ABSTRACT

HONEYCUTT, JAMIE LYNN. (née MANKIEWICZ) Environmental and Endocrine Regulators of Stress Effects in Teleost Fishes (Under the direction of Dr. Russell J. Borski).

The adaptive stress response is mediated by the endocrine stress axis whereby catecholamines and glucocorticoids promote production of energy substrates essential for re-establishing homeostasis. Here we utilize two teleost fish models to examine two overarching research goals: 1) the role catecholamines, cortisol, and glucose might play in regulating leptin (LepA), an important hormone controlling energy homeostasis in the Mozambique tilapia (*Oreochromis mossambicus*), and 2) the impact of environmental stressors, namely temperature, on nursery habitat sex ratios and sex determination in southern flounder (*Paralichthys lethostigma*), a species that exhibits both genetic and environmental sex determination.

Leptin is a cytokine critical for regulating energy expenditure in vertebrates, yet it is unclear how the hormone interacts with the endocrine stress axis, particularly in fishes. We evaluated the actions of epinephrine, cortisol, and glucose in regulating leptin in the liver, the primary site of hormone production in the tilapia. Both cortisol and epinephrine stimulate LepA secretion from hepatocytes. While epinephrine had no effect on mRNA, cortisol suppresses *lepa* mRNA levels. Epinephrine stimulates both leptin synthesis and secretion *in vivo*. Leptin is directly sensitive to glucose as hepatic synthesis and secretion declines as ambient glucose levels rise. By contrast, glucose injection increases *lepa* mRNA levels by 14-fold, indicating that there are likely other systemic factors regulated by glucose that may enhance *lepa* mRNA. These data show that tilapia LepA is negatively regulated by rises in extracellular glucose at the level of the hepatocyte but stimulated by hyperglycemia *in vivo*. The increase in LepA in response to cortisol

and epinephrine suggests that classic stress hormones may augment leptin to help promote glucose mobilization as part of the integrated, adaptive stress response.

Southern flounder is a valuable commercial and recreational species found in the southeast United States. The fisheries are dependent on females due to sexually dimorphic growth, with females growing larger and faster relative to males. Southern flounder like other Paralichthids exhibit environmental sex determination (ESD), where factors in the environment such as temperature and background color, can influence phenotypic sex and masculinize populations. Impacts of the environment on sex occurs during early juvenile development and sex reversal is limited to the female genotype. To evaluate the effects of the environment and ESD on wild juvenile southern flounder populations, we examined spatial and temporal variation in sex ratios across a range of nursery habitats in North Carolina. Data show that northern habitats averaged optimal temperatures near 23°C during the sex determining window and produced the greatest abundance of females, while southern habitats consistently produced male skewed sex ratios (up to 94% male) and were associated with warmer temperatures. Other water quality parameters such as salinity and dissolved oxygen did not appear to have a correlation with male skewed sex ratios, suggesting that temperature is likely the key factor influencing sex ratio bias across habitats.

Temperature profiles recorded in different nursery areas experienced similar patterns of temperature fluctuation over time yet showed relatively consistent differences in temperature that averaged a maximum difference near 4°C among sites. In one northern nursery habitat that produced 1:1 sex ratios, the average temperature over the sex determination period was 23.2°C, consistent with the permissive temperature that yields 1:1 sex ratios in the laboratory. We

mimicked this temperature profile ± 4°C and show that the patterns of temperature variation observed in NC nursery habitats produce similar sex ratios under controlled laboratory conditions. These studies suggest that warmer water temperatures found in some nursery habitats produce male-biased sex ratios, which could impact the management of this important fishery that largely depends on females.

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Environmental and Endocrine Regulators of Stress Effects in Teleost Fishes

by
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DEDICATION

To my husband and my mother, for all your encouragement and love.

And to my late father, for sharing with me your love of the ocean.

BIOGRAPHY

Jamie Lynn Honeycutt (formerly Jamie Lynn Mankiewicz) was born in Annapolis, MD on September 16, 1980 to Frances Louise and Robert John Mankiewicz. She spent much of her childhood playing around the waters of the Chesapeake Bay with her two older brothers, Ryan and Keith. After graduating from Severna Park High School in 1998 she entered a two-year program at Anne Arundel Community College, where after a few biology courses and a scuba certification, she realized her passion for marine science. From there she made the move down to NC to get her Bachelor of Science degree from the University of North Carolina at Wilmington and graduated in 2005 with a B.S. in Marine Biology and a minor in Chemistry. It was here at UNCW where she met a wonderful and eccentric man who shared similar interests in biology. This man, Sam Honeycutt, would turn out to be the love of her life and they would marry just a short 13 years later. As an undergraduate, she conducted research in a marine ornamental aquaculture lab where she studied clownfish breeding techniques under the direction of Dr. Ileana Clavijo. It was this research which sparked an interest in aquaculture and following graduation worked briefly at a salmon hatchery in Esther Island, AK. She returned to NC and found work as a researcher in a molecular genetics lab at Duke University with Dr. Michel Bagnat on zebrafish development, and it was here where she reconnected with academia. In the fall of 2011, she enrolled as a master's student at North Carolina State University under the direction of Dr. Russell Borski. The following year she transitioned into a Ph.D. program in Biology with a minor in Biotechnology. Her studies focused on fish development and physiology, conducting research both in the laboratory and in the field.

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CHAPTER I

Review of leptins interactions with the endocrine stress axis and role of cortisol and background color on sex determination in southern flounder [1,2]

Research in this chapter has been published in the following journals:

[1]Deck, C.A., Honeycutt, J.L., Cheung, E., Reynolds, H.M., and Borski, R.J. 2017. Assessing the Functional Role of Leptin in Energy Homeostasis and the Stress Response in Vertebrates. *Front Endocrinol* 8, 63.

[2]Mankiewicz, J.L., Godwin, J., Holler, B.L., Turner, P.M., Murashige, R., Shamey, R., Daniels, H.V., and Borski, R.J. 2013. Masculinizing Effect of Background Color and Cortisol in a Flatfish Environmental Sex-Determination. *Integr Comp Biol* 53(4): 755-65.

Abstract

Stress is implicated in virtually all facets of vertebrate physiology. The key hormones of the endocrine stress response, namely catecholamines and glucocorticoids (cortisol or corticosterone) are critical in maintaining homeostasis during periods of stress. Leptin is a pleiotropic hormone that is important in regulating appetite, energy metabolism, growth, and immune function across vertebrate groups. In mammals, it has been classically described as an adipostat, relaying information regarding energy status to the brain. While retaining poor sequence conservation with mammalian leptin, teleostean leptin exhibits a number of similar regulatory properties, although current evidence suggests that it does not function as an adipostat in this group of vertebrates. Teleostean leptin also exhibits functionally divergent properties, however, possibly playing a role in glucoregulation, as we have found with the Mozambique tilapia (*Oreochromis mossambicus*). Further, leptin has been recently implicated as a mediator of immune function and the endocrine stress response in teleosts. The first portion of this chapter provides an overview of the endocrine stress response and reviews what we know to date about the interactions with leptin and key hormones of the endocrine-stress axis in vertebrates.

The second part of this chapter discusses environmental sex determination (ESD), a phenomenon likely mediated by glucocorticoids. Environmental sex determination occurs when environmental factors regulate phenotypic sex, typically occurring during a critical period of early development. Southern flounder (*Paralichthys lethostigma*) have been shown to exhibit temperature dependent sex determination that appears to be restricted to the presumed XX female genotype with temperatures, both high (28°C) and low (18°C), skewing sex ratios toward males. Background color and exogenous cortisol were evaluated to observe any influence these factors may have on

sex determination. Blue tanks showed significant male-biased sex ratios (95 and 75% male) compared with black and gray tanks. Additionally, whole body levels of cortisol, the primary stress hormone in teleosts, were higher in those fish reared in blue tanks during the period of sex determination. These results suggest that background color represents an environmental variable that regulates sex determination in flounder, with blue background color serving as a potential stressor that produces male-skewed sex ratios in culture conditions. To examine the effects of cortisol on sex determination in southern flounder, an all XX population of fish were fed cortisol gelatin coated feed at 0, 100, or 300 mg/kg cortisol, intermittently prior to, and just through, the period of sex determination. Control fish showed female-biased sex ratios approaching 100%, whereas treatment with 100 mg/kg and 300 mg/kg cortisol produced 28.6% and 13.3% females, respectively. These data suggest that cortisol is a critical mediator of sex determination in southern flounder by promoting masculinization. This linkage between the endocrine stress axis and conserved sex determination pathways may provide a mechanism for adaptive modification of sex ratio in a spatially and temporally variable environment.

Leptin Function in Teleost Fishes

Leptin is a 16 kDa cytokine hormone encoded by the *obese* (*ob*) gene that is involved in regulating numerous physiological processes, including cell differentiation and growth, metabolism, immunity, angiogenesis, and reproduction (Zhang et al. 1994, Ahima and Flier 2000, Londraville et al. 2014). Its best characterized and perhaps most dominant function is in the control of energy homeostasis. Despite extensive research into its role in regulating energy homeostasis in mammals, particularly with regard to obesity, an understanding of how leptin regulates metabolism in non-mammalian vertebrates is lacking (Ahima and Flier 2000, Copeland et al. 2011). In mammals, leptin acts as an adipostat, where it circulates in proportion to fat stores, and exerts potent anorexigenic and lipolytic actions to prevent excessive lipid accumulation (Siegrist-Kaiser et al. 1997). In ectothermic vertebrates such as fishes, where the caloric inputs, basal energy requirements, and metabolic regulation can differ substantially from that of homeotherms (5-8 fold) of equivalent body size (Else and Hulbert 1981, Silva 2006), there is growing evidence that leptin may play a greater role as a glucoregulatory hormone than as an adipostat.

Administration of recombinant leptin A (LepA), the dominant paralog in fishes, increases plasma glucose and concomitantly reduces liver glycogen in tilapia (*Oreochromis mossambicus*; Baltzegar et al. 2014) *in vivo*, suggesting LepA exerts both hyperglycemic and glycogenolytic effects. Similar responses to leptin were observed in rainbow trout (*Oncorhynchus mykiss*; Aguilar et al. 2010) and lizards (*Podarcis sicula*; Paolucci et al. 2006). In tilapia, homologous LepA suppresses hepatic gene expression of lipoprotein lipase and hormone-sensitive lipase, a response that is inconsistent with a function of LepA in lipolysis (Baltzegar et al. 2014). In grass

carp (*Ctenopharyngodon idellus*), however, homologous hormone induces glycerol liberation in fatty degenerated (i.e. triglyceride enhanced) hepatocytes, raising the possibility that leptin may be lipolytic (Lu et al. 2012). It is unclear whether the response is observed in intact animals or in normal hepatocytes. Collectively, it appears leptin serves to enhance energy expenditure in vertebrates, at least in part through stimulation of either lipid or carbohydrate catabolism. Interestingly, circulating levels and/or hepatic gene expression of the hormone rise with fasting when lipid stores decline in tilapia and most other teleosts (Kling et al. 2009, Douros et al. 2017, see Deck et al. 2017 for review), suggesting the hormone is not an adipostat and may have more limited function in controlling lipid metabolism in teleosts. This raises the possibility that the adipo-regulatory function of leptin in mammals may reflect a more derived state that evolved with homeothermy and the prominence of adipose tissue in organisms that must maintain a consistent internal body temperature regardless of external influences. The hormone appears to function more as a carbohydrate catabolic hormone in teleosts, and perhaps other ectotherms, mobilizing glucose during periods of energy expenditure or stress. Consistent with this, the hormone is upregulated during periods of catabolic stress such as hypoxia, hyperosmotic (seawater) challenge, and fasting (Chu et al. 2010, Baltzegar et al. 2014, Douros et al. 2017, Deck et al. 2017). Little is known about the mechanisms governing stress-induced leptin production and regulation in fishes and other ectotherms.

Endocrine Stress Response

It is apparent that leptin is a catabolic hormone in vertebrates that enhances energy mobilization and suppresses appetite, two processes often linked to stress responses. Stress impacts virtually all aspects of vertebrate physiology including immunity, reproduction, hydromineral balance,

and energy homeostasis (Chrousos and Gold 1992, Bonga 1997, Barton 2002). The adrenergic (humoral and neuronal) and hypothalamic–pituitary–adrenal [interrenal in fish; HPA/hypothalamic-pituitary interrenal (HPI)] axes are central components of the vertebrate stress response and ultimately aid in restoration of homeostasis when disrupted. The two axes release catecholamines (epinephrine/norepinephrine) and glucocorticoids (cortisol/corticosterone), respectively, to allow for the mobilization of energy stores (Bonga 1997, Reid et al. 1998, Gorissen and Flik 2016).

Upon the perception of a stressor, sympathetic nerve fibers release acetylcholine onto chromaffin cells within the adrenal medulla (mammals) or interrenal tissue (teleosts) to stimulate the secretion of catecholamines and allow for the rapid mobilization of energy stores from peripheral tissues (Nilsson et al 1976, Mazeaud et al 1977, Axelrod and Reisine 1984, Reid et al 1998). Simultaneously, the hypothalamus releases corticotropin-releasing factor (CRF), which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH then triggers the production and release of glucocorticoids from the adrenal cortex (mammals) or interrenal cells of the head kidney (teleosts) (Axelrod and Reisine 1984, Bonga 1997, Barton 2002). These glucocorticoids then elicit a myriad of metabolic effects such as inducing lipid and protein catabolism and stimulating gluconeogenesis to increase plasma glucose levels (Bonga 1997, Mommsen et al. 1999). In a classic negative feedback pathway, the increase in circulating cortisol then inhibits further release of CRF and ACTH, attenuating the stress response.

Catecholamines and Leptin

Epinephrine is thought to be the primary hormone of the humoral adrenergic system in most fishes (Reid et al. 1998, Barton 2002). As part of the "fight or flight" response, catecholamines exert numerous actions that include rapid mobilization of glucose and free fatty acids through enhanced glycogenolysis and lipolysis, respectively, as well as regulation of respiration and blood flow (Bonga 1997, Reid et al. 1998, Fabbri et al. 1998). Leptin is also critical for regulating energy expenditure in vertebrates and responds to various stressors, yet little is known about how the hormone interacts with components of the endocrine stress axis, particularly in non-mammalian vertebrates (Copeland et al. 2011, Figure 1). To date, the majority of studies examining the relationship between leptin and the endocrine stress axis come from work in mammals (Ahima and Flier 2000, Copeland et al. 2011). Leptin stimulates the release of catecholamines in both porcine (Takekoshi et al. 1999) and bovine (Utsunomiya et al. 2001) adrenal medullary cells. In addition, leptin increases mRNA levels of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine production (Takekoshi et al. 1999). This suggests a synergistic relationship between leptin and catecholamines wherein leptin mobilizes energy from lipids while simultaneously stimulating the release of catecholamines to mobilize glucose during periods of stress (Trayhurn et al. 1998). Interestingly, other studies utilizing human chromaffin cells have shown no significant change in catecholamine release with leptin treatment (Glasow et al. 1998). The contradictory responses observed between human and other mammalian models could possibly be due to differences in methodology (isolated cells vs. whole adrenal tissue), the leptin concentrations used, or simply species differences (Takekoshi et al. 1999). The regulation of catecholamines by leptin in fishes has not been well characterized. In goldfish (*Carassius auratus*), chronic leptin treatment (injected for 10 days) had little effect on hypothalamic

catecholamines (de Pedro et al. 2006). Leptin regulation of the humoral adrenergic system or of circulating catecholamines has yet to be examined.

While leptin exerts a stimulatory effect on catecholamine release in mammals, epinephrine has been shown to directly inhibit leptin secretion (Kosaki et al. 1996, Mantzoros et al. 1996, Fritsche et al. 1998, Carulli et al. 1999). In addition, increases in intracellular cAMP in medullary cells, one of the second messengers involved in adrenergic signaling, downregulate leptin mRNA (Slieker et al. 1996). Thus, epinephrine that increases in response to leptin exposure could act in a negative feedback loop to inhibit further leptin release. One theory behind this inhibition is that it is not advantageous for catecholamines to stimulate leptin during acute stress as obtaining energy from lipolysis is too slow for a "fight or flight" response, however, it may play a role in mediating the response to chronic stress (Copeland et al. 2011). The regulation of leptin by catecholamines in fishes and other ectotherms is still unclear. However, both leptin and epinephrine exhibit glycogenolytic and/or lipolytic actions and have been shown to increase during times of stress in fishes (Bonga 1997, Baltzegar et al. 2014, Deck et al. 2017).

Glucocorticoids and Leptin

The human *ob* promoter region possesses glucocorticoid response elements, suggesting that cortisol may elicit some of its actions through regulation of leptin transcription (Gong et al. 1996, Leal-Cerro et al. 2001). Indeed, glucocorticoids elicited a stimulatory effect on leptin synthesis and secretion in rats (de Vos et al. 1995), humans (Newcomer et al. 1998), and cultured human adipocytes (Wabitsch et al. 1996, Figure 1). In addition, the synthetic glucocorticoid

dexamethasone increased mRNA levels and stimulated leptin secretion from rat adipocytes (Murakami et al. 1995, Slieker et al. 1996). Initial evidence indicates cortisol may also increase hepatic leptin mRNA levels in teleosts *in vivo* and *in vitro*, at least based on effects observed in rainbow trout (*O. mykiss*; Madison et al. 2015). Whether a similar response occurs in other, more derived teleosts (e.g., Perciformes) or with leptin secretion generally remains unknown. It has been speculated that since cortisol release is slower than that of catecholamines, the prolonged stressors that elicit cortisol actions would also benefit from the catabolic effects of leptin on lipids in mammals and/or carbohydrates reported in fishes, particularly in the liver where leptin is produced and may act locally (Copeland et al. 2011, Lu et al. 2012, Baltzegar et al. 2014, Douros et al. 2014, Song et al. 2015, Wu et al. 2017).

Leptin, in turn, has an overall inhibitory effect on the HPA axis in mammals (Leal-Cerro et al. 2001), inhibiting CRF release from the hypothalamus in mice (Heiman et al. 1997) and suppressing cortisol secretion from adrenal cells (Bornstein et al. 1997, Pralong et al. 1998, Roubos et al. 2012, Figure 1). In contrast, leptin has no effect on ACTH secretion from the pituitary, suggesting that it regulates glucocorticoid release indirectly *via* the hypothalamus and directly by acting on the adrenal gland (Heiman et al. 1997). When human adrenocortical cells are incubated with leptin, a dose-dependent decrease in ACTH-stimulated cortisol secretion is observed (Glasow et al. 1998), while in leptin knockout mice (*ob/ob*), circulating levels of glucocorticoids are 85% higher than basal. Injecting these knockouts with leptin, however, reduced the level of glucocorticoids by 40% (Ahima et al. 1998, Davis et al. 2015).

Initial investigations suggest a similar downregulation of the HPI axis and glucocorticoids may occur in teleosts. In the common carp (*Cyprinus carpio*), leptin inhibited ACTH-stimulated cortisol secretion *in vivo* and caused a dose-dependent decrease in CRF-induced ACTH secretion from the pituitary *in vitro* (Gorissen et al. 2012, Gorissen and Flik, 2014). No changes in circulating cortisol were observed in leptin-injected goldfish (*C. auratus*; Vivas et al. 2011), however, it is possible that leptin only inhibits glucocorticoid production when the HPI axis has been activated and circulating cortisol levels are elevated. In general, we do know that teleost pituitary glands are responsive to leptin (Tipsmark et al. 2008, Gorissen and Flik, 2014, Douros et al. 2014; 2017), and as such, it has been postulated that leptin may regulate the stress axis at the level of the pituitary in teleosts (Bernier et al. 2012, Gorissen and Flik, 2014). Further studies are warranted to assess interactions between leptin and glucocorticoids in regulating the overall stress response in teleosts.

Conclusions – Leptin and Stress

It is apparent that leptin is a catabolic hormone in vertebrates that enhances energy mobilization and suppresses appetite. In teleost fishes and ectotherms generally, there is much that remains to be described about the role of leptin in energy homeostasis. The hormone appears to act as a glucoregulatory factor in teleosts, with stimulation of hyperglycemia in tilapia and salmonids (Aguilar et al. 2010, Baltzegar et al. 2014). Whether leptin responds to alterations in glucose in teleosts is unclear, although increases in *lepa* mRNA levels with rises in glucose have been observed in trout hepatocytes (Lu et al. 2015). Leptin may exert lipolytic effects as well (Lu et al. 2012, Song et al. 2015, Deck et al. 2017, Wu et al. 2017), despite there being no evidence to suggest it acts as an adipostat in fishes. In mammals, leptin has been implicated in regulating the

metabolism of both glucose and lipids, suggesting some conservation of function between the two groups, perhaps sharing roles in promoting glucose uptake and glycolysis (Douros 2015, Deck et al. 2017).

To date, the studies investigating the involvement of leptin in regulating the adaptive stress response suggest that this role may be conserved within vertebrates. However, it is currently unclear by what means metabolic energy stores might be preferentially mobilized by leptin upon exposure to acute and chronic stressors, such as osmotic stress or hypoxia. Further, it remains to be determined how multiple endocrine signals (e.g., catecholamines, glucocorticoids) might integrate with leptin signaling to achieve the appropriate physiological response under such conditions. Investigating the interactions between leptin and the key hormones of the stress axis in tilapia are integral components of this dissertation. Studies in teleosts, or other ectotherms, may shed light on potential new functions of leptin that are conserved in the vertebrate lineage.

Considering the hormone responds to various stressors and is likely to induce the mobilization of energy stores similar to that observed for catecholamines and glucocorticoids, the goals of Chapter 2 in this dissertation are to:

Objective 1. Evaluate whether the production or secretion of leptin might be sensitive to glucocorticoids and catecholamines in tilapia (*O. mossambicus*), key hormones known to mediate the vertebrate endocrine stress response; and

Objective 2. Determine if glucose might directly modulate leptin production and secretion from the liver, the main source of circulating hormone in the tilapia both *in vitro* and *in vivo*. Despite its potent hyperglycemic action, little is known on how leptin responds to glucose in fishes or ectotherms generally.

Environmental Sex Determination in Southern Flounder

Gonadal sex is a central component of an organism's mating strategy but is not always thought of as an adaptively plastic component of reproduction. Even in the familiar and well-studied mammals and birds, adaptive adjustment of the sex ratio is prevalent (Capel 2017). When key aspects of the environment an offspring will inhabit cannot be predicted by cues available to parents, plasticity in sex determination in those offspring can be selectively advantageous (Charnov and Bull 1977). This appears to be the case for many teleost fishes, particularly in the marine environment where the habitats ultimately occupied by juveniles and adults often are determined after long range dispersal by a planktonic larval stage. Gonadal development may also be considerably delayed following settlement of larvae into their newly found habitat. If there is variation across habitats in terms of relative reproductive success expected for males and females developing in those habitats and cues from the physical or social environments predict these differences, then the delay between larval settlement and gonadal differentiation may be viewed as a ‘‘window of opportunity’’ for selectively advantageous sex determination. These are the general conditions postulated by Charnov and Bull (1977) to select for environmental sex determination (ESD).

Teleost fishes display a wide range of sexual patterns including ESD, genotypic sex determination, and gradations between these patterns in response to variation both in the physical and social environments (Ospina-Álvarez and Piferrer 2008). We have been focusing on ESD in the southern flounder (*Paralichthys lethostigma*), an ecologically and economically important species in the southeastern USA. Southern flounder exhibit sexually dimorphic growth, with females growing faster and larger relative to males. Like their Japanese congener *Paralichthys olivaceus* and a number of other flatfish species, southern flounder exhibit temperature dependent sex determination (Luckenbach et al. 2009). In Japanese and southern flounder, this is restricted to the presumed XX female genotype with the extremes of temperature favoring development of males (Yamamoto 1999; Luckenbach et al. 2003). For southern flounder, high (28°C) and low (18°C) temperatures produce a preponderance of males, while a mid-range temperature (23°C) favors 1:1 female: male sex ratio (Luckenbach et al. 2003). The window of sex determination in which the gonad is sensitive to temperature is thought to be around 30 to 65 mm total length (TL) or even earlier (Montalvo et al. 2012) with gonadal differentiation occurring between 75 and 120 mm TL in southern flounder (Luckenbach et al. 2005). The precise mechanisms that transduce thermal cues into determination of sex are not known for any species, but accumulating evidence suggests that the endocrine stress axis, i.e. glucocorticoids, plays a critical role in this process (Hattori et al. 2009, Yamaguchi et al. 2010, Fernandino et al. 2012). From this perspective, any environmental cue that may reflect a stressor such as temperature, background color, dissolved oxygen, and pH, could affect sex determination and hence sex ratios in populations.

Flounders are benthic predators that lay on the substrate and rely on camouflage for ambushing prey. Considering their cryptic nature, these fishes may be particularly sensitive to background coloration, which has been previously shown to regulate the glucocorticoid stress axis in fishes (Papoutsoglou et al. 2000, Rotllant et al. 2003, Merighe et al. 2004, Barcellos et al. 2009). We postulate that background color may represent an additional environmental cue that influences sex determination in fishes, possibly mediated by glucocorticoids. For improving the culture of flounder, it is important to identify those background (tank) colors that might regulate sex and that are often used in commercial operations to maximize the production of faster growing females.

Effects of Tank color on Sex Determination in Southern Flounder

To examine the effects of background color on sex determination, southern flounder were reared in triplicate in either black, gray, or blue tanks. Fish were grown out to 120 mm TL to perform gonadal histology and establish sex ratios from each background color. When early rearing takes place at temperatures of 23°C, post-metamorphic flounder fingerlings should develop as 50% males and 50% females, whereas early rearing at 18°C should produce sex ratios biased toward males (Luckenbach et al. 2003). In an initial experiment evaluating effects of varying background colors at 23°C water temperature, both gray and black tanks produced flounder with sex ratios of 50:50, whereas blue tanks produced strongly male-biased (95% male) sex ratios (Figure 2A). In a subsequent experiment on background color, fish were raised at an average temperature of 18.8°C for the first 50 days and thereafter raised at 23.0°C during the time when gonads were already differentiating. As predicted, all three background colors yielded a proportion of males greater than 50%. Interestingly, the blue tanks yielded sex ratios that were

significantly more male-biased (74%) than did the gray or black tanks (Figure 2B). These two experiments with different cohorts of offspring provide strong evidence that background color influences sex determination in southern flounder. It is not clear what aspect of color, overall lightness (L*), or perhaps the color itself (a*, b*; explained below), is responsible for this effect.

To evaluate the different aspects of background color, an additional study was conducted (data unpublished) with additional tank colors to those tested previously (blue, black, and gray) to better determine what component (L, a, or b) of background color (lightness or hue) might regulate sex determination. We tested blue (L = 76, a = -16, and b = -3) as a positive control for sex reversal, dark gray (L = 42, a = -0.9, and b = -2.9) as a negative control for no sex reversal, light gray (L = 78, a = -0.8, and b = -5.5) as a test for lightness L, and yellow (L = 78, a = 7.6, and b = 39) as a test for lightness and hue. For this experiment, an all XX population was used and produced from temperature sex reversed, meiogynogenic male broodstock (XX males) and standard female broodstock. Larvae were raised through metamorphosis by a local commercial producer (Carolina Flounder) prior to acquisition of juveniles for the trial. The trial was performed in triplicate at a stocking density of 500 fish/m^2 with salinity gradually reduced from 15 to 4.0 ppt. Sex of individuals was determined by gene expression biomarkers of gonadal differentiation once flounder reached an average TL of 90 mm. Müllerian inhibiting substance (*mis*) was used as a marker for male development, and P450 aromatase (*cyp19a1a*) and Forkhead transcription factor L2 (*foxl2*) were used as markers for female gonad differentiation. The data show the proportion of females was 35% in dark gray, 29% in light gray, 25% in yellow and 22% in blue tanks (Figure 3). Although, the blue tanks had the highest level of masculinization and dark gray tanks produced the most females (blue vs. dark gray tanks Chi-square; $P < 0.002$),

all color treatments produced sex ratios significantly different than that of the expected sex ratio of 100% female for these all XX fish (Chi-square; $P < 0.0001$). The underlying cause for the lack of production of a high percentage of females in all XX populations of flounder even in those tank colors (dark gray) that maximize generation females is uncertain. However, it is possible that additional environmental cues, as yet to be defined, may have skewed sex ratios. It is also possible that female broodstock may show individual variation with susceptibility to ESD. Any future work should use multiple female broodstock to reduce the possibility of producing progeny derived from broodstock that might have particularly high sensitivity to ESD.

