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Mechanisms of growth and sex determination in the Atlantic silverside, *Menidia menidia*, and the spatial scale of local adaptation

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Abstract of the Dissertation

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Local adaptation in widely-dispersed marine species may result from strong selection on multiple life history traits that differ among populations. This results in unique sets of traits that maximize fitness of individuals within populations along the species' range. The Atlantic silverside, *Menidia menidia*, is a fish that exhibits high gene flow potential, but differences in growth and sexual differentiation among populations demonstrate strong local adaptation. To better understand the spatial pattern of local adaptation in this species, I compared the form of sex determination, which shifts from genetic to environmental sex determination, across *M. menidia*'s range and quantified the rate of gonad development across populations. Strong local selection in this species

drives genetic differences among populations, but few of the mechanisms that control these differences have been studied. Therefore, I also quantified the activity of a major sex-determining gene during gonad differentiation in populations with different forms of sex determination and assessed the role of a major growth protein in structuring growth differences among populations with unique life-history traits. Finally, widely-dispersed marine species come into contact with many anthropogenic stressors within their ranges, and there may be differential interactions between these stressors and local populations. I addressed how locally-adapted differences in sex determination contribute to population-level susceptibility to estrogenic contaminants in the wild.

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Introduction

Local adaptation in the marine environment, until recently, was considered to be the result of behavioral isolation or reduced gene flow owing to oceanographic or geographic barriers (Conover *et al.*, 2006). Adaptive divergence is common in freshwater organisms, primarily due to differential selection pressures across isolated environments. However, isolation is comparatively rarer in the marine environment leading to the belief that gene flow is high in most marine organisms, especially in those with planktonic embryo or larval stages (Conover, 1998; Conover *et al.*, 2006). For many years, high gene flow was expected to swamp the effects of local selection. Yet, local selection has been demonstrated to create major genetic differences across panmictic populations and many of these cases of local adaptation occur across steep environmental gradients that cross latitudinal gradients. This is now well recognized in many marine fish species (Holt and Gaines, 1992; Warner, 1997; Conover, 1998; Conover *et al.*, 2006). Where gene flow is high, differences in quantitative traits such as growth rate, fecundity and thermal tolerances among fish populations may be the result of strong local selection maintaining local adaptation.

Differential selection along an environmental gradient may lead to two well-recognized phenotype patterns; cogradient and countergradient variation (Conover *et al.*, 2009). Co-gradient variation (CoGV) results when environmental influence and genetic components of traits co-vary positively along a gradient, resulting in phenotypes that reflect expected differences across an environmental gradient. For example, changes in the shell morphology in a marine gastropod, *Bembicium vittatum* (Parsons, 1997) reflect wave exposure: strong wave action results in short, flat shells while low-energy environments select for individuals with conical, larger shell shapes. Vertebral number in the Atlantic silverside, *Menidia menidia* (Billerbeck *et al.*, 1997) displays a similar pattern where high latitude populations have more vertebrae than southern conspecifics when reared both in the lab and in the wild. Countergradient variation (CnGV) results in a different pattern, where wild phenotypes look more similar across an organisms' range, despite sharp environmental gradients (Levinton, 1983; Conover and Schultz, 1995). This is due to negative covariance between the environment and genetic factors, which minimizes differences in phenotypes among wild populations. However, these

differences are readily apparent when populations are reared in a common garden environment, revealing genetic differences among populations (Conover *et al.*, 2009). Fish often exhibit CnGV in growth and developmental rates, and populations in high latitude environments with short growing seasons grow faster than their southern counterparts. CnGV has been documented in at least 60 species (Conover *et al.*, 2009) and many of these are teleosts including *M. menidia* (Conover and Schultz, 1995), Atlantic halibut, *Hippoglossus hippoglossus* (Jonassen *et al.*, 2000), Atlantic cod, *Gadus morhua* (Salvanes *et al.*, 2004) and others (Conover *et al.*, 2009). For *M. menidia*, this trend is hypothesized to be the result of adaptation to a reduced growing season which produces adult phenotypes that are similar in size along its range, masking differences in growth rate in the wild. Both CoGV and CnGV are patterns of local adaptation, and are likely to be maintained by different selective pressures. Local adaptation has been documented in numerous fish species (Conover *et al.*, 2009), and a significant body of work has documented numerous traits in *M. menidia* that exhibit local adaptation.

Many marine fish have life-histories that are dispersive and species' ranges often extend over steep environmental gradients. Because of limited physical barriers, marine organisms adapted to a particular environment may not be able to choose an environment where fitness is optimal and may be carried into suboptimal environments by physical oceanographic processes (Hare *et al.*, 2005). Phenotypic plasticity allows organisms to compensate for reduced fitness in suboptimal environments (Bull, 1987). Environmental sex determination (ESD) is a form of plastic sex determination documented in 50+ fish species, including *M. menidia*, and allows the optimal phenotypic sex to be produced. Environmental sex determination occurs when the environment an individual experiences early in life determines phenotypic sex (Bull, 1981). This environmental cue for sex determination could be pH, salinity or other abiotic factors, but is more common to be the result of temperature in teleosts (Strussmann and Nakamura, 2002; Conover, 2004). This type of ESD is termed temperature-dependent or thermolabile sex determination (TSD). In *M. menidia*, cold water temperatures early in the season produce females whose reproductive success depends more on adult body size than it does in males. Males are produced later in the season as water temperatures warm (Conover, 1984). Charnov and Bull (1989) proposed that ESD evolves in a species when: 1) a given environment favors

the fitness of one sex over the other and 2) offspring have no control over which environment they are produced in. *M. menidia* life history meets these requirements and TSD is therefore likely to be an adaptive life-history trait (Conover, 1984).

M. menidia exhibits clinal variation in growth rate, level of environmental sex determination and vertebral number (Conover and Heins, 1987A; Conover and Schultz, 1995; Billerbeck *et al.*, 1997). Recent research shows that a considerable amount of gene flow exists within the range of this species, but this exchange among populations may be more restricted where major oceanographic barriers exist, such as the Gulf Stream. Clarke (2007) found that northern adult *M. menidia* may migrate up to 740 km (the maximum distance between sites in this study) from their natal estuary to other estuaries following overwintering on the continental shelf, although a majority of their movement was restricted to within 50 km. Therefore, this high potential for gene flow indicates that strong local selection must necessarily occur to maintain the cline in these traits, which otherwise would be expected to disappear (Holt and Gaines, 1992; Lagomarsino and Conover, 1993). The degree of selection for each trait most likely changes along spatial, and possibly temporal, scales and may be unique for each phenotypic trait. Therefore, local adaptation is a balance between migration and selection (Conover *et al.*, 2006). Understanding the pattern in life-history traits, such as level of TSD, along a latitudinal gradient can help researchers understand the selective forces that produce patterns of local adaptation in *M. menidia*.

Artificial and natural selection target the phenotype of a population, which is a combination of additive genetic variance, dominance variance, environment influence and gene by environment interactions (G X E) (Falconer and Mackay, 1996). Therefore, strong selection is likely to cause changes in the genetic mechanisms that contribute to phenotypic variation within a population. The endocrine system may be particularly susceptible to selection because it produces functional hormones that are necessary for growth and reproduction in fish (Devlin and Nagahama, 2002). Selection acting on an aspect of growth and/or reproduction is likely to alter endocrine system regulation of these traits, and is therefore measureable at the level of genetic regulation or regulation of gene products (Naish and Haird, 2008). Identification of the proximate molecular mechanisms that control population-level differences in some quantitative traits is one

way to better understand how selection acts on those traits. This may also pinpoint which part(s) of the endocrine system may be under selection in response to domestication or artificial selection (Fleming *et al.*, 2002; Lankford and Weber, 2006) or in response to the processes shaping local adaptation.

Understanding the selective pressures that produce a particular life-history is necessary to predict and understand the response to anthropogenic alteration of the environment. Environmental endocrine disruptors and shifts in temperature due to climate change can rapidly alter the environments to which organisms are adapted. Plasticity in life-history characters will ensure that some organisms can adapt to a changing environment, but the rate of environmental change will play an important role in this process (Ojaveer and Kalejs, 2005; Eldredge *et al.*, 2005). The clinal trend in life-history characters in silversides may be altered by anthropogenic influences over time. For example, exponential human population growth on coastlines results in millions of gallons of wastewater, both municipal and industrial, being dumped into bays and estuaries. Many common chemicals in wastewater that are not eliminated during treatment have the potential to impair reproduction and affect overall fitness in marine invertebrates and vertebrates (Sumpter, 1997; Oberdorster and Cheek, 2000; Mills and Chichester, 2005). The presence of environmental endocrine disruptors may act as a strong selection pressure for alternative life-history strategies and affect populations with different life-history strategies in different ways.

Menidia menidia is an excellent species to answer questions regarding the effects of selective pressures, local adaptation, phenotypic plasticity in multiple life-history traits and response to anthropogenic impacts in fish. Because this species is easy to manipulate in the laboratory, common garden experiments can tell us a great deal about the genetic basis of a trait of interest and artificial selection experiments allow us to understand how these traits respond to selection. Phenotypic plasticity in traits among populations makes it ideal for studying differential responses within a single species. *M. menidia* inhabits a wide gradient of environments along the eastern coast of North America and thus, application of this species for questions regarding inter-population differences in life-history strategies is appropriate.

I carried out the following studies to answer three major questions about the life-

history characteristics of *M. menidia*. First, I define the spatial scale of adaptive variation in sex determination and relate this to gonadal development rate among species with different life-history characteristics. Secondly, I assessed two mechanisms that contribute to differences in the form of sex determination and growth rate in this species. Finally, I addressed how these life-history traits contribute to the response to anthropogenic influences in this model species.

Chapter 1: The geographic pattern of sex determination in *Menidia menidia*

Abstract

One of the steepest gradients in sea surface temperature in the world occurs along the east coast of North America. The Atlantic silverside, *Menidia menidia*, exhibits local adaptation along this environmental gradient and several life history traits change with latitude. One of these traits is sex determination. The form of sex determination changes from temperature-dependent sex determination (TSD) to genetic sex determination (GSD) across this cline. To determine the geographic pattern in the mode of sex determination, the level of TSD was determined for 31 sites along the species' range using common garden experiments. The current work demonstrates that the level of TSD is high in the southern end of the range through New Jersey then declines toward full GSD throughout the mid-Atlantic into the Gulf of Maine where GSD predominates. Additionally, a population with mixed TSD and GSD persists in the Gulf of St. Lawrence. Level of TSD clearly demonstrates two major shifts in the level of TSD across areas of environmental change. These breaks occur between Maryland and New Jersey (N 38^{o}- N 39^{o}) and in the northern Gulf of Maine and into the Bay of Fundy (N 44^{o}). Between these two locations the level of TSD declines linearly with increasing latitude. The relationship between the level of TSD and length of the growing season shows a positive linear relationship for growing seasons less than 220 days. In populations with growing seasons greater than 220 days, the level of TSD remains constant at around 0.8. This indicates that growing season length above 220 days does not significantly contribute to increased TSD, and that some genetic influence on sex determination persists in southern populations.

Introduction

Steep environmental clines associated with latitude are strong forces that shape life-history evolution. One of the steepest environmental clines of this nature in the marine environment is associated with the east coast of North America (Bower *et al.*, 1985; Wares, 2002) where rapid changes in the abiotic environment structure biota, often leading to local adaptation (Conover, 1998). Despite high gene flow across abrupt environmental gradients, some marine species exhibit local adaptation (Conover *et al.*, 2009; Nielsen *et al.*, 2009). In these species, local selection produces genotypes whose fitness is maximized in an environment. Therefore, steep shifts in the selective factors that maintain local adaptation may give rise to patterns of life-history traits that change rapidly over a short distance (Conover *et al.*, 2006). Identifying the geographic pattern of local adaptation can first provide information about how populations are structured in space, but also which selective factors dominate to create the pattern in life-history traits as the environment changes.

Local adaptation has been rigorously studied in the Atlantic silverside, *Menidia menidia*, an estuarine species common to the northwest Atlantic. Conover and Heins (1987A) identified a latitudinal gradient in sex determination in *M. menidia* that ranges from predominantly temperature-dependent sex determination (TSD) in southern populations to genetic sex determination (GSD) in northern environments. In fish from South Carolina where TSD prevails, sex determination is under polygenic control, with temperature sensitive genetic factors interacting with the environment (G X E interaction) shortly after hatch to determine an individual's phenotypic sex (Conover and Heins, 1987B; Lagomarsino and Conover, 1993). Fish from the Annapolis River in Nova Scotia, Canada have sex determination that is controlled by major genetic factors that are temperature-insensitive. Intermediate populations between these two environments and populations within the Gulf of St. Lawrence exhibit sex determination that is a mixture of TSD and GSD. The major selective force shaping this pattern is hypothesized to be the length of the growing season, which Conover and Heins (1987A,B) found to be positively correlated to the level of TSD. Despite this overarching gradient, environments in close proximity may exhibit stark differences in selective pressures due to rapid changes in the physical or biological environment (Stockwell *et al.*, 2003;

Conover *et al.*, 2006). Therefore, addressing the pattern of sex determination at a fine scale along *M. menidia*'s range will allow us to see where shifts in the form of sex determination occur and to understand the selective pressures contributing to this pattern.

Menidia menidia represents the only fish species where the adaptive significance of TSD has been rigorously studied. TSD has been identified in over 50 species of fish, but for most of these, little attention has been paid to understanding its benefit (Conover, 2004). TSD is adaptive in southern populations of *M. menidia* because it differentially maximizes the fitness of each sex in their given environment (Bull, 1981; Charnov and Bull, 1985). Stable environments with protracted growing seasons in southern latitudes select for TSD because the temperature a larvae experiences early in development is a good predictor of the length of the growing season it will experience (Conover and Heins, 1987A). Females are favorably produced in colder temperatures early in the spawning season and can therefore attain a larger body size that maximizes their reproductive fitness (Conover and Kynard, 1981; Conover, 1984). Males are produced later in the season under warmer conditions and attain a smaller body size relative to females produced in the same environment. Alternatively, growing seasons are considerably shortened in northern locations and there are sharp fluctuations in interannual temperatures which select for GSD (Conover and Heins, 1987A).

The goal of this study is to define the spatial pattern of the sex determining mechanism across the range of *Menidia menidia*. Additionally, season length was hypothesized by Conover and Heins (1987A) to predict the level of TSD. However, that study was limited to seven locations along the species' range and only assessed the pattern in the level of TSD in one year. More complete coverage of the range of *M. menidia*, combined with a multi-year approach, allows us to test this hypothesis more rigorously. This study provides evidence as to the selective factors that influence sex determination in *M. menidia* along a latitudinal cline. Addressing the pattern of sex determination at a fine scale along *M. menidia*'s range allows us to see where shifts in the form of sex determination occur and to understand the selective pressures contributing to this pattern.

Methods

Determining the latitudinal distribution of sex determination

To determine the latitudinal distribution of sex determination, embryos from *M. menidia* were collected from 31 locations along the species' range from Amelia Island, FL (N 30° 31.13', W 081° 27.78') to the Magdalen Islands, Quebec, Canada (N 47° 23.60', W 061° 50.76') (Table 1.1). Collections were carried out from April through July yearly from 2005-2007. Fish were collected from approximately 10-12 locations per year, and in all years collections spanned the majority of the range of *M. menidia*. This resulted in collection sites that averaged 60-80 km from each other for complete coverage of *M. menidia*'s range (Figure 1.1).

Spawning adult *M. menidia* were collected with a 100' seine net and stripped spawned onto four 10 X 10 cm mesh screens following the methods employed by Lagomarsino and Conover (1993). Six to eight adults of each sex were used for each screen to ensure mixed parentage. Once embryos had water hardened, they were moved into aerated coolers kept at 15°C and transported to Flax Pond Laboratory in Old Field, NY. Multiple mesh screens from each site were suspended in aerated 18 L flow-through containers at 21°C until embryos hatched. After yolk absorption, fry were fed *Artemia* sp. nauplii (Brine Shrimp Direct, Ogden, UT) supplemented with larval pelleted food (250 µm) then switched to 360-620 µm food as size increased (Otohime Hirami, Japan).

Fry were randomly allocated into 15°C or 28°C temperature treatments one week post-hatch by slowly adjusting temperature. Temperature baths were held at constant temperatures with heater and chiller units. When fry reached an average of 8-10 mm total length (TL), density in these containers was adjusted to 70 fish per container, in triplicate. Following two weeks of growth, density in each container was standardized to 50 fish. Individuals removed at this point were euthanized with tricaine methane sulphonate (MS-222; Western Chemicals Inc., Ferndale WA) and measured for growth rate (Hice, 2010). The remaining 50 fish were fed *ad libitum* and reared until they reached a minimum of 21-23 mm TL. Time to reach this size ranged from one to six months, depending on growth rate and temperature treatment. Fish were then euthanized with a lethal dose of MS-222 and stored in 10% neutral buffered formalin (NBF) until processing.

Sex was determined macroscopically with a dissecting microscope and total lengths were recorded. Females were identified by opaque or lightly pigmented fragile ovaries, as compared to thin, translucent and flexible testes in males (Conover and Fleisher, 1986). Any individuals with ambiguous gender (< 21mm) were classified as unknown. Some mortality occurred throughout the larval period and mortalities were sexed where possible, although this did not appear to be sex-biased mortality. An average of 228 $\pm$ 36 (standard deviation, S.D.) fish were sexed from each location. In the first year of the study, fish in 15°C treatments from southern latitudes were terminated before gonads in all individuals were fully developed, so sex ratios could not be accurately assessed. Therefore, these sites were removed from analysis and only five northern sites were included in the 2005 data.

Statistical analyses

Level of TSD was calculated as the difference in sex ratio of larval *M. menidia* reared at feminizing (15°C) and masculinizing (28°C) temperatures (defined as, [F/(F+M) at 15°C]-[F/(F+M) at 28°C], Conover and Heins, 1987B). Sex ratios were weighted by sample size for each temperature treatment. Piecewise linear regression was applied to the latitudinal distribution of sex determining mechanism to determine breakpoints in the form of sex determination. The validity of five models (1 to 5 segments) was assessed using Akaike's Information Criteria with the following equation:

$$AIC = [\left(-\frac{n}{2}\right) * (\ln\frac{RSS}{n}) - (\frac{n}{2})] - K$$

where *n* is the sample size, *RSS* is the residual sum of squares and *K* is the number of parameters in the model. AIC weights (w_i) were calculated for each model (Burnham and Anderson, 2002).

Sea surface temperature (SST) data was collected from the NOAA National Buoy Data Center archives for sites within the U.S.A. and from the Oceanographic Database maintained by the Dept. of Fisheries and Oceans for Canadian sites. Season length was calculated as the mean number of days that SST within the estuary was $\geq$ 12^{o}C, averaged over 2-3 years between 2004 and 2007. Conover and Heins (1987A) defined season

length in this manner because somatic growth is approximately zero below 12^{o}C. Data for season length within coastal estuaries was available for 19 of the 31 sites.

Results

Latitudinal distribution of sex determination

Sex ratios were calculated by weighting the sex ratio from each replicate by the number of individuals. The pattern of sex ratios for the 15^{o}C and 28^{o}C treatments mirrored each other, and in all populations, 15^{o}C treatments produced more females than males (Figure 1.2). However, there were non-significant differences between the number of males and females produced in locations between N 44^{o} and 46^{o}, indicating GSD. From this data, the level of TSD was calculated for 31 sites along the range of *M. menidia* spanning almost 18 degrees of latitude (Figure 1.3). Level of TSD was consistently found to be around 0.7 between latitudes N 30°-N 38°, then decreased between latitudes N 38° and N 45°. GSD predominated in five populations in the northern Gulf of Maine and Bay of Fundy where level of TSD did not differ significantly from zero, or complete GSD (G-test, $p \geq 0.21$). The three populations assessed from the Gulf of St. Lawrence also exhibited moderate TSD (0.4-0.6), as expected. Additionally, the latitudinal pattern of level of TSD held true across all years of the study, indicating that this pattern is temporally stable.

Breakpoint analysis of the latitudinal cline revealed two major shifts or breakpoints in the form of sex determining mechanism (AIC weight, $w_i = 0.7$, Table 1.2). These occurred between Maryland and New Jersey (N 38° - N 39°) and in the northern Gulf of Maine into the Bay of Fundy (N 44° - N 45°). Length of the growing season, as measured by the days when mean SST $\geq$ 12^{o}C, declines with increasing latitude. Breakpoint analysis of this data revealed 4 minor, but significant breakpoints (5 segment breakpoint regression, $w_i = 0.7$, Figure 1.4). Two of these breakpoints occur between Maryland and southern New Jersey (N 38° - N 38.5°). One breakpoint occurs around N 35° and the fourth breakpoint occurs at N 46°.

The relationship between level of TSD and season length was plotted to determine the impact of season length on the form of sex determination. A breakpoint analysis was also applied to this relationship with a best-fit model producing a single breakpoint

occurring around a season length of 220 days. The level of TSD exhibits a positive linear relationship with season length up to ~220 days, but then plateaus at a value where increased season length appears to have little effect on the level of TSD in a population (Figure 1.5).

Discussion

Latitudinal distribution of sex determination

M. menidia represents the first teleost in which the spatial pattern of TSD has been rigorously assessed, both spatially and temporally. This is one of few teleosts that exhibits a gradient between TSD and GSD along a latitudinal range. In this study, this gradient is demonstrated at a fine spatial scale (~ 60 km) across almost 18 degrees of latitude. The mechanism of sex determination shifts abruptly at two locations; between MD and NJ and a second region between the northern Gulf of Maine and Bay of Fundy. Further, TSD appears to be only partly explained by season length and the level of TSD does not increase above a season length of ~220 days.

The sex ratio patterns from both temperature treatments illustrate that all populations have a genetic component to sex determination that is not responsive to temperature. First, the patterns of sex ratio between masculinizing and feminizing treatments mirror each other, but incompletely. In low latitudes (N 30° through N 40°), the temperature treatments lead to stark differences in sex ratio, as is expected in populations with high TSD. High temperature, 28^{o}C treatments frequently result in sex ratios near zero (100% males) in these low latitude populations. However, 15^{o}C treatments never produce 100% females, indicating there is a consistent genetic component to sex determination. Based on this observation, it is possible that low temperatures are not sufficient to overwhelm the genetic component to sex ratio as they may at high temperatures. Alternatively, this asymmetric response to temperature treatments also supports the idea that different genes may be involved in sex determination only in a subset of temperatures (Conover *et al.*, 1992). Therefore, the genes that contribute to sex determination at low temperatures would involve a subset of one to many genes that are non-responsive to temperature.

Population sex ratios at 15^{o}C and 28^{o}C were used to calculate the level of TSD at each location (Conover and Heins, 1987B). Populations in the southern latitudes exhibit maximal levels of TSD, which are ~0.7-0.8, indicating that temperature has little to no impact on sex ratio in 20-30% of the population. This is consistent with TSD in other teleosts where G X E interactions infrequently produce a 100% shift in sex ratio under environmentally relevant temperatures (Conover, 2004). Level of TSD clearly shifts between N 38° (northern MD) through N 40° (New Jersey) and then declines linearly with latitude to pure GSD between N 40° and N 44° (northern Gulf of Maine). Levels of TSD are low in five of the populations within these latitudes which indicates that these five populations have nearly pure GSD (i.e. level of TSD is not significantly different from zero). In these populations where season length is short (<140 days) and interannual variability in SST is greatest, GSD is favored over TSD (Conover and Heins, 1987A,B). The northernmost breakpoint in the level of TSD occurs in the northern Gulf of Maine and Bay of Fundy where GSD is favored, then increases in the more isolated populations that exist in the shallow, warm environments of the Gulf of St. Lawrence.

Season length appears to only partly explain the form of sex determination in *M. menidia*. The addition of 19 sites to Conover and Heins' (1987A) data (Figure 1.3) demonstrates that this relationship is not linear as was predicted based on the 7 sites previously evaluated. In the present study, the level of TSD is positively correlated with season length for growing seasons shorter than ~220 days. Above this "threshold", TSD shows minimal increase. Season lengths of >220 days are common from Florida through Virginia (N 30°-38°) and the shift in the level of TSD occurs just north of this "threshold value" of TSD. Additionally, the temperature range between winter and summer may prove to be important in structuring the pattern of TSD. For example, the mean temperature range (i.e. where the temperature fluctuation between seasons is greatest) is largest in Sandy Point, MD (N 39.0°), which coincides with the location in the shift from a high level of TSD toward GSD. The length of the growing season also demonstrates two major breakpoints between N 38° and N40°, coinciding with the area between northern Maryland and central New Jersey. Analysis of multiple environmental variables would be necessary to predict the selective pressures structuring this cline.

Level of TSD in *M. menidia* does not exceed 0.8 which means that at least 20% of the individuals in a population have sex determination that is not influenced by temperature, regardless of season length (Conover and Heins, 1987A,B). This poses the question, what selective factors are maintaining temperature insensitive genotypes in these southern populations? Environments in these latitudes are fairly stable in a given year, growing seasons are protracted, and temperatures warm at a slower rate than northern estuaries, all of which favor TSD. Maintenance of thermal insensitive genotypes may be adaptive in these populations because it prevents extreme swings in sex ratio. Population mixing between southern estuaries may be lower than in northern sites where populations exhibit strong mixing following offshore migration (Clarke, 2007). Because *M. menidia* do not overwinter offshore in southern latitudes (Shultz *et al.*, 1998), mixing may be reduced and therefore strong sex ratio skews could occur under the right conditions (for example, a shortened spawning season) if populations exhibited full TSD (100% male or female sex ratios in a given environment). Fisherian selection would then result in maintenance of some temperature insensitive genotypes (Charnov and Bull, 1985; Conover and Heins, 1987A; Conover *et al.*, 1992). Most fish that exhibit TSD also display a mild to moderate genetic component to sex determination that functions irrespective of the environment, with the exception of pejerrey, *Odontesthes bonariensis*, where sex ratio skews of 100% occur at environmentally relevant temperatures (Strussmann *et al.*, 1996; Conover, 2004). The occurrence of temperature insensitive genotypes in these populations is likely to provide an adaptive benefit that depends on the selection pressures and patterns of gene flow in southern environments.

Intermediate environments presumably have environmental forces that select for mixed TSD/ GSD sex determination due to moderate interannual stability in temperatures and medium-length growing seasons, which favor flexibility in sex determination. Therefore, some TSD is favorable under certain environmental conditions and is maintained at a reduced prevalence. Conover *et al.* (1992) showed that the form of sex ratio is capable of evolving by losing ESD or by the population shifting its response to the environmental temperature. Interestingly, the shift in population sex ratios across these intermediate latitudes (Figure 1.2) appears to differ between temperature treatments. For example, the magnitude of the slopes of population sex ratio regressed

onto latitude between N 40° and N 44° differs. The slope for the 28°C treatment is 0.078, while the slope of the 15°C treatments is -0.019. The absolute values of the slopes indicate that the response to 28°C shifts more rapidly across these latitudes than does the response to 15°C temperatures, indicating a difference in temperature response across these latitudes. This may help to explain the cline in the level of TSD across these mid-latitudes.

Several environmental variables demonstrate steep declines across the same latitudes that coincide with the shift from TSD to GSD. Steep shifts in the environment across these intermediate latitudes could select for changes in the frequency of temperature sensitive genes, lead to differences in the response to temperature across populations and/or lead to behavioral changes, all of which may help to structure this cline. For example, maximum yearly temperature declines from 31°C to 17°C between N 38° and N 44° (data not shown). Additionally, breeding season length may change rapidly across these latitudes. Populations south of this cline may behave differently by overwintering within estuaries and near coastlines in these locations (Shenker and Dean, 1979), while northern populations more frequently migrate onto the continental shelf to overwinter (Conover and Murawski, '82, Conover and Ross, '82). Therefore, this location between MD and NJ may indicate a break in the prevalence of overwinter mortality, which would select for populations with GSD. Without experimental confirmation, single environmental or behavioral variables cannot be attributed to this shift in sex determining mechanism, but these data do suggest that this region is an area of steep environmental and thus, likely behavioral change. Year to year trends in TSD across this range show the same pattern each year (2005-2007) indicating that this pattern is not anomalous.

