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Investigation of the sex determination mechanisms in summer flounder (Paralichthys dentatus) and black sea bass (Centropristis striata)

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INVESTIGATION OF THE SEX DETERMINATION MECHANISMS IN SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) AND BLACK SEA BASS (*CENTROPRISTIS STRIATA*)

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ABSTRACT

INVESTIGATION OF THE SEX DETERMINATION MECHANISMS IN SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) AND BLACK SEA BASS (*CENTROPRISTIS STRIATA*)

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In many cultured finfish species, sexually dimorphic growth is observed, therefore, understanding the mechanisms of differentiation in fishes is necessary for successful aquaculture. Summer flounder (*Paralichthys dentatus*) and black sea bass (*Centropristis striata*) are good candidate species for aquaculture because they easily adapt to culture conditions and have high market value. The production of monosex populations is advantageous in species exhibiting sexually dimorphic growth rates. The purpose of this research was to gain an understanding of the mechanisms involved in sex differentiation of summer flounder and black sea bass. In summer flounder, monosex female culture is beneficial because females grow two to three times larger than males. Like many teleosts with an XX/XY sex chromosome system, Paralichthid females are homogametic (XX) and males heterogametic (XY). Only homogametic individuals can be phenotypically sex reversed during sex differentiation. In the first part of this study, meiogynogenesis and temperature-dependent sex

determination were used in the first steps towards the production of monosex female cultures of summer flounder. Meiogynogens were produced by applying a 6 minute pressure shock (8,500 psi) 2 minutes post-fertilization resulting in the production of 1,100 juveniles. For temperature-dependent sex determination, meiogynogens and control diploids were reared at a low temperature regime (12-20 °C), 21, and 26 °C for 376 days post hatch (DPH). Females were primarily produced at the low temperature regime (62.5 % in meiogynogens and 22.6 % in controls). The second part of this study examined the disruption of female-specific sex differentiation in black sea bass. Metamorphosed juveniles were reared at 17, 21, and 25 °C for 279 DPH. While there was not a significant difference in sex ratios among treatments, the expected outcome of 100 % female was not obtained in this protogynous species. Additionally, males were significantly larger than females by 275 DPH. This may provide a mechanism for future production of monosex (male) populations of this protogynous species. Understanding the control of sex in this species is crucial for broodstock maintenance. This research will increase our understanding of the reproductive biology of these species and improve their culture.

INTRODUCTION

The need for aquaculture increases as the world population continues to grow. This need is increased further by the decrease in fisheries landings. According to the Agricultural Research Service, global aquaculture production will have to increase by 500 % by the year 2025 to meet the needs of a projected world population of 8.5 billion. Currently, the United States imports 40 % of its seafood and this trade deficit is second only to that of petroleum (ARS 2003). Despite the vast number of endemic species in the Northeastern United States, only one species, the Atlantic salmon (*Salmo salar*), is cultured in significant quantities. The salmon industry faces many challenges, including decreasing market values due to competition from imported fish. The need for new aquaculture species is critical for the expansion of the United States industry.

When choosing new species for aquaculture, several qualities must be considered. The species must command a premium price, have high consumer demand, and easily adapt to domestication at high densities. Previous experiments have shown promise for the culture of black sea bass (*Centropristis striata*) because they meet all of these criteria. Adult black sea bass acclimate well to hatchery conditions (Copeland et al. 2003) and have been spawned in captivity (Berlinsky et al. 2005), while larvae can be cultured over a variety of salinities and are easily weaned to formulated feeds (Berlinsky et al. 2000). Limited commercial production of black sea bass has been initiated at Great Bay Aquaculture LLC (GBA), a collaborator of the University of New Hampshire.

Summer flounder (*Paralichthys dentatus*) have also shown promise for aquaculture. In Asia, South America, and Central America Paralichthid flounder are currently being cultured successfully. Asia currently produces 573,000 tonnes of Paralichthid flounder annually (Lungren et al. 2006). Summer flounder have also been found to command competitive prices (Waters 1996), suggesting profitable culture of this species. Currently, GBA is the only company producing summer flounder juveniles in the United States. In species that exhibit sexually dimorphic growth rates, the production of monosex populations is essential to profitable culture.

Controlling sex differentiation in summer flounder will allow for the manipulation of sex ratios, which is important because females grow considerably faster and larger than males (Morse 1981; Dery 1988; Terceiro 1998; King and Nardi 2001). In one study, King and Nardi (2001) found that during routine production, female summer flounder grew 1.4 times larger than males at 15 months post hatch and were projected to be twice as large by harvest at 23 months. Therefore, aquaculture of this species would benefit from the production of all female stocks. Monosex female culture would increase production efficiency by reducing: size heterogeneity, mortality due to cannibalism, length of grow out period, and labor costs due to size grading (Luckenbach et al. 2002; Borski et al. 2003).

Similarly, controlling sex differentiation in black sea bass may allow for the manipulation of sex ratios. Although this species is a protogynous hermaphrodite in the wild, female-specific differentiation has been found to be disrupted under

culture conditions (Benton 2005). This finding may be important for commercial production, as black sea bass may exhibit sexually dimorphic growth rates, with males growing faster than females (Alexander 1981). Consequently, it is necessary to understand factors involved in sex differentiation in this species.

Reproductive strategies vary greatly in teleosts. Many teleosts are gonochorists, which develop as male or female and remain so throughout their lives. In contrast, hermaphroditic fish can produce both male and female gametes at some point in their lives. Synchronous hermaphrodites produce both male and female gametes at the same time and some are capable of self-fertilization. Sequential hermaphrodites, on the other hand, produce one gamete type then reverse sex and produce the other. There are two types of sequential hermaphrodites, protandrous and protogynous. Protandrous hermaphrodites are those which develop as male first while protogynous hermaphrodites mature as female first (Atz 1964). Protogynous species may be further divided into two forms. Males in monandrous species develop from previously matured females, while males in diandrous species can differentiate directly as males or sex reverse from females.

The differentiation of sex depends on both genotypic and environmental factors. In many higher vertebrates genotypic sex leads to phenotypic sex development, but in many lower vertebrates phenotypic sex can be altered by environmental factors (Hunter and Donaldson 1983; Piferrer 2001).

Sex determining mechanisms vary greatly among vertebrate groups, although some mechanisms remain relatively conserved (Manolakou et al. 2006).

There are three primary sex determining mechanisms: chromosomal, polygenic, and genotypic-environmental interaction (Piferrer 2001). In chromosomal and polygenic sex determination the sex is determined at fertilization. Chromosomal sex determination involves inheritance of highly evolved sex chromosomes which contain most of the genes responsible for the development of sex. In the XX/XY system the female is the homogametic sex, while in the WZ/ZZ system the male is the homogametic sex. In polygenic sex determination, the genes controlling sex are located on other chromosomes (autosomes) and sex is the result of the cumulative effect of their expression (Hunter and Donaldson 1983; Piferrer 2001). Finally, in genotypic-environmental sex determination, environmental factors may alter genotypic sex. These environmental factors include pH (Rubin 1985), salinity (Prevedelli and Simonini 2000; Saillant et al. 2003), nutrition (Prevedelli and Simonini 2000), density (Lindsey 1962), social cues (Francis and Barlow 1993; Liu and Sadovy 2004), and temperature (Conover and Kynard 1981; Sullivan and Schultz 1986).

Temperature is the most common environmental factor affecting sex differentiation. Temperature-dependent sex determination (TSD) was first found in reptiles (Crews 1996) and amphibians (Hayes 1998), but has since been demonstrated in many teleost species (Conover and Kynard 1981; Middaugh and Hemmer 1987; Schultz 1993; Baroiller et al. 1996; Romer and Beisenherz 1996; Kwon et al. 2000; Kitano 2002; Mylonas et al. 2003; Nan et al. 2005). TSD has not been found in mammals and birds as embryonic development in these species occurs under controlled temperature conditions (homeothermy;

Strussmann and Nakamura 2002). Patterns of TSD are (1) males at high and low temperatures and females at intermediate temperatures, (2) males at high temperatures and females at low temperatures, or (3) females at high temperatures and males at low temperatures (Valenzuela and Lance 2004). It is likely that TSD works through the modification of development due to differential expression of genes controlled by a temperature sensitive process (Spotila et al. 1994).

The molecular events that occur during sex differentiation in mammals have been well studied. Sex determination in mammals is chromosomal, where individuals carrying a Y chromosome will develop as males and those without will develop as females. Specifically the *SRY* gene found on the short arm of the Y chromosome is responsible for male differentiation (Waters et al. 2007). There are a number of other genes involved in sex differentiation that are not sex specific but are sexually dimorphic. These genes include *SOX*-9, *DAX*-1, *SF*-1, and *WT*-1. *SOX*-9 is believed to be important in male differentiation (Moreno-Mendoza et al. 2004; Barrioneuvo et al. 2006) while *DAX*-1, located on the X chromosome, is likely important in ovarian differentiation (Manolakao et al. 2006). *SF*-1 acts as a global mediator of steroidogenesis (Caron et al. 1997). *WT*-1 encodes a transcription factor used during kidney and gonadal development (Hastie 1994; Kojima et al. 2008). However, the exact roles of these genes during sex differentiation have not been established.

The sex determining mechanism in birds is also chromosomal, with females being heterogametic (ZW) and males homogametic (ZZ). It is assumed

that there is a comparable W-linked gene responsible for ovarian development in birds (Short 1998), but it has yet to be identified. There are two proposed mechanisms for sex differentiation in birds. The first suggests that the testis is the default gonad, and genes on the W chromosome invoke ovarian development, similar to *SRY* in mammals. The second model suggests there is a dose-dependent mechanism based on the Z chromosome, where a double dose is required for male differentiation (Clinton 1998; Mittwoch 1998). Other sex determining genes, such as *SOX*-9 and *DMRT*1 have also been identified in birds (Wibbels et al. 1998; Ferguson-Smith 2007).

Reptiles possess a variety of sex-determining mechanisms from genetic sex determination (GSD; XX/XY or WZ/ZZ) to TSD (Viets et al. 1994; Wibbels et al. 1994). In some crocodilians and turtles there are no obvious sex chromosomes, and gonadal development is influenced by temperature and/or hormones (Spotila et al. 1998). The *SOX*-9 and *DMRT*1 genes have also been identified in reptiles (Jeyasuria and Place 1998; Spotila et al. 1998; Wibbels et al. 1998; Ferguson-Smith 2007).

