THE EFFECTS OF TEMPERATURE ON CYP19A1A, FOXL2, DMRT1 AND AMH EXPRESSION DURING SEX DIFFERENTIATION IN SUMMER FLOUNDER (PARALICHTHYS DENTATUS)

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Abstract
Female summer flounder grow considerably faster and larger than males, and a tremendous increase in performance can therefore be realized through production of monosex female populations. Rearing temperature has been shown to affect sex differentiation in other teleost species by influencing expression of genes encoding transcription factors, hormones or enzymes involved in endocrine function such as cyp19a1a, foxl2, dmrt1 and amh. These genes have been linked to female (cyp19a1a, foxl2) or male (dmrt1, amh) development, and exhibit sexually dimorphic expression in some species. In the present study, summer flounder (37 days post hatch; DPH) were raised at 13°C, 16°C or 19°C. Fish from all three treatments were sampled during early development (38-66 DPH), and fish from 13 and 16°C treatments were sampled through later juvenile development (191 DPH). A partial summer flounder cDNA sequence for cyp19a1a was identified, verified, and submitted to the NCBI GenBank database. Partial summer flounder sequences were also identified for foxl2, dmrt1 and amh, but these single sequence reads were not verified or submitted to NCBI. Summer flounder samples were analyzed in qPCR to determine cyp19a1a, foxl2, dmrt1 and amh gene expression levels. Sex ratios of additional fish grown to > 150 mm at each temperature treatment were determined. Low female production was achieved overall (26.9, 17.6 and 0% at 13, 16 and 19°C, respectively). Cyp19a1a expression was significantly lower at 52 DPH (~15 mm total length) at the male producing temperature (19°C), and increased to similar levels as other treatments at 66 DPH. Cyp19a1a expression levels later in juvenile development (66-191 DPH) largely decreased with fish size. No clear trend in gene expression levels was present for foxl2, dmrt1 or amh. The period of sex differentiation in summer flounder remains unknown but cyp19a1a expression patterns suggest that it may occur earlier in development than that of congeneric, and that cyp19a1a may be a more robust sex marker in summer flounder than foxl2, dmrt1 or amh. Further research is necessary to understand the sex-determining mechanisms in this species before sexually dimorphic growth can be used to achieve economic advantages in commercial production.

Keywords
cyp19a1a, ovarian aromatase, Paralichthys dentatus, sex determination, sex differentiation, summer flounder, Biology, Zoology

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BY

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B.A., Biology, Wellesley College, 2010

THESIS

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of Master of Science In Zoology September, 2015
This thesis has been examined and approved in partial fulfillment of the requirements for the degree of Master of Science in Zoology by:

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On July 31, 2015

Original approval signatures are on file with the University of New Hampshire Graduate School.
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DEDICATION

TO MY PARENTS
Frank Caruso and Paula Caruso

As long ago I carried to your knees
The tales and treasures of eventful days,
Knowing no deed too humble for your praise,
Nor any gift too trivial to please,
So still I bring with older smiles, my dears
This gift with which to claim the old, dear right;
Your faith throughout life’s many days and nights,
Your love still close and watching through the years.

TO MY GRANDMOTHER
Catherine Angela Delores Sancinito Curro

Who always believed in me the most, and encouraged me to never stop writing.
We Catherines have to stick together.
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ABSTRACT

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By
Catherine Curro Caruso

University of New Hampshire, September 2015

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Cyp19a1a expression was significantly lower at 52 DPH (~15 mm total length) at the male producing temperature (19°C), and increased to similar levels as other treatments at 66 DPH. Cyp19a1a expression levels later in juvenile development (66-191 DPH) largely decreased with fish size. No clear trend in gene expression levels was present for foxl2, dmrt1 or amh. The period of sex differentiation in summer flounder remains unknown but cyp19a1a expression patterns suggest that it may occur earlier in development than that of congenerics, and that cyp19a1a may be a more robust sex marker in summer flounder than foxl2, dmrt1 or amh. Further research is necessary to understand the sex-determining mechanisms in this species before sexually dimorphic growth can be used to achieve economic advantages in commercial production.
CHAPTER 1

INTRODUCTION

Global seafood demands have drastically increased over the past century, and will continue to do so as the world population grows. However, wild fisheries are overexploited worldwide, with fisheries capture production plateauing in the 1980s, and unlikely to increase in the near future. Consequently, aquaculture development is the most realistic avenue for increasing global seafood production to meet demands, while reducing dependence on wild fisheries (NOAA FishWatch Aquaculture). In fact, according to the Agricultural Research Service, global aquaculture production must increase 500% by 2025 to meet the needs of a projected 8.5 billion world population (USDA Agricultural Research Service 2014). Currently the United States imports 91% of its seafood, 40% of which is from aquaculture, resulting in a 10.4 billion deficit in seafood imports that is second only to petroleum (Van Voorhees and Lowther 2012). There are a large number of native marine species in the northeast United States that are popular for human consumption, yet only the Atlantic salmon (Salmo salar) is cultured in significant quantities. However, the salmon industry faces many challenges, including market competition from imported fish, and competition with wild fisheries (NOAA FishWatch Atlantic Salmon). Thus, it is critical that new marine aquaculture species are developed to expand US seafood production, and decrease dependence on imported seafood and wild fisheries.

Much research is needed on which species are most suitable for aquaculture, and how aquaculture can be optimized to maximize production and economic benefits. When choosing a new marine finfish species for culture, there are many qualities to consider. The species must
have a high market price, high consumer demand, and the ability to thrive in a wide range of culture conditions. Summer flounder (*Paralichthys dentatus*) meet these criteria, which gives them a great deal of potential for aquaculture. Summer flounder can tolerate a range of salinities, temperatures and dissolved oxygen levels, and can be cultured at high densities (Daniels et al. 1996; Packer et al. 1999). They are native to the eastern coast of the US, from Gulf of Maine to South Carolina, and spend the summer months in shallow, coastal, estuarine environments. In the fall/winter months, summer flounder migrate offshore to the warmer water of the outer continental shelf, where they spawn (Packer et al. 1999). Summer flounder achieve sexual maturity around two years of age, and the L50 (size at which 50% of individuals are sexually mature) is 24.6 cm total length (TL) for males and 32.2 cm TL for females (Packer et al. 1999). Females produce pelagic, buoyant eggs that hatch into 2 mm pelagic larvae. Like other Paralichthyd species, summer flounder larvae are symmetrical and free-swimming in the water column. Pelagic larvae remain on the continental shelf for about a month before migrating inshore to coastal and estuarine nursery areas. During inshore migration, larvae undergo metamorphosis (around 13 mm TL), which involves migration of the right eye across the top of the head. Once they reach coastal/estuary habitats, they settle to the bottom, having achieved their asymmetrical, demersal, adult form (Packer et al. 1999). Summer flounder are popular for human consumption, and are fished commercially and recreationally year round, with a US fishery valued at over $28.6 million in 2010 (Packer et al. 1999; NOAA FishWatch Summer Flounder 2014). They also have a high market price, with 2014 filet prices increasing to upwards of $10/pound in the northeastern United States, in part due to an overall shortage of groundfish (FishChoice Summer Flounder 2014). Total annual summer flounder landings in the US peaked in 1983 at 26,100 metric tons (mt), and have been much lower in recent years, averaging 8,197
Paralichthyd flounder are currently being cultured in South America, Central America and Asia, which produces over 78,000 tonnes annually (Lymer et al. 2010). By contrast, little research and development has been done on flounder culture in the United States.

Summer flounder are sexually dimorphic, with females that grow more quickly and achieve larger overall sizes than males. Previous research found that cultured summer flounder females were 1.4 times larger than males at 15 months post hatch, and were twice as large as males by harvest at 23 months (King et al. 2001). There is also evidence that females have higher natural survival rates than males (Morse 1981). Therefore, rearing female dominated populations in an aquaculture setting economically advantageous. Summer flounder are unable to tolerate cold coastal water temperatures in the fall/winter, which makes them unsuitable for year-round outdoor culture in the Northeast US. Closed, recirculating aquaculture systems are a viable alternative for year-round summer flounder culture, but these systems are expensive due to high construction and operating costs. Monosex female populations will reach market size in less time, reducing size heterogeneity, mortality due to cannibalism, length of “grow out” period, labor costs, and increasing the likelihood of successful commercial culture in recirculating systems (Luckenbach et al. 2002). However, in order to culture faster growing female fish, it is necessary to understand and control summer flounder sex determination and sex differentiation.

Vertebrates are highly diverse in their mechanisms for sex determination and sex differentiation, two closely linked processes that relate to whether an individual becomes male or female. Sex determination refers to the genetic and environmental processes and factors that influence sex differentiation, while sex differentiation is the physical development of male or female gonads during maturation (Devlin and Nagahama 2002). In many higher vertebrates such
as mammals, genotypic sex controls phenotypic development, but many lower vertebrates such as herptiles and fish have an environmental component to sex determination that can alter phenotypic sex (Piferrer 2001). Sex determining mechanisms vary among vertebrate groups, but there are three relatively conserved sex determining mechanisms; chromosomal, polygenic, and genotypic-environmental interaction (Piferrer 2001). In chromosomal and polygenic sex determination, sex is determined at fertilization. In chromosomal sex determination, individuals inherit highly evolved sex chromosomes that contain most genes necessary for sex development. There are two main chromosomal systems, the XX/XY system (homogametic females), and the WZ/ZZ system (homogametic males). In polygenic sex determination, sex is controlled by sex-determining genes distributed among the other chromosomes (autosomes). Thus, the sex of an individual is determined by whether more male or female sex factors are expressed (Piferrer 2001). In genotypic-environmental sex determination, environmental factors such as pH, salinity, nutrition, density, social cues, temperature, and stress may alter genotypic sex, and result in a phenotype that differs from chromosomal sex (Prevedelli and Simonini 2000; Saillant et al. 2003; Lindsey 1962; Francis and Barlow 1993; Liu and Sadovy 2004; Conover and Kynard 1981; Sullivan and Schultz 1986; Mankiewicz et al. 2013).

Temperature-dependent sex determination (TSD) is the most common type of environmental sex determination (ESD). It has been established in reptiles and amphibians, and has been documented in some teleost species (Crews 1996; Hayes; 1998; Conover and Kynard 1981; Middaugh and Hemmer 1987; Schultz 1993; Baroiller et al. 1996; Römer and Beisenherz 1996; Kwon et al. 2000; Mylonas et al. 2003; Ospina-Álvarez and Piferrer 2008). TSD species have no chromosomal system, and experience shifts in sex ratios in response to exposure to temperatures that fall within their natural range (Ospina-Álvarez and Piferrer 2008). TSD does
not occur in mammals and birds, which undergo embryonic development in controlled temperature conditions (Shen and Wang 2014).

Some teleosts possess a genotypic system that can be influenced by temperature, and are classified as genotypic sex determination + temperature effects (GSD + TE). The sex of GSD + TE species is dictated by the interaction between a chromosomal system (consistent genetic differences between sexes), and the water temperature that fish experience during the sex-determining period of development. Recent studies suggest that many teleost species previously characterized as TSD including Japanese flounder are in fact GSD + TE (Ospina-Alvarez and Piferrer 2008). Three main patterns of temperature-dependent sex determination have been identified. In pattern 1 there are more males at high temperatures, and more females at low temperatures, while in pattern 2 there are more females at high temperatures and more males at low temperatures. In pattern 3, there are more males at both high and low temperatures, and a 1:1 sex ratio at intermediate temperatures (Luckenbach et al. 2009). Recent research suggests that true TSD species only exhibit pattern 1, and other patterns may be indicative of GSD + TE species (Ospina-Alvarez and Piferrer 2008).

Figure 1. Temperature induced patterns of sex determination in teleosts, adapted from Luckenbach et al. 2009.

