ABSTRACT

LUCKENBACH, JOHN ADAM. Breeding Biotechnology, Sex Determination, and Growth in Southern flounder, *Paralichthys lethostigma*. (Under the direction of Dr. John Godwin and Dr. Russell J. Borski).

Southern flounder (*Paralichthys lethostigma*) support valuable, but declining US fisheries. This species is therefore a strong candidate for aquaculture to mitigate fishing impacts and stabilize seafood supply. Because female flounder reach substantially larger sizes than males, all-female culture is desirable for commercial aquaculture. Hence, a thorough understanding of sexual development, its timing and regulation by temperature is essential for optimization of flounder aquaculture.

To better understand ovarian and testicular development in southern flounder, structural and cellular sex-distinguishing markers were studied using histological methods. We found that histologically discernible sex differentiation occurs in southern flounder at ~75-120 mm TL and that early differentiation is considerably delayed relative to its Japanese congener, *P. olivaceus*. High (28°C) and low (18°C) water temperatures, produced a higher proportion of males (96% and 78% males, respectively). The sex ratio at a mid-range (23°C) temperature was not different from 1:1. This suggests that southern flounder possess a temperature sensitive mechanism of sex determination. Growth was also affected by temperature with the temperature that maximized females inducing better growth.

Aromatase cytochrome P450 ($P450_{arom}$) is responsible for estrogen biosynthesis

and plays a critical role in ovarian differentiation. We cloned ovarian $P450_{arom}$ and developed a qRT-PCR for assessment of early sex differentiation. The deduced amino acid sequence for southern flounder $P450_{arom}$ is very similar to $P450_{arom}$ in other teleosts. Comparison of $P450_{arom}$ intron sequences of southern flounder within and between different populations revealed substantial inter-individual variation that may affect sex determination responses. Ovary and spleen exhibited high levels of $P450_{arom}$ mRNA, while $P450_{arom}$ was weakly detected in testis, brain, and gill. Gonads sampled from wild flounder spanning the period of sex differentiation initially exhibited a low level of $P450_{arom}$ gene (fish 40-55 mm TL) followed by bifurcation into two clearly segregated groups beginning at ~65 mm TL. Through histology we show high and low $P450_{arom}$ mRNA relates to ovarian and testicular differentiation, respectively. This work imparts a powerful tool for better understanding mechanisms of sex determination and rapidly defining conditions that influence sex.

Effective methods for induction of diploid gynogenesis in southern flounder are needed to initiate all-female culture. To test methods for inducing gynogenesis in this species, four treatments, named for their expected outcome, were employed: haploid, diploid, triploid, and gynogenetic diploid. Diploid gynogenesis was induced by activating egg development with UV-irradiated flounder sperm (70 J/cm^2) and then 'cold shocking' the eggs to prevent second polar body extrusion. Control treatments omitted one or more of these steps to separately assess the effectiveness of UV irradiation and cold shock. Haploid larvae exhibited abnormal external morphology while diploid, gynogenetic diploid, and triploid larvae appeared normal. Erythrocyte nuclear sizes for treatment groups corresponded to predicted ploidy (triploid > diploid > haploid) and did

not differ between normal diploids and gynogenetic diploids, suggesting that our procedures were successful. To eliminate any possible paternal genetic contribution during gynogenesis, activation of flounder eggs with striped mullet (*Mugil cephalus*) sperm was also tested. Induction of diploid gynogenesis was successful when flounder eggs were fertilized with irradiated mullet sperm and cold shocked. This work provides procedures for induction of diploid gynogenesis in southern flounder and validates a method for verification of ploidy in larval fishes.

These studies establish the schedule of southern flounder sex differentiation based on results of gonadal histology and patterns of $P450_{arom}$ gene expression. We also demonstrate that sex determination in this species is temperature sensitive. This knowledge, along with methods for induction of gynogenesis, can be utilized for creation of all-female southern flounder populations for aquaculture. These findings may also benefit fisheries management by providing methods for sex assessment and guiding stock enhancement.

BREEDING BIOTECHNOLOGY, SEX DETERMINATION, AND GROWTH IN SOUTHERN FLOUNDER, *Paralichthys lethostigma*

by

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DEDICATION

To my late mother Ann Luckenbach and my father Roy Luckenbach who have wholly supported and guided throughout my life and its many endeavors, always keeping my focus on making a difference in the world.

BIOGRAPHY

John Adam Luckenbach was born March 1, 1975 in the small town of Asheboro, North Carolina, the last of four children and one could say a little unexpected. From an early age he had a strong interest in critters, particularly the ones with fins, as well as a love of plants and gardening. This passion for animals and plants escalated over the years and by fourteen years of age his mother's home and surrounding property was teaming with animal life. While living in Asheboro, Adam attended McCrary Elementary, North Asheboro Middle School, and graduated from Asheboro High School in 1993. He attended college at his father's alma mater, North Carolina State University, located in Raleigh, North Carolina, where he graduated in 1998 with a Bachelor of Science degree in Zoology. Almost fatefully, just after graduation and before his next move was concrete, Adam crossed paths with one of his previous college professors, Dr. Russell Borski. A discussion with Dr. Borski that morning led to Adam taking a Research Biologist position in Dr. Borski's laboratory at NC State University. This opportunity sparked a keen interest in fish physiology and endocrinology and in the spring of 1999 he began Graduate School in the Department of Zoology at NC State University. Thereafter, Adam has been conducting his doctoral research under the supervision of Associate Professors of Zoology, Drs. Russell Borski and John Godwin. After completing his Ph.D. and marrying the love of his life, Ms. Paula Vuncannon, in December 2004, he plans to begin postdoctoral research related to fish reproductive endocrinology at the University of Maryland, Center of Marine Biotechnology in Baltimore.

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I must first acknowledge that these research accomplishments would not have been possible without the input and assistance of numerous dedicated colleagues at North Carolina State University and abroad.

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CHAPTER I

Introduction[1]

[1] Based on an article published in World Aquaculture 33: 40-45 (2002) by J. Adam Luckenbach, John Godwin, Harry V. Daniels, and Russell J. Borski entitled, Optimization of North American flounder culture: a controlled breeding scheme.

ABSTRACT

Southern and summer flounder of the genus *Paralichthys* support valuable North American fisheries and show promise for aquaculture. Fisheries for these species remain below historic levels along the entire U.S. Atlantic coast. The annual loss of revenue to commercial and recreational fisheries is in the millions of dollars. Currently there are only a few operations producing flounder in the United States, but commercial production is well established in Japan. Large-scale production of flounder for mariculture and stock enhancement can help reduce impacts on capture fisheries and stabilize supply, but these goals require the development of mariculture technologies. Flounder biology presents both challenges and opportunities. Female flounder grow faster and achieve much larger adult sizes than males. Thus, aquaculture techniques that generate all female stocks would be beneficial for commercial operations. An interesting phenomenon seen in few fish species, but one observed in some flounder, is temperature-dependent sex determination. The larger sizes reached by females coupled with the influence of temperature on sex allows design of a scheme for controlled breeding and production of only female flounder. In order to effectively control sex in southern and summer flounder, it is critical to determine the markers and timing of sexual differentiation, define the specific rearing temperatures that induce male and female development, and characterize the developmental period or 'window' during which temperature can influence sex. The ultimate goal is to develop reliable and economical methods for producing faster-growing female flounder necessary to optimize mariculture production. An understanding of

environmental influences on sex ratios is also essential to sound fishery enhancement in these species.

BACKGROUND AND RATIONALE

Seafood consumption is increasing rapidly in the United States, but yields from capture fishing are now well below figures from a decade ago. This has resulted in economic losses to recreational and commercial fisheries worth millions of dollars annually. Evidence suggests summer flounder (*Paralichthys dentatus*) stocks, found mainly in areas ranging from Canada to the mid-Atlantic U.S. coast are recovering from lows reached in the early 1990's (Figure 1). This is due to tighter fishing restrictions, but it is believed that the stock is still over exploited. Recently, there has been concern for southern flounder (*Paralichthys lethostigma*) populations, which range from Maryland to Florida's East Coast and throughout the Gulf of Mexico (North Carolina Division of Marine Fisheries, 2001). Data suggests the age distribution for southern flounder is skewed toward younger fish, leading to poor reproduction (Grist, 2004). Therefore, restocking programs could positively impact flounder fisheries along the entire eastern seaboard. Developing biotechnological approaches to enhance the mariculture of southern and summer flounder could benefit this effort and improve stability and profitability of commercial operations.

Establishing large-scale commercial farming operations for marine fish like southern and summer flounder is an important national priority. Realizing this goal could have several benefits, such as offsetting serious trade imbalances for seafood, ensuring availability and safety of our seafood supply in the future, and diversification and increased profitability of mariculture. In Japan, half of the Japanese flounder or hirame (*Paralichthys olivaceus*)

consumed are produced through commercial aquaculture. The natural fisheries of Japan are also supported by the annual release of 23 million juvenile flounder produced in hatcheries (Copeland et al., 1998). Success in management of the hirame, a relative of the southern and summer flounder suggests promise for similar approaches in North America. Both summer and southern flounder show promise as mariculture species (National Research Council, 1992). These species are high in value with strong economic potential for farming. Importantly, females grow to three times the size of males (Copeland et al., 1996). Monosex female culture will therefore reduce size difference between individuals during growout, lessen mortality due to cannibalism, decrease labor costs for size grading, and give a higher return on investments for feed and infrastructure. Most female flounder mature at 2 years of age, after the typical growout phase in aquaculture. Production of all-female flounder stocks for aquaculture will prevent unwanted reproduction, thereby shifting more energy to growth.

The pattern of sex determination of flounder of the genus *Paralichthys* presents an unusual, yet valuable opportunity to control sex and breed only female fish. The sex of flounder is determined partly as it is in humans, by the XX (female) or XY (male) sex chromosomes (or genotype). However, sex is also influenced by temperature in XX individuals in at least some *Paralichthys* species (Yamamoto et al., 1999), a phenomenon termed temperature-dependent sex determination that is best known from reptiles like alligators and sea turtles. Importantly, the XX female genotype can be easily manipulated to produce broodstock of the opposite sex with functional testes (XX-male broodstock). This feature of flounder biology allows for crossing sex reversed XX-male broodstock with normal females to

produce monosex female flounder populations. Moreover, the temperature sensitivity of sex determination in flounder allows for production of male broodstock by simple manipulation of rearing temperature during early development. This is an attractive alternative to the hormone treatments, like methyl-testosterone or estrogen that are commonly used to achieve phenotypic sex reversal in other cultured fish species.

BROODSTOCK MANAGEMENT AND LARVAL PRODUCTION

Summer and southern flounder larvae are obtained from breeding of conditioned adult broodstock (Figure 2; reviewed in Morse, 1981; Smith et al., 1999). Ideally, broodstock are domesticated, produced in captivity and hatchery reared. However, wild-caught broodstock can be quite productive if well acclimated. The broodstock are maintained in temperature and photoperiod conditions typical of the normal seasonal fluctuations experienced by wild populations, with the exception that simulation of photothermal conditions can be used to trigger reproductive responses when desired. Implants of GnRHa, an analog of the naturally produced reproductive hormone that stimulates the ovary, have been used together with appropriate light cycles to optimize broodstock-spawning results (Berlinsky et al., 1997). Conditioned females are commonly selected for spawning based on oocyte diameter in biopsy samples and induced to ovulate for *in vitro* fertilization (strip spawning). The fish may also spawn freely in tanks (Smith et al., 1999). When strip spawning flounder, eggs of eligible

females and milt from spermiating males are pooled together in seawater. Unfertilized and inviable eggs sink in seawater and are discarded before fertilized eggs are incubated. For tank spawning, floating eggs are collected in mesh bags and assessed for fertility prior to incubation.

LARVAE AND JUVENILE REARING METHODS

Larval and juvenile rearing methods for flounder are similar to those used for other marine finfish (Daniels et al., 1996; Bengtson, 1999). Fertilized eggs are incubated in seawater with gentle aeration until hatching (2-3 days). Sac fry are stocked at 20-40 fish/liter to induce first feeding. Aeration is provided as well as overhead illumination. A 12 hour light:12 hour dark photoperiod with low light intensity (500-1000 lx) is commonly used during larval rearing. Algae, such as *Tetraselmis suecica* are added to the water to increase the lifespan of the zooplankton, which are fed to the young flounder. Larvae are fed rotifers, such as *Brachionus plicatilis* beginning 3-4 days post-hatch. Hatched flounder larvae are free-swimming and symmetric in body form, but later metamorphose into bottom-dwelling, asymmetric juveniles. Brine shrimp (*Artemia* spp.) are provided beginning 18 days post-hatch and continuing until metamorphosis is complete, usually around 45 days post-hatch. Post-metamorphic flounder are gradually weaned from *Artemia* to high protein pelleted feed. Juvenile culture is maintained in open or closed recirculating fish culture systems. A unique feature of southern flounder is that they can be cultured in fresh water (0 ppt) and grow at rates similar to fish reared in

seawater (Daniels and Borski, 1998). Thus, southern flounder could be cultured in areas other than coastal regions where land costs are typically high.

SEX DETERMINATION: DEVELOPMENTAL TIMING AND EFFECTS OF TEMPERATURE

The influence of environmental temperature on sex determination has been characterized and extensively studied in reptiles, including lizards, alligators, and turtles (Bull and Vogt, 1979; Ferguson and Joanen, 1983; Wibbels et al., 1994). A variety of temperature effects on male to female sex ratios have been identified. Only a few species of fish have been shown to exhibit temperature-dependent sex determination (see Baroiller and D'Cotta, 2001; Devlin and Nagahama, 2002), with the hirame and other flounder species among them (Yamamoto et al., 1999; Goto et al., 1999). The hirame (*Paralichthys olivaceus*) shows an interesting trend of temperature-dependent sex determination in comparison to that shown for lizards and turtles (Figure 3). Studies have shown that sex differentiation occurs between 27 and 37 mm total length in this Japanese species (Tanaka, 1987). Temperature dramatically alters sex in juvenile hirame during a transient period preceding sexual differentiation. Raising juvenile hirame at a moderate temperature (18-20°C) during the thermosensitive period produces 50% females and 50% males, but higher temperatures yield a majority of male juveniles (> 90% at 25-27°C). Even in genetically all-female (XX) stocks, female development appears unstable in culture.

Moderate rearing temperatures induce female determination, while up to 95% of the juveniles develop as males at higher rearing temperatures (Yamamoto, 1999). This phenomenon presents an opportunity for a controlled breeding scheme where only the larger and faster growing sex is produced. This sexual plasticity in relation to temperature could also pose problems for fishery restocking programs, where temperature-induced sex reversal could skew sex ratios in release habitats or fingerling production facilities. Considering these possibilities, critical temperature thresholds and the developmental 'window' for sex determination must be identified for North American flounder.

The pivotal temperatures influencing sex vary across latitudinal gradients in several species that exhibit temperature-dependent sex determination. This appears to represent an adaptation to the typical ambient environmental temperatures experienced when juveniles are undergoing sex determination. The significance of this variation from the perspective of rearing flounder is the likelihood of similar latitudinal variation in the pivotal temperatures that induce either male or female development. Summer flounder populations form at least two or three distinct stocks over a latitudinal range in the Western Atlantic (Wilks et al., 1980; Jones and Quattro, 1996). Because of physical barriers imposed by the Florida peninsula, southern flounder populations of the Atlantic Coast appear to be geographically isolated from those of the Gulf of Mexico (Ginsburg, 1953). Genetic analyses of these populations suggest that genetic structuring is present, but do not suggest that these populations are completely independent stocks (Blandon et al., 2001). Based on evidence of genetic structuring in these populations, the

temperature-dependent sex determination response in populations at the northern end of the species range could potentially be adapted to colder pivotal temperatures than more southerly populations. If different flounder stocks do vary in the pivotal temperature inducing male determination, rearing conditions should be optimized for different regions during the critical period for temperature influences on sex.

The challenges to efficient, large-scale monosex production of North American flounder are to clearly define temperatures that induce male and female development, the developmental window over which sex differentiation occurs, and temperatures that produce normal and skewed sex ratios. We have conducted studies to describe the pattern of sexual development in southern flounder, compare these patterns to those of the hirame (*P. olivaceus*), and determine whether this species exhibits temperature-dependent sex determination. We examine the gonads microscopically to assess sex differentiation and determination. In this technique gonads are infiltrated with paraffin wax, thinly sectioned, laid on microscope slides, and stained for visual analysis with a light microscope. After identifying structural and cellular sex-distinguishing characteristics (Tanaka, 1987; Patiño et al., 1996; Nakamura et al., 1998), specific stages of ovarian development in females and testicular development in males are described. Our findings show that sex differentiation occurs after metamorphosis and is sensitive to temperature in southern flounder. It appears that southern flounder are considerably delayed in their development of early sex-distinguishing markers relative to the hirame and barfin flounder of Japan (see Chapter II). This evidence suggests that the thermosensitive period of sex

determination may also be substantially delayed relative to that shown for the hirame. This possible relative delay is supported by patterns of $P450_{arom}$ expression during sex differentiation in juvenile southern flounder (Chapter III).

Temperature-dependent sex determination was tested in southern flounder with rearing trials of varying temperatures (Chapter II). Rearing temperatures causing significant deviations from 1:1 (male/female) sex ratios were considered effective at inducing sex change. Our results demonstrate that extreme rearing temperatures, low or high, can skew sex ratios in southern flounder, producing predominantly male fish as seen with the hirame.

Optimizing growout for southern and summer flounder represents an additional challenge. We postulate that moderate temperatures induce female sex determination, delay metamorphosis and slow growth relative to warmer temperatures. The need for cooler temperatures to induce female determination and warmer temperatures later to maximize juvenile growth, makes it necessary to precisely define the temperature sensitive window for sex determination. After that period, the sex of the fish should be irreversible. To be useful, cooler water exposure must be limited to the critical period for female development, thus allowing for rapid growout outside this period at a higher temperature, or at any temperature that is convenient to manage for hatchery, pond, or net-pen culture.

ROLE OF P450 AROMATASE IN SEX DIFFERENTIATION AND DETERMINATION

Ovarian differentiation can be induced in many fishes, including the hirame, by the application of exogenous estrogens (Yamamoto et al., 1999; Piferrer, 2001). Biosynthesis of the primary estrogen in fishes, estradiol-17β (E_2), during sex determination in fishes is dependent upon activity of cytochrome aromatase P450 ($P450_{arom}$). This rate-limiting enzyme is responsible for conversion of androgens to E_2. In the hirame, $P450_{arom}$ levels rise significantly during female differentiation and then remain elevated, suggesting this gene is sex-specific and therefore potentially a reliable biomarker of female sex differentiation (Kitano et al., 1999). Because histological analyses are labor intensive and long trials are required for fish to reach a size where sex can be easily assessed, development of $P450_{arom}$ as a biomarker for female development would be a valuable tool for early assessment of the direction of sex differentiation in southern flounder in the laboratory and field. To initiate our study of the role of $P450_{arom}$ in the process of sex differentiation in southern flounder we cloned the ovarian form of the southern flounder $P450_{arom}$ gene. Furthermore, through development of a custom-designed quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay we aim to assess the utility of $P450_{arom}$ as a biomarker for early female differentiation in southern flounder. This method is effective in detecting $P450_{arom}$ mRNA in flounder and can therefore be employed to determine feminizing or masculinizing effects of temperature in cultured and wild populations.

GYNOGENESIS: BIOTECHNOLOGY FOR PRODUCTION OF XX-MALE BROODSTOCK

Some flounder species are unusual among fishes in that the female genetic constitution (XX) that provides optimal growth is also the genotype that may be manipulated with temperature to produce adults with the functionality of the opposite genetic sex. By rearing 'known' XX individuals at a warm temperature during early development, production of valuable XX male flounder is possible (Tabata, 1991; Yamamoto, 1999). These XX males are valuable because, once grown to reproductive maturity, they can be routinely crossed with female broodstock to produce all-XX offspring. Because both parents are 'XX' genotype, both the male and female parents can contribute only X-sex chromosomes so there is no chance of offspring inheriting the male Y chromosome. Therefore, these crosses will result in monosex female stocks in just one generation under appropriate rearing conditions. During the early rearing period these fish would be maintained at a moderate temperature, ensuring the female phenotype during growout (Figure 4). The use of temperature for induction of sex reversal to generate monosex stocks has strong advantages over the hormone-based methods used in many cultured fish species. Because the fish produced through these manipulations are like naturally occurring XX flounder there should also be little concern about problems arising from accidental introduction of these fish into the wild (see Induction of Gynogenesis section below for details on gynogen production).

Two methods can be used for induction of sex-reversal in gynogenetic female offspring to produce phenotypic males with the XX genotype. Prior to onset of gonadal differentiation rearing temperatures can be elevated to the point critical for sex reversal. Based on studies of hirame and experiments with southern flounder this appears to be the simplest and most effective method for making XX males. These XX males should be sexually mature in approximately 10-12 months, and could then be bred to normal or gynogenetic XX. If necessary, sex reversal of gynogens (XX) into functional males can be accomplished through administration of methyl-testosterone in the feed.

Though production of XX offspring that can be sex reversed to produce male broodstock is a highly technical process, it would not be necessary to routinely make gynogens required for monosex culture. Rather, gynogenesis would only be necessary for the initial creation of the XX offspring. Afterward, generation of XX-male broodstock could be sustained by simply sex reversing some the definitive XX offspring as needed. This novel biotechnology can provide essential strategies that marine hatcheries can use in production of other commercially valuable flatfish, as well as other species exhibiting sexually dimorphic growth.

Southern and summer flounder are high value fish with well-established markets. Though some commercial operations are producing these species, supply is not great enough to alleviate fishing pressure and meet current demands. Large-scale flounder culture is already a reality in Japan, generating millions of adults and juveniles for mariculture and stock enhancement purposes. Development and refinement of technologies for optimized production

of southern and summer flounder is necessary to make them widely desirable and profitable for mariculture in North America. The peculiar biology of flounder, exhibiting sex-specific growth and temperature-dependent sex determination creates valuable opportunities for controlled breeding not possible for most fishes. Production of gynogens is essential for the profitability of 'natural' monosex culture of flounder. Following sex reversal, XX males may be crossed with female broodstock generating all-XX flounder, which when reared at a moderate temperature should yield only larger and faster-growing females. Establishing these technologies in mariculture of North American flounder should continually provide better return on investment for commercial operations.

INDUCTION OF DIPLOID GYNOGENESIS

As further described in Chapter IV, gynogenetic, all-XX flounder stocks are initially made through a combination of egg ploidy manipulation and fertilization with null sperm. Ovulated eggs are activated using flounder sperm, or that of an unrelated species, irradiated with ultraviolet (UV) light. Just after fertilization the eggs are subjected to cold (0-4°C) shock to cause retention of the second polar body, one of the two female haploid set of chromosomes usually extruded from the egg just after fertilization. The procedure of fertilizing cold-shocked eggs with UV-irradiated sperm prevents contribution of genetic material, including sex chromosomes, from the male parent while doubling the genetic contribution from the female

parent. It produces gynogenetic, all-female (XX) offspring. UV-irradiated sperm contain inactive paternal chromosomes, but should retain full motility and the capacity to activate egg development. Sperm from another species may also activate egg development, but should not contribute genetic material to offspring. Eggs from these crosses are hatched and the larvae are raised just as normal flounder larvae.

The success of chromosome set manipulations to produce gynogens can be demonstrated experimentally and have been for southern flounder (see Chapter IV). Experimental treatments include: a gamete quality group produced using ovulated eggs and sperm stripped from normal flounder, a haploid (inviable) group produced by activating normal eggs with UV-irradiated sperm, and a triploid group made using normal sperm and cold shocked eggs. These control groups verify quality of gametes from donor fish (viability control), blockage of Y-chromosome transfer (haploid control), and diploidy of eggs following cold shock (triploid control). Haploid offspring are expected to be inviable and should be short-lived, while the triploids should be viable. Triploidy and haploidy (should haploids survive) in the offspring can be confirmed by particle size analysis with a Coulter Counter, comparing red blood cell volumes in those individuals to volumes of normal diploids. We took another approach with southern flounder where larvae were sectioned and erythrocyte cross-sectional areas measured to determine ploidy (Chapter IV). If heterologous sperm from an alternate (heterospecific) species is used and successful hatch takes place, then the offspring can only be gynogenetic diploids.

Compared with other animals, it is difficult to distinguish the chromosomal makeup of fish species using classical karyotyping (chromosome identification) procedures. Success of gynogenesis can be evaluated in flounder, however, by examining the sex of a subsample of putative gynogenetic offspring, which should be approximately 100% female under rearing temperatures that induce female sex determination.

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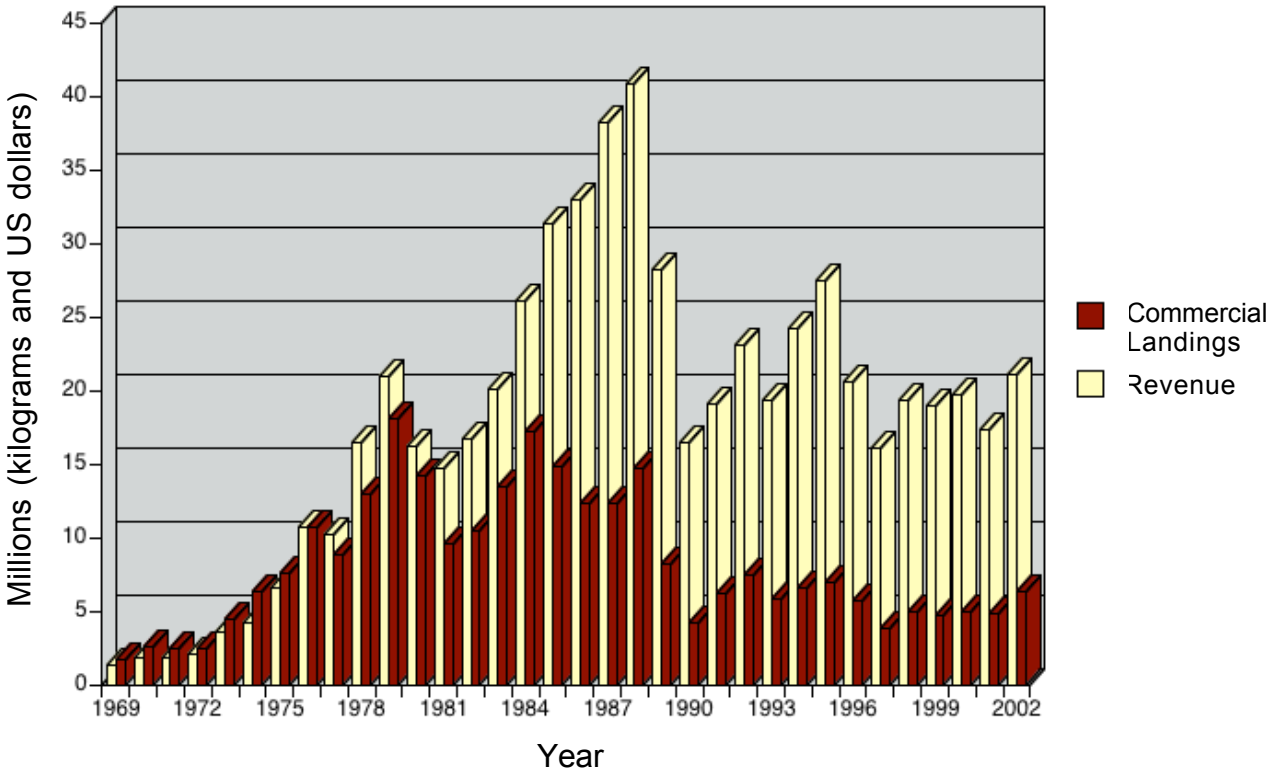

Figure 1. Annual commercial landings (kilograms) and revenue (U.S. dollars) for summer and southern flounder from 1969 to 2002 (National Marine Fisheries Service, 2004). Note the sharp decline in landings in the early1990's, following improved capture-fishing techniques and increased demand.

