Investigation of sex change, sex differentiation and stress responses in black seabass (Centropristis striata)

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INVESTIGATION OF SEX CHANGE, SEX DIFFERENTIATION AND STRESS RESPONSES IN BLACK SEA BASS (CENTROPRISTIS STRIATA)

BY

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B.S. Marine Biology, University of Rhode Island, 2007

THESIS

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ABSTRACT

INVESTIGATION OF SEX CHANGE, SEX DIFFERENTIATION AND STRESS RESPONSES IN BLACK SEA BASS (CENTROPRISTIS STRIATA)

by

Danielle C. Duquette

University of New Hampshire, May 2013

Black sea bass (Centropristis striata) have been the focus of research as an aquaculture species for several years due to their high consumer demand and limited seasonable availability. As protogynous hermaphrodites, black sea bass initially develop as females in the wild, and undergo sex reversal between 2 and 6 years of age. Previous studies demonstrated that in captivity, however, a significant number of fish differentiate initially as males, and sex reversal is hastened in females. Full control of reproduction is required for successful commercial culture. Since captive environments pose several sources of stress upon the cultured species, this research investigates stress and effects upon sex differentiation and sex change in black sea bass. The first study measured production of the stress hormone cortisol relative to stocking density, a common source of stress in the culture environment. Black sea bass juveniles were held in high (3.66g fish/L), medium (2.56g fish/L), low (1.4g fish/L) densities, in triplicate for 84 days, after which blood was collected for cortisol radioimmunoassay. Fish in the highest density treatment were found to have significantly (p=0.0003 Wilcoxon/Kruskal-Wallis Test) lower plasma cortisol concentrations (0.419ng/ml) than those in the medium (3.243ng/ml) and low-density (3.232ng/ml) groups. Higher cortisol levels may have resulted from fish maintaining and defending territories in the lower density treatments.
The second study investigates the process of sex change in adult black sea bass and interactions caused by either an acute physical stressor or exogenously administered cortisol. A preliminary trial measured plasma cortisol at 6 time points (0, 1, 6, 12, 24, 48 hours) after an acute stressor or exogenous application of cortisol through an infused feed. Both trials showed plasma cortisol peaks at 6 hours post feed/stressor. To assess effects upon sex change adult fish in triplicate tanks were fed a cortisol-infused diet (100 mg/kg feed), exposed to an acute stressor (net chasing, 30 seconds), or fed a control diet and not exposed to an acute stressor (control). After 84 days, blood was drawn for cortisol radioimmunoassay and sex was assessed by ovarian biopsy, abdominal massage or gonadal histology. A greater number of females in the cortisol-fed (30%) and chased (28%) treatments underwent sex change compared to those in the control groups (11%), but treatment effects were not significant (p=0.5089 One-Way ANOVA) due to small sample sizes. Although not statistically significant, these results would still be functionally significant in an aquaculture setting. The third study investigated the effect of exogenously administered cortisol upon sex differentiation in juvenile black sea bass. Fish (n=270) were distributed among 9 151L aquaria (30 fish per tank) and fed a cortisol infused diet (control: 0 mg/kg, high: 100 mg/kg, low :10 mg/kg) daily to apparent satiation. After 175, 189 and 217 days in treatment individuals were euthanized and gonads removed for histology to determine sex. Fish which differentiated as female represented 51.85%, 79.19%, and 17.84% of the control, low and high treatments respectively (p=0.0007, One-Way Analysis of Variance, Tukey's Test). Fully differentiated spermatozoa or spermiation was detected in 36.4%, 16.7% and 56.5% of the control, low and high treatments fish, respectively. The low treatment average length
(13.64cm) and weight (39.93g) was significantly higher (Length: p=<0.0001, Weight: p=0.0002, One-Way Analysis of Variance, Tukey’s Test). The precocious puberty and affected growth and may be linked to previously unperceived stressors in the culture environment. This research will increase our understanding of the physiological processes of black sea bass and help to advance successful commercial propagation.
INTRODUCTION

The trade deficit associated with seafood ranks second behind oil and exceeds $9 billion annually. The demand for seafood is anticipated to rise as the US population increases and fishery landings remain stable or decline. Globally, finfish aquaculture has proven successful for a number of marine species including Atlantic salmon (Salmo salar), Paralichthid founder and European sea bass (Dicentrarchus labrax). In addition to consumer appeal and high market value, new candidates for aquaculture must fulfill a number of requirements to attain profitability. Among these are rapid growth to a marketable size, a good feed to muscle conversion ratio, disease resistance, and the ability to adapt to commercial conditions. Black sea bass (BSB; Centropristis striata) have been the focus of research as an aquaculture species for several years due to their high consumer demand and limited seasonal availability (Copeland et al. 2005).

Life History

This species (C. striata striata), is considered a temperate reef fish that inhabits coastal waters from the Gulf of Maine to Florida and supports important commercial and recreational fisheries throughout its range. A subspecies is also found throughout the Gulf of Mexico (C. striata melana). On the Atlantic coast, BSBs undertake seasonal migrations to deeper water during the winter months and return to shallow coastal areas in the spring to spawn (Musick and Mercer 1977). These fish are harvested commercially with traps in shallow water during the summer and with otter trawls when aggregated in deeper water in the late fall (Frame and Pearce 1973). The breeding season of BSBs progresses seasonally from south to north, with spawning occurring from
February-April in Florida and from late May-August in New England (Hardy 1978). A second, minor spawning period, during September, was described in fish captured from the South Atlantic Bight (Wenner et al. 1986). In the wild, females have been shown to grow faster than males and mature between years 1 and 2 (Lavenda 1949, Mercer 1978, Wenner et al. 1986). Spawning occurs in coastal waters with temperatures 18-20°C and salinities >15 ppt. Eggs and larvae have been collected from waters 12-24°C with salinity levels 30-35 ppt (Steimle et al. 1999). Juveniles remain in the estuaries throughout the summer, feeding on small benthic crustaceans, fish, and polychaete worms (Steimle et al. 1999).

In the wild, black sea bass, like most of the Serranidae, are protogynous hermaphrodites. The majority of individuals develop first as females, remain females for 3-5 years and then undergo sexual succession to become functional males (Lavenda 1949, Mercer 1978). In juvenile surveys, less than one percent of fish have been found to develop directly as males (primary males: McGovern et al. 2002). In culture conditions, however, sex change can be accelerated, and high rates of direct phenotypic male differentiation have been shown to occur (Howell et al. 2003, Benton and Berlinsky 2006, Colburn et al. 2009). The reasons for hastened sex reversal in captivity are not understood, but for aquaculture necessitate frequent collection of wild individuals to replenish female broodstock.

Aquaculture Studies

Previous aquaculture studies with this species have focused on induced spawning, larviculture, dietary needs and establishing environmental conditions conducive for

Spawning wild-captured fish was first reported by Earll (1884) and followed shortly afterwards by descriptions of embryonic and larval development (Wilson 1889). Methods were developed for hormonally induced ovulation using human chorionic hormone (Hoff 1978, Roberts et al. 1976, Hettler and Clements 1978), and later refined using analogs of gonadotropin releasing hormone (Berlinsky et al. 2005, Denson et. al. 2007). Protocols for both volitional tank spawning and manual (strip) spawning were also developed and the initiation and spawning duration were manipulated by photothermal conditioning (Chappell et al. 2001, Watanabe et al. 2002, Howell et al. 2003).

A significant amount of work on nursery culture of BSB has also been conducted including determining the optimal and limiting effects of temperature and salinity. Black sea bass eggs hatch over a wide range of temperatures (15-30 °C) and salinity levels (10-35 ppt), and larval black sea bass are euryhaline and eurythermal (Berlinsky et al. 2002). The optimal salinity for juvenile growth is 23.4 g/L with a low lethal tolerance of 10 g/L and juveniles grown at salinities of 20 and 30 g/L grew at a similar rate (Atwood et al. 2001, 2003, Cotton et al. 2003). Highest juvenile growth rates were found at 25 °C (Atwood et al. 2001, 2003), and fish reared at this temperature grew faster than those reared at 20 and 30 or 16 °C (Cotton et al. 2003). Low and high thermal tolerance limits of 3.7 °C and 33 °C, respectively, were also established (Atwood et al. 2001). Juvenile BSBs were found to accept a wide variety of commercially prepared diets (Berlinsky et
al. 2000, Copeland et al. 2002) and recent studies have shown that fishmeal can be replaced with high amounts of terrestrial plant proteins without affecting growth (Alam et al. 2012, Uyan et al. 2006). In captivity, production methods often result in populations of juveniles with sex ratios heavily skewed toward males or mono-sex (male) populations. As these juveniles may initiate spermatogenesis at very small sizes (< 50 g), somatic growth is compromised at the expense of reproductive effort (Colburn et al. 2009).

For commercial aquaculture, fish must be grown at relatively high densities to maximize space efficiency. In some species, the maximum population density is asymptotic, and if exceeded, can initiate a stress response and depressed somatic growth. Few studies, however, have examined the optimal density at which to raise BSBs for commercial aquaculture, but since they are a reef dwelling species and may not occupy all layers of the water column (Stuart and Smith 2003), novel rearing systems may be required for optimal growth. Further, the rearing density may also influence initial sex determination, as found in other species such as *Thalasoma bifascidtum* (Guerrer-Estevez and Moreno-Mendoza 2010).

**Stress Responses**

The physiological reactions that occur in response to external stressors are necessary for organism’s attempt to maintain homeostasis. Hans Selye developed the concept of General Adaptation Syndrome (GAS) as the first set of bodily responses in reaction to stress (1936). These initial reactions may be followed by prolonged physiological reactions induced by chronic stress. Due to a more intimate connection to
changes in their environment such as pollutants, the integrated stress response of teleost fish may be more variable and sensitive than terrestrial organisms (Wendelaar Bonga 1997). A host of physiological changes can occur in response to social factors, temperature changes, hypoxia, crowding, predation, and handling practices. The physiological cascade of responses to stress appears to be species-specific and may have evolved relative to environmental cues such as temperature (Wendelaar Bonga 1997, Barton et al. 1998) and is influenced by genotype (Fevolden et al. 1991, Fevolden et al. 1993, Barton 2000, Cnaani and McLean 2009). For instance, within a species of fish (salmonids, Morone saxatilis), some strains react greater to specific stressors (high responders) than others (low responders) (Castranova et al. 2005, Wang et al. 2004).

The primary stress response initiated in acute situations is regulated by the hypothalamic-sympathetic-chromaffin cell axis. External stressors are perceived sensory receptors, conveyed to brain centers that stimulate sympathetic nerves of the autonomic nervous system. These preganglionic cholinergic fibers subsequently stimulate (neural, postganglionic) chromaffin cells located in the head-kidney of teleosts. Chromaffin cells are analogous to the adrenal medulla of mammals, and release the catecholamines (CAs): epinephrine and norepinephrine. Release of CAs elicit a rapid “fight or flight” response, resulting in increased ventilation, heart rate and blood flow, higher hematocrit, and stimulation of hepatic glycogenolysis (Wendelaar Bonga 1997, Barton 2002, Mommsen et al. 1999).

The secondary and tertiary stress responses are slower, and target more systemic, non-neural tissues. Cortisol is the primary glucocorticoid hormone responsible for the physiological adjustments during chronic stress events. Cortisol release is initiated in the
hypothalamus and many factors including atrial natriuretic factor, angiotensin II, growth hormone, thyroxin, neuropeptide Y, and arginine vasotocin have stimulatory corticotropic influences (Wendelaar Bonga 1997). Regulation of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which directly initiates cortisol release, is also species-specific and influenced by several environmental factors (Barton, 2002, Davis 2004). Similar to the adrenal cortex in mammals, the interrenal cells of the teleost head-kidney secrete cortisol, which has a broad array of systemic effects. Prolonged release of cortisol can have detrimental effects on somatic growth, immune function and reproductive capacity (Head and Malison 2000, Small et al. 2008, Campbell et al. 1992, Cleary et al. 2000, Morgan et al. 1999, Pankhurst and Van Der Kraak 2000, Soso et al. 2008). In some cases, lifelong exposure to environmental toxins can heighten circulating cortisol levels to the point where the interrenal cells atrophy, ACTH receptors are downregulated, and the individual is no longer responsive to acute stressors (Wendelaar Bonga 1997).

