Fall 12-2010

Reproductive Life History of *Fundulus jenkensi* and Comparative Development of Five Sympatric Fundulid Species

Eric Thomas Lang
University of Southern Mississippi

The University of Southern Mississippi

REPRODUCTIVE LIFE HISTORY OF *FUNDULUS JENKINSI* AND COMPARATIVE DEVELOPMENT OF FIVE SYMPATRIC FUNDULID SPECIES

by

Erik Thomas Lang

A Thesis
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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Director

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Dean of the Graduate School

December 2010

ABSTRACT

REPRODUCTIVE LIFE HISTORY OF *FUNDULUS JENKINSI* AND COMPARATIVE DEVELOPMENT OF FIVE SYMPATRIC FUNDULID SPECIES

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Fundulus jenkinsi is recognized federally and within the state of Mississippi as a Species of Concern. Little is known about the life history of this coastal killifish, but a detailed reproductive histology study of *F. jenkinsi* and a diagnostic key of the early life stages of select members of Fundulidae can provide the foundation needed to accurately identify it and quantify reproductive parameters in this rare species in need of conservation. Monthly gonadosomatic index (GSI) of male and female *F. jenkinsi* were documented, and spawning phases and oocyte stages were examined using reproductive histology. In addition, various stages of coastal *Fundulus* spp. and *Adinia xenica* have been illustrated and their morphometrics and meristics recorded. While GSI indicated a *F. jenkinsi* spawning season from April through August, the ovarian histology suggested March through August was a more accurate season. The composition of the ovaries also suggested spawns occur multiple times in a single tidal cycle within a population and on the individual level. The diagnostic key reveals that branchiostegal rays are essential to separate young fundulids into two groups that can then be identified by pigment patterns and morphometrics. This work contributes an estimation of *F. jenkinsi* spawning frequency and an early development diagnostic key that allows for accurate identification of young fundulid species.

ACKNOWLEDGMENTS

My experience here at the Gulf Coast Research Laboratory has taken me to another level of cognitive thought that I did not think was possible for me. First, I have to thank my advisor, Dr. Mark S. Peterson, for his support and willingness to the take time to teach at the drop of a hat. I would also like to thank his wife, Nancy Brown-Peterson, for her patience in guiding me through the skills needed to become a reproductive fish biologist/ecologist. Thanks also to Dr. Todd Slack, whom I admire for his attention to detail and humor, which are two qualities that behooves any ichthyologist. In addition, without Dr. Bruce Comyns I would have never known I could illustrate anything, much less larval fish. A special thanks to Steve Manning, who not only let me take over a piece of his space, but also imparted a great deal of knowledge. This research was approved and performed by The University of Southern Mississippi IACUC protocol # 10040802. I would also like to thank all my lab mates and others who helped me in my thesis work including J.D. Lopez, Paul Grammar, Mike Lowe, Jean-Marie Havrylkoff, and Mike Andres. Thanks to Dr. Andre Landry, who inspired me to not only chase a career in fisheries biology, but also to be damn good at it someday. I could not forget my parents; no one has supported me more in every facet of life than they have. I was lucky enough to be born into one of the most supportive, intelligent, and fun families on this earth. They have all made me who I am today, and I am forever grateful. Finally, I could not live without my wife, Hannah, whose constant love, support, and goofiness get me through every day with a smile on my face; no one makes me happier.

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CHAPTER I

BACKGROUND

The order cyprinodontiformes consists of three families in the United States: Fundulidae (killifishes), Cyprinodontidae (pupfishes), and Poeciliidae (livebearers). Killifish are oviparous spawners, but freshwater fundulids attach their eggs to submerged aquatic vegetation (Taylor and Burr 1997), whereas saltwater killifish spawn on high marsh during spring tides and strand the eggs to be incubated in air in the salt marsh. The poeciliids exhibit ovoviviparity, and occupy brackish and freshwater habitats. Pupfish also are found in brackish and freshwater, but their eggs are fertilized and gestate outside of their body (Boschung and Mayden 2004). The family Fundulidae is the focus of this study, in particular the fundulids along the Gulf of Mexico (GOM) coast. The most prominent GOM coastal fundulid is *Fundulus grandis*, which inhabits salt marsh in various salinity regimes. The salinity in the natural environment where this common fundulid is found ranges from 0.05-76.1 (Griffith 1974). Although *Fundulus similis* can tolerate low salinity, it is mostly found on sandy banks and beaches in meso-stenohaline environments (Griffith 1974; Greeley et al. 1986). Another GOM fundulid, *Adinia xenica*, most often occupies salinities in the 20-30 range, but can also live in meso-oligohaline environments (Hastings and Yerger 1971), while *Fundulus pulvereus* and *Fundulus jenkinsi* prefer a meso-oligohaline environment (Griffith 1974; Peterson et al. 2003; Lopez et al. 2010a). All of these fundulids except for *F. similis* are heavily associated with salt marsh habitat and will use the tide to seek refuge and find food in the marsh vegetation. Fundulids are an important component of the coastal food web by acting as a means of trophic transfer from the terrestrial to the marine environment when

eaten by larger estuarine fish (Rozas and LaSalle 1990; Kneib and Knowlton 1995). However, *F. jenkinsi* is of particular concern due to its federal recognition as a Species of Concern (NOAA). Thompson (1999) sites the lack of information on diet, habitat, reproduction, and development of this species. Lopez et al. (2010a, b) has addressed issues of diet, distribution, habitat preference, size at maturity, and sexual dimorphism of the saltmarsh topminnow. It is important that the lack of reproductive life history and development information on this species be addressed in order to form a better conservation plan for *F. jenkinsi* (Thompson 1999; Peterson et al. 2003).

CHAPTER II

REPRODUCTION

Introduction

The genus *Fundulus* comprises a large group of freshwater and saltwater killifishes (family Fundulidae). Coastal killifish predominantly use the tides to reproduce in areas that are only inundated during or close to spring tide events, which allows the eggs to incubate in air to receive more oxygen as well as providing protection from predators (Taylor et al. 1977; Taylor 1984; DeMartini 1999). Along the east coast, fundulid reproduction is thought to occur in a semi-lunar pattern according to the moon phase cycles, which is in sync with the semi-diurnal (twice daily) tidal cycle. *Fundulus* on the east coast has been shown to spawn during a spring tide when there is either a full or new moon (Taylor and DiMichele 1980; Taylor 1991). However, in the northern Gulf of Mexico (nGOM) tides are diurnal (once daily), and the tides coincide with the moon declination rather than lunar phase (Greeley and MacGregor 1983). Therefore, spring tides do not always occur on full or new moons. Thus, it is unclear if fundulids found along the coast of the nGOM use the same semi-lunar spawning pattern as most Atlantic coast *Fundulus*.

The saltmarsh topminnow (*Fundulus jenkinsi*) is a NOAA Species of Concern and inhabits the *Spartina* and *Juncus* saltmarshes of the nGOM (Thompson 1999; NOAA 2009). Little is known about this small killifish's life history, and a key component to its survival is reproduction. Lopez et al. (2010b), using oocyte diameters, noted that *F. jenkinsi* spawn in the spring through the summer, but no other data are available on reproductive habits. Reproductive histology is required for a complete understanding of

the *F. jenkinsi* reproductive life history to expand on that preliminary work. A detailed study of ovarian development through the spawning season will indicate how this species prepares for and completes oogenesis.

Although detailed information on *F. jenkinsi* reproduction is lacking, reproductive histology has been completed on *Fundulus grandis* (gulf killifish), a common nGOM fundulid species; Greeley et al. (1988) described *F. grandis* as having a "semilunar reproductive cycle" (p. 105). They noted that the killifish invested more energy in a spawning peak at the beginning of the spawning period so that a significant amount of the young-of-the-year (YOY) from that spawning cycle will develop during summer conditions. This is due to an almost daily spawning regiment at the beginning of the season whereas a more semi-lunar approach becomes evident towards the end of the season (Greeley et al. 1988). Numerous reports note that coastal killifish from the Atlantic coast and the nGOM reproduce daily in a cluster of three to six days (Greeley and MacGregor 1983; Greeley et al. 1986; Shimizu 1997; Petersen et al. 2010). For example, *Fundulus heteroclitus* (mummichog), has the capability to spawn daily in captivity, regardless of the semilunar cycle (Shimizu 1997). Although the fish were in aquaria exposed to ambient temperature and natural moon phases, the daily spawning observed had no tidal influence. Petersen et al. (2010) observed a lack of semilunar spawning in *F. heteroclitus* from a population in Mount Desert Island, Maine, while Taylor (1991) observed semilunar spawning periods without tidal influence in *F. heteroclitus* from Delaware Bay. Kneib and Stiven (1978) also observed a bimodal peak in spawning in *F. heteroclitus* with a larger peak in the spring and a smaller peak in mid-summer. These inconsistencies may indicate a difference in reproductive behavior

amongst various wild populations. In wild conditions, *F. grandis*, *Fundulus similis* (longnose killifish), *F. heteroclitus*, and *Fundulus luciae* have exhibited a spawning season from spring to early fall (Kneib and Stiven 1978; Kneib 1978; Greeley and MacGregor 1983).