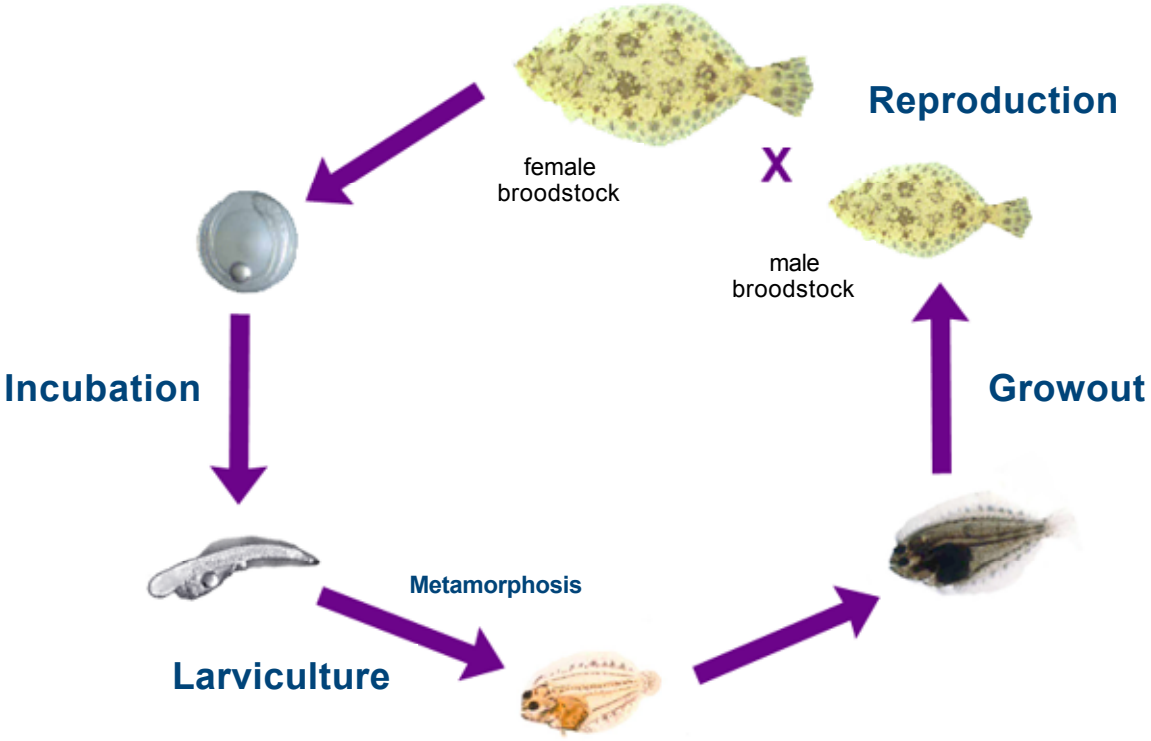

Figure 2. General hatchery scheme for production of flounder. Begins with crossing male and female broodstock. Fertilized eggs are incubated until hatching (2-3 days). Larvae undergo a metamorphosis into a benthic flatfish. Juveniles are prepared for market or future brooding during the growout phase.

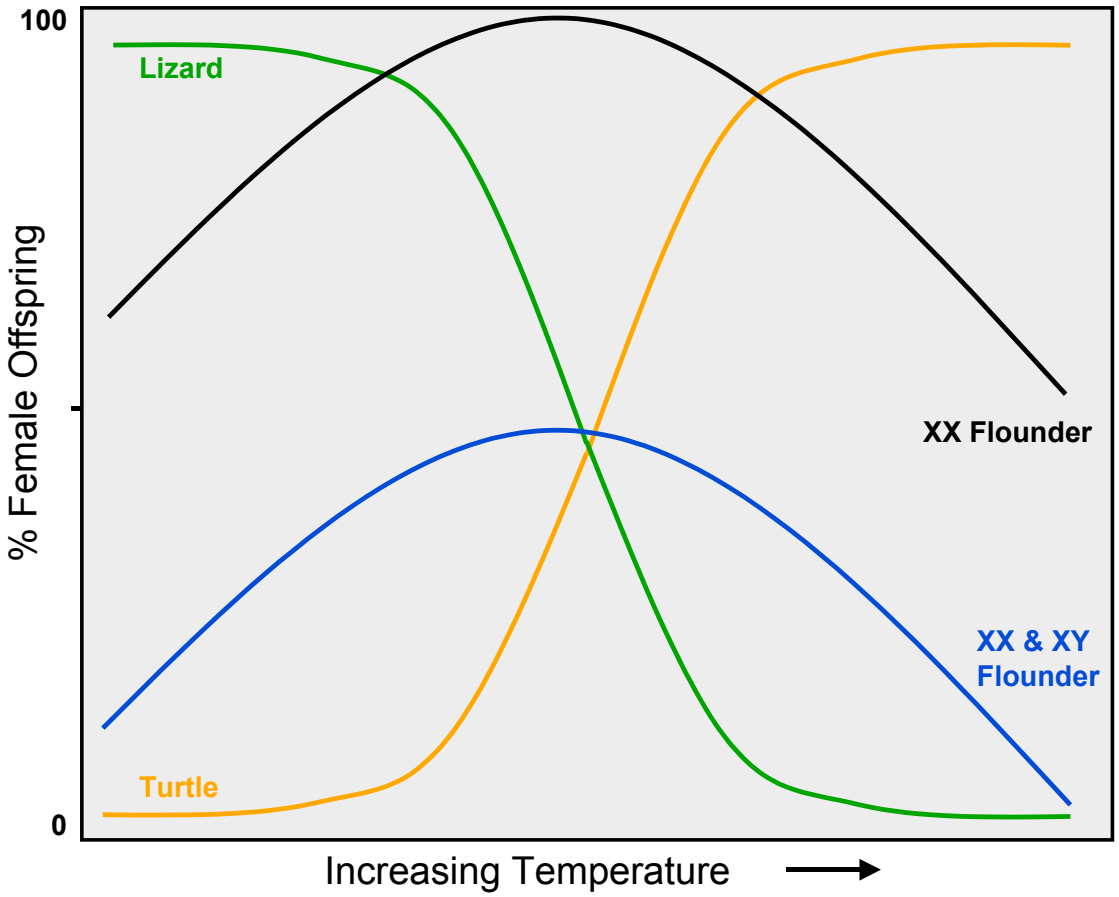

Figure 3. Trends in sex determination with varying incubation/rearing temperature. This figure shows general trends, and does not precisely reflect patterns of individual species.

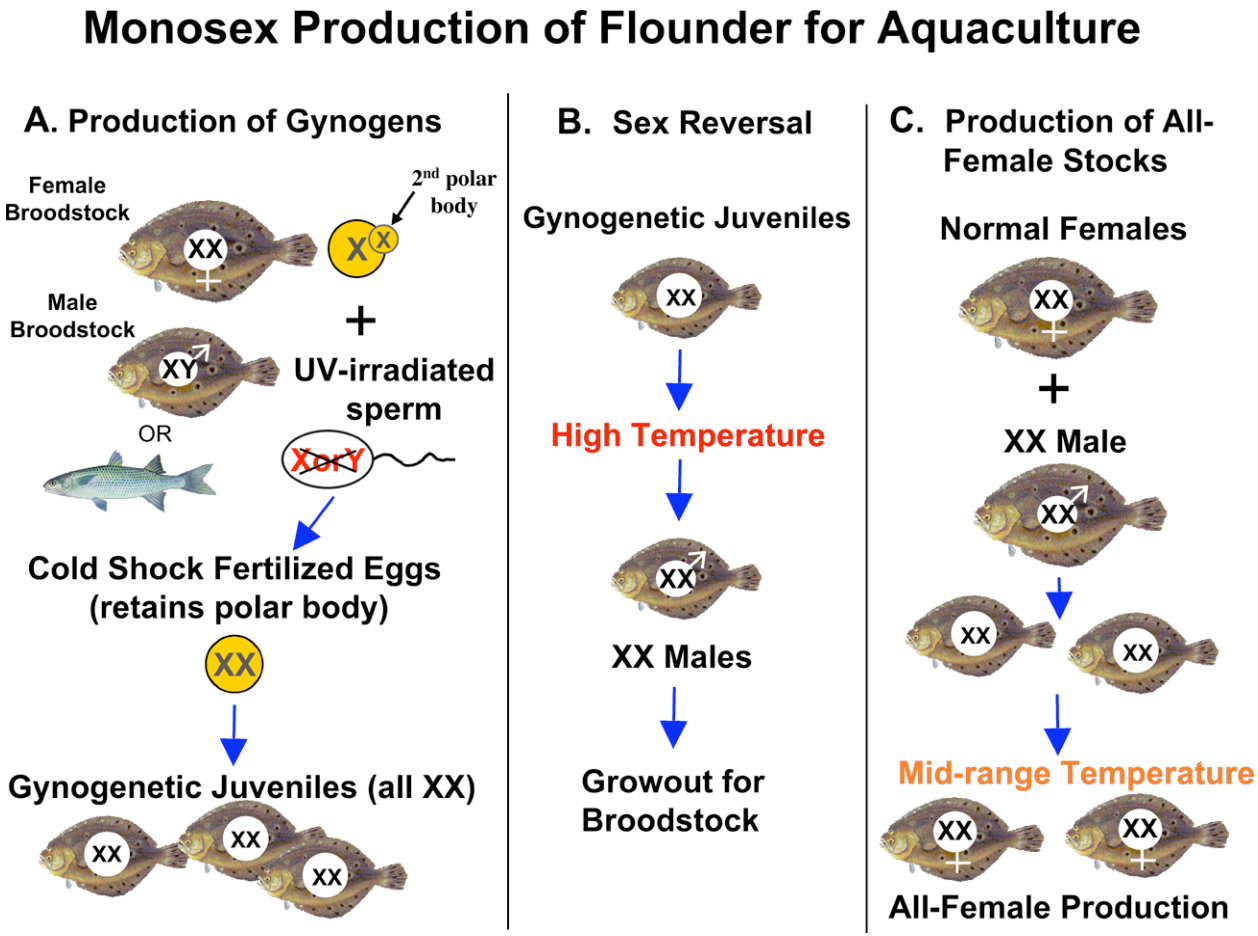

Figure 4. Schematic overview of monosex (all-female) production of flounder. Shows methods for (A) producing gynogenetic, all-XX flounder, (B) sex reversal of gynogenetic juveniles into XX males for future brooding, and (C) routine production of all-female stocks by crossing female broodstock with XX-male broodstock.

CHAPTER II

Gonadal Differentiation and Effects of Temperature on Sex Determination in Southern Flounder (*Paralichthys lethostigma*)[1]

[1] Published in Aquaculture 216: 315-327 (2003) by J. Adam Luckenbach, John Godwin, Harry V. Daniels, and Russell J. Borski.

ABSTRACT

Southern flounder (*Paralichthys lethostigma*) support valuable North American fisheries and show great promise for aquaculture. Because females grow faster and reach larger adult sizes than males, monosex culture of females is desirable for commercial operations. A detailed understanding of sexual development and its timing is critical to control sex and optimize culture. Structural and cellular sex-distinguishing markers were identified histologically, and then used to describe ovarian development in female and testicular development in male flounder. In presumptive ovaries of southern flounder, development of an ovarian cavity first occurs in fish ranging from 75-100 mm total length (TL). This is considerably delayed relative to that observed in the Japanese congener, *Paralichthys olivaceus*, where an ovarian cavity is seen in fish as small as 40 mm TL. The smallest southern flounder that possessed primary oocytes in the early perinucleolus stage was 115 mm TL. In presumptive testes, the formation of seminiferous tubules first occurs in fish of approximately 100 mm TL. Spermatogonia remained quiescent until most fish were over 100 mm TL. Overall, gonads from southern flounder greater than 120 mm TL commonly possess gonial cells undergoing meiosis, clearly differentiating sex. The effect of temperature on sex determination in southern flounder was addressed in a separate experiment. Juvenile southern flounder were grown at 18, 23, or 28°C for 245 days. High and low temperatures induced phenotypic sex reversal in juvenile southern flounder, producing a higher proportion of males (96% males at high temperature, $P<0.001$; 78% males at low temperature, $P<0.01$). Raising southern flounder at the mid-

range temperature held sex ratios close to 1:1. Sex ratios from these trials suggest that southern flounder possess a temperature sensitive mechanism of sex determination similar to that shown for *P. olivaceus*, but possibly shifted towards warmer temperatures. These findings indicate that sex differentiation in southern flounder is distinguishable in most fish by 100-120 mm TL and that sex determination is sensitive to temperature. This information is critical to development of strategies to maximize the number of faster-growing females for commercial flounder culture.

INTRODUCTION

The southern flounder (*Paralichthys lethostigma*) is a high-value North American flatfish prized by both commercial and recreational fishers. This euryhaline species is commonly found in estuarine and shelf waters throughout the southeastern United States. Southern flounder show promise for aquaculture due to their ability to grow well in fresh or saline water and substantial market appeal (Daniels and Borski, 1998; Smith et al., 1999a; Benetti et al., 2001). While there are only a few commercial operations for flounder in the United States, production is well established in Japan, generating millions of fish for consumption and fishery stock enhancement (Copeland et al., 1998). Recent concern for North American *Paralichthys* flounder fisheries has heightened the need for large-scale commercial production of flounder to reduce fishing pressure, meet the rising demand for seafood, and provide fingerlings for stock enhancement (National Research Council, 1992). Interestingly, female flounder grow faster and reach an adult size that is three times larger than males (Fitzhugh et al., 1996; Monaghan and Armstrong, 2000; King et al., 2001). Hence, culture of primarily females would help optimize flounder production, providing better return on investment for present and future commercial operations.

In the majority of vertebrates, sex is determined at fertilization by the inheritance of sex-specific chromosomes. However, many turtles, lizards, and crocodiles exhibit an unusual form of sex determination in which incubation temperature experienced during

early development influences the phenotypic sex of hatchlings (reviewed in Bull, 1980; Wibbels et al., 1994). A similar plasticity in sex determination is also seen in some gonochoristic fish species (Francis, 1992; Crews, 1996). Sex determination in certain fish may be influenced by a single variable, while multiple stimuli have a combinatorial effect in other species (Nakamura et al., 1998; Pandian and Koteeswaran, 1999; Baroiller et al., 1999). Temperature, pH, steroid hormones, and social cues are some exogenous factors that influence sex ratios in fish.

Temperature has been shown to affect female:male sex ratios in only a few fishes (reviewed in Strüssmann and Patiño, 1995; Patiño et al., 1996). One of the most dramatic examples of temperature effects on sex determination occurs in the Japanese flounder or hirame, *Paralichthys olivaceus*. During early development temperature can influence sex in XX individuals. High (25 and 27.5°C) and low (15°C) temperatures produce a greater proportion of males, rather than females (Yamamoto, 1995; Yamamoto, 1999). Another Japanese species of a more northerly distribution, the barfin flounder of the genus *Verasper*, also exhibits temperature-dependent sex determination (TSD) (Goto et al., 1999). Sex in XY individuals is not affected by temperature in either of these Japanese species. In that aspect, patterns of TSD in flounder differ from those characterized in reptiles and fishes like the Atlantic silverside, *Menidia menidia* (Conover and Kynard, 1981; Conover and Heins, 1987). The unusual biology of sex determination in Japanese flounders creates the need for a sound understanding of sexual development in other flatfish, including commercially important North American *Paralichthys* species.

Considering the commercial value of southern and summer flounder (*Paralichthys dentatus*) recent technological advances in their culture, and the dramatic differences in growth rates between sexes it has become imperative to develop methods to control sex with the ultimate aim of producing monosex populations of only faster-growing females. Since nothing is known of the timing and control of sex differentiation in southern and summer flounder, the present investigations were undertaken to define the schedule and effects of temperature on sex differentiation in southern flounder.

MATERIALS AND METHODS

Defining the Schedule of Sex Differentiation

Wild adult southern flounder were captured by commercial pound net in the Pamlico Sound, North Carolina during September and October 1999. The fish were transported to the North Carolina Department of Agriculture and Consumer Services, Tidewater Research Station in Plymouth, NC. The fish were conditioned as broodstock using established photoperiod, salinity, and temperature control protocols (Smith et al., 1999b; Watanabe et al., 2001). Fish were fed three times weekly with frozen lake smelt, squid, and bloodworms. GnRHa implants were used to stimulate the ovaries of females prior to strip spawning in January 2000 (Berlinsky et al., 1996). Eggs and sperm were collected from multiple broodstock and pooled for *in vitro* fertilization. Larvae were

cultured in seawater according to Daniels et al. (1996) and gradually weaned beginning on day 55 post-hatch from a natural diet (rotifers/*Artemia* nauplii) to a mixture of dry diets (INVE, 600-800 microns, Salt Lake City, Utah and Biokyowa feeds, 1000-1200 microns, St. Louis, Missouri) through metamorphosis (Daniels and Hodson, 1999). Juveniles were fed a 50% protein pelleted feed (1.5-4 mm, Corey Feed Mills Ltd., New Brunswick, Canada) 3-6 times daily. Flounder were held at 18-23°C (depending on natural fluctuations) on a 12:12 L:D photoperiod. Fish were sampled at approximately 20 mm TL size intervals following metamorphosis for paraffin and plastic histology. Tissues were preserved in Bouin's fixative for at least 48 hours, washed in 50% ethanol, and stored in 70% ethanol until histological processing. Samples were infiltrated and embedded with paraffin and serially sectioned at 2-6 micron thickness (2-4 microns for plastic, 4-6 microns for paraffin preparations). Sections were stained with hematoxylin and counterstained with eosin. Structural and cellular sex-distinguishing markers were identified, and then used to describe stages of oogenesis in females and spermatogenesis in males.

Temperature-dependent Sex Determination Trials

Southern flounder fry were produced and weaned by the methods described above. Post-metamorphic fish were held at 18 ± 1°C and gradually acclimated from sea to fresh water until reaching a mean total length of approximately 40 mm (157 dph). At the initiation of the study, fish were randomly designated for each treatment and then stocked into duplicate tanks (700 L, 1.22 m diameter) at similar densities (46-49 fish /tank/treatment; mean total length and

weight: 42.85 ± 1.07 mm [mean ± SEM] and 0.79 ± 0.04 g; range of sizes in a subsample: 33-55 mm TL and 0.49-1.33 g). Fish were maintained in identical freshwater recirculating systems equipped with UV-sterilization and biofiltration and maintained at one of three temperatures with titanium heaters or chiller units. Calcium chloride ($CaCl_2$) and sodium bicarbonate ($NaHCO_3$) were added to each system to maintain water hardness at 150 mg/L, alkalinity at 200 mg/L, and salinity at 0 ppt. The 'low' target temperature was 18°C (actual mean temperature was 18.13°C ± 0.04, mean ± SEM), the 'mid-range' target temperature was 23°C (actual mean temperature was 22.78°C ± 0.07), and the 'high' target temperature was 28°C (actual mean temperature 27.56°C ± 0.11). Temperature was recorded twice daily. Throughout the course of the experiment, the fish were fed three times daily *ad libitum* and held on a 12:12 photoperiod. Cannibalism and aggressive interactions necessitated grading the fish into groups of larger and smaller individuals within each temperature treatment. Densities remained similar among treatments.

The temperature trials were maintained for 245 days. Some individuals that reached a body length of >160 mm (7-9 fish/treatment) before 245 days were terminated to reduce cannibalism of smaller fish. We could be confident that sex would be histologically discernible in fish of this size and early termination would prevent potential mortalities. At 245 days (402 dph), the remaining fish from each treatment were measured, weighed, and gonads were removed and preserved in Bouin's fixative for paraffin histology (23°C: 176.47 ± 3.28 mm [mean ± SEM] and 71.36 ± 3.92 g; 18°C: 158.38 ± 3.74 mm and 47.08 ± 2.70 g; 28°C: 125.83 ± 3.74 mm and 28.21 ± 2.44 g; N=45, 37, and 42, respectively). Serial sections were taken at 4-

6 microns. Slides were stained with hematoxylin and eosin and numerically coded. Sex was then assessed for all individuals by an observer blind to treatment using established sex-distinguishing characteristics (Nagahama, 1983; Tanaka, 1987; Yamamoto, 1995, Nakamura et al., 1998). Sex-specific features of gonadogenesis and gametogenesis were identified and recorded for each fish. Sex ratios were calculated and then statistically analyzed using X^2 goodness of fit tests, where the hypothesized composition of a normal population is 1:1 female:male regardless of water temperature (Zar, 1996). Sex was not assessed in a small number of fish from each treatment due either to problems in histological processing or a lack of clear sex differentiation in small specimens (<120 mm TL).

RESULTS

Defining the Schedule of Sex Differentiation

To determine the schedule of sex differentiation, histological criteria were used to evaluate sexual development in southern flounder of different sizes. In premetamorphic larvae, primordial germ cells were located between the mesonephric ducts and the gut (Figure 1). In post-metamorphic juveniles, paired, ribbon-like gonads were found at the posterior end of the abdominal cavity, beneath the ventral surface of the renal region. Germ cells gradually multiplied in number by mitosis amongst somatic cells and stromal

tissue. Gonad size and the number of germ cells increased dramatically in fish between 50-70 mm TL.

In presumptive ovaries, diffuse clusters of gonial cells commonly remained inactive until fish were greater than 100 mm TL. Primary oocytes were first observed in a 96 mm TL fish and were distinguished from oogonia by their larger size and more darkly stained peripheral cytoplasm. The presence of an ovarian cavity, an early sign of female sex differentiation, was first seen in southern flounder ranging from 86-100 mm TL (Figure 2). However, the presence of an ovarian cavity did not always precede the onset of meiosis. Some fish possessed primary oocytes in early perinucleolus stage, but lacked a discernible ovarian cavity. In other cases, formation of the ovarian cavity appeared to be just beginning while meiotic divisions were well advanced. The smallest fish that possessed primary oocytes in the chromatin-nucleolus and early perinucleolus stages was 115 mm TL, while others exhibiting these stages were generally greater than 150 mm TL. The timing of ovarian differentiation relative to fish size is illustrated in Figure 3.

In presumptive testes, structural changes associated with testicular maturation included the formation of densely clustered seminal lobules containing gonial cells, separated by a layer of fibrous connective tissue (Figure 4). Blood vessels were often located between the seminal lobules. Distally, the gonads consisted primarily of connective tissue forming tubules leading to the sperm ducts. These structural changes began in fish of approximately 100 mm TL. Spermatogonia remained inactive until most

fish were over 100 mm TL. Active spermatogenesis beyond the spermatogonial stage was first observed in a 96 mm TL fish. By 120 mm TL, many male fish showed advanced stages of testicular development including the presence of primary and secondary spermatocytes, spermatids, and spermatozoa. The smallest fish observed with spermatozoa was 112 mm TL, although males greater than 150 mm TL commonly possessed tubules and collecting ducts with pools of spermatozoa.

Temperature-dependent Sex Determination Trials

Based on histological criteria for developing gonads established in the sex differentiation study described above, the sex of fish raised at 18, 23, or 28°C was assessed. Sex ratios were similar among replicates within each temperature. The mid-range temperature, 23°C treatment, produced a sex ratio that did not differ from 1:1 (Figure 5). In contrast, both low and high temperatures produced skewed sex ratios. Exposure to 18°C produced a male-biased sex ratio ($P<0.01$), with 78% of the total population showing clear morphological features characteristic of testicular development. At the higher temperature, 28°C, 96% of fish were male ($P<0.001$), an effect more pronounced than that of the 18°C treatment. Survival rates ranged from 68-72.8% across treatments. The timing and pattern of gonadal development in animals from this temperature study was similar to that in the animals used for defining the schedule of sex differentiation. Ninety-three percent of fish greater than 120 mm TL had gonads that possessed meiotic gonial cells, versus only 36.4% in fish less than 120 mm TL.

DISCUSSION

In this study, we describe the pattern of sexual development in southern flounder based on sex-specific histological criteria. Prior to initiation of gonadal differentiation germ and somatic cells gradually multiply in number. Formation of an ovarian cavity, a sex distinguishing feature never observed during testicular development, appears to occur between 75-100 mm TL. However, the presence of a discernible ovarian cavity was not always associated with the onset of meiosis in oocytes. Because we observed some larger females that possessed meiotic oocytes, but no discernible ovarian cavity, we believe that the presence or absence of an ovarian cavity does not provide a reliable early marker of sex in this species. The sex of southern flounder greater than 120 mm TL can usually be unambiguously assessed in a high proportion of fish based on observation of meiotic divisions and other structural changes. From this we conclude that sex differentiation generally occurs between the sizes of 75-120 mm TL in southern flounder.