Intermediate TSD is maintained throughout the Gulf of St. Lawrence, possibly due to environmental similarity to NY/MA latitudes (Conover *et al.*, 1992; Conover and Heins, 1987A). The Gulf of St. Lawrence is a wide, shallow basin that warms rapidly over the summer, despite its location in high latitudes (Conover and Heins, 1987A). However, analysis of the SST regime in this location is not possible at this time because temperature data only exists for open or deep (>3m) water. However, *M. menidia* are likely to school in the upper few meters of water column, above the thermocline. The

areas around Prince Edward Island and the Magdalen Islands provide small pockets of warm water in enclosed bays and small estuaries that are warmer than surrounding waters and thus create an environment that more closely resembles an intermediate latitude location (Conover and Heins, 1987A). Due to lack of data within these warm areas where *M. menidia* are located, accurate measures of growing season cannot be used in this analysis.

Very little is known about the adaptive benefit of TSD in the 50+ fishes it has been identified in. Yamahira and Conover (2003) identified the pattern of TSD across a latitudinal gradient in a southern congener, *M. peninsulae*. Across *M. peninsulae*'s range, the species shifts from an annual breeding season in the north (mid-Florida, N 29^{o}) to a semiannual season in the southern end of its range, which extends to Veracruz, Mexico (N 22^{o}) (Middaugh and Hemmer, 1987; Yamahira and Conover, 2003). In this species, the adaptive benefit of TSD exists in the southern population(s), where temperature is a good cue for length of the growing season an individual will experience. In semiannual populations, the link between temperature, the time of larval development and the benefit of becoming one sex over the other is broken because breeding occurs year round. Therefore, GSD is the optimal strategy in these populations. This pattern in TSD and GSD and the explanation that growing season length is an important selective force driving the pattern is consistent to what we have observed in *M. menidia*. Both short growing seasons (in *M. menidia*) and long growing seasons (in *M. peninsulae*) select for GSD. Therefore, growing season may play an important role in explaining the adaptive significance of environmental sex determination in many fishes that have this form of sex determination.

The latitudinal pattern of the form of sex determination in *M. menidia* is a complex pattern that appears to only be partly structured by season length. Level of TSD clearly represents some major shifts across areas of environmental change, but appears to be optimal for each local population. *M. menidia* represents an interesting species for addressing these patterns along a spatial scale, but further work must be carried out to address the relative importance in the life-history characteristics that ultimately determine timing of sex differentiation.

Table 1.1. Sites and years of *M. menidia* collection for the level of temperature-dependent sex determination

Site no.	Location	Latitude	Year
1	Amelia Isl., FL	30.52	2007
2	Jekyll Isl., GA	31.02	2006
3	Edisto River, SC	32.65	2007
4	Pawleys Isl., SC	33.40	2006
5	Topsail, NC	34.37	2007
6	Hatteras Inlet, NC	35.18	2006
7	Oregon Inlet, NC	35.77	2006
8	Norfolk, VA	36.97	2007
9	Chincoteague Isl., VA	37.93	2007
10	Solomons, MD	38.38	2006
11	Cape May, NJ	38.95	2007
12	Sandy Pt., MD	39.00	2006
13	Rutgers, NJ	39.56	2007
14	Sandy Hook, NJ	40.40	2006
15	Patchogue, NY	40.75	2005
16	Southold, NY	41.08	2007
17	Waquiot, MA	41.55	2005
18	Naragansett, RI	41.63	2006
19	Duxbury, MA	42.05	2007
20	Essex, MA	42.63	2007
21	Kittery Pt., ME	43.08	2006
22	Freeport, ME	43.83	2007
23	Broad Cove, ME	44.03	2005
24	Bar Harbor, ME	44.42	2006
25	Halifax, NS	44.65	2007
26	St.Andrews,NB	45.08	2005
27	Minas Basin, NS	45.20	2007
28	Joggins, NS	45.50	2005
29	Brule, NS	45.75	2007
30	Alberton, PEI	46.78	2005
31	Magdalen Isl., QE	47.40	2006

Table 1.2. Akaike's Information Criteria (AIC) and respective weights (W_i) for best-fit piecewise linear regression of TSD data. The 3 segment model is the best approximation, given the data.

Model	K	RSS	AIC	Δ_{AIC}	W_i
5 seg.	11	0.36	42.44	-4.35	0.10
4 seg.	9	0.38	43.62	-5.53	0.17
3 seg.	7	0.36	46.42	-8.34	**0.70**
2 seg.	5	0.71	38.09	0.00	0.01
1 seg.	3	0.74	39.37	-1.29	0.02

K represents the number of parameters in the model and RSS is the residual sum of squares.

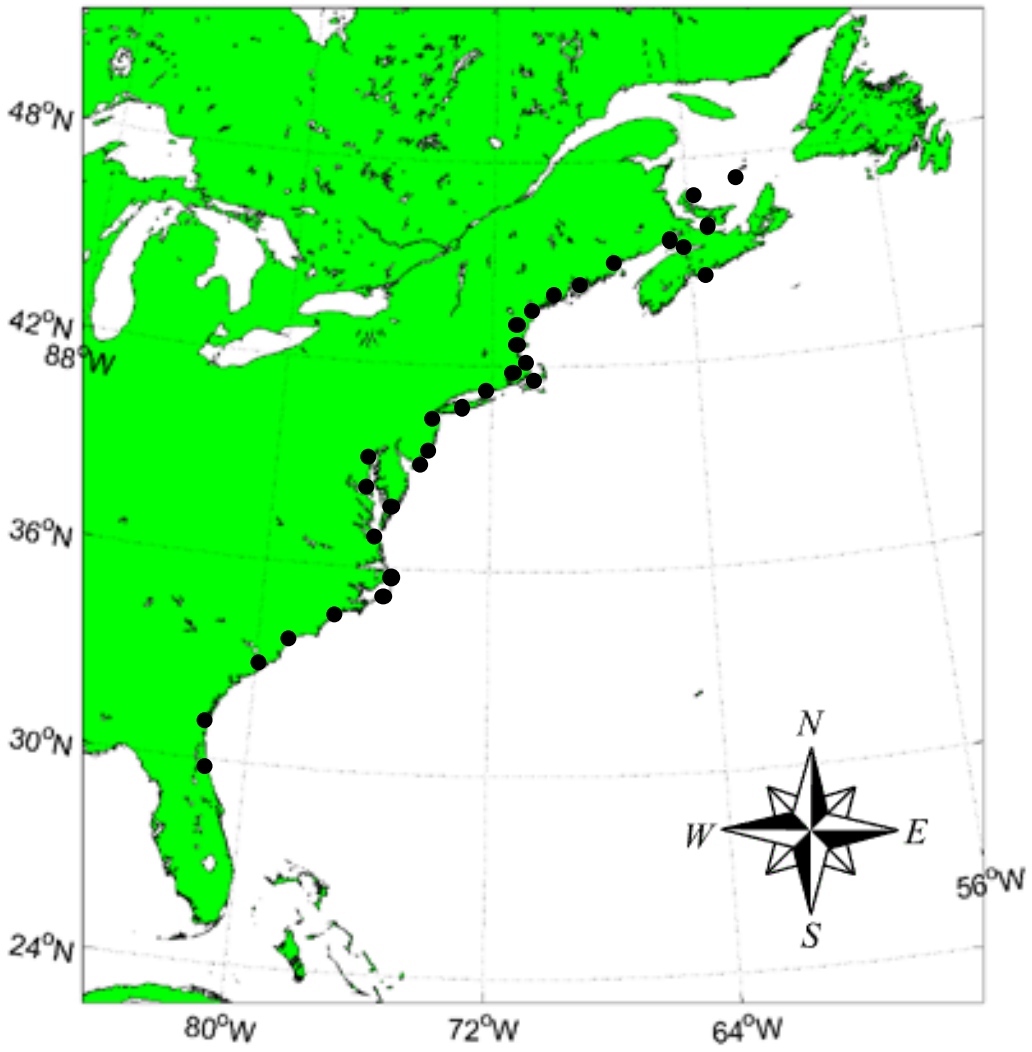

Figure 1.1. Map of *Menidia menidia* collection sites (2005-2007). Populations used for histological comparisons of development rate are in white.

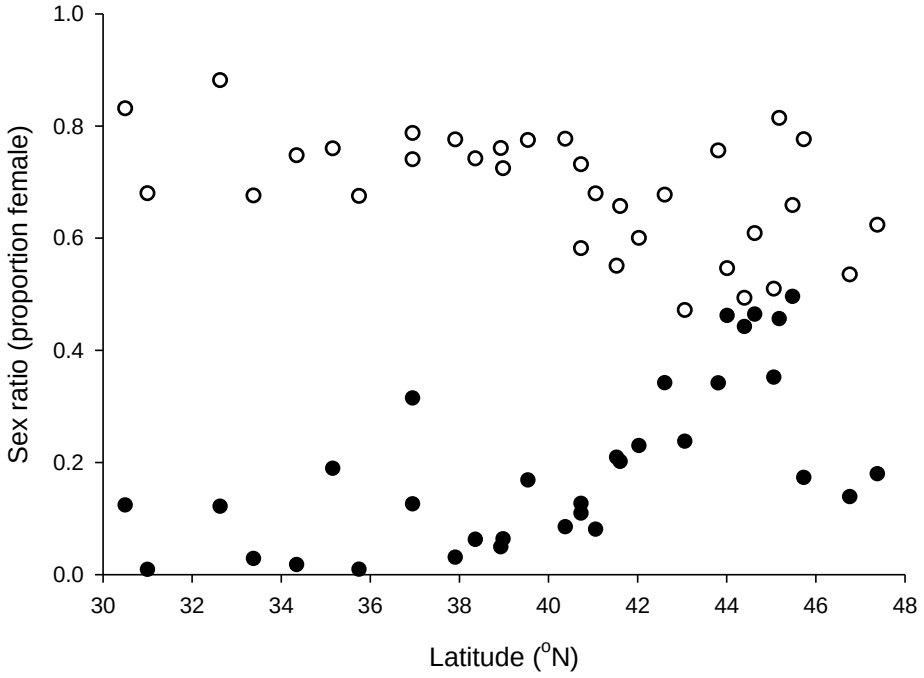

Figure 1.2. Mean replicate sex ratios from 15°C treatment (white circles) and 28°C treatments (black circles) from each location. Replicate means are weighted by the number of fish from each replicate.

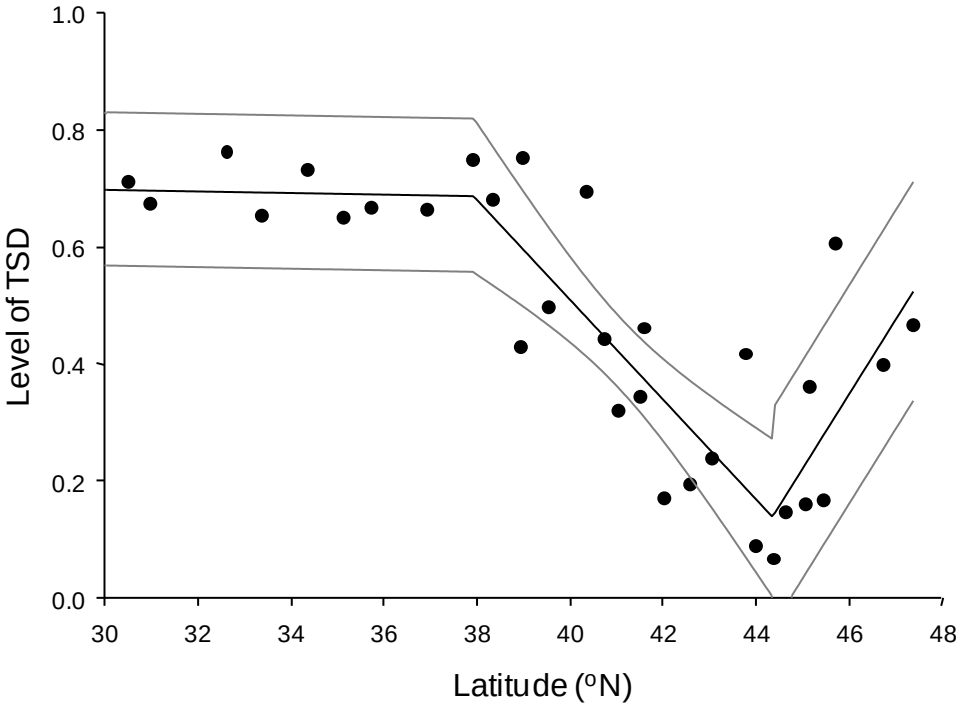

Figure 1.3. Variation in the level of temperature-dependent sex determination (TSD) with latitude. The solid line is the best fit piecewise linear regression model (3 segments) with the 95% confidence interval in grey.

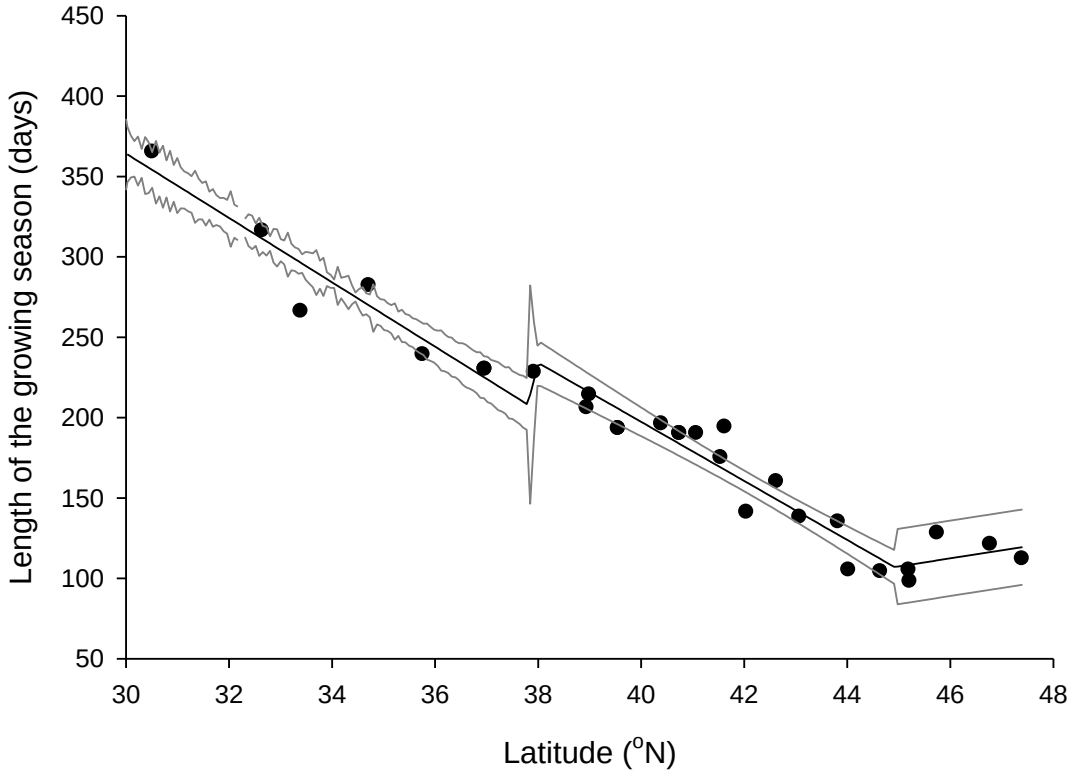

Figure 1.4. The relationship between length of the growing season (defined as mean number of days where SST $\geq$ 12°C) with latitude. The solid line is the best fit piecewise linear regression model (4 segments, AIC weight=0.7) with the 95% confidence interval in grey.

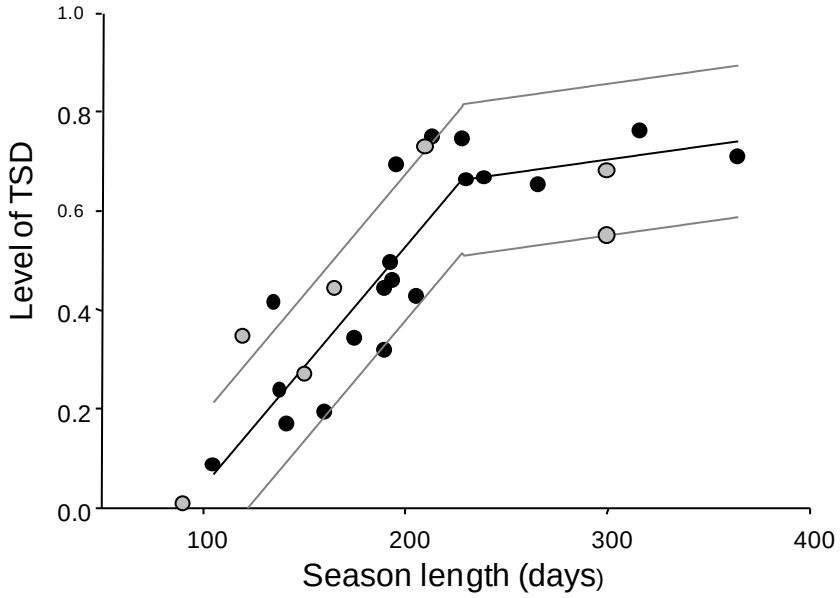

Figure 1.5. Variation in the level of TSD related to season length (mean number of days where SST $\geq$ 12°C). The solid line is the best fit piecewise linear regression model (2 segments) with the 95% confidence interval in grey. Data from Conover and Heins (1987A) are superimposed over this pattern in grey circles.

Chapter 2: Local adaptation in *Menidia menidia* determines gonadal development rate along a latitudinal cline

Abstract

The Atlantic silverside, *Menidia menidia*, exhibits local adaptation in the form of sex determination and growth rate throughout its range, spanning from Florida, U.S.A. to the Gulf of St. Lawrence, Canada. Along this steep environmental gradient, the form of sex determination shifts from temperature-dependent sex determination (TSD) to genetic sex determination (GSD). Additionally, *M. menidia* exhibits countergradient variation (CnGV) in growth rate with higher latitude populations exhibiting intrinsic growth rates that exceed those seen in southern populations. Southern *M. menidia* do develop morphologically distinct gonads at a later size (~2-3mm longer) than northern conspecifics, indicating that life-history traits that differ among populations could be guiding the timing of differentiation and development. To determine if the form of sex determination or growth rate has a predominant influence on gonad differentiation and development, larval and juvenile gonads from four populations, representing four different combinations of growth rate and level of TSD, were assessed for histological features that indicate sexual differentiation and development. Patterns of gonad differentiation do not demonstrate a clear latitudinal trend, but development among populations indicates a latitudinal trend indicating that growth rate may play a bigger role in development. However, these data suggest that multiple life-history traits, including form of sex determination and growth rate, are likely to play significant roles in structuring the timing of sex differentiation. Further work must be carried out to address the relative importance in the life history characteristics that ultimately determine timing of sex differentiation.

Introduction

Tissue differentiation and development is an integrated process in vertebrates. Individuals must have tissue and organ systems that develop at similar rates so that all processes are coordinated within an individual (Adkins, 2008). However, there is disagreement in the literature over whether certain life-history strategies such as fast growth necessarily constrain developmental rates within an organism or if additional life-history traits in the same organism will override these constraints and allow for developmental flexibility (Smith *et al.*, 1985; Kavanagh, 2003; Adkins, 2008). Life-history traits such as sex determination, age at maturity and growth are highly plastic in teleost fishes and can be strongly influenced by environmental parameters, even in fish that have genetic sex determination (Baroillier and D'Cotta, 2001). Traits like somatic growth and the form of sex determination may, in tandem, influence differentiation and development of distinct gonads. However, it is possible that selection optimizes the influence of these life-history traits on rates of differentiation and development, leading to a scenario where either the growth rate or the form of sex determination is the primary determinant of patterns of gonad development.

The Atlantic silverside, *Menidia menidia*, is an estuarine species that is common to the northwest Atlantic, from Florida, U.S.A. to the Gulf of St. Lawrence, Canada. *Menidia menidia* exhibit a latitudinal gradient in sex determination that ranges from predominantly temperature-dependent sex determination (TSD) in southern populations to genetic sex determination (GSD) in northern environments (Conover and Heins, 1987A). In fish from South Carolina where TSD prevails, sex determination is determined by temperature sensitive genetic factors that respond to environmental cues (temperature) shortly after hatch (G X E interaction) (Conover and Heins, 1987B; Lagomarsino and Conover, 1993). Fish from the Annapolis River in Nova Scotia, Canada have GSD owed to major genetic factors that are temperature-insensitive. Intermediate populations between these two environments and populations within the Gulf of St. Lawrence exhibit sex determination that is a mixture of TSD and GSD. The major selective force shaping this pattern is hypothesized to be the length of the growing season, which is positively correlated to the level of TSD (Conover and Heins, 1987A).

Growth rate also exhibits a positive latitudinal cline in *M. menidia* (Conover and Present, 1990). Fast growth maximizes body size at the end of a shortened growing season at higher latitudes, a pattern called countergradient variation (CnGV) (Conover and Schultz, 1995; Conover *et al.*, 2009). CnGV results when genetic traits oppose environmental influences, resulting in a higher or lower trait value than would be predicted based on plasticity alone (Conover *et al.*, 2009). Fast rates of tissue differentiation (here identified as the fate of primordial germ cells to develop into a particular cell type) and proliferation (the replication and growth of a particular cell type) often co-occur with fast somatic growth. Timing of tissue differentiation and development rate often parallel overall somatic growth in an organism since many tissue types, organs and structures are needed to support growth. However, fast growth often results in tradeoffs like reduced skeletal ossification relative to size in fast-growing bluegill sunfish, *Lepomis gibbosus* (Arendt and Wilson, 2000). Frequently, locally adapted populations exhibit development rates that are genetically based and counter the environmental gradient (CnGV) (DiMichele and Westerman, 1997; Laugen *et al.*, 2003; Sanford *et al.*, 2006). Most of these studies focus on the development rate of a whole organism (i.e. embryonic development, time to metamorphosis) and do not focus on differentiation of individual tissue types or organs. Addressing development at the level fo the tissue may provide a more informative platform for understanding tradeoffs from the adoption of one life-history trait over another.

Reproductive development in *M. menidia* is dependent upon population, as fish from southern latitudes (i.e. Florida to Maryland) do not develop morphologically distinct gonads as early as northern fish when reared at the same temperature. Further, a larger body size (i.e. 2-3 mm longer) must be attained in southern fish before sex can be determined macroscopically (Conover and Duffy, personal observation). Presumably, sex differentiation that occurs a few days earlier or later provides little or no adaptive benefit since all individuals mature at year one. Therefore, differences in timing of differentiation may be constrained or "dragged" along by selection on other life-history traits.

Bull (1983) argued that TSD is a physiological constraint to early sex differentiation because individuals must wait for a cue(s) from the environment to initiate

sex differentiation. This strategy results in a high degree of plasticity in both the timing and outcome of gonad differentiation and development. Populations with GSD are expected to demonstrate considerably less plasticity because sex is determined in the embryo and therefore, differentiation could begin earlier than in TSD populations. Alternatively, Kavanagh (2003) contends that development is fixed by a "molecular switch" that due to inherent differences in growth rate. Because he patterns of growth rate and sex determination in *M. menidia* oppose one another, this presents an opportunity to determine whether the form of sex determination or growth rate more strongly influences the timing of gonad differentiation and subsequent rates of development.

To compared gonad differentiation and development, I used four populations that exhibit different combinations of growth rates and forms of sex determination. I identified major structures in developing gonads to determine both the timing of appearance and the absolute sizes of these structures in populations that were exposed to feminizing (15°C) and masculinizing (28°C) temperatures then compared these to the pattern of growth rate and form of sex determination. Based on previous observations, both gonad differentiation and development were expected to occur sooner and at smaller sizes in the fast-growing, northern populations relative to the southern populations.

Methods

Gonad differentiation in relation to growth and type of sex determination

Embryos were collected from four *M. menidia* populations in 2007, spanning the species' range. Embryos were collected from spawning adults from Amelia, Florida (FL), Patchogue, New York (NY), Minas Basin, Nova Scotia (MB) and Brule, Nova Scotia (BR) (Table 2.1). These sites were chosen to represent four unique populations; 1) high TSD, slow growth, 2) intermediate TSD, intermediate growth, 3) fast growth, pure GSD and 4) fast growth, intermediate TSD, respectively. Spawning adults were collected with a 100' seine net and stripped spawned onto four 10 X 10 cm mesh screens following Lagomarsino and Conover (1993). Six to eight adults of each sex were used for each screen to ensure mixed parentage. Once embryos had water hardened, they were moved into aerated, coolers kept at 15°C on ice and transported to Bluepoint Marine Laboratory,

West Sayville, NY. Multiple mesh screens from each site were suspended in aerated 18 L, flow-through containers at 21°C until embryos hatched. After yolk absorption, fry were fed *Artemia* sp. nauplii (Brine Shrimp Direct, Ogden, UT) supplemented with larvae/fry pelleted food (250 µm) (Otohime Hirami, Japan).

When fry reached approximately 7 mm TL, forty to sixty larvae were acclimated to 15°C or 28°C treatment containers. All rearing was carried out as previously mentioned. Larvae and juveniles were collected for histological analysis throughout the period of sex differentiation, which occurs when fish are between 8 and 21 mm TL in fish from New York (Conover and Fleisher, 1986) to ensure that a range of fish sizes could be used for interpopulation comparisons. In the 28°C treatment, six to eight fish were collected every two to three days, euthanized in MS-222 and stored in 10% NBF. Individuals from the 15°C treatments were collected approximately once per week reflecting slower growth rates.

For histological processing, individuals were grouped by length and the head and peduncle were trimmed off leaving the abdomen with developing gonads intact. Groups of individuals of equal length were prepared for sectioning and coded for blind-reading. Abdomens were embedded in paraffin and sectioned to a thickness of 6 µm with a microtome. Transverse sections of the abdomens were stained with hematoxylin and counterstained with eosin (H & E) and several sections (6-30 µm apart from each other) were placed on the same slide to ensure multiple views of gonadal cross sections (Dana-Farber/Harvard Cancer Center, Specialized Histopathology Services, Boston, MA).

Gonadal cross-sections were viewed with a Nikon Eclipse E400 microscope. Digital photomicrographs were taken of representative developing gonads at 400X magnification. Multiple features of developing gonads were scored blindly using numerical codes for each group of fish. Features were scored as present/absent and the gonad cross section size, area of connective tissue, area of vascularization, number of germ cells, and number of somatic cells were calculated using Image J software (NIH, USA).

Gonads were scored for the presence or absence of several key features in development. These included germinal epithelium, vascularization, germ cell mitosis, germ cells, somatic cells, aggregations of stromal cells, somatic elongations, ovarian

lumen, oogonium, sperm ducts and testicular lobules. Additionally, number of integumentous connections was observed but not included in analyses because histological sectioning tended to rupture these connections. Germ cell meiosis was also observed infrequently and therefore not included in analyses. Additionally, gonad surface area, percentage of the gonad that was vascularized or had connective tissue and the germ cell/somatic cell ratio were also calculated. Connective tissue was observed in larger developing gonads by the presence of light pink, stringy or spongy nondescript tissue often contained in the center of the developing gonad radiating toward the gonad periphery.

Differences in the timing of appearance of some structures were observed and these were modeled as binomial data (presence/absence) using binary logistic regression with the SPSS statistical software package (SPSS, Inc.). The logistic regression function (Θ) estimating the probability of development was modeled as:

$$\theta = \frac{e^{(a+\beta_1 x_1)}}{1 + e^{(a+\beta_1 x_1)}}$$

where α is the model constant, x is length and β is coefficient of the predictor variable. Comparisons among populations were carried out with population in the model as a categorical variable. Predicted probabilities of development of a given trait were significant at $p<0.05$. Both gonads within an individual were scored if possible, and all developing gonads were treated independently for analysis because gonad development is partially independent within an organism.

Comparisons between temperature treatments were carried out using ANCOVA to compare regression line slopes. Data collected using Image J (gonad surface area, (percent total surface area vascularized, percent total surface area with connective tissue and the germ/somatic cell ratio) was analyzed for differences among populations using 1-way ANOVA. Linear regression was used to test for significant increases in germ cell numbers with size. These statistical analyses were carried out using SigmaStat software package (Systat software, Inc.).

