

ABSTRACT

HOLLER, BRITTANY LYNNE. Effect of Cortisol and Stocking Density on Sex Determination and Growth in Southern Flounder (*Paralichthys lethostigma*). (Under the direction of Russell John Borski).

Environmental sex determination (ESD) is the phenomenon by which environmental factors regulate sex determination, typically occurring during a critical period of early development. Previous studies have shown that southern flounder (*Paralichthys lethostigma*) exhibit temperature-dependent sex determination, a form of ESD. The ESD response appears to be restricted to the presumed XX female genotype, as high or low temperatures skew sex ratios toward males. Although meiogynogenesis may yield all genotypic female (XX) progeny in XX/XY species, our work in *P. lethostigma* has yet to definitively establish that this species demonstrates this sex chromosome system. The purpose of this study was to evaluate the influence of stocking density and cortisol on sex determination using all XX populations of *P. lethostigma*. The stress corticosteroid, cortisol, was investigated as it may mediate sex determining processes associated with environmental variables.

Cortisol was applied at 0, 100 or 300 mg/kg of gelatin-coated feed. Fish were fed intermittently prior to and just through the period of sex determination. Gonadal P450 aromatase (*cyp19a*) and forkhead transcription factor L2 (*FoxL2*) mRNA levels, expression of which rise in ovarian development, were measured as molecular markers for female differentiation. Müllerian inhibiting substance (MIS) was used as a marker of male gonadal development, as its expression rises in testes. Partial sequences of *FoxL2* and MIS genes from *P. lethostigma* were cloned. The *FoxL2* translated sequence was shown to be 100 % identical to that of Japanese flounder (*Paralichthys olivaceus*), 87 % identical to clawed frog (*Xenopus laevis*), 83 % identical to chicken (*Gallus gallus*), 79 % identical to rat (*Rattus*

norvegicus) and 78 % identical to human (*Homo sapiens*). The southern flounder MIS translated sequence was 98 % identical to the published *P. olivaceus* MIS sequence.

We found that control gynogens fed no cortisol showed female-biased sex ratios approaching 100 %, suggesting the species follows an XX/XY system of genotypic sex determination. Cortisol treatment yielded male biased sex ratios by measures of gonadal markers. This was confirmed by gonadal histology from a limited number of fish. Fish held at a density of 250 fish/m² and provided control feed with no added cortisol had populations consisting of 90.9 % females (N = 11). Chi square analyses indicate that cortisol produced a dose-dependent skewing of sex ratios highly biased toward males (P < 0.0001). Fish in the low cortisol treatment yielded 28.57 % females (N = 28), while those treated with 300 mg cortisol/kg feed showed a sex ratio of only 13.33 % females (N=15). Early exposure to cortisol did not significantly alter growth of fish over the 78-day trial.

We then evaluated the effects of stocking density on growth and sex determination in southern flounder. In this study, fish were grown for 78 days at densities of 250, 500, 1000, and 1500 fish/m² in duplicate. Density had little effect on daily weight gain or daily length gain over the course of the experiment. Stocking density did not significantly alter final sex ratios (P = 0.3336, Chi-Square analysis). A density of 250 fish/m² produced 90.91 % females (control for the cortisol experiment, N = 11), 500 fish/m² yielded 86.67 % females (N = 15), and 1000 fish/m² yielded 100 % females (N = 16). The highest density of 1500 fish/m² produced the most masculinization (18.75 % males: 81.25 % females, N = 16). These results suggest that cortisol is a critical mediator of sex determination in southern flounder, promoting masculinization at concentrations and exposure rates that do not impact growth. Stocking density does not appear to affect sex determination, and densities as high

as 1000 fish/m² may be optimal for promoting the faster-growing female phenotype with little impact on growth rate.

Effect of Cortisol and Stocking Density on Sex Determination and Growth in
Southern Flounder (*Paralichthys lethostigma*)

by
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DEDICATION

To my Nana, Josephine, to whom love was the highest law and for whom family was everything.

BIOGRAPHY

Brittany Lynne Holler grew up in California as the second child of Dale and Katheryn. She and her older brother Alan and their younger sister Nicole enjoyed a childhood of adventures together, with much love from their family. She graduated from William Smith College in Geneva, New York in 2008 with Latin praise, earning her bachelor's degree in Environmental Studies and a minor in biology. Brittany decided to attend North Carolina State University to pursue a master's degree in physiology that incorporated her interests of environment and biology. She will be staying in Raleigh to attend the College of Veterinary Medicine.

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INTRODUCTION

Southern flounder (*Paralichthys lethostigma*) is a euryhaline species native to the United States. They are endemic along the south east coast, including the estuarine waters of North Carolina. Southern flounder are adept at converting feed protein into body mass, with conversion ratios below 2:1 (Daniels 2000). They can grow up to 1.3 mm/day (Reichert and Van Der Veer 1991). Once they have undergone metamorphosis, juveniles move to lower saline environments including riverbeds making this fish suitable for growth in low saline or freshwater environments (Peters and Kjelson 1975, Guindon and Miller 1995, Daniels *et al.* 1996). The attributes of fast growth, good food conversion and their ability to thrive in low salinity environments make southern flounder an ideal species for aquaculture, especially in the coastal plain of North Carolina.

Commercial catch of flounder in the United States (including southern flounder, among other varieties) totaled 20 billion tons in the 1980s (Daniels *et al.* 2010). Due to a collapse in fisheries, flounder has been subsequently deemed as overfished. Environmental control measures instituted in the offshore waters of the United States limit commercial capture. The Magnuson-Stevens Fisheries Conservation Act was renewed in 2007, serving as a major legal influence in reduction of overfishing in United States coastal waters (Review of Fisheries 2010). Currently, the maximum harvest of flounder in the U.S. is capped at 5 billion tons (Daniels *et al.* 2010). In 2006, flatfish represented 19 % of the total value of commercial landings, which was greater than either tuna (9 %) or salmon (17 %) (Review of Fisheries 2010). Decreased fishing activity along with increased consumer preferences for healthy protein sources has increased demand for seafoods, particularly of high-value marine

finfish. Indeed, from 2000 to 2010, imports of seafood have increased 40 % in the U.S. (Review of Fisheries 2010). Some of this market demand must eventually be met through domestic aquaculture production. Total worldwide fish consumption in 2006 was 110.4 MT, including both captured and aquaculture sources (State of World Fisheries 2009). These trends suggest a significant market to support the growth of aquaculture of southern flounder and other species. To meet this growth, practical research on aquaculture of southern flounder and other piscine species must be carried out.

While in some species, males are valued because they grow larger or faster than females, the opposite is true of southern flounder. In this sexually dimorphic species, females grow three times larger than males, while males may never reach market size (Morse 1981, Matlock 1991, Fitzhugh *et al.* 1996; Borski *et al.* 2010). Because of this, production of female flounder is more profitable than males in aquaculture. Profits can therefore be maximized if monosex female populations can be realized. For this reason, it is important that the sex of flounder be controlled or manipulated to produce the highest proportion of females.

Sex Determination

Sex determination is related to, but slightly different from sex differentiation. Sex determination includes the mechanisms that may direct sex differentiation (Baroiller *et al.* 1999). Sex differentiation is the process of development of testes or ovaries from an undifferentiated or bipotential gonad (Baroiller *et al.* 1999). Once sex has been determined, southern flounder do not switch sex, and thus the species is gonochoristic. Sex determination

in southern flounder is under both genetic and environmental influences (Godwin *et al.* 2003, Luckenbach *et al.* 2009, Borski *et al.* 2010). Genes play a part in determining the sex of southern flounder as genotypic XY males will become male regardless of the environment whereas genotypic XX females are susceptible to environmental influences like temperature and can differentiate as males. The developmental period of sex determination is thought to be complete by the time fish are 75 mm TL, although it is still difficult to distinguish sex by gonadal histology at this stage (Luckenbach *et al.* 2004). Gonadal sex of southern flounder is more readily distinguished by histology when fish have attained 150 mm TL (Luckenbach *et al.* 2003). Once the window of sex determination has passed and gonads begin to differentiate, flounder can no longer change sex even if exposed to unfavorable conditions. Use of sex-specific molecular biomarkers enables observation of sex ratios in southern flounder at the end of sex determination, precluding the need to conduct extended growout trials of animals required to distinguish sex by histology.

Meiogynogenesis and Production of Faster-Growing All Female Southern Flounder

Chromosomal set manipulations through the process of meiogynogenesis have been established in southern flounder (Luckenbach *et al.* 2004, Morgan *et al.* 2006). This allows exclusive production of progeny with only the presumed XX female genotype. Briefly, homologous or heterologous sperm is UV-inactivated to eliminate contributions of the paternal Y or X chromosomes (Valcárcel *et al.* 1994; Luckenbach *et al.* 2004; Morgan *et al.* 2006). The sperm remains functionally mobile and capable of activating early developmental processes. Stripped eggs are then activated by the UV-irradiated sperm, and

briefly cold-shocked (Luckenbach *et al.* 2004) or pressure-shocked (Morgan *et al.* 2006) to retain the polar body (an X chromosome) that is typically extruded with normal fertilization (Felip *et al.* 1999). The outcome is generation of diploid eggs composed entirely of the XX genotype, which should develop as females. In the subsequent (F1) generation, diploid meiogynogens (XX progeny) resulting from these procedures are sex reversed to males using masculinizing temperature (see below) and raised to sexual maturity to serve as broodstock. Sperm from male XX broodstock fish, which contain an X chromosome, are then used to fertilize eggs from females. This breeding scheme produces progeny composed entirely of the XX genotype that should develop as females. Meiogynogenesis has been successfully achieved in southern flounder (Luckenbach *et al.* 2004, Morgan *et al.* 2006), summer flounder (*Paralichthys dentatus*, Colburn *et al.* 2009) and Japanese flounder (*Paralichthys olivaceus*, Yamamoto 1999).

Genetic Patterns of Sex Determination

Previous studies in the Japanese flounder (*P. olivaceus*) using gynogens (all XX genetic offspring) and progeny testing demonstrate that this species has the XX/XY system of genetic sex determination (Yamamoto 1999), in which the homogametic sex is female (XX) and the heterogametic sex is male (XY). Sex may be genetically determined by the ZW/ZZ system instead of the XX/XY system. For such species, males are homogametic and females are heterogametic, therefore the paternal parent contributes the “Z” sex chromosome to all offspring, and the maternal parent contributes either a W or a Z sex chromosome (Ezaz *et al.* 2006). In this system, females are actually heterogametic (ZW) and males are

homogametic (ZZ), in contrast to the XX/XY sex determination system described above (Ezaz *et al.* 2006). This only occurs in a few species of fish, and is not likely the mechanism of genetic sex determination found in southern flounder. Indeed, initial evidence suggests that *P. lethostigma* follows the XX/XY system of genetic sex determination. This is because all XX genotypic populations of southern flounder, generated through meiogynogenesis protocols, yield a higher proportion (> 50 %) of phenotypic females while a ZW/ZZ system should yield a maximum of 50 % females (unpublished observations). Previously, we have not been able to produce near 100 % phenotypic females despite the use of all XX genotypic female flounder populations and the presumed XX/XY systems of genetic sex determination. This is likely due to the influence of as yet undefined environmental variables that can masculinize a portion of the XX populations of southern flounder. A critical element to confirming that southern flounder follow the XX/XY system of genetic sex determination is to demonstrate that nearly 100 % females can be produced from a population of fish composed entirely of the XX genotype.

Environmental Sex Determination (ESD) in Flounder

Sex determination in fishes that exhibit ESD may be influenced by temperature, social hierarchy, and pH, among other factors (Baroiller *et al.* 1999). Thermosensitive sex determination is well studied in birds and amphibians. In the European pond turtle (*Emys orbicularis*), incubation of eggs at temperatures of 30 to 35 °C increased the levels of aromatase, an enzyme critical to estradiol-17 β production, and yielded higher proportions of females than incubation at 25 to 30 °C (Desvages and Pieau 1992). In some fish species, the

effect of temperature on sex determination may be the opposite, where greater proportions of males result from higher temperatures (Baroiller *et al.* 1999). Atlantic silversides (*Menidia menidia*) displaying temperature-dependent sex determination show increased levels of aromatase expression at 15 °C compared to expression levels at 28 °C (Duffy *et al.* 2010). Temperature is a critical factor affecting ESD in southern flounder and other flounder species. Thermal stress increases male differentiation above or below an optimum temperature in flounder species (Luckenbach *et al.* 2003, Luckenbach *et al.* 2009 for review). The temperature that yields equal proportions of male and female southern flounder is 23 °C within normal populations (Luckenbach *et al.* 2003). However, at lower (18 °C) or higher (28 °C) temperatures, a male biased sex ratio was observed, resulting in a U-shaped curve in temperature-dependent sex determination (Luckenbach *et al.* 2009). This is similar to a pattern seen in *P. olivaceus* where water temperatures of 20 °C produce 50:50 female: male sex ratios, while temperatures above 23 °C or below 15 °C produce > 90 % males (Yamamoto 1999, Luckenbach *et al.* 2009). High temperature has also been shown to masculinize summer flounder (*P. dentatus*) (Colburn *et al.* 2009). Therefore, temperature has been isolated as a major environmental parameter regulating sex determination in flounder species (genus *Paralichthys*), in which non-permissive temperatures can cause masculinization of genotypic females. As mentioned previously, the sensitivity of sex determination to temperature has been exploited for producing gynogenetic male broodstock with the XX genotype.

Fish are naturally found in many different aquatic environments at various depths and light levels. Light intensity may regulate sex determination in fish species (Baroiller *et al.*

1999). Light intensity of 100 and 1500 lux had little effect on sex ratios of southern flounder, while a very low light intensity of 5 lux caused a significant masculinization and deviation from the 50:50 female: male ratio (Turner 2008).