Many fish species also posses GSD systems ranging from XX/XY to WZ/ZZ (Piferrer 2001) as well as TSD. *SRY* equivalents (*DMY* and *DMRT*1) have been found in fish (Strussmann and Nakamura 2002). In fish where GSD occurs, environmental factors may override GSD, and *SF-1* is likely involved in this pathway (Short 1998). Environmental and social factors likely moderate sex differentiation in fish through the hypothalamo-pituitary-gonadal (HPG) axis (Devlin and Nagahama 2002). This axis consists of the gonadotropin-releasing

hormone (GnRH) neurons of the hypothalamus, the gonadotropin (GtH) producing pituitary gland, and the gonad where sex steroid biosynthesis occurs (Figure 1). Gonadotropin I (GtH I) is analogous to mammalian follicle stimulating hormone and promotes steroid synthesis and gonadal differentiation. Gonadotropin II (GtH II) is analogous to mammalian luteinizing hormone and is produced prior to sexual maturation (Devlin and Nagahama 2002). The HPG axis is important in converting environmental stimuli to gonad differentiation information (Francis 1992; Strussmann and Nakamura 2002; Godwin et al. 2003) while gonadotropins have been found to play a critical role in sex differentiation in hermaphroditic fish (Devlin and Nagahama 2002).

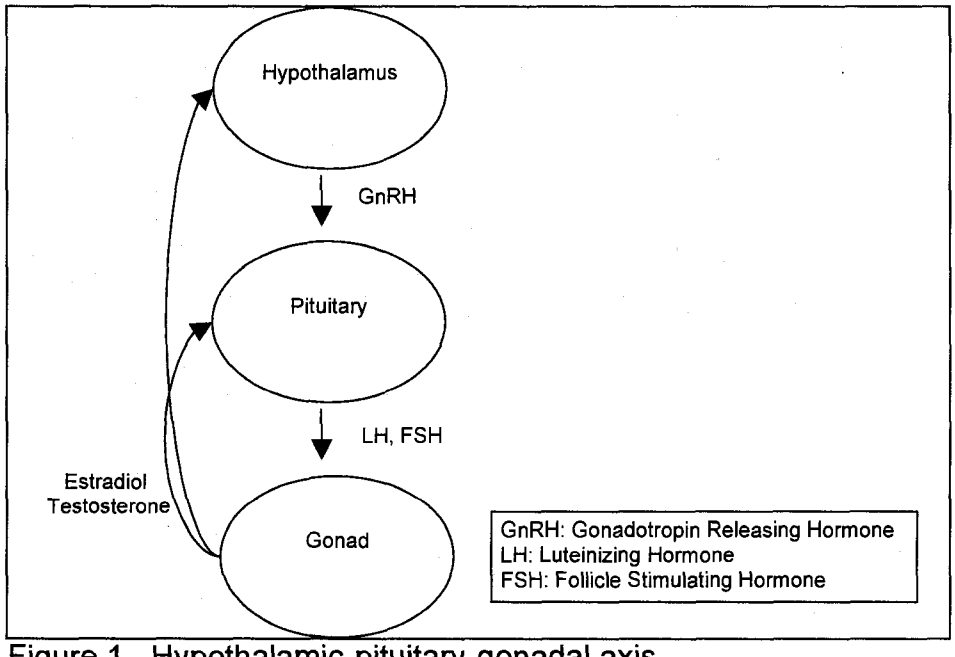

Figure 1. Hypothalamic-pituitary-gonadal axis.

In fish, sex differentiation is very "plastic" and may be altered by steroid hormones (Cardwell and Liley 1991). Estradiol has been found in higher levels in females and is believed to be the major sex steroid responsible for ovarian development while 11-ketotestosterone is found in males and is responsible for

testis development (Yamamoto 1969; Nakamura et al. 1998; Nagahama 1999). Numerous experiments have been performed on fish in which the sex was altered by administration of exogenous estrogens or androgens (Hunter and Donaldson 1983; Cardwell and Liley 1991; Piferrer 2001; Devlin and Nagahama 2002). Species that rely primarily on endogenous sex steroid production for gonad differentiation may be more susceptible to environmental sex determination. Androgen and estrogen receptors have been found in both ovaries and testes, suggesting exogenous steroids are capable of affecting both sexes (Strussmann and Nakamura 2002). In mammals sex steroids are believed to play a role in shaping the sexually dimorphic structures during sexual development rather than having a role in deciding the fate of the gonad (Strussmann and Nakamura 2002). Short (1998) suggested viviparous animals have evolved mechanisms to protect male embryos from becoming feminized by maternal estrogens, and therefore, sex determination is not affected by exogenous sex steroids in these species.

More recent studies have examined the role of enzymes responsible for steroid biosynthesis in sex differentiation. Cytochrome P450 aromatase (aromatase) is the enzyme responsible for the conversion of androgens to estrogens (Figure 2). Aromatase is part of a superfamily of heme proteins, the cytochrome P450s, that function as oxygenases (Stegeman 1993). Aromatase is a membrane bound enzyme associated with NADPH (Place et al. 2001) and uses the reductive equivalents from NADPH to convert androgens (C_{19}) to estrogens (C_{18}) by the removal of the methyl group and aromatization of the

steroid A ring (Simpson et al. 1997). Estrogens are critical for the processes of sex determination and differentiation, therefore, it is believed that aromatase is a key enzyme in these processes in fishes.

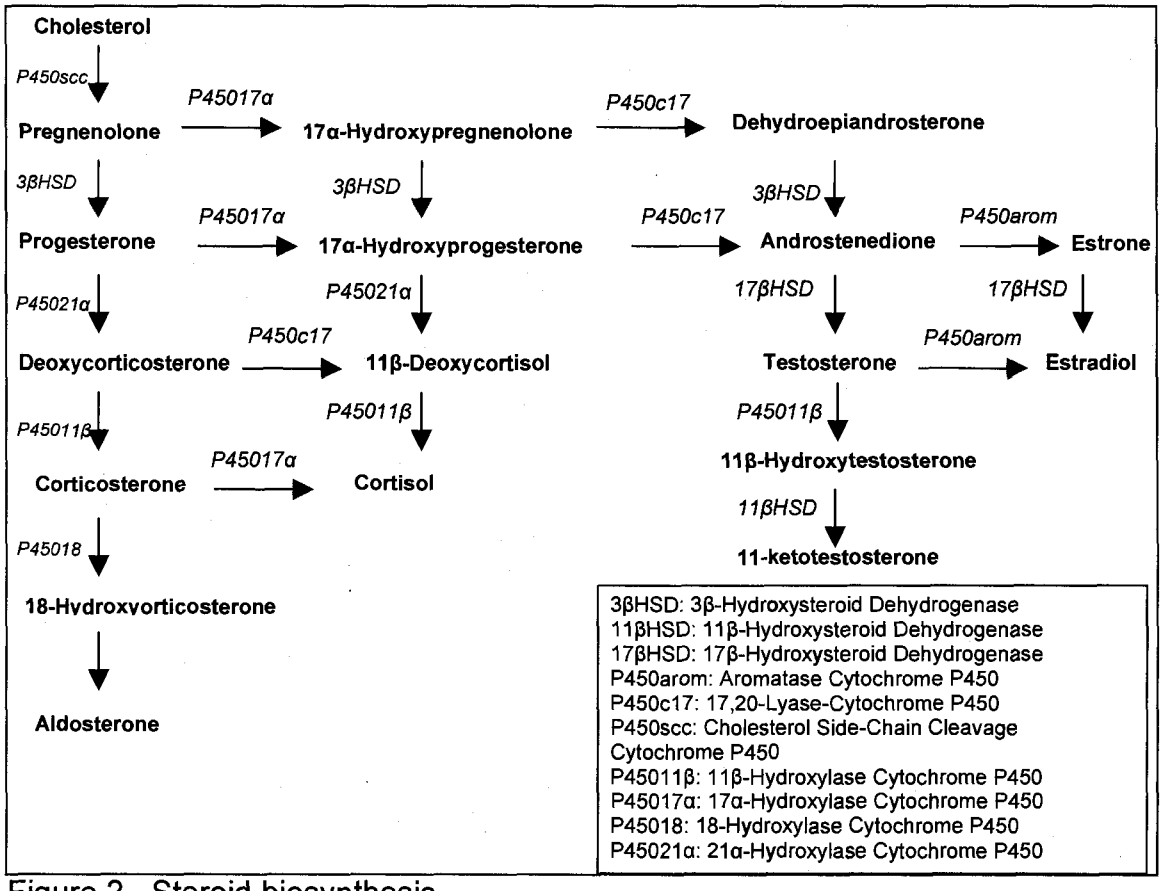

Figure 2. Steroid biosynthesis.

Aromatase is encoded by the *CYP*19 gene. There are two isoforms of this gene in fish, the ovarian *CYP*19a and the brain *CYP*19b (Gelinas et al. 1998; Tchoudakova and Callard 1998; Chiang et al. 2001; Kishida and Callard 2001; Kwon et al. 2001; Zhao et al. 2001; Blazquez and Piferrer 2004; Chang et al. 2005; Greytak et al. 2005). Previous studies have demonstrated that aromatase isoforms have different tissue distributions, responses to exogenous estrogen, and expression patterns during gonad ontogeny (Chang et al. 2005). *CYP*19a is primarily expressed in the follicular cells lining vitellogenic oocytes in the ovary

during vitellogenesis. *CYP*19b is expressed abundantly in the brain, specifically in the hypothalamus and ventral telencephalon, extending to the olfactory bulb (Chiang et al. 2001). The expression of *CYP*19a, and subsequent aromatase activity is responsible for estradiol synthesis in the gonads and, therefore, mediates ovarian differentiation (Guiguen et al. 1999; Kitano et al. 1999; Suzuki et al. 2004; Chang et al. 2005). The inhibition of *CYP*19a prevents estradiol biosynthesis, resulting in testicular differentiation (Guiguen et al. 1999; Kitano et al. 1999; Kwon et al. 2001; Uchida et al. 2004). Administration of aromatase inhibitors, such as Fadrozole (Novartis Pharma Ltd., Basil, Switzerland), during sex differentiation leads to reduced aromatase expression and testis formation in numerous fish species (Kitano et al. 2000; Kroon and Liley 2000; Kwon et al. 2000; Kitano 2002; Lee et al. 2003; Bhandari et al. 2004; Uchida et al. 2004; Li et al. 2006). These studies suggest that gonadal aromatase expression directs sex differentiation, with high levels of aromatase activity leading to ovarian development and low levels leading to testicular development. Brain aromatase, however, appears to be mainly involved in neural estrogen synthesis (Chang et al. 2005).