Japanese flounder, the most well studied Paralichthyd species, exhibit GSD + TE, with an XX-XY chromosomal system that can be influenced by temperature. In Japanese flounder, XY
fish always develop into males, while genotypic (XX) females can be masculinized by exposure to certain water temperatures. Previous work has shown that at 18°C, Japanese flounder develop into their genotypic sex, resulting in a 1:1 sex ratio (Kitano et al. 1999). This was confirmed in another study, where genotypic females developed into males when raised at 25-27.58°C or 15.8°C, but developed into females when raised at 17.5-22.58°C (Yamamoto et al. 1999). Cumulatively, these studies indicate that Japanese flounder follow pattern 3 of sex differentiation, a U-shaped pattern with high male production at high and low temperatures, and a 1:1 sex ratio that aligns with genotypic sex at intermediate temperatures. Japanese flounder raised at 15°C complete metamorphosis 25-50 DPH, at ~17.5 mm TL (11.4mm SL) (Fukuhara 1986). They undergo sex differentiation at 32 mm TL, while their ovarian cavity is not present until ~40 mm TL (Yamamoto 1999; Goto et al. 1999; Kitano et al. 1999).

The sex determination pattern for Southern flounder (*Paralichthys lethostigma*), another congeneric species, is less clear. A previous study using North Carolina fish produced 78% males at 18°C, 96% males at 28°C, and a 1:1 sex ratio at 23°C, which suggests a U-shaped pattern of sex differentiation similar to that of Japanese flounder (pattern 3; Luckenbach et al. 2003). However, a more recent study on fish from the Texas Gulf Coast found that the proportion of females decreased with increasing water temperatures between 18.2°C and 29.6°C, which is consistent with pattern 1 of sex differentiation (Montalvo et al. 2012). The contradiction between these two studies may be attributed to adaptive variation due to geographical differences between Southern flounder populations, but certainly merits additional research. Since neither a chromosomal system nor a consistent pattern of sex differentiation has been confirmed in this species, it is unknown if they can be classified as GSD-TE. Southern flounder raised at 17-18°C undergo metamorphosis at ~30 DPH, and complete the process at ~45
DPH, when they are 10-16 mm TL (Daniels et al. 1996; Daniels 2000). Sex differentiation does not occur until ~65 mm total length, while the ovarian cavity and early-stage meiotic oocytes become visible at ~75-100 mm (Luckenbach et al. 2005).

Summer flounder exhibit primary gonochorism, which means that gonadal primordium in juveniles develops directly into either testes or ovaries, causing them to become males or females. Once an individual becomes male or female, it remains the same sex throughout life (Piferrer 2001). It is unknown whether or not summer flounder possess an XX/XY chromosomal system similar to that of Japanese flounder, but it has been established that more females are produced at lower temperatures, and more males occur at higher temperatures. Previous experiments on summer flounder juveniles produced 22.9% females at 12°C, 0% females at 21°C and 3.9% females at 26°C, and commercial production achieved 26% females at 18°C (Colburn et al. 2009; Colburn et al. 2015). Among meiogynogenetic individuals, 62.5% females were produced at 12°C and 0% females were produced at 21°C and 26°C (Colburn et al. 2009). Previous studies on summer flounder have been unable to achieve a 1:1 sex ratio, and it is unclear when the critical period for temperature exposure in summer flounder occurs, and whether this critical period is driven by fish size or fish age. However, it is known that summer flounder metamorphose at 40 DPH, when fish are 14-20 mm TL (when raised at 17-18°C), and definitive gonadal development occurs long after metamorphosis, at ~100-150 mm TL (Colburn et al. 2009).

Previous studies have also established a relationship between stress and masculinization of Paralichthys species. Juvenile Japanese flounder raised at 27°C exhibited higher cortisol levels and higher rates of masculinization than juveniles raised at 18°C, which suggests a link between temperature-induced masculinization of Japanese flounder and elevated cortisol levels.
(Yamaguchi et al. 2010). Southern flounder exhibited significant masculinization and elevated cortisol levels when raised in blue tanks as opposed to black or gray tanks, indicating that stress due to tank color can also result in higher male production (Mankiewicz et al. 2013). Summer flounder may exhibit similar stress-induced masculinization in response to environmental factors, though it is unknown whether temperature or some other stressor has the most significant effect on sex differentiation.

Species with a temperature component to sex differentiation are particularly vulnerable to global climate change, which is causing long term alterations in ocean temperatures worldwide (EPA Climate Change Indicators 2014). Climate change models have established the potential for female dominated loggerhead turtle (Caretta caretta) populations, and a recent study on teleost fishes confirms that climate change may cause skewed sex ratios in temperature-dependent species (Hawkes et al., 2007; Ospina-Álvarez and Piferrer 2008). However, it is unclear how much changing ocean temperatures will affect sex ratios in species such as summer flounder that may possess a genotypic-environmental sex determination system rather than a solely TSD system. Recent NOAA surveys on juvenile summer flounder found that wild populations are dominated by males (Powell and Morson 2008). Ocean warming is more rapid in the Northern hemisphere, particularly in the north Atlantic region that summer flounder inhabit (Rhein et al. 2013). Rising ocean temperatures along the eastern coast of the US could further impact the temperature sensitive sex differentiation of summer flounder by causing high rates of masculinization, resulting in wild populations with unusually high male production, and reduced reproductive success. Consequently, it is essential to understand the relationship between temperature and sex differentiation in summer flounder in order to predict how changing ocean temperatures may affect wild populations.
The sequence of molecular events during sex differentiation in mammals has been well established. Mammals have a chromosomal system of sex differentiation where XY individuals develop into males, and those without a Y chromosome (XX) develop into females. More specifically, the \textit{SRY} gene on the short arm of the Y chromosome is responsible for male differentiation (Waters et al. 2007). There are a number of genes involved in sex differentiation in mammals such as \textit{SOX9}, \textit{DAX1}, \textit{SF1} and \textit{WT1} that are not sex specific, but do demonstrate sexually dimorphic expression. \textit{SOX9} is important in male differentiation (Moreno-Mendoza et al. 2004; Barrioneuvo et al. 2006; Kojima et al. 2008) while \textit{DAX1}, located on the X chromosome, is important in ovarian differentiation (Manolakou et al. 2006). \textit{SF1} is an essential factor in reproduction, based on its involvement in the hypothalamic-pituitary-steroidogenic organ axis (Caron et al. 1997), and \textit{WT1} encodes a transcription factor involved in kidney and gonadal development (Davies et al. 1999). However, little is known about the exact roles of these genes during sex differentiation.

Birds also have a chromosomal sex determining mechanism, with heterogametic females (ZW) and homogametic males (ZZ). There is likely a comparable W-linked gene responsible for ovarian development in birds, but it has not been identified (Short 1998). There are several possible mechanisms for sex differentiation in birds. One mechanism suggests that the testis is the default gonad, and genes on the W chromosome induce ovarian development, much like the \textit{SRY} gene in mammals. Another model suggests that there is a dosage mechanism where males receive two doses of a particular gene product, while females receive only one (Clinton 1998). \textit{Dmrt1} may be involved in the dosage mechanism, where a higher dosage results in testicular differentiation in males, activating \textit{sox9} expression (a gene linked to male development) and reducing expression of genes linked to female development (Smith et al. 2009). There is also
evidence that in females, testis growth is accelerated at the beginning of development, but is overtaken by ovarian growth, which is mediated by the presence of estrogen rather than the presence of a particular gene (Mittwoch 1998).

Reptiles are diverse in their sex determining mechanisms, which range from genetic sex determination (GSD; XX/XY or WZ ZZ) to TSD (Viets et al. 1994; Wibbels et al. 1994). Some crocodilians and turtles have no obvious sex chromosomes, with gonadal developmental influenced by temperature and/or hormones (Spotila et al. 1998). The WTI, SOX9 and DMRT1 genes have also been identified in reptiles (Jeyasuria and Place 1998; Spotila et al. 1998; Wibbels et al. 1998; Ferguson-Smith 2007).

In teleosts, ovarian development generally occurs before testes development, and many species possess sex determination systems that involve a combination of GSD and ESD (Piferrer 2001; Guerrero-Estévez and Moreno-Mendoza 2010). In some species, environmental factors, most commonly water temperature (TSD or TE), can override genetics, which may involve the SFI gene (Short 1998). It is likely that environmental factors moderate sex differentiation in fish via the hypothalamic-pituitary-gonadal (HPG) axis (Devlin and Nagahama 2002). In this axis, environmental stimuli cause neurons in the hypothalamus to release gonadotropin-releasing hormone (GnRH). GnRH stimulates the pituitary gland to produce Gonadotropin I (GtH I), which is analogous to mammalian follicle stimulating hormone and promotes steroidogenesis and gonadal differentiation, and gonadotropin II (GtH II), which is analogous to mammalian luteinizing hormone and is produced before sexual maturation (Devlin and Nagahama 2002). GtH I and GtHII cause the gonad to initiate sex steroidogenesis, which results in differentiation of either ovaries or testes. Sex steroids then become part of a positive or negative feedback loop until sex differentiation is complete. In species with TSD or TE, water temperature is the
environmental cue that influences the HPG pathway, thus affecting whether fish differentiate into males or females (Francis 1992; Devlin and Nagahama 2002; Strüssmann and Nakamura 2002; Godwin et al. 2003).

Sex differentiation in fish is highly sensitive to steroid hormones (Cardwell and Liley 1991; Strüssmann and Nakamura 2002). Across species, females exhibit higher levels of estradiol, which is the major sex steroid responsible for ovarian development, and males have higher 11-ketotestosterone levels, an androgen steroid responsible for testis development (Yamamoto 1969; Nakamura et al. 1998). There have been a number of experiments where fish sex was altered by administration of exogenous estrogens or androgens (Cardwell and Liley 1991; Piferrer 2001; Devlin and Nagahama 2002). Plasticity of sex differentiation in fish varies by species, and species that utilize endogenous sex steroid production for gonad differentiation may be more influenced by environmental factors. These species undergo gonadal steroidogenesis before sex differentiation, as opposed to fish with strong genotypic sex determination, which do not undergo steroidogenesis until after sex differentiation. Androgen and estrogen receptors have been identified in both ovaries and testes, which suggests that exogenous steroids can affect both sexes (Strüssmann and Nakamura 2002). By contrast, sex steroids in mammals are not involved in gonad differentiation, but rather are responsible for shaping the sexually dimorphic structures during sexual development after sex differentiation has occurred (Strüssmann and Nakamura 2002). This may be because viviparous species have evolved mechanisms to prevent maternal estrogens from feminizing male embryos, which consequently prevents exogenous sex steroid from affecting sex determination (Short 1998).

In teleosts, there are a number of genes that play important roles in sex differentiation by producing hormones, enzymes and transcription factors linked to male or female development.
Cyp19a1a and forkhead transcriptional factor 2 (foxl2) are genes associated with female development, while doublesex/mab-3 related transcription factor 1 (dmrt1) and anti-Müllerian hormone (amh) are important in male differentiation. These genes have been established in other teleost species, including Paralichthys, and thus are likely involved in sex differentiation of summer flounder (Kitano et al. 1999; Yoshinaga et al. 2004; Luckenbach et al. 2005; Nakamoto et al. 2006; Smith et al. 2013).