The overall goal of this project is to describe the reproductive habits of *F. jenkinsi*, which is vital to a better understanding of this rare species that requires conservation measures. Thus, the specific objectives were to:

1. quantify the spawning season in male and female *F. jenkinsi* using the gonadosomatic index;
2. describe ovarian development of *F. jenkinsi* with detailed reproductive histology; and
3. determine if *F. jenkinsi* is a semi-lunar spawner.

Methods

Seasonal Collection

Fundulus jenkinsi were collected in the Pascagoula River, Grand Bay National Estuarine Research Reserve (NERR), Simmons Bayou, Davis Bayou, and Fort Bayou along the Mississippi Coast twice monthly during spring tides from April through October 2008 using standard minnow traps baited with blue crab (*Callinectes sapidus*). The spring tides in February and March of 2009 were also sampled to assure representation of the full reproductive season. Fish were collected leaving the flooded marsh as the tide receded and were immediately fixed in 10% neutral buffered formalin. The wet weight (WW, mg), total length (TL, mm), and gonad weight (GW, mg) were measured for each fish, and the ovaries were placed into individually labeled plastic

cassettes and reimmersed in 10% neutral buffered formalin from a week to a month for reproductive histology analysis.

Semilunar Collection

Sampling was conducted from 14 to 25 June 2010 to determine *F. jenkinsi* spawning frequency dynamics within a single tidal cycle. High tides were sampled five days between two spring tide events. Early research has indicated that two nodes are present in the Gulf of Mexico, one near the Florida Strait and one between the Mississippi River delta and the Yucatan Peninsula, from which tidal processes arise (Zetler and Hansen 1971; Seim et al. 1987). The unexplained nature in which these two seiche waves interact may explain why summer diurnal high tides usually occur in daylight hours on the Mississippi coast (www.freetidetables.com). Standard minnow traps were used for collection and all fish were fixed in 10% neutral buffered formalin. The predicted high tide level of each day for the 2010 samples was taken from tide charts specific to the sampled area (www.freetidetables.com).

Histological Preparation

Standard histological techniques were performed according to Luna (1960) and Prophet et al. (1992). Casssettes containing formalin fixed tissues were rinsed overnight in running tap water to remove the fixative. Partial dehydration was accomplished by soaking the cassettes for two hours in each of the following solutions: 60% ethanol, 70% ethanol, and then 70% ethanol again. The gonads were processed in a Shandon Hypercenter 2 or Citadel 1000 tissue processor, where they were soaked for one hour in each of the following solutions 70% ethanol, 80% ethanol, two treatments of 95% ethanol, three treatment of 100% ethanol, and three treatments of Thermo Scientific

xylene substitute. The last steps of the processor were two, one hour treatments of paraplast plus paraffin, under vacuum, which aided in the paraffin impregnation of the gonadal tissue. The cassettes were transferred to liquid paraplast extra in a Shandon embedding center. The gonads were taken out of their cassettes and placed in a square metal histology mold. The mold was filled with paraffin (McCormick Paraplast X-tra) and the bottom of the cassette, containing the sample number, was placed on top of the mold. The molds were cooled to form a solid block of paraffin containing the gonad for sectioning. Extra paraffin was trimmed from the block so it fit into the chuck of the microtome and the block was sectioned with an AO rotary microtome at 4μm. All paraffin ribbons were placed in a warm water bath (35°C) containing Sta-on (Surgipath) and then each section was mounted onto a slide. After mounting, the slides were labeled and placed on a slide warmer at low heat for two hours in preparation for staining.

Slides were stained to visualize proteins, lipids and yolk material. Before staining, the slides were cleared with xylene substitute and rehydrated in ethanol of descending concentrations. Slides were then soaked in Hematoxylin 2 (Richard Allen) for 5-6 min to stain the basophilic nucleus proteins. Afterwards, the sectioned gonads were exposed to acid water for 2 dips and Blueing water for 30 sec to enhance the color of Hematoxylin for better visualization of oocyte stages. Slides were then counterstained with Eosin-Y (Richard Allen) from 45 sec to 2 min (Appendix A). Afterwards, the slides were dehydrated with ethanol and cleared using xylene substitute and then coverslips were placed on the slides with Richard Allen Mounting Media and allowed to dry. Once dry, they were scraped, cleaned, and ready to observe.

Analysis

The gonadosomatic index (GSI) was used to estimate *F. jenkinsi* spawning preparedness. The equation used was obtained from Greeley et al. (1986):

$$\left(GW \middle/ (WW - GW)\right) \times 100 \ .$$

All GSI data were arcsin square-root transformed prior to analysis. Linear regression of arcsin square-root transformed GSI values versus gonad-free body weight (GFBW) was conducted separately for males and females to determine if the GSI was influenced by GFBW. A One-Way ANOVA was used to determine if there is a significant difference among monthly mean GSI values. If a significant F-value was calculated, a post-hoc Sidak test followed. Homogeneity of variance, using Levene's statistic, and normality, using the Kolmogorov-Smirnov test, were tested prior to analysis. If arcsin square-root transformed GSI data proved to be heterogeneous, a Games-Howell (GH) test was used instead of a Sidak for pairwise comparisons. All p-values ≤ 0.05 were considered significant and statistics were processed using SPSS software (ver. 15).

Fundulus jenkinsi ovaries were classified into reproductive phases as in Brown-Peterson et al. (2011) and oocyte terminology was adapted from Grier et al. (2009). The entire ovarian section on each slide was photographed using a Jenopik ProgRes C5 dissection microscope camera and loaded into the ProgRes Capture 2.5 Software. The software allows for calibration of 1 mm measurements at various magnifications so proper estimation of the area can be made. The gonad tissues were traced and the area (mm^2) obtained for each stage of oocyte to the nearest 0.00001 mm^2. The total area of the gonad was the summation of the areas for all oocyte stages; empty spaces between oocytes were not included in these calculations. The area of each oocyte stage was

expressed as a percentage of the total area of the gonad. The percent area occupied by various oocyte stages in the ovary was compared with a stacked bar histogram.

Results

Seasonal Collection

A total of 435 female and 176 male *F. jenkinsi* were collected along the course of the study with the largest female reaching 70.3 mm TL and the largest male reaching 60.3 mm TL (Table 1).

Table 1 Number, total length (mm) range, and mean GSI values of *Fundulus jenkinsi* collected by month

	Female				Male			
			Total Length (mm)				Total Length (mm)	
Date	*n*	Mean GSI	Min	Max	*n*	Mean GSI	Min	Max
Feburary	56	1.24	26.6	65.8	15	0.28	29.0	48.1
March	45	3.05	28.3	51.4	27	0.36	25.3	53.5
April	81	5.69	35.6	69.6	16	0.33	30.0	52.3
May	95	5.92	42.4	66.4	24	0.33	40.0	60.3
June	45	4.91	42.5	70.3	18	0.26	40.8	59.9
July	28	5.79	48.1	63.7	11	0.34	41.4	52.0
August	45	6.45	43.3	61.0	40	0.36	34.0	52.7
September	25	0.74	40.7	54.3	17	0.14	37.9	48.7
October	15	0.34	29.7	56.5	8	0.09	41.4	49.2

Male body weight was not correlated to GSI ($r^2 = 0.018$, $p = 0.074$) whereas female GSI values were correlated with body weight ($r^2 = 0.074$, $p < 0.0001$), but the relationship explained only 7.4% of the variance in GSI and was considered not biologically significant. Mean female GSI among months was significantly different (ANOVA: $F_{8, 426} = 88.018$, $p < 0.0001$). The GH test suggested a spawning season of April through August, all of which were statistically different than February, March, September, and October (GH; $p < 0.05$). However, whereas February, September and October were all statistically equal, March was found to be intermediate between February and April-August values. Mean *F. jenkinsi* male GSI was found to be

significantly different between months (ANOVA: $F_{8, 164} = 18.636$, $p < 0.0001$). The males seem to be capable of spawning earlier because GSI values were statistically equal from February through August (GH; $p > 0.05$), followed by a sharp decrease in September and October (GH; $p < 0.05$) (Fig. 1).

