TRANSCRIPTOMICS ANALYSIS GENE EXPRESSION AND EFFECTS OF ACUTE EXPOSURE TO EXPERIMENTAL TEMPERATURES AND SALINITIES ON THE SURVIVAL OF SOUTHERN FLOUNDER (*Paralichthys lethostigma*) PREMETAMORPHIC LARVAE SPAWNED BY PARENTS COLLECTED ALONG THE ENVIRONMENTAL CLINE OF THE TEXAS COAST

A Dissertation

by

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Southern flounder (*Paralichthys lethostigma*) provides important commercial and recreational fisheries opportunities on the Texas coast. The economic impact of this fishery is negatively affected by recent declines in harvest throughout the geographic range of this species. Environmental variables that may be important determinants of Southern flounder abundance and fishery productivity include freshwater inflows, extent and type of the nursery habitat and changes in temperature regimes related to climate change. Despite progressive harvest regulations to manage the population decline and protect the adult spawning migration to the Gulf of Mexico, the Southern flounder population has not recovered to its historical level. The Texas Parks and Wildlife Department is implementing a stock enhancement program for Southern flounder to supplement natural populations on the Texas bays. A critical phase of the program is to validate at large scale the larval and juvenile production protocols available for Southern flounder to optimize physical environmental parameters that influence survival during larval rearing. Temperature and salinity are two ubiquitous abiotic factors that influence all biological functions of these two early life history stages and survival. I exposed Southern flounder eggs and premetamorphic larvae to different temperature and salinities found on the environmental cline of the Texas coast to evaluate the effect of these conditions on survival of individuals spawned by broodstock collected from different environments of coastal Texas. RNA next generation sequencing was used to generate more than 29,000 sequences that were assembled in a de novo transcriptome.

Temperature was the most important factor affecting the survival of Southern flounder at these two stages of development. Synergy between temperature, salinity, and site of collection of the parents also played a role in fish survival in response to the temperature and salinity challenges.

From the sequences, 96% were mapped and used to identify proteins, genes and biological gene functions. The number of up-regulated genes and gene expression was higher for temperatures and salinity in Sabine and Aransas fish than in Galveston.

DEDICATION

I dedicate this work to the great people of the State of Texas and their natural resources. During an important segment of my professional life Texas's natural resources have been a source of inspiration and motivation for my work.

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I am humble with this experience and my love and thanksgiving goes to God. Max thank you for walking at my side on this trail with such patience and love. My sons Carlos Jose and

Omar Jose and my family always have been the foundation of my innermost strength. I feel blessed with their love and prayers.

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This work was supervised by a dissertation committee consisting of Dr. Frances Gelwick and Dr. Delbert Gatlin (Wildlife Fisheries Sciences), Dr. James Cai (Veterinary Medicine and Biomedical Sciences) and Dr. Robert R. Vega (Texas Parks and Wildlife Department Coastal Fisheries). Dr. Divhya Arassapan, Dr. Ence Yang and Benny Goetz (University of Texas at Austin) assisted with the transcriptome assemblage.

All other work conducted for the dissertation was completed by the student independently.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Southern flounder is an important commercial species with a combined Atlantic and Gulf fishery for all flatfish species of approximately 12,000 metric tons annually (Pritchard 2009), and a recreational harvest of approximately 700 metric tons (Gulf States Marine Fishery Council 2000). The economic impact of this fishery was negatively affected by recent declines in harvest throughout the geographic range of Southern flounder (Daniels 2000; Gulf States Marine Fishery Council 2000). Possible reasons for these declines are over-fishing, by-catch mortality, and habitat degradation (Fisher 2000, Takade- Heumacher and Batsavage 2009). Some of the environmental variables that may be important determinants of Southern flounder abundance and fishery productivity include freshwater inflows, extent and type of nursery habitat (Burke et al. 1991, Craig et al. 2015, Del Toro et al. 2008, Furey and Rooker 2013, Glass et al. 2008, Nañez-James et al. 2009), and changes in temperature regimes related to climate change. Southern flounder populations in Texas and Florida are near the southern limits of the species' range. This species is currently rare in northern Mexico, south of the Baja Laguna Madre del Sur, and absent in Florida, south of the Loxahatchee River on the Atlantic Coast and the Caloosahatchee River on the Gulf coast (Corey et al.2017, Murphy et al. 1994). The present distribution of this species may reflect temperature constraints for larval or juvenile survival (Houde 1989). This premise is supported by a data series collected over 35 years from the Texas Parks and Wildlife Department (TPWD) that suggests a higher relative abundance of southern flounder juveniles after colder winter years in Texas (Fernando Martinez, personal communication; TPWD 2017).

The TPWD has implemented several management strategies to assist in the recovery of southern flounder populations: recent elimination of gigging during the month of November to reduce mortalities during the fall spawning migration, reductions of bag and size limits, implementation of a shrimp vessel license buy-back program to reduce by-catch, and application of by-catch reduction devices (BRD) to trawl nets. Nevertheless, flounder populations have not recovered to historical levels as rapidly as expected (Martinez -Andrade 2018, Fisher 2000). Analyses of long-term trends in monitoring data for southern flounder in Texas coastal waters indicate low but stable recruitment of juveniles; however, trends display continued declines in survival rates of post-juvenile Southern flounder and adults (Froeschke et al. 2011, Martinez-Andrade 2018, Nañez et al. 2009).

In an attempt to supplement natural stocks, TPWD initiated a southern flounder stock enhancement program. The TPWD Coastal Fisheries Division stock enhancement group is developing protocols and strategies for the artificial culture of Southern flounder at marine hatcheries (Vega, 2009). Moreover, several states in the United States also are interested in stock enhancement and aquaculture activities for this species (Carroll et al. 2005, Daniels 2000). Preliminary studies of artificial propagation of Southern flounder were developed on the East coast (Benetti et al. 2001, Daniels and Borski 1998, Jenkins et al.1997, Mangino and Watanabe.2007, Smith et al. 1999) and transferring these rearing techniques seems feasible, but it may require refinement of protocols to increase proficiency in production of juveniles. Genetic differences reported in the Gulf of Mexico and the Atlantic coast, including clinal genetic variations along the Texas coast, are possibly related to environmental adaptations (Anderson et al. 2012, Anderson and Karel 2012, Blandon et al. 2001, Wang et al. 2015). Spatial genetic structure of some marine species continues to be unclear, especially regarding genetic structure associated with

environmental variation along marked environmental clines. Previous research has shown that seasonal temperature records from spring, fall, and winter strongly correlate with clinal genetic structure (Oomen et al. 2015) apparently influenced by phenotypic plasticity associated with environmental variation and the spawning time for groups of adults of the same species with respect to particular temperatures (Canale and Henry 2010). In North Carolina, researchers have found temperature to be critical during incubation of embryos and larval rearing, and vital for the successful propagation of Southern flounder (Alam et al. 2013, Carroll et al. 2005, Van Maaren and Daniels 2001, Watanabe et al.2010). Differences in thermal optima and tolerances of Southern flounder from different localities may imply that temperature protocols used in hatcheries in North Carolina or Florida may not be as successful in Texas (Benetti et al. 2001, Kaiser et al. 2012). Furthermore, protocols may need to be optimized for extensive coastal reaches of the Texas coast that encompass different ecosystems (EPA United States Environmental Protection Agency, Gulf of Mexico Review 2018). For example, bays of the lower Texas coast are shallow and have limited freshwater inflow as well as limited exchange with the Gulf of Mexico, causing them to be warm throughout much of the year. The lower coast includes the upper Laguna Madre, a very shallow, warm, hypersaline lagoon (Tunnell 2012). In contrast, bays of the upper coast are comparatively deeper, have greater freshwater inflow, greater exchange with Gulf of Mexico waters, and more variable temperatures among seasons. Overall, the Texas coast exhibits a complex interplay between salinity and temperature gradients (Bodner and Sharp. 1988, Dupuis and Anis, 2013). This environmental mosaic is reflected in the current distribution of southern flounder in Texas, with their greatest abundance in the northern coast from Sabine Pass to Port Aransas, and the lowest catch rate for the entire Texas coast in the upper and lower Laguna Madre of the southern coast (Matlock 1982;1991).

The State of Texas stock enhancement activities for other species integrate genetic differences among bays and estuaries along the coast (Vega 2009). The Gulf of Mexico waters and the lower Texas coast have experienced increasing temperatures during the last 15 years accompanied by limited rainfall with consequent increases in salinity for some coastal areas (Castillo et al.2014, Chapman 1966, Coleman and Koening, 2010, Joseph et al. 2013). The successful culture and stocking of southern flounder in Texas waters requires a comprehensive understanding of the role of temperature and salinity in the life history and ecology of this species (Furey and Rooker 2013, Glass et al. 2008, Guindon and Miller 1995, Nañez et al. 2009, Taylor et al. 2009, Van Mareen et al. 2009). This is especially true in the context of a changing marine environment due to global warming (Chollet et al. 2012).

Intergovernmental Panel on Climate Change (2007, Liang and Kunkel, 2001) and the unknown resilience of southern flounder to these changes. In general, fishes have evolved physiologically to live within a specific range of environmental variation; their continued existence outside of this range can be stressful or highly restricted (Blanchard et al. 2012, Campos et al. 2013, Cavieres et al. 2016, Claireaux et al. 1995, Dell et al 2011). One of the expected effects of climate change is an increase in mean ocean temperatures (Hansen et al. 2010, Hori et al. 2010). Higher oceanic temperatures are expected to have deleterious effects on many marine organisms (Dietz and Somero 1993; Fangue et al. 2006; Rodriguez-Lanetty et al. 2009, Somero 2002); especially those near the ecological limits of their distribution, which for southern flounder is in south Texas and Florida. The tolerance of individuals in spatially separated populations of a single species can be used as an indicator of the species ability to persist or go extinct in response to climate change (Cascade et al. 2011). Developing precise information on physical parameters needed for southern flounder to survive, specifically for temperature and salinity, should assist

managers in their decisions with regard to appropriate locations for the production facilities on the Texas coast, and cost-effective culture of southern flounder for stock enhancement activities, including refinement of rearing and stocking protocols.

In the natural environment, water temperature is the factor most frequently associated to recruitment variability of temperate marine fishes, with most studies concentrated on the viability and development of embryos and larvae during their pelagic phase, which is when most of the recruitment mortality occurs (Edwards and Steele. 1968, Van der Veer et al. 2000) Temperature affects biochemical and physiological functions such as metabolism and respiration (Boltaña et al. 2017, Norin and Malte 2012). Thus, important aspects of the life history of many fishes are temperature dependent, including egg development, larval phase duration, survival, growth, sexual maturation, sex determination, and sex ratios (Conover and Kynard. 1981, Devlin and Nagahama. 2002, Ospina -Alvarez Piferrer. 2008, O'Connor et al. 2007). Early developmental stages of southern flounder are especially sensitive to temperature effects, which implies their importance for aquaculture (Carroll et al. 2005) and stock enhancement. In addition to playing a critical role in survival and growth (Carroll et al 2005, Faulk et al. 2007; Taylor et al. 2009), temperature is crucial to sex determination in southern flounder (Devlin and Nagahama. 2002 Luckenbach 2003; Luckenbach 2009; Montalvo 2012). Environmental factors may be altered by spatial or temporal inter-annual changes, environmental clines, and climate change (Auth et al. 2011, Paul et al. 2012). Temperature differences between Sabine Pass and the Lower Laguna Madre are approximately 10°C, and temperature differences among all Texas bays range from 10°C to 20°C (National Oceanic and Atmospheric Administration (NOAA), National Centers for Environmental Information 2018).

A salinity cline along the Texas coastline is formed by various freshwater effluents and topographic features that contribute to their physical diversity, including one of the few hypersaline lagoons in the world, the Laguna Madre de Tamaulipas (Tunnell and Jude 2012). The salinity cline on the Texas coast has been reported to range from 5 ppt in Sabine Lake near the border with Louisiana up to more than 55 ppt in the Lower Laguna Madre near the border with Mexico (Montagna et al. 2002). Salinity is another critical environmental factor that affects the survival of fish larvae and thus influences their distribution at different developmental stages (Geffen et al. 2007, Glass et al 2008, Yañez- Arancibia and Day 2004), including yolk sack absorption, and larval and juvenile growth (Faulk and Holt 2007 Henne and Watanabe 2007, Wang et al. 2013). However, there is limited information regarding the tolerance of southern flounder pre-metamorphic larvae at this critical stage of development when larvae settle into the estuary at the completion of metamorphosis. At this time, they are exposed to acute local changes in temperature and salinity that accompany morphological, physiological and behavioral changes during their transition between pelagic and benthic life; this stage is also generally associated with hatchery mortalities (Chavez. 2018, Texas Parks and Wildlife Department personal communication).

Estuarine species are often euryhaline or adapted to tolerate fluctuating salinity; whereas, marine species are stenohaline and more limited by their narrower range of physiological tolerance. For some species, salinity is a severe environmental stressor (Gelwick et al. 2001, Gong et al 2018, Kultz 2015). Salinities at the limits or outside the range of tolerance of a species will prevent species' occurrences, influence their behavior, and limit growth, reproduction, fitness, and survival (Nissling et al. 2002, Vallin and Nissling 2000). Adults of species inhabiting environments with variable salinities, such as estuaries, are considered to be equipped to occupy

novel osmotic niches (Burke et al. 1991, Capossela et al. 2013, Craig et al. 2015, Smith 2009). However, early life stages of those species, such as their eggs and settled larvae, are generally fragile and vulnerable to drastic environmental changes of salinity or temperature (Burggren 2009, Drost et al. 2014, Perrichon et al. 2017).

Salinity is a determining factor for the function, structure and assemblage of organisms in aquatic ecosystems (Alber 2002, Ritcher et al. 2003, Wetz et al. 2011). Effects of excess freshwater effluents or saltwater influxes can modify local salinity regimes and depends on the assimilative capacity of both the environment and the species of interest. For example, hypo- and hyper-osmotic environments provide physiological challenges for aquatic organisms in which an active flux of ions is transported among tissues, such as the gills, the gut, or skin epithelia (Kultz 2015, Lachman et al. 1996). However, very few studies have been concentrated on the effects of acute exposure to drastic changes of temperature and salinity, which can be encountered by southern flounder during final larval stages of pre-metamorphosed, and by settled early-juveniles, in the heterogeneous and variable environment of bays, estuaries, and rivers. The introduction and widespread application of high-throughput next generation RNA (Joubert et al. 2010, Lockwood and Somero 2011) sequencing provides an opportunity to apply a novel approach to studying pre-metamorphic larvae of southern flounder, a non-model organism, during its settlement stage, and to compare developmental outcomes, as well as related gene activity, under normal and stressed conditions.

CHAPTER II

EFFECTS ON DEVELOPMENT AND SURVIVAL OF NEWLY-HATCHED LARVAE OF SOUTHERN FLOUNDER (*PARALICHTHYS ETHOSTIGMA)* FROM ARANSAS, TEXAS UNDER CONDITIONS OF ACUTE EXPOSURE TO EXPERIMENTAL TEMPERATURE AND SALINITY TREATMENT COMBINATIONS

Introduction

The Southern flounder (Paralichthys lethostigma) is an estuarine-dependent species that supports important commercial and recreational fisheries across its biogeographical range from the Baja Laguna Madre Del Sur in the western Gulf of Mexico (GOM) to North Carolina on the Atlantic Coast of the United States of America (USA) (Hubbs et al. 1991). Its life history is complex and involves migration in late fall and winter of adult fish from shallow estuaries to deeper water inshore for spawning (Corey et al. 2017, Craig 2015, Furey et al. 2013, Glass et al. 2008). Eggs and early larvae drift and develop in the relatively stable nearshore environment until they are transported by currents and tides into the estuary (Glass et al. 2008, Liang and Kunkel 2005, Passalacqua et al. 2016). Advanced larvae and juvenile stages settle and inhabit coastal bays, estuaries, and rivers where environmental conditions are more variable (Furey et al. 2013, Glass et al. 2008, Guindon and Miller 1995, Nanez-James et al. 2009).

Reports of drastic declines in southern flounder populations have prompted conservation groups and government agencies in several Atlantic and Gulf Coast states to evaluate the feasibility of hatchery-based stock enhancement programs to supplement wild stocks (Daniels and Borsky1998, Watanabe and Daniels 2010). These programs include the release of hatchery-reared juveniles into the natural environment for the purpose of augmenting wild populations by

protecting vulnerable early life stages from adverse environmental conditions, starvation, and predation, and thus, overcoming potential recruitment limitations (Lorenzen 2005, 2008, Lorenzen et al. 2010, Lorenzen et al. 2012). Stock enhancement programs are considered to be expensive and, in some cases, controversial. As such, these types of conservation efforts are often under the scrutiny of fisheries managers and the scientific community (Laikre et al. 2010, Blankenship and Leber 1995, Lorenzen 2005, 2008, 2012). The effectiveness of stock enhancement programs is generally based on the survival rates of juveniles released into the wild, the number of hatchery-reared adults entering the fishery, and the biological reliability of individuals produced under hatchery conditions (Kitada, Kitada and Kishino 2006).

The hatchery component of stock enhancement activities involves utilizing large numbers of high-quality fertilized eggs for larval culture (Mazorra et al. 2003, Emata and Borlongan 2003). Robust larvae are essential for the production of substantial quantities of healthy juveniles for release into coastal waters (Kitada and Kishino 2006). Southern flounder females are obtained from the wild, acclimated to captive hatchery conditions, and induced to spawn using a photoperiod-temperature maturation cycle and/or hormone injection treatment (Benetti et al. 2001, Berlinsky et al. 1997, Daniels and Borski 1998, Daniels et al. 2000, Kaiser et al. 2012, Watanabe 1999). Flounder culture generally involves intensive culture under hatchery conditions from egg stage until the completion of metamorphosis (Daniels and Borski 1998, Okada et al. 2003). The high market value of southern flounder also makes this species an attractive candidate for aquaculture (Berlinski et al. 1996, Daniels and Borski 1998). Flounder hatcheries and grow-out ponds have been located in different geographic regions along the Atlantic and GOM, USA across various environmental conditions, but large-scale success of culturing flounder has been limited.

Costs of research into modifications of environmental parameters to optimize specific temperature and salinity conditions for incubation of southern flounder eggs may be costly for commercial aquaculture entities (Crisp et al. 2017); however, testing effects of temperature and salinity treatment combinations is a basic step towards culture of a novel species in order to evaluate the subsequent development of embryos and larvae. Previous flounder research in laboratory settings has focused on evaluating the survival of embryos and larvae tested against single environmental parameters (Smith et al. 1999, Van Mareen and Daniels 2001). However, the synergistic effects of temperature and salinity combinations, and their significance for survival of southern flounder eggs, embryonic development, and large-scale aquaculture production of larvae have not been fully assessed (Song et al. 2013).

Knowledge of their early life stages is critical to understanding the subsequent biology and ecology of adult fishes in natural environments (Fuiman and Werner 2002). Some marine species (including Southern flounder), have a complicated early life history during which they are exposed to interannual and spatial changes in suitability of habitats (Laurel and Blood 2008, Souza et al. 2015, Van der Veer et al. 2000). Poor environmental conditions can follow unfavorable meteorological phenomena such as winds and heavy storms influencing the hydrodynamics of marine currents, temperature and salinity inversions, and warm-water eddies, (Bowen et al. 1989, Carrillo et al. 2017, Cherian and Brink 2016, Chollet et al. 2012, Meltzer et al. 2017). From an ecological perspective, global climate change is predicted to affect the survival of marine organisms across all life stages (Kunkel and Liang 2005, Hammond and Hofmann 2010, Hansen et al. 2010, Jobling 1997). Laboratory data related to species-specific differences in preferred and tolerable extremes of temperature and salinity across developmental stages can provide base-line information on a species' resilience and persistence in the face of climate changes, while also

providing potential explanations for year class failures observed in the natural environment (Bowen et al. 1989, Corey et al.2017). Inter-annual fluctuations in recruitment strength and relative abundance of southern flounder juveniles have been reported; however, the cause-effect relationships of these outcomes, vis-à-vis interactions of abiotic and biotic factors, have not been fully explained (Craig et al. 2015, Laurel et al. 2008, Mueter et al. 2011, Van der Veer et al. 2000).

Although some studies (Benetti et al. 2001, Daniels and Borski 1998, Laurel and Blood 2011, Powell and Henley, 1995) investigating individual temperature or salinity effects have been conducted for this species and other flatfish, information regarding early life stages is sparse or lacking. Early developmental stages of southern flounder are especially sensitive to temperature and salinity effects that can imply success or failure in efforts to culture this species (Carroll et al. 2005, Van Mareen and Daniels 2001, Watanabe and Daniels 2010), as well as for their growth and ultimate survival in the wild (Faulk et al. 2007, Taylor et al. 2009). Temperature and salinity affect physical, biochemical, and physiological functions in southern flounder, as they do for other aquatic organisms, especially during early developmental stages (Song et al. 2013, Thepot and Jerry 2015, Yang and Chen 2005). Effects include egg buoyancy, morphological deformities, rate of metabolism, protein stability and activity, cell membrane structure and permeability, consumption of nutritional reserves in eggs and early larvae, as well as patterns in development, growth, and survival during these vulnerable stages (Tsalafouta et al. 2015, Van Mareen and Daniels 2001, Watanabe 2001). Consequently, to facilitate juvenile hatchery production with high survival and normal development of southern flounder eggs and larvae, protocols that include optimal interactions between temperature and salinity need to be established. The objective of this study was to evaluate the combined effects of various combinations of temperature and salinity on

morphological development and survival of southern flounder eggs and larvae that promote hatching success and healthy growth characteristics of these early stages.

Materials and Methods

Captive Flounder Maintenance and Spawning

Wild-caught southern flounder were collected during the fall November 2012, December 2013 from Aransas Bay, Texas. Fish were spotted from the collection boat, then slowly approached by wading, then trapped with a soft rubber dip-net and transported to the CCA Marine Development Center in Corpus Christi, Texas within 2-3 h. This method provided a high-quality fish that was less stressed as compared to other capture methods. At arrival to the laboratory, fish were dipped in de-chlorinated freshwater, treated with 5 mL of oxytetracycline (OTC) per liter and injected with 1mL of OTC per Kg. The captive southern flounder broodstock (150 individuals) were maintained in 12,000-L fiberglass tanks. Three times a week fish were fed a combination of beef liver, squid, shrimp and mackerel, supplemented once a week with pellets prepared with fishmeal, menhaden oil, minerals, vitamins and Algamac bonded with gelatin (Alejandro Buentello, Texas A and M University, 2012, personal communication). Southern flounder broodstock were subjected to a photoperiod and seawater temperature regime (18ºC; 10 h light: 14 h dark) designed to simulate spawning conditions in a period of 12 months. A conspicuous distended abdomen in females from behind the stomach to the caudal area was interpreted as a sign of hydration of their ovaries. This proxy was used to determine the suitability of the females to receive a hormone injection to induce spawning instead of a biopsy of ovaries to measure the diameter of the eggs (Berlinski et al. 1996). Southern flounder viable females were treated with 0.1 ml per 0.5 kg of weight (Daniels and Watanabe 2010) of Gonadotropin-Releasing Hormone analogue (Western Chemicals Inc., Ferndale, WA, USA). Females and males were strip-spawned

48 h after hormone treatment. Different male and female broodstock from Aransas Bay were maintained and spawned on each of three occasions (a complete replicate on each occasion), following methods described by Kaiser et al. (2012) and Watanabe et al. (1999). Eggs were collected and mixed with sperm for 1 minute; sterilized seawater (1 L at 32 ppt of salinity) was used to induce motility (Kaiser et al. 2012). Eggs were transferred to 1-L graduated cylinders; eggs floating in the water column were considered viable, enumerated, and transferred to a circular, 250-L incubator tank.

Egg Incubation

A total of 3 spawns were obtained for this trial. Eggs were incubated in aerated filtered (125 µ) seawater and maintained at previously established optimal hatchery conditions (18 ºC, 32 ppt, 5-6 ppm DO, and pH 8.1) using heaters, chillers and temperature controllers in a static system for approximately 24 h post-fertilization. Eggs were sampled and viewed through a microscope (Micromaster-Fisher Scientific, Houston, TX, USA) fitted to a digital camera. The software MICRON 1 (Mill Creek, WA, USA) was used to observe the eggs until they reached the neurula stage. A total of 50 eggs from each spawn were measured and photo-documented.

Egg Development under Different Temperature and Salinity Combinations

A total of 100 fertilized eggs at neurula stage were counted under the microscope and collected. Embryos were maintained with gentle aeration and transferred to 1-L clear plastic mesocosms to facilitate water exchange and oxygenation. Mesocosms used for the trials were 1-L containers, 20-cm diameter, with bottom and side openings covered by 125-µ nylon mesh screens to facilitate water flow through them. Salinity-temperature trials were conducted for a period of 24 h. Salinity treatments were randomly assigned to experimental units (40-L aquaria) that contained filtered seawater obtained from a common reservoir. Treatments for temperature

and salinity combinations were prepared with three replicates each. Salinity treatments used for the trials were 25, 30, 32, 35, 40, 45 and 50 ppt; temperature treatments were 8, 14, 15 ,16, 17, 18, 19, 28, and 30°C. Optimal levels of pH and dissolved oxygen (8.1 pH and >5.00 mg/L DO) were maintained for all treatments. Temperature treatments were achieved by using heaters, chillers, temperature controllers, and oxygen saturators. Salinity challenge treatments were achieved by mixing natural pre-filtered seawater (32 ppt) at ambient temperature (22°C) with artificial seawater salts, sodium chloride, and distilled-deionized water in proportions recommended by the manufacturer (Fritz, Aquatic Ecosystems, Florida). .The control treatment was designated as 32 ppt and 18ºC. After the 24-h experimental run, eggs and larvae were counted under the microscope to record final counts of individuals among categories as either normal, live, deformed, dead larvae or undeveloped eggs (Liew et al.2006). .A subsample (5-10 larvae) from each mesocosm was examined using a microscope to measure developmental characteristics of larvae (total length TL, yolk sack and oil globule diameter) recorded to the nearest mm.

Statistical Analysis

To assess the treatment effects of salinity and temperature combinations on embryonic mortality (whether due to larval mortality or the embryos failing to develop), a standardize least squares linear model was constructed using a binomial error distribution and the logit link function. Backwards regression was used to eliminate variables unimportant to explaining larval mortality (P values > 0.05). To test for effects of salinity and temperature combinations on the developmental progress of larvae, a multinomial logistic regression was used to differentiate among seven categories as follows: normal, deformities, (scoliosis, lordosis, swollen larvae), not developed, and dead.

To understand the effects of salinity and temperature combinations on the measured characteristics such as total length of the surviving larvae, the size of the yolk sack, and the size of the oil globule, multiple regressions were performed using backwards elimination to remove non-significant effects ($P < 0.00001$). Aquarium experimental units were treated as a random effect influencing the intercept of the model to account for unmeasured differences inherent to the split-plot design utilized here to avoid pseudo replication. The significance ($P < 0.00001$) of the amount of variance explained by the random factor (aquarium unit) was determined using the likelihood ratio test to compare the model incorporating the random factor versus an identical model without the random factor. To assess the global significance of the fixed effects retained in the model after backwards regression, a likelihood ratio test was used to compare the final model of the retained fixed effects to a null model with only the random effect of aquarium included.

Results

Temperature alone, had a quadratic effect on larval mortality, with extreme values leading to reduced survival; whereas, the effect of salinity alone on survival was not significant ($\chi2(3) = 209.55$, McFadden's pseudo r2 = 0.0495, $p << 0.00001$, Table 1, Fig. 1). Combinations of temperature and salinity affected the development of southern flounder embryos over the course of the 24-h exposure to treatment ($\chi2(12) = 352.24$, $p << 0.00001$, Table 2). Low temperatures (8, 14, 15 °C) caused non-development or dead embryos over the 24-h treatment; higher temperatures (28, 30 °C) led to near 100 % larval mortality. Also, embryos exposed to both low salinities (25, 30 ppt) and low temperatures were more likely to exhibit edema or were dead after exposure to a hypotonic environment due to the embryos to becoming swollen.

Table 1. Coefficients and significance of logistic regression model of larval mortality.