Cyp19a, the most well studied of these genes, has two isoforms in fish, an ovarian form (cyp19a1a) and a brain form (cyp19a1b; Gelinas et al. 1998; Tchoudokova and Callard 1998; Chiang et al. 2001; Kishida and Callard 2001; Kwon et al. 2001; Zhao et al. 2001; Blazquez and Piferrer 2004; Chang et al. 2005; Greytak et al. 2005). These aromatase isoforms differ in their tissue distribution, responses to exogenous estrogen, and expression pathways during gonad ontogeny (Chang et al. 2005). Cyp19a1b is primarily expressed in the brain, specifically in the hypothalamus and ventral telencephalon, extending to the olfactory bulb (Chiang et al 2001). It encodes for brain aromatase, which is mainly involved in neural estrogen synthesis (Chang et al. 2005). Cyp19a1a is primarily expressed in the follicular cells lining vitellogenic oocytes in the ovary during vitellogenesis. Cyp19a1a encodes for cytochrome P450 aromatase (P450 ovarian aromatase), a steroidogenic enzyme that converts testosterone into E2 (17β estradiol; Figure 2; Kitano et al. 1999; Liu et al. 2010). P450 aromatase is part of a superfamily of heme proteins, the cytochromes P450, which function as oxygenases (Werck-Reichhart and Feyereisen 2000). It is a membrane bound enzyme associated with NADPH (Place et al. 2001) that uses the reductive equivalents from NADPH to convert androgens (C19) into estrogens (C18) by the removal of the methyl group (demethylation) and aromatization of the steroid A ring (Simpson et al. 1994; Werck-Reichhart and Feyereisen 2000; Navarro-Martín et al. 2011). Because estrogens are
needed for sex determination and differentiation, it is believed that cyp19a1a expression, and subsequent P450 aromatase activity is responsible for estradiol synthesis, and thus plays a key role in ovarian differentiation and development (Guiguen et al. 1999; Kitano et al. 1999; Suzuki et al. 2004; Chang et al. 2005). Conversely, cyp19a1a inhibition prevents estradiol biosynthesis, which results in testicular differentiation (Guiguen et al. 1999; Kitano et al. 1999; Kwon et al. 2001; Uchida et al. 2004). Recent research on European sea bass found that higher water temperatures increase methylation of a cyp19a1a promoter, which suppresses cyp19a1a expression, and results in masculinization of genotypic females Navarro-Martín et al. 2011. This result was confirmed in Japanese flounder, where high levels of cyp19a1a promotor methylation were found in Japanese flounder testis and correlated with low cyp19a1a expression compared to that expressed in ovarian tissue (Wen et al. 2014). These studies provide the first evidence of an epigenetic mechanism that connects water temperature, cyp19a1a expression, and sex ratios in teleosts.

![Figure 2.](image)

Figure 2. P450 aromatase converts testosterone into estradiol through removal of a methyl group and aromatization of the steroid A ring

Many fish species exhibit sexually dimorphic cyp19a1a expression, though the timing of expression in relation to sex differentiation varies. In Nile tilapia (Oreochromis niloticus), cyp19a1a can be detected five days post hatch (DPH), well before sex differentiation (Ijiri et al. 2008). Zebrafish (Danio rerio) cyp19a1a expression occurs at a slightly later stage, during sex differentiation (Rodriguez-Mari et al. 2005) while medaka (Ozyrias latipes) cyp19a1a expression
is detectable between 4 and 10 DPH, after sex differentiation is complete (Suzuki et al. 2004). In congeneric Paralichthys species, Japanese flounder and Southern flounder, cyp19a1a expression coincides with sex differentiation, and has been used as an early predictor of fish sex (Kitano et al. 1999; Luckenbach et al. 2005). Japanese flounder begin to exhibit sexually dimorphic cyp19a1a expression at 32 mm TL, and can be masculinized by water temperatures until the completion of sex differentiation at ~40 mm TL (Kitano et al. 1999; Yamamoto 1999; Yamaguchi et al. 2010). By contrast, cyp19a1a expression in southern flounder does not become a reliable biomarker of sex until 65 mm TL, and fish smaller than 90 mm TL can be masculinized by exposure to water temperature (Luckenbach et al. 2005). In numerous fish species, the administration of aromatase inhibitors such as Fadrozole (Novartis Pharma Ltd., Basil, Switzerland) or 17α-Methyltestosterone (Sigma-Aldrich Co. LLC., St. Louis, MO) during sex differentiation leads to reduced P450 aromatase expression and testis formation. These studies with exogenous steroids confirm that gonadal cyp19a1a expression drives sex differentiation, with high expression leading to ovarian development, and low expression resulting in testicular development (Kitano et al. 2000; Lee et al. 2003; Bhandari et al. 2004; Uchida et al. 2004; Li et al. 2006).

Forkhead transcriptional factor 2 (foxl2) is a member of the winged helix/forkhead group of transcription factors, which is highly conserved in vertebrates (Nakamoto et al. 2006; Kobayashi et al. 2010). It has been shown to play a role in ovarian development of mammals, chickens, turtles and various fish species, and demonstrates sexually dimorphic gonadal expression in these species (Loffler et al. 2003; Baron et al. 2005). Foxl2 is recognized as the earliest known sex marker expressed during development in fish, and its expression is closely linked to P450 aromatase production. Within the ovary, foxl2 is expressed in the granulosa cells
that surround the previtellogenic and vitellogenic follicles, but it has not been detected in mature oocytes (Nakamoto et al. 2006; Kobayashi et al. 2010). It encodes a transcription factor that directly binds to a promoter region of cyp19a1a through its forkhead domain, which activates the transcription of P450 aromatase (Pannetier et al. 2006; Wang et al. 2007; Yamaguchi et al. 2007; Ijiri et al. 2008; Guiguen et al. 2010).

Foxl2 expression precedes that of cyp19a1a in a number of fish species including medaka, Japanese flounder and Nile tilapia, which is evidence of its role in activating gene transcription in the ovary (Nakamoto et al. 2006; Yamaguchi et al. 2007). Foxl2 mRNA is co-localized with cyp19a1a mRNA in these species, and foxl2 expression correlates with cyp19a in both sexual dimorphism (high expression in developing ovaries, low expression in developing testes), and response to water temperature (Smith et al. 2013; Wang et al. 2004; Nakamoto et al. 2006; Yamaguchi et al. 2007; Kobayashi et al. 2010). In Japanese flounder, sexually dimorphic foxl2 expression occurs prior to morphological sex differentiation (at 50 DPH), and continues throughout sex differentiation, and certain water temperatures suppress foxl2 expression, resulting in increased levels of masculinization (Yamaguchi et al. 2007). However, some teleost species do exhibit differences between foxl2 and cyp19a1a expression patterns. In sablefish (Anoplopoma fimbria), cyp19a1a has more pronounced sexual dimorphic expression than foxl2, while foxl2 is more broadly expressed in a greater number of tissue types, and is most highly expressed in the pituitary (Smith et al. 2013). Foxl2 has also been detected in the brain, pituitary, gills and gonads of Nile tilapia and the brain, pituitary, eye, gills and liver of the three-spot wrasse (Halichoeres trimaculatus), which suggests that it may be involved in the hypothalamic-pituitary-gonadal axis, likely through transcriptional regulation of the GnRH-GtH-sex steroid pathway (Wang et al., 2004; Kobayashi et al. 2010). In rainbow trout (Oncorhynchus mykiss)
two foxl2 paralogs have been identified that demonstrate differential expression. Foxl2a is expressed early in development, and highly correlates with cyp19a1a expression, while foxl2b is expressed later in development, and does not correlate with cyp19a1a expression (Baron et al. 2004; Baron et al. 2005). Overall these studies suggest that foxl2 plays an important role in activating cyp19a1a expression, and thus initiating female differentiation in fish, but foxl2 expression is not as reliable as an indicator of fish sex.

Doublesex/mab-3 related transcription factor 1 (dmrt1) is a gene that encodes a protein containing a DM-domain, which is a zinc fingerlike DNA binding motif (Ohmuro-Matsuyama et al. 2003; Kobayashi et al. 2008). Dmrt1 is highly conserved in both vertebrates and invertebrates, and plays a role in male development (Kobayashi et al. 2008). In mammals it has been linked to testis differentiation (Raymond et al. 2000), and in birds there is evidence that it may be the master sex-determining gene (Smith et al. 2009).

In fish, dmrt1 is more highly expressed in testes than in ovaries for a number of different species including Japanese flounder (Johnsen and Anderson 2012; Kobayashi et al. 2004; Kobayashi et al. 2008; Herpin and Schartl 2011). A recent study on Japanese flounder found high levels of dmrt1 promoter methylation and low dmrt1 expression in ovaries, while no dmrt1 promoter methylation and high dmrt1 expression occurred in testes tissue (Wen et al. 2014). This further suggests that methylation of gene promoters may be the epigenetic mechanism by which water temperature influences sex ratios. The timing and location of dmrt1 expression varies greatly by teleost species (Johnsen and Anderson 2012; Kobayashi et al. 2004; Kobayashi et al. 2008; Herpin and Schartl 2011). In Atlantic Cod (Gadus morhua) and orange-spotted grouper (Epinephelus coioides), dmrt1 expression is higher in the testes, and only occurs in spermatogonia (Xia et al. 2007; Johnsen et al. 2010; Johnsen and Anderson 2012). In medaka,
dmrt1 expression occurs after sex differentiation, (20-30 DPH), and is involved in regulating spermatogenesis in the testes (Kobayashi et al. 2004). Other studies on medaka have identified dmy (dmrt1b(y)) as a homolog of dmrt1 that is the master sex determining gene. Dmy occurs in the sex determining region of the Y chromosome, and its expression precedes that of dmrt1 (Matsuda et al. 2002; Matsuda et al. 2007). However, dmy is only present in two out of twenty closely related Ozyrias species, which suggests that dmy is not the master sex determining gene in other fish species, and demonstrates that mechanisms of sex differentiation can vary even among congenerics (Kondo et al. 2003). Species such as rainbow trout, Gobiocypris rarus, zebrafish (Danio rerio) and Nile tilapia exhibit sexually dimorphic dmrt1 expression very early in development before any histological change in the gonads, with males exhibiting high dmrt1 expression that coincides with low cyp19a1a expression (Marchand et al. 2000; Cao et al. 2012; Jorgensen et al. 2008; Kobayashi et al. 2008; Ijiri et al. 2008). In zebrafish, three different dmrt1 isoforms have been identified. Dmrt1 expression peaks before gonadal differentiation (10 DPH), but appears to be important in testis and ovary development (Guo et al. 2005). In tilapia, dmrt1 expression can be detected in the testes at 6 DPH, and increases throughout male development, but is never detectable in developing females. In developing Nile tilapia males, dmrt1 inhibits cyp19a1a gene transcription and estrogen production, which represses the expression of the female pathway and allows testes to develop (Ijiri et al. 2008; Jorgensen et al 2008; Kobayashi et al. 2008; Wang et al. 2010). In Nile tilapia and rainbow trout females sex reversed by hormones or medaka females sex reversed by high temperature exposure, high dmrt1 expression occurs in the testes, which is further evidence of its importance in male development (Marchand et al. 2000; Hattori et al. 2007; Kobayashi et al. 2008). Cumulatively these findings suggest that dmrt1
plays an important role in male differentiation of fish species, though the exact mechanism is unknown.

The *amh* gene codes for anti-Müllerian hormone (*amh*), also known as Müllerian inhibiting substance (*mis*), which is a glycoprotein that belongs to the transforming growth factor β (TGF-β) superfamily (Yoshinaga et al. 2004). *Amh* has been well studied in mammals, where it is expressed in all individuals early in development, but is up-regulated in developing testes, and down-regulated in developing ovaries. In males, it causes the regression of Müllerian ducts, which would become the oviducts and uterus, while in females, it is weakly expressed in the granulosa cells surrounding the oocytes. When mammalian ovaries are exposed to *amh*, they experience inhibited *cyp19a1a* expression, which promotes male development (Cate et al. 1986; Swain and Lovell-Badge 1999). In lower vertebrates, *amh* has been linked to the downregulation of *foxl2* and *cyp19a1a*, and exhibits elevated expression in Sertoli cells during testis differentiation (Western et al. 1999; Rodríguez-Marí et al. 2005; Baroiller et al. 2009; Hattori et al. 2012).