Cytochrome P450 11β-hydroxylase (11β-hydroxylase) is an important steroidogenic enzyme in the biosynthesis of glucocorticoids and mineralocorticoids (Kusakabe et al. 2002). It is also a member of the cytochrome P450 superfamily. 11β-hydroxylase is encoded by *CYP*11B (Stegeman 1993) and converts 11-deoxycortisol to cortisol (Perry and Grober 2003). 11β-hydroxylase is also important in teleosts in the production of 11-ketotestosterone

(11-KT) in males (Kusakabe et al. 2002). 11-KT production requires the oxygenation of testosterone by 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase. 11β-hydroxlase converts testosterone to 11β-hydroxytestosterone, while 11β-hydroxysteroid dehydrogenase converts 11-hydroxytestosterone to 11-KT (Perry and Grober 2003), the androgen needed for spermatogenesis (Jiang et al. 1996). A study on Nile tilapia (*Oreochromis niloticus*) found that 11β-hydroxylase expression was elevated at male producing temperatures (35 °C) compared to controls (27 °C), which suggests masculinizing temperatures may affect gene expression (D'Cotta et al. 2001).

Often endocrine changes occur before the gonads are visible in differentiating fish. It is believed that steroids begin to influence sex differentiation before clear histological differences can be observed (Baroiller et al. 1999). Enzymes have an optimal temperature at which they function and gene expression can also be thermally sensitive. Decreased gene expression and/or enzyme activity at critical temperatures provides likely mechanisms for TSD.

A final mechanism of sex determination that is less conventional is gynogenesis, the process of uniparental inheritance in which offspring obtain only maternal DNA. This may happen naturally or through experimental manipulation, although it is rare in nature. There are two types of gynogenesis: mitotic gynogenesis and meiotic gynogenesis. In mitotic gynogenesis, diploidy is obtained through the suppression of the first mitotic cleavage. In meiotic gynogenesis, the second polar body is retained during meiosis (Ihssen et al.

1990). Meiotic gynogens are not homozygous at all loci due to recombination that occurs during crossing over of chromosome pairs (Devlin and Nagahama 2002). Gynogenesis has been used to help identify sex-determining mechanisms in species in which it is unknown and also to create monosex populations for aquaculture. In order to achieve gynogenesis, the egg must be activated by spermatozoa to reinitiate meiosis, without pronuclear contribution. This may be performed by using inactivated sperm (ionizing, ultraviolet, chemical), sperm from a heterologous species, or a combination of both. Ultraviolet irradiation doses between 180 and 330 mJ/cm^2 have been used to deactivate sperm (Felip et al. 2001). The result of this process is the production of a gynogenetic haploid. Diploidy is then established by exposing activated eggs to an environmental stressor, such as thermal, chemical, or hydrostatic pressure treatments to prevent expulsion of the second polar body (Chourrout 1984; Gheyas et al. 2001). Pressure shocks have been used successfully in many culture species and are often the preferred method of choice (Ihssen et al. 1990; Benfey et al. 2000). Previous studies have found that pressure shocks between 492 and 703 kg/cm^2 (~7000-9000 psi) are effective in suppressing the second meiotic division. Pressure shocks of 592 kg/cm^2 (~8500 psi) applied for 5-10 minutes have been successfully used to induce meiogynogenesis in rainbow trout (*Salmo gairdneri*; Chourrout 1984), muskellunge (*Esox masquinongy*; Garcia-Abiado et al. 2001), red sea bream (*Pagrus major*; Kato et al. 2002), shortnose sturgeon (*Acipenser brevirostrum*; Flynn et al. 2006), and southern flounder (*Paralichthys lethostigma*; Morgan et al. 2006). The timing of

the pressure shock is very important and must be applied prior to anaphase II or meiosis cannot be suppressed. Shocks are generally applied immediately following fertilization to 20 minutes post-fertilization (Ihssen et al. 1990; Flynn et al. 2006). Viable gynogenetic diploids have been successfully produced in many species of fish (Garcia-Abiado et al. 2001; Gheyas et al. 2001; Flynn et al. 2006), including some flatfish, such as the Atlantic halibut (*Hippoglossus hippoglossus*; Tvedt et al. 2006).

By taking advantage of the temperature-dependent sex determination mechanism exhibited in many species of fish, it is possible to raise diploid gynogens at appropriate temperatures to create broodstock that are phenotypic males with an XX genotype. These XX males may then be bred with normal XX females to produce monosex female populations. This protocol has been effective in the commercial production of all-female populations of salmonids (Devlin and Nagahama 2002). A major advantage to the production of monosex cultures is that energy is diverted from reproduction to somatic growth.

The production of monosex female cultures of southern flounder has been performed by Borski et al. (2003). Researchers were able to create diploid gynogens by fertilizing eggs with UV-irradiated sperm and applying a thermal shock. The diploid gynogens were subsequently reared at a high-temperature to obtain XX male broodstock, which were then bred with normal females to obtain an all-female populations (Borski et al. 2003). Similar experiments were performed by Luckenbach et al. (2002) to obtain all-female stocks of southern flounder using pressure shocks.

While monosex male production has not been considered previously in black sea bass, the fact that female-specific differentiation is disrupted in culture conditions (Benton 2005) and juvenile males may grow larger than females (Alexander 1981) suggests that this may be desirable and feasible. Considerable differences in reproductive patterns, growth rates, and life history characteristics have been reported for black sea bass along their geographic range, reflecting adaptations to different environmental conditions, such as temperature. Black sea bass are found from Cape Cod, Massachusetts to Cape Canaveral, Florida and in the Gulf of Mexico (Wenner et al. 1986; Vaughan et al. 1995). The black sea bass found in the Gulf of Mexico are considered to be a separate subspecies (Hood et al. 1994), while those found in the Atlantic Ocean may be divided into two populations, separated by Cape Hatteras, North Carolina (Mercer 1978; Wenner et al. 1986; Vaughan et al. 1995). The northern Atlantic population (Mid-Atlantic Bight, MAB) migrates seasonally from shallow waters along the middle Atlantic and southern New England coasts during the summer to deeper water in the southern part of the middle Atlantic Bight during the winter (Musick and Mercer 1977). Southern populations (Southern Atlantic Bight, SAB) remain associated with hard substrates such as pilings and reefs year-round (Mercer 1978; Wenner et al. 1986). The spawning season progresses from south to north, occurring in January to April in the south and in March through June in northern waters (Mercer 1978; Wenner et al. 1986). A second, less significant spawning event occurs in the fall between September and November (Wenner et al. 1986; McGovern et al. 2002). The average size of fish changes

with latitude. Fish from Florida were on average 13-20 mm larger (standard length, SL) than fish from North Carolina (McGovern et al. 2002). In general, females predominate the size intervals less than 200 mm SL and ages less than 4 years old, while males are found mostly at sizes greater than 220 mm SL and ages greater than 4 years (Hood et al. 1994; McGovern et al. 2002). Age and size of maturity increase with increasing latitude as well. In Florida, males occurred most frequently at sizes greater than 220 mm SL and ages greater than 4, while in North Carolina they were found at sizes greater than 240 mm SL and ages greater than 5 (McGovern et al. 2002). A study examining the morphological and meristic differences between northern and southern populations of black sea bass found differences which suggest the division of stocks into multiple components (Shepherd 1991).

Differences in size, age at maturity, reproduction, and behavior of black sea bass in coastal U.S. waters are likely due to temperature differences across latitudes. Shepherd (1991) suggested that meristic differences in populations may reflect differences in the geographic origins of the larvae. He proposed two hypotheses about black sea bass: (1) they are a single stock that varies morphologically along a latitudinal cline, or (2) local spawning groups maintain adequate temporal, spatial, and reproductive isolation to sustain the characteristics of separate stocks. Different populations of black sea bass are exposed to different environmental conditions, therefore, it is likely that they adapt to their environment. As discussed above, the control of sex is primarily genetic in most vertebrates, but both genetic and environmental factors can

influence differentiation in many fish species. Although black sea bass are protogynous hermaphrodites, populations may have adapted to allow primary male differentiation in some individuals. Smith (1967) described the adaptive significance of various forms of hermaphroditism. In protogynous species, fish may either all change sex at the same age (completely metagonous) or part of the population may change sex at any given time interval (incompletely metagonous). Incomplete metagonous forms are most common as it allows the population to contain males and females at all times. The majority of protogynous species are found in tropical waters (Choat and Robertson 1975). The plasticity in reproductive strategies in closely related species suggests that they may evolve easily through subtle changes in the pathways to gonadal development. Hermaphroditism evolved to increase reproductive output, and the diversity of mechanisms "reflects the variation in habitats, the diversity of life histories, and the complexity of demographic interactions" (Devlin and Nagahama 2002).

To examine sex differentiation in summer flounder and black sea bass, two studies were conducted. The first involved the production of meiogynogens and the influence of temperature-dependent sex determination in summer flounder. The second experiment examined temperature-dependent sex determination as a possible mechanism involved in the disruption of female-specific differentiation of black sea bass.

CHAPTER I

INDUCED MEIOTIC GYNOGENESIS AND SEX DETERMINATION IN SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) *

Abstract

Meiogynogenesis and temperature-dependent sex determination were used to produce XX male summer flounder broodstock for future production of monosex (all female) populations. Meiogynogens were produced by mixing summer flounder eggs with ultraviolet irradiated (null) black sea bass sperm and applying a 6 minute pressure shock (8,500 psi) 2 minutes post-fertilization. Manually spawning four females resulted in production of 136,000 eggs, of which 95.6 $\pm$ 1.8 % were viable, 51.0 $\pm$ 13.0 % fertilized and 8.9 $\pm$ 6.0 % hatched. Following metamorphosis meiogynogens and controls were raised under a low temperature regime (12-20 °C), 21, and 26 °C for up to 376 days post hatch (DPH). Female sex determination was greater in meiogynogens (62.5 %) and control fingerlings (22.6 %) raised under a low temperature regime compared to those raised at the higher rearing temperatures: 0 % at 21 °C, and 0 and 3.9 % at 26 °C in meiogynogens and controls, respectively. These results suggest that summer flounder exhibit temperature-dependent sex determination, whereby low temperature during the critical phase preceding gonadal development is

* Chapter 1 is an article submitted for publication to Aquaculture by Heidi R. Colburn, George C. Nardi, Russell J. Borski, and David L. Berlinsky entitled, Induced Meiotic Gynogenesis and Sex Determination in Summer Flounder (*Paralichthys dentatus*).

necessary for female differentiation while higher temperatures favor male differentiation.

Keywords: Summer flounder; *Paralichthys dentatus*; Meiogynogenesis; Temperature-dependent sex determination

Introduction

The summer flounder (*Paralichthys dentatus*) inhabits coastal waters from Maine to Florida (Gutherz 1967; Collette and Klein-MacPhee 2002) and supports important recreational and commercial fisheries throughout the Mid-Atlantic Bight. Total annual landings of summer flounder peaked at 18,000 metric tons (mt) in 1980, but recent (1990-2006) landings have been much lower, fluctuating from 4,000 mt to 6,300 mt (NOAA 2008). Due to their high value and demand, considerable research has been conducted to establish culture conditions for this species (Deubler and White 1962; Burke et al. 1999; Watanabe and Carroll 2001; Gaylord et al. 2004) and limited commercial production has been underway for over a decade (Bengtson 1999).