Background color regulates cortisol levels in a variety of fish species (Papoutsoglou et al. 2000, Rotllant et al. 2003, Merighe et al. 2004, Barcellos et al. 2009). The masculinizing effect observed with blue background from the initial tank color study was associated with elevations in whole body cortisol levels in southern flounder (Mankiewicz et al. 2013). Cortisol levels increased relative to baseline values (0 days post stocking, dps) in southern flounder from blue tanks at 34 dps, but not at subsequent sampling periods, while no such increase was observed for gray or black tanks (Figure 4). Importantly, these fish were an average size of 59 mm TL at 34 dps, which is just before the 65 mm size when some juveniles (presumptive females) begin to display elevations in mRNA for aromatase (*cyp19a1a*), the cytochrome P450 enzyme that converts testosterone to estradiol-17B, both in the laboratory and in field populations (Luckenbach et al. 2005). It is hypothesized that cortisol can inhibit conversion of testosterone into estrogens (and therefore inhibit normal differentiation into females), possibly by suppression of aromatase (Kitano et al. 2000, Perry and Grober 2003, Yamaguchi et al. 2010). It is also known that treatment with estrogen can rescue the masculinizing effect of cortisol in medaka

(Kitano et al. 2012). These observations from other species and our finding of elevated cortisol in blue tanks suggest that this steroid may be interfering in the biosynthesis of estrogens and in female sex determination in southern flounder as well. Consistent with this hypothesis, we also show a masculinizing effect of exogenous cortisol treatment on sex determination in southern flounder based on expression of conserved molecular markers of sex determination (see below).

Molecular Biomarkers for Sexing Juvenile Southern Flounder

Previous work shows that FoxL2 promotes transcription of aromatase and is expressed primarily in the gonads of females and not in males during sexual differentiation (Pannetier et al. 2006, Alam et al. 2008, Yamaguchi et al. 2007; 2010). Likewise, aromatase is causally linked to female sex differentiation in Japanese flounder (Kitano et al. 1999; 2000) and its ovarian gene expression rises and remains elevated during sexual differentiation of southern flounder females, but not that of males (Luckenbach et al. 2005). Japanese flounder, black porgy (*Acanthopagrus schlegeli*), and pejerrey (*Odontesthes bonariensis*) show a sexually dimorphic pattern in *mis* expression, increasing during testicular development and declining with ovarian differentiation (Yoshinaga et al. 2004, Fernandino et al. 2008, Wu et al. 2010, Yamaguchi et al. 2010). We evaluated the differential patterns of expression of these genes and of aromatase to determine the sex of individuals exposed to cortisol. In most gonads that were evaluated, there was a clear dimorphism in expression of *foxl2* and *cyp19a1a* relative to *mis* mRNA levels, but some individuals had low levels of expression for all three genes, suggesting an undifferentiated state. Based on these results and on patterns in other species discussed above, we classified individuals with elevated gonadal *foxl2* and *cyp19a1a* mRNA and low expression of *mis* as females and those exhibiting the opposite pattern as males. Additionally, our previous work on southern

flounder shows that expression of gonadal aromatase mRNA, *cyp19a1a*, is a good predictor of gonadal phenotype (Luckenbach et al. 2005). Determinations of sex ratios using this marker produce results identical to those obtained by gonadal histology (Luckenbach et al. 2005). Interestingly, we found that the difference in magnitude of *foxl2* expression between the sexes is 28 times greater than that for aromatase, suggesting it to be an even more robust biomarker for predicting sex.

Effects of Cortisol on Sex Determination in Southern Flounder

To examine the effects of cortisol on sex determination in southern flounder, an all XX population of fish were fed cortisol gelatin coated feed at 0, 100, or 300 mg/kg cortisol, intermittently prior to, and just through, the period of sex determination. The data show that early and periodic application of cortisol dramatically masculinized fish in a dose-dependent fashion. Indeed, the proportion of females declined to 29% and 13% in those fish receiving 100 and 300 mg/kg cortisol, respectively, while the control cohort was 91% female (Figure 5). This effect on sex determination does not appear to be due to strongly negative effects on health as treatment with cortisol did not influence somatic or skeletal growth (data not shown) or significantly affect mortality (79–98% survivorship in cortisol treatments relative to 93% for controls). Thus, it would appear that the doses and temporal manner in which cortisol was applied likely yielded concentrations of hormone reflecting responses associated with a brief period of moderate stress, rather than one representing a chronic stress, or one associated with application of pharmacological steroid exposures that suppresses appetite and growth in fish (Barton and Iwama 1991, Bonga 1997, Lawrence 2007). Our results are in agreement with those found for Japanese flounder, in which cortisol was shown to masculinize fish during continuous treatment

with steroids over a 70-day period (Yamaguchi et al. 2010). This group also established that levels of whole-body cortisol increase in fish exposed to masculinizing temperatures, consistent with the hormone-mediating, temperature-sensitive effects on sex-determination. The data from this study and the emerging view from other studies of ESD in fishes suggest that cortisol may be the physiological mediator not only of the sex determining effects of temperature, but also of background color and perhaps other, as yet undefined, environmental variables. The mechanisms whereby cortisol might masculinize fish during the window of sex differentiation are uncertain, although evidence suggests the hormone could inhibit aromatase expression and instead activate pathways to development of males (Gardner et al. 2004). In Japanese flounder, a FoxL2 site, and response elements for estrogen receptor and cAMP lie upstream of the aromatase promoter (Yamaguchi et al. 2007) and cortisol has been shown to directly interfere with cAMP-dependent activation of aromatase transcription (Yamaguchi et al. 2010). Alternatively, as suggested by Fernandino et al. (2012), cortisol instead may directly affect masculinization of the gonad, with suppression of aromatase expression being a consequence, rather than a cause, of this masculinization. These authors found that cortisol dose-dependently stimulated 11-KT production and increased expression of a key enzyme in androgen biosynthesis, *hsd11b2*, in pejerrey testes *in vitro* (Fernandino et al. 2012). The stimulation of 11-KT production could be suppressed by treatment with RU486, an antagonist of the glucocorticoid receptor. This suggests an alternate hypothesis: that cortisol may be masculinizing the gonad, with decreases in aromatase expression being a consequence, rather than cause, of differentiation of males (Fernandino et al. 2012). Studies addressing the effects of cortisol both on *foxl2* expression and on *mis* expression could help address this possibility of direct masculinization.

Although not a focus of this work, it was not certain that southern flounder exhibit a XX/XY system of sex determination like that in the congeneric Japanese flounder (Yamamoto 1999). However, although we are not able to karyotype our male broodstock, our results are consistent with those from Japanese flounders. There was a very strong female biased sex ratio observed in the progeny of meiogynogenetically derived male southern flounder (all XX genotypic populations) in the absence of cortisol treatment (91% female, Figure 5). Overall, these observations argue strongly that our meiogynogenetically derived male broodstock have an XX genotype and that the southern flounder exhibit the XX-XY sex determining system.

Conclusions – ESD in Southern Flounder

In summary, these studies on ESD in southern flounder show that intermittent application of cortisol results in a significant male bias without affecting growth, suggesting that even short-term stress during the critical period of sex determination can cause masculinization of genetic females in this species. Additionally, we show that blue background color accompanied by increased cortisol during the window of sex determination, skewed sex ratios toward males. Background color, in addition to temperature, has now been shown to significantly affect ESD in southern flounder and our findings suggest cortisol mediates these environmental effects. There is a range of other environmental factors, yet to be examined, that potentially could impact sex determination. If cortisol is a key mediator of ESD, then other environmental variables that represent stressors with impacts on glucocorticoid signaling during sex determination could impact population sex ratios and should be investigated. Despite this research indicating environmental parameters can regulate sex determination, there is no evidence to date that suggests natural variations in temperature or other habitat variables might control sex ratios in

the wild. Evaluating sex ratios of wild North Carolina juvenile southern flounder populations is therefore a key component of this dissertation. The goals of Chapters 3 and 4 in this dissertation are to:

Objective 3. Evaluate whether sex ratios vary in young-of-the-year flounder juveniles in coastal nursery habitats in North Carolina and how the variation may be linked to temperatures or other variables experienced during the sex determination period; and

Objective 4. Test the hypothesis that patterns of temperature variation observed in productive NC nursery habitats that might produce male-biased juvenile sex ratios are sufficient (by themselves) to generate skewed sex ratios under controlled laboratory conditions.

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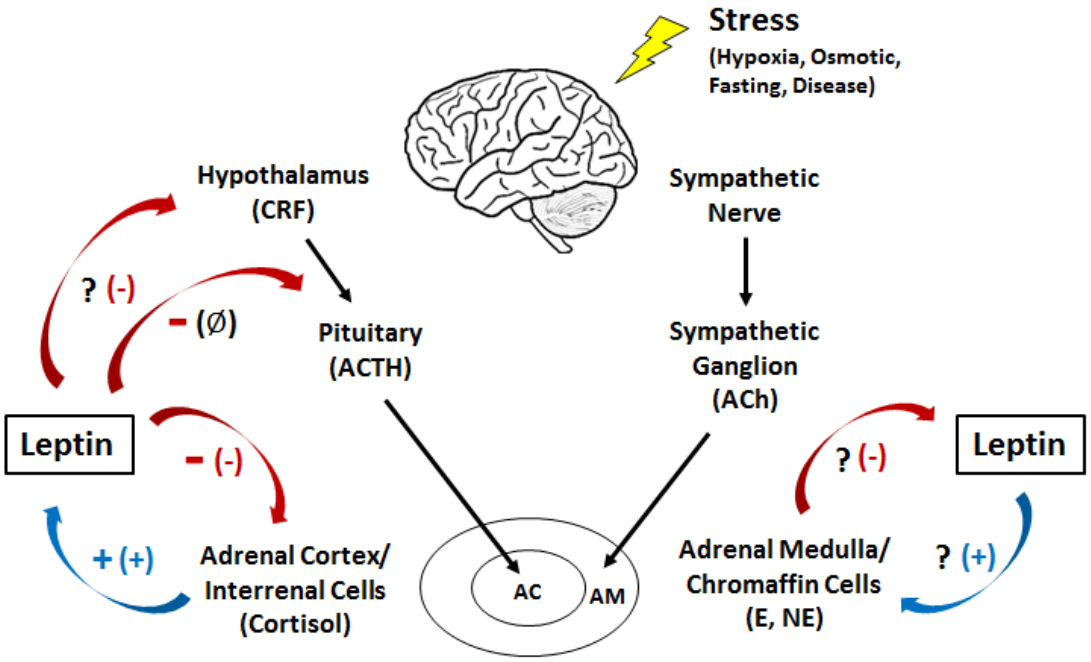

Figure 1. Interactions between leptin and the humoral adrenergic and hypothalamic–pituitary–adrenal/interrenal axes in teleosts and mammals. +, stimulation; -, inhibition; Ø, no effect; ?, an unknown relationship. The mammalian response is represented by the symbol in parentheses. Knowledge on the interactions of teleost leptin with the humoral adrenergic and glucocorticoid HPI axis is unknown or poorly understood. CRF, corticotropin-releasing factor; ACTH, adrenocorticotropic hormone; Ach, acetylcholine; E, epinephrine; NE, norepinephrine; AC, adrenal cortex; AM, adrenal medulla.

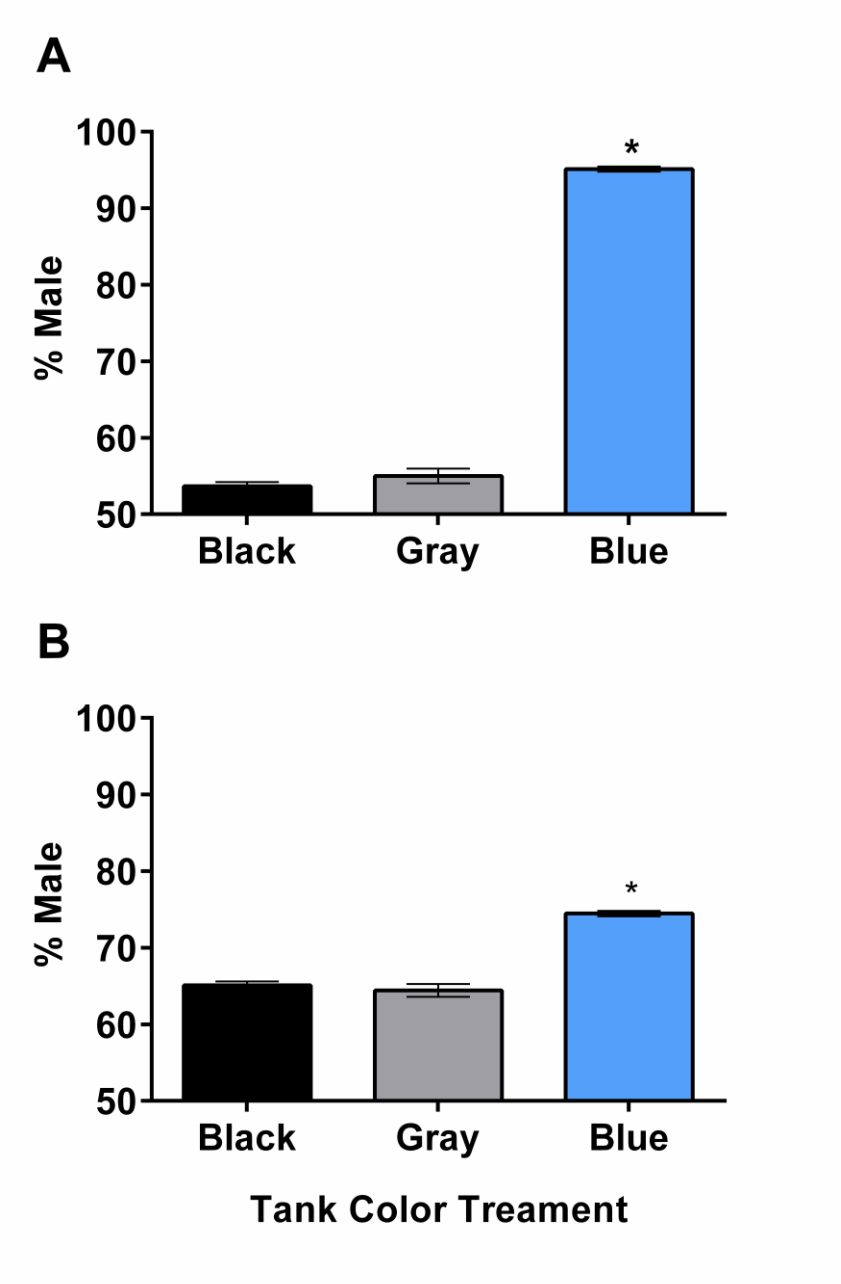

Figure 2. Effect of gray, black, and blue background colors on sex determination in southern flounder from two separate experiments. (**A**) Fish were raised at 23°C throughout the trial (n = 40–41 per treatment; *$P < 0.0001$ represent significant deviations from a 50:50 sex ratio). (**B**) Fish were raised at 19°C until 50 dps and then at 23°C thereafter (n = 45–47 per treatment group; $P < 0.001$ represent significant deviations from a 50:50 sex ratio).

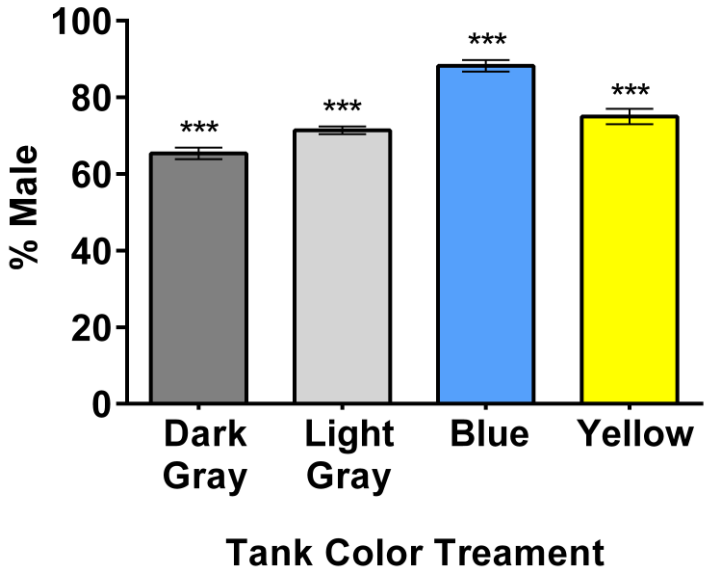

Figure 3. Effect of dark gray, light gray, blue, and yellow background colors on sex determination in southern flounder. All fish were of XX genotype and raised at 23°C throughout the trial. Dark gray tanks resulted in 65% male, light gray with 71% male, blue with 88% male, and yellow with 75% male (n = 14–26 per treatment; $P < 0.0001$ represent significant deviations from a 100% female sex ratio).

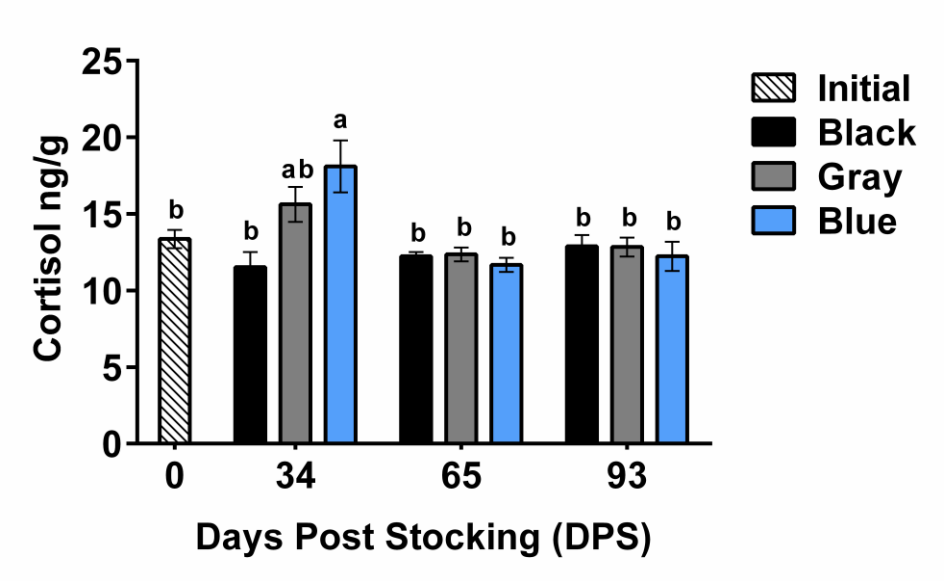

Figure 4. Effect of gray, black, and blue background colors on concentrations of whole-body cortisol in southern flounder raised at 19°C until 50 dps and then at 23°C thereafter (n = 30 per tank color per timepoint). Significant differences between treatments are indicated by different letters; $P < 0.001$.

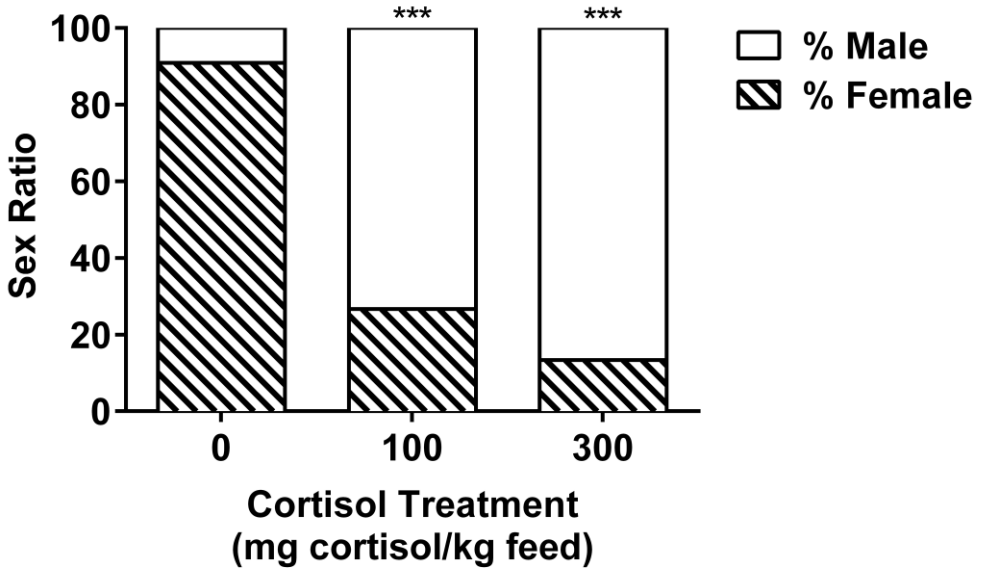

Figure 5. Sex ratios of cortisol-treated southern flounder that were of XX genotype. Control treatment (0 mg/kg cortisol) resulted in 91% females, whereas both groups that were fed cortisol show a male-biased ratio; 100 mg/kg cortisol treatment resulted in 29% females and 71% males; 300 mg/kg cortisol resulted in 13% females and 87% males. Both cortisol-treated groups were found to be significantly different than controls and from the expected 100% female sex ratio (n = 11–28 per treatment; $P < 0.0001$).

CHAPTER II

Regulation of leptin by glucose and key hormones of the endocrine stress axis in tilapia (*Oreochromis mossambicus*)

Abstract

Stress can impact nearly all aspects of physiology including hydromineral balance, immunity, reproduction, and many other biological functions in vertebrates. Stress is regulated through the sympathetic adrenergic and hypothalamic-pituitary-adrenal (interrenal in fish) (HPA or HPI in fish) axis whereby catecholamines and glucocorticoids are principal elements of the endocrine stress response. Leptin is a cytokine critical for regulating energy expenditure in vertebrates, yet little is known about how the hormone interacts with the endocrine stress axis, particularly in fishes and other ectotherms. The hormone is stimulated with various stressors in fish including fasting, hyperosmotic challenge, and hypoxia. Previous studies in tilapia have shown that leptin A (LepA) is the dominant form of leptin and that it increases plasma glucose and reduces liver glycogen *in vivo*, indicating a role in stimulating glycogenolysis. These data suggest that LepA may be involved in the adaptive stress response by mobilizing energy reserves, particularly carbohydrates. Currently, the regulatory interactions between the classical stress hormones (e.g. cortisol, epinephrine), metabolites (e.g. glucose), and leptin in fishes is unclear. We evaluated the actions of epinephrine, cortisol, and glucose in regulating LepA in the liver, the major site of leptin hormone production in the tilapia (*Oreochromis mossambicus*). Using hepatocyte incubations and a homologous LepA ELISA, we show that LepA synthesis and secretion declined as ambient glucose levels increased (10-25 mM). By contrast, bolus glucose administration in tilapia increased *lepa* mRNA levels 14-fold at 6 hours, suggesting systemic factors regulated by glucose may counteract the direct inhibitory effects of glucose on hepatic *lepa* mRNA observed *in vitro*. Cortisol at physiological concentrations (100 nM) stimulated LepA protein secretion from hepatocytes at all timepoints becoming significant by 6 hours. Interestingly, *lepa* mRNA levels were suppressed, showing discordant regulation between

synthesis and secretion of leptin by cortisol *in vitro*. This suppression of leptin synthesis by cortisol was attenuated by RU486, a glucocorticoid receptor antagonist. Lastly epinephrine, a major adrenergic stress hormone, stimulated LepA secretion from hepatocytes in a dose-dependent fashion within 15 minutes but had little effect on *lepa* mRNA levels. The response was accompanied by increases in glucose release likely indicating a classical glycogenolytic effect exerted by epinephrine. An *in vivo* injection of epinephrine into tilapia stimulated a rapid rise in blood glucose which was followed by a 4-fold increase in hepatic *lepa* mRNA levels at 2.5 and 6 hours. Plasma LepA was also elevated by 6 hours relative to controls. The results demonstrate that tilapia LepA is negatively regulated by rises in extracellular glucose at the level of the hepatocyte but stimulated by hyperglycemia *in vivo*. Cortisol and epinephrine increase LepA, suggesting the classical stress hormones may modulate glucose homeostasis in part through their regulation of hepatic leptin.

Introduction

Stress has been defined as any threat or disruption to an organism's homeostasis (Chrousos and Gold 1992). The magnitude and way in which the threat is perceived is subjective, however, counteracting these pressures with adaptive physical, behavioral, biological, and physiological changes, is essential for the organism to re-establish homeostasis (Chrousos and Gold 1992, Bonga 1997). Stress can impact nearly all facets of physiology including hydromineral balance, immunity, reproduction, and many other biological functions in vertebrates (Bonga 1997, Barton 2002). Acute and chronic stress events are mediated through the sympathetic adrenergic and hypothalamic-pituitary-adrenal (interrenal in fish) (HPA or HPI in fish) axes whereby catecholamines and glucocorticoids are principal elements of the endocrine stress response. In fishes, the primary glucocorticoid, cortisol, and the key hormone of the humoral adrenergic system, epinephrine/norepinephrine, are critical for the mobilization of energy resources during these times of stress (Bonga 1997, Reid et al. 1998, Mommsen et al. 1999, Barton 2002). Catecholamines exert numerous actions as part of the "fight or flight" aspect of the stress response. These actions can include stimulation of glycogenolysis, gluconeogenesis, and lipolysis, resulting in rapid hyperglycemia and mobilization of free fatty acids to provide energy for coping with the stressor (Fabbri et al. 1998, Reid et al. 1998). Cortisol can also elicit a variety of metabolic effects. It induces fat and protein catabolism, stimulates gluconeogenesis, and is immune suppressive, among other actions (Mommsen et al. 1999).

Leptin is a 16 kDa pleiotropic cytokine critical for regulating energy expenditure in vertebrates, yet little is known about how the hormone interacts with the endocrine stress axis, particularly in fishes and other ectotherms (Copeland et al. 2011, Deck et al. 2017). Leptin is associated with

numerous physiological processes, including energy homeostasis, cell differentiation and growth, metabolism, immunity, and reproduction (Zhang et al. 1994, Ahima and Flier 2000a, Londraville et al. 2014). In mammals, leptin is an adipostat, circulating in proportion to fat stores and exerting potent anorexigenic and lipolytic actions (Siegrist-Kaiser et al. 1997, Ahima and Flier 2000b). Some studies suggest that while leptin acts as a key metabolic regulator during stress in all vertebrates, it may primarily control carbohydrate energy resources (as opposed to lipids) in non-mammalian vertebrates, including fishes (Baltzegar et al. 2014, Deck et al. 2017). To date, the majority of studies examining the relationship between leptin and the stress axis have been performed in mammals (Ahima and Flier 2000a, Copeland et al. 2011). Little is known about the mechanisms of stress-induced leptin actions in fishes and other ectotherms and it is still unclear how the classical stress hormones, cortisol and epinephrine, might interact with leptin in regulating energy homeostasis. However, responses of leptin observed during stress events in fish include hypoxia (Chu et al. 2010, Cao et al. 2011, Bernier et al. 2012), infection (MacDonald et al. 2014), hyperosmotic stress (Baltzegar et al. 2014), and fasting (Kling et al. 2009, Fuentes et al. 2012, Douros et al. 2017) suggest an important link between leptin signaling, stress, and metabolic energy expenditure. Thus, it is possible that leptin acts as a key metabolic regulator during stress in all vertebrates.

In mammals, leptin stimulates the release of catecholamines (Takekoshi et al. 1999, Utsunomiya et al. 2001). In addition, leptin increased mRNA levels of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine production (Takekoshi et al. 1999). The regulation of catecholamines by leptin in fishes has not been well characterized. In goldfish (*Carassius auratus*), chronic leptin treatment (injected daily for 10 days) had little effect on hypothalamic catecholamines (de

Pedro et al. 2006). Leptin regulation of the humoral adrenergic system (i.e., circulating catecholamines) has yet to be examined. In mammals, epinephrine directly inhibits leptin secretion (Kosaki et al. 1996, Mantzoros et al. 1996, Fritsche et al. 1998, Carulli et al. 1999) and increases in intracellular cAMP (Slieker et al. 1996), one of the second messengers involved in adrenergic signaling, downregulate leptin mRNA. Currently there are no studies that examine the regulation of leptin by catecholamines in fishes or other ectotherms, and the relationship between these hormones is therefore still unclear. However, both leptin and epinephrine exhibit glycogenolytic actions and have been shown to increase during times of stress in fishes (Bonga, 1997, Baltzegar et al. 2014, Deck et al. 2017).