Preference of environmental color also varies among species (Marshall and Vorobyev 2003). Tank color has previously been shown to affect sex determination in southern flounder (Turner 2008). Grey and black tank colors do not significantly alter sex ratios from the normal 50:50 female: male distribution in southern flounder, while light blue colored tanks produced male - biased sex ratios (Turner 2008). Southern flounder raised in blue tanks also displayed elevated cortisol during the window of sex determination relative to fish raised in grey and black tanks, suggesting this stress steroid might mediate the masculinizing effects of background color (Turner 2008). With regard to the visible color spectrum, studies show that fish see color in a similar way to humans (Vanleeuwen *et al.* 2007). Fish species inhabiting marine and freshwater environments experience color vision differently from one another. Marine fishes tend to see blue and yellow colors, whereas freshwater fishes tend to see red and green (Marshall and Vorobyev 2003). Summer flounder (*P. dentatus*) showed neural responsiveness to colors in the blue to yellow wavelength range, but not to red wavelengths (Horodysky *et al.* 2007). As its congener, *P. lethostigma* is likely to have a similar responsiveness to color, and it is possible that blue color evokes a stress response that causes an increase in cortisol, which in turn may alter key genes involved in sex determination and gonadal differentiation. Further studies are required to assess if cortisol regulates sex determination and the color attribute (lightness or hue) that may be responsible

for controlling sex differentiation in *P. lethostigma*. The effects of cortisol on sex determination in fishes will be further discussed below.

Density and ESD

Density and other social factors have been shown to affect ESD in several species of fish (see Baroiller *et al.* 1999 for review). When densities become too low in some species, for example the gonostomatid (*Gonostoma bathyphylum*), some individuals undergo sex inversion (Badcock 1986). In other species such as the Mediterranean rainbow wrasse (*Coris julis*), sex inversion occurs when densities become too high (Lejeune 1987). Isolation leads to male differentiation in the paradise fish *Macropodus opercularis*, and increasing the number of fish increases the proportion of females (Francis 1984). The American eel (*Anguilla rostrata*) is sensitive to rearing density as well, and differentiates into males at higher densities (Krueger and Oliviera 1999).

Density has been shown to increase cortisol levels in many species, indicating social stress. Stocking density was shown to increase cortisol secretion in brook char (*Salvelinus fontinalis*) (Vijayan and Leatherland 1990). Other species displaying elevated cortisol levels at higher stocking densities include sea bream (*Sparus aurata*) (Tort *et al.* 1996), Japanese flounder (*P. olivaceus*) (Bolasina *et al.* 2006), shortnose sturgeon (*Acipenser brevirostrum*) (Wuertz *et al.* 2006), carp (*Cyprinus carpio*) (Ruane *et al.* 2002), rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) (Ramsay *et al.* 2006). Sex ratios in zebrafish are determined by growth rates resulting from differential feed consumption and density is thought to indirectly affect sex determination by altering the amount of feed

consumed by each fish (Lawrence 2007). It has been proposed that crowding causes stress in European eels, increasing cortisol secretion and delaying differentiation so that the number of males increases (Roncarati *et al.* 1997). Crowding has been shown to reduce growth rates and increase mortalities as well as cause leucopenia or suboptimal white blood cell levels in salmonids (Pickering and Pottinger 1987a). Overall, it appears that crowding stress can increase cortisol and cause male differentiation in a variety of fish species. Whether stocking density elicits a stress response, and whether this response regulates sex determination in flounder species is unknown and will be a focus of these investigations. It is also critical to determine the range of stocking densities that can be used to maximize production of females in southern flounder aquaculture.

Cortisol as a Marker of Stress and its Role in ESD

Stress can be defined as a response reaction by a fish that may alter the individual's state of homeostasis (Barton and Iwama 1991). Cortisol is one of the major hormones involved in stress adaptation where it regulates gluconeogenesis and other metabolic processes critical for providing glucose as an energy substrate. In fish, this steroid hormone is produced by the interrenal gland in response to adrenocorticotropin hormone (ACTH) released from the pituitary (Barry *et al.* 1995a). Cortisol is classically used as an indicator of stress response in fish (Takahashi *et al.* 1985, Barry *et al.* 1995a, Barton and Iwama 1991, Seth and Brown 1978, Sink *et al.* 2007). Handling, sorting, and transport have long been studied as sources of stress (Barton and Iwama 1991). Unfavorable environmental

conditions, for instance suboptimal temperatures and densities, may be perceived as stressful and result in enhanced production of cortisol.

Temperature shock has been demonstrated to cause acute or chronic stress as evident by elevated cortisol levels. Low (5 °C) or high (up to 35 °C) temperatures have been used to induce an acute cortisol spike in many species of fish (Pickering *et al.* 1987a, Barton and Iwama 1991). Non-permissive temperatures have been shown to increase cortisol concentrations in *P. olivaceus* (Yamaguchi *et al.* 2010) and pejerrey (*Odontesthes bonariensis*) (Hattori *et al.* 2009). In the pejerrey, elevated temperatures augmented whole body cortisol levels and this was correlated with an increased percentage of males (Hattori *et al.* 2009). Not all species show a male-biased sex ratio under stressed conditions. The common carp (*Cyprinus carpio* L.) shows reduced 11-ketotestosterone levels in response to prolonged feeding of cortisol in pubertal fish (Consten *et al.* 2002). Chronic stress causes decreased levels of both estrogen and testosterone *in vitro* in rainbow trout (*Salmo gairdneri*) (Carragher and Sumpter 1990). Male Brown trout (*Salmo trutta* L.) experienced a decline in circulating testosterone levels following a month of chronic confinement stress (Pickering *et al.* 1987b). As mentioned previously, temperatures above 23 °C or below 18 °C in southern flounder cause male bias in sex ratios (Luckenbach *et al.* 2003). Therefore, it is possible that low and high temperature may elicit rises in cortisol in southern flounder causing male-biased sex ratios.

Stocking density increases cortisol concentrations in some flounder species. Winter flounder have increased cortisol levels at increased stocking densities (Sulikowski *et al.* 2006). Cortisol levels rise during the period of sexual differentiation in *P. olivaceus* raised at

a high temperature where a higher proportion of males is produced (Yamaguchi *et al.* 2010). The present studies will establish if cortisol may alter sex determination in southern flounder, as a mediator or causative factor regulating sex determination responses observed with various environmental factors.

Foxl2 and Aromatase

Aromatase (Cytochrome P450 aromatase or *cyp19a1a*) produced in the gonads and the brain (*cyp19a1b*) catalyzes the conversion of testosterone into estradiol 17- β . Its under- or over- expression in the gonads directly affects the amount of estradiol synthesized by an individual (Baroiller *et al.* 1999), and hence the subsequent sex of individuals. Its expression levels are higher in females than males. Early studies in reptile ESD have noted that reduced aromatase activity, which leads to reduced estradiol levels, favors male development (Vogt and Bull 1982, Bull and Vogt 1979). It has been demonstrated that aromatase levels are predictive of sex in flounder species; males express very low levels, and females express significantly higher levels of aromatase during the period of sex determination and differentiation (Kitano *et al.* 1999, Luckenbach *et al.* 2005, Yamaguchi *et al.* 2010). Application of an aromatase inhibitor or testosterone caused male differentiation of *P. olivaceus* (Kitano *et al.* 2000). Luckenbach *et al.* (2005) used aromatase expression to distinguish female and male southern flounder as small as 70 mm TL, which is below the size at which this can be observed by gonadal histology.

Forkhead transcription factor L2 (FoxL2) is expressed earlier in gonads than aromatase and promotes aromatase transcription in the goat (*Capra hircus*), and in the grouper

(*Epinephelus merra*) (Pannetier *et al.* 2006, Alam *et al.* 2008). Because it is only associated with female development, its expression does not have to be quantified, and its presence alone may serve as an indicator of female sex determination (Yamaguchi *et al.* 2007). In mice, FoxL2 ablation causes male differentiation, indicating its necessity in enhancing aromatase gene expression and subsequent female development (Uhlenhaut *et al.* 2009). Yamaguchi *et al.* (2010) recently demonstrated a much cleaner distinction in expression of FoxL2 compared with expression of aromatase during gonadal sex differentiation in Japanese flounder. For these reasons, it may serve as an additional and perhaps a better biomarker than aromatase for determination of the gonadal sex in ESD studies of southern flounder, precluding the need to conduct lengthy growout trials of animals required for distinguishing gonadal sex by histology.

Müllerian Inhibiting Substance (MIS)

A male specific marker that can be used to positively identify males during sexual differentiation is müllerian inhibiting substance (MIS), also known as anti-müllerian hormone (AMH). MIS is a member of the transforming growth factor β superfamily and binds the Type II serine-threonine kinase receptor (Yoshinaga *et al.* 2004, de Caestecker 2004, Shiraishi *et al.* 2010). Ligands binding to the type II receptor enable the type I receptor to form a stable ternary complex and facilitate phosphorylation of downstream elements (de Caestecker 2004). In mammalian males, MIS causes regression of the müllerian ducts and is involved in male differentiation (Blázquez *et al.* 2009, Fernandino *et al.* 2008, Guerrero-Estevaz *et al.* 2010, Paibomesai *et al.* 2010, Pala *et al.* 2008, Shiraishi *et al.* 2010). Teleosts

do not have müllerian ducts. However, this hormone is involved in regulating male development. MIS shows an expression pattern that is opposite to that of aromatase (Fernandino *et al.* 2008). In juvenile fish, MIS is found in testes. It is not expressed in brain, heart, liver, spleen, or ovary (Yoshinaga *et al.* 2004, Yamaguchi *et al.* 2010). MIS is expressed during sexual differentiation in Japanese flounder, among other species (Wu *et al.* 2010). In *P. olivaceus*, MIS is first expressed in undifferentiated gonads as early as 50 days after hatching (DAH), and present in testes at 60 DAH but is severely down-regulated in ovaries from this time forward (Yoshinaga *et al.* 2004). As with FoxL2, MIS expression precedes sexual maturation and its transcription can be measured prior to histologically discernable sexual differentiation. Evidence also suggests that MIS is involved in suppressing estrogen production and preventing female differentiation, as it lies upstream of genes encoding FoxL2 and aromatase (Fernandino *et al.* 2008). During follicular atresia, MIS may be transcribed in the ovaries of some species, which prevents aromatase transcription from occurring, blocking conversion of testosterone into estrogen. Expression of MIS varies among teleost species; it appears to be expressed differentially in mature males and females, or it may be expressed in gonads of both sexes (Fernandino *et al.* 2008). Measuring gene expression of MIS is therefore useful as a male molecular marker during early sexual development, but may not be effective in mature fish of all species.

The effect of Cortisol on Aromatase Expression

Cortisol has been shown to reduce sex steroid levels in several species by various approaches. Cultured rainbow trout ovarian follicles show suppression of estradiol and

testosterone levels in a dose dependent manner in response to repeated cortisol exposure (Carragher and Sumpter 1990). However, the mechanism of action is not completely known. Like cortisol, estrogen and testosterone are steroid hormones, and high levels of one steroid can affect levels of another hormone along the same pathway (Hadley and Levine 2007). There is some evidence that steroid response elements are located upstream of the aromatase gene (Luckenbach *et al.* 2009). Glucocorticoid response elements have been identified upstream of aromatase in the protogynous goby (*Gobiodon histrio*) (Gardner *et al.* 2004). *P. olivaceus* has an estrogen response element in the promoter region of the aromatase gene (Yamaguchi *et al.* 2007). FoxL2 binds to a forkhead transcription site in the promoter region of aromatase, and a cAMP response element is downstream of the FoxL2 binding site (Yamaguchi *et al.* 2007). Exogenous cortisol application has been shown to cause downregulation of glucocorticoid receptors in hepatocytes, leukocytes, and gill tissue (Mommsen *et al.* 1999, Vijayan *et al.* 2003, Maule and Schreck 1990, Pottinger and Pickering 1990, Shrimpton and McCormick 1999, Shrimpton and Randall 1994). Cortisol administration also downregulates estradiol receptors in the liver and plasma in juvenile rainbow trout (Pottinger and Pickering 1990). Direct binding of excess cortisol to other p450 enzymes, such as aromatase, may cause steric hindrance, preventing testosterone from binding, and limiting the production of estrogen. Reduced expression of p450 enzymes, as well as steric hindrance of the enzymes expressed would cause a buildup of testosterone and lower estrogen levels. This in turn would result in excess testosterone and cause male development.

GOALS AND OBJECTIVES

Southern flounder and other *Paralichthys* species show sexual dimorphism in growth whereby females attain sizes two to three times greater than males. Therefore, it is critical to maximize production of females for aquaculture. Meiogynogenetic protocols now allow for production of pure populations of flounder with the XX female genotype. However, several environmental factors have been shown to masculinize genotypic females, causing production of slower growing, less desirable male flounder. Although stocking density is known to regulate ESD processes in some fishes, nothing is known about its effect on sex determination in flounder species. Moreover, cortisol is thought to be a primary mediator of stress responses in vertebrates and its upregulation may be a crucial mechanistic link in mediating the effects of suboptimal environmental variables known to skew sex ratios in flounder. Therefore, we are interested in determining if this steroid regulates sex determination and growth in southern flounder.

The objectives of this thesis were to:

- 1) Evaluate whether stocking density regulates sex determination and growth of all XX female genotypic populations of southern flounder. We will use a range of stocking densities that fall below and above those typically used (750 fish/m²) for growout of southern flounder.

- 2) Determine if periodic cortisol treatment prior to and during the window of sex determination can exert masculinizing effects and alter growth rate of all XX populations of flounder
- 3) Establish whether aromatase, FoxL2 and MIS gene expression can be effectively used in combination to assess sex of flounder well before histologically discernable sex differentiation. For these studies FoxL2 and MIS genes were sequenced in southern flounder and mRNA was measured along with aromatase in gonads of individuals treated with cortisol and stocked at different densities. Identity of females and males based on expression of gonadal markers was confirmed with histological analyses of gonads.