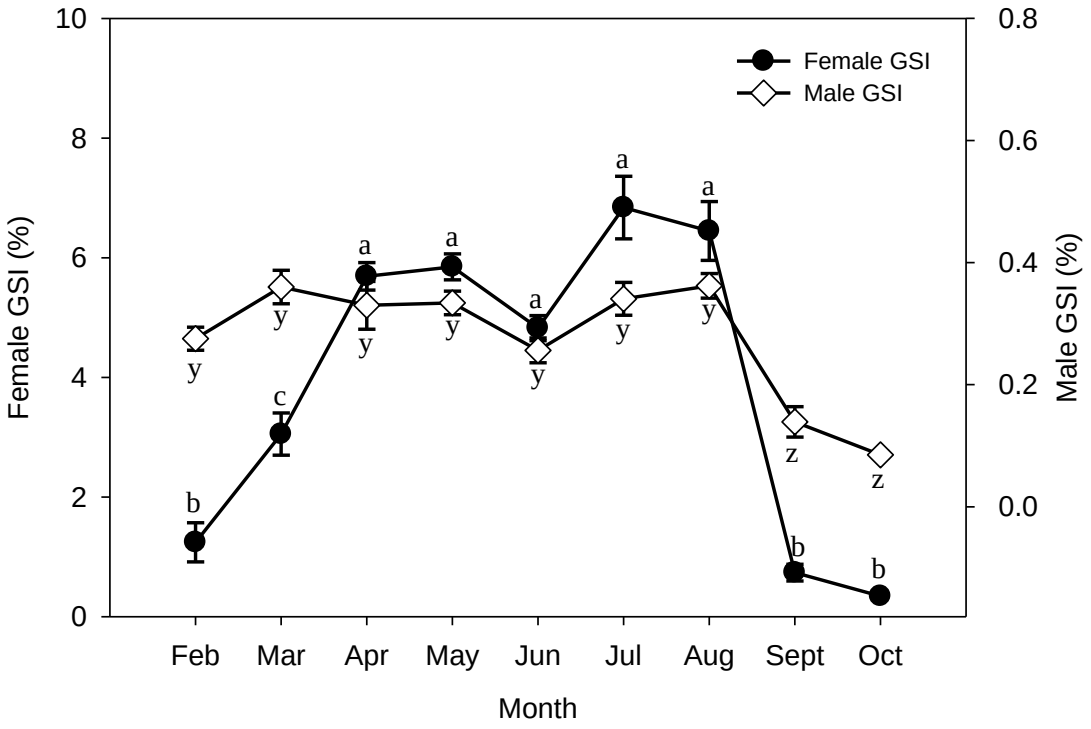

Fig. 1 Plot of mean (± SE) female and male gonadosomatic index (GSI) by month for *Fundulus jenkinsi*. In October, error bars were so small they were hidden by the data point symbol. Data points are significantly different ($p \leq 0.05$) if letter labels are not the same, and not significantly different ($p > 0.05$) if the letter labels are the same.

Although GSI works well as a general seasonal pattern of spawning preparedness, reproductive histology produces a more accurate examination of spawning condition. Appearance of the Developing phase signals entry into the annual reproductive cycle. The Developing phase of an ovary is demarcated by the lack of postovulatory follicles (POF), and no vitellogenic oocytes beyond early secondary growth (SGe). Primary

growth (PG) oocytes and cortical alveoli (CA) also can occupy a developing ovary (Fig. 2A). The Spawning Capable phase is denoted by more vitellogenin uptake in the oocytes and sometimes can include post ovulatory follicles (POF; Fig. 2B). The appearance of late secondary growth (SGl) and full grown (SGfg) oocytes classify an ovary as Spawning Capable.

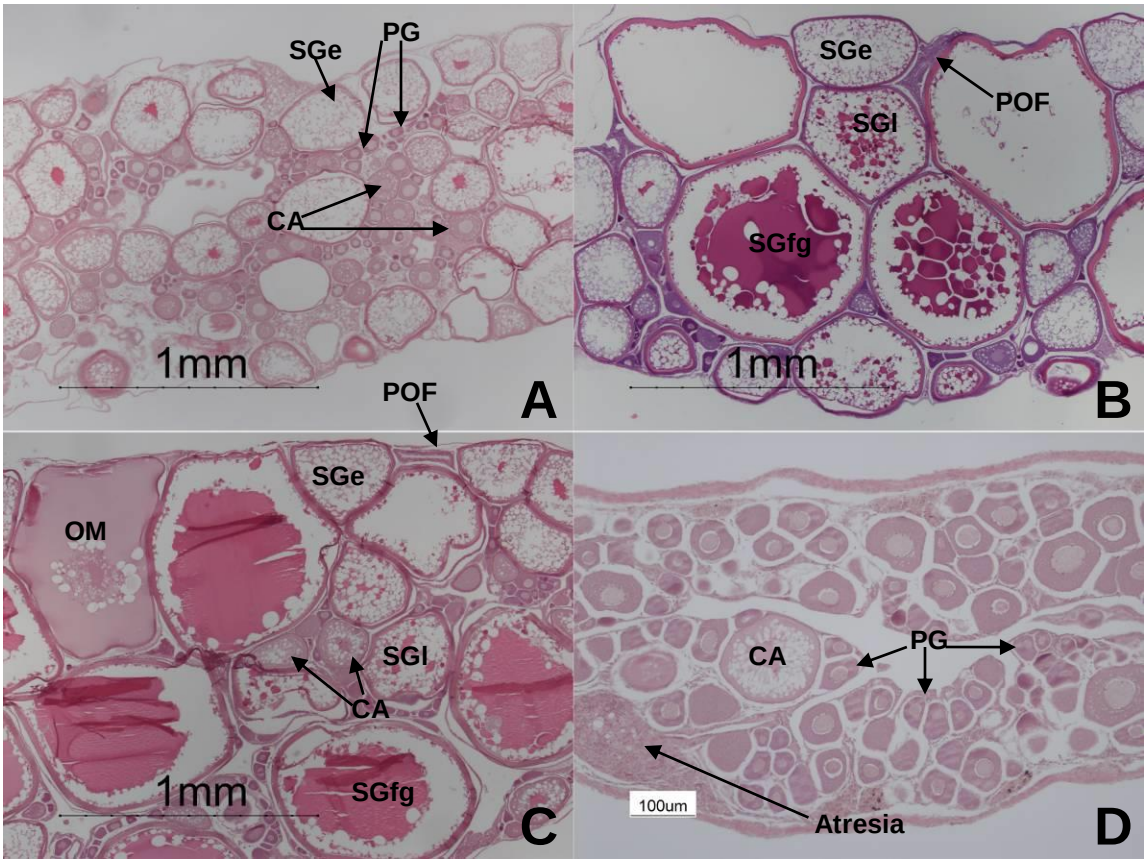

Fig. 2 Photographs illustrating spawning phases of female *Fundulus jenkinsi*: (A) Developing, (B) Spawning Capable, (C) Actively Spawning, and (D) Regressing. Oocyte stages are labeled as primary growth (PG), cortical alveoli (CA), early secondary growth (SGe), late secondary growth (SGl), full secondary growth (SGfg), Oocyte maturation (OM), postovulatory follicles (POF), and atresia.

Actively Spawning is a subphase of Spawning Capable and this subphase indicates spawning is imminent. However, actively spawning ovaries of *F. jenkinsi* can include all other oocyte stages, including POFs (Fig. 2C). Oocyte maturation indicates that the fish would have spawned that day. Fish in the Regressing phase are found at the end of the

spawning season, and ovaries are mostly comprised of PG oocytes with some atresia and a small amount of CA oocytes (Fig. 2D). No fish in the Regenerating phase were sampled during this study.

Histological analysis of the ovaries revealed that in February all females were in the Developing phase. By March the majority of females were in the Spawning Capable phase and some of these were in the Actively Spawning subphase (Table 2A). Overall, 25% of all females captured in March were Actively Spawning. All females were Spawning Capable from April through August, and the percentage of females in the Actively Spawning subphase varied from 39 to 65% during these months (Table 2A, Fig. 3A).

Table 2 *Fundulus jenkinsi* reproductive phase (A) and oocyte stage (B) percentages by month used to determine spawning season

A

	Month of Capture								
Spawning Phase (%)	February (*n*=4)	March (*n*=16)	April (*n*=33)	May (*n*=72)	June (*n*=39)	July (*n*=17)	August (*n*=16)	September (*n*=8)	October (*n*=9)
Developing	100	25	0	0	0	0	0	0	0
Spawning Capable	0	50	35	61	45	59	56	25	0
Actively Spawning	0	25	65	39	55	41	44	0	0
Regressing	0	0	0	0	0	0	0	75	100

B

	Month of Capture								
Oocyte Stage (%)	February (*n*=4)	March (*n*=16)	April (*n*=33)	May (*n*=72)	June (*n*=39)	July (*n*=17)	August (*n*=16)	September (*n*=8)	October (*n*=9)
Primary Growth	32	29	3	1	2	2	2	26	74
Cortical Alveoli	9	7	2	2	2	2	2	10	15
Early and Late Secondary Growth	59	40	36	41	31	39	45	18	0
Full Grown	0	20	28	37	44	42	30	4	0
Oocyte Maturation	0	3	27	17	15	10	10	0	0
Post-Ovulatory Follicles	0	0	4	3	6	5	12	0	0
Atresia	0	0	0	0	0	0	0	42	11

Although 25% of females were Spawning Capable in September, none were Actively Spawning. The high percentage of fish in the Regressing phase in September indicated the end of the spawning season; all females were in the Regressing phase in October (Table 2A, Fig. 3A).