Sexual development in southern flounder shares strong similarities to that of hirame (*Paralichthys olivaceus*), although critical differences exist which may be important for culture. A common early sign of female sex differentiation in teleost fishes is the formation of a presumptive ovarian cavity where the dorsal and ventral edges of the gonad expand laterally and eventually fuse, leaving a gap (Nakamura et al., 1998). Based on this criterion of sex differentiation, sex determination in hirame occurs in fish ranging from 27-37 mm TL (Yamamoto, 1999). Similarly, based on the presence of an ovarian

cavity, studies in the barfin flounder (*Verasper moseri*) of Japan also indicate that sex differentiation occurs around 35 mm TL (Goto et al., 1999). In contrast, we found that neither an ovarian cavity nor early meiotic oocytes are commonly present in southern flounder before 75-100 mm TL. These findings indicate sexual development in southern flounder is substantially delayed relative to these Japanese species. Our preliminary studies and work by King et al. (2001) indicates a similar delay in the development of sex-distinguishing markers in summer flounder (*P. dentatus*). It is uncertain why *Paralichthys* flounders of the Eastern United States show delayed sex differentiation relative to *Paralichthys* and *Verasper* species of Japan. Differences in seasonal temperature fluctuations between the two regions or the timing of spawning may account for these observations, particularly considering all of these flounder species exhibit TSD (see below).

Temperature profoundly influences sex determination in southern flounder. Southern flounder exposed to high and low temperature show male-biased sex ratios, while those exposed to a mid-range temperature exhibited a sex ratio approaching 1:1. In this study, fish experienced temperatures well within their natural range, as wild southern flounder have been found in temperatures from 5 to 35°C (Reagan and Wingo, 1985). The existence of a pattern of temperature-induced sex determination in southern flounder is further supported by a similar effect in hirame, although the temperature response of southern flounder may be shifted slightly towards warmer temperatures relative to that of its Japanese congener (15°C-20.1, 20°C-49.2, 25°C-31.5, and 27.5°C-6.1% female; data adapted from Yamamoto, 1995).

However, the temperatures used were slightly different, so this has not been directly assessed. Future studies with a greater number of temperatures are required to firmly establish if temperature thresholds of sex determination in southern flounder differ from hirame. In both species, exposing fish to a high or low temperature during the period of sex determination appears to induce many genotypic females to develop as phenotypic males, while a mid-range temperature holds sex ratios closest to 1:1. Research on the effects of temperature on sex ratios in barfin flounder showed that maintaining fish at 14°C produced a sex ratio of 1:1, while shifting fish to 18°C for 62 days prior to sex differentiation resulted in an all male population (Goto et al., 1999). Only 'low' and 'high' temperatures were tested. Since the barfin flounder is a northern Japanese species, the low (14°C) temperature tested may actually reflect a typical 'mid-range' temperature seen in the wild. Therefore, it is possible barfin flounder may exhibit a pattern of TSD similar to that of hirame and southern flounder. Sex reversal from genetic males into phenotypic females does not appear to occur in southern flounder, hirame, or barfin flounder (Yamamoto, 1999; Goto et al., 1999). The strong effects of temperature on sex determination observed in this study, as well as that shown for hirame, suggests temperature is a prominent regulator of sex in *Paralichthys* flounders and possibly other flatfish species.

Since sex differentiation is substantially delayed in southern flounder compared with Japanese species, it would appear that the period of sex determination and its influence by temperature is likely to occur during a later window. The results of the temperature study provide further evidence that sex determination is delayed in southern flounder since the fish were stocked at approximately 40 mm TL, a size after the reported period of sex differentiation

in Japanese flounder species. However, it has not yet been shown for any flounder species that initial sex determination is irreversible. Temperature shift experiments will be necessary to address this question.

Initiation of gonadal differentiation in fishes is typically related to body size or age (Hunter and Donaldson, 1983). In species of fish with long sexually undifferentiated periods, body size is considered the most important determinant of the timing of gonadal differentiation (Francis and Barlow, 1993; Blázquez et al., 1999), although age may also play a role. To assess potential effects of age and body size on timing of sex differentiation in southern flounder, we conducted a separate investigation comparing timing of sexual differentiation of 9-month old fish to 12-month old growth-stunted fish from the same cohort. These fish were raised at 18 ± 1°C and 0 ppt salinity following metamorphosis. Despite their greater age the 12-month old growth-stunted fish did not mature at an appreciably smaller size (unpublished results). Thus, it would appear body size is a significant determinant of the timing of gonadal differentiation.

One of the primary goals of this study was to characterize sex determination responses to temperature. In order to maximize sample numbers for gonad examination and prevent losses of smaller individuals to cannibalism, we sampled some large individuals prior to terminating all remaining fish at day 245 of the study. Although this variation in time of sampling prevents this from being a fully valid growth study, we found that the remaining fish at experiment termination were largest at 23°C (23°C:

176.47 ± 3.28 mm [mean ± SEM] and 71.36 ± 3.92 g; 18°C: 158.38 ± 3.74 mm and 47.08 ± 2.70 g; 28°C: 125.83 ± 3.74 mm and 28.21 ± 2.44 g; N=45, 37, and 42, respectively). Interestingly, one theory regarding the evolution of temperature-dependent sex determination suggests that if growth potential varies across temperatures then individuals developing at temperatures that best promote growth should become the sex that most benefits from attaining large body size (Charnov and Bull, 1977). Since female southern flounder grow much larger than males (Fitzhugh et al., 1996; Monaghan and Armstrong, 2000, King et al., 2001), more rapid growth at female-determining temperatures may provide support for this theory. Future studies should therefore rigorously assess temperature effects on juvenile growth in this species and others that exhibit TSD. Growth efficiency is a critical variable to establishing viable commercial production of flounder. We show the rearing temperature (23°C) that ideally promotes female sex determination, may also allow for rapid 'growout' after the period of sex determination.

Southern flounder are a high-value, high-demand product. Because females grow faster and larger than males, a sound understanding of sexual development and sex control is required for monosex female culture. Southern flounder appear to be considerably delayed in their development of early sex distinguishing markers relative to their congener, the hirame. Research in both hirame, and now the present study in southern flounder, indicates that a mid-range temperature promotes female differentiation while both higher and lower temperatures promote male development. Information on the schedule of sex

differentiation and influences of temperature on sex determination can be directly implemented into existing and future commercial flounder operations in the United States and may provide useful management tools for optimal production.

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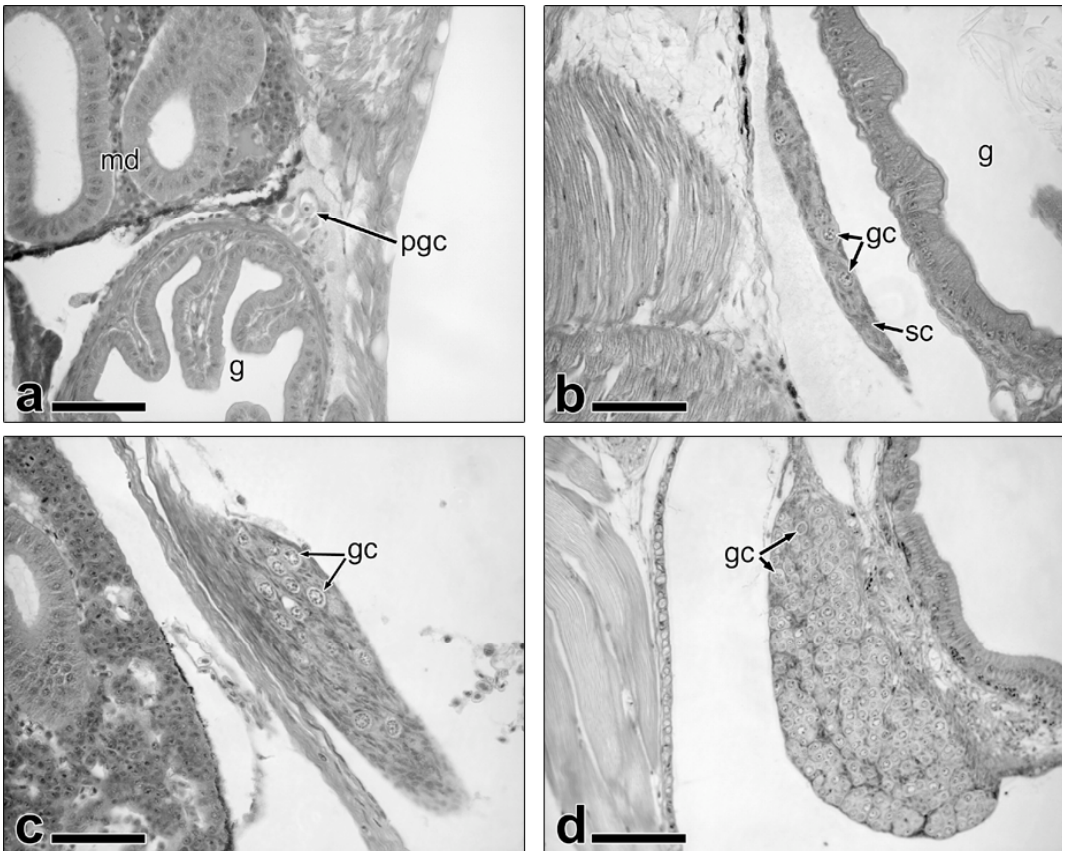

Figure 1. Undifferentiated gonads of southern flounder. (a) Cross-section of an 8.4 mm TL larva, (b) sagittal section of a 13 mm TL larva and a (c) 43 mm TL fish, and (d) cross-section of a 71 mm TL fish. pgc, primordial germ cell; md, mesonephric ducts; g, gut; gc, germ cell; sc, somatic cells. Bar=50 µm for plates a, b, and c and 100 µm for plate d.

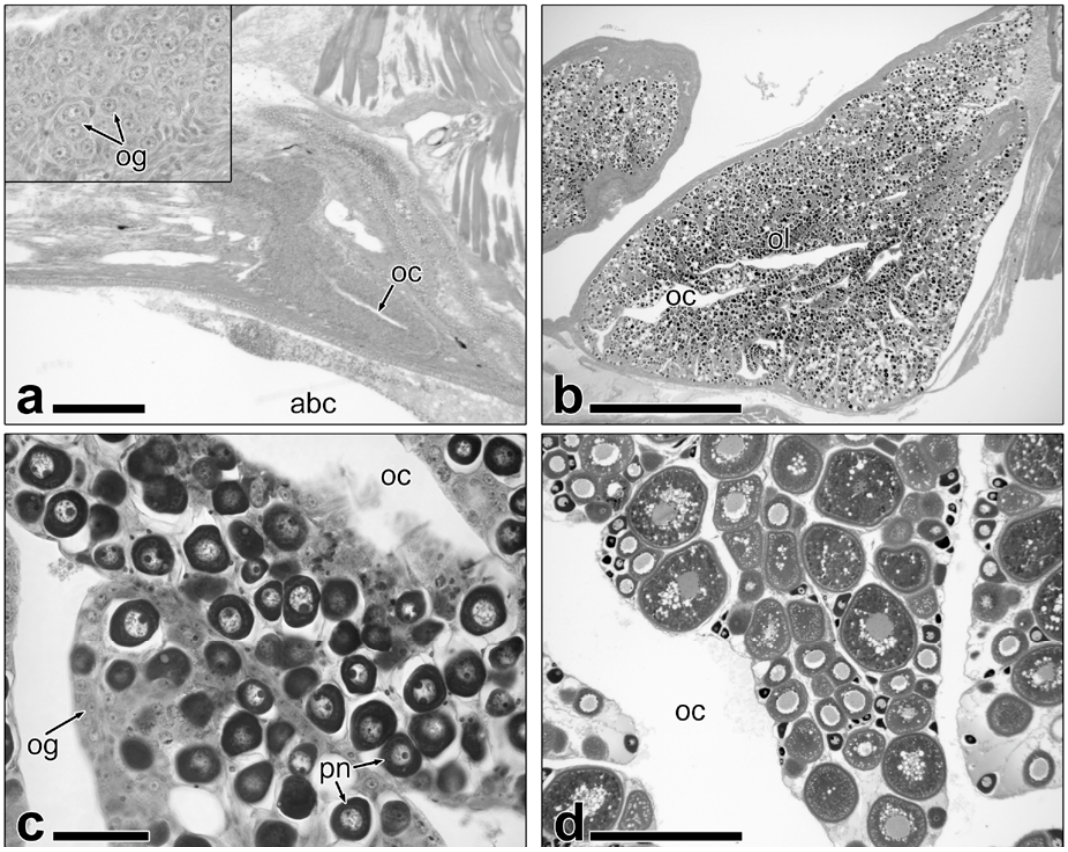

Figure 2. Ovarian development in southern flounder. (a) Sagittal section of an 86 mm TL fish (Bar=250 µm). Inset shows higher magnification. Cross-section of a 119 mm TL fish at (b) lower (bar=1000 µm) and (c) higher (bar=50 µm) magnification. oc, ovarian cavity; abc, abdominal cavity; og, oogonium; ol, ovarian lamella; pn, early perinucleolus stage oocytes. (d) Cross-section of an ovary from a 450 mm TL fish composed of oocytes at various stages, including perinucleolus, oil droplet, yolk, and migratory nucleus (bar=500 µm).

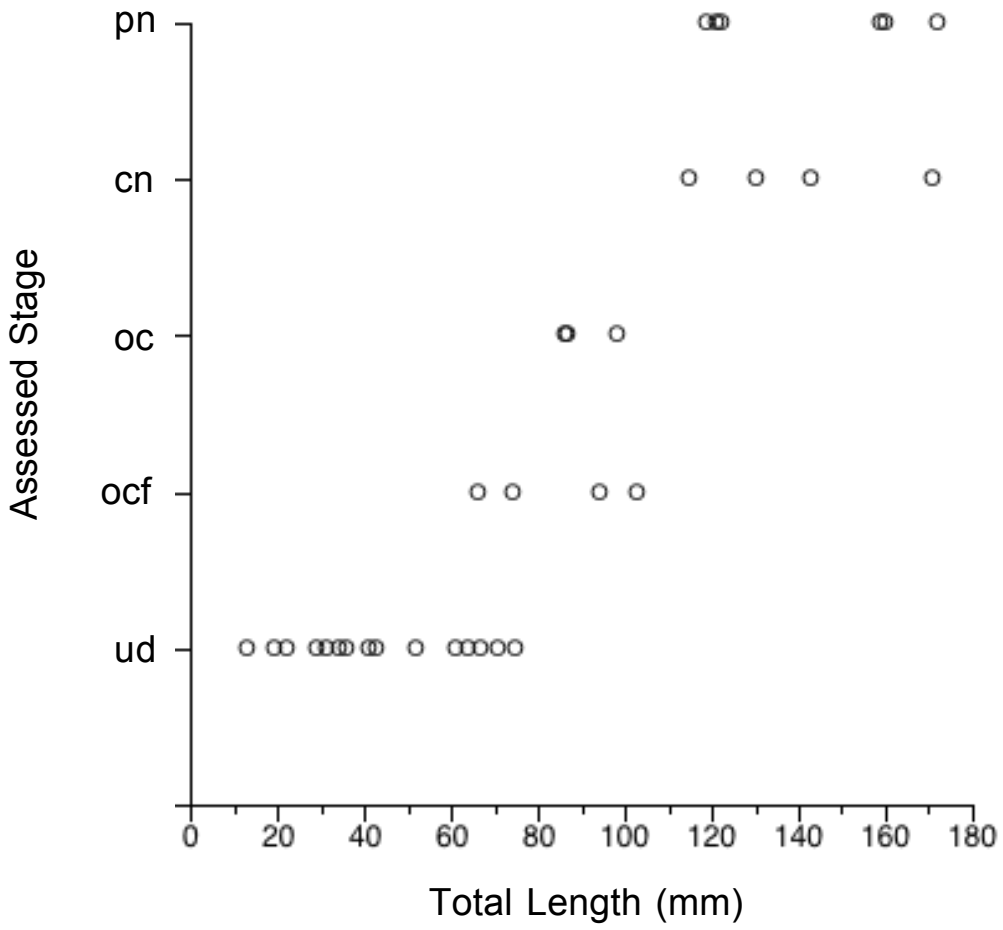

Figure 3. Schedule of ovarian differentiation versus body total length (mm) in southern flounder. Stages are: ud, undifferentiated; ocf, ovarian cavity forming; oc, ovarian cavity present; cn, chromatin-nucleolus stage oocytes; pn, perinucleolus stage oocytes. Female sex differentiation commonly occurs from 75 to 120 mm TL.

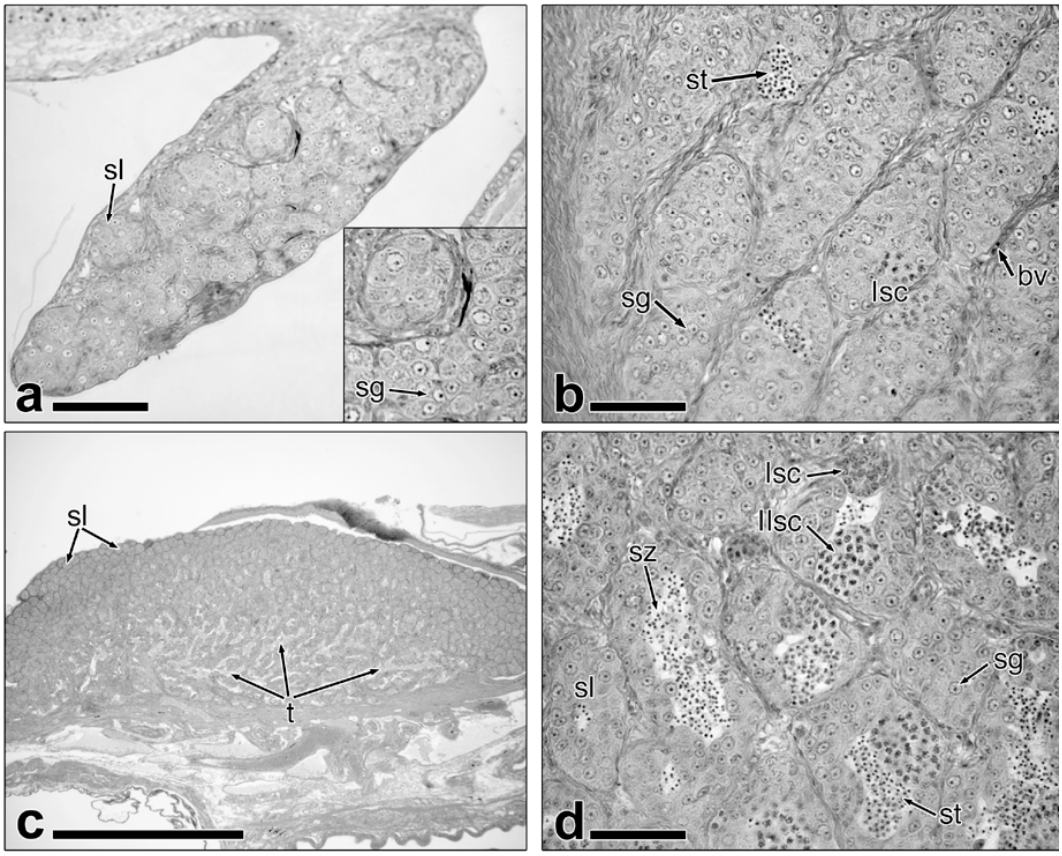

Figure 4. Testis development in southern flounder. (a) Cross-section of a 95 mm TL fish (bar=250 µm). Inset shows higher magnification. (b) Cross-section of a 135 mm TL fish (bar=50 µm). Cross-section of a 183 mm TL fish at (c) lower (bar=1000 µm) and (d) higher magnification (bar=50 µm). sl, seminal lobule; sg, spermatogonium; Isc, primary spermatocytes; st, spermatids; bv, blood vessel; t, tubules leading to the sperm duct; IIsc, secondary spermatocytes; sz, spermatozoa.

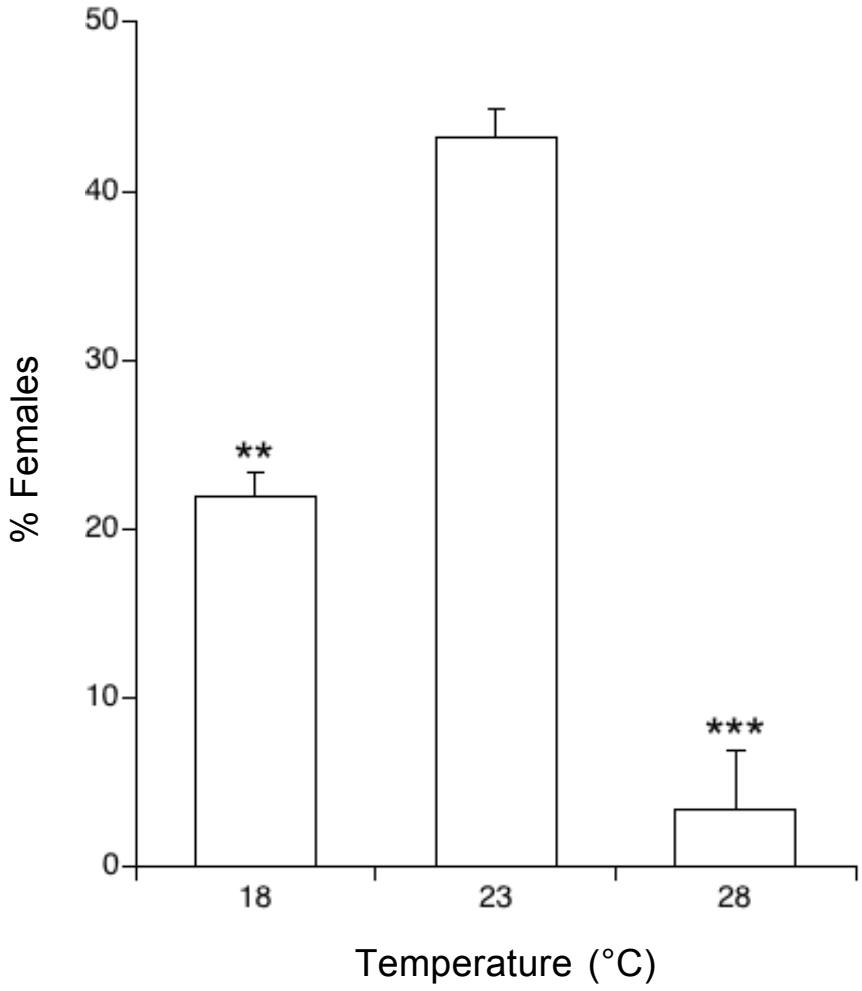

Figure 5. Effect of temperature on sex determination in southern flounder. Percentage of female southern flounder produced from replicate experiments (mean ± S.E.M.) at temperatures of 18, 23, and 28°C (*N*=64, 53, and 49, respectively, with 20-34 fish/temperature/replicate; ***P*<0.01 and ****P*<0.001 represent significant deviations from a 1:1 male/female sex ratio).

CHAPTER III

Aromatase Cytochrome P450: Cloning, Intron Variation, and Ontogeny of Gene Expression in Southern Flounder (*Paralichthys lethostigma*)[1]

[1] In preparation for submission to Journal of Experimental Zoology. Authors of the manuscript are J. Adam Luckenbach, Lea W. Early, Ashlee A. Rowe, Russell J. Borski, Harry V. Daniels, and John Godwin.

ABSTRACT

Aromatase cytochrome P450 ($P450_{arom}$) is the enzyme complex responsible for conversion of androgens to estrogens in vertebrates. Consequently, in fishes its activity appears critical to ovarian differentiation. The southern flounder (*Paralichthys lethostigma*) is a commercially important flatfish in which females grow larger than males. Sex determination in this species is temperature-sensitive and may be influenced by high and low water temperatures. Through cloning of the *cyp19a*-gene encoding $P450_{arom}$ and quantitative RT-PCR, we developed a functional biomarker for early female differentiation in southern flounder. The deduced amino acid sequence for southern flounder $P450_{arom}$ shares a high degree of identity with other teleost fishes. Comparison of $P450_{arom}$ introns from fish of different populations (mid-Atlantic Ocean and Gulf of Mexico) revealed substantial inter-individual variation that may affect sex determination responses. Adult ovarian tissue exhibited high levels of $P450_{arom}$ mRNA, while $P450_{arom}$ was detected only at low levels in testes. Liver, intestine, kidney, muscle, heart, and brain showed little or no $P450_{arom}$ expression, whereas relatively high levels were detected in the spleen. Gonads sampled from wild juvenile flounder spanning the period of sex differentiation initially exhibited a low level of $P450_{arom}$ gene (fish 40-55 mm in total length) followed by increases in some individuals and bifurcation into two clearly segregated groups (i.e., putative males and females) beginning at ~65 mm in length. The same pattern of $P450_{arom}$ expression was also observed in a hatchery-produced flounder population. Predictions of sex based on $P450_{arom}$ expression in wild and hatchery-produced flounder agreed precisely with those from gonadal histology, clearly demonstrating that the

pattern of $P450_{arom}$ expression observed relates to sex-specific differentiation. This research represents a unique application of a molecular assay to study of sex differentiation in a natural population. Furthermore, we provide a powerful technique for better understanding mechanisms of flounder sex determination and rapidly defining conditions for controlling sex for aquaculture.

INTRODUCTION

Estrogens play an instrumental role in reproduction of female vertebrates. In lower vertebrates, such as reptiles and fishes, estrogens are often critically involved in the processes of both sex determination and differentiation. Cytochrome aromatase P450 ($P450_{arom}$) is an important rate-limiting enzyme responsible for conversion of C_{19} steroids (e.g., testosterone) to estrogens; the primary estrogen in fishes is estradiol-17β (E_2). The gene that encodes $P450_{arom}$ is termed, *cyp19*. Two *cyp19* isoforms have been identified in fishes, *cyp19a* or the ovarian-derived $P450_{arom}$ and *cyp19b* or brain-derived $P450_{arom}$. Ovarian $P450_{arom}$ expression (*cyp19a*) and subsequent $P450_{arom}$ activity is responsible for endogenous E_2 biosynthesis and thereby mediates the process of ovarian differentiation in several fishes (e.g., Guiguen et al. 1999; Kitano et al., 1999; D'Cotta et al., 2001). Previous studies also suggest that suppression of $P450_{arom}$ gene expression, $P450_{arom}$ activity, and E_2 biosynthesis may be necessary for testicular differentiation (e.g., Guiguen et al. 1999; Kitano et al., 1999; D'Cotta et al., 2001; Kwon et al., 2001; Uchida et al., 2004). Consistent with this hypothesized role, dietary application of specific aromatase inhibitors, such as Fadrozole, prior to and during the period of sex differentiation induces testis formation in both gonochoristic and sequentially hermaphroditic fishes (reviewed by Devlin and Nagahama, 2002; Strüssman and Nakamura, 2002; see also Bhandari et al., 2004). This is true even in genetically all-female populations [e.g., hirame *Paralichthys olivaceus* (Kitano et al., 2000a) and zebrafish *Danio rerio* (Uchida et al., 2004)]. Taken together, results from a diversity of teleosts, albeit not all teleosts, suggest that $P450_{arom}$

expression and activity in morphologically undifferentiated gonads directs the process of sex determination, with high and low $P450_{arom}$ levels leading to ovarian and testicular development respectively.