MATERIALS AND METHODS

Animals

Male XX broodstock were produced through meiogynogenesis (Morgan *et al.* 2006) and masculinized with high temperature (Luckenbach *et al.* 2003) at the Lake Wheeler fish laboratory at North Carolina State University. Fish were subsequently grown out to maturity and held in the laboratory of our collaborator, Dr. Wade Watanabe at University of North Carolina Wilmington. Sperm from these fish was used for fertilization of eggs stripped from female fish. All XX progeny were grown out to approximately 43 mm TL by Keith Hairr of Carolina Flounder, LLC (Wallace, NC). Fish were then transported to the Lake Wheeler fish laboratory, bulk weighed and counted and stocked into three identical recirculating systems each equipped with four 100 – L grey tanks. The four tanks in one system were stocked with fish at a density of 250 fish/m² (125 fish/tank) and served as groups fed cortisol at 100 and 300 mg/kg feed (N= 2 tanks/group). Fish were stocked in the other two systems in duplicate at four different densities; 250, 500, 1000 and 1500 fish/m². The group containing 250 fish/m² also served as controls for the cortisol treatment groups. Activated charcoal was used to absorb any cortisol liberated in culture water. Charcoal was rinsed thoroughly and held in fine mesh bags (Aquatic Ecosystems, part no. MB1) attached to the water inlet spigots in each tank as well as to the sump of each recirculating system. A subsample of individuals were weighed and measured for total length prior to the start of the experiment (day 0 of the study). Fish were fed *ad libitum* four times daily with a commercial feed (Skretting Feeds; New Brunswick, Canada). Feed consumption was monitored beginning 14 days after initiation of the study. The recirculating systems were regularly backwashed and replaced

with fresh culture water. Pre-mixed salt (Crystal Sea Marine mix; Baltimore, MD) was added as necessary to maintain similar salinities among the three systems. Salinity was decreased equally among all systems from 24 ppt to 4 ppt over the duration of the study. Photoperiod was maintained on a 12L: 12D schedule. Light intensity was maintained at approximately 100 lux and temperature at 23.5 °C. These environmental parameters along with grey tank color are those previously shown to maximize production of females (Luckenbach *et al.* 2003, Turner 2008). Salinity, dissolved oxygen, and water temperature were monitored daily. Water hardness, alkalinity, ammonia, nitrites and nitrates were monitored weekly and maintained at similar levels across the recirculating systems. Water quality parameters fell well within the tolerance levels for flounder.

Cortisol Feed

Feed was prepared according to King and Berlinsky (2006). Cortisol was dissolved in 1 mL of ethanol, then mixed into unflavored Knox gelatin prepared according to package directions. This mixture was spread evenly over 0.5 kg of feed. Treatment groups included 0, 100 and 300 mg cortisol/kg feed. Fish were fed cortisol treated feed (and gelatin control feed) once a day every three days for two weeks up to 43.8 mm TL (0 to 14 days), then given a two week respite to prevent chronic or pharmacological steroid effects and potential receptor downregulation, and then fed cortisol again once a day every three days for two weeks (from 55.2 mm at 26 days) until they reached the point of molecularly discernable sex determination with a body size of 76.51 mm TL at 42 days.

For terminal sampling, fish were anesthetized with a lethal dose (300 mg/L) of MS - 222 dissolved in oxygenated tank water and buffered with sodium bicarbonate. Fish in cortisol treated tanks were collected every 24 hours for the first two weeks (six fish/tank). These samples were unfortunately lost due to equipment failure. Gonads were also collected from fish at 14, 27, 42, 69, and 78 days post stocking (equal to 90 to 189 days post hatch, DPH). This periodic sampling allowed maintenance of a similar total biomass throughout the experiment. For gonad sampling, an incision was made encircling the body cavity, and gonads were carefully removed with forceps and placed in a labeled microcentrifuge tube and snap frozen in liquid nitrogen. Samples were stored at -80 °C until processing. Fish weight and total length were measured at each sampling point. Gonadal tissues from 30 fish from each tank were also collected at the termination of the study (78 days post stocking) for assessment of sex-specific mRNA markers and determination of sex ratios. Fish were subsequently grouped by treatment and moved to larger tanks for further grow out. Twelve fish were sampled for gonads from each treatment for histological analysis.

Histology

Gonads were fixed in 10 % neutral buffered formalin for 48 to 72 hours. After this time gonads were washed in two rinses of 50 % ethanol, and then washed in 70 % ethanol before being stored in 70 % ethanol until processing. Gonads were processed and embedded by the histopathology department at North Carolina State University College of Veterinary Medicine. Paraffin blocks were placed on ice to cool for 20 minutes. Tissues were then sectioned at 5 µm thickness. Sections were melted on a glass slide in a 42 °C, clean water

bath and then dried for 15 minutes at 60 °C. Sections were deparaffinized by immersion into Safeclear II (xylene substitute) for 5 minutes. They were rehydrated for 5 minutes in 100 % ethanol, then 5 minutes in 70 % ethanol, and finally 1 minute in tap water.

Gonadal sections were stained with hematoxylin and counterstained with eosin according to Fisher *et al.* (2007). Rehydrated sections were stained in hematoxylin for 30 seconds, then rinsed in two changes of tap water for 30 seconds. Slides were placed in 1 % aqueous eosin Y for 10 to 30 seconds, then rinsed in two changes of tap water for 30 seconds. They were then dehydrated with two changes of 95 % ethanol, followed by two changes of 100 % ethanol for 30 seconds each. Alcohols were extracted with two changes of Safeclear II. One drop of permount was added to each slide and a coverslip was placed on top. Slides were permitted to dry at room temperature for at least 24 hours before handling. Sex of gonads was identified according to Luckenbach *et al.* (2003).

Isolation of RNA

Tissues were homogenized in 1 mL of TriReagent (Molecular Research Center, Inc, Cat. No. TR 118), using 6 µL poly acryl carrier (Molecular Research Center, Inc, Cat. No. PC 152) in a bead homogenizer. Once thoroughly homogenized, samples were incubated at room temperature for 5 minutes. 100 µL of 1-bromo-3-chloropropane (BCP) (Molecular Research Center, Cat. No. BP 151) was added to each tube, and they were shaken for 15 seconds. Homogenate was incubated at room temperature for 15 minutes and then centrifuged at 12,000 g, at 4 °C for 15 minutes. 400 µL of the clear, aqueous layer was transferred to a microcentrifuge tube containing 500 µL isopropanol. Tubes were mixed by

inversion and incubated at room temperature for 10 minutes. Samples were then centrifuged at 8,000 g, at 4 °C for 8 minutes. Isopropanol was aspirated from tubes, and precipitated pellets were washed twice in 1 mL of chilled 75 % ethanol with 0.3 M sodium acetate. Ethanol was removed by aspiration and micropipette. Pellets were resuspended in 20 µL of nuclease free water. RNA samples were quantified using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and qualified by 1 % gel electrophoresis. Any samples not showing clearly discernible 18S and 28S ribosomal bands were discarded. Samples were stored at -80 °C for a few days until RNA from all samples had been isolated. Samples were diluted to 500 ng/µL and DNase treated (DNA Free, Ambion, Cat. No. AM1907). Sample RNA concentration was determined in triplicate using a NanoDrop ND-1000 spectrophotometer prior to and after DNase treatment.

Sample RNA was adjusted to 250 ng/µL and cDNA was generated using a high capacity cDNA RT kit according to the manufacturer's directions (Applied Biosystems, Carlsbad, CA). Reaction plates containing cDNA were stored at -20 °C until qRT-PCR analyses.

FoxL2 and MIS Cloning and Sequencing

A partial coding sequence for southern flounder FoxL2 was obtained by PCR of genomic DNA derived from pooled ovaries of mature adults. Genomic DNA was homogenized as described above for RNA isolation and purified using TriReagent according to the manufacturer's protocol. A partial coding sequence for southern flounder MIS was obtained by PCR of cDNA synthesized from total RNA isolated from adult testes. Primers

were designed from *Paralichthys olivaceus* FoxL2 (AB303854) and MIS (AB166791; Yamaguchi *et al.* 2010) sequences using Primer Express 3.0 software (Applied Biosystems; Foster City, CA). All primers used in the characterization of southern flounder FoxL2 and MIS are shown in Table 1. All PCR reactions were performed using GoTaq® polymerase and buffers, 10 mM dNTP mix, 10 µM forward and reverse primer concentrations, and 50 ng of cDNA or genomic DNA (Promega; Madison, WI). The PCR thermal cycling parameters were as follows: 1 cycle at 95 °C for 2 minutes; 40 cycles at 95 °C for 30 seconds, a temperature gradient of 58 to 68 °C for 30 seconds, and 72 °C for 1 minute; and one cycle of final extension at 72 °C for 10 minutes. Products were run on a 1 % agarose gel to verify size of amplicons. Bands corresponding to the PCR amplicons for FoxL2 and MIS were excised and purified by a Qiaquick PCR purification kit, according to manufacturer's protocols (Qiagen; Valencia, CA). Purified product for the MIS amplicon was submitted directly to University of Chicago for forward and reverse sequencing. Isolated amplicons for FoxL2 were cloned into the PGEM TEasy Vector and transformed into JM109 series competent cells (Promega; Madison, WI). Two selected clones were submitted to the University of Chicago Cancer Research Center for sequencing.

Deduced nucleotide sequences were translated to amino acid sequences using the ExPASy Proteomic Server's translation tool (Gasteiger *et al.* 2003). ClustalW (v1.83) was employed to align southern flounder FoxL2 and MIS with known sequences from other species (Thompson *et al.* 2002). The multiple alignment parameters were as follows with no gap penalty: delay divergent sequences = 30 %, DNA transition weight = 0.50, similarity matrix = gonnet. Visual enhancement of amino acid alignments was performed using

BOXSHADE v 3.21. Sequence identity was determined using Sequence Identities and Similarities (SIAS) software available from the Immunomedicine group at Complutense University of Madrid, Spain.

Quantitative Real-Time PCR for Measurement of mRNA

Specific primers were designed from resulting sequence for FoxL2 using Primer Express 3.0 software and ordered from Invitrogen (Life Technologies; Carlsbad, CA). Primers were also designed based on published aromatase sequence (Luckenbach *et al.* 2004) using this software. Primers were verified using a standard curve with pooled cDNA in 8 - fold dilutions on a qRT-PCR.

Total RNA from gonads was isolated as outlined above. Gene-specific quantitative RT-PCR (qRT-PCR) primers were designed from *P. lethostigma* FoxL2, MIS and aromatase (Luckenbach *et al.* 2004) sequences using Primer Express gene detection software (Table 1). Real-time qRT-PCR analysis was performed on an ABI 7900 HT Sequence Detection System, using Brilliant® III SYBR Green master mix (Stratagene; La Jolla, CA), 1.5 μ M forward and reverse primers, and 1 μ g cDNA in a total reaction volume of 20 μ L. The qRT-PCR cycling parameters were an initial denaturing step at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 30 seconds and 60 °C for 1 minutes. The absence of genomic DNA contamination was assessed using two negative controls: sterile water used as template (No-Template Control; NTC) and DNase-treated RNA used as template (No-Amplification Control, NAC). Primer specificity was verified and only a single-gene product was observed from melting curves generated by qRT-PCR. Cycle threshold (Ct) values for experimental

samples were transformed using a standard curve of serially diluted cDNA versus Ct values ($R^2 = 0.93 - 0.98$) and normalized to reflect the amount of cDNA template per nanograms of total RNA.

Samples showing high expression of MIS and low expression of FoxL2 and Aromatase were identified as male individuals (Figures 10-11). Females were identified from samples showing high expression of both FoxL2 and Aromatase and low expression of MIS.

Data Analysis

Average daily growth rate in length and weight (DGR) was calculated for each tank as $(\text{mean final weight} - \text{mean initial weight}) / (\text{final time} - \text{initial time})$. Two-way ANOVA was used to evaluate the effect of treatment (density or cortisol) and time on length, weight, and DGR (GraphPad Prism). Bonferroni post hoc test was used to assess differences in means among treatment groups. Statistics were analyzed by GraphPad Prism Software. Average weight and length within a group are presented as mean \pm SEM. Average daily growth rate (DGR) among replicate groups is presented as mean \pm SD. $P < 0.05$ were deemed significant.

For each gonadal sample, females were identified as those gonads that had a combination of elevated values of aromatase and FoxL2 expression (≥ 0.35 ng cDNA/ng total RNA) and reduced MIS expression. Samples showing elevated expression of MIS (≥ 0.35 ng cDNA/ng total RNA) and minimal expression of FoxL2 and aromatase were counted as males. These threshold values (≥ 0.35 ng cDNA/ng total RNA) for FoxL2 and aromatase

for females and MIS for males represent the lowest value of an eight-point, two-fold standard curve of pooled cDNA for each of the genes, which ranged from 0.35 ng/uL cDNA – 54 ng/uL cDNA. Sex was not determined in gonads that showed low expression (< 0.35 cDNA/ng RNA) of all three genes. Individuals showing elevation of MIS and either aromatase or FoxL2 were also excluded. Sex ratios were determined for each tank, and for each treatment. Chi Square analysis was used to differentiate significant differences among groups (GraphPad Prism; San Diego, CA).

RESULTS

Cloning of FoxL2 and MIS

Partial cDNA coding sequences of 575 base pairs (bp) for FoxL2 and 238 bp for MIS was characterized for *P. lethostigma* by PCR. Based on translation of the partial FoxL2 sequence (e.g. 190 amino acids) southern flounder displays highest amino acid identity (identical amino acids) with *P. olivaceus* (100 %), *X. laevis* (87.43 %), *G. gallus* (84.29 %) followed by *R. norvegicus* (78.53 %) and *H. sapiens* (78.01 %) (Table 2). The southern flounder MIS translated sequence of 90 amino acids (Figure 2), which only reflects approximately 15 % of full-length protein sequence, also showed high amino acid identity (98.7 %) to the Japanese flounder sequence.