Results

Histological analysis of gonad development

Histological analysis of developing gonads from 10-20 mm TL revealed key differences between temperature treatments as well as differences among populations in development rate. A total of 244 individual fish were used in the analyses and a total of 364 individual gonads were scored individually for various developmental and differentiation features in the gonad. Both developing gonads were present in 67% of the fish surveyed. One gonad was visible in sections from the other 23%. No abnormal development was observed and no intersex individuals were identified. Level of TSD in the four populations compared ranged from 0.36 to 0.71 and growth rate at 28ºC ranged from 0.6 to 0.93 mm d^{-1}(Table 2.1). Growth rate at 15ºC was depressed in all four populations and ranged from 0.1 to 0.23 mm d^{-1}.

Gonad development of both male and female fish followed similar patterns to that of documented in other teleosts. Developing gonads were located attached to the mesentery along the dorsal peritoneum (Figure 2.1). Gonadal development originated from primordial germ cells (PGCs; identified by a round, prominent nucleus), which increased in number by migration and mitosis (Figure 2.2 A), leading to a gonad with germ cells accompanied by somatic cells. In presumptive females, somatic elongations formed in fish beginning around 12 mm TL in the 28°C treatment. These elongations consisted of projections of somatic cells which eventually formed the ovarian cavity (Figure 2.2 B). Increase in the number of germ cells is hypothesized to be a general indicator of ovarian development. However, in this study, the number of germ cells did not increase overall in either temperature treatment. Florida (FL) at 15ºC was the only population to exhibit a positive increase in the number of germ cells with increasing fish size (linear regression, $p < 0.001$, $r^2=0.47$).

All individuals were considered undifferentiated until either a fully formed ovarian lumen, often coinciding with the presence of oogonia, was observed in presumptive females, or testicular lobules or sperm ducts were observed in presumptive males. Additionally, presumptive females often were observed with two points of attachment of the gonad to the dorsal mesentery, while presumptive males displayed only one attachment point (Paull *et al.*, 2008). Gonad shape was often indicative of

developing sex. Males had club- or triangular-shaped testes and female ovaries were generally round or oval in shape (Strussmann *et al.*, 1996; van Aerle *et al.*, 2004), although this was not used to distinguish differentiated from undifferentiated fish. Fully differentiated individuals with either primary oocytes or spermatocytes were not observed in this study, but several larger individuals were cross-sectioned for comparison with smaller fish (Figure 2.2 C). No intersex individuals were observed in this study.

Two major structural differences were noted between the gonads of individuals from the feminizing (15°C) and masculinizing (28°C) treatments (Figure 2.3). First, the average percentage of gonad that contained connective tissue increased with fish length in both temperature treatments (linear regression, 15°C treatment $p<0.001$, 28°C treatment $p =0.012$), but this increase was steeper for the 15°C treatment (ANCOVA, $p=0.019$, Figure 2.3 A). Therefore, fish reared at 15°C had higher percentage of connective tissue than equally sized fish at 28°C. The other major difference observed between temperature treatments was in the percentage of gonad surface area that was vascularized, which was defined specifically as the proportion of gonad surface area that contained blood vessels. The percentage of the gonad that was vascularized did not increase in the 15°C treatment (linear regression, $p=0.987$), but did increase in the 28°C treatment ($p=0.041$). Additionally, the slopes of these regression lines differed significantly (ANCOVA, $p=0.035$), indicating a significant increase in vascularization in the high temperature treatment (Figure 2.3 B).

In general, development of individuals at 15°C was suppressed and very few (≤ 3) individuals in each population exhibited presumptive male or female development. For example, individuals in FL exhibited small gonads that consisted of small clusters of somatic and germ cells, but few other developmental features indicating sexual differentiation. Comparisons of the predicted development of vascularization, germinal epithelium, mitotic germ cells and stromal aggregations were limited by few individuals containing these features in the largest size classes. Additionally, gonad surface area (μm), % vascularization and germ/somatic cell ratio comparisons revealed no significant differences among populations. Major structural features differed among populations and are illustrated in Figure 2.4. Populations differed in % connective tissue within the gonad, with NY having the highest amount of connective tissue but the other populations

exhibited no differences (data not shown). Due to the limited differentiation and development of individuals in this temperature treatment, further analyses are focused mostly on the 28°C treatment.

Primordial germ cells were observed in few individuals from either temperature treatment, and were generally observed in fish less than 12 mm TL. A minimum of two PGCs or germ cells (GCs) were always present, indicating that germ cell migration into the gonad had already begun in most of the individuals. Therefore, analyses on the appearance of structural features focused on germinal epithelium, vascularization, mitotic germ cells and stromal cell aggregations. Plots of the probability of observing a particular trait revealed that the appearance of traits became more frequent with increased length (Figure 2.5). Interpopulation comparisons indicated that these structures were generally first seen in NY, followed by BR or MB and occurred latest in FL (Figure 2.5). Additionally, the predicted probability of 50% of the individuals having a particular feature routinely differed by $\leq$ 10 mm between the fast (NY) and slow (FL) differentiating populations. No observations of either mitotic germ cells or somatic cell aggregations were made in gonads in fish from FL, so these fish are therefore assumed to be delayed relative to the other three populations (Figure 2.5 C,D). The BR and MB populations differed significantly for all four traits, with BR generally developing these traits faster than MB. The general trend for appearance of these traits was NY > BR > MB > FL.

Additionally, I compared the mean gonad surface area (μm), % of area vascularized, % with connective tissue and the germ/somatic cell ratio among populations (Figure 2.6). For these comparisons, the mean of these traits were calculated from fish that were 17-20mm TL in the 28°C treatment. These comparisons revealed significant differences among populations, with gonad surface area and % of connective tissue being largest in BR and MB, respectively and the largest germ/somatic cell ratios present in MB and BR (Figure 2.6 A,C and D). Although non-significant, % vascularization presented a similar trend (Figure 2.6 B). These comparisons indicate that MB/BR > NY/FL for development of gonadal structures.

Discussion

Histological analysis of gonad development

Gonadal differentiation displayed differences among the four populations with differentiation occurring earlier in northern populations than in FL, but these data could not be statistically compared due to few individuals with morphologically differentiated gonads in the larger size classes. However, in all observations, the FL population demonstrated the slowest differentiation and development under both temperatures. Development of major morphological features that were not exclusive to one sex provided the best platform for interpopulation comparisons. These comparisons demonstrated that development does not clearly follow the gradient in either TSD or growth rate. These four populations spanned the gradient in growth rates (slowest in FL to fastest in BR), but no population exhibited full GSD. Therefore, this constrains the interpretation considerably. Gonadal structures indicating differentiation tended to first appear in NY, followed by BR, MB then FL at 28°C. However, once these features appear in gonads, this study demonstrated that several of these structures (connective tissue area, area of vascularization, gonad surface area and germ cell/somatic cell ratio) display a latitudinal trend and are greatest in BR or MB during the later part of the thermosensitive window.

Both differentiation and development were slowest in the 15°C treatments due to depressed growth rates at this temperature (Table 2.1). Two temperature treatments were used in this study to compare differentiation and development between males and females among populations, assuming that sex ratios would be skewed in populations with TSD. Presumably, the 15°C treatment would have provided the greatest number of examples of ovarian development. However, given the few individual gonads that appeared to be differentiated at this low temperature, comparisons are limited to some general qualitative observations of differentiation. Differentiation and development in the 28^{o}C treatments were markedly faster than in the 15°C treatments. This is consistent with work by Conover and Fleisher (1986), who demonstrated that rearing temperature has a significant impact on the length of the window of sex determination in *M. menidia*. Cold temperature (15^{o}C) significantly increased the duration of the window of sex determination, which would explain why differentiation and development were delayed

in low temperature treatments. The largest individuals collected in this study were 20 mm, but the window of temperature sensitivity, and thus differentiation may extend well beyond this size if reared at 15°C. Additionally, *O. bonariensis*, a South American silverside, also exhibits delayed differentiation at low experimental temperatures, demonstrating that temperature (Ito *et al.*, 2005). Most of the comparisons in this study were made with populations reared at 28°C, although observations of differentiation were also limited to a few individuals in the largest size classes.

The two most notable differences between temperature treatments were in connective tissue and blood vessel area within the developing gonads. It is important to note that the temperatures used in this study represent values that are close to the thermal extremes for positive growth in *M. menidia*. While these fish commonly experience temperatures below 15°C in the winter, growth is severely stunted (Conover and Heins, 1987A). Additionally, 28°C represents a temperature that larvae may experience occasionally during the summer months, and above this temperature growth is not increased indicating it is near their thermal maxima (Conover and Present, 1990). Although extreme, these temperatures were chosen to ensure that both female and male development could be compared, especially in the populations with TSD.

Thin, spongy connective tissue was observed in some developing gonads and appeared to be linked to limited gonad development. Connective tissue comprised more of the gonad in the 15ºC treated fish and the relative amount of connective tissue increased more steeply with size in the 15ºC treatment than in the 28ºC treatment. Connective tissue has been documented in both male and female gonads and connective tissue hypertrophy is often associated with endocrine disruption in male testes (Gill *et al.*, 2002; Hano *et al.*, 2007). Excessive connective tissue has been observed in male medaka, *Oryzias latipes,* exposed to a high dose synthetic estrogen alpha ethinylestradiol (EE_2) and in several flounder species from an urbanized river with high concentrations of estrogenic contaminants (Gill *et al.*, 2002; Hano *et al.*, 2007). Low temperatures in this study may induce the production of high levels of natural estrogens in undifferentiated males and females, leading to the production of excessive connective tissue prior to complete differentiation. Another more likely explanation for excess connective tissue could be due to depressed PGC migration into the gonad and mitosis at low temperatures

(Braat, 1999). Additionally, PGC death can occur from a lack of differentiation of cell types and limited somatic cells to support PGCs (Richardson and Lehman, 2010). Increased connective tissue may result from compensation for the lack of germ and somatic cell structure in cold temperature conditions.

Blood vessel area also exhibits different patterns between temperature treatments, with increased vascularization in the 28ºC treatments, but no change in blood vessel area with length at 15ºC. Increased vascularization is expected in many tissue types at high temperatures, due to increased growth rate and metabolic demand at high temperatures. Increase in vascularization is also consistent with patterns of blood vessel development in the gonads of *O. bonariensis* exposed to different temperature treatments. In this species, blood vessels first appear in higher temperature treatments and increase in area with size of individuals (Ito *et al.*, 2005).

Gonad differentiation in *M. menidia* is comparable to other teleosts. The first signs of ovarian development in teleosts are generally found to be an increase in germ cells followed by development of an ovarian cavity (Nakamura *et al.*, 1998; Ito *et al.*, 2005; Lewis *et al.*, 2008; Gao *et al.*, 2009). Germ cell increase was only observed in the FL population and therefore did not provide a consistent measure of ovarian differentiation in *M. menidia*. In this study, I used a conservative approach suggested by Gao *et al.* (2009) to determine ovarian differentiation only when the ovarian cavity is present with either meiotic germ cells or presumptive oogonia. In most histological studies, male differentiation is determined by the formation of a sperm duct and/or seminal lobules (Rasmussen *et al.*, 2006). These features were consistent with male differentiation in *M. menidia*.

In many studies, an individual's sex is distinguishable in histological sections earlier than can be deduced by the morphology of the developing gonad (Nakamura *et al.*, 1998). Conover and Fleisher (1986) demonstrated that the window of sex determination in *M. menidia* from New York occurs between 8 and 21mm TL and that gonads are morphologically distinct as soon as most fish reach 21 mm. Histological structures distinguishing males from females were absent in many of the larger fish used in this study (18-20 mm). There are some possible explanations for this. In *O. bonariensis*, another Atheriniform with TSD, differentiation occurs in an anterior to posterior direction

(Strussmann and Ito, 2005). *M. menidia* abdomens were serially cross-sectioned, covering only about 600 µm of the developing gonad. Therefore, in larger animals, it is possible that an undifferentiated portion of the gonad was sectioned, missing the histological features inherent to male or female differentiation. Male differentiation is delayed relative to female differentiation (Nakamura *et al.*, 1998), and this may limit the number of differentiated individuals that were observed between 18 and 20 mm. Additionally, the window of sex determination shifts with temperature treatments (Conover and Fleisher, 1986), but it may also be shifted among populations, suggested by gene expression of cytochrome P450aromatase in developing *M. menidia* (Duffy *et al.*, 2010). The gene cytochrome P450 aromatase was depressed in fish from South Carolina until presumptive females reached approximately 15 mm, indicating that the window of sex determination is shifted in southern populations, relative to increased expression in a population from Nova Scotia at 8 mm. Differentiation may not be completed in most populations until fish reach larger sizes (>21 mm), but these sized fish were not analyzed in this study.

Interestingly, the probability of structures appearing early in development of the gonad did not increase directly with either the form of sex determination or an increase in growth rate. In all comparisons, blood vessels, mitotic germ cells and stromal cell aggregations first appeared in the mid-latitude population, NY. Similarly, germinal epithelium appeared in NY first, but this was not statistically different from MB. If growth rate were structuring this pattern, we would expect structures to appear first in MB followed by NY and BR, with FL developing last. In fact, the fastest growing population (MB at 28ºC) repeatedly demonstrated a delay in development of these structures relative to NY and BR, which displays relatively slower growth rates than MB. As expected, FL shows delayed development for all metrics assessed.

The effect of the experimental temperatures used in this study may help to explain this interesting pattern in gonad development. *M. menidia* from peripheral regions (FL and BR) are less likely to experience consistent extreme temperatures in their natural environment and would therefore be metabolically stressed (i.e. FL fish wouldn't experience 15^{o}C for an extended length of time). However, NY fish are likely to commonly experience both temperatures used in this study and thus, may be better

adapted to extended periods of low and high temperatures. Maniscalco (2006) found that *M. menidia* from central latitudes allocated more energy to immune system function, rather than somatic growth, than northern and southern populations. Thus, since growth is moderate at this latitude, energy may be shunted into other developmental processes, like gonad differentiation, along with immune function. Therefore, enhanced differentiation in a mid-latitude population may be explained by depressed overall growth in low-latitude populations, resulting in limits to rates of differentiation. In northern populations, trade-offs between growth and other life-history characteristics such as predator avoidance could also limit energy used for gonad differentiation. Therefore, this pattern may be more reflective of constraints in peripheral populations, relative to enhanced rates of differentiation in a mid-latitude population.

The relative size of structures within the gonad however, showed a latitudinal pattern with one or both of the high latitude populations exhibiting the largest gonadal cross section size, and area of blood vessels and connective tissue across populations. This indicates that once structures appear, they grow fastest in high latitude populations. Also, the germ/somatic cell ratio was highest in the two fastest growing populations. Taken together, this indicates that differentiation may be fastest in a mid-latitude population, but development and proliferation are greatest at high latitudes. The analysis here is limited by a lack of fully differentiated individuals in the larger size classes, but development of other features in the gonad is likely to estimate overall rates of development, including male or female-specific structures (Gao *et al.*, 2009). The NY population might demonstrate early differentiation due to an optimal combination of life history traits that favors fast differentiation. Alternatively, maternal effects could also contribute to the pattern in differentiation early in life in this study since embryos were collected from wild adults (Marshall *et al.*, 2008).

Several histological studies have determined that growth rate appears to be related more to size than age (Nakamura *et al.*, 1998; Blazquez *et al.*, 1999; Gao *et al.*, 2009), indicating that growth rate is likely to be an important factor in structuring gonadal development rate. Relationships between growth rate and the timing of development of reproductive organs have been assessed for bluegill sunfish, *Lepomis macrochirus*, and the roach, *Rutilus rutilus*, and both studies found that size and not age, was the principal

determinant in gonad differentiation. Unfortunately, in these studies growth was manipulated experimentally by a combination of food limitation and differences in density rather than using wild populations that exhibit genetically different growth rates. Kavanagh (2003) suggested that growth rate plays a role in determining gonad differentiation, but this has only been demonstrated when growth rate is manipulated by the environment. Patterns of CnGV in growth rates in wild organisms provide excellent examples to compare patterns of development in reproductive systems in relation to growth.

Timing of differentiation and development is clearly delayed in FL relative to the other populations, but is this necessarily costly? *M. menidia* is an annual species that spawns in estuaries along its range following its first winter (Conover and Ross, 1982; Huber and Bengtson, 1999). Therefore, differentiation must occur early in life. Morphological variation between male and female gonad is established by ~23 mm in all fish, regardless of population of origin (Conover and Duffy, personal observation). Therefore, the timing of differentiation differs between 4 and 10 mm across populations. It is unlikely that this small size difference in the timing of differentiation has long-lasting effects on reproductive fitness unless development continues to lag behind somatic growth throughout life. In general, *M. menidia* growth is almost entirely invested into somatic growth during its first growing season, then energy is invested into reproduction when it reaches sexual maturity (Conover and Ross, 1982; Huber and Bengtson, 1999). More than likely, a lag in differentiation and development in FL populations is probably compensated for over a long growing season and during periods of rapid gonadal growth prior to initial spawning. Therefore, slight delays in differentiation are probably minimal. Delayed differentiation may improve fitness for populations with TSD. Because the environment must be a reliable cue for which sex to become, a delayed and/or protracted window of temperature sensitivity may better allow the individual to get an accurate temperature cue from the environment. If a delay in differentiation or a prolonged window of temperature sensitivity reduced reproductive fitness for either sex, it is likely that TSD would have been selected against because the relative fitness benefit for females would be lost.

Growth rate exhibits CnGV leading to the fastest growth occurring in high latitude populations (Conover and Present, 1990; Hice, 2010), but the level of TSD does not exhibit a simple linear latitudinal trend. Therefore, the unique geographic patterns of growth rate and the form of sex determination demonstrate that selection pressures acting on these populations are unique (i.e. the selection pressure creating an almost linear trend in growth rate is likely not the same selective agent creating the more complex pattern of the level of TSD). Gonad differentiation is plastic in fish regardless of the form of sex determination (Nakamura *et al.*, 1998; Blazquez *et al.*, 1999) and the environment can act at several levels of differentiation to fine tune fitness. For gonad development to occur, the fish must respond to environmental cues which are then regulated at the level of the gene and then translated into sex steroids that shape differentiation (Nakamura *et al.* 1998; Godwin *et al.*, 2003). From there, gonad structures can form as the sex of the whole animal is determined.

There are many steps along the path of sex differentiation and sex determination is not as simple as a single molecular switch (Mittwoch, 2000). In all populations, regardless of the form of sex determination, the environment plays a role in the rate of gonadal development, from controlling growth rate to influencing genes involved in the cascade of sex determination. From this study, it is not clear if the form of sex determination or if growth rate has a larger role in structuring differentiation and development in *M. menidia*, but these data indicate that they both play a role in differentiation and development at what is likely to be several levels of organization. Additionally, patterns of gonad differentiation and development do not demonstrate a clear latitudinal trend, indicating that multiple life-history traits, including form of sex determination and growth rate probably play significant roles in structuring the timing of sex differentiation among locally adapted species.

Table 2.1. Level of TSD and growth rate for *Menidia menidia* at 28°C and 15°C. Growth rate data is taken from Hice (2010).

Population	Latitude (degrees N)	Level TSD (± S.D.)	Growth rate at 28°C ($mm\ d^{-1}$ ± S.D.)	Growth rate at 15°C ($mm\ d^{-1}$ ± S.D.)
Amelia, FL (FL)	30.52	0.71 ± 0.16	0.60 ± 0.05	0.10 ± 0.00
Patchogue, NY (NY)	40.75	0.44 ± 0.11	0.86 ± 0.07	0.23 ± 0.03
Minas Basin, NS (MB)	45.20	0.36 ± 0.20	0.93 ± 0.07	0.22 ± 0.01
Brule, NS (BR)	45.75	0.60 ± 0.10	0.86 ± 0.25	0.21 ± 0.01

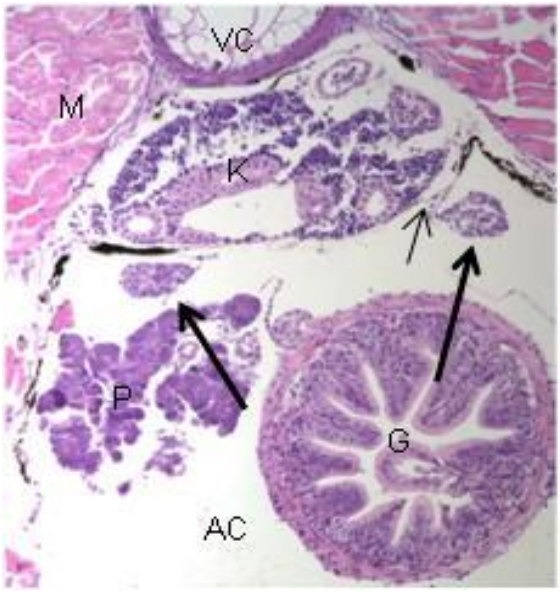

Figure 2.1. Developing gonads are located dorsally in the abdominal cavity (AC). Gonads are attached to the abdominal cavity mesentery, indicated by the thin arrow. Thick arrows indicate the position of gonads (here presumptive ovaries). M-muscle, VC-vertebral column, K-kidney, P-pancreas and G-gut. (200x magnification).

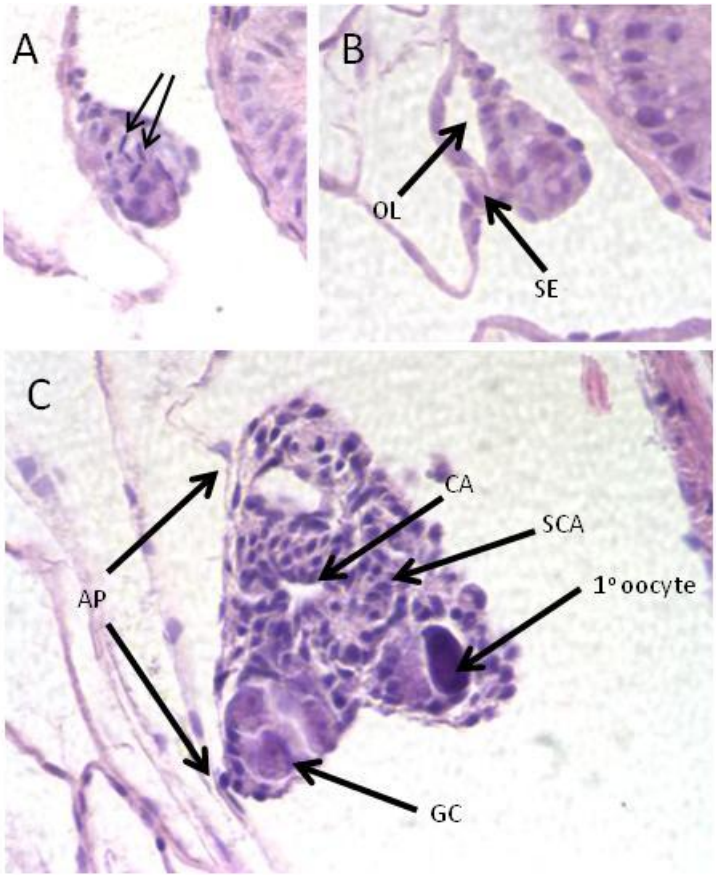

Figure 2.2. Ovarian differentiation in *M. menidia* from NY. A) Mitotic figures (arrows) seen in a 10 mm individual, B) somatic elongations of stromal cells forming an ovarian cavity (14mm) and C) a differentiated female (24mm) with a presumptive primary oocytes. SCA-stromal cell aggregation, CA-central arteriole, AP-attachment point and GC-germ cells (400x magnification).

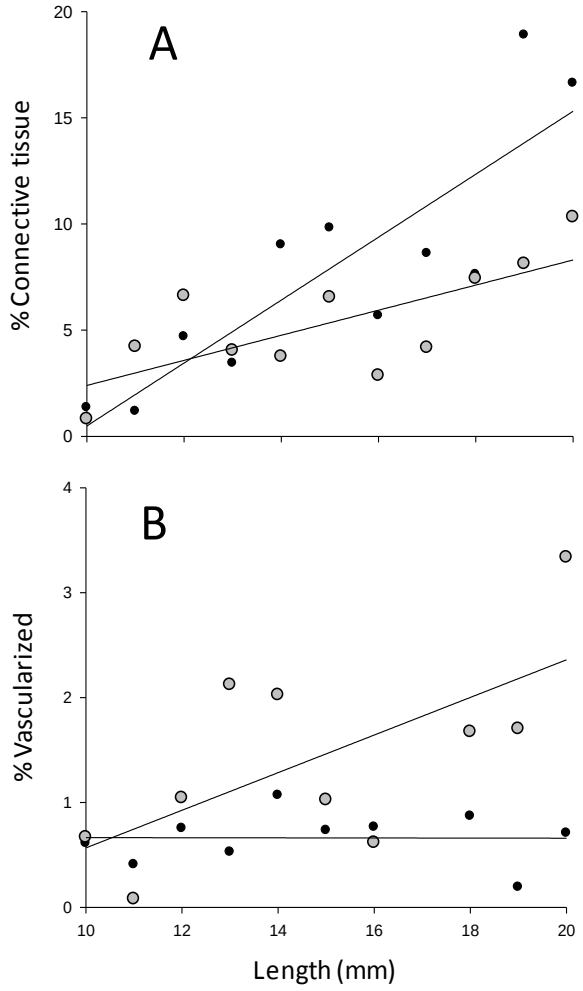

Figure 2.3. Mean change in (A) % connective tissue and B) % vascularization in the developing gonad. Regression lines for each trait are significantly different from each other (ANCOVA 15°C, p=0.019 and 28°C, p=0.035). Black circles are the mean values for 15°C treatments, grey circles are mean values for the 28°C treatments.

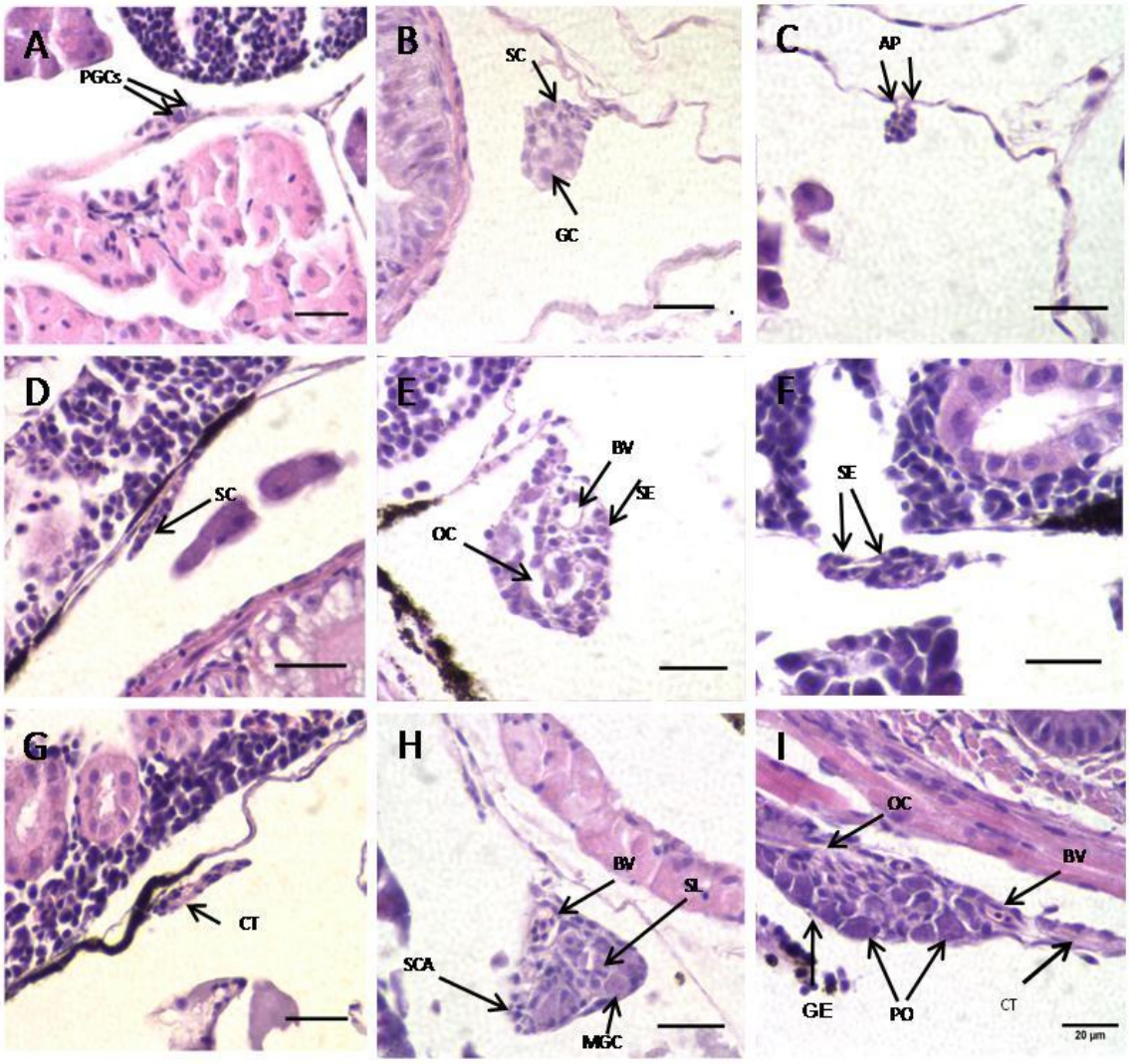

Figure 2.4. Sexual differentiation among fish reared at 28°C. Columns represent three of the four populations and rows represent different size classes. Column 1 (A,D,G) is the Amelia Island, Florida (FL) population, column 2 (B,E,H) is the Patchogue, New York (NY) population, and column 3 (C,F,I) is from Brule, Nova Scotia (BR). Individuals in row 1 (A,B,C) are 10-11mm, Row 2 (D,E,F) are 14-15mm and row 3 (G,H,I) are 19-20mm. PGCs –primordial germ cells, SC-somatic cells, GC-germ cells, AP-attachment point, OC-ovarian cavity, BV-blood vessel, SE-somatic elongation, CT-connective tissue, SCA-somatic cell aggregation, SL-seminal lobule, MGC-mitotic germ cell, GE-germinal epithelium, PO-presumptive oogonium. All scale bars are 20 µm.