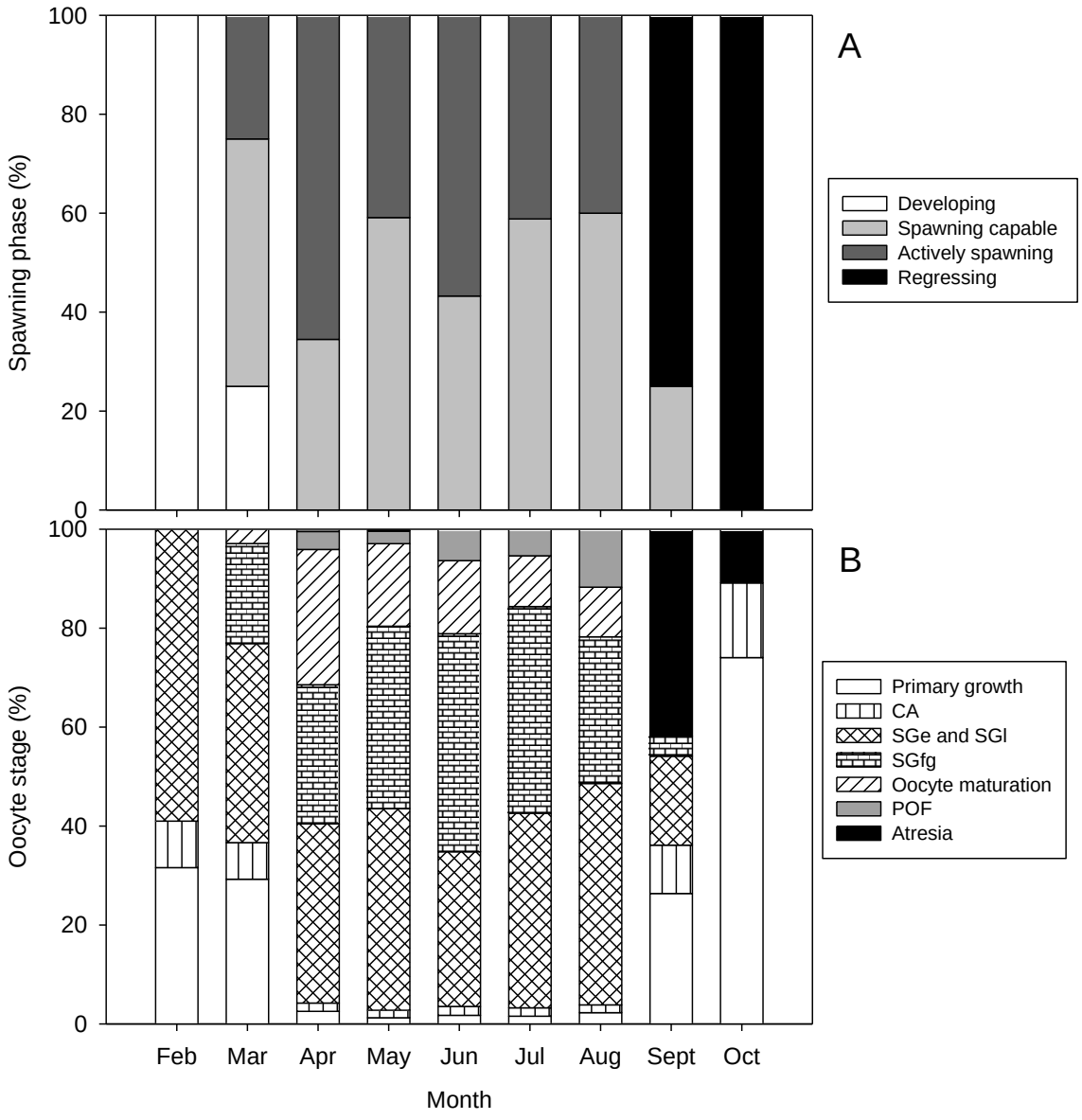

Fig. 3 Percent *Fundulus jenkinsi* in each reproductive phase (A) and percent of oocytes in each stage (B) by month. Oocyte stages present were primary growth, cortical alveoli (CA), early and late secondary growth (SGe and SGl), full grown (SGfg), oocyte maturation, post-ovulatory follicles (POF),and atresia.

Although vitellogenic oocytes predominated in February, full grown oocytes did not appear until March and were present through September (Table 2B, Fig. 3B). In September, a large percentage of atretic oocytes were present in most of the ovaries and October was comprised mostly of the pre-vitellogenic oocyte stages with some atresia still occurring. Although the female GSI values indicated that spawning started in April, the reproductive histology showed fish in the Actively Spawning subphase in March. However, the lack of OM in September indicates the spawning season appears to end in August, which was indicated by the GSI data. Therefore, *F. jenkinsi* appears to spawn from March through August in Mississippi waters.

Semilunar Collection

A total of 58 females were collected over five dates between two spring tides in June 2010. Fish ranged in size from 41.2 to 64.6 mm TL (Table 3).

Table 3 Number and total length (mm) of *Fundulus jenkinsi* by date with GSI values used to determine spawning periodicity

	Female			
			Total Length (mm)	
Date	*n*	Mean GSI	Min	Max
6/14/2010	17	3.97	43.6	64.6
6/16/2010	7	1.95	48.1	53.5
6/18/2010	15	1.86	44.5	60.5
6/21/2010	5	2.41	50.7	54.1
6/25/2010	14	6.01	41.2	64.0

Arcsin square-root transformed female GSI values from 14 to 25 June 2010 were not explained by GFBW ($r^2 = 0.025$, $p = 0.233$). Transformed female GSI values were normally distributed ($p > 0.05$), but were heteroscedastic ($p < 0.05$). The transformed GSI values varied over the tidal cycle (ANOVA: $F_{4,53} = 18.164$, $p < 0.0001$), with higher values on spring tide and lower values during neap tide (Fig. 4). The GH test showed there was no difference between 16, 18, and 21 June 2010 GSI values (GH: $p > 0.05$),

and 14 and 21 June 2010 were also not different (GH: $p > 0.05$). The 25 June 2010 GSI values were greater than all other GSI values (Fig. 4), suggesting *F. jenkinsi* GSI values were significantly higher during the peak spring tides.

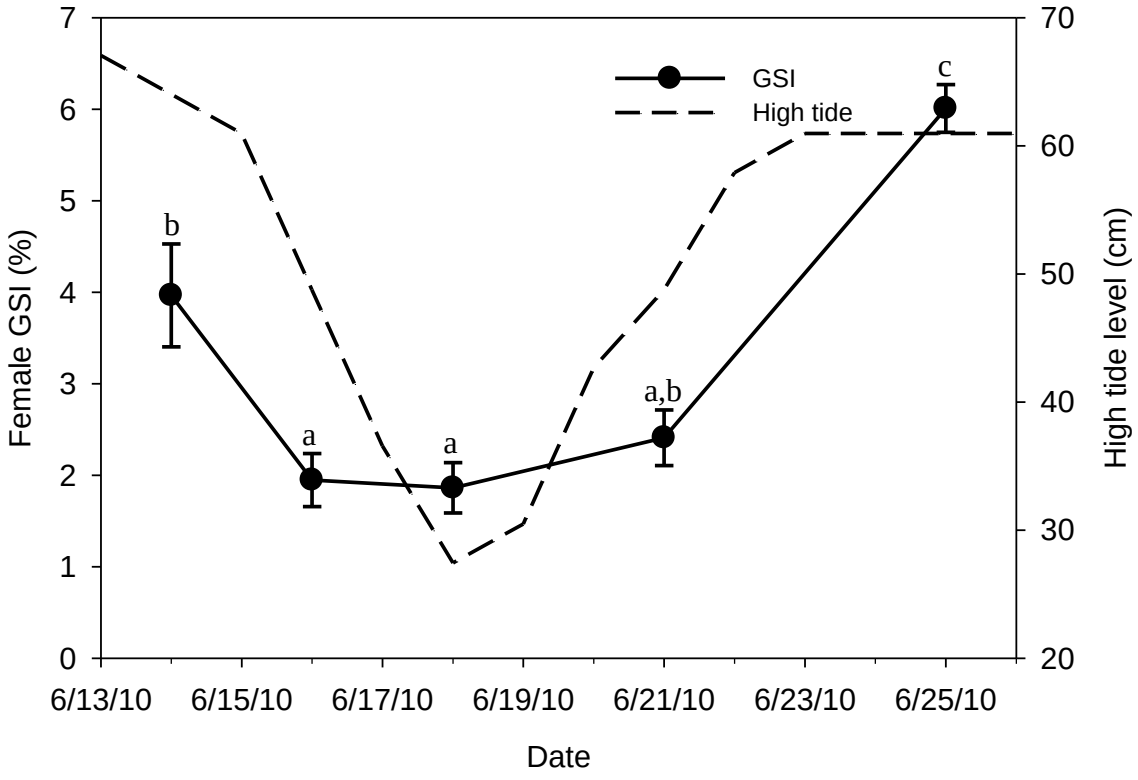

Fig. 4 Plot of mean (± SE) female *Fundulus jenkinsi* gonadosomatic index (GSI) between two spring tides with high tide (cm) by date. Data points are statistically different ($p \leq 0.05$) if letter labels are not the same, and statistically similar ($p > 0.05$) if the letter labels are the same.

Spawning phases and oocyte stages were also examined from spring tide to successive spring tide. This analysis indicated that, even within the spawning season, females enter a spawning phase just before and during neap tide that resembles the Developing phase. Because a fish cannot move backward in the spawning cycle, this phase was classified as Redeveloping and placed as a new sub-phase under Spawning Capable. Peak spawning occurs on spring tide as indicated by the high percentage of

females in the Actively Spawning subphase, and no spawning takes place on a neap tide (Fig. 5A). However, some Actively Spawning females are found several days before and after the spring tide (Fig. 5A, Table 4A). The newly defined Redeveloping sub-phase was only found immediately preceding and during neap tide.