Fish raised in aquaculture facilities may experience a wide range of anthropogenic stressors such as netting and inoculation, but may also be subjected to adverse social interactions such as bullying and territorialism, due to suboptimal stocking densities. Depending on the species, these social encounters may elicit strong physiological responses (Earley et al. 2006, Mileva et al. 2009, Overli et al. 2004, Parikh et al. 2006). Aggressive behavior has shown to increase plasma cortisol and associated metabolites in both dominant and submissive individuals (Clement et al. 2005). As stocking density is generally a long-term condition, the constant release of cortisol from these interactions may have deleterious effects on health and growth. Very little research
has been conducted to determine optimal rearing densities for BSBs, and these studies are necessary to optimize reproduction of broodstock and juvenile growth.

Reproduction

Sex Determination

Teleosts, with their diverse life histories and environmental ranges, employ a number of different reproductive strategies. Sexual determination, like that of reptiles, has a genetic component, but in many cases can be influenced by environmental factors. Sex determination and differentiation in many fishes is defined as gonochoristic, where individuals develop as males or females and remain so for the duration of their lives (Devlin and Nagahama 2002). Alternatively, fish may be hermaphroditic, and develop gonadal tissue of both (ovaries and testis) genders at some point during their lives. Synchronous or simultaneous hermaphrodites are capable of producing both male and female gametes during the same reproductive event, and some species can even self-fertilize (*Fundulus diaphanus*: Porter and Fivizzani 1983). Sequential hermaphrodites initially develop as one sex, and change to the other, thereby producing only one gamete at a time. Protandrous and protogynous species develop initially as males and females, respectively (Devlin and Nagahama 2002, Guerrer-Estevez and Moreno-Mendoza 2010, Nakamura et al. 1998, Penman and Piferrer 2008, Strussmann and Nakamura 2002).

In vertebrates, sex determination and (phenotypic) differentiation may be due entirely to genetic factors, such as with mammals, or also be influenced by environmental factors, as in the case of lower vertebrates such as some reptiles and fishes. Among
vertebrates, genetic determination may be entirely due to specific sex chromosomes, or involve polygenetic interactions from autosomes.

Chromosomal sex determination relies on highly evolved sex chromosomes, in which males (XX/XY; mammals) or females (WZ/ZZ; birds) can be heterogametic. Located on these sex chromosomes are specific genes that are responsible for initiating a genetic/hormonal cascades resulting in final sex differentiation (Guerrer-Estevez and Moreno-Mendoza 2010, Devlin and Nagahama 2002). The SRY gene, located on the Y sex chromosome has been shown to initiate male differentiation in mammals (Tiersch et al. 1992, Penman and Piferrer 2008). Expression of this gene regulates the expression of "down stream" genes including SOX-9 and AMH (Anti-Mullerian hormone) which collectively cause differentiation of sertoli cells in the developing embryonic testis. While the SRY sequence has only been found in mammals, a second vertebrate sex-determining gene, DMRT1/DMY, was recently found in the teleost fish Medaka (Oryzias latipes). The expression of DMRT1/DMY likely acts in a manner similar to SRY in mammals to stimulate a hormonal/ enzymatic cascade resulting in male differentiation (Ferguson-Smith 2007, Penman and Piferrer 2008, Matsuda et al. 2003. Matsuda et al. 2007, Suzuki et al. 2005, Ferguson-Smith 2007). Female-determining genes DAX1 and WNT4 have also been found in mammals, but no such analogs have been found in any other vertebrates.

In fishes sex determination can be quite labile, due to the interaction of multiple environmental factors with sexual genotype. These environmental factors include salinity, pH, nutrition, temperature and social cues (Francis and Barlow 1993, Nakamura et al. 1998, Godwin et al. 2003, Fairchild et al. 2007, Guerrero-Estevez and Moreno-
Mendoza 2010, Penman and Piferrer 2008). For instance, in studies with the European sea bass (*Dicentrachus labrax*), a euryhaline marine fish, a higher percentage of males resulted when larvae were transferred from low to high salinities 93 days post-fertilization (Saillant et al. 2003). One of the most prevalent and widely studied environmental factors that influences sex determination is temperature. Temperature-dependent sex determination (TSD) has been studied in reptiles, amphibians and multiple species of teleost fishes (Colburn et al. 2009, Godwin et al. 2003, Yamaguchi et al. 2010, Penman and Piferrer 2008, Hayes et al. 1998, Pieau et al. 1999). For the majority of species studied, higher rearing temperatures often resulted in male-skewed populations and higher female-skewed ratios at intermediate and lower temperatures (Strussmann and Nakamura 2002, Hattori et al 2009). In many teleost species with an “XX/XY” sex chromosome system, homogametic individuals can be phenotypically sex reversed by exposure to high water temperatures or exogenous steroids (Kitano et al. 1999, Kitano 2002). Exposure to estrogens and androgens during the sex-determining period can result in monosex populations of females and males, respectively. The likely mechanism underlying TSD, is the temperature sensitivity of the mitochondrial enzyme Cytochrome P450 Aromatase (aromatase) that converts the steroid testosterone to estrogen (Godwin et al. 2003, Penman and Piferrer 2008).

If aromatase is upregulated by temperature or other factors, testosterone is converted to estrogen and female phenotypes result. If aromatase is downregulated, and estrogen not synthesized, male phenotypes result (Pieau et al. 1999, Strussmann and Nakamura 2002). The up and down regulation of aromatase, and resulting steroid profile,
has also been shown to influence sex change in hermaphroditic species (Suzuki et al. 2004, Pieau et al. 1999, Strussmann and Nakamura 2002).

Recent studies of the Japanese flounder (Paralichthys olivaceus) and the Pejerrey (Odontesthes bonariensis) have shown relationships between cortisol and TSD, particularly at male rearing temperatures. Japanese flounder undergo TSD with males differentiating at higher temperatures. Yamaguchi et al. (2010) found cortisol inhibited CYP19a1 (aromatase coding gene) mRNA expression by interfering with cAMP activation. Similarly, application of a cortisol synthesis inhibitor (metrapone) inhibited masculinization of flounder grown at higher rearing temperatures. The pejerrey (Odontesthes bonariensis) also undergoes TSD with increased male differentiation at higher temperatures, and Hattori et al. (2009) found fish raised at male-determining temperatures had higher cortisol levels. Fish treated with cortisol also showed higher
levels of 11-ketotestosterone, testosterone, upregulation of the AMH (Anti-Mullerian hormone) and downregulation of CYP19a1. Collectively, this recent evidence suggests a connection between cortisol and male differentiation, and may influence other aspects of fish reproduction such as sex change.

Teleost Reproduction

In vertebrates, reproduction is controlled by a complex series of hormones and feedback mechanisms, all of which are not completely understood, operating through the hypothalamo-pituitary-gonadal (HPG) axis. The neurons of the hypothalamus, located under the thalamus of the brain, forms the ventral portion of the diencephalon, and release gonadotropin-releasing hormones (GnRHs), well conserved 10 amino acid peptides. GnRHs bind to receptors on cells in the anterior pituitary gland (gonadotropes) to cause the synthesis and release of gonadotropic hormones, gonadotropins I and II or follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In mammals FSH acts on cells in the gonads to stimulate sex steroid biosynthesis and gametogenesis. LH primarily acts during the final stages of gametogenesis to prepare the gametes for eventual release and fertilization.

Specifically, in females, Gth-I stimulates synthesis of androstenedione (an androgen) from theca cells that form the outer layer of follicle cells surrounding the oocyte. Androstenedione is further converted by aromatase to 17-β estradiol, in the inner layer of follicle cells (granulose cells) surrounding the oocyte of the ovary (Devlin and Nagahama 2002, Nakamura et al. 1998, Guerrer-Estevez and Moreno-Mendoza 2010).
In males, gonadotropin stimulation primarily leads to androgen synthesis, particularly 11-ketotestosterone, by upregulating key steroidogenic enzymes.

**Primary Follicle**

![Diagram of Primary Ovarian Follicle](image)

As with sex differentiation, the ultimate synthesis of estrogen or androgens, by up or down regulation of aromatase, has also been shown to drive sex reversal in hermaphroditic species, including black sea bass (Lee et al. 2003, Bhandari et al. 2004, Kwon et al. 2002, Devlin and Nagahama 2002, Guiguen et al. 1999, Suzuki et al. 2004, Benton and Berlinsky 2006). For instance, exogenous application of aromatase inhibitors such as Fadrozole, or 11-ketotestosterone have been shown to induce sex reversal in BSBs in as little as 1 week (Benton and Berlinsky 2006). Dietary administration of Fadrozole or exposure of female zebrafish (*Danio rerio*) to high water temperatures
resulted in oocyte apoptosis, lower aromatase activity and spermatogonia differentiation (Uchida et al. 2004). During ovarian development, the expression of aromatase mRNA was required for the formation of the ovarian cavity in medaka (*Oryzias latipes*; Suzuki et al. 2004). In other species, such as the saddleback wrasse (*Thalassoma duperrey*: Nakamura et al. 2005), goby (*Lythrypnus dalli*: Black et al. 2005) and red spotted groupers (*Epinephelus akaara*: Li et al. 2006) high and low levels of aromatase corresponded to female and male phenotypes, respectively. The CYP19 gene expresses the aromatase enzyme and studies have shown two isoforms of this gene, CYP19a and CYP19b, are expressed in the ovaries and brains of fishes, respectively. A study on the Japanese flounder (*Paralichthys olivaceus*) has shown suppression of CYP19a mRNA expression triggers male differentiation, particularly at higher rearing temperatures (Yamaguchi et al. 2010) but its exact roles in sex determination and reversal is unknown (Tchoudakova and Callard 1998, Guiguen et al. 1999, Suzuki et al. 2004). It is also unclear what environmental and social factors regulate aromatase expression during sex change in most teleost species. Sex change in many coral-reef fishes is dependent on social cues and the relative size of conspecifics. In protogynous examples, such as saddleback wrasse (*Thalassoma duperrey*: Ross et al. 1983), bluehead wrasse (*Thalassoma bifasciatum*: Warner and Swearer 1991), bluebanded goby (*Lythrypnus dalli*: Rodgers et al. 2005) *Cephalophis boenak* (Liu and Sadovy 2004), and black sea bass (*Centropristis striata*: Benton and Berlinsky 2006) removal of the largest or dominant male and other social cues cause females to initiate sex change. Similar findings exist for the protandrous anemonefish (*Amphiprion melanopus*), where subordinate males undergo sex-reversal when females were removed from the social
group (Godwin and Thomas 1993). The best-studied example of social structure influencing sex change is with the protogynous bluehead wrasse (*Thalassoma bifasciatum*). In this species, dominant males guard harems of females and inhibit sex change through aggressive interactions (Perry and Grober 2003). Loss of these aggressive terminal phase males then initiates the sex change of the initial phase females (Warner and Swearer 1991). Profiles of the neuropeptide arginine vasotocin (AVT) in sex-changing females and terminal phase males was much higher than subordinate females, with an approximately two-fold increase of AVT mRNA levels when fish acquired dominance over a spawning site (Semsar and Godwin 2003). Additionally, blocking AVT action through a receptor antagonist prevented behavioral sex change in females who were made experimentally socially dominant (Semsar and Godwin 2004).