Effect of Cortisol on Growth

ANOVA shows an effect of time on weight ($P < 0.0001$), length ($P < 0.001$), and daily growth rate (DGR) in weight ($P = 0.0742$) and length ($P < 0.01$) indicating fish grew throughout the study. However, cortisol did not affect growth of southern flounder in weight, length or daily weight and length gain (Figures 3-6). There was no significant interaction of treatment and time.

Effect of Cortisol on Sex Determination

The mRNA levels of MIS, FoxL2, and aromatase were measured in gonads of fish treated early on with cortisol and in those raised under different stocking densities. The expression of these three genes was highest in 75 mm TL through 120 mm TL flounder

(Figures 7-9). MIS appeared to be strongly expressed between 75 mm TL and 115 mm TL, and was downregulated in fish larger than that (Figure 7). Both FoxL2 and aromatase were strongly expressed between 75 mm TL and 130 mm TL (Figures 8-9). MIS mRNA was expressed lowest among the three genes examined, with highest expression values around 60 ng cDNA/ ng total RNA, compared with aromatase which attained values up to 180 ng cDNA/ ng total RNA and FoxL2 which was expressed up to 5080 ng cDNA/ng total RNA.

In a comparative analysis of the expression levels of these three genes, individuals that show the lowest levels of MIS mRNA (range = 0.01 – 2.578, mean \pm SEM = 0.1781 \pm 0.0378 cDNA/ng RNA) also have elevated FoxL2 (range = 0.3769 – 5084, mean \pm SEM = 288.1 \pm 122.0 cDNA/ng RNA) and aromatase (range = 0.3267 – 183.5, mean \pm SEM = 22.17 \pm 4.242 cDNA/ng RNA) mRNA in their gonads. Conversely, other individuals with elevated gonadal MIS mRNA values (range = 0.3506 – 62.51, mean \pm SEM = 15.44 \pm 2.637 cDNA/ng RNA) have the lowest level of FoxL2 (range = 0.01– 2.857, mean \pm SEM = 0.2119 \pm 0.0474 cDNA/ng RNA) and aromatase (range = 0.01 – 12.87, mean \pm SEM = 0.6151 \pm 0.2624 cDNA/ng RNA) mRNA. Based on these measurements and the demonstrated role of MIS in male development and FoxL2 and aromatase in female development, relative expression of these genes was used to assess the sex of individuals. Fish with low MIS and high FoxL2 and aromatase mRNA were deemed females and individuals with high MIS and low FoxL2 and aromatase expression levels were considered males. This pattern is also shown via logarithmic transformations (Figures 10-11).

Based on the relative abundance of gonadal MIS, FoxL2, and aromatase mRNA, the influence of cortisol on southern flounder sex determination was assessed. The mean

percentage of females produced from replicate groups provided the control diet (no added cortisol) was 91 % (Figure 12). By contrast, cortisol caused a significant dose-dependent masculinization of treated groups ($P < 0.0001$). The low cortisol treatment group (100 mg cortisol/kg feed) had a sex ratio of 28.57 % females: 71.43 % males, while the high cortisol group (300 mg cortisol/kg feed) had a ratio of only 13.33 % females: 86.67 % males. These results were confirmed with histological analyses of gonads. Figures 13 and 14 show a representative picture of an hematoxylin/ eosin stained section of an ovary and testis in southern flounder. Based on histological analyses, the control group had a much higher percentage of females (75 %) compared with the cortisol fed groups. Low cortisol treatment yielded 33 % females and high cortisol concentrations produced only 15 % females (Figure 15). Because fish were sampled prior to attaining 150 mm TL, many of the gonads analyzed by histology did not show morphological features that clearly distinguished them as ovaries or testes. Hence, the sample size of fish from the treatment groups was small: four fish from the control (0 mg/kg cortisol), 13 from the 100 mg/kg, and nine from the 300 mg/kg cortisol treated group. This sample size was too low for Chi-square analysis, so these data were not statistically analyzed.

Effect of Stocking Density on Growth and Sex Determination

There was no overall effect of density on average body weight (Figure 16) or on daily length gain (Figure 19) over the course of the experiment. Average length was affected by density ($P < 0.01$, 2-way ANOVA). Average length of fish at the 1500 fish/m² stocking density was lower than the 250 fish/m² stocking density ($P < 0.05$), but did not differ from

500 or 1000 fish/m² densities at the end of the experiment (day 78; Figure 17). Fish stocked at 1000 fish/m² had a significantly higher daily weight gain than other densities during the 70 to 78 day time interval ($P < 0.05$; Figure 18). However, overall daily weight gain over the course of the experiment (day 14 to 78) was not affected by stocking density.

Based on measures of gonadal MIS, FoxL2 and aromatase mRNA levels, we found no significant difference in sex ratios of fish populations stocked at different densities. A stocking density of 250 fish/m² produced 91 % females, 500 fish/m² yielded 87 % females, and 1000 fish/m² resulted in 100 % females. Stocking densities of 1500 fish/m² yielded 81 % females, the lowest proportion of females among the different densities. None of these densities differed from the expected 100:0 female: male sex ratios. Histological analyses showed population consisting of 75 %, 40 %, 63 % and 75 % females with densities of 250, 500, 1000 and 1500 fish/m², respectively. This pattern was similar to that observed with the gonadal markers of sex determination, but with overall lower percentages of females. Again, the number of individuals whose sex could be discerned by gonadal histology was limited (N = 4 to 8 individuals/group), precluding statistical analyses.

DISCUSSION

These studies were undertaken to further establish those environmental variables and potential mechanisms that may underlie ESD in southern flounder, a species whose sex may be determined by genotype (Godwin *et al.* 2003, Luckenbach *et al.* 2003, Borski *et al.* 2010). Indeed, only potential females of the presumed XX genotype appear susceptible to ESD, and can be readily masculinized by temperature and other environmental conditions. Previous work has established meiogynogenetic protocols (Luckenbach *et al.* 2004, Morgan *et al.* 2006) and utilized temperature-sensitive sex determination of southern flounder to produce functional male broodstock with the female XX genotype. When these males are bred with normal females, all XX progeny can be produced. Based on the XX/XY female/male genetic system of sex determination, one would predict that progeny of the XX genotype would lead to production of a nearly pure population of females. If southern flounder had the WZ/ZZ female/male genetic pattern of sex determination that is observed with some fish such as the tongue sole (*Cynoglossus semilarvatus*) (Chen *et al.* 2008, Luckenbach *et al.* 2009) then the breeding of male gynogens (male broodstock with the WZ genotype) with females would at best yield 50: 50 sex ratios. The XX/XY system of sex determination is known to occur in Japanese flounder (Yamamoto 1999) and our own observations suggests that it may apply to southern flounder as well, although direct evidence for it has been lacking since production of populations approaching 100 % females has not been realized. The present investigations show for the first time that a nearly pure population of females can be produced in a population consisting entirely of the putative XX genotype (Figure 20). Fish from these

studies were raised under permissive conditions known to maximize the female phenotype in XX/XY populations of flounder; grey tank color, water temperatures of 23 °C, and light intensity of 100 lux (Luckenbach *et al.* 2003, Turner 2008).

Previous work shows that FoxL2 promotes transcription of aromatase and is expressed primarily in the gonads of females and not males during sexual differentiation (Pannetier *et al.* 2006, Alam *et al.* 2008, Yamaguchi *et al.* 2007; Yamaguchi *et al.* 2010). Likewise, aromatase is causally linked to female sex differentiation in Japanese flounder (Kitano *et al.* 1999, 2000) and its ovarian expression rises and remains elevated during sexual differentiation of southern flounder females, but not males (Luckenbach *et al.* 2005). MIS shows an expression pattern reciprocal to aromatase (Fernandino *et al.* 2008). *P. olivaceus*, black porgy (*Acanthopagrus schlegeli*) and Pejerrey (*Odontesthes bonariensis*) shows a sexual dimorphic pattern in MIS expression, increasing during testicular and declining with ovarian differentiation (Yoshinaga *et al.* 2004, Yamaguchi *et al.* 2010, Wu *et al.* 2010). We obtained partial sequences of FoxL2 and MIS in southern flounder that show high amino acid identity to other vertebrate sequences and nearly 100 % identity to *P. olivaceus*. We evaluated the differential patterns of expression of these genes and of aromatase (Luckenbach *et al.* 2005) to determine sex of individuals under different stocking densities and exposure to cortisol. In nearly 72 % of gonads evaluated there was a clear dimorphism in expression of FoxL2 and aromatase relative to MIS mRNA levels. Average gonadal MIS mRNA levels were 87 times lower, while FoxL2 and aromatase were 839 and 28 times higher, respectively, in a subset of gonads while the opposite pattern occurred for most other tissues analyzed. Some had low levels of all three genes suggesting an undifferentiated state. Based on these results and other

published research (see above) it is concluded that individuals with elevated gonadal FoxL2 and aromatase mRNA and low expression of MIS are females, while the opposite pattern reflects males. This conclusion is further supported by histological analyses whereby the patterns in sex ratios determined from gonadal gene expression profiles, albeit limited, generally parallels that determined by gonadal histology (Figures 12, 15). Additionally, our previous work in southern flounder shows that gonadal aromatase mRNA expression is a good predictor of gonadal phenotype and sex ratio determinations producing results identical to that shown with gonad histology (Luckenbach *et al.* 2005). Interestingly, we found that the difference in magnitude of FoxL2 expression between the sexes is 28 times greater than that for aromatase, suggesting it is an even better indicator than aromatase in predicting sex or for identifying female individuals. Overall, analyses of gonadal markers of sex determination (MIS, FoxL2 and aromatase mRNA) provides a method for predicting sex of southern flounder at around 70 to 80 mm TL, eliminating the need to grow fish out to around 150 mm when sex can be reliably determined by histological methods. These biomarkers of gonadal sex differentiation should prove useful for evaluating the suite of environmental factors that may regulate sex determination in southern flounder as well as other fish species.

Two environmental factors were evaluated as potential regulators of sex determination. One factor was exogenous cortisol and the second was stocking density. Cortisol causes mobilization of energy stores, which is useful in adaptation to short term stress, but long term cortisol exposure increases metabolism, reduces the ability to assimilate food and negatively affects growth rates (reviewed by Bonga 1997). We found that early, periodic application of cortisol dramatically masculinized fish in a dose-dependent fashion.

Indeed, the proportion of females declined to 27 and 13 % in those fish receiving 100 mg/kg and 300 mg/kg cortisol, while population receiving gelatin coated feed alone had 91 % females. We found that cortisol had no effect on somatic or skeletal growth, nor did it result in significant mortalities (79 to 98 % survivorship relative to 93 % for controls). Thus, it would appear that the doses and temporal manner in which cortisol was applied likely yielded concentrations of hormone reflecting responses associated with a brief period of moderate stress; rather than one representing a chronic stress or one associated with pharmacological steroid application that suppresses appetite and growth in fish (Barton and Iwama 1991, Bonga 1997, Lawrence 2007). Our results are in agreement with that found for Japanese flounder, where cortisol was shown to masculinize fish during continuous steroid treatment over a 70 – day period (Yamaguchi *et al.* 2010). This group also established that whole-body cortisol levels increase in fish exposed to masculinizing temperatures, indicating that the hormone is likely mediating temperature-sensitive sex determination responses. Future studies should evaluate if temperature may also alter endogenous cortisol in southern flounder. Nevertheless, raising southern flounder in light blue versus grey or black tanks caused a brief 2-fold increase in whole body cortisol during sex differentiation and significantly masculinized fish in mixed XX/XY populations (Turner 2008). These results along with the masculinizing effects of cortisol demonstrated here, suggest that the steroid may mediate not only the sex determining effects of temperature, but also of tank color and perhaps other, as yet undefined environmental variables.

The mechanisms by which cortisol might masculinize fish during the window of sex differentiation is uncertain, although evidence suggests glucocorticoid response elements

(GRE) lie upstream of the aromatase gene in the protogynous goby *Gobiodon histrio* (Gardner *et al.* 2004), raising the possibility the hormone could negatively modulate aromatase expression and activate pathways toward male development. In *P. olivaceus*, FoxL2 site and estrogen receptor and cAMP response elements lie upstream of the aromatase promoter (Yamaguchi *et al.* 2007) and cortisol has been shown to directly interfere with cAMP-dependent activation of aromatase transcription (Yamaguchi *et al.* 2010). Further studies are clearly required to assess if sex determining genes regulated upstream of aromatase, *e.g.* FoxL2 and MIS are modulated by glucocorticoids.

Elevated density increases cortisol levels in many species including brook char (*Salvelinus fontinalis*) (Vijayan and Leatherland 1990), sea bream (*Sparus aurata*), carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) (see Lawrence 2007). Stocking density also correlates with increased cortisol levels in winter flounder (Sulikowski *et al.* 2006). In the present studies we found no major effect of stocking densities ranging from 250 and 1500 fish/m² on southern flounder growth, although there was a slight decline in average length and weight of fish at the 1500 fish/m² stocking density. We also did not see a significant effect of stocking density on sex ratios in this study although fish at the highest density had slightly lower proportion of females than the other groups. Based on these studies we would recommend that fish be stocked at densities up to 1000 fish/m² to maximize production of faster growing females.