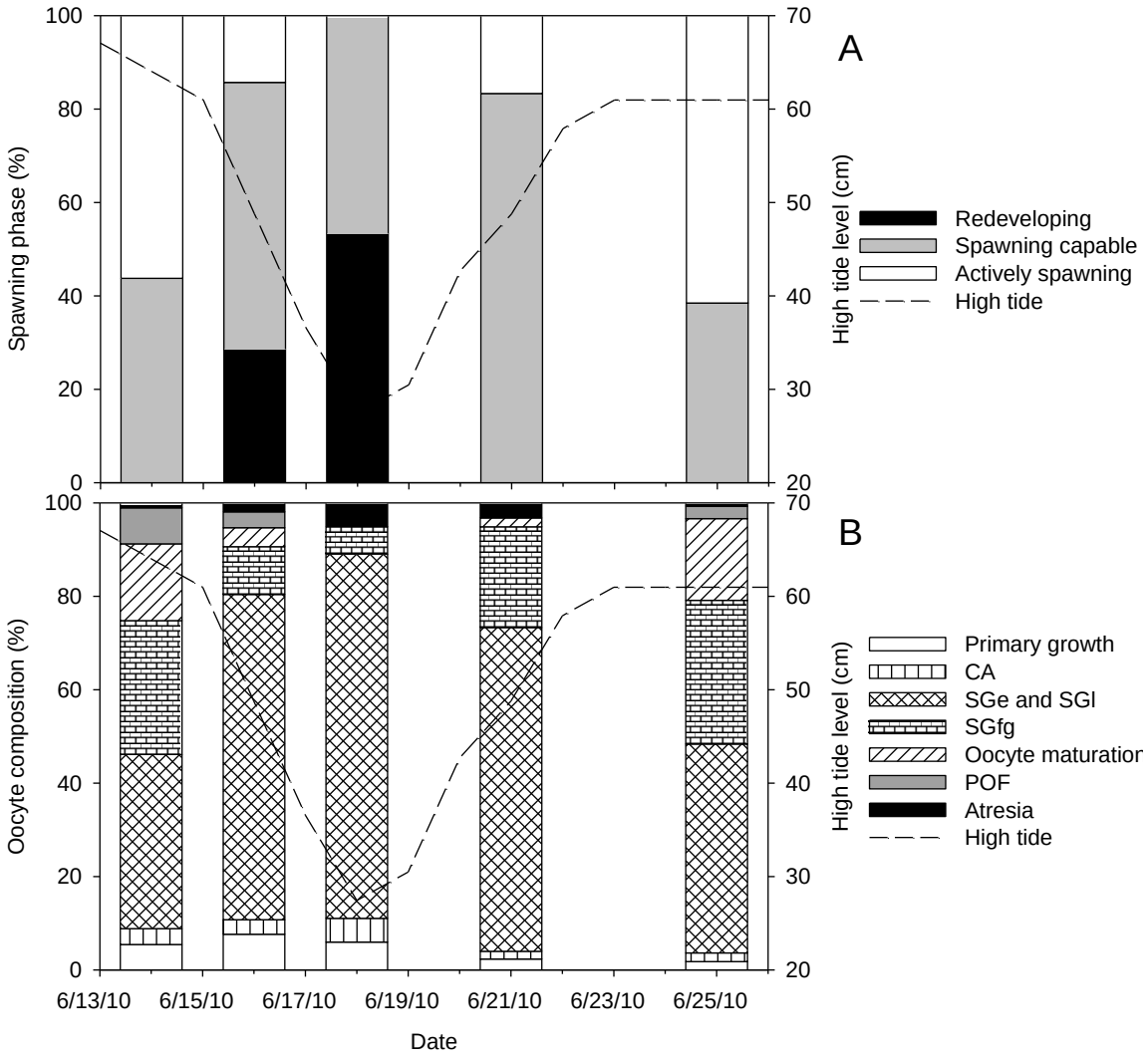

Fig. 5 Ovarian spawning phases (A) and oocyte stages (B) of *Fundulus jenkinsi* with high tide (cm) by date.

Observation of oocyte stages in the ovary mirrored the reproductive phases with OM present on the days after and before but never during the neap tide period (Fig.5B;

Table 4B). The ovaries of fish during neap tide consisted mainly of early secondary growth oocytes although some full grown, primary growth, and CA oocytes were also present. There was also a relatively high percentage of atretic oocytes during the neap tide period (Fig. 5B, Table 4B).

Table 4 *Fundulus jenkinsi* spawning phase (A) and oocyte stage (B) percentages by date used to determine spawning periodicity.

A

	Date of Capture				
Spawning Phase (%)	6/14/2010 (*n*=16)	6/16/2010 (*n*=7)	6/18/2010 (*n*=15)	6/21/2010 (*n*=6)	6/25/2010 (*n*=13)
Re-developing	0	29	53	0	0
Spawning Capable	44	57	47	83	38
Actively Spawning	56	14	0	17	62

B

	Date of Capture				
Oocyte Stage (%)	6/14/2010 (*n*=16)	6/16/2010 (*n*=7)	6/18/2010 (*n*=15)	6/21/2010 (*n*=6)	6/25/2010 (*n*=13)
Primary Growth	5	8	6	2	2
Cortical Alveoli	3	3	5	2	2
Early and Late Secondary Growth	37	70	78	69	45
Full Grown	29	10	6	22	31
Oocyte Maturation	16	4	0	2	17
Post-Ovulatory Follicles	8	3	0	0	3
Atresia	1	2	5	3	1

A closer look at POFs and OM shows no fish were captured during neap tide with OM or POF, which are indicative of imminent or recent spawning (Fig. 6). However, spawning did occur on and the three days following spring tide. The presence of OM but lack of POFs on 21 June 2010 indicates that spawning resumed on that date after the neap tide on 18 June 2010. This suggests that, within a population, spawning occurs several days before and after the spring tide, but ceases during the neap tide period.

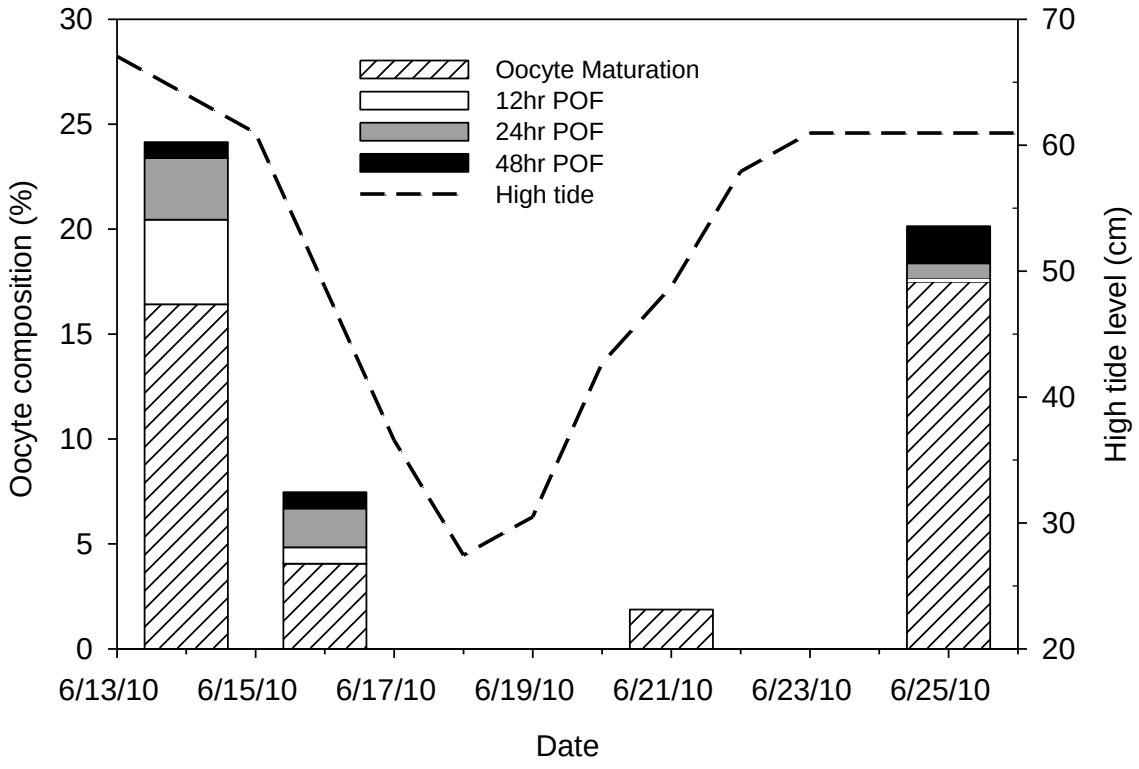

Fig. 6 Oocyte maturation and postovulatory follicles (POF) displayed with high tide (cm) by date.

Oocyte maturation (OM) in the same ovary as 12 hr and 48 hr POF suggest that one fish can spawn multiple times within the same tidal cycle (Fig. 7). There were numerous *F. jenkinsi* exhibiting this same pattern. Of the actively spawning fish collected, over 88% spawned either 24 or 48 hrs before they were collected in March through August, based on the presence of POF.