Apart from aromatase, other cytochrome enzymes also appear to influence the reproductive development. P450 11-β hydroxylase is responsible for conjugating 11deoxycortisol to cortisol and converting testosterone to 11-ketotestosterone (11-KT), a hormone important for establishment of male reproductive characteristics in fishes (Miura et al. 2008, Yazawa et al. 2008, Blazquez et al. 2009, Arterbery et al. 2010). This enzyme is coded by the cyp11b gene, and correlations have been found between not only the abundance of cyp11b gene expression but 11-KT production in differentiating European sea bass (*Dicentrachus labrax*: Balazquez et al. 2009) and prodandrous sex changing anemonefish (*Amphiprion clarkii*: Miura et al. 2008). The connection between 11β-hydroxylase and both glucocorticoid and androgen synthesis indicates the possibility of interactions between responses to stress and reproductive development.
CHAPTER I

DENSITY DEPENDENT STRESS IN BLACK SEA BASS (CENTROPRISTIS STRIATA)

Introduction

As global fishery stocks continue to decrease, alternatives are necessary to meet the growing demand for seafood. Finfish aquaculture has proven successful for many species, most notably salmonids. New candidates for aquaculture must not only hold a high demand and price at market but also adapt to the high production of a commercial hatchery. Black sea bass (BSB; Centropristis striata) have been the focus of research as an aquaculture species for several years due to their high consumer demand and limited seasonal availability (Copeland et al. 2005). This species meets the culture requirements by acclimating well to domesticated rearing conditions, spawns in captivity, and produces larvae capable of surviving in a range of conditions and commercial feed formulations (Shepherd and Idoine 1993, Jirsa et al. 2009, Berlinsky et al. 2000, Atwood et al. 2003, Berlinsky et al. 2004, Copeland et al. 2002, Copeland et al. 2003, Watanabe et al. 2003).

Fish raised in culture may experience a wide range of anthropogenic stressors such as netting and handling, but also encounter many antagonistic social cues from conspecifics. Stocking density is one possible source of stress, which also directly affects overall profits. Previous studies investigating the effects of high rearing densities demonstrated a decrease in growth (Schram et al. 2006, Papoutsoglou et al. 2006, Irwin et al. 1999), variability in weights (Irwin et al. 1999), increased feed competition (Papoutsoglou et al. 2006) and upregulation of the S-enolase gene, which has been linked
with stress responses (Ribas et al. 2004). Research investigating acute stress responses after a period of high rearing density or crowding, showed increased plasma cortisol in common carp (Cyprinus carpio, Ruane et al. 2002), European sea bass (Dicentrarchus labrax, Santos et al. 2010), Senegalese sole (Sole senegalensis, Costas et al. 2008), great sturgeon (Huso huso, Falahatkar et al. 2009) and winter flounder (Pseudopleuronectes americanus, Sulikowsky et al. 2006). Cortisol is the primary glucocorticoid responsible for the physiological adjustments during chronic stress events. Prolonged release of cortisol can have detrimental effects on somatic growth, immune function and reproductive capacity (Head and Malison 2000, Small et al. 2008, Campbell et al. 1992, Cleary et al. 2000, Morgan et al. 1999, Pankhurst and Van Der Kraak 2000, Soso et al. 2008). Previous work with other species have shown negative effects of stress or application of cortisol on reproduction including lower levels of reproductive steroids (Cleary et al. 2000, Pankhurst and VanDerKraak 2000, Reddy et al. 1999, Soso et al 2008), altered timing of reproduction (Campbell et al. 1992, Morgan et al 1999, Schoech et al. 2009), reduced number of gametes (Campbell et al. 1992, Cleary et al. 2000, Soso et al 2008) and increased larvae mortalities/abnormalities (Campbell et al. 1992, Morgan et al. 1999, Soso et al 2008).

Due to a more intimate connection to changes in their environment, the integrated stress response of teleost fish is more variable and sensitive than terrestrial organisms (Wendelaar Bonga 1997). A host of physiological changes can take place because of social factors, temperature changes, hypoxia, crowding, predation, or handling practices. The physiological cascade of responses to stress appears to be species-specific and influenced by environmental conditions such as temperature (Wendelaar Bonga 1997,
Barton et al. 1998, Fevolden et al. 1991, Fevolden et al. 1993 Barton 2000, Cnaani and McLean 2009). The following study investigated this possible link between rearing density and plasma cortisol production in black sea bass. Better understanding of the relationship between growing conditions and stress could greatly increase the profitability of rearing this species.

**Materials and Methods**

**Broodstock Acquisition**

Black sea bass broodstock were captured by hook and line or fish pots in coastal Rhode Island waters, transported to Great Bay Aquaculture LLC (GBA; Portsmouth, NH, USA), and maintained in captivity in flow-through seawater (28-30 ppt salinity) systems comprised of 6,000 L insulated fiberglass tanks, photothermal control, and UV sterilization. Each fish was implanted with a passive integrated transponder (PIT) tag (Biomark, Boise, ID, USA) for identification. Fish were maintained at 12-18 °C and fed a commercial ration (9.0 mm, Burris, Franklinton, LA, USA) to apparent satiation 2-3 times per week. Photoperiod and temperature were adjusted weekly to simulate the natural conditions, with half-hour crepuscular periods. Light intensity, measured with a light meter (Sper Scientific, Scottsdale, AZ, USA), ranged from 5 lux (dawn/dusk) to 30 lux (day) at the water surface during the light period. Water temperature and dissolved oxygen were measured daily (Oxyguard Handy Gamma, Birkerod, Denmark) and total ammonia-nitrogen and nitrite-nitrogen were monitored weekly (HACH®, Loveland, CO USA). Fish were maintained at GBA for approximately 1-2 years before study was conducted.
Broodstock Spawning

During all procedures broodstock were anesthetized with 70 mg/L MS-222 (Tricaine-S, Tricaine Methanesulfonate, Western Chemical Inc., Scottsdale, AZ, USA). Two weeks prior to anticipated spawning, ovarian development was assessed by ovarian biopsy. Those fish containing vitellogenic oocytes were induced to spawn with luteinizing hormone releasing hormone analogue (LHRHa) as per methods of Berlinsky et al. (2005). Females were checked daily for evidence of ovulation by exerting gentle abdominal pressure to express ovulated eggs until a sufficient number of individuals (3-4) with high quality eggs ovulated. Ovulated eggs were expressed into a 500 ml polypropylene beaker and their total volume recorded. A subsample of eggs (n = 200) was examined to assess quality. Eggs were retained for fertilization if the majority appeared to be of high quality. Eggs from 3-4 females were pooled prior to fertilization. Manually expressed milt was collected into 3 ml syringes from 2-3 anesthetized males, pooled in a 10 mL polypropylene beaker, and used immediately or held on ice for no longer than 1 h prior to use. Pooled milt (0.5 mL) was added to the beaker containing eggs and activated with 150 mL seawater (35 ppt). The egg and sperm mixture was gently swirled during a two-minute fertilization period, after which they were transferred to a separatory funnel to determine the amount of buoyant (viable) and sinking (nonviable) eggs. The viable eggs were then incubated in 100 L conical incubators at 17-19 °C until hatch. Black sea bass larvae were raised through metamorphosis at GBA in 2,000 L fiberglass tanks on a flow-through system at 26-29 ppt salinity and 17-19 °C.
Seawater was filtered (10 microns) and UV sterilized prior to use. The fish were fed live prey according to standard GBA protocols and were weaned onto a commercial diet (Otohime B1; Reed Mariculture, California, USA) by 40 days post hatch.

**Juvenile Husbandry**

Black sea bass juveniles (n=315, 80-140g) were obtained from Great Bay Aquaculture LLC (GBA; Portsmouth, NH, USA) and transported to the Ritzman Laboratory at the University of New Hampshire. Nine -1500L insulated tanks were integrated into a recirculating aquaculture system with temperature (17-22 °C), salinity (25-30 ppt), dissolved oxygen (7.0-9.0 mg/L) and ambient photoperiod maintained daily. Total ammonia nitrogen, nitrite and pH were monitored on a weekly basis using a water quality test kit with DR/850 colorimeter (HACH®, Loveland, CO USA). Systems were equipped with biological and mechanical filtration, foam fractionation and UV sterilization. With densities comparable to previous literature (Copeland et. al 2003), fish were placed into tanks at random, in three densities; high-50 fish per tank (3.66g fish/L), medium-35 fish per tank (2.56g fish/L), low-25 fish per tank (1.4g fish/L). Tank densities were obtained empirically according to tank bottom surface area and average fish total length, as black sea bass are a reef dwelling species and do not predominantly occupy the water column. All tanks were fed a 4.0mm pellet (Skretting, Vancouver, British Columbia, Canada) to apparent satiation once daily and average daily feed intake recorded to monitor any lack of interest as a result of stress.
Sampling Procedure

After 84 days in treatment, six individuals from each tank were quickly netted, anesthetized in metomidate hydrochloride (5mg/L Aquacalm™, Western Chemical Inc., Scottsdale, AZ) and 1mL blood samples drawn from the caudal vasculature using heparinized 3mL syringes fitted with 22-gauge needles. Blood was then transferred to heparinized microfuge tubes containing 40 μl of aprotinin (SIGMA) and kept on ice until analysis. Length (to the nearest mm) and mass (to the nearest 0.001g) were determined using a measuring board and analytical balance (Adventurer; Ohaus Corporation, Pine Brook, NJ).

Blood samples kept on ice were then centrifuged at 10,000 rpm at 4°C for 7 minutes. Plasma was then decanted using Pasteur pipets into 1.5 Eppendorf microfuge tubes and stored at -20°C until extraction for radioimmunoassay.

Radioimmunoassay Analysis

Plasma was analyzed by radioimmunoassay (RIA) to determine cortisol levels using protocol modified from King and Berlinsky, 2006 (See Appendix A). Cortisol antibody (UC Davis) was diluted to a 1:4000 dilution with standard diluent. Cortisol radiolabel was diluted to a concentration of 12577 CPM (counts per minute) per 100μl. The assay was conducted with 31% binding, 6% non-specific binding and 5.83% inter-assay variation. To reduce interference due to non-specific binding in the RIA, tritium labeled cortisol stock was purified prior, using a C-18 SPE Sep Pak (Waters, Milford, MA) to remove free tritium from stock solution. (Protocol provided by Dr. Stacia Sower and associates. See Appendix B).
Results

Statistical Analyses

Plasma cortisol data was not normally distributed, and non-parametric statistics were used. All other data were normally distributed. The average plasma cortisol levels (4.19ng/ml) produced by the fish in the high density treatment was significantly lower (Fig. 4. p=0.0003, Wilcoxon/Kruskal-Wallis test) than the medium (32.43ng/ml) or low (32.33ng/ml) density treatments. There were no significant differences between treatments for growth (average total length, p=0.194 and weight, p=0.3781) or average daily feed intake as a percent of final body weight (p=0.6802). Figure 6 shows the top-down view of BSBs evenly distributed on the tank bottom for the high and low densities. In the high density, a small percentage of individuals are distributed in the water column.

Mean Plasma Cortisol by Density
Fig. 3. Mean plasma cortisol by relative density treatment with standard error bars. Different letter superscripts denote significant differences. (*p=0.0003, Wilcoxon/Kruskal-Wallis Test)

Fig. 4. Mean final length (p = 0.194, One-Way ANOVA) and weight (p = 0.378, One-Way ANOVA) with standard error bars among the density treatments after 84 days.
Mean Daily Feed Intake as a Percent of Final Mean Body Weight

Fig. 5. Mean daily feed intake expressed as a percentage of final mean body weight among three density treatments with standard error bars (p = 0.68, One-Way ANOVA)

Fig. 6. View of bottom spatial distributions for densities (A) high and (B) low.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Mean Weight (g)</td>
<td>143.9 ± 7.5</td>
<td>140.9 ± 4.6</td>
<td>153.7 ± 7.6</td>
<td>0.3781</td>
</tr>
<tr>
<td>Change in Weight (g)</td>
<td>36.2 ± 7.5</td>
<td>33.2 ± 4.6</td>
<td>46 ± 7.6</td>
<td>0.3781</td>
</tr>
<tr>
<td>Final Mean Length (cm)</td>
<td>20.7 ± 0.27</td>
<td>20.6 ± 0.20</td>
<td>21.3 ± 0.32</td>
<td>0.1944</td>
</tr>
<tr>
<td>Change in Length (cm)</td>
<td>2.5 ± 0.27</td>
<td>2.4 ± 0.2</td>
<td>3.1 ± 0.32</td>
<td>0.1944</td>
</tr>
<tr>
<td>Mean Daily Feed Intake as % of Final Body Weight</td>
<td>0.32 ± 0.08</td>
<td>0.28 ± 0.03</td>
<td>0.32 ± 0.03</td>
<td>0.6802</td>
</tr>
<tr>
<td>Mean Plasma Cortisol (ng/ml)</td>
<td>4.19 ± 1.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.3 ± 6.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.4 ± 7.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Table 1.: Growth, fed intake and plasma cortisol data by treatment with probability of statistical significance with standard error.

