in scoring or simply remained unlinked. Linkage mapping revealed 32 rainbow trout linkage groups covering a total of 1378.4cM with an average marker interval distance of 8.0cM. A microsatellite marker was placed on each of the 4 linkage groups having significant QTL in order to confirm correspondence with previously published maps.

CIM revealed two significant ($p < 0.05$) QTL for post-stress body cortisol levels (linkage groups WA-I, WA-XIV) (Fig 2 and Table 1). Together the two QTL identified for cortisol explained 23.9% of the overall variation in cortisol levels. Opposing additive effects were seen as the wild WR allele was associated with decreased cortisol levels at *cort-1*, and increased levels at *cort-2*. Linkage group WA-XIV also showed a significant body length QTL (*bodylength-1*) at the same locus at *cort-2*. This QTL together with *bodylength-2* found on WA-XXVII explained 26.8% of the body length variance in the doubled haploids. Each of the body length QTL has negative additive effect, meaning the WR allele was associated with shorter body length. A final QTL (bodymass-1) was found on WA-VI and explained 14.3% of the body weight variation.

4. Discussion

The WR x AR doubled haploid population showed a wide range in phenotypic variation for post-stress body cortisol concentrations (fig 1a). The highly domesticated AR parental line has been previously characterized as having a reduced stress response with an average cortisol concentration of approximately 10ng/g (Drew *et al.*, 2007). The

WR x AR doubled haploid population here had an average of 23.0ng/g likely indicating presence of high stress response in the wild WR parental line. Broad phenotypic distributions were also seen in body mass (fig 1b) and body length (fig 1c). A negative relationship between cortisol levels and growth has been previously reported (Jentoft *et al.*, 2005; Weil *et al.*, 2001). Linear regression analysis of the WR x AR doubled haploid population is consistent with those findings (Figure 3a,b). High post-stress cortisol levels were associated with decreased body length (r^2=0.131, p=0.001) and body mass (r^2=0.099, p=0.005). Pearson correlation analysis also revealed a negative correlation between cortisol and body length (-0.362) and mass (-0.312).

Domesticated rainbow trout and other salmonids commonly have reduced stress responses to crowding and handling compared to their wild counterparts (Mazur, Iwama, 1993; Shrimpton *et al.*, 1994; Woodward, Strange, 1987). Three QTL were identified for size traits, two for body length (*bodylength-1* and *2*) and one for body mass (*bodymass-1*). In all three cases the allele of the wild WR line showed association with smaller body size. Two significant QTL for post-stress cortisol levels were identified (Table 1). QTL *cort-2* on WA-XIV had a large positive additive effect, meaning the allele of the wild WR clone is associated with increased stress response. Strikingly the *cort-2* QTL associated with increased cortisol in WR and the *bodylength-1* QTL associated with decreased body length in WR mapped to the same location on WA-XIV (Figure 2 and Table 1). Each of these QTL explained approximately 14% of the respective trait variation (Table 1). This is not the first study to detect co-localization of cortisol and growth QTL in rainbow trout. Drew et al. identified QTL for both body mass and post-

stress log transformed cortisol levels on a common locus on rainbow trout linkage group XXVII, however the correlation between the two traits was positive (Drew *et al.*, 2007).

QTL for rainbow trout cortisol and growth-related traits have been identified in a number of studies (Drew, 2006; Drew *et al.*, 2007; Martyniuk *et al.*, 2003; O'Malley *et al.*, 2003; Perry *et al.*, 2005; Robison *et al.*, 2001). Many of the QTL identified fall on analogous linkage groups. Table 2 describes all QTL localized to the same linkage group as those in this study. This correspondence of growth-related and cortisol QTL localizing to common linkage groups among these studies is interesting. Exact QTL location cannot be determined due to lack of common markers. Traits such as body length, body mass and specific growth rate are undoubtedly under complex control of many genes. Therefore it is not unexpected to identify many QTL associated with these traits. The association of cortisol QTL with these traits suggests that stress response may be a contributing factor to growth variation. To our knowledge, the co-localization of stress and growth QTL having a negative correlation is among the first strong evidence supporting genetic linkage between stress and growth in rainbow trout. This result along with the correlations of QTL to common linkage groups described in table 2 contributes to the growing body of evidence for underlying genetic factors linked to both variation in body size and stress responsiveness.