The southern flounder (*Paralichthys lethostigma*) is a flatfish found along the southeastern and Gulf of Mexico coasts of the United States. Females of this species grow faster and often reach a three-fold larger adult body size than males (Fitzhugh et al., 1996; Monaghan and Armstrong, 2000). Southern flounder exhibit a plasticity of sex determination, referred to as temperature-dependent sex determination (TSD), where water temperature can influence sex determination during early development (Luckenbach et al., 2003). In juvenile flounder, both low and high water temperatures can greatly affect the female:male sex ratio, inducing male-skewed populations in cultured animals. The female proportion, however, is never greater than approximately 50%. Based on the proposed XX-XY system of sex determination for flounder (Yamamoto et al., 1999), sex determination in genetically male flounder is not temperature sensitive, whereas the environment may influence sex determination in the homogametic genotype. Although much progress has been made in this area of research, the detailed mechanisms of TSD and modes by which environmental stimuli are transduced are not known for flounder, and are still lacking for vertebrates that exhibit TSD generally, including well-studied reptilian models (Godwin et al., 2003; Crews, 2003).

Endocrine changes associated with sex differentiation often begin well before morphological changes are apparent in the gonads of fishes. Indeed, exogenous steroid

hormones have been found to elicit a stronger effect when fish are treated just prior to any clear histological sex differentiation of the gonads (Baroiller and D'Cotta, 2001; Piferrer, 2001). Thus it appears that steroids begin to influence the direction of sex differentiation in fish prior to clear morphological/histological sex differentiation of the gonads. In southern flounder, the period of gonadal differentiation has been previously assessed only by histological methods (Luckenbach et al., 2003). The first signs of histological sex differentiation do not appear until at least ~75 mm in total length (TL). However, to clearly distinguish the sex of a high percentage of animals (93%) they are typically raised to a body size of 120-150 mm TL. Within this range, meiotic divisions are apparent within the gonads and the ovarian cavity is well formed. Since the cascade of endocrinological events leading to gonadal differentiation likely begins prior to clear histological sex differentiation and exogenous steroids elicit their strongest effects in histologically undifferentiated fish (Baroiller et al., 1999; Piferrer, 2001), molecular and/or biochemical approaches are needed to further investigate early sex differentiation and the 'window' during which temperature can influence this process in southern flounder.

In this study, we cloned and sequenced southern flounder $P450_{arom}$ cDNA derived from ovary and studied the genomic organization of the gene and potential variation at the $P450_{arom}$ locus among individuals and across two populations, the western mid-Atlantic and Gulf of Mexico stocks. These populations appear to be geographically isolated (Ginsburg, 1952), but genetic differentiation across the populations does not appear to be extensive (Blandon et al., 2001). Prior to this work, no studies have addressed genetic differences between these

populations as it relates to genes involved in sex differentiation or the TSD response. We also developed a 'real-time' quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay for measuring $P450_{arom}$ mRNA in southern flounder. Using this approach, we addressed the tissue-specific expression of $P450_{arom}$ in southern flounder, the ontogeny of $P450_{arom}$ expression in southern flounder gonads during the process of sex differentiation, and determined if $P450_{arom}$ expression could be used as a biomarker for early assessment of the direction of sex differentiation in both wild and cultured southern flounder populations. We conclude that high expression of $P450_{arom}$ is associated with the process of ovarian differentiation and low $P450_{arom}$ expression is associated with testicular differentiation, suggesting a likely role of this gene in these processes, and perhaps sex determination in southern flounder. Furthermore, we demonstrate that $P450_{arom}$ expression is a useful biomarker for distinguishing the sex of juvenile southern flounder much earlier than previously possible with histology. Thus, this work provides a powerful tool for future study of the mechanisms of TSD in wild and hatchery-produced flounder that should be easily adaptable for work in other species.

MATERIALS AND METHODS

Cloning of $P450_{arom}$ cDNA

Ovarian tissue was collected from an 0.5 m adult female southern flounder, frozen with dry ice, and stored at –80°C. Total mRNA was isolated using the Straight A's™ mRNA Isolation System (Novagen, Madison, WI, USA). Single-stranded cDNA was synthesized from 1 μg of ovarian mRNA using oligo (dT) primers as the template for reverse transcription in 20 μl reactions (Omniscript™ RT kit, Qiagen Inc., Valencia, CA, USA). To partially clone $P450_{arom}$ cDNA in southern flounder, multiple oligonucleotide primer sets for PCR were designed within relatively conserved regions of $P450_{arom}$ among fishes [*Paralichthys olivaceus* (GenBank Accession no. AB017182); *Oreochromis niloticus* (P70091); *Oryzias latipes* (Q92087); *Ictalurus punctatus* (Q92111)] using Oligo 6.6 (Molecular Biology Insights Inc., Cascade, CO, USA) and MacVector™ 7.2 (Accelrys Inc., San Diego, CA, USA) software. The first PCR utilized degenerate primers, Arom F1 and Arom R1 (Table 1). The product of this reaction was then used as template in a nested PCR with degenerate primers, Arom N1 and Arom N2. The three PCRs that followed utilized primer pairs, Arom F2/Arom R2, Arom F3/Arom R3, and Arom F4/Arom R4, respectively (see Table 1). For cloning the 3′ end of the gene, the rapid amplification of cDNA ends (RACE) method was used (FirstChoice™ RLM-RACE Kit, Ambion Inc., Austin, TX, USA). For these reactions, southern flounder-specific forward primers, 3RACE F1 and 3RACE F2, were used for nested PCR in conjunction with reverse primers, 3RACE R1 and 3RACE R2, provided with the kit.

All standard PCRs were carried out in a Hybaid thermal cycler (Thermo Electron Corp., Waltham, MA, USA) using the HotStar Taq™ DNA polymerase kit (Qiagen Inc.). Reactions consisted of 100 ng cDNA template, 1X PCR buffer (1.5 mM $MgCl_2$), 200 µM of each dNTP, 0.2 µM gene-specific forward and reverse primers, and 2.5 units of DNA polymerase for a total reaction volume of 50 µl. General thermal cycling conditions were as follows: initial activation at 95°C for 15 min, 30 cycles of PCR at 94°C for 1 min, 53-67°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 15 min.

Amplified products were separated by 1.5% agarose gel electrophoresis. PCR products with an amplicon of the predicted size were cloned into pCR®II vector using the TA Cloning® Kit and transformed in INVαF´ competent cells following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Twenty positive clones were selected for each reaction, subcultured, plasmid DNA extracted, and 3-6 clones with inserts of the predicted size were sequenced. Resulting sequences were compared to those of other species at the National Center for Biotechnology Information (NCBI) website using the BLAST search service. Sequence alignments and comparisons to other $P450_{arom}$ sequences were performed using MacVector™ software.

Cloning of $P450_{arom}$ intron regions and comparisons between two populations

Adult southern flounder representative of the western mid-Atlantic and Gulf of Mexico populations and captured in North Carolina (NC) and Texas (TX) coastal waters, respectively, were purchased. Muscle tissue was collected from six fish per population

and frozen on dry ice. Nuclear DNA was extracted from ~1 g of muscle for each animal. Frozen tissues were homogenized in a 0.1 mM Tris-EDTA grinding buffer with 1% SDS and 0.1% proteinase K, incubated at 65°C for 30 min, and centrifuged at 12,000 x g for 10 min. DNA was isolated using a phenol/chloroform isoamyl-alcohol extraction procedure where samples were mixed with 0.3M sodium acetate and DNA was precipitated in ethanol. DNA pellets were resuspended in 1X Tris-EDTA and stored at –20°C until needed.

Southern flounder $P450_{arom}$ -specific primers were designed within separate exon regions, predicted based on splicing junctions reported for the medaka, *Oryzias latipes* (GenBank Accession no. D82969, Tanaka et al. 1995), so putative intron regions would be bridged during PCR amplification. Targeted southern flounder intron regions corresponded to introns II-VI in medaka. Primer pairs and specific targets in southern flounder were as follows: Arom F2 /Arom R2 for introns II and III, Arom F5 /Arom R5 for introns IV and V, and Arom F6/Arom R6 for intron VI. Putative flounder introns II and III were sequenced in NC and TX animals (N=6/population) and compared across individuals and populations, while introns IV, V, and VI were cloned from a single NC specimen. PCR conditions and cloning procedures were generally as described above. Resultant clones for introns II and III were sequenced four times in each direction and consensus sequences were compiled for each fish. For introns IV-VI, clones were sequenced two times in each direction.

Measurement of $P450_{arom}$ mRNA via qRT-PCR

For measures of $P450_{arom}$ mRNA in adults, body tissues were collected from wild-caught southern flounder, snap frozen in liquid nitrogen, and stored at –80°C. To study the ontogeny of $P450_{arom}$ expression in a naturally occurring population of southern flounder, juvenile fish were collected from estuarine nursery habitats in the Pamlico Sound, NC from mid-May to late June 2003 during a larval recruitment survey conducted by the North Carolina Division of Marine Fisheries. Location of capture, water depths, and bottom temperatures and salinities were recorded for the three flounder collections represented in this study (Table 2). The two-seam otter trawl net used in these collections had a 3.2 m headrope and 0.64 cm bar-mesh; the tailbag was 0.32 cm bar-mesh. Trawling speed and duration for each collection were 2.25 knots and 1 min, respectively. The juvenile flounder captured ranged from 40-175 mm TL. Fish were identified as southern flounder (field methods in Gutherz, 1967) and their length and weight recorded before being deeply anaesthetized and gonads carefully dissected and removed. Gonads of a subset of larger juveniles (100-175 mm TL) were further divided into two pieces. One lobe was snap frozen in liquid nitrogen and stored at –80°C for qRT-PCR, while the other section was placed in Bouin's fixative for paraffin histology (methods in Luckenbach et al., 2003). This approach allowed us to morphologically identify the sex of juvenile flounder via previously established histological criteria and measure $P450_{arom}$ mRNA abundance in the same animals.

For measurement of $P450_{arom}$ mRNA abundance in a hatchery-produced population of southern flounder undergoing sex differentiation, a random sample of fish (N=25) ranging in size from 57-97 mm TL was collected 101 days after hatching from a commercial-scale flounder study being conducted at the Lake Wheeler Field Laboratory, North Carolina State University. Fish were handled and gonads dissected as described above. The actual sex ratio of this captive population of flounder was evaluated approximately 3 months later (185 days after hatching) by macroscopic examination of the gonads and/or gonadal histology, when necessary, in 41 randomly selected fish. Similar to Kitano and coworkers (2000b), the actual sex ratio was then compared to the sex ratio predicted for this population by the $P450_{arom}$ expression profile.

Total RNA was extracted for all tissue samples using Tri-Reagent® (Molecular Research Center Inc., Cincinnati, OH, USA). For isolation of RNA in small tissues (i.e., gonads of juvenile flounder), 1 ml of Tri-Reagent® was supplemented with 6 µl of Polyacryl Carrier™ (Molecular Research Center Inc.). Extracted RNA for all samples was treated with DNA-free™ (Ambion Inc., Austin, TX, USA) to remove any genomic DNA contamination following the manufacturer's protocol, except that the volume of DNase-I enzyme was increased to 2 µl (i.e., 4 units) per reaction and the incubation time was extended to 1 hour. RNA concentration was determined in triplicate for each sample using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purity (A260/A280) ranged from 1.7-2.1. Synthesis of cDNA was as described above, with 1 µg of total RNA reverse transcribed per sample.

To allow design of specific qRT-PCR primers for two candidate internal control or housekeeping genes, southern flounder glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor-1 alpha (EF-1α) were first partially cloned by RT-PCR using 100 ng of ovarian poly $(A)^+$ mRNA as template. Primers for RT-PCR were designed using the known sequence of GAPDH and EF-1α in hirame, *P. olivaceus* (Genbank accession numbers AB029337 and AB017183). Degenerate primers GAP F1 and GAP R1 (Table 1), corresponding to nucleotides 191-208 and 553-570 in hirame (Aoki et al., 2000), were used to partially clone southern flounder GAPDH. Primers for cloning EF-1α in southern flounder, EF F1 and EF R1, were identical to those used by Kitano and coworkers (1999) for semi-quantitative PCR and corresponded to nucleotides 4-23 and 562-581 in hirame. Clones for southern flounder GAPDH and EF-1α were sequenced and their identity verified using BLAST.

Abundance of $P450_{arom}$ and EF-1α mRNA was quantified in various adult tissues and gonads of juvenile southern flounder by qRT-PCR. Abundance of GAPDH mRNA was assessed only in adult tissues. Southern flounder gene-specific primers and dual-labeled FRET (Fluorescent Resonance Energy Transfer) probes (Table 3) for qRT-PCR were designed within cDNA sequences obtained in the present study using Primer Express® 2.0 software (Applied Biosystems, Foster City, CA, USA) and recommendations from the literature (Bustin, 2000). The selected reporter and quencher dyes for the dual-labeled probes were FAM and TAMRA, respectively. All reactions

were performed in duplicate. The total volume of each reaction was 25 µl and final concentrations were as follows: 1X TaqMan® Universal PCR master mix (Applied Biosystems), 100 ng of cDNA template, 900 nM forward and reverse primers, and 250 nM FRET probe. A 'no amplification' control (NAC) was run in duplicate for all samples. Instead of cDNA, these reactions contained template RNA that was not reverse transcribed, but was otherwise handled identically. These reactions should reveal genomic DNA contamination in sample preparations. A no template control (NTC) was also run in duplicate for each gene and contained no cDNA or RNA template. This negative control should reveal PCR carryover contamination in reagents if present.

All qRT-PCRs were performed with a GeneAmp 5700 Sequence Detection System (Applied Biosystems) using the following thermal cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 65°C for 1 min. Results for $P450_{arom}$ and EF-1α were optimized by manually setting the baseline to 6-15 cycles and 3-12 cycles and the threshold to 0.04 and 0.05, respectively. Standard curves for $P450_{arom}$ and EF-1α were generated by serially diluting a pool of ovarian cDNA (concentrations ranged from 0.01-100 ng) and were run in duplicate for each plate/reaction. To control for inter-assay variation and loading differences, Ct-values for $P450_{arom}$ were normalized to those of EF-1α (for more details see Relative Standard Curve Method; ABI Prism 7700 Sequence Detection System, User Bulletin #2, P/N 4303859 Rev. A, Stock No. 777802-001).

Following qRT-PCR, a standard curve sample for each gene was electrophoresed on a 1.5% gel to confirm product size and an aliquot was directly sequenced from PCR in both directions. Resulting sequences were compared to those for other species using BLAST.

RESULTS

Cloning of $P450_{arom}$ cDNA

The partial $P450_{arom}$ cDNA sequence for southern flounder is 1985 nucleotides, of which, 1550 nucleotides are part of the open reading frame encoding $P450_{arom}$ (Figure 1). This constitutes an estimated 99.6% of the coding region and 97.0% of the cDNA. An ATG (methionine) initiation codon located in the open reading frame encodes a putative $P450_{arom}$ protein of 508 amino acid residues. We note that the 3′-untranslated region, composed of 432 nucleotides, contains 7 single nucleotide polymorphisms (see Figure 1). At these positions, two possible nucleotides were observed within clones generated from the single female fish utilized in this work. A polyadenylation signal is located 18 nucleotides from the beginning of an 11-nucleotide poly $(A)^+$ tail.

When compared to other fishes, the partial southern flounder $P450_{arom}$ cDNA sequence showed a high degree of sequence homology. The sequence was most similar to

$P450_{arom}$ in hirame (*P. olivaceus*), with the cDNA and coding region showing 93% and 97% identity, respectively. The deduced ovarian $P450_{arom}$ cDNA sequence for southern flounder was also very similar to other teleosts (Figure 2).

Cloning of $P450_{arom}$ intron regions and comparisons between two populations

Five southern flounder $P450_{arom}$ intron regions were successfully cloned from nuclear DNA extracted from muscle. These introns correspond to introns II-VI in the medaka (*O. latipes*) and European sea bass (*D. labrax*) based on identical splicing junctions. Donor and acceptor sites of the five flounder introns conformed to the GT/AG rule for splicing, as each intron sequence began with the nucleotides GT and ended with AG. Introns II-VI in the flounder differed in size when compared to corresponding introns in these other fishes (Table 4). Comparison of $P450_{arom}$ introns II and III in flounder from different populations (i.e., NC and TX) revealed substantial variation among individuals (Figure 3). Intron III exhibited greater overall sequence variation and ranged from 82-88 nucleotides in length. A short, 6-nucleotide insertion/deletion polymorphism was discovered in intron III beginning 69 bases into the sequence. If considered an insert, this polymorphism has principally arisen in NC fish (3 of 6 fish), whereas only a single TX fish possessed the insertion. Multiple nucleotide polymorphisms were found for introns II and III within and across individual animals. This indicates the occurrence of distinct alleles at these positions and establishes that genetic variation does exist at the $P450_{arom}$ locus in this species. Phylogenies based on

consensus sequences for introns II and III for NC and TX flounder indicated no clear or obvious population differentiation (Figure 4).

qRT-PCR assay validation

Relative abundances of $P450_{arom}$, GAPDH, and EF-1α transcripts in tissues of adult southern flounder were determined using qRT-PCR. Direct sequencing of qRT-PCR products for each gene confirmed that the short amplicons for each gene were 100% identical to respective southern flounder cDNA sequences. Standard curves generated with a serially diluted pool of ovarian cDNA and included in each plate/reaction demonstrated a strong correlation of Ct-value to amount of template delivered to the reaction (R^2 values=0.99 and 1.00 for $P450_{arom}$ and EF-1α, respectively). This strong correlative relationship gives confidence that gene-specific mRNA measures with this assay are accurate. Negative controls (NTC and NAC) included in each reaction showed no detectable amplification over 40 cycles of qRT-PCR.

$P450_{arom}$ mRNA abundance in adult tissues

In adult tissues of southern flounder, $P450_{arom}$ mRNA was most abundant in the ovary and spleen (Figure 5). This was maintained when $P450_{arom}$ values were normalized to EF-1α mRNA. $P450_{arom}$ mRNA was detected at much lower levels in the brain, testis, gill, and liver and was not detected in muscle, heart, intestine, or kidney. GAPDH and EF-1α gene expression were detected in all adult tissues tested, but cycle-threshold values

(Ct values) for GAPDH varied greatly across tissues (18.14 for muscle-34.66 for testis) with the same amount of starting RNA/cDNA. EF-1α expression (Ct values) was more consistent across tissues (ranged from 13.76-17.54), indicating this housekeeping gene is a more reliable control for normalization of $P450_{arom}$ gene. More importantly, for an assay that is proposed for assessment of sex differentiation, GAPDH measurements for pooled ovary samples differed considerably from testis samples (Ct-value=24.58 for ovary versus 34.66 for testis, a >30-fold difference in GAPDH mRNA abundance).

$P450_{arom}$ mRNA abundance in gonads of wild juvenile flounder

Relative $P450_{arom}$ mRNA abundance was quantified in gonad samples collected from wild-caught juvenile flounder ranging from 40-175 mm TL and values were normalized to relative EF-1α mRNA. As above, standard curves generated using pooled ovarian cDNA for these reactions showed a strong correlation of Ct-value to amount of template delivered (R^2 values ranged from 0.99-1.00 for $P450_{arom}$ and EF-1α). EF-1α mRNA abundance was similar across samples with Ct values ranging from 15.35-18.41. In juveniles, $P450_{arom}$ mRNA was detectable at low levels in most flounder ranging from ~40-55 mm TL (Figure 6). However, at larger body sizes, individuals exhibited dramatically elevated $P450_{arom}$ mRNA levels (putative developing females) or $P450_{arom}$ expression remained low (putative developing males). This clear divergence in $P450_{arom}$ /EF-1α expression from basal levels appears to occur by ~65 mm TL, is more pronounced by 80 mm TL, and is maintained throughout the size range that defines the period of histological sex differentiation. Based on $P450_{arom}$ mRNA abundance in fish ≥65

mm TL, the predicted sex ratio of this wild flounder sample would be 16 females:15 males, which is ~1:1. Bottom water temperatures at the capture locations were from 23-24°C during collection 1, but had climbed to 29°C by collection 3 (see Table 2). Fish sampled during the third collection and exposed to this high water temperature ranged from 77-175 mm TL.

A portion of the gonads of a subset of wild juveniles (100-175 mm TL) assayed for $P450_{arom}$, were processed for histology. In all cases, high levels of $P450_{arom}$ expression were associated with the presence of the ovarian cavity and/or developing oocytes (Luckenbach et al., 2003). Low levels of $P450_{arom}$ mRNA were associated with signs of testicular differentiation, such as seminal lobules and/or stages of spermatogenesis.

$P450_{arom}$ mRNA abundance in gonads of hatchery-produced juvenile flounder

$P450_{arom}$ abundance was evaluated in a hatchery-produced population of southern flounder for a comparison of the ontogeny of expression to wild fish and to further test the utility of this assay for early and accurate assessment of the direction of sex differentiation. Gonads of randomly selected hatchery-produced juveniles undergoing sex differentiation exhibited a pattern of $P450_{arom}$ expression that very closely matched the pattern observed in wild juvenile flounder of similar body sizes (Figure 7). Clear segregation of $P450_{arom}$ mRNA into characteristically high and low levels appears to have occurred by 65 mm TL. Prior to this body size, clear high and low expression groups are not apparent. Of flounder ≥65 mm TL, 12 fish show high $P450_{arom}$ mRNA levels

(putative females) and 7 fish show low $P450_{arom}$ mRNA levels (putative males). Therefore, the predicted sex ratio for this hatchery-produced population would be 63.2% female and 36.8% male.

The actual sex of randomly selected fish (range of sizes was147-205 mm TL) from this captive population was assessed by macroscopic examination of the gonads and/or gonadal histology. We found that of 41 fish, 26 were female and 15 were male, which is 63.4% female and 36.6% male. The actual sex ratio of this flounder population strongly agrees with our predicted sex ratio (63.2% female:36.8% male) based on $P450_{arom}$ expression data for flounder ≥65 mm TL and demonstrates the utility of this technique for early assessment of the direction of sex differentiation in juvenile southern flounder.

DISCUSSION

We have isolated and characterized a large portion of $P450_{arom}$ cDNA derived from ovary of southern flounder. The $P450_{arom}$ nucleotide and deduced amino acid sequences are highly similar to $P450_{arom}$ (i.e., *cyp19a*) of other teleosts. One potential start codon, encoding a protein of 508 residues, was present in the southern flounder $P450_{arom}$ sequence, although we could not successfully sequence the entire 5′ region of the gene to determine if alternate start sites might exist. Based on the high degree of homology of this

southern flounder sequence to $P450_{arom}$ in hirame (*P. olivaceus*), it is likely that a second possible initiation codon exists just upstream from the site that we have identified (Kitano et al., 1999). The presence of two $P450_{arom}$ initiation codons has also been reported in several other teleosts, including rainbow trout (Tanaka et al., 1992), medaka (Tanaka et al., 1995), Nile tilapia (Chang et al., 1997), goldfish (Tchoudakova and Callard, 1998), and sea bass (Dalla Valle et al., 2002). A third possible initiation codon has been found thus far only in the catfish (Trant, 1994).

The 3′-untranslated region of southern flounder $P450_{arom}$ determined by RACE was 432 bases and within this region we found several nucleotide polymorphisms. The poly $(A)^+$ tail was 11 nucleotides in length, 10 nucleotides shorter than that reported for hirame $P450_{arom}$ cDNA, which was isolated from an ovarian-derived library. This difference was apparent for all positive clones in southern flounder. However, this difference could reflect an artifact of the annealing site of the 3′-RACE adapter.

The medaka (*Oryzias latipes*) is the first teleost fish in which the structural gene encoding $P450_{arom}$ was isolated and characterized (Tanaka et al., 1995). The medaka $P450_{arom}$ gene consists of nine exon and eight intron regions. More recently, genomic organization of $P450_{arom}$ has been studied in the European sea bass, *Dicentrarchus labrax* (Dalla Valle et al., 2002). These studies show that the *cyp19* gene encoding $P450_{arom}$ is relatively small in fishes, measuring only ~3 kb, whereas mammalian *cyp19* is >50 kb due to the presence of large intron regions. We cloned and sequenced five southern flounder

$P450_{arom}$ intron regions from nuclear DNA and found that the splicing junctions for these introns are identical to those in the medaka and European sea bass and that all introns conform to the GT/AG rule for RNA splicing. However, the size of the southern flounder introns differed from corresponding introns in the medaka and sea bass, which also differed from one another.

Within a gene, intron regions are likely to be more variable than exons, which encode the amino acids. Thus, analysis of introns across individuals and populations is a promising approach to studying genetic polymorphisms (Hillis et al., 1996). Comparison of putative $P450_{arom}$ introns II and III in southern flounder from different geographic populations (i.e., NC and TX) revealed substantial inter-individual variation. Clearly different alleles exist within and across individual animals at this locus. These observed allelic differences did not clearly segregate by population, however, when a phylogenetic reconstruction was performed. The lack of clear segregation at the $P450_{arom}$ locus across these southern flounder populations agrees with prior genetic work conducted by Blandon and coworkers (2001), which suggests that these are not independent populations. It is well documented in atherinid fishes, such as the Atlantic silverside (*Menidia menidia*), that TSD responses vary across populations and among families (Conover and Kynard, 1981; Lagomarsino and Conover, 1993). Sex ratios of Atlantic silverside progeny from different female parents crossed with the same male parent show natural variation and variation in their response to rearing temperature. Similarly, among-family variation in the TSD response has been documented in the American alligator

(*Alligator mississipiensis*) and common snapping turtle (*Chelydra serpentina*) (Rhen and Lang, 1998). In hirame (*P. olivaceus*), sex ratios of fish cohorts reared at the same water temperature appear to exhibit variation in the TSD response (see Yamamoto, 1995; 1999). However, to our knowledge, among-family variation has not been experimentally assessed in this species. Our analysis of southern flounder $P450_{arom}$ clearly establishes that variation exists at the $P450_{arom}$ locus. Variation in introns (and 3´-untranslated region) observed among and within individuals could be correlated with variation in other regions of this gene, most especially the promoter region. It is possible that such variation could produce differences in $P450_{arom}$ gene expression and potentially the TSD response. Further research will be necessary to test if variation in the $P450_{arom}$ gene relates to any potential differences in its regulation.