Growth rate remains the dominant factor controlling profitability of land-based culture of this species, and the costs associated with juvenile growth to market size must be reduced to gain competitiveness on the global market (King and Nardi 2001). Summer flounder, like other Paralichthid species, exhibit sexually dimorphic growth rates with females growing considerably faster and larger than males (Morse 1981; King and Nardi 2001). A tremendous increase in growth performance can therefore be realized through the production of all-

female populations of fingerlings. In one study, King and Nardi (2001) found that during routine production, female summer flounder grew 1.4 times larger than males at 15 months post hatch, and were projected to be twice as large by harvest at 23 months. Monosex populations of Japanese flounder (*P. olivaceus*) have been produced commercially in Japan and Korea since 1990 and 1995, respectively (Yamamoto 1999; Seikai 2000).

Like many teleosts with an XX/XY sex chromosome system, Paralichthid females are homogametic (XX) and males heterogametic (XY) (Tabata 1991; Yamamoto 1999; Luckenbach 2004). During the sex-determining period of development, however, homogametic individuals can be phenotypically sex reversed by exposure to high water temperatures or exogenous steroids (Kitano et al. 1999; Kitano 2002). These XX-males can then be reared to maturity, distinguished from XY males in the population by progeny testing, and crossed with normal XX females to produce monosex populations (Hattori et al. 2007). To avoid the time and expense associated with progeny testing, populations of fish possessing only XX, maternal genotypes have been produced by diploid gynogenesis and then sex-reversed (Hulata 2001; Devlin and Nagahama 2002; Luckenbach et al. 2002; Borski et al. 2003).

Diploid gynogenesis has been accomplished for several fish species, including southern flounder (*P. lethostigma*; Luckenbach et al. 2004; Morgan et al. 2006), Atlantic halibut (*Hippoglossus hippoglossus*; Tvedt et al. 2006), Japanese flounder (Tabata et al. 1986; Tabata 1991), and sole (*Solea solea*; Howell et al. 1995), and involves a two-step process. Initially, oogenesis is

reinitiated by fertilizing eggs with genetically-inactivated, but motile spermatozoa. Ultraviolet (UV) irradiation has been successfully used in a number of fish species to crosslink paternal DNA and produce genetically-inactivated sperm (Felip et al. 2001; Morgan et al. 2006). The use of untreated or UV-irradiated, heterologous sperm further ensures that no parental genetic contribution is possible and that all surviving larvae were produced by gynogenesis. The second step involves re-establishing diploidy by blocking expulsion of the second polar body (meiotic gynogenesis or meiogynogenesis) or preventing the first embryonic cell division (mitotic gynogenesis or mitogynogenesis) with the use of thermal or physical shock shortly after fertilization (Ihssen et al. 1990). These procedures, coupled with exposure to proper sex-determining environmental conditions, have been used to sex-reverse broodstock of many important aquaculture species for production of monosex populations (Ihssen et al. 1990; Garcia-Abiado et al. 2001; Devlin and Nagahama 2002; Morgan et al. 2006).

The objectives of the present study were to induce meiogynogenesis in summer flounder using UV-irradiated heterologous sperm from black sea bass (*Centropristis striata*). The survival, growth, and sex determination of the meiogynogens were compared to control diploid summer flounder raised in recirculating systems under three temperature regimes.

Materials and Methods

Fish Husbandry

Summer flounder were captured in the coastal waters of Virginia and held at Great Bay Aquaculture LLC (GBA; Portsmouth, NH, USA) for two years prior to the start of the experiments. Each fish was implanted with a passive integrated transponder (PIT) tag for individual identification and averaged 5 years of age and 1.3 kg at the time of spawning. The fish were held in 6,000 L fiberglass rectangular tanks within a recirculating seawater (28-30 ppt salinity) system. Systems were equipped with biological and mechanical filtration, ultra-violet sterilization, foam fractionation, and photothermal control. Half-hour crepuscular periods were provided with 100 W incandescent bulbs to simulate dawn and dusk. 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 light period. Water temperature and dissolved oxygen were measured daily (Oxyguard Handy Gamma, Birkerod, Denmark) and total ammonia nitrogen and nitrite were monitored weekly (HACH®; Loveland, CO, USA). Water quality within the culture tanks remained within ranges suitable for rearing this species (Watanabe et al. 1998). The fish were fed a commercial ration (9 mm pellet, 50 % protein, 15 % fat, Burris, Franklinton, LA, USA) to apparent satiation 2-3 times per week. As summer flounder naturally spawn in the fall, photoperiod and temperature were maintained at 12L:12D and 19 $\pm$ 1 °C until two months prior to desired spawning and adjusted to 8L:16D and 14 °C, respectively (Watanabe et al. 1998; Bengtson 1999).

Mature male black sea bass were captured in commercial traps in the coastal waters of Rhode Island and held at GBA, under the conditions described above for two years prior to the start of the experiments. The sea bass were maintained on a phase-shifted photoperiod, and temperature was adjusted from 12 to 18 °C to initiate autumn spawning.

Spawning Procedures and Meiogynogen Production

During all procedures fish 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 visually assessed with the aid of a light table (Watanabe and Carroll 2001; Luckenbach et al. 2002). Those fish containing vitellogenic stage oocytes were induced to spawn with daily injections of carp pituitary extract (CPE, 2 mg/kg; Stoller Fisheries, Spirit Lake, IA, USA; Smigielski 1975; Berlinsky et al. 1997). Females were checked for evidence of ovulation daily by exerting gentle abdominal pressure. If ovulation did not occur, the degree of ovarian development was visually assessed and CPE was re-administered. This procedure was repeated daily (~3-4 days) until a sufficient number of individuals (3-4) with high quality eggs (> 50 mL) ovulated.

Ovulated eggs were collected into a 500 mL polypropylene beaker and their total volume recorded. A subsample of eggs (n = 200) was examined to assess quality. High quality (fertilizable) eggs from marine teleosts are generally clear, buoyant, spherical, and lack a perivitelline space prior to fertilization

(McEvoy 1984; Kjorsvik et al. 1990). An estimate of the number of eggs exhibiting these characteristics was determined. If most of the eggs appeared to be of high quality, the batch was retained for fertilization.

Manually expressed flounder and black sea bass milt was collected into 3 mL polypropylene syringes from 2-3 anesthetized males (0.5-2.5 mL/fish). Flounder milt was pooled in a 10 mL beaker and used immediately or held on ice no longer than 1 h prior to use. Pooled milt from black sea bass was diluted 1:10 with Ringer's solution (0.1 M KCl, 0.003 M $CaCl_2$, 0.003 M NaCl). Diluted milt (1 mL) was spread thinly over a Petri dish (85 mm diameter) and UV irradiated at 70 mJ/cm^2 at a distance of 10 cm from the UV source, as per methods of Morgan et al. (2006). Prior to use, spermatozoa were activated with seawater and their motility confirmed using a compound microscope (Olympus CH, Melville, NY, USA; 400 X).

Large quantities of meiogynogens and controls were produced in two trials over two days. Ovulation was induced as described above, and eggs from two females were collected in a 2 L polypropylene beaker. These eggs were divided into "meiogynogen" (treatment) and "diploid" (control) groups. Meiogynogens were produced by fertilizing the eggs with UV-irradiated black sea bass milt (0.4-0.6 mL) and 20-40 mL of filtered seawater (34 ppt). After mixing 2 min, eggs were transferred to a pressure shock chamber (TRC Hydraulics, Inc., Dieppe, NB, Canada) containing 25 ppt seawater and exposed to 8,500 psi for 6 min according to the procedures of Morgan et al. (2006). Controls were produced by fertilizing eggs with untreated summer flounder milt. Both control and

meiogynogen eggs were then transferred to a calibrated separatory funnel containing 700-800 mL seawater and statically incubated for 15 min to allow the buoyant (viable) and sinking (non viable) eggs to separate. The volumes of both groups of eggs were recorded. The number of eggs/mL was estimated (1,200 eggs/mL) based on previous findings (Berlinsky et al. 1997). The percentage of fertilized eggs was determined after 2 h (4-8 cell stage) by microscopic examination of approximately 200 eggs. The viable eggs were then incubated at GBA in 100 L conical tanks at 15-17 °C and 35 ppt salinity until hatch.

Duration of Pressure Shock

To determine if meiogynogen production could be improved by altering the pressure shock duration, the eggs from two summer flounder were pooled and held at 18 °C prior to fertilization and pressure shock treatment. Forty mL aliquots of eggs were transferred to 1 L beakers and fertilized with UV-irradiated black sea bass sperm, as above. After the two minute fertilization, the egg and sperm mixture was transferred to the pressure shock chamber containing 25 ppt seawater. The eggs were pressure-shocked at 8,500 psi for 4, 6, or 8 minutes (in triplicate) and the order of fertilization and pressure shock duration was randomized to normalize any pre-fertilization incubation effects.

Following pressure shock, the eggs were transferred to a separatory funnel as described above. Buoyant eggs were returned to the 1 L beaker and brought to 600 mL seawater (35 ppt) with supplemental aeration and incubated between 15 and 17 °C until hatch. On days one and two post-fertilization, eggs

were transferred to the separatory funnel to determine the volume of buoyant and sinking eggs and to perform a water change (35 ppt, 17 °C). Only buoyant eggs were retained and pre-hatch viability (beating hearts) was assessed on days three and four post-fertilization.

Following hatching, the meiogynogenetic and haploid larvae were enumerated. Haploid larvae are easily distinguished by abnormal characteristics ('haploid syndrome') that include kyphosis (bowing of the body), body shortening, underdevelopment of the head, and tail deformities (Purdom 1969; Chourrout and Quillet 1982; Thorgaard 1983; Luckenbach et al. 2004). All fertility and viability calculations were based on total initial egg volumes.

Temperature-Dependent Sex Determination

The control and meiogynogenetic larvae produced above were raised separately through metamorphosis at GBA in 800 L tanks in a recirculating system with filtered (10 micron) and UV sterilized seawater at 26-29 ppt salinity and 17-19 °C. The fish were fed according to standard GBA protocols with supplemental microalgae (*Nanochloropsis* sp., ~$3x10^8$/mL) provided during the first 14 days. All fish were weaned onto a commercial diet (Otohime B1; Reed Mariculture, CA, USA) by 34 days post hatch (DPH).