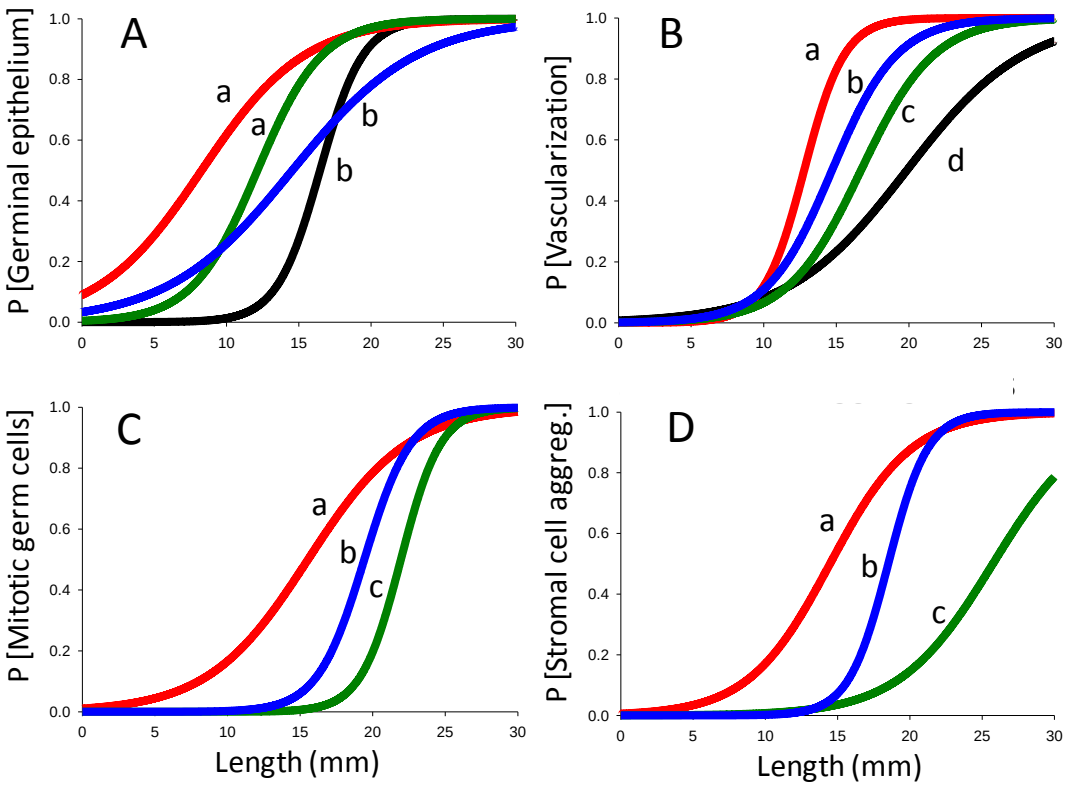

Figure 2.5. Modeled probability of developmental indicators versus length in 28°C treatments. Indicators are A) Germinal epithelium, B) vascularization, C) mitotic germ cells and D) stromal cell aggregations. Probability curves are color coded; FL-black, NY-red, MB- green and BR-blue. Probability curves of FL data are not depicted in graphs C-D because no individuals were observed to have mitotic germ cells or stromal cell aggregations. Unique letters represent significant differences at $p<0.05$, predicted by categorical comparisons within the binomial logistic regression model.

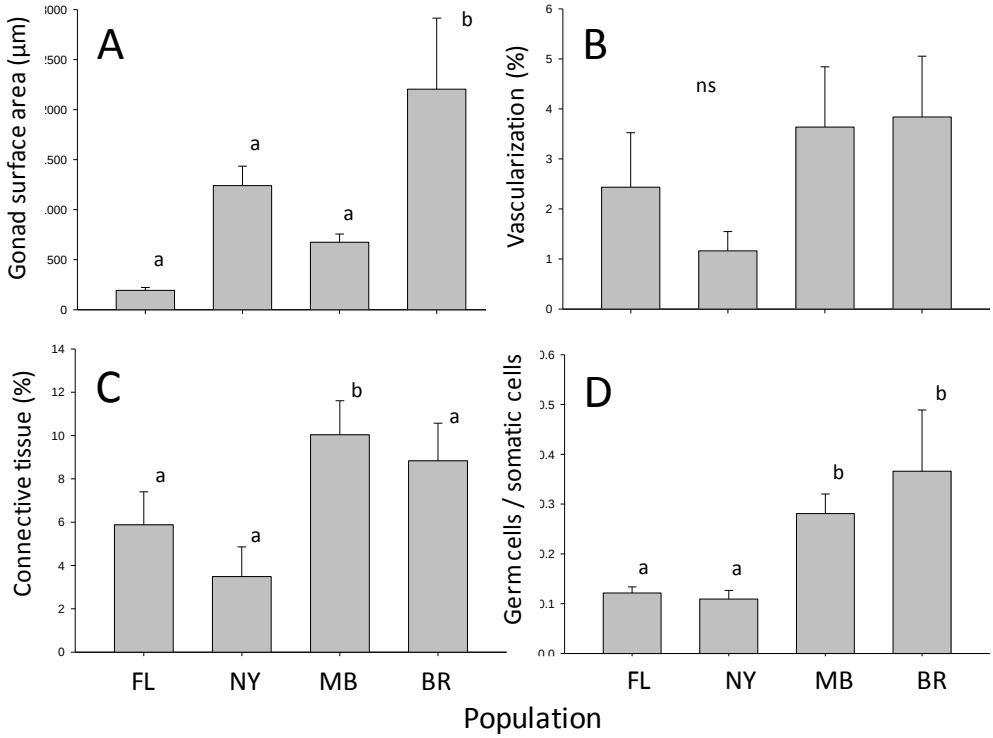

Figure 2.6. Mean trait value for developing juveniles 17-20 mm TL reared at 28°C for A) gonad surface area, B) vascularization (% of total surface area), C) connective tissue (% of total surface area) and D) germ/somatic cell ratio. Error bars represent standard error. Significant differences ($p<0.05$) are indicated by unique letters, ns indicates no significant differences among populations.

Chapter 3: Ontogenesis of gonadal aromatase gene expression in Atlantic silverside (*Menidia menidia*) populations with genetic and temperature-dependent sex determination

Abstract

Cytochrome P450 aromatase (P450arom), an enzyme that converts testosterone to 17β-estradiol, is an important mediator of sex determination in teleosts with genetic sex determination (GSD) and temperature-dependent sex determination (TSD). I compared the ontogenetic expression of P450arom in two populations of Atlantic silversides, *Menidia menidia*, that exhibit TSD (South Carolina, U.S.A.) or GSD (Nova Scotia, Canada) using quantitative, real-time polymerase chain reaction (qRT-PCR). Embryos and newly-hatched larvae were reared at an intermediate sex ratio producing-temperature (21°C) and older larvae and juveniles were reared at temperatures that feminize (15°C) and masculinize (28°C) to assess the temperature response of P450arom during development. Prior to sex determination, embryos and 6mm larvae displayed negligible P450arom expression, indicating minimal upregulation of this gene prior to sex determination. Gene expression increased in both populations during sex differentiation. Nova Scotia fish with GSD exhibited presumptive male- and female-like expression levels during early sex differentiation that were not influenced by temperature treatments. South Carolina fish with TSD displayed low levels of expression at 28^{o}C with significantly heightened expression in some individuals at 15^{o}C, indicating that P450arom is temperature sensitive in the population with TSD. Populations also differed in both the timing and maximal levels of P450arom expression, with fish from Nova Scotia exhibiting both the highest and earliest increase in expression in presumptive females. These results support the hypothesis that P450arom is involved in female sex differentiation in this species, but is only responsive to temperature in *M. menidia* populations that exhibit TSD.

Introduction

Teleosts possess all known forms of sex determination that occur in vertebrates (Manolakou *et al.*, 2006; Penman and Piferrer, 2008). Regardless of the form a species displays, gonad differentiation occurs *via* similar pathways (Strussmann and Nakamura, 2002). In teleosts, female development is mediated primarily through the conversion of the sex steroid 11 keto-testosterone (T) to 17-β estradiol (E_2) via the enzyme aromatase (Crews, 1996; Devlin and Nagahama, 2002; Strussmann and Nakamura, 2002). The gonadal form of the cytochrome P450 aromatase gene (P450arom, also called cyp19a or cyp19a1) is responsible for aromatase regulation in gonad differentiation and development (Devlin and Nagahama, 2002; Piferrer and Guiguen, 2008). Increased P450arom activity is correlated with ovarian differentiation and this relationship has been documented in species that possess the two most prevalent forms of sex determination, genetic sex determination (GSD) and temperature-dependent sex determination (TSD) (Strussmann and Nakamura, 2002; Luckenbach *et al.* '09). P450arom regulation in species with TSD is cued by environmental temperature, making sex determination a plastic process (Karlin and Lessard, 1986; Strussmann and Nakamura, 2002; Godwin *et al.*, 2003). However, genetic, behavioral or other environmental factors may influence P450arom expression in both TSD and GSD systems (Crews, 1996; Devlin and Nagahama, 2002; Baroiller *et al.*, 2009). The extent to which environmental cues act in concert with the mechanisms that control sex determination may vary both within and among species (Charnov and Bull, 1977; Conover *et al.*, 1992).

Temperature-dependent sex determination is the process whereby environmental temperature, acting in concert with genetic factors, irreversibly determines an organism's sex during development, and is adaptive if one sex gains a fitness advantage (Charnov and Bull, 1977; Bull, 1983). In many species with TSD, P450arom expression is influenced by the environment, providing a mechanistic link between temperature and steroid hormone regulation via P450arom (Strussmann and Nakamura, 2002; Penmann and Piferrer, 2008; Baroiller *et al.*, 2009). Environmental temperature is postulated to act primarily as a suppressor of P450arom under conditions that favor male development (Penman and Piferrer, 2008). Thus, low P450arom expression is often a clear indicator of male development, while elevated P450arom is associated with feminization in a diverse

group of fishes with TSD including southern flounder, *Paralichthys lethostigma* (Luckenbach *et al.*, 2005), Nile tilapia, *Oreochromis niloticus* (D'Cotta *et al.*, 2001) and pejerrey, *Odontesthes bonariensis* (Karube *et al.*, 2007) as well as in species with GSD such as rainbow trout, *Oncorhynchus mykiss* (Vizziano *et al.*, 2007) and Atlantic halibut, *Hippoglossus hippoglossus* (Matsuoka *et al.*, 2006).

Comparisons of the structure and function of P450arom have been used to understand the role of aromatase in gonad differentiation among and within populations with different life-history characteristics. However, few comparisons have linked mechanistic or genetic differences among populations to differential response to environmental conditions (Piferrer and Guiguen, 2008). Luckenbach *et al.* (2005) found significant variation in the P450arom locus among individuals of southern flounder, *Paralichthys lethostigma*, but this variation was not present when Gulf of Mexico and mid-Atlantic stocks were compared. He *et al.* (2008) demonstrated that single nucleotide polymorphisms (SNPs) in P450arom exons were correlated with differences in circulating E_2 within a single population of Japanese flounder, *P. olivaceus*. Selection for resistance to estrogenic compounds in heavily urbanized estuaries may induce polymorphisms in the alleles that mediate estrogenic responses as may be the case in killifish, *Fundulus heteroclitus*, from the New Bedford Harbor, MA superfund site (Hahn *et al.*, 2004). The P450arom gene clearly plays an important role in reproductive development, and may therefore be regulated differently among populations with uniquely different life history characteristics.

Menidia menidia (Atherinidae) is an estuarine teleost that exhibits locally adapted levels of GSD and TSD throughout its range (Conover and Heins, 1987A) which extends along the east coast of North America from Volusia County, FL to the Gulf of St. Lawrence, Nova Scotia, Canada (Gosline, 1948; Johnson, 1975). It is one of few teleosts known to exhibit a cline in the mode of sex determination and may exhibit inter-population differences in the way P450arom responds to temperature. Conover and Heins (1987A) assessed the level of TSD, which is the proportion of individuals in a population whose sex is determined by temperature, in seven populations across the fish's range. They found that the level of TSD is positively correlated with growing season length, resulting in a gradient consisting of GSD in northern populations, mixed

TSD/GSD at intermediate latitudes and predominantly TSD in southern populations. TSD is adaptive for silversides where the growing season is long because it maximizes the relative fitness of individuals entering seasonally different environments by producing a sexual dimorphism in body size. Cooler spring temperatures produce a higher proportion of females, ensuring a longer growing season and larger size at reproduction, maximizing reproductive fitness. Males are produced under warmer conditions, but the protracted growing season does not significantly impair reproductive fitness in the smaller males (Conover, 1984; Conover and Heins, 1987A). The Atlantic silverside is an excellent model to compare P450arom regulation within a single species with both forms of sex determination.

I cloned and partially sequenced the ovarian form of cytochrome P450arom (cyp19a) in order to compare expression in *M. menidia* exhibiting TSD and GSD prior to and during gonad differentiation. A qRT-PCR assay was used to measure P450arom mRNA in two populations of *M. menidia* reared under laboratory conditions; one population from the Annapolis River in Nova Scotia, Canada with pure GSD and a second from the Edisto River in South Carolina with 70% TSD (Conover and Heins, 1987A). To understand the role of P450arom in sex determination, I reared embryos, larvae and juveniles in feminizing (15°C), masculinizing (28°C) and balanced sex ratio (21°C) temperatures and assessed the ontogeny of P450arom expression prior to and during sex differentiation. Additionally, I assessed P450arom regulation in different tissues from older juveniles to assess the relative contribution of each tissue to P450arom expression.

Materials and Methods

Animal Collection and Experimental Design

Embryos of the *M. menidia* were collected from the Edisto River in South Carolina in the spring of 2004 using procedures similar to Lagomarsino and Conover (1993). They were then transported to Bluepoint Marine Laboratory, West Sayville, NY where they were reared at 21^{o}C and a salinity of 26-27 ppt. Upon hatch, larvae were fed *Artemia* sp. nauplii (Brine Shrimp Direct, Ogden, UT) then switched to pelleted food (Otohime Hirami, Japan), frozen brine shrimp (San Francisco Bay Brand, Newark CA)

and frozen mysid shrimp (Hikari, Hayward CA). A second population of juvenile *M. menidia* was sampled from the Annapolis River in Nova Scotia in the summer of 2005 and embryos were transported to Bluepoint Marine Laboratory and reared as previously mentioned. Both populations were reared on a 15 light:9 dark photoperiod and adults were induced to spawn on yarn mops at 9 months of age by photoperiod manipulation as described in Conover and Fleisher (1986).

Second and third generation, lab-reared offspring were used in this study to minimize maternal effects and tricaine methane sulphonate (MS-222; Western Chemicals Inc., Ferndale WA) was used to euthanize all fish prior to sacrifice. Ovarian tissue was collected from six juvenile *M. menidia* (82.0 $\pm$ 7.7 mm) for P450arom cloning. Additionally, samples were dissected from juvenile fish to compare P450arom expression in different tissues between Nova Scotia and South Carolina populations. Three fish from each population were taken from 21°C baths and euthanized, then the ovary, brain, liver, viscera (gut, adipose and other internal organs including heart) and muscle (peduncle) were removed. All tissues were immediately frozen on dry ice and stored at -80^{o}C.

Yarn mops with attached embryos were collected from tanks of spawning adults every two-three days and placed into five gallon flow-through hatching containers at 21^{o}C. Full-term embryos (seven days post fertilization) and newly hatched larvae (one day post hatch, approx. five to six mm total length, TL) were sampled from 21°C containers prior to sex differentiation. Between six and eight embryos were combined per sample and single, newly hatched larvae were collected directly from hatching containers. Multiple batches of embryos and newly hatched larvae were collected throughout the spawning period to ensure offspring collected were derived from multiple parents.

To determine the response of P450arom to temperature, two day post-hatch larvae were acclimated to 15°C or 28°C and stocked in five-gallon containers at 55 fish per container. Each container was sampled at one of three target lengths (8, 15 and 21mm TL), bracketing the window of gonad differentiation (Conover and Fleisher, 1986). Once fish in each container reached the average target length, a subsample of fish were removed, euthanized in MS-222 and individually snap-frozen in liquid nitrogen. No

container was sampled more than once. Any dead fish were immediately removed from each container, but mortality was minimal and no consistent pattern between treatments was observed. The experimental design involved two treatments (15°C and 28°C), two populations (NS and SC) and three mean body sizes at collection (8, 15, and 21 mm), resulting in a two X two X three factorial design. All treatments were carried out in duplicate yielding 24 experimental containers. The protocols used in this research were approved by Stony Brook University's Institutional Animal Care and Use Committee.

Cloning P450arom in M. menidia

Total RNA was isolated from frozen juvenile ovaries using a TriReagent protocol (Molecular Research Center; Cincinnati, OH). RNA quality was checked by electrophoresis, while concentration and purity of RNA were determined with a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE) before and after DNase treatment (DNA-Free, Ambion, Austin, TX). Complimentary DNA was synthesized from 1 μg RNA in 20 μl reactions using a High-Capacity cDNA Reverse Transcription kit with cycling conditions of 25^{o}C for 10 min., 37^{o}C for 120 min. and 85^{o}C for 5 sec. (Applied Biosystems, Foster City, CA).

A partial coding sequence (cds) for *M. menidia* P450arom was obtained using degenerate reverse transcription PCR primers (Forward: 5'-TAYTTTGACACVTGGCAGAS-3'; Reverse: 5'-TTCATCATCACCATRGCKAT-3'), designed from gonadal aromatase nucleotide sequences (Vector NTI, Invitrogen, Carlsbad, CA) (Li and Moriyama, 2004) found in the NCBI GenBank database; *Carassius auratus* (AB009336), *Rutilus rutilus* (AB190291), *Kryptolebias marmoratus* (AB251460), *Fundulus heteroclitus* (AY428665), *Oryzias latipes* (ORZP450A), *Odontesthes hatcheri* (EF051123), *Epinephelus coioides* (AY510711), *Pseudolabrus japonicus* (DQ298135), *Mugil cephalus* (AY859425) and *Oreochromis mossambicus* (AF135851). PCR reactions were performed using a GoTaq® kit and protocol (Promega; Madison, WI) with 1μg cDNA template, 10 mM dNTP mix and 10 μM primer concentration under the following cycling conditions: 1 cycle at 95 for 2 min; 35 cycles at 95°C for 30 s, 51-53°C for 30 s, 72°C for 2 min; and one final extension cycle at 72°C for 5 min. PCR resulted in a single amplicon of approximately 700 bp, which was cloned

into a pCR 2.1 (Invitrogen) vector and transformed into JM109 competent cells (Promega). Plasmids were purified from two positive clone colonies using a QIAprep Miniprep kit (Qiagen, Valencia, CA) and sent to the University of Chicago Cancer Research Center for forward and reverse sequencing using M13 primers. The partial cds of ovarian P450arom in *M. menidia* is listed as GenBank accession number FJ861317.

qRTPCR Primer design and validation

Primers for qRT-PCR were designed for *M. menidia* P450arom and the house-keeping gene, elongation factor-1α (EF-1α, Luckenbach *et al.*, 2005) using conserved GenBank sequences of >18 teleost species (Applied Biosystems Primer Express Software 3.0, Applied Biosystems). P450arom primers were designed specifically using sequence regions conserved within the gonadal form and not the brain form of P450arom. Primer sets for qRT-PCR were chosen for P450arom (Forward: 5'- AGATACGGTCGTA GGTGAGAGACG-3'; Reverse: 5'- GCCTTTGGGCACCCTGTAG-3') and EF-1α (Forward: 5'-TCAACAAGATGGACTCCA-3'; Reverse: 5'-GGTTGTAGCCGATCT TCTTGATGTA-3'). EF-1α was chosen as the housekeeping gene for its relative stability during normal development in numerous teleosts (Luckenbach *et al.*, 2005; Olsvik *et al.*, 2005; McCurley and Callard, 2008). Standard curves for both primer sets yielded r^2=0.99. To verify primers, RNA was extracted from a pool of 21 mm fish, DNase treated and reversed transcribed as described above. Quantitative, real-time expression was performed in these five fish using Brilliant® II SYBR® Green QPCR Master Mix (Stratagene, Agilent Technologies, La Jolla, CA) in 20 µl reactions. This assay was highly transferrable and all ontogenetic analyses were carried out using an ABI 5700 Sequence Detection System (Applied Biosystems), with later tissue analysis done using an Eppendorf Mastercycler ep Realplex^4 1.5 thermal cycler (Eppendorf, Westbury, NY). Forward and reverse primers were used in 1.5 µM concentrations and the qRT-PCR reaction was carried out as follows: 95^{o}C for 10 minutes followed by 40 cycles of 95^{o}C for 30 sec., 60^{o}C for 1 min. and 72^{o}C for 1 min. A dissociation step of 95^{o}C for 15 sec, 60^{o}C for 15 sec. and 95^{o}C for 15 sec. was added at the end to further confirm detection of a single gene product. The melting curves for both P450arom and EF-1α each yielded a single peak and therefore a single PCR amplicon. Confirmation that this

primer set was not detecting the brain isoform of P450arom (cytochrome P450b or 19a1b) was carried out through sequencing the qRT-PCR product from the head and trunk (Applied Biosystems 3730XL 96-capillary sequencer, Univ. of Chicago Cancer Research Center).

qRT-PCR measurement of P450arom mRNA

Immediately before RNA extraction, larvae collected during the thermosensitive window (8-21 mm TL) were removed from -80^{o}C storage, placed on dry ice and the heads removed with a clean razor blade and discarded. Total RNA was immediately isolated from trunks and whole larvae and embryos. RNA concentrations were measured in duplicate and diluted to equal concentrations as previously mentioned. RNA purity (A_{260}/A_{280}) ranged from 1.67 to 2.05. Genomic DNA contamination was removed and cDNA was synthesized as described previously, using 1 μg of RNA per sample.

For ontogenetic P450arom and EF-1α expression analyses, qRT-PCRs were carried out as previously mentioned. All samples, including a 'no amplification control' (NAC) containing template RNA *in lieu* of cDNA and a 'no template control' (NTC) containing neither cDNA nor RNA, were run in duplicate. Both the NAC and NTC displayed no detectable amplification over 40 qRT-PCR cycles. Duplicate Ct values for a given sample that did not agree by more than 0.6 cycles and were more than two standard deviations (S.D.) from the group mean were considered statistical outliers. Such outliers were removed from the analysis. Less than 3% of all data were discarded in this manner. P450arom abundance was normalized to EF-1α (P450arom/EF-1α mRNA) for analyses.

Statistical Analysis

P450arom values for the tissue expression comparison were log-transformed for normality and compared with a two-way ANOVA and *post hoc* Holm-Sidak multiple pairwise comparisons. Normalized P450arom values in embryos and newly-hatched larvae were compared in the same manner.

Ontogenetic expression during the thermosensitive window was analyzed using an analysis of covariance (ANCOVA) to compare the slopes of regression lines for temperature treatments. Because no temperature interaction was expected for Nova

Scotia fish, a linear increase in expression for females and a linear, depressed expression pattern throughout development for males was assumed. Therefore, the male- and female-like regression lines were split visually. For Nova Scotia fish, all individuals greater than 12mm in length with P450arom values less than 0.8 ng P450arom normalized to EF-1α were assumed to be males and fish with P450arom values greater than 0.8 ng P450arom normalized to EF-1α were considered presumptive females based on bimodal segregation of expression beginning prior to 12mm. All fish ≤12mm in length were included in both male and female regression lines because no clear expression pattern was apparent. A strong thermal component was anticipated for South Carolina fish, so regression lines were based on the complete data set for each temperature treatment. This approach allowed the comparison of the rates of ontogenetic P450arom expression both within and among populations.

Results

Cloning of P450arom

A partial P450arom cDNA coding sequence was cloned from M. menidia ovaries from Nova Scotia, yielding a sequence of 683 nucleotides (Figure 3.1). This partial sequence for P450arom was closely homologous to ovarian P450arom (cyp19a1a) of other teleosts in the order Atheriniformes, showing 89% identity to *Odontesthes hatcheri* (EF051123) and 88% identity to *O. bonariensis* (EF030342). This sequence also showed 88% and 85% identity to ovarian P450arom of *Fundulus heteroclitus* (AY428665) and *Dicentrarchus labrax* (AJ298290), respectively. Sequencing of the P450arom amplicon from both trunk and head of juvenile *M. menidia* revealed 98% homology (163/165 bp) between sequences from each region. This verified that the primers for P450arom were not amplifying the brain form. Amplification of the brain form was not expected given that brain and gonadal P450arom are generally 60% homologous within a single species of fish (Pellegrini *et al.*, 2005).

Expression of the housekeeping gene, EF-1α, was consistent among all samples and did not vary between embryo and newly hatched larvae. There was no significant effect of either temperature or average length within each population (two-way ANOVA, $p \geq 0.22$ for all). However, a small but significant difference in mean EF-1α expression

was found between the two populations (Nova Scotia; 15.7 $\pm$ 0.37 S. D., South Carolina; 16.3 $\pm$ 0.56 S. D., Mann-Whitney Rank Sum test, $p<0.05$). Therefore, analyses using P450arom values normalized to EF-1α alone were limited to comparisons within a population.

Tissue distribution of P450arom

Tissue distribution analysis in 65mm juveniles revealed differential expression among tissue types in both populations (two-way ANOVA, $p<0.001$, Figure 3.2 A,B). P450arom (normalized to EF-1α) was expressed predominantly in the gonad and there were no differences between populations in gonadal expression (*post hoc* pairwise comparison; $p=0.377$). Other tissues showed low or negligible expression of the ovarian P450arom. Despite low-level expression, differences in tissue-specific P450arom RNA between populations were observed for muscle, with the Nova Scotia population exhibiting heightened expression ($p=0.017$, Figure 3.2). Comparisons among brain, liver, muscle and viscera (internal organs including gut, adipose and kidney) expression in Nova Scotia individuals revealed no significant differences ($p<0.05$), but significantly higher levels of P450arom were found in the brain relative to the liver ($p=0.001$) and muscle ($p=0.001$) in the population from South Carolina.

P450arom expression during development

P450arom mRNA normalized to EF-1α was assessed in 6-9 embryos and larvae from both populations at 21^{o}C and for 25-35 fish per temperature treatment from each population ranging from 7.5 mm to 24.0 mm TL. Standard curves were run in duplicate. Ct values of the standard curves were positively correlated with template (0.1-50 ng), resulting in r^2 values for P450arom and EF-1α of 0.99 and 0.98, respectively for embryo, larval and juvenile samples. Additionally, P450arom qRT-PCR product (165bp) from ovarian tissues from the two populations were sequenced and demonstrated 99% homology (164/165 bp).