Real-time qRT-PCR is an increasingly popular technique for measuring gene-specific mRNA abundance. Benefits of this PCR-based approach to measuring mRNA transcript abundance include high specificity of detection, particularly when a probe-based method is employed, and the requirement of only small starting quantities of RNA (Bustin, 2000). This allows for measurement of transcript abundance in extremely small tissues, such as juvenile flounder gonads in our case. A principal difference between qRT-PCR and standard or semi-quantitative PCR methods is that with qRT-PCR relative sequence detection occurs during the log phase of amplification and not at the end point of analysis when amplification may have reached the plateau phase (Overturf et al., 2001). If the number of cycles selected for standard PCR enters the plateau phase of

amplification for some or all of the experimental samples, inaccuracies in relative gene expression may arise.

Internal control or housekeeping genes (e.g., 18S, GAPDH, and β-actin) are commonly used for normalization of target gene values following qRT-PCR, theoretically correcting for loading differences across samples. GAPDH has been frequently utilized as an internal control for qRT-PCR. However, the validity of GAPDH as a control has been questioned based on inconsistencies of expression in some studies (see Bustin, 2000; 2002, for review). GAPDH was ubiquitously expressed in southern flounder. However, as noted in other studies, its abundance varied greatly across tissues, particularly testis and ovary. We concluded that GAPDH is a poor internal control for this qRT-PCR assay and its use was discontinued. Expression of EF-1α mRNA in southern flounder was also ubiquitous, but demonstrated greater consistency across tissues and individual animals. Therefore, EF-1α appeared to be a reliable internal control for this assay and was employed in our subsequent work.

Using a validated qRT-PCR assay designed for detection of southern flounder $P450_{arom}$ mRNA, we found high levels of $P450_{arom}$ mRNA in the adult ovary and spleen. $P450_{arom}$ expression was detectable only at low levels in the brain, testis and gill and was negligible in other body tissues tested. Two $P450_{arom}$ isoforms, ovarian and brain $P450_{arom}$, have been identified in several fishes [e.g., the goldfish (Tchoudakova and Callard, 1998; Kishida and Callard, 2001), zebrafish (Trant et al., 2001), tilapia (Kwon et

al., 2001), sea bass (Dalla Valle et al., 2002; Blázquez and Piferrer, 2004), and goby (Kobayashi et al., 2004)]. Sequence identity of these $P450_{arom}$ isoforms in fishes is approximately 55%. To date, a brain-derived $P450_{arom}$ has not been identified in flounder. However, since brain $P450_{arom}$ activity is typically high in fishes (Tchoudakova et al., 2001) and ovarian $P450_{arom}$ was not detected in appreciable amounts in the adult (Figure 5) or juvenile (data not shown) southern flounder brain, it is likely that a brain-derived $P450_{arom}$ exists in flounder. Brain-derived $P450_{arom}$ appears to be involved in the process of sex differentiation in some fishes, including zebrafish (Trant et al., 2001). Thus, the existence of a brain-derived $P450_{arom}$ in flounder and its possible role in sex differentiation should be addressed in future studies.

The tissue distribution of southern flounder $P450_{arom}$ in adult southern flounder generally agrees with findings in other teleosts (e.g., Kitano et al., 1999; Kwon et al., 2001; Dalla Valle et al., 2002; Kobayashi et al., 2004). One particularly interesting discovery in southern flounder was the relatively high levels of $P450_{arom}$ mRNA present in the spleen. $P450_{arom}$ expression in the spleen has been previously reported in hirame (Kitano et al., 1999), Nile tilapia (Kwon et al., 2001), a goby (*Trimma okinawae*, Kobayashi et al., 2004) and in the human fetus (Price et al., 1992). These findings suggest that the spleen may produce estrogens via actions of $P450_{arom}$. However, the role of $P450_{arom}$ expression and any potential sexual dimorphism in spleen function remains unclear.

By developing and validating a $P450_{arom}$ biomarker, we defined the ontogenic pattern of $P450_{arom}$ expression in the gonads of wild and hatchery-produced southern flounder during sex differentiation. $P450_{arom}$ expression was initially undetectable or detected at low levels in post-metamorphic southern flounder. Thereafter, $P450_{arom}$ mRNA remained low in gonads of putative males and was markedly increased in gonads of putative females, beginning at roughly 65 mm TL. Analysis of $P450_{arom}$ mRNA abundance in one lobe of the southern flounder gonads coupled with histological examination of the other lobe in a subset of these fish indicated complete concordance between expression of $P450_{arom}$ and the presence of ovarian features. Specifically, high $P450_{arom}$ was always correlated with signs of ovarian development (e.g., ovarian cavity and oocytes) and low $P450_{arom}$ expression with testicular development (e.g., seminal lobules and spermatogenesis).

Relative to other flounder species studied, southern flounder exhibit a substantial delay in early sexual development (Luckenbach et al., 2003). The ovarian cavity and early-stage meiotic oocytes are not histologically discernible until fish reach ~75-100 mm TL. Shortly before this (65 mm TL), we found a sex-specific divergence in $P450_{arom}$ abundance. Suzuki and coworkers (2004) demonstrated in the medaka (*O. latipes*) that $P450_{arom}$ activity and subsequent endogenous E_2 biosynthesis are necessary for formation of the ovarian cavity. In Japanese flounder species (hirame and barfin flounder), the ovarian cavity is present in females by ~40 mm TL (Yamamoto et al. 1999; Goto et al. 1999). In hirame, at least, the timing of histological signs of ovarian differentiation is

consistent with increases in $P450_{arom}$ expression, which occur by ~32 mm TL (Kitano et al., 1999). The ontogeny of $P450_{arom}$ expression in juvenile southern flounder relative to the appearance of ovarian morphology is also consistent with patterns in various other vertebrates [e.g., *Pleurodeles waltl* (Chardard et al., 1995), the chicken (Yoshida et al., 1996), diamondback terrapin *Malaclemys terrapin* (Jeyasuria and Place, 1997), zebra finch (Freking et al., 2000), *Xenopus* tadpole (Miyashita et al., 2000), and red-eared slider turtle *Trachemys scripta* (Murdock and Wibbels, 2003)]. Further studies will be required to determine if high $P450_{arom}$ expression and the cascade of endocrine events that likely follow are responsible for ovarian cavity formation and moreover, development of the undifferentiated gonad into a functional ovary.

The ontogeny of $P450_{arom}$ expression in juvenile southern flounder provides a framework for assessment of the thermosensitive period of sex determination. The pattern of $P450_{arom}$ expression in juveniles indicates that the direction of sex differentiation can be clearly assessed using the $P450_{arom}$ biomarker in animals beginning at a body size of 65 mm TL. Since $P450_{arom}$ profiles in juveniles have separated into male and female groups by this body size, it appears that the path of sex differentiation is clearly established by this point. Therefore, the beginning of the period during which sex can be affected by rearing temperature in southern flounder is likely prior to 65 mm TL. The body size at which southern flounder are no longer sensitive to the masculinizing effects of temperature appears to be 90 mm or smaller. This is based on the fact that 8 of 15 wild flounder captured at high temperatures (i.e., collection 3, Figure 6), ranging in size from 90-115 mm TL, exhibited high $P450_{arom}$ expression and ovarian

morphologies despite experiencing high water temperatures that have previously generated male-skewed sex ratios in captive southern flounder populations when applied beginning at ~40 mm TL (Luckenbach et al., 2003). Future studies will better define sensitivity of southern flounder to masculinization during this developmental period.

This research establishes valuable tools for further study of the $P450_{arom}$ gene and its role in the processes of sex differentiation and sex determination of southern flounder. Since flounder exhibit dimorphic growth with females growing substantially larger than males, the overwhelming majority (estimated at 90%) of the commercial harvest for this species consists of females (Grist, 2004). Marine aquaculturists also target production of primarily female flounder due to better growth potential and associated higher return on investment (Luckenbach et al., 2002; 2004). Hence, gaining a better knowledge of the process of female sex differentiation in this species is of importance to fisheries managers/conservationists and the emerging field of flounder aquaculture in North and South America. In this study, we establish that variation exists at the $P450_{arom}$ locus in southern flounder, which may relate to heritable differences in sex determination responses. The qRT-PCR assay developed here allows for rapid and reliable assessment of the direction of sex differentiation in wild and hatchery-produced southern flounder, well before they reach the body size required for accurate histological assessment of sex. Through study of naturally occurring and cultured southern flounder populations, we demonstrate that $P450_{arom}$ is a useful biomarker for studies of sex determination and differentiation in the field and hatchery. This biomarker will be particularly useful in testing acute environmental effects on these processes. Furthermore, this unique

approach to studying sex differentiation in a teleost should be easily amenable to studies in other vertebrates.

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Table 1
Primers used for cloning southern flounder $P450_{arom}$ cDNA and introns and GAPDH and EF-1α cDNA

Primer name	Primer sequence	Direction	Use
Arom F1	GGNYTNGTNCARTTYGT	Sense	$P450_{arom}$ cDNA cloning
Arom R1	ACRCANACYTCNACNGTYTT	Antisense	$P450_{arom}$ cDNA cloning
Arom N1	AYMGGATHCCNGCNTGYGAY	Sense	$P450_{arom}$ cDNA cloning
Arom N2	TGGCTGWTGCTCKGCWGARG	Antisense	$P450_{arom}$ cDNA cloning
Arom F2	GGTTTGGGGCCACTTCTCTCATATGT	Sense	$P450_{arom}$ cDNA & introns II/III cloning
Arom R2	GGAGACGCAGACCTCCACTGTCTT	Antisense	$P450_{arom}$ cDNA & introns II/III cloning
Arom F3	CATCCTCAGCAGAGCATCAGCCATTTAC	Sense	$P450_{arom}$ cDNA cloning
Arom R3	GGGCGCCGCGATCACCATCTC	Antisense	$P450_{arom}$ cDNA cloning
Arom F4	TTGTGGAACAGAAGCGGAGAGAC	Sense	$P450_{arom}$ cDNA cloning
Arom R4	TTTGCCAGCTTCCTCTCTGTCTG	Antisense	$P450_{arom}$ cDNA cloning
3RACE F1	GCTCAAGCAGAATCCAGATG	Sense	$P450_{arom}$ 3' RACE
3RACE R1	GCGAGCACAGAATTAATACGACT	Antisense	$P450_{arom}$ 3' RACE (from kit)
3RACE N1	GCACAGAGTTTTTCCGCAAGC	Sense	$P450_{arom}$ 3' RACE
3RACE N2	CGCGGATCCGAATTAATACGACTCACTATAGG	Antisense	$P450_{arom}$ 3' RACE (from kit)
Arom F5	GCTGCACCGTGGTCGACATC	Sense	$P450_{arom}$ introns IV and V cloning
Arom R5	ACGTCTCTCCGCTTCTGTTC	Antisense	$P450_{arom}$ introns IV and V cloning
Arom F6	TTGTGGAACAGAAGCGGAGAGAC	Sense	$P450_{arom}$ intron VI cloning
Arom R6	GGGCGCCGCGATCACCATCTC	Antisense	$P450_{arom}$ intron VI cloning
GAP F1	TACATGGTYTACATGTTC	Sense	GAPDH cDNA cloning
GAP R1	AAGTTGTCRTKGATGACC	Antisense	GAPDH cDNA cloning
EF F1	AGTTCGAGAAAGAAGCTGCC	Sense	EF-1α cDNA cloning
EF R1	ATCCAGAGCATCCAGCAGTG	Antisense	EF-1α cDNA cloning

For degenerate primers above:
N=A+C+T+G, R=A+G, Y=C+T, M=A+C, K=T+G, W=A+T, H=A+T+C

Table 2
Date, capture location, bottom temperature and salinity, and water depth for collections of wild southern flounder in the Pamlico Sound, North Carolina

Collection	Date	Location	Temp (°C)	Salinity (ppt)	Capture Depth (m)
1	May 13, 2003	Pungo River	23.2-24.1	5.1-6.2	1.1-1.2
2	June 16, 2003	Long Shoal River	24.6-26.7	6.2-8.3	1.2-1.8
		Far Creek	24.7-25.3	2.5-7.2	0.8-1.1
		Middleton Creek	26.2	5.4	0.9
3	June 30, 2003	Rose Bay	29.1	5.6	2.9
		Swanquarter Bay	29.3	6.7	2.7

Table 3
Gene-specific forward and reverse primers and FRET probes (5′ to 3′ orientation) used for qRT-PCR

Gene	Forward Primer	Reverse Primer	FRET Probe
$P450_{arom}$	CAGCAGAGCATCAGCCATTTAC	TCTCTCATACATGCCGATGCA	TCCCGAAACGTGACGTGTAATGTCCAT
EF-1α	CGAGAAAGAAGCTGCCGAGAT	CGCTCGGCCTTCAGTTTGT	CAAGGGCTCCTTCAAGTACGCCTGG
GAPDH	GTCTTCACCACCATTGAGAAAGC	GGGTGCAGAGATGATGACTTTCTT	GCGCCACCCTTCAAGTGAGCAGA

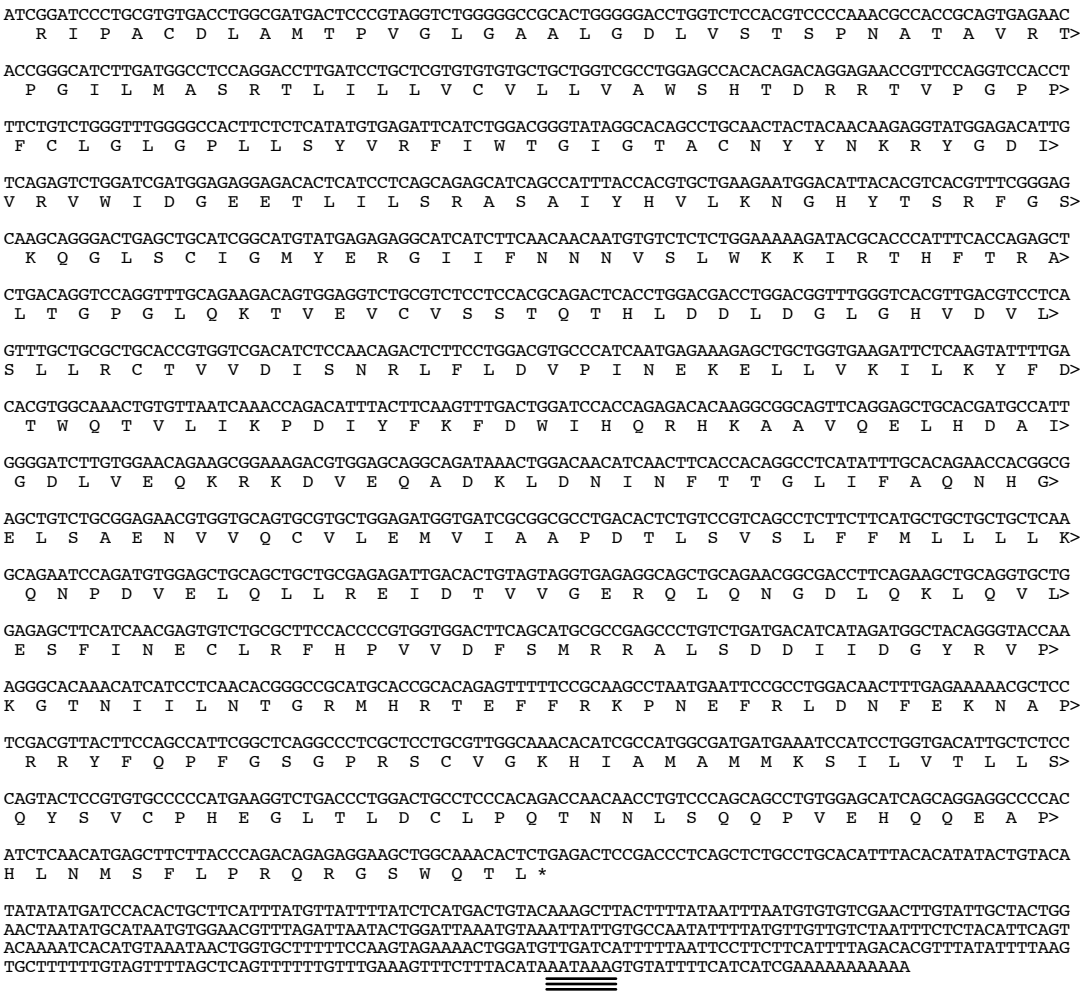

Figure 1. Southern flounder P450$_{arom}$ gene (*cyp19*) partial cDNA sequence (top) and deduced amino acid sequence for the coding region (below). This partial sequence of 1985 bases codes for 516 amino acid residues and potentially encodes a 508-residue protein. The stop codon is denoted by an asterisk (*) and the polyadenylation signal is underlined three times.

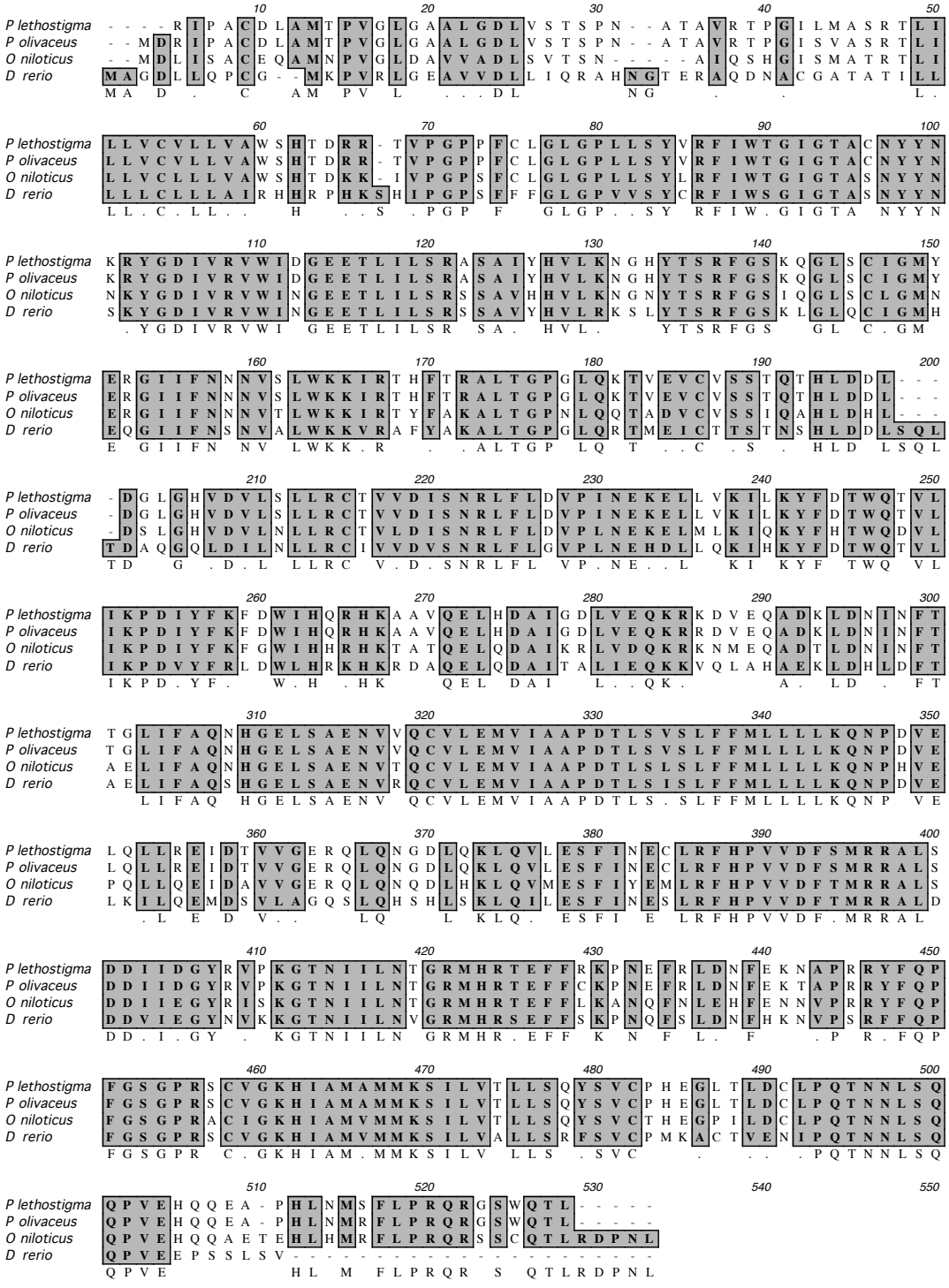

Figure 2. Deduced amino acid sequence for $P450_{arom}$ in southern flounder (*P. lethostigma*) aligned with $P450_{arom}$ of other fishes. The deduced amino acid sequence for southern flounder $P450_{arom}$ has 99% identity to hirame (*P. olivaceus*), 76% to tilapia (*O. niloticus*), 63% to zebrafish (*D. rerio*) and goldfish, 61% to catfish, and 49% to human.

Table 4
Total size (nucleotides) of $P450_{arom}$ gene introns II-VI in the flounder, sea bass and medaka

Intron	Flounder	Seabass	Medaka
II	90	96	75
III	82-88	89	75
IV	146	121	90
V	83	116	79
VI	75	99	110

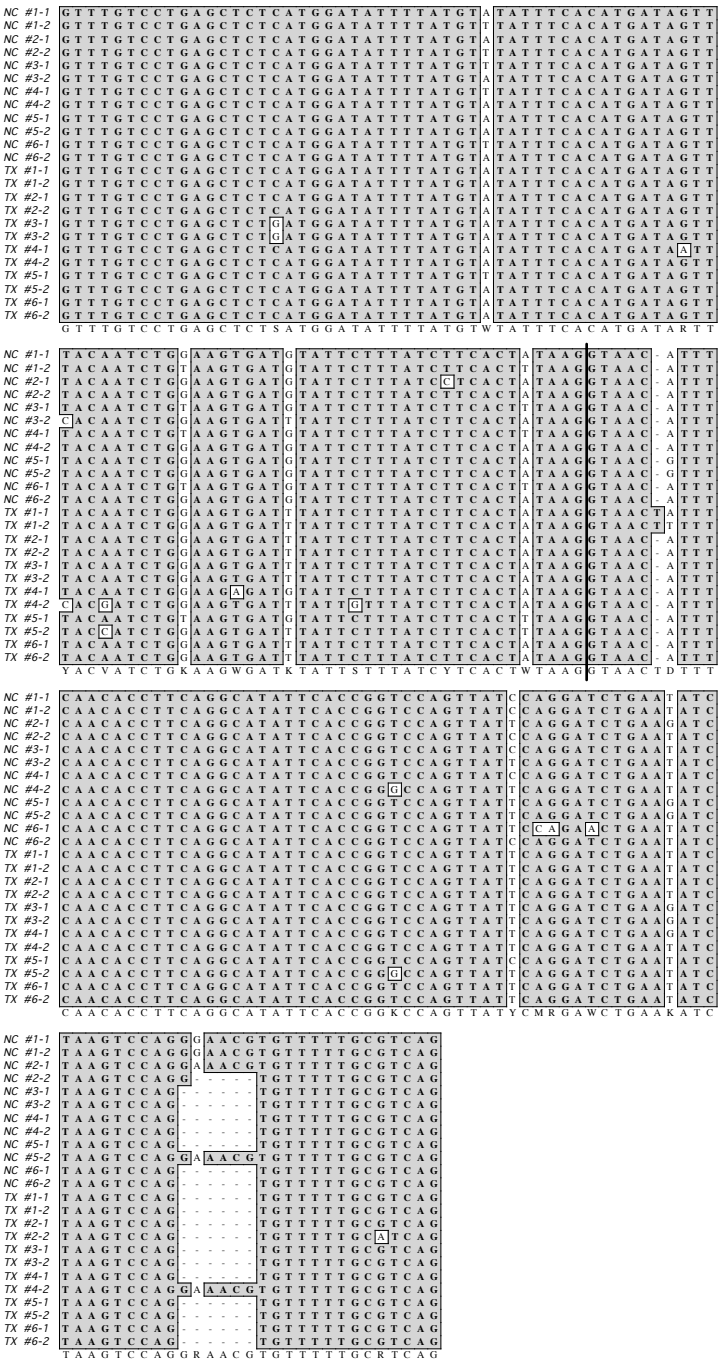

Figure 3. Consensus nucleotide sequence comparison for putative southern flounder $P450_{arom}$ introns II and III of fish from the mid-Atlantic and Gulf of Mexico stocks. Labels denote the flounder stock (NC or TX), fish number (1-6), and consensus sequence number (1 or 2) for each fish. Nucleotide polymorphisms occur at multiple positions in these intron regions. Introns II and III are separated by a solid vertical line.

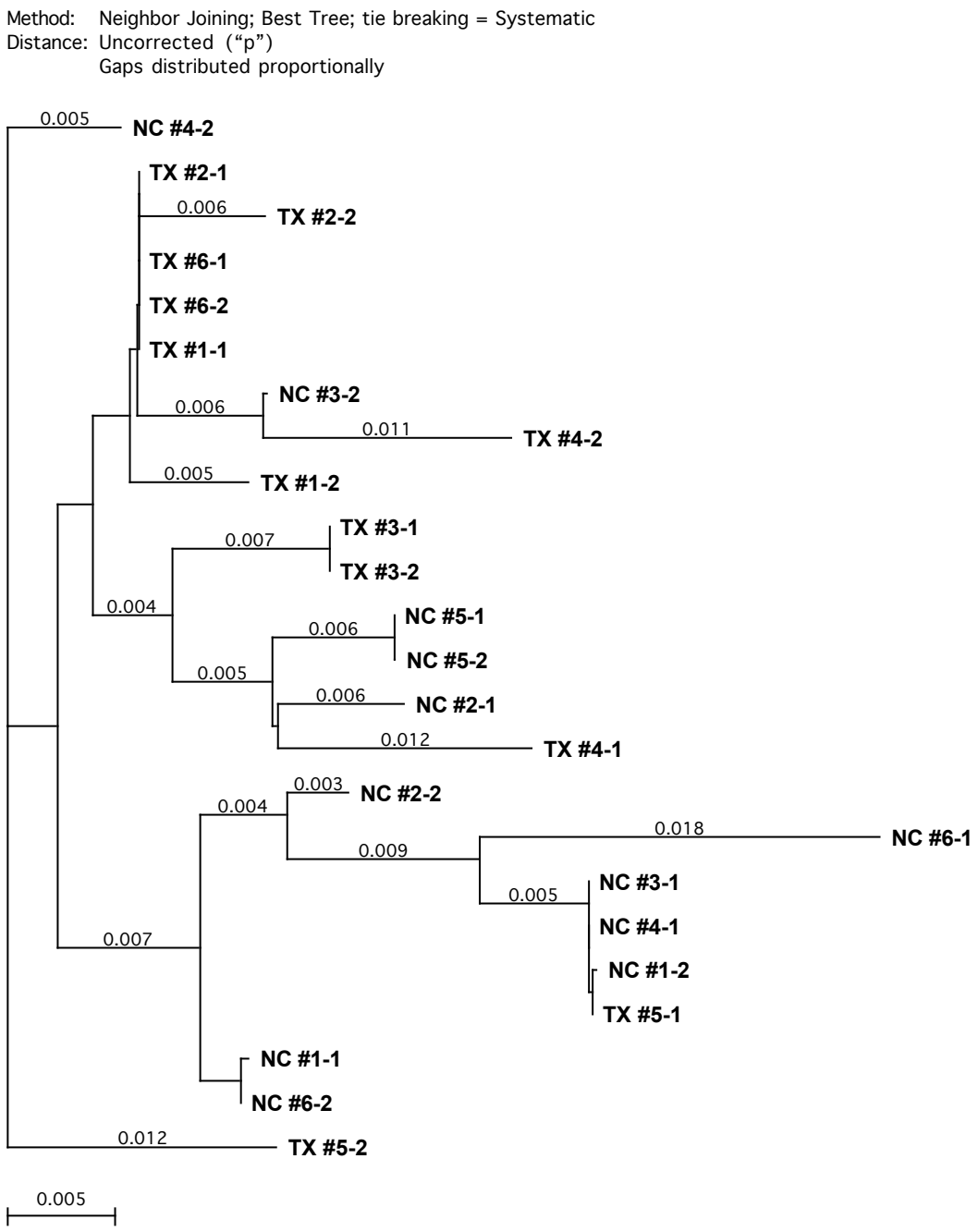

Figure 4. Phylogenetic reconstruction of putative $P450_{arom}$ consensus sequences for introns II and III in southern flounder from the mid-Atlantic and Gulf of Mexico stocks. Labels denote the flounder stock (NC or TX), fish number (1-6), and consensus sequence number (1 or 2) for each fish. Branch lengths are proportional to the degree of sequence divergence and numbers along branches indicate the calculated relative differences between the nucleotide sequences.