	Estimate	Standard Error	Z	P
Salinity	0.023	0.047	0.489	0.62500
Temperature	0.770	0.233	3.3060	0.00095
Temperature^2	-1.551	0.155	-10.024	<0.00001

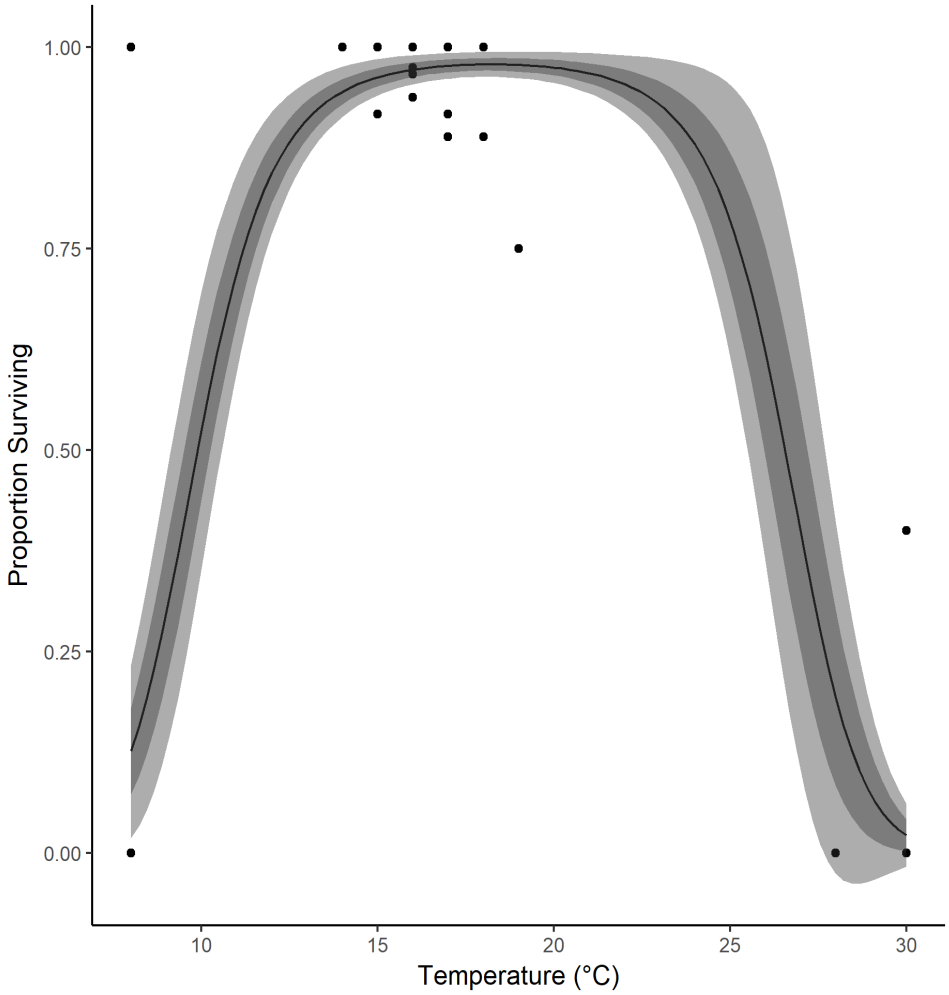

Fig. 1. Plot of the effect of temperature (°C) on larval survival. Points show proportion of larvae surviving at each temperature treatment. The line represents the generalized linear model with binomial error distribution. Darker shaded area represents one standard deviation of the predicted mean survival and lighter shaded area represents two standard deviations of predicted mean survival.

The random factor aquarium used to hatch the eggs, explained 24.3% of the variation observed in larval total length ($\chi^2_{(1)} = 23.18$, $p << 0.00001$). Temperature and salinity had additive negative effects on larval length based on an alpha of 0.1, with only temperature being significant with alpha of 0.05 ($\chi^2_{(2)} = 6.57$, conditional $r^2 = 0.260$, marginal $r^2 = 0.0495$, $p = 0.0374$, Table 2, Fig. 2).

Table 2. Coefficients of multinomial regression model showing the effect of salinity, temperature and their interaction effects on larval condition during development.

Condition	Independent Factor	Estimate	Standard Error	Z	P
Deformed	Salinity	0.0296	0.028	1.052	0.2930
	Temperature	0.0029	0.051	0.057	0.9540
	Temperature x Salinity	0.0017	0.002	0.812	0.4170
Swollen	Salinity	-2.9270	0.072	- 40.81	<0.0001
	Temperature	-6.5660	0.154	-42.61	<0.0001
	Temperature x Salinity	0.1520	0.003	44.398	<0.0001
Not Developed	Salinity	3.6990	0.003	1355.81	<0.0001
	Temperature	-0.3650	0.931	-0.392	0.6950
	Temperature x Salinity	-0.5210	0.022	-23.87	<0.0001
Dead	Salinity	0.034	0.034	0.993	0.3200
	Temperature	0.492	0.085	5.773	<0.0001
	Temperature x Salinity	-0.001	0.003	-0.429	0.6680

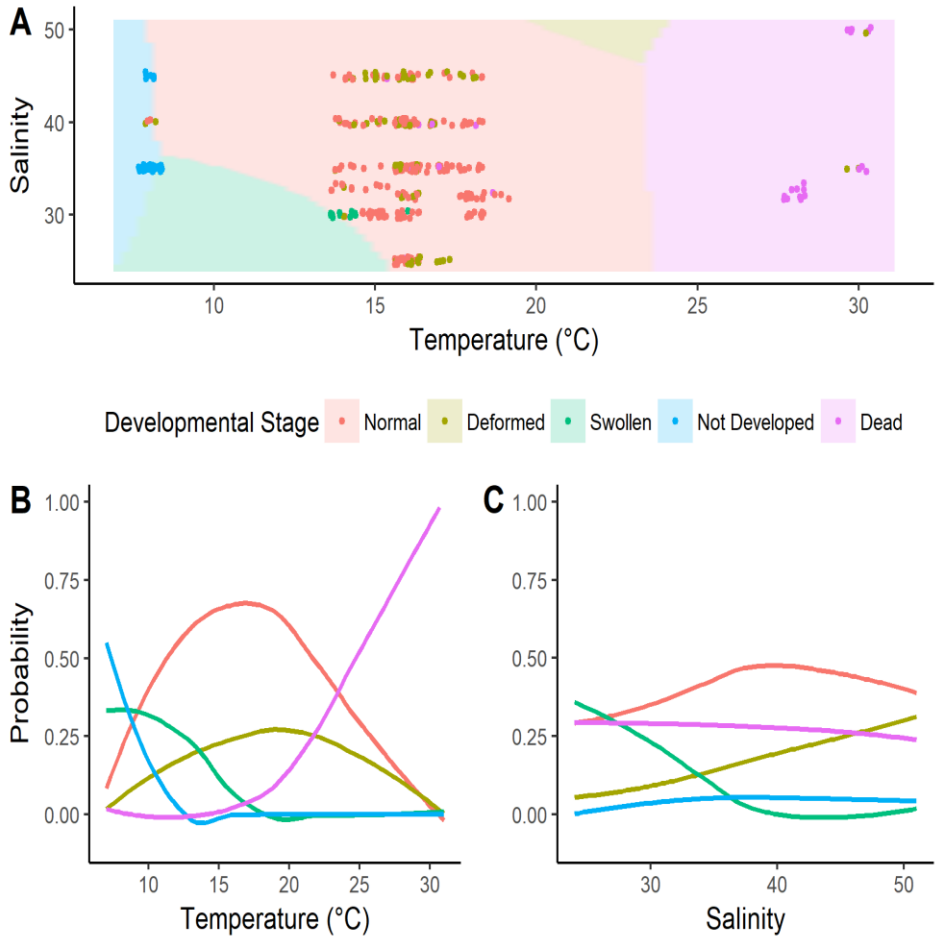

Fig. 2. Results of multinomial model to predict the condition observed during development of larval under test combinations of temperature and salinity. Panel A shows the most probable condition observed at test combinations of temperature and salinity. Panels B and C show the probability of observing each developmental condition under a single test factor (temperature or salinity, respectively), at the median test value of the other factor.

Table 3. ANOVA Table showing factors retained after backwards regression in explaining the size-total length of surviving individual larvae based on temperature and salinity treatments.

	Sum of Squares	Mean Squares	Numerator DF	Denominator DF	F	P
Salinity	0.241	0.241	1	69.588	.061	0.085
Temperature	0.339	0.339	1	53.695	4.301	0.043

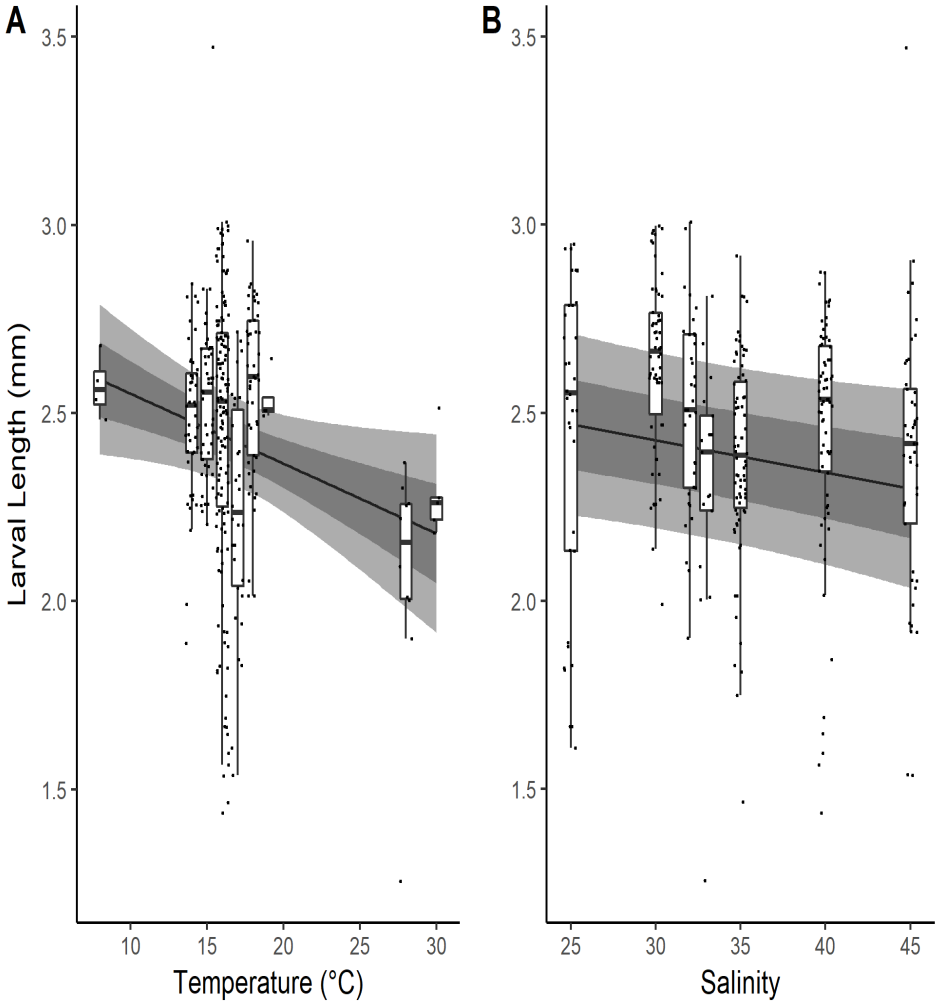

Fig. 3. Plot of the effect of temperature(A) and salinity (B) on the total length of surviving larvae. The line represents the predicted mean from the multiple regression model. Darker shaded area represents one standard deviation of the predicted mean length and lighter shaded area represents two standard deviations of predicted mean length.

The random effect of aquarium unit in which embryos were incubated and hatched explained 17.3% of the variation observed in larval total length ($\chi^2_{(1)}$ = 22.95, p << 0.00001). Temperature had a significant negative effect on yolk size ($\chi^2_{(1)}$ = 11.43, conditional r^2 = 0.217, marginal r^2 = 0.0535, p = 0.0007, (Table 4, Fig. 4).

Table 4. ANOVA showing factors retained after backwards regression in explaining the yolk sack size of surviving individuals based on different temperature treatments.

	Sum of Squares	Mean Squares	Numerator DF	Denominator DF	F	P
Temperature	0.276	0.276	1	110.69	11.945	0.00078

Neither salinity nor temperature were found to significantly influence globule size ($\chi^2_{(3)}$ = 2.23, p = 0.526, Table. 5).

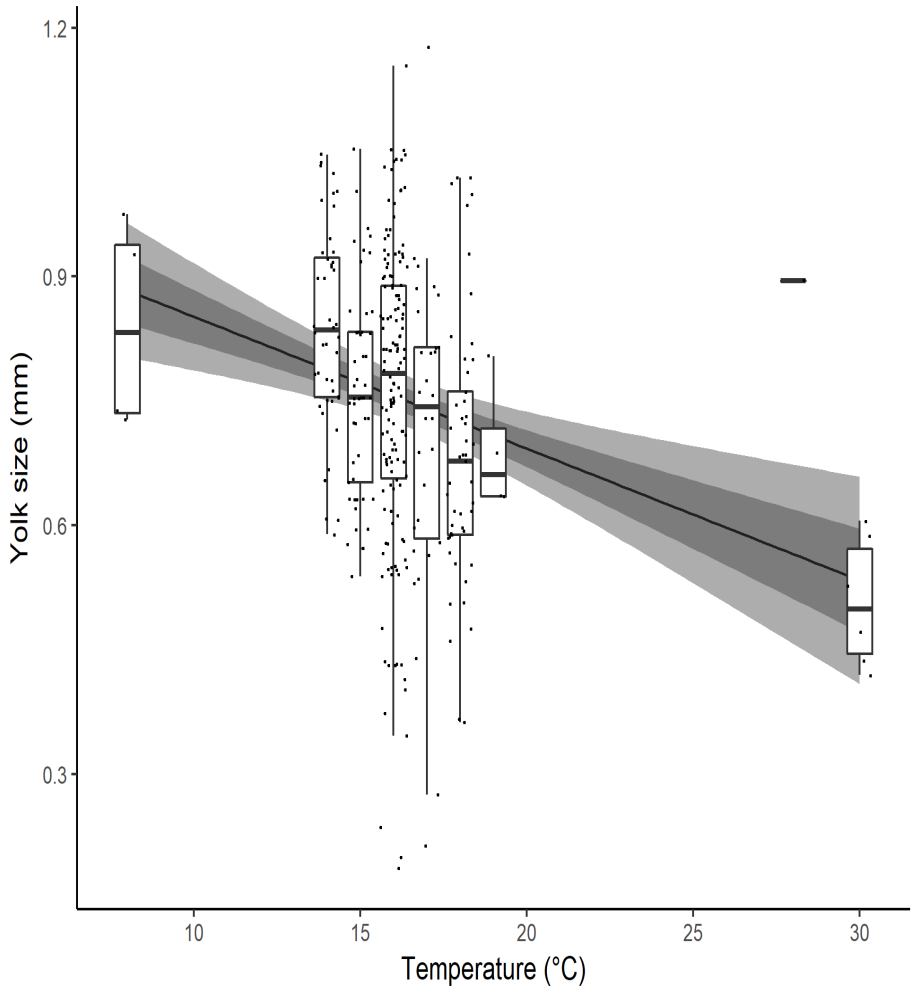

Fig. 4. Plot of the effect of temperature on the size of the yolk sack of surviving larvae. The line represents the predicted mean from the multiple regression model. Darker shaded area represents one standard deviation of the predicted mean length and lighter shaded area represents two standard deviations of predicted mean length.

Table 5. ANOVA table showing factors in the full model explaining the globule diameter of surviving individuals based on individual and combined temperature and salinity treatments. backwards regression removed all variables and the fixed effects were found to be non-significant.

	Sum of Squares	Mean Squares	Numerator DF	Denominator DF		P
Temperature	0.0012	0.0012	1	241.79	97	0.161
Salinity	0.0012	0.0012	1	253.61	99	0.159
Temperature x Salinity	0.0011	0.0011	1	269.16	83	0.177

Discussion

Temperature and salinity are two critical abiotic factors known to affect the survival of embryos and larvae by disturbing the normal development of these early life history stages (Elliot and Elliot 2010, Laurel and Blood 2011, Nash and Geffen 2012, Yan and Cheng 2005). In my experiments, the best survival and normal larval development was obtained at 17-18 C° and 32-40 ppt. Among experimental combinations of temperature and salinity, those outside of this range had some type of negative effect on larval development. Overall, temperature had the greatest effect on survival of the embryos and larvae. Lower temperatures impaired development and prevented embryos from hatching; whereas, higher temperatures caused higher probability of mortality and deformities. Among life stages, temperature tolerance is considerably narrower during embryonic development, especially during activation, cleavage and gastrulation (Yang and Chen 2005, Song et al. 2013, Liew et al. 2006). For this set of experiments, I used southern flounder eggs at gastrula stage to evaluate the ability of the embryos to tolerate abrupt changes of temperature and salinity

combinations as development was progressing and ending in newly hatched larvae (Alami-Durante et al. 2006). Embryonic development is an intricate process in which cellular differentiation, proliferation, migration and organogenesis occurs concurrently (Iwamatsu 2004, Liew et al. 2006, Cucchi et al. 2012). The formation of organs and somatic growth are mainly controlled by enzymes (Zanbonino-Infante and Cahu 1994, Cahu et al. 2004, Savoie et al. 2011) that are affected by environmental temperature (Crisp et al. 2017, Thepot and Jerry 2015). The Intergovernmental Panel on Climate Change (2015) predicts an increase in global mean temperature between 1.5-4.5 ^{o}C degrees that is expected to cause populations of many fish species (including southern flounder) to decline in the natural environment (Kunkel and Liang 2005, Hansen et al. 2010, Hammond and Hofmann 2010, Fernando Martinez, Texas Parks and Wildlife Department Coastal Fisheries Division 2017, personal communication). This concern has increased the interest in aquaculture and stock enhancement as one way to moderate fishing pressure and to supplement natural populations (Vega 2009). Consequently, knowledge on the effect of temperature and salinity combinations on embryonic forms and larval development is a prerequisite for a successful hatchery operation (Benetti et al. 2001, Carroll et al. 2005, Crisp 2017), while also contributing to the understanding of year class failure and instantaneous egg mortality rates in the wild (Koslow et al. 1985, Leggett and Deblois 1994, Garrido et al. 2015).

As a single factor, temperature is considered most critical in determining survival, development and growth of fish larvae. Motani and Wainwright (2015) reported temperature tolerance to be considerably narrower during embryonic development, especially during activation, cleavage and gastrulation stages. For my experiments, larvae at the sensitive, gastrula stage, were specifically used to evaluate the impact of salinity and temperature combinations on embryonic development.

My experiments supported this based on the fact that larval mortality showed a quadratic response to temperature; whereas, salinity had no apparent effect (Table 1, Figure 1). Both temperature and salinity were individually important for the viability of southern flounder embryos (Geffen, 2002, Houde, 1989, Jobling, 1997, Koslow, 1985). Low temperatures caused the embryos to not develop and to die during the lapse of 24 h; whereas, higher temperatures resulted in almost a 100% larval mortality. The best range for temperature was 16-18C°; those treatments produced normal larvae and the best survival (90-100%) when combined with salinities ranging from 32-42 ppt.

Under experimental conditions of sub-optimum salinity and temperature, eggs became swollen (edema from absorption of water)presumably caused by osmotic disequilibrium. The multinomial model used to predict the developmental condition of larvae that were hatched under a particular combination of temperature and salinity, accounting for mean temperature and salinity conditions (Figure 2, Panel A) shows the most likely outcome (highest probability) for each category of condition for larvae hatched at particular test combinations of temperature and salinity. Panels B and C show the probability of observing each developmental stage under a given temperature or salinity regime, respectively. Temperature affected the size of the yolk sack of surviving larvae but have no effect on the size of the oil globule at this stage of development.

This study showed the effects of temperature and salinity interactions on hatching success and developmental condition of southern flounder from Aransas bay, Texas. The interaction of temperature and salinity influenced the viability of southern flounder larvae, and optimal morphological conditions of the larvae after hatching. Temperature displayed the most negative effect on survival and normal larval development of this species.

CHAPTER III

EFFECTS OF EXPOSURE TO TEMPERATURE AND SALINITY COMBINATIONS ON THE SURVIVAL OF SOUTHERN FLOUNDER (*PARALICHTHYS LETHOSTIGMA*) PREMETAMORPHIC LARVAE SPAWNED BY PARENTS COLLECTED ALONG THE ENVIRONMENTAL CLINE OF THE TEXAS COAST

Introduction

The southern flounder (Paralichthys lethostigma) provides important commercial and recreational fisheries across it range from the Baja Laguna Madre del Sur in the western Gulf of Mexico to North Carolina on the East Coast of the United States of America (Darnell and Kleypas 1987, Ginsburg 1952, Hubbs et al. 1991, Matlock 1991, Powell and Henley 1995). Southern flounder adults migrate from the estuaries to deeper inshore waters of the Gulf of Mexico and Atlantic coast to spawn at depths of 20 to 60 m (Hoese and Moore 1998, Stokes. 1977). In the natural environment, eggs and larvae are transported by marine currents, therefore embryonic development occurs in a relatively stable marine environment (Passalacqua et al. 2005). Southern flounder spawn during late fall and winter months and settle in the estuaries as pre-metamorphic larvae or early post-metamorphosed juveniles (Burke et al. 1991, Glass et al. 2008, Nañez-James et al. 2009).The survival of eggs, embryos, larvae, and ultimate recruitment, depends on the environmental conditions of the nearshore, and coastal zones of the Gulf of Mexico and Atlantic (Meltzer and Sbrahmanyam 2016, Passalacqua et al. 2016) (Guindon et al.1995, Gutherz 1967, Furey and Rooker 2013, Jenkins et al. 1997, Miller et al. 1991).

During the last 20 years, meteorological studies have reported progressively warmer winter temperatures in the Gulf of Mexico (Chollett et al. 2012, Hansen et al. 2010, NOAA 2018,

Passalacqua et al. 2016). Salinity extremes in this area are associated with either abundant freshwater inflow from river basins (Antonov et al. 2010, Love et al. 2013, NOAA 2013) or higher than normal salinity, at the surface of the eddies or in coastal areas, due to excessive evaporation. In their natural environment, fishes are exposed to a wide range of environmental stressors very early in life; responses to adverse environmental conditions are considered fundamental to their survival, and include the development of protective biochemical mechanisms associated with protein folding and immune responses (Huang et al. 2015, Hu et al. 2014, Perrichon et al. 2017, Sudhagar et al. 2018). Abrupt fluctuations of environmental conditions also can impact the early developmental stages of southern flounder in their natural environment (Taylor et al. 2009, Van der Veer et al. 2000). Southern flounder larvae settle in the estuaries as pre-metamorphic larvae or early post-metamorphosed juveniles (Burke et al. 1991, Glass et al. 2008, Nañez-James et al. 2009). Populations of Southern Flounder have continued to decline and actually this species is listed as near threatened by the IUCN (Munroe, T. 2015. *Paralichthys lethostigma* htp//dx.doi.org/10.2305/IUCN.UK.2015-4RLTS.T202632A46958684.enww.iucnredlist.org/details/202632/0).

Despite the economic importance of southern flounder, comprehensive information on the early life history stages for this species, including temperature and salinity tolerance, acclimation or adaptations along environmental clines is not available. Therefore, I designed this study to investigate the survival of southern flounder pre-metamorphic larvae after acute experimental exposure under laboratory conditions to critical environmental factors (especially temperature and salinity) for individuals spawned by parents from three ecological regions located along the strong temperature and salinity cline of Texas coastal bays (Fig. 5).

Materials and Methods

Adult Fish Collection, Laboratory Maintenance and Spawning Conditions

Southern flounder adults (males and females) were collected in Sabine Lake Bay, Galveston Bay, and Aransas Bay, TX during their spawning migration in each of three consecutive years (2012, 2013, 2014) utilizing a 2-inch soft rubber dip net with a large bag. Aransas Bay fish were transported to the Marine Development Center in Corpus Christi, Texas; Sabine and Galveston fish were transported to Sea Center of Texas in Lake Jackson, Texas. All fish were transported in ambient seawater from their respective bays in hauling tanks with aeration. These broodstock fish were maintained in 1,600-L fiberglass tanks for one year to expose them to a temperature and photoperiod regime (18ºC and 10 h light:14 h dark) designed to induce sexual maturation of females and males (Watanabe and Daniels 2010) At the end of this period, adult females were treated with salmon gonadotropin releasing hormone (Western Chemicals Inc, Ferndale, WA, USA) at the dose of 0.1 ml per 0.5 kg of weight. Females and males were strip spawned 48 h after hormone treatment.

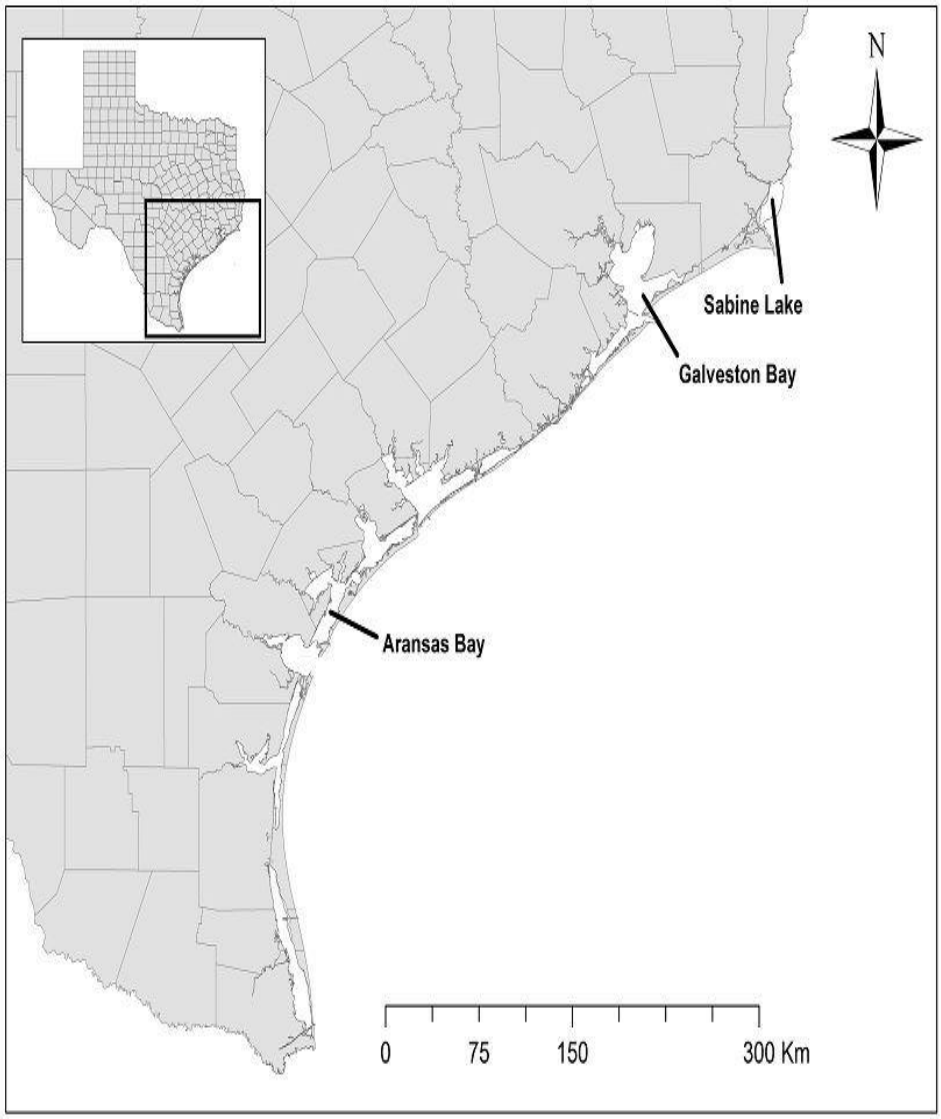

Fig. 5. Map of the Texas coast showing the collection localities for broodstock along the Texas coastal environmental cline. (Sabine 5 ppt, Galveston 33 ppt, Aransas nearby upper Laguna Madre 50 ppt)

Eggs were collected and mixed with sperm for 1.5 minute; sterilized seawater (1 L at 32 ppt of salinity) was used to induce motility (Benetti et al. 2001, Watanabe et al. 1999, Kaiser et al. 2012). Eggs floating at the surface were considered fertile, enumerated and separated from sinking unfertile eggs.