*Amh* plays a role in testis differentiation and development in various fish species. However, unlike mammals, fish do not have Müllerian ducts, and few details are known about the specific pathway (Yoshinaga et al. 2004; Rodríguez-Marí et al. 2005; Ijiri et al. 2008; Siegfried 2010). In zebrafish, *amh* expression occurs in presumptive Sertoli cells prior to sex differentiation. Adult testes exhibit high *amh* expression, while adult ovaries exhibit decreasing *amh* expression in granulosa cells of developing oocytes that negatively correlates with increasing *cyp19a1a* expression (Rodríguez-Marí et al. 2005). Japanese flounder and Nile tilapia show similarly dimorphic *amh* expression. In these species, *amh* is expressed in all undifferentiated fish, but males demonstrate up-regulated *amh* expression during testicular
differentiation that correlates with inhibited \textit{cyp19a1a} expression, while females exhibit the inverse pattern (Yoshinaga et al. 2004; Kitano et al. 2007; Ijiri et al. 2008; Baroiller et al. 2009). This expression pattern occurs in all Japanese flounder males, whether genetic (XY), or produced by masculinizing genotypic females (XX) through exposure to water temperatures or the anti-estrogen tamoxifen (Sigma-Aldrich Co. LLC., St. Louis, MO) (Yoshinaga et al. 2004; Kitano et al. 2007). In fact, there is evidence that \textit{amly}, an early expressed homolog of \textit{amh} is the master sex determining gene in the Patagonian perjerrey (\textit{Odontesthes hatcheri}), and in sablefish it is more sexually dimorphic than \textit{dmrt1} (Hattori et al. 2012; Smith et al. 2013). By contrast, sexually dimorphic \textit{amh} expression has not been established in the medaka, and there appears to be no correlation between \textit{amh} and \textit{cyp19a1a} expression in this species (Klüver et al. 2007). In addition, \textit{amh} expression in the Patagonian perjerrey is not influenced by temperature (Shen and Wang 2014). Overall there is evidence that \textit{amh} is an important gene for male differentiation in many fish species, though more research is needed to establish the specific role.

Previous research indicates that \textit{cyp19a1a}, \textit{foxl2}, \textit{dmrt1} and \textit{amh} are robust sex markers in a number of fish species including Japanese flounder, and exhibit sexually dimorphic expression in ovaries and testes (Kitano et al. 1999; Yoshinaga et al. 2004; Luckenbach et al. 2005; Cao et al. 2012; Smith et al. 2013). Endocrine changes often occur before the gonads are visible in differentiating fish, which makes it likely that enzymes begin to influence sex differentiation before clear histological differences between males and females are observed (Baroiller et al. 1999). Enzymes have optimal temperatures, and gene expression can also be thermally sensitive. Species such as summer flounder that have a temperature component to sex differentiation may experience increased or decreased gene expression and/or enzyme activity during a thermally sensitive period of sex differentiation. The combination of temperature
Sensitive sex differentiation and sexually dimorphic growth in summer flounder has important implications for aquaculture and the wild flounder fishery. In summer flounder aquaculture, water temperature could be used to control sex differentiation, and manipulate phenotypic sex ratios. Lower water temperatures could be used to create populations dominated by faster and larger growing females, thereby increasing commercial aquaculture production efficiency (Luckenbach et al. 2003). Commercial aquaculture of summer flounder in the US would provide a stable source of a popular finfish species while reducing dependence on imports and wild caught fish. As ocean temperatures rise, wild summer flounder populations may experience skewed sex ratios, resulting in male-dominated populations, and reduced reproductive success (Ospina-Álvarez and Piferrer 2008).

The present studies use a combination of molecular and histological techniques to investigate the environmental component of sex change in summer flounder by 1). assessing the resulting sex ratios of summer flounder raised at 13°C, 16°C and 19°C 2). determining expression levels of cyp19a1a, foxl2, dmrt1 and amh, four genes that demonstrate sexually dimorphic expression in other teleost species 3). establishing relationships in timing and magnitude of expression levels of these four genes, and 4). using gene expression levels to identify the period of sex determination when summer flounder may be sensitive to temperature effects.

**Literature Cited**


Guiguen Y, Baroiller J-F, Ricordel M-F, Iseki K, McMeel OM, Martin SAM, Fostier A (1999) Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (Oncorhynchus mykiss) and a tilapia (Oreochromis niloticus). Molecular Reproduction and Development 54:154-162


Herpin A, Schartl M (2011) Dmrt1 genes at the crossroads: A widespread and central class of sexual development factors in fish. FEBS Journal 278:1010-1019


Johnsen H, Andersen O (2012) Sex dimorphic expression of five dmrt genes identified in the Atlantic cod genome. The fish-specific dmrt2b diverged from dmrt2a before the fish whole-genome duplication. Gene 505:221-232


King NJ, Nardi GC, Jones CJ (2001) Sex-linked growth divergence of summer flounder from a commercial farm: are males worth the effort? Journal of Applied Aquaculture 11(1/2):77-88


Lymer D, Funge-Smith S, Miao W (2010) Status and potential of fisheries and aquaculture in Asia and the Pacific. FAO Regional Office for Asia and the Pacific. RAP Publication 85pp


http://www.fishwatch.gov/farmed_seafood/in_the_us.htm


http://www.fishwatch.gov/seafood_profiles/species/flounder/species_pages/summer_flounder.htm


CHAPTER 2

THE EFFECTS OF TEMPERATURE ON OVARIAN AROMATASE (CYP19A1A) EXPRESSION AND SEX DIFFERENTIATION IN SUMMER FLOUNDER (PARALICHTHYS DENTATUS)\(^1\)

Introduction

Paralicthyd flounder species are cultured throughout the world, particularly in Asia where Japanese flounder (Paralichthys olivaceus) culture represents one of the most significant marine aquaculture industries (Bolasina et al. 2006). In the United States, considerable research has been conducted to develop protocols for summer (Paralichthys dentatus) and southern flounder (P. lethostigma) aquaculture, and limited commercial production has been occurring for over a decade, primarily in land-based, recirculating aquaculture systems (Bengtson 1999; Watanabe and Carroll 2001). While earlier research focused on fundamental aspects of aquaculture such as methods to improve spawning, larviculture, nutrition and health management, more recent studies have focused on improving juvenile growth (Watanabe and Carroll 2001; Gaylord et al. 2003).

Growth rate is a primary factor that determines the profitability of land-based flounder culture, and the costs of growing fish to market size must be reduced to offset high production costs associated with construction and operation of recirculating systems (King et al. 2001). All Paralicthyd species studied to date exhibit sexually dimorphic growth, with females growing

\(^1\) Chapter 1 was submitted for publication by Catherine C. Caruso, Timothy S. Breton, and David L. Berlinsky, entitled, The effects of temperature on ovarian aromatase (cyp19a1a) expression and sex differentiation in summer flounder (Paralichthys dentatus).
faster and larger than males (Morse 1981; Yamamoto 1999; Daniels 2000; King et al. 2001; Yoneda et al. 2007). A tremendous increase in growth performance can therefore be realized through production of monosex female populations, and Japanese flounder have been commercially produced by this method in Japan and Korea since 1990 and 1996, respectively (Yamamoto 1999; Bai and Lee 2010).

Of the Paralichthyd species, the sex determining mechanisms are best known for Japanese flounder. This species, like many teleosts, exhibits genotypic sex determination (GSD), with homogametic (XX) females and heterogametic (XY) males (Tabata 1991; Yamamoto 1999; Luckenbach et al. 2009). However, GSD can be overridden by environmental (temperature) effects in female Japanese flounder (genotypic sex determination + temperature effects; GSD + TE), if fish are exposed to masculinizing temperatures during the sex-determining period of development (Kitano et al. 1999; Ospina-Álvarez and Piferrer 2008). Specifically, XX Japanese flounder will develop into phenotypic males if they are exposed to high (25-27.5°C) or low (15°C) water temperatures during sex differentiation (Yamamoto 1999) and a similar system has been described for southern flounder (Luckenbach et al. 2003). Less is known about the mechanism or timing of sex determination in summer flounder, but previous studies have shown a propensity towards male differentiation when exposed to a wide range of temperatures (12-21°C; Colburn et al. 2009; 2015).

A number of genes that code for transcription factors or enzymes involved in endocrine function are expressed during teleost sex determination (Luckenbach et al. 2005; Ijiri et al. 2008; Mankiewicz et al. 2013; Smith et al. 2013). Of these, cyp19a1a is the most well-studied, and has been shown to play a role in the regulation of ovarian development. Cyp19a1a encodes for cytochrome P450 ovarian aromatase, a steroidogenic enzyme that converts testosterone into
estrogen (17β estradiol; E2; Kitano et al. 1999; Liu et al. 2010). A number of teleosts, including Japanese flounder, exhibit sexually dimorphic cyp19a1a expression (Kitano et al. 1999; Wen et al. 2014), and in some species it has been established as a robust sex marker (Smith et al. 2013). Cyp19a1a expression has also been shown to be influenced by water temperature and is likely responsible for GSD + TE sex determination in some species (Kitano et al. 1999; Yoshinaga et al. 2004; Hattori et al. 2007; Yamaguchi et al. 2007; Baroiller et al. 2009; Wen et al. 2014).

The purpose of the present study was to investigate the timing and magnitude of cyp19a, expression during sex differentiation in summer flounder reared at three experimental temperatures (13°C, 16°C and 19°C), and assess the sex ratios resulting from each temperature treatment. To accomplish this, partial summer flounder cDNA sequences for cyp19a1a and eef1α (a common reference gene), were identified and their expression patterns assessed using real time quantitative PCR.

**Materials and methods**

**Broodstock culture and juvenile rearing**

Summer flounder broodstock were captured from Rhode Island coastal waters and held at GreatBay Aquaculture LLC (GBA; Portsmouth, NH) for at least two years prior to the start of the experiment. Fish were maintained in recirculating aquaculture systems (28-30 ppt salinity) equipped with biological and mechanical filtration, ultraviolet sterilization, foam fractionation, and photothermal control. Water quality within the culture tanks remained within ranges suitable for rearing this species (Watanabe et al. 1998). The fish were fed a commercial broodstock ration (Breed-M, Inve, Salt Lake City, UT) to apparent satiation 2-3 times per week. Photoperiod and temperature were maintained at 12L: 12D and 19 ± 1 °C until two months prior to spawning (~5 years, 1.3 kg), when they were adjusted to 8L: 16D and 14 °C (Watanabe et al. 1998; Bengtson
To obtain ovarian tissue for partial cDNA identification, an adult female broodstock was euthanized via immersion in 200 mg/L tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA), and ovaries were immediately dissected, preserved in RNA Later® Solution (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY), and stored at \(-70^\circ\)C for later RNA extractions.

Summer flounder broodstock were spawned at GBA, and larvae were raised to the beginning of metamorphosis in 800 L tanks in a recirculating system with filtered (10 µm) and UV sterilized seawater (26-29 ppt) at 17-19°C. Larvae were fed supplemental microalgae (Nanochloropsis sp., \(\sim 3 \times 10^8/\text{mL}\)) until 14 days post hatch (DPH). Fish were fed rotifers, *Brachionus plicatilis*, and brine shrimp, *Artemia*, nauplii during larval culture and weaned onto a commercial diet (Gemma Micro 300 µm; Skretting USA, Tooele, UT) by 34 DPH.