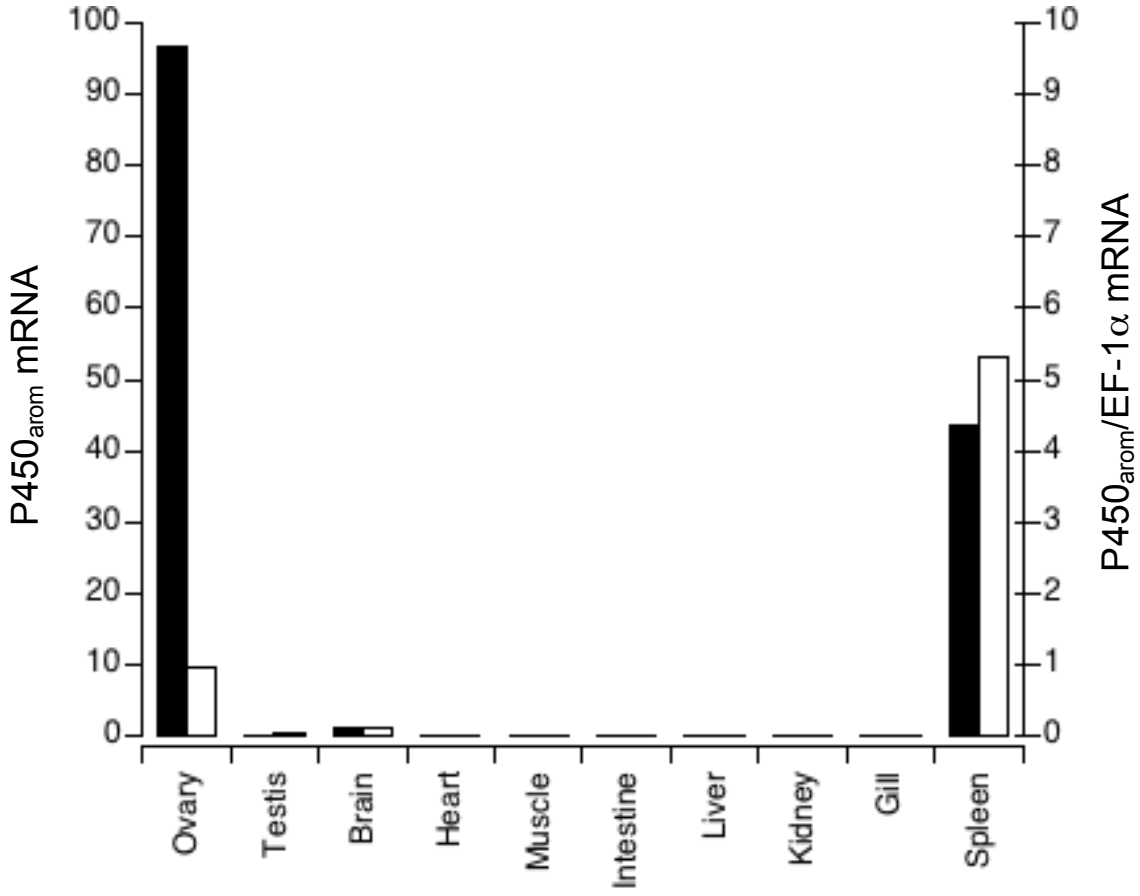

Figure 5. $P450_{arom}$ mRNA and $P450_{arom}$ normalized to EF-1α in various adult body tissues of southern flounder. Each bar represents the mean $P450_{arom}$ mRNA abundance for an RNA pool of three animals (filled bars) or this measure corrected for EF-1α abundance in the same sample (unfilled bars). Samples were measured in duplicate.

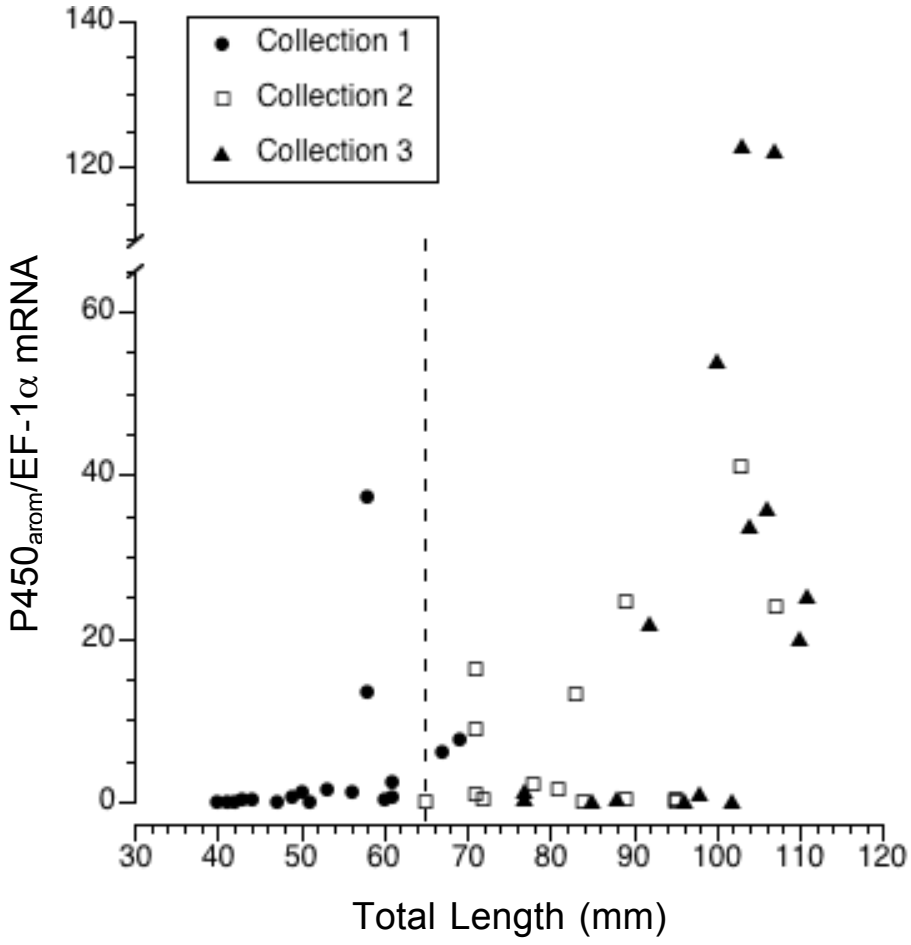

Figure 6. $P450_{arom}$ mRNA abundance in gonads of wild juvenile southern flounder collected from May to June 2003 (see Table 2 for collection data). Points represent the mean mRNA measurement in duplicate samples for each animal. The range of bottom temperatures at the capture sites for flounder collections 1-3 were 23.2-24.1°C, 24.6-26.7°C, and 29.1-29.3°C, respectively. The dashed line signifies the body size (65 mm) at which $P450_{arom}$ values begin to bifurcate into putative male and female groups.

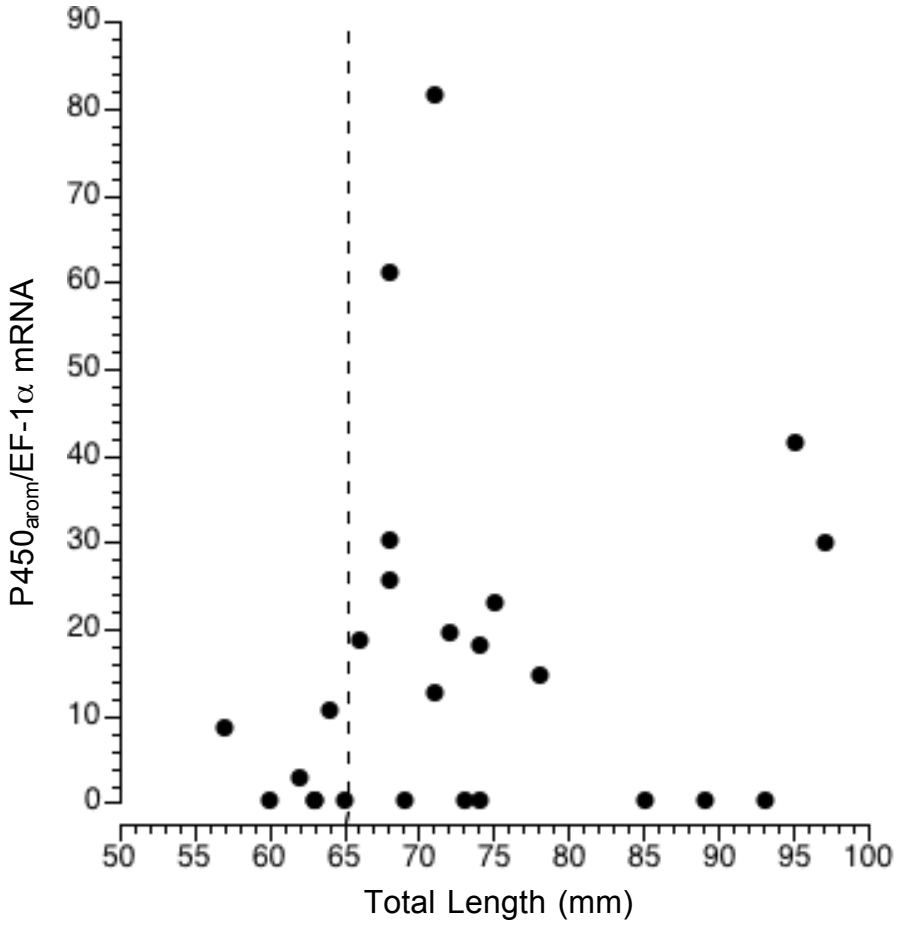

Figure 7. $P450_{arom}$ mRNA abundance in gonads of randomly selected juvenile southern flounder produced in the hatchery. Points represent the mean mRNA measurement in duplicate samples for each animal. Measures of $P450_{arom}$ in these fish show a similar $P450_{arom}$ profile to wild caught juvenile flounder and serve to test the utility of this assay for accurately predicting sex ratios of flounder populations. The dashed line signifies the body size (65 mm) at which $P450_{arom}$ levels appear to have clearly segregated into female- and male-typical expression.

CHAPTER IV

Induction of Diploid Gynogenesis in Southern Flounder (*Paralichthys lethostigma*) with Homologous and Heterologous Sperm[1]

[1] Published in Aquaculture 237: 499-516 (2004) by J. Adam Luckenbach, John Godwin, Harry V. Daniels, Jessica M. Beasley, Craig V. Sullivan, and Russell J. Borski

ABSTRACT

Effective methods for induction of diploid gynogenesis in North American flounder of the genus *Paralichthys* are needed to initiate monosex culture, which will allow growers to take advantage of the more rapid growth and larger size attained by females. To test methods for inducing diploid gynogenesis in southern flounder (*Paralichthys lethostigma*) using homologous sperm, four treatments, named for their expected outcome, were employed: haploid, diploid, triploid, and gynogenetic diploid. Diploid gynogenesis was induced by activating egg development with UV-irradiated flounder sperm (70 J/cm^2) for 3 to 4 min in seawater, and then subjecting the eggs to cold shock in 0-2ºC seawater for 45-50 min. Cold shock was used to prevent extrusion of the second polar body. Control treatments omitted one or more of these steps to separately assess the effectiveness of UV irradiation and cold shock. Larvae were observed for physical abnormalities and then histologically processed for ploidy determination. Haploid larvae exhibited abnormal external morphology while diploid, gynogenetic diploid, and triploid larvae showed normal morphologies. Cross-sectional areas of erythrocyte nuclei were measured for larvae in each treatment group and significant differences were found. Nuclear areas for treatment groups corresponded to predicted ploidy (triploid > diploid > haploid) and did not differ between normal diploid controls and gynogenetic diploids. These results suggest that the procedures of sperm irradiation and egg cold shock successfully generated gynogenetic diploids. Due to the low volumes of semen produced by male flounder, and to eliminate any potential genetic contribution by homologous

sperm, activation of flounder eggs with heterologous sperm was also investigated. Induction of diploid gynogenesis was successful when flounder eggs were fertilized with irradiated (50 J/cm^2) sperm from striped mullet (*Mugil cephalus*), and then cold shocked. This work provides procedures for induction of diploid gynogenesis in southern flounder using homologous and heterologous sperm, and validates a method for verification of ploidy in larval fish.

INTRODUCTION

North American flounders of the genus *Paralichthys* have recently been subject to intense investigation as potential candidates for commercial aquaculture production. Southern flounder, *Paralichthys lethostigma,* are believed to be particularly well suited for aquaculture because of their ability to grow well across a wide range of salinities (Daniels and Borski, 1998; Smith et al., 1999a) and their established presence in the marketplace (Smith et al., 1999b). Like other flatfishes, *Paralichthys* species exhibit sexually dimorphic growth, with females growing faster and reaching larger adult sizes than males (Morse, 1981; Yamamoto, 1995; Fitzhugh et al., 1996). Thus, development of techniques for preferentially producing females is necessary to optimize production of these species (King et al., 2001; Luckenbach et al., 2002; 2003). Monosex culture of fish is often used for prevention of unwanted reproduction and precocious sexual maturation (Yamazaki, 1983; Donaldson, 1996). Due to the faster growth of female flounder, monosex culture would be especially advantageous to producers, potentially decreasing size differences between fish during growout, reducing mortality due to cannibalism, minimizing labor required for size grading, and ultimately providing higher return on investment.

As in many fishes, *Paralichthys* species possess an 'XX/XY' sex chromosome system; females are homogametic (XX) and males are heterogametic (XY) (Yamamoto, 1995; 1999 for *P. olivaceus*). Early in development, homogametic individuals can be sex-reversed by exposure to high water temperature (Yamamoto, 1995; Godwin et al., 2003;

Luckenbach et al., 2003) or exogenous androgens, resulting in production of phenotypic males (Tabata, 1991; Yamamoto, 1995). Sex-reversed, homogametic (XX) males are valuable as broodstock because they can be mated with normal females to produce all-female populations (Hunter and Donaldson, 1983; Yamazaki, 1983; Yamamoto, 1995), employed as founders of isogenic and inbred lines for genome mapping or genetic linkage and variance studies, or otherwise exploited in investigations of fish genetics (Thorgaard, 1986; Ihssen et al., 1990; Thorgaard et al., 1992; Arai, 2001; Lutz, 2001). Induction of diploid gynogenesis is a method used with female homogametic species to generate populations of fish solely possessing the female genotype (see reviews by Ihssen et al., 1990; Pandian and Koteeswaran, 1998; Felip et al., 2001). Diploid gynogenesis has been achieved for certain flatfishes including in an early study of plaice, *Pleuronectes platessa,* and European flounder, *Platichthys flesus* (Purdom, 1969), and more recently for the hirame, *Paralichthys olivaceus* (Tabata et al., 1986; Tabata and Gorie, 1988), and common sole, *Solea solea* (Howell et al., 1995). Triploidy has recently been investigated in the turbot, *Scophthalmus maximus* (Piferrer et al., 2000; 2003).

Induction of diploid gynogenesis involves two steps: (1) gynogenesis is typically induced through activation of eggs by genetically inactivated spermatozoa. (2) extrusion of the second polar body is blocked (meiotic gynogenesis or meiogynogenesis) or the first embryonic cleavage is prevented (mitotic gynogenesis or mitogynogenesis) by application of an external shock (thermal, pressure, or chemical). Irradiation of spermatozoa with ultraviolet (UV) light has been successfully used to crosslink paternal DNA and produce

genetically inactivated sperm for induction of gynogenesis in a number of fishes (Chourrout, 1984; Ihssen et al., 1990; Felip et al., 2001). These genetically inactivated sperm serve to trigger completion of meiosis by fertilized eggs and the ensuing early stages of embryonic cleavage, but are unable to contribute genetically to offspring. In some studies, untreated or UV-irradiated heterologous sperm (from a different species) has been used to initiate gynogenesis in eggs, although reports on its use in flatfishes are limited (Arai, 2001; Felip et al., 2001). Makino and Ozima (1943) first showed in fish that a thermal shock can cause retention of the second polar body, a full haploid set of chromosomes that is normally extruded from eggs following completion of the second meiotic division shortly after fertilization. When sperm are genetically inactivated and extrusion of the second polar body is blocked, gynogenetic diploids (gynogens) inherit both chromosome sets from the female parent and none from the male parent. Hence, this procedure generates all-homogametic offspring in species that exhibit female homogamety.

The primary objective of the present study was to establish methods for inducing diploid gynogenesis in the southern flounder, a strong candidate for commercial aquaculture in the United States. In addition to gynogens, three control groups (haploid, normal diploid, and triploid) were included in our experiments to test the effectiveness of various procedures of chromosomal manipulation used to produce gynogens with flounder sperm. Larval measures of erythrocyte nuclear area by histological analyses provided a means to assess success of ploidy manipulations during early development, precluding the need for raising animals to adequate body sizes for blood extraction and

traditional measures of erythrocytes (i.e., particle size analysis or flow cytometry). We also sought to design protocols for inducing diploid gynogenesis in southern flounder using heterologous sperm from striped mullet, *Mugil cephalus*. Use of mullet sperm would assure any progeny resulting from future applications of this technique are gynogens.

MATERIALS AND METHODS

Broodstock source and conditioning

Female southern flounder were captured in the Pamlico Sound, North Carolina and transported to the hatchery. Male flounder were either wild-caught as above or hatchery produced at the Tidewater Research Station in Plymouth, North Carolina and held indoors for 3 years. Fish were conditioned for spawning for at least 3 months using established protocols for manipulating photoperiod, salinity, and temperature (Smith et al., 1999b; Watanabe et al., 2001). Broodstock were fed with frozen lake smelt (*Osmerus mordax*), squid (*Illex* spp.), and a high-protein pelleted feed (7 mm floating, Melick Aquafeed Inc., Catawissa, Pennsylvania, USA). Final maturation and ovulation of females were induced by implanting females with pellets containing a synthetic analogue of mammalian gonadotropin-releasing hormone (GnRHa), as described previously (Berlinsky et al., 1996). Striped mullet were captured in coastal ponds at the Pamlico

Aquaculture Field Laboratory in Aurora, North Carolina and were held in outdoor tanks under ambient conditions until spawning trials were initiated, at which time they were moved indoors, held under photothermal conditions similar to flounder, and injected with silastic implants containing 17α-methyltestosterone (Steraloids Inc., Newport, Rhode Island, USA) to stimulate spermiation (Lee et al., 1986; 1992).

Analysis of sperm motility: effects of prediluent and UV irradiation

Male flounder and mullet were anaesthetized with clove oil (eugenol, Sigma Chemical Corp., St. Louis, Missouri, USA). Semen was stripped by applying gentle pressure to the abdomen and collected using glass Pasteur pipets. Contact of semen with water or urine was avoided. Semen from 2-3 individuals was pooled and stored on ice until use. The concentration of flounder and mullet sperm was determined by diluting samples (1:1000) in a physiological Ringer's solution (0.75% NaCl, 0.04% $CaCl_2$, 0.02% KCl in distilled water) that does not activate sperm motility, mixing aliquots of the dilute semen with eosin stain, and then counting quadruplicate aliquots of spermatozoa using a hemacytometer (Hausser Scientific, Horsham, Pennsylvania, USA). To evaluate the effects of semen dilution and UV irradiation on sperm motility, a two-step technique was employed where semen samples were diluted in Ringer's solution prior to further dilution with artificial seawater (33 ppt; Crystal Sea Marinemix, Marine Enterprises International, Baltimore, Maryland, USA) for activation of sperm motility (Billard and Cosson, 1992). Semen was diluted to a concentration of 1:50 in Ringer's solution at 3ºC and then a 2 µl aliquot was placed on a microscope slide coated with 0.25% BSA solution to prevent

sperm from adhering to the glass (Detweiler and Thomas, 1998). Slides were then irradiated in a microprocessor-controlled UV crosslinker (FisherBiotech FB-UVXL-1000, Pittsburgh, Pennsylvania, USA) at a distance of 10 cm from the UV source (5, 8-watt 254 nm UV tubes, irradiance display resolution: ± 5 $\mu W/cm^2$). The range of UV exposures tested was 0-130 J/cm^2 (0-13,000 $ergs/mm^2$). A built-in UV integrator assured delivery of the desired energy level. Sperm was activated with 40 µl (1:20 dilution) of 17 ± 1°C artificial seawater, mixed, coverslipped, magnified 400 times using a compound light microscope (Olympus BH-2), and motility recorded using a CCD video camera (Sony XC-77) connected to a cassette recorder (Panasonic Omnivision VHS model PV-7450) and monitor (Panasonic CT-1383). To evaluate the degree and duration of sperm motility, each session was recorded until sperm motility ceased, beginning immediately after addition of seawater. Semen stored in Ringer's solution was checked for changes in motility to evaluate potential degradation of sperm during storage. Since changes in temperature have been shown to affect the duration of sperm motility in fish (Billard and Cosson, 1992), room temperature was monitored and maintained at 18-19°C throughout the tests.

Optimization of the UV dosage for induction of gynogenesis

The goal of these experiments was to determine the UV dosage that genetically inactivates flounder sperm, but retains the ability to trigger egg development. Ovulated eggs were collected from 3 females, pooled, and 4-ml aliquots were placed into eight, 250-ml glass beakers. Semen was collected from 3-5 flounder and pooled. Separate experiments tested

semen volumes of 0.3 and 0.9 ml for their ability to activate development in a fixed egg volume. In each trial semen was diluted with Ringer's as described above, equally divided into 8 chilled glass Petri dishes (10 cm diameter), and exposed to UV dosages ranging from 0-190 J/cm^2. Each batch of semen was poured into the beakers described above and approximately 100 ml of artificial seawater at 16 ± 1ºC was added to the eggs and semen. After 5 min of gentle swirling the contents of each beaker were individually poured into a separatory funnel to collect the viable floating eggs, which were then divided among three, 1-L glass jars containing 700 ml of artificial seawater. Gentle aeration was provided and containers were maintained at a temperature of 18 ± 1ºC. Percent fertility was determined 3 to 4 hours later by examining 100-200 eggs for cell cleavage. Three days after fertilization (egg activation) the percentage of hatched larvae and larvae exhibiting haploid syndrome (i.e., morphological deformities) was estimated from 50-ml samples. Survival of larvae was estimated 36 hours later. Triplicate estimates of percent fertility, hatch, haploid syndrome, and survival were averaged for each replicate.

Chromosomal manipulation using homologous sperm

The experiment involved production of four different types of larvae and the experimental treatments were named for these types, as follows: gynogenetic diploid, haploid, normal diploid, and triploid (Figure 1). Control procedures in which one or more experimental steps were omitted served to test the efficacy of individual elements of the protocol for inducing diploid gynogenesis. These elements were effectiveness of UV dose

(haploid control), gamete quality (normal diploid control), and cold-shock procedures (triploid control).

Ovulated eggs were stripped from multiple females, pooled (total egg volume per experiment was 100-120 ml or ~100,000 eggs), and divided among four, 800-ml glass beakers. Semen (~1 ml) was diluted to a concentration of 1:50 in Ringer's solution and half the sample was spread thinly on the bottom of chilled glass Petri dishes (10 cm diameter) and UV irradiated as described above at an intensity of 70 J/cm^2. Untreated semen was handled similarly, but not exposed to UV light. Irradiated and untreated semen were each divided equally into two beakers containing eggs. Approximately 100 ml of seawater (16 ± 1ºC) was simultaneously added to each of 4 beakers to activate the sperm. After 3 to 4 min of gentle swirling, eggs were added to either 0-2ºC (cold shock) or 16ºC seawater (control) and held for 45-50 min, as follows. Temperature was maintained with a recirculating water bath (Model T-2, Brinkman Instruments, Westbury, New York, USA) and monitored using a digital microcomputer thermometer (Model 9060, Hanna Instruments, Woonsocket, Rhode Island, USA). One beaker containing eggs and irradiated sperm, and one containing eggs and untreated sperm, were cold shocked by gently pouring the eggs into a 500 µm mesh bag for transfer to 0-2ºC seawater. These treatments were intended to produce gynogenetic diploids and triploid controls, respectively. The remaining beakers containing eggs and irradiated sperm, and eggs and untreated sperm, were not cold shocked, but instead were transferred to seawater maintained at 16ºC. These treatments were intended to generate haploid and normal

diploid controls, respectively. After these treatments, cold-shocked eggs were slowly acclimated to 16 ± 1ºC and all groups were incubated separately in the same flow-through seawater system (70-L center-drain, fiberglass tanks) with gentle aeration. Percent fertility was estimated 4 hours after egg activation by checking for embryonic cell cleavage of 50-100 eggs per group (Watanabe et al., 2001). The percentage of hatched embryos was estimated ~3 days after fertilization. Values are expressed as a percentage of that measured in normal diploid controls.