Culture and Long-Term Acclimation of Experimental Larvae

The number of fertilized eggs from each broodstock pair (based on the bay system where they were collected) was volumetrically estimated (using a graduated cylinder) and percent fertilization was determined via microscopic examination. Eggs from each bay's broodstock were transferred to a separate circular 250-L rearing tank filled with filtered (125 µ) seawater. Eggs were incubated with gentle aeration in a closed static system during the first 5 days. All tanks were equipped with aerators, heaters, and chillers attached to a temperature controller to maintain environmental parameters for long-term acclimation to experimental control values (18 ºC temperature, 32 ppt salinity, 5 mg/L DO, and 8.1 pH). Environmental parameters were monitored four times daily (8:00 AM, 12:00 PM, 4:00 PM and 10:00 PM) using a multi-probe meter YSI 556 MPS (YSI, Brannum Lane, Yellowsprings, OH, USA). Ammonia and nitrite were monitored three times daily using a spectrophotometer DRL 5000 (HACH, Loveland, CO, USA). For 30 days, ambient experimental conditions included continuous fluorescent light (100 lux) (Henne 2007) and air conditioning to stabilize room-air temperature (18-20 ºC). Water exchanges in the rearing tanks were performed at equal volumes four times a day with water previously adjusted to the control values of the experiment, salinity of filtered (125 µ) Laguna Madre water was adjusted with dechlorinated tap water, temperature was maintained at 18 ºC in the water reservoir located in the same room as the larval rearing tanks with a drop chiller fitted to a temperature controller. DO was maintained with a saturator fitted to an oxygen tank.

Larvae commonly hatch at 48-72 h post-fertilization; during this time flounder eggs and embryos were monitored every 6 h to verify normal embryonic developmental stages. Each stage was documented during the first week of culture by viewing larvae through a microscope lens (Micromaster-Fisher Scientific, Houston, TX, USA) fitted to a digital camera. The software

MICRON 1 (Mill Creek, WA, USA) was used to take measurements of the images. After absorption of the yolk sack (5 days, post hatch) southern flounder larvae were fed omega-3-fatty–acid-enriched rotifers (Bio-marine Algamac, Aquatic Ecosystems, Florida, USA). During the first 2 weeks, a total rotifer density of 40/ml was maintained in each rearing tank by enumeration three times a day and adjustment as needed to targeted density. After 15 days the diet was gradually changed to omega-3-fatty-acid-enriched Artemia sp nauplii (10/ml) supplemented three times daily (Alam et al. 2013) and formulated larval feed (Otohime, Japan). Development was monitored via stereo microscopic viewing to determine when the larvae had reached the pre-metamorphic stage (25-30 day/post hatch, and 8-10 mm TL).

Acute Temperature Challenges

A subsample of 30 pre-metamorphic larvae from each of the bay rearing tanks was placed into individual mesocosms (replicate experimental units). These were 1-L, 20-cm diameter, clear plastic baskets with bottom and side openings covered by 125-µ nylon mesh screens in order to retain the larvae, yet allow water and prey items to circulate in the mesocosm and larvae to continue feeding during the experimental treatment (4 h). A set of subsamples (comprising one mesocosm from each bay rearing tank) were randomly allocated to each of three 40-L aquaria as separate experimental replicates (larvae from each bay location within each temperature treatment).

Control temperature treatments: larvae were held at 18 °C and processed identically to the temperature challenge treatment groups (van Mareen and Daniels 2001, Kaiser et al.2012)

Temperature challenge treatments: Target temperature treatments were 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 °C. For each target temperature treatment, other environmental conditions were monitored using a multi-probe meter YSI 556 MPS (YSI, Brannum Lane, Yellow springs, OH) at the beginning and end of the 4-h temperature experiment. Ammonia and nitrite

were monitored at the beginning and end of the experiment using a spectrophotometer DRL 5000 (HACH, Loveland, CO, USA). Experimental aquaria were maintained at optimum values for other water conditions (32 ppt salinity, 5 mg/L DO, and 8.1 pH) by using heaters, chillers, temperature controllers and oxygen saturators during 48 h prior to beginning the experiment. Experimental treatments were maintained for 4 h to avoid total mortality, as determined by pilot studies. During the experiment, individuals were visually monitored for survival. Survival rates were calculated as the proportion of individuals surviving at the end of the 4-h experiment.

Acute Salinity Challenges

Salinity challenge experiments were conducted with pre-metamorphic larvae reared using the same methods, experimental design, and 4-h exposure time as described for the temperature challenge experiments; other ambient environmental conditions were maintained at the optimum values (18°C, 5 mg/L DO, and 8.1 pH) by using heaters, chillers, temperature controllers and oxygen saturators as previously described. Salinity values selected for the experiments as well as control condition represent the salinity cline observed on the Texas coast (NOOA-National Oceanic and Atmospheric Administration-Gulf of Mexico salinities data,2018).

Control salinity treatments were held at 32 ppt and processed identically to the salinity challenge treatment group. Salinity challenge treatment: Target salinity treatments were 5, 32, 35, 40, and 45 ppt, achieved by mixing filtered (125 µ) seawater at 32 ppt salinity with artificial seawater salts, sodium chloride and distilled-deionized water in proportions recommended by the manufacturer (Fritz, Aquatic Ecosystems, Florida) and maintained at experimental condition for 48 h prior to beginning the experiment. Southern flounder larvae survival was calculated at the end of the 4-h experiment as for the temperature challenge, with the exception that larvae from Galveston were not available for inclusion in the 5 ppt challenge treatment.

Experimental Design and Statistics

The survival of southern flounder pre-metamorphic larvae was calculated for experiments in each of three brood production seasons (January 2012, January 2013 and November 2013) for different sets of parents in each season from each bay location (Sabine, Galveston, and Aransas), for three replicates (mesocosms) per experimental treatment (Temperature and Salinity). A total of 32 aquaria were available for each experimental run. Therefore, temperature challenges for all combinations of temperature and bay location (under control salinity of 32 ppt) for a brood production season were run during one day. Then the aquaria were drained and cleaned within 24 h so that the salinity challenges for all combinations of salinity and bay location (under control temperature of 18oC) could be run using larvae from the same brood production season. Initial exploratory graphing analysis indicated that DO was also influencing survival (Figure2. Matrix Scatter Plot); therefore, I included DO in the model. A standardized least squares linear model was fit for the effects of target temperature (6, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 °C, under control salinity of 32 ppt), target salinity (5, 32, 35, 40, and 45 ppt, under control temperature of 18oC), and initial DO, as a full factorial model with up to three degrees for interactions, and with bay location as a by variable to fit separate models for each of the three bays (Aransas, Galveston and Sabine). Only up to three-way interactions were included in the model because higher order effects are less-easily interpreted.

Results

Environmental conditions during larvae rearing showed no stress to experimental animals as conditions remained within acceptable values (Ammonia 0.001- 0.0001 and Nitrite 0.003+0.0002 mg/L). During experiments, ranges for initial and final DO were acceptable (4.0 -

8.7 mg/L) and did not increased more than 2.0 mg/L during the experiment. The strongest effects on survival were for temperature, salinity, and their interaction with location (Fig. 6).

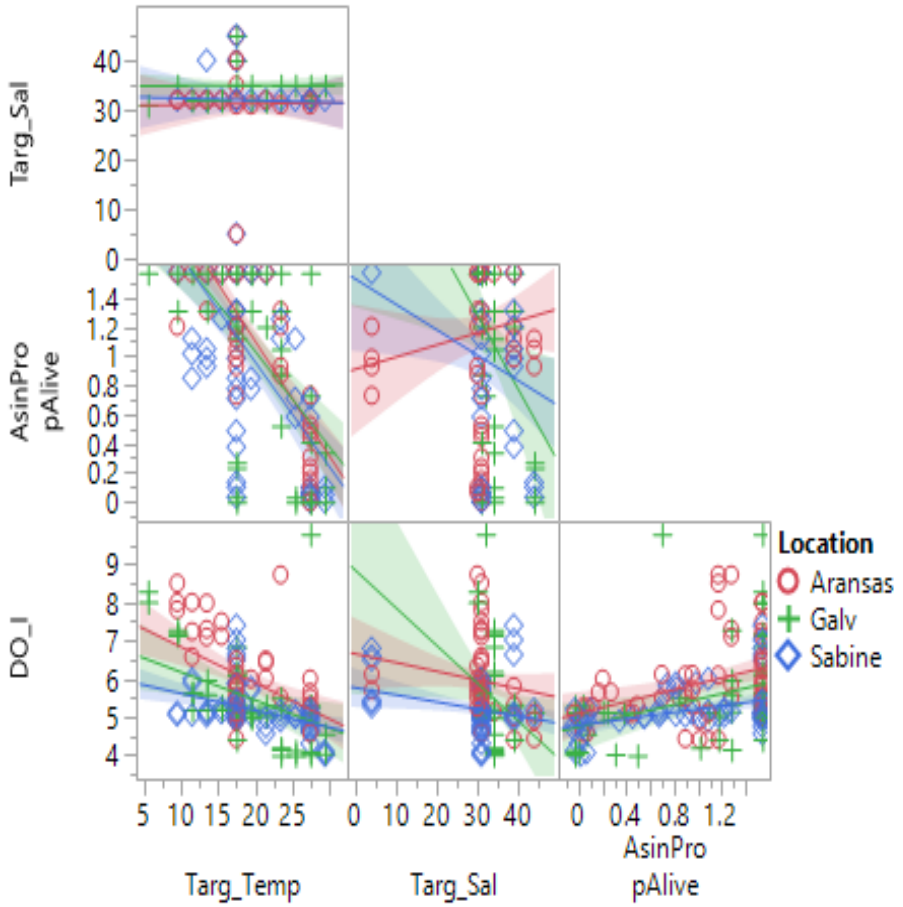

Fig. 6. Scatter matrix representing the data exploration and interactions of salinity, temperature and DO with location and the Arcsine proportion of pre-metamorphic Southern flounder larvae alive.

All locations had significant ANOVA models (Table 6). The model fit ranged from 0.84 (Aransas), to 0.61 (Galveston) and 0.52 (Sabine) (Table7).

Table 6. Analysis of variance showing results of the Standardized Least Squares Linear Model for location (Aransas, Galveston and Sabine).

Aransas					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	7	17.5284	2.5041	58.392	<.0001
Error	68	2.9161	0.0429		
C. Total	75	20.4445			
Galveston					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	7	16.1170	2.3024	14.771	<.0001
Error	54	8.4174	0.1559		
C. Total	61	24.5344			
Sabine					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Model	7	14.7555	2.1079	12.207	<.0001
Error	64	11.0519	0.1727		
C. Total	71	25.8074			

Table7.
Model Fit

	Aransas	Galveston	Sabine
Rsquare	0.8574	0.6569	0.5718
RSquare Adj	0.8427	0.6124	0.5249
Root Mean Square Error	0.2071	0.3948	0.4156
Mean of Response	1.16	1.0816	0.9898
Observations (or Sum Wgts)	76	62	72

Table 8. Parameters estimates for Standardize Least Squares Linear Model for Location

Aransas				
Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.1257	0.8115	1.39	0.1699
Targ_Temp	-0.1016	0.0082	-12.42	< **0.0001**
Targ_Sal	0.0638	0.024	2.66	**0.0096**
DO_I	0.0388	0.0387	1	0.3197
(Targ_Temp-19.8158) *(Targ_Sal-31.2105)	0.0246	0.0129	1.9	0.0611
(Targ_Temp-19.8158) *(DO_I-5.9625)	0.0357	0.0075	4.75	< **0.0001**
(Targ_Sal-31.2105) *(DO_I-5.9625)	0.0732	0.0225	3.26	**0.0017**
(Targ_Temp-19.8158) *(Targ_Sal-31.2105) *(DO_I-5.9625)	0.0181	0.0131	1.38	0.1723
Galveston				
Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	4.933	1.6618	2.97	**0.0045**
Targ_Temp	-0.0835	0.0177	-4.72	< **0.0001**
Targ_Sal	-0.0562	0.03	-1.87	0.0668
DO_I	-0.0215	0.083	-0.26	0.7968
(Targ_Temp-19.7742) *(Targ_Sal-34.9516)	-0.0024	0.0078	-0.3	0.7638
(Targ_Temp-19.7742) *(DO_I-5.49726)	0.0326	0.0107	3.05	0.0035
(Targ_Sal-34.9516) *(DO_I-5.49726)	0.0405	0.0448	0.9	0.3701
(Targ_Temp-19.7742)*(Targ_Sal-34.9516)*(DO_I-5.49726)	0.0053	0.0046	1.16	0.2492

Table 8. Continued Term	Estimate	Std Error	t Ratio	Prob>|t|
Sabine				
Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.8102	1.7987	1.01	0.318
Targ_Temp	-0.0599	0.0118	-5.06	< **0.0001**
Targ_Sal	-0.0048	0.0561	-0.09	0.9325
DO_I	0.1209	0.0937	1.29	0.2018
(Targ_Temp-20.1389) *(Targ_Sal-31.9583)	0.0095	0.0257	0.37	0.7144
(Targ_Temp-20.1389) *(DO_I-5.18806)	0.0437	0.022	1.99	0.051
(Targ_Sal-31.9583) *(DO_I-5.18806)	0.0307	0.3458	0.09	0.9295
(Targ_Temp-20.1389)*(Targ_Sal-31.9583)*(DO_I-5.18806)	0.0083	0.1616	0.05	0.9594

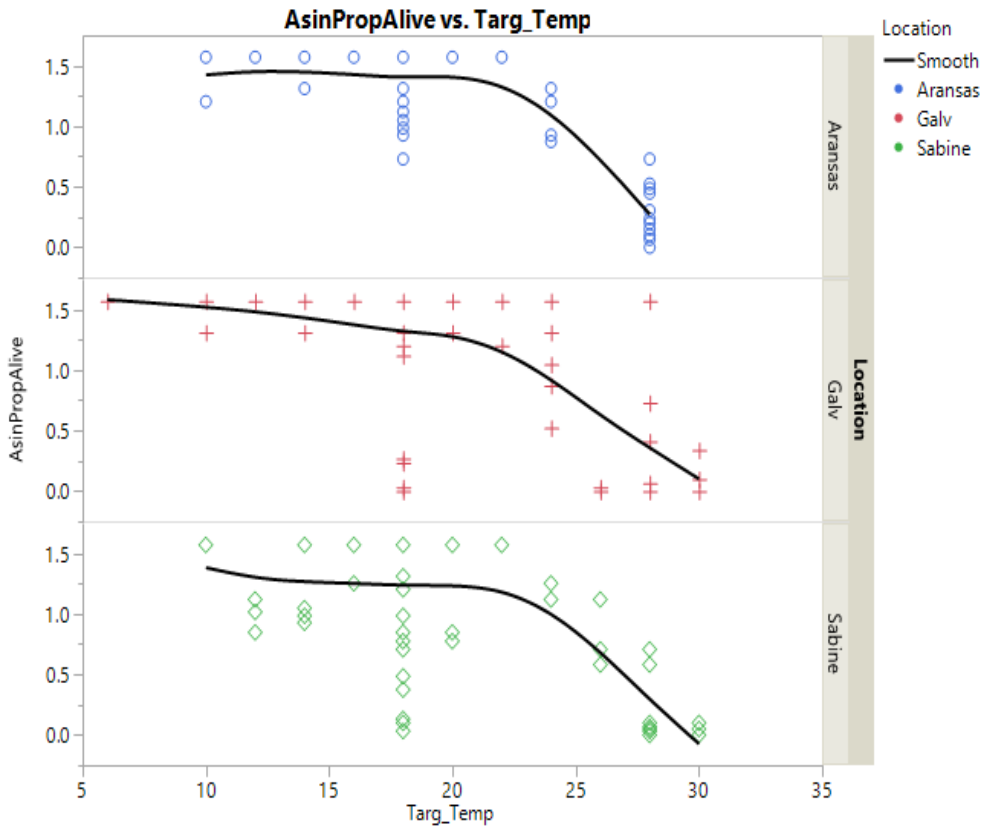

Fig. 7. Response representing the interaction of temperature, salinity and broodstock collection site as Arcsine proportion of southern flounder pre-metamorphic larvae alive after exposure to temperature and salinity challenges. Lines represent the smoothing function for the response of Arcsine proportion alive across replicates.

Temperature and its interaction with initial levels of DO had a significant effect on survival of pre-metamorphic larvae for all three locations; only Aransas larvae showed significant effects of salinity and salinity x DO (Table 3). Survival declined most rapidly in larvae from the three locations Aransas, Galveston and Sabine as temperatures increased above 24oC (Fig.7).

The best survival occurred for all three groups between 16-18 C°. Lower survival (< 75%) began to occur as Temperature increased above 24oC and DO declined below 7 ppm (Fig. 8). In reference to DO and salinity, larvae from Aransas had overall highest survival (> 75%) with an initial DO below 5 ppm and salinity increased above 35 ppt (Fig.9). In contrast, larvae from Galveston showed higher survival when salinity was approximately 35 ppt and DO was above 5 ppm, and survival for Sabine larvae was higher only when DO was above 5 ppm and salinity was at 15 ppt (Fig. 9).

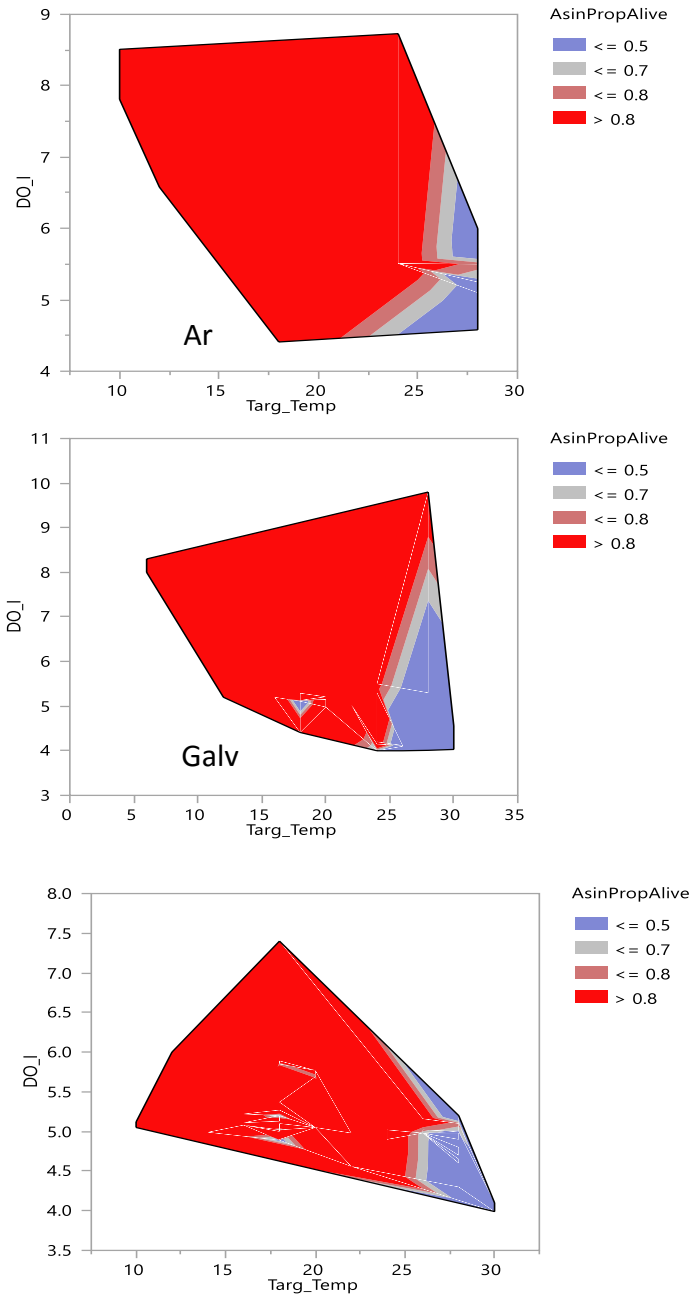

Fig. 8. Contour plot of Arcsine proportion alive for target temperature and initial DO values. Arcsine 0.5 = approximately 50% survival, Arcsine 0.7 = approximately 65%, Arcsine 0.8 = approximately 75% survival.

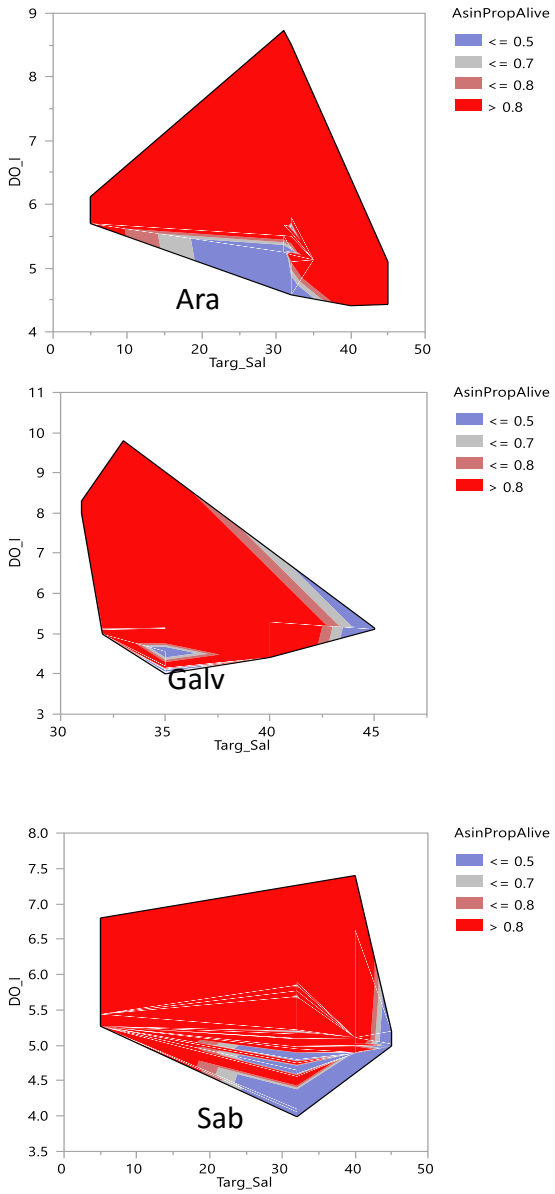

Fig. 9. Contour plot of Arcsine alive for target salinity and initial DO values. Arcsine 0.5 = approximately 50% survival, Arcsine 0.7 = approximately 65%, Arcsine 0.8 = approximately 75% survival.

Discussion

The Texas coast displays a complex interaction between salinity, temperature, and environmental gradients (Bodner and Sharp 1988, Dupuis and Anis 2013). This environmental mosaic is reflected in the distribution of southern flounder in Texas, with the greatest abundance in the northern coast from Sabine Pass to Port Aransas, and the lowest catch rate of the Texas coast in the lower Laguna Madre (Martinez-Andrade 2018). Additionally, correlation analysis of the occurrence of southern flounder juveniles and temperatures registered during winter and spring along the Texas coast revealed that during warmer winters, flounder recruitment was diminished, based on TPWD's data collected for more than 35 years (Martinez -Andrade 2018). Temperature is recognized as the most important abiotic parameter affecting survival and therefore recruitment of marine fish (Koenker et al.2018, Akin et al. 2003) in the natural environment. Differences in temperature between Sabine Lake and Aransas Bay can vary during winter from 6-10 °C and >30 ppt of fluctuating salinity (NOAA 2018).

The present study suggests differences in temperature, salinity responses, tolerances, and survival of pre-metamorphic larvae spawned by parents collected from different sites along the environmental cline of temperature and salinity of the Texas coast. The data showed the best survival for the three groups of southern flounder at this phase of development occurs at the 16 °C treatment, estimating that optimal temperature range for pre-metamorphic larvae is between 16-17°C and not 18°C, the target temperature, used as control for these experiments. The target temperature and salinity used to efficiently grow all the larvae for the experiments is also currently used to rear larvae at the Marine Development Center in Corpus Christi. Larvae from the three parental groups have been used for production but elevated larval mortalities on the Aransas fish were noticed with temperature increases of only 1°C (19-19.5 °C) above the target temperature

(Chavez, Texas Parks and Wildlife Department personal communication 2018). These data suggest that a higher survival for all three groups of larval until the pre-metamorphic stage of development can be achieved at lower temperatures of 16-17°C for Sabine, Galveston and Aransas larvae. It is possible that 18°C is the upper temperature tolerance limit for prolonged exposure of Aransas and Galveston Bay larvae, because this study also showed that Galveston Bay larvae tolerated similar temperatures as Aransas Bay fish during the pre-metamorphic phase. Conversely, Sabine's larvae displayed the same optimal temperature for survival of 16°C as Galveston and Aransas Bays, but Sabine larvae exhibited better tolerance to warmer temperatures. This difference in temperature response and survival may be explained by > 15°C differences in temperature recorded during winter between the geographical location of Sabine Lake and Aransas Bay, but only 3°C difference in temperature between Galveston and Aransas (NOAA National Centers for Environmental Information). Interestedly, Furey and Rooker (2015) calculated 16°C as the average sea surface temperature for the Texas coast during winter based on NOAA's data bank, but predicted with a model that newly settled post-metamorphic juveniles in Galveston Bay would be more likely to occur in areas with temperatures higher than 16°C.

Salinity is another important abiotic factor influencing survival of this species (Nims and Walter 2014, Breves et al. 2010). The results of this study showed differences in survival and tolerances of pre-metamorphic larvae to higher salinities (35, 40, 45 ppt). Aransas Bay larvae displayed the best survival for all treatments and even survived t 40 and 45 ppt salinity. However, larvae from Sabine and Galveston showed less tolerance to the high salinity treatments of 40 and 45 ppt. The lowest survival for the Sabine Lake and Galveston Bay pre-metamorphic larvae was recorded at 45 ppt. Nims and Walter (2014) revealed with their study of microelement signatures in southern flounder otoliths the existence of two groups, or contingents, of southern flounder in

the South Texas coast; one contingent using the freshwater-low salinity habitat, another using exclusively saltwater marine environment. That study also highlighted that use of the oligohaline habitat appeared to be facultative of the south Texas population and that could be a local adaptation to inter-annual drought cycles and variable freshwater inputs in this area. This study provides some support for the observed intraspecific differences in salinity tolerances and survival between Galveston Bay, Sabine Lake and Aransas Bay larvae. Additional support is furnished by differential gene expression distinguished between Galveston Bay, Sabine Lake and Aransas Bay southern flounder pre-metamorphic larvae (Blandon unpublished data 2018).

Parental effects, including transgenerational plasticity, in an ecological context of phenotypic plasticity, are important for population persistence in a changing environment. These are factors that may explain differences in physiological responses or adaptation (Jablonka et al. 1995, Kuijper at al. 2014, Uller 2008).

Genetic studies of southern flounder populations in Texas utilizing neutral microsatellite markers and mt-DNA show a very subtle, if not an absence, of genetic structure (Anderson et al. 2012, Anderson and Karel 2012) or clinal genetic variation (Blandon et al. 2001) based on allozymes. Population genetic studies of southern flounder on the Atlantic coast utilizing mt-DNA and amplified fragment length polymorphism (AFLP) revealed similar results, an absence of genetic differentiation at a broad spatial scale in the Atlantic basin, but in contrast genetic structure was detected at a finer spatial scale between estuaries (Wang 2013).

Tagging studies in North Carolina reported that southern flounder adults spawn offshore but return to near the site of tagging, or their estuarine residency (homing behavior), and that during the period of residency in the estuary their movements were limited (Craig and Rice 2008, Craig et al. 2015). Similar behavior was described for tagged southern flounder juveniles (Craig and Rice

2008). In opposition, recent tagging studies in the Atlantic coast revealed that southern flounder exhibit complex migratory patterns with some adults remaining offshore through late spring and summer months (Taylor et al. 2009). Movement of post-migratory adults along the US South Atlantic coast suggested a strong potential for mixing along different estuaries in different states of the US Atlantic Coast (Wang 2015.). The migratory habits of southern flounder and pelagic earlier life history may be a disadvantage for this species because groups spawning offshore and uncontrollable dispersal of the larvae may cause larvae with different tolerances to salinity and temperature to be transported to unfavorable environments that may represent an additional challenge for the persistence of this species.

In the face of climate change, the future of a species and their geographic distribution will depend on the ability of the species to respond to environmental alteration (Planque and Fredou 1999). According with this study, warmer winters in Texas may continue to impact recruitment of this species on the Texas coast as current trend data shows a direct correlation between winter temperatures and southern flounder relative abundance (Martinez 2018).

The intraspecific differences in temperature tolerance and survival of southern flounder larvae from Sabine Lake, Galveston Bay and Aransas Bay may be important for hatcheries in their efforts to increase survival of southern flounder larvae during rearing in tanks and ponds specifically during larval and pre-metamorphic stages. This information may also be significant for the design of juvenile's stockings sites to match the location of broodstock collection including best efforts to procure males and females exclusively from the same bay (Conover 1998). Broodstock numbers should be large for males and females to prevent reductions of genetic variation. Annual supplementation of new broodstock to the different hatcheries may also assist attempts to maintain genetic diversity.