Leptin has an overall inhibitory effect on the HPA axis in mammals (Leal-Cerro et al. 2001), inhibiting CRF release from the hypothalamus in mice (Heiman et al. 1997) and suppressing cortisol secretion from adrenal cells (Bornstein et al. 1997, Pralong et al. 1998, Roubos et al. 2012). In addition, leptin knockout mice (*ob/ob*) have circulating levels of glucocorticoids that are 85% above basal and injecting these knockouts with leptin reduced the level of glucocorticoids by 40% (Ahima et al. 1998). Similar trends have been observed in teleosts, suggesting that interactions between leptin and glucocorticoids may in part fit the mammalian paradigm. In the common carp (*Cyprinus carpio*), leptin inhibited ACTH-stimulated cortisol secretion *in vivo* and caused a dose-dependent decrease in CRF-induced ACTH secretion from the pituitary *in vitro* (Gorissen et al. 2012, Gorissen and Flik, 2014). No changes in circulating cortisol were observed in leptin injected goldfish (*C. auratus*; Vivas et al. 2011), however, it is possible that leptin only inhibits glucocorticoid production when the HPI axis has been activated and circulating cortisol levels are elevated. In contrast to leptin's inhibitory effects on the HPA

axis, glucocorticoids elicit a stimulatory effect on leptin synthesis and secretion in mammals (de Vos et al. 1995, Wabitsch et al. 1996, Newcomer et al. 1998). Similar results have been observed in teleosts, with cortisol increasing hepatic leptin mRNA levels in rainbow trout (*Oncorhynchus mykiss*) both *in vivo* and *in vitro* (Madison et al. 2015). Additionally, when trout hepatocytes were treated simultaneously with cortisol and RU486, a glucocorticoid receptor antagonist, the increase in leptin mRNA was attenuated (Madison et al. 2015). It has been speculated that since cortisol release is slower than that of catecholamines, the prolonged stressors that elicit cortisol actions would also benefit from the catabolic effects of leptin on lipids in mammals and/or carbohydrates reported in fishes, particularly in the liver where leptin is produced and may act locally (Copeland et al. 2011, Baltzegar et al. 2014, Douros et al. 2014, Lu et al. 2012, Song et al. 2015, Wu et al. 2017).

Glucose mobilization is a vital component of an organism's adaptive response to stress. Both cortisol and epinephrine are glucoregulatory hormones and increase glucose during a stress response via gluconeogenesis or glycogenolysis (Bonga 1997, Barton 2002). Leptin induced hyperglycemia in tilapia (*O. mossambicus*; Baltzegar et al. 2014), rainbow trout (*O. mykiss*, Aguilar et al. 2010), and the Italian wall lizard (*Podarcis siculus*, Paolucci et al. 2006) and concurrently reduced liver glycogen, suggesting the hormone may stimulate glycogenolysis. On the other hand, heterologous leptin exerted either a hyperglycemic (Vivas et al. 2011) or hypoglycemic effect (de Pedro et al. 2006) in goldfish (*C. auratus*). The disparity between these responses in goldfish could be due to the time of day when the hormone is administered, as the hyperglycemic effect was only observed when leptin was injected during the daytime (Vivas et al. 2011). In mammals, leptin may also regulate carbohydrate metabolism, where it has been

implicated in the regulation of cellular glucose uptake (Frühbeck and Salvador 2000). Accordingly, glucose was found to stimulate leptin in mammals (Mueller et al. 1998, Wang et al. 1998, Levy et al. 2000). Leptin regulation by glucose in ectotherms is poorly understood, whose energy requirements and caloric intake may differ substantially from homeotherms, (Silva et al. 2006, Deck et al 2017). A study in grass carp (*Ctenopharyngodon idella*) suggests glucose may increase *lepa* mRNA in hepatocytes (Lu et al. 2015). Further studies are required to define how the metabolite alters leptin and its function in regulating glucose in vertebrates.

The purpose of the studies here was to assess whether glucose and the classic stress hormones, epinephrine and cortisol, might regulate LepA in tilapia (*Oreochromis mossambicus*). The goal was an improved understanding of how leptin might integrate with the classic endocrine stress hormones to control energy homeostasis and the adaptive stress response. The regulatory actions of glucose, epinephrine, and cortisol on the synthesis and secretion of hepatic LepA, the dominant leptin paralog found in tilapia and that is produced by the liver, were evaluated *in vitro* and *in vivo*.

Methods

Hepatocyte Incubations

All studies presented were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC). tilapia were maintained in recirculating systems with water quality parameters described below for injection studies. tilapia were given a lethal dose of Tricaine/MS-222 (Pentair Aquatic Eco-Systems, Apopka, FL, USA) buffered with $NaHCO_3$. Hepatocytes were harvested from the livers of tilapia as previously described (Pierce et al. 2004,

Douros et al. 2014). The liver tissue was carefully excised and placed in a calcium-free Hank's balanced salt solution (HBSS) containing 0.3 mg/mL type IV collagenase (Sigma-Aldrich, St. Louis, MO). Tissue was finely chopped with a razor blade and incubated in the HBSS-collagenase solution for 30–45 minutes at room temperature with gentle agitation. The digested liver tissue was gently massaged through a 210 µm mesh filter to remove structural tissue, and then allowed to drip freely through a second 60 µm mesh into an ice-cold beaker (ELKO Filtering LLC). Filtered hepatocytes were then transferred to 15 mL culture tubes. Cells were collected with slow speed centrifugation at 70 x g for 4 minutes at 15°C and washed through a series of 2-minute spins at 60 x g in HBSS containing 3 mM $CaCl_2$, 1 % BSA, and 1X essential and non-essential MEM amino acid solutions (Gibco, Carlsbad, CA) to remove erythrocytes, then allowed to recover on ice for 1 hour. Hepatocytes were resuspended in RPMI 1640 growth media containing L-glutamine, 25 mM HEPES, 5 mM $NaHCO_3$, 5 mM glucose, and 1% penicillin-streptomycin (Gibco, Carlsbad, CA). Cell viability was determined via a Trypan Blue exclusion test. The hepatocytes were incubated in 24-well Falcon Primaria plates at a density of 1 x 10^6 cells/mL. Cells were allowed to form a monolayer and acclimate for 4 hours at 25°C, after which the media was removed and replaced with fresh media containing the hormone or metabolite being examined. For control wells, media was replaced with fresh media alone containing 5 mM glucose unless otherwise specified. Doses and time points for incubations were: epinephrine at 0, 5, and 50 nM for 15, 30 and 60 minutes; cortisol time course at 0 and 100 nM for 1, 6, 12, and 18 hours; cortisol dose response at 0, 5, 10, 50, 100 nM, 1000 nM RU486 (mifepristone; Sigma, St. Louis, MO) alone, and 1000 nM RU486 + 100 nM cortisol for 1 hour; glucose at 0.25, 2.5, 10, and 25 mM for 1 hour. Stock cortisol and RU486 were initially dissolved in 100% ethanol and diluted in media to the appropriate concentrations. Control media

received similar final concentrations of ethanol (0.02%). At the termination of the culture, media was removed and stored at -20°C until analysis of glucose (Abcam, Cambridge, MA) and LepA secretion using a previously validated homologous ELISA (Douros et al. 2014). Cells were taken up in Tri-Reagent (Molecular Research Center, Cincinnati, OH) for gene expression of *lepa* mRNA.

Epinephrine Injection

Adult male tilapia (135.7 ± 2.4 g mean body weight, BW) were allowed to acclimate for 2 weeks in recirculating tank systems (salinity 5 ppt, hardness 112–126 mg/L, alkalinity 168–182 mg/L, pH 7.2, temperature 23–25°C, photoperiod 12 h light:12 h darkness) and fed daily 2% BW (40% protein/10% fat; Ziegler Brothers, Gardner, PA, USA). Fish were fasted for 24 hours prior to the start of the experiment. Fish were anesthetized using buffered MS-222, weighed, and administered an i.p. injection of 5.0 µg/g BW of epinephrine hydrochloride in 0.9% saline (Sigma-Aldrich, St. Louis, MO) or 0.9% saline as a control injection. Fish (N = 90; n = 10 fish/group) were returned to tanks separated by treatment and timepoint, and then sampled at 0, 0.5, 1, 2.5, and 6 hours post-injection. Blood was collected from the caudal vein using heparinized syringes and tubes. Glucose was measured immediately after blood collection using a Bayer Contour Blood Glucose Meter (Bayer Healthcare LLC, Mishawaka, IN). Blood samples were spun at 3000 x g for 7 min to collect plasma and stored at -20°C until analysis. Liver tissue was collected (~100 mg), placed in 1 mL of RNAlater (Invitrogen), kept overnight at 4°C, and then stored at -20°C until extractions.

Glucose Injection

Adult male tilapia (92.8 ± 1.5 g mean body weight, BW) were allowed to acclimate for 1 week in recirculating tank systems with the same parameters as previously described for the epinephrine injection study. Feed was restricted 24 hours prior to injections. Fish were anesthetized using buffered MS-222, weighed, and administered an i.p. injection of 600 µg/g BW of glucose (Sigma-Aldrich) or 0.9% saline as a control before being returned to the tanks. Fish (N = 128; n = 13 - 16 fish/group) were sampled at 0, 1, 2, 6, and 24 hours post-injection. Blood was collected and blood glucose measured immediately with a glucometer as previously described. Plasma was isolated by centrifugation and stored at -20°C. Liver tissue was collected (~100 mg), placed in 1 mL of RNAlater, kept overnight at 4°C, and then stored at -20°C until extractions.

RNA Isolation and Quantitative Real-time PCR

Total RNA was isolated from hepatocytes and liver tissue samples using Tri-Reagent and RNA purification Direct-zol miniprep spin columns with DNase treatment using standard methods from the manufacturer (Zymo Research, Irvine, CA). RNA quality was assessed by presence of 18S and 28S ribosomal RNA bands using gel electrophoresis, and then quantified by absorbance OD 260:280 ratio using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Total RNA (1 µg) was used in a cDNA synthesis reaction via reverse transcription following the manufacturer's instructions (High Capacity cDNA Synthesis, Applied Biosystems).

The mRNA levels of *lepa* were determined by quantitative real-time PCR (qPCR) using gene-specific primers previously described (Baltzegar et al. 2014; GenBank accession number KC354702). Sequences of primers used for qPCR are as follows: *lepa* forward primer (FP): GGGTCTCCCAGATCAAGTACGA, *lepa* reverse primer (RP): TGCCGCCACAGATGAATG,

18S FP: ATTACTGGCTACACCGAGCG, and *18S* RP: AGTCCTGACTCTCCTCCGTC. All reactions were run in triplicate and performed on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems), with Brilliant II SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA), using 1.5 µM primers, and 2 µL of 1:6 diluted cDNA in a total reaction volume of 10 µL. The qPCR cycling parameters were 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. A dissociation melt curve step at the end was performed to verify a single PCR product. The absence of genomic DNA contamination was confirmed using water (No Template Control; NTC) and DNase treated RNA with no reverse transcriptase enzyme (No-Amplification Control; NAC) as negative controls. Cycle threshold (Ct) values for samples were transformed using a standard curve of serially diluted pooled cDNA versus Ct values (R^2 = 0.97-0.98). Samples were then normalized to reflect the amount of template cDNA per ng total RNA loaded into each reaction (cDNA/ng total RNA) and/or samples were normalized to the expression levels of *18S* ribosomal RNA. The expression of *18S* has previously been validated as a reference gene in tilapia liver (Douros et al. 2017). The values are expressed as fold change relative to the mean of the initial baseline group as indicated in the figure legends.

Glucose and Leptin Assays

For injection studies, blood glucose measurements were taken immediately after blood collection using a Bayer Contour Blood Glucose Meter. Media glucose from the epinephrine hepatocyte incubation was measured in duplicate with a colorimetric glucose assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's protocol. 2 µL of media was loaded into each well and the volume was adjusted to 50 µL total well volume with glucose assay buffer. Glucose

standards were generated from stock solution ($R^2 = 0.98$). The procedure for LepA measurements was previously validated in Douros et al. 2014. Costar 3590 RIA/ELISA 96-well polystyrene plates (Corning Life Sciences, Tewksbury, MA) were coated with 200 µL 1:1 *v/v* plasma diluted with PBS, or 200 µL culture media, and/or 200 µL of tilapia LepA standard antigen (GenScript: 177060) for 4 hours at room temperature (~ 23°C). Standards were created from a serial dilution of the tilapia LepA antigen ranging from 0.01 to 1000 nM and were run in triplicate on each plate. Plain culture media and 1X PBS were used as blanks to calculate background and were subtracted from all samples. Coated wells were washed once with 300 µL of 1X PBS and then blocked with 300 µL of 5% *w/v* non-fat milk in 1X PBS for 2 hours at room temperature. Wells were washed 4X with 300 µL of 1X PBS and then incubated for 2 hours with 200 µL of the anti-tLepA primary antibody (GenScript: 177060) diluted 1:300 in 1X PBS. The wells were washed again 4X and then incubated for 1 hour with 200 µL of goat anti-rabbit IgG HRP-conjugated antibody (sc-2004, Santa Cruz Biotechnology Inc., Dallas, TX) diluted 1:5000 in 1X PBS. Following incubation, wells were washed again 4X before addition of 200 µL of O-phenylenediamine dihydrochloride (OPD, Thermo Fisher Scientific) solution (1 µL 30% H_2O_2 and 1 mg OPD per mL of OPD Buffer: 0.05M sodium citrate and 0.05M sodium phosphate in 1X PBS at pH 5). Wells were allowed to develop for 15 minutes and absorbance readings were measured at 15, 25, 30, and 40 minutes to track development over time and saturation. Detection of absorbances for both assays was performed on an ELx800 microplate reader (BioTek Instruments, Winooski, VT) with a 570 nm filter for glucose and a 450 nm filter for LepA. Samples were analyzed using non-linear regression and GraphPad Prism 6 (GraphPad, La Jolla, CA). All samples were interpolated from a sigmoidal curve generated from standards on each plate.

Statistical Analyses

The data utilizing a single timepoint from hepatocyte incubations, were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc to test for differences of treatment groups from controls. For the glucose hepatocyte incubation, the 2.5 mM dose was used as the control calibrator as it is the closest to endogenous glucose levels in tilapia (around 2 mM; see results for baseline values from injection studies). Those hepatocyte cultures that had multiple timepoints and all injection studies were analyzed by two-way ANOVA (treatment x time) and were analyzed for significance at each time point with Fisher's Least Significant Difference (LSD) test and also differences over time from control groups within treatments with Dunnett's post-hoc. All analyses were performed using JMP (Pro v13, SAS Institute, Cary, NC) and/or GraphPad Prism 6. The level set for statistical significance for all analyses was $P < 0.05$ and data are shown as mean values ± SEM.

Results

Cortisol Regulation of Leptin *in vitro*

Cortisol at 100 nM significantly decreased *lepa* mRNA levels in hepatocytes at 1 hour ($P = 0.002$), 6 hours ($P < 0.0001$), and 12 hours ($P = 0.007$; Figure 1A) relative to the 1-hour control. In contrast, cortisol treatment (100 nM) stimulated LepA secretion in the same culture from 0.53 ± 0.07 nM (0 nM cortisol) to 1.75 ± 0.09 nM at 6 hours ($P = 0.003$) and from 1.08 ± 0.24 nM (0 nM cortisol) to 1.86 ± 0.22 nM at 18 hours ($P = 0.005$; Figure 1B). Control wells also showed an increase in LepA secretion over time as the hormone accumulated in the media, as well as a decrease in *lepa* levels in hepatocytes. A second experiment was designed to assess the dose response effect of cortisol and whether the actions are blocked by the intracellular glucocorticoid

receptor antagonist, RU486. Cortisol reduced *lepa* mRNA levels in a dose dependent fashion after 1 hour, becoming significant at 50 nM ($P = 0.02$) and 100 nM ($P = 0.0003$, Figure 2). RU486 impeded the decline in *lepa* mRNA observed with cortisol alone, indicating that the effects observed are the result of signaling through the classical glucocorticoid receptor.

Epinephrine Regulation of Leptin *in vitro*

Epinephrine stimulated glucose release into the media from 3.86 ± 1.01 mM (0 nM epinephrine) to 9.06 ± 0.53 mM (50 nM epinephrine) at 15 minutes ($P = 0.0009$) and from 5.70 ± 0.75 mM (0 nM epinephrine) to 8.93 ± 1.10 mM (50 nM epinephrine) at 30 minutes ($P = 0.03$), likely indicating a classical glycogenolytic effect of the adrenergic hormone (Figure 3A). There was no significant effect of epinephrine on *lepa* mRNA levels in hepatocytes (Figure 3B). However, epinephrine at 50 nM stimulated LepA secretion from 3.16 ± 0.48 nM (0 nM epinephrine) to levels of 9.26 ± 2.08 nM at 15 minutes ($P = 0.001$) and from 3.86 ± 1.01 mM (0 nM epinephrine) to 2.62 ± 0.14 nM at 30 minutes ($P = 0.04$). Although the 5 nM dose increased LepA secretion, there was no significance compared to the 0 nM epinephrine within each timepoint (Figure 3C).

Epinephrine Regulation of Leptin *in vivo*

When epinephrine at 5 µg/g BW was injected into tilapia, it showed a similar stimulatory effect as in hepatocytes treated with epinephrine *in vitro*. Epinephrine significantly increased blood glucose at all time points, with a maximum increase from 1.03 ± 0.08 mM (saline) to 6.24 mM at 6 hours (0.5 hour: $P = 0.002$, 2.5 and 6 hours: $P < 0.0001$; Figure 4A). There was no change in *lepa* mRNA levels at 0.5 or 1 hour, however, there was an approximate 4-fold increase at 2.5 and 6 hours relative to controls (2.5 hours: $P = 0.0005$, 6 hours: $P < 0.0001$; Figure 4B). LepA

secretion was significantly elevated by 6 hours at 4.86 ± 1.05 nM compared to saline controls at 2.27 ± 0.38 nM (P = 0.001; Figure 4C). There was an increase in plasma LepA for both saline (0.90 ± 0.24 nM to 3.50 ± 0.71 nM) and epinephrine (0.62 ± 0.14 nM to 3.55 ± 0.98 nM) injected groups between the 1 and 2.5-hour timepoints, although there was no difference between treatments.

Glucose Regulation of Leptin *in vitro*

Glucose had an overall inhibitory effect on leptin synthesis and secretion in hepatocytes after 1 hour of incubation. All treatments were compared with the 2.5 mM glucose group (0.79 ± 0.04 nM LepA) as this glucose concentration reflects the baseline range observed in tilapia *in vivo* (Baltzegar et al. 2014 and Figure 6A). The levels of *lepa* mRNA decreased by approximately 50% in the 10 mM (P = 0.01) and 25 mM (P = 0.02) treatments (Figure 5A). LepA secretion was also significantly lower with 10 mM (P = 0.01) and 25 mM glucose (P = 0.0008) measuring 0.53 ± 0.04 nM and 0.41 ± 0.09 nM LepA respectively (Figure 5B).

Glucose Regulation of Leptin *in vivo*

tilapia received a bolus i.p. glucose injection of 600 µg/g BW and as anticipated blood glucose was significantly elevated at 1 and 2 hours (P < 0.0001) as well as at 6 hours (P = 0.02) post-injection (Figure 6A). Glucose had returned to control levels by 24-hours post-injection. Saline injected fish averaged blood glucose levels of 1.8 ± 0.08 mM across all timepoints. The highest blood glucose readings were measured at 1 hour (8.38 ± 0.41 mM) and 2 hours (6.45 ± 0.36 mM). The levels of *lepa* mRNA were increased 9-fold at 1 hour (P = 0.0009), 6-fold at 2 hours (P = 0.02), and 14-fold 6 hours post-injection relative to controls (Figure 6B; P < 0.0001). By

24-hours *lepa* mRNA levels had returned to baseline and were no longer significantly different from saline-injected controls.

Discussion

These studies show that leptin is directly regulated by glucose and the key hormones of the stress response, epinephrine and cortisol. All three hormones rise with stress events in tilapia or other fishes (Bonga, 1997, Reid et al. 1998, Baltzegar et al. 2014, Deck et al. 2017). More specifically, hepatic *lepa* mRNA levels, leptin receptor (*lepr*), and plasma glucose increased rapidly within 4 hours, upon challenge of tilapia to seawater (Baltzegar et al. 2014). Similarly, hepatic *lepa* and *lepr* mRNA increased within 1 day of fasting (earliest time point measured), which was followed by a more delayed rise in circulating hormone (Douros et al. 2017). Other studies show hepatic glucocorticoid receptors, interrenal cortisol production and/or systemic cortisol may rise with seawater perturbations, handling, or fasting, events that require the mobilization of energy stores necessary for the organism to adjust and survive (Bonga 1997, Morgan et al. 1997, Barton 2002, Dean et al. 2003, Peterson and Small 2004). Both cortisol and recombinant tilapia leptin enhance plasma glucose in tilapia, suggesting they may work in concert to mobilize energy stores (Baltzegar et al. 2014). Cortisol has been shown to stimulate hepatic *lepa* mRNA in rainbow trout both *in vivo* and *in vitro* (Madison et al. 2015). Whether this action is accompanied by increased secretion is unknown. Here, we found cortisol stimulated LepA secretion from tilapia hepatocytes in a dose-dependent fashion. The response was accompanied by concomitant declines in *lepa* mRNA which was exacerbated with longer exposure periods. The discordant regulation of *lepa* mRNA levels and LepA secretion by cortisol suggests the steroid may enhance translation of leptin mRNA transcripts and subsequent release of the hormone, as glucocorticoids

are known to enhance translation rates as part of their molecular mechanisms of actions (Roewekamp et al. 1976). Whether the hormone may also impair transcription of *lepa* gene is unknown and requires further investigation. Alternatively, it is possible that increased levels of LepA itself might suppress accumulation of its own mRNA *in vitro* as has been demonstrated in rat adipocytes (Sliker et al. 1996). Collectively, our studies suggest cortisol is a direct stimulator of hepatic LepA secretion and may conversely suppress steady state *lepa* mRNA levels in tilapia hepatocytes. The actions of cortisol appear to be mediated through the classical glucocorticoid receptor, as RU486 was effective in ameliorating the inhibitory actions of cortisol on *lepa* mRNA accumulation. In tilapia, a single cortisol dosage had little effect on *lepa* mRNA levels *in vivo* in tilapia (supplementary data, Baltzegar et al. 2014). Further studies will be required to assess whether sustained treatment with cortisol *in vivo* might alter LepA, particularly its secretion in tilapia.

Epinephrine is released rapidly in response to a stress increasing ventilation rates, heart rate and the mobilization of energy reserves (Fabbri et al. 1998, Reid et al. 1998). In tilapia hepatocyte incubations, epinephrine stimulated glucose within 15 minutes, likely resulting from the acute glycogenolysis (Figure 3A). The hormone had little effect on *lepa* mRNA levels, but increased LepA release by 3-fold within 15 minutes (Figure 3C), a response that subsequently subsided by 60 minutes. The response on LepA secretion observed *in vitro* was also demonstrated *in vivo* with epinephrine injection. Epinephrine increased plasma glucose by 3-fold within 2.5 hours, as well as hepatic *lepa* mRNA levels by 4-fold at 2.5 and 6 hours and induced a rise in circulating LepA at 6 hours. These results suggest for the first time in teleosts that epinephrine stimulates LepA secretion, and that its actions are likely to occur, at least in part, directly at the level of the

hepatocyte. Considering the well-established effect of epinephrine in stimulating glycogenolysis and recent evidence that homologous and heterologous leptin induces hyperglycemia and depletes hepatic glycogen stores in tilapia *in vivo*, suggests the two hormones may work in concert to mobilize glucose (Fabbri et al. 1998, Aguilar et al. 2010, Vivas et al. 2011, Baltzegar et al. 2014).

The response to epinephrine we observed in tilapia contrasts that seen in mammals, where catecholamines have been shown to inhibit leptin (Kosaki et al. 1996, Mantzoros et al. 1996, Fritsche et al. 1998, Carulli et al. 1999). One possible explanation for this disparity is the different metabolic actions exerted by leptin in these two groups of vertebrates. In mammals, leptin stimulates lipolysis and fatty acid oxidation (Siegrist-Kaiser et al. 1997, Wang et al. 1999) while in tilapia, salmonids, and lizards the hormone appears to stimulate peripheral glycogenolysis (Paolucci et al. 2006, Aguilar et al. 2010, Baltzegar et al. 2014). It may not be advantageous for catecholamines to stimulate leptin during acute stress in mammals as obtaining energy from lipolysis is likely to slow for an acute "fight or flight" response, whereas in fish a stimulation of leptin by catecholamines could promote more rapid mobilization of glucose from carbohydrate stores (Copeland et al. 2011, Baltzegar et al. 2014, Deck et al. 2017).

Baseline circulating glucose levels in tilapia are typically around 2 mM with circulating levels increasing as high as 12 mM with salinity challenge (see Figure 6 this paper, Baltzegar et al. 2014). Since leptin induces hyperglycemia in tilapia and other teleosts and vertebrates (Aguilar et al. 2010, Vivas et al. 2011, Baltzegar et al. 2014), we postulated hormone may be sensitive to modulation by ambient glucose. Baseline levels (2.5 mM) and lower concentrations of glucose

(0.25 mM) had no effect, while elevated glucose (10 and 25 mM) significantly reduced *lepa* mRNA levels in hepatocytes and LepA released into the media (Figure 5). These data suggest that increased glucose may suppress hepatic synthesis and secretion of LepA. Based on these *in vitro* responses, we postulate that glucose may act as a negative feedback regulator of leptin, whereby leptin stimulates hyperglycemia (via glycogenolysis) and elevated ambient glucose subsequently suppresses further leptin synthesis and secretion by the liver. When sufficient glucose is present, there may be less need for leptin to mobilize the metabolite.

Interestingly, when a bolus dose of glucose was injected into tilapia, *lepa* mRNA levels were significantly elevated, up to 14-fold higher than saline injected fish (Figure 6). This increase in *lepa* gene expression corresponded with periods of hyperglycemia although a slight hysteresis was observed. Circulating levels of glucose were elevated within 1-hour post-injection, reaching levels as high as 8 mM and then declining to near baseline at 6 hours, while *lepa* levels reached their peak by 6 hours. This response is more similar to what is observed in mammals where leptin is stimulated by hyperglycemia, to aid in tissue glucose uptake (Mueller et al. 1998, Wang et al. 1998, Levy et al. 2000). It is well established that blood glucose clearance is substantially more delayed in fish than mammals, often referred to as insulin-resistance (Riley et al. 2009). It is possible, therefore, that leptin may induce hyperglycemia to mobilize glucose during stress and be more sensitive to elevated glucose *in vivo* to enhance tissue glucose uptake in fish.

The disparity between leptin responses *in vitro* and *in vivo* from glucose treatment suggest there are likely other systemic factors involved when glucose is injected into the animal. The hyperglycemic conditions achieved from a bolus glucose injection likely stimulated other

metabolic hormones, but how other glucoregulatory hormones, such as insulin, glucagon, or glucagon-like peptides might regulate leptin is still unclear. Insulin is a key metabolic hormone that is released in response to hyperglycemia. Circulating leptin is elevated by insulin secretion in birds and rats, but not in humans, where it is more responsive to direct changes in glucose metabolism rather than insulin signaling (Dagogo-Jack et al. 1996, Barr et al. 1997, Ashwell et al. 1999). Insulin also increased leptin mRNA in grass carp hepatocytes (Lu et al. 2015). It is conceivable that the induced hyperglycemia stimulated insulin release which in turn upregulated leptin mRNA in the tilapia as is the case in other vertebrates. However, additional studies need to be conducted to determine what interactions might be at play between leptin and these other glucoregulatory hormones.

In summary, we demonstrate that tilapia LepA increases in response to cortisol and epinephrine, is negatively regulated by rises in extracellular glucose at the level of the hepatocyte but is stimulated by hyperglycemia *in vivo*. The increase in LepA in response to cortisol and epinephrine suggests that classic stress hormones may augment leptin to help promote glucose mobilization as part of the integrated, adaptive stress response. As studies on leptin in non-mammalian species advance, it is clear that this hormone is not simply a regulator of appetite but is also undoubtedly a regulator of energy expenditure and a mediator of stress in conjunction with other key components of the stress axis.