Following metamorphosis, the juveniles (41 DPH) were transferred to the Aquaculture Research Center (ARC) at the University of New Hampshire (Durham, NH, USA) where they were housed in three recirculating systems. Each system consisted of four 235 L cylindrical tanks, biological and mechanical

filtration, ultraviolet sterilization, foam fractionation, and photothermal control. One-hundred and fifty control and meiogynogen fish were transferred to two tanks of each system. The three systems were maintained on a 12L:12D photocycle, with 30 min crepuscular intervals. Light intensities were 0, 15, and 40 lux during dark, crepuscular, and light periods, respectively. The fish in all systems were acclimated for one week at 18 °C before being heated or cooled one degree per day to reach final temperatures of 12, 21, and 26 °C. To simulate rising spring temperatures, the temperature in the 12 °C treatment was raised by one degree at 178, 180, 182, 184, 226, 264, 278, and 310 DPH to a final temperature of 20 °C. Temperature and dissolved oxygen were monitored daily (OxyGuard Handy Gamma, Birkerod, Denmark) and nitrates, total ammonia nitrogen, and pH were monitored weekly (HACH®, Loveland, CO, USA). The fish were fed an appropriately-sized marine diet according to GBA protocol in excess four times daily and uneaten feed was removed by siphon.

For length measurements, 6 fish were sampled from each control tank at 73, 101, 115, 143, and 192 DPH and 12 fish were sampled at 59 and 87 DPH. Total lengths were measured using a digital caliper (ProMax Ultra-Cal IV; Newton, MA, USA) until fish reached 100 mm, after which they were measured using a metric ruler (Aquatic Eco-Systems, Inc., Apopka, FL, USA). For routine histology 6 fish were sampled from each control tank at 227 and 336-376 DPH and 6 meiogynogens were sampled at 336-376 DPH. Fish were euthanized with an overdose of MS-222 and the gonads dissected and preserved in Bouin's fixative. The samples were paraffin-embedded, sectioned at 5 µm, stained with

hemotoxylin and eosin, and examined under a compound microscope (Zeiss AxioCam MRm, Carl Zeiss Inc., Thornwood, NY, USA). Gender classification was based on gonad characteristics previously described (Tanaka 1987; Tabata 1991; Nakamura et al. 1998; Goto et al. 1999; Luckenbach et al. 2003; Luckenbach et al. 2004; Flynn et al. 2006). Fish with gonads containing seminal lobules or in various stages of spermatogenesis were designated as male (Figure 1a) and those with lamellate structure or clear oogonia were designated as female (Figure 1b). The fish was classified as undifferentiated if germ cells were not distinguishable as spermatogonia or oogonia (Figure 1c) and sterile if gonadal tissue had no apparent germ cells (Figure 1d). Due to the small size of some sampled fish, insufficient gonadal tissue was obtained for unambiguous classification. Sex ratios were calculated from differentiated fish only.

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) using JMP IN 5.1 software (SAS Institute, Inc., Cary, NC, USA). Percent data were arcsine transformed prior to analysis. Differences between means with a probability (p) less than 0.05 were considered significant and subjected to a Tukey's HSD post hoc test to distinguish differences among treatments. Differences with a p less than 0.10 were considered trends and are discussed as such. Results are expressed as means plus or minus standard error of the mean (SEM).

Results

Spawning Procedures and Meiogynogen Production

In the first production of meiogynogens (64,000 eggs) viability (buoyancy) was 94.3 % of which 38.0 % were fertilized. From this, 2.9 % hatched for a total of 1,750 larvae. In the second meiogynogen production (74,000 eggs) viability was 96.8 %, of which 64.0 % were fertilized. From this, 14.9 % hatched for a total of 10,750 larvae. From the pooled larvae 1,100 meiogynogens (8.8 %) survived through metamorphosis. Percent viability and fertilization were not different in the control treatment but hatching ranged from 50-60 %.

Duration of Pressure Shock

There was no significant difference among pressure shock durations on the number of buoyant eggs at 0 or 1 days post fertilization (DPF). There was, however, a significant decline in the number of buoyant eggs at 2 DPF with the 8 versus the 4 min duration, but none between the 6 min and 4 or 8 min durations. There was no significant difference among treatments in development prior to hatching at 3 or 4 DPF or the number of diploid versus haploid larvae hatched. There was a decreasing trend in the number of diploid versus haploid larvae hatched with longer duration of pressure shock ($p = 0.0862$; Table 1).

Temperature-Dependent Sex Determination

Throughout the study there were 180, 226, and 215 observed mortalities at 12, 21, and 26 °C treatments, respectively, which did not differ among

treatments. Gynogens experienced higher mortality than control diploids at each treatment (Table 2). The number of observed mortalities was highest within the first 100 DPH (65 %), then dropped to low levels for the remainder of the study. The number of fish unaccounted for at the end of the study (0-56) did not differ among treatments and may have been due to cannibalism.

The growth rates varied among temperature treatments, with fish at 21 °C growing fastest and those at low temperature regimes the slowest. Lengths of fish were not significantly different among treatments prior to 87 DPH. By 101 DPH the fish at the 21 °C and 26 °C treatment were not significantly different in length, although they were significantly larger than those at 12 °C. This pattern continued through the end of the study. In addition, at the last sampling event (336-376 DPH), meiogynogens were significantly smaller than control diploids at all three temperature treatments (Table 3).

Sex differentiation of fish reared under different temperature regimes is shown in Table 2. A greater number of females were produced with the low temperature regime (controls = 22.6 %, meiogynogens = 62.5 %), while a substantially higher proportion of males or only males was produced at the higher temperatures (96.1-100 %). The one intersex (both testicular and ovarian tissue) and six sterile fish were not included in the sex ratio calculations.

Discussion

In the present study, meiogynogenesis and temperature-dependent sex determination were used to produce XX males for use as future broodstock.

Using conditions developed for the production of southern flounder meiogynogens (Morgan et al. 2006) greatly facilitated efforts with summer flounder. Although the survival of meiotic gynogens was low, it was similar to that reported in other flatfish species, including Atlantic halibut (Tvedt et al. 2006), European flounder (*Platichthys flusus*; Purdom 1969), Japanese flounder (Tabata 1991) and southern flounder (Luckenbach et al. 2003) and was likely due to increased homozygosity and expression of deleterious recessive alleles. As only two females were used for meiogynogen production and juveniles were pooled following metamorphosis, it was not determined if significant variation in survival exits among females. Assuming normal fertility of the sex-reversed fish, as found in other species, more than enough meiogynogen broodstock were produced in this study for the commencement of commercial production of monosex populations.

During meiogynogen production diploidy is reestablished following activation of the ova with genetically inactivated sperm by disrupting the microtubule assembly during the second meiotic or first embryonic mitotic division. For this purpose, both mechanical (pressure) and thermal (heat or cold) shock have been employed, with varying degrees of success. In addition to the type of shock, some variables contributing to success include the magnitude and duration of the shock and the time post-fertilization that it is applied. Pressure shocks of 800-8,500 psi, applied for 4-6 min have been successfully used to induce meiogynogens in a number of species including striped bass (*Morone saxatilis*; Leclerc et al. 1996), sole (Howell et al. 1995), Atlantic halibut (Tvedt et

al. 2006), and shortnose sturgeon (*Acipenser brevirostrum*; Flynn et al. 2006). Morgan et al. (2006) used these procedures to produce southern flounder meiogynogens, and found similar results if the shock was applied 1, 2 or 3 minutes post-fertilization. Results from the present study indicate a trend towards improved meiogynogen survival with pressure shocks of shorter duration. Further studies are necessary to determine if significant improvement can be gained by further shortening the pressure shock duration, such as that used to produce European sea bass (*Dicentrarchus labrax*) meiogynogens (Peruzzi et al. 2004).

In many fishes, exposure to high water temperature during the period of sex determination can masculinize XX individuals through suppression of cytochrome P450 aromatase (aromatase) gene expression in the gonad (temperature-dependent sex determination, TSD; Kitano et al. 2000; Piferrer and Blazquez 2005). Aromatase is the enzyme responsible for conversion of C_{19} androgens (e.g. testosterone) to estrogens, and its expression has been shown to precede gonad development in Paralichthid species (Luckenbach et al. 2005). Furthermore, phenotypic sex reversal was demonstrated in XX Japanese flounder exposed to an aromatase inhibitor (Fadrozole; Kitano et al. 2000). TSD has been found in all Paralichthid species examined to date, and sex-determining temperatures are species-specific. For instance, in Japanese flounder low (15 °C) or high (25-27.5 °C) temperatures produce a greater number of males, while 1:1 sex ratios occur at moderate temperatures (20 °C; Yamamoto 1999). Similar

results were found in the southern flounder with a balanced sex ratio occurring at 23 °C and male-skewed at 18 and 28 °C (Luckenbach et al. 2003).

In the wild, summer flounder spawn over the continental shelf in the fall through the winter, when water temperatures are low (12-19 °C; Smith 1973). Following hatching, the larvae are transported by water currents toward coastal estuarine areas that serve as nursery grounds during the warming spring months (Able et al. 1990; Szedlmayer et al. 1992; Szedlmayer and Able 1993). Under natural conditions, summer flounder generally produce a balanced sex ratio (Morse 1981), and are likely exposed to relatively low temperatures (9-18 °C; Smith 1973) during the sex-determining period (~100-150 mm). In the present study, female phenotypes were primarily produced under the low temperature regime, that most simulated natural conditions. As only XX individuals can develop female phenotypes, irrespective of environmental conditions, about 50 % of the control fish and all of the meiogynogens could theoretically have differentiated as females. The fact that only 22.6 and 62.5 % of the controls and meiogynogens, respectively, developed as females suggests that either the exact female-determining temperature was not provided and/or other sex determining environmental influences were present. Male phenotypes were also observed in Japanese flounder reared at female-inducing temperatures (Yamamoto 1999). Also in the current study, two control fish, reared at a male-determining temperature (26 °C) developed as females, a phenomenon also observed in studies on southern flounder (Luckenbach et al. 2003). These two females were among the largest fish sampled (top 10), and one was the largest. These

findings may indicate resistance to aromatase suppression in some individuals, but the underlying mechanism responsible is currently unknown.

Fish size has been shown to be one of the most important determinants in gonadal differentiation (Hunter and Donaldson 1983). In southern flounder, body size had a greater effect than age in gonadal differentiation, as stunted 12-month-old fish differentiated at a similar size to fast growing, 9-month-old cohorts (Luckenbach et al. 2003). This was also shown in the present study where most gonadal differentiation was complete in fish reared at 21 and 26 °C by 227 DPH (~150 mm) but not until 336-376 DPH in those raised at colder temperatures (~160 mm). Three fish greater than 150 mm remained undifferentiated, however, and six fish, primarily from the high temperature treatment, appeared to be sterile with no germ cells present in their gonadal tissue. It has previously been found that high water temperature can induce sterility in two species of pejerrey (*Odontesthes bonariensis* and *Patagonina hatcheri*; Strussmann et al. 1998) and that a low percent of hatchery-reared summer flounder do not undergo sexual differentiation (G. Nardi unpublished data). Further research is necessary to determine the conditions that induce sterility in this species and its subsequent effect on somatic growth. Although lower temperature regimes appear requisite for feminization in summer flounder, they also exhibit slow growth and can reduce or eliminate the advantage of creating monosex populations. Therefore, determining the minimum water temperature and exposure duration required for feminization (or to reduce masculinization) is necessary before the full economic advantages associated with sexually dimorphic growth can be realized.