The major objective of QTL studies is to identify regions of the genome influencing a trait (Mackay, 2001). Once identified, these regions can be searched for specific genes, single nucleotide polymorphisms, promoter sequences or other underlying genetic factors controlling phenotypic variation. Close marker association with such genetic differences can be applied in marker assisted selection (Thorgaard *et al.*, 2006).

In rainbow trout one study has reported localization of a candidate gene with QTL location, but these instances are rare (Leder *et al.*, 2006).

The peak LOD score for *cort-1* fell in very close proximity to AFLP marker Eaccagt121a (figure 2). In a previously published rainbow trout map Eaccagt121a mapped within 3.4cM of the carbonyl reductase (CBR1) gene, a distance well within the 1-LOD interval for *cort-1* (Nichols *et al.*, 2003b). In rainbow trout the carbonyl reductase enzyme has some analogous functions to 20beta-hydroxysteroid dehydrogenase. Although very little is known about factors involved with cortisol metabolism and clearance in fish, 20beta-hydroxysteroid dehydrogenase has been consistently identified with the process (Mommsen *et al.*, 1999; Truscott, 1979) and to date is the only gene known to be involved with the cortisol pathway that has been mapped in rainbow trout. Metabolism and degradation of cortisol removes it from circulation. Once removed from circulation and metabolized, cortisol can no longer have the detrimental effects discussed above. In fact, rainbow trout with faster metabolism (quicker reduction) of post stress cortisol levels have been shown to exhibit increased growth rate compared to those with slower cortisol clearance (Weil *et al.*, 2001). This makes the CBR1 gene a highly attractive candidate for pursuit in understanding the genetic control of stress response.

The *cort-1* QTL found to be in close association with CBR1 gene had a negative additive effect. This means that, contrary to prediction, the AR allele was associated with an increase in stress response. A precursor for selection is availability of alleles associated with variance in phenotype. We must remember that clonal populations represent only a single haploid genome of one individual from a population. If multiple

alleles still exist at the *cort-1* locus in the AR hatchery population, the individual used to create the clonal line may possess the allele for high stress response.

Like all complex traits, stress response is undoubtedly controlled by many contributing genetic loci. Therefore the probability of finding one or two loci with major effect on the traits may be low. Although the doubled haploid design is powerful for detecting QTL (Martinez *et al.*, 2002), the small sample size in this experiment may have limited the ability to detect QTL of low to moderate effect (Melchinger *et al.*, 1998).

5. Conclusion

Our results suggest a negative correlation between stress-induced cortisol levels and size in phenotypic and genetic analyses. These results are in accordance with a number of previously published works in rainbow trout (Jentoft *et al.*, 2005; Weil *et al.*, 2001). Genetic loci influencing on both the cortisol stress response and body size , as well as those associated with stress-related genes offer avenues for continued research. Traditional selective breeding methods or marker-assisted selective breeding may allow production of broodstocks with reduced stress response and increased growth. However this outcome is not certain. In one study, rainbow trout selectively bred for high stress response displayed higher body mass compared to low stress response fish (Pottinger, Carrick, 2000). The management of stress in aquaculture is complex and many inconsistencies have been reported for the association of stress and performance characteristics (for review see Davis 2006). Stress response and other important aquaculture characteristics have shown heritable components (Fevolden *et al.*, 1999; Pottinger, Carrick, 1999). Continued use of genetics as a tool for decreasing stress

response is likely to result in increased performance characteristics and thus benefit fish production (Davis, 2006).