Several important advancements in the development of southern flounder aquaculture are shown here. Overall, this study demonstrates that *Paralichthys lethostigma* inherits sex via an XX/XY system, and that genotypic XX fish stocks can be used to create nearly 100 %

females under permissive conditions that maximize retention of this phenotype. Secondly, cortisol applied intermittently was shown to result in a significant male bias without affecting growth, suggesting that even short term stress during the critical period of sex determination can cause masculinization of genetic females in this species. Furthermore, MIS and FoxL2 sequence fragments were cloned and the expression of these molecular markers was used to predict phenotypic sex of individuals. MIS is expressed at higher levels in males compared to females, and both FoxL2 and aromatase is expressed at higher levels in females compared to males. Sex differences in FoxL2 expression are magnitudes higher than aromatase, and hence it may serve as a more robust predictor of female differentiation than aromatase. Finally, stocking densities as high as 1000 fish/m² were found to maximize production of females with no negative impact on growth. Collectively, these studies provide tools that can be used to assess environmental parameters that promote female development and the conditions required to produce populations of faster-growing females needed to enhance the efficiency and profitability of flounder aquaculture.

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Table 1. Primer sequences used for initial coding, partial cloning, and qRT-PCR.

Primer Name		Forward Primer 5' to 3'	Use	Accession No.
P. olivaceus MIS	Forward Primer	TGACCCGTACCTACGAGC TG	Coding sequence	AB1667182
	Reverse Primer	TCGTCCACGTTCTCGCTCT C		
P. olivaceus FoxL2	Forward Primer	TCATCAGCAAGTTCCCCT TC	Partial cloning	AB303855
	Reverse Primer	TCCGTTGCTGGAGGAGAC C		
P. lethostigma MIS 02	Forward Primer	CTGCCGAGGCTCTTGCA	qRT- PCR	
	Reverse Primer	CAGGACGGCATGGTTGAT G		
P. lethostigma Arom 02	Forward Primer	GGAGCCACACAGACAGG AGAA	qRT- PCR	
	Reverse Primer	GGCCCCAAACCCAGACA		
P. leth FoxL2 02	Forward Primer	GTCCCCGCCCAAGTACCT	qRT- PCR	
	Reverse Primer	GGCCGAGCGACCATGAG		

Table 2. Sequence identity of FoxL2 from southern flounder (*Paralichthys lethostigma*), Japanese flounder (*Paralichthys olivaceus*; AB303854.1), clawed frog (*Xenopus laevis*; NM001134784), chicken (*Gallus gallus*; AY487165.1), rat (*Rattus norvegicus*; XM345975), and human (*Homo sapiens*; NM023067).

Southern Flounder	100					
Japanese Flounder	100	100				
Clawed Frog	87.43	80.71	100			
Chicken	84.29	81.63	79.01	100		
Rat	78.53	73.2	71.42	76.06	100	
Human	78.01	73.52	70.45	76.72	96.25	100
	Southern Flounder	Japanese Flounder	Clawed Frog	Chicken	Rat	Human

Figure 1. ClustalW (v1.83) alignment of FoxL2 amino acid sequences. Sequences used include Southern flounder (*Paralichthys lethostigma*), Japanese flounder (*Paralichthys olivaceus*; AB303854.1), clawed frog (*Xenopus laevis*; NM001134784), rat (*Rattus norvegicus*; XM345975), chicken (*Gallus gallus*; AY487165.1), and human (*Homo sapiens*; NM023067).

Figure 2. ClustalW (v1.83) alignment of MIS amino acid sequences. Sequences used include Southern flounder (*Paralichthys lethostigma*), Japanese flounder (*Paralichthys olivaceus*; AB166791), rat (*Rattus norvegicus*; NM012902), chicken (*Gallus gallus*; U61754), and human (*Homo sapiens*; NM000479).

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Rat      ---MQGPHLSL L L L L L A-----TMGAVLQADTV---EELTNTR-----GLIFL
Human   ---MRDLP L T S L A L V L S-----ALGALLGTEALRAE E PAVGTS-----GLIFR
Chicken MKAAALGVLLP Y L V L L L R S T A L P R K G T P G A E A V L E Q S L S E E M G L G A K R E N V S R A R P G L A V R
S_Flounder
J_Flounder
consensus
.....

Rat      EDG-----VW P P S S P P E P L C L V A V R G E G D T S K A S L T V V G G L H
Human   EDL-----D W P P G S P Q E P L C L V A L G G D S N G S S P L R V V G A L S
Chicken PKMTAASRL L P E P A A S C T O A D G S S L Q P W P L G G L E G P V C R L K M D R N - E V P P S H L E V V G V L T
S_Flounder
J_Flounder
consensus
-----MPVVNVFCCGALVLCWTC A A L T V S H P S P H L A P C Y V D D
.....

Rat      S Y E H A F L E A V Q E S R W G P Q D L A T F G V C S T - D S Q T L P A L Q R L G A W L G E T G E - - C Q L L V L H L
Human   A Y E O A F L G A V Q R A R W G P R D L A T F G V C N T G D R Q A A L P S L R R L G A W L R D P G G - - Q R L V L H L
Chicken R Y E S A F I K V L R - A G W E H R S P E T F G L C P G G D A Q T V L R P L Q C L Q E H L A G P G M G L C R F L I L H L
S_Flounder
J_Flounder
consensus
I F A A L R E G V G A D G E L A S S S F A L F G V C A V S D S S S G S V L L E L A K E T S R S Q R R G L E V L H P T G V
.....

Rat      A E V I W E P Q L L L K F Q E P P P G G A S R W E - - - C A L L V L Y P G P G P - - - - - Q V T V T G A G L Q G
Human   E E V I W E P T P S L R F Q E P P P G G A G P E - - - L A L L V L Y P G P G P - - - - - E V T V T R A G L P G
Chicken E E V K W E A Q P R L W F R L P F H A E V G R L L G E L H V A L L L F Y P G S P T G R A E G R Q E L L V E G T G L T Q
S_Flounder
J_Flounder
consensus
L V T E E D D R G A L L E L T F D L P Q S P L L R L S - - - P V L L L A F Q S P L K G D D L N - - - V T F S S L S L Q P H
.....

Rat      T Q S L C P T R D T R Y L V L T V H F P A G A W S G S - - - G L A L T L Q P S K E G - A T L T I A Q L C A F L F G S D
Human   A Q S L C P S R D T R Y L V L A V D R P A G A W R G S - - - G L A L T L Q P R G E D - S R L S T A R L C A L L F G D D
Chicken E Q S L C F T R D T Q Y L L L R A S V A A V S S S S K E L S F Q L S L T I R H H G D G G A P L S L M E T C Q L L F G S D
S_Flounder
J_Flounder
consensus
T Q S V C I S A E T Q Y I L L T G N A S D A S F V Q K - - - - - W R I S V E A K S P D L K P N L K D M L I G E K
.....

Rat      S R C F T R M T P T L V L L P P T G P - - - - - T P - - - Q P A H G Q L D T V P F P Q P G L - - - - - S L E P - - - - -
Human   H R C F T R M T P A L L L L P R S E P - - - - - A P - - - L P A H G Q L D T V P F P P P R P - - - - - S A E L - - - - -
Chicken E K C F T R M T P V L L L L A G S P P E G D A A A P S S Y L S A S G V D V A P Y P Q L S P P H P G T A E L P P H T T A
S_Flounder
J_Flounder
consensus
L G S G I N I S P L L L F S G G T E T - - - - -
.....

Rat      - - - - - E D L P H S A D P F L E T L T R L V R A L R G P - - - L T R A S N T R L A L D P G A L A S F P Q G L V N L S
Human   - - - - - E E S P P S A D P F L E T L T R L V R A L R V P - - - P A R A S A P R L A L D P D A L A G F P Q G L V N L S
Chicken S P A N T T S P T P G D S G Q F L G V L T L F I R R V L S P R S E P P T Q P S S Q H W L D F Q M M E T L P H Q L L N L S
S_Flounder
J_Flounder
consensus
- - - - - R R V S G S T S S S S C T F S F L C E L R R F L C D V L P Q D R P E S P Q L Q L D S L Q S M P Q L S L G L S
.....

Rat      D P V A L G R - L L D G E E P L L L L S P A A A T V G E P M R L H S P T S A P W A A G L A R R V A V E L Q A A A S E L
Human   D P A A L E R - L L D G E E P L L L L R P T A A T T G D P A P L H D P T S A P W A T A L A R R V A A E L Q A A A A E L
Chicken E T A A L E R - L V Q S E E P S V L L F P Q E G G A G - - - L E Q H F G D W O P E G T V L Q L M G - K L Q A V I H E L
S_Flounder
J_Flounder
consensus
S S E T L L A G L I N S S S P T V F S F S R W G S M L Q - - V H H Q L A L S P S L L E E L R Q R V E Q T E M Q M M E V
.....

Rat      R D L P G L P P T A P P L L S R L L A L C - - P N D S R S A G D P - - - - - L R A L L L L K A L Q G L R A E W R G R E -
Human   R S L P G L P P A T A P L L A R L L A L C - - P G G P G G L G D P - - - - - L R A L L L L K A L Q G L R V E W R G R D P
Chicken R D I P E F Q A N T G - L F Q H L L S F C Y H P P G P A A G G O P P G S G K L H A L L L L K A L Q T V R A R W Q E R R -
S_Flounder
J_Flounder
consensus
I R G K E V G S R A T E R L G R L K E L S A L P E K E P A A G E S - - - - - Q Y R A F L L L K A L Q T V T R T Y E L Q I G
.....

Rat      - G R G R A G R S K G - T G T D G L C A L R E L S V D L R A E R S V L I P E T Y Q A N N C Q G A C A W P C S D R N P R Y
Human   R G P G R A Q R S A G A T A A D G P C A L R E L S V D L R A E R S V L I P E T Y Q A N N C Q G V C G W P C S D R N P R Y
Chicken - - - - - K V L R Q N R S A R H Q A H C R L Q E L T I D L R D R N F I V M P T Y Y E A N N C E G P C R L P L S T R V P G Y
S_Flounder
J_Flounder
consensus
- - - - - V R N T R A G S N D P A R G N I C S L R S L T V S L - - E R H L V G P N T A V I N N C R G S C T F P L F N A I N - -
.....

Rat      G N H V V L L L K M Q A R G A A L G R L P C C V P T A Y T G K L L I S L S E E H I S A H H V P N M V A T E C G C R
Human   G N H V V L L L K M Q A R G A A L A R P P C C V P T A Y A G K L L I S L S E E R I S A H H V P N M V A T E C G C R
Chicken H S H T V L L L M Q E R G S P L O P L P C C V P V R Y S D Q L I I S V L A E G L E V R K F P N M V A E C G C R
S_Flounder
J_Flounder
consensus
- - - - - H A V L L N S H I E S E N V D - - - - -
- - - - - H A V L L N S H I E S E N V D E R A P C C V P V A Y E A L E V V D L N E H G T Y L S V K P D V V A K E C G C R
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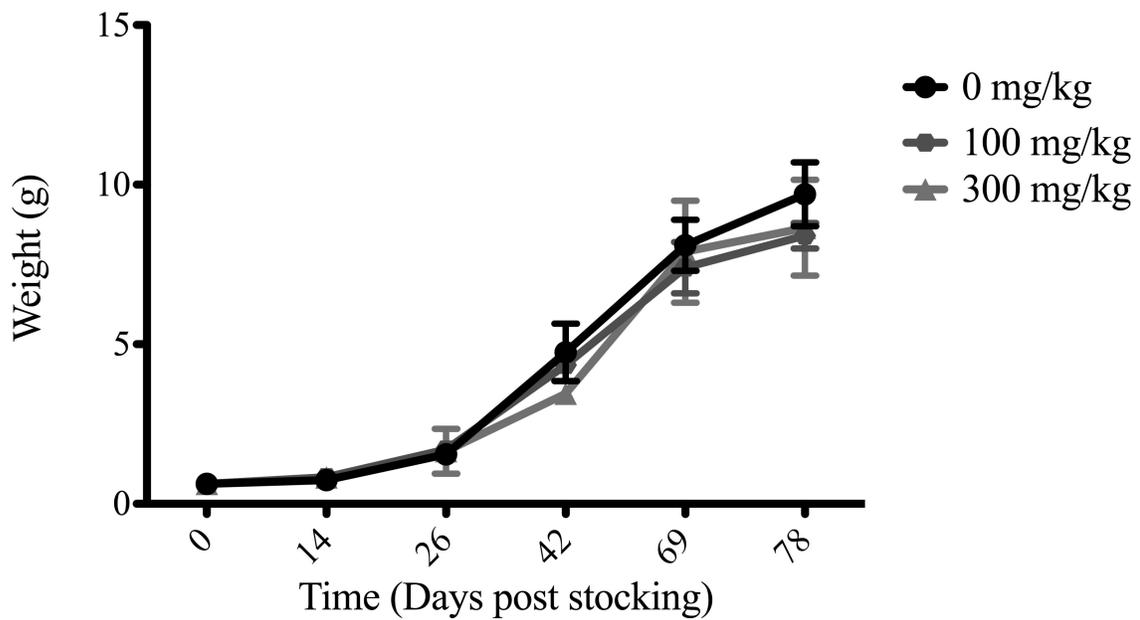


Figure 3. Average body weight of cortisol treated southern flounder over a 78-day growth period. Cortisol treated feed was offered every third day prior to sex determination between day 0 -14, and between day 26 – 42. Cortisol did not significantly alter growth (mean \pm STD, 2 tanks/treatment).