**Discussion**

Optimizing stocking density is critically important for profitable, commercial aquaculture and can influence both growth and feed conversion (Papoutsoglou et al. 1998). Previous studies have shown that overly high rearing densities can influence many aspects of fish health and growth (Irwin et al. 1999 Head and Malison 2000, Schram et al. 2006, Papoutsoglou et al. 2006), Circulating cortisol can decrease fish growth by increasing protein and lipid catabolism, decreasing feed efficiency and decreasing feed intake (Small et al. 2008, Mommsen et al. 1999, Peterson and Brown-Peterson 1992). Recent investigations suggest that cortisol may decrease the circulating levels of insulin-like growth factor-1 (IGF-1) in gilthead seabream (*Sparus aurata*, Saera-Vila et al. 2009) and channel catfish (*Ictalurus punctatus*, Peterson and Small 2005).
IGF-1, produced by the liver mediates the effects of growth hormone to promote somatic growth. Cortisol also has other metabolic effects such as increasing plasma glucose by hepatic glycogenolysis in Nile tilapia (*Oreochromis nilotcus*, Barreto and Volpato 2006) and gilthead seabream (*Sparus aurata*, Saera-Vila et al. 2009). Although significant differences in growth were not observed among treatment in the present (84 day) study, overall growth may not have been optimal due to depressed rearing temperature. Optimal rearing temperature for black sea bass juveniles has been estimated to be 25 °C (Atwood et al. 2003) but temperatures in the present study ranged between 17-22 °C. Further research is necessary to fully elicit the optimal stocking densities in this species.

High stocking densities can elicit a stress response in cultured fish, as demonstrated in common carp (*Cyprinus carpio*, Ruane et al. 2002), European sea bass (*Dicentrarchus labrax*, Santos et al. 2010), Senegalese sole (*Sole senegalensis*, Costas et al. 2008), great sturgeon (*Huso huso*, Falahatkar et al. 2009) and winter flounder (*Pseudopleuronectes americanus*, Sulikowsky et al. 2006). In the present study, however, higher rearing densities resulted in lower circulating cortisol levels over a 84-day period. The levels measured in fish reared at the medium and low densities (32.3 ng/ml, 32.4 ng/ml) were slightly lower than those observed following a stressor in a previous study with this species (50-155 ng/ml, King et al. 2005). In contrast, the fish in the high density treatment shows a significantly lower plasma cortisol level (mean 4.19 ng/ml). In a study with gilthead sea bream (*Sparus aurata*), juveniles were subjected to a high density (chronic stressor), followed by an acute stressor. Following the acute stressor, the fish held in the high-density treatment exhibited plasma cortisol levels 50% lower than those in the control treatment (Barton et al. 2005). Similarly, African catfish (*Clarias*
gariepinus) subjected to both long-term high and low densities followed by an acute stressor, showed little or no increase in plasma cortisol (van de Niewegiessen et al. 2008). Vijayan et al. (1990) found that brook char (Salvelinus fontinalis) grown in high densities had a lower sensitivity to application of ACTH than control. Collectively, these studies demonstrate negative feedback regulation of cortisol release and/or down-regulation of its receptors (Wendelaar Bonga 1997).

Social interactions from aggression are exhibited in many forms, ranging from maternal protection (Iguchi et al. 2004, Rubenstain and Wikelski 2005), to male competition and territoriality (Kudoh and Yamaoka 2004, Picciulin et al. 2006, Blanchet et al 2006, Earley et al. 2006, Francis 1984). Territory defense and aggressive events can elicit a stress response in both the aggressor and submissive party (Clement et al. 2005). As a temperate reef fish, black sea bass defend territories situated on substrate, and in culture primarily reside on the tank bottom (Stuart and Smith 2003, Copeland et al. 2003, Able et al. 1995, Weaver 1996). As illustrated in Figure 6, the fish in this study spaced themselves relatively equidistantly from each other, and defended territories at the low and medium densities, but were unable to do so at the high densities. Since other stressors such as poor water quality and handling, were avoided in the study, the elevated cortisol levels observed were likely due to social influences. A similar response was found in African catfish (Clarias gariepinus), in which higher incidence of aggressive interactions were observed at low rearing densities, compared to higher stocking densities (van de Niewegiessen et al. 2008). There is likely an asymptotic relationship between stocking density and stress and future studies should explore the upper limits of tank density that minimizes cortisol production while maximizing profit.
References


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Papoutsoglou, S. E., Tziha, G., Vrettos, X. and Athanasiou, A. 1998. Effects of stocking density on behavior and growth rate of European sea bass (Dicentrarchus labrax)
juveniles reared in closed circulated system. Aquaculture Engineering, 18(2), 135-144.


CHAPTER II

SEX CHANGE AND STRESS IN BLACK SEA BASS (CENTROPRISTIS STRIATA)

Introduction

In the wild, black sea bass, like most of the Serranidae, are protogynous hermaphrodites, the majority of which develop first as females, and undergo sexual succession to become functional males between 3-5 years of life (Lavenda 1949, Mercer 1978). In juvenile surveys, less than one percent of fish have been found to develop directly as primary males (McGovern et al. 2002). In culture conditions, however, sex change can be accelerated, and high rates of direct phenotypic male differentiation have been shown to occur (Howell et al. 2003, Benton and Berlinsky 2006, Colburn et al. 2009). Relative sex ratios in rearing tanks have been shown to affect the rate of sex change, with higher sex reversal in the absence of males (Benton and Berlinsky 2006). The exact trigger or feedback mechanism that regulates sex change in black sea bass is unknown, but the higher rate in culture suggests an environmental or nutritional cause. Aquaculture presents many situations involving both acute and chronic anthropogenic stressors that fish may experience, such as netting and handling. Cortisol is the primary glucocorticoid responsible for the physiological adjustments during chronic stress events and prolonged release can have detrimental effects on somatic growth, immune function and reproductive capacity (Head and Malison 2000, Small et al. 2008, Campbell et al. 1992, Cleary et al. 2000, Morgan et al. 1999, Pankhurst and Van Der Kraak 2000, Soso et al. 2008). Previous studies with other fish species have shown negative effects on reproduction caused by stress or cortisol application, including lower sex steroid levels.

The objectives of this study were to determine the effects of physical stressors, typical of those that fish would experience in an aquaculture environment, and exogenous cortisol administration on sex reversal in adult black sea bass.

**Materials and Methods**

**Fish Husbandry**

Black sea bass adults were captured by hook and line or fish pots in coastal Rhode Island waters, transported to Great Bay Aquaculture LLC (GBA; Portsmouth, NH, USA), and maintained in captivity in flow-through seawater (28-30 ppt salinity) systems comprised of 6,000 L insulated fiberglass tanks, photothermal control, and UV sterilization. Each fish was implanted with a passive integrated transponder (PIT) tag (Biomark, Boise, ID, USA) for identification. Fish were maintained at 12-18 °C and fed a commercial ration (9.0 mm, Burris, Franklinton, LA, USA) to apparent satiation 2-3 times per week. Photoperiod and temperature were adjusted weekly to simulate the natural conditions, with half-hour crepuscular periods. Light intensity, measured with a light meter (Sper Scientific, Scottsdale, AZ, USA), ranged from 5 lux (dawn/dusk) to 30 lux (day) at the water surface during the light period. Water temperature and dissolved oxygen were measured daily (Oxyguard Handy Gamma, Birkerod, Denmark) and total
ammonia-nitrogen and nitrite-nitrogen were monitored weekly (HACH®, Loveland, CO USA).

Black sea bass adults were then transported from Great Bay Aquaculture LLC (GBA; Portsmouth, NH, USA) to the University of New Hampshire Ritzman Laboratory and Aquaculture Research Center (ARC). Individuals were held or underwent experimental procedures in 1500L insulated tanks integrated into a recirculating aquaculture system with temperature (17-22 °C), salinity (25-30 ppt), dissolved oxygen (7.0-9.0 mg/L) and ambient photoperiod maintained daily. Total ammonia nitrogen, nitrite and pH were also monitored on a weekly basis using a water quality test kit with DR/850 colorimeter (HACH®, Loveland, CO USA). Systems were equipped with biological and mechanical filtration, foam fractionation and UV sterilization.

Cortisol-Infused Diet Formulation

Two test diets were produced: control (0 mg/kg feed) and cortisol-infused (100 mg/kg feed). Cortisol was first dissolved in 100% ethanol with 100 mg hydrocortisone dissolved in 1 mL ethanol to prepare the cortisol working stock. Ethanol alone was used to prepare the control diets. The gelatin was prepared according to instructions on the package (Knox Original Unflavored Gelatin). To prepare gelatin necessary for 1kg of feed, 8g of powdered gelatin was added to a mixture of 62mL cold deionized (DI) water: 188mL boiling DI water.

For 1 kg of feed, 1 mL of the appropriate cortisol working stock or 100% ethanol was added to 250 mL of liquid gelatin and mixed. This solution was poured over the feed (9.0mm pellets, Skretting, Vancouver, British Columbia, Canada), mixed continuously
(shaken in a plastic bag) and dried at room temperature. All feed was kept at 4 °C until use.

**Preliminary Time Trial Procedure**

To assess the temporal effect of physical stress and ingested cortisol on black sea bass adults, a cortisol time course study was performed. Six adult black sea bass were placed in six 1500L tanks incorporated into recirculating system at the Aquaculture Research Center in the University of New Hampshire. The fish were acclimated for 21 days prior to each time trial.

The first time trial examined plasma cortisol levels after an acute physical stressor; vigorous chasing with a net for thirty seconds. Following chasing, all individuals in a tank were anesthetized in metomidate hydrochloride (6.5 mg/L Aquacalm™, Western Chemical Inc., Scottsdale, AZ) and 1-2mL of blood obtained from the caudal vasculature using heparinized 3mL syringes fitted with 22-gauge needles. The fish were bled at 1, 6, 12, 24, and 48 hours post-chasing and 1 tank of fish was not chased and served as a control. The blood was transferred to 1.5 ml heparinized Eppendorf microfuge tubes containing 40 µl of aprotinin (SIGMA) and kept on ice until analysis.

A separate time trial was conducted for fish fed the cortisol infused diet after a 21-day acclimation period. Five tanks of fish (n = 6 fish/tank) were fed to apparent satiation and sampled at the time points indicated above with control fish unfed. Individuals were anesthetized and bled as in previous trial.
The blood samples were kept on ice until centrifuged at 10,000 rpm at 4 °C for 7 minutes. Plasma was then decanted using Pasteur pipets into Eppendorf microfuge tubes and stored at -20 °C until extraction for radioimmunoassay.

Radioimmunoassay Analysis

Plasma was analyzed by radioimmunoassay (RIA) to determine cortisol levels using protocol modified from King and Berlinsky, 2006 (See Appendix A). Cortisol antibody (UC Davis) was diluted to a 1:4000 dilution with standard diluent. Cortisol radiolabel was diluted to a concentration of 12577 CPM (counts per minute) per 100μl. Assay presented with 31% binding, 6% non-specific binding and 5.83% inter-assay variation. To reduce interference due to non-specific binding in the RIA, tritium labeled cortisol stock was filtered prior, using a C-18 SPE Sep Pak (Waters, Milford, MA) to remove free tritium from stock solution. (Protocol provided by Dr. Stacia Sower and associates. See Appendix B).