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QTL	Linkage Group	Position[a]	LOD[b]	a[c]	r^{2d}
cort-1	WA-I (Eaccagt121a)	36.7	2.6	-5.151	0.099
bodymass-1	WA-VI (OMM1046) and cluster	11.0	3.0	-0.153	0.143
cort-2	WA-XIV (Eaccaca14a)	83.0	3.5	6.312	0.140
bodylength-1	WA-XIV (Eaccaca14a)	78.7	2.9	-2.044	0.146
bodylength-2	WA-XXVII (Eacgaga05w)	9.7	2.8	-1.869	0.122

Table 1 List of QTL identified in WR x AR doubled haploid rainbow trout at a significance level of $p<0.05$.

[a] Position is the QTL distance form the top of the linkage group in kosambi cM. Markers corresponding to QTL are listed in parentheses.

[b] LOD is the log of odds that the additive effect is significantly different from zero. Permutations were calculated with 1000 replications and QTL were significant ($P < 0.05$)

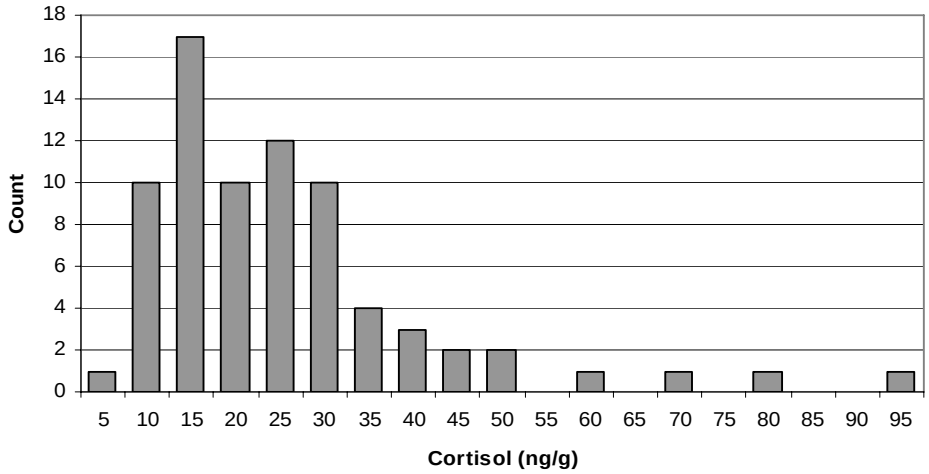
[c] Additive effect, or effect caused by presence of the WR allele at the locus.

[d] r^2 is the percentage of the total trait variance explained by the QTL.

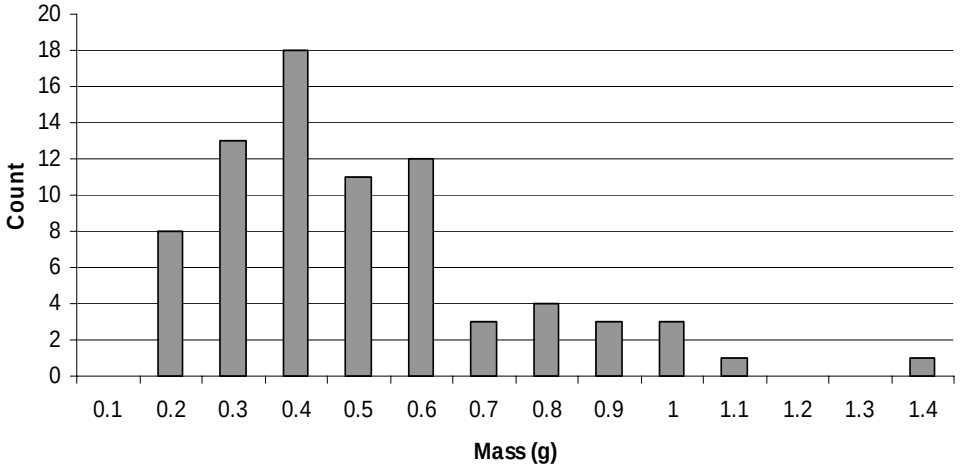
Rainbow trout linkage group	Linkage group name	Trait	Source
27	OA-XXVII	log cortisol	(Drew *et al.*, 2007)
	OA-XXVII	body mass	(Drew *et al.*, 2007)
	OS-27	body mass	(Drew, 2006)
	15	body mass	(O'Malley *et al.*, 2003)
	WA-XXVII	body length	this study
1	WA-I	cortisol	this study
	OS-1	specific growth rate[s]	(Drew, 2006)
6	WA-VI	body mass	this study
	S	body mass[s]	(O'Malley *et al.*, 2003)
14	WA-XVI	cortisol	this study
	WA-XVI	body length	this study
	OS-14	body mass	(Drew, 2006)
	OS-14	condition factor	(Drew, 2006)
	OS-14	specific growth rate	(Drew, 2006)