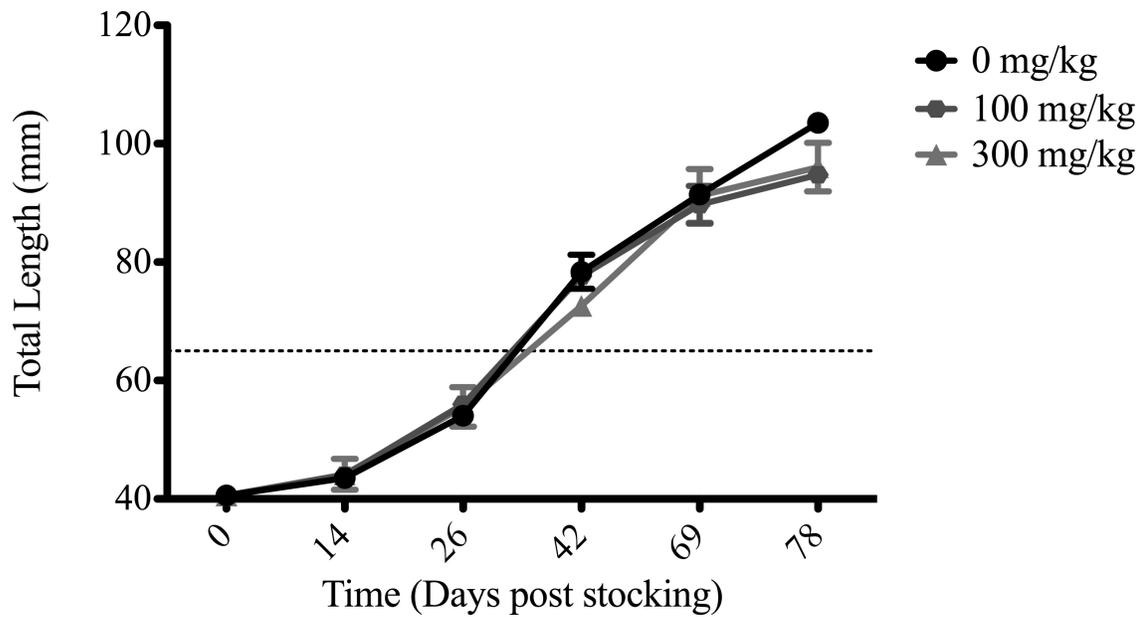


Figure 4. Average length of cortisol treated southern flounder over a 78-day growth period. Cortisol treated feed was offered every third day prior to sex determination between day 0 to 14, and between day 26 to 42. Growth did not differ among groups (mean \pm STD, 2 tanks/treatment). Dashed line indicates the size at which individuals begin to undergo sex differentiation (Luckenbach *et al.* 2003).

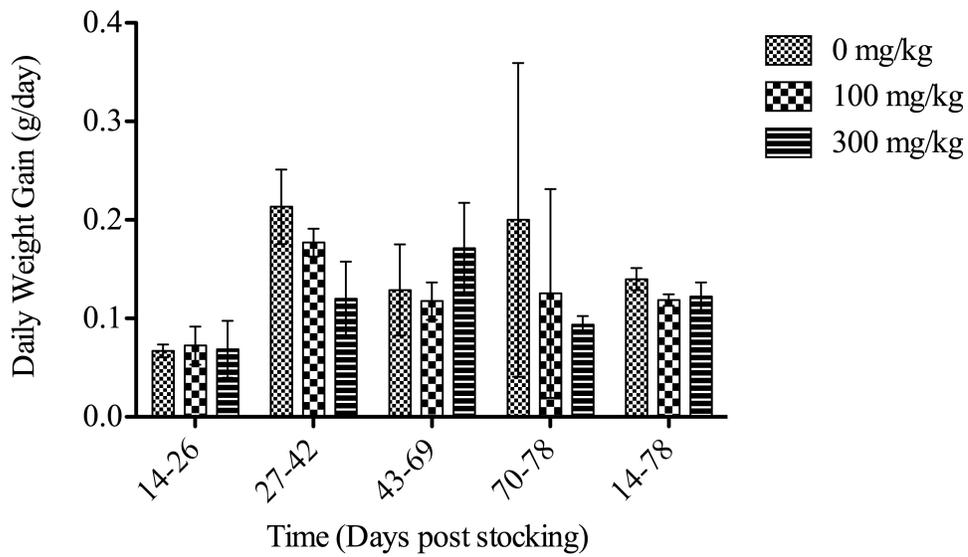


Figure 5. Daily weight gain (g/day) of cortisol treated southern flounder over a 78-day growth period. Cortisol treated feed was offered every third day prior to sex determination between day 0 to 14, and between day 26 to 42. Daily weight gain did not differ among groups (mean \pm STD, 2 tanks/treatment).

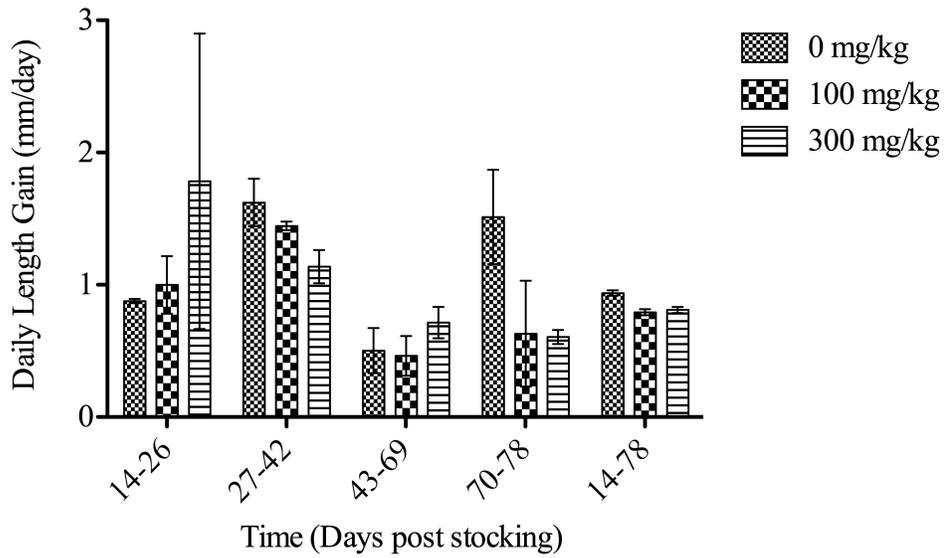


Figure 6. Daily length gain (mm/day) of cortisol treated southern flounder over a 78-day growth period. Cortisol treated feed was offered every third day prior to sex determination between day 0 to 14, and between day 26 to 42. Daily length gain did not differ among groups (mean \pm STD, 2 tanks/treatment).

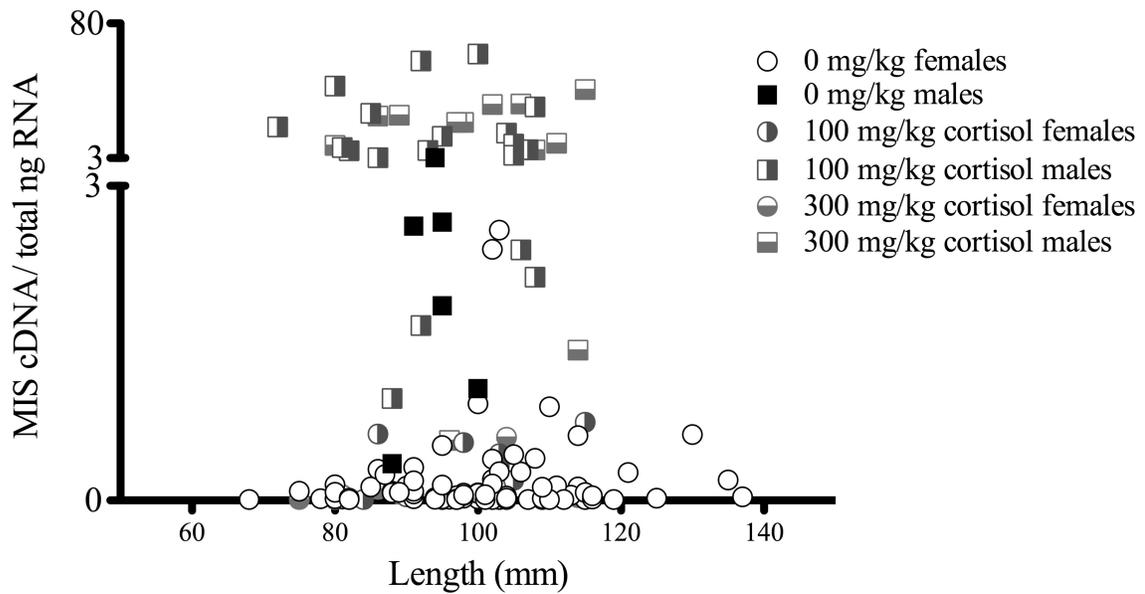


Figure 7. Gonadal MIS expression levels (cDNA/total ng RNA) as a function of total body length (mm). Females are indicated by circles and males are indicated by squares. Male expression of MIS ranged from 0.3506 - 62.5 cDNA/total ng RNA (mean \pm SEM = 15.44 ± 2.637). Female expression of MIS ranged from 0.01 - 2.57 cDNA/total ng RNA; (mean \pm SEM = 0.1770 ± 0.0378). As shown above, males showed considerably higher MIS mRNA values than females, and cortisol treated males showed significantly higher levels than non-treated individuals.

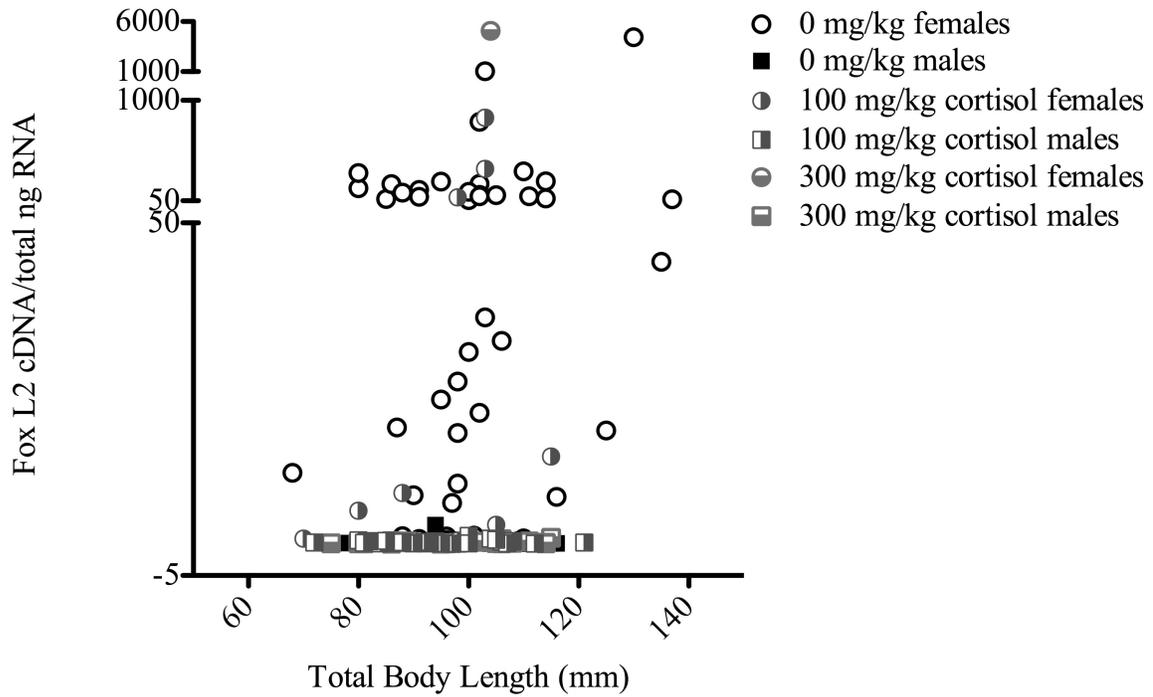


Figure 8. Gonadal FoxL2 expression levels (cDNA/total ng RNA) as a function of total body length (mm). Females are indicated by circles and males are indicated by squares. Female expression of FoxL2 ranged from 0.01 – 5084 cDNA/total ng RNA (mean \pm SEM = 288.1 ± 122.0). Male expression of FoxL2 ranged from 0.01 - 2.857 cDNA/total ng RNA (mean \pm SEM = 0.2119 ± 0.0474). As shown above, females showed considerably higher FoxL2 mRNA values than males.

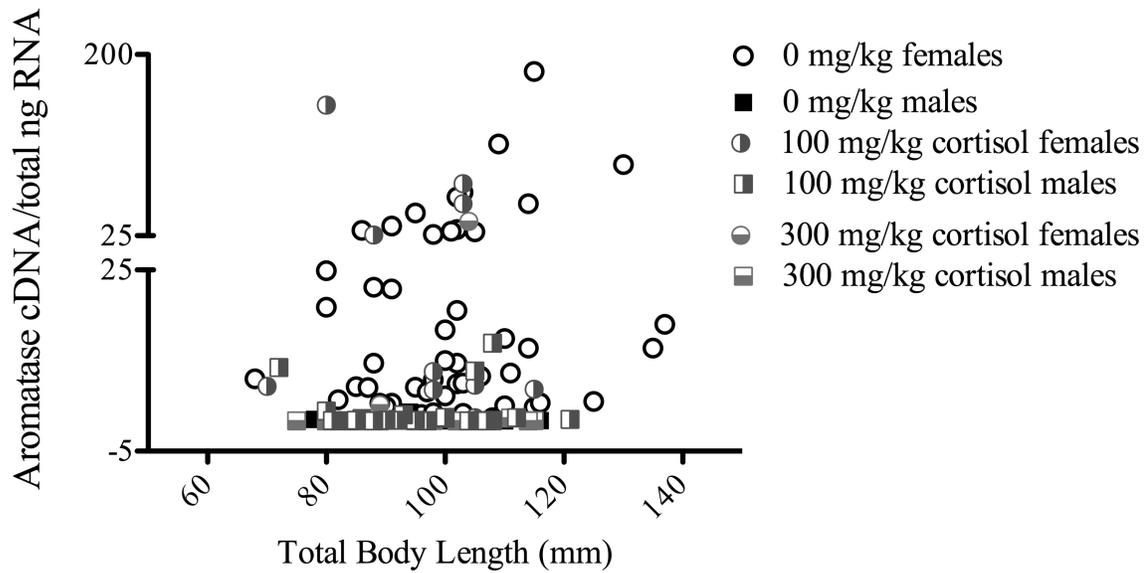


Figure 9. Gonadal aromatase expression levels (cDNA/total ng RNA) as a function of total body length (mm). Females are indicated by circles and males are indicated by squares. Female expression of aromatase ranged from 0.3267 – 183.5 cDNA/total ng RNA (mean \pm SEM = 22.17 ± 4.242). Male expression of aromatase ranged from 0.01 - 12.87 cDNA/total ng RNA (mean \pm SEM = 0.6151 ± 0.2625). As shown above, females showed considerably higher aromatase mRNA values than males.