Sex Change Experimental Procedure

Adult black sea bass (n=72) obtained from Great Bay Aquaculture were placed in 9 1500L insulated tanks at the Ritzman Laboratory facility. Sex of all individuals was initially assessed by ovarian biopsy (Shehadeh et al. 1973) and presence of sperm following abdominal massage. The fish were individually identified with passive integrated transponder tags and housed in 1:3 ratio, 2 males : 6 females. The fish in the acute stressor experiment were chased with a net for 30 seconds biweekly and no net chasing was applied to the control group. In the cortisol-fed experiment, the fish received
the cortisol infused diet (9.0mm (Skretting, 100mg cortisol/Kg feed) as described previously. The fish were fed to apparent satiation two times/week and fish fed a gelatin-coated diet served as controls. All diets were weighed to record feed intake per tank. Each experiment lasted for 84 days.

**Sampling Procedure**

Upon conclusion of the experiment, all initially female individuals were anesthetized in metomidate hydrochloride (6.5 mg/L Aquacalm™, Western Chemical Inc., Scottsdale, AZ) and sex was assessed by abdominal massage and ovarian biopsy. If biopsy was not possible, individuals were euthanized by anesthetic overdose and samples of gonad removed for histological analysis. Due to size of adult gonad, seven cross-sections were taken at the proximal, central and distal portions of both left and right lobes with the final section taken at the fork of both lobes near site of attachment. The dissected gonads were fixed in 10 % formalin for 24 h, and then transferred to 70 % ethanol. Preserved samples were embedded in paraffin, sectioned at 5 μm, stained with hemotoxylin and eosin, and examined under a light microscope at 100-400X magnification. Gender classification was based on gonad characteristics previously described (Lavenda 1949; Nakamura et al. 1998; Benton & Berlinsky 2006). Sex ratios were arcsin square root transformed and analyzed by one-way ANOVA using JMP software (SAS Institute Inc).
Results

Preliminary Time Trial

The baseline cortisol level (time zero) for cortisol-fed fish averaged 29.71 ng/mL. One hour post-feeding levels increased to 200.4 ng/mL, plateaued at 242.94 ng/mL six hours, and returned to baseline levels (59.53-35.58 ng/mL) 24-48 hours (Figure 7).

Baseline cortisol levels at time zero for the acutely-stressed treatment fish were 53.77 ng/mL and remained at baseline one hour post-stressor (52.92 ng/mL). Levels peaked at 88.01 ng/mL at six hours post-stressor and returned to baseline levels (49.2 ng/mL) by 12 hours (Figure 7).

![Mean Plasma Cortisol: 48 hour Time Trial](image)

**Fig. 7.** Mean plasma cortisol levels for acutely-stressed and cortisol-fed time trial treatments at Hours 0, 1, 6, 12, 24 and 48 post-feeding/stressor with standard error bars.
Plasma Cortisol (ng/ml)

<table>
<thead>
<tr>
<th>Hour Post Stressor/Feeding</th>
<th>Acutely-Stressed</th>
<th>Cortisol-Fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>53.77 ± 12.7</td>
<td>29.71 ± 11.2</td>
</tr>
<tr>
<td>1</td>
<td>52.92 ± 8.52</td>
<td>200.40 ± 70.4</td>
</tr>
<tr>
<td>6</td>
<td>88.01 ± 13.06</td>
<td>242.94 ± 80.08</td>
</tr>
<tr>
<td>12</td>
<td>49.20 ± 17.63</td>
<td>141.29 ± 36.06</td>
</tr>
<tr>
<td>24</td>
<td>45.68 ± 10.22</td>
<td>59.53 ± 14.63</td>
</tr>
<tr>
<td>48</td>
<td>11.65 ± 2.11</td>
<td>35.58 ± 6.14</td>
</tr>
</tbody>
</table>

Table 2.: Mean plasma cortisol levels for acutely-stressed and cortisol-fed time trial treatments at Hours 0, 1, 6, 12, 24 and 48 post-feeding/stressor with standard error.

**Sex Change Experiment**

In the control treatment where individuals were not exposed to a physical stressor nor fed a cortisol-infused diet, 11.11% of female fish became spermiating males. In the acutely-stressed treatment, 30% of the females reversed sex and 26.67% did so in the cortisol-fed treatment. One-Way Analysis of Variance p=0.5089 (Figure 8).

The average feed intake (87.29g) during biweekly feeding periods was higher in control treatment compared to the acutely-stressed (42.34g) or cortisol-fed (54.84g) treatments ((p=0.0128; Figure 9).
Fig. 8. Percent of fish that underwent sex-reversal during the 84-day study separated by treatment.
Fig. 9. Mean feed intake during the 84-day cortisol-induction study with standard error bars. Significant differences are signified by letter superscripts. (*p=0.0128 One-Way ANOVA Tukey’s Test)
Table 3.: Percent of fish that underwent sex change and mean biweekly feed intake per tank by treatment with standard error. (control, acutely-stressed and cortisol-fed).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Acutely-Stressed</th>
<th>Cortisol-Fed</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent of Fish that</td>
<td>88.89</td>
<td>70.00</td>
<td>73.33</td>
<td>0.5281</td>
</tr>
<tr>
<td>Underwent Sex Change</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Biweekly Feed Intake Per</td>
<td>87.29 ±</td>
<td>42.34 ± 8.07</td>
<td>54.84 ± 6.47</td>
<td>0.0128</td>
</tr>
<tr>
<td>Tank (g)</td>
<td>7.58</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Stress and increased circulating cortisol levels have been shown to affect several aspects of teleost reproduction. Following acute stressors, estradiol levels were lower in snapper (Pagrus auratus, Cleary et al. 2000), jundia (Rhamdia quelen, Soso et al. 2008), Arctic char (Salvelinus apinus, Berg et al. 2003) and rainbow trout (Oncorhynchus mykiss, Reddy et al. 1999). Elevated cortisol has also been correlated with reduced egg size (Oncorhynchus mykiss, Campbell et al. 1992) increased ovarian atresia (Pagrus auratus, Cleary et al. 2000), and decreased/altered courtships in Atlantic cod (Gadus morhua, Morgan et al. 1999). Elevated cortisol can also influence final gamete maturation (Semenkova et al. 2006), larval survival, and incidence of larval abnormalities (Campbell et al. 1992, Soso et al. 2008, Morgan et al. 1999). Few studies, however have investigated the effects of stress and elevated cortisol on the incidence of sex change in hermaphroditic species.

In the present study 11% to 30% of the fish experienced sex change in the experimental control and “stressed” treatments, respectively. The lack of statistical
significance of these results may have been due to the small sample sizes, but the trend observed may have implications for commercial aquaculture. Though the 84-day experimental period was sufficient for individuals to complete sex change, higher concentrations of cortisol may be necessary to initiate the process. A study performed by Peterson and Small (2005) fed cortisol infused diets daily at two levels (400 and 200 mg/kg feed) to channel catfish (*Ictalurus punctatus*). Fish fed the cortisol diets weighed 50% less than controls after 28 days. These treatments also exhibited lower levels of plasma IGF-1 and an increase in ~20 kDa IGF binding proteins which are known to inhibit circulation of IGF-1 and energetically costly functions during times of stress such as starvation. These results signify considerable physiological changes due to the cortisol infused diet in a short period of time. Since the feed production methods of the current and previous studies were similar, differing only in cortisol concentrations, higher concentrations may be required to elicit statistically significant physiological changes in black sea bass as seen in the channel catfish.

Fish may be subjected to both acute and chronic stressors in aquaculture settings and cortisol may play a role in the reproductive changes observed in cultured back sea bass. The cortisol levels observed in the acutely-stressed trial fish were similar in amplitude to those observed previously in this (King et al. 2005) and other species (cobia, *Rachycentron canadum*; Cnaani and McLean 2009), (paddlefish, *Polyodon spathula*; Barton et al. 1998), (striped bass, *Marone saxatilis*; Wang et al. 2004) and (trout, *Oncorhynchus mykiss, Salvelinus fontinalis, Salvelinus namaycush, Salmo trutta*; Barton et al. 2000). The peak levels observed in the cortisol-fed trial, however, were nearly 100 ng/ml higher than previously measured in black sea bass (King et al. 2005).
Although the amplitudes were different, the timing of the cortisol peaks occurred 6 hours after ingestion or induction (e.g. chasing) of cortisol. Previous studies have shown that the timing of the cortisol peak is species-specific and is also dependent on the severity of the stressor, but often occurs 1-6 hours after application of the stressor.

There have been few studies that compared the effects of chronic and acute stressors on aquacultured fish species. In studies of chronic stress, habituation may occur, resulting in downregulation of cortisol or ACTH receptors due to negative feedback mechanisms (Wendelaar Bonga 1997). For example, brook char (Salvelinus fontinalis) grown in high densities had a lower sensitivity to exogenous ACTH application compared to controls (Vijayan and Leatherland 1990). The repetitive application of chronic stressors in the present study may have resulted in a chronically stressed state, possibly exacerbated by other unintentional factors present in the aquaculture environment (e.g. noise, lighting, social aggression). Additional studies are necessary to determine the magnitude of specific stressors and determine compound effects that can occur due to aquaculture practices.

In aquaculture, the social environment experienced by cultured fish is much different from that found in natural habitats. Since social interactions are intimate and more frequent, the role of social cues may influence initiation of sex change in black sea bass faster than in the wild. Previous studies have shown removal of males from a culture cohort will cause females to undergo sex reversal (Benton and Berlinsky 2006). Although the sex ratio in the current study was chosen to prevent this type of social feedback, additional social cues such as body size or pheromone signaling (Godwin et al. 2003) may have impacted the final sex ratio. Neuropeptides have also shown to
influence sex change behavior. Neuropeptide Y (NPY), expressed in the hypothalamus, is thought to regulate GnRH secretion and initiate sex reversal in *Thalassoma bifasciatum* (Kramer and Imbriano 1997). Similarly, arginine vasotocin (AVT), is also released from the preoptic area of the hypothalamus, (Godwin et al. 2003), and is upregulated in sex changing females (Godwin et al. 2000, Semsar and Godwin 2003, Perry and Grober 2003). Behavioral studies have shown increased and decreased courtship displays with exogenous application and inhibition of this neuropeptide, respectively (Semsar et al. 2001). Although the reproductive behavior and social structure of black sea bass are currently unknown, it is possible that since they are a temperate reef fish, dominance hierarchies and reproductive strategies may be similar to tropical reef species. Further investigations should focus on the social structure and reproductive behavior of this species in conjunction with the neuroendocrine basis of sex change.

**References**


CHAPTER III
CORTISOL AND SEX DIFFERENTIATION IN BLACK SEA BASS

(*CENTROPRISTIS STRIATA*)

**Introduction**

Black sea bass, like many members of the Serranidae family, are protogynous hermaphrodites with the majority of individuals developing first as females, and undergoing sex reversal to become functional males at 3-5 years of age (Lavenda 1949, Mercer 1978). In juvenile surveys, less than one percent of fish have been found to develop directly as primary males (McGovern et al. 2002). In culture conditions, however, high rates of direct phenotypic male differentiation have been shown to occur (Howell et al. 2003, Benton and Berlinsky 2006, Colburn et al. 2009).

In many teleost species, genetic mechanisms of sex determination can be influenced or overridden by environmental stimuli. Temperature, in particular has been shown to influence sex differentiation (temperature-dependent sex determination; TSD) in a number of teleosts and other lower vertebrates. Although the exact environmental influences on sex determination in black sea bass have not been identified, previous studies suggest that temperature alone is not the sole determinant (Colburn et al. 2009).