Table 2 Rainbow trout cortisol and growth QTL comparison among 4 independent studies. Linkage group symmetry for this study and O'Malley et al. 2003 was determined by microsatellite markers common to (Sakamoto *et al.*, 2000) or Nichols et al. 2003. Drew 2006 and Drew et al. 2007 confirmed linkage by matched AFLP markers to Nichols et al. 2003. Suggested QTL are followed labeled with [s], significant QTL are unlabeled.

a.


Count
18
16
14
12
10
8
6
4
2
0
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95
Cortisol (ng/g)

b.


Count
20
18
16
14
12
10
8
6
4
2
0
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1 1.1 1.2 1.3 1.4
Mass (g)

c.

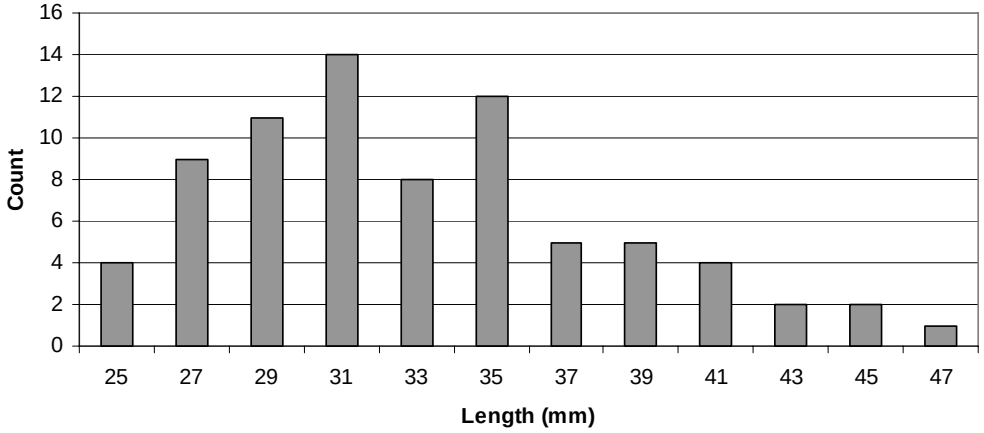

Count
16
14
12
10
8
6
4
2
0
25 27 29 31 33 35 37 39 41 43 45 47
Length (mm)

Figure 1 Histograms of (a) whole body cortisol levels (b) body mass and (c) body length WR x AR doubled haploids.