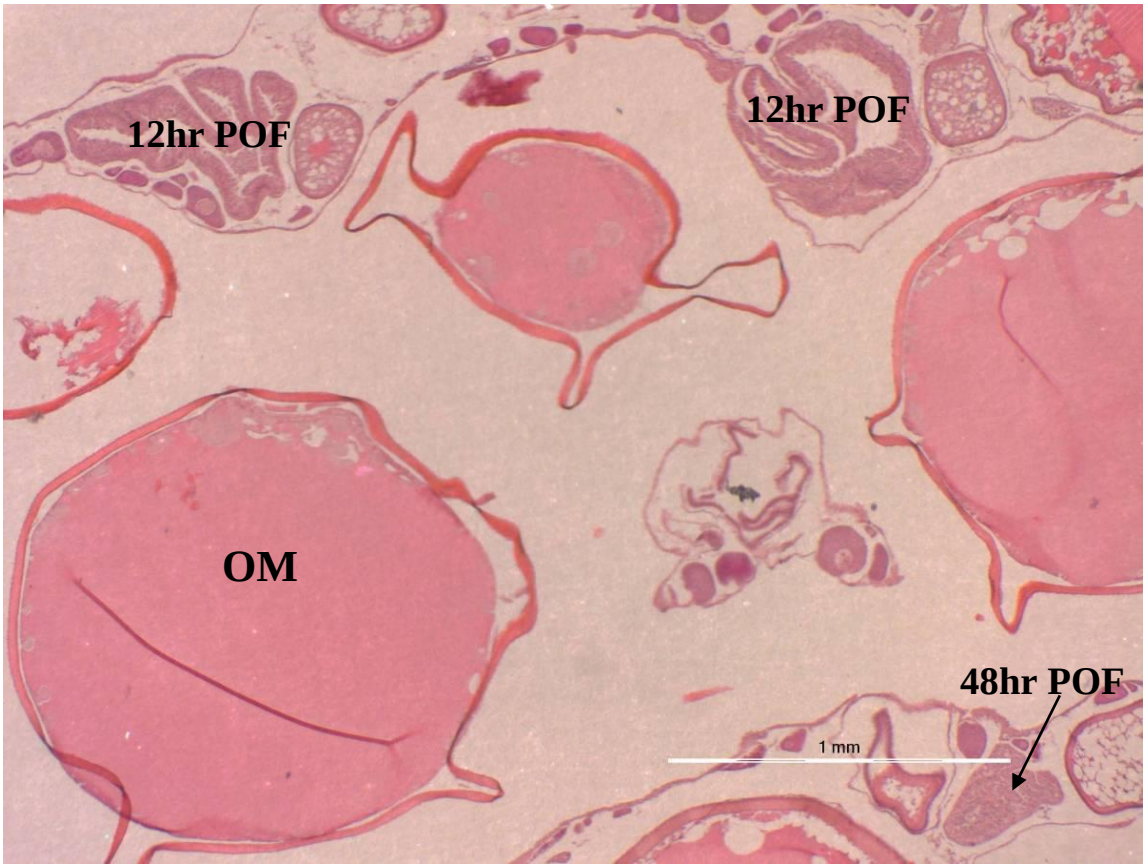

Fig. 7 Image of a *Fundulus jenkinsi* (52.3 mm TL) ovarian histological section showing various post ovulatory follicles (POF) along with oocyte maturation (OM) stages.

Discussion

Fundulus jenkinsi is a patchily distributed species throughout its range and, until recently, little data was available on its life history and ecology (Peterson et al. 2003; Lopez et al. 2010a,b), warranting its listing as a Species of Concern by numerous state and federal agencies (Gilbert and Relyea 1992; MMNS 2005; NOAA 2009). Information on all aspects of a species' life history allows for the development of a comprehensive conservation plan, especially important are details of the spawning season and ovarian dynamics.

Fundulus jenkinsi female GSI values reflected a definite spawning period from April through August, which generally coincides with other known fundulid spawning

seasons. For example, a spawning season of March through August was characterized by GSIs for *F. grandis* (Greeley and MacGregor 1983), whereas the spawning season of *F. similis* extends from mid-March through August (Greeley et al. 1986). *Fundulus jenkinsi* males exhibited spawning coloration before and after the spawning season and GSIs indicated males were spawning-capable in February, but females were not yet prepared for reproduction. Therefore, the beginning of the spawning season cannot completely be assumed by the appearance of male sexual coloration because females are not yet carrying eggs. Although the *F. jenkinsi* GSI dip in June was not significantly lower than the rest of the spawning season GSI values, other fundulids have exhibited more exaggerated bimodal spawning peaks (Kneib and Stiven 1978). Harrington (1959) suggested the mid-summer pause in spawning of *Fundulus confluentus* (marsh killifish) may be due to a high temperature threshold in the shallow, intertidal waters of Florida. Methods for determining spawning readiness other than GSI, such as quantifying oocyte diameters, have been used for freshwater (DuRant et al. 1979; Taylor and Burr 1997) and marine (Hastings and Yerger 1971; Byrne 1978; Kneib 1978) killifishes. In addition, reproductive histology is a more in-depth method of observing spawning condition and provides more detail on the timing of reproduction (Wallace and Selman 1981; Greeley et al. 1988).

The histological sections of *F. jenkinsi* ovaries show the large oocytes common in fundulids that are characteristic of demersal spawning fishes. The spawning phases reveal *F. jenkinsi* actively spawning in March, which would indicate an earlier start of the spawning season than the GSI values suggested. However, the end of the spawning season was consistent with both histological and GSI results. It has become common

knowledge that GSI cannot alone accurately determine spawning readiness, and reproductive histology provides direct biological information that confirms or refines the GSI results (Jons and Miranda 1997). The results of *F. jenkinsi* spawning from March through August conflict with the findings of Lopez et al. (2010b), who presented only data from oocyte diameters that suggested a spring and summer spawning period, but also suggested about 35% of *F. jenkinsi* collected in the winter were Spawning Capable. My data suggests there are no Spawning Capable female *F. jenkinsi* in the winter as 100% of the February ovaries were in the Developing phase and 100% of October ovaries were in the Regressing phase. The *F. jenkinsi* spawning season is consistent with all other recorded gulf coast fundulids except for *F. similis*, which starts in April (Gunter 1945; Hastings and Yerger 1971; Greeley and MacGregor 1983; Greeley et al. 1986). *Fundulus parvipinnis*, a west coast fundulid, spawns from April through September (Fritz 1975), and, on the east coast, *F. heteroclitus* and *F. luciae* spawn from March through August (Byrne 1978; Kneib and Stiven 1978; Kneib 1978). All estuarine fundulids seem to be spring to summer batch spawners that use high tides to deposit their eggs in a secure marsh environment.

Fundulus jenkinsi also spawns multiple times between two spring tides. Not only is OM displayed on numerous days between spring tides, but POFs are also indicative of spawning before those days. Extrapolating from the data, spawning is estimated to occur about nine of the 14 days present between spring tides. While multiple spawns do occur between spring tides, spawning intensity also seems to increase with tide height. For instance, when the tide was above 45 cm, the percent area of the ovary occupied by oocytes undergoing OM was above 15%, but this percentage decreased when the tide fell

below the 45 cm mark. Although lunar phase or declination has a key influence on tides, *F. jenkinsi* seem to use the tide height as a spawning indicator and spawned when the marsh vegetation was inundated. The ability of *F. jenkinsi* to spawn through a cluster of days is similar to *F. grandis* populations from Alabama (Greeley and MacGregor 1983; Greeley et al. 1988). However, *F. grandis* and *F. similis* spawning was reported to only occur on late ascending tides and spring tides (Greeley and MaccGregor 1983; Greeley et al. 1986), while *F. jenkinsi* also spawned on early descending tides. Despite the ability of *F. grandis* and *F. similis* to spawn on ascending high tide in addition to spring tides, Greeley et al. (1986) and Greeley et al. (1988) contended that both species spawned in sync with moon declination and spring tides more in the middle and late months of the spawning season and therefore stated that *F. grandis* and *F. similis* were semi-lunar spawners. In contrast, Waas and Strawn (1983) contended that there was a "lack of lunar effect" on the spawning of *F. grandis* in Galveston Bay, Texas and attributed the lack of lunar synchronization to wind overriding the lunar tides (p. 138). Petersen et al. (2010) showed a lack of semi-lunar spawning in *F. heteroclitus*, and attributed the behavior to the shorter spawning season due to the boreal climate of Maine. The semi-lunar spawning cycle exhibited by *F. heteroclitus* in Delaware indicates that *Fundulus* spawning may vary along the east coast (Taylor 1984; Taylor and DiMichele 1980). The wind-driven tides and small tidal differences of the nGOM coast lead to marsh inundation periods being of longer duration but aperiodic compared to the Atlantic coast, which may have resulted in an adaptation for spawning on consecutive days (Kneib 1997). Over time fundulids in the nGOM that use multiple reproductive cues, such as temperature, day length, and tide patterns, have probably been naturally selected for, and because certain

populations of *F. heteroclitus* live in areas where day length and moon phases coincide with optimal reproduction factors, they do not have to adapt (Greeley and MacGregor 1983; Waas and Strawn 1983). The unique ability of *F. jenkinsi* to spawn on descending tides in addition to the ascending and spring tides may be due to its shallow water depth preference compared to sympatric fundulids (Peterson et al. 2003; Lopez et al. 2010a).