Stock enhancement efforts have the potential to be more effective for this species by incorporating different approaches including physiological responses, phenotypic plasticity, and differential survival caused by environmental stress (Blankenship and Leber 1995, Cascade 2011). These data sets in addition to population genetics data would be instrumental to facilitate the survival of hatchery-raised individuals in the wild (Kitada and Kishino, 2006, Kitada, 1999, Laikre, et al. 2010)

Declines in the natural environment might be related to the life history characteristics of southern flounder, their behavioral migration from the bays to spawn offshore, and high dispersal of eggs and larvae that are transported by currents, eddies and tides across relatively large spatial scales. All these factors may influence the survival of the larvae or newly settled juveniles procreated by parents who may be locally adapted to particular environmental conditions but whose offspring are transported and settled in an environment dissimilar to the environment of residency of the parents.

This study shows that southern flounder premetamorphic larvae spawned by broodstock collected from Sabine Lake, Galveston and Aransas bay exhibited different responses in terms of survival to environmental challenges related to salinity and temperature. Aransas and Galveston Bay premetamorphic larvae raised under the same temperature showed less tolerance to high temperature. Meanwhile, Sabine Lake larvae presented a higher tolerance to elevated temperature treatments included in the experiment at premetamorphic stage. The differences in surface water temperatures between Sabine Lake and Aransas Bay can be up to >10 °C different during winter.

The present study suggests that environmental variabilities on the Texas coast combined with climate change may be contributing to the decline of southern flounder. In addition, other factors such as fishing pressure, drastic environmental changes (drought), habitat alterations and

loses (coastal development) and unexpected unfavorable environmental conditions (storms, toxic algae blooms) in areas of the Texas coastline may be contributing to this decline. Conservation efforts for this species may be limited by environmental constraints.

CHAPTER IV

TRANSCRIPTOMIC ANALYSIS OF PHYSIOLOGICAL RESPONSES OF SOUTHERN FLOUNDER (*PARALICHTHYS LETHOSTIGMA*) PREMETAMORPHIC LARVAE EXPOSED TO HIGH TEMPERATURE AND SALINITY

Introduction

Climate change is predicted to affect the survival of many marine organisms at all life stages. In the case of marine fish, global warming is expected to affect species-specific responses at the population, community, and ecosystem level (Hoffman and Sgro 2011, Jesus et al. 2017, Somero 2010, Somero 2002,) Tomanek 2010). Moreover, current environmental conditions caused by drought, limited freshwater inflows and other anthropological disturbances could be exacerbated by climate change. Genetic information on environmental adaptations and transcriptomic analysis coupled with eco-physiological responses may be useful to investigate extreme temperature limits and tolerance ranges within and among fish species (Buckley et al. 2001; Fangue et al. 2006, Limborg et al. 2011). Data on such species-specific differences will provide information revealing potential resilience and persistence of fishes during global warming. An important step in determining the response of species in shallow coastal ecosystems to temperature, and related changes in salinity, is to experimentally evaluate their tolerance to such changes (Rodriguez-Lanetty et al. 2009, Geffen 2002, Hansen et al. 2010, Hemmer Hansen et al. 2007, Iwama et al. 1998). The study of vulnerable early-life stages, such as larval fish, can provide clues about the distribution and abundance of adults and the resilience of species to rising ocean and coastal ecosystem temperatures (Einum and Fleming 2000, Elmer et al. 2011, Long et al. 2012).

Commercial and recreational marine species have been the focus of studies to assess the sustainability of these economically important fisheries under climate change scenarios (Kishi et al. 2010). Evaluations of physiological limitations of fish in natural populations can identify functional genes of adaptive significance, including genes that are linked to physiological stress related to environmental conditions or to depressed immunological responses (Basu et al. 2002, Cooke et al. 2008, Smith et al. 2013). Changes in distribution and range in fish have been linked to population genetic structure and environmental adaptations of species such as the European flounder *Platichthys flesus* (Larsen et al. 2008, Laurel et al. 2008*)* and Atlantic salmon *Salmo trutta* (Larsen et al. 2008). Information on the role and limits of adaptation associated with genotypic and phenotypic traits can be useful in the management and conservation of propagated as well as wild fish populations (Araki et al. 2008; Carlson et al. 2008, Fangue et al. 2006, Hohenlohe et al. 2010, Hohenhole et al. 2012). Genetic mechanisms include adaptive stress responses via phenotypic plasticity, which is the capacity of a genotype to modify phenotypic expression in response to changes in the environment (Mazurais et al. 2011, Podrabsky and Somero 2004, Roelofs et al. 2010). Southern flounder is a poikilothermic organism with physiological functions directly affected by the ambient water temperature (Carroll et al. 2005, Montalvo et al. 2012, Kaiser et al. 2012). This species inhabits shallow waters of estuaries that, in some cases, are exposed to drastic changes in temperature (Matlock 1991).

The southern flounder is an important commercial and recreational fishery across it range from the Baja Laguna Madre Del Sur in the western Gulf of Mexico to North Carolina on the East Coast (Hubbs et al. 1991). Southern flounder populations in Texas and Florida are near the southern limits of the species range, being rare or absent in Mexico south of the Baja Laguna Madre del Sur, and in Florida, south of the Loxahatchee River on the Atlantic Coast and the

Caloosahatchee River on the Gulf Coast (Murphy et al. 1994). This distribution may reflect temperature constraints to larval or juvenile survival (Houde 1989). This premise is supported by data series collected over 35 years by the Texas Parks and Wildlife Department (TPWD) that suggests a higher relative abundance of juvenile southern flounder in colder winter years in Texas (Fernando Martinez, personal communication; TPWD 2008).

The TPWD has implemented several management strategies to assist in the recovery of southern flounder populations. Nevertheless, flounder populations have not recovered as rapidly as expected (Martinez-Andrade 2018). Analyses of long-term trends in monitoring data for southern flounder in Texas coastal waters indicate stable recruitment of juveniles; however, trends show continued population declines in survival rates of post-juvenile southern flounder and adults (Froeschke et al. 2011).

Thus, to supplement natural stocks, TPWD initiated a southern flounder stock enhancement program, and is currently developing protocols and strategies for the artificial culture of southern flounder (Robert Vega, Director of the Marine Enhancement Program, personal communication). Preliminary studies of artificial propagation of southern flounder in North Carolina (Carroll et al. 2005) have found that incubation temperatures and larval rearing temperatures are critical for the successful propagation of this species (van Maaren and Daniels 2001), especially for early developmental stages (Faulk et al. 2007; Taylor et al. 2009). Differences in thermal optima and tolerances of southern flounder from different localities may imply that temperature protocols used in hatcheries in North Carolina or Florida are not applicable to Texas (Benetti et al. 2001, Carroll et al. 2005, Kaiser et al. 2012,). Moreover, temperature is crucial to sex determination in southern flounder (Luckenbach 2003; Luckenbach 2009; Montalvo 2012), in addition to playing a critical role in survival and growth.

Higher oceanic temperatures are expected to have deleterious effects on many marine organisms, especially those near the ecological limits of their distribution (Somero 2002; Dietz and Somero 1993; Fangue et al. 2006; Rodriguez-Lanetty et al. 2009). Southern flounder in Texas are within their most southern latitudinal range, but across Texas bays and estuaries, a complex mosaic of environmental conditions encompasses an extreme environmental gradient of temperature and especially salinity. Compared to bays of the upper coast, those of the lower coast are shallow, have limited freshwater inflow, limited exchange with the Gulf of Mexico (GOM), and are consistently warm and become hypersaline throughout much of the year (Tunnell 2001). The greatest relative abundances (catch-per-unit-of-effort) are recorded along the upper coast from Sabine Pass to Port Aransas, and lowest are in the Laguna Madre of the lower coast (Matlock 1982; Matlock 1991). Previous population genetic studies conducted across the species' distribution (Blandon et al. 2001, Anderson. et al. 2012) using a limited number of samples and microsatellite markers, have shown population divergence between southern flounder populations in the GOM and the Atlantic coast of the U. S. A. and genetic differences between the upper and lower Texas coast. One of the studies suggests clinal genetic variation of protein loci for southern flounder along the Texas coast (Blandon et al. 2001). Next Generation RNA Sequencing technology has been successfully used among populations and species to identify functional genes in studies of transgenerational plasticity, acclimation and adaptive capacity, epigenetics mechanisms, and local adaptation responses to a changing environment and resolution of genetic structure (Davey et al. 2011, 2010, Ekblom and Galindo 2011, Ferraresso et al. 2013, Nielsen et al. 2010). Discovering genes to be applied as markers for diseases, immune response, sex determination, metabolism and growth at early stages of development will be advantageous for culture activities to manipulate environmental conditions to study these important biological

processes (Luckenback et at.2009, Montalvo et al. 2012). Some other potential applications of molecular markers are the identification of hormones and proteins up-regulated at the onset of metamorphosis (Fijii et al. 1989, Infante et al. 2008, Schreiber 2001). Other applications of the markers are evaluation of growth, enhanced diets tests, and investigations of tolerance to higher or lower temperature or salinity in the natural environment, as well as applications for stock enhancement activities and aquaculture (Holopainen et al. 2012, Watanabe al et. 2010).

I designed this study to identify genes and quantify gene expression by pre-metamorphic larvae of southern flounder in response to acute salinity and temperature challenges. I compared responses of larvae produced by broodstock pairs collected from bays of the upper and lower coast (Sabine Lake, Galveston Bay in the upper coast and Aransas Bay in the lower coast). A “De Novo" transcriptome was assembled with RNA transcripts generated via Next Generation RNA Sequencing technology.

Materials and Methods

Adult Fish Maintenance and Spawning Conditions

Adult male and female southern flounder were collected in Sabine Lake, Galveston Bay, and Aransas Bay, TX during the spawning migration utilizing a dip net with a large bag (Fig. 1) Aransas Bay fish were transported to the CCA Marine Development Center in Corpus Christi, Texas; Sabine Lake and Galveston Bay fish were transported to Sea Center of Texas in Lake Jackson, Texas. All fish were transported in ambient seawater from their respective bays in hauling tanks with aeration. Broodstock fish were maintained in 1,600-L fiberglass tanks for 150 days to expose them to a temperature and photoperiod regime of (15-22 C° and 10 h light:14 h dark) designed to induce sexual maturation of females and males. At the end of this cycle, adult females were treated with salmon gonadotropin releasing hormone (Western Chemicals Inc,

Ferndale, WA, USA) at the dose of 0.1 ml per 0.5 kg of weight. Females and males were strip spawned 48 h after hormone treatment. Eggs were collected and mixed with sperm for 1.0 minute; sterilized seawater (1 L at 32 ppt of salinity) was used to induce motility (Kayser et al. 2012). Eggs floating at the surface were considered fertile, enumerated and separated from sinking unfertile eggs.

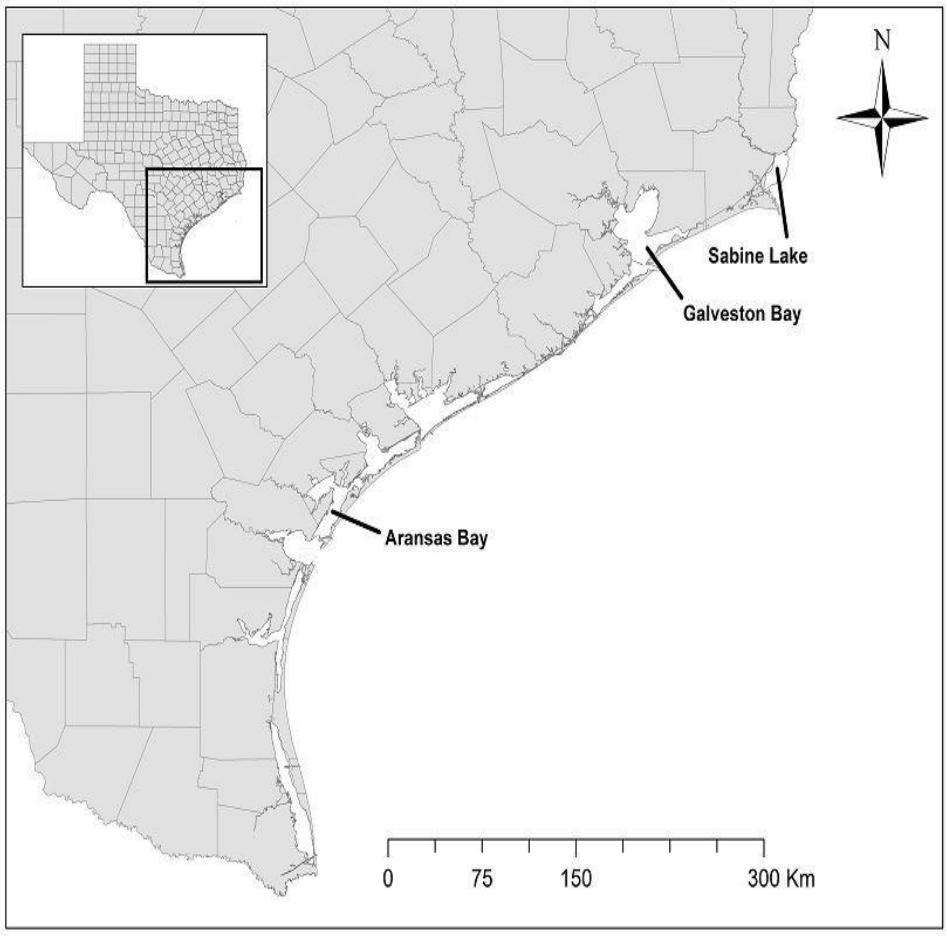

Fig. 10. Map of the Texas coast showing the collection localities for broodstock along the Texas coastal environmental cline. (Sabine 5 ppt, Galveston 33 ppt, Aransas nearby upper Laguna Madre 50 ppt).

Larvae Culture and Long-Term Acclimation of Experimental Larvae.

The number of fertilized eggs from each broodstock pair (bay) was volumetrically estimated (using a graduated cylinder) and percent fertilization was determined via microscopic examination. Eggs from each bay were transferred to a separate circular 250-L rearing tank filled with filtered (125 µ) seawater. Eggs were incubated with gentle aeration in a closed static system during the first 3 days. All tanks were equipped with aerators, heaters, and chillers attached to a temperature controller to maintain environmental parameters for long-term acclimation to Control values (18 ºC temperature, 32 ppt salinity, 5 mg/L DO, and 8.1 pH). Environmental parameters were monitored four times a day (8:00 AM, 12:00, 4:00 PM and 10:00 PM) using a multi-probe meter YSI 556 MPS (YSI, Brannum Lane, Yellowsprings, OH, USA). Ammonia and nitrite were monitored three times a day using a spectrophotometer DRL 5000 (HACH, Loveland, CO, USA). Ambient experimental conditions included fluorescent light (100 lux) 24 h a day for 30 days (Henne 2003) and a stable room-air temperature (20-22 ºC) using air conditioning.

Larvae hatched at 72 h post-fertilization, during which flounder eggs and embryos were monitored every 6 h to verify normal embryonic development. Each stage of development was documented during the first week of culture by viewing larvae through a microscope lens (Micromaster-Fisher Scientific, Houston, TX, USA) fitted to a digital camera. The software MICRON 1 (Mill Creek, WA, USA) was used to take measurements on the images. After absorption of the yolk sack (5 days, post hatch) southern flounder larvae were fed enriched rotifers (Bio-marine Algamac, Aquatic Ecosystems, Florida, USA). A total rotifer density of 40/ml was maintained to feed larvae during the first 2 weeks by enumeration three times a day and adjusted to targeted rotifer density in each rearing tank. After 15 days, the diet was gradually changed to omega-3-fatty-acid-enriched *Artemia sp* nauplii (10/ml) supplemented three times a day (Alam et

al. 2013) and formulated larval feed (Otohime, Japan). Development was monitored via stereoscopic examination to determine when the larvae had reached the pre-metamorphic stage (25-30 day/post hatch, and 9-12 mm TL).

Acute Temperature Challenges

A subsample of 30 pre-metamorphic larvae from each bay tank were placed into an individual mesocosm. Mesocosms were 1-L, 20-cm diameter, clear plastic baskets with bottom and side openings covered by 125-µ nylon mesh screens in order to retain the larvae, yet allow water and prey items to circulate in the mesocosm and larvae to continue feeding during the experiment (4 h). A set of subsamples (comprising one mesocosm from each bay rearing tank) were randomly allocated to each of three, 40-L aquaria as separate experimental replicates of larvae from each bay within each temperature treatment. Control temperature treatments: were held at 18 °C and processed identically to the temperature challenge treatment group. Temperature challenge treatment: 8°C. For each temperature treatment, other environmental conditions were monitored using a multi-probe meter YSI 556 MPS (YSI, Brannum Lane, Yellow springs, OH) at the beginning and end of the temperature experiment. Ammonia and nitrite were monitored at the beginning and end of the experiment using a spectrophotometer DRL 5000 (HACH, Loveland, CO, USA). Experimental aquaria were maintained at optimum values (32 ppt salinity, 4-5 mg/L DO, and 8.1 pH) by using heaters, chillers, temperature controllers and oxygen saturators for 48 h prior to beginning the experiment. Experimental treatments were maintained for 4 h to avoid total mortality, as determined by pilot studies. Oxygen was maintained at optimal levels (4-6 ppm) with aerators connected to a saturator attached to an oxygen tank.

Temperature, salinity, dissolved oxygen and pH were measured with a multi-probe meter YSI 556 MPS (YSI, Brannum Lane, Yellow springs, OH) at the beginning and end of each salinity

and temperature experiment. Ammonia and nitrite were monitored at the beginning and end of the experiment with a spectrophotometer DRL 5000 (HACH, Loveland, CO, USA).

During the experiment, individuals were visually monitored for survival. Survival rates for control and experimental groups were compared using inferential statistics. Larvae were anaesthetized with ice, counted and placed in a test tube with disposable RNA se free sterile pipettes in RNA later tissue preservative (Ambion, Austin, Texas, USA). Tissues were exposed to liquid nitrogen vapors to flash freeze them. Larval tissues were transferred to a -80 °C freezer for storage.

Acute Salinity Challenges

Salinity experiments were conducted with pre-metamorphic larvae reared using the same methods, experimental design and procedures described for the temperature challenge experiments, and exposure time (4 h) and other ambient environmental conditions were maintained at optimum values (18°C, 5 mg/L DO, and 8.1 pH) by using heaters, chillers, temperature controllers and oxygen saturators. Control salinity treatments: were held at 32 ppt and processed identically to the salinity challenge treatment group. Salinity challenge treatment: 40 ppt, was achieved by mixing filtered (125 µ) seawater at 32 ppt salinity with artificial seawater salts, sodium chloride and distilled-deionized water in proportions recommended by the manufacturer (Fritz, Aquatic Ecosystems, Florida) and maintained at experimental condition for 48 h prior to beginning the experiment. Southern flounder larvae survival was monitored as was done for the temperature challenge experiment, and findings similarly analyzed.

Three biological replicates of each combination of salinity and temperature (control at 18 ºC and high salinity at 40 ppt) for each of two broodstock collection sites (Galveston and Aransas Bays) were run for 4 h (i.e., 30 larvae per mesocosm per bay, within one 40-L aquarium for each

temperature and salinity combination, (Table 9) and were used to generate a set of samples to build 18 libraries for RNA Next-generation sequencing (three replicates per each of three salinity-temperature combinations for each of two bays). Experimental temperature and salinity treatment combinations were randomly assigned to experimental units (40-L aquaria). Each aquarium was filled initially with filtered seawater (150 µ) at 32 ppt of salinity, then experimental combinations of salinity and temperature were achieved with heaters or chillers fitted to temperature controllers and maintained at each experimental condition for 48 h prior to beginning the experiment. A second set of experiments was set the following year to include Sabine Lake samples in the analysis because samples from this group did not yield the quality and quantity of RNA needed for the Illumina sequencing.

After the completion of the experiment, live larvae were drained through a fine,250-µ nylon net fitted to a plastic frame. The fine mesh was replaced for every sample to prevent cross-contamination of RNA. The larvae were transferred with sterile RNAse-free plastic pipettes into RNA later solution (Ambion, Austin, TX, USA) and flash frozen in liquid nitrogen. Samples were stored in a -80 ºC freezer until total RNA isolation.

Total RNA Isolation, Library Preparation, and RNA Sequencing.

Total RNA samples were isolated from flash frozen pooled larvae (30 larvae/sample) by homogenizing and lysing the tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and sterile pestles, attached to a tissue homogenizer. RNA was extracted with chloroform and ethanol. Final purification was completed with RNAeasy Mini Spin Columns (Qiagen Sciences, Valencia, CA, USA) following manufacturer's protocols. All RNA samples were stored at -80ºC.

Table 9. Experimental conditions used for acute exposure (4 h) of southern flounder pre-metamorphic larvae to different temperature and salinities to generate RNA samples for next generation RNAseq.

Experimental Group	Temperature (°C)	Salinity (ppt)	Replicates	Number of Larvae
Aransas Bay Control	18	32	3	90
Aransas Bay High Temperature	28	32	3	90
Aransas Bay High Salinity	18	40	3	90
Galveston Bay Control	18	32	3	90
Galveston Bay High Temperature	28	32	3	90
Galveston Bay High Salinity	18	40	3	90
*Sabine Lake High Temperature	28	32	4	90
*Sabine Lake High Salinity	18	40	3	90

Total RNA was measured with a NanoDrop 1,000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, NC, USA). Quality of RNA samples were analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the RNA Nano Bioanalysis chip. All processed samples exhibited RNA integrity numbers between 8.9 and 9.8 and a 260/280 absorbance between 1.8 and 2.2. The extracted RNA samples were used for

cDNA synthesis at the Texas A&M University, College Station, Agrilife Molecular Laboratory. Poly A RNA was isolated with dt beads (Qiagen Sciences, Valencia, CA, USA). RNA samples were fragmented. Fragments were ligated to two adaptors (P1 and P2). The P1 sequencing primer contained a 6 bp unique sequence as an identifier. The P2 adapter contained an Illumina (Illumina, Inc, San Diego, CA) primer for pair end-sequencing. After the enrichment and adapter ligations, samples were evaluated by size using gel electrophoresis. cDNA extraction

was conducted with a Mini Elute Gel Extraction kit (Qiagen, Valencia, CA, USA). Only cDNA 200 ± 25 bp fragments were purified and enriched via PCR to construct 21 cDNA libraries. External RNA, ERCCExFold RNA Spike-In Mixes, as standards (Life Technologies, MA, USA) were used to quantify gene expression across temperature and salinity experiments (Sunde et al. 2018, Forsman 2015). Next Generation RNA Sequencing was performed in three lanes on an Illumina Hi Seq2000 (Illumina, Inc., San Diego, CA, USA) platform as a paired-end 100 bp reads.

Bioinformatics of Hi Seq RNA

Sequences were visualized with FastxToolkit, the quality was assessed with FastQC (version 10.0 http://www.bioinformatics.babraham.ac.uk/project/fastqc), and finally they were trimmed with FastQC/A clipper. The screened high-quality short sequence reads produced by the Illumina RNA sequencing were then aligned with the ultrafast short-read mapping program, Bowtie (http://bowtie-bio.sourceforge.net/index.shtml) (Langmead et al 2009, Li et al. 2009). The software Top Hat (http:// tophat.cbcb.umd.edu/) was used to align short sequences from southern flounder and aligned to the human (*Homo sapiens*), three spine stickleback (*Gasterosteus aculeatus*), Japanese puffer fish (*Takifugu rubripes*), and zebrafish (*Danio rerio*) genomes to identify exon-exon splice junctions. The software Cufflinks (http:// cufflinks.cbcb.umd.edu/) was used to assemble the transcripts generated under two or more experimental conditions. The software feature Cuffdif (4http:// cufflinks.cbcb.umd.edu/) was used to report genes and transcripts that were differentially expressed. The application CummeRbund was use to apply the statistical package R Bioconductor (Trapnell et. al. 2012) to prepare differentially-expressed transcript plots. The transcriptome "de novo" assembler Trinity version r20140717 (Grabherr et. al. 2011, Hass et. al 2013) was used to assemble the southern flounder transcripts. Trinity assemblage pipeline is formed by three consecutive modules: Inchworm, Chrysalis, and Butterfly. The overlapping k-

mer values were extracted from the RNA sequence reads. The module Inchworm examined each k-mer in decreasing order of abundance, generating transcripts contigs, to report portions of alternatively spliced transcripts. Chrysalis was used to cluster the generated transcripts of paired end raw reads. This computation clustered together regions of closely related gene families and also partitioned reads among clusters. This module reported full length transcripts for alternatively spliced isoforms included in cDNA sequences. Multiple sequences for different experimental conditions were concatenated with this module into individual single files. The software BUSCO version 3(http://busco.ezlab.org/) was used to provide quantitative measures to assess the genome assembly, gene set, and transcriptome completeness, based on evolutionary-informed expectations of gene content from near-universal single copy orthologues selected from OrthoDB version 9. The annotation of the assembled contigs was used for gene identifications and gene function determinations that were conducted with BLASTX (http://www.BLASTX.org) by comparing the southern flounder transcriptome to protein sequences of the human genome, the three-spined stickleback genome (BROADS1) (https://www.broadinstitute.org/stickleback/stickleback-genome-project), the zebrafish genome (GRCz10) (https://www.ncbi.nlm.nih.gov/grc/zebrafish) and the green puffer fish genome(FUGU4.0) (https://genome.jgi.doe.gov/Takru4.home.html). Functional annotation of genes was conducted with the program Blast2go (http://www.BLAST2go.org). Fst values were calculated with the R Bioconductor. The software HMMER version 3.1b2 (http://hmmer.org/) was used to find homology with the EggNOG orthologous groups database version 4.5 (http://eggnogdb.embl.de/#/app/home), especially fiNOG (http://eggnogdb.embl.de/download/eggnog_4.5/data/fiNOG/), the database for fish. Each assemblage was given two annotations: one from a BLAST result, the second from the HMMER

result. The model Transdecoder from the Trinity project was used to predict the longest ORFs in each assembled contig. We then used HMMscan to compare the ORFs against the fiNOG database.

Differential gene expression analysis was conducted with DESeq 2 from the Trinity differential expression pipeline (a comparison for each sample and for each gene identified a calculation of the log 2, fold change of the log 2, the Standard error, T statistics, P value and adjusted P value). This data was used to prepare MA and Volcano plots for a graphical representation of each sample comparison. Heat map graphics were generated by comparisons of the top 1,000 differentially expressed transcripts and the TMM normalized values. The EdgeR module of the software R (McCarthy et al. 2012, Robinson et al. 2010) was used to identify differentially expressed transcripts by conducting pairwise comparisons for each sample. The red data points in a Volcano plot corresponds to all the transcripts that were identified as being highly significant with a False discovery rate (FDR <= 0.05). FDR is a method used to evaluate the rate of type I errors in a null hypothesis testing when conducting a multiple comparison (Storey and Tibshirani, 2003). Trinity was used for extracting transcripts that were above some statistical significance (FDR- threshold) and fold-change in expression. These files were used to generate the heat map plots of differentially expressed (DE) transcripts that were at least 4-fold different. These files were then filtered by their P-values (FDR values) that were set at 0.001, and fold change (C) were set to 2^ (2) or 4-fold. The data files were used to prepare a matrix of the differentially expressed transcripts as a subset of the FPKM matrix.

Genes and gene products such as proteins are considered up-regulated when an organism synthesizes and increases specific cellular components or gene product (proteins, hormones, enzymes) in response to an external stimulus. If the organism decreases the amount of specific component in response to an external stimulus, this process is named down-regulation.

Comparisons of up-regulation are established by comparing the sample for a particular stressor against control conditions.

Results

All larvae used in the experiment were produced by wild broodstock from Sabine Lake, Galveston Bay, and Aransas Bay. During the larval rearing process, all three groups were maintained strictly under the same environmental parameters including temperature, salinity, oxygen and pH (temperature 18°C±0.5, salinity 32 ± 1 ppt, oxygen 5±1 mg/L, pH 8.1±0.5). Water quality was monitored a minimum of three times a day and ammonia fluctuated from (0.01 to 0.0052 mg/L). Nitrite levels were maintained very low (0.001-0.0003 mg/L) during larval rearing, with water exchanges of 30-50%, three times a day. The water source was maintained in a reservoir located in the same room of the rearing tanks to keep optimal environmental parameters, including temperature, for water exchanges.