Newly hatched larvae and eggs from Nova Scotia and South Carolina exhibited very low P450arom expression (Figure 3.3). Because significant differences in EF-1α existed between populations early in development (two-way ANOVA, $p=0.002$),

P450arom within each population was compared using total P450arom cDNA/ng cDNA loaded into each qRT-PCR reaction. No significant differences between embryos or newly hatched larvae (two-way ANOVA, $p=0.85$) or between populations ($p=0.23$) were found.

Conversely, P450arom patterns during the window of sex differentiation were strikingly different between the Nova Scotia and South Carolina populations. Individuals from the Nova Scotia population appeared to differentiate into putative female and male levels of expression as early as 10-12 mm TL, and these putative differences were clearly apparent beginning around 15mm TL (Figure 3.4 A). Throughout the window of sex differentiation in Nova Scotia fish, expression either increased (putative females, linear regression; $p<0.001$) or was depressed (putative males, $p=0.131$), resulting in a clearly bimodal distribution for both populations, regardless of temperature treatment. No statistically significant differences were detected between temperature treatments when the slopes of putative females (ANCOVA; $p=0.99$) or putative males ($p=0.557$) within the Nova Scotia population were compared.

Fish from South Carolina showed a different pattern, with depressed expression in both treatments until the middle of the thermosensitive window. Expression increased in most individuals by 15 mm TL in the 15^{o}C (feminizing) treatment (Figure 3.4 B). In contrast, fish from the 28^{o}C (masculinizing) treatment displayed relatively depressed expression throughout the period of sex differentiation. Overall, temperature treatments led to statistically different expression patterns in South Carolina fish (ANCOVA; $p=0.005$). Additionally, the slopes of regression lines of putative females from Nova Scotia (from both temperatures) and putative females from South Carolina (from 15^{o}C) were compared with ANCOVA. Results revealed significant differences in the slopes ($p<0.001$) with a steeper slope in the Nova Scotia (0.39) *versus* South Carolina (0.15) population and with higher overall P450arom expression values in Nova Scotia fish compared to those from South Carolina (Figure 3.4 A,B).

Discussion

These results support the hypothesis that the gonadal form of P450arom is involved in gonad differentiation in *M. menidia* and that this gene is differentially

influenced by temperature between populations with different forms of sex determination. P450arom is unresponsive to temperature in the population with GSD, exhibiting clear and biomodal male- and female-like expression patterns regardless of temperature. Conversely, P450arom expression in the population with TSD is significantly influenced by temperature. Additionally, P450arom appears to be upregulated in presumptive females with GSD earlier within the window of gonad differentiation than in presumptive females with TSD.

Tissue analysis of expression in older, sexually differentiated juveniles revealed that most of the P450arom is expressed within the gonad at this stage, with negligible expression occurring in other organs. The small size of the gonads of fish used in this study prohibited evaluation of the tissue-specific expression during the early stages of differentiation. Based on work addressing tissue-specific expression of P450arom in other teleosts, it is likely that expression was localized within the developing ovary (Barney *et al.*, 2008; Esterhyuse *et al.*, 2008). Additionally, male- and female-like differences in expression of P450arom are pronounced in differentiating teleosts and can be used to predict the sex of a developing individual (Luckenbach *et al.*, 2005; Piferrer and Blázquez, 2005).

Both populations of *M. menidia* show patterns similar to aromatase expression observed in other fish. In teleosts, the gonad form of P450arom is depressed in embryos and larvae, and is generally not expressed until just prior to histological observation of gonad differentiation (Devlin and Nagahama, 2002; Strussmann and Nakamura, 2002). Expression in *M. menidia* is lowest in embryos and newly-hatched larvae (Figure 3.3), similar to patterns of depressed P450arom expression in Japanese medaka, *Oryzias latipes* (Patil and Gunasekera, 2008), Mozambique tilapia, *Oreochromis mossambicus* (Esterhuyse *et al.*, 2008) rainbow trout, *Onchorhyncus mykiss* (Vizziano *et al.*, 2007) and southern flounder (Luckenbach *et al.*, 2005) prior to differentiation. There were no significant differences between the embryos or newly-hatched larvae from Nova Scotia and South Carolina populations, indicating that aromatase is not up-regulated during these developmental periods. These results agree with previous work on an intermediate latitude population (New York) of *M. menidia* where sex determination and differentiation occurred between 8 and 21mm TL and temperature had little effect on the

phenotypic sex in fish prior to this window of development and differentiation (Conover and Fleisher, 1986). The results of this study indicate that little differentiation occurs in the embryo or early larval stages of both populations, regardless of the form of sex determination.

However, P450arom expression patterns change significantly during gonad differentiation. The population with GSD (Nova Scotia) displays increased P450arom expression in approximately half of the experimental animals (presumptive females), regardless of temperature treatment. This indicates that P450arom is not regulated by temperature in this population. Furthermore, expression is bimodal, and clear differences in presumptive female and male expression as early as 8mm is evident, with clearly defined sex-specific expression between 14 and 24 mm TL. Because the whole trunks of fish were used, the sex of individual fish is unknown and therefore cannot be compared to P450arom expression. However, the sex ratios expected for these populations under these temperature treatments (Conover and Heins, 1987A; Lagomarsino and Conover, 1993) closely parallel those observed with the P450arom expression pattern. This is exemplified in the Nova Scotia population, whose predicted sex ratio (1:1 sex ratio regardless of temperature), is consistent with the pattern of P450arom expression, particularly late in the thermosensitive window.

Conversely, the population with TSD (South Carolina) appears to be strongly influenced by environmental temperature, displaying significantly different trends in the expression pattern of P450arom throughout development. P450arom increases during the middle of the thermosensitive window for the feminizing treatment around 15 mm TL, as is expected with female development. Elevated gonadal aromatase mRNA levels during sex differentiation in southern flounder (*P. lethostigma*) with TSD are a good predictor of female development and therefore sex ratios of flounder populations (Luckenbach *et al.* 2005, 2009).

In this study, both populations exhibited bimodal P450arom expression patterns with segregation between female and male-like expression, but this pattern was evident much later in the thermosensitive window (at sizes 3-7 mm TL larger) in the South Carolina population than in the Nova Scotia population (Figure 3.4). Macroscopic observation of gonad development in *M. menidia* shows that gonads in South Carolina

fish are only distinguishable when the fish are an average 2-3 mm longer than Nova Scotia conspecifics reared at the same temperature (D. Conover and T. Duffy, personal observation). Delayed onset of P450arom regulation as well as delayed gonadal development in the TSD population indicate that aromatase is tightly coupled with ovarian development, which is consistent with findings in many other teleosts (Nakamura *et al.*, 1998; Strussmann and Nakamura, 2002; Piferrer and Guiguen, 2008). An increase in P450arom expression preceding or coinciding with histological observation of putative ovaries has been reported in *P. olivaceus* (Kitano *et al.*, 1999), *O. bonariensis* (Karube *et al.*, 2007), *O. mossambicus* (Esterhuyse *et al.*, 2008) and other fish.

Timing of gonad development is a highly labile process both within (Maack *et al.*, 2003; Melia *et al.*, 2006; Wang *et al.*, 2007) and among species (Devlin and Nagahama, 2002; Chiasson *et al.*, 2008). Plasticity in the timing of gonad differentiation may be due to differences among strains (e.g. Zebrafish, *Danio rerio,* Wang *et al.*, 2007; Maack and Segner, 2003) or directed by life-history or environmental cues (e.g. European eel, *Anguilla anguilla*, Melia *et al.*, 2006). Close relatives of *M. menidia*, *Patagonia odontesthes* and *P. hatcherii* (strong TSD and GSD, respectively) display differences in the onset of histological gonad differentiation, with differentiation occurring later in the species with TSD (Strussmann *et al.*, 1996). Upregulation of P450arom occurs earlier in development in Japanese flounder (*P. olivaceus*) compared to summer flounder (*P. dentatus*), and this coincides with earlier gonadal differentiation (30-37 mm TL) in *P. olivaceus* than *P. dentatus* (85 mm TL), indicating the tight coupling between aromatase regulation and the timing of gonadal development (Borski *et al.*, 2010). Growth rates may also contribute significantly to the development rate of sex organs in fish (Kavanagh, 2003). Therefore, the significantly reduced capacity for larval and juvenile growth in the South Carolina population, relative to Nova Scotia (Conover and Present, 1990) may contribute to the delay in gonad development. Delayed differentiation and development in South Carolina may be a fitness cost of TSD, but further examination of this relationship will be necessary to resolve this question.

Despite the significant effects of temperature, neither feminizing nor masculinizing treatment produced a clear 100% shift in sex ratio in the South Carolina population, in strong contrast to abrupt temperature-induced sex ratio shifts observed in

reptiles with TSD (Bull, 1980; Valenzuela, 2008A,B). Of the teleosts with TSD studied to date, few display complete environmental control over sex ratio at environmentally relevant temperatures; genetic factors play a role in the phenotypic sex, at least in part (Conover, 2004). P450arom expression in *M. menidia* with TSD resembles that of a closely related fish with TSD, the pejerrey, *O. bonariensis.* When exposed to masculinizing and feminizing temperatures during gonad differentiation, most pejerrey exhibit depressed or up-regulated P450arom, respectively, but some individuals with a strong genetic component to sex differentiation display expression that is characteristic of the sex opposite to that predicted by temperature alone (Karube *et al.*, 2007).

Control of sex in *M. menidia* varies among populations; Nova Scotia silversides exhibit sex determination dominated by major genetic factors (TSD=0), while fish from South Carolina exhibit sex determination that is a combination of polygenic factors and temperature effects (TSD=0.7) (Lagomarsino and Conover, 1993). In NS, these genetic factors are presumably overriding environmental control of P450arom and other genes involved in differentiation (Lagomarsino and Conover, 1993). Therefore, it is likely that the low frequency of temperature insensitive genotypes in South Carolina fish allows P450arom to be responsive to environmental temperature in roughly 70% of the population. Conover *et al.* (1992) showed that the form of sex determination is capable of evolving by experimentally biasing sex ratios in three *M. menidia* populations with extreme temperature over several generations. They found that TSD evolved by two mechanisms that are not mutually exclusive; selection for genotypes that are non-responsive to temperature which override temperature-sensitive genes and secondly by a shift in the sensitivity of the sex-determining gene itself, possibly P450arom. Therefore, either scenario could explain the pattern in sex ratio from previous studies and P450arom from this study.

In this study, the partial coding sequence of ovarian P450arom was determined from the temperature-insensitive, GSD population. Since P450arom was detected in both populations, it is likely that the differential responses to temperature are due to factors that regulate gene expression, rather than in direct changes to the P450arom coding sequence itself (Nielsen *et al.*, 2009). However, differences in coding sequences cannot be ruled out. Additionally, transcription factors, SNPs in promoter regions and novel

vigilin-like proteins could act to regulate mRNA in this system, causing the differences in regulation between populations (Piferrer and Blázquez, 2005). A detailed look at the structure and function of p450arom and other genes involved in sex determination is needed in order to understand how these two forms of sex determination have evolved in *M. menidia* to produce the pattern observed in this study.

In Nova Scotia fish, P450arom expression clearly segregates between male- or female-like expression during the thermosensitive window, but a bimodal pattern is less pronounced in the South Carolina population (Figure 3.4). These patterns are understandable in the context of genotype-by-environment (G X E) interaction which is present in South Carolina fish, but relatively absent in Nova Scotia fish (Lagomarsino and Conover, 1993). Temperature-dependent sex determination is a threshold trait, such that small changes in temperature interacting with genes involved in sex determination are capable of shifting the balance between male and female differentiation within an individual (Conover and Kynard, 1981; Karube *et al.* 2007; Hattori *et al.*, 2007). In contrast to reptiles with TSD that have a distinct threshold temperature, fishes usually exhibit shifts in sex ratio over a broad range of temperatures due to a significant genetic component to sex determination (Conover, 2004). Therefore, the fact that maximal presumptive female expression in South Carolina fish is one-third to one-half that of Nova Scotia fish could be due to the strong influence of other sex determining genes on P450arom.

Continued research addressing the specific mechanisms by which P450arom is regulated in this system could contribute to understanding of the evolution of TSD in teleosts. In organisms possessing TSD, it is unclear if temperature regulates the gene of interest directly or indirectly and/or if temperature might also modify aromatase levels or activity, and hence E_2 levels in fish (Piferrer and Blázquez, 2005). Additionally, it is possible the brain P450 isoform could play a role in sexual differentiation in *M. menidia*, particularly at the neural and behavioral levels (Pellegrini *et al.*, 2005). Brain P450arom activity is upregulated in a related Atheriniform fish, *O. bonariensis*, prior to sex differentiation (Strobl-Mazzulla *et al.*, 2008). Whether brain P450arom might play a significant role in sex differentiation in *M. menidia* and other TSD systems is uncertain and requires further studies.

This latitudinal cline in TSD and GSD in *M. menidia* provides a unique system for intra-species comparison of P450arom mRNA regulation during gonad development. In this species, P450arom appears to be an important component of sex differentiation and development in populations with both TSD and GSD. Regulation of this gene is clearly temperature-dependent in South Carolina fish and independent of temperature in fish from Nova Scotia. Whether P450arom is directly regulated by temperature or by the influence of other genes remains unknown, but these results are consistent with what is known about the genetic control of sex ratio in these populations. To my knowledge, this represents the first intraspecific comparison of P450arom- regulated gonad differentiation among populations with different forms of sex determination.

```
M. menidia       1   TACTTTGACACGTGGCAGACCGTGCTGATCAAACCCGACATCTACTTCAAGTTTGGCTGGATTCATCAGA
O. bonariensis   1   ..T........A........T..............T.....................T.............
F. heteroclitus  1   ..T.................T..............................C.CA.......C..C..A.
D. labrax        1   ..T........A........T..............A.....T.............A.........C....

M. menidia       71  GGCACAAGACGGCAGCCCAGGAGCTGCAAGATGCCATAGAAGTCCTTGTCGACCAAAAGAGAAGAGACAT
O. bonariensis   71  ..........A...........A...................AG......A..A.....A..GG....A..
F. heteroclitus  71  .A.........................A.GG...........G.G...C..G..G........G..GC.A..
D. labrax        71  ....T.....A............T................GAGT.....A..A..G.....G.....T..

M. menidia       141 GGAGCAGGCGGATAAGCTGGACACCATCAATTTCACATCAGAGCTCATTTTTGCACAAAACCACGGCGAG
O. bonariensis   141 T..A.....A.............A......C......G.G........A.....................
F. heteroclitus  141 .........A.............---....C......G....C.....A.....................
D. labrax        141 .........A.....A.......---....C.....TG....C.....A.........G....GT.....A

M. menidia       211 CTGTCCGCCGACAACGTGAGGCAGTGCGTGCTGGAGATGGTGATCGCGGCGCCGGACACTCTGTCCATCA
O. bonariensis   211 .....T..T..G.....CC.......T...T.................A..A........G..........
F. heteroclitus  208 .....G..G..G.......C...A.....................................T.........
D. labrax        208 ...A.T..T..G..T........A..T...T.............T..A..A..A...........TG...

M. menidia       281 GCCTCTTCTTCATGCTGCTGCTCCTCAAGCAGAACCCGCATGTGGAGCTGCAGCTGCTGCAGGAGATAGA
O. bonariensis   281 ............................A..A..T..A....................A...........
F. heteroclitus  278 ............................A........C..C..............................
D. labrax        278 ..........T.................A.....T..AG.C..............................

M. menidia       351 TACGGTCGTAGGTGAGAGACGGGTTGAGAACGGGGACCTTCAGAAGCTGCAGGTGCTGGAGAGCTTTATA
O. bonariensis   351 C..T..T...........G.A.C..A....T..............AT....A..............C...
F. heteroclitus  348 C.A....A.......C...GA.C.GC...........................T........T..C..C
D. labrax        348 C..T..T........A....A.C..C...................G.T...................CG.C

M. menidia       421 AACGAGTGCCTCCGCTTCCACCCAGTGGTGGACTTCACCATGCGTCGGGCCCTTTCTGATGATGTCATCG
O. bonariensis   421 ..T........T.....................................T..............CA....A.
F. heteroclitus  418 ...........G.................................A..C.....G..A..C.........A.
D. labrax        418 .....A...T.G...........T........T..............A.....G.....C..CA....A.

M. menidia       491 ACGGCTACAGGGTGCCCAAAGGCACCAACATAATCCTGAACACTGGCCGCATGCACCGGACAGAGTTTTT
O. bonariensis   491 .T...........A.....G.....A..T..............C..T.......G...A...........
F. heteroclitus  488 ............C..G..G.....A..T..C...........G.....T..................C..
D. labrax        488 .T...........A..G..G.....A..T.....T........C....A....................

M. menidia       561 CCAAAAAGCAAACGAATTTAGTCTGGATAACCTCAAGAAAAAAGCTCCTCGTCGTTACTTCCAGCCGTTC
O. bonariensis   561 .........C..T..............G...T...GA.....T.......................C..T
F. heteroclitus  558 ...C..G..CG.................G...T..C.......TA.......C..............A...
D. labrax        558 ..TC...C.C..T.......A......C...T.T..A.....TC.......C..C...........A...

M. menidia       631 GGTTCGGGCCCTCGCGCCTGCGTTGGCAAGCACATCGCCATGGTGATGATGAA
O. bonariensis   631 .....A........G.G............A.....A..................
F. heteroclitus  628 ..C..A.................C..............................
D. labrax        628 .....A..............T.....T..........................
```

Figure 3.1. Comparative alignment of the partial coding sequence of cytochrome P450aromatase (gonadal form) from *Menidia menidia* and three additional teleosts. Periods (.) indicate identical nucleotides.

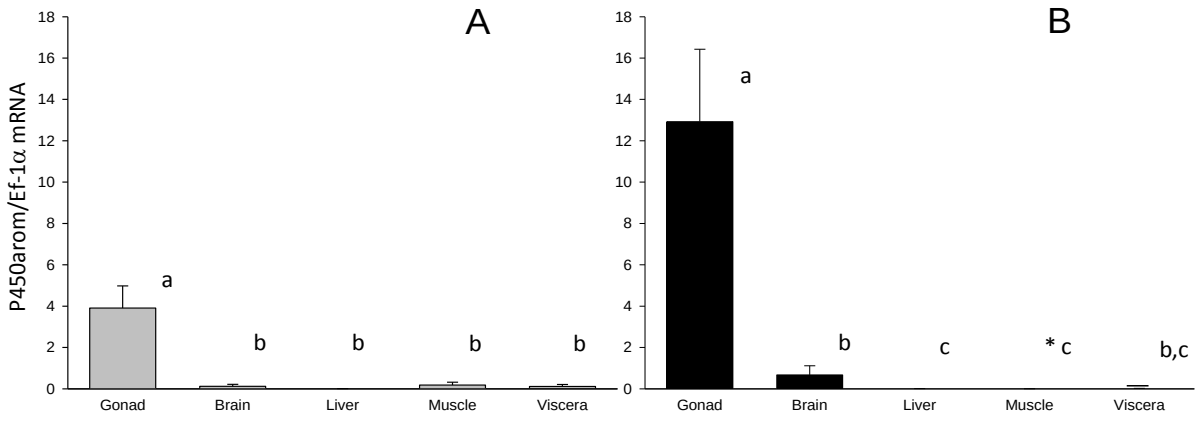

Figure 3.2. P450arom relative expression in different tissues of juvenile (~65mm) *Menidia menidia* reared at 21°C from A) Nova Scotia and B) South Carolina. All samples are pooled from n=3 fish. Significant differences among tissue types are denoted by different letters and the asterisk represents differences in expression of a given tissue between the two populations.

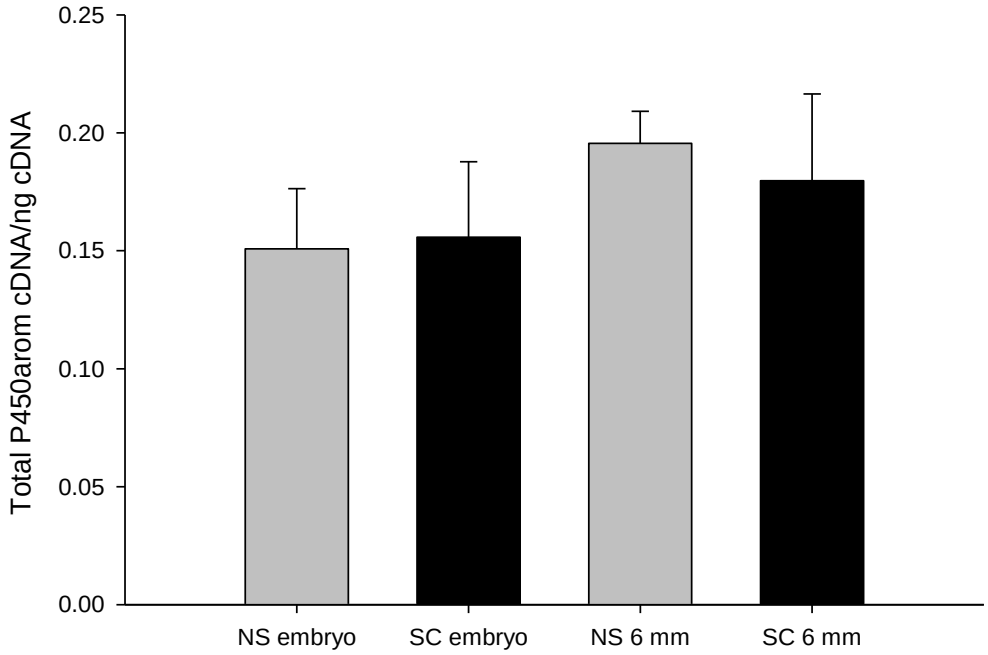

Figure 3.3. P450arom expression normalized to total cDNA in embryos and newly hatched larvae from Nova Scotia (NS) and South Carolina (SC) collected at 21°C prior to the window of sex determination**.**

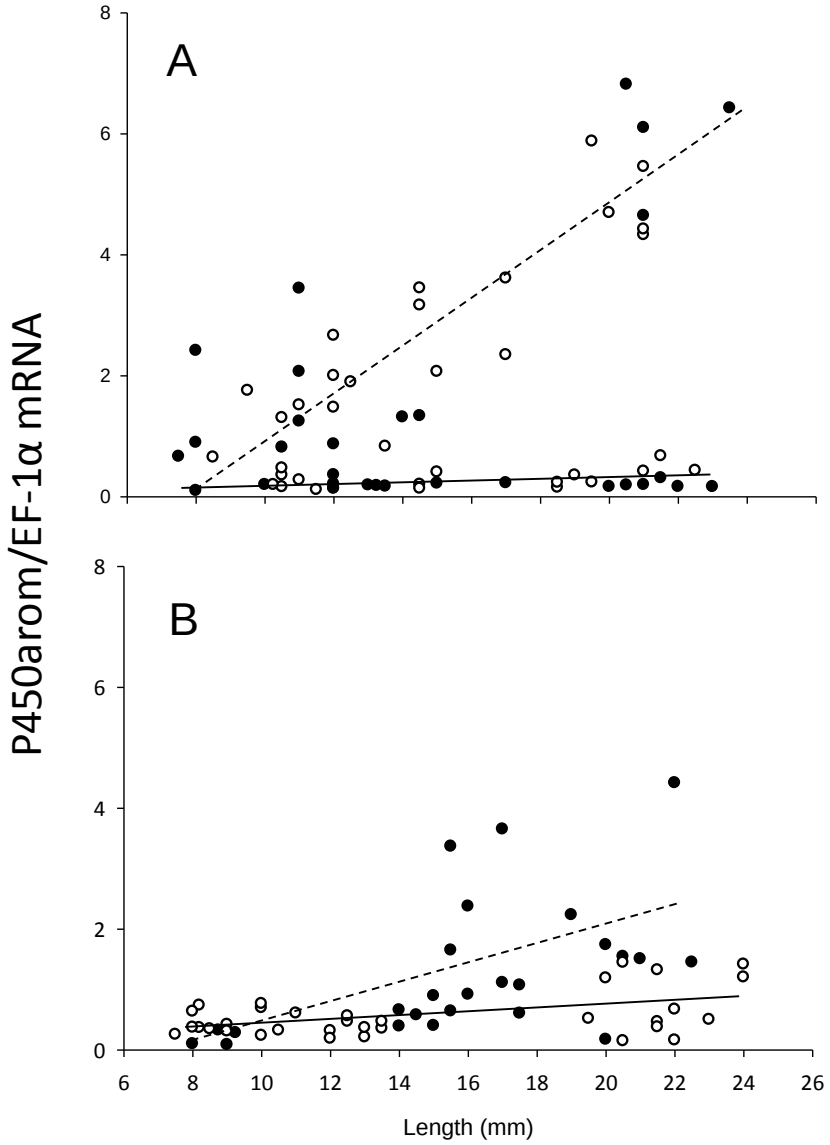

Figure 3.4. Cytochrome P450aromatase mRNA normalized to the housekeeping gene, EF-1α during the window of sex determination in A) Nova Scotia and B) South Carolina. Open circles represent 28°C (masculinizing) treatments, black circles represent 15°C (feminizing) treatments. Dashed lines represent the presumptive female regression line, solid lines represents the presumptive male expression trend (see text for explanation). Sample sizes are A) n=31 and n=35 for 15°C and 28°C, respectively and B) n=25 and n=33, 15°C and 28°C respectively.

Chapter 4: Variable susceptibility and response to estrogenic chemicals in *Menidia menidia*

Abstract

Environmental endocrine disruptors can profoundly impact sex ratios of fish populations, especially if the fish exhibits plasticity in sex determination. The Atlantic silverside, *Menidia menidia*, exhibits a genetically-based latitudinal cline in numerous traits including temperature-dependent sex determination (TSD), which varies from high thermal influence on sex determination in southern fish to pure genetic sex determination (GSD) with no thermal influence in northern fish. This gradient in TSD/GSD was used to test for differential population susceptibility to a common municipal wastewater constituent, 17 β-estradiol (E_2), among four populations with varying levels of TSD. Larvae were exposed to E_2 during sex differentiation and assessed for sex ratios. Sex ratio in southern populations was more responsive to E_2 than in northern populations, indicating differential population sensitivity. To assess the potential sensitivity of wild populations to estrogenic compounds, sex ratios in 12 *M. menidia* populations were measured over a three-year period along a pronounced longitudinal gradient in sewage effluent and urbanization across Long Island. Water temperatures were measured to account for thermal influence on sex ratio. I found a significant trend toward female-biased sex ratios in more urbanized estuaries with lower proportions of females in less impacted estuaries. Temperature was ruled out as the primary cause. Because sewage effluent often carries high concentrations of estrogenic compounds, and these experiments confirmed a heightened sensitivity to E_2 in fish with TSD, the most likely reason for the longitudinal trend in sex ratio is a gradient in the concentration of estrogenic compounds associated with urbanization.

Introduction

Endocrine disrupting chemicals (EDCs) are known to impair the fitness of freshwater fish, but impacts at the population level in marine and estuarine fish are relatively unknown (Oberdorster and Cheek 2001, Matthiessen 2003). Included in this group are a number of organic compounds associated with municipal and industrial wastewater effluent and are known to feminize fish in environments receiving effluent in large quantities (Sumpter 1997, Desbrow *et al.* 1998, Sumpter 2005). Three major estrogenic compounds have been identified in sewage effluent; the natural steroids estrone (E_1) and 17 β-estradiol (E_2), and the synthetic estrogen, 17 alpha-ethinylestradiol (EE_2) (Desbrow *et al.* 1998). Additionally, less potent, but often more concentrated estrogenic compounds such as nonylphenol are also present in effluent (Servos *et al.* 2003). Routine wastewater treatment does not completely remove these compounds (Johnson and Sumpter 2001), and urban effluents contain EDCs in concentrations reported to induce changes in behavior, growth and reproduction of marine and freshwater fish that utilize heavily urbanized coastlines (Oberdorster and Cheek 2001, Matthiessen 2003).