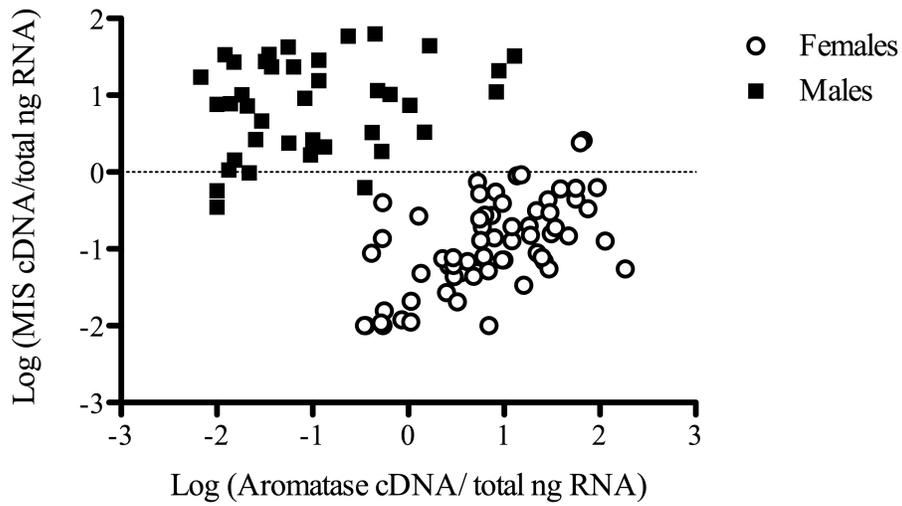


Figure 10. Gonadal MIS expression (Log [MIS cDNA/total ng RNA]) as a function of gonadal aromatase expression (Log [aromatase cDNA/total ng RNA]). Females are indicated by circles and males are indicated by squares. Shown above are dimorphic expression patterns for each marker.

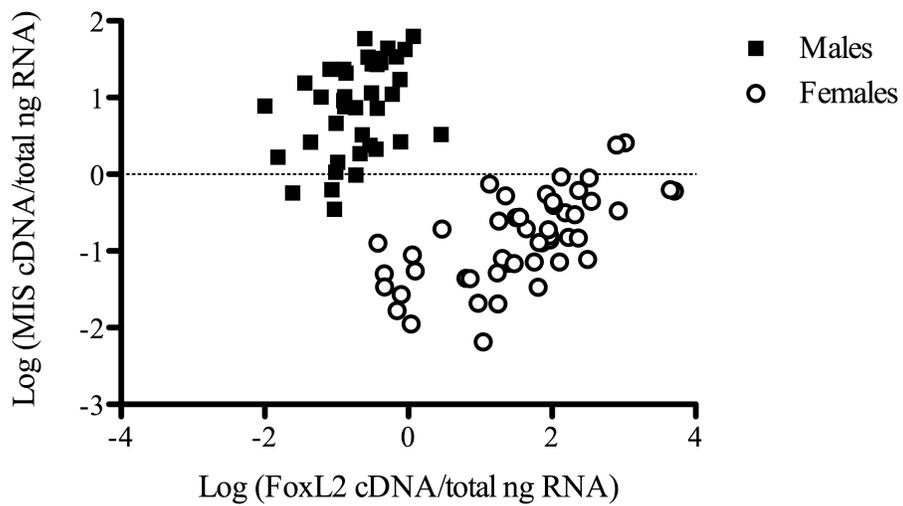


Figure 11. Gonadal MIS expression (Log [MIS cDNA/total ng RNA]) as a function of gonadal FoxL2 expression (Log [FoxL2 cDNA/total ng RNA]). Females are indicated by circles and males are indicated by squares. Shown above are dimorphic expression patterns for each marker.

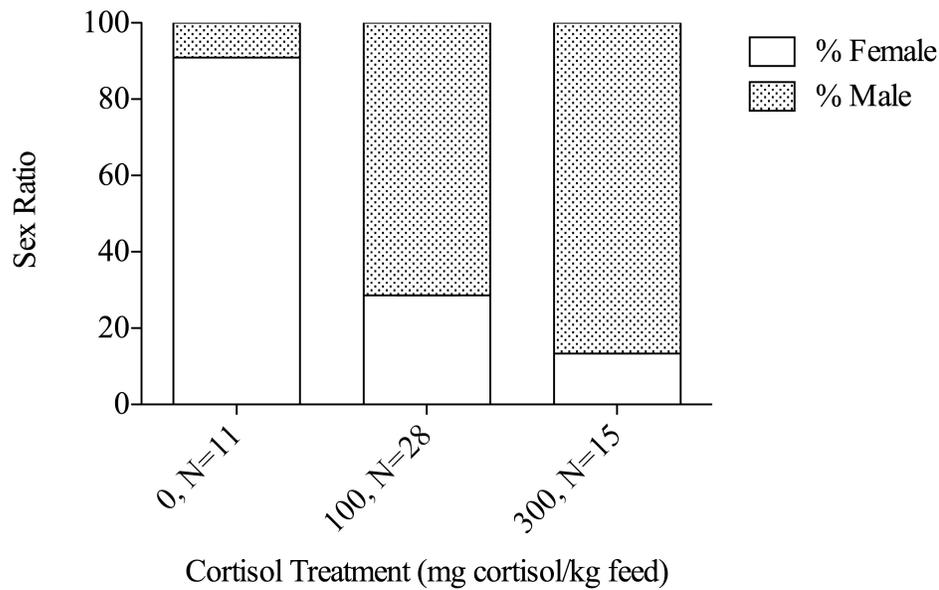


Figure 12. Sex ratios of cortisol-treated southern flounder with the XX female genotype. Female and male sex differentiation was distinguished by the expression profiles of gonadal MIS, aromatase, and FoxL2. Control treatment (0 mg/kg cortisol) resulted in 91 % female, whereas both groups fed cortisol show a male biased ratio; 100 mg/kg cortisol treatment resulted in 29 % female and 71 % male, and 300 mg/kg cortisol resulted in 13 % female and 87 % male. Both cortisol-treated groups were found to be significantly different than controls (Chi Square, $P < 0.0001$).

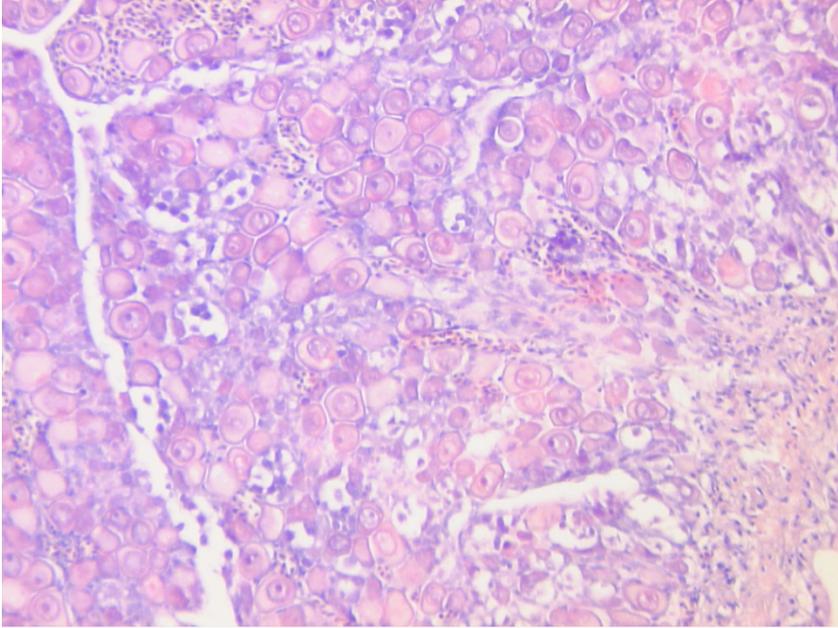


Figure 13. Ovary of a southern flounder in the early perinucleus stage, showing oögonia. Ovarian cavity visible (white division within photograph). Total body length was 107 mm TL. 200X magnification.

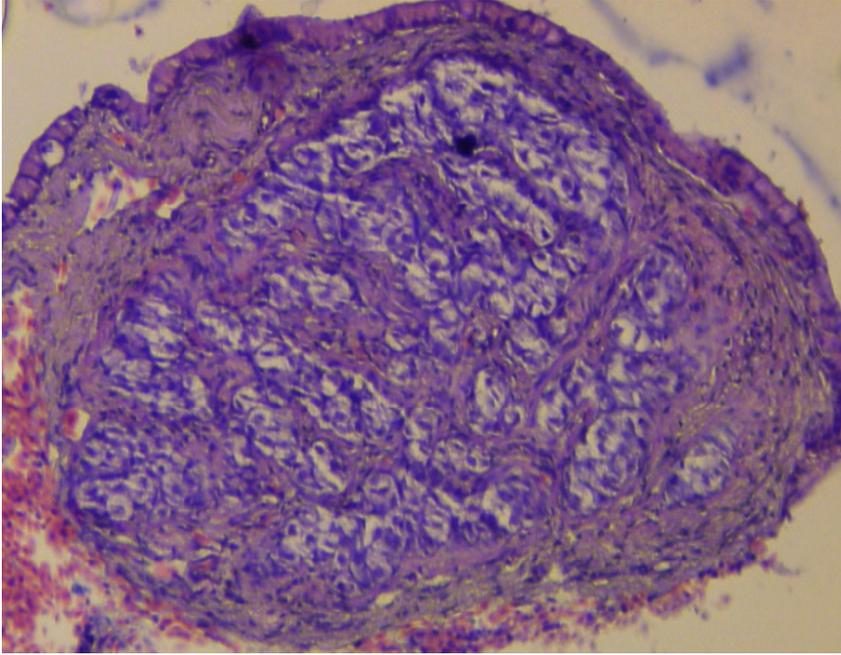


Figure 14. Testes of a southern flounder showing early stage (A) spermatogonia. Total body length was 109 mm TL. 200X magnification.

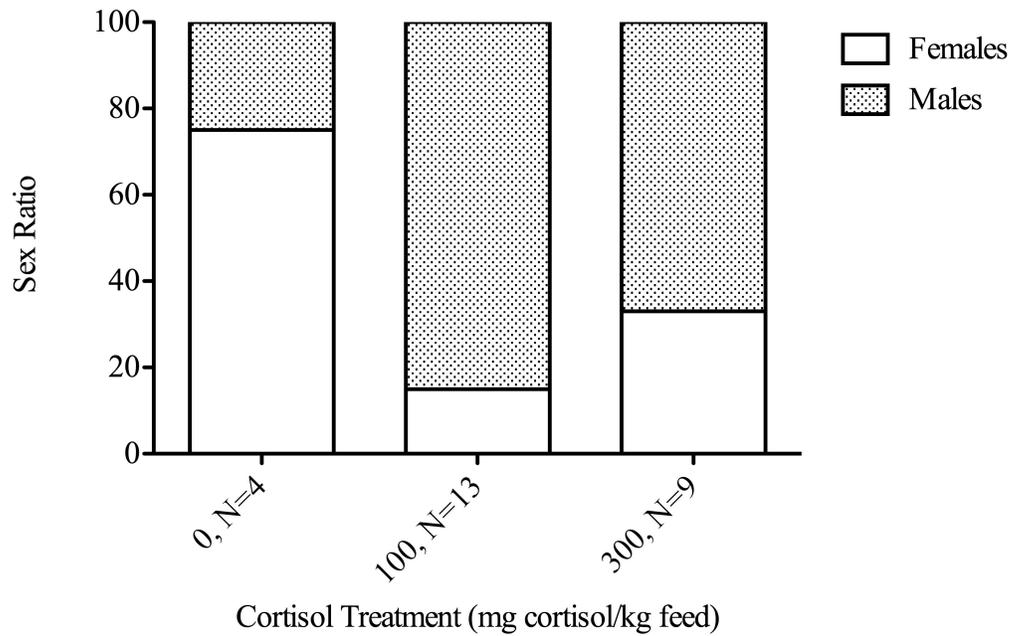


Figure 15. Sex ratios of cortisol treated southern flounder with the XX genotype. Female and male sex differentiation was assessed by gonadal histology. Controls are mostly female (75 %), and both cortisol treatment groups resulted in a male biased ratio (100 mg/kg cortisol – 15 % females, 85 % males, 300 mg/kg cortisol – 33 % females, 67 % males).