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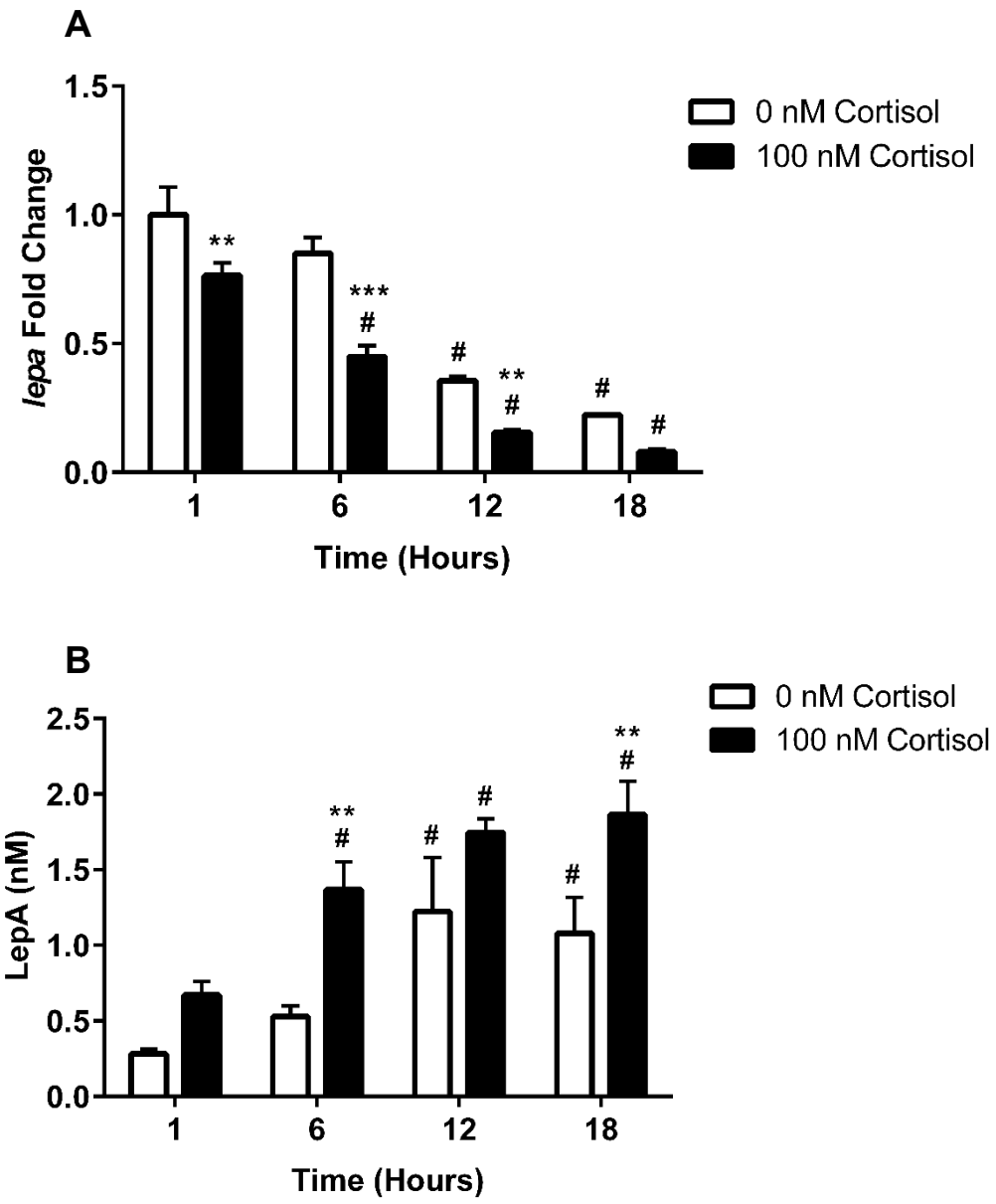

Figure 1. Effects of *in vitro* hepatocyte incubation with cortisol over time: (**A**) *lepa* mRNA levels (shown as fold change from 0 nM cortisol at 1 hour) and (**B**) nM LepA secretion. Values reported as means ± SEM. # denote differences over time within treatments from 1-hour time point. * denote significant differences between treatments at each time point. (n = 6, $^{**}P \leq 0.007$, $^{***}P < 0.0001$).

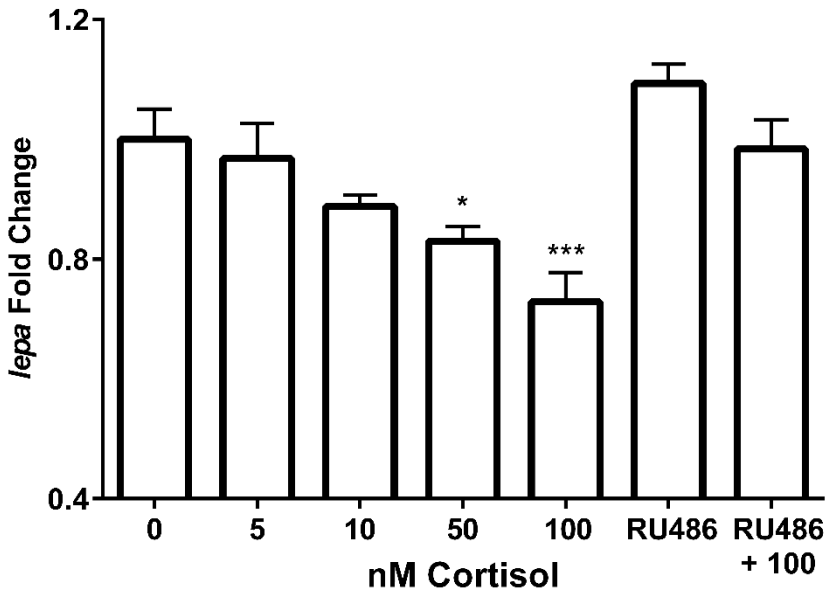

Figure 2. Effects of *in vitro* cortisol treatment in hepatocytes for 1 hour on *lepa* mRNA levels (shown as fold change from 0 nM cortisol). * indicates significance from 0 nM cortisol. (n = 6 – 9, **P* = 0.02, ****P* = 0.0003). Values reported as means ± SEM.

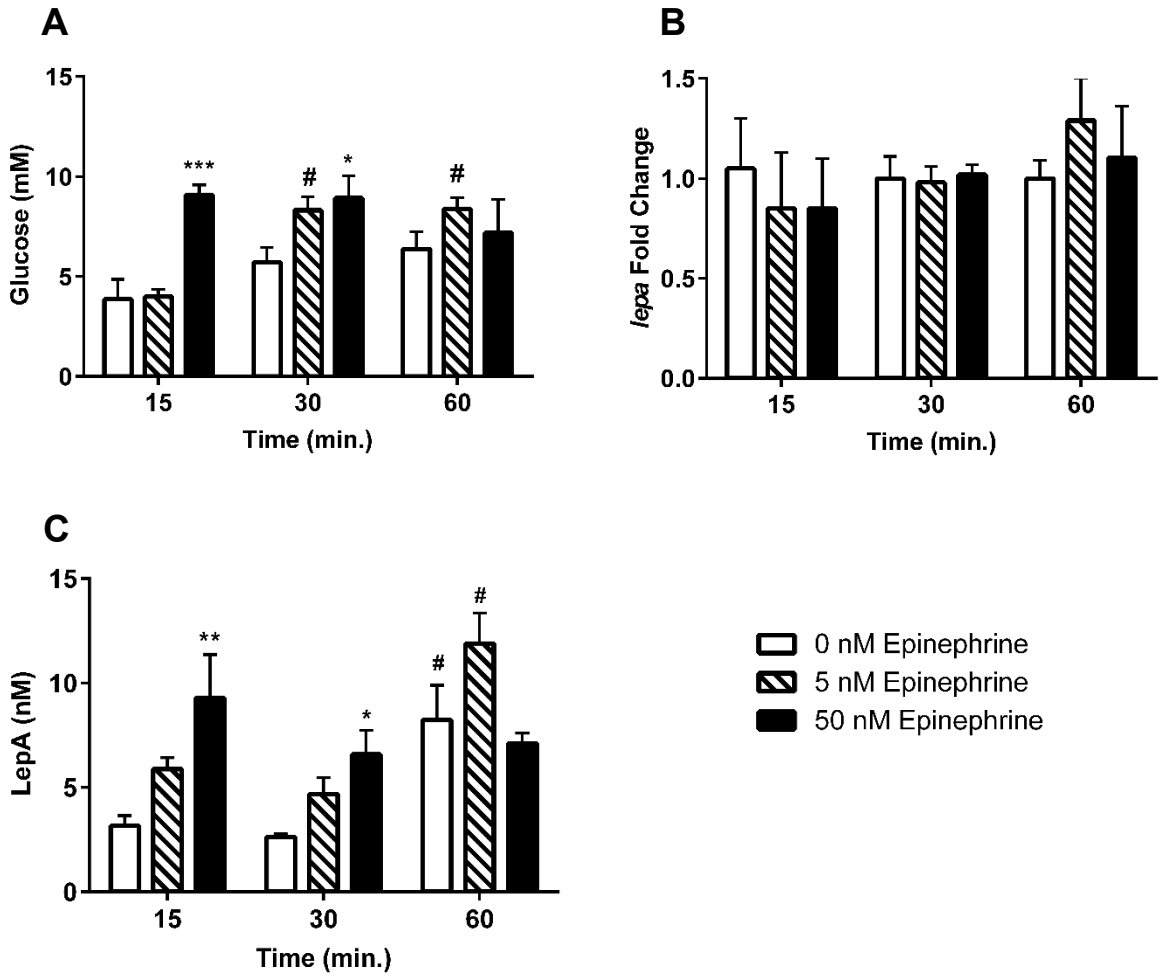

Figure 3. Effects of *in vitro* hepatocyte incubation with epinephrine on (**A**) media glucose (mM), (**B**) *lepa* mRNA levels (shown as fold change from 0 nM epinephrine at 15 minutes), and (**C**) nM LepA secretion. Hepatocytes were incubated with 0, 5, and 50 nM epinephrine for 15, 30, and 60 minutes. Values reported as means ± SEM. # denote differences over time within treatments from 15-minute timepoint. * denote significant differences between treatments at each time point. (n = 4, *$P \leq 0.04$, **$P = 0.001$, ***$P = 0.0009$).

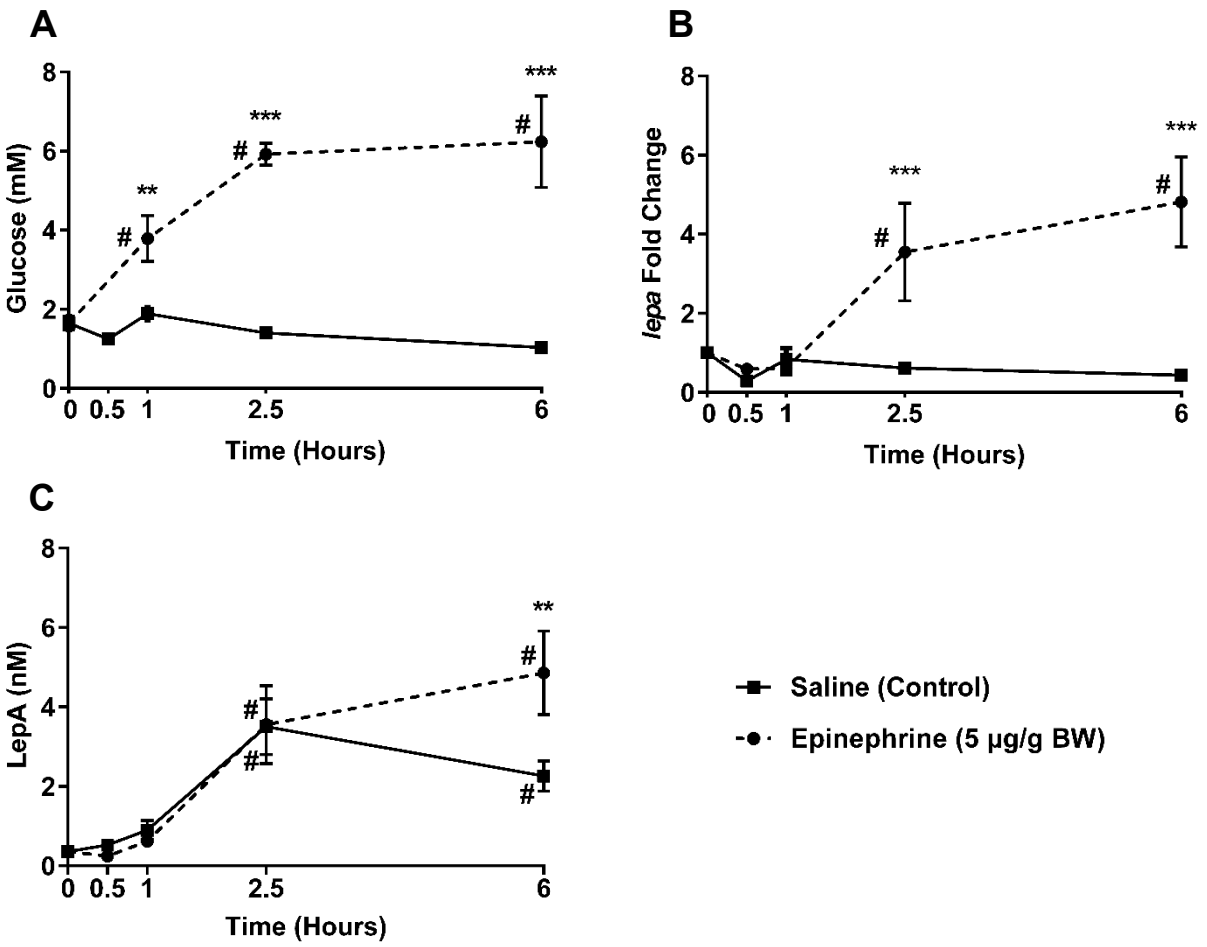

Figure 4. Effects of *in vivo* epinephrine i.p. injection on (**A**) blood glucose (mM), (**B**) hepatic *lepa* mRNA levels (shown as fold change from uninjected time 0), and (**C**) plasma LepA (nM). Fish received either an injection of saline (control) or 5 µg/g BW epinephrine and were sampled at 0.5, 1, 2.5, and 6 hours post-injection. Values reported as means ± SEM. # denote differences over time within treatments from initial time 0. * denote significant differences between treatments at each time point. (n = 8 - 10, ** P = 0.002, *** $P \leq 0.0005$).

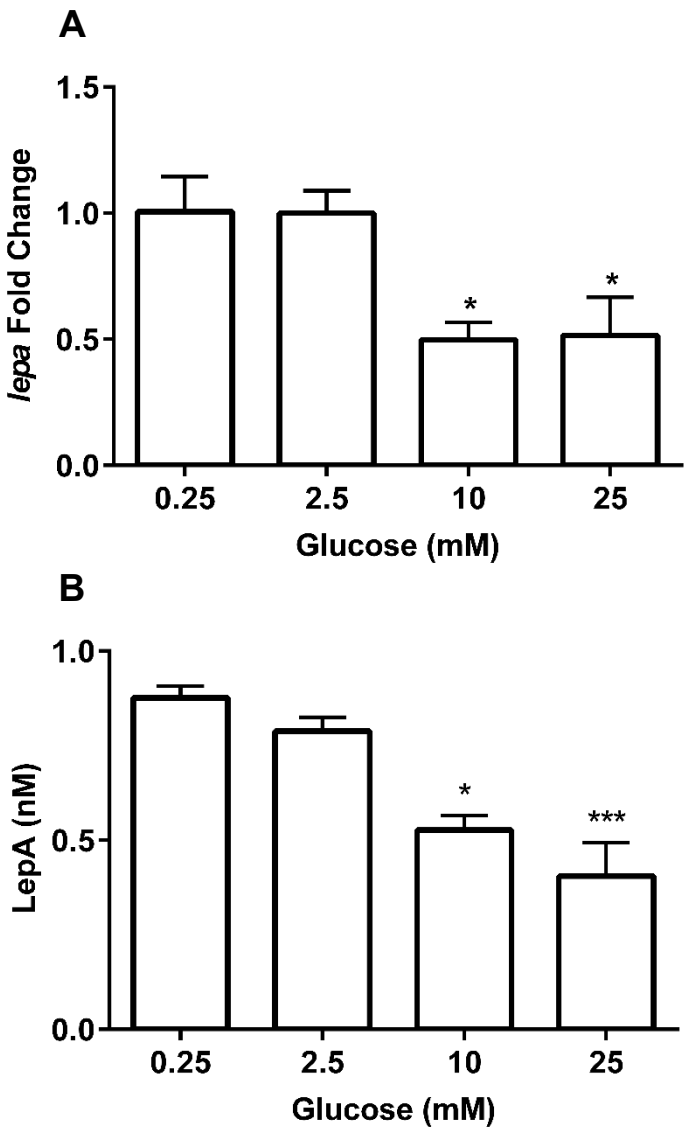

Figure 5. Effects of *in vitro* glucose treatment in hepatocytes for 1 hour on (**A**) *lepa* mRNA levels (shown as fold change from 0 nM cortisol), and (**B**) nM LepA secretion. * indicates a significant difference from 2.5 mM glucose. Values reported as means ± SEM. (n = 4, *$P \leq 0.02$, ***$P = 0.0008$).

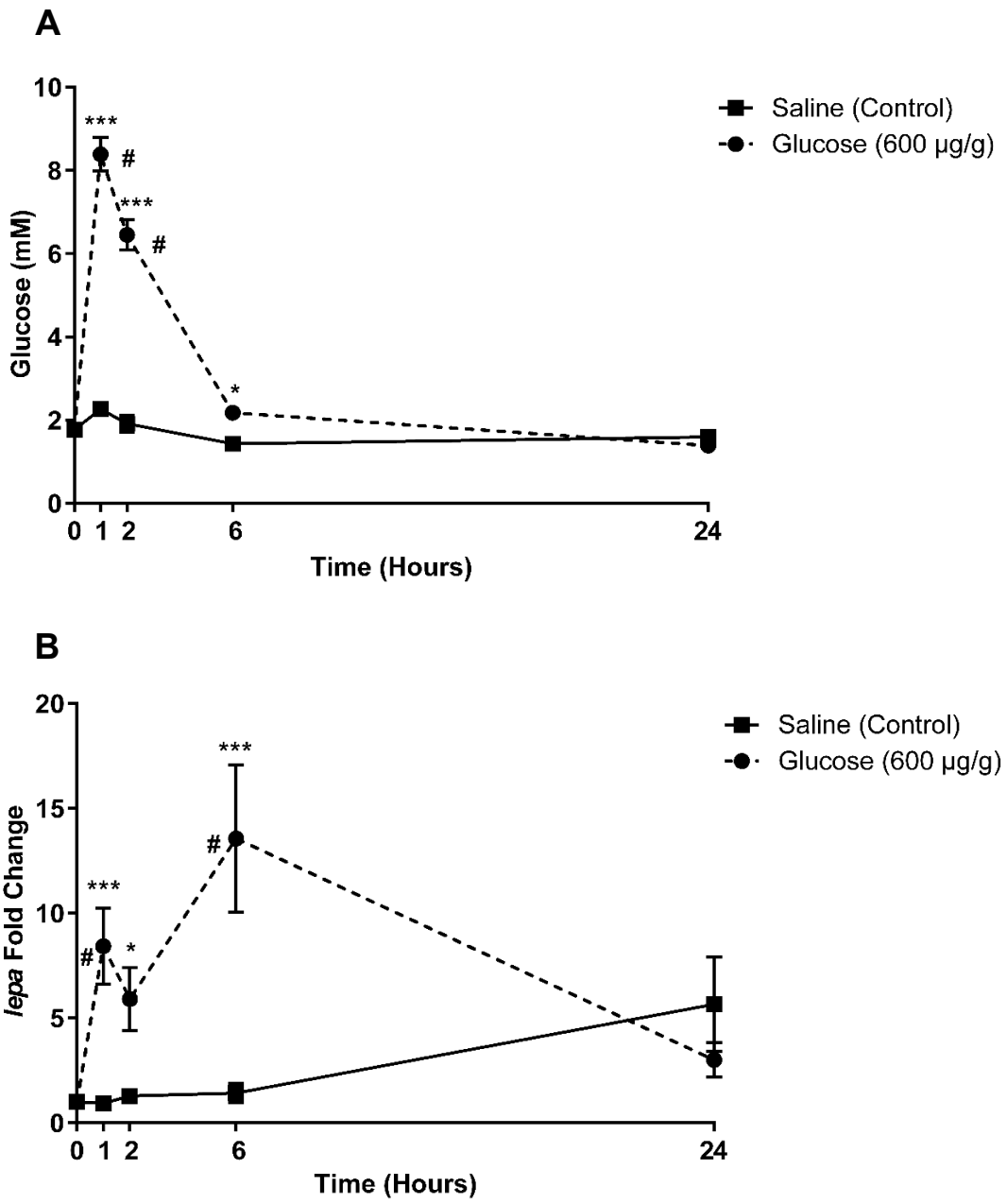

Figure 6. Effects of *in vivo* glucose i.p. injection on (**A**) blood glucose (mM) and (**B**) hepatic *lepa* mRNA levels (shown as fold change from uninjected time 0). Fish received either an injection of saline (control) or 600 µg/g BW bolus glucose and were sampled at 1, 2, 6, and 24 hours post-injection. Values reported as means ± SEM. # denote differences over time within treatments from initial time 0. ***** denote significant differences between treatments at each time point. (n = 11 - 15, *$P \leq$ 0.02, ***$P \leq 0.0009$).

CHAPTER III

The effects of habitat temperatures on sex ratios of juvenile wild southern flounder

Abstract

Southern flounder (*Paralichthys lethostigma*) is a valuable commercial and recreational species found in the southeast United States. The fisheries are dependent on females due to sexually dimorphic growth, with females growing larger and faster, and relatively few males reaching harvestable size. Southern flounder and other Paralichthids exhibit environmental sex determination (ESD), where factors in the environment can influence sex and masculinize populations. Impacts of the environment on sex occur during early juvenile development (35 - 65 mm total length) and environmental effects on sex are limited to the XX genotype (female). Variation in environmental factors such as temperature and background color can influence sex determination under captive rearing conditions. Ideal conditions will produce at most 50% females, with much lower proportions of females possible through masculinization of presumptive females. Hence it is possible that the environment, particularly warmer waters, may masculinize flounder populations in the wild and limit the number of females that enter the fishery. To evaluate the possibility of environmental effects on sex determination in wild southern flounder populations, we examined spatial and temporal variation in sex ratios of juvenile flounder and monitored temperature and water quality parameters across a range of nursery habitats in North Carolina, USA. We obtained temperature profiles over four years in up to 14 different juvenile southern flounder nursery habitats in a season. Although temperature varied annually, the most southerly located habitats consistently exhibited the warmest temperatures. Additionally, dissolved oxygen (D.O.) and salinity data for 5 of these locations were collected in 2016. Mean salinity ranged from 3.6 ppt (Hancock Creek) to 24.8 ppt (Mill Creek) and the mean D.O. ranged from 5.14 mg/L (Clubfoot Creek) to 8.81 mg/L (Germantown Bay). To assess the sex of juveniles, expression of biomarkers for sex was measured in gonads

from field-caught individuals where mRNA was extracted from gonadal tissue and key sex determining genes; *mis*, *foxl2*, and *cyp19a1a*, were measured via quantitative real-time PCR (qPCR). Samples were organized into three regions: Pamlico (north), Neuse (intermediate), and south of the New River (south). Samples from northern sampling sites in the Pamlico River area exhibited near 50:50 sex ratios in 2014 (52% male), slightly female-biased ratios in 2015 (37% male), and slightly male biased in 2016 (61% male) and 2017 (66% male). The Neuse River, an intermediate location, produced male-biased sex ratios from 2012 to 2017 (88%, 82%, 76%, 59%, 82%, 78% male). In the southern sampling locations, south of the New River, sex ratios were significantly male-biased over all sampling years from 2014 to 2017 (88%, 86%, 81%, and 94% male). The northern habitats exhibited average temperatures near the 23°C conditions that promote 50:50 sex ratios in captive rearing and also produced the greatest proportions of female juveniles. By contrast, the habitats that consistently produced male-biased sex ratios were associated with mean temperatures that were on average 2.2 to 3.1°C warmer over all sampling years. These data suggest that variation in nursery habitat water temperatures may be a critical factor driving regional differences in juvenile southern flounder sex ratios. With a fishery dependent on females and global ocean temperatures projected to significantly increase, these temperature effects on sex ratios could be a significant concern for wild flounder stocks. These are the first studies to provide evidence of temperature-dependent sex determination affecting a key demographic parameter of an important fisheries species. We document both geographic and interannual variation in sex ratios associated with nursery habitat temperatures with the southernmost habitats sampled consistently producing the most male juveniles.

Introduction

Southern flounder (*Paralichthys lethostigma*) is a species of benthic flatfish that supports major commercial and recreational fisheries in the southeast United States and is a high value finfish fishery in North Carolina (NC). The range for southern flounder extends along the mid-Atlantic and Gulf coasts, although the population is discontinuous around the Florida peninsula (Gilbert 1986, Daniels 2000). Adults migrate out from estuaries to coastal waters to spawn in late fall and early winter. The fertilized eggs are buoyant and will float for two to three days until hatching. After another 30 to 60 days of development in offshore waters, the larvae will undergo metamorphosis before finally migrating into rivers and settling into estuarine nursery habitats where they gain protection and grow before eventually moving back out to cooler, deeper waters (Gilbert 1986, Daniels 2000). Juvenile southern flounder habitats typically include estuarine waters with aquatic vegetation, shells, and mud bottoms along saltmarsh edges (Walsh et al. 1999, NCDMF 2005).

Southern flounder exhibit sexually dimorphic growth, with female flounder growing larger and faster than males (Fitzhugh et al. 1996). Due to the use of a minimum size limit as a management strategy, the southern flounder fishery is heavily dependent on females, with males rarely growing to a harvestable size (Takade-Heumaker and Batsavage 2009). A reduction in the proportion of females translates directly into a reduction in recruitment to the fishery. Additionally, models used to estimate fishing mortality and spawning stock biomass (SSB) rely on indices of year class strength that are based on abundance of age-0 flounder. Most age-0 fish cannot be sexed macroscopically so the assessment assumes a 1:1 sex ratio. If the juvenile sex ratio is actually biased toward males, the year class strength of female flounder will be

overestimated, resulting in erroneous model estimates. Based on survey data, there are decreasing trends in juvenile and adult abundances, and southern flounder are under "concerned" status (Takade-Heumacher and Batsavage 2009; NCDMF 2013, 2017, Figure 1). Although not without controversy, recent assessments have indicated that southern flounder are overfished, overfishing is still occurring, the fishery is depleted, and the species was updated to "near threatened" on the International Union for Conservation of Nature (IUCN) Red List in 2015 (Munroe 2015).

Many species of fish exhibit temperature-dependent sex determination (TSD), a phenomenon that occurs when an organism's sex can be permanently influenced by the temperature in the surrounding environment, typically occurring during a critical period of early development (Conover 1984, Ospina-Álvarez and Piferrer 2008, McNair et al. 2015). When factors other than temperature in the environment can affect an organism's sex, it is termed environmental sex determination (ESD) (Charnov and Bull 1977, Capel 2017). Fishes exhibit a great deal of plasticity in sex differentiation and sexual strategies, and as such have been shown to display variations in ESD where social and physical factors (i.e., temperature, background color, exogenous chemicals, etc.) can influence sex and produce biased sex ratios (Luckenbach et al. 2009, Mank et al. 2006, Mankiewicz et al. 2013). When the relative fitness of males and females differs across environments, flexibility in sex determination becomes advantageous (Charnov and Bull 1977, Conover 1984).

Southern flounder and other Paralichthyds exhibit environmental sex determination (Yamamoto 1999, Luckenbach et al. 2003, Colburn et al. 2009, Mankiewicz et al. 2013). These species

utilize an XX/XY genetic sex determining system and the effects of the environment on sex appear to be limited to the XX (female) genotype. If an individual is of XY genotype, they will develop as phenotypic males regardless of environmental conditions. However, those XX genetic females may differentiate into phenotypic males (XX males) based on the environmental parameters they are exposed. When juveniles are exposed to cooler or warmer water temperatures (18°C and 28°C; Luckenbach et al. 2003) or blue background color in tanks (Mankiewicz et al. 2013) during a critical early developmental stage (estimated to be 35 – 65 mm total length, TL; Montalvo et al. 2012), there is sex reversal of genetic females which leads to masculinized sex ratios. Thus, the maximum proportion of females that can occur is approximately 50%, with male-biased sex ratios possible if temperature or other variables lead to masculinization of XX females.

The primary stress hormone in fish, cortisol, has been implicated in mediating ESD effects in a variety of fish species (Hattori et al. 2009, Yamaguchi et al. 2010, Kitano et al. 2012, Mankiewicz et al. 2013, Fernandino et al. 2012, Goikoetxea et al. 2017). Rearing southern flounder in blue tanks can significantly bias sex ratios towards males and this effect is associated with elevations of whole-body cortisol levels during the developmental sex determining period (Mankiewicz et al. 2013). Exogenous cortisol administered intermittently to southern flounder during the window of sex determination also masculinizes fish (Mankiewicz et al. 2013). Although the mechanisms behind ESD are still poorly understood, glucocorticoid signaling appears to be involved by possibly interfering with aromatase and thus estrogen production (Yamaguchi et al. 2010) or by upregulating male markers like 11-KT and *hsd11b2* (Fernandino et al. 2012). It is well established that various “suboptimal” environmental conditions, e.g.

temperature, low food availability, hypoxia, rapid salinity fluctuations, pollutants, heavy metals, etc., may elicit stress responses in fishes (Bonga 1997). Therefore, it is possible that variables that regulate glucocorticoid signaling can transduce environmental influences into sex determination responses in southern flounder.

Fish are strongly influenced by climate variability with regards to reproduction, productivity, food availability, and recruitment (Walther et al. 2002). Importantly with respect to temperature-dependent sex determination, global temperatures are on the rise and sea surface temperatures are projected to increase at an average rate of 0.18°C per decade (IPCC 2014). Understanding the consequences of climate change on those species that exhibit TSD/ESD and are sensitive to temperature in their environment is critical. To date, no studies have investigated the effects of environment on juvenile sex ratios of southern flounder populations in the wild. If these fish are exposed to undesirable conditions during the sex determination window, then wild stocks could be at risk of masculinization which would negatively impact an already declining female-dependent fishery. It is currently unknown if wild juvenile southern flounder exhibit annual or geographic variation in sex ratios. Here, we examine the sex ratios of juvenile southern flounder populations across several years and in a number of nursery locations on the coast of North Carolina, USA that show consistent differences in water temperatures.