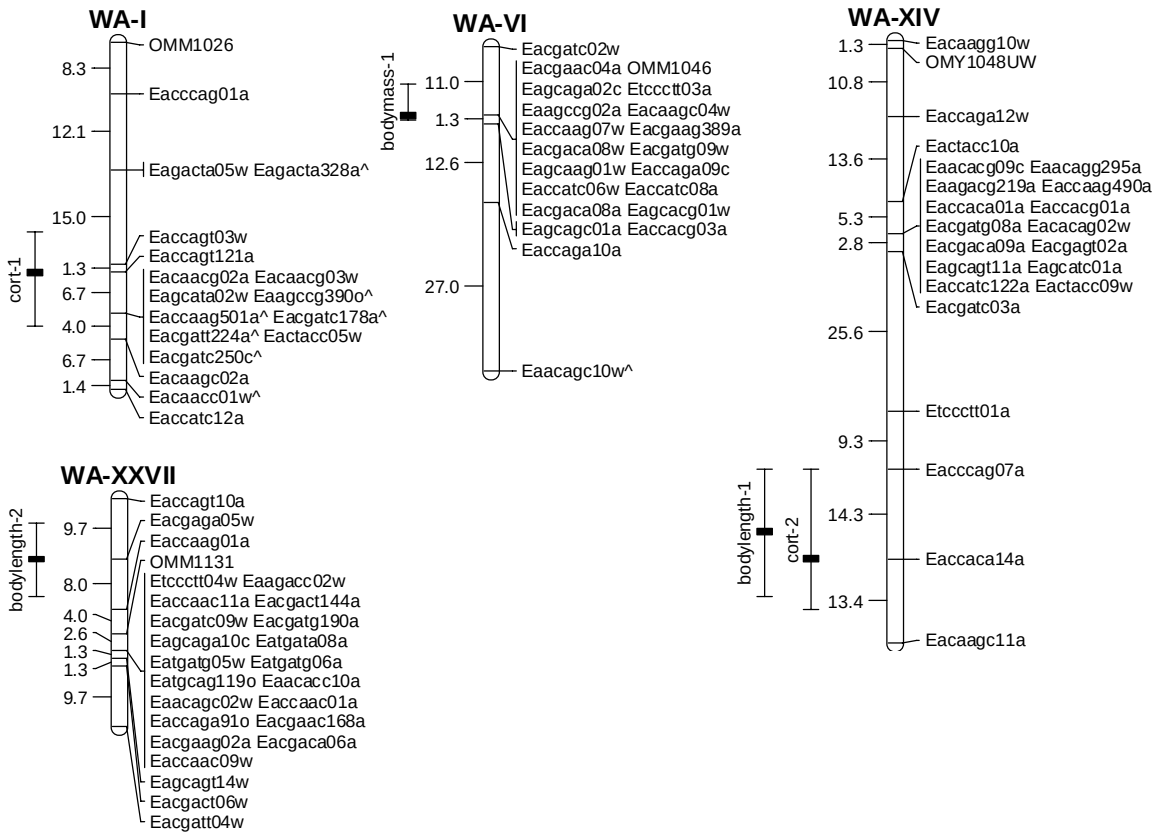
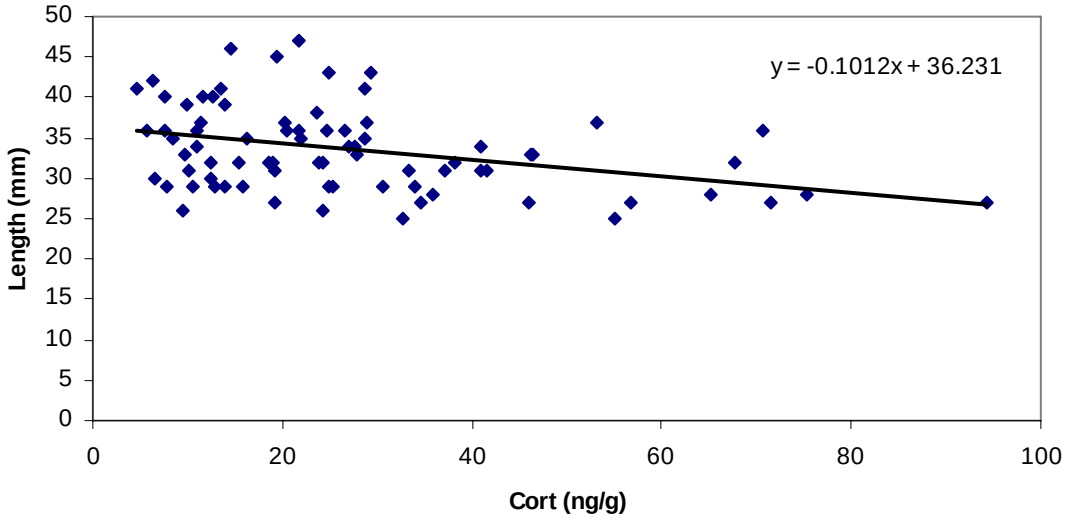


Figure 2 WR x AR linkage groups with significant QTL for cortisol, body weight and body length. Linkage group names correspond to those in the rainbow trout reference map (Nichols et al., 2003). Interval distances in (kosambi cM) are shown on the right of the linkage group along with QTL name and position (dark bars) and 1-LOD significance interval (wiskers).

a.



b.