Larval development of southern flounder reared from egg to pre-metamorphic stage (30 days post hatch) was documented. Embryonic development, larval, and pre-metamorphic larvae were normal. Larvae produced by the different broodstock used for the trials ranged in size from 8-10 mm TL at 30 days post hatch.

Next generation RNA sequencing performed on an Illumina HighSeq 2000 platform of 21 libraries, prepared with RNA from southern flounder pre-metamorphic larvae under different experimental conditions, generated 289,367,813 raw nucleotide reads. The total GC count of the sequences was 129,294,048 base pairs. A total of 329,198 sequences were produced after trimming. The sequences were assembled in N25: 3,049 (bp) and N:50 1,640 (bp). From the total amount of short read sequences, 96.48% were mapped to assemble a “De novo southern flounder pre-metamorphic larvae transcriptome”. The quantitative measures were conducted with Bench

Universal Single Copy Orthologs BUSCO v3 (Zdobnov et al. 2017) for the assessment of the transcriptome assemblage and completeness based on evolutionary informed expectations of gene content from near universal single copy orthologs selected by OrthoDB v9 (Lee et al. 2016). These analyses indicated that 81.3% of the transcriptome assemblages were complete, 7.7 % were fragmented and 11.0 % were missing. The exploratory cluster analysis showed the grouping of the data for the different treatments and groups of fish included in the study (Fig. 11). Galveston high temperature 28°C formed a cluster of three biological replicates, similar results were obtained with Aransas high salinity 40ppt and Aransas and Aransas control 18°C as far as the other conditions one Aransas sample for high temperature 28 °C exhibited some degree of similarity with Galveston 40 ppt but yet but also with the Aransas samples for high temperature 28 °C.

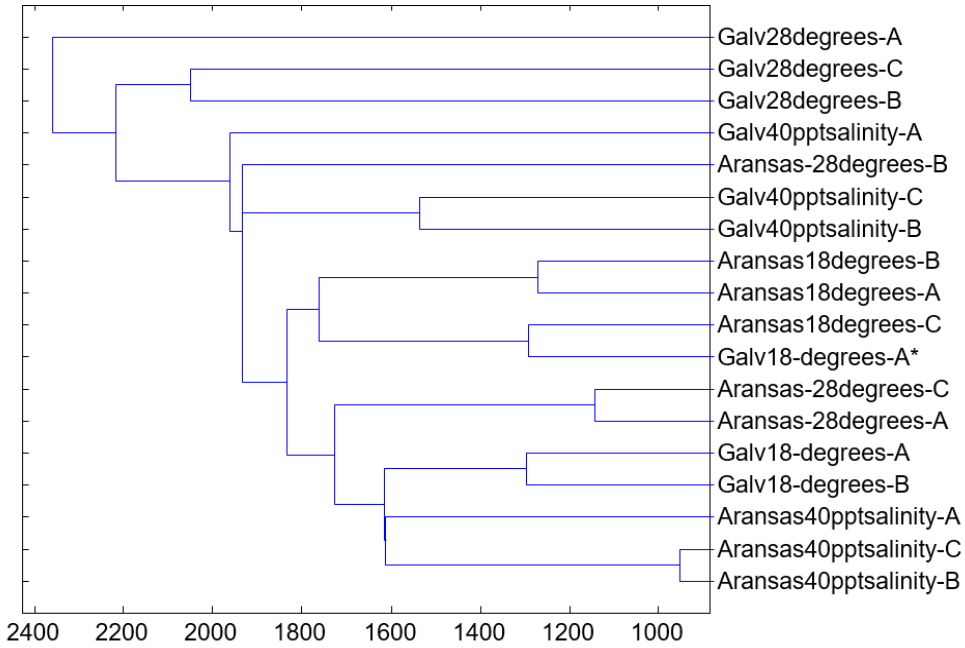

Fig. 11. Cluster analysis of RNA sequencing data generated from southern flounder pre-metamorphic larvae from Aransas Bay and Galveston Bay exposed to 28°C and 32 ppt salinity, and larvae from the same cohorts exposed to 18°C 40 ppt salinity compared to control conditions 18°C and 32 ppt salinity in both sets of samples.

Temperature Challenge Results

All tests to expose the larvae from the three groups of experimental fish lasted only 4.0 h. Galveston Bay RNA sequences produced under the high temperature treatment of 28°C yielded 3,856 up-regulated genes and only 7 down-regulated genes (Table. 10). In contrast, Aransas Bay samples generated under the same high temperature treatment of 28°C expressed a total of 11,923 up-regulated genes and 332 down-regulated genes (Table.10).

Table 10. Temperature and salinity treatments used on the transcriptome study, number of replicates and number of genes up-regulated and down regulated under each treatment

Samples	Experimental Condition	Genes up-regulated	Genes down-regulated	Biological Replicates
Galveston	Temperature 28°C- Salinity 32 ppt	3856	7	3
Galveston	Temperature 18°C - Salinity 40 ppt	6485	7	3
Aransas	Temperature 28°C - Salinity 32 ppt	11923	332	3
Aransas	Temperature 18°C - Salinity 40 ppt	5946	101	3
Sabine	Temperature 28°C - Salinity 32 ppt	377	921	4
Sabine	Temperature 18°C - Salinity 40 ppt	27626	302	1
Aransas	Temperature 18°C - Salinity 5 ppt	4,263	1,687	1

The Sabine Lake samples produced 377 up-regulated genes and 921 down-regulated (Table. 10) on the high temperature treatment (28°C and 32 ppt). Sabine Lake samples only yielded results for four biological replicates of the high temperature (28°C) experimental condition. All the control (18°C and 32 ppt) samples for Sabine Lake did not yield enough RNA for the Illumina sequence; therefore, I compared the high temperature (28°C and 32 ppt) samples from Sabine Lake to temperature control samples from Galveston Bay, the geographically adjacent group with a full complement of replicated-samples for control. A graphic representation of the results for Sabine Lake versus Galveston Bay as the control, are depicted on the MA and Volcano plots (Fig. 12). These results are plotted as a binary logarithm of fold-change on the x-axis versus statistical significance (negative base 10 logarithm of the p-value) showed the statistically significant (P value 0.001) expressed genes.

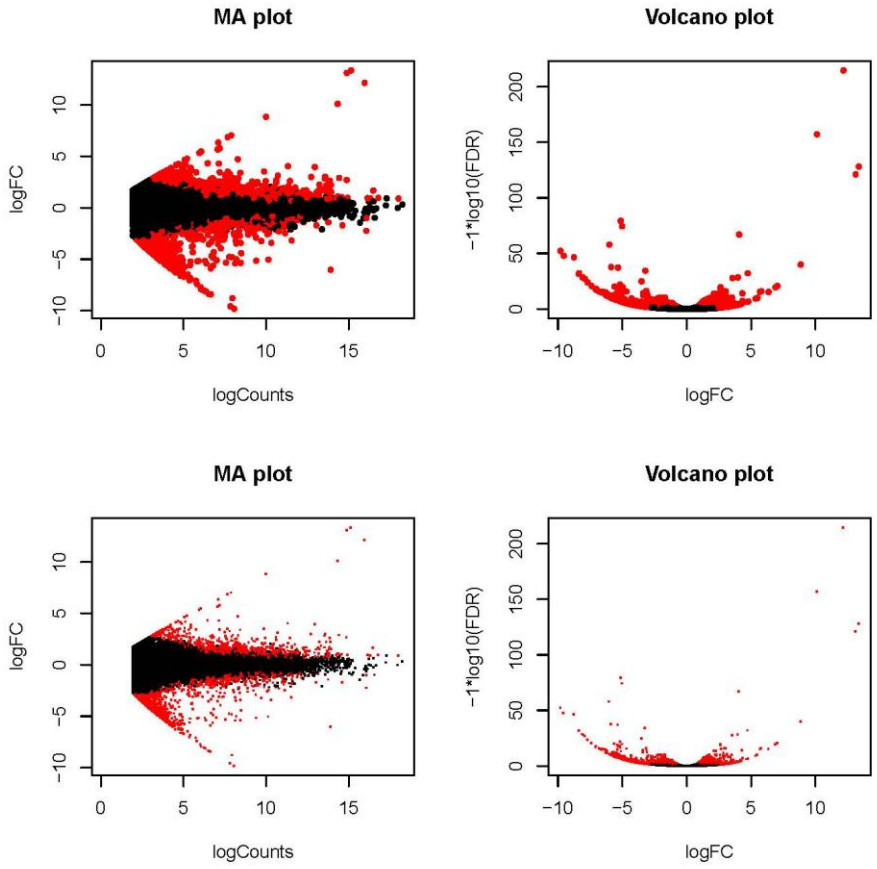

Fig. 12. Sabine Lake Volcano plots and MA representing gene expression control 18°C and 32 ppt from Galveston and Sabine high temperature 28°C under constant salinity 32 ppt. Genes with higher gene expression are located away from the center of the graphic and marked in red.

The Volcano plots representing gene expression in the replicated data for Galveston and Aransas Bays are shown in Figures 13 and 14.

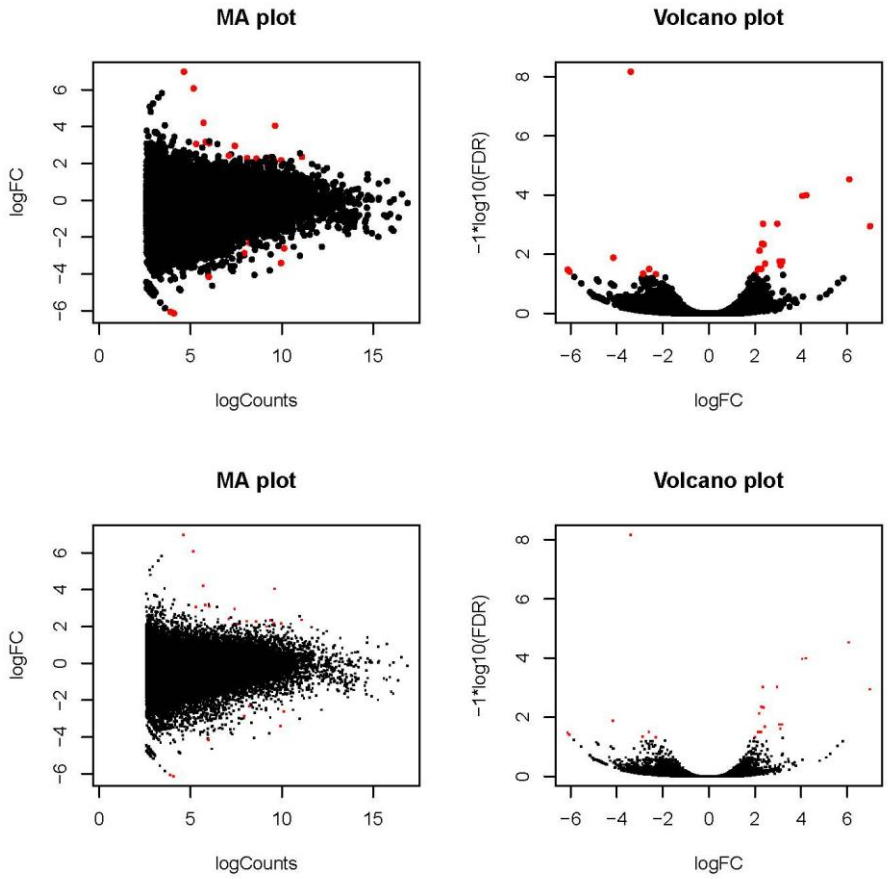

Fig. 13. Galveston Bay Volcano plots and MA representing gene expression at control 18°C and high temperature 28°C under constant salinity 32 ppt. Genes with higher gene expression are located away from the center of the graphic and marked in red.

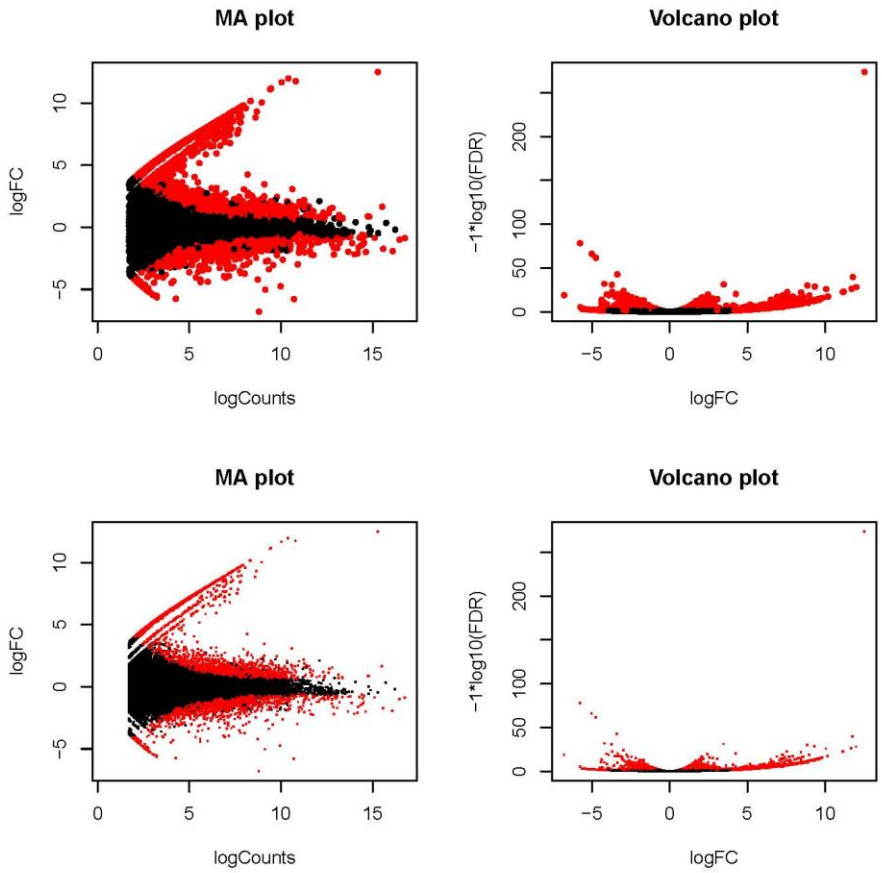

Fig. 14. Aransas Bay Volcano plots and MA representing gene expression at control 18°C and 32 ppt salinity high temperature 28°C under constant salinity. Genes with higher gene expression are located away from the center of the graphic and marked in red.

Temperature Results-Genes Identified

Thermal shock induces a denaturation of proteins, which can become cytotoxic by forming aggregates. Differentially expressed genes identified in the high temperature treatment (28°C) were numerous chaperone genes (Table. 11).

Table 11. Up-regulated genes in response to acute thermal stress (28°C at 32 ppt) compared to control conditions (18°C at 32 ppt) for 4 h for Southern flounder *P. lethostigma* premetamorphic larvae (30 day/post hatch).

Gene ID	Gene Name and Pathway
c13677	HSF1 Heat shock transcription factor
c158450	TPC1 subunit 2 Chaperonin beta cct2
c161248	TPC1 subunit 3Chaperonin gamma cct3
c16389	HSPB Heat shock 27 k (associated protein 1
c107303	HSPA 4 a Heat shock protein 4a
c105650	HSPA 4 b Heat shock protein 4b
c129981	HSPA 5 Heat shock protein family A
c16676	HSPB 7 OR HSP 20 alpha crystallin family
c114953	HSPA 8 Heat shock protein 8
c104955	HSPA 9 Heat shock protein 9
c12793	HSP10 Heat shock protein 10
c135424	HSPB11-Alpha-crystallin family HSP 20
c32716	HRSP12 Heat responsive protein 12
c101402	HSP14 Heat shock protein 14
c193759	HSPB 15 Heat shock protein alpha c
c129181	DnaJ Heat shock protein 40 member 13
c138654	HSPD 1 Heat shock 60 protein 1
c127526	HSP 70 kd Heat shock 90 proteins
c100581	HSP 70.1 kd Tandem duplicate cognate 70-kd
c102056	HSP 90 b1 Heat shock 90 protein beta 1
c100960	AGO1 Argonaute RISC catalytic t 1. Protein involved in gene silencing
c129359	rplp1 Ribosomal protein, large, P1
c101276	ttc4 Tetratricopeptide repeat domain 4
c100182	hnrnpm Heterogeneous nuclear ribonucleoprotein stress response heat stress
c100814	uap1l1 UPD-N-acetylglucosamine pyrophosphorylase 1, li
c147625	CANX Calnexin
c109278	pdia3 Protein disulfide isomerase family A, member 3
c122981	pdia4 Protein disulfide isomerase family A, member 4
c134	pdia5 Protein disulfide isomerase family A, member 5
c136875	pdia6 Protein disulfide isomerase family A, member 6
c101164	ASZ1 Ankyrin repeat, SAM basic leucine zipper c protein folding
c151215	ctsd Cathepsin D
c101309	ctsl.1 Cathepsin L
c136372	ctsz Cathepsin Z
c135048	dpp4 Dipeptidyl-peptidase IV-N terminal

Table 11 Continued	
Gene ID	**Gene Name and Pathway**
c107996	lgmn Legumain
c11103	sqstm1 Sequestosome 1
c10681	psmg1 Proteasome (prosome, macropain) assembly chaperone 1.
c109249	psmf1 Proteasome inhibitor subunit 1
c107903	psmf1 Proteasome subunit alpha 8
c117007	psme4b Proteasome activator subunit 4b
c110222	psmb5 Proteasome subunit beta 5 -Multicatalytic enzyme to break peptides
c111933	psme3 Proteasome activator subunit 3
c15653	psma5 Proteasome activator alpha5
c172018	psma1 Proteasome subunit alpha 1
c177616	psme4a Proteasome subunit alpha 4a
c181149	psma4 Proteasome subunit alpha 4
c182178	ube3a Ubiquitin protein ligase E3A
c185	ube2d1b Ubiquitin-conjugating enzyme E2D 1b
c186215	uchl5 Ubiquitin carboxyl-terminal hydrolase L5
c187877	ube2t Ubiquitin-conjugating enzyme E2T (putative)
c101182	RPP30. Ribonuclease ribonucleoproteins metabolism of RNA molecules.
c101183	MARS Methionyl-tRNA synthetase. Oxidative aspartate aminotransferase
c101184	PRIM1 Primase, DNA, replication and RNA transcription.
	Protein Biosynthesis
c202019	bxdc Brix domain containing 2 biogenesis of ribosomes
c127575	eef1a1b Eukaryotic translation elongation factor 1 alpha 1b
c33624	eif4g3a Eukaryotic translation initiation factor 4 gamma, 3a
c105	hmgb2a High mobility group box 2a
c3041	hmgb2 High mobility group box 2b
c52446	lsm5 LSM5 homolog, U6 small nuclear RNA, mRNA degradation.
c73075	lsm6 LSM6 homolog, U6 small nuclear RNA and mRNA degradation
c134993	rpl13 Ribosomal protein L13 60S ribosomal protein
c74649	rpl8a Ribosomal protein L18a 60S ribosomal protein
c1034	rpl27 Ribosomal protein L27 60S ribosomal protein
c210804	SMU1 DNA replication regulator and spliceosomal factor
c110266	snrpf Small nuclear ribonucleoprotein polypeptide F
c117467	snrpe Small nuclear ribonucleoprotein polypeptide E
c154253	snrpe Small nuclear ribonucleoprotein polypeptides B and B1
c81700	stat1a Signal transducer and activator of transcription 1a
c145408	tho5 THO complex 5

Table 11 Continued	
Gene ID	**Gene Name and Pathway**
c69228	zcchc17 Zinc finger, CCHC domain containing 17 Nucleoral protein of 40kDa
c101925	GBF1 resistance factor novel AMPK, phosphorylation, protein transport.
c101987	SZT2 Seizure threshold 2 homolog
c107087	STIP1 Stress-induced phosphoprotein 1. DNA repair.
c101994	BRCA2 and CDKN1A interacting protein.
c79160	Transcriptional regulator cardiac
c100938	DICER1, dicer 1, ribonuclease type II, dicer 1, role in synthesis microRNA
	Energy Metabolism Respiration
c170113	gpia Glucose-6-phosphate isomerase a
c161549	gpib Glucose-6-phosphate isomerase b
c51700	ldha Lactate dehydrogenase A4
c1599641	ckba Creatine kinase, brain a
c161425	ISYNA1 Inositol-3-phosphate synthase 1
c108998	GANAB Glucosidase, alpha; neutral AB
c120937	cmc2 C-x (9)-C motif containing 2 COX assembly mitochondrial protein2
c56629	cox7a2a Cytochrome c oxidase subunit VIIa polypeptide 2a (liver)
c26452	mt-nd1 NADH dehydrogenase 1, mitochondrial
c135558	mt-nd2 NADH dehydrogenase 2, mitochondrial respiration
c17836	apoa1bp Apolipoprotein A-I binding protein catalyzes the epimerization
c73453	hsd3b7 Hydroxy-delta-5-steroid dehydrogenase, 3 beta- steroid delta-isomerase
c13057	hexa Hexosaminidase A (alpha polypeptide)
c103033	SMOX Spermine oxidase
c129657	tdh L-threonine dehydrogenase
c100581	RPTP1Ribosomal protein, Stress trans generation acclimation to warming temp.
c100205	CAT Catalase, aerobically respiring protects cells toxic effects of peroxide.
c100342	ABC4a.ATP-binding cassette, sub-family G, member 4a. Energy translocation.
c100526	PKN2 Protein kinase N2, Cortisol stress gene, Insulin metabolism muscle.
c10059	RAPGEF, Rap guanine nucleotide fac Neuroendocrine pancreatic b cells-insulin
c100817	PNPT1, Polyribonucleotide nucleotidyltransferase. respiration, oxidation.
c101107	OGDH1, Oxoglutarate dehydrogenase. Enzyme bioenergetics, Krebs cycle
	Molecule and Ion Transport
c73976	fabp1.1 Fatty acid binding protein 1b, tandem duplicate 1
c51660	fabp11b Fatty acid binding protein. 11b Lipocalin/cytosolic fatty acid binding
c56728	Hemoglobin beta globin (LOC100174873), mRNA
c80366	rcn3 Reticulocalbin 3, EF-hand calcium binding domain
c73435	slc2a3b Calcium binding
c73886	slc2a3b Calcium binding protein A11

Table 11 continued	
Gene ID	**Gene Name and Pathway**
c154652	slc2a3b Ferritin, heavy polypeptide 1a stores iron in a soluble,
c78631	ceruloplasmin. Coagulation factor V-proaccelerin labile factor
c100884	RALGAPA 1, Ral GTPase activating protein, alpha subunit 1 (catalytic)
c110224	SV2, Synaptic vesicle glycoprotein 2, Sugar transporter
c103647	XPO5, Nuclear export signal receptor
c97972	XPR1b, Xenotropic and polytrophic retrovirus receptor 1b, cell transport
c100813	HIAT1a, Hippocampus abundant transcript 1a, Major Facilitator Superfamily
	Cytoskeletal Reorganization
c74412	G3BP2 GTPase activating protein (SH3 domain) binding protein 2
c72600	SGK1 Serum/glucocorticoid regulated kinase 1
c128581	ACT1 Actin, alpha, cardiac muscle 1
c136981	actn1 Actinin, alpha 1
c81659	cldn1 Claudin 1
c60913	cldna Claudin a
c61861	cldne Claudin e Claudin 5
c64868	tpm4a Tropomyosin 4a
c105174	tnnc2 Troponin C type 2 (fast)
c59525	tnni1a Troponin I type 1a (skeletal, slow)
c101311	ttna Titin a myosin light chain. Thermal acclimation of the heart muscle.
c168524	MYO19 Myosin XIX
c68031	GABARAPL GABA type A receptor associated protein1
c178628	GABARAPL GABA type A receptor associated protein like 2
c68425	mmp9 Matrix metallopeptidase 9, gelatinaseB,92kDa, collagenase IV
c68821	mmp13b Matrix metallopeptidase 13 b or metallopeptidase 20
c130046	tubb5 Tubulin, beta 5
c118574	plod1a Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1a
c110022	plod1a Collagen, type XV, alpha 1b
c100772	adam8a ADAM metallopeptidase domain 8a
c100771	Adamts ADAM metallopeptidase with thrombospondin type 1 motif 1
c100717	DDR2a, Discoidin domain receptor tyrosine kinase 2, protein collagen receptors
c101897	THBS 3a, Thrombospondin 3a, -3 glycoproteins, tumor metastasis, tissue repair.
c101839	GLB1, Galactosidase, beta 1-like, Enzyme in lysosome, elastin protein interacts neuroaminase 1
	Apoptosis and Cell Division
c106021	CDC27 Cell division cycle 27
c106334	FAIMA, Fas apoptotic inhibitory molecule a
c101168	PPP1R27b, Protein phosphatase 1, regulatory subunit 27b, myocardial contractility, Alzheimer
c10157	CASP6, Caspase 6, apoptosis-related cysteine peptidase. Apoptosis in fish.