Juveniles (37 DPH) in various stages of metamorphosis (pelagic, symmetrical larvae to demersal juveniles; Packer et al. 1999) were transferred to the Aquaculture Research Center (ARC) at the University of New Hampshire (UNH; Durham, NH), where they were maintained in three separate recirculating systems. Each system consisted of four replicate 235 L cylindrical tanks (400 fish/tank) equipped biological and mechanical filtration, ultraviolet sterilization, foam fractionation, photothermal control and temperature control. The three systems were maintained on a 12L: 12D photocycle (40 lx and 0 lx, respectively), with 30 minute crepuscular intervals (15 lx). Salinity and dissolved oxygen were monitored daily (OxyGuard Handy Gamma), along with pH (Ecosense® PH and temperature (Temp Pen, Pentair Aquatic Ecosystems, Apopka, FL). Nitrates and total ammonia were monitored weekly (HACH®). Fish were fed an appropriately sized marine diet in excess (Skretting, USA).

**Juvenile summer flounder sampling**
To provide an initial assessment prior to exposure to different temperatures, fish at 38 DPH (n = 5 fish/system) were weighed on an analytical balance (Mettler Toledo International Inc., Leicester, UK), and measured (standard length (mm); fish snout to tail base) using a digital caliper (Mitutoyo America, Aurora, IL). Due to small fish sizes, gonads could not be accurately dissected from each individual. Rather, heads and tails were removed using sterile razor blades, and gonadal regions were excised (Figure 3; Goto et al. 1999; Winkler et al. 2004), preserved in RNA Later® Solution (Life Technologies, Thermo Fisher Scientific Inc., Grand Island, NY), and stored at -70°C for later RNA extractions. All fish were acclimated at 19°C for one week (38-44 DPH). Fish in one system were then maintained at 19°C, while the two other systems were slowly cooled (1°C/day) to reach final temperatures of 13 and 16°C. Fish from all three temperature treatments were sampled again as described above, after all experimental temperatures were attained (52 DPH, n = 4 fish/treatment). Fish (n = 9 fish/treatment) were sampled again at 66 DPH.

Figure 3. Juvenile summer flounder (38 DPH) sampled for cyp9a1a gene expression analyses. Dotted lines represent larval dissections made using razor blades to excise summer flounder gonads. Area indicated by the arrow represents the gonadal region (GR) that was preserved for later RNA extraction.

To further investigate juvenile growth and expression patterns during later development, additional fish from the 13 and 16°C treatments were sampled at 105, 133, and 171 DPH (n = 5, 8, or 9 fish/treatment). Due to overall slow growth in the 13°C treatment, an additional 12 fish were sampled at 191 DPH. All fish were weighed, measured, and gonadal regions were
removed. Additional muscle tissue in larger fish was trimmed from gonadal regions prior to preservation (RNA Later® Solution) and storage at -70°C. Fish from the 19°C treatment were not sampled after 66 DPH, due to relatively low numbers of fish in the replicate tanks, and the remainder were retained to determine temperature-dependent sex ratios.

Remaining fish were maintained at their respective temperatures until 217 DPH, at which point all treatments were slowly increased to 21°C over two weeks (<1°C/day), to both simulate spring temperatures and accelerate juvenile growout (Colburn et al. 2009, 2015). Summer flounder do not undergo morphological gonadal differentiation until late in juvenile development (≥150 mm total length (TL); Colburn et al. 2009; 2015). At this point, approximately 50 fish from each treatment were euthanized with MS-222 and gonads were visually assessed, to determine sex ratios (Colburn et al. 2015). Fish that could not be sexed visually were fixed in 10% formalin for 24 hours, and transferred to 70% ethanol for histological processing. Preserved samples were embedded in paraffin, sectioned at 5µm, stained with hemotoxylin and eosin, and examined under a compound light microscope (UNICO M250, UNICO, Dayton, NJ). Sex classification was based on gonad characteristics previously described (Nakamura et al. 1998; Goto et al. 1999; Luckenbach et al. 2003). Fish with germ cells forming clusters in seminal lobules or gonads in various stages of spermatogenesis were designated as male. Fish with gonads with lamellate structure or clear oogonia were designated as female (Colburn et al. 2009).

Partial cDNA identification

Total RNA was extracted from adult summer flounder ovary tissue. Samples were removed from RNA Later® Solution, blotted on Whatman #1 filter paper (Whatman Inc., Sanford, ME), weighed and added to 500µL cold TRIzol Reagent (Life Technologies, Grand Island, NY). The tissue was homogenized using microtubes and pestles (Fisher Scientific,
Pittsburgh, PA) and brought to 1000µL with TRIzol Reagent. RNA was extracted using standard phenol/chloroform techniques (Molecular Research Center, Cincinnati, OH). mRNA was extracted using 20 µg of the total RNA in an Ambion MicroPoly (A) Purist kit (Life Technologies, Grand Island, NY). Synthesis of cDNA was performed using 500 µg mRNA and Superscript III reverse transcriptase.

Gene-specific primers were designed to amplify 400-600 base pair (bp) partial sequences for *eef1a* (eukaryotic elongation factor 1 alpha) and *cyp19a1a* (P450 aromatase) in polymerase chain reaction (PCR). Primer sets (Table 1) were designed in Geneious 4.7.6 (Biomatters Ltd., San Francsisco, CA) from consensus alignments of closely related fish species. The consensus sequence for *eef1a* was generated from coding domain sequence (cds) alignments of four teleost species including *Paralichthys olivaceus* (AB017183.1), *Hippoglossus hippocircus* (EU561357.1), *Sparus aurata* (AF184170.1) and *Oreochromis niloticus* (AB075952.1). The consensus sequence for *cyp19a1a* was generated from coding domain sequence (cds) alignments of *Paralichthys olivaceus* (AB017182.1), *Hippoglossus hippocircus* (AJ410171.1), *Oryzias latipes* (D82968.1) and *Sebastes schegelii* (FJ594995.2).

Primers and ovarian cDNA were used in the Clontech Advantage 2 PCR kit (Clonetech Laboratories Inc., Mountain View, CA), with 5µL template (1/100, 1/10), 20 µL total volumes (Clonetech Laboratories Inc., Mountain View, CA), and standard thermocycling conditions (95°C for 1 min, followed by 34 cycles of denaturing for 30 seconds at 95°C, annealing for 30 sec at 60°C and extension for 1 min at 68°C, and a 1-cycle final extension for 1 min at 68°C, then held at 4°C). PCR products were electrophoresed in a 2% agarose gel, and bands at the expected base pair (bp) size were excised from the gel. PCR products were extracted from agarose gels using a QIAGEN Qiaex II gel extraction kit (QIAGEN, Valencia, CA). PCR
products were sequenced at the UNH Hubbard Center for Genome Studies using the dideoxy chain termination method in an ABI 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA), with the forward and reverse primer for each gene. Chromatograms were quality trimmed and analyzed using: 1) Blastn against NCBI general nucleotide database and 2) Blastx against NCBI protein database (n/r).

RNA extractions and cDNA synthesis of juvenile samples

Juvenile summer flounder gonadal regions were used in RNA extractions, using similar techniques as described above. RNA was quantified using an ND 1000 NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). For each sample, 2.5 μg RNA was treated with 2.5μL DNase, and reverse transcribed into cDNA using 2.5 μM oligo dT primer (20mer), and 200 units of SuperScript III reverse transcriptase according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Juvenile summer flounder cDNA was stored at -20°C prior to relative quantification with real time quantitative PCR.

Real-Time Quantitative PCR (QPCR)

*Eef1a* and *cyp19a1a* primer sets for QPCR were designed in NCBI Primer-Blast to amplify 80-150 bp fragments (Table 1). Relative quantification SyberGreenER QPCR (Invitrogen) assays were performed using an ABI 7500 Fast System (Applied Biosystems, Foster City, CA) with 1000 nM primer concentrations and standard cycling conditions (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min), followed by dissociation curve analysis. Samples were performed in duplicate (1/10 dilution). Triplicate relative standard curves (1/5-1/640) were generated from dilutions of pooled cDNA from eight individuals per gene. Optimized assays consisted of eight point linear standard curves with approximately 100-110% PCR efficiency. Standard QPCR negative controls (no template and no reverse
transcriptase) were performed in duplicate for each assay and exhibited no contamination <10 Ct from samples (Luckenbach et al. 2009). Both gene assays displayed a single peak in dissociation curve analysis.

Table 1. GenBank accession numbers, primer sequences and product sizes (bp) for partial cds identification and QPCR assays, with percent efficiency (% eff) and mean cycle threshold (Ct) values for QPCR assays. Mean Ct values refer to the mean 1/10 diluted standard curve point for each assay. Higher Ct values indicate lower mRNA abundance.

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Statistical Analysis

Fisher-Exact tests were run to determine if male:female ratios of fish grown to ≥ 150 mm differed significantly by temperature treatment. Pairwise comparisons were Bonferroni-corrected (p<0.017) and used to assess which temperature treatments were significantly different. PCR results were analyzed using the Pfaffl method for relative quantification (Pfaffl 2001). Individual expression levels of cyp19a1a were calibrated to the 38 DPH (initial) group mean and then normalized to eef1α, to compensate for differences in cDNA synthesis efficiency among samples. All data were evaluated for normality and heteroscedasticity prior to analysis. Relative expression values were normalized to the 38 DPH (initial) group mean and log-transformed prior to analyses to satisfy assumptions of equal variance among groups. One-way analyses of
variance (ANOVARs) were conducted in JMP Pro 11 (SAS Institute Inc., Cary, NC) to assess significant differences in cyp19a1a expression and fish growth (p<0.05). Each ANOVA was followed by a Tukey’s HSD means separation test to identify pairwise significant differences. All numerical data are represented as the mean ± standard error. To identify correlations between fish size and cyp19a1a expression, linear regression analyses were performed in JMP Pro 11.

**Results**

**Partial cDNA fragments**

The 471 bp fragment corresponding to eef1α exhibited high nucleotide sequence identity with other teleost species, including Paralichthys lethostigma (99%), Paralichthys olivaceus (99%) and Hippoglossus hippoglossus (95%). The translated protein sequence exhibited high identity match with Paralichthys lethostigma (99%), Paralichthys olivaceus (99%) and Hippoglossus hippoglossus (96%). The 540 bp fragment corresponding to cyp19a1a was quality trimmed from the expected fragment size (597 bp). It exhibited high nucleotide sequence identity with other teleost species including Paralichthys lethostigma (98%), Paralichthys olivaceus (98%), Verasper moseri (96%) and Hippoglossus hippoglossus (95%). The translated protein sequence exhibited high identity match with Paralichthys lethostigma (98%), Paralichthys olivaceus (98%) Verasper moseri (95%) and Hippoglossus hippoglossus (94%). The eef1α and cyp19a1a partial cDNA fragments were both submitted to the NCBI GenBank database (KR061538 and KR061539, respectively).

**Cyp19a1a expression during juvenile development**

Most juvenile flounder at 38, 52 and 66 DPH exhibited overall stable cyp19a1a expression, except for those reared at 19°C at 52 DPH, which significantly decreased in expression (4.5 fold; p = 0.0016; Figure 4A). At 52 DPH, fish acclimated to 13°C exhibited
significantly higher (6 fold) expression than fish reared at 19°C. After the growth period, the final sex ratios differed by temperature treatment, and largely mirrored differences in cyp19a1a expression present at 52 DPH (Figure 4B). Significantly more females were produced at the low (13°C) and intermediate (16°C) temperature regimes (females = 26.9 and 17.6% respectively) than at the high (19°C) temperature regime (females = 0%; p<0.0001), but there was no significant difference in the percentage of females in the 13°C and 16°C treatments.