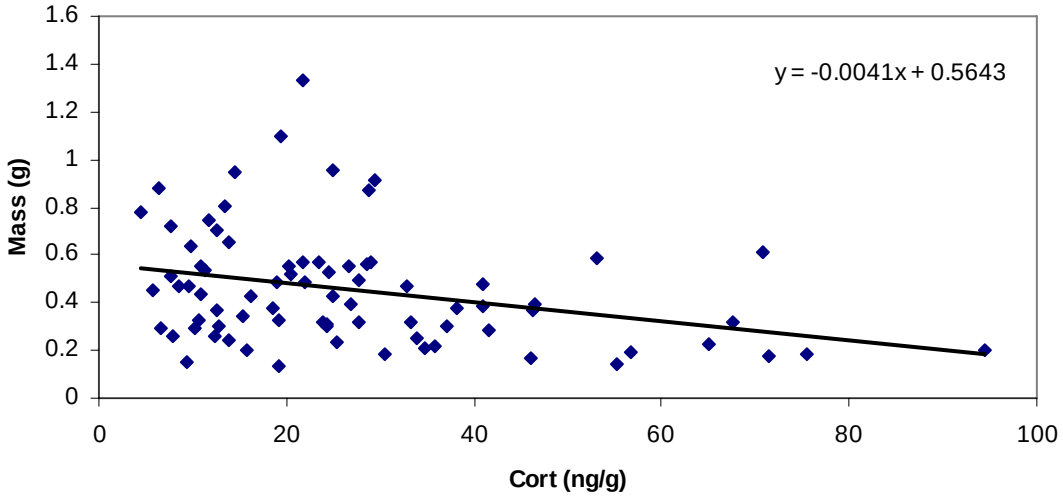


Figure 3 Relationship between cortisol and (a) body length and (b) body mass for WR x AR doubled haploids. Linear regression equations are shown in the figures. The r^2 values and associated p-values are (a) $r^2 = 0.131$, $p = 0.001$ and (b) $r^2 = 0.099$, $p = 0.005$.

CHAPTER THREE

FUTURE DIRECTIONS

A QTL study is used to identify regions of the genome having influence on a particular trait. Traits of interest in intensive animal culture are typically those influencing production efficiency including meat quality, reproduction, immune response, food conversion efficiency and stress response. Conducting QTL studies locating genomic regions influencing such traits is one step toward the ultimate goal of modifying trait prevalence through selective breeding. Once QTL have been identified, the regions can be searched for the underlying causes of variation. This primarily includes searching for and identifying genes involved with the trait. This approach has been successful in identifying loci underlying disease traits in humans and pigs (see Rothschild 1996). Genes can then be searched for mutations in coding regions which may be responsible for phenotypic variations. Development of efficient genotyping methods for such polymorphisms could then lead to marker assisted-selection, a method used to create broodstocks exhibiting desirable traits.

This study focused on identification of QTL for post-stress body cortisol levels in rainbow trout. Negative correlations with cortisol levels and traits of economic importance have been reported by Jentoft *et al.*, 2005; Weil *et al.*, 2001 and this study. Here, two cortisol QTL were identified in a wild by domestic rainbow trout cross. One of the QTL (*cort-1*) was found on the rainbow trout linkage group 1, and the other (*cort-2*) on linkage group 14.