Local adaptation is well-known in marine fish and it allows populations to respond totheir local environment in a way that maximizes fitness (Conover 1998, Kawecki and Ebert 2004, Schulte 2007). Additionally, if selection acts on reproductive, metabolic or behavioral traits to produce unique, locally adapted populations, it is likely that these populations will respond to EDCs in unique ways. In particular, differences in traits relating to regulation of growth, sex determination, gonad development and reproduction in fish may be indicative of how a fish may respond to contaminants (Schulte 2007). Species that display local adaptation in traits related to sex development and maintenance may be particularly useful to determine how these traits contribute to susceptibility to contaminants that compete with reproductive hormones and disrupt endocrine function (Sumpter 1997).

Under appropriate conditions endocrine disruptors can manipulate the phenotypic sex of fish, primarily when exposed early in development (Strussman and Nakamura 2002, Mills and Chichester 2005). Gonadal differentiation in fishes usually occurs in the late larval or early juveniles stages when the male or female gonad develops from bi-

potential germ cells (Devlin and Nagahama 2002). Exposure to endocrine disruptors during sex differentiation can override endogenous signals, leading to skewed sex ratios. Brion *et al.* (2004) compared sex ratios of zebrafish, *Danio rerio*, exposed to E_2 for three weeks during three distinct developmental stages. The clearest shift in sex ratio to females occurred in the fish exposed prior to sex differentiation. Additionally, significant feminization of genetic males occurred when fish were exposed as juveniles, during gonadal development. Similarly, treatment of *D. rerio* with EE_2, a potent synthetic estrogen, significantly biased the sex ratio toward females only when exposed during gonad differentiation (Maack and Segner 2004). Liney *et al.* (2005) exposed roach, *Rutilus rutilus*, to a gradient of wastewater effluent and found little response in post-spawning males but 100% feminization in fish exposed during the embryonic through juvenile period. Stronger responses to E_2 during differentiation as compared to other developmental periods have been demonstrated in medaka, *Oryzias latipe*s (Koger *et al.* 2000) and the fathead minnow, *Pimephales promelas* (van Aerle *et al.* 2002).

Many fish display a highly labile form of gonad differentiation known as environmental sex determination (ESD). The most prevalent form of ESD in teleosts is temperature-dependent sex determination (TSD) (Strussmann and Nakamura 2002, Conover 2004), which Charnov and Bull (1977) defined as the irreversible determination of sex by environmental temperature. Temperature dependent sex determination was first demonstrated as a locally adapted trait in the Atlantic silverside, *Menidia menidia,* (Atherinidae) (Conover and Kynard 1981) and TSD has since been extensively studied in this species (Conover 1984, Conover and Fleisher 1986, Conover and Heins 1987A). Temperature dependent sex determination is widespread; occurring in at least 54 species of fish, across eleven families (Conover 2004).

Temperature-dependent sex determination reflects an increased plasticity of sex determination relative to that of a population with genetic sex determination (GSD) and small shifts in sex hormones can shift the sex of an individual with TSD (Charnov and Bull 1977, Bulmer and Bull 1982, Conover 2004). Therefore, fish with TSD may be more labile in their response to environmental factors during the period of sex differentiation than fish with GSD where sex is controlled by major genetic factors. Exogenous estrogens, for example, readily feminize fish with TSD, as in the Argentinean

silverside, *Odontesthes bonariensis*, when exposed during the sex determining period (Strussmann *et al.* 1996). In several species of turtles that possess TSD, the application of E_2 during gonad differentiation causes feminization even at masculinizing temperatures (Merchant-Larios *et al.* 1997, Pieau and Dorizzi 2004, Freedberg *et al.* 2006). To my knowledge, however, no study has compared the relative sensitivity of sex differentiation to estrogen exposure in fish with TSD *vs*. those with GSD.

If fish with highly plastic sex determination are more sensitive to environmental endocrine disruptors, can these effects be seen at the population level? While the impacts of these compounds are still relatively unknown for marine fish, skewed sex ratio and gender abnormalities have been described in numerous freshwater lake and riverine species (Mills and Chichester 2005, Cheek 2006). Feminization is documented in populations adjacent to municipal wastewater discharge containing feminizing compounds, while fish exposed to paper mill effluent are often masculinized (Larsson *et al.* 2000). Evidence of endocrine disruption in the marine environment is mounting, but many of these studies measure biomarkers of estrogenic compounds but not the sex ratio (Oberdorster and Cheek 2001, Mills and Chichester 2005). Severely skewed sex ratios can often lead to a change in the reproductive capacity and therefore fitness of populations.

Here I investigate the effect of EDCs in fish populations with and without TSD. *Menidia menidia* was chosen as a model for this purpose because local populations differ greatly with latitude in the mode of sex determination (see above). First, I tested the hypothesis that the magnitude of sex ratio change will be greatest in populations with TSD. To do so, I conducted lab experiments comparing the response of sex ratio to varying concentrations of exogenous estrogen among populations with varying levels of TSD. Second, I determined whether the sex ratio of wild *M. menidia* is correlated with an urban gradient in population density and wastewater effluents. I compared population sex ratios of *M. menidia* across a pronounced urban to non-urban gradient. I hypothesized that sex ratios in the more urbanized estuaries would be significantly more female biased than populations from estuaries receiving less wastewater discharge.

Methods

Study species

The Atlantic silverside, *Menidia menidia* is one of the most common fish in the western North Atlantic with a distribution from Volusia County, Florida to the Magdalen Islands, Quebec, Canada (Gosline 1948, Johnson 1975). *M. menidia* has an annual life cycle and spawns repeatedly throughout spring and early summer. Larvae and juveniles presumably stay in natal estuaries until large enough to migrate in the fall (Middaugh 1981, Conover and Ross 1982, Conover and Kynard 1984).

Menidia menidia exhibits a latitudinal cline in level of temperature-dependent sex determination, which is positively correlated with the length of the growing season (Conover and Heins 1987A). The level of TSD is defined as the difference in sex ratio of larval fish reared at 15^{o}C and 28^{o}C (Conover and Heins 1987B) and ranges from 0 in northern populations to 0.75 in southern populations (Duffy and Conover, unpublished data). *M. menidia* at intermediate latitudes such as New York exhibit an intermediate level of TSD, showing partial response to temperature in addition to the contribution of genetic factors to sex ratio (Lagomarsino and Conover 1993). A level of TSD of 0.75 indicates that a minimum of 75% of the fish have sex determination that is thermally plastic (i.e. temperature influences the phenotypic gender). Conversely a level of TSD close to zero is evidence that environmental temperature exerts little influence on phenotypic sex of fish reared at temperatures that larval fish would be exposed to in nature.

TSD is adaptive for silversides that experience longer growing seasons; females are produced early when water temperatures are lower, which gives them a size-advantage over males produced in later in the season. Therefore, population sex ratios are variable throughout the spawning season, reaching a balanced sex ratio at the end of the breeding season (Conover and Kynard 1981). The latitudinal cline in TSD allowed for comparison of sensitivity to exogenous estrogen as a function of the level of the level of TSD within a single species. I used four populations of *Menidia menidia*, each with different levels of TSD and GSD. A preliminary exposure of first generation (f0) larvae from wild adults was conducted on two populations and a second study using two lab-reared populations was conducted using third generation (f3) larvae.

Experiments on f0 fish

Adult *Menidia menidia* in spawning condition were collected in the spring of 2005 from Broad Cove, Maine (ME) where fish were expected to have a low level of TSD (<0.03) and Patchogue, New York (NY) where fish had an expected level of TSD around 0.45 (Conover and Heins 1987A). *M. menidia* were strip-spawned according to Lagomarsino and Conover (1993) and embryos were transported to Flax Pond Laboratory in Old Field, New York. Upon hatch at 21^{o}C, fish (f0) larvae were reared on *Artemia* sp. nauplii (Brine Shrimp Direct, Ogden, UT) in 18 L containers with screens to allow water exchange with the bath in which they were submerged.

When fish reached approximately 7 mm they were stocked at random into closed, 12 liter containers (replicates) at a density of 60 fish per container with 10 L of ambient water. This density was chosen based on previous observations of larval survival at different densities. Each treatment was carried out in three containers (replicates). However, a fourth control replicate was included for New York because of an excess of similar-sized larvae. Buckets were immersed in a water bath and maintained at room temperature 21 $\pm$ 0.5 ^{o}C (mean $\pm$ standard deviation, unless otherwise noted). Treatments were initiated when average size of fish within containers reached 8 mm total length (TL). This size is the beginning of the thermosensitive window of sex differentiation, which occurs as fish grow from 8 to 21 mm TL (Conover and Fleisher 1986). Larvae generally reached an average of 8mm after 24-36 hours after stocking.

Treatments of E_2 (Sigma Chemical Co., St. Louis MO) in dimethyl sulfoxide (DMSO, Baker, Phillipsburg NJ) were added to the containers daily for a final container concentration of 100 ng l^{-1} E_2. An equal volume of DMSO without E_2 (0 ng l^{-1} E_2, final concentration) was added to control containers. Addition of E_2 mix or DMSO was carried out following daily renewal of 80% water exchange at ambient temperature. Excess food and waste were also removed daily, prior to dosing. Mortalities were noted and removed daily.

Because larval growth rates differ slightly between these populations, treatments were continued until the slower-growing (New York) population reached 21 mm, the length at which sex is irreversibly determined (Conover and Fleisher 1986). Thus, both

populations were dosed for an equal period of 29 days. Following the end of treatment, fish were returned to 18L containers and growth was allowed to occur over an additional seven to ten days to ensure all fish had reached a size at which sex could be determined by dissection. Fish were euthanized with tricaine methane sulphonate (MS-222; Western Chemicals Inc., Ferndale WA) and preserved in 10% sodium borate buffered formalin (Fisher Scientific, USA). Sex was determined with a dissecting microscope. Females were identified by the presence of large, opaque and fragile ovaries and males identified by the presence of thin, translucent, flexible and threadlike testes (Conover and Fleisher 1986).

Experiments on f3 fish

Menidia menidia were collected from the Edisto River, South Carolina in May, 2004 and from the Annapolis River in Nova Scotia, Canada in September, 2004. These populations were chosen because South Carolina fish show strong thermal dependence of sex ratio with a level of TSD around 0.7 and the Nova Scotia population displays predominantly GSD with a level of TSD near zero (Conover and Heins 1987A). Embryos were collected from spawning adults collected in South Carolina by strip-spawning as previously mentioned and transported to Flax Pond Laboratory. Larvae were reared on *Artemia* nauplii and later switched to an adult diet of pellet food (Otohime Hirami, Japan), frozen brine shrimp (San Francisco Bay Brand, Newark CA) and frozen mysid shrimp (Hikari, Hayward CA). Because of the time of collection, only juveniles could be collected from Nova Scotia and these were transported to Flax Pond Laboratory and reared on the adult diet. When both populations reached a large enough size, they were induced to spawn on yarn mops with photoperiod manipulation as in Conover and Fleisher (1986). Third generation larvae (f3) were collected from parents that had been held in the laboratory for two generations to minimize maternal influences.

Larvae averaging five to six mm were randomly assigned to 12L containers at a density of 60 fish per 10 L of water. For this experiment, larvae were collected from spawning adults and grouped by hatch dates within 5 days of each other. In the laboratory, adult *Menidia menidia* produce relatively low numbers of eggs over an extended period of months (Conover, pers. observ.). Therefore, it was impossible to

collect enough larvae at one time to carry out all replicates within the same period. Replicates had incomplete temporal overlap so they were analyzed as separate trials rather than pooled for analysis. Additional treatments in this experiment necessitated the use of two baths for temperature control. Fish were acclimated for 48-72 hours before beginning treatments when larvae averaged 8mm.

The f3 dosing study was carried out similar to the f0 study. A total of four logarithmic concentrations were chosen; 0 ng l^{-1} (control), 1 ng l^{-1} and 10 ng l^{-1} , representing measurements of E_2 in receiving waters and sewage treatment effluent (Reddy and Brownawell 2005, Desbrow *et al.* 1998), and 100ng l^{-1} representing a much higher dose than expected in nature . Each treatment received an aliquot of E_2 dissolved in DMSO so the final container concentration reflected the treatment concentration. Dosing encompassed the 8-21 mm period of sex differentiation, and was terminated after the slower-growing, South Carolina population reached an average of 21 mm in all treatments (34 days). Average temperature between trials (21.0 $\pm$ 0.5 ^{o}C) and between baths (20.9 $\pm$ 0.6^{o}C) did not differ significantly (T-Test, $p > 0.05$ for both). Daily static renewal was consistent with the first experiment.

Fish were allowed to grow and depurate for 10-14 days until sizes attained were sufficient to easily assess gender and then euthanized in MS-222 and fixed in 10% buffered formalin as described above.

Field study

Long Island, NY represents a longitudinal gradient of urbanization with heavy municipal wastewater discharge in western estuaries adjacent to New York City, decreasing exponentially in eastern estuaries. *Menidia menidia* were collected across Long Island from 10 sites yearly from 2005-2007. For logistical reasons, some sites were replaced with others throughout the study period, such that a total of 12 sites were sampled over the three year period (Figure 4.1).

Fish were collected using multiple tows of a 33-66 m beach seine at each site between late July and early September. Collection occurred as soon as the smallest fish in a given water body exceeded 21 mm which is needed for accurate sex identification (Conover and Fleisher 1986) and to ensure that all size classes were caught, minimizing

gear selectivity and thus, sex ratio bias. Timing of collection was chosen to precede size-dependent migration from estuaries, which occurs in autumn as fish get larger (Conover and Ross 1982). Therefore, fish were presumed to have been collected in their natal estuary. A subsample of 100-135 fish from each site were collected for sex identification, euthanized with MS-222, stored on ice for transport and then preserved in 70% ethanol.

Because temperature might explain sex ratio differences observed among sampling locations, environmental temperature was recorded throughout the summer of 2006 for all ten sites and at five sites in 2007 using Hobo Pendant Temperature Loggers (Onset Computer Corp.). Loggers were placed 0.3 m below the lowest low water (LLW) mark for each embayment and secured to a PVC pipe driven into the sediment. These were placed in the field between late April and early May and removed during specimen collection in late summer. Temperature was recorded six times daily.

Statistical Analyses

F0 exposure-A replicated G-Test of independence for frequency data was used to confirm that sex ratios in replicate samples were not significantly different from each other. A planned, one-tailed G-Test (Sokal and Rohlf 1995) was then used to compare pooled treatments.

F3 exposure-Sex ratios for individual trials were compared to the control from the same trial using a planned, one-tailed G-Test. Replicates were not combined because of incomplete temporal overlap of experimental replicates.

Field Study – Sex ratio was arcsine transformed to achieve normality for all statistical tests. An ANCOVA did not reveal significant differences among the adjusted means and slopes of arcsine sex ratio regressed onto longitude for 2005, 2006 and 2007 ($p > 0.9$) (Sokal and Rohlf 1995). Therefore, data from three years was pooled for analysis. The relationship between sex ratio and longitude was analyzed by linear regression.

Fish collected at all sites were highly variable in size (mean = 58.6 $\pm$ 17 mm), likely due to the protracted breeding season (two months). Therefore, fish spawned on different dates and likely experienced different temperatures during the period of sex differentiation. To estimate the mean temperature that fish experienced during larval

development, a growth rate of 1mm d^{-1} throughout the growing season was assumed based on prior studies fish from New York (Schultz *et al.* 2002). Individuals were grouped by size (within 5 mm) and average temperatures during the thermosensitive window for each population were calculated by using the temperature experienced by each size-class of fish and weighting this by the number of fish within each size-class. The static growth rate was used as a correction factor to determine the period of sex differentiation (8-21 mm). The relationship between temperature and sex ratio was analyzed by linear regression to evaluate potential thermal influence on sex ratio.

Results

Experiments on f0 fish

Sex ratios for control groups (Figure 4.2) were similar to the predicted sex ratio at 21^{o}C based on previous work: i.e., a sex ratio of 0.5 was expected for the Maine population and a low proportion of females (<0.2) was expected for the New York population (Conover and Heins 1987A). Both populations experienced significant ($p < 0.001$) increases in the proportion of females when dosed with 100ng l^{-1} E_2, as compared to the control groups. However, sex ratio was skewed to a greater extent in the New York population, with an 84 % increase in proportion of females (Figure 4.2 A) as compared to a 34% increase in proportion of females for the Maine population (Figure 4.2 B). Some mortality occurred early in each replicate, but there were no consistent differences in mortality between control and dosed groups. Average length at the end of the grow-out period was 39.7 $\pm$ 4.6 mm for New York and 39.5 $\pm$ 5.1 mm for Maine fish.

Experiments on f3 fish

Both the NS and SC populations showed significant ($p < 0.05$) increases in the proportion of females in the 100 ng l^{-1} dosed group as compared to each trial's respective control (Figure 4.3). The proportion of NS females increased significantly ($p < 0.05$) in response to the 100 ng l^{-1} E_2 dose in both trials, but did not increase significantly at the lower concentrations (Figure 4.3 A,B).

In contrast, a significant change in sex ratio was observed with the lower concentration treatments in the fish from South Carolina. Sex ratios at 1 ng l^{-1} and 10 ng

l^{-1} were significantly different from the control ($p < 0.01$) for the first trial of SC fish (Figure 4.3 C) and a highly significant ($p < 0.001$) increase in the proportion of females was seen at the 10 ng l^{-1} dose in the second trial (Figure 4.3 C,D). The proportion of females in the 100 ng l^{-1} dose, as compared to the controls, increased substantially more for the SC population (68%) than in the NS population (32%). Water temperature between trials and length of dosing period did not differ significantly. Some mortality occurred early in each trial, but there were no consistent differences in mortality between control and dosed groups. Average length at the end of the grow-out period was 25.9 $\pm$ 3.9 mm for South Carolina and 30.3 $\pm$ 4.2 mm for Nova Scotia fish.

Field study

Sex ratio was significantly correlated with longitude (Figure 4.4, $p < 0.001$, $r^2 = 0.478$) for each year analyzed separately and all years combined. Population sex ratios in *Menidia menidia* from estuaries closest to New York City were significantly more female biased than those from eastern Long Island where the sex ratio was approximately 1:1.

Temperature was measured in the second and third year of the study to determine if the gradient in sex ratio was correlated with a gradient in temperature across locations. No significant trend was found between arcsine-transformed sex ratios and the average temperature during the thermosensitive period ($r^2 = 0.03$, $p = 0.55$). Additionally, a linear regression of temperature and longitude was not significant ($r^2 = 0.058$, $p > 0.1$).

Discussion

The results from the exposure study indicate differential susceptibility to E_2 among four populations of *Menidia menidia* that differ in the level of TSD. Fish from all four locations responded to the highest dose of E_2 with a significantly increased proportion of individuals differentiating into females. In both dosing experiments, however, southern populations exhibited the largest change in sex ratio with E_2 treatment relative to northern populations. However, the magnitude of this change may be reflective of the male-biased sex ratio in the southern population control group. Nevertheless, the population from South Carolina with a highest level of TSD responded at lower concentrations of E_2 than did the Nova Scotia with complete GSD. This suggests

that TSD is associated with an individual's susceptibility to E_2 exposure. This is especially likely given that the endocrine system is highly sensitive to cues from the environment during the early life history (Sumpter 1997, Devlin and Nagahama 2002, Strussmann and Nakamura 2002). However, other intrinsic differences in the physiology of *M. menidia* could also play a role.

Fish from higher latitudes (Maine and Nova Scotia) exhibit significantly faster intrinsic growth rates than southern counterparts (Conover and Present 1990). These genetically determined differences in growth rates are the result of differences in metabolic rates, food conversion efficiencies and food consumption (Conover and Present 1990, Present and Conover 1992), and may contribute to differential response of sex differentiation to E_2. Faster growth rates in northern latitude fish may mean that they are able to metabolize and remove E_2 as waste in a more efficient manner than southern fish. Additionally, faster growth rates may abbreviate the window of sex differentiation, thus decreasing the period when fish may be most susceptible to E_2. However, sex differentiation is known to be tightly controlled by the regulation of E_2 and other steroidal hormones. The most direct and therefore parsimonious explanation is that the presence of E_2 overrode the thermal effects on sex determination (Devlin and Nagahama 2002, Strussmann and Nakamura 2002, Burger *et al.* 2007). However, indirect effects of other intrinsic characteristics that vary with latitude such as growth rate (Present and Conover 1992) cannot be ruled out.

Champlin *et al.* (2002) treated larval *Menidia menidia* collected from Rhode Island (intermediate level of TSD, Duffy and Conover, unpublished data) with E_2 for 35 days and found moderate to complete feminization when exposed to10 ng l^{-1} and 50 ng l^{-1} E_2. These results are comparable to Champlin *et al.* (2008) and differences in sex ratio likely result from differences in the mode, length and timing of exposure. Direct comparison of sex ratios from laboratory studies is difficult due to protocol differences, but the magnitude of feminization observed in *M. menidia* in response to E_2 exposure is similar to that observed in other species such as *Oryzias latipes* (Nimrod and Benson 1998, Koger *et al.* 2000).

A majority of studies that address responses of fish to EDCs reflect short-term exposure to single compounds and address changes in sex ratio, physiological and

biochemical processes and behavior (Mills and Chichester 2005). Nash *et al.* (2004) compared the effect of 5 ng l^{-1} EE_2 aqueous exposure on *Danio rerio* reproduction during a short-term (26 day) and life-long exposure. The 26-day exposure elicited no changes in egg production, viability and embryo mortality but the prolonged exposure resulted in 100% reproductive failure. Robinson *et al.* (2007) exposed the marine sand goby, *Pomatoschistus minutus*, to a range of E_2 concentrations over an eight month period. A comparison of several indices of reproductive health such as gonadosomatic index, vitellogenin production (a biomarker of male exposure to feminizing compounds) and fertility revealed impairment to some of these parameters at intermediate concentrations (16-97 ng l^{-1} E_2) and complete reproductive failure at the highest concentration (669 ng l^{-1} E_2). These studies demonstrate that exposure to EDCs in the wild has the potential to significantly alter reproductive fitness, especially for species that are exposed at critically sensitive periods or for long periods of time (Sumpter 1997, Arcand-Hoy and Benson 1998, Oberdorster and Cheek 2001).

Results from the field study demonstrate population-level differences along an urban gradient of wastewater effluent. This is evidenced by highly female-biased *Menidia menidia* sex ratios from urbanized estuaries of western Long Island compared to the approximately 1:1 sex ratios in eastern Long Island. This trend was significant in each of the three years sampled in this study. Additionally, the non-significant relationship between sex ratio and temperature appears to rule out thermal differences among estuaries as a major factor determining this pattern. These data are consistent with the hypothesis that EDCs are sufficiently concentrated in urban estuaries so as to cause a sex ratio skew favoring females. This interpretation is consistent with the experimental results, and also those on other species where a female-biased sex ratio is a characteristic response to estrogenic compounds (Arcand-Hoy and Benson 1998, Sumpter 2005, Cheek 2006). Further studies should explore other indices and impacts of EDCs including biomarkers of exposure at the molecular level, reproductive impairment such as gonad pathology or fecundity, or reduced fitness in the form of diminished growth or recruitment (Mills and Chichester 2005).

I chose to use sex ratio as a potential indicator of estrogen and estrogen mimics because it is a simple and ecologically important population parameter in fish. The

ubiquitous presence of silversides in estuaries that vary in contaminant loading, their limited movement during their larval and juvenile stages and their presumed heightened susceptibility to endocrine disruptors during gonad differentiation makes them a good indicator species for detecting the impact of EDCs in the environment.

Human population density and thus wastewater discharge, is markedly different between New York City and eastern Long Island. New York City (encompassing five counties) produces an average of 1,300 million gallons per day (MGD) of sewage effluent. Nassau County, (immediately east of New York City) produces an average of 140 MGD and Suffolk County (eastern half of Long Island) discharges only 30 MGD of effluent (IEC 2008). *Clostridium perfringens* spores, reliable tracers of sewage effluent, demonstrate a strong positive correlation between human population density and wastewater effluent along a longitudinal gradient in Long Island Sound (Buchholtz ten Brink *et al.* 2000). Unfortunately, a comprehensive survey directly measuring estrogenic compounds in waters around Long Island has never been conducted, so correlation of sex ratio changes with known estrogenic sewage-derived contaminants is not possible at this time.

Previous work has demonstrated that effluent from several New York City sewage treatment plants with concentration of E_1 and E_2 of 5-13 ng l^{-1} and estrogenic nonylphenol ethoxylates is estrogenic to larval hybrid striped bass (Todorov *et al.* 2002, McArdle *et al.* 2000). Furthermore, surveys of juvenile winter flounder, *Pseudopleuronectes americanus*, have demonstrated highly female biased sex ratios in Jamaica Bay in western Long Island, as opposed to a sex ratio of 0.5 in Shinnecock Bay on eastern Long Island (McElroy *et al.* 2006). Sex ratios of *Menidia menidia* from this study mirror those of *P. americanus* from the same study sites. In particular, Jamaica Bay is highly urbanized and receives most of its freshwater input from wastewater (Swanson *et al.* 1992) and sediments contain high levels of estrogenic compounds such as E_2 (< 0.53 ng g^{-1}) nonylphenol ethoxylates (> 50 μg g^{-1}) (Ferguson *et al.* 2003, Reddy and Brownawell 2005). Collectively, these studies indicate that feminization of fish in urban estuaries around New York City may be a widespread phenomenon. Although these data are consistent with environmental estrogenic chemicals playing an important role in sex ratio of *M. menidia*, more research is needed to determine the causative agent(s).

Any number of hormonal and physiological traits may contribute to population susceptibility to estrogenic chemicals. Kidd *et al.* (2007) carried out a seven year study by adding EE_2, a more potent form of E_2 (Desbrow *et al.* 1998), to a lake in Canada. EE_2 induced severe reproductive abnormalities in the fathead minnow, *Pimephales promelas*, as evidenced by abnormal and arrested testicular development in males and depressed oogenesis in females. Chronic exposure resulted in reduced fecundity and an eventual population crash not seen in a nearby control lake. The authors conclude that the short lifespan of this species led to its swift collapse, relative to a healthy population of longer-lived pearl dace in the same lake.

Bull (1983) proposed that temperature-dependent sex determination is adaptive if the environment an individual experiences early in life disproportionately favors the fitness of one sex over the other and an individual has no control over which type of environment it enters. Endocrine disruptors in the aquatic environment may counteract the adaptive benefit of TSD by altering the sex ratio and creating individuals that are less fit in the environment, all else being equal. Hirai *et al.* (2006A,B) found that continuous exposure to E_2 from hatch through reproduction resulted in significantly reduced fecundity and number of spawning events by sex-reversed (XY) female Japanese medaka. Reproductive success of fish that are phenotypically females, but would have otherwise become a male in the absence of EDC exposure, is poorly understood and needs further evaluation to understand population response as a whole. Moreover, populations with severely skewed sex ratios exhibit reduced relative fitness in the majority sex (Leimar *et al.* 2004), due in part to competition for mates (Berec and Boukal 2004), and increased relative fitness of the minority sex. This occurs because the minority sex produces more offspring per capita than does the majority sex and so its genes spread more quickly in subsequent generations. Because members of the minority sex are likely to possess higher levels of GSD, strong skews in sex ratio may cause the evolution of GSD over TSD (Bulmer and Bull 1982). This process has been demonstrated through selection experiments in *M. menidia* (Conover and Van Voorhees 1990). Hence, the sex ratio skew caused by EDCs could influence the evolution of the sex determination in populations subject to EDCs.

These results suggest that Atlantic silversides from southern populations are

especially sensitive to environmental estrogens, and further indicate that the form of sex determination (TSD) may be a factor that contributes to this susceptibility. This study also provides evidence of population-level alteration in sex ratio in *Menidia menidia* in the wild, which may be attributed to a pronounced gradient of wastewater effluent. Because *M. menidia* exhibits local adaptation in TSD and GSD among populations, it represents a unique opportunity to determine how endocrine disrupting chemicals in the environment interact with the genotype of an individual to determine sex. Because TSD is common in fishes, there may be numerous other species that also possess heightened susceptibility to endocrine disrupting compounds. Hence, an understanding of such genotype by environment interactions is important to predicting the overall effect of EDCs on natural populations of fishes.