Figure 4. (A) Relative cyp19a1a mRNA abundances, normalized to eef1α, during early juvenile development at initial sampling (38 DPH, 19°C), and at 52 and 66 DPH (13, 16 and 19°C). Each bar represents the mean ± standard error. (B) Summer flounder sex ratios determined after temperature treatments (44-217 DPH) and growout period (21°C, ≥150 mm total length). Sex was assessed by visual examination during gonadal dissection or histology. Different letters indicate significant differences among treatments and ages (p < 0.05).
Juvenile summer flounder reared at 16°C exhibited significantly greater growth than those at 13°C by 133 DPH (Figure 5A). In contrast, flounder reared at 16°C exhibited lower \textit{cyp19a1a} levels at 105 and 171 DPH (p = 0.0288 and 0.0419, respectively; Figure 5B). In both 13 and 16°C temperature treatments, aromatase expression largely decreased after 66 DPH, and an inverse relationship was evident between \textit{cyp19a1a} expression and fish size (p < 0.0001; Figure 5C).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Summer flounder (A) growth and (B) relative \textit{cyp19a1a} mRNA abundances (normalized to \textit{eef1a}) during juvenile development at 13 and 16°C (38-191 DPH). Asterisks denote significant differences between treatments (*p < 0.05; ** p < 0.01; *** p < 0.001). (C) Relationship between relative \textit{cyp19a1a} mRNA abundance (logarithmic scale) and standard lengths (mm) of fish raised at 13 (p < 0.0001) and 16°C (p < 0.0001).}
\end{figure}
**Discussion**

The process of sex determination in fishes is highly variable among species and may be influenced by both genetic and environmental factors (Devlin and Nagahama 2002). In mammals, the presence of the *Sry* gene on the Y chromosome generally results in formation of testes, but the sex-determining gene(s) in females are less well understood (Waters et al. 2007). In teleosts, the sex determining genes remain largely unknown, and vary widely in the few species in which they have been identified, such as the medaka (*Oryzias latipes; dmy*), the Patagonian pejerrey (*Odontesthes hatcheri; amhy*), *Oryzias luzonensis* (*Gsdf*), fugu (*amhr2*) and rainbow trout (*Oncorhynchus mykiss; sdY*) (Kobayashi et al. 2004; Kikuchi and Hamaguchi 2013).

More research has focused on downstream genes involved in sex differentiation including *cyp19a1a, foxl2, dmrt1* and *amh* (Luckenbach et al. 2005; Nakamoto et al. 2006; Ijiri et al. 2008; Herpin and Schartl 2011; Smith et al. 2013). *Cyp19a1a*, in particular, plays a pivotal role in teleost sex determination, and relatively high or low expression occurs during female and male sex differentiation, respectively (Kitano et al. 1999; Kitano et al. 2000; Lee et al. 2003; Luckenbach et al. 2005). During the critical period of sex differentiation, several factors, including water temperature, can influence *cyp19a1a* expression levels, and therefore the resulting phenotypic sex of the developing fish (Kitano et al. 1999; Kitano et al. 2000; Lee et al. 2003; Luckenbach et al. 2005). Stress can also affect expression levels, as seen in studies on Japanese and southern flounder where increased cortisol in response to temperature and culture conditions suppressed *cyp19a* expression, and led to higher proportions of male individuals (Yamaguchi et al. 2010; Mankiewicz et al. 2013). A recent study on European sea bass found that higher water temperatures increased methylation of a *cyp19a1a* promoter, which suppressed expression and resulted in masculinization of genotypic females (Navarro-Martín et al. 2011). A
similar result was achieved in Japanese flounder, where high levels of \textit{cyp19a1a} promotor methylation correlated with low expression in testes, while no methylation and high \textit{cyp19a1a} expression occurred in ovarian tissue (Wen et al. 2014). In the present study, more female summer flounder resulted from culture at lower temperatures, but the proportion was low overall among treatments (0-26.9%). These results are similar to those of previous studies with cultured summer flounder where high male differentiation occurred, even in meiogynogenetic individuals (Colburn et al. 2009; 2015). Male-skewed sex ratios have also been reported in wild populations, but it is not known if this was due to sampling biases or differential mortality rates (Powell and Morson 2008).

Japanese flounder, the most well studied Paralichthyd, have a confirmed XX/XY chromosomal system that is influenced by water temperature (GSD+TE). They exhibit a U-shaped pattern of sex differentiation where intermediate temperatures (20°C) produce a 1:1 sex ratio that aligns with chromosomal sex, while high (25-27.5°C) and low (15°C) temperatures cause sex-reversal of genotypic females, resulting in male-dominated populations (Kitano et al. 1999; Yamamoto et al. 1999; Ospina-Álvarez and Piferrer 2008). The system of sex differentiation in southern flounder is less clear. A chromosomal system has not been confirmed, but one study on Atlantic coast fish (NC) found a U-shaped pattern of temperature-influenced sex differentiation similar to that of Japanese flounder (Luckenbach et al. 2003). Research on fish from the Texas Gulf Coast found that the proportion of females decreased with increasing water temperatures, similar to the differentiation pattern in summer flounder (Montalvo et al. 2012). These differences may be explained by adaptive variation due to geographical differences between southern flounder populations, but additional research is necessary before either summer or southern flounder can be definitively classified as GSD+TE. It is possible that
summer flounder do not produce a balanced sex ratio because they possess a sex differentiation system that differs fundamentally from that of Japanese flounder. Such differences have been established in other congeneric teleost species (e.g. XX/XY vs. ZZ/ZW; *Oreochromis, Mystus, Clarias*; Devlin and Nagahama 2002), and even between subpopulations within a species (Penman and Piferrer 2008).

Japanese and southern flounder both undergo sex differentiation well after metamorphosis, but differ in their timing. Japanese flounder metamorphose at ~17.5 mm TL (25-30 DPH; Fukuhara 1986) and undergo sex differentiation at 32 mm TL, when sexually dimorphic *cyp19a1a* expression occurs (Kitano et al. 1999). Southern flounder metamorphose at a comparable age and size (~30-45 DPH; 10-16 mm TL; Daniels et al. 1996; 2000), but do not initiate sex differentiation until ~65 mm TL (Luckenbach et al. 2005). While summer flounder also metamorphose at a similar age and size (13 mm TL; 39 DPH; Gavlik et al. 2002), it is possible that they undergo sex differentiation earlier than congeneric. At the onset of the present study, the treatment fish were a combination of pelagic larvae, and settled, post-metamorphic juveniles (~14 mm TL; 37 DPH). Fourteen days later, after brief exposure to experimental temperature treatments (~15 mm TL; 52 DPH), fish exhibited differential *cyp19a1a* expression by treatment, and this may correspond to the window of sex determination in this species. Fish reared at a temperature known to produce all male populations (19° C; Colburn et al. 2015) exhibited a decrease in *cyp19a1a* expression, while female-producing temperatures (13 and 16°C) exhibited stable expression. This contrasts with other Paralichthyd species where males exhibit stable expression, and females demonstrate elevated *cyp19a1a* levels (Luckenbach et al. 2005; Smith et al. 2013). The 13° C treatment, however, only produced 26.9% females, and mean expression levels at 13 and 16°C likely reflect high proportional contributions of male *cyp19a1a*
expression. Conditions that may produce higher proportions of females could result in dramatic increases in mean cyp19a1a expression, similar to that of other Paralichthys species. By 66 DPH, expression levels in fish reared at 19°C increased to levels similar to other treatments, and expression levels in the 13 and 16°C treatments largely decreased with increasing fish size. There was no clear sexual dimorphic cyp19a1a expression at the ages and sizes where sex-specific gene expression differences occur in other Paralichthys (Kitano et al. 1999; Luckenbach et al. 2005). Therefore, the window of sex determination in this species (~15 mm TL; 52 DPH) appears to be earlier than that of congers, and may occur at or soon after metamorphosis. The apparent increase in cyp19a1a expression in the male-producing temperature (19°C) after 52 DPH contrasts with patterns in other Paralichthys, where male expression remains low throughout juvenile development (Kitano et al. 1999; Luckenbach et al. 2005). These expression increases in male juvenile summer flounder may indicate that estrogens are involved in testicular differentiation, as in black porgy (Acanthopagrus schlegelii) and rainbow trout (Wu et al. 2010; Delalande et al. 2015). Therefore, in summer flounder, sex-specific differences in cyp19a1a during early gonadal development may be limited to a temporally short window of sex determination.

The relatively small cyp19a1a expressional changes observed in this study may have resulted from the use of gonadal regions in RNA extractions, which included non-gonadal tissues, and likely reduced gonad-specific expression patterns. The inverse relationship between cyp19a1a expression and fish size may also reflect the relative contribution of non-gonadal tissue as the fish grew. A more accurate assessment of expressional changes during juvenile summer flounder development may require gonadal micro-dissection, if possible, from early developmental stages, including pre-metamorphosis.
In conclusion, the conditions necessary to produce a balanced sex ratio were not obtained in this study, and predominantly male phenotypes resulted from the experimental culture temperatures employed. Summer flounder may need to be exposed to temperature regimes earlier than congenerics to promote female differentiation. Differential cyp19a1a expression patterns were observed at ~15 mm TL (52 DPH) which may correspond to the window of sex determination in this species. Additional research is necessary to fully evaluate gonadal cyp19a1a expression patterns, and other genes involved in sex differentiation, during early development in this species.

**Literature Cited**


King NJ, Nardi GC, Jones CJ (2001) Sex-linked growth divergence of summer flounder from a commercial farm: are males worth the effort? Journal of Applied Aquaculture 11(1/2):77-88


CHAPTER 3

PRELIMINARY INVESTIGATIONS INTO FOXL2, DMRT1 AND AMH EXPRESSION DURING SEX DIFFERENTIATION IN SUMMER FLOUNDER (PARALICHTHYS DENTATUS)

Introduction

Previous studies on Paralichthyds including Japanese, southern, and summer flounder have established a critical period during juvenile development when sex determination is sensitive to temperature effects (Kitano et al. 1999; Luckenbach et al. 2003). There are a number of genes that play a role in sex determination in fishes by producing hormones, enzymes and transcription factors involved in male or female development. Cyp19a1a, the most well studied, was investigated in summer flounder in Chapter 2, but others including foxl2, dmrt1 and amh have also been connected to sex determining processes in teleost species. These genes exhibit sexually dimorphic expression patterns during male and female development, and interact with cyp19a1a in various ways (Luckenbach et al. 2005; Nakamoto et al. 2006; Ijiri et al. 2008; Herpin and Schartl 2011; Smith et al. 2013). In order to more fully characterize the sex determining window in summer flounder, additional work is needed on expression patterns of foxl2, dmrt1 and amh in this species.

Foxl2 (Forkhead transcriptional factor 2) is a transcription factor in the winged helix/forkhead group (Nakamoto et al. 2006; Kobayashi et al. 2010). It is highly conserved in vertebrates, and in teleosts it plays a role in ovarian development. More specifically, it encodes a transcription factor that binds to a promoter region of cyp19a1a, which activates P450 ovarian
aromatase transcription (Pannetier et al. 2006; Wang et al. 2007; Yamaguchi et al. 2007; Ijiri et al. 2008; Guiguen et al. 2010). *Foxl2* is the earliest known sex marker in teleosts, and in females it has been shown to exhibit increased expression before or concurrently with increased *cyp19a1a* expression, and demonstrates a similar response to water temperature (Smith et al. 2013; Wang et al. 2004; Nakamoto et al. 2006; Yamaguchi et al. 2007; Kobayashi et al. 2010). However, it exhibits less pronounced dimorphic expression than *cyp19a1a*, perhaps because it is more broadly expressed in a greater number of tissue types (Smith et al. 2013).