Methods

Sampling Wild Juvenile Southern Flounder

Juvenile southern flounder were collected with the assistance of North Carolina Division of Marine Fisheries (NCDMF) biologists during the routine juvenile monitoring Program 120

(P120), the North Carolina Estuarine Trawl Survey. This survey samples shallow, upper estuary locations typically in May and June using an otter trawl with a 3.2 m headrope, 6.4 mm bar mesh wings and body, and 3.2 mm bar mesh cod end. At each station, the trawl is towed for 1 min at 1.1 m/s covering ~ 69 m. We supplemented NCDMF southern flounder collections with our own sampling efforts using similar gear (otter trawl) or using a 2 m wide beam trawl depending on the habitat. Sampling was focused on collecting young of the year (YOY) age-0 southern flounder 50 mm TL and larger (this is the body size at which sex-specific molecular biomarkers begin to be expressed). These fish were collected over a wide spatial area to test the null hypothesis that there is no effect of region on sex ratio. Sampling regions included: 1) the Pamlico region, the most northern of our sampling sites including the Pamlico River and tributaries, 2) the Neuse River; an intermediate region including three sites, and 3) south of the New River, the most southern region, primarily sampled from Mill Creek and then supplemented with fish from two other nearby creeks (Figure 2, large circles indicate the three regions). We aimed to capture a minimum of 50 flounder from each of the three study regions in each of the sampling years, 2014 to 2017. Samples were also obtained in 2012 and 2013, but only from the Neuse region for these years. Fish were kept alive with a battery-operated aerator until sampling could be completed. Immediately prior to dissection, the fish were euthanized using a lethal dose of buffered MS-222 (Pentair Aquatic Eco-Systems), and then weighed (g), and measured for total length (mm TL). Whole liver tissue was removed and weighed to obtain the hepatosomatic index (HSI; (liver weight/total body weight) X 100), a useful indicator of fish health and overall energy stores reflective of recent food consumption (Busacker et al. 1990). Gonadal tissue was dissected, placed in 0.5 mL of RNAlater (Invitrogen) overnight at 4°C and then stored at -20°C

until RNA extractions and later analysis for expression of sex-specific biomarkers via quantitative real-time PCR (qPCR) to determine sex ratios.

Habitat Water Quality Monitoring

Water temperature was recorded every 2 hours using iButton temperature loggers (DS1922L - 40 to 85°C Thermochron, iButtonLink Technology, Whitewater, WI) enclosed in a waterproof housing. Temperature loggers were deployed in sampling locations and juvenile southern flounder habitat (GPS coordinates in Table 1) throughout the spring and summer (typically late March to early July) from 2014 to 2017 (Figure 2, black stars). Moored YSI datasondes (YSI models 6600 EDS or 600 XLM, Xylem, Yellow Springs, OH) were used in 2016 to monitor salinity and dissolved oxygen (D.O.) every hour in habitats located in each of our three sampling regions; Pamlico River area (Swanquarter and Germantown Bays), Neuse River area (Clubfoot and Hancock Creeks), and south of the New River (Mill and Virginia Creeks). For D.O. measurements, datasondes were maintained and cleaned of fouling every 10-14 days, at which time the data were downloaded, and instruments were re-calibrated to real-time measurements recorded from a hand-held YSI ProDSS unit. To determine if later data adjustments were necessary due to fouling or drift, the hand-held YSI was used to take D.O. readings just before servicing. This real-time data was compared to the readings from the datasonde just prior to maintenance and with the first readings after the datasonde had equilibrated after servicing. If the difference between the hand-held reading and either of the measurements prior to or after maintenance was > 0.3 mg/L (Wagner et al. 2006), then the data were adjusted using Aquarius 2.0 software (Aquatic Informatics, Vancouver, British Columbia). The Aquarius software generated a linear drift correction over time for the entire length of time the datasonde was

recording for that period. All negative values were disregarded and if any corrected D.O. values were > 2.0 mg/L away from the real-time measurements with the hand-held YSI, then those points were excluded. All data for temperature, salinity, and D.O. were averaged over a 24-hour period to generate an average daily measurement for each habitat. Temperature, dissolved oxygen, and salinity measurements were also taken at the time of juvenile fish sampling by NCDMF and our crew.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from all gonadal tissue with 1 mL of TRI-reagent and 6 µl of Polyacryl carrier (Molecular Research Center, Cincinnati, OH) using standard methods from the manufacturer, and pellets were resuspended in 20 µl of nuclease-free water. RNA was quantified by absorbance OD 260:280 ratio using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was assessed when there was adequate sample volume by presence of 18S and 28S ribosomal RNA bands using gel electrophoresis. All RNA was DNase treated (Turbo DNA-free, Invitrogen) to eliminate genomic DNA, then re-quantified and diluted to 100 ng/µl unless concentrations were already below 100 ng/µl. The size of the gonadal tissue samples varied based on the age and size of the fish, so the quantity of RNA extracted varied between individuals. Therefore, a range of 0.4 to 1.0 µg of total RNA was used in a cDNA synthesis reaction via reverse transcription (High Capacity cDNA Synthesis Kit, Applied Biosystems).

Quantitative Real-time PCR

Expression of gonadal biomarkers was used to sex individual southern flounder using qPCR and had previously been validated in this species (Luckenbach et al. 2005, Mankiewicz et al. 2013). Müllerian-inhibiting substance (*mis*) was used as a marker for male development, Forkhead transcription factor L2 (*foxl2*) and P450 aromatase (*cyp19a1a*) were used as female markers, and elongation factor-1 alpha (*ef-1α*) was used as a reference gene. Gene specific primers were used to measure the expression of these markers (GenBank accession numbers: *foxl2*: KF534720, *mis*: KF534719, *cyp19a1a*: AY902192, and *ef-1α*: AY884199). Sequences of primers used for qPCR are as follows: *foxl2* forward primer (FP): GTCCCCGCCCAAGTACCT, *foxl2* reverse primer (RP): GGCCGAGCGACCATGAG, *mis* FP: CTGCCGAGGCTCTTGCA, *mis* RP: CAGGACGGCATGGTTGATG, *cyp19a1a* FP: GGAGCCACACAGACAGGAGAA, *cyp19a1a* RP: GGCCCCAAACCCAGACA, *ef-1α* FP: CGAGAAAGAAGCTGCCGAGAT, *ef-1α* RP: CGCTCGGCCTTCAGTTTGT. qPCR analyses were performed on a BioRad CFX384 instrument using Brilliant II SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA), 1.5 µM primers, and 2 µl of 1:6 diluted cDNA in a total reaction volume of 10 µL. The qPCR cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. A dissociation melt curve step at the end was performed to verify a single PCR product. The absence of genomic DNA contamination was confirmed using water (No Template Control; NTC) and DNase-treated RNA (No-Amplification Control; NAC) as negative control templates setup during cDNA synthesis. Cycle threshold (Ct) values for samples were transformed using a standard curve of serially diluted pooled cDNA versus Ct values (R^2 = 0.96-0.99). Samples were then normalized to reflect the amount of template cDNA per ng total RNA loaded into each reaction (cDNA/ng total RNA). Additionally, we quantified expression levels of

ef-1α RNA as a reference gene, as the expression of *ef-1α* had previously been shown not to vary significantly across gonadal tissues (validated as a reference gene for southern flounder gonadal mRNA measures in Luckenbach et al. 2005). As such all data were normalized using the two methods, both to the expression of *ef-1α* and to the cDNA/ng total RNA. Both normalization methods produced similar results.

Determining Sex ratios and Statistical Analyses

All samples were analyzed for expression of *foxl2, cyp19a1a,* and *mis* using qPCR. A ratio of the levels of the three genes were established in each individual sample. Samples with relative high expression of *foxl2* and *cyp19a1a* and with low *mis* expression were assessed to be female, a sample with relative high expression of *mis* and with low levels of *aromatase* and *foxl2* was assessed to be male, and those samples with low or uncharacteristic expression of all sex biomarkers was classified as undetermined. All sex ratios were analyzed by region and compared against a predicted 1:1 sex ratio using Chi-square goodness-of-fit tests. HSI and annual temperature were analyzed using a two-way analysis of variance, ANOVA, with multiple comparisons (region by year and across years) followed by Tukey's honest significant difference (HSD) post hoc for multiple comparisons within years (HSI) and across years (annual temperature). Salinity and D.O. data for 2016 were analyzed with one-way ANOVA followed by Tukey's HSD post-hoc test. All analyses were performed using JMP (Pro v13, SAS Institute, Cary, NC) and/or GraphPad Prism 6 (GraphPad, La Jolla, CA). The threshold set for statistical significance for all analyses was $P < 0.05$.

Results

Water Quality: Temperature, Salinity, and D.O.

Temperature loggers were placed in representative NC juvenile southern flounder nursery habitats across four years, 2014 (11 probes), 2015 (13 probes), 2016 (14 probes) and 2017 (13 probes) (Figure 2). Temperature readings fluctuated daily and annually as anticipated (Figure 3). The average daily temperature across the three regions between 4/16 - 6/30 in each sampling year were: Pamlico: 2014 = 24.8 °C, 2015 = 25.6 °C, 2016 = 24.5 °C, 2017 = 25.7 °C, Neuse: 2015 = 25.6 °C, 2016 = 25.0 °C, 2017 = 26.1 °C, and South of the New River: 2015 = 27.1 °C, 2016 = 26.7 °C, 2017 = 27.4 °C (Table 2). The average maximum difference in temperature recorded between habitats was 2.8°C in 2014, 3.7°C in 2015 and 2016, and 3.3°C in 2017. Across years the habitats that consistently recorded the warmest temperatures were in the region south of the New River, i.e. Mill and Virginia Creeks (see red series in Figure 3). When average annual water temperature from two representative creeks from each region, Swanquarter and Germantown in the Pamlico, Clubfoot and Hancock in the Neuse, Virginia and Mill in the South, are analyzed from 4/16 to 6/30 from 2015 to 2017, the Pamlico region produced the coolest temperatures, the Neuse produced intermediate temperatures, and south of the New River produced the warmest temperatures (Figure 4, $P< 0.0001$). With all habitats combined, the effect of year shows the temperature for 2016 was cooler than 2015 or 2017 (Figure 4, $P< 0.0001$). In 2014, temperature loggers were not deployed early enough in all habitats to be included in analyses. Additionally, Swanquarter Bay, a site in the Pamlico region, the recorded temperature in 2014 was on average 23.2°C, in 2015 was 21.2°C, 2016 was 20.7°C, and in 2017 was 23.6°C during the period when the flounder would be in the critical sex determining window. This was based on daily growth rate calculations determined from fish sampled at two time points and

then extrapolated back to when fish would have been 35 mm (the beginning of the sex determination window). In contrast, Mill Creek, the site farthest south located in the south of the New River region, recorded average temperatures through the presumed sex determining period of 24.0°C in 2015, 24.5°C in 2016, and 26.2°C in 2017.

Salinity fluctuated throughout the 2016 season, however, Mill Creek, south of the New River, consistently recorded the highest salinity while the habitats in the Pamlico and the Neuse regions logged salinities that were more comparable (Figure 5). Mean salinity readings ranged from: Pamlico: Swanquarter at 7.8 ppt and Germantown at 8.2 ppt, Neuse: Hancock Creek at 3.6 ppt and Clubfoot Creek at 9.5 ppt, South: Mill Creek at 24.8 ppt between 3/28/16 to 6/30/16 (Figure 6A). Clubfoot Creek had significantly lower salinity and Mill Creek had the highest salinity out of all five habitats (All $P < 0.0001$; except for Germantown/Clubfoot $P = 0.004$ ANOVA). The datasonde in Virginia Creek in the southern region was not recovered and hence salinity and D.O. could not be assessed in this location. Due to fouling issues and possible mechanical concerns, after drift corrections, much of the D.O. data set is inconclusive (Figure 7). However, when the present data sets are averaged to obtain an overall mean D.O. reading for each habitat, all locations produce readings significantly different from each other ($P < 0.0001$). The D.O. ranged from Pamlico: Swanquarter at 7.76 mg/L and Germantown at 8.81 mg/L, Neuse: Hancock Creek at 7.12 mg/L and Clubfoot Creek at 5.14 mg/L, South: Mill Creek at 6.29 mg/L between 3/28/16 to 6/30/16 (Figure 6B).

Flounder HSI and Total Length

HSI was calculated as the liver weight relative to total body weight of an individual (HSI = (Liver weight/Total body weight) X 100). Values were calculated for individual samples and then tabulated into regional HSI for each year. The regional HSI values ranged from (expressed as mean ± SEM): Pamlico: 2014 = 0.87 ± 0.08, 2015 = 0.93 ± 0.05, 2016 = 0.94 ± 0.02, 2017 = 0.90 ± 0.01, Neuse: 2014 = 0.83 ± 0.03, 2015 = 1.06 ± 0.03, 2016 = 1.00 ± 0.05, 2017 = 0.97 ± 0.02, South: 2014 = 1.21 ± 0.05, 2015 = 1.42 ± 0.08, 2016 = 1.25 ± 0.02, 2017 = 1.16 ± 0.03. Over all sampling years, the region south of the New River steadily produced those flounder with the greatest HSI (Figure 8, $P < 0.0001$). In addition, the flounder collected over all sampling years from south of the New River were consistently the smallest fish over the same sampling months, with only June 2017 as an exception (Table 3). They were on average 12.2 mm TL smaller than flounder collected in the Pamlico or Neuse over all years, with a maximum difference of 20.4 mm TL smaller than the Neuse flounder in May of 2016 and a minimum of only 1.5 mm TL larger than the Neuse flounder in June of 2017. We observed two distinct size groupings of flounder in two sampling years south of the New River in Mill Creek. In May 2016, of the 57 southern flounder captured, 51 fish (37 males, 1 female, and 12 undetermined) were on average 50.1 mm TL, five fish were on average 120.0 mm TL (5 females) and one male that was 235 mm TL. In June 2016, of the 49 total southern flounder captured, 39 of fish (34 males, 2 females, and 3 undetermined) were on average 56.1 mm TL, and 10 fish (2 males and 8 females) that were on average 145.6 mm TL. Similarly, in Virginia Creek in June of 2016 there were eight fish that were on average 50.8 mm TL (7 males and 1 female), and two fish (2 females) that were on average 146.0 mm TL. In June of 2017 there were 49 fish captured that were on average 69.7 mm TL (48 males and 1 female), four fish that were on average 146.0 mm TL (2 males and 2

females), and two fish (1 male and 1 undetermined) that were on average 254.5 mm TL and were likely not age-0.

Sex Ratios of Wild Southern Flounder

All flounder were sexed first as individuals and then were grouped by region. We expect 1:1 sex ratios in all habitats if there is no effect from the environment, so all sex ratios were compared to the expected 50% male. There were significant differences from 1:1 sex ratios across regions over all years of data collection with 10 out 12 comparisons (Figure 9, Chi-square). In 2012 (n = 55) and 2013 (n = 73), samples were only collected from the Neuse river and produced sex ratios that were significantly biased toward males (88% males in 2012, 82% in 2013; $P < 0.0001$, data not shown). In 2014, Pamlico (n = 40), Neuse (n = 92), south of New River (n = 17), where 52%, 76% ($P < 0.0001$), and 88% ($P < 0.0001$) respectively were classified as male. In 2015, Pamlico (n = 76), Neuse (n = 70), south of the New River (n = 14), where 37% ($P < 0.02$), 59%, and 86% ($P < 0.007$) respectively were classified as male. In 2016, Pamlico (n = 112), Neuse (n = 99), south of New River (n = 99), where 61% ($P < 0.02$), 82% ($P < 0.0001$), and 81% ($P < 0.0001$) respectively were classified as male. In 2017, Pamlico (n = 189), Neuse (n = 105), south of New River (n = 65), where 66%, 78%, and 94% respectively were classified as male ($P < 0.0001$).

Discussion

This is the first study to show consistent and significant geographic variation in juvenile sex ratios in southern flounder from productive nursery habitats. Importantly, those habitats producing the highest proportions of males also had consistently warmer water temperatures than those with lower proportions of males. As anticipated, temperature in nursery habitats varied

annually. Temperatures recorded over an equivalent spring timeframe in all habitats were significantly higher in 2015 and 2017 compared to 2016. However, this disparity in temperature did not appear to directly correlate to differences in overall sex ratios observed in those years. The proportion of males was higher in the Pamlico and Neuse regions in 2016 despite recording a lower average annual temperature. Although all three regions produced a greater number of males in 2017, this was not the case for 2015, a year that provided a higher abundance of females in the Pamlico and Neuse regions. Temperature is a cue for spawning in southern flounder, with the cooler water temperatures of fall and winter initiating an emigration to offshore waters (Gilbert 1986, Daniels 2000). Variable temperatures during spawning could lead to interannual differences in sex ratios as it could lead to changes in the time of spawning and when larvae settle into nursery habitats, and ultimately what temperatures they are exposed to as juveniles (Lagomarsino and Conover 1993).

Temperature also varied across sampling habitats at different latitudes, with the most northern sites in the Pamlico region producing the coolest temperatures, and to south of the New River recording the warmest temperatures over all sampling years. Interestingly, the Pamlico region averaged a temperature close to the 23°C that was found to produce the highest proportion of females in laboratory trials (Luckenbach et al. 2003) during the period of sex determination (i.e. when flounder would be expected to be 35-65 mm TL). This region produced the greatest proportion of females each year, achieving the closest to 50:50 sex ratios in 2014 and was even slightly female biased in 2015. The Neuse River was more variable with male-biased sex ratios produced most years (up to 88% male) except for in 2015 when there was 59% male. The region south of the New River consistently produced male-biased sex ratios over all years (81% to 94%

male). Some of the first studies to examine TSD in fish were completed in the Atlantic silverside (*Menidia menidia*) and to date, effects of the environment on sex have been extensively studied in this species in the laboratory and the field (Conover and Kynard 1981). Studies show a correlation between latitude and the degree of ESD observed in *M. menidia*, with ESD more prevalent in northerly latitudes where the length of the growing season declines (Conover and Heins 1987, Lagomarsino and Conover 1993). Spawning for southern flounder begins earlier at more northerly latitudes and moves southward (Gilbert 1986). This would suggest that flounder larvae would settle earlier into those northern environments like the Pamlico, compared with the southern habitats. Although the fish in these studies were not aged, potential evidence for this pattern may be the overall smaller body size in the southern region, suggesting that they could hypothetically be younger and would be reaching the critical sex determining window later in the summer when water temperatures are much higher and less likely to promote female development, a similar phenomenon previously shown to occur for *M. menidia* (Conover and Kynard, 1981). Discriminating both the age and sex of YOY juvenile southern flounder spatially and temporally could more accurately determine the timing and impact of nursery habitat temperature on sex determination.

Another possibility for the smaller size of the flounder collected south of the New River is that the fish in this region could grow more slowly, thereby extending the window over which they are sensitive to their environment. The growth rates observed in NC populations of southern flounder can range wildly, from 0.35 to 1.5 mm per day and can vary regionally (Fitzhugh et al. 1996). The differential growth observed between juveniles across regions could be due to food size and availability, although southern flounder that spawned earlier were not shown to be

larger or faster growing than those spawned later in the season (Fitzhugh et al. 1996). Female southern flounder have been shown to grow faster and larger than males, but typically this sexual dimorphic growth is not apparent until later when body sizes are around 200 mm TL (Fitzhugh et al. 1996). Therefore, sex differences in growth would not account for size disparities of smaller juveniles. Although smaller in body size, the fish in our southern study region did appear to be healthy and eating well based on higher calculated HSI's than observed in other regions over all sampling years. Fish store energy in the form of glycogen in their liver tissue, which can be utilized when food sources are scarce or can be rapidly mobilized in times of stress (Bonga 1997). Therefore, it seems unlikely that the lack of food availability is responsible for the smaller body size of fish in southern regions.

Age-0 southern flounder in South Carolina (SC) have been shown to have bimodal distributions in length at 50 and 140 mm TL in June (Wenner et al. 1990). This phenomenon is similar to what we observed over multiple years south of the New River and could suggest the timing of spawning and juvenile growth in this region are more comparable to that of SC populations. For example, in 2016 we captured two size groups of southern flounder and they were on average 56.1 mm TL and 145.6 mm TL. Although we did capture larger age-0 flounder in the Pamlico and the Neuse regions, there was not the clear presence of two distinct size groupings as observed in the south. Previous studies show bimodal length distributions in age-0 NC southern flounder were shown to be independent of sex, generally occurred around 75 to 100 mm TL, and was likely due to a change to a piscivorous diet (Fitzhugh et al. 1996). Interestingly, female flounder were more prevalent in the larger size grouping of fish we captured south of the New River, suggesting these fish determined sex at a more optimal time for female development.

Hypoxia and rapid salinity changes can induce the stress response in fish (reviewed in Bonga 1997). Shallow estuarine nursery habitats such as those in the Neuse River can be susceptible to hypoxia, especially during hot summer months (Paerl et al. 1998). Although our D.O. readings over time produced inconclusive data sets, the mean D.O. values varied across the locations measured and there was no apparent correlation between D.O. and variations in regional sex ratios. However, this is a parameter that should be re-evaluated in the future. Southern flounder is a euryhaline species and can tolerate a wide range of salinities (Daniels et al. 1996, Smith et al. 1999). Larviculture of southern flounder typically occurs with near full-strength seawater (33 ppt) and can be slowly dropped after metamorphosis (Daniels and Watanabe 2002). Previous studies on ESD with post-metamorphic southern flounder utilized varying salinity profiles including 0 ppt (Luckenbach et al. 2003) and gradual declines from 22 to 0.7 ppt and from 24 to 4 ppt (Mankiewicz et al. 2013). None of these salinity regimes significantly altered juvenile sex ratios. South of the New River salinity was significantly higher (24.8 ppt) compared to the Pamlico and Neuse regions. This is not surprising given the habitats south of the New River have a closer proximity to the Atlantic Ocean, while the Neuse and Pamlico habitats have the expansive barrier of the Pamlico Sound between them and the higher saline waters of the ocean. Salinity in the Pamlico habitats did not exhibit major fluctuations and did not show significant differences between the habitats with mean salinity measurements of Swanquarter at 7.8 ppt and Germantown at 8.2 ppt. The locations in the Neuse recorded salinities that were lowest in Hancock Creek at 3.6 ppt, and in Clubfoot Creek the salinity was higher with an average of 9.5 ppt, but also had greater variability. Clubfoot and Hancock Creeks in the Neuse both produced male-biased sex ratios and have similar temperature regimes, yet different salinity profiles. Overall, we found that environments with high, low, and fluctuating salinities all produced male-

biased sex ratios. This suggests that any salinity profile alone likely does not account for the regional differences we observed in sex ratios or that the effects of temperature likely outweighed those of salinity.

The sex ratio of the spawning population may be influenced by ESD not just in NC nurseries, but in habitats across regions (and states) that contribute to the spawning stock of the population. The southern flounder fishery has long been exploited and overfished, and complex coastwide management strategies have been implemented in an attempt to save this lucrative fishery (NCDMF 2017). An increase in the number of males developing with fewer reproductive females could contribute to a decline in the breeding population. The southern flounder fishery may be especially susceptible to decline since it is dependent on the larger growing females. The male biases we observed in juvenile populations therefore present a potential problem with management of the fishery. This may be particularly true because the current population models of spawning stock biomass assume 50:50 sex ratios (Takade-Heumaker and Batsavage 2009).

Warm temperatures have been strongly correlated to masculinized sex ratios in southern flounder in fish reared in tanks (Luckenbach et al. 2003) and now in natural populations. With global temperatures rising due to climate change many species will potentially be impacted, especially those that exhibit TSD (Ospina-Álvarez and Piferrer 2008, Kallimanis 2010). Loss of suitable habitat, possible shifts in distribution and larval dispersal, and changes to larval development time have been suggested (Perry et al. 2005, Ospina-Álvarez and Piferrer 2008, IPCC 2014). These types of shifts can already be observed in NC, where rising water temperature and salinity have been implicated in altering the distribution of juvenile Bull Sharks, with an increased usage

of nursery habitats farther north in the Pamlico Sound in North Carolina (Bangley et al. 2018). The potential impacts of climate change on reptiles, of whom many species also exhibit TSD, have been studied and suggested that even increases in mean temperatures of less than 2°C could severely skew sex ratios (Janzen 1994). For the case of sea turtles, cool temperatures tend produce males and warm temperatures produce more females, so global warming could feminize sea turtle populations (Janzen 1994). A recent study on sex ratios of sea turtles near the Great Barrier Reef showed variation in sex ratios across regions, with mild female-biased ratios in cooler, southern beaches and extreme female-biased sex ratios in warmer more northern nesting sites (Jensen et al. 2018). This latitudinal variation in sex ratios of natural turtle populations shows a response due to TSD in a warming environment similar to what we observed across a temperature cline in southern flounder. This phenomenon may not be limited to turtles and southern flounder and should be investigated for other species that exhibit TSD.

Overall, our findings indicate there is consistent variation in the water temperatures across nursery habitats in North Carolina that are inhabited by juvenile southern flounder during the critical period of development for ESD/TSD. These temperature differences are associated with variation in juvenile sex ratios across regions. Warmer nursery habitats in southern NC produced significantly more male southern flounder compared to those environments farther north. These data suggest that variation in nursery habitat water temperatures may be a critical factor driving regional differences in juvenile southern flounder sex ratios. With a fishery dependent on females and global ocean temperatures projected to significantly increase, these temperature effects on sex ratios could be a significant concern for wild flounder stocks. These are the first

studies to provide evidence of temperature-dependent sex determination affecting a key demographic parameter in wild populations of an important fishery species.

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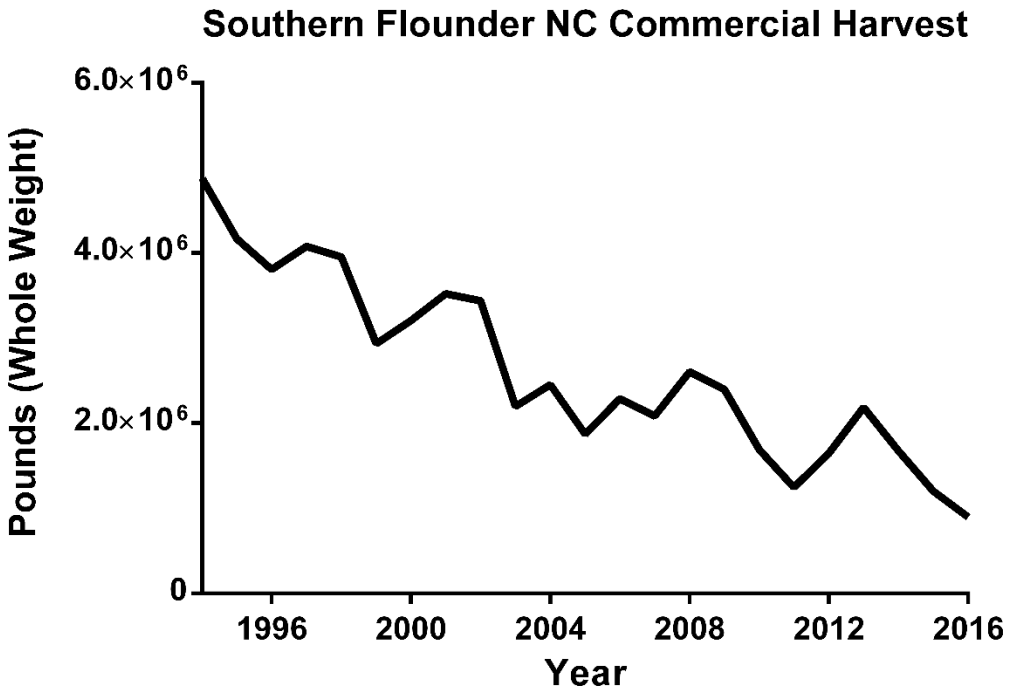

Figure 1. Southern flounder commercial harvest amounts in pounds caught (whole weight) in North Carolina from 1994 to 2016 (Data from North Carolina Division of Marine Fisheries, NCDMF 2016).