Another interesting result from this study was that some female *F. jenkinsi* had no late secondary growth oocytes before and during neap tide. Because fish in this condition were present in the middle of the spawning season and fish cannot go backwards from the Spawning Capable to the Developing phase, this ovarian condition was characterized as a new sub-phase of Spawning Capable called Redeveloping. A similar situation was reported in *Sardina pilchardus* in which 39% of mature females exhibited this Redeveloping sub-phase in peak spawning months (personal comm., Konstantinos Ganias, Aristotle University of Thessaloniki, Greece).

The use of GSI to estimate spawning preparedness in fishes is a standard technique in fisheries science; however, detailed reproductive histology determines when a fish could spawn or has already spawned, which is valuable in terms of estimating reproductive habits at the individual level. The presence of multiple oocyte stages not only supports *F. jenkinsi* asynchronous oocyte development, but also the ability for a single *F. jenkinsi* individual to spawn over successive days. Other members of the family Fundulidae (*F. grandis* and *F. heteroclitus*) are known to spawn in a cluster of three to six days as a population (Greeley et al. 1988; Petersen et al. 2010), but only in *F. heteroclitus* has previous literature shown an individual capable of spawning daily (Shimizu, 1997). In contrast, the reproductive histology reported here indicates that *F.*

jenkinsi spawns over successive days in the wild, with ovarian histology showing OM along with 12, 24, and/or 48 hr POFs present. This pattern is repeated in more than 88% of the actively spawning fish and is present from April through August, suggesting this ovarian condition is common in *F. jenkinsi*. An actively spawning fish with POFs displays evidence of a cluster of spawning days not only through the population, but within an individual. In conclusion, *F. jenkinsi* individuals spawn multiple days in a row throughout the season, unlike *F. grandis*. With an individual *F. jenkinsi* able to spawn at least four days successively, it not only sheds new light on the reproductive strategy of the fish, but also adds information necessary for potential total fecundity and population sustainability estimates.

The quantification of the reproductive strategy of this rare fundulid provides additional data concerning the linkage of life history with critical salt marsh habitat. This data extends and enhances the data found in Lopez et al. (2010a,b), which collectively supports development of a detailed conservation plan for *F. jenkinsi* across the range of its distribution.

CHAPTER III

COMPARATIVE DEVELOPMENT

Introduction

The genus *Fundulus* comprises a large group of freshwater and saltwater killifishes (family Fundulidae); the saltwater group is a key component of an intertidal estuarine trophic web (Peterson and Peterson 1979; Kneib 1997). Intertidal estuarine habitats provide a number of services ranging from storm protection and pollution abatement to nursery habitat, protection from predators, and areas for foraging and spawning (Beck et al. 2001; Peterson 2003; Rountree and Able 2007). Fundulids are adapted to intertidal life with large eggs that produce motile, large larvae that emerge and survive in the dynamic intertidal environment (Greeley et al. 1988; Boehlert and Mundy 1988). Lopez et al. (2010b) recently quantified this habitat linkage in *Fundulus jenkinsi* (saltmarsh topminnow) in terms of feeding and, to a lesser degree, spawning. Anthropogenic habitat alteration along the banks of estuarine marsh complexes threatens access by a myriad of taxa (Peterson and Lowe 2009), thus preventing or reducing breeding, protection from predators, and vital nursery functions.

Fundulus jenkinsi is a northern Gulf of Mexico (nGOM) coastal fundulid that is recognized both federally (NOAA 2009), as well in a number of states (Gilbert and Relyea 1992; MMNS 2005), as a Species of Concern. It is generally believed that *F. jenkinsi* is rare, patchily distributed, found in low salinity, and uses *Spartina* marsh explicitly (Thompson 1980, 1999). However, it has been recently shown that this species is more abundant than previously thought, mainly in habitats where salinity < 16 (Peterson et al. 2003; Lopez et al. 2010a). *Fundulus jenkinsi* appears to behaviorally most

closely resemble *Fundulus luciae* (spotfin killifish) from the east coast, and both Byrne (1978) and Kneib (1978) have documented that this small intertidal fundulid uses high marsh more than the other east coast fundulid species. Ross (2001) stated that *F. jenkinsi* is one of the smallest nGOM coastal fundulids and suggests it swims with the tide higher into the marsh than other fundulids, much the same as *F. luciae* does.

Although few studies have been completed on *F. jenkinsi* life history, recent work on distribution and habitat use (Peterson et al. 2003; Lopez at al. 2010a), sexual dimorphism, trophic ecology, and reproduction (Lopez et al. 2010b) has produced a significant increase in understanding relative to its conservation. However, there is a need to accurately identify larvae and post-larvae of this patchy and rare fundulid as a number of museum specimens have been mis-identified (Thompson 1999). The first step in achieving that goal is to produce an early developmental key of fundulids in the regional nGOM species pool.

Most comparative keys focus on large juveniles and adults (Brown 1957; Relyea 1983; Ross 2001; Boschung and Mayden 2004), but earlier developmental stages of other members of the family Fundulidae have been documented on the east coast. For example, early stages of *Fundulus heteroclitus* (mummichog) and *Fundulus majalis* (longnose killifish) have already been diagnostically compared from specimens in the Quinnipiac River, Connecticut (Richards and McBean 1966). Fin counts, vertebral counts, branchiostegal ray counts, and body measurement ratios of *F. luciae* from North Carolina have also been documented throughout development (Byrne 1978). Descriptions of life history and comparative development have been collected within a key for each of the three east coast fundulids (Hardy 1978; Able and Fahay 1998). The development of

Adinia xenica (diamond killifish) and *Fundulus grandis* (gulf killifish) from Florida and Mississippi, respectively, have been described (Hastings and Yerger 1971; Koenig and Livingston 1976; Vivian 2005), but no detailed study compares larval and postlarval stages of common fundulids in the nGOM. There are six species along the nGOM that comprise Fundulidae; *A. xenica*, *F. grandis*, *Fundulus confluentus* (marsh killifish), *F. jenkinsi*, *Fundulus pulvereus* (bayou killifish), and *Fundulus similis* (striped killifish) (Brown 1957). *Fundulus confluentus* resides on the nGOM coast, but its western boundary is Big Lake in Gulf Shores, AL (Brown 1957; Ross 2001; Boschung and Mayden 2004), and developmental stage diagnostics of this species are available (Hardy 1978).

To aid in proper developmental-stage identification and in conservation of *F. jenkinsi*, a comparative key of common, co-occuring fundulids is required for species in the nGOM. Thus, the specific objective of this study was to

1. produce detailed drawings of larval and post-larval stages of *A. xenica*, *F. jenkinsi*, *F. grandis*, *F. similis*, and *F. pulvereus* up to 15mm TL based on the laboratory spawning of known adults, and
2. use these, plus meristics and morpometric measurements, to develop a dichotomous key.

Methods

Starting in the reproductive season of 2009, all native Mississippi estuarine *Fundulus* spp. and *A. xenica* were collected using standard minnow traps to extract eggs and sperm. Collection sites included Bayou Cumbest (Grand Bay NERR), Old Fort Bayou, and Tchoutacabouffa River in Mississippi. The only known parents that did not

come from the Mississippi coast were the *F. grandis*, which were the fourth generation of a brood stock originally housed in the Shoemaker Toxicology Laboratory at the Gulf Coast Research Laboratory but were initially collected from the Alabama coast. All other fundulid adults collected from the Mississippi coast were placed into heated buckets of seawater on a 12L:12D artificial light cycle with a salinity of 15 and a temperature of 30ºC to be stripped of gametes as soon as possible.

Fundulid eggs were stripped into 1500 ml Carolina culture dishes filled with seawater at a salinity of 15, then sperm from males was extruded into a 6 ml Carolina culture dish and the milt was added to the females' extracted eggs. If the males did not milt, their testis were removed and macerated in a 6 ml Carolina culture dish. If females did not produce a sufficient amount of viable eggs, 50-100 µl of human chorionic gonadotropin (HCG) (Sigma Aldrich), depending on the size of the fish, was injected into the adult fish for two days in order to induce a higher yield of eggs ready for fertilization. Gametes were mixed 24 hrs after the last injection of HCG. All fertilized eggs gestated in the culture dishes filled with seawater, which were held at a 12L:12D artificial light cycle. Immediately after hatching, the larvae were transferred to species-specific 19 L aquaria with the same artificial light cycle and were fed brine shrimp (*Artemia* spp.) (Sorgeloos et al. 1980). There was no filtration on the aquaria, but litter and feces in the tank were removed with a pipette every other day. The young fundulids were fixed in 10% formalin for 24 hrs then transferred to 70% ethanol for preservation. If available, at least three fish were preserved for each of four size classes: 0-6.99 mm (size class one), 7-9.99 mm (size class two), 10-11.99 mm (size class three), and 12-15 mm (size class four). Size class was determined by grouping the sizes together based on the least amount

of allometry prior to marked changes in body dimensions within a species. The fish were then photographed at various focal points with a Jenopik C5 digital camera attached to a Meji dissecting scope and the images were imported into Adobe Photoshop CS3 to achieve a uniformly focused picture through overlain images. The individuals < 15 mm TL were traced on a computer screen with Adobe CS3 on a 9 x 12 Wacom Intuos drawing tablet, and at least four specimens were illustrated for each species, one for each size class. Each size class of fish was described by meristics, pigment patterns, and morphometrics that were expected to differentiate the larvae. These included ratios of TL (mm) to body depth (BD, mm), TL to caudal peduncle width (CPW, mm), head length (HL, mm) to snout length (SNL, mm), HL to eye diameter (ED, mm) (Richards and McBean 1966), TL to HL, and dorsal fin, anal fin, and branchiostegal ray counts (Hubbs and Lagler 1958). All body morphometrics ($\overline{x} \pm 1$ standard deviation [stdev]) and meristic counts were tabulated by size class.