*Dmrt1* (Doublesex/mab-3 related transcription factor 1) is a highly conserved gene that encodes a protein involved in regulating male development (Kobayashi et al. 2008). In mammals it has been linked to testis differentiation (Raymond et al. 2000), and in various teleost species, including Japanese flounder, *dmy* is more highly expressed in developing testes than in ovaries, which may be due to high levels of *dmrt1* promoter methylation in ovaries, and no such methylation in testes (Wen et al. 2014). However, the timing and location of *dmrt1* expression varies greatly by species (Johnsen and Anderson 2012; Kobayashi et al. 2004; Kobayashi et al. 2008; Herpin and Schartl 2011). A homolog of *dmrt1, dmy(dmrt1b(y)) has been identified as the master sex determining gene in the medaka, but it only occurs in two out of twenty closely related *Ozyrias* species, which suggests that it is not the master sex determining gene in other fish species (Matsuda et al. 2002; 2007).

*Amh*, also known as *mis* (Müllerian inhibiting substance) is a gene that codes for anti-Müllerian hormone, a glycoprotein in the transforming growth factor β (TGF-β) superfamily (Yoshinaga et al. 2004). In mammals it is up-regulated in developing testes and down-regulated in developing ovaries (Cate et al. 1986; Swain and Lovell-Badge 1999). In lower vertebrates, *amh* is involved in the downregulation of *foxl2* and *cyp19a1a* (Western et al. 1999; Rodríguez-
Amh exhibits sexually dimorphic expression in various fish species, and has been shown to play a role in testis differentiation and development (Yoshinaga et al. 2004; Rodríguez-Marí et al. 2005; Ijiri et al. 2008; Siegfried 2010). In sablefish it is a more robust male sex marker than dmrt1, and in the Patagonian perjerrey (Odontesthes hatcheri), there is evidence that amhy, a homolog of amh, is the master sex determining gene (Hattori et al. 2012; Smith et al. 2013). However, few specifics are known about how amh regulates male development, and additional research is needed.

To identify the sex determining window in summer flounder, and better understand the mechanisms involved in sex determination, it is necessary to look at the expressional patterns of these additional sex determining genes. Foxl2, dmrt1 and amh have previously been studied in other teleosts, including Japanese flounder, and understanding their expression in summer flounder will provide a clearer picture of the sex determination process in this species. The objective of this preliminary work was to investigate the timing and magnitude of foxl2, dmrt1 and amh expression in summer flounder reared at three different experimental temperatures. This was achieved by identifying partial summer flounder sequences for these three genes, and assessing their expression patterns in real time quantitative PCR, using summer flounder juveniles raised at 13, 16, and 19°C (Chapter 2).

**Materials and methods**

**Broodstock culture and juvenile rearing**

Summer flounder broodstock were captured from Rhode Island coastal waters and held at GreatBay Aquaculture LLC (GBA; Portsmouth, NH) for at least two years prior to the start of the experiment. Fish were maintained in recirculating aquaculture systems until spawning (~5 years, 1.3 kg) as described in Chapter 2. To obtain ovarian and testis tissue for partial cDNA
identification, one adult female and one adult male broodstock were euthanized via immersion in MS-222, and ovaries and testis were immediately dissected, preserved in RNA Later® Solution, and stored at -70°C for later RNA extractions.

Summer flounder broodstock were spawned at GBA, and larvae were raised to the beginning of metamorphosis (Chapter 2). Juveniles (37 DPH) in various stages of metamorphosis (Figure 6) were transferred to the Aquaculture Research Center (ARC) at the University of New Hampshire (UNH; Durham, NH), where they were maintained in three separate recirculating systems as described in Chapter 2.

![Image of summer flounder larvae](image)

**Figure 6.** Summer flounder in various stages of metamorphosis during transfer to the Aquaculture Research Center (ARC) at 37 DPH. Individuals range from pelagic, symmetrical larvae to demersal juveniles that have undergone eye migration and settled to the bottom.

**Juvenile summer flounder sampling**

To provide an initial assessment prior to exposure to different temperatures, fish at 38 DPH (n = 5 fish/system) were weighed and measured (SL). Gonadal regions (Figure 3) were excised, preserved in RNA Later® Solution, and stored at -70°C for later RNA extractions. All fish were acclimated at 19°C for one week (38-44 DPH). Fish in one system were then maintained at 19°C, while the two other systems were slowly cooled (1°C/day) to reach final
temperatures of 13 and 16°C. Fish from all three temperature treatments were sampled as described above at 52 DPH (n = 4 fish/treatment), and again at 66 DPH (n = 9 fish/treatment).

To further investigate juvenile growth and expression patterns during later development, additional fish from the 13 and 16°C treatments were sampled at 105, 133, and 171 DPH (n = 5, 8, or 9 fish/treatment), and an additional 12 fish from 13°C were sampled at 191 DPH. All fish were weighed, measured (SL until 105 DPH, SL and total length (TL) starting at 133 DPH), and gonadal regions were removed.

As outlined in Chapter 2, remaining fish were maintained at their respective temperatures until 217 DPH, at which point all treatments were slowly increased to 21°C over two weeks (<1°C/day). Remaining fish were grown to ≥150 mm TL, at which point approximately 50 fish from each treatment were euthanized with MS-222 and gonads were visually assessed, to determine sex ratios (Colburn et al. 2015). Fish that could not be sexed visually were sexed using histological techniques and gonad characteristics (Chapter 2).

Partial cDNA identification

Total RNA was extracted from adult summer flounder ovary and testis tissues and reverse transcribed into cDNA using the techniques described in Chapter 2. Gene-specific primers were designed to amplify 400-600 base pair (bp) partial sequences for foxl2, dmrt1 and amh. For foxl2 a primer set based on the partial foxl2 sequence for Paralichthys lethostigma (KF534720.1) was designed using NCBI primer-BLAST. For dmrt1 a consensus alignment of Anoplopoma fimbria (KC112917), Paralichthys olivaceus (EU490514.1), Epinephelus coioides (EF017802.1), Dicentrarchus labrax (AM993096.1), and Oreochromis niloticus (AF203489.1) partial sequences was used to design primers Geneious 4.7.6. For amh a primer set based on the
partial amh sequence for Paralichthys olivaceus (AB166791.1) was designed using NCBI primer-BLAST.

Primers and gonadal cDNA (ovary for foxl2, testis for dmrt1 and amh) were used in the Clontech Advantage 2 PCR kit and standard PCR thermocycling conditions (see Chapter 2). PCR products were electrophoresed, and bands at the expected base pair (bp) size were excised from the gel. PCR products were extracted from agarose gels, and sequenced at the UNH Hubbard Center for Genome Studies (described in Chapter 2) with the forward primers for foxl2 and dmrt1, and the reverse primer for amh. Chromatograms were quality trimmed and analyzed using: 1) Blastn against NCBI general nucleotide database and 2) Blastx against NCBI protein database (n/r). For each gene, the resulting nucleotide sequence was a 90-100% identity match with at least two closely related fish species, and the translated protein sequence was a 90-100% match with at least two closely related fish species, which indicates that the correct gene product was identified.

RNA extractions and cDNA synthesis of juvenile samples

Juvenile summer flounder gonadal regions were used in RNA extractions, using techniques described above and in Chapter 2. RNA was quantified, reverse transcribed, and juvenile summer flounder cDNA was stored at -20°C prior to relative quantification with real time quantitative PCR.

Real-Time Quantitative PCR (QPCR)

Foxl2, dmrt1 and amh primer sets for QPCR were designed in NCBI Primer-Blast to amplify 80-150 bp fragments (Table 2). Relative quantification assays were performed with 1000 nM primer concentrations for foxl2 and amh, and 800 nM primer concentrations for amh in standard cycling conditions, followed by dissociation curve analysis. Samples were performed in
duplicate, and triplicate relative standard curves were generated from dilutions of pooled cDNA from eight individuals per gene. Optimized assays consisted of 6-8 point linear standard curves with approximately 95-110% PCR efficiency. Standard QPCR negative controls were performed in duplicate for each assay and exhibited no contamination. Both gene assays displayed a single peak in dissociation curve analysis (complete QPCR techniques described in Chapter 2).

Table 2. Primer sequences and product sizes (bp) for partial cds identification and QPCR assays, with percent efficiency (% eff) and mean cycle threshold (Ct) values for QPCR assays. Mean Ct values refer to the mean 1/10 diluted standard curve point for each assay. Higher Ct values indicate lower mRNA abundance.

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**Statistical Analysis**

PCR results were analyzed using the Pfaffl method for relative quantification (Pfaffl 2001), described in Chapter 2. One-way analyses of variance (ANOVARAs) were conducted in JMP.
Pro 11 (SAS Institute Inc., Cary, NC) to assess significant differences in gene expression and fish growth (p<0.05) for foxl2, dmrt1 and amh and. Each ANOVA was followed by a Tukey's HSD means separation test to identify pairwise significant differences. All numerical data are represented as the mean ± standard error. Linear regression analyses were performed in JMP Pro 11 to identify correlations between fish size and foxl2, dmrt1 and amh expression.

Results

Partial cDNA fragments

The 401 bp fragment corresponding to foxl2 was quality trimmed from the expected fragment size (466 bp). It exhibited high nucleotide sequence identity with other teleost species including Paralichthys lethostigma (99%) and Paralichthys olivaceus (99%), while the translated protein sequence exhibited high identity match with Paralichthys olivaceus (100%) and Paralichthys lethostigma (100%). The 154 bp fragment corresponding to dmrt1 was quality trimmed from the expected fragment size (498 bp). It exhibited high nucleotide sequence identity with other teleost species including Paralichthys olivaceus (98%) and Hippoglossus hippoglossus (96%). The translated protein sequence exhibited high identity match with Paralichthys olivaceus (100%) and Hippoglossus hippoglossus (93%). The 154 bp fragment corresponding to amh was quality trimmed from the expected fragment size (532 bp). It exhibited high nucleotide sequence identity with other teleost species including Paralichthys olivaceus (99%) and Hippoglossus hippoglossus (92%). The translated protein sequence exhibited high identity match with Paralichthys olivaceus (100%) and Hippoglossus hippoglossus (92%). The foxl2, dmrt1, and amh partial cds sequences were not verified using additional sequencing reactions (>1), and were not submitted to the NCBI GenBank database (Table 3).

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Table 3. Quality trimmed gene sequences and product size (bp) for summer flounder *foxl2*, *dmrt1*, and *amh*. Individual fish from all three temperature treatments (13°C, 16°C and 19°C) were sampled throughout development for gene expression analyses.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Trimmed sequence</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>foxl2</em></td>
<td>TTCGAGAAGGGGAACTACAGGAGACGCAGCGAATGAAGCGGCCGTTCAGACCTCCGCCGACGCACTTCCAGCCGGGGAAGTCGTTATTCGGAGGGGACGGCTACGGTTACCTGTCCCCGCCCAAGTACCTGCAGTCTAGCTTCATGAACAACTCATGGTCGCTCGGCCAGCCACCCACCCCGATGTCCTACACGTCCTGTCAGATGGCCAGTGGCAACGTGAGTCCCGTGAACGTCAAGGGACTGTCACCCGCTTCCCTCTCTTATAACCCTTACTCCCGGTTGCGAGACATGGCGCTGCCCAGCATGGTGAACTCTTACAACGGTATGGGTCACCATCACCACCCCGCGCATCCCACCATGCAGCAACTGAGCCCGGCACCACGCCGAGCCGCCGCCCCGTT</td>
<td></td>
</tr>
<tr>
<td><em>dmrt1</em></td>
<td>TCGCTCTGCATCATCCTCCAGCCGCATCGGGCGCCTTAGGGGTCACTGAGGCCCGCAGCTCCGCCGACCTGATGGGATACTTACAACGGTATGGGTCACCATCACCACCCCGCGCATCCCACCATGCAGCAACTGAGCCCGGCACCACGCCGAGCCGCCGCCCCGTT</td>
<td>154</td>
</tr>
<tr>
<td><em>amh</em></td>
<td>ACAAGCCCAGCGATAGCTGGGGCATGGACTGTAGGGAGTCCAGCTGGAGCTGAGGGGACTCAGGGCGGTCCTGAGGCAAGACATCGCACAGGAAGCGCCTCAGCTCACACAGGAAGGAGATCTGTGATGAGGGGGAGGTTGATCCTGAAAC</td>
<td>154</td>
</tr>
</tbody>
</table>

*Foxl2, dmrt1* and *amh* expression during juvenile development

During early juvenile development, when *cyp19a1a* expression significantly differed by treatment (Chapter 2), *foxl2* and *amh* expression remained stable in all treatments at 38, 52 and 66 DPH, while *dmrt1* expression was significantly higher (3.5 fold) in the 13°C treatment at 66 DPH, than in the initial (48 DPH) group (p = 0.0298).