Conclusion

The results of this study indicate that meiogynogenesis and temperature-dependent sex determination may be used to produce XX-male broodstock for future production of monosex summer flounder populations. Meiogynogens were successfully produced by fertilizing eggs with UV-irradiated (70 mJ/cm^2) black sea bass sperm and exposing them to pressure shocks of 8,500 psi for 6 minutes applied two min post fertilization. Under these conditions there was 51.0 % fertilization, 8.9 % hatch, and 8.8 % survival through metamorphosis. Rearing fish at relatively high temperatures (21 and 26 °C) sex reversed most individuals. Although the exact female-determining temperatures were not determined, the low temperature treatment resulted in the greatest proportion of female phenotypes.

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Figures and Tables

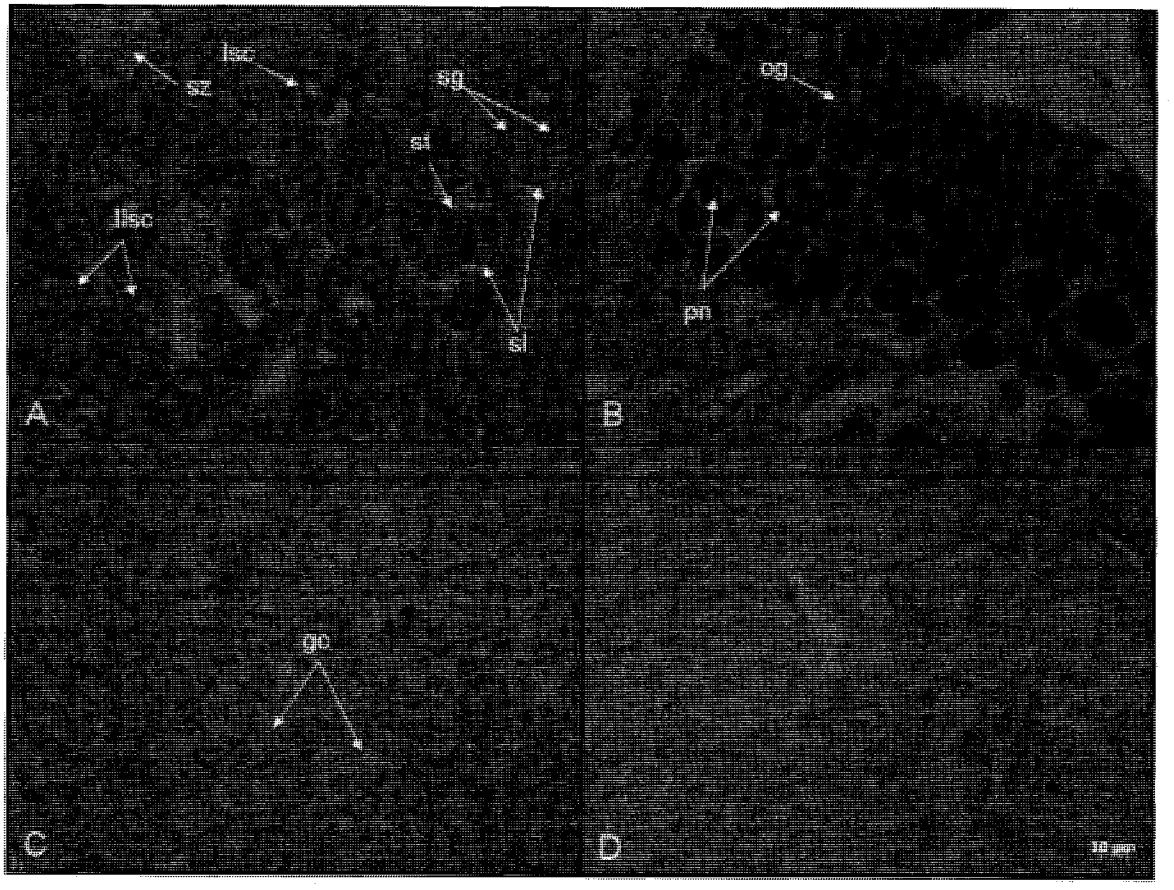

Figure 1. Histomicrographs of summer flounder gonads at 336-376 DPH depicting: A) a differentiating male with cells at various stages of spermatogenesis in seminal lobules, B) an ovary with primary oocytes, C) an undifferentiated gonad with gonial cells, and D) a gonad with germ cell deficiency (sterile). sg: spermatogonia; Isc: primary spermatocytes; IIsc: secondary spermatocytes; st: spermatids; sz: spermatozoa; sl: seminal lobules; og: oogonia/germ cells; pn: early perinucleolus stage oocytes; gc: undifferentiated gonial cells.

Table 1. The effect of pressure shock duration on meiogynogen production. Results are calculated from the total number of eggs at the start of each treatment. Superscript letters reflect significant differences among treatments ($p \leq 0.05$) within a column. Percent buoyant eggs were calculated on 0-2 DPH and percent prehatch on 3-4 DPH.

Treatment (minutes)	0 DPF	1 DPF	2 DPF	3 DPF	4 DPF	% Haploid
4	52.6 ± 3.7^{a}	46.9 ± 5.6^{a}	$5.7 + 0.7^{a}$	1.6 ± 0.6^{a}	0.5 ± 0.1^{a}	0.3 ± 0.1^{a}
6	46.7 ± 3.6^{a}	44.7 ± 1.7^{a}	4.4 ± 0.8^{ab}	0.9 ± 0.4^{a}	0.4 ± 0.1^{a}	0.2 ± 0.1^{a}
8	43.9 ± 4.0^{a}	44.1 ± 1.3^{a}	2.9 ± 0.2^{b}	0.4 ± 0.1^{a}	0.1 ± 0.1^{a}	0.0 ± 0.0^{a}

Table 2. Mean length (mm) and percent female summer flounder at 336-349 DPH. Percent female values are expressed as percent of differentiated fish only. Percent mortality is throughout the study. UD: undifferentiated.

Treatment	Male Length (mm)	Female Length (mm)	UD Length (mm)	Sterile Length (mm)	Intersex Length (mm)	Ambiguous Length (mm)	% Female	% Mortality
12 °C Control	193.1±4.2 (n=48)	193.1±4.7 (n=14)	181.0±0 (n=1)	122.0±0 (n=1)	194.0±0 (n=1)		22.6	15.3
12 °C Gynogen	174.0±2.0 (n=3)	152.6±9.9 (n=5)	116.0±0 (n=1)			95.3±5.3 (n=6)	62.5	44.7
21 °C Control	234.1±6.3 (n=15)						0.0	34.3
21 °C Gynogen	202.8±6.2 (n=13)		117.0±0 (n=1)			174.0±0 (n=1)	0.0	41.0
26 °C Control	221.3±3.4 (n=49)	278.0±24.0 (n=2)	167.5±7.5 (n=2)	217.6±14.9 (n=5)		103.0±0 (n=1)	3.9	17.7
26 °C Gynogen	195.0±7.8 (n=23)			119.0±0 (n=1)		126.0±0 (n=1)	0.0	54.0

Table 3. Length of summer flounder (mm) reared under different temperature regimes. Superscript letters reflect significant differences among treatments ($p \leq 0.05$) within a column.

Treatment	59 DPH	73 DPH	87 DPH	101 DPH	115 DPH	143 DPH	192 DPH	227 DPH	336-376 DPH
12 °C Control	20.7±0.7 (n=24) [a]	25.7±1.3 (n=12) [a]	28.5±1.1 (n=24) [a]	33.5±2.0 (n=12) [a]	39.6±1.6 (n=12) [a]	49.7±3.1 (n=12) [a]	62.3±3.3 (n=12) [a]	86.2±3.3 (n=24) [a]	191.4±3.4 (n=66) [b]
12 °C Gynogen									129.1±9.8 (n=14) [a]
21 °C Control	19.3±1.0 (n=25) [a]	26.5±2.4 (n=12) [a]	37.4±1.9 (n=24) [b]	47.8±3.0 (n=12) [b]	66.3±3.7 (n=12) [b]	88.2±4.1 (n=12) [b]	125.1±3.7 (n=12) [b]	151.6±13.5 (n=12) [b]	230.6±6.1 (n=17) [d]
21 °C Gynogen									195.1±8.0 (n=15) [bc]
26 °C Control	21.2±1.0 (n=24) [a]	31.7±1.9 (n=12) [a]	44.5±1.6 (n=24) [c]	55.4±2.5 (n=12) [b]	63.7±2.0 (n=12) [b]	84.8±2.6 (n=12) [b]	112.3±5.2 (n=12) [b]	143.7±6.6 (n=24) [b]	218.7±4.1 (n=60) [cd]
26 °C Gynogen									189.2±8.2 (n=25) [b]

CHAPTER II

THE EFFECTS OF TEMPERATURE ON SEX DIFFERENTIATION IN BLACK SEA BASS (*CENTROPRISTIS STRIATA*) *

Abstract

To examine the effects of temperature on sex differentiation in the black sea bass (*Centropristis striata*), a protogynous hermaphrodite, juveniles (~0.5 g) were cultured in recirculating systems at 17, 21, or 25 °C. Growth was assessed at 155, 182, 241, and 275 days post hatch (DPH) and sex differentiation was determined histologically. No differences were found in the sex ratios of fish reared at different temperatures, but only 55-64 % developed as females. Growth was significantly greater in males across all temperature treatments. These results suggest that black sea bass exhibit sexually dimorphic growth patterns and that female-specific sex determination can be disrupted in culture.

Keywords: Black sea bass; *Centropristis striata*; Sex determination; Sex-specific growth

* Chapter 2 is an article submitted for publication to Aquaculture Research by Heidi R. Colburn, Abigail B. Walker, and David L. Berlinsky entitled The Effects of Temperature on Sex Differentiation in Black Sea Bass (*Centropristis striata*).

Introduction

The black sea bass (*Centropristis striata*) is a high value teleost species that inhabits the U.S. Atlantic coast from the Gulf of Maine to Florida (Wenner et al. 1986; Vaughan et al. 1995). Due to high consumer demand and limited seasonal supply, this species has been the subject of numerous investigations to determine conditions conducive for its culture. These studies have focused on environmental preferences and tolerances of larvae and juveniles (Berlinsky et al. 2000; Atwood et al. 2001; 2003; Cotton et al. 2003), system designs (Copeland et al. 2002; Stuart and Smith 2003; Bender et al. 2004), reproduction (Howell et al. 2003; Denson et al. 2007), and nutritional influences on growth (Cotton and Walker 2005). As a result of these investigations, limited commercial production has been initiated.