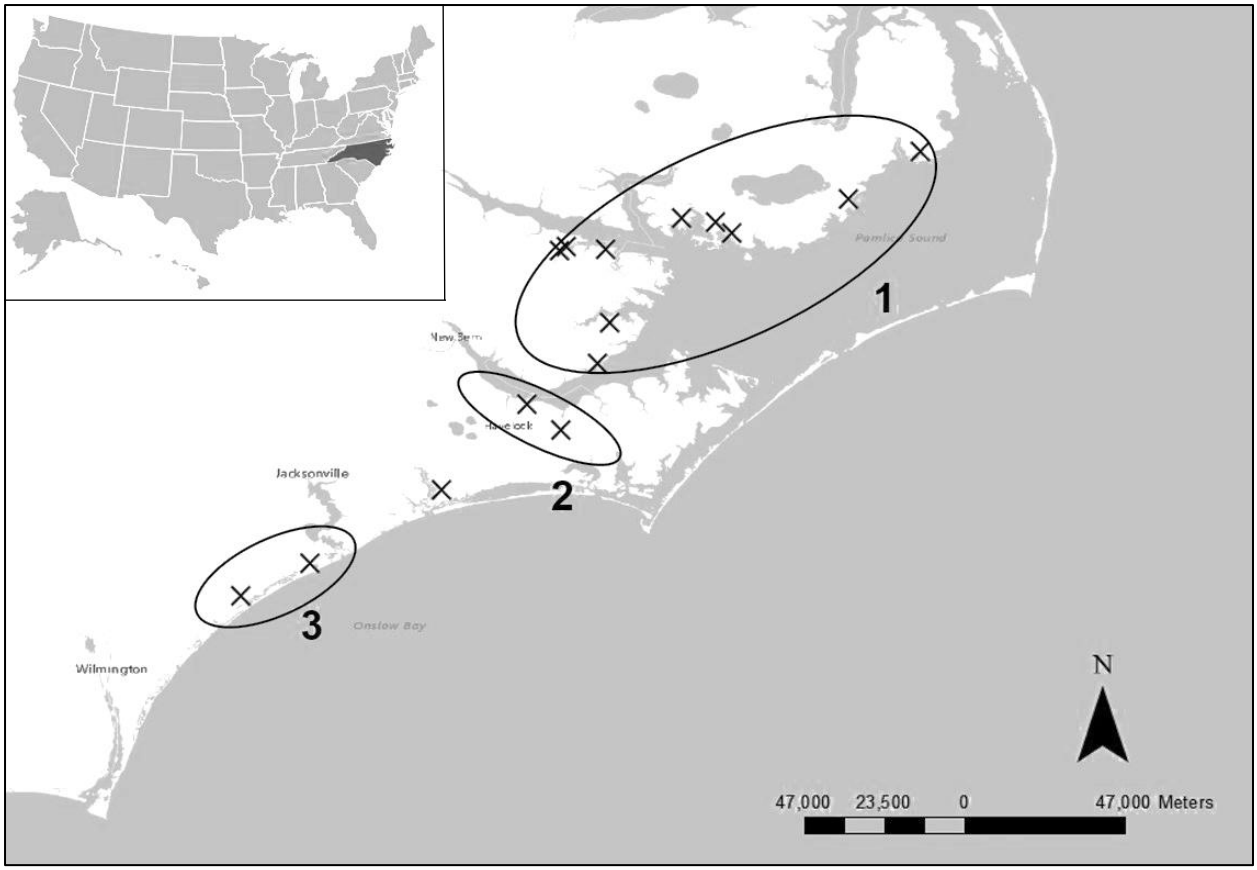

Figure 2. Map showing the location of temperature probes deployed in North Carolina juvenile southern flounder nursery habitats from 2014 to 2017. "X" marks the location of a single temperature logger. The circles indicate the three regions that sites were grouped: 1) Pamlico (northern), 2) Neuse (intermediate), 3) south of the New River (south).

Table 1. GPS Coordinates for temperature probes deployed in juvenile southern flounder habitats in North Carolina from 2014 to 2017.

Location	NCDMF Station #	Region	Water Body	Latitude	Longitude
Middletown Creek	FC3	Pamlico	Far Creek	35.474	-76.008
Pains Bay	LSR5	Pamlico	Long Shoal River	35.598	-75.818
Germantown Bay	SB5	Pamlico	Germantown Bay	35.422	-76.446
Swanquarter Bay	SQB3	Pamlico	Swanquarter Bay	35.385	-76.312
Shingle Creek	SQB1	Pamlico	Swanquarter Bay	35.414	-76.357
Strawhorn Creek	PAR16	Pamlico	South Creek	35.340	-76.644
Tooley Creek	PAR27	Pamlico	South Creek	35.347	-76.749
Jacobs Creek	PAR31	Pamlico	South Creek	35.336	-76.765
Ball Creek	CS2	Pamlico	Bay River	35.148	-76.636
Pierce Creek	F3N	Pamlico/Neuse	McCotter Bay	35.043	-76.666
Clubfoot Creek	H2	Neuse River	Neuse River	34.865	-76.762
Hancock Creek	NR11	Neuse River	Neuse River	34.935	-76.852
White Oak Narrow	CC1	White Oak River	White Oak River	34.709	-77.076
VA Creek	VC1	South of the New River	Virginia Creek	34.433	-77.606
Mill Creek	SSI1	South of the New River	Mill Creek	34.518	-77.424

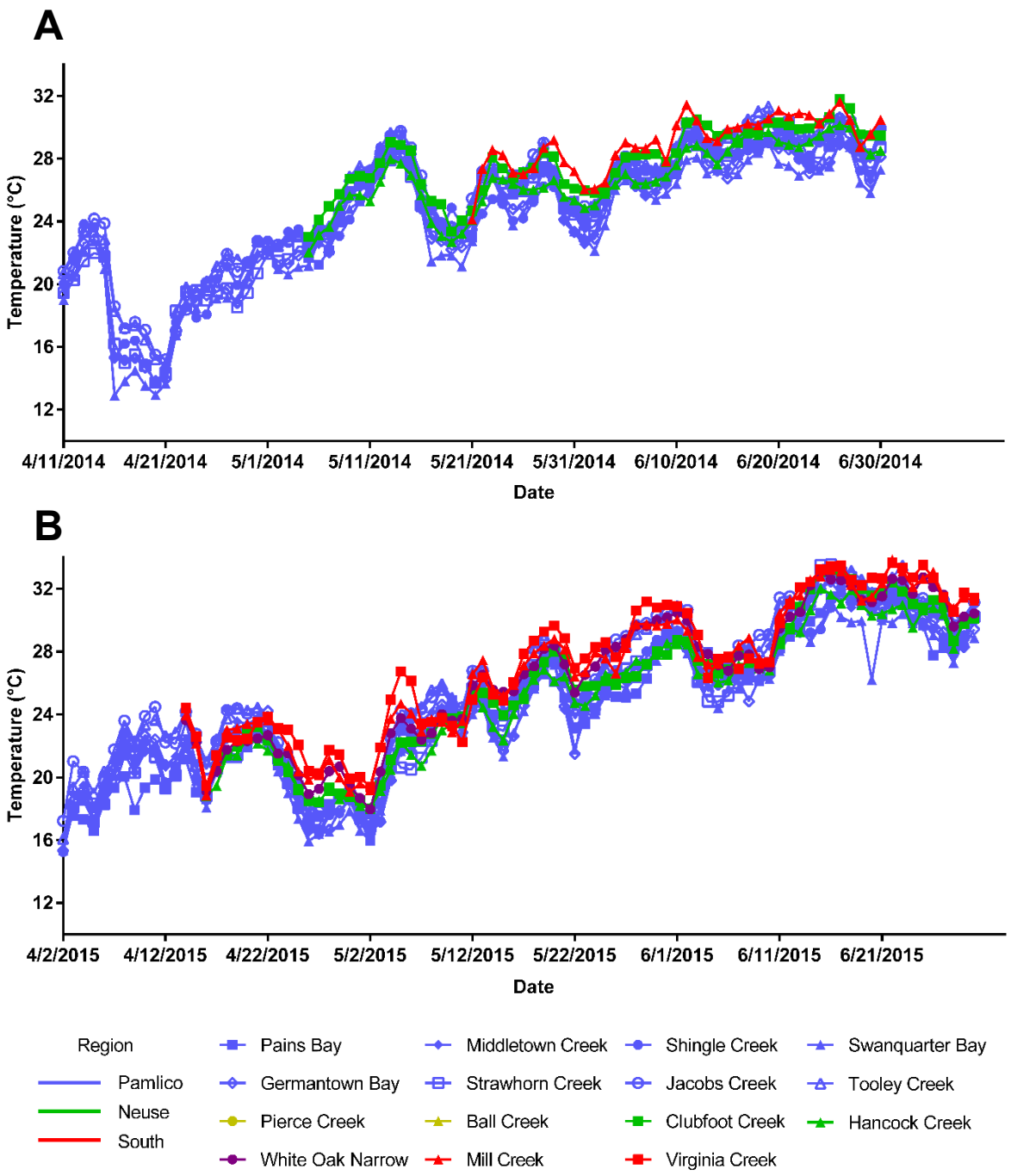

Figure 3. Average daily water temperature from juvenile southern flounder habitats over four years, (**A**) 2014 (4/11/14 – 6/30/14), (**B**) 2015 (4/2/15 – 6/30/15), (**C**) 2016 (3/23/16 – 6/30/16) and (**D**) 2017 (3/30/17 – 6/30/17). Data was recorded with iButton data logger every 2 hours and temperature readings were averaged over a 24-hour period.

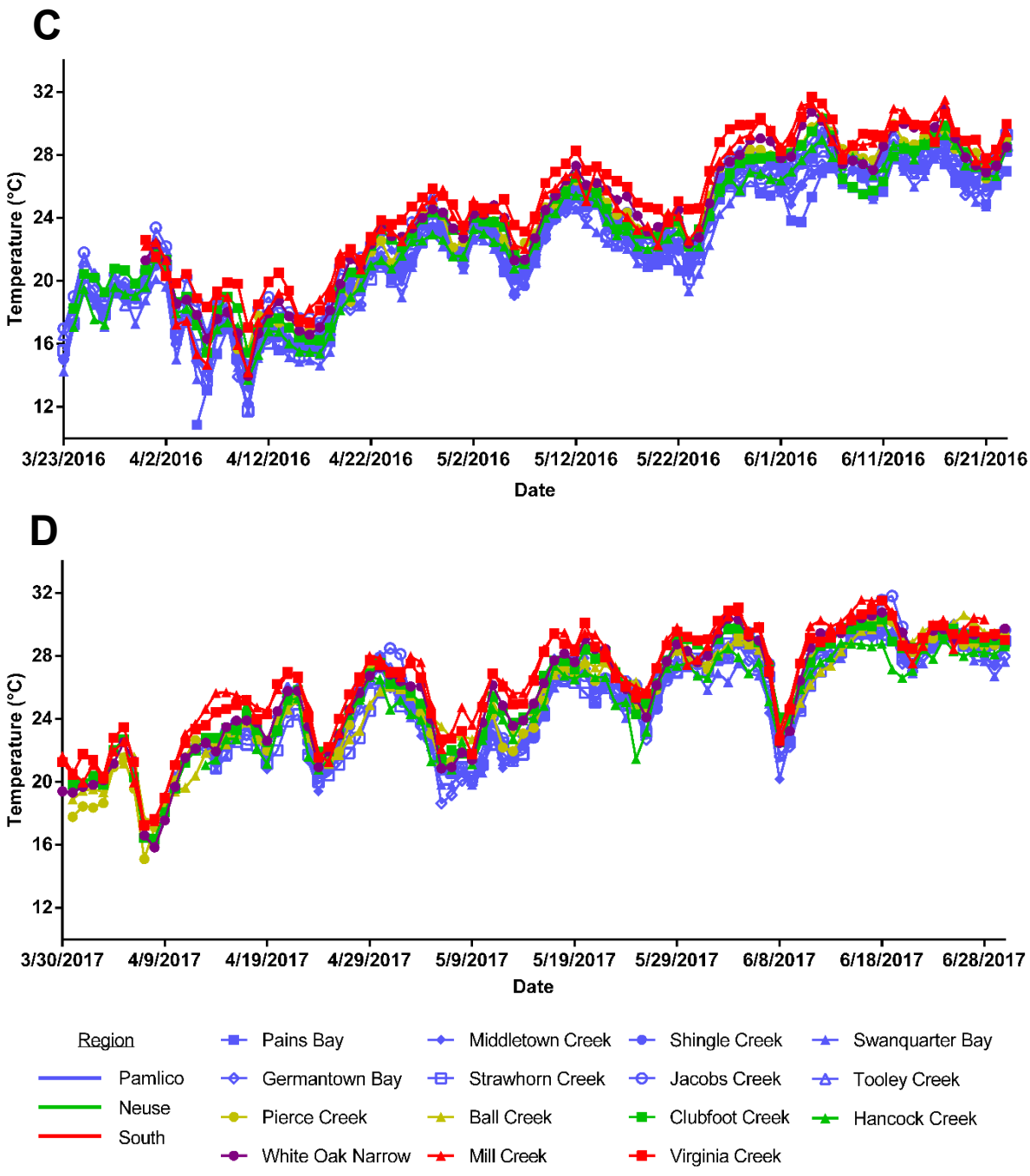

Figure 3 continued. Average daily water temperature from juvenile southern flounder habitats over four years, (**A**) 2014 (4/11/14 – 6/30/14), (**B**) 2015 (4/2/15 – 6/30/15), (**C**) 2016 (3/23/16 – 6/30/16) and (**D**) 2017 (3/30/17 – 6/30/17). Data was recorded with iButton data logger every 2 hours and temperature readings were averaged over a 24-hour period.

Table 2. Average daily temperature readings from various NC southern flounder nursery habitats from 4/16 – 6/30 from 2014 to 2017. For 2014, many of the sites were not included as the loggers were deployed later in the season so the average temperature did not span the entire period from 4/16 – 6/30.

Location	**Avg. temperature across years (°C)**			
	2014	**2015**	**2016**	**2017**
Pains Bay	—	25.2	23.9	—
Middletown Creek	—	25.8	24.9	25.9
Shingle Creek	24.7	25.4	24.5	—
Swanquarter Bay	23.8	24.6	23.7	25.2
Germantown Bay	24.2	25.2	24.3	25.4
Strawhorn Creek	24.8	25.6	24.7	25.4
Jacobs Creek	25.5	26.7	25.3	26.1
Tooley Creek	25.6	26.6	25.0	26.0
Pierce Creek	—	—	25.6	26.2
Ball Creek	—	—	—	26.3
Clubfoot Creek	—	25.9	25.4	26.6
Hancock Creek	—	25.4	24.7	25.5
White Oak Narrow	—	26.5	25.9	26.7
Mill Creek	—	27.3	26.9	27.5
Virginia Creek	—	27.0	26.5	27.3
Pamlico Avg.	24.8	25.6	24.5	25.7
Neuse Avg.	—	25.6	25.0	26.1
South Avg.	—	27.1	26.7	27.4

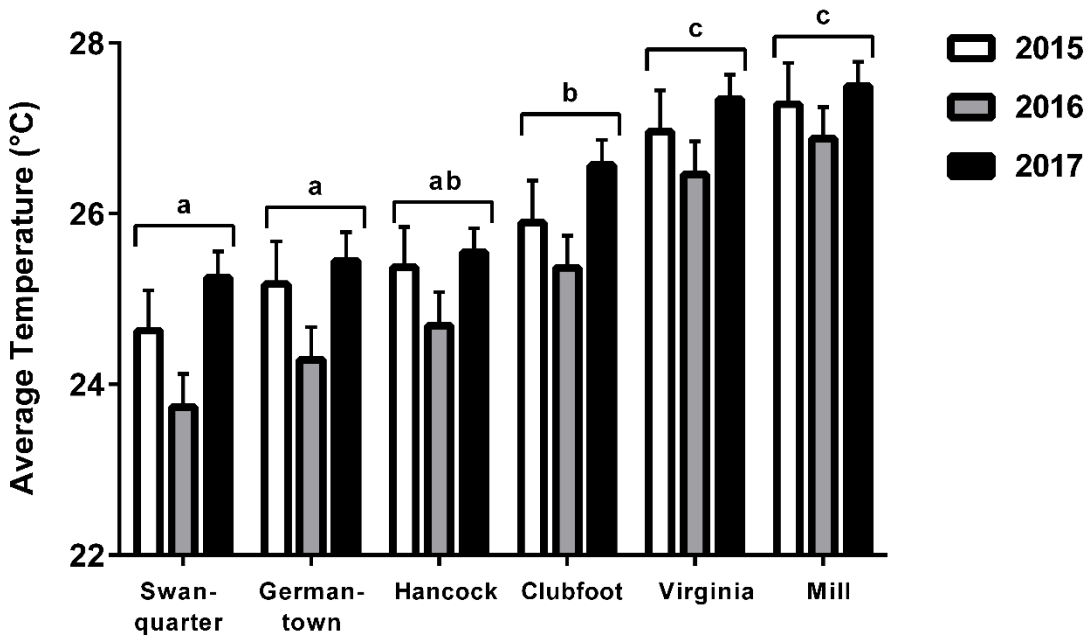

Figure 4. Average annual water temperature from representative NC juvenile southern flounder habitats in years 2015, 2016, and 2017 (4/16 to 6/30). Temperature was recorded every 2 hours and readings were averaged for each 24-hour period. Letters indicate differences between habitats over the three years taken together ($P < 0.0001$). With all habitats combined the effect of year was significant for 2016 ($P < 0.0001$).

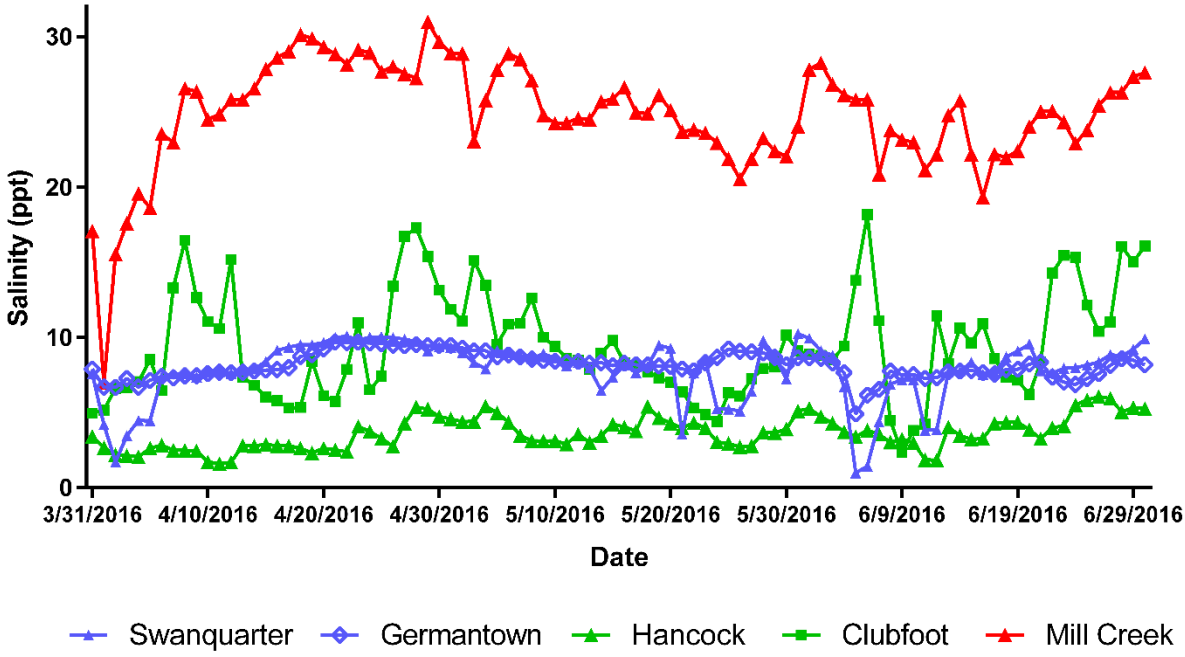

Figure 5. Average daily salinity (ppt) measurements recorded hourly with in five NC juvenile southern flounder habitats from 3/31/16 to 6/30/16.

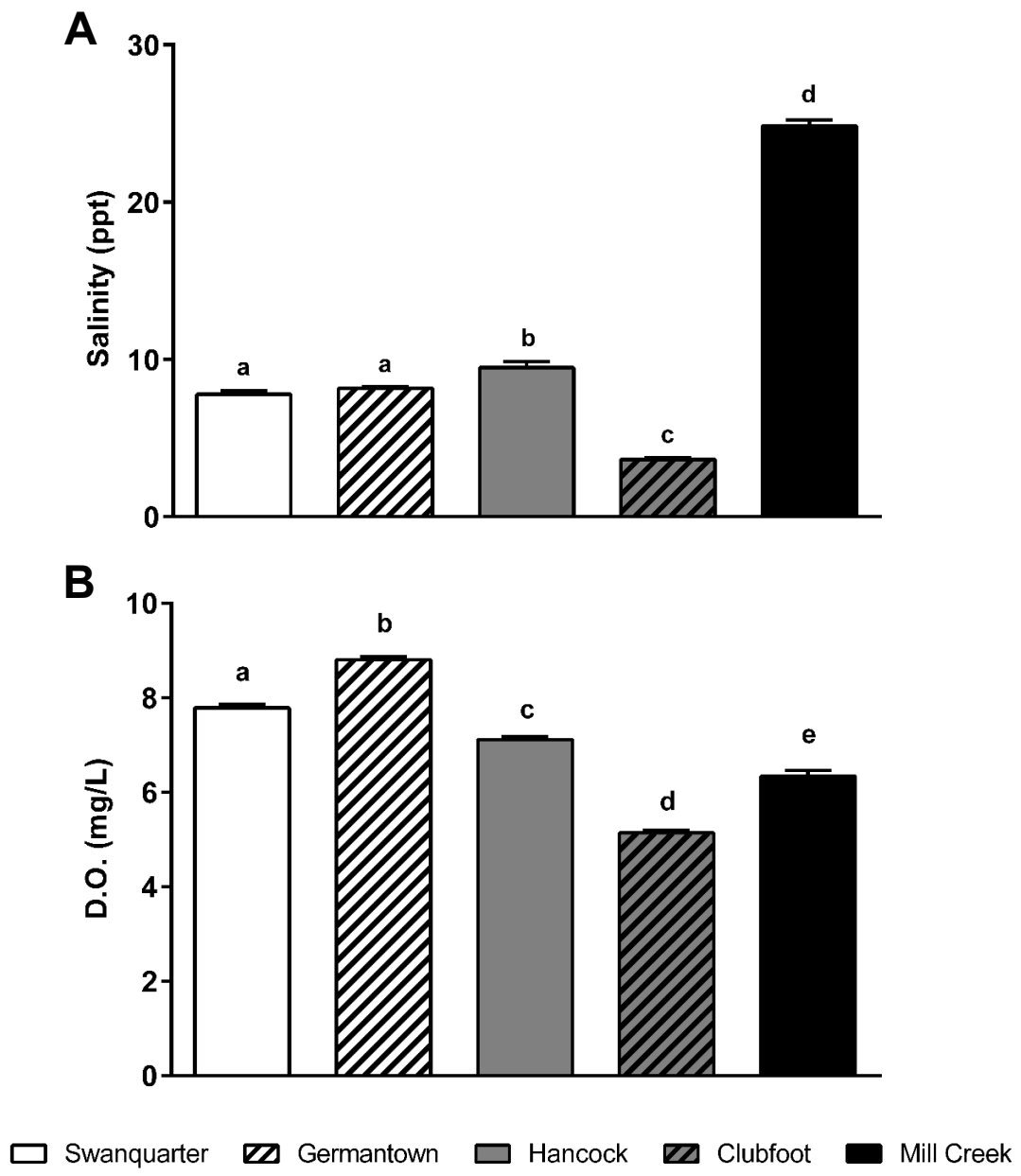

Figure 6. Averaged water quality from juvenile southern flounder habitats in 2016. All measurements recorded hourly with in five NC juvenile southern flounder habitats, Pamlico region: Swanquarter and Germantown, Neuse region: Hancock and Clubfoot, South region: Mill Creek, from 3/28/16 to 6/30/16 and then averaged daily and over the season. (**A**) Average daily salinity (ppt) and (**B**) average daily dissolved oxygen, D.O. (mg/L). Letters indicate significance between regions (All $P < 0.0001$; except salinity for Germantown/Clubfoot $P = 0.004$).

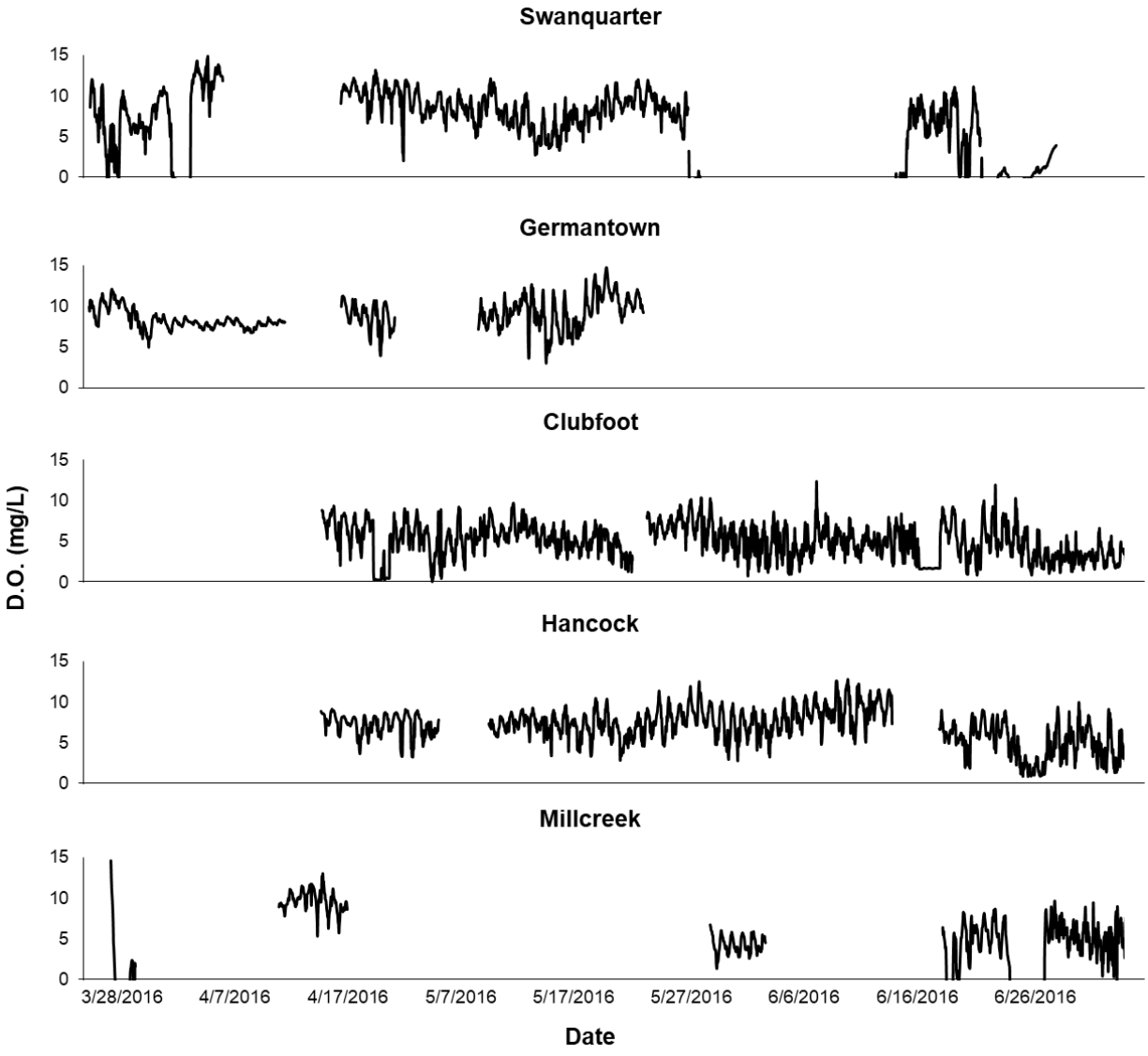

Figure 7. Average daily dissolved oxygen (D.O., mg/L) measurements recorded hourly in five NC juvenile southern flounder habitats from 3/31/16 to 6/30/16

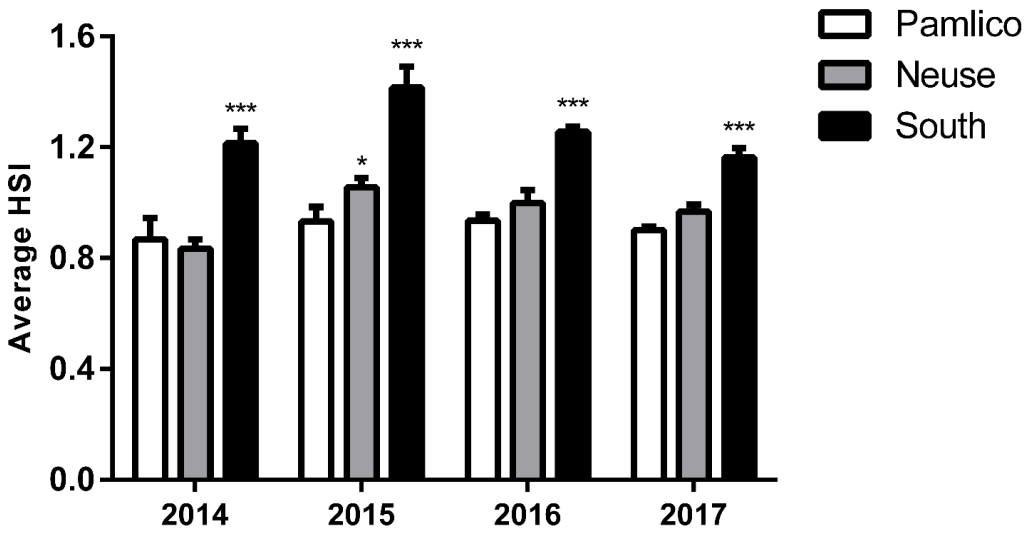

Figure 8. Average hepatosomatic index (HSI) for NC juvenile southern flounder across three sampling regions; Pamlico (northern), Neuse (intermediate), and South, over four years from 2014 to 2017 (*P = 0.02, ***$P \leq 0.0003$). HSI = (Liver weight/Total body weight) X 100.