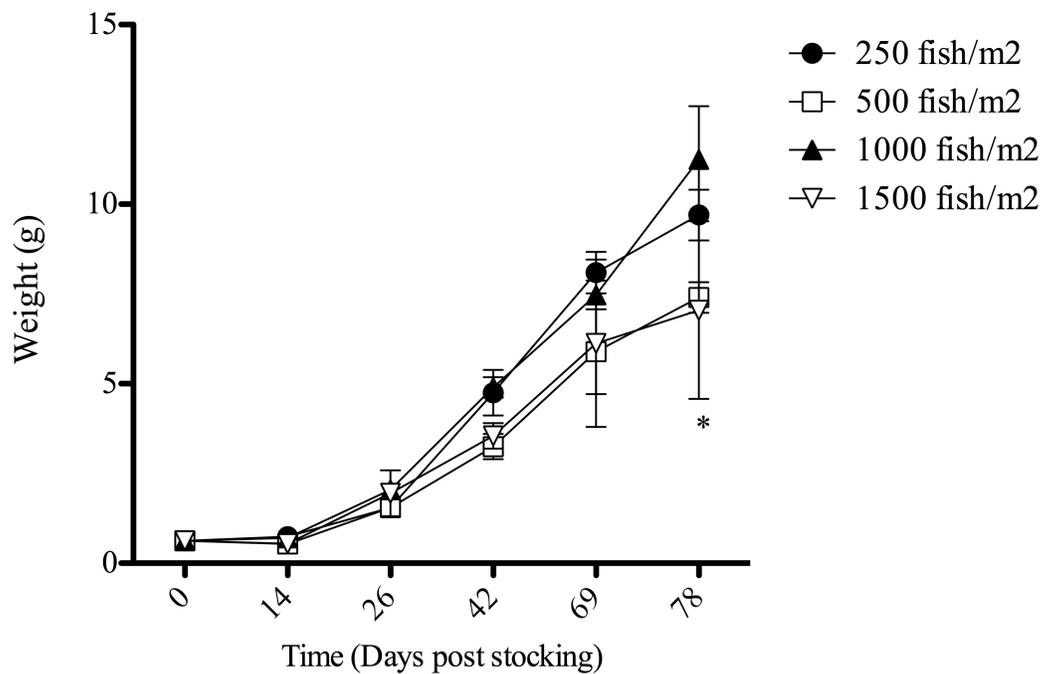


Figure 16. Average body weight of Southern flounder stocked at different densities over a 78-day growth period. Tanks were stocked at four densities (250 fish/m², 500 fish/m², 1000 fish/m², 1500 fish/m²) and biomass was maintained over the course of the experiment. A significant difference, as indicated by asterisk (*) was seen at 78 days between both 1500 fish/m² and 500 fish/m² compared to groups stocked at 1000 fish/m² and 250 fish/m² (mean ± STD, 2 tanks/treatment).

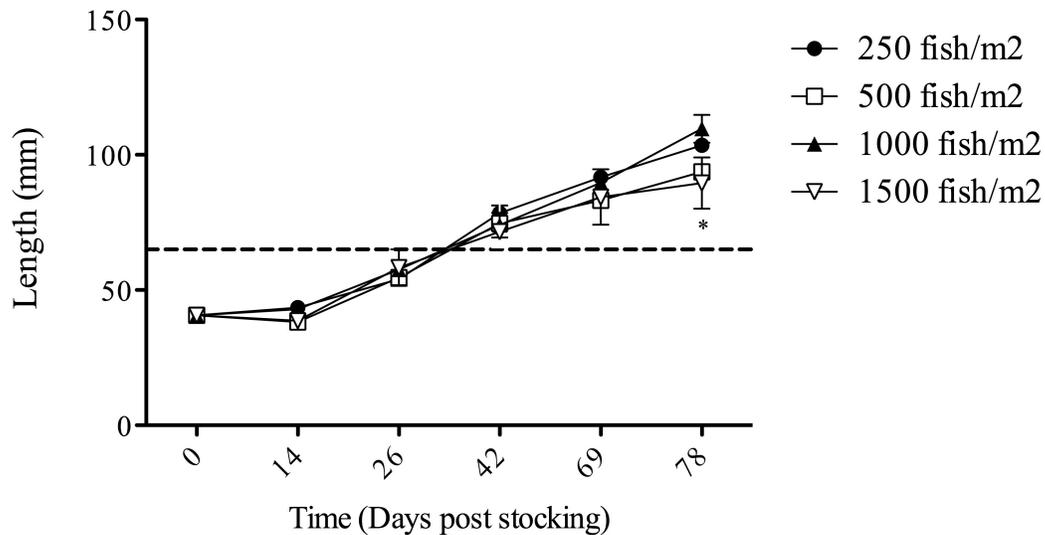


Figure 17. Average total body length of Southern flounder stocked at different densities over a 78-day growth period. Tanks were stocked at four densities (250, 500, 1000 and 1500 fish/m²) and biomass was maintained over the course of the experiment. Average length (mean \pm STD, 2 tanks/treatment) was affected by density ($P < 0.01$, 2-way ANOVA). Average length of fish stocked at 1500 fish/m² was significantly lower, as indicated by asterisk (*) than length of fish at 250 fish/m² stocking density ($P < 0.05$, Bonferroni post-hoc test), but did not differ from 500 or 1000 fish/m² densities at the end of the experiment (day 78). Dashed line indicates size at beginning of sex determination (Luckenbach *et al.* 2003).

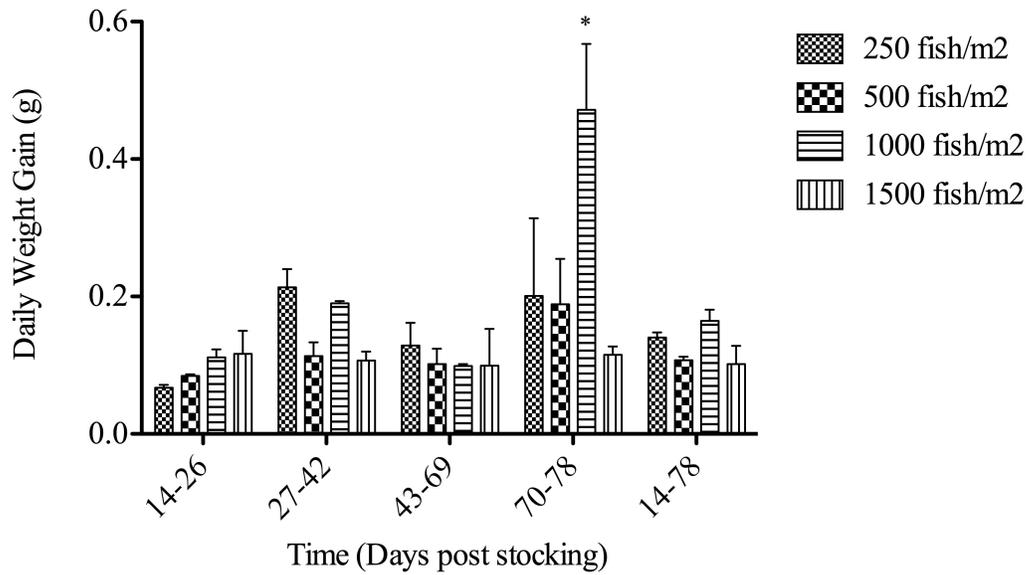


Figure 18. Daily weight gain (g/day) of Southern flounder stocked at different densities over a 78-day growth period (mean \pm STD, 2 tanks/treatment). Density was found to cause a significant difference in daily weight gain among groups ($P < 0.01$, 2-way ANOVA). This difference occurred in tanks stocked at 1000 fish/m² as shown by asterisk (*) between 70 and 78 days ($p < 0.05$, Bonferroni post-hoc test), but there was no overall effect throughout the course of the experiment (14 to 78 days).

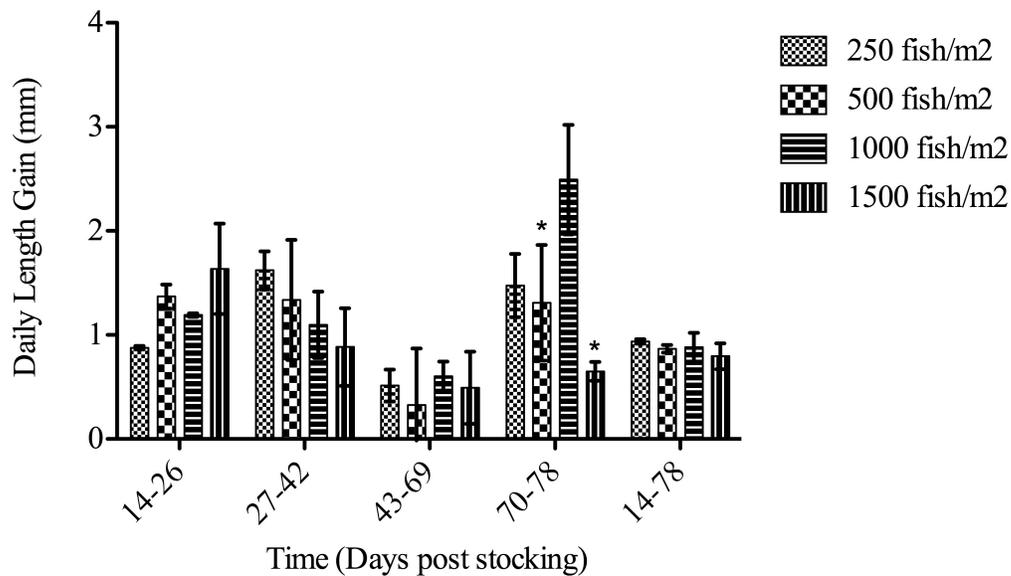


Figure 19. Daily length gain (mm/day) of Southern flounder stocked at different densities over a 78-day growth period. Density was found to cause a significant difference in daily weight gain among groups ($p < 0.01$, 2-way ANOVA). Between 70 and 78 days, daily length gain of both 500 fish/m² and 1500 fish/m² densities were significantly different than 1000 fish/m², however no groups differed from 250 fish/m² ($p < 0.05$, Bonferroni post-hoc test). However, daily length gain did not differ among groups throughout the study (14 to 78 days) (mean \pm STD, 2 tanks/treatment).

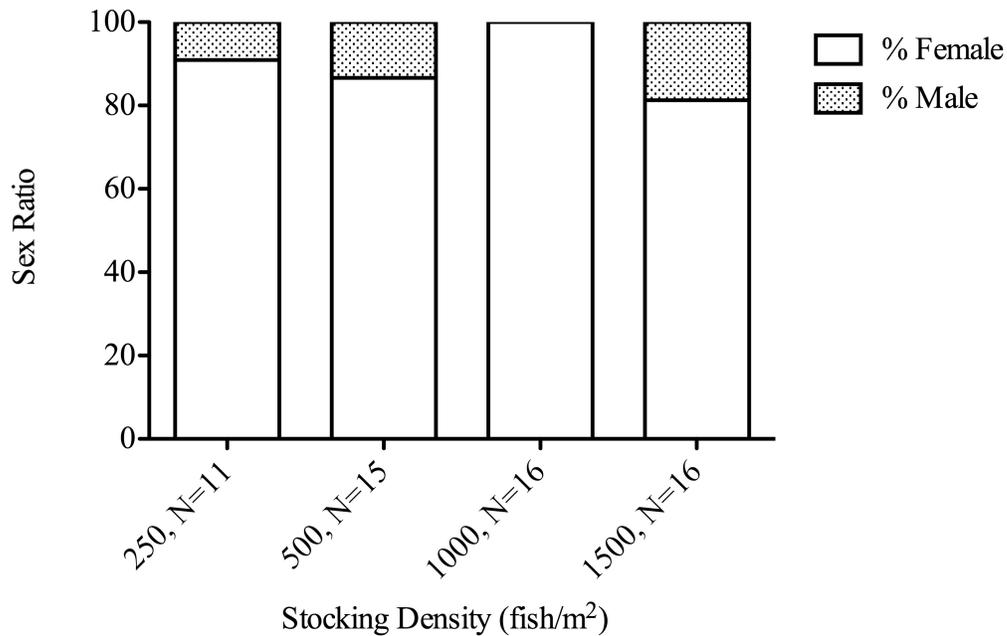


Figure 20. Sex ratios of XX genotype Southern flounder stocked at different densities. Sex was determined by gene expression of aromatase, FoxL2, and MIS. The low stocking density (250 fish/m²) caused negligible masculinization (91 % females, 9 % males), whereas medium stocking density (1000 fish/m²) caused no masculinization (100 % females, 0 % males). Stocking densities of 500 fish/m² resulted in slight masculinization (87 % females, 13 % males), as did stocking densities of 1500 fish/m² (81 % females, 19 % males). Sex ratios did not differ significantly among groups from expected 100: 0 female: male proportions (Chi-square analysis).

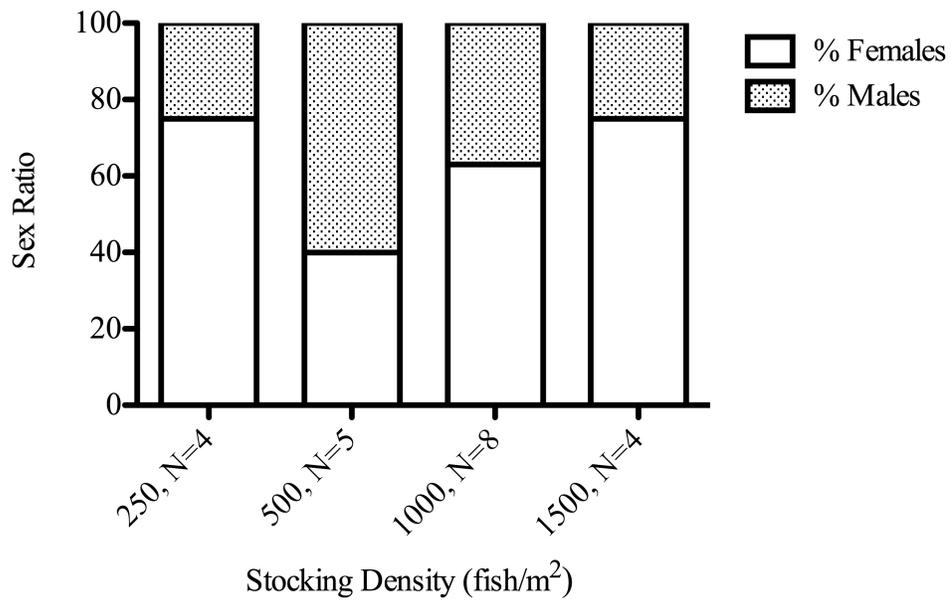


Figure 21. Sex ratios of XX genotype Southern flounder stocked at different densities. Sex was determined by histological analysis. Stocking densities of 250 fish/m² resulted in 75 % females, 500 fish/m² resulted in 40 % females, 1000 fish/m² resulted in 63 % females, and 1500 fish/m² resulted in 75 % females.