Table 11 Continued	
Gene ID	**Gene Name and Pathway**
	Immune Response
c101899	MMD, Monocyte to macrophage receptors to sense environmental change
c111262	reacts inflammatory or anti-inflammatory.
c111262	MMD 2a, Monocyte to macrophage differentiation receptors to sense environment.
c1060	ISLR2, Immunoglobulin superfamily containing leucine-rich repeat 2.
c100814	UAP1-N-acetylglucosamine pyrophosphorylase 1, Thermal stress, low immune
c110574	Response. Oncogene protein glaucoma breast cancer
c110574	TITIN a, Immunoglobulin domain
c100004	PTPRFb, Protein tyrosine phosphatase, receptor type, f, b, Immunoglobulin, cancer suppressor
c110286	IL4I1, Interleukin 4 induced 1
c110589	ULK unc-51 like autophagy activating kinase 2
c110967	ICA1, Islet cell autoantigen 1, important during development, Islet cell activity.
c111434	IRF4a, Interferon regulatory factor 4a, virus infection adaptive immune system
c100004	PTPRFb, Protein tyrosine phosphatase, receptor, f, oncogene of immunoglobulin.
c100306	TSSC1, Tumor suppressing sub transferable candidate 1. Tumor suppressing cell protection-stress
c100608	PTPN12, Protein tyrosine phosphatase, non-receptor type 12, protein cell growth oncogenic formation.
c107475	HIC1, Hypermethylated in cancer 1, Zinc finger, C2H2 type
c110324	Fli-1 proto-oncogene, ETS transcription factor b
c110946	SART 3, Squamous cell carcinoma antigen recognized by T cells 3
c100306	TSSC1, Tumor suppressing subtransferable candidate. Tumor suppressing cell protection-stress
c101373	Idh 3b, Isocitrate dehydrogenase 3 (NAD+) beta, oncogene human cancers.
c10057	THOC, THO complex 1, immune response mRNA transcription.
c100618	MELK, maternal embryonic leucine zipper kinase protein. Cell division cancer resistance maternal mRNA
c97987	HIST2H3C, Histone cluster 2, H3c, Oncogene associated with glaucoma and breast cancer
c100995	DCC, DCC netrin 1 receptor, Immunoglobulin, angiogenesis Netrin promoting or inhibiting
c100149	PDE8A, phosphodiesterase 8A. This gene binds to the N-terminus of the oncogenic protein C-MYC.
c100771	ADAMTS 1 metallopeptidase with thrombospondin type 1 motif 1
c101484	SNRPA, Small nuclear ribonucleoprotein polypeptide A, Lupus, rheumatism autoimmune disease
c100550	MBOAT 7membrane bound O-acyltransferase, enzymatic oxidation of arachidonic acid.
c10184	SVEP1, sushi, von Willebrand factor type A. Lymphatic vessel. Breast cancer antibody.
c101937	SSNA1, Nuclear autoantigen 1, Sjogren syndrome, lacrimal, salivary glands autoimmune disease.
c101785	THEMIS2, thymocyte selection associated family member 2. Thymus cell to support T cell
c102178	FYN b proto-oncogene, Src family tyrosine kinase b, Stress response of the cell has developed.
c103671	HIST2H2AB, histone cluster 2, H2ab
c101866	XPNPEP1, X-prolyl aminopeptidase 1. Protein inflammation, autoimmune angioneurosis edema.
c100878	RNF41ring finger protein 41-Interferon, immune response, apoptosis, DNA repair.
	Stress Response

Table 11 Continued	
Gene ID	**Gene Name and Pathway**
c100165	TAOK2b, TAO kinase 2b, T stress activate in cells to protect DNA from damage,
c100182	involved in cell signaling microtubule organization, stability and apoptosis.
c100182	HNRNPM, heterogeneous nuclear ribonucleoprotein M, Myelin expression factor 2
c10098	KDAprotein involved in splicing as well as in early stress, expressed at heat shock.
c68732	Aromatase inhibitor (cytP19 B), vitellogenin and transferrin, stress, due to temperature.
c108883	GLRA3, glycine receptor, alpha 3, Neurotransmitter-gated ion-channel expressed during stress.
c10179	Neurotransmission for audition, glutamate, GABA, dopamine, norepinephrine, serotonin.
c10179	si:ch211-163l21.7
c110601	NBEAA, neurobeachin a
c110823	NMUR3, neuromedin U receptor 3, Serpentine type 7TM GPCR chemoreceptor Srsx
c111500	SNAP25a, synaptosomal-associated protein, 25a
c111597	NRXN3a, neurexin 3a
c100696	ABCC4, ATP binding cassette subfamily C member 4, stress glucocorticoids function
c100867	transport across membranes, maternal cortisol in stickleback embryos
c100867	TRDMT1, tRNA aspartic acid methyltransferase 1, stress response to abiotic stressor,
c10188	IARS, isoleucyl-tRNA synthetase, edits proteins that lead to brain, heart pathologies in adult fish.
c100748	INTS2, integrator complex subunit 2
c100749	WDR1, WD repeat domain 1stress response, enzyme involved in DNA repair
c100750	USP15, ubiquitin specific peptidase 15, stress response protein intracellular protein breakdown
c100770	APEX 2nuclease (apurinic/apyrimidinic endonuclease) 2, stress response, enzyme for DNA repair.
c100526	PKN2, protein kinase N2, Cortisol level fasting, stress gene
	Odor
c101070	TRPM3, transient receptor potential cation channel, subfamily M, member 3,
c101156	cellular sensors detecting environmental cues, temperature and osmolality, odor, vision, taste.
c101156	ITPR1b, Inositol 1,4,5-trisphosphate receptor, type 1b, odor activated nervous channel
c102206	MYEF2, myelin expression factor 2
c10462	IRS2b, Insulin receptor substrate 2bI, insulin like growth factor 1, plays a role in development
	Circadian Cycle
c131008	HTR6, 5-hydroxytryptamine (serotonin) receptor 6
c100352	CDK2, cyclin-dependent kinase 2. Circadian cycles
c100395	SKVC, SKI2 homolog, superkiller viralicidic activity 2-like, mRNA, RNA helicase circadian cycle
	Muscle Proteins
c9947	MYO15ab, myosin XVab
c10001	MYhb Myosin N-terminal SH3-like domain, myosin heavy chain, heart muscle contractions
c10001	MYH13, myosin protein
c100075	MAPKAPK3, mitogen-activated protein kinase-activated protein kinase 3
c10001	Myhb, Myosin N-terminal SH3-like domain

Table 11 Continued	
Gene ID	Gene Name and Pathway
	Cell Growth and Proliferation
c100510	EPS15L1, epidermal growth factor receptor pathway substrate 15-like.
c69948	cDNA and primary stucture of pre-growth hormone of three species of silver carp
	Sex Determination
c68732	Aromatase inhibitor (cytP19 B), vitellogenin and transferrin, caused by temperature stress
c65115	ADI1 -MTND
c76734	TGF-B gonadal differentiation.
c68894	Thyroid hormone and androgens differentially regulate gene expression in testes and ovaries
c65312	Piwi-like protein 4, plays a role during spermatogenesis
c100034	MYCBP-associated, testis expressed 1 maats1
c10061	Boule-like, this gene regulates male and female sex in Medaka and Stickleback
c100635	DDX-1, DEAD (Asp-Glu-Ala-Asp) box helicase 1, male sex determination in catfish
c100691	ADAMTS like2, ovary and follicle pre-ovulatory protease, involved in cell migration.
c100273	Calmegin spermatogenesis testis specific chaperone. Exosome helicase, involved in sperm fertility.
c10191	OVGP1, oviductal glycoprotein
c102339	PAQR 7-b Progestin and adipoQ receptor member VII-b, steroid membrane receptor progestin binding. MAPK
c105932	Missing oocyte meiosis regulation
c100510	HGNC, oocyte maturation, yolk, progestin, epidermal growth receptor, substrate 15-like1,
c100805	DDX 23, DEAD polypeptide 23, cellular growth spermatogenesis
c101056	TRPM-1b. Thermosensitive, ion channel to sperm in teleosts. Transient receptor potential cation channel
c106673	SPEF1 or HGNC sperm flagellar 1
c109295	TOPAZ domain 1 testis and ovary specific
c109363	PGR progesterone receptor
c109612	SOX 4-b or SRY Sex determining region Y
c112104	SPATA 22 Spermatogenesis associated 22
c112908	GTSF1 gametocyte specific factor 1
c112888	SPAG 9-a
c115935	PGRMC1 Progesterone receptor membrane 1
c119835	ODF 312 Outer dense fiber of sperm tails 3B
c120384	ESRRGA Estrogen related receptor gamma
c127613	SPEF2 Sperm flagellar 2
c133640	SPATA 4 Spermatogenesis associated 4
c130827	SPATA 5 Spermatogenesis associated 5
c134302	SPATS2 Spermatogenesis associated serine rich 2
c138597	SPAG9-a Luteinizing hormone/choriogonadotropin receptor
c149995	GMC like 1Germ cell less spermatogenesis associated 1
c156729	FNIP 1 Folliculin interacting protein 1

Table 11 Continued	
Gene ID	**Gene Name and Pathway**
c157250	MOSPD 2 Motile sperm contain 2
c167260	ZPCX Zona pellucid containing protein C
c172591	SPAM 1 Sperm adhesion protein 1
c65846	GGNBP2, gametogenin binding protein2. Zhou et al. 2007.Identification of a novel testicular germ cell protein.
c213717	VTG5 Vitellogenin 5
c76734	NELL 1 Sex chromosome XY gdf6Y encoding TGF-B gonadal differentiation. Ravi et al. 20114.
	Antifreeze Proteins
c58596	G3PDH Glycerol-3-phosphate dehydrogenase enzyme stored by fish in high levels as antifreeze protein
c61875	Antifreeze glycoprotein found in Rock cod and Nothotenia fish
c766769	C type lectin, carbohydrate binding. Freeze preventing protein in *Clupea harengus*
C79160	AFPIII Antifreeze protein related to genetic adaptation to cold environment in fish
c89198	AFPIV Antifreeze protein type IV related to genetic adaptation to cold environment in fish
c57691	Tetranectin, -Lectin Calcium dependent Nectin, novel fish antifreeze protein related to immunity
c10433	ATP50, ATP synthase, H transporting mitochondrial F1 complex, O is present in cold adapted fish
c101175	LRRC 47. Leucine rich repeat containing 47. Antifreeze protein, low temp tolerant fish glycoprotein
c64584	Kelch domain 9 protein related to glycosylation -glycan group gametocyte surface protein

These molecules are critical for protein folding and stabilization (Iwama 1998, Hammond and Hoffman 2010), forming part of a molecular control system to restore proteins that become misfolded when cells are exposed to different forms of stress, including thermal shock (Buckley and Owen 2001, Farrell, 2009, Wang et al. 2015). The southern flounder larval RNA sequencing revealed many copies or sequences of these genes and other genes, such as chaperonins, that were up-regulated. Chaperonins are considered essential for maintaining myofibril and cardiac actins organization (Melkani et al. 2017) and proper heart function. Another group of up-regulated genes identified were related to protein degradation and protein biosynthesis. Eukaryotic cells control catabolic and anabolic processes to maintain cells homeostasis (Zhang et al. 2014). Heat induces the use of nutrients to obtain the energy to cope with thermal stress. Hormones involved in growth,

such as growth hormone receptor and insulin-like growth factor, were present and up-regulated in the high temperature treatment (Nakano et al. 2013, Pierce et al. 2010). Additionally, heat stress has severe impact on the health of the fish, compromising immune function. The immune response up-regulated genes identified in southern flounder were immunoglobulins, interleukins, interleukin receptors, oncogenes, tumor suppressing, interferon-cytokines, and antibodies as immune responses to pathogens usually found in saltwater such as *Vibrio Sp.* Monocytes to macrophage differentiation receptors were also identified. Chaperonin molecules sense environmental change and respond with inflammation (Table.11). Several interleukins were present in the transcriptome, including the interleukin 17 (IL-17), a member of the family of cytokines that are responsible for inflammation as a mechanism of protection against pathogens. Heat stress induces responses involving three regulatory systems: neural, endocrine and immune. When the stressor is as in this study, the response pattern is stimulatory and the immune response shows an activating phase to enhance the response.

I found up-regulated genes related to the circadian cycle, the biological process of diurnal and nocturnal activities, displayed by the biological clock of 24 h of every organism. I maintained lights (100 Lux) over the larval rearing tanks 24 h. Light is an environmental parameter known to affect levels of serotonin, 5 hydroxitryptamine (5HT), a neurotransmitter, and several receptors that are related to light exposure, vasoconstriction and vasodilatation, growth and spinal motor - neuron regeneration in zebrafish. This molecule and associated receptors are also involved in healing (Barreiro-Iglesias et al. 2015, Gosh and Pearse 2015)

Other group of up-regulated genes linked to temperature in southern flounder were genes related to gonadal differentiation, among others the thyroid hormone, Piwi and Boule like, ADASMTS, HGNC, and aromatase inhibitor (Table.11). I was able to recognize molecular

markers for gonads of males and females. The temperature used to grow the southern flounder larvae was 18°C. At this temperature, the ratio of male: female flounder has been reported to be 50:50 for fish in Texas (Montalvo 2012). In this study, we identified gonadal aromatase (CYP 19a1) and histones. The CYP 19a1 is an enzyme that irreversibly catalyzes androgens into estrogen. The gonadal sex of several fish species with sex chromosomes (XX, XY), as is the case of southern flounder, can be influenced by exposure to extreme temperature which produces a skewed sex ratio (Fan et al. 2014, Matsumoto et al. 2016, Montalvo et al. 2012, Luckenback et al. 2012).

I am reporting the presence of several antifreeze proteins present in southern flounder. Some antifreeze proteins have been reported for other flatfishes from Northern latitudes such as the Japanese flounder (*Paralichthys olivaceous),* winter flounder, as well as, Antarctic teleosts fishes (Cheng and Detrich, 2007, Graham et al. 2013). These proteins have not been reported yet for southern flounder (Table. 11).

Salinity Challenges Results

Galveston Bay samples exposed to the high salinity treatment of 40 ppt and constant temperature of 18°C yielded 6,485 up-regulated genes and 7 down-regulated genes (Table. 10). However, Aransas Bay samples produced a total of 5,946 up-regulated genes and 101 down-regulated genes for the high salinity treatment of 40 ppt and constant temperature 18°C experimental condition. Meanwhile the number of genes up-regulated in a single Sabine Lake sample under high salinity 40 ppt, 18°C temperature, compared with Galveston control treatment for salinity, produced 27,626 up-regulated genes and 302 down-regulated. A single sample from Aransas Bay exposed to 5 ppt salinity produced only 4,263 up-regulated and 1,687 down-regulated transcripts (Table.10).

The Volcano plots (Figs. 15 and 16) represent gene expression in the replicated data for Galveston and Aransas Bays versus controls, plotted as a binary logarithm of fold-change on the x-axis versus statistical significance (negative base 10 logarithm of the p-value) showed the statistically significant (P value 0.001) expressed genes.

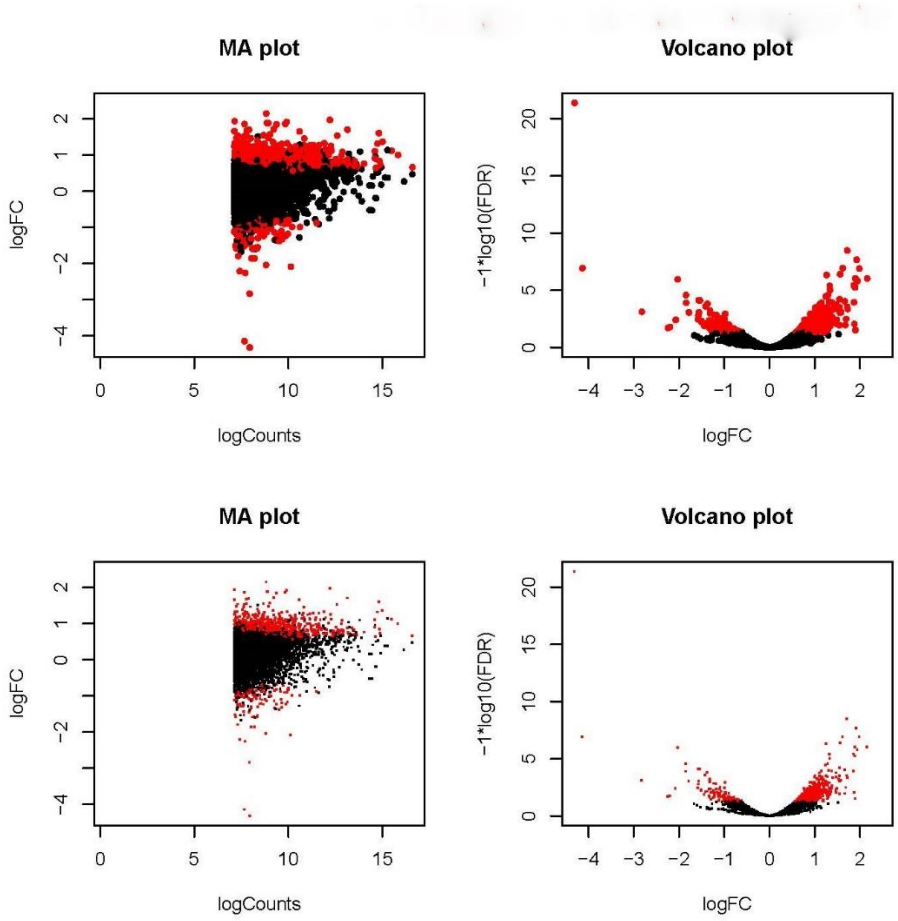

Fig. 15. Galveston Bay Volcano plots and MA representing gene expression at control 18°Cand 32 ppt salinity high salinity 40 ppt under constant temperature 18°C. Genes with higher gene expression are located away from the center of the graphic and marked in red.

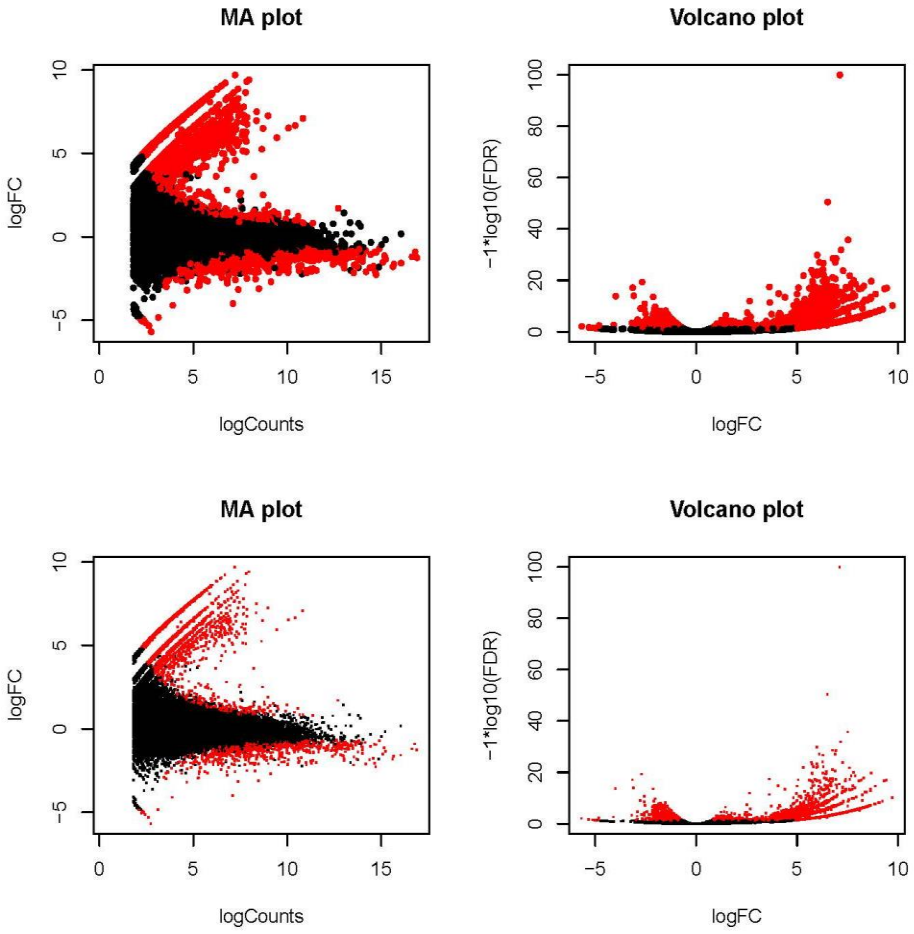

Fig. 16. Aransas Bay Volcano plots and MA representing gene expression at control 18°C and 32 ppt salinity high salinity 40 ppt under constant temperature 18°C. Genes with higher gene expression are located away from the center of the graphic and marked in red.

Gene expression of 1,000 genes from every set of samples analyzed were compared with their corresponding control and were used to prepare heat maps with centered genes (Figs. 17,18, and 19). Heat Maps were prepared to graphically represent individual values of gene expression of up-regulated and down regulated genes.

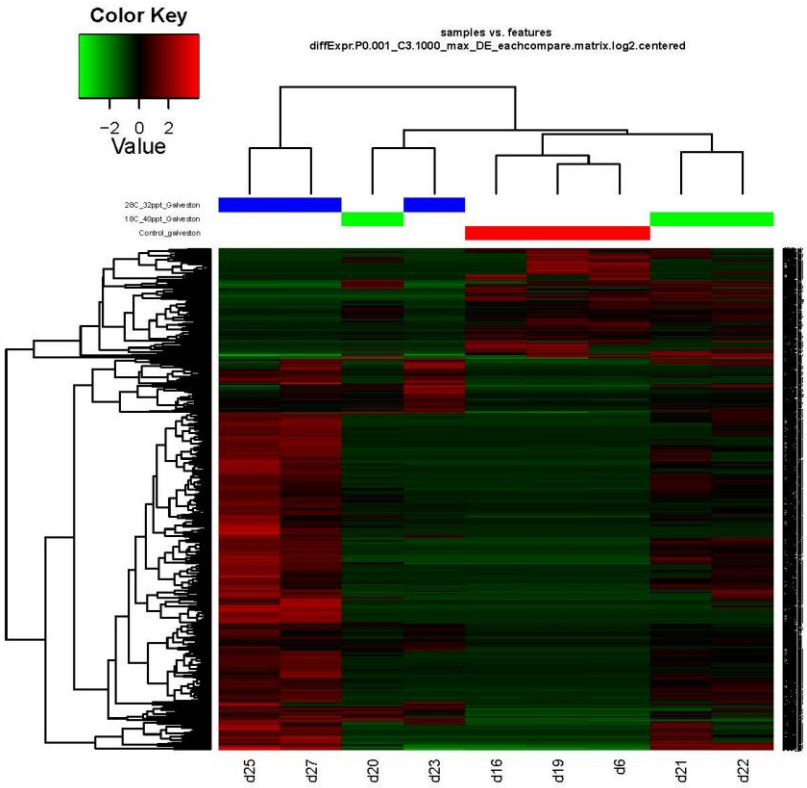

Fig. 17. Heat map graphic representing 1,000 differentially expressed transcripts at a P value of 0.001 by Galveston southern flounder pre-metamorphic larvae exposed to 28°C and 32 ppt, and larvae from the same cohort exposed to 18°C salinity and 40 ppt, compared to Control Galveston temperature of 18°C and 32 ppt.

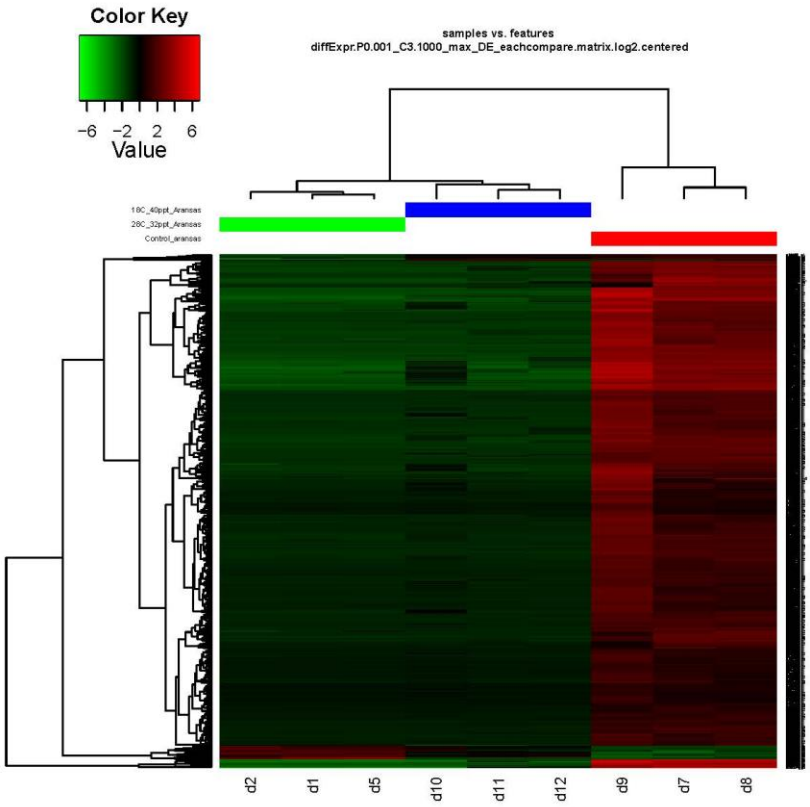

Fig.18. Heat map graphic representing 1,000 differentially expressed transcripts with a P. value (0.001) generated by Aransas bay southern flounder pre-metamorphic larvae exposed to temperature 28°C salinity 32 ppt and larvae from the same cohort exposed to temperature 18°C salinity 40 ppt, compared to Aransas control temperature 18°C salinity 32ppt.

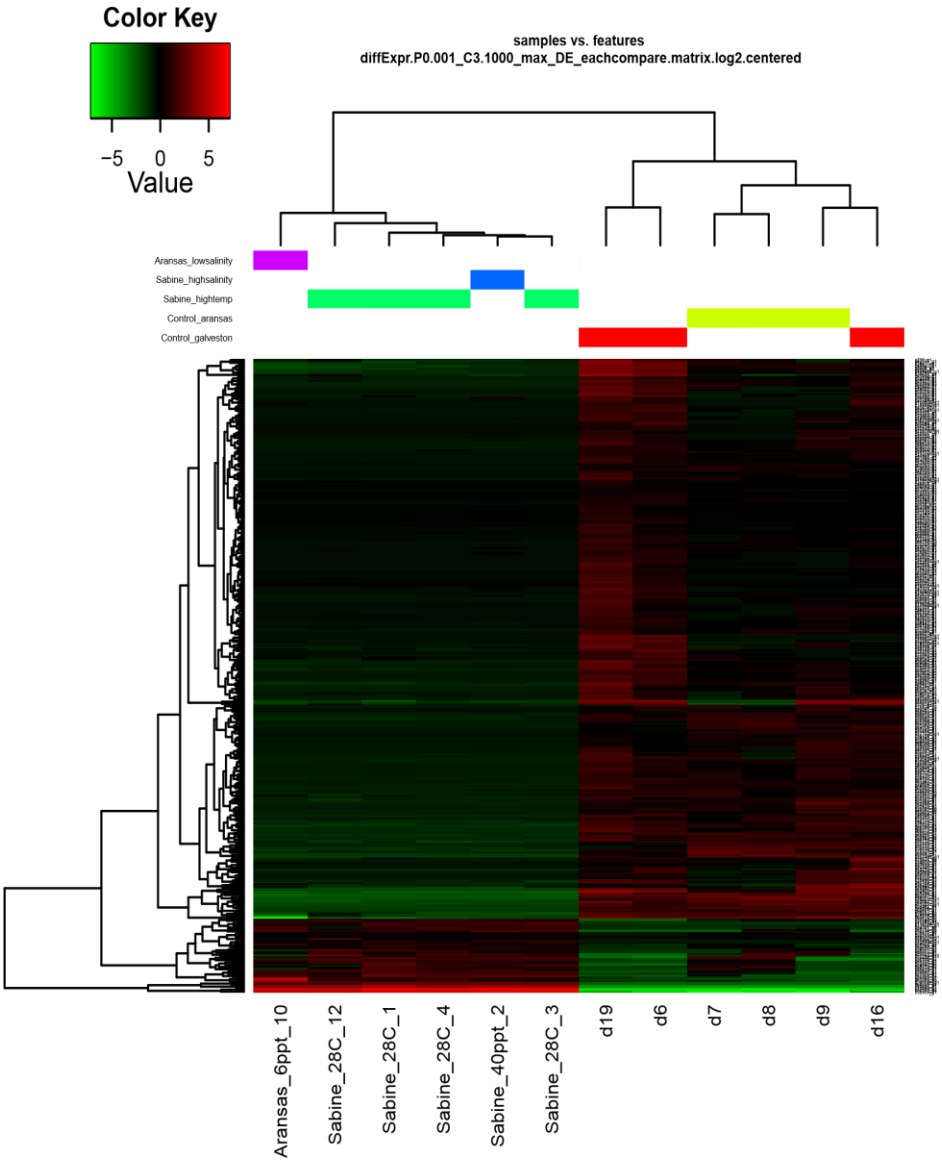

Fig. 19. Heat map graphic representing 1,000 genes with a P. value of 0.01 for Sabine 28°C and 32 ppt compared with Galveston-Control temperature (18°C) and 32 ppt. A single sample of Sabine exposed to 18°C and 40 ppt was compared to a single sample from Aransas at 18°C and 6 ppt.

Discussion

I observed differences in the number of up-regulated genes and gene expression between southern flounder larvae, spawned by parents collected in different locations along the environmental cline of temperature and salinity of the Texas coast. The flow of information from DNA to RNA to protein provides the cell with potential control for self- regulating its function by adjusting the amount and type of proteins synthetized (Murray et al. 2004, Pockley 2002). All living organisms, including larval fish, possess the ability to sense and respond to stress conditions (Descombes et al. 2015, Jesus et al. 2016). Biological processes related to stress responses play an important role in normal development and homeostasis of organisms. Therefore, organisms have evolved systems to sense and respond to stress. Stress is an important factor to induce diseases and mortality in cultured fishes (Lafferty 2009, Schade et al. 2014) and to contribute to population declines in the natural environment (Eliason et al. 2011, Auth et al. 2011). Understanding stress in the semi-controlled environment of culture systems could assist to explain the decline of environmentally sensitive species, like southern flounder, confronting climate change and increased temperature challenges in the natural environment. Physiological adaptations related to differences in thermal responses among sockeye salmon populations have been suggested to occur at a local scale (Brander 2005, Eliason et al. 2011, Keefer et al. 2018, Narum and Campbell 2015).

In the case of this study, transcriptomic analysis of larvae produced by parents collected in different environments of the Texas coast and exposed to environmental challenges of temperature or salinity responded differently to these challenges. This study suggests that environmental conditions experienced by the broodstock-parents were related to the number of transcripts and up-regulated genes, gene expression generated by the larvae when exposed to different types of stressors. The highest number of up-regulated genes, levels of gene expression for the temperature

challenges, were observed in the Aransas larvae (Table 10, Fig. 14), the Texas coastal area with shallow bays in which seasonal changes of temperature are marked, also the location closest to the southern-most distribution of this species in the Gulf of Mexico. The number of up-regulated genes in the Galveston fish under temperature stress were not as abundant as for Aransas. Sabine was compared with Galveston due to the lack of RNA samples for control from Sabine. Responses in terms of the number of genes and gene expression were also obtained for the higher salinity (40 ppt) for the Galveston larvae and were higher than for the Aransas group of fish (Table 10, Fig. 13). I refer to these groups of fish because they had a full set of replicates and control treatments. The Aransas fish responded with a lower number of up-regulated genes than Galveston when exposed to a higher salinity treatment, possibly reflecting a higher tolerance to salinity related to the environmental salinity cline of the Texas coast (Table 10, Fig.16). The single sample from Sabine fish exposed to 40 ppt yielded a total of 27,626 transcripts. Sabine is the group of fish in which parents inhabit an almost freshwater environment (Table. 10).