Table 3. Average length (mm TL) and number of juvenile southern flounder collected from 2014 to 2017 in three regions: Pamlico (north), Neuse (intermediate), and South.

Region	Date	# Fish	Avg. mm TL
Pamlico	June, 2014	41	83.8
Neuse	June, 2014	53	88.0
Neuse	July, 2014	41	105.9
South	June, 2014	18	74.2
Pamlico	May, 2015	85	68.2
Neuse	May, 2015	80	68.3
Neuse	June, 2015	73	84.1
South	June, 2015	15	68.0
Pamlico	May, 2016	59	81.2
Pamlico	June, 2016	58	85.8
Pamlico	July, 2016	4	98.5
Neuse	May, 2016	60	85.4
Neuse	June, 2016	40	93.3
Neuse	July, 2016	39	87.7
South	May, 2016	67	65.0
South	June, 2016	49	74.4
Pamlico	May, 2017	63	76.5
Pamlico	June, 2017	66	85.7
Pamlico	July, 2017	32	79.4
Neuse	May, 2017	28	77.2
Neuse	June, 2017	81	80.5
South	May, 2017	11	64.1
South	June, 2017	55	82.0

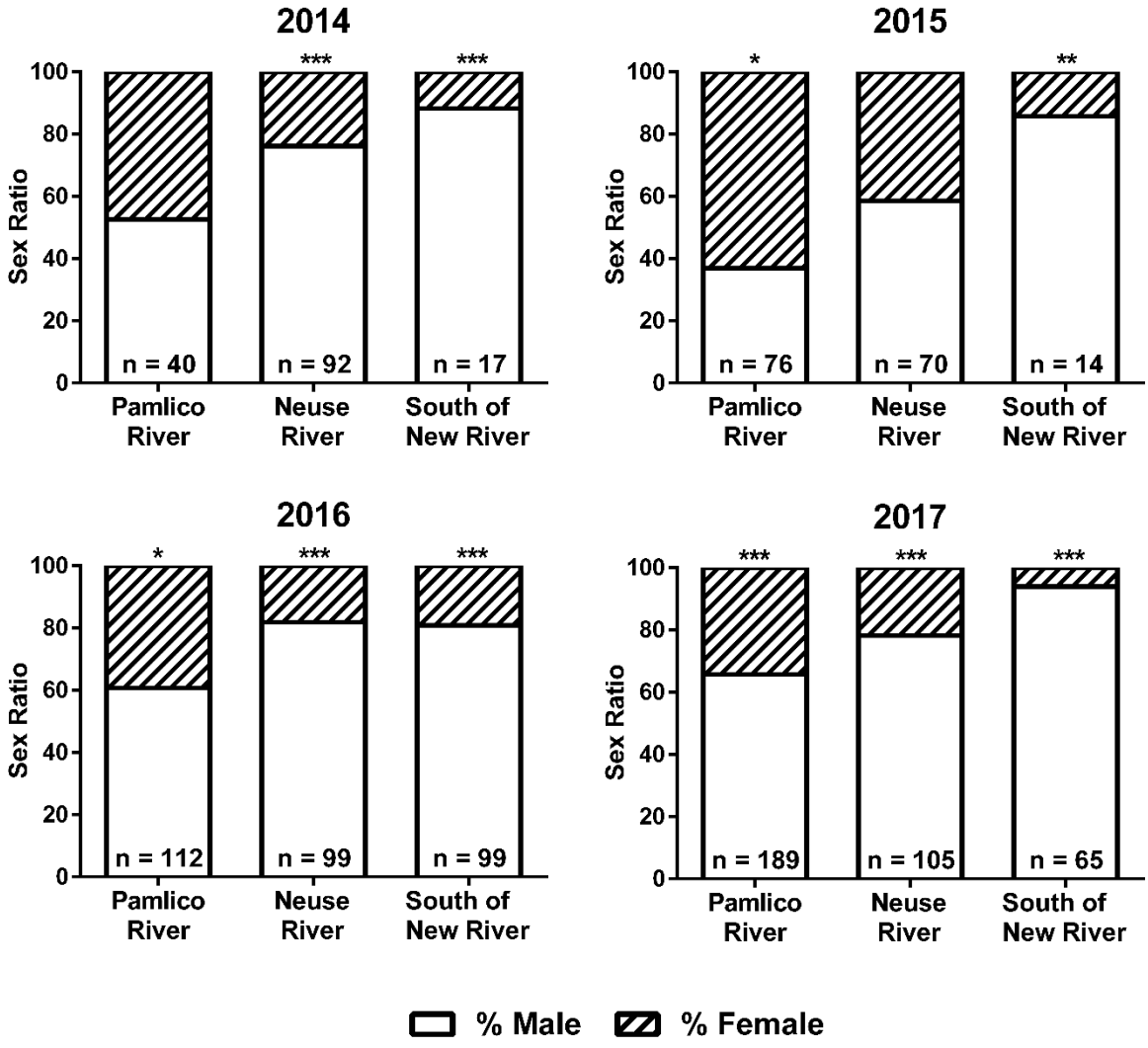

Figure 9. Sex ratios of juvenile southern flounder from different sampling regions Pamlico River (northern), Neuse River (intermediate), and South of the New River (southern) in NC, from 2014 to 2017. Sex ratios were determined by measuring gonadal gene expression of male (*mis*) and female (*cyp19a1a* and *foxl2*) via qPCR. 2014 ***$P < 0.0001$, 2015 *$P = 0.02$, **$P = 0.007$, 2016 *$P = 0.02$, ***$P < 0.0001$, and 2017 ***$P < 0.0001$; all P represent significant deviations from a 50:50 sex ratio.

CHAPTER IV

Effects of fluctuating temperatures on sex ratios of juvenile southern flounder

Abstract

Southern flounder (*Paralichthys lethostigma*) exhibit environmental sex determination (ESD), where environmental factors can influence phenotypic sex during early juvenile development. Temperature extremes and background color can masculinize fish with ideal conditions at 23°C producing at most 50% females and is limited to the XX genotype (female). Females grow faster and to larger size due to sexually dimorphic growth, so southern flounder fisheries are consequently dependent upon females. With climate change and global temperatures on the rise, wild populations could be at risk of masculinization because of implications from ESD. Previous studies evaluated the effects of habitat and temperature on wild populations of juvenile southern flounder in North Carolina, USA, from 2014 to 2017. Findings show that while northern habitats averaged optimal temperatures near 23°C during the sex-determining window and produced the greatest abundance of females, southern habitats consistently produced male-biased sex ratios (up to 94% male) and were associated with warmer temperatures. Temperature profiles recorded in different nursery areas experienced similar patterns of temperature fluctuation over time within sites yet showed relatively consistent differences in temperature that averaged a maximum difference near 4°C among sites. Other water quality parameters such as salinity and dissolved oxygen did not appear to have a correlation with male skewed sex ratios of wild populations, suggesting that temperature is likely the key factor influencing sex ratio bias across habitats. In one northern nursery habitat that produced 1:1 sex ratios, Swanquarter Bay, the average temperature over the sex determination period was 23.2°C, consistent with the permissive temperature that yields 1:1 sex ratios in the laboratory under constant rearing temperatures. Therefore, we tested the hypothesis that patterns of temperature variation observed in NC nursery habitats that produce male-biased juvenile sex ratios are sufficient (by themselves) to

generate the skewed sex ratios observed. We tested this by mimicking these temperature profiles under controlled laboratory conditions while other variables were held constant. We tested a 4°C increase or decrease under the naturally fluctuating temperature profile of the 2014 Swanquarter nursery habitat (23°C). Treatments included fluctuating temperatures that averaged 19°C, 23°C, and 27°C, as well as a 27°C constant temperature. Using a gonadal biomarker technique previously validated, we determined sex ratios produced by these treatments were 83%, 65%, 100% male for the 19°C, 23°C, and 27°C fluctuating temperatures respectively. The 27°C constant temperature system produced a sex ratio that was 98% male. (n = 48 per treatment, Chi-square: 23°C $*P = 0.04$, all others $***P < 0.0001$; all *P* represent significant deviations from a 50:50 sex ratio). Although the sex ratio observed in the 23°C fluctuating treatment was mildly male biased (65% male), it produced more females than any other treatment, and is comparable to what was observed in wild populations of similar temperature profiles. The 27°C fluctuating system produced 100% male flounder which is consistent with sex ratios observed in southern sampling habitats that produced sex ratios of up to 94%. Specific growth rates (SGR) did not differ between the 23°C and 27°C fluctuating systems, however, the 19°C system had the lowest SGR and the constant 27°C had the highest SGR. Juvenile female southern flounder were on average 12 mm longer in total length (TL) than males across all treatments, however, the SGR from those treatments that reared females did not differ significantly from males. These studies show that when southern flounder juveniles are reared in experimental temperature regimes mimicking the natural profiles of juvenile habitats, the variation in sex ratios is consistent with that observed in natural habitats. This result is consistent with temperature being the key factor influencing male-biased sex ratios in wild nursery habitats.

Introduction

Southern flounder (*Paralichthys lethostigma*) exhibit a phenomenon called environmental sex determination (ESD), where phenotypic sex can be influenced by factors in the environment during a critical period of early development (Charnov and Bull 1977, Conover 1984, Luckenbach et al. 2009). Teleost fishes display a great deal of sexual plasticity and wide range of sex determination patterns including those in which sex is determined by cues from the physical and social environments (Ghislen 1967, Devlin and Nagahama 2002, Mank and Avise 2009, Liu et al. 2017). There are variations of ESD and temperature-dependent sex determination (TSD), where animals can exhibit TSD/ESD or genetic sex determination (GSD) with temperature effects (GSD + TE) (Ospina-Álvarez and Piferrer 2008, Shen and Wang 2014, Capel 2017). Southern flounder, like other Paralichthyds, appear to use a combination of genotypic and environmentally determined systems, where an animal with an XY genotype is a genetic male and XX is female, however, phenotypic sex in these XX fish can be influenced by the environment (Yamamoto 1999, Luckenbach et al. 2003, Colburn et al. 2009, Mankiewicz et al. 2013). If a flounder is of XY genotype, then they will develop as a male regardless of environmental conditions. However, if an individual is of XX genotype and they are exposed to conditions unfavorable for female development, they instead can differentiate into phenotypic males (Luckenbach et al. 2009). Previous studies have shown that the primary stress hormone in these fish, cortisol, is likely regulating the sex reversal (Yamaguchi et al. 2010, Mankiewicz et al. 2013). If exposed to conditions that would stimulate the stress axis and thus cortisol during the sex determining window (35 – 65 mm total length, TL), then masculinization of XX females can occur. When rearing occurs with optimal parameters at 23°C, mixed XX/XY populations can achieve at most 50% females. Conditions not favorable for female development include

temperature extremes (18°C and 28°C; Luckenbach et al. 2003), blue background color (Mankiewicz et al. 2013), or likely any environmental parameter that would activate the stress axis. It is well established that numerous environmental conditions such as temperature, low food availability, hypoxia, and rapid salinity fluctuations may elicit stress responses in fishes (Bonga 1997). In addition to temperature, both PH (cichlids, Römer and Beisenherz 1996) and hypoxia (zebrafish, Shang et al. 2006) have also been implicated in regulating sex determination and differentiation in fish.

Southern flounder display sexually dimorphic growth, with female flounder growing faster and larger relative to males (Fitzhugh et al. 1996). Minimum size limits are used as a management strategy for the southern flounder commercial and recreational fisheries, and thus these fisheries are primarily dependent upon the largest female fish (Takade-Heumaker and Batsavage 2009). Southern flounder fisheries have been deemed overfished and near-threatened (NCDMF 2005, Munroe 2015). If wild populations of southern flounder are sensitive to their environment and susceptible to sex reversal via ESD, then flounder stocks could become masculinized with exposure to warm water temperatures and directly impact the declining female-dependent fisheries. From previous studies, we show significant masculinization of North Carolina (NC) young of the year (YOY) juvenile southern flounder populations consistently over years (2014 to 2017; see Chapter 3), with the most southern habitats recording the warmest temperatures and producing the greatest number of males. Although the sex determining window is thought to occur between 35 and 65 mm TL (Montalvo et al. 2012), gonadal differentiation generally occurs between 75 and 120 mm TL, and fish cannot be accurately sexed via histological methods until 120 mm TL (Luckenbach et al. 2003). Sex of juvenile flounder is typically assessed by visually

inspecting the gonad and sexing them macroscopically. Given this challenge, it is difficult to sex flounder under 120 mm TL to determine sex ratios of juvenile populations by traditional methods. Because of this difficulty in assessing juvenile sex ratios, most fishery models assume a 1:1 juvenile sex ratio. We have previously developed and validated a molecular gonadal biomarker technique that allows us to sex individuals at much smaller sizes, eliminating additional time and cost of grow-out periods in experiments (Mankiewicz et al. 2013). In addition, using this approach southern flounder can be captured and sexed from their juvenile habitats, prior to the body size when they move out and mix with other populations in deeper waters. This allows sex ratios to be evaluated for juveniles across a range of nursery habitats.

When temperature is held constant in tanks, southern flounder exhibit sex reversal to males at both 18°C and 28°C, while 23°C is the optimal temperature for female development (Luckenbach et al. 2003). Nevertheless, water temperature is a dynamic variable and does not remain constant in natural environments, instead fluctuating both daily and annually. It has been suggested that studies where temperatures are held constant may not be ideal for modeling natural environments (Kingsolver et al. 2015). As previously described, we observed significant masculinization of juvenile southern flounder in natural nursery habitats and a latitudinal variation in sex ratios, with northern habitats producing the most females and the most southern and warmest habitats producing the greatest number of males (Chapter 3). Temperature profiles recorded in different nursery areas exhibited similar patterns of temperature fluctuation over time yet showed relatively consistent differences in temperature that averaged a maximum difference near 4°C among sites. In one northern nursery habitat site that produced 1:1 sex ratios, Swanquarter Bay, the average temperature over the sex determination period in 2014 was

23.2°C, consistent with the permissive temperature that yields 1:1 sex ratios in the laboratory (Luckenbach et al. 2003). Other water quality parameters such as salinity and dissolved oxygen did not appear to have a correlation with male skewed sex ratios of wild populations, suggesting that temperature is likely the key factor influencing sex ratio bias across habitats. Therefore, we tested the hypothesis that patterns of temperature variation observed in NC nursery habitats that produce male-biased juvenile sex ratios are sufficient (by themselves) to generate these skewed sex ratios. By mimicking these temperature profiles under controlled laboratory conditions while other variables are held constant, we examined the effects of fluctuating temperature alone. We experimentally assessed whether a 4°C increase or decrease from the naturally fluctuating temperature profile we observed in the Swanquarter nursery habitat could produce male-biased sex ratios in flounder reared in recirculating systems.

Methods

Experimental Design

To test whether temperature profiles of NC nursery habitats that produced male-biased juvenile sex ratios are sufficient to generate these sex ratios, we mimicked these temperature profiles under controlled laboratory conditions while holding other variables constant. Southern flounder were spawned at the University of North Carolina at Wilmington, Center for Marine Science (UNCW-CMS) in Wilmington, NC, and then reared at North Carolina State University (NCSU) in Raleigh, NC. Wild-caught southern flounder were maintained at UNCW-CMS and eggs and sperm were collected by strip-spawning from multiple broodstock and pooled for *in vitro* fertilization (Daniels and Watanabe 2002). Fertilized eggs were transported to the NCSU Lake Wheeler laboratory in Raleigh, NC and larvae were reared in a 3000-L recirculating artificial

seawater system until 60 days post hatch (dph) (Daniels and Watanabe 2002). Larvae were fed a diet of live feed (rotifers and Artemia) and gradually weaned onto a diet of high-protein dry feed (Reed Mariculture, Campbell, CA, USA) that was continued through metamorphosis. All procedures and research were approved by Institutional Animal Care and Use Committees at the University of North Carolina at Wilmington and North Carolina State University.

Post-metamorphic flounder at an average size of 24.6 ± 0.8 mm total length (TL) were stocked in triplicate in 100 L round fiberglass tanks on three separate recirculating aquaculture systems at a density of 250 fish/m^2 for fluctuating temperature systems and at the same density in duplicate 340 L tanks in another system for the 27°C control. All tanks were gray, salinity started at ~29 ppt and was gradually decreased to and maintained at 5 ppt. Photoperiod was maintained on a 12L:12D schedule using artificial lighting, all conditions previously shown to produce 1:1 sex ratios of flounder (Holler 2011, Mankiewicz et al. 2013). Fish were fed a commercial diet 3-5 times daily and any unconsumed food was siphoned out of tanks (Reed Mariculture, Otohime, range of sizes, ~ 51% crude protein, 11% crude fat). Temperatures were controlled to produce natural fluctuations in temperatures similar to what we observed in nursery habitats (rising over the course of spring season) but with average daily temperature deviating by 4°C from control groups, i.e. average temperature of 19°C, 23°C, and 27°C over the course of the experiment (Figure 1A). The 23°C temperature profile is based on that recorded at the Swanquarter bay location from 2014 (Latitude: 35.385, Longitude: -76.312), a habitat in the Pamlico region that produced 50:50 sex ratios that season. Additionally, when the average daily temperature is considered across all nursery habitats, the greatest mean difference recorded between any creeks was near 4°C, so treatments were varied by this amount. Actual water temperatures in the

systems were recorded every 2 hours using iButton temperature loggers (DS1922L - 40 to 85°C Thermochron, iButtonLink Technology, Whitewater, WI) enclosed in a waterproof housing, and secured to a standpipe in one of the tanks on each treatment system.

Temperature was maintained and adjusted daily to fit the assigned profile through a combination of submersible heaters and ¼ hp drop-in titanium chillers (CY-3-CWCT, Aqualogic Inc., San Diego, CA) for each of the three systems. Animals were grown out to an average of 102 mm TL, which is beyond the presumed window of sex determination (35-65 mm TL) and fish could be accurately sexed using our gonadal biomarker approach. At the termination of the experiment in a system and immediately prior to dissection, the fish were euthanized using a lethal dose of buffered MS-222, and then weighed (g), and measured for length (mm TL). Gonadal tissue was dissected, placed in 0.5 mL of RNAlater overnight at 4°C and then stored at -20°C until later analysis for expression of sex-specific biomarkers.

Flounder were sampled periodically throughout the study to obtain length (mm TL) and weight (g) measurements. As fish were growing at different rates across systems, fish were not sampled on the same day for all systems to ensure similar densities. The 27°C fluctuating system failed 28 days into the study and was restarted three days later with flounder transferred from the 19°C system as they were still on average 31.3 ± 1.3 mm TL, prior to the start of the window of sex determination (35 – 65 mm TL). Due to the limited number of flounder to stock in the new system and to ensure sufficient number of fish were left to obtain final sex ratios, the periodic sampling was eliminated for the 27°C fluctuating treatment and as such was limited to initial and final growth data. A final sampling of each system occurred when the average size of flounder

was approaching 100 mm TL in that system. Specific growth rate (SGR) was calculated for each treatment for length (mm TL) and weight (g) based on initial size, the size at final sampling, and the number of days between the initial and final sampling.

SGR = [(log final – log initial)/ time (days)] x 100

RNA Isolation and cDNA Synthesis

Total RNA was extracted from all gonadal tissue with 1 mL of TRI-reagent and 6 µL of Polyacryl carrier (Molecular Research Center, Cincinnati, OH) using standard methods from the manufacturer, and pellets were resuspended in 20 µL of nuclease-free water. RNA quality was assessed when there was adequate sample volume by presence of 18S and 28S ribosomal RNA bands using gel electrophoresis, and then quantified by absorbance OD 260:280 ratio using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). All RNA was DNase treated (Turbo DNA-free, Invitrogen, Waltham, MA) to remove genomic DNA, then re-quantified and diluted to 100 ng/µL or not if concentrations was already below 100 ng/µL. Total RNA (1.0 µg) was used to synthesize cDNA via reverse transcription (High Capacity cDNA Synthesis Kit, Applied Biosystems, Waltham, MA).

Real-time Quantitative PCR

Expression of gonadal biomarkers were used to sex individual southern flounder using real-time quantitative PCR (qPCR) and had previously been validated in this species (Mankiewicz et al. 2013). Müllerian-inhibiting substance (*mis*) was used as a marker for male development, Forkhead transcription factor L2 (*foxl2*) and P450 aromatase (*cyp19a1a*) were used as female

markers, and elongation factor-1 alpha (*ef-1α*) was used as a reference gene. Gene specific primers were used to measure the expression of these markers (GenBank accession numbers: *foxl2*: KF534720, *mis*: KF534719, *cyp19a1a*: AY902192, and *ef-1α*: AY884199). Sequences of primers used for qPCR are as follows: *foxl2* forward primer (FP): GTCCCCGCCCAAGTACCT, *foxl2* reverse primer (RP): GGCCGAGCGACCATGAG, *mis* FP: CTGCCGAGGCTCTTGCA, *mis* RP: CAGGACGGCATGGTTGATG, *cyp19a1a* FP: GGAGCCACACAGACAGGAGAA, *cyp19a1a* RP: GGCCCCAAACCCAGACA, *ef-1α* FP: CGAGAAAGAAGCTGCCGAGAT, *ef-1α* RP: CGCTCGGCCTTCAGTTTGT. qPCR analyses were performed on a BioRad CFX384 instrument, using Brilliant II SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA), using 1.5 µM primers, and 2 µL of 1:6 diluted cDNA in a total reaction volume of 10 µL. The qPCR cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. A final melt curve step was performed to verify a primer specificity for each gene with the presence of a single peak. The absence of genomic DNA contamination was confirmed using water (No Template Control; NTC) and DNase-treated RNA (No-Amplification Control; NAC) as negative control templates setup during cDNA synthesis. Cycle threshold (Ct) values for samples were transformed using a standard curve of serially diluted pooled cDNA versus Ct values (R^2 = 0.96-0.99). Samples were then normalized to reflect the amount of template cDNA per ng total RNA loaded into each reaction (cDNA/ng total RNA). Additionally, we quantified expression levels of *ef-1α* RNA as a reference gene, as the expression of *ef-1α* had previously been shown not to vary significantly across tissues (validated as a reference gene in Luckenbach et al. 2005). As such all data were normalized using the two methods, both to the expression of *ef-1α* and the cDNA/ng total RNA, and then compared. Both normalization methods produced similar results (Figure 2).

Determining Sex Ratios and Statistical Analysis

All samples were analyzed for expression of *foxl2*, *cyp19a1a*, and *mis* using qPCR. Ratios of the levels of the three genes were established in each individual sample. A sample with relative high expression of *foxl2* and *cyp19a1a* and with low *mis* expression was identified as female, a sample with relative high expression of *mis* and with low levels of *aromatase* and *foxl2* was identified as male, and those samples with low or uncharacteristic expression of all sex biomarkers was classified as undetermined. This was completed utilizing normalization methods to *ef-1α* and Total RNA (Figure 2). All sex ratios were analyzed by treatment and compared against a predicted 1:1 sex ratio using Chi-square goodness-of-fit tests. Growth rates were analyzed using a one-way analysis of variance, ANOVA, followed by Tukey's honest significant difference (HSD) post hoc for multiple comparisons. Length between males and females was analyzed using an unpaired t-test to test for significant differences in size. All analyses were performed using JMP (Pro v13, SAS Institute, Cary, NC) and/or GraphPad Prism 6 (GraphPad, La Jolla, CA). The level set for statistical significance for all analyses was $P < 0.05$. Data are expressed as the mean ± SEM.

Results

Temperature

We reared southern flounder in different fluctuating temperature regimes mimicking natural temperature profiles from juvenile southern flounder nursery habitats in North Carolina. Experimental treatments were temperatures fluctuating and averaging 19°C, 23°C, and 27°C throughout sex determination (Figure 1A). As a positive control for sex reversal, there was an additional system held constant at 27°C (Luckenbach et al. 2003). There were two dates, days 16

and 49 post-stocking, where the heater breaker tripped for the 27°C constant system so the temperature dropped to an average of 24.4°C on day 16 and 25.6°C on day 49, though the water temperature averaged 27.5°C over the course of the experiment. All systems had similar starting temperatures of 19.8°C, 18.8°C, 20.0°C, and 20.1°C for 19°C, 23°C, 27°C and constant 27°C respectively. Actual average daily temperatures of each system during the sex determination window (i.e. when fish were measuring below 65 mm TL) were 18.3°C, 21.8°C, 26.8°C, and 27.1°C respectively (Figure 1B). Over the entire study the average daily temperatures for each system were 20.5°C, 23.5°C, 27.5°C, for the 19°C, 23°C, 27°C treatments respectively.

Growth

Flounder were sampled periodically (every ~15 mm TL) throughout the study to obtain length (mm TL) and weight (g) measurements. A final sampling of each system occurred when the average size of flounder was approaching 100 mm TL in that system. The final sampling of each system occurred on days 95, 71, 64, and 53, and flounder were on average (mean ± SEM) 103.0 ± 1.7, 106.2 ± 1.1, 97.0 ± 2.3, and 103.6 ± 1.2 mm TL for the 19°C, 23°C, 27°C and constant 27°C treatments respectively (Figure 3A). The average weight of the flounder at the last sampling for each system were 12.46 ± 0.57, 11.86 ± 0.37, 10.36 ± 0.65, and 11.20 ± 0.40 g, for the 19°C, 23°C, 27°C and constant 27°C treatments respectively (Figure 3B). The SGR for length (% mm TL/day) and weight (body weight g/day) was significantly higher for the 27°C constant system compared to all other treatments (Figure 4, all $P < 0.0001$). For both length and weight, the SGR was the lowest in the 19°C treatment, and SGR increased with warmer fluctuating temperatures, although there was no difference between 23°C and 27°C fluctuating groups. There was a total number of 26 females compared to 166 males total across all treatment

groups. At the end of the study, those 26 females had an overall mean length of 113.88 ± 1.80 mm TL, which was on average 12.01 mm TL larger than the males which had a mean length of 101.87 ± 1.12 mm TL (Figure 5A, $P < 0.0001$). There were not enough females produced across all treatments to detect significant differences in the mean length of flounder by sex within treatment. However, for the treatments that reared females, they were on average larger than the males in the same treatment group (Figure 5B). Consequently, the larger females also had marginally higher SGR's across treatments, although were comparable to the male growth rates in their group (Figure 5C).

Sex Ratios

Sex ratios were calculated using gonadal biomarkers for *foxl2*, *cyp19a1a*, and *mis* (Figure 2). Normalization was completed utilizing *ef-1α* as a reference gene (Figure 2A and 2B) and also using the cDNA/ng total RNA (Figure 2C and 2D). Individuals were sexed utilizing both of these methods and compared. Although there was some variation in the relative mRNA abundance of biomarkers, the sex ratios did not differ between methods. The sex ratios for each treatment were 83%, 65%, 100% male for the 19°C, 23°C, and 27°C fluctuating temperatures respectively. The 27°C constant temperature system produced 98% males (Figure 6, n = 48 per treatment, Chi-square: 23°C $P = 0.04$, all others $P < 0.0001$; all P represent significant deviations from a 50:50 sex ratio). Although the 23°C fluctuating treatment was mildly male biased (65% male), it produced more females than any other treatment, followed by the 19°C group (83% male). The 27°C fluctuating system produced only one female, and the 27°C constant system produced zero females. All treatments produced sex ratios that were significantly different from the 65% male

of the 23°C fluctuating system (19°C $P = 0.004$; 27°C constant and fluctuating $P < 0.0001$). There were no individuals that were deemed undetermined from any treatment.