Fish raised in culture may experience a wide range of anthropogenic stressors such as netting and handling while also encountering many antagonistic social cues from conspecifics. Cortisol is the primary glucocorticoid responsible for the physiological adjustments during stress events, and prolonged release can have detrimental effects on somatic growth, immune function and reproductive capacity (Head and Malison 2000,
Small et al. 2008, Campbell et al. 1992, Cleary et al. 2000, Morgan et al. 1999, Pankhurst and Van Der Kraak 2000, Soso et al. 2008). Recent studies of Japanese flounder (*Paralichthys olivaceus*) and the pejerrey (*Odontesthes bonariensis*) demonstrated an interaction between cortisol and TSD. Yamaguchi and colleagues (2010) found cortisol inhibited mRNA expression of the aromatase encoding gene CYP19a1, by interfering with cAMP activation in Japanese flounder. Similarly, application of a cortisol synthesis inhibitor (metrapone) inhibited masculinization of flounder grown at higher rearing temperatures. Hattori and colleagues (2009) found that pejerrey (*Odontesthes bonariensis*) raised at higher, male-determining temperatures also had elevated cortisol levels relative to females reared at lower temperatures. Fish treated with cortisol also showed higher levels of 11-ketotestosterone, testosterone, upregulation of the AMH (Anti-Mullerian hormone) and downregulation of CYP19a1.

The purpose of this study was to examine the effects of exogenously administered cortisol on sex differentiation in juvenile black sea bass. Understanding the mechanisms of sex determination in this species will lead to more effective management practices for commercial culturists.

**Materials and Methods**

**Broodstock Acquisition**

Black sea bass broodstock were captured by hook and line or fish pots in coastal Rhode Island waters, transported to Great Bay Aquaculture LLC (GBA; Portsmouth, NH, USA), and maintained in captivity in flow-through seawater (28-30 ppt salinity) systems comprised of 6,000 L insulated fiberglass tanks, photothermal control, and UV
sterilization. Each fish was implanted with a passive integrated transponder (PIT) tag (Biomark, Boise, ID, USA) for identification. Fish were maintained at 12-18 °C and fed a commercial ration (9.0 mm, Burris, Franklinton, LA, USA) to apparent satiation 2-3 times per week. Photoperiod and temperature were adjusted weekly to simulate the natural conditions, with half-hour crepuscular periods. Light intensity, measured with a light meter (Sper Scientific, Scottsdale, AZ, USA), ranged from 5 lux (dawn/dusk) to 30 lux (day) at the water surface during the light period. Water temperature and dissolved oxygen were measured daily (Oxyguard Handy Gamma, Birkerod, Denmark) and total ammonia-nitrogen and nitrite-nitrogen were monitored weekly (HACH®, Loveland, CO USA). Fish were maintained at GBA for approximately 1-2 years before study was conducted.

Broodstock Spawning

During all procedures broodstock were anesthetized with 70 mg/L MS-222 (Tricaine-S, Tricaine Methanesulfonate, Western Chemical Inc., Scottsdale, AZ, USA). Two weeks prior to anticipated spawning, ovarian development was assessed by ovarian biopsy. Those fish containing vitellogenic oocytes were induced to spawn with luteinizing hormone releasing hormone analogue (LHRHa) as per methods of Berlinsky et al. (2005). Females were checked daily for evidence of ovulation by exerting gentle abdominal pressure to express ovulated eggs until a sufficient number of individuals (3-4) with high quality eggs ovulated. Ovulated eggs were expressed into a 500 ml polypropylene beaker and their total volume recorded. A subsample of eggs (n = 200) were examined to assess quality. Eggs were retained for fertilization if the majority
appeared to be of high quality. Eggs from 3-4 females were pooled prior to fertilization. Manually expressed milt was collected into 3 ml syringes from 2-3 anesthetized males, pooled in a 10 mL polypropylene beaker, and used immediately or held on ice for no longer than 1 h prior to use. Pooled milt (0.5 mL) was added to the beaker containing eggs and activated with 150 mL seawater (35 ppt). The egg and sperm mixture was gently swirled during a two minute fertilization period, after which they were transferred to a separatory funnel to determine the amount of buoyant (viable) and sinking (nonviable) eggs. The viable eggs were then incubated in 100 L conical incubators at 17-19 °C until hatch. Black sea bass larvae were raised through metamorphosis at GBA in 2,000 L fiberglass tanks on a flow-through system at 26-29 ppt salinity and 17-19 °C. Seawater was filtered (10 microns) and UV sterilized prior to use. The fish were fed live prey according to standard GBA protocols and were weaned onto a commercial diet (Otohime B1; Reed Mariculture, California, USA) by 40 days post hatch.

**Juvenile Husbandry**

Black sea bass juveniles (n=270) were obtained from Great Bay Aquaculture (GBA; Portsmouth, NH, USA) and then transported to the Aquaculture Research Center (ARC) at the University of New Hampshire. Individuals were evenly distributed among 9 visually isolated 151L aquaria (30 fish per tank). Average weight of individuals was 8.1g and 7.7 cm total length. These nine aquaria were incorporated into three separate recirculating systems with three tanks connected to a sump containing mechanical and biological filtration. Each system also contained in-line UV sterilization, foam fractionation, and a side-mounted power filter (Aquaclear® Rolf C. Hagen Inc.) Each
A recirculating system was maintained with temperature (17-22 °C), salinity (25-30 ppt), dissolved oxygen (7.0-9.0 mg/L) and ambient photo period maintained daily. Total ammonia nitrogen, nitrite and pH were monitored on a weekly basis using a water quality test kit with DR/850 colorimeter (HACH®, Loveland, CO USA). Each system of three aquaria and was fed one experimental diet. These diets consisted of a control (0 mg cortisol/kg) high treatment (100 mg cortisol/kg) and low treatment (10 mg cortisol/kg). The diets were fed to apparent satiation once daily and feed containers weighed to record daily intake.

Cortisol-Infused Diet Formulation

Three test diets were produced: control (0 mg/kg feed), low cortisol (10 mg/kg feed), and high cortisol (100 mg/kg feed). Cortisol was first dissolved in 100% ethanol with 100 mg hydrocortisone dissolved in 1 mL ethanol to prepare the high cortisol working stock. To prepare the low cortisol working stock, 100 µL of the high cortisol working stock was dissolved in 900 µL 100% ethanol. Ethanol alone was used to prepare the control diets. The gelatin was prepared according to instructions on the package (Knox Original Unflavored Gelatin). To prepare gelatin necessary for 1kg of feed, 8g of powdered gelatin was added to a mixture of 62mL cold deionized (DI) water: 188mL boiling DI water.

For 1 kg of feed, 1 mL of the appropriate cortisol working stock or 100% ethanol was added to 250 mL of liquid gelatin and mixed. This solution was poured over the feed (Skretting Europa 15, 2 mm, Skretting, Vancouver, British Columbia, Canada), mixed
continuously (shaken in a plastic bag) and dried at room temperature. All feed was kept at 4 °C until use.

**Sampling Procedure**

After 175 and 189 days in their respective treatments, six individuals per aquaria were euthanized in an overdose of MS-222 (Tricaine Methanesulfonate, Western Chemical Inc., Scottsdale, AZ), length (to the nearest mm) and mass (to the nearest 0.001g) were determined using a measuring board and Adventurer balance (Ohaus Corporation, Pine Brook, NJ) and gonads were removed for histological analysis to determine if differentiation had occurred. After 217 days in treatment all individuals were euthanized length and mass determined and gonads removed. Of these final samples, six individuals per tank were prepared for histology. The dissected gonads were fixed in 10 % formalin for 24 h, and then transferred to 70 % ethanol. Preserved samples were embedded in paraffin, sectioned at 5 μm, stained with hemotoxylin and eosin, and examined under a light microscope. Gender classification was based on gonad characteristics previously described (Lavenda 1949; Nakamura et al. 1998; Benton & Berlinsky 2006). Male differentiation was also determined by expressing milt using abdominal massage and spermiating individuals were classified as males in sex ratio calculations.

**Sex Analysis Procedure**

For each histological slide, gender was assigned if either ovarian or testicular tissue clearly covered > 60% of the differentiated tissue. For individuals where both
tissue types were present in significant amounts, photographic mosaics of histological section were analyzed digitally. This was accomplished by systematically photographing the entire gonad with a light microscope at 240X magnification and AxioVison software (Carl Zeiss MicroImaging, Germany). Photographs were overlapped by ~ 20-50% and were imported into a digital stitching program, Calico (Kekus Digital, LLC) that combined all photomicrographs into one high resolution image (See Appendix C). This composite image was then imported into ImageJ (S. National Institutes of Health, Bethesda, Maryland, USA) and ovarian and testicular percent was determined using a grid. Sex ratios were arcsin square root transformed and analyzed by one-way ANOVA using JMP software (SAS Institute Inc).

Within gender categories, males, females and intersex fish were further classified by the degree of reproductive development. Mature/Running males were characterized by the presence of secondary spermatocytes or running milt. Primary males were characterized by the presence of primary spermatocytes in addition to undifferentiated gonadal tissue. Vitellogenic females were characterized by the presence of oocytes with vitellogenin granules. Intersex fish (both ovarian and testicular tissue) were classified as either >60% males or >60% females if >60% of the gonadal tissue was testicular or ovarian, respectively.

Results

After 175, 189 and 217 days fed experimental diets, over 96% of gonads sampled contained differentiated male or female tissue. Of the fish fed the control diet during the experimental period, 51.85% (n=53) differentiated as female. Greater numbers of fish
fed the low (10 mg/kg) cortisol-infused diet differentiated as females (79.19%; n=54) compared to those fed the high (100 mg/kg) treatment diet (17.84%; n=49). All female differentiation percentages were statistically significant from each other (p=0.0007, One-Way Analysis of Variance, Tukey’s Test). Fully differentiated spermatozoa or spermiation was detected in 36.4%, 16.7% and 56.5% of the control, low and high treatments fish, respectively (Figure 10). Undifferentiated gametic tissue and primary spermatocytes were found in 39.4%, 41.7% and 24.2% of males in control, low and high treatments, respectively. Vitellogenic oocytes were observed in control (14.8% of females) and high (22.2% of females) treatments. Samples which contained both male and female gametic tissue (Intersex) represented 23.3%, 11.1% and 23.9% in the control, low, and high treatments. Within the intersex group, greater amounts of testicular tissue (>60 %) was found in all treatments; 57.1% in Control, 83.3% in Low and 70.6% in High (Figure 13).

Across all sampling dates, the average length (13.64cm) and weight (39.93g) for the low (10mg/kg) cortisol-infused diet treatment (n=79) was significantly higher (Length: p=<0.0001, Weight: p=0.0002, One-Way Analysis of Variance, Tukey’s Test) than the control (Length: 12.15cm, Weight: 31.2g, n=80) or high (100mg/kg) cortisol-infused diet treatment (Length: 12.35cm, Weight: 31.42g, n=79) (Figure 11).