With regard to stress response in rainbow trout only one gene, carbonyl reductase 1 (CBR1), has been mapped to date (Nichols *et al.*, 2003) . Carbonyl reductase is

analogous to 20β-hydroxysteroid dehydrogenase and is know to be involved with cortisol metabolism and clearance (Mommsen *et al.*, 1999; Truscott, 1979). In our study, the cort-1 QTL mapped in close association to the CBR1 gene.

In pigs, the estrogen receptor (ER) gene is known to have influence on litter size (Rothschild *et al.*, 1996). Investigations revealed a map-able polymorphism within the gene between the Meishan and Large White breeds of pigs and allelic association was made to litter size (Rothschild *et al.*, 1996). The AA homozygote pigs produced on average 2.3 more pigs in their first litter than the homozygote BB individuals (Rothschild *et al.*, 1996). Identification of mutational polymorphisms on genes, such as the one described above, do not conclusively mean that that gene is responsible for the observed phenotypic changes. They do, however, provide a valuable tool for increasing the prevalence or magnitude of desirable traits.

The same principle can be applied to the CBR1 gene for stress response in rainbow trout. Mutational changes in the CBR1 gene coding region can be detected by cDNA sequencing of the WR and AR parental lines. If mutations are detected, the doubled haploid mapping population can be genotyped. Once scoring of the mapping population is complete, a correlation can be made between an allele and phenotype. If one allele is strongly associated with decreased stress response it can then be implemented in marker-assisted selection to create a low stress line of fish. It has been reported that body mass in rainbow trout is more closely associated with prolonged elevation of cortisol levels rather than with peak post stress levels (Weil *et al.*, 2001). Fish with variations in the CBR1 gene may also display variations in cortisol clearance after a stress event. Identification of alleles associated with more rapid cortisol decreases

may be possible. Using marker-assisted selection, lines divergent in cortisol metabolism rate could be created. Cortisol clearance rates could be tested by exposing fish to a stressor and measuring cortisol levels at various times post stress . This would allow testing for associations of cortisol clearance rate and economically important traits.

To date, 4 QTL for post stress cortisol levels have been identified in rainbow trout, two in this study and two by Drew et al. (2007). Only one of these (cort-1 mentioned above) has been associated with a gene. For the others, the candidate gene method should be employed to identify genes associated with stress response. In essence "the candidate gene approach is an educated guess based on physiological knowledge of the underlying biology of a trait" (p233) (Silverstein *et al.*, 2006). This approach is based on understanding the physiology behind an important trait, and identifying genes important in the process. Stress response is a highly complex trait involving many genes and regulatory steps, see (Wendelaar Bonga, 1997). In fishes, after perception of a stressor, corticotrophin releasing hormone (CRH) is released from the hypothalamus and stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), α-melanophore-stimulating hormone (α-MSH) and β-endorphin (β-end). These then stimulate the interrenal cells to produce and release cortisol. Many other genes coding for cortisol production and inactivation are also potential candidate genes. The ones listed would be of top priority for testing association with QTL loci.

For continued research on the control and effects of stress response in fish, production of two lines divergent for stress response would be invaluable. Identifying easily scoreable markers, such as microsatellites, that track with the cortisol QTL found in this study and Drew et al (2007) would allow marker-assisted selection and creation of

divergent lines. Fish divergent for stress response would allow testing for any number of traits that may be influenced by stress. Traits of main interest may include body mass, immune response, reproduction, disease resistance and food conversion efficiency.

The detection of QTL influencing stress response in a commercially important aquaculture species has large potential benefits. The intensive culture of fishes is relatively new compared to other forms of animal husbandry practices. The process of domestication involves evolution of metabolic, physiological and behavioral traits best suited for the new environment. Persistence of strong stress response in intensive culture environments has reported negative effects. Understanding the underlying genetics of stress response has potential for drastic improvements in the efficiency of production. The QTL study performed here is one of many steps leading to the understanding needed for the enhancement of rainbow trout culture. Co-localization of candidate genes with QTL would is the next step in understanding the complex control of stress response. Eventual creation of lines divergent for longevity of, or peak stress response would be a powerful system for understanding the relationship of stress and economically important traits.

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