Measurement of erythrocyte nuclear area

To assess the effectiveness of the ploidy manipulations, erythrocyte nuclear areas were measured in 7-12 larvae from each of the putative haploid, diploid, triploid, and gynogenetic diploid groups. Hatched larvae were sampled from each group, assessed for morphological abnormalities, photographed, and fixed in neutral buffered formalin (0.4% NaH_2PO_4, 0.65% Na_2HPO_4 in 10% formalin). Larvae were embedded in a sagittal orientation in paraffin and serially sectioned at 5-6 μm. Sections were stained with hematoxylin and eosin. Sharply defined, darkly staining, 'larval-type' erythrocyte nuclei (Miwa and Inui, 1991) were located in vascular tissue, magnified 1000 times under oil immersion, traced, and their areas calculated using Brain 3.0 software (Drexel University Computer Imaging and Vision Center, Philadelphia, Pennsylvania, USA). The round shape of larval flounder erythrocyte nuclei eliminates the need to define and measure the major and minor axes of elliptical-shaped erythrocytes present in juvenile and adult fish (Miwa and Inui, 1991). Areas from 5-7 nuclei were averaged for each individual larva to

account for potential variation in the cross-sectional plane at which erythrocytes were cut.

Induction of diploid gynogenesis using heterologous sperm

Untreated heterologous sperm has been successfully used for induction of diploid gynogenesis in some fishes (e.g., Peruzzi et al., 1993; Howell et al., 1995). Therefore, in our first trial, untreated mullet sperm was used for activation of flounder eggs. This was followed by a 0-2ºC cold shock of eggs as described above. In a second set of trials, 0.50-0.75 ml of mullet semen was diluted 1:50 as before, spread thinly onto chilled glass Petri dishes, and irradiated at 50 J/cm^2. Pooled eggs from two females (volumes ranged from 98-230 ml in N=7 experiments) were collected in an 800-ml beaker, fertilized for 2.5-4.0 min, strained of some water using a separatory funnel, and then split between two, 1-L Erlenmeyer flasks containing ~800 ml of -1.2 to -0.5ºC seawater (allowed for a 0-2ºC shock in each flask). The duration of cold shock was 45-50 min. During cold shock, the water was gently aerated to keep the eggs suspended in the water column. To confirm the quality of eggs used for the heterologous sperm trials, some eggs from each lot were fertilized with flounder semen. Following cold shock, eggs were incubated in the recirculating seawater system described above, but at 21 ± 1°C. Fertility estimates were made 5-6 hours after fertilization by examining 100-200 eggs for cell cleavage. Nonviable eggs or those in which cell cleavage was not clearly discernible were considered unfertilized. After hatching (~2 days after fertilization) larvae were assessed for morphological abnormalities and percent hatch. Larvae were reared according to

published methods with rotifers provided as a food source beginning 4 days after hatching (Daniels and Watanabe, 2002). Survival of larvae beyond the critical period of first feeding was assessed 11 days after hatching.

Statistical Analyses

Sperm motility (percent and duration) and average larval erythrocyte nuclear area were statistically analyzed using one-way analysis of variance and a Tukey-Kramer HSD *post-hoc* test. The correlation between predicted ploidy state and average erythrocyte nuclear area was determined using linear regression analysis. All statistical analyses were performed using JMP 5.0 software (SAS Institute Inc., Cary, North Carolina, USA). Data are expressed as mean ± S.E.M unless otherwise indicated.

RESULTS

Analysis of sperm motility: effects of prediluent and UV irradiation

Southern flounder semen contained $8.4 \times 10^9 \pm 6.2 \times 10^7$ spermatozoa/ml while mullet semen was more concentrated, containing $15 \times 10^9 \pm 2.6 \times 10^8$ spermatozoa/ml. Compared with undiluted semen, the prediluent used in the study reduced the motility of southern flounder and mullet sperm by 31.5 ± 5.6% and 25.0 ± 2.0% (N=4), respectively, following addition of seawater. Duration of storage in the prediluent had only a minor

effect on sperm motility. Motility was reduced by 5.3 ± 1.9% in flounder (N=4) and 4.5 ± 3.5% in mullet (N=2, mean ± S.D.) following 60 min of storage at 4ºC in the prediluent, relative to that seen with freshly diluted semen. Undiluted flounder and mullet semen can be stored on ice for as long as 5 days with little decline in motility. UV irradiation caused a dramatic, dose-dependent reduction in the motility of both southern flounder and striped mullet sperm (Figure 2). Duration of sperm motility also declines in a dose-related manner in response to UV exposure in both species (Table 1). Sperm motility was reduced by ~90% from control levels at UV dosages of 70 J/cm^2 and 50 J/cm^2 in flounder and mullet, respectively.

Optimization of the UV dosage for induction of gynogenesis

Non-irradiated semen (0 J/cm^2) induced the highest fertility and survival rates in both experiments (Figure 3). Fertility and survival rates generally decreased with increasing UV exposure of semen (ranging from 10-190 J/cm^2), whereas the percentage of larvae exhibiting haploid syndrome increased. Hatch rates declined rapidly compared to non-irradiated controls when semen was exposed to a UV dose of 10 J/cm^2. However, hatch rates showed some recovery at higher UV dosages denoting our elucidation of the 'Hertwig effect'. The lowest UV dosage that produced optimum results was 70 J/cm^2 regardless of the semen to egg ratio. Specifically, at this dose all hatched embryos exhibited haploid syndrome and did not survive post-hatch, however, egg activation and hatch rates were maximized.

Chromosomal manipulation using homologous sperm

The general body morphology of larvae differed among certain ploidy groups. All larvae from the haploid control group showed a combination of abnormal external morphologies, including kyphosis (bowing of the body) and tail and head deformities (Figure 4). The body conformation and appearance of gynogenetic diploid and triploid larvae were typically indiscernible from normal diploid controls, although a low proportion (~15%) of the hatched larvae in these groups exhibited kyphosis. Mean fertility values (N=4 trials) relative to those of normal diploid controls were 68.8 ± 12.0%, 33.8 ± 9.9%, and 21.3 ± 10.1% for haploids, triploids, and gynogens, respectively. Mean percentage of hatched embryos relative to diploid controls were 60.5 ± 12.1, 19.3 ± 6.5, and 2.1 ± 1.3 for haploids, triploids, and gynogens, respectively. Haploid larvae did not survive beyond one day post-hatch. Other groups were maintained to first feeding, but did not perform well during this period due to low larval densities.

Larval erythrocyte nuclear areas showed a strong linear relationship with predicted ploidy state, increasing with the expected chromosomal constitution (R^2=0.72; $P < 0.0001$). Erythrocyte nuclear areas of putative triploid larvae were significantly larger than putative haploid, normal diploid, and gynogenetic diploid larvae (Figure 5), while those of putative haploid larvae were significantly smaller than all other groups. Specifically, each incremental increase or decrease in ploidy level of 1N approximately

equals a nuclear area of 3.12 μm^2. There were no differences in erythrocyte nuclear areas between normal and gynogenetic diploids.

Induction of diploid gynogenesis using heterologous sperm

In an initial experiment, untreated mullet sperm was found to activate the development of flounder eggs. Although some embryos reached the 164 cell-stage, all died prior to hatching. In preliminary studies testing a range of UV exposures for mullet semen we found that 50 J/cm^2 produced the highest hatch rates (data not shown). Therefore, mullet semen was treated with this UV dosage in subsequent gynogenesis trials. Fertility and hatch rates ranged from 16.7-38.4% and 1.6-11.4%, respectively (Table 2). Survival rates beyond the period of first feeding ranged from 0 to 30%. A gamete-quality control procedure involving use of untreated flounder sperm for normal fertilization yielded fertility rates of 30-56%. Larvae hatched 2 days after fertilization, similar to larvae produced with flounder sperm. Over 90% of hatched larvae showed normal morphology and, following metamorphosis, juveniles exhibited the typical body morphology of southern flounder (van Maaren and Daniels, 2000).

DISCUSSION

In this study, methods for inducing diploid gynogenesis in southern flounder using homologous and heterologous sperm were developed and validated. Evaluation of the erythrocyte nuclear areas of larvae from the various treatment groups (haploid, diploid, and triploid) provides strong evidence that chromosomal manipulations performed using flounder sperm were successful in inducing diploid gynogenesis. Attempts to activate flounder eggs and produce gynogens using UV-irradiated striped mullet sperm were also successful.

The effectiveness of treatments used to induce diploid gynogenesis in trials using homologous sperm was supported by examinations of external morphology, as well as measurements of erythrocyte nuclear area. Larvae produced by gynogenesis using irradiated sperm, and predicted to be haploid, exhibited the suite of gross deformities referred to as 'haploid syndrome' (Purdom, 1969; Cherfas, 1981; Arai, 2001), whereas predicted diploid and triploid larvae exhibited normal body morphology. In the hirame, *Paralichthys olivaceus*, a congener of southern flounder, irradiation of sperm for induction of gynogenesis also produced abnormal, putative haploid larvae that did not survive after hatching (Tabata et al., 1986; Tabata and Gorie, 1988). However, external morphology did not aid in distinguishing larvae of higher levels of ploidy (i.e., diploids versus triploids) as has been demonstrated here.

Erythrocyte nuclear or cellular dimensions have previously been correlated with ploidy in fishes (e.g., Swarup, 1959; Purdom, 1972; Wolters et al., 1982; Beck and Biggers, 1983; Lincoln and Scott, 1983; Ueno, 1984). Theoretically, triploid animals should have 50% more genetic material than diploids, and haploids should have 50% less than diploids (Fankhauser, 1945). Particle size analysis and flow cytometry are typically used for measurements of erythrocyte dimensions or direct measurement of DNA (Thorgaard et al., 1981; Benfey et al. 1984; Wattendorf, 1986; Goudie et al., 1995). While these techniques have been adapted for use with larvae (e.g., Ewing et al., 1991), the histological approach demonstrated here may be accessible to more investigators since less specialized equipment is required. Flounder larvae were processed and examined using routine histological procedures, involving embedment of the fish in paraffin for sectioning, in order to expose erythrocytes for nuclear measurement. A similar approach with cartilage cell nuclei was successful at ploidy determinations in late-stage brown trout embryos (Purdom 1969). Through our measurements of erythrocyte nuclei, we verified the success of chromosomal manipulations used to generate southern flounder gynogens. Nuclear areas for the triploid control group were significantly larger than those for the other groups, indicating that the cold-shock procedure was successful at preventing extrusion of the second polar body. In addition, erythrocyte nuclear areas of putative haploid larvae were significantly smaller than those of larvae from the diploid and triploid treatment groups, indicating UV irradiation successfully nullifies DNA contribution by sperm. Most importantly in terms of supporting the diploid status of the gynogens, erythrocyte nuclear areas of diploid controls did not differ from those of putative

gynogenetic diploids. The correlation between ploidy state and erythrocyte area provides strong evidence that our intended chromosomal manipulations were successful in this study. This histological approach to measuring erythrocyte nuclear area of larval fish should prove useful for validation of ploidy manipulations in other fishes.

It is well established that the precise timing and specific technique used for each element of gynogenesis is critically important and tends to vary widely among fishes (Ihssen et al., 1990; Lutz, 2001). Therefore, these elements required investigation in southern flounder. We found that the prediluent (Ringer's solution) used for semen dilution was adequate for flounder and mullet since sperm remained inactive during exposure to Ringer's solution and responded rapidly upon introduction of seawater. However, we observed reduced sperm motility in diluted compared with undiluted ("dry") semen as shown in some other studies (Suquet et al., 1992; Detweiler and Thomas, 1998; Yao et al., 1999). We also found that thinly spread semen diluted 1:50 provided for adequate penetration of UV light when irradiating spermatozoa, perhaps by ensuring exposure of a high proportion of spermatozoa to UV light. The highest dosage (130 J/cm^2) examined eliminated all activity of flounder and mullet sperm, while lower treatment levels significantly decreased sperm motility in a dose-dependent manner. The Hertwig effect associates greater survival of gynogens with application of higher doses of radiation, probably due to complete inactivation of sperm chromatin at high UV doses versus only partial sperm inactivation at lower doses (Ihssen et al., 1990). Our analyses of sperm motility and optimization of the UV dosage for induction of gynogenesis

demonstrate that a UV dosage of 70 J/cm^2, which reduces flounder sperm motility to ~10% (50 J/cm^2 for mullet), genetically inactivates sperm while maximizing its ability to activate egg development.

To our knowledge, there has been one study that examined the effects of UV irradiation on duration of sperm motility in teleosts (Felip et al., 1999). The duration of sperm motility in flounder and mullet was reduced by UV exposure. The dose-related effect observed with these species may not apply to all fish, however, as the duration of sperm motility was unresponsive to UV dosage in the sea bass, *Dicentrarchus labrax* (Felip et al., 1999). The UV treatment used to induce gynogenesis in flounder allowed sperm activity to persist for ~2 min, allowing time for activation of egg development prior to cold shock.

The utility of heterologous sperm for induction of gynogenesis has been demonstrated for several fish species (see Pandian and Koteeswaran, 1998; Arai, 2001; Felip et al., 2001, for review). When successful, this approach provides an additional source of sperm for induction of gynogenesis. This can be important for species like southern flounder that produce limited quantities of semen and for which procedures for hormonal induction of spermiation are not fully developed (Berlinsky et al., 1996; Watanabe and Carroll, 2001; Luckenbach and Sullivan, submitted). Other captive species, such as mullet, may produce greater quantities of semen with higher densities of more motile spermatozoa. More importantly, utilization of heterologous sperm exploits the

very low probability of survival of hybrids between distantly related species, ensuring that surviving larvae are indeed gynogens (Chourrout, 1982; Peruzzi et al., 1993; Váradi et al., 1999; Arai, 2001). For these reasons, we tested striped mullet sperm for its utility in induction of gynogenesis in southern flounder. Mullet were considered good candidates for gynogenesis trials in southern flounder because these species coinhabit coastal and estuarine waters of the United States and their natural spawning seasons closely coincide.

UV-irradiated mullet sperm was effective in producing viable gynogenetic flounder that could be raised through metamorphosis. These results suggest that sperm from mullet, and possibly from other marine species, can be genetically inactivated by treatment with UV light while retaining sufficient motility for inducing gynogenesis in southern flounder. In summary, for successful induction of gynogenesis in southern flounder, semen from flounder or mullet is diluted 1:50 with Ringer's solution, spread thinly onto dishes, treated with an appropriate dose of UV light (70 J/cm^2, flounder; 50 J/cm^2, mullet), and then exposed to seawater for 2.5-4 min to activate motility and trigger embryonic development.

To induce retention of the second polar body in southern flounder, cold shocks of 0-2ºC were applied after fertilization for 45-50 min. This is similar to the temperature range and time period of cold shock used in other marine teleosts (Felip et al., 2001). We found that straining the water used for fertilization of eggs through a mesh bag and then releasing the eggs to the cold-shock bath resulted in shearing or other damage to eggs (data

not shown). Accordingly, our current working protocol for cold shock involves draining the majority of seawater used for fertilization via a separatory funnel and then simply releasing the floating eggs and other contents directly into the cold-shock flask. Even with reduced handling, a large number of eggs that underwent artificial gynogenesis were damaged by the harsh conditions of the protocol. High levels of mortality were evident at hatching and during transition of the larvae to live feed, as has been reported in other studies of chromosomal manipulation of fishes (Cherfas, 1981; Felip et al., 1999). We therefore recommend using large numbers of high quality gametes for induction of diploid gynogenesis in southern flounder.

This study validates procedures for chromosome set manipulation of southern flounder. These include the induction of diploid gynogenesis using either homologous or heterologous sperm. This study also incorporates a straightforward method of measuring erythrocyte nuclear area in larval flatfish that may be applied to ploidy analyses in other finfish species as well. It should be possible to combine gynogenesis with the temperature-dependent sex determination properties of southern flounder (Luckenbach et al., 2003) to create homogametic (XX) male broodstock. Since these gynogenetic, sex-reversed broodstock can be mated with females to produce all-homogametic offspring, gynogenesis is a critical component to the generation of monosex populations of faster-growing female southern flounder.

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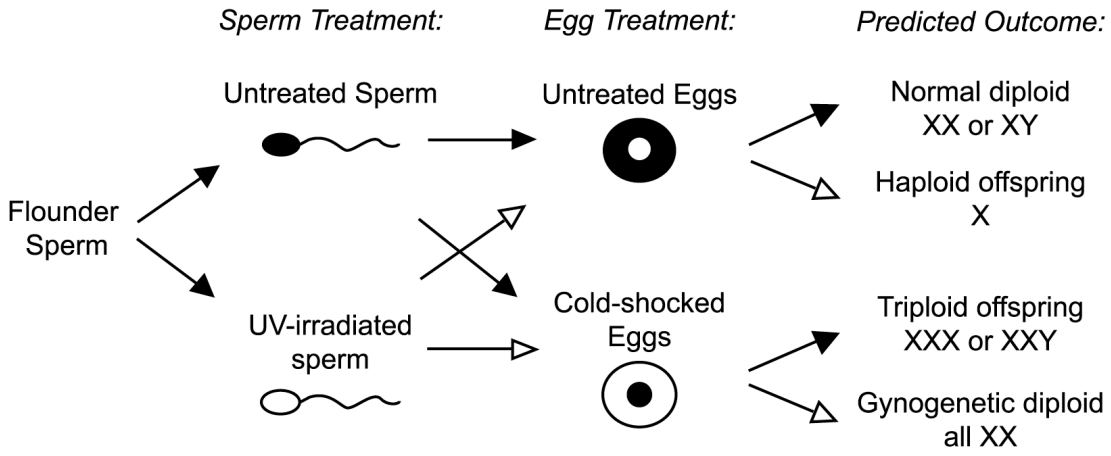

Figure 1. Schematic representation of the experimental design for validation and production of gynogenetic diploid southern flounder using homologous sperm. The production of offspring with different chromosomal constitutions serves to determine the effectiveness of sperm irradiation (haploid) and egg cold shock (triploid) in the induction of diploid gynogenesis. Production of normal diploids serves as a control for overall gamete quality.

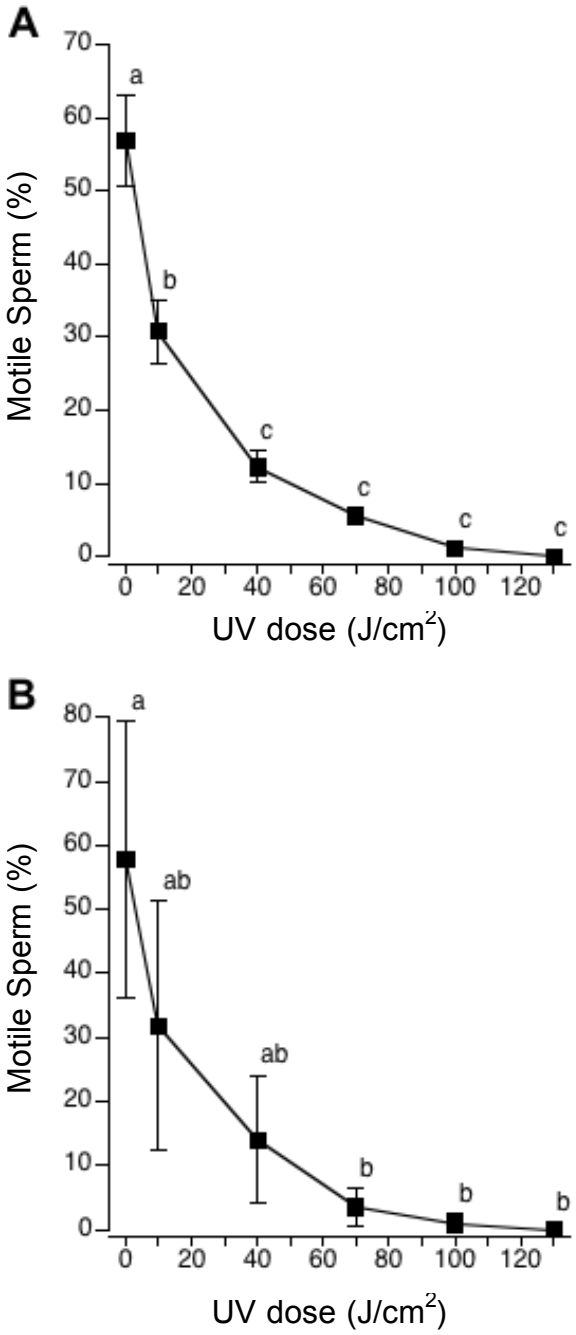

Figure 2. Motility of southern flounder (Panel A) and striped mullet (Panel B) spermatozoa exposed to UV dosages ranging from 0-130 J/cm^2. Sperm motility was evaluated immediately following activation by exposure to seawater. Points represent the average sperm motility from replicate experiments in which semen was pooled from 2-3 male fish (N=4 experiments for flounder, mean ± S.E.M.; N=2 experiments for mullet, mean ± S.D.). Data points not connected by the same superscript letter are significantly different ($P<0.05$).

Table 1
Duration of sperm motility (min) in southern flounder and striped mullet semen diluted with Ringer's solution, UV irradiated (0-130 J/cm^2), and activated with seawater

UV-dose (J/cm^2)	Southern flounder	Striped mullet
0 (control)	6.04 ± 0.33^{a}	4.28 ± 0.79^{a}
10	5.00 ± 0.41^{a}	3.78 ± 1.63^{a}
40	2.63 ± 0.21^{b}	2.44 ± 0.80^{a,b}
70	1.43 ± 0.27^{b,c}	1.62 ± 0.73^{a,b}
100	0.37 ± 0.33^{c,d}	0.20 ± 0.28^{b}
130	0^{d}	0^{b}

Values shown indicate the activity for sperm pooled from 2-3 males in replicate experiments (N=4, mean ± S.E.M., flounder; N=2, mean ± S.D., mullet). Values not connected by the same superscript letter are significantly different ($P<0.05$).

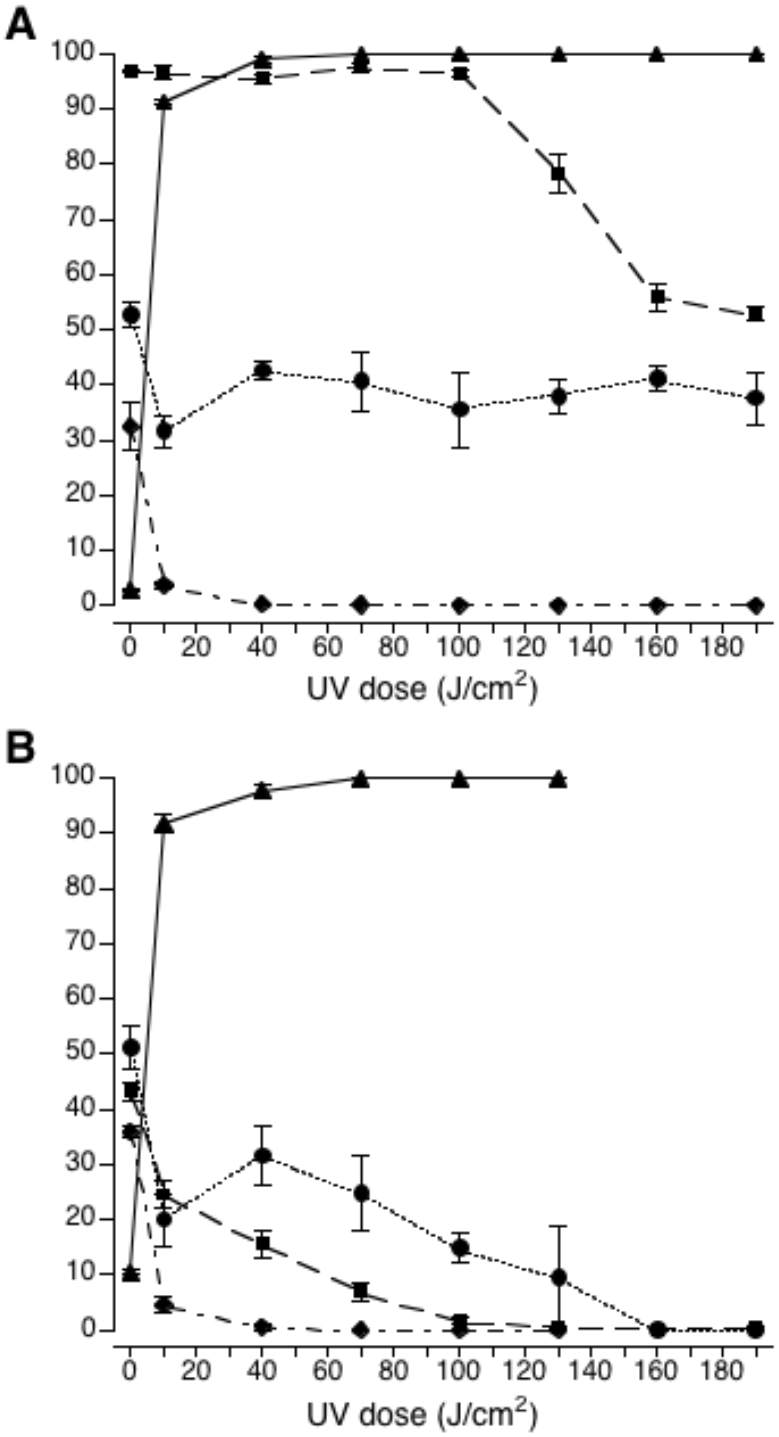

Figure 3. Optimization of the UV dose for induction of gynogenesis in southern flounder eggs. Flounder semen was pooled from 3-5 males, diluted with Ringer's, and UV irradiated at dosages ranging from 0-190 J/cm^2. High (Panel A) and low (Panel B) semen to egg ratios (0.9:4.0 ml versus 0.3:4.0 ml) were tested in separate experiments. Data represent triplicate determinations of percent fertility (■), hatch (●), haploid syndrome (▲), and survival ~36 hours after hatching (◆) for each treatment.