Transgenerational plasticity, or maternal effects, can influence the phenotype of the offspring, serving as a protective mechanism for environmental change (Burgess and Marshall, 2011). This study suggests that southern flounder pre-metamorphic larvae showed signatures of maternal effects in response to temperature and salinity challenges, altering the phenotype that the offspring are likely to experience in the natural environment (Narum and Campbell 2015, Mackenzie and Hofmann 2010, Primmer et al. 2011).

Aquaculture of sensitive species sometimes requires a strict environmental control to reduce economic losses and to prevent the production of biologically defective organisms (deformities, altered pigmentation, reduced growth) induced by environmental stressors (Boltaña et al. 2017, Burggren 2009, Basu et al. 2002). The study of candidate genes for stress response

induced by osmotic and thermal stress could assist aquaculturists to monitor stress in sensitive species or critical early life stages. This is also valid to understand processes affecting wild populations in the natural environment. Stress responses are regulated by neural, endocrine and immune systems. The primary function of these mechanisms is to equilibrate the organism's biological systems to manage the energy required by the fish to maintain homeostasis (Morrison and Nakamura 2018, Misra et al 1989). Metabolic pathways are involved in the stress responses of several organs involved in production of hormones, neurotransmitters and proteins (Aluru and Vijayan 2009, Tort 2011).

Temperature is considered a prevalent abiotic factor that influences all metabolic activities, and the life history of fish (Oomen and Hutchings, 2015, Okada et al. 2003, Oshlack et al. 2011). The thermal environment experienced by an organism often changes dramatically during ontogeny; these changes may reflect seasonal warming or cooling and/or changes of habitat during development. Most fish species are unable to regulate their body temperature and have developed a complex set of molecular mechanisms such as membranes, lipid layer composition, protein folding, methylation, and antifreeze proteins to assist them to survive rapid and drastic changes in daily and seasonal temperatures (Beitinger et al. 2000). Further, in the face of climate change, some species that are heat sensitive, like southern flounder, are affected at early life history stages during larval development, metamorphosis and settlement into the estuary. Despite obvious effects on recruitment and widespread population declines, environmental temperature varies in time and space, presenting the organism with continuous challenges across their life that force them to regulate and function in different environments (Cossins and Crawford 2005, Ju et al. 2002, Podrabsky and Somero, 2004, Nickells and Browder, 1988). In this study, I was able to detect a number of sexes determining genes for females and males and a few markers presented a transition

between both sexes. The temperature used was 18 C° for larval rearing and has been reported to produce males and females at a 50:50 ratios in Texas (Montalvo 2012). This information is important for the stock enhancement program because sex ratios in wild population of southern flounder in Texas are altered 6:1 (Stunz et al. 2000). We should make our best efforts to produce a close value to the 50:50 sex ratio in juvenile southern flounder produced at the hatchery for stock enhancement.

Sex Determination

Fishes are one of the most diverse vertebrate groups and also one of the most interesting groups regarding sex determination (Devlin and Nagahama, 2002). Southern flounder sex is determined genetically (XX or XY) and environmentally. Female sex can be disrupted by temperature to environmentally become a male (Luckenback 2009). Genetic males always developed into males but females can change sex if they are exposed to different temperatures. Water temperature that will induce the formation of the 50:50 ratios is 18°C in Texas (Montalvo et al. 2012) and 23°Cin North Carolina (Luckenback et al. 2009).

The Southern flounder transcriptome revealed the presence of antifreeze proteins (AFP), specifically antifreeze glycoproteins (AFGP), in the treatment temperature. I reduced the activity of the larvae to enumerate survivors accurately before euthanizing them with ice and exposing the tissues during processing to liquid nitrogen vapors to freeze the whole larvae for preservation at -80C°. These types of proteins are characterized by their ability to prevent ice crystal formation in the cells at low temperature by lowering the freezing point of marine fish plasma in a non-colligative way by adsorbing to surface ice crystals, modifying their structure, inhibiting or stopping crystal growth. Thermal hysteresis is the difference between the freezing and melting point of pure water. Dissolved salts, such as sodium chloride (NaCl) and proteins, can increase the

hysteresis freezing point or the temperature that causes the development of ice crystals in the cells (Chen et al. 2007, Kristiansen and Zachariassen 2005) by the adsorption of antifreeze proteins to very small crystal surfaces (Cziko et al. 2014). Antifreeze glycoprotein, or AFGPs, are found in Antarctic notothenioids and northern cod (Star et al. 2011, Fletcher et al. 2001). They are 2.6-3.3 kD and evolved separately in notothenioids and northern cod. In notothenioids, the AFGP gene arose from an ancestral trypsinogen-like serine protease gene (Pogson and Fevolden 2003). Type I AFP is found in winter flounder, longhorn sculpin, and shorthorn sculpin. It is the best documented AFP because it was the first to have its three-dimensional structure determined. Type I AFP consists of a single, long, amphipathic alpha helix, about 3.3-4.5 kD in size. There are three faces to the 3D structure: the hydrophobic, hydrophilic, and Thr-Asx face. Type I-hyp AFP (where hyp stands for hyperactive) are found in several righteye flounders (Fletcher et al. 2001). It is approximately 32 kD (two 17 kD dimeric molecules). The protein was isolated from the blood plasma of winter flounder. It is considerably better at depressing freezing temperature than most fish AFPs. In Texas, southern flounder larvae survival has been reported in rearing ponds at very low temperatures of 4°C (Kaiser et al. 2012, MacDonald et al. 2016, Taylor et al. 2000). Under laboratory conditions, two sizes of juvenile southern flounder (small and large) have endured also 4°C with a high survival of 89-100 % for the larger juveniles of approximately 20 mm TL. Lower survival rate was reported at 30% for pre-metamorphic larvae approximately 9.8 mm TL. Antifreeze proteins, similar to the ones found in flounder, have been detected in other species of flatfish from colder regions and in fish from the Artic area (Fletcher et al. 2001, Nicodemus et al. 2011).

Salinity, Proteins, Enzymes, Hormones.

Euryhaline fish species have the ability to inhabit a wide range of salinity environments, including estuaries, in which salinity change occurs daily and in migratory species that occupy the freshwater or seawater during a particular stage of their lives. Osmoregulation is one of the biological mechanisms to cope with environmental changes (Lópetz and Martinez 2002, Lee et al. 2006, Eissa et al.2017). This process consists in the ability to maintain body fluids and osmolality.

The endocrine system in these species is critical to maintain homeostatic regulation (Takei 2008,2009, Liu et al. 2006). Several hormones play an important role in sustaining body fluid balance in euryhaline fish species during abrupt salinity changes in the environment (Chasiotis and Kelly 2011). Some hormones act very fast, such as angiotensin, natriuretic peptides, guanylins, neurophysisal, urotensins, and adrenomedulin. The slow acting hormones found were prolactin, insulin-like growth factor (IGF), mineralocorticoids, and thyroid and sex steroid hormones. The neuroendocrine system reacts to osmotic and ionic changes by signaling specific hormonal changes to regulate water and ion fluxes (Breves et al. 2010). Hormone systems are rapidly released to manage salinity challenges by consuming saltwater and activating ion transport in the gills, gastrointestinal tract and kidney (Kosaka et al. 2003, Nobata and Takei 2011, Takei and Balment 2009). Other hormones, such as prolactin and insulin growth factor-1(IGF), organize the body for long term acclimation by changing the levels of ion transport proteins and differentiation of ionocytes and other osmoregulatory cells (Marshall 2003). Fish acclimate to a hyperosmotic environment by drinking saltwater from the environment, accompanied with water absorption by the intestine, to balance water loss from the body surface (Lockwood and Somero 2011). Excess mono-valent ions (Na^+ and Cl^-) are eliminated by the chloride cells-ionocytes in the gills, opercula epithelium, and intestine. Osmoregulation to the freshwater environment is attained by limiting

water influx across body surfaces and excreting the excess water through the kidney (Marshall et al. 2003). The degree of tolerance to abrupt changes of salinity is different for some fish species and changes across ontogeny (Burggren and Blank 2009, Campos et al. 2013). The nervous system activates the release of a hormonal system formed by fast acting amine or oligopeptide hormones. This sends a signal to the brain to induce salt water drinking. Meanwhile, the production of transport molecules, channels, and cell adhesion proteins trigger the activation of the slow acting hormones to induce re-organization of the epithelial cell membrane and intercellular junctions by differentiation of stem cells. This results in proliferation of cells to reverse the direction of ion and water fluxes from their body (LeBlanc et al. 2010).

Transcriptomic analysis of southern flounder pre-metamorphic larvae can be a useful tool to study physiological processes during early life stages for this species and at the critical stage of metamorphosis. This study also provides insights regarding sex determination, these two critical phases of the life history of Southern flounder (Elmer and Meyer,2011). Information concerning this study is useful for determining optimal conditions to culture of southern flounder in an aquaculture setting and for purposes of stock enhancement (Lorenzen 2012,2008, Lorenzen et al 2010). Providing additional data for southern flounder broodstock collection, larval rearing, and stocking protocols. From the ecological perspective, we generated quantitative information to correlate environmental stress conditions and survival of southern flounder at the pre-metamorphic stage.

This study demonstrated different patterns of responses to hyper-thermal and osmotic stressors in groups of southern flounder larvae spawned by parents collected along an environmental cline of temperature and salinity on the Texas coast(McKhenie et al. 2016, Megrey et al. 2007) Results of gene expression indicated different reactions between groups of larvae

included in the study, suggesting conspecific differences in phenotypic plasticity related to parental effects (Fangue et al. 2006, Jablonka et al. 1995, Kuijper et al. 2014, Oomen and Hutchings 2015). These results suggest acclimation of the parents to different environmental conditions and a transgenerational plasticity effect detectable in the progeny. However, these effects will be advantageous only if the phenotype of the progeny matches the environment of the parents. Many genes related to morphological, physiological and neuroendocrine changes related to the metamorphosis of southern founder were found in the transcriptome. Furthermore, many genes identified in the Southern flounder transcriptome have also being identified in other species of fish subjected to environmental stress.

CHAPTER V

CONCLUSIONS

Climate change is predicted to affect many marine species. Southern flounder and other cold water adapted species are showing population declines across their geographical distribution. Despite progressively increased management regulations in Texas, the southern flounder trends continue showing a decline. Southern flounder populations trends in Texas exhibited a higher recruitment rate and relative abundance of Southern flounder on the coast after colder winters based on the analysis of serial data collected over the last 35 years on the Texas coast (Martinez-Andrade 2018). The Texas coast is characterized by an environmental cline with a North to South temperature and salinity gradient. These studies show that temperature is the most critical factor influencing the viability of the early life stages of Southern flounder affecting larvae survival, total length, and yolk sac size, Interactions of salinity and temperature also prevented normal development. Similarly, interactions of temperature and salinity and dissolved oxygen affected premetamorphic larvae survival as well. Transcriptomic analysis corroborated the trends and relative abundance of southern flounder along the Texas coast. The transcriptomic analysis showed a larger number of genes expressed and higher gene expression for the temperature treatment 28 °C with control salinity 32 ppt by the Sabine and Aransas premetamorphic larvae in contrast the Galveston samples expressed a more reduced number of genes. The samples from Aransas and Galveston expressed similar number of genes and gene expression in response to the higher salinity treatment of 40 ppt at control temperature 18°C. A single high salinity (40 ppt and 18 °C) sample from Sabine yielded more than 27,000 genes under these conditions. Texas is implementing a stock enhancement program to supplement natural Southern flounder populations. These early life history studies can contribute to a fine scale refinement of current protocols for southern flounder

and will be applicable to integrate the environmental variability of the Texas coast into stock enhancement strategies related to broodstock collection and stocking design, including environmental conditions that will facilitate survival of southern flounder during larval and pre-metamorphic stages. The protein and hormone molecular markers found in this study will be useful for monitoring sex proportions of fish produced by the hatchery as a routine. to study environmental adaptations for salinity and the role of temperature on sex determination of hatchery produced fish prior to stocking. Other markers related to biological processes such as growth, protein biosynthesis metamorphosis, circadian cycle could be applicable to aquaculture, stock enhancement as well.

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APPENDIX A

SOUTHERN FLOUNDER REARING PROTOCOL

Procedures Used to Grow the Experimental Southern Flounder
For the Southern Flounder Transcriptome and Physiological Responses
Texas Parks and Wildlife Department
CCA Marine Development Center, Corpus Christi, Texas.

Note: Methods were researched and adapted from the literature and adjusted to the MDC hatchery setting with procedures described by Kayser et al. 2012, Daniels andWatanabe 2009.

Procedures for Culturing Southern Flounder (*Paralichthys lethostigma*)

BROODFISH TANK SYSTEMS

Equipment needed for broodstock accommodation and husbandry

A Broodfish Tank System for Southern Flounder culture should be equipped with a minimum of two powerful chillers one to be used and another as a back up

An independent indoor reservoir connected to a chiller, is required to store water for water exchanges to match the temperature of the broodstock tanks

A sturdy aeration line should be connected to a powerful blower to provide aeration to each tank

Light 100 lux-long bulb, should be installed to illuminate across the tank fitted with automatic timers, to be used for photoperiod schedule. A computer program could be used to control the changes of light.

Circular fiberglass tanks of approximately 3,000 L. Each of these tanks could accommodate the biomass of 35-40 fish of approximately 1 kg each or 35 Kg of fish in 3,000 L of water

Tanks should have an incoming water line and a central drain, with a venturi (internal and external standpipe) between 75 mm and 100 mm in diameter (Red Ewald Fiberglass Company TM, Karnes City, Texas).

The broodstock tank should have a smooth flat bottom, with a soft slope to facilitate drainage and elimination of extra feed and organic waste. The tanks should be connected to a recirculation pump connected to a power supply system

- Each circular fiberglass tank should have a 5 feet radius, X 5 feet height
- A minimum of 100-150 individuals should be maintained in each 4-tank module

Another alternative could be long raceways or cement tanks with polished bottoms. Flounder spend a great amount of time at the bottom of the tank and tanks with smooth bottoms will prevent skin abrasions.

It is recommended the tanks to be painted with gel coating and the color of the tank should be light grey or bluish.

Flounder are stimulated to feed with good illumination.

A glass or acrylic window should be installed at least in one face of the tanks to observe the fish during feeding and ovary development.

The broodstock tank system should be connected to a sand –gravel filter for water filtration and to reduce the bio-load of suspended solids in the tanks

The Biofilter should have a large surface area to facilitate bacterial colonies to establish in the filter and enough porosity to allow oxygen to diffuse for reduction of ammonia and nitrite.

The Biofilter and sand filter should be inoculated with Nitrosomonas and Nitrobacterium (Proline water conditioning for Saltwater-Aquatic Ecosystems, Florida, USA) at least a month before moving any fish into the tanks.

Ambient temperature water >24 C° helps the bacteria to colonize the filter faster than cold water temperature.

Add an ammonia substrate to increase the number of bacteria.

The filter should be treated with 10 g of Ammonium Chloride, diluted in 1L of DI water, this will "feed" the population of bacterium.

Thereafter, monitor the levels of ammonia, nitrite and nitrate to verify the functionality of the Biofilter. Ammonium levels should decrease and nitrate levels should be higher.

Filtration should be continuous to facilitate colonization of the biofilter and sand filter.

Filtration area of 12.16 ft2, filtration flow rate of 182 gallons per minute (gpm), and filtration rate of between 10-15 gpm.

Incubator tank systems are diverse in terms of the type of system selected by each aquaculture facility (e.g., recirculating, flow-through, water bath, static system with periodical water exchanges). 300 L tanks are use at MDC fitted to a reservoir with water at 16-18 C° and the water in the incubator is maintained at this temperature with a drop-in chiller.

PVC pipes deliver water from the reservoir that is fitted to water movement throughout the hatchery. The incubator tank system is typically comprised of several incubator units, several filters (e.g. Sand filters, carbon filter), chillers, storage reservoirs, UV light system, Ozonation system

Biofilter maintenance

Sand filters should be back-washed after every flushing to prevent buildup of decomposing organic material and the formation of pockets of Hydrogen Sulfide in the system. Backwash also will help to unpack the sand in the filter and this will improve aeration-oxygenation.

Additionally, nematodes, large copepods, and fish lice can be parasitic to southern flounder and reductions of organic matter that serves as food for these organisms will benefit the health of the fish

Rinse the sand filter by unpacking the sand with abundant water at least once a month. This will reduce the amount of organic matter and sanitize the filter gently.

Procedures for Culturing Southern Flounder *(Paralichthys lethostigma*)

Water Treatment

Biological filtration can be used to reduce the number of suspended solids with sand filters.

Recirculation of water from the tanks should be 24hr. through a biofilter with adequate surface to be colonized by beneficial bacterium. This will assist with removal of organics, ammonium and nitrite.

Ozonation in addition to biological filtration, other methods can be used to

treat water to oxidize harmful algae (red tide, brown tide, Amyloodinium), and to kill pathogenic bacterium. Ozonation, is the application of ultraviolet radiation light, and

filters. Ozonation can be harmful to the fish due to the capacity of oxidizing important tissues as the gills and it is toxic to eggs and larvae at very low concentrations (5 μg/L to fish)

Ozone is produced utilizing the Corona Discharge Method, in which

Ozone (O3) is generated by passing Oxygen across a high-energy electric

field.

Ozone must be removed from the treated water that is used into the broodstock system, as it can be lethal to broodfish.

Ozone is completely removed through aeration of the water or by irradiation with Ultraviolet light and in combination with activated carbon.

A residual Ozone test must be used to verify that residual Ozone is not present in the water that will be used for water exchanges in broodstock tanks

Water is also treated with Sodium Thiosulfate to eliminate residual Chlorine.

Chlorination. Water can be chlorinated and dechlorinated prior to being used. This will serve the same purpose of sanitation, oxidation of harmful algae, pathogens and parasites as the Ozone treatment

Back up emergency power is needed in the event of a power outage

Power outages can be caused by storms or high winds.

It is essential to provide an alternate or backup source of

Electrical power to ensure that tank aeration and circulation are not altered to prevent broodfish stress and mortality.

Generators are a common source of backup electrical power and are used by

TPWD MDC. Generators must be operated outside due to the

noise and carbon monoxide generated

Water Supply A treated water reservoir holding between 2-3-day supply of water

Should be maintained at all times, as emergencies or unexpected events can arise.

Water should be stored in reservoirs or tank systems with aeration at the same temperature as the broodfish tanks

Water exchanges should be conducted three times a week, in all of the Southern flounder broodfish tanks.

The water level of the broodfish tanks should be lowered approximately 30 % of the tank. After draining the tank to a desired level, replace the volume with clean treated water from a reservoir.

Dechlorinated city water can be used to adjust salinities to optimum or as a mean to disrupt the life cycle of a suspected or confirmed parasite.

pH level should be adjusted in the egg collectors or recirculation pumps by adding hydrated soda ash, slowly into the collectors or the pump. Drastic changes of pH could cause serious damage in the gill tissue of the fish, impairing osmoregulation and exchange of gases across the gill lamella.

Procedures for Culturing Southern Flounder (*Paralichthys lethostigma*)

Broodfish Collection and Transport

Southern flounder broodstock can be collected in a number of different ways.

Flounder are prone to post -capture stress, best efforts should be made to reduce the level of stress due to capture and handling. Temperature shock, pH shock or low DO should be prevented during transportation in a vessel or trailer.

The best methods to collect flounder are:

1. Using dip nets made out of very soft rubber to avoid damaging the delicate skin or dislodging the scales of the fish. Using a boat with lights illuminate the shores of sandbanks looking for flounder nests "beds" circular depressions in the sand use by flounder to hide while waiting for prey items to swim by. The fish collector walks in the shallow water to the fish at night and drops the net with a sturdy large frame on top of the flounder, gently picking up the fish in the net or using another net to prompt the fish into the capture net
2. Thereafter, the fish are transferred to a basin kept in the boat, filled with oxygenated clean bay water. If necessary, the water can be exchanged several times during the collection to maintain the fish in acceptable water quality.
3. The fish are then transferred to a trailer full with clean water, at the same temperature and salinity, the same as the bay of origin of the fish, pH 8.0 and oxygen at 7-8 mg/L. Rapid transport to the hatchery will ensure higher survival by preventing water quality deterioration.

Prophylactic Treatment with Antibiotics and Freshwater

At arrival to the hatchery, the fish are dipped in oxygenated, dechlorinated fresh water with 1.0 mL /L of Oxytetracycline (OTC). Every fish is injected with 0.5 mL of OTC per /Kg with a new, sterile needle.

It is recommended to lower the water temperature to reduce stress and to prevent harmful bacteria and algae to flourish in the tank's water. Temperature should be lowered at approximately 2-3 C°/day

Water can also be treated after the capture of the fish with 20 mls/3,000 L of Copper Sulfate to treat or prevent Ammylodium and other harmful organisms

A good time to collect broodstock is typically during Fall after a Northern frontal system which is when broodstock migrate to the Gulf of Mexico to spawn out of passes and inlets of bays and estuaries.

After moving the fish to the broodfish tanks, water quality should be monitored and water exchanges done as appropriate to prevent ammonia build up. Moribund, injured or dead fish should be removed promptly from the system to prevent water/tanks/filters contamination with diseases.

4. By rod and reel. Skillful, recreational and commercial fishermen sometimes assist in this duty and donate the fish to the hatchery.
5. Southern flounder broodstock should be separated by bay of origin

Sexing Southern flounder

1. Southern flounder is sexually dimorphic with females attaining larger sizes than males
2. Females display a large ovary and their abdomen becomes distended during sexual maturation and hydration of eggs during the spawning season.

3. One method to differentiate a female from a male is by using an acrylic tray or a transparent glass sheet and with a hand-held light illuminate the abdomen of the fish. The female ovaries will be more conspicuous. The male organs are not easy to observe.
4. Another method is using a sonogram, or even a more complicated method can be the detection of hormones in the urine of the females and males using an ELISA test, but a baseline data for hormones profiles is needed to differentiate the levels of hormone, before, during and after the spawning season.
5. For the experience eye looking at the gonadopores (males display one opening and females two openings)
6. The male opening is situated below the pectoral fin; you will need to raised up gently with a sterile tweezer the fin and gently press the abdomen from back to front. Observe if the male is releasing a clear or whitish color fluid. Males should be always handled with gloves, sanitized rubber nets only, very gentle care.
7. Sometimes the sperm is packed and dense, to increase the volume of sperm a very small amount of Ovaprim can be injected to the male (< less than 0.1ml). The males will hydrate, and finish spermatogenesis, the sperm will flow better and last longer in the spawning season.
8. After handling the males, they should be injected with a very small amount of OTC, below the dorsal fin and before returned to broodstock tank. Thereafter observe if the fish feed, if they are feeding feed them brownies or a small multivitamin pill.

Separation of Broodfish in tanks

Male and female southern flounder are sexually dimorphic, with females attaining larger sizes than males. In Texas, southern flounder males and females attain sexual maturity at 2-3 years of age.

Broodfish tanks should be stocked by fish size. Larger females should be separated from smaller females to reduce competition for feed and aggression.

The smaller fish should be grouped together, this group should contain most of the males

We recommend to maintain a few males with the females to fertilize spontaneous natural spawns that may occur in the tanks, but males in captivity seem to be reproductively asynchronous with female southern flounder.

Males most likely will not fertilize the eggs released in the tanks or the volume of sperm is so small that may become diluted in the tank water volume.

A potential solution for this situation is to maintain a large number of small southern flounder males by themselves to facilitate access to flowing males at the moment of fertilization.

Male southern flounder produces a limited amount of sperm, and sometimes after the stripping become lethargic and do not feed do to stress.

Best efforts should be made to collect a large number of males to maintain genetic variability and to facilitate an effective fertilization of large numbers of eggs for incubation.

Fertilizing large number of eggs will promote an effective use of space, time, live feed, and produce uniform cohorts of larvae for rearing.

Quarantine is the process by which the newly acquired broodfish are separated from the group of fish of the same species already established at the hatchery, until the fish are accustomed to the hatchery to prevent the establishment of pathogens or parasites into the hatchery tank

systems. A reasonable period for quarantine for southern flounder is three months. The can be acclimated to the conditions of their holding tank after they routinely feed.

Temperature and TPWD MDC keeps the target culture water temperature at
Photoperiod 16°C in January, which is then increased to 17°C in February,
Regimes followed by an increase to 18°C about March. This 18°C target
water temperature should be maintained through April 15th, when
the target water temperature should be increased to 20°C. The
temperature is then increased up to 22°C by May, after which the
temperature is increased to no higher than 24°C throughout the
summer months (e.g., May through August). The target waters
temperature is decreased starting in September at 21°C, decreasing
to 20°C in October, then to 17°C in November, and down to 16°C
in December. This water quality factor, temperature, in
combination with photoperiod, assists with the success in the
hormone-induced strip spawning of southern flounder broodstock.

Photoperiod used to encourage ovaries and testis development for the production of eggs and sperm

reproduction during southern flounder season is 14 hours of light/
10 hours of darkness in August, 13 hours of light/ 11 hours of
darkness in October, and 10 hours of light/ 14 hours of dark
consistently from December through May.

Feeding Flounder Broodstock

Feeding and accustoming new "wild" broodstock to the frozen feed use at the hatchery is challenging.

Feeding should start as soon as possible after capture to prevent digestive enzymes to decrease, as well as to stimulate appetite and maintain general health condition.

Wild fish are used to feed on live prey, to stimulate the wild southern flounder to feed, use live shrimp, previously dipped for 2 min in fresh dechlorinated water with 1 mL of OTC/ L with aeration to maintain the shrimp alive, combine the live shrimp and offer a few squid heads with tentacles

Remove the uneaten feed and clean the baskets in the recirculating pumps

Flounder have the tendency to stop feeding if rotten feed or a dead animal is in the system

Flounder consume less feed when salinity is high or pH is low. Stressed fish displays a hyper-pigmentation around their heads and sometimes the whole body.

After the fish began to consume the frozen feed, the fish should be fed between 2-3% of the body weight. Cut feed for broodstock should include squid (*Loligo* sp.) heads with the tentacles attached, Atlantic mackerel (*Scomber* sp.) center steaks and tail portions, beef liver cut into about 1 inch by 1-inch size pieces, and frozen shrimp.

Flounder should be fed every day or at least 3 times a week

Supplemental feed -Mazuris (Aquatic Gel Diet for Carnivorous Fish, Ontario, Canada), or brownies should be provided a minimum of 6 months prior to spawning.

Adult multivitamins can be inserted in fish steaks to facilitate the consumption of extra nutrients.

Squid heads with the tentacles are fed first, as squid heads are especially nutritious in promoting healthy egg production for female broodstock. This feed item also glides, simulating a natural prey movement.

Drop 1 piece of feed into the tank at a time, making sure the feed is consumed before dropping in

additional pieces of feed. Patience is important to feed southern flounder broodstock.

Feeding behavior should be observed closely, and recorded as fish that do not

eat may be an indication of possible bacterial infection, disease, or parasitism.

Remove the uneaten feed promptly with elongated dip nets attached via PVC pipe to prevent the buildup of nitrogenous wastes in the tank, causing poor water quality and diseases.

In addition to the cut feed, some vitamins and minerals should be added to the diet to boost immunity and provide for all macronutrients and micronutrients required in the diet in adequate

quantities.

Southern flounder need between 50-55% protein in their diets and between 12-15% lipids or fat in terms of nutrients

Providing these nutrients in adequate quantities, providing a feed that contains most or all of these nutrients, is essential in proper growth and development, as well as promoting

healthy egg production on female broodstock.

Water Quality

Water quality factors include dissolved oxygen, temperature,

salinity, and pH. Each of these water quality factors should be

measured three times a day (morning at 7 AM, afternoon at 12:00

PM, and night at 10:00 PM) by a standard YSI Pro-Plus Multiprobe (YSI, Yellow Springs, Ohio).

Accurate records collected and maintained

Dissolved oxygen should be maintained between 6-8 mg/L. If O2 drops below 4 mg/L, an O2 bottle with aerator -diffuser should be added to the tank to raise the DO

Temperatures should follow carefully the temperature photoperiod protocol to ensure the sexual maturation of the flounder.

Temperatures, oxygen levels, pH and salinity should be measured daily- 3 times a day.

TPWD MDC kept the temperature at about 18°C from

January through mid-April, during which the temperature was

increased up to 21°C from April 15th through April 17th, after

which was increased to 22°C and maintained at that temperature.

pH is another important water quality factor. pH is typically maintained as close as possible to 8.0. pH units which is close to the pH of the natural seawater 8.1 pH units.

Adjust the pH slowly by preparing a solution of 500g Sodium bicarbonate - soda ash to treat 12,000 gallons or the volume of 4 tanks in a module.