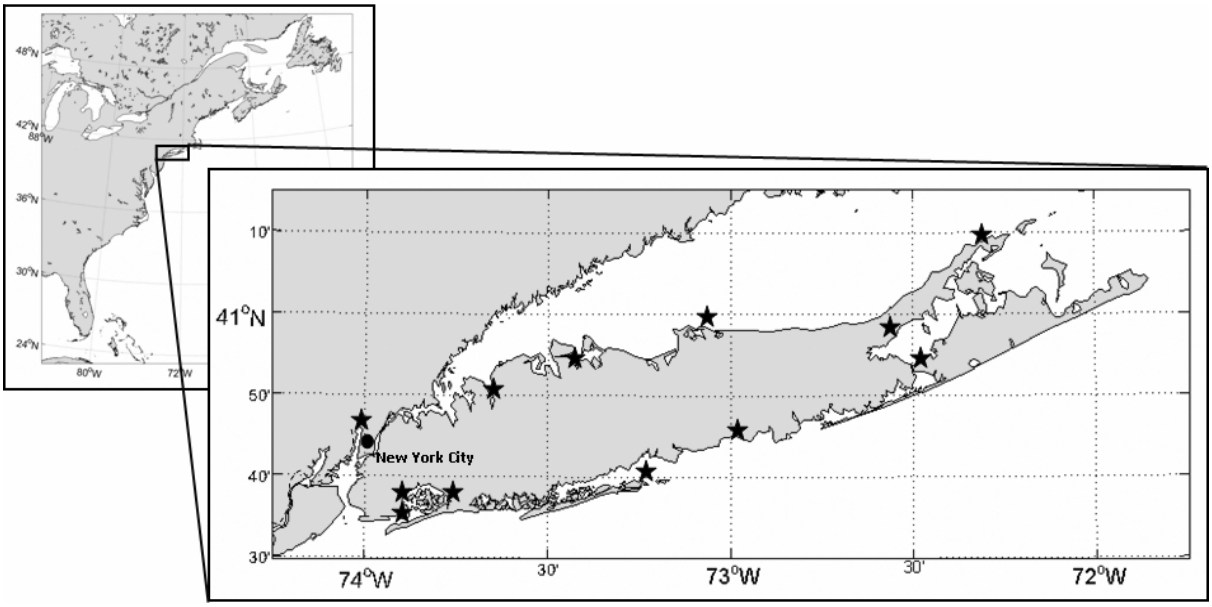

Figure 4.1. Sites of fish collection, Long Island, NY for 2005-2007. Map courtesy of O. Doherty.

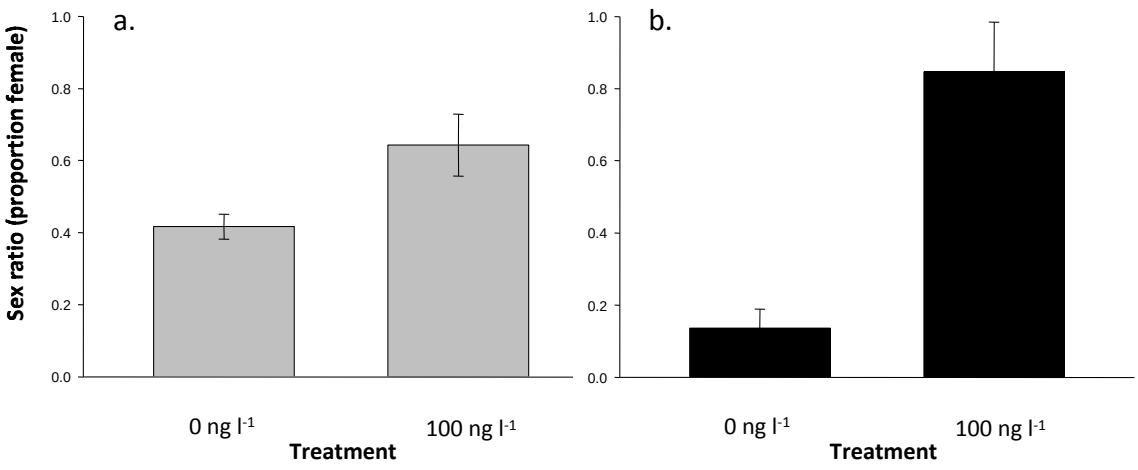

Figure 4.2. Sex ratio (as proportion of females) for pooled replicates from a. Maine and b. New York fish (mean $\pm$ SD). Pooled treatment replicates (100 ng/l) are significantly different from respective control ($p < 0.001$) in both populations.

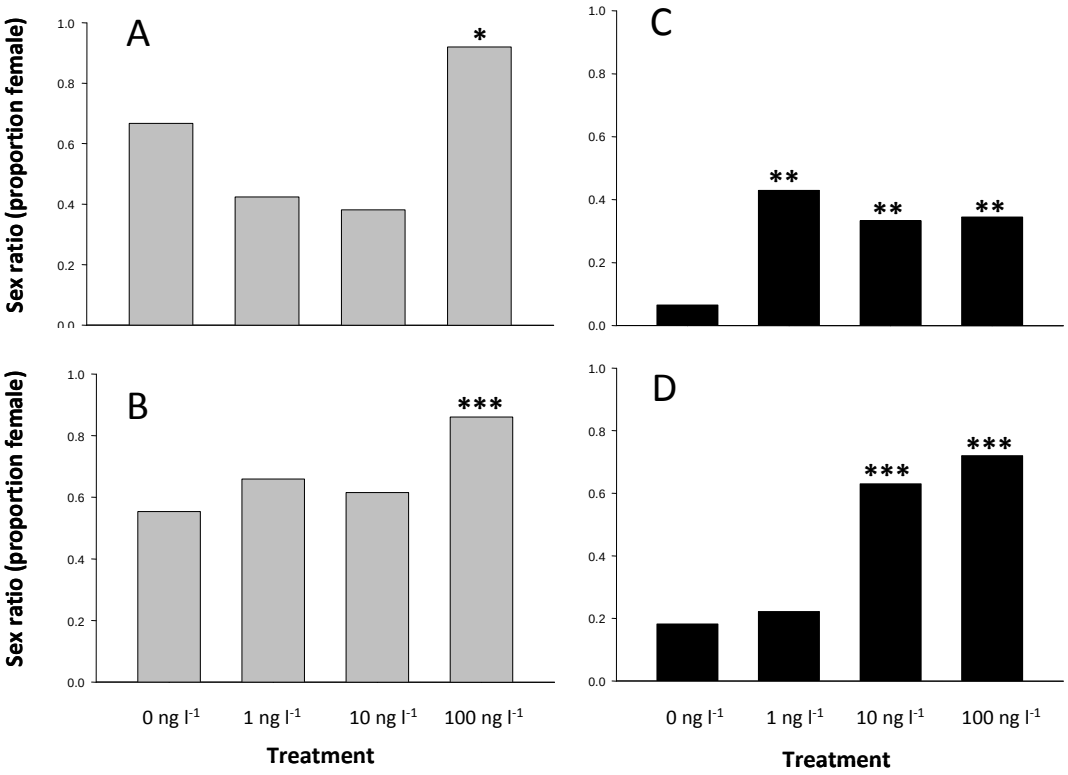

Figure 4.3. Sex ratio (proportion of females) for individual f3 trials from Nova Scotia (A,B-grey) and South Carolina (C,D-black). Asterisks indicate level of significant difference compared to respective control (* $p<0.05$, ** $p < 0.01$, *** $p < 0.001$).

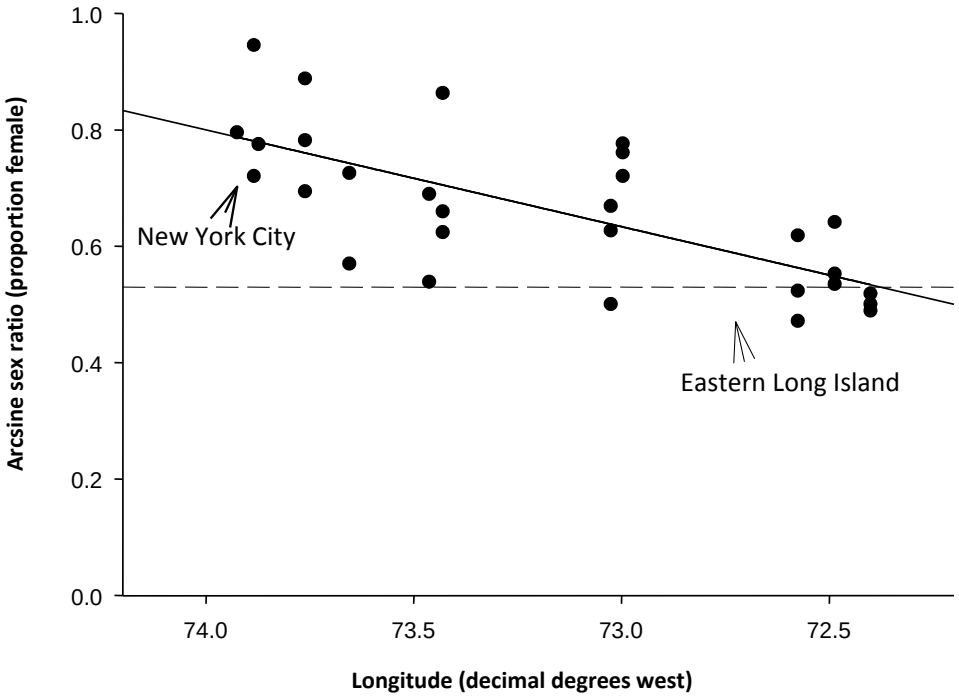

Figure 4.4. Sex ratio (arcsine transformed for normality) of *Menidia menidia* collected along a longitudinal urban gradient ($p < 0.001$, adj. $r^2 = 0.478$). Solid line represents the best-fit linear regression; dotted line represents a 1:1 sex ratio.

Chapter 5: Artificial selection for fast growth results in reduced plasma IGF-1 in *Menidia menidia*

Abstract

Selection for fast growth in domesticated fishes often results in predictable and measurable changes within the GH-IGF (growth hormone-insulin-like growth factor) axis. However, little is known about the mechanisms controlling changes in growth capacity resulting from fishery-induced evolution. The Atlantic silverside, *Menidia menidia*, provides an excellent system for addressing changes in the GH-IGF axis. I took advantage of a long-term study where *M. menidia* experienced multiple generations of strong selection on body size to mimic fisheries-induced selection. This selection regime produced populations with major differences in growth rate; fast and slow growth and a third under random selection representing the reference population. Plasma IGF-1 was measured in these populations as a proxy for growth potential. IGF-1 was significantly correlated with length and mass as well as growth rate (g d^{-1}), indicating it may be an appropriate indicator of growth capacity. However, when the effect of increased IGF-1 with length/weight was removed, slow-growing fish exhibited the highest IGF-1 levels relative to the depressed IGF-1 seen in fast-growing individuals. I offer possible explanations for this unusual pattern and argue that somatic growth is likely to be under the control of a mechanism that is downstream to the production of IGF-1. In addition, IGF-1 was compared between populations of *M. menidia* that exhibit different growth rates (South Carolina and a hybridized South Carolina X Nova Scotia population). Little evidence for relationships between IGF-1 and growth patterns was seen in these populations. IGF-1 may be a useful biomarker of growth potential in this species, but it is likely to be population-dependent and strongly influenced by the type of selection that is exerted on growth.

Introduction

Selection for enhanced growth capacity in domesticated fish often results in measurable and profound changes in growth rate and other growth-related traits (Gjedrem, 2001; Allendorf *et al.*, 2008; Tymchuk *et al.*, 2009). These changes in phenotype likely correspond to changes in the growth hormone-insulin-like growth factor-1 (GH-IGF) axis (Perez-Sanchez and Le Bail, 1999; Fleming *et al.*, 2002; Wood *et al.*, 2005). GH and IGF-1 are major polypeptides that regulate growth, development and differentiation through various autocrine, paracrine and endocrine actions (Wood *et al.*, 2005; Tymchuk *et al.*, 2009). During anabolic states, GH is released from the pituitary and binds to hepatic GH receptors. This in turn stimulates release of IGF-1 into the blood stream which acts as a negative feedback on GH release. IGF-1 controls major growth processes like cell differentiation and cell growth, leading to somatic growth via its receptor, IGF-1 receptor (IGF-1R) (Wood *et al.*, 2005; Picha *et al.*, 2008A). Strong selection on growth-related traits is likely to alter the function of the major regulators of growth such as GH and IGF-1 (Perez-Sanchez and Le Bail, 1999; Fleming *et al.*, 2002; Wood *et al.*, 2005). Salmonid breeding programs provide excellent evidence for changes in plasma GH and IGF-1 following artificial selection for increased growth (Fleming *et al.*, 2002; Lankford and Weber, 2006; Taylor *et al.*, 2008; Tymchuk *et al.*, 2009), but changes to the GH-IGF axis in most domesticated fish are not yet fully understood.

Strong human-imposed selection for growth-related traits is common in aquaculture breeding programs (De-Santis and Jerry, 2007; Picha *et al.*, 2008A, Tymchuk *et al.*, 2009). However, it is now widely accepted that fisheries may impose strong selective pressures that cause genetic changes in traits like growth rate, age at maturity and behavior (Edeline *et al.*, 2007; Swain *et al.*, 2007; Conover *et al.*, 2009). Several researchers have proposed that IGF-1 may be an ideal biomarker for measuring growth capacity in domesticated fishes (Fleming *et al.*, 2002; Dyer *et al.*, 2004; Wood *et al.*, 2005), but this utility has not yet been tested in wild fishes in response to size-selective harvest. IGF-1 was shown to be a good candidate for measuring growth capacity in domesticated salmonids (Lankford and Weber, 2006; Tymchuk *et al.*, 2009), and may be a promising biomarker in channel catfish (Peterson *et al.*, 2008), hybrid striped bass (Picha *et al.*, 2008B) and other species (De-Santis and Jerry, 2007; Picha *et al.*, 2008A).

Circulating IGF-1 may therefore be a candidate, non-lethal polypeptide that can be used to address changes in growth potential in fishes that have undergone strong selection in the wild. Further understanding of the changes within the GH-IGF axis may help to elucidate the specific mechanisms that contribute to changes in life-history characters as a result of strong human-induced evolution.

Conover and Munch (2002) provided one of the first empirical cases of change in growth-related traits in a fish in response to simulated size-selective harvest. Over five generations, they simulated fisheries-induced selection on size in lab-reared populations of the Atlantic silverside, *Menidia menidia*, by removing the largest or smallest individuals, allowing the smallest or largest 10%, respectively, to reproduce. Growth rates and population biomass diverged rapidly as compared to a randomly-harvested control population. Additionally, Walsh *et al.* (2006) found that after five generations of selection, multiple characters such as food conversion efficiency, larval survival and egg volume also shifted in predictable directions. Following generation five, Conover *et al.* (2009) relaxed selection for an additional five generations to see if growth rate and other traits were likely to rebound in the absence of selection. Some recovery occurred in the large-harvested, slow-growing population. These fish showed a tendency toward increasing size at age, and thus growth rate, while the small-harvested, fast-growing population showed no change in growth rates after selection was relaxed. The authors concluded that an additional five generations would be needed for growth rates in the large-harvested population to fully recover. Despite partial recovery, growth rates differed significantly among the lines in generation 10, and therefore provide an opportunity to address changes with IGF-1 regulation between populations with different growth capacities.

Menidia menidia represents an interesting species to address growth rate differences within the experiment above, but also in wild populations. The Atlantic silverside has a broad geographic distribution from Florida to the Gulf of St. Lawrence, Canada and exhibits strong local adaptation in growth rate (Conover and Present, 1990). Intrinsic growth rate is positively correlated with latitude and therefore, different populations exhibit genetically-based growth rate differences. Based on growth differences among populations, regulation of IGF-1 and/or other endocrine regulators of

growth may be different among populations. Therefore, IGF-1 can be compared within a species that has undergone natural selection for growth to determine the role of IGF-1 in contributing to the latitudinal pattern of growth rates in *M. menidia*.

In this study, I compare circulating plasma IGF-1 profiles in juvenile *M. menidia* populations that have undergone strong artificial selection for growth-related traits. The objective of this work is to see if changes within the GH-IGF axis (here, plasma IGF-1) may help to explain the divergence in growth rates induced by intentional selection of growth traits in *M. menidia*. IGF-1 was measured in this study, rather than GH, in this study because 1) changes in circulating IGF-1 often correspond to changes in growth rate in domesticated fish (Picha *et al.*, 2008A), 2) IGF-1 is demonstrated to be a mediator of the effects of circulating GH (Wood *et al.*, 2005), and 3) plasma IGF-1 is a relatively stable proxy of growth (Yakar *et al.*, 2002; Wood *et al.*, 2005). I hypothesize that elevated growth rates correspond to elevated plasma IGF-1. Secondly, IGF-1 profiles in a slow-growing population of *M. menidia* were compared to those in a faster-growing hybridized population to see if IGF-1 may also contribute to growth differences in these populations.

Methods

Fish Collection

Menidia menidia originating from New York were experimentally manipulated to simulate size-selective fishing pressure for five generations. Three treatments were carried out in duplicate; selection for large and small body size and a no selection control. Selection for large and small body size was done by removing 90% of the smallest and largest individuals, respectively, prior to spawning. A third treatment representing random selection was carried out by similar methods, but fish were randomly selected regardless of size (see Conover and Munch 2002 for details). Beginning in the sixth generation, selection was relaxed by treating all populations like the control treatment for an additional five generations to assess potential rebounding of growth rate and size-related traits that changed under the selection regimes applied during the first five generations (Conover *et al.*, 2009).

Wild *M. menidia* embryos were collected from the Edisto River in South Carolina, USA and the Annapolis River in Nova Scotia, Canada in the spring of 2007 (see Lagomarsino and Conover, 1993 for methods) and transported to Bluepoint Marine Laboratory in Patchogue, NY. Larval *M. menidia* were reared on *Artemia* sp. nauplii (Brine Shrimp Direct, Ogden, UT) then switched to pelleted food (Otohime Hirami, Japan), frozen brine shrimp (San Francisco Bay Brand, Newark CA) and frozen mysid shrimp (Hikari, Hayward CA) as juveniles and adults. Several Nova Scotia and South Carolina fish were hybridized by allowing spawning to occur between these two populations (confirmed by vertebral counts, Hice, 2010). For this analysis, South Carolina juveniles were compared with the faster-growing, hybridized population.

Experimental setup

Tenth generation, adult fish from identical duplicate treatment populations (Conover *et al.*, 2009) were combined in 220 gallon recirculating tanks and were induced to spawn on yarn mops by photoperiod manipulation (see Conover and Fleisher, 1986 for methods). Embryos (generation 11) were collected two days after spawning occurred and were held at 21^{o}C in five gallon flow-through containers and reared as mentioned previously. Upon reaching 30 mm, juveniles with fertilization dates occurring within a two week period were moved into 220 gallon tanks at a density of 175 fish. Fish were kept on a continuous 15:9 light:dark cycle at 24-27psu and 21^{o}C and fed *ad libitum* throughout the sampling period.

Plasma was collected at 5 timepoints between 160-240 days post hatch (dph) for the small harvested and random harvested population. Due to slow growth rates and small sizes, the large harvested population was sampled at day 270 instead of day 240 to ensure a broad range of sizes for comparison. Density in each of the three tanks was adjusted after each sampling timepoint. Fish were fed ad libitum 15 hours prior to sampling, which occurred prior to the morning feeding, between 8am and 11am. Additionally, sampling was carried out by mixing the order in which populations were collected to minimize the effect of diel IGF-1 cycling. Due to small plasma volumes, between 2 and 5 fish (mean= 2.8 $\pm$ 0.75 SD (standard deviations)) were used per sample and were size-matched to within 10 mm where possible. To minimize handling at

collection time, a small sample of fish were removed from the tanks and placed in a holding bucket. Prior to collection, fish were anesthetized in tricaine methane sulphonate (MS-222; Western Chemicals Inc., Ferndale WA), weighed to the nearest milligram and measured for length. The caudal fin was immediately severed with a clean scalpel blade and blood was collected in a clean heparinized microcapillary tube. Blood was immediately put into microcentrifuge tubes on ice and equal volumes of blood were combined within a tube. Whole blood was centrifuged at 11,000 x *g* for four minutes. Plasma was removed with a sterile pipette and immediately frozen on dry ice and stored at -80^{o}C prior to analysis.

The second experiment utilizing SC and hybrid fish was carried out as above with the following changes. Recirculating systems were stocked at 90 fish per tank that were spawned over five weeks due to the low egg production of both stocks. Fish were sampled over three time points from day 115-160 corresponding to the smallest sizes at which plasma can be obtained in sufficient quantities when growth rates are expected to be maximal.

RIA analysis

Circulating levels of total IGF-I were measured from acid/ethanol extracted plasma by radioimmunoassay (RIA) using recombinant barramundi IGF-I as tracer and standard, rabbit anti-barramundi IGF-I primary antibody (Novozymes GroPep; Adelaide, Australia) and goat anti-rabbit secondary antibody (Sigma; St. Louis, Missouri) according to previously described methods (Shimizu *et al.* 2000). Barramundi IGF-I was iodinated using the chloramine-T method and purified by column chromatography. Tracer (^{125}I-barramundi IGF-I) was diluted to 20,000 cpm for each assay tube. Serially diluted Atlantic silverside plasma was shown to produce a displacement curve that parallels that of the standard, verifying its validity for measures of circulating IGF-I in this species (Figure 5.1).

Analysis and statistics

Mean growth rates in mm d^{-1} and g d^{-1} were calculated from average lengths and weights of fish immediately prior to plasma sampling. Average weight at the day of

sampling was compared for both sets of fish using a two-way ANOVA with Tukey comparisons to account for non-parametric data. Linear regression was used to test for relationships between plasma IGF-1 and weight and IGF-1 and length. Because these relationships were significant for populations in the selection experiment (see below), interpopulation IGF-1 was compared using residuals from a linear regressions of all IGF-1 data and either length or weight. This allowed me to compare across populations with individuals of different sizes by normalizing IGF-1 values for length or weight. Normalized population IGF-1 values were compared among populations with an ANCOVA. Additionally, linear regression was used to detect relationships between raw plasma IGF-1 and growth rate in length and weight. All non-normal data was natural log transformed for normality. All comparisons carried out with the selection experiment were duplicated for the hybrid-South Carolina populations.

Results

NY Selection Comparison

Comparisons of mean growth rates among experimental populations revealed significant differences (one-way ANOVA with Bonferroni post hoc comparisons, $p<0.001$ for all) with SH fish experiencing maximal growth rates. Further two-way ANOVA revealed significant differences in mean weights at a given age between populations ($p<0.001$) as well as significant population by age effects ($p<0.001$) (Figure 5.2). Tukey-Cramer pairwise comparisons revealed significant differences among populations within a given age. The final timepoint was not included in this analysis because ages did not overlap (240 vs. 270 days) and cannot be directly compared.

In all populations, plasma IGF-1 increased with both mass ($p\leq0.02$, Figure 5.3) and length ($p\leq0.02$). Linear regression revealed a significant positive relationship between growth rate in mass (g/d) and IGF-1 (ng/ml) for the small-harvested and control population ($p<0.001$, $r^2=0.53$ and $p=0.041$, r2=0.136, respectively) but no significant relationship between growth in mass and plasma IGF-1 in the large-harvested population (Figure 5.4 A). Conversely, no significant relationship between growth in length and plasma IGF-1 was found in any of the populations ($p>0.05$, data not shown).

Mass-corrected plasma IGF-1 values were significantly higher overall in the large-harvested (slow-growing) fish than the small-harvested (fast-growing) fish (Figure 5.4 B, 1-way ANOVA, p=0.026). Bonferroni multiple pairwise comparisons revealed no differences in other comparisons (RA not significantly different from LH or SH). Similarly, regression lines of IGF-1 residuals and length were compared with an ANCOVA (due to equality of regression lines) and large-harvested fish were found to have significantly elevated IGF-1 overall than the small-harvested fish (p=0.002).

Nova Scotia (hybrid) and South Carolina comparison

Mean population growth rates measured in both mm d^{-1} and g d^{-1} were significantly different between populations (t-test, df=27, $p<0.001$ for growth rate in mm/d and g/d). Additionally, average mass was significantly different between populations at all sampling points (Figure 5.5). In the Nova Scotia population, plasma IGF-1 increased with both length (linear regression, p=0.015) and mass (p=0.006), but this relationship was non-significant in the South Carolina population ($p>0.3$ for both relationships). Further, no significant relationship between IGF-1 and growth rate in mass (SC, p=0.36; NS, p=0.156, Figure 5.6 A) or length (mm/d) (SC, p=0.303; NS, p=0.515, not shown) was found. Additionally, neither mass-corrected nor length-corrected linear regressions revealed significant differences in plasma IGF-1 among populations (ANCOVA, p=0.260 for length, p=0.149 for mass, Figure 5.6 B).

Discussion

NY Selection comparison

In this experiment, circulating plasma IGF-1 showed a positive relationship with both weight and length in *M. menidia*. Therefore, circulating IGF-1 may predict current mass or length. This relationship presumably reflects the involvement of IGF-1 in a physiological function in growth, but what specific role it has in *M. menidia* is unknown. It is important to note that because IGF-1 changes with length (and therefore age), comparisons across populations should be done with similar aged individuals or the length effect must be removed (Shimizu *et al.*, 2009). External cues such as photoperiod and temperature are seasonal cues that have strong influences on IGF-1 regulation (Wood

et al., 2005; Picha *et al.*, 2008A). Plasma IGF-1 responds to seasonal fluctuations in many species such as rainbow trout (Taylor *et al.*, 2008) and seabream (Mingarro *et al.*, 2002). In this study, photoperiod and temperature were held constant, indicating that increases in IGF-1 is likely due to internal endogenous regulation of IGF-1 or by subtle external cues from a laboratory environment that we could not measure (i.e. subtle changes in water chemistry). The increase in IGF-1 with age in all lines warrants further examination.

Another possible explanation for the increase in IGF-1 with size is a potential relationship between the GH-IGF axis and reproduction. *M. menidia* has an annual life cycle (Conover and Ross, 1982) and usually reach maturity around nine months of age. In laboratory populations, fish are reared for eight months and then photoperiod is manipulated to induce vitellogenesis (Conover and Fleisher, 1986). *M. menidia* may have been undergoing endogenous changes to prepare for reproduction, reflected in the increase in IGF-1 over a period of four months. The GH-IGF-1 tightly regulates growth in teleosts and may contribute to reproductive development prior to puberty as energy is directed from somatic to gonadal growth (Wood *et al.* 2005). Several studies have demonstrated that IGF-1 plays a functional role in puberty in vertebrates. IGF-1 promotes oocyte maturation in amphibians and mammals (review by Le Gac *et al.*, 1993). Weber and Sullivan (2000) demonstrated that IGF-1 induces *in vitro* maturation of striped bass oocytes and other studies show that IGF-1 increases prior to reproduction *in vivo* (Moriyama *et al.*, 1997; Taylor *et al.*, 2008). The mechanisms by which IGF-1 may influence puberty are still largely unknown and therefore the link between increased IGF-1 with size in *M. menidia* is speculative.

Growth rate (g d^{-1}) was also positively correlated with IGF-1in the fast growing and control populations, but no significant relationship was found in the slow-growing group. These results suggest that IGF-1 may be useful for indicating growth potential in this experimental system. Despite these significant results, no relationship between IGF-1 and growth in length was found in any population. IGF-1 appears to be a poor biological predictor of growth in length during this phase of growth in *M. menidia*. However, IGF-1 may play a functionally significant role in growth in weight, and therefore may useful for this relationship. Growth is a highly coordinated process with

many factors involved including the stress (Bonga 1997; Lankford and Weber, 2006) and thyroid hormone axes (Power *et al.*, 2000), which demonstrate that overall growth is the sum of all these factors (Wood *et al.*, 2005). IGF-1 has many functional roles including coordination of skeletal and somatic growth, cell differentiation, puberty and responses to changes in salinity (Le Gac *et al.*, 1993; Wood *et al.*, 2005; Picha *et al.*, 2008A). The role of IGF-1in coordinating these processes may differ between selection regimes, because selection could be acting, for example, on the GH-IGF axis in the large harvested line and on the stress axis in the small harvested line. Use of IGF-1 to predict growth potential in this species should be further studied to understand the utility of this polypeptide as a growth biomarker in *M. menidia*.