Average daily feed intake as a percentage of final body weight (g) was not significantly different across treatments (p=0.8915, One-Way Analysis of Variance) and ranged from 0.52-0.54% of body weight (Figure 12).
Final Sex Ratio: 175 - 217 Days

Fig. 10. Final gender of differentiated individuals fed control (0mg/kg), low (10mg/kg) or high (100mg/kg) cortisol infused diets over 175-217 days. Different superscript letters denote significant differences ($p=0.0007$, One-Way ANOVA, Tukey's Test).
Fig. 11. Mean final length (cm) and weight (g) of individuals fed Control (n=80), Low (n=77) or High (n=79) cortisol-infused diets over 175-217 days in treatment with standard error bars. Different superscript letters denote significant differences (*p=0.0002, **p=<0.0001, One-Way ANOVA, Tukey’s Test).
Mean Daily Feed Intake as Percent of Final Body Weight

Fig. 12. Mean daily feed intake as a percentage of final body weight per cortisol-infused diet treatment with standard error bars (p=0.8915).
<table>
<thead>
<tr>
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<th>Treatment</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (0mg/kg)</td>
<td>Low (10mg/kg)</td>
<td>High (100mg/kg)</td>
<td>p-Value</td>
<td></td>
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<tr>
<td>Final Mean Weight (g)</td>
<td>31.19 ± 1.42b</td>
<td>39.93 ± 1.93a</td>
<td>31.42 ± 1.56b</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Change in Weight (g)</td>
<td>23.09 ± 1.42b</td>
<td>31.83 ± 1.93a</td>
<td>23.32 ± 1.56b</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Final Mean Length (cm)</td>
<td>12.15 ± 0.22b</td>
<td>13.64 ± 0.22a</td>
<td>12.35 ± 0.19b</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Change in Length (cm)</td>
<td>4.43 ± 0.22b</td>
<td>5.93 ± 0.22a</td>
<td>4.64 ± 0.19b</td>
<td>&lt;0.0001</td>
<td></td>
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<tr>
<td>Mean Daily Feed Intake as a % of Final Body Weight</td>
<td>0.54 ± 0.045</td>
<td>0.53 ± 0.0014</td>
<td>0.53 ± 0.018</td>
<td>0.8915</td>
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<table>
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<th>Final % Sex: 25-31 Weeks</th>
<th></th>
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<tr>
<td>Female</td>
<td>51.85a</td>
<td>79.19b</td>
<td>17.84c</td>
<td>0.0007</td>
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<tr>
<td>Male</td>
<td>48.15a</td>
<td>20.81b</td>
<td>82.16c</td>
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</tbody>
</table>

Table 4.: Final growth, feed intake and sex ratio data after 175 to 217 days in treatment with probability of statistical significance and standard error.
Reproductive Development by Treatment

Fig. 13. Reproductive development by treatment in cortisol – induction study.
Table 5.: Counts of samples displaying differing reproductive development by treatment.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low</th>
<th>High</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percent by Gender</td>
<td>Number</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mature/Running</td>
<td>33</td>
<td>36.4</td>
<td>12</td>
</tr>
<tr>
<td>Primary</td>
<td>13</td>
<td>39.4</td>
<td>2</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitellogenic</td>
<td>27</td>
<td>14.8</td>
<td>42</td>
</tr>
<tr>
<td>Intersex</td>
<td>4</td>
<td>14.8</td>
<td>0</td>
</tr>
<tr>
<td>&gt;60% Male</td>
<td>14</td>
<td>57.1</td>
<td>6</td>
</tr>
<tr>
<td>&gt;60% Female</td>
<td>8</td>
<td>57.1</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>23.3</td>
<td></td>
<td>11.1</td>
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Discussion

Glucocorticoids have wide-ranging physiological effects on multiple systems, including general metabolism, that often complicate interpretation of treatment effects. The most surprising findings of this study were the prevalence of male differentiation and advanced reproductive development in these juvenile fish. In the wild, direct male differentiation is rare and spawning doesn’t occur until 2-3 years of age. (Howell et al. 2003, Benton and Berlinsky 2006, Colburn et al. 2009). Fish in all treatment groups demonstrated significant male differentiation; 48.1%, 20.8% and 82.2% in the control, low and high treatments respectively. Of the male individuals, the percentage of samples that exhibited advanced spermatogenesis or expressible milt ranged from 16.7% in the low treatment to 56.5% in the high treatment. A small percentage of females in the control (14.8%) and high (22.2%) treatments also exhibited evidence of vitellogenic oocytes. These advanced stages of reproductive development indicate precocial puberty,
a problem common to several domesticated species. For example, European sea bass 
(Dicentrarchus labrax) reared at warmer temperatures in culture exhibit increased 
incidences of precocious males (Navarro-Martín et al. 2009). Although differences in 
male differentiation were not observed to correlate with dosages of exogenous cortisol, 
baseline cortisol levels may have already been elevated due to inherent stressors in the 
culture environment.

Cortisol often has a negative effect on growth resulting from several inter-related 
processes. Increased plasma cortisol can decrease fish growth by increasing protein 
catabolism, increasing lipolysis, decreasing feed efficiency and decreasing feed intake 
(Small et al. 2008, Mommsen et al. 1999, Peterson and Brown-Peterson 1992, 
Wendelaar-Bonga 1997). Increased levels of catecholamines and gulcocorticoids after a 
stressor also increase metabolic rate with subsequent decreases in growth. For instance, 
in largemouth bass (Micropterus salmonides), increased plasma glucose following a 
stressor was correlated with 22 and 42% decreased growth with 10 and 20% increases in 
metabolism, respectively (Rice 1990). In the current study, paradoxically, significantly 
greater growth was demonstrated in the low cortisol (10 mg/kg feed) treatment group 
compared to control and high (100 mg/kg feed) treatment groups. Fish in the low 
treatment group, however, also exhibited the lowest incidence of precocious puberty 
(running males, vitellogenic females) relative to the control and high treatment groups. 
As reproductive development often occurs at the expense of somatic growth this would 
explain the results obtained. Further studies are necessary to determine the dose-
dependent effects of cortisol on reproduction in this species.
Many past studies have shown the importance of the aromatase and 11β-hydroxylase on female or male differentiation respectively. CYP19a is the determining gene for aromatase, that converts testosterone to estradiol. Inhibitors of aromatase, like the breast cancer treatment drug Fadrozole*, have been shown to increase male differentiation in rainbow trout (*Oncorhynchus mykiss*: Guiguen et al. 1999), tilapia (*Oreochromis niloticus*: Kwon et al. 2002) and black sea bass: Benton and Berlinsky 2006). In medaka (*Oryzias latipes*), inhibition of aromatase suppressed the development of the ovarian cavity (Suzuki et al. 2004). 11β-hydroxylase is coded by the gene CYP11B and is responsible for synthesis of 11-ketotestosterone, the dominant androgen in teleost fishes. A recent study found an upregulation of CYP19a and CYP11B in differentiating female and male European sea bass (*Dicentrarchus labrax*), respectively (Balazquez et al. 2009). Recent evidence has shown the function of these genes may be regulated by cortisol. Yamaguchi et al. (2010) reported that Japanese flounder (*Paralichthys olivaceus*), a species that exhibits temperature-dependent sex determination, had lower CYP19a mRNA production when treated with cortisol. Higher circulating cortisol levels were observed when fish were reared at higher (male-determining) temperatures, while undifferentiated fish treated a cortisol inhibitor (metyrapone) differentiated as females. Similar results were observed in pejerrey (*Odontesthes bonariensis*: Hattori et al. 2009) where elevated cortisol, 11-KT and T were observed at male-producing temperatures. An exogenous application of cortisol also increased 11-KT, T, AMH while down regulating CYP19a. Aromatase inhibitors produced a similar effect to that of high male-determining temperatures (Uchida et al. 2005).
Collectively, these data demonstrate the inhibition of aromatase, and inhibition of female sex differentiation by cortisol in teleosts.

Although the influence of cortisol on black sea bass sex differentiation was not fully explained in this study, the reproductive development observed differed greatly from that observed in wild fish. Further investigations are necessary to determine what factors in aquaculture act as stressors to this species and if growth can be significantly influenced by husbandry practices.

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CONCLUSIONS AND FUTURE WORK

The current studies investigated many aspects of black sea bass physiology, behavior and candidacy as an aquaculture species. Since this species is a temperate reef fish, consideration has to be taken into the husbandry conditions, which may be different than other cultured groups such as salmonids or flounders. The social aspects of reef dwelling fish are much different than schooling or benthic teleosts, and displays of territoriality could impact their survival in culture. Additional research on the behavioral patterns, and neuroendocrine signals produced by BSBs in both the wild and in culture would help to understand the physiological differences seen in captivity. The first study presented here showed a link between density and production of cortisol. At the highest density tested, the lowest plasma cortisol levels were found indicating a correlation between holding and defending territories and increases in cortisol. Since antagonistic interactions are known to increase cortisol, higher densities may be beneficial. This benefit can be asymptotic however, and future studies may look at the optimal density which controls not only cortisol production, but other aspects of fish heath such as water quality. Also, investigating the regulation of ACTH may clarify what is causing the lower concentrations in plasma cortisol at higher densities. Since density may act as a chronic stressor, hormonal cascades and receptors might undergo downregulation and produce the levels of cortisol seen in this study.

There are many well-known negative effects of stress upon reproduction. Cortisol in teleosts has been seen to effect egg quality, larval survival and courtship behavior. For protogynous hermaphrodites like black sea bass, sex change is another critical step for optimal reproductive fitness. In culture, control of this sex change is also critical to
increase oocyte yield and maintain a healthy number of functional broodstock. Since the culture environment is quite different from their natural habitat, it is reasonable to assume stressors may arise and effect the normal reproductive progression of this species. The second study looked to make connections between physical stressors found in culture, cortisol and sex change. The results of the time trials showed the exogenous application of cortisol through an infused feed elicited a similar temporal plasma cortisol response as the physical net stressor. Though the cortisol-fed trial did produce a peak plasma cortisol higher than seen in any of the current studies or previous literature, studies with other species utilized two to four times higher cortisol concentrations in their infused feeds. Future work may repeat the current study with more individuals and higher concentrations of cortisol infused feed. With these changes, results may yield statistical significance, though the 11-30% sex reversal seen in females would be functionally significant in a true commercial setting. Since we know the importance of social interactions in this reef species, future studies could investigate other compounds important in behavior like AVT, or factors higher up the hypothalamic-pituitary-gonadal axis such as GnRH.

Controlling sex differentiation in a cultured species is also beneficial for commercial grow-out and profits. Not only do some sexes grow faster than their counterparts, accelerated reproductive development is detrimental to all growth. Since growth to market size and production of end filet is the ultimate goal of any aquaculture endeavor, precocious puberty is detrimental. In the third study, two treatments exhibited this accelerated reproductive development and sacrificed overall somatic growth. Additional studies may also perform proximate analysis on sampled tissue to tease out
the molecular composition of growth to see if cortisol has an effect upon muscle or lipid production in the filet. Since the control treatment also exhibited reproductive development, the results could not be directly correlated to the cortisol-infused diets. However, many untested possible stressors may be present in the culture environment and contributed to the accelerated puberty. Since the final samples of this study showed such advanced reproductive traits it is also difficult to ascertain if the sex determined by histology was initial differentiation or if sex change had already occurred. Future studies may repeat these methods but with periodic sampling to determine the window of differentiation in these precocious individuals.

Investigating the integrated roles of stress, growth, reproduction and behavior, we are moving closer to understanding the physiological processes of black sea bass. Once we fully comprehend the effects of rearing these fish in culture we can then proceed to ramp up production and provide a highly desirable and sustainable food source.
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APPENDIX A

RADIOIMMUNOASSAY PROCEDURE

Solutions

Phosphate Buffer for RIA (PBS-A), 0.1 M, pH 7.6

18.24 g Potassium Phosphate, diabasic, trihydrate (K₂HPO₄·3H₂O)
2.76 g Sodium Phosphate Monobasic (NaH₂PO₄)
8.76 g Sodium Chloride (NaCl)
1.00 g Sodium Azide (NaN₃)

Bring to 1000 mL with DI water. Adjust pH to 7.6. Store at 4 °C in RIA lab.

Phosphate Buffer for Homogenates (PBS-HOMO), 0.1 M, pH 7.3

1.78 g Sodium Phosphate, 2-basic, anhydrous (Na₂HPO₄)
1.38 g Sodium Phosphate Monobasic (NaH₂PO₄)
8.18 g Sodium Chloride (NaCl)

Bring up to 1000 mL with DI water. Adjust pH to 7.3. Store at 4 °C in RIA lab.