Apply the solution after the exothermic reaction of the Sodium bicarbonate when mixes with water is completed (solution is not feeling warm) and apply the slurry to the circulation pump or the egg collector of the tanks

If the pH is measured below 8.0 units apply sodium bicarbonate (Na2CO3) to treat the water at about 50 mg/L concentration as a buffering agent and/or perform a 30% water exchange. Abrupt changes of pH can damage the gill tissues, do not apply directly to the tank.

Salinity is another critical parameter and usually is maintained between 28-32 ppt, this will allow hydration during the spawning season.

Adjustments of salinity with city water and Ozone treatment has the tendency to lower pH, similar treatment can be applied to the water source.

Diseases and Parasites

Diseases in aquaculture, diseases can occur periodically during the period of the higher temperature or caused or aggravated by water quality issues, stress, lack of biosecurity, poor disinfection or low-quality nutrition can cause diseases to appear. Stress lowers the immune system of the fish facilitating disease development.

The best practice to avoid diseases is to maintain good water quality, excellent nutrition and reduce stress in the fish.

Southern flounder broodfish are affected by various pathogenic

bacterial species, fungal species, and viruses.

Bacterial diseases include *Aeromonas hydrophila*, and *Vibrio sp.* Southern flounder are also likely impacted by the nervous necrosis, epidermal hyperplasia or the herpes virus.

Several external and internal signs of disease are included below

- Skin lesions, blistering
- Loss of pigmentation
- Exophthalmia
- Inflammation and reddening
- Necrotic ulcer
- Mouth lesions and tumors

Parasites

- Broodstock stop feeding.
- Broodstock show higher rate of breathing, or increased

Opercular movement

- Broodfish demonstrate flashing or “scratching” behavior against either the

tank walls or on the bottom.

Amyloodinium *ocellatum*, which is a dinoflagellate, with an infective dinospore stage, *Amyloodinium ocellatum* primarily infects the gills of southern

flounder, but can additionally be found on the skin-velvety golden appearance in the skin and eyes must be

treated promptly with copper sulfate upon detection because infections can lead to rapid mortality.

Other parasites include sea lice (*Argulus* sp.), and *Cryptocaryon* sp., intestinal worms, leeches, nematodes, and parasitic copepods (*Ergasilus* sp.)

Pathogens Aeromonas, Vibrio sp,

Prevention of diseases

1.Water disinfection and removal of extra feed in the tanks

2. Periodical cleaning of the filters to eliminate excess organic matter and on extreme case disposal of the sand and sterilization of the biofilter material with chlorine and total desiccation. 3. The filter material and sand harbors parasites and pathogens. Followed by sterilization of the tank with chlorine solution at 10%-tank scrubbing and treating the 3,000 L of the tank with 10% volume: volume chlorine

Use of hydrogen peroxide at 10 mls/L to kill *Argulus*

Use of 8 mls/3000 L of DYLOX to treat the water for *Argulus, Cryptocaryon and Ergasilus*

Reducing the salinity of the tank will create an unhospitable environment

Applying copper sulfate to treat the tank water or use of approved antibiotics

Antibiotic treatment of oxytetracycline or other approved
medication can be injected into individual piece of feed before
feeding the piece into the tank.

In the case of oxytetracycline, 1 mL of oxytetracycline can be injected into individual pieces of feed before feeding the broodfish tank.

Evaluation of female's ovary maturation by evaluating and grading the gonadal
Broodstock development in female broodstock. The four stages of maturity
Females graded at either Stage 3 or Stage 4 indicate good candidates for strip
spawning (Bonnot et al. 2013):

- Stage 1: Little to no detectable gonadal development.
- Stage 2: Minor abdominal swelling.
- Stage 3: Conspicuous abdominal swelling, with the gonads more than ¾
  ¾ in the abdominal cavity next to the anal fin.
- Stage 4: The gonads are completely extended, and there is a
  clear or colorless area around the oviduct region.

Biopsy of the ovary.

To obtain a sample of eggs, sedate the fish with MS222 and using a sterile fine rigid catheter attached to syringe, rinse with abundant distilled, sterile water the vent area of the fish.

Carefully insert the catheter into the oviduct and gently pull a sample by pulling the plunger of the syringe.

Set the egg sample on a glass slide and measure the eggs under a light microscope, decide if the female should be injected or not based on the size and appearance of the egg (500 µ) and transparent

Calibrate the objective lenses of the microscope before measure the eggs.

Hormone injection

Select the females for hormone treatment and transport them with oxygen from the broodstock module into the incubation room. Hormone

The hormone used by TPWD is Ovaprim.

Ovaprim. Induces the female broodstock ovary to hydrate between 36 to 48 hours before strip spawning.

Ovaprim is manufactured by (Western Chemical Incorporated, Ferndale, Washington)

Female southern flounder broodstock with oocyte diameters measuring approximately 500 μm are given a dosage of Ovaprim according with their weight.

Hormone-induced strip spawning involves the evaluation of sexually mature male and female southern flounder broodstock. It is advisable to collect the broodstock 12 months in advance to provide enough time to quarantine and establish the temperature -photoperiod cycle of 150 days

Females are evaluated by the development of the ovary and the size and condition of the eggs the flounder eggs should look under the microscope clear, transparent and contain a single oil globule.

For the female to be eligible for hormone treatment the diameter of the eggs should be 500 μ for hormone inducement.

Females can be injected at the dose of …. Per Kg with Ovaprim (Western Laboratories, Seattle, Washington). I

Injection of gonadotropin-releasing hormone analogue (GnRHa) hormone into eligible females is to induce ovulation and complete vitellogenesis or egg development.

The dose of hormone is based on the weight of the fish.

Females are spotted in the tanks early in the morning before the room lights turned on. By shinning a flashlight on the fish in the dark background provides a good look to hydrated females with distended abdomens.

Prior to the collection of eligible fish, an incubator should be filled with water between 16-18 C° and set into recirculation.

Aeration in the tank should be vigorous but not excessive (Air-Bubble disease)

A separate container fitted with a lid should be filled with approximately 3 L of seawater from the incubator room that will be use to incubate the eggs.

The container with the lid should be treated with 2g of MS222-Tricaine as anesthetic.

Using a balance tare, the container with a net that will be use to transfer the fish, full with water and the lid.

Place the fish in the net in this container and install the lid. Record the weight of the fish and determine the amount of hormone needed.

After the fish is sedated and calm, inject the fish.

Using a scanner scan the dorsal area of the fish to detect a PIT tag. If a PIT tag is detected by the reader, record this number.

If the PIT tag is not present, scan a PIT tag and use a with a clean- sanitized applicator to insert the PIT tag into the dorsal muscle of the fish. This data will be useful to track fecundity (number of eggs produced by this female, fertilization rate and number of times this female have been spawned.

Ovaprim is a gonadotropin releasing hormone analogue hormone (GnRHa hormone)

A minimum of 3-4 females should be induced at the same time to obtain enough eggs to conform a least a couple of incubators.

Hormone treated females are maintained in for approximately 48 hr. prior to the strip spawning.

Preparation for strip spawn.

Table with a cushion and a clean glass bowl, a feather, clean or sterile seawater

The females are anesthetized by dipping the female with a net in a water bath with MS222 in a container with a lid. Close the lid until the fish is calm.

The dorsal area of the fish needs to be scanned to read the PIT tag.

The verified tag ID (PIT tag #)/female should be located in the data sheet.

The females should be handled in the net to prevent an accidental sliding of the fish in the floor.

The glass bowl is placed below the vent of the female and eggs are stripped from the female's abdomen by a gently massage towards the vent area, and collected in a glass bowl.

Eggs should flow freely, and should be transparent, the ovary should be soft to the touch.

Eggs collected should be mixed with fresh neat sperm with a bird feather vigorously for 90 seconds. Use sperm from different males if possible.

After this time gently pour a 100 ml of seawater from the incubator or sterile saltwater to induce sperm motility, swirl to cover all the eggs, and transfer the eggs to a graduate cylinder to verify the volume of floating-live eggs. Wait until all floating eggs travel to the upper part of the cylinder.

The egg volume is multiplying by 1,700, this number corresponds to the number of fertile eggs form the spawned female. Record the volume of live buoyant eggs and number of sinking eggs as well.

Record the number of floating/ fertilized eggs in the data sheet and in the incubator board under the correspondent date and incubator identification number and the correspondent female PIT tag

The female will then be injected with OTC and placed into the ice chest with water from the incubator room to be returned to broodstock tank of origin.

All materials including nets, cushion, ice chest, should be rinsed before cleaned, disinfected with alcohol and prepared for the next female

After 36 to 48 hrs. post-injection, eggs are stripped from the injected females. The same container

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Hormone treatment for the females

Using a rubber bag net and a container with water and covered with a lid, tare a balance to 0. Add a small amount (2g) MS222 diluted in 3 liters of seawater.

Pull a fish that previously received hormone treatment with the net used to tare the container.

Place the fish secured in the net in the container with MS222 and close the lid.

Scan the fish with the PIT Tag reader, if the PIT tag is not present scan a new tag, record the number and tag the fish by inserting the tag in the dorsal part of the fish.

Clean with 95 % alcohol to sanitized the PIT tag applicator needle after tagging every fish

Record the tag below the dorsal fin deep in the muscle, scan the tag to verify the tag number and record the weight. Calculate the dosage of Ovaprim, inject the fish in the muscle gently. Always handle the fish in the net and use gloves. Thereafter transfer the fish to the mating-holding tank

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Ovulation should occur between 48 -72 hours after hormone application. If males and females have been together in the mating tank, and a presence of eggs have been detected, verify if fertilization has occurred by collecting a sample of eggs to observe under the microscope if cell division occurred.

List of materials needed for strip spawn

Sperm collection

Flash light

Bottle/s with distilled water

Paper towels to blot and clean vent area of the male

Blunt pair of sterile tweezers

Plastic syringes or pipettes

A container with lid

MS222

Rubber nets-cleaned previously sanitized with chlorine water and rinsed with abundant tap water.

Rinse the net with saltwater before use the net to capture the males in the tank to eliminate residual chlorine

OTC injection

Pitcher or container with ice

Females strip spawning

a. Pencil

b. Broodstock spawning waterproof sheet containing, PIT tag number, date of hormone inducement information.

PIT tag applicator- disinfected

PIT tag reader

PIT tags

d. Bird feather

f. Large, 12-quart glass mixing bowl

g. Two plastic pipette tips

h. Tricaine MS-222

1. Prepare a large ice chest with lid and oxygen fitting to recover and transport the females after the collection of the eggs. Fill the ice chest to 50% of the volume with water from the broodfish incubator tank water to prevent temperature shock

2. Add in the appropriate amount of MS-222 to a container with a lid

3. Obtain a female from the incubator holding-mating tank.

4. Using short black nets, with a deep bag transfer one female to the lid container with MS222.

Tricaine MS-222 should sedate the female after two or three minutes.

Males can be evaluated by lifting the anal fin and pressing gently the vent. The sperm should flow freely and be clear or whitish in color. One drop of sperm can be pulled with a pipette tip for microscopic examination.

The spermatozoa cells should be motile after activation with saltwater under the microscope. Sometimes the sperm is dense and packed, a very small amount of

Ovaprim will help with to increase sperm volume by hydration as well as to complete spermatogenesis. Excess Ovaprim could cause impairment in the production of sperm

Sperm is collected from the male's minutes prior to fertilization of the eggs by cleaning the vent area with DI water, blotting the area and hand stripping the male.

The sperm should be collected with a sterile syringe made out of plastic not reactive to biological material-medical grade.

The sperm can be kept in ice and maintained for a few minutes in ice. The spermatozoa will die if kept too long or out of the ice

Sperm should be collected from several males and use small amounts from every syringe available to fertilize the eggs from several females to increase genetic variability.

Strip spawning

6. Remove the female from the mating tank, and place

this female on the table or flat surface.

7. Strip the eggs from the female southern flounder massage gently the abdomen of the female by gently pressing from the back towards the vent area opening. The eggs should be transparent and flow freely with a watery ovarian fluid present until the flow stops into a 12-quart glass mixing.

8. Add a few drops of sperm from the syringes, if possible, from more than one male

9. The eggs and sperm are then mixed using a bird feather

in a 12-quart glass mixing bowl. Mix the sperm and eggs with the feather for 90 seconds

Add 200 mls of seawater preferably autoclaved, or from the incubator used by the females gently swirl the eggs and sperm for a minute to induce motility.

Eggs are transferred to a 1000-mL volume glass graduated cylinder, the volume should be completed to a liter with the same water used for fertilization, allow floating- fertilized, viable eggs to float and nonviable eggs to settled on the bottom.

12.The viable floating eggs volume is measured and multiply X 1700. The number resulting from this calculation should be recorded in the broodstock notebook and the incubator board under the PIT TAG number of the female. Record also the number of dead eggs in the broodstock notebook.

13. The eggs are then transferred into a prepared 450-L volume

circular fiberglass tank, with vigorous of aeration in the incubator. Initiate the flow through and record the temperature, monitor the incubator temperature every hour and adjust the flow through accordly.

The female/s should be injected with 1ml of OTC /Kg, before the females are returned to the broodstock tank of origin in clean water. This is a prophylactic step will help to prevent the transfer of diseases from stressed individuals after the strip spawn. The temperature of the broodstock tank should be measured and adjusted before the transfer of the fish.

Acclimated the fish if the water in the broodfish tank is cooler or warmer that the incubator room by slowly adding water from the broodfish tank into the transporting ice chest until the temperature equilibrates.

Monitor development with a microscope two hrs. after the strip spawn the fertile eggs should be two cells developing to 4 cells depending on the temperature of the tank

Procedures for Culturing Southern Flounder (*Paralichthys lethostigma*)

Larval Culture

TPWD MDC employs a flow-through system for southern flounder larval culture operations. Water should flow in the following order

- Incoming water is pumped in from the intake canal.
- Water is pumped through an Astral High Rate Sand Filter Model 06805 located outside of the aquaculture facility.
- Water flows by gravity from the outdoor reservoir into the Astral High Rate Sand Filter Model 06805 located outside of the incubator Room.
- Water from the sand filter flows through a closed Ultraviolet Filter system.
- After the water runs through the Ultraviolet Filter, it is transferred into a storage reservoir inside of the incubation room.
- When water is needed for water exchanges Chilled new water is dripped from the storage reservoir into the incubation tanks.

The second sand filter serves as a second bio filtration for the removal of large organic matter, retention of algae, and other planktonic organisms including Parasites.

This sand filter should receive periodic cleaning every few weeks.

Activated carbon can be use in the sand filter to adhere molecules that may be toxic to the larval fish (domoic acid and brevitoxins from dinoflagellates.

Backwashing the filter daily should prevent anaerobic conditions in the sand filter, as well as accumulation of Hydrogen sulfide.

The treatment with ultraviolet filter is an enclosed reactor to irradiate the water to kill pathogens, and fungi.

Incubators

Circular fiberglass constructed tanks containing a central drain and standpipe measuring between 75 and 100 mm in diameter Volume of the tank 300 L

- Each circular fiberglass tank has a 1.5-foot radius from the edge of the tank to the center drain. This results in a total diameter of 3 feet.
- The height of the incubator tank is about 1.5 feet, with the water level maintained at about 1.4 feet, depending on the amount of water present in each incubator.

• The volume of water in each tank measures about 300 L.
Since the incubator room system is designed as a flow-through system, water discharged from the incubator tanks are then released into the environment, following regulations and standards established by the Texas Commission on Environmental Quality and the State of Texas (Total suspended solids,

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Temperature TPWD MDC uses individual chillers for each incubation tank.
Control A ½ horsepower- Cyclone Pro Trimline Series Drop-In Coil Water
Chiller Model Number TLC-5 (Aqualogics, San Diego, California is used to chill the incubator tank water. The chiller is set up between a temperature range of 17° C° and will oscillate + or – 1 C° in a way that the minimum temperature should be 16 C° and the maximum temperature 18C°

Electrical cords and extension cords are maintained height above the culture tank for safety.
The coil should be properly attached to the chilling unit, and the temperature controller set appropriately next to the culture tank.

Photoperiod
Southern flounder larvae at TPWD MDC receive 24 hours of light (100 Lux fluorescent light)

Incubation of eggs
Incubators tank with volume capacity of 300L are used in the incubator room
The incubators should be filled with clean filtered seawater filled to 85% the capacity of the tank, aeration stones should be distributed throughout the tank standpipe and all around to facilitate good oxygenation to prevent the eggs and embryos to die.

Organic debris should be removed from the water if present before the strip spawning
procedures are performed. Water in the egg's incubator should match the broodstock tank (mating tank).

Incubation of newly fertilized southern flounder eggs are stocked into
300 L volume circular fiberglass incubator tanks.
Aeration should be provided in ample quantities, with four air stones extended outward from the standpipe region of the tank, and four additional air stones are distributed around the periphery of the incubator tank as well.
This is to ensure adequate aeration required for proper egg hatch.

Low dissolved oxygen levels induce the eggs to hatch prematurely without proper development Water quality factors such as pH, salinity, and temperature are critical for proper development of Southern flounder

Eggs are incubated with dissolved oxygen levels of at least 7.5 mg/L, pH 8.0, salinity 32 ppt, and water temperature around 17°C.

Depending on environmental water quality factors such as water temperature, southern flounder eggs hatch between 48 and 55 hours of incubation.

Water in the reservoir should have the same (temperature, ph, salinity as the water used for flow through volume of water exchanged 2L/min during eggs incubation, dissolved oxygen should be a minimum of 6mg/L.

Once southern flounder eggs hatch, the aeration needs to be reduced to prevent the delicate larvae to roll and tumble and get damage due to excess aeration.

As soon as possible syphon the egg cases out of the tank to prevent colonization of the egg cases by pathogens.

Gently and slowly syphon the bottom of the incubator to remove the egg cases and organic debris in a bucket.

Wait until the organic debris settles at the bottom of the bucket, the live larvae will continue in the water column. Carefully using a flashlight and a beaker collect the larvae from the bucket and return the larvae to the incubator of origin. Do not mix the contents of different tanks in the same bucket because the larvae may be at different stages of development/age

Check the larvae daily under the microscope to monitor the absorption of the yolk sac and determine when the first feeding will occur.

Introduce a small number of enriched rotifers to the tank when the yolk sac is 75% consumed and the mouth furrow forms in the larvae. This will help the larvae to detect the odor of the prey item-rotifers.

Continue the monitoring of the larvae and look at flexion and rotifer consumption.

Excellent water quality is required when rearing southern

• Dissolved oxygen provided should be at a minimum of 6.0 mg/L, salinity 32 ppt, ph 8

Southern flounder larvae will continue to feed omega 3 enriched rotifers until approximately 15-20 days. The feeding will be done by hand and a peristaltic pump will deliver 1 L of fed/enriched rotifers every hour-/24 hrs. A batch of rinsed /enriched rotifers will be set every morning in a bucket and the peristaltic pump will deliver the feed into the tank.

After the 20 days mixed small amounts of recently hatched Artemia enriched with omega 3 fatty acids (Selco,) at the rate of 1mL/dissolved in 100 mL of DI sterile water, enrich rotifers and Artemia for 1 hour. Observe the feeding behavior, the larvae should be orange in color if they are feeding on Artemia. Prey item density in the tank should be 40-60 rotifers/L at first feeding and thereafter the density of prey can be combined with Artemia

Do a water exchange prior by syphoning /draining the tank from behind the stand pipe to feeding in the morning since metabolites are accumulated at night and the live prey (rotifers and Artemia will produce ammonia and nitrite accumulation can occur)

At 25 days introduce sprinkles of Otohime or very small amounts with an automatic feeder

Syphon the tanks at least twice every day, collect live larvae and returned to the tank of origin

Feed and syphon/clean the tank in this way until metamorphosis

Periodic tank cleaning of each incubator is essential, as waste and debris accumulating at the bottom of the tank.

For larvae that are less than 20 days old post hatch and or have not undergone complete metamorphosis, tank cleaning is performed by siphoning the bottom of the tank into a clean, white, 5-gallon volume bucket.

A custom-made siphon comprised of 1 cm diameter tubing with

about 3 ft in total length is used at the TPWD MDC. Upon siphoning a tank, the bucket is allowed to sit between 10 and 15 minutes to allow the waste and debris to settle out to the bottom. Larvae are then scooped out from the surface or middle of the water column and transferred gently back into the tank.
The center standpipe should be siphoned as well. Hydrogen sulfide can develop on the bottom of the center standpipe, and potentially foul water quality, and cause mortality of larvae. Siphoning occurs in the same method as described above, only at and around the bottom of the center standpipe.

Water quality is essential in larval rearing operations, since poor water quality can lead to stress, disease, or mortality.
Maintaining a good, high water quality is also essential during development providing a healthy environment for southern flounder to grow and develop. Southern flounder requires optimal environmental conditions to survive and grow development, and reproduction.
Dissolved oxygen is perhaps the most important water quality factor as dissolved oxygen supports the respiration of southern flounder. Dissolved oxygen from egg hatch through fingerling culture should maintain at least 6.0 mg/L O2 as a minimum requirement. Temperature is perhaps the second most important water quality factor, especially for southern flounder, as excessive temperature can lead to stress, disease problems, or even mortality. As stated, above, temperature at initial stocking into the incubator tank (Hour 0) through 72 hours should be maintained at 16°C. After 72 hours, this temperature (16°C) should be slowly increased over time to between 17°C and 20°C over the next 40 days.
Salinity is another important water quality factor. As stated above, southern flounder eggs hatch at 32 or 33 ppt, and larvae should be maintained at about 32 ppt through the fingerling stage.
pH is another important water quality factor. pH should be maintained between 7.5 and 8.5 on the pH scale from 1-14.
Ammonia and nitrite are both important water quality factors, as excessive nitrite can cause brown blood disease and excessive ammonia can instigate ammonia toxicity. This is one reason Larviculture tanks should be routinely siphoned, since the buildup of ammonia and other waste products can foul water quality and cause stress or mortality of larvae. Ammonia should be kept to no more than 0.1 mg/L NH_3- N, while nitrite should be kept to no more than 0.01 mg/L NO_2- N.
Alkalinity and calcium hardness are two other water quality factors. Both alkalinity and calcium hardness should be equal to or

greater than 200 mg/L CaCO3.

Procedures for Culturing Southern Flounder

Nutrition is an essential component to southern flounder Rotifers, *Brachionus plicatilis*., are cultured at the Texas Parks and Wildlife Department Coastal Conservation Association Marine Development Center in Flour Bluff, Texas. Rotifers are cultured in cylindrical fiberglass cones constructed byRed Ewald company. Rotifers are the first feed for southern flounder larvae. Southern flounder larvae do not feed immediately after post-hatch, but once larvae have completely absorbed their yolk sac at about Day 4 post-hatch, first feeding shortly follows. It is essential to note that southern flounder larval growth and development depends on environmental conditions such as water temperature, and thus larvae often develop at a slower rate at lower water temperatures. Larval development must be closely monitored and checked by looking at development under a compound light microscope perhaps at least once a day.

At TPWD MDC, rotifers are enriched with Orione rotifer Enrichment diet, a powder diet produced by Skretting a Nutrico Company based out of Italy. This is prepared by measuring out about 10 grams of Orione Rotifer Enrichment (Skretting a Nutrico Company), transferring the Orione into a 5 L beaker, and then adding 1000 mL of water. This is stirred for an hour, after which is transferred into two 1000-mL plastic graduated cylinders. One of the tubes are connected to the MITY FLEX Peristaltic Pumps by Anko are placed in each graduated cylinder. The Digital Recycling Timer Sentinel is set to “Day ON Time” setting, and then the Orione is fed out over a period of 24 hours, with a rate of 1 L/ hour.

Rotifers are fed out to the larvae by first rinsing the 5-gallon white buckets with freshwater, and cleaning the tubes to remove debris. Then, 5 L of treated water are transferred into each of the 5-gallon buckets. A 5 L beaker is used to transfer the number of rotifers to be harvested to the larvae into the buckets. At TPWD MDC, 5 L of rotifers are harvested from one of the cones per day, depending on the number of larvae currently in culture, approximately 15-20 million rotifers are needed every day. The rotifers are directly distributed to the incubator tank, around the border of the tank ensuring all areas of the tank, receive rotifers.

Artemia is the second live feed to be fed the southern flounder larvae. *Artemia* should be fed to southern flounder larvae starting about Day 25 post-hatch, when the mouths are more developed and metamorphosis should be in progress. Development must be

closely monitored, as the larvae mouth size has to be able to easily consume *Artemia.* Over time, starting about Day 25, depending on environmental conditions, southern flounder larvae start consuming *Artemia* and are weaned onto *Artemia* over a period of a few weeks.

Artemia are cultured in three plastic cones. Frozen *Artemia* cysts are used in *Artemia* culture, and should be stored in a freezer onsite. After removing the *Artemia* from the freezer, 10 grams of frozen *Artemia* cysts are weighed and measured out on a scale. About 50 mL of freshwater is added to the *Artemia*, mixed, and allowed to sit for some time.

Afterwards, the *Artemia* cone is then prepared by first closing the valve at the bottom, and filling the *Artemia* cone with saltwater. Two air stones are added to the *Artemia* cone, the aeration turned on to high, and the prepared *Artemia* cysts are transferred into the *Artemia* cone. The *Artemia* hatch in 24 hours, which is when the *Artemia* should be harvested out to feed to the older southern flounder larvae. Over time, the *Artemia* can become too large in size for the southern flounder larvae to consume, so *Artemia* should be fed out soon after 24 hours of hatching out.

Once the *Artemia* hatch, the *Artemia* cone is harvested by first removing the debris and dead *Artemia* gathered at the bottom in a 5-gallon volume bucket. A plastic 1 L volume beaker containing a specialized 50- micron net on the bottom is held above a bucket. The water and the *Artemia* drain first into the specialized beaker, with debris going straight down to the bottom past the filter. After the water level drains down about 3 inches close to the bottom, the specialized beaker is removed and a separate 5-gallon bucket is placed to collect the last of the *Artemia.*

A separate 1 L plastic beaker is obtained, filled partially with treated saltwater, and then the *Artemia* in the net is transferred to the 1 L beaker. The *Artemia* in the bucket is allowed to settle for about 5 minutes, and then the live *Artemia* at the top of the water column is transferred into a 5-L volume plastic beaker. The separate 1 L plastic beaker containing the *Artemia* is also transferred into this 5-L beaker.

The highly unsaturated fatty acids (HUFA) enrichment emulsion should be prepared and diluted prior to adding to a 5-gallon bucket with rotifers or a 5 L beaker with *Artemia.* Liquid Selco HUFA (PentairTM) is used as the HUFA enrichment, and freshwater is used as the diluting factor. The HUFA is prepared and diluted by adding 10 grams of the Selco Liquid HUFA (PentairTM) to 100 mL of freshwater. Afterwards, the enrichment is stored in the refrigerator until applied to rotifers or *Artemia*, the live feed used to feed the larvae. The HUFA enrichment is fed out to the rotifers

or *Artemia* by first gently shaking the mixture and creating an equal suspension, and then pipetting 12 mL of enrichment into the rotifers or *Artemia* culture. After this, allow an hour for the rotifers or *Artemia* to absorb the HUFA prior to feeding the larvae.

Pond Culture Southern flounder are stocked into ponds when southern flounder complete metamorphosis from being bilaterally symmetrical and swimming upright in the water column to settling out of the water column, flattening out, and becoming benthic. Southern flounder typically start metamorphosing about 25 days' post hatch and complete metamorphosis by 40 days' post hatch. This is depending on environmental conditions, especially water temperature. With a colder water temperature or a colder winter, southern flounder grow slower and start metamorphosis later.

Pond culture of southern flounder fingerlings has been successful at Texas Parks and Wildlife Department Coastal Conservation Association Marine Development Center in Flour Bluff, Texas, with a 90 to 95% survival rate.

Fingerlings should be siphoned one or two at a time into a small, plastic box in which they are counted. A 100-gallon volume Igloo ice chest is prepared, in which the ice chest is partially filled up about 35% full and an air stone or oxygen ring is placed inside to provide oxygen. Oxygen must be provided, either in the form of an air stone or an oxygen ring. From there, fingerlings are gently transferred into the ice chest using a 1 L plastic beaker to scoop up larvae and then gently release them into the ice chest. Once completed, the ice chest is then placed onto a 2018 Kawasaki Mule and driven out to the ponds.

The ice chest is placed on the edge of the pond. Using the plastic 1 L beaker, roughly 3 or 4 scoops of pond water is added to the ice chest every 10 minutes. The estimated acclimation period prior to entering the pond is roughly an hour, after which the fingerlings are released into the pond.

Once stocked into ponds, grow-out operations of the southern flounder fingerlings commence.