Discussion

In this study, we show that when southern flounder are reared in fluctuating temperatures in the laboratory intended to mirror those of natural temperature regimes, the sex ratio differences observed between these natural habitats are recapitulated. Previous studies show that in addition to inter-annual variation, that productive juvenile southern flounder nursery habitats displayed strong and significant male biased sex ratios (up to 94% male) and these environments also exhibited with the warmest average temperatures (Chapter 3). Temperature profiles across different nursery habitats experienced similar patterns of fluctuations, although between any two habitats temperature could differ in mean recordings upwards of 4°C. In addition, Swanquarter Bay, a northern nursery habitat in the Pamlico Sound that produced 1:1 sex ratios, the average temperature over the sex determination period was 23.2°C, consistent with the observed female optimal temperature of 23°C that yields 1:1 sex ratios in the laboratory (Luckenbach et al. 2003). In the current study, when the temperature profile of this habitat is reproduced in a recirculating system to obtain temperature fluctuations that similarly average 23°C during sex determination, this regime produced the greatest proportion of female flounder (35% female) and significantly higher than those of 19°C (17% female) or 27°C (0% female) treatments (Figure 6). Although the 23°C fluctuating treatment yielded the greatest proportion of females, it was still mildly male biased at 65% male. This is comparable to what was observed for the northern Pamlico region for 2016 and 2017, where sex ratios were 61% and 66% respectively, while other sampling habitats farther south produced a greater proportion of males both years (82% and 94% male).

The 27°C fluctuating treatment that produced 100% males, was representative of the southern sampling habitats, south of the New River, that also displayed significantly male skewed sex ratios in field collections. Although previous field sampling did not include a habitat comparable to the 19°C treatment, it could be representative of nursery environments farther north towards the Albemarle Sound, a region where juvenile southern flounder are also known to inhabit (NCDMF 2005). Other physical parameters (i.e. salinity, oxygen, light, etc.) were similar across treatments, suggesting that temperature is likely the predominant factor that is influencing sex determination in natural habitats.

Temperature has been shown to be a critical factor controlling growth in flounder and other flatfishes (Yamashita et al. 2001). In ectotherms, the relationship between growth and temperature is typically non-linear, with an optimal intermediate temperature and extremes displaying negative effects (Kingsolver et al. 2015). Previous studies in southern flounder support this and showed higher growth at 23°C compared to fish reared at 18°C and 28°C. However, growth rates did not differ until flounder were approaching 100 mm TL, after which the flounder maintained at 23°C experienced a divergence as their growth increased (Luckenbach et al. 2003, 2007). Thus, the growth rates during the first 100 mm TL were similar overall between rearing temperatures (Luckenbach et al. 2007). In the present study, we sampled all fish around 100 mm TL, before the divergence in growth rates described in previous studies would have occurred. Our data show a similar trend in growth with no difference in SGR for length or weight between fluctuating temperatures at 23°C and 27°C, however, there was a marked decrease in growth rates at cooler temperatures fluctuating at 19°C (Figure 4). The fish were exposed to temperatures much colder than the average for the treatments in the early portion of

this study. The 19°C system dropping down to 12°C on day 12, and the in the 23°C system dropping to 14°C on day 11, mimicking springtime dips in temperature in NC juvenile southern flounder habitats. Once temperature in the systems began to warm, as natural habitat temperatures do with the progress of spring the fish grew more rapidly in both treatments, although the 19°C fish did not exhibit a substantial increase in growth until after day 50. Interestingly, we did observe a higher SGR at 27°C constant temperature compared to any fluctuating system. This suggests that constant temperatures produce superior growth rates compared to fluctuating temperatures in southern flounder. However, some species of fish grow more rapidly under fluctuating temperatures than at either high or low extreme of the cycle held constant (*Micropterus salmoides*, Diana 1983). Both yellow perch (*Perca flavescens*) and walleye (*Sander vitreus*), two species with similar thermal preferences had different growth responses to fluctuating temperatures (Coulter et al. 2016). These studies suggest that different species have optimal temperature regimes for growth.

Sexually dimorphic growth in southern flounder is thought not to commence until flounder are larger in size, around 200 mm TL (Fitzhugh et al. 1996). Growth rates appear to be similar between sexes during early stages of development and the divergence in growth after 100 mm TL could be associated with an ontogenetic shift in metabolism correlated with the onset of piscivory (Fitzhugh et al. 1996, Luckenbach et al. 2007). Although the fish from the current study were only grown-out to 100 mm TL, female flounder grew significantly larger than males across all temperature treatments (Figure 5A). There were not enough females propagated within all temperature treatments to test significant differences of the size between sex. However, for the treatments that produced females, they were on average larger than the males (Figure 5B).

Similar patterns between males and females were seen with SGR, albeit differences were not significant (Figure 5C). The temperatures that produce the best growth rates should in turn maximize production of females as this is the sex that would benefit most from rapid growth (Luckenbach et al. 2009). We observed the best growth at 27°C constant temperature compared to any fluctuating system and this treatment also produced a high preponderance of males at 98%. Based on previous studies, it could be hypothesized that a 23°C constant treatment would produce 50:50 sex ratios and would have equivalent growth rates to that of the 27°C constant treatment since fish were around 100 mm TL. However, if flounder were grown-out to larger sizes we would expect to see enhanced growth rates at 23°C (Luckenbach et al. 2003, 2007). In contrast, when barfin flounder were reared with increasing temperature throughout sex determination it resulted in significant masculinization but also enhanced growth (Goto et al. 1999), suggesting that the correlation between temperature, growth and sex is species specific.

In this study, we show that fluctuating temperatures around 23°C yield the greatest number of females, producing similar trends in sex ratios to previous studies of constant temperatures and to flounder nursery habitats of a similar temperature profile. By contrast, both 27°C fluctuating and 27°C constant produced significantly male-biased sex ratios, with only one female between both treatments. The highest growth was recorded at constant 27°C temperature, however, growth did not differ between 23°C and 27°C fluctuating flounder. Although female flounder were on average larger than males, it was a small difference that did not translate to substantial disparity in SGR. When fish are reared in temperature regimes mimicking the natural profiles of juvenile southern flounder habitats they produce similar sex ratios, indicating that temperature is likely the key factor influencing male-biased sex ratios in nursery habitats.

Fluctuating temperatures have been shown to impact sex ratios in fish and turtles that exhibit TSD/ESD (*Menidia menidia*, Conover and Kynard 1981; *Carassius auratus,* Goto-Kazeto et al. 2006; *Pimephales promelas*, Coulter et al. 2015; *Trachemys scripta*, Carter et al. 2018). Although there are many environmental parameters that could potentially induce masculinization in southern flounder, temperature appears to be a primary factor. With global temperatures rising, this is cause for concern for wild populations of southern flounder and other species that exhibit TSD/ESD (Ospina-Álvarez and Piferrer 2008, Kallimanis 2010). Water temperatures are projected to increase 0.18°C per decade and could prove to be unfortunate for marine organisms where water temperature drives patterns in life history (Walther et al. 2002, IPCC 2014). The present results show that warmer temperatures mimicking natural fluctuations in the environment produce a greater proportion of males, which could impact the management of this important fishery that largely depends on females.

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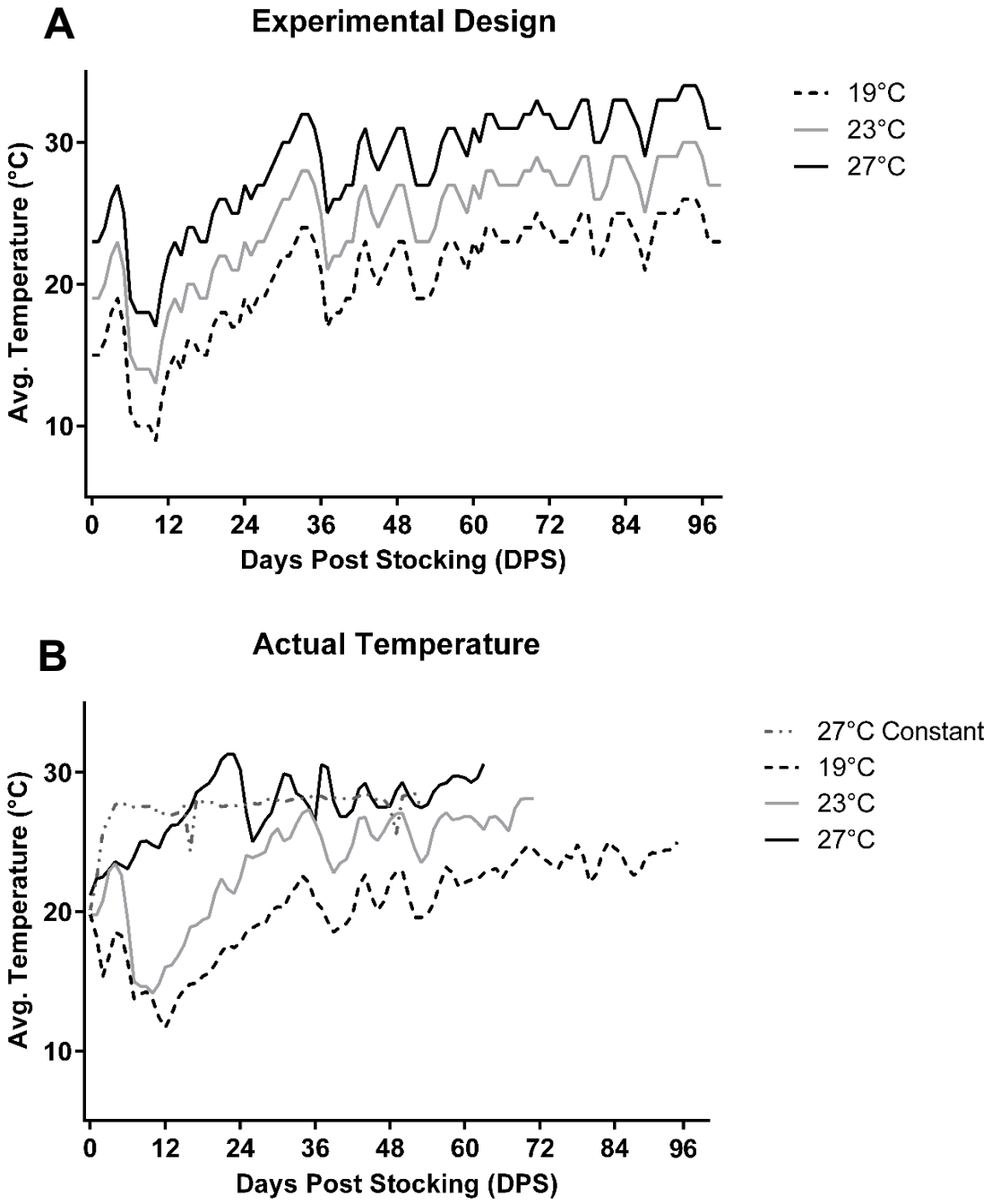

Figure 1. Temperature profiles from an experiment with juvenile southern flounder that were reared in different fluctuating temperature profiles that deviate by 4°C throughout the period of sex determination. (**A**) The experimental design temperature profiles for 19°C, 23°C, and 27°C average temperature treatments. (**B**) The actual recorded daily average temperature (°C). Actual average temperatures of each system recorded during the sex determination window were 18.3°C, 21.8°C, and 26.8°C respectively. Additionally, there was a 27°C constant treatment with an actual average temperature of 27.1°C.

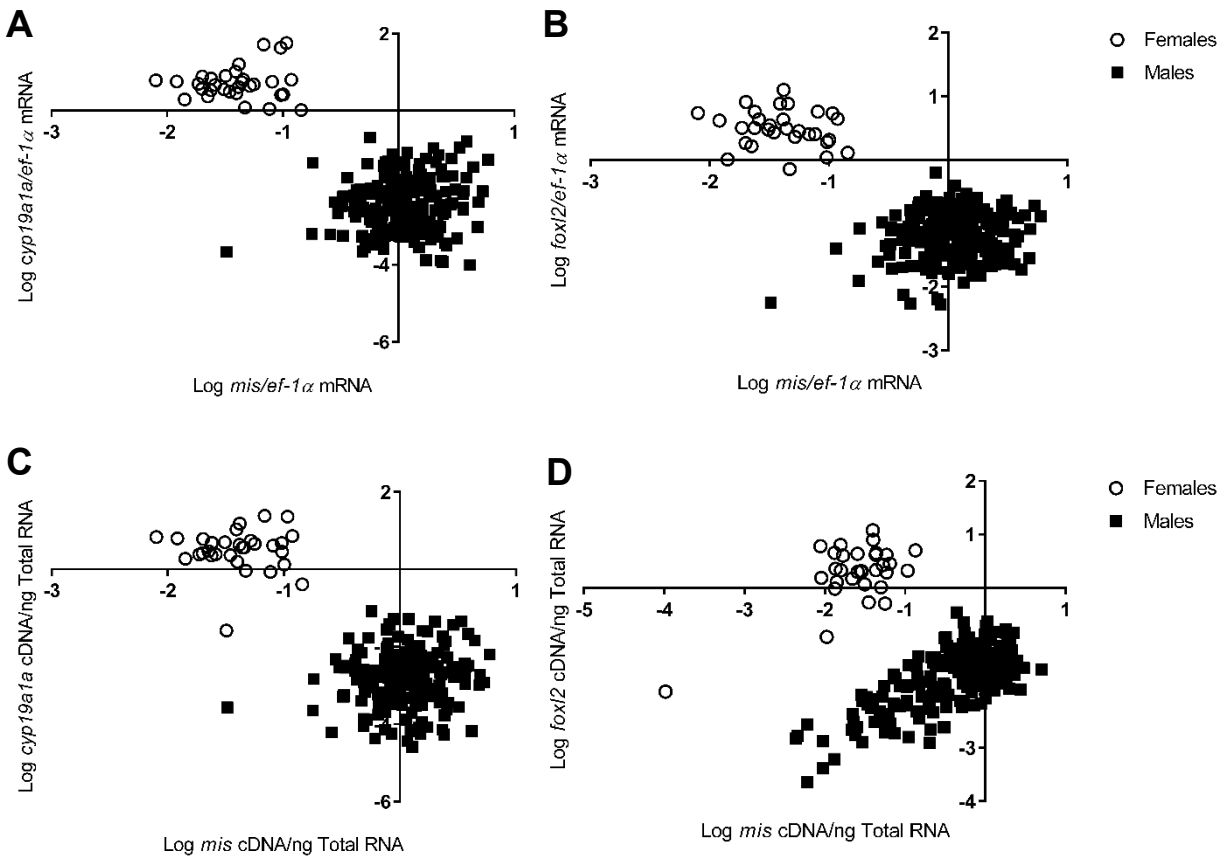

Figure 2. Log-transformed levels of gonadal mRNA expression of sex biomarker genes as functions of each other normalized to both *ef-1α* (gene of interest/*ef-1α*) and to cDNA/ng total RNA, from juvenile southern flounder reared in tanks. (**A**) c*yp19a1a* vs. *mis* / *ef-1α* mRNA (**B**) *foxl2* vs. *mis* / *ef-1α* mRNA (**C**) c*yp19a1a* vs. *mis* / cDNA/ng total RNA (**D**) *foxl2* vs. *mis* / cDNA/ng total RNA. White circles indicate females and black squares indicate males.

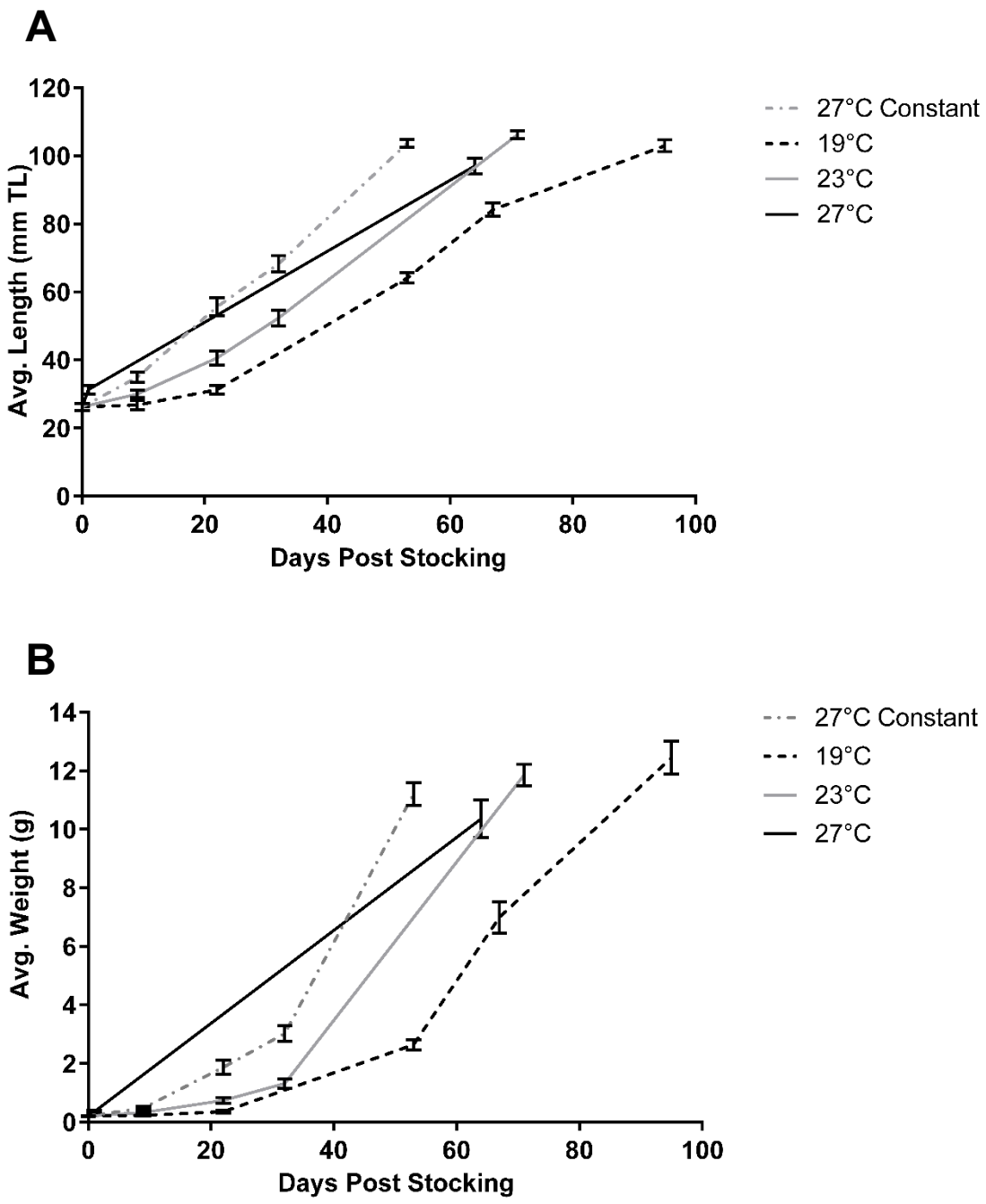

Figure 3. Juvenile southern flounder were reared in different fluctuating temperature profiles that deviate by 4°C throughout the period of sex determination. (**A**) The average total length (mm TL) and (**B**) the average weight (g) from each fluctuating temperature treatment and the constant 27°C group. Flounder were periodically sampled throughout the study to collect length and weight.

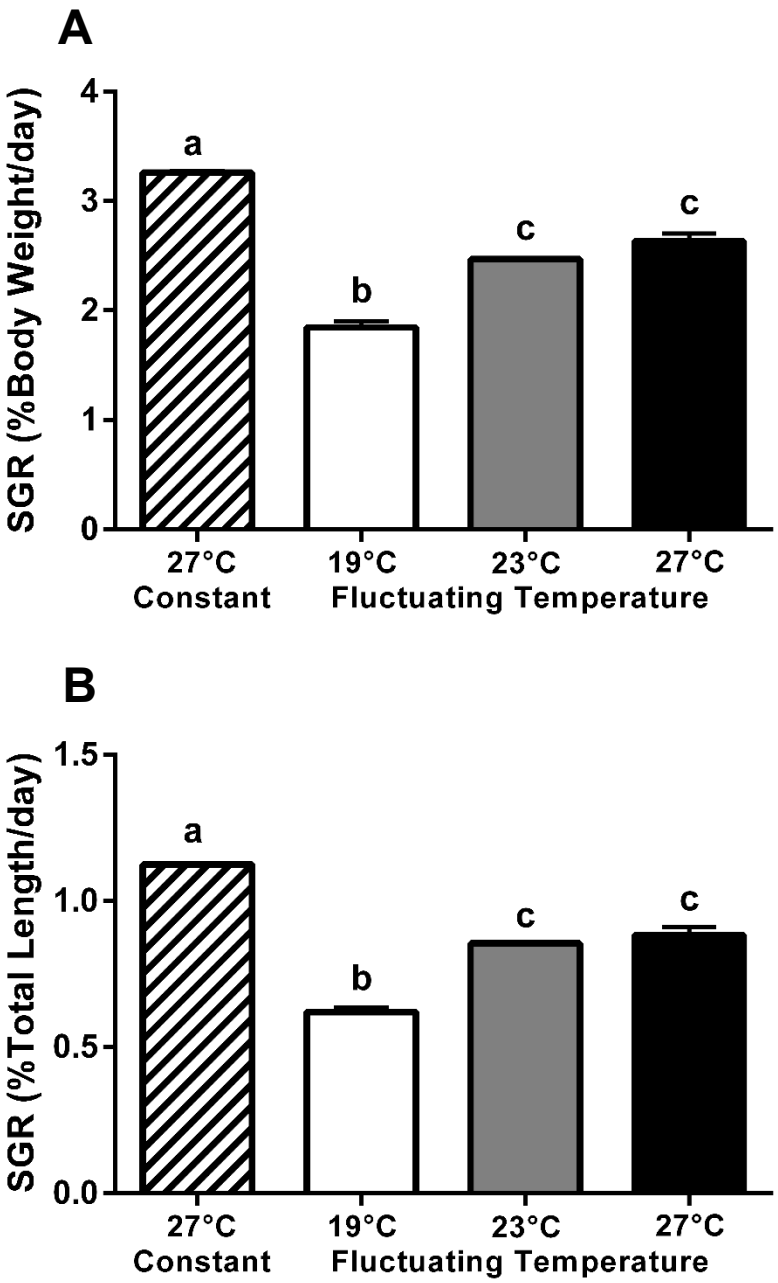

Figure 4. Specific growth rate (SGR) of juvenile southern flounder were reared in different fluctuating temperature profiles that deviate by 4°C throughout the period of sex determination. (**A**) SGR % body weight per day and (**B**) SGR % total length per day from each fluctuating temperature treatment. Letters indicate significance between treatments (All $P < 0.0001$).

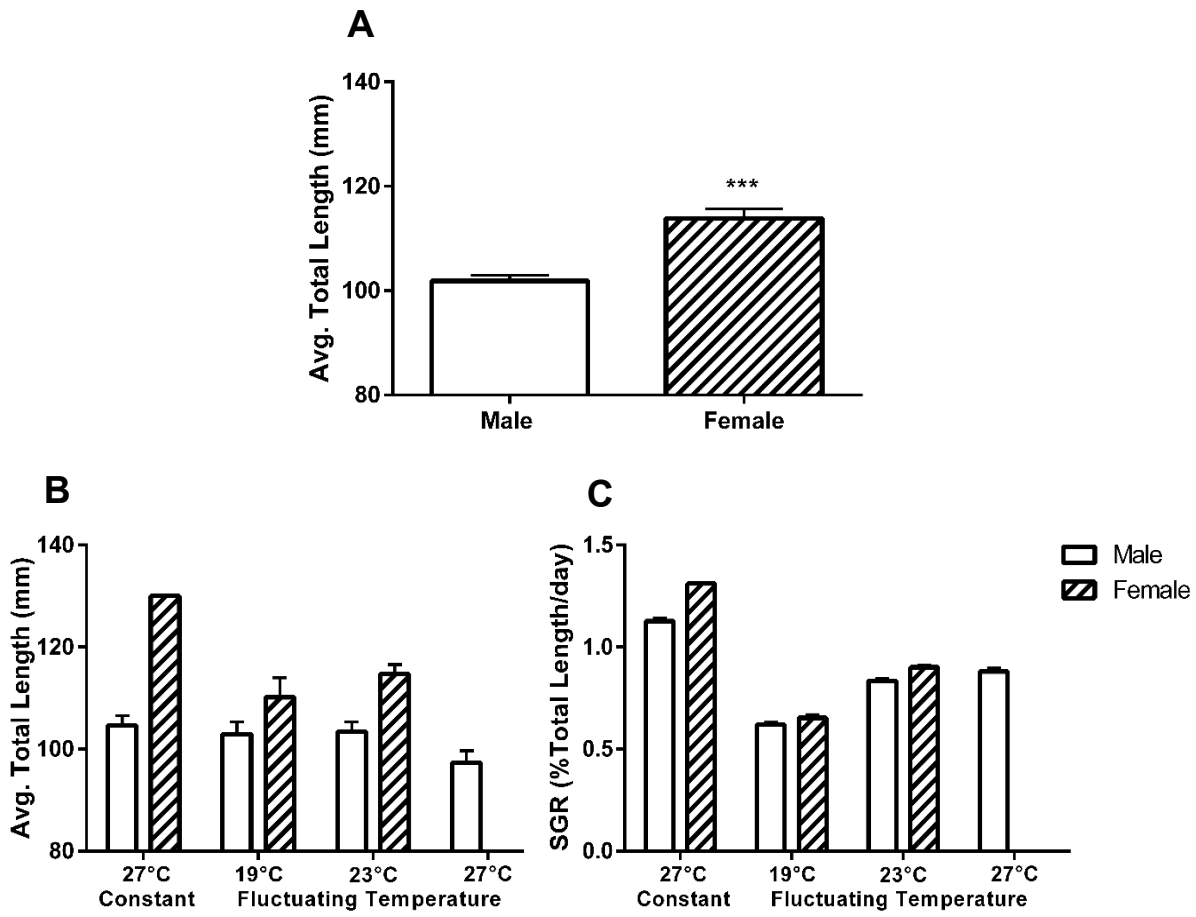

Figure 5. Mean total length (mm) of juvenile southern flounder by sex (**A**) the average total length (mm) of all males (101.9 mm TL, n = 166) and females (113.9 mm TL, n = 26) from all treatments ($P < 0.0001$), (**B**) the average total length (mm) of male and female flounder by treatment. 27°C constant: 47 males - 104.6 mm TL and 1 female - 130.0 mm TL; 19°C: 40 males - 102.9 mm TL and 9 females - 110.1 mm TL; 23°C: 31 males - 103.4 mm TL and 17 females - 114.7 mm TL; 27°C: 48 males – 97.3 mm TL and 0 females, and (**C**) specific growth rate (SGR) as SGR % total length per day from each fluctuating temperature treatment by sex.

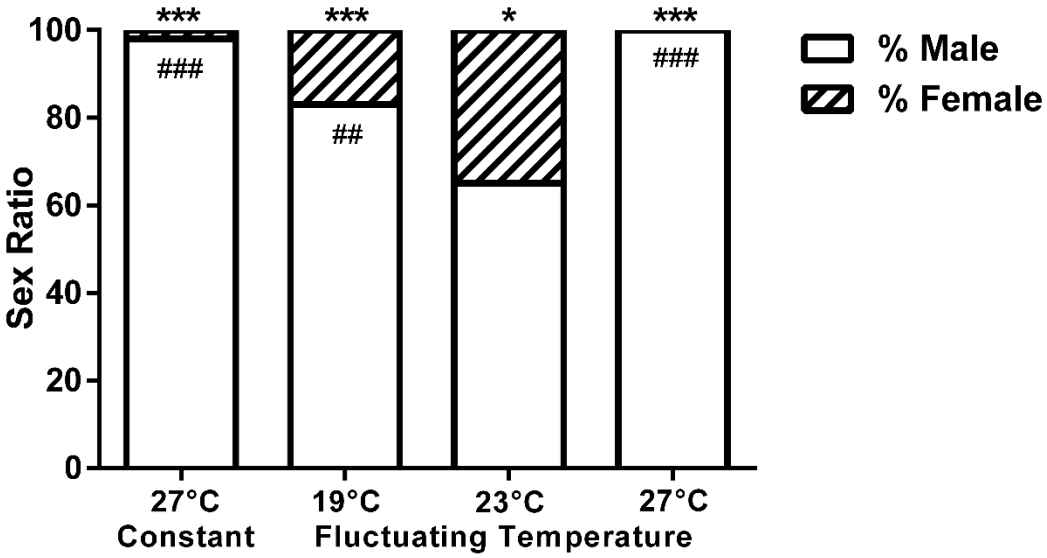

Figure 6. Sex ratios of southern flounder reared in different fluctuating temperature regimes mimicking natural North Carolina temperature profiles from juvenile southern flounder nursery habitats. Treatments averaged temperatures of 19°C, 23°C, and 27°C throughout sex determination and sex ratios were 83%, 65%, 100% male respectively. The 27°C constant system produced 98% male. (n = 48 per treatment, $*P = 0.04$, $***P < 0.0001$; all P represent significant deviations from a 50:50 sex ratio). The proportion of males was significantly higher for all treatments relative to the 23°C fluctuating group ($^{\#\#}P = 0.004$ for 19°C and $^{\#\#\#}P < 0.0001$ for 27°C fluctuating and 27°C constant temperature groups; P represents significant deviations from the 65:35 male:female sex ratio).