Standard Diluent, pH 7.4

100 mL PBS-A
900 mL DI water
7.6 g Sodium Chloride (NaCl)
10.0 g Bovine Serum Albumin (BSA)

Adjust pH to 7.4. Store at 4 °C in RIA lab.
3H Cortisol Working Stock

Allow the high stock (from manufacturer) to come to room temperature. Add 5 µL of high stock label to 50 mL standard diluent. Check the resulting solution by adding 100 µL to 800 µL standard diluent in a scintillation vial containing 4 mL ScintiVerse. Vortex and count. Adjust to obtain ~10,000-12,000 CPM/100 µL. Label the working stock bottle with the CPM/100 µL, the volume of the standard diluent, the activity (µCi) added (1 µL = 1 µCi), date, and your initials. Store at 4 °C in RIA lab.

Cortisol Antisera

Dilute high stock (UC Davis) 1:4000 in standard diluent. Store in glass vials at -20 °C.

Charcoal Dextran Solution

100 mL PBS-A
1 g Norit A Charcoal
0.1 g Dextran (MW ~70000) – stored at 4 °C

Allow the dextran to come to room temperature. Weigh and add dextran to PBS-A. Stir vigorously (on stir plate). Add charcoal. Stir until dissolved. Cover with parafilm and store at 4 °C in RIA lab.

Standards

Stock A: 0.01 g hydrocortisone in 250 mL 100% ethanol
Stock B: 1 mL stock A in 99 mL 100% ethanol
2000 pg/100 µL: 1 mL stock B in 19 mL 100% ethanol
1000 pg/100 μL: 10 mL 2000 stock in 10 mL 100% ethanol
500 pg/100 μL: 10 mL 1000 stock in 10 mL 100% ethanol
250 pg/100 μL: 10 mL 500 stock in 10 mL 100% ethanol
125 pg/100 μL: 10 mL 250 stock in 10 mL 100% ethanol
62.5 pg/100 μL: 10 mL 125 stock in 10 mL 100% ethanol
31.3 pg/100 μL: 10 mL 62.5 stock in 10 mL 100% ethanol
15.6 pg/100 μL: 10 mL 31.3 stock in 10 mL 100% ethanol
7.8 pg/100 μL: 10 mL 15.6 stock in 10 mL 100% ethanol
0.0 pg/100 μL: 10 mL 100% ethanol

Store all stocks and standards in 20 mL glass vials with parafilm at -20 °C. Bring to room temperature before opening.

**Standard Curve**

1. Bring solutions (³H cortisol, standards, & standard diluent) to room temperature before use. Turn on water bath (37 °C).

2. Label 24 - 12x75 glass tubes (#1-24).
   a. 1-3: Total Counts
   b. 4-6: NSB
   c. 7-8: 0 pg/100 μL standard
   d. 9-10: 7.8 pg/100 μL standard
   e. 11-12: 15.6 pg/100 μL standard
   f. 13-14: 31.3 pg/100 μL standard
   g. 15-16: 62.5 pg/100 μL standard
h. 17-18: 125 pg/100 μL standard  
i. 19-20: 250 pg/100 μL standard  
j. 21-22: 500 pg/100 μL standard  
k. 23-24: 1000 pg/100 μL standard  

3. Add 100 μL of the appropriate standard to its corresponding tube. (Add nothing to tubes 1-6).

4. Evaporate the ethanol under a stream of nitrogen at 37 °C (~45 min).
   a. During this time take antibody out of freezer.
   b. Take out repeat pipettor & 2-100 μL tips.
   c. Put away standards.

5. Add 200 μL standard diluent to all tubes using repeat pipettor (set at 2).

6. Vortex on max for 10 sec.

7. Add 100 μL standard diluent to tubes 1-6 using repeat pipettor (100 μL set at 1).

8. Add 100 μL antibody to tubes 7-24 using repeat pipettor (100 μL set at 1).

9. Add 100 μL ³H cortisol to all tubes using ³H repeat pipettor (100 μL set at 1).

10. Vortex all tubes gently (setting 4.5) for 3 sec.

11. Cover with parafilm and incubate at room temperature for 2 hours.
    a. During this time put away standard diluent, antibody, and ³H cortisol.
    b. Turn on centrifuge (4 °C) 30 min before use.
    c. Prepare an ice bath and place PBS-A in it to chill. Place charcoal dextran in ice on stir plate.
    d. Take out repeat pipettor and 3 tips – 100 μL and 250 μL.

12. Incubate assay tubes in ice bath for 5 min.
13. Add 500 μL cold PBS-A to tubes 1-3 using repeat pipettor (100 μL set at 5).
14. Add 500 μL cold charcoal dextran to tubes 4-24 using repeat pipettor (250 μL set at 2).
15. Vortex the tubes for 2 sec (setting 4.5).
16. Incubate on ice for 20 min.
   a. During this time, put away charcoal and PBS-A.
   b. Set up extra tubes to balance centrifuge (900 μL water).
17. Centrifuge the tubes at 4 °C and 3000 rpm for 15 min.
   a. During this time set up scintillation vials (label & fill with 4 mL scintillation fluid).
   b. Put ³H solids trash can next to bench.
18. Decant samples into scintillation vials.
19. Invert tubes once to mix and vortex for 5 sec.
20. Count each vial for 5 min (User 1 program).

Antibody Dilution Curve

Prepare the following antibody dilutions: 1:100, 1:500, 1:1,000, 1:5,000, 1:10,000, 1:20,000, and 1:50,000.

1. Bring solutions (³H cortisol, antibodies, & standard diluent) to room temperature before use. Take out repeat pipettor & 1-100 μL tip.
2. Label 27 – 12x75 glass tubes (#1-24).
   a. 1-3: Total Counts
   b. 4-6: NSB
c. 7-9: 0 pg/100 μL standard – 1:100 AB

d. 10-12: 0 pg/100 μL standard – 1:500 AB

e. 13-15: 0 pg/100 μL standard – 1:1,000 AB

f. 16-18: 0 pg/100 μL standard – 1:5,000 AB

g. 19-21: 0 pg/100 μL standard – 1:10,000 AB

h. 22-24: 0 pg/100 μL standard – 1:20,000 AB

i. 25-27: 0 pg/100 μL standard – 1:50,000 AB

3. Add 200 μL standard diluent to all tubes using repeat pipettor (set at 2).

4. Vortex on max for 10 sec.

5. Add 100 μL standard diluent to tubes 1-6 using repeat pipettor (100 μL set at 1).

6. Add 100 μL appropriate antibody to tubes 7-24.

7. Add 100 μL 3H cortisol to all tubes using 3H repeat pipettor (100 μL set at 1).

8. Vortex all tubes gently (setting 4.5) for 3 sec.

9. Cover with parafilm and incubate at room temperature for 2 hours.

   j. During this time put away standard diluent, antibody, and 3H cortisol.

   k. Turn on centrifuge (4 °C) 30 min before use.

   l. Prepare an ice bath and place PBS-A in it to chill. Place charcoal dextran in ice on stir plate.

   m. Take out repeat pipettor and 3 tips – 100 μL and 250 μL.

10. Incubate assay tubes in ice bath for 5 min.

11. Add 500 μL cold PBS-A to tubes 1-3 using repeat pipettor (100 μL set at 5).
12. Add 500 μL cold charcoal dextran to tubes 4-24 using repeat pipettor (250 μL set at 2).

13. Vortex the tubes for 2 sec (setting 4.5).

14. Incubate on ice for 20 min.

   n. During this time, put away charcoal and PBS-A.
   o. Set up extra tubes to balance centrifuge (900 μL water).

15. Centrifuge the tubes at 4 °C and 3000 rpm for 15 min.

   p. During this time set up scintillation vials (label & fill with 4 mL scintillation fluid).
   q. Put ³H solids trash can next to bench.

16. Decant samples into scintillation vials.

17. Invert tubes once to mix and vortex for 5 sec.

18. Count each vial for 5 min (User 1 program).

To determine which AB dilution to use, do the following calculations. First, average all replicates. Then, subtract the NSB value from each of the others. Then, divide the value for each 0 std - AB dilution into the total counts to obtain percent binding for each. Choose the AB dilution that produces 30-50% binding.
APPENDIX B:

PURIFICATION OF $^3$H CORTISOL STOCK USING SEP-PAK

Supplies:

Sep-Pak Plus C18 cartridges

Methanol (HPLC grade)

mQ water

0.15 M NaCl

10 cc syringe

10 mL pipette

Pump

Manostat tubing (1/16 x 1/32)

Water bath (37 °C)

Procedure:

• Set up apparatus accordingly:

• Precondition Sep-Pak C-18:

  o Wash sep-pak with the following:

    1. 10 mL 100% MeOH
    2. 10 mL mQ water
    3. 5 mL 0.15 M NaCl

  o Remove the plunger before attaching syringe to Sep-pak. Attach the syringe to the long end of the Sep-pak. Always load on this end. Never pull up on the plunger of the syringe if the Sep-pak is attached to it. Use a
10 cc syringe to load the above solution washes onto the Sep-pak in order.

Use a 10 mL pipette to transfer washes into syringe barrel. Load the Sep-pak slowly and consistently.

- Depending on amount of $^3$H stock label to be purified, the peristaltic pump set to 60 may be used to elute stock through sep-pak, or the 10 cc syringe.

- Load all $^3$H stock label onto the pre-conditioned Sep-pak using a 10 cc syringe or through a peristaltic pump set to 60 depending on the amount of label to be purified. Save the effluent to count.

- Wash with 10 mL 0.15 M NaCl. Save the wash to count. (This contains free $^3$H. Dispose of 3H waste in liquid waste can).

- Wash with 25 mL mQ water. Save the wash to count. (This contains free 3H. Dispose of 3H waste in liquid waste can).

- Elute the steroid label from the Sep-pak with 15 mL 100% MeOH. The elution, which may look cloudy, should be collected in a ground glass bottle that will hold ~200 mL.

- Dry the MeOH fraction under nitrogen or air in a warm water bath (37 °C), ~30 min.

- Add 100 mL RIA standard diluent buffer to reconstitute. Count 100 µL and adjust to 10,000 CPM/100 µL.

- Count 100 µL of each fraction saved above.
  - Add 100 µL of each solution to 800 µL standard diluent in a scintillation vial containing 4 mL ScintiVerse.
  - Vortex & count.
• To adjust the final solution to 10,000-12,000 CPM/100 μL:
  o \( \frac{\text{Obtained CPMs/100 mL}}{\text{Desired CPMs/100 mL}} \times \text{Volume} = X \)
  o \( X - \text{Volume} = \text{Volume to add} \)
  o Example: \( \frac{12,000}{10,000} \times 100\text{mL} = 120 \text{ mL} \)
    
    \( 120 \text{ mL} - 100 \text{ mL} = 20 \text{ mL} \)
  o Add slightly less than 20 mL buffer to be conservative and you should get
    10,000 CPM/100mL.
APPENDIX C:

GONAD PHOTOMOSAIC GALLERY – Chapter III - Cortisol and Sex Differentiation in Black Sea Bass

Final Determined Sex, Cortisol Treatment, (Mass (g), Total Length (cm))

Male, Control treatment (29.6g, 12cm).
Female, Control treatment (35g, 13cm).

Male, Control treatment (34.8g, 13.25cm).
Male, Control treatment (22g, 11.6cm).
Female, Control treatment (6.9g, 7.8cm).
Male, Low treatment (15.9g, 10.25cm).
Male, Low treatment (29.1g, 12.5cm).
Male, Low treatment (29.6g, 12cm).
Male, High treatment (48.2g, 15cm).
Female, High treatment (18.8g, 10.25cm).
Male, High treatment (25.5g, 12.25cm).
Male, High treatment (31.1g, 12cm).
Male, High treatment (15.9g, 10.25cm).
Male, High treatment (35g, 12.9cm).
Male, Control treatment (29.6g, 12cm).
APPENDIX D
BLACK SEA BASS INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
APPROVAL DOCUMENTATION
The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a report with regard to the involvement of animals in this study before that date. If your study is still active, you may apply for extension of IACUC approval through this office.

The appropriate use and care of animals in your study is an ongoing process for which you hold primary responsibility. Changes in your protocol must be submitted to the IACUC for review and approval prior to their implementation.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Glad Porsche, UNH Health Services.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File