In the wild, black sea bass are protogynous hermaphrodites that change sex from female to male between two and five years of age (Lavenda 1949; Hardy 1978). In captivity, however, a number of individuals may differentiate directly as males (Benton 2005) and sex change may be accelerated (Howell et al. 2003). These disruptions in the timing of sequential hermaphroditism may be due to environmental and/or social factors such as water temperature (Conover and Kynard 1981; Sullivan and Schultz 1986), nutrition (Prevedelli and Simonini 2000), density (Lindsey 1962; Kuhlmann 1975), and sex ratios (Francis and Barlow 1993; Liu and Sadovy 2004; Benton and Berlinsky 2006) that have been shown to influence reproductive development in other species.

Water temperature in particular has been shown to influence phenotypic sex determination in many commercially important teleost species including Paralichthid flounders (Kitano et al. 1999; Borski et al. 2003; Luckenbach et al. 2003), Atlantic halibut (*Hippoglossus hippoglossus*; Van Nes and Andersen 2006), barfin flounder (*Verasper moseri*; Goto et al. 1999), European sea bass (*Dicentrarchus labrax*; Mylonas et al. 2003), and Nile tilapia (*Oreochromis niloticus*; Bezault et al. 2007; Rougeot et al. 2007; Rougeot et al. 2008). Temperature-dependent sex determination (TSD) occurs during a critical period of gonadal differentiation and is mediated through the enzyme cytochrome P450 aromatase (aromatase). The expression of the aromatase gene (*CYP*19), and subsequent enzyme activity, results in the conversion of testosterone to estradiol which mediates ovarian differentiation (Guiguen et al. 1999; Kitano et al. 1999; Suzuki et al. 2004; Chang et al. 2005). Disruption of aromatase gene expression or enzyme function prevents estradiol biosynthesis, and testicular differentiation results (Guiguen et al. 1999; Kitano et al. 1999; Kwon et al. 2001; Uchida et al. 2004). *CYP*19 has been shown to be thermally-sensitive, and temperature perturbation during the critical period of gonad development is thought to cause TSD.

Many fish species exhibit sexually dimorphic growth (Davis et al. 2007) and this has also been suggested to be the case for black sea bass (Alexander 1981). The objectives of the present study were to examine the effects of temperature on sex determination in black sea bass and to determine if they exhibit sexually dimorphic growth.

Materials and Methods

Animals and Systems

Black sea bass 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 for two years 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 individual identification. Fish were maintained at 12-18 °C and fed a commercial ration (9.0 mm, 50 % protein, 15 % fat; Burris, Franklinton, LA, USA) to apparent satiation 2-3 times per week. Photoperiod and temperature were adjusted weekly to simulate the natural conditions (Portsmouth, NH, USA), 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). Water quality parameters remained within ranges suitable for rearing this species (Watanabe et al. 1998; Copeland et al. 2003; Atwood et al. 2004).

Spawning

During all procedures fish 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. High quality (fertilizable) eggs from marine teleosts are generally clear, buoyant, spherical and lack a perivitelline space prior to fertilization (Fahay 1983; McEvoy 1984; Kjorsvik et al. 1990). 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 (DPH).

Temperature-Dependent Sex Determination

Following metamorphosis, 1,350 juveniles (63 DPH) were transferred to the Aquaculture Research Center (ARC) at the University of New Hampshire (UNH; Durham, NH, USA) where they were housed in nine 75 L aquaria (150 fish each) with biological and mechanical filtration, temperature control, and aeration. An additional 450 juveniles remained at GBA in the flow-through systems described above for 48 days before being transferred to UNH. Temperature and dissolved oxygen were monitored daily (OxyGuard Handy Gamma, Birkerod, Denmark) and total ammonia-nitrogen, nitrite-nitrogen, and pH were monitored weekly (HACH®, Loveland, CO, USA). Juveniles were fed in excess four times daily with a commercially marine grower diet and uneaten feed was siphoned.

The juveniles at UNH were allowed to acclimate for one week before the tanks were adjusted one degree Celsius per day to treatment temperatures of 17, 21, or 25 °C. Each temperature treatment was represented in triplicate. At 107

DPH, the juveniles were transferred to recirculating systems corresponding to their respective temperature treatments. Each system consisted of four 235 L cylindrical tanks, biological and mechanical filtration, ultraviolet sterilization, foam fractionation, and photothermal control. The three systems were maintained on a 12L:12D photoperiod with 30 minute crepuscular intervals. Light intensities were 0, 15, and 50 lux during dark, crepuscular, and light periods, respectively.

At 111 DPH the 450 juveniles maintained at GBA at 17-19 °C were transported to UNH, added to the fourth tank in each system, and gradually brought to temperature over 2 days. At 155 DPH the number of fish in each tank was reduced to 90 individuals. On days 182, 241, and 275 DPH, 12-15 juveniles were removed from each tank and euthanized with an overdose of MS-222 (Tricaine-S, Tricaine Methanesulfonate, Western Chemical Inc., Scottsdale, AZ). The gonads were dissected from each fish and fixed in 10 % formalin for 24 h, and then transferred to 70 % ethanol for histological processing. Preserved samples were embedded in paraffin, sectioned at 5 µm, stained with hemotoxylin and eosin, and examined under a light microscope (Zeiss AxioCam MRm, Carl Zeiss Inc., Thornwood, NY, USA). Gender classification was based on gonad characteristics previously described (Lavenda 1949; Nakamura et al. 1998; Benton and Berlinsky 2006). If germ cells were not distinguishable as spermatogonia or oogonia, the fish were classified as undifferentiated (Figure 1a). Fish with germ cells forming clusters in seminal lobules or gonads in various stages of spermatogenesis were designated as male (Figure 1b). Fish with gonads with lamellate structure or clear oogonia were designated as female

(Figure 1c). Fish with gonads containing both oogonia and spermatogonia were designated as intersex (Figure 1d).

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) using JMP IN 5.1 software (SAS Institute, Inc., Cary, NC, USA). Percent data were arcsine transformed prior to analysis. Differences among treatments with a probability (p) less than 0.05 were considered significant and subjected to a post hoc Tukey's HSD test. Results are expressed as means plus or minus standard error of the mean.

Results

While in aquaria, there was a significant difference in mortality between the 17 (23.3 $\pm$ 2.8 %) and 21 °C (11.8 $\pm$ 1.2 %) treatments, but that in the 25 °C treatment (20.2 $\pm$ 3.2 %) was not significantly different from the others. After transfer to the recirculating systems there was no difference in mortality among treatments (0.9 $\pm$ 0.3, 1.6 $\pm$ 1.0, and 0.8 $\pm$ 0.2 % in the 17, 21, and 25 °C treatments, respectively). The number of mortalities was highest in the first 100 DPH, and then dropped to low levels for the remainder of the study.

The growth rates varied between temperature treatments, with fish in the 25 °C treatment growing fastest, and 17 °C slowest. Weights of fish differed significantly among all treatments at both 155 and 182 DPH. Beginning 241 DPH and continuing through the end of the study (275 DPH), fish were significantly

smaller in the 17 °C treatment than in the 21 °C and 25 °C treatments, which were not different from each other (Table 1).

The final sex ratios (275 DPH) of differentiated fish for each temperature treatment are shown in Table 2. The sex ratios of the fish that were maintained at GBA for an additional 48 DPH were not different from those initially reared in aquaria, and were combined in the final analysis. No significant differences were found in final sex ratios among treatments. Among the differentiated fish sampled at 275 DPH there was a significant difference in weight between males and females at each temperature treatment (Table 3).

Discussion

Considerable differences in reproductive patterns, growth rates, and life history characteristics have been reported for black sea bass along their geographic range, and sub-populations may separate at Cape Hatteras, North Carolina. The northern population inhabiting the Mid-Atlantic Bight (MAB) is thought to undertake more extensive off-shore winter migrations and spawn later than that inhabiting the Southern Atlantic Bight (SAB; Mercer 1978; Wenner et al. 1986). In an extensive study conducted throughout the SAB, fish from the more southern latitudes (Florida and Georgia) were found to be larger at a given age, and underwent sex reversal at younger ages and smaller sizes than those from the more northern latitudes (North Carolina; McGovern et al. 2002). Morphometric and meristic variation and differential growth rates were also found among fish within the MAB (Shepherd 1991). These differences may reflect

adaptations to different environmental conditions, such as temperature, which may be important in evaluating and selecting fish for site-specific culture.

In two short-term studies (6 and 8 weeks), Atwood et al. (2003) and Cotton et al. (2003) reported greatest growth in SAB black sea bass juveniles (1.8 and 9.2 g) grown at 25 °C, compared to warmer or cooler culture temperatures. In the present study, using juveniles from the northern extreme of their range, fish grown at 21 and 25 °C were significantly larger than those grown at 17 °C throughout the study, but no differences in growth were found between those grown at the 2 warmer temperatures after 185 DPH. These results compare favorably with the prior studies during the earlier sampling periods, when the fish were of similar sizes, but further studies are required to determine if temperature preferenda differ among larger fish originating from different geographical regions.

In the present study, the weights of male black sea bass were greater than females across all temperature treatments. As neither sex exhibited precocious puberty, somatic growth wasn't compromised by reproductive development. In other commercially important species, such as channel catfish (*Ictalurus punctatus*; Davis et al. 2007) and Nile tilapia (Bhandari et al. 2006), in which males have a growth advantage, efforts have been made to create monosex populations to fully capture the growth advantage. Endogenous steroids have been used to induce sex change in protogynous species but culture conditions have been shown to disrupt female-specific development (Benton 2005; Present study).

At least 25 % of the fish differentiated as males (primary males) in this study though no differences were found among temperature treatments. Therefore, no conclusions can be drawn about the existence of TSD in this species, as some other environmental or social factor(s), such as rearing density, may have contributed to male differentiation. In the wild, small (< 119 mm), age-1 males were captured in low frequency in the SAB (McGovern et al. 2002) and primary males (≤ 1 %) were observed in samples collected in the southern portion of the MAB (R. Pemberton personal communication). These observations suggest that not all black sea bass differentiate as females in the wild, though the pervasiveness of this occurrence is unknown. Further research is necessary to determine factors that affect sex differentiation before gains from sex-specific growth can be realized.

Conclusion

The results of this study suggest that black sea bass undergo some form of environmental sex determination, at least under culture conditions. In the wild, black sea bass are protogynous hermaphrodites, although the degree of primary males within the population may vary with latitude. The exact mechanism controlling differentiation in this species is not fully understood and should be examined further. Additionally, juvenile males were found to be significantly larger than females, suggesting monosex male production in this protogynous hermaphrodite may be desirable for improved culture efficiency.