Interestingly, the slowest-growing population (LH) displayed the higher plasma IGF-1 levels than the fast-growing (SH) population. However, circulating IGF-1 in both populations were not significantly different from the control population. Therefore, it is unknown whether the differences seen between the large-harvested and small-harvested lines are reflective of IGF-1 regulation shifting in either direction in one of the lines, or shifting in opposite directions simultaneously. The non-significant differences between the lines selected for rapid and slow growth may indicate that IGF-1 is converging back to its original state (plasma IGF-1 in the control population) or that IGF-1 is acting in concert with other mechanistic factors to cause significant growth differences among lines. Conover *et al.* (2009) demonstrated that length at day 90 and 190 showed signs of reversal following six generations of relaxed selection in the large-harvested population, but not in the small-harvested population. Additionally, several of the traits related to growth that were measured by Walsh *et al.* (2006) show evidence of full recovery in the tenth generation (Salinas *et al.*, In prep). Higher normalized IGF-1 in the slowest growing line may be indicative of an evolutionary rebound in some traits regulated by IGF-1 or that IGF-1 regulation is highly plastic and responsive to relaxed selection. However, these possibilities suggest that other factors in the growth axis may play a considerable role in controlling somatic growth.

The relationship of length normalized IGF-1 among lines is opposite to that predicted by previous patterns of IGF-1 regulation found in domesticated fish species. Several studies have demonstrated positive relationships between plasma IGF-1 or IGF-1

gene expression (which may be indicative of plasma IGF-1 concentration) and growth rate (Lankford and Weber, 2006; Magdeldin *et al.*, 2007; Tymchuck *et al.*, 2009), while other studies find little evidence for relationships between growth and plasma IGF-1 or IGF-1 expression (Fleming *et al.*, 2002; Frantzen *et al.*, 2004; Peterson *et al.*, 2008). To my knowledge, this is the first demonstrated case of enhanced IGF-1 in a fish species that has been selected for slow growth and subsequent reduced IGF-1 in a fast-growing population. Therefore, several plausible explanations for this observation are provided.

IGF-1 is a regulator of growth, primarily by mediating the actions of GH (Wood *et al.*, 2005; Kajimura and Duan, 2007). In turn, IGF-1 is regulated both by the number and type of IGF binding proteins (BP) (Dupont and LeRoith, 2001; Shimizu *et al.*, 2009) and the ratio of free versus bound IGF-1 can also be an important mediator of growth processes and energy homeostasis (Shimizu *et al.*, 2009). These binding proteins are necessary for carrying IGF-1 to cell surface IGF-1 receptors, which mediate the physiological actions of IGF-1 (Wood *et al.*, 2005). To date, six IGFBPs (termed BP 1 through 6) have been isolated in mammals, and five in fish (Shimizu *et al.*, 2009). Of these forms, IGFBP-1 appears to be a major carrier and mediator of IGF-1. Excess IGFBP-1 causes growth retardation and hyperglycemia in mammalian and fish (zebrafish) models and in one case in mice, coincided with no change to circulating IGF-1 relative to control animals (Kajimura and Duan, 2007). One plausible explanation for enhanced growth with decreased plasma IGF-1 in this system may be that selection for growth altered the normal function of IGFBPs, resulting in excess IGF-1 production, but reduced IGF-1 activity in developing *M. menidia*. Interruption in IGF-1 regulation via IGFBP production results in severe dwarfism in mice (Rajkumar *et al.*, 1995; Wood *et al.*, 2005; Kajimura and Duan, 2007). IGFBPs are the primary regulators of IGF-1 plasma concentrations, and can have both stimulatory and inhibitory effects on growth (Kajimura and Duan, 2007). Therefore, alteration in BP regulation via artificial selection could explain the shift in IGF-1 and growth rate in one or both of the size-harvested populations.

Inhibitory actions of BPs may be moderated through the binding of the IGF-1 ligand to its receptor, IGF-1R. Mohseni-Zadeh and Binoux (1997) found that IGFBP-3 interfered with the ability for IGF-1 to bind to its receptor in mouse embryo fibroblasts

and myoblasts *in vitro*, potentially reducing growth capacity in whole animals. Duan *et al.* (1999) showed that IGFBP-2 inhibits growth in zebrafish cells lines downstream from the GH-IGF axis, acting as a growth inhibitory protein, effectively cancelling the growth enhancing effects of IGF-1. These inhibitory relationships could be the reason for slowed growth in the large-harvested population while presenting high IGF-1 levels.

IGF-1 produces physiological effects when binding proteins transport IGF-1 to receptors on cell surfaces, which are ubiquitous throughout tissue types (LeRoith and Yakar, 2007). Receptor availability is plastic within animals and can strongly affect whole-animal or tissue specific sensitivity to circulating IGF-1. This relationship has been illustrated under catabolic conditions in juvenile rainbow trout, when fasting animals display increased IGF-1R gene expression (Norbeck *et al.*, 2007). Additionally, IGF-1 and insulin (I) have distinct and overlapping functions via their respective receptors (IGF-1R and IR) with high specificity (Hernandez-Sanchez *et al.*, 2008). It is plausible that insulin signaling pathways have been altered in size-selected fish, potentially mediating some of the physiological growth processes that would otherwise have been mediated by the IGF-1-receptor complex. The role of IGF-1R and other receptors or IGF-1 clearance in regulating growth in response to size-selective harvest is poorly understood, but may provide explanations for the altered IGF-1 relationships in this system.

Additional research is needed to elucidate other patterns within the GH-IGF axis in this system. Several possible explanations for the patterns seen in *M. menidia* are provided. However, these explanations are not mutually exclusive, nor are they the only possible explanations. Growth is a complex process that is regulated at multiple levels (Norbeck *et al.*, 2007), and a comprehensive review of all growth parameters would be needed to understand how IGF-1 is regulated in this system. The discussion has purposefully been limited to the mechanistic regulation of IGF-1 that occurs downstream, regulating IGF-1 actions after it IGF-1 is produced, to explain the apparent reversed relationship between plasma IGF-1 and growth potential. Because IGF-1 is inversely correlated to growth capacity between large and small-harvested lines, it is possible that selection has reduced the growth-promoting effects of IGF-1 in the large-harvested

population. Therefore, IGF-1 may be regulated differently among populations and that the largest impacts on IGF-1 activity are mediated by downstream mechanisms.

Nova Scotia (hybrid) and South Carolina interpopulation comparison of IGF-1

In this comparison, the slow-growing wild population from South Carolina shows no significant relationship between plasma IGF-1 and length or weight, but this relationship is positive in the faster-growing hybridized population. No relationships between growth rate in either length and weight were observed for either population. Together, this indicates that IGF-1 is not a good indicator of growth potential in these populations, but that IGF-1 may be associated with growth in the hybridized population, possibly due to the genetic contributions from wild Nova Scotia populations.

Based on significantly faster growth rates in the hybridized population relative to wild South Carolina, I hypothesized that significant relationships between growth and IGF-1 as well as enhanced circulating IGF-1 in the hybridized population would be apparent. These results suggest that no simple relationship between IGF-1 and growth capacity exists in wild *M. menidia* that have undergone natural selection for growth or that the pattern seen in the wild is masked in experimental fish used in this study due to hybridization of the northern, fast-growing population. Additionally, fish were pooled by average dates of fertilization that spanned five weeks due to low spawning production of parents. Therefore, any patterns that may have emerged may be masked by differences in IGF-1 production with age, as noted in the selection comparisons above. Comparisons of wild fast and slow-growing populations would demonstrate the true relationship between IGF-1 and growth.

Conclusions

This study provides evidence that strong size-selective harvest can induce changes in IGF-1 in *M. menidia*. Interestingly, the pattern of IGF-1 regulation in fish harvested for large and small body size is the opposite of that predicted from growth scenarios in other domesticated fish species (Perez-Sanchez and Le Bail, 1999; Picha *et al.*, 2008A). Here, fast-growing fish have significantly reduced IGF-1 relative to the slow-growing individuals with heightened IGF-1. The results from this study suggest that regulation of

IGF-1 is unusual in this system and that regulation of overall growth may be regulated by mechanisms 'downstream' of IGF-1 production, potentially by the actions of IGFBPs or IGF-1 receptors. In addition, it is possible that selection for size has altered IGF-1 regulation through different mechanisms in the large- and small-harvested populations. This paper provides some of the first evidence for patterns in IGF-1 that result from four generations of strong selection resembling intense size-selective harvest, followed by multiple generations that mimic a moratorium in that fishery. Thus, circulating IGF-1 appears to have been reduced in fish selected for fast growth and heightened in those selected for slow growth.

I have provided several possible explanations for the patterns observed in this study. The mechanisms by which artificial selection influences fish growth have been well documented in domesticated species, but relatively few studies have addressed the mechanisms that alter growth potential in wild fisheries (Nielsen *et al.*, 2009). Model systems which simulate the impact of fisheries may provide excellent means for understanding changes to the growth axis, and are able to disentangle genetic changes from environmental influences on the phenotype (Conover and Baumann, 2009). Understanding the mechanisms that control life-history changes in response to fishing is important for both identifying changes as they occur in a population and for predicting the potential for rebound in wild fisheries.

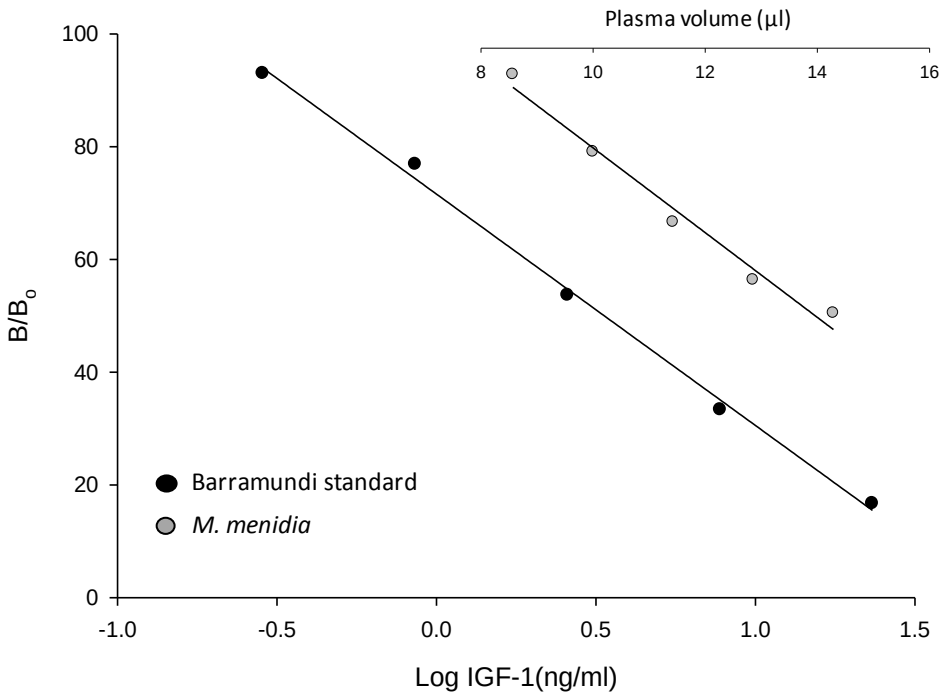

Figure 5.1. Validation of the RIA assay in *M. menidia* using Barramundi IGF-1 standard. Percent ^{125}I IGF-1 binding (B/B_o) plotted against $\log_{10}$ IGF-1 (ng/ml) for barramundi standard and *M. menidia* plasma volume (µl), denoted by small axis. r^2=0.99 for both standard curves.

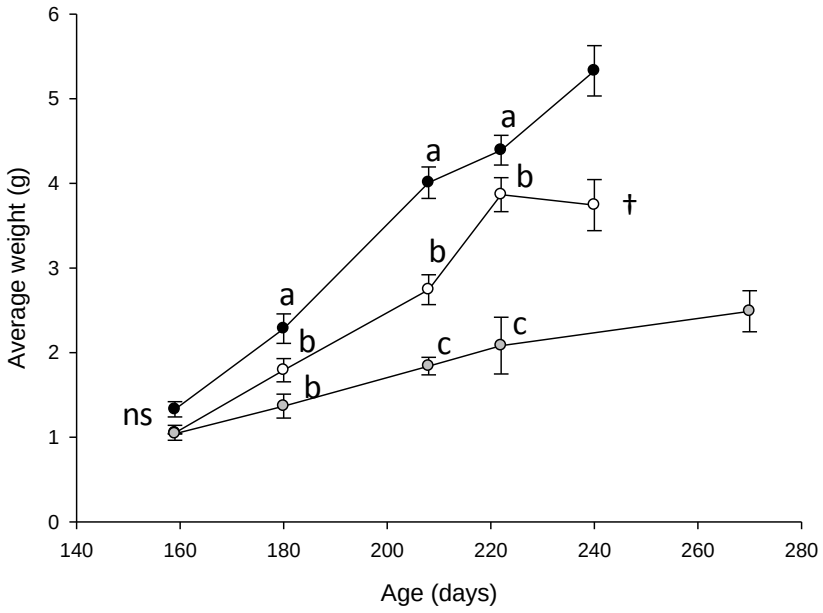

Figure 5.2. Average weight (g) for small-harvested (SH; black circles), random-harvested (RA; white circles) and large-harvested (LH; grey circles) *Menidia menidia* at sampling. Mean weights are plotted with standard error. Letters represent statistically significant differences (2-way ANOVA, Tukey-Kramer multiple comparisons, $p<0.05$) for comparisons of a given age. Weights at the final timepoint (day 240 for SH and RA and day 270 for LH) were excluded from analysis due to non-overlapping ages, denoted by †.

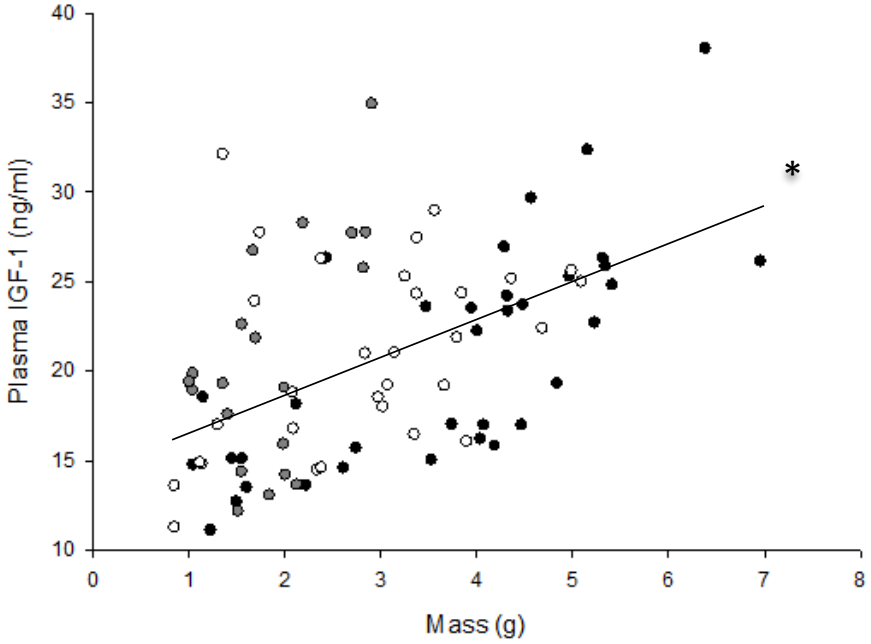

Figure 5.3. All populations from the selection experiment exhibited a significant, positive relationship between plasma IGF-1 (ng/ml) and mass (overall regression; $p<0.0001$, $r^2= 0.26$).

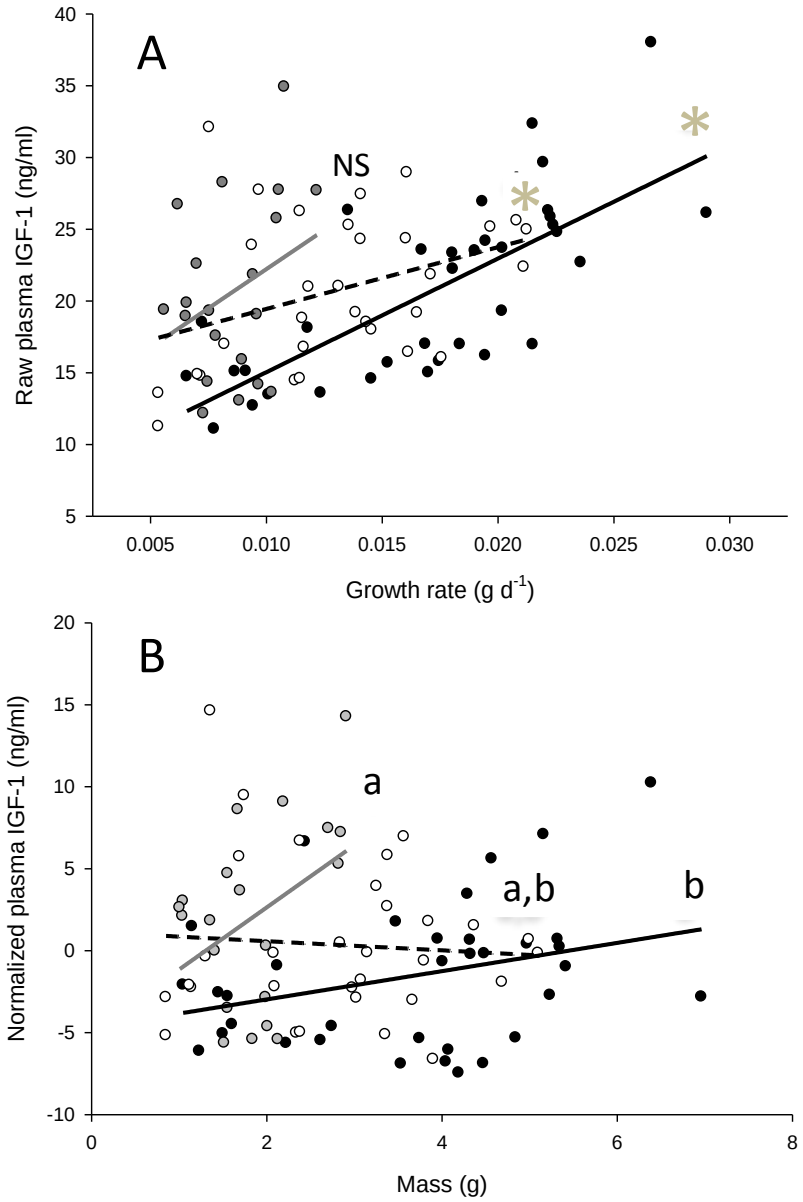

Figure 5.4. Relationships between A) raw plasma IGF-1 concentration plotted as a function of mean growth rate (g d^{-1}) and B) mass and normalized (mass-corrected with residuals) plasma IGF-1 concentration. In A), asterisks (*) represent a positive linear relationship, a non-significant relationship is denoted with NS and B) dissimilar letters represent significant differences (1-way ANOVA). . Large-harvested (slow-growing) fish are depicted in grey, random-harvested (control fish) are depicted in open circles and black circles represent small-harvested (fast-growing) individuals. Lines of matching color are the linear regression line.

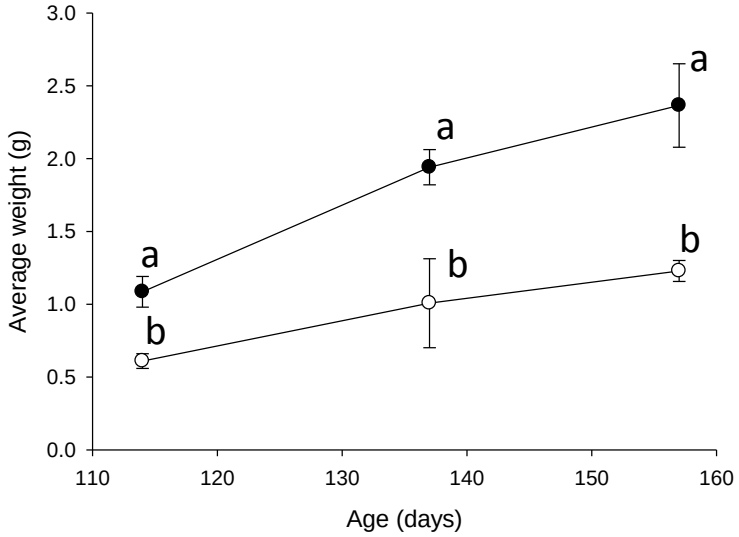

Figure 5.5. Average weight (g) for Nova Scotia hybrids (NS; black circles) and South Carolina (SC; open circles) at sampling. Mean weights are plotted with standard error. Letters represent statistically significant differences (2-way ANOVA, Tukey-Kramer multiple comparisons, $p<0.05$) for comparisons of a given age.

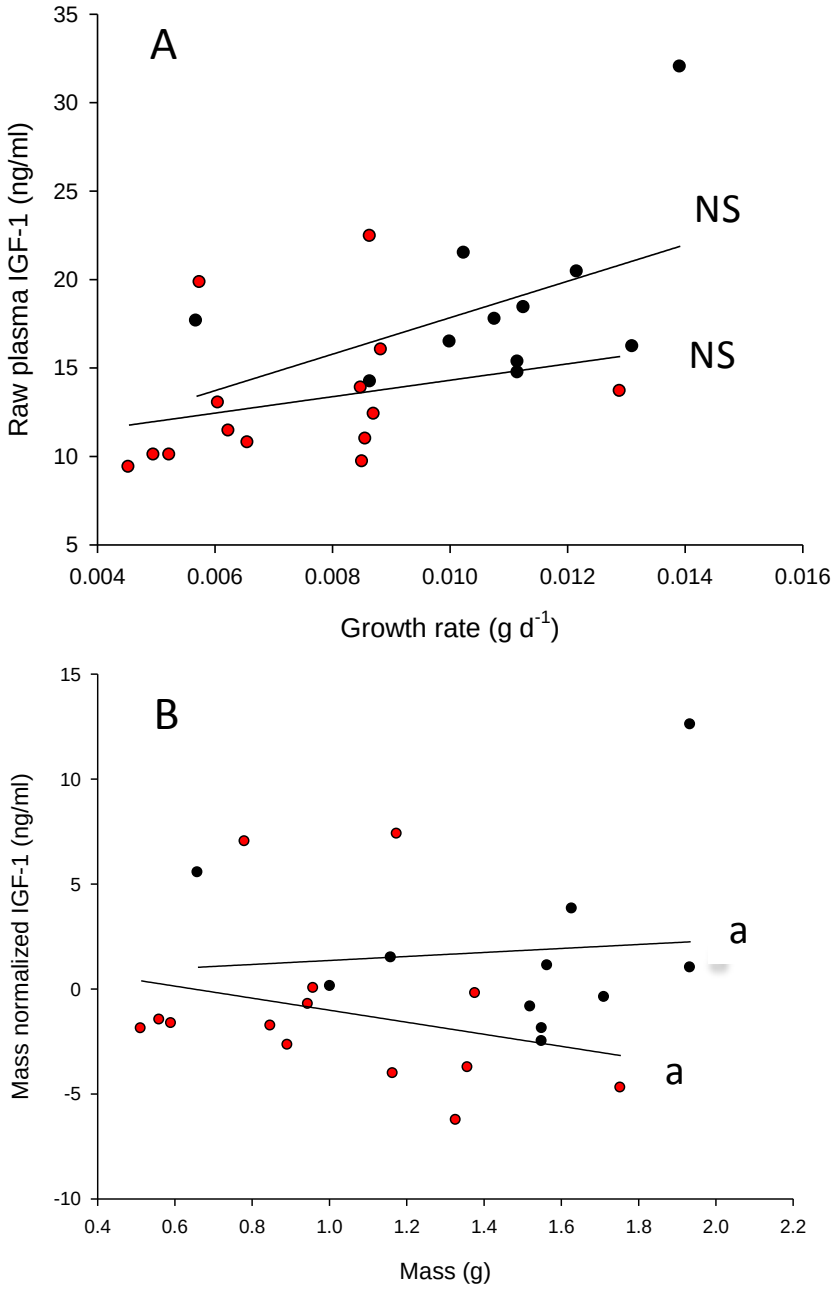

Figure 5.6. Relationships between A) mass and normalized (mass-corrected with residuals) plasma IGF-1 concentration and B) raw plasma IGF-1 concentration plotted as a function of growth rate (g d^{-1}). Nova Scotia (fast-growing) fish are depicted in black, and South Carolina (slow-growing) fish are depicted in red. Solid lines are the linear regression lines. Regression lines in panel A are not significantly different from zero and in panel B, regression lines are not significantly different (ANCOVA, $p<0.05$).

Summary

In this dissertation, I used *Menidia menidia* as an excellent model species to understand the patterns of local adaptation, the mechanisms that contribute to patterns of local adaptation and the responses of an organism to anthropogenic influences or selective pressures. In the first chapter, I refined the previously reported latitudinal pattern of the form of sex determination and demonstrate that the season length, as previously thought, cannot be the only selection pressure creating this gradient. Further, I assessed gonadal differentiation rate to see if either the form of sex determination or growth rate, two life history traits that vary with latitude, controlled the patterns of differentiation and development within the gonad itself. While gonad development did vary among populations, the ultimate mechanism causing the pattern is unclear. Locally adapted organisms, especially those displaying differences in growth rate, are ideal for determining trade-offs between life-history traits and developmental constraints.

Understanding the molecular mechanisms for sex determination and growth may allow us to better understand the evolutionary relationship between TSD and GSD. The latitudinal cline in TSD and GSD in *M. menidia* provided an excellent system for intra-species comparison of one particular gene involved in female sex differentiation, P450aromatase. P450aromatase appears to be an important component of sex differentiation and development in populations with both TSD and GSD and regulation is clearly temperature-dependent in a population with TSD and insensitive in another with GSD. Whether P450arom is directly regulated by temperature or by the influence of other genes remains unknown and opens questions for further work on sex determination in *M. menidia*. To my knowledge, this represents the first intraspecific comparison of P450aromatase-regulated gonad differentiation among populations with different forms of sex determination, a life-history trait gradient that may be unique to this species.

The results from chapter three suggest *M. menidia* from southern populations are more sensitive to environmental estrogens than northern conspecifics, and this work indicated that the form of sex determination (TSD) may be a factor that contributes to this susceptibility. This study provides evidence of sex ratio alteration in *Menidia menidia* along a pronounced gradient of urbanization across Long Island. Again, because *M. menidia* exhibits local adaptation in TSD and GSD among populations, it represented a

good model in which to determine how endocrine disrupting chemicals in the environment interact with the genotype of an individual to determine sex. Numerous other species may possess heightened susceptibility to endocrine disrupting compounds, especially for those that have environmental sex determination of some form. Hence, an understanding of such genotype by environment interactions is important to predicting the overall effect of endocrine disrupting chemicals on locally adapted populations.

Chapter four provides evidence that strong size-selective harvest can induce changes in regulation of major growth proteins. Interestingly, the pattern of the major growth protein I focused on, IGF-1, demonstrated a pattern opposite to that predicted from growth scenarios in other domesticated fish species. Fast-growing fish displayed significantly reduced IGF-1 concentrations relative to slow-growing individuals with heightened IGF-1. The results from this study suggest human-induced, size-selective harvest can cause major differences in the regulation of the IGF-1 system. Major regulators of IGF-1 and other polypeptides could be causing the mismatched growth rate and protein levels and opens up several questions for future study. In addition, it is possible that selection for size has altered IGF-1 regulation through different mechanisms in the large- and small-harvested populations. The mechanisms by which artificial selection influences fish growth have been well documented in domesticated species, but relatively few studies have addressed the mechanisms that alter growth potential in wild fisheries (Nielsen *et al.*, 2009). Model systems which simulate the impact of fisheries may provide excellent means for understanding changes to the growth axis, and are able to disentangle genetic changes from environmental influences on the phenotype (Conover and Baumann, 2009). Understanding the mechanisms that control life-history changes in response to fishing is important for both identifying changes as they occur in a population and for predicting the potential for rebound in wild fisheries.

Throughout this work, I have demonstrated that local adaptation is a complex pattern resulting from multiple selection pressures at many levels of organization, from regulation at the level of the gene to the population. *M. menidia* is an excellent model teleost for answering questions about the mechanisms that contribute to local adaptation in marine species, and how these mechanisms shift in response to anthropogenic alteration. The work presented here has answered a few of the questions that are

discussed among evolutionary biologists and ecologists today and *M. menidia* is a model system that may help to answer many more questions in the future.

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