When juvenile development was assessed on a broader scale (38-191 DPH), *foxl2* expression was significantly higher in the 13°C treatment at 171 DPH than in 1) the 13°C treatment at 191 DPH, or 2) the 16°C treatment at 66 DPH, while *foxl2* expression was significantly lower in the 13°C treatment at 191 DPH than in the 16°C treatment at 171 DPH (p =
0.0008). *Dmrt1* expression was also significantly higher in the 13°C treatment at 191 DPH than in the initial (48 DPH) group, or in the 16°C treatment at 171 DPH (p=0.0035). No significant differences in *amh* expression were present among ages or treatments.

Figure 7. Relative (A) *foxl2* (B) *dmrt1* and (C) *amh* mRNA abundances (normalized to *eef1a*), during early juvenile development at initial sampling (38 DPH, 19°C), and at 52 and 66 DPH (13, 16 and 19°C). Each bar represents the mean ± standard error. Different letters indicate significant differences among treatments and ages (p < 0.05).
Figure 8. Summer flounder relative (A) foxl2, (B) dmrt1 and (C) amh mRNA abundances (normalized to eef1a) during juvenile development at 13 and 16°C (38-191 DPH). Different letters indicate significant differences among treatments and ages (p < 0.05). All unlabeled time point refer to ABC designations for pairwise comparisons.

There was no relationship between fish size and foxl2 expression at 13°C, but there was a positive correlation between fish size and foxl2 expression at 16°C (p < 0.0001). There was no relationship between fish size and dmrt1 expression at 13 or 16°C, nor was there a relationship
between fish size and *amh* expression at 16°C. There was a positive correlation between fish size and *amh* expression at 13°C (*p* = 0.0039).

![Figure 9](image)

**Figure 9.** Relationship between relative (A) *foxl2*, (B) *dmrt1* and (C) *amh* mRNA abundance (logarithmic scale) and standard length (mm) of fish raised at 13 and 16°C. Asterisks denote significant correlations (*p* < 0.05).
Discussion

Fishes vary greatly in their processes of sex determination, and cyp19a1a has been studied extensively as an important gene that influences male or female development (Kitano et al. 1999; Luckenbach et al. 2005). However, genes such as foxl2, dmrt1 and amh have also been shown to play an important role in teleost sex determination, and exhibit sexually dimorphic expression during male or female differentiation of various species (Luckenbach et al. 2005; Nakamoto et al. 2006; Ijiri et al. 2008; Herpin and Schartl 2011; Smith et al. 2013). These genes, like cyp19a1a, have been established as robust sex markers in other teleosts, including Japanese flounder, and are similarly sensitive to temperature effects during a critical period of juvenile development (Kitano et al. 1999; Luckenbach et al. 2005; Smith et al. 2013). However, results in the present study found little relationship between foxl2, dmrt1 or amh expression and summer flounder ages, sizes, or temperature treatments, which suggests that they may be less robust sex markers in summer flounder than cyp19a1a (discussed in Chapter 2).

Cyp19a1a is predominantly expressed in ovarian tissue to convert testosterone to 17β-estradiol (Kitano et al. 1999; Luckenbach et al. 2005). By contrast, foxl2, dmrt1 and amh encode for products that are present in a wider range of tissues and play more diverse roles other than sex determination during juvenile development (Ijiri et al. 2008; Smith et al. 2013; Shen and Wang 2014), which may result in divergent expressional patterns.

In several teleost species, foxl2 gene expression precedes that of cyp19a1a and activates P450 aromatase production (Nakamoto et al. 2006; Wang et al. 2007; Yamaguchi et al. 2007; Ijiri et al. 2008). While it is linked to female development and shows sexually dimorphic expression, foxl2 is considered a less robust sex marker than cyp19a1a in sablefish (Smith et al. 2013). Previous studies demonstrated that foxl2 is more broadly expressed in a greater number of
tissue types than *cyp19a1a*, which is predominately expressed in the gonad. Extra-gonadal *foxl2* expression has been observed in sablefish (*Anaplopoma fimbria*; pituitary; Smith et al. 2013), three-spotted wrasse (*Halichoeres trimaculatus*; brain, pituitary, eyes, gills and liver; Kobayashi et al. 2010) and Nile tilapia (*Oreochromis niloticus*; brain, pituitary and gills; Wang et al. 2004). Additionally, a study on rainbow trout identified two paralogs of *foxl2*, one expressed early in development that correlates with *cyp19a1a* expression (*foxl2a*), and another expressed later in development, that has no relationship with *cyp19a1a* expression (*foxl2b*; Baron et al. 2004; 2005).

In the present study there was a positive correlation between *foxl2* expression and fish size at 16°C, and no correlation at 13°C. The small size of summer flounder juveniles sampled necessitated the use of “gonadal regions” for gene expression analysis which contained non-gonadal tissue. In addition, it is unknown if multiple *foxl2* paralogs are present in summer flounder. The absence of a consistent relationship between fish size and *foxl2* expression may be due to mRNA contributions from extra-gonadal tissue, or unknown *foxl2* paralogs.

Less is known about the role of *dmrt1* and *amh* in sex differentiation of teleosts, though both have been linked to male development, and exhibit sexually dimorphic expression in some species, and no trend in others (Kobayashi et al. 2004; Guo et al. 2005; Hattori et al. 2007; Kobayashi et al. 2008; Johnsen et al. 2010; Herpin and Schartl 2011; Johnsen and Andersen 2012). In zebrafish, three isoforms of *dmrt1* have been identified (*dmrt1a, dmrt1b* and *dmrt1c*). *Dmrt1a* exhibits much higher expression in the testis than the other isoforms, and cumulatively these isoforms produce transcription factors that play diverse roles in regulating sex determination. *Dmrt1a* is expressed at a lower level in the ovaries, and may also be important in ovary differentiation (Guo et al. 2005). In the present study, there was no clear trend in *dmrt1*
gene expression levels based on age, size, or temperature treatment. The broad nature of dmrt1 functioning and the potential for isoforms may explain why an expressional trend was not present in summer flounder gonadal regions.

Amh is a more robust male sex marker than dmrt1 in sablefish (Smith et al. 2013), and may be a homolog of amhy, the master sex determining gene in the Patagonian perjerrey (Hattori et al. 2012). However, amh expression levels in the Patagonian perjerrey are not influenced by temperature, and amh shows no sexually dimorphic expression in the medaka (Klüver et al. 2007; Shen and Wang 2014). In the present study, there was no trend in amh expression by size, age or treatment, which may be explained by the variations in amh temperature sensitivity, expression patterns and role in male sex determination that are present among different species. It is also possible that expression levels for dmrt1 and amh genes were affected by the presence of extra-gonadal tissue in the gonad regions used for gene expression analyses, as suggested for foxl2.

The present study found no clear foxl2, dmrt1 or amh gene expression patterns in summer flounder, which may reflect the use of gonadal regions for expression analyses, or the presence of unidentified gene paralogs and/or isoforms. It is also possible that foxl2, dmrt1 and amh are less robust markers than cyp19a1a for sex determination in this species. However, closely related Japanese flounder exhibit clear sexually dimorphic foxl2, dmrt1 and amh expression patterns (Yoshinaga et al. 2004; Kitano et al. 2007; Herpin and Schartl 2011; Wen et al. 2014). Future studies on foxl2, dmrt1 and amh expression in summer flounder should include earlier juveniles stages, including pre-metamorphic larvae, and focus on dissected gonads rather than gonad regions. Future work should also determine if multiple gene paralogs/isoforms exist, and how their expression patterns coincide with other genes involved in sex determination. Such research
will provide a better assessment of how the timing and/or mechanism of sex determination in summer flounder differs from other Paralichthyd species.

**Literature Cited**


Colburn HR, Nardi GC, Borski RJ, Berlisnky DL (2009) Induced meiotic gynogenesis and sex differentiation in summer flounder (*Paralichthys dentatus*). Aquaculture 289:175-180


Herpin A, Schartl M (2011) Dmrt1 genes at the crossroads: A widespread and central class of sexual development factors in fish. FEBS Journal 278:1010-1019


Johnsen H, Andersen O (2012) Sex dimorphic expression of five dmr genes identified in the Atlantic cod genome. The fish-specific dmrt2b diverged from dmrt2a before the fish whole-genome duplication. Gene 505:221-232


CHAPTER 4

CONCLUSIONS

The present study provides progress towards understanding the sex determining process in summer flounder, by focusing on temperature effects and gene expression patterns during juvenile development. Low female production was achieved overall, regardless of temperature, but cyp19a1a expression results suggest that the window of temperature sensitivity for sex determination in summer flounder may occur earlier than it does in congeneric Japanese and southern flounder. No consistent trend in foxl2, dmrt1 and amh gene expression occurred based on fish size, fish age, or temperature treatment.

Further research should take a more fine-scale approach to gene expression work, by focusing on summer flounder very early in development. Future studies should expose pre-metamorphic, larval summer flounder to differential temperature treatments, and sample fish starting in this pelagic stage before metamorphosis has been initiated. Frequent sampling for gene expression analyses will ensure that the window of temperature sensitivity during sex determination can be pinpointed. In many fish species foxl2 expression precedes that of cyp19a1a, which further necessitates early developmental summer flounder sampling to establish the relationship between these genes. Future work should also focus on dissected gonads rather than gonadal regions, to eliminate the mRNA contributions of non-gonadal tissue that likely affected apparent gene expression levels in the present study. It is possible that foxl2, dmrt1 and amh are less robust sex markers in summer flounder than cyp19a1a, but additional research is needed to more clearly identify processes related to sex differentiation in this species.
If summer flounder do possess a much earlier window of temperature sensitivity during sex determination than congenerics, then this is an important development for the aquaculture potential of this species. Thus far all previous studies have achieved low female production regardless of temperature (Colburn et al. 2009; 2015). It is possible that culturing at even lower temperatures could further increase female production, but from a practical aquaculture standpoint the additional growth gained from higher female production at lower temperatures would be offset by slow growth, a prolonged, labor-intensive pelagic phase, and delayed weaning to commercial diets (Gavlik and Specker 2004; Katersky et al. 2008). However, if summer flounder exhibit very early sex determination, and a brief window of temperature sensitivity during this period, early exposure to low temperatures could increase female production. Once fish develop past the sex determination window, temperatures could be increased to promote faster growth, which would allow commercial aquaculture productions to profit from the growth gains associated with female dominated populations.
APPENDIX A. ANIMAL CARE AND USE APPROVAL DOCUMENTATION

University of New Hampshire
Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

23-Feb-2011

Berlinsky, David L
Biological Sciences, Rudman Hall
Durham, NH 03824

IACUC #: 090302
Project: The Use of Technology to Improve Flounder Growth
Category: B
Next Review Date: 25-Mar-2012

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a report with regard to the involvement of animals in this study before that date. If your study is still active, you may apply for extension of IACUC approval through this office.

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

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

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

For the IACUC,

Dean Elder, D.V.M.
Vice Chair

cc: File