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Figures and Tables

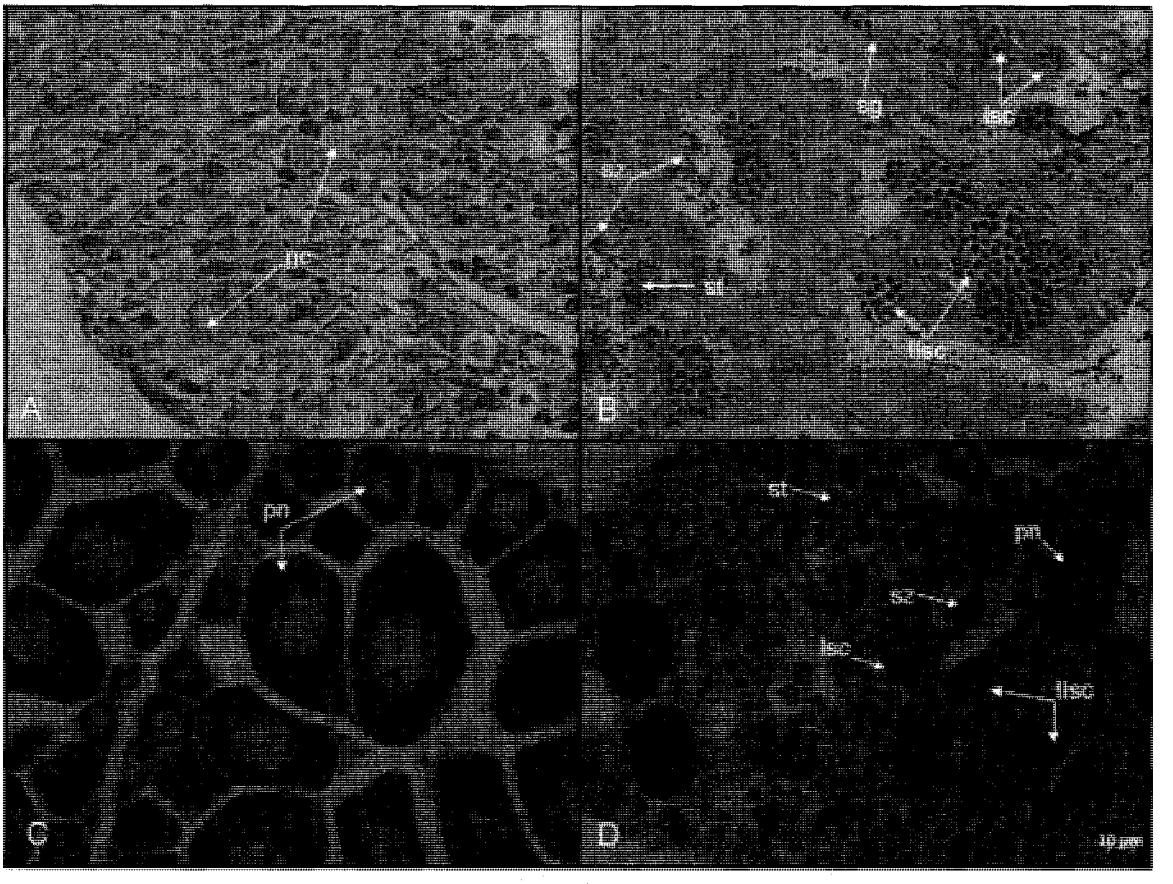

Figure 1. Histomicrographs of black sea bass gonads at 275 DPH depicting: A) an undifferentiated gonad with gonial cells, B) a differentiating male with cells at various stages of spermatogenesis, C) an ovary with primary oocytes, and D) an intersex gonad. sg: spermatogonia; lsc: primary spermatocytes; llsc: secondary spermatocytes; st: spermatids; sz: spermatozoa; pn: early perinculeolus stage oocytes; gc: undifferentiated gonial cells.

Table 1. Mean growth of black sea bass during temperature-dependent sex determination. Mean weights of fish (g) by temperature treatment. Superscript letters reflect significant differences among treatments ($p \leq 0.05$) within a column.

Treatment	155 DPH	182 DPH	241 DPH	275 DPH
17 °C	6.3 ± 0.4 (n=60) [a]	12.1 ± 0.7 (n=60) [a]	23.2 ± 1.8 (n=48) [a]	37.6 ± 1.2 (n=210) [a]
21 °C	14.0 ± 0.7 (n=134) [b]	22.5 ± 1.2 (n=60) [b]	58.2 ± 2.9 (n=48) [b]	78.8 ± 2.1 (n=200) [b]
25 °C	17.3 ± 0.7 (n=122) [c]	32.6 ± 1.6 (n=60) [c]	61.2 ± 4.0 (n=48) [b]	84.1 ± 2.4 (n=222) [b]

Table 2. Sex differentiation of black sea bass at 275 DPH. Values are expressed as percent of differentiated fish only.

Treatment	% Male	% Female	% Intersex
17 °C (n=71)	25.0 ± 4.6	54.6 ± 6.7	20.4 ± 7.4
21 °C (n=61)	29.5 ± 1.7	62.5 ± 5.0	8.0 ± 4.8
25 °C (n=66)	25.3 ± 4.3	64.2 ± 6.4	10.6 ± 5.3

Table 3. Mean weight (g) of black sea bass by sex at 275 DPH. Superscript letters reflect significant differences between sexes ($p \leq 0.05$) within a row.

Treatment	Male	Female
17 °C	47.6 ± 3.4 (n=18) [a]	33.9 ± 2.7 (n=39) [b]
21 °C	85.7 ± 6.4 (n=17) [a]	67.5 ± 4.4 (n=38) [b]
25 °C	106.6 ± 9.6 (n=17) [a]	82.2 ± 5.6 (n=42) [b]

CONCLUSION

In the first study with summer flounder, the first step towards monosex female production was performed. Meiogynogenetic diploids (XX) were sex reversed at high temperatures (21 and 26 °C) to produce XX males. Upon sexual maturity, these XX males may be crossed with normal females to produce monosex female populations of summer flounder. This is advantageous in this species because females grow significantly larger and faster than males. Similarly, a step towards monosex male production of black sea bass was realized in the second study. In the present study, this protogynous hermaphrodite experienced a disruption of female-specific sex determination, with up to 30 % males produced. Additionally, the males were found to grow significantly larger than females, suggesting monosex male populations may be beneficial to profitable culture of this species.

The production of monosex populations is a technique that is used in a variety of aquaculture species that exhibit sexually dimorphic growth rates. The advantages of monosex production include: faster growth rates, elimination of reproduction and reproductive behavior, reduction in size variation, and reduced costs due to size grading. The methods for producing monosex populations vary from the very basic sorting of fingerlings by hand or mechanically, to a more complex genetical approach. Other methods include hormonal sex reversal and inter-specific hybridization (Beardmore et al. 2001). In the majority of species in

which monosex culture is practiced the female is the more economically attractive sex due to differential growth rates, although in few species it is the male. Currently, tilapia (*Oreochromis spp.*) is the primary species in which monosex production is used commercially (Beardmore et al. 2001). In tilapia, monosex production is desirable to prevent precocious sexual maturity that occurs in pond culture, and as males grow faster than females, they are the preferred sex. In stinging catfish (*Heteropneustes fossilis*; Gheyas et al. 2001), Atlantic halibut (*Hippoglossus hippoglossus*; Tvedt et al. 2006), and southern flounder (*Paralichthys lethostigma*; Luckenbach et al. 2004) monosex female production is obtained through meiogynogenesis. In many species steroid hormones have been used to alter sex. Estrogens have been used to manipulate sex differentiation in at least 56 species of teleosts from 24 families. The majority of these are gonochoristic species (91 %) although some work has been done with hermaphrodites (review by Piferrer 2001). The use of monosex production in aquaculture must be considered before the economic advantages associated with sexually dimorphic growth can be realized.

Sex differentiation has been found to be correlated to fish size in many species (Hunter and Donaldson 1983). In both summer flounder and black sea bass, slow-growing fish reared at colder temperatures differentiated at a similar size to faster growing cohorts reared at warmer temperatures. Size, rather than age, appears to be limiting factor for gonadal sex differentiation. This has been shown in southern flounder as well (Luckenbach et al. 2003). Understanding factors that affect sex differentiation is necessary for successful aquaculture in

many species. While the genetic basis of sex determination involves activation of steroid hormones, several environmental and social factors can influence phenotypic sex differentiation. These factors include pH (Rubin 1985), population density (Francis 1984), relative fish size (Francis and Barlow 1993), and temperature (Conover and Kynard 1981). The exact initiation and duration of the critical period during which gonadal sex may be manipulated is unknown for most species but is believed to coincide with the period of steroid-sensitive gonad differentiation, which has been documented in several cultured species (Blazquez et al. 1998; Place et al. 2001). Importantly, the window during which sex determination occurs varies across species. Determining the window when gonadal sex may be manipulated is necessary to increase profitability in aquaculture. As the temperature necessary to produce female summer flounder is lower than the optimal temperature for growth, there is an increased need to determine the timeframe of sex differentiation in order to minimize the time spent at cold temperatures. Further studies should be conducted to pinpoint the exact window of sex determination in this species and to determine the optimal temperature for female production. In black sea bass, however, we found skewed sex ratios, unrelated to temperature. It is likely that the skewed sex ratios were due to other environmental or social factors such as density or light intensity. Future studies should examine the effects of these factors on sex differentiation on black sea bass. Additionally, the idea that primary males may exist in this species should be examined further through histological studies.

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APPENDICES

APPENDIX A

SUMMER FLOUNDER INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL DOCUMENTATION

UNIVERSITY of NEW HAMPSHIRE

March 27, 2006

Berlinsky, David
Zoology, Spaulding Life Science Center
Durham, NH 03824

IACUC #: 060304
Approval Date: 03/24/2006
Review Level: B
Project: The Use of Reproductive Technology to Improve Flounder Growth

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category B on Page 4 of the Application for Review of Vertebrate Animal Use in Research or Instruction - *the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.* The IACUC made the following comments on this protocol:

1. *The Committee approved this study with the understanding that it needs to inspect the Great Bay Aquaculture facilities and that if that inspection raises any problems, the Committee will be in contact with the investigator.*

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

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. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File

Research Conduct and Compliance Services, Office of Sponsored Research, Service Building, 51 College Road, Durham, NH 03824-3585 * Fax: 603-862-3564

APPENDIX B

BLACK SEA BASS INSTITUTIONAL ANIMAL CARE AND USE

COMMITTEE APPROVAL DOCUMENTATION

University of New Hampshire

Research Conduct and Compliance Services, Office of Sponsored Research
Service Building, 51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

04-Dec-2007

Berlinsky, David
Zoology, Spaulding Life Science Center
Durham, NH 03824

IACUC #: 061103
Project: Sex Determination in Black Sea Bass
Category: B
Next Review Date: 14-Dec-2008

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. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File