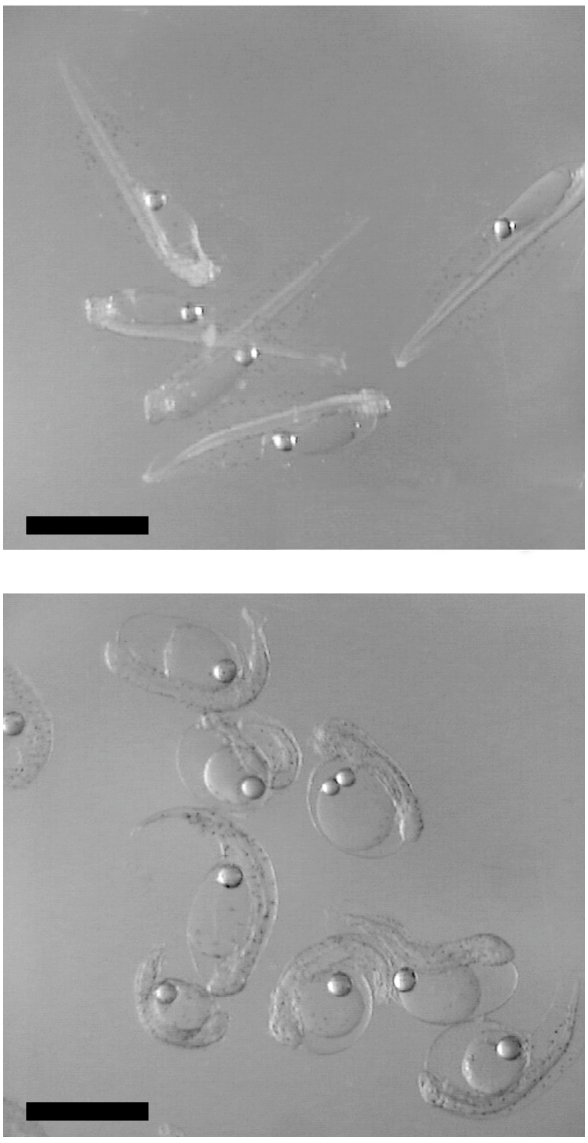

Figure 4. Southern flounder larvae (0 days post-hatch) derived from eggs fertilized with either untreated flounder sperm (Upper panel) or sperm irradiated at 70 J/cm^2 (Lower panel). Note the normal larval morphology in diploid (Upper panel) compared to the morphological deformities of putative haploid (Lower panel) offspring. Bar=1 mm.

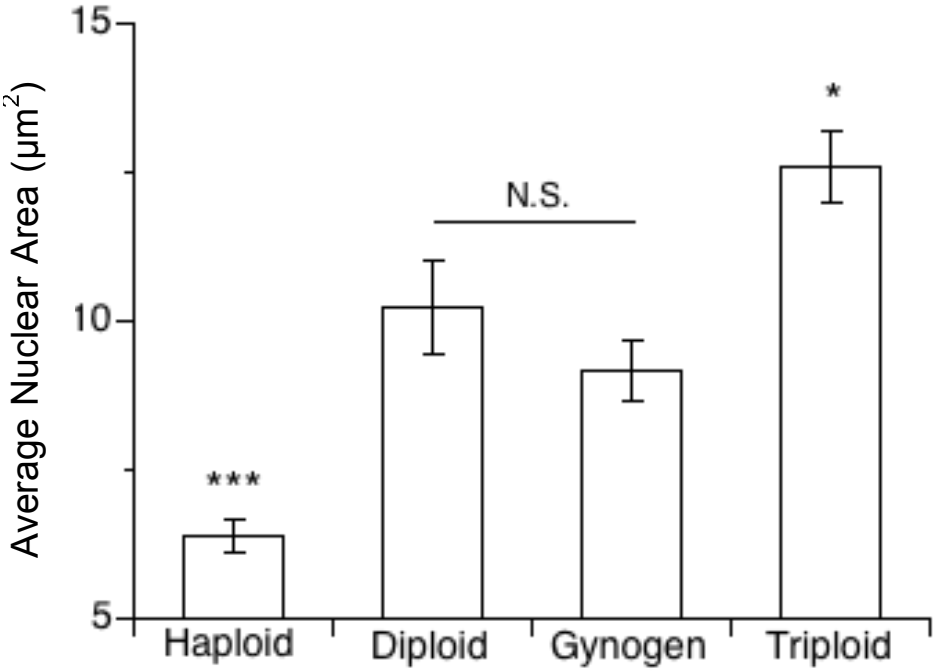

Figure 5. Average erythrocyte nuclear area of larvae from the haploid, normal diploid, gynogenetic diploid, and triploid treatment groups (*N*=7-12 fish with 5-7 separate measures per fish). Asterisks indicate significant differences from normal diploids (**P*<0.05 and ****P*<0.001). N.S. denotes no statistical difference between groups.

Table 2
The percentage of fertilized eggs, hatched embryos, and embryos surviving beyond first feeding (i.e., 11 days post-hatch) in seven heterologous fertilization trials using UV-irradiated striped mullet semen for induction of gynogenesis in southern flounder eggs.

Trial	% Fertility	% Hatch	% Survival
1	16.7	3.2	0
2	33.3	3.6	7.0
3	36.5	3.6	30.0
4	37.1	1.6	25.6
5	38.4	1.8	0
6	37.0	1.8	3.0
7	30.3	11.4	26.2

CHAPTER V

Conclusions and Future Directions[2]

[2] A portion of this chapter is modified from an article published in Evolution and Development 5: 40-49 (2003) by John Godwin, J. Adam Luckenbach, and Russell J. Borski entitled, Ecology meets endocrinology: environmental sex determination in fishes.

Prior to these studies of southern flounder (*Paralichthys lethostigma*), little or no research related to sexual development had been conducted on this species or any North American flounder of this genus. The work included in this dissertation firmly establishes the schedule of gonadal differentiation in southern flounder through histology and ontogeny of cytochrome P450 aromatase expression during the process of sex differentiation. The research also demonstrates a highly temperature-sensitive mechanism of sex determination in southern flounder and effective methods for induction of diploid gynogenesis with homologous and heterologous sperm. Overall, this work provides critical information and technologies required to generate predictable sex ratios of flounder for future stock enhancement efforts and generation of monosex stocks of faster-growing female fish for aquaculture, and a genetic biomarker for evaluating environmental control of sex determination in both hatchery and wild southern flounder populations. Below, I outline conclusions drawn from data collected in these studies and discuss possible implications and future directions of this research.

TEMPERATURE-DEPENDENT SEX DETERMINATION AND GROWTH

Temperature appears to be the primary environmental determinant of sex in many species of fishes (Baroiller and D'Cotta, 2001). Generally three types of temperature responses have now been documented in fishes: 1) In the majority of temperature-

sensitive fishes, the proportion of males increases with exposure to higher water temperatures and female differentiation is induced by low temperatures. This pattern of TSD is seen in some atherinids, poecilids, cichlids, pleuronectids, and cyprinids (Baroiller et al., 1999). 2) Conversely, in a few species (e.g., channel catfish, *Ictalurus punctatus*), higher temperatures induce female differentiation and low temperatures promote male-skewed sex ratios (Patiño et al., 1996). 3) Flounder of the genus *Paralichthys* are unique in that male-skewed sex ratios are generated at both high and low rearing temperatures while an intermediate temperature yields a 1:1 sex ratio (Yamamoto 1999; Luckenbach et al. 2003). Figure 1 summarizes the pattern of TSD across rearing temperatures for three flounder species. These findings in flounder suggest that sex determination in XX-genotype individuals is influenced by temperature, while XY-genotype individuals exhibit purely genetic sex determination (GSD). This is supported by experiments with gynogenetic *P. olivaceus*. A similar genotype-environment interaction is seen in Atlantic silversides (*Menidia menidia*). Silverside populations of northerly latitudes exhibit a low frequency of TSD (i.e., mainly GSD) while southern populations exhibit a high frequency of TSD (Conover and Kynard, 1981; Conover, 1984; Lagomarsino and Conover, 1993). Hence, northern silverside populations appear to possess a major sex-determining gene or multiple genes that is/are unresponsive to temperature, whereas this genetic component appears to be lacking or is easily overridden by environmental influences in fish originating from southern populations.

Of the Japanese flounder species that have been investigated for TSD, the barfin flounder (*Verasper moseri*) has a more northerly distribution, inhabiting waters of Japan that are influenced by the cool Liman and Oyashio currents rather than the warmer Tsushima and Kuroshio currents that southern Japan experiences (Figure 2). The cold-shifted TSD response shown by barfin flounder (Goto et al., 1999) relative to hirame (*P. olivaceus*) may represent an adaptation to these cooler water temperatures. Based on observed latitudinal differences in the TSD response across populations of Atlantic silversides and what appears to be adaptation to local temperatures in the TSD response of different flounder species, it is possible that TSD responses may vary with latitude in flounder. Potential latitudinal variation in the TSD response of flounder has not been addressed. However, this would certainly be an interesting future direction of research.

Mechanisms of environmental sex determination are becoming better understood in a few model systems. However, the means by which environmental stimuli are transduced into changes in these pathways remains obscure. For example, it is not known how a temperature signal leads to genetic sex reversal in some fishes. With regard to southern flounder, it is clear from research presented in this dissertation and our unpublished findings that the homogametic-female (XX) genotype is highly unstable and prone to sex reverse, at least in the culture environment. Our preliminary data in southern flounder and studies in hirame (Kitano et al., 1999; 2000) suggest that suppression of P450 aromatase gene expression is associated with sex reversal of genetic female flounder into functional males. To further complicate matters, it is also quite possible that among-family variation in the TSD response exists in these species

where families (i.e., cohorts) exhibit variation in temperature sensitivities (see Chapter IV). In hirame, sex ratios of different cohorts reared at the same temperature appear to show variation in the TSD response (see Yamamoto, 1995; 1999). Future research should further address the thermosensitive period of southern flounder sex determination, as well as expand upon our current knowledge of TSD in this species to develop a detailed and reliable rearing schedule for maintaining the female phenotype or preventing sex reversal of genetically female flounder. It is important to note that although the adaptive role of TSD is well established in Atlantic silversides (Conover, 1984), it remains unclear in most other fishes. In fact, few studies of environmental sex determination (ESD) have addressed these environmental effects on wild populations and therefore many observations of ESD may be artifacts of fish culture (Baroiller and D'Cotta, 2001). Numerous studies of ESD have exposed fish to conditions that would not be encountered by wild populations (e.g., extremely high temperatures) or constant environmental regimes that do not mimic natural conditions. To better understand the evolution of this unusual mechanism of sex determination in flounders, future studies should rigorously assess the occurrence of TSD in wild flounder populations. The southern flounder P450 aromatase biomarker developed here will likely play an instrumental role in testing possible effects of temperature on sex determination in critical flounder nursery habitats.

Our research in southern flounder indicates that in addition to effects on sex determination, water temperature also greatly affects growth. In an initial study (see Chapter II), we found that flounder reared at 23°C were significantly larger after one year than fish reared at either 18 or 28°C (Figure 3). Charnov and Bull (1977) proposed that

ESD is favored in species when the environments in which early development will occur vary in terms of growth potential such that expected individual fitness under these varying growth conditions differs between the sexes. Since female flounder reach larger adult sizes than males, faster growth observed at the temperature that produces the greatest proportion of females is consistent with Charnov and Bull's hypothesis because females should benefit more from rapid growth than males. It is also relevant that observations of southern flounder spawning in tanks suggest little competitive or aggressive interaction between males in which large size might provide an advantage (Smith et al., 1999). Importantly, the same initial study of southern flounder suggests that the body size of males and females does not differ within rearing temperatures (Figure 3). This mirrors findings from the Atlantic silverside where female-determining temperatures also produced faster growth, but no male-female size difference was found within a rearing temperature (Conover, 1984). The lack of observed sex differences in growth within temperatures in both species is consistent with the Charnov and Bull hypothesis since it indicates that temperature rather than sex is the critical variable regulating growth (Rhen and Lang, 1998). Findings in southern flounder and silversides contrast with work in barfin flounder where moving juveniles from 14 to 18°C significantly enhanced growth in the first 150 days following fertilization, despite 18°C inducing more males to develop (Goto et al., 1999).

Additional southern flounder growth studies have been conducted among several laboratories at North Carolina State University. Our most recent studies of temperature

effects on growth agree with results from the initial study and suggest that a mid-range temperature (23°C) optimizes the rate of growth in juvenile flounder when compared to a high temperature (28°C). Also in agreement with this previous study, growth rate at the mid-range temperature is greater regardless of the sex of the flounder. These temperature studies were initiated in southern flounder at ~30, 40, and 50 mm TL. However, improved growth rate at 23°C versus 28°C did not begin to occur until fish reach a body size of ~100 mm TL or ~12 g. Prior to this body size, growth at both temperatures is similar. The significance of growth divergence at this body size between these temperature treatments remains unclear. However, growth status of southern flounder at these rearing temperatures has been correlated with blood circulating levels of a critical growth-regulating hormone, insulin-like growth factor-I (IGF-I). Better growth is linked with significantly higher circulating IGF-I levels when compared to fish exhibiting slower growth. To date, little is known about regulation of IGF-I under different states of growth in flounder. However, recent studies in southern flounder have assessed growth regulation through IGF-I in fed versus feed-deprived fish (Ueda, 2004). We specifically sought to determine whether alterations in circulating IGF-I and hepatic IGF-I mRNA accompany changes in growth under these different nutritional states. Before initiating this study, we partially cloned southern flounder IGF-I cDNA to enable design of primers and a probe for quantitative RT-PCR. Feed-deprived fish exhibited a significant decline in circulating IGF-I levels relative to controls during the starvation period, and upon refeeding, levels were completely restored to that of control fish. Blood circulating IGF-I levels were positively correlated to changes in specific growth rate. Interestingly,

hepatic IGF-I mRNA abundance did not differ between control and the feed-deprived animals, although an increasing trend in IGF-I mRNA was observed after refeeding flounder in the feed-deprived treatment groups. Taken together, data collected in these growth studies suggest that measures of IGF-I could provide an instantaneous indicator of growth status of southern flounder in the field, hatchery, or commercial production facility, thereby permitting rapid assessment of environmental conditions (salinity, photoperiod, etc.) that best promote growth.

BREEDING BIOTECHNOLOGY IN SOUTHERN FLOUNDER

As discussed above, undifferentiated or bipotential gonads of fishes are generally very labile with respect to sex determination (Pandian and Koteeswaran, 1999). Although, in gonochoristic fishes like the southern flounder, once a path of sex differentiation is initiated either through expression of sex-determining genes or exogenous factors such as hormones, the condition is perpetuated throughout subsequent gonadal development (Devlin and Nagahama, 2002). Several monofactoral sex-determination systems have been conceptualized for gonochoristic fishes, but due to the potential influence of environmental factors and minor genetic elements these models often provide only a framework of the mechanism of sex determination (Lutz, 2001). Most fishes possess an XX-XY type of sex chromosome system where females are the homogametic

sex (XX) and males are heterogametic (XY). Other fishes including species of the family's cyprinidae, cyprinodontidae, anguillidae and pleuronectidae utilize what is termed a ZZ-ZW system where females are the heterogametic sex (ZW) and males are homogametic (ZZ).

Induction of diploid gynogenesis has proven to be a valuable tool for assessing the mechanism of sex determination in fishes (Lutz, 2001; Piferrer, 2001). Sex ratios of gynogenetic offspring, which through the process of gynogenesis should inherit only maternal chromosomes, have been useful in elucidating the mechanism of sex determination in several fishes (Lutz, 2001). For instance, if 100% female offspring are produced through diploid gynogenesis, it suggests an XX-XY system of sex determination, whereas a 1:1 sex ratio would suggest a ZZ-ZW system. Perplexing results of gynogenetic studies in some species have provided evidence for the existence of minor sex-determining genes that function independently of the major sex-determining system (i.e., XX-XY or ZZ-ZW). This conclusion is based on unusual sex ratios obtained either through gynogenesis or following second-generation crosses of gynogens with normal and/or sex-reversed individuals. Actions of minor genetic factors have been documented in common carp (*Cyprinus carpio*), species of the genus *Oreochromis* (Lutz, 2001), among other fishes. Studies in the hirame (*P. olivaceus*), have established that flounder of the genus *Paralichthys* exhibit an XX-XY sex chromosome system (Yamamoto, 1995; 1999). However, due to the incidence of TSD in this species, the mode of sex determination was initially difficult to elucidate (Yamamoto, 1999). In fishes

that exhibit an XX-XY system, induction of diploid gynogenesis is a critical step toward production of all-female populations for aquaculture.

We have developed reliable methods for induction of diploid gynogenesis in southern flounder with homologous or heterologous sources of semen. Our primary objective in undertaking this research was for future production of faster-growing all-female flounder populations. However, also of importance is the utility of these fish and subsequent crosses from them with other broodstock in developing isogenic or inbred southern flounder lines. Due to the high degree of homozygosity, these lines could prove valuable for genetic selection and propagation of desirable flounder traits (e.g., high rates of growth, disease resistance, and stable sex determination).

Using methods similar to those reported here for induction of diploid gynogenesis in southern flounder, our research group, now led in this area by NC State University M.S. student Andrew J. Morgan, has successfully produced several relatively large cohorts of diploid gynogenetic flounder. We have attempted sex reversal in these animals via high rearing temperatures early in development and hope to use these putative XX-male broodstock in crosses with normal female flounder for creation of all-XX flounder populations. If successful, this would represent the first genetically all-female (XX) southern flounder population for aquaculture in the world. We are excited about the future applications of this research.

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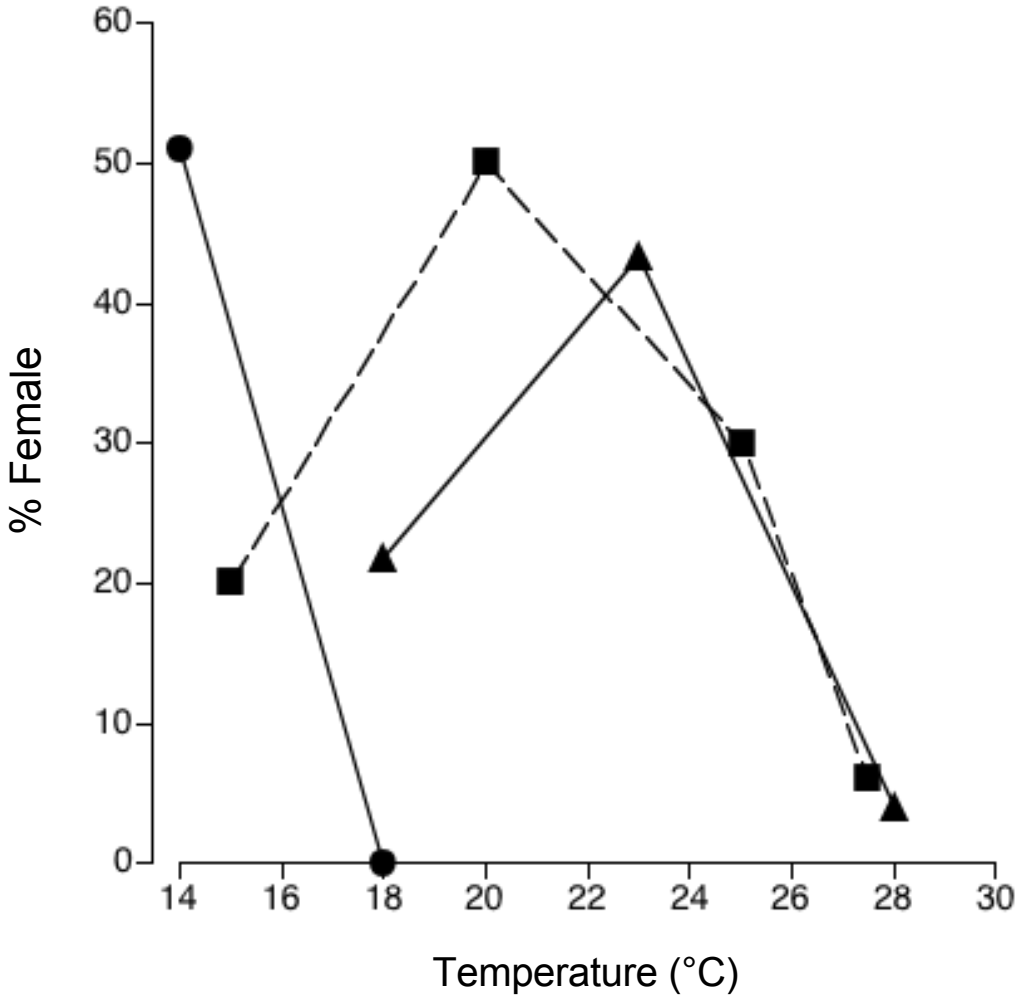

Figure 1. Flounder sex ratios resulting from development at different temperatures. Depicted are data from barfin flounder (●, solid line), hirame (■, dashed line), and southern flounder (▲, solid line). References are in text. Hirame values are means calculated from Yamamoto (1999).

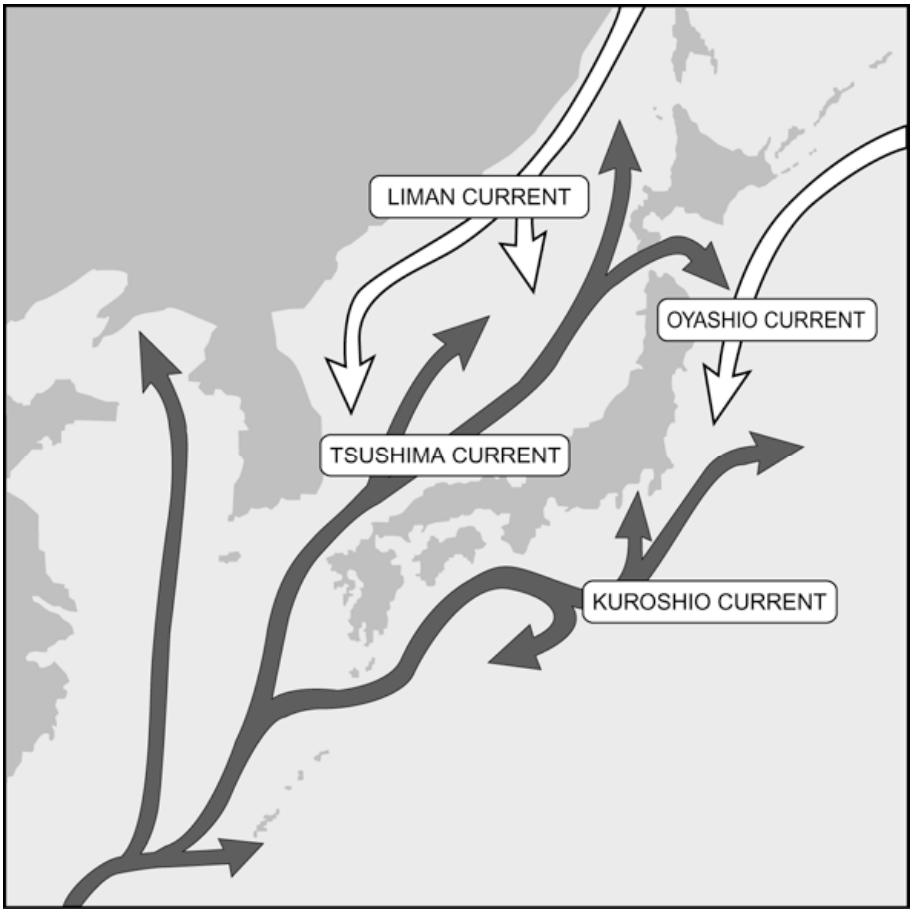

Figure 2. Warm and cold currents in the area of Japan. The range of the hirame (*Paralichthys olivaceus*) extends from northern Japan south to Hong Kong. The range of the barfin flounder (*Verasper moseri*) extends from the Kuril Islands north of Japan south to approximately the point where the Oyahsio and Kuroshio currents are shown meeting.

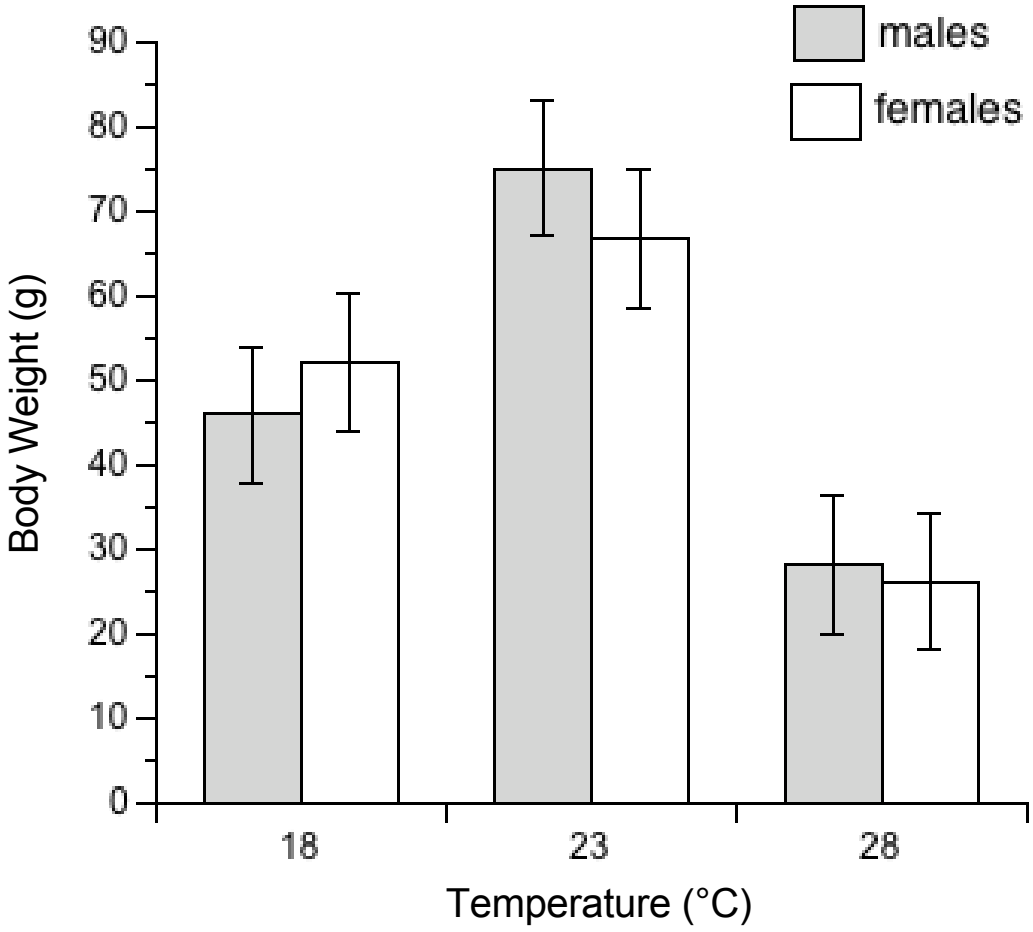

Figure 3. Body sizes of southern flounder grown at different temperatures in captivity for 1 year (mean ± S.E.M.). Fish grown at 23°C were significantly larger than fish grown at either 18°C or 28°C, but no sex difference in size was found in any of the three temperatures.