An ANCOVA was run on each body morphometric with either TL or HL as the covariate and species as the fixed factor. Data were tested for normality with the Kolmogorov-Smirnov 1-sample test and variances were tested for homogeneity with Levene's statistic. If the ANCOVA displayed an interaction between the fixed factor and the covariate (non-homogeneous slopes), then the covariate was separated into a low value ($= \overline{x}$ - 1 stdev), a mean value ($\overline{x}$), and a high value ($= \overline{x}$ + 1 stdev) for an ANOVA and post-hoc Sidak test adjusted for the covariate (Green and Salkind 2008). If there was no interaction, then the original marginal adjusted means were compared using an ANOVA and a Sidak post-hoc test. All statistics were processed using SPSS (ver. 15) and all p-values ≤ 0.05 were considered significant.

Results and Discussion

The broodstock collected for every species consisted of at least five males and five females that ranged in size from 30 to 100 mm TL. The smallest larvae were less than 6 hrs old whereas the largest postlarvae were no older than 14 days. The largest newly hatched larva on average was *F. similis*, whereas the smallest was *F. pulvereus*. In an attempt to eliminate lab bias in the morphometric and meristic measurements, certain methods in raising the developing fundulids to eliminate stunted growth and malnourishment were used. For example, no more than 30 fish were raised in a 19 L aquaria, and temperature was kept at a steady 30ºC (Moser et al. 1984; Blaxter 1988). The small fish were also fed *Artemia* sp. to insure they received the exogenous enzymes needed to aid in digestion and normal growth (Sorgeloos et al. 1980; Blaxter 1988). Unfortunately, shrinkage due to fixation and preservation could not be completely avoided. However, Blaxter (1988) stated that a thinner more fragile fish may be more susceptible to shrink in the preservation process, whereas fundulids tend to be hearty when they hatch because of their breeding strategy. Shrinkage is thought to be about 5-10% in younger fish and around 2% as they grow larger (Blaxter 1988). The fundulids would probably shrink less than many other species because larvae are large and well developed at hatch.

Meristics and Body Morphometrics

All morphometric data were found to be normally distributed ($p > 0.05$) and variances were homogeneous ($p > 0.05$). The only two morphometrics found to have a significant ANCOVA interaction term were BD ($F_{4, 83} = 3.466$, $p = 0.011$) and CPW ($F_{4, 83} = 7.993$, $p < 0.0001$) adjusted for TL among species. The remaining morphometrics

adjusted for TL or HL did not show significant interaction terms ($p > 0.05$). Head length adjusted for TL was significantly different ($F_{4, 83} = 11.346$, $p < 0.0001$) among species (Fig. 8). Adjusted HL was significantly smaller in *F. grandis* (Sidak, $p < 0.05$) compared to *F. jenkinsi*, *F. pulvereus*, and *F. similis*, whereas the adjusted HL of *A. xenica* did not differ (Sidak, $p > 0.05$) from any species (Fig. 8). *Fundulus jenkinsi* had a HL similar to *F. similis* and *F. pulvereus*, but the HL of *F. pulvereus* was greater than *F. similis* (Sidak, $p < 0.05$).

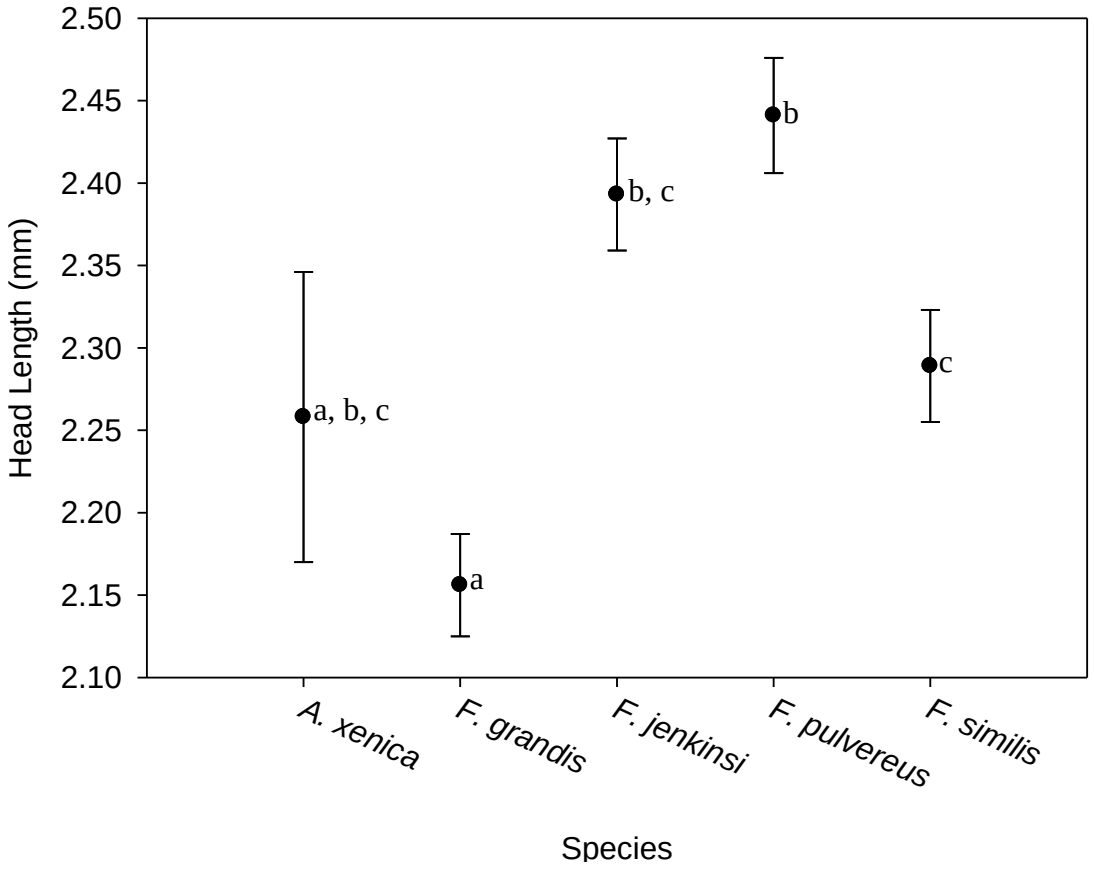

Fig. 8 Plot of head length (mm; $\bar{x} \pm 1$ SE) adjusted for total length (mm) by species. Data points are significantly different ($p \leq 0.05$) if letter labels are not the same, and and not significantly defferent ($p > 0.05$) if the letter labels are the same.

Body depth and CPW differed significantly among species ($p < 0.05$). However, because CPW ($F_{4, 83} = 7.993$, $p < 0.0001$) and BD ($F_{4, 83} = 3.466$, $p = 0.011$) displayed a significant interaction term, TL (the covariate) had to be split into low, mean, and high values for the ANOVA and pairwise comparisons (Green and Salkind 2008). None of the adjusted BD for TL_{low} (6.81 mm) values were significantly different among species ($F_{4,83} = 1.851$, $p = 0.127$), but the adjusted BD for TL_{mean} (9.91 mm) values ($F_{4,83} = 11.920$, $p < 0.0001$) and adjusted BD for TL_{high} (13.01mm) values ($F_{4,83} = 13.046$, $p < 0.0001$) were different among species (Fig. 9A). For the TL_{mean} and TL_{high} values, BD in *A. xenica* and *F. pulvereus* were not different (Sidak, $p > 0.05$), but both were significantly larger than *F. grandis*, *F. jenkinsi*, and *F. similis*, which were also not different from each other (Sidak, $p > 0.05$) (Fig. 9A).

Caudal peduncle width adjusted for the TL_{low} ($F_{4,83} = 10.543$, $p < 0.0001$), TL_{mean} ($F_{4,83} = 31.921$, $p < 0.0001$) and TL_{high} ($F_{4,83} = 28.611$, $p < 0.0001$) values were each significantly different among species (Fig. 9B). For CPW means adjusted to the TL_{low} value, the similar *A. xenica* and *F. pulvereus* CPW values were both significantly larger (Sidak, $p > 0.05$) than *F. grandis* and *F. similis*, which were also not different from each other (Sidak, $p > 0.05$). In addition, while *F. jenkinsi* had a larger CPW than *F. grandis*, its CPW was not different than the other fundulids. All CPWs adjusted for the TL_{mean} were significantly different among species (Sidak, $p < 0.05$). However, when CPW was adjusted for the TL_{high} value *F. jenkinsi* was not different (Sidak, $p > 0.05$) than *F. grandis* and *F. pulvereus*, but all other species comparisons were significantly different ($p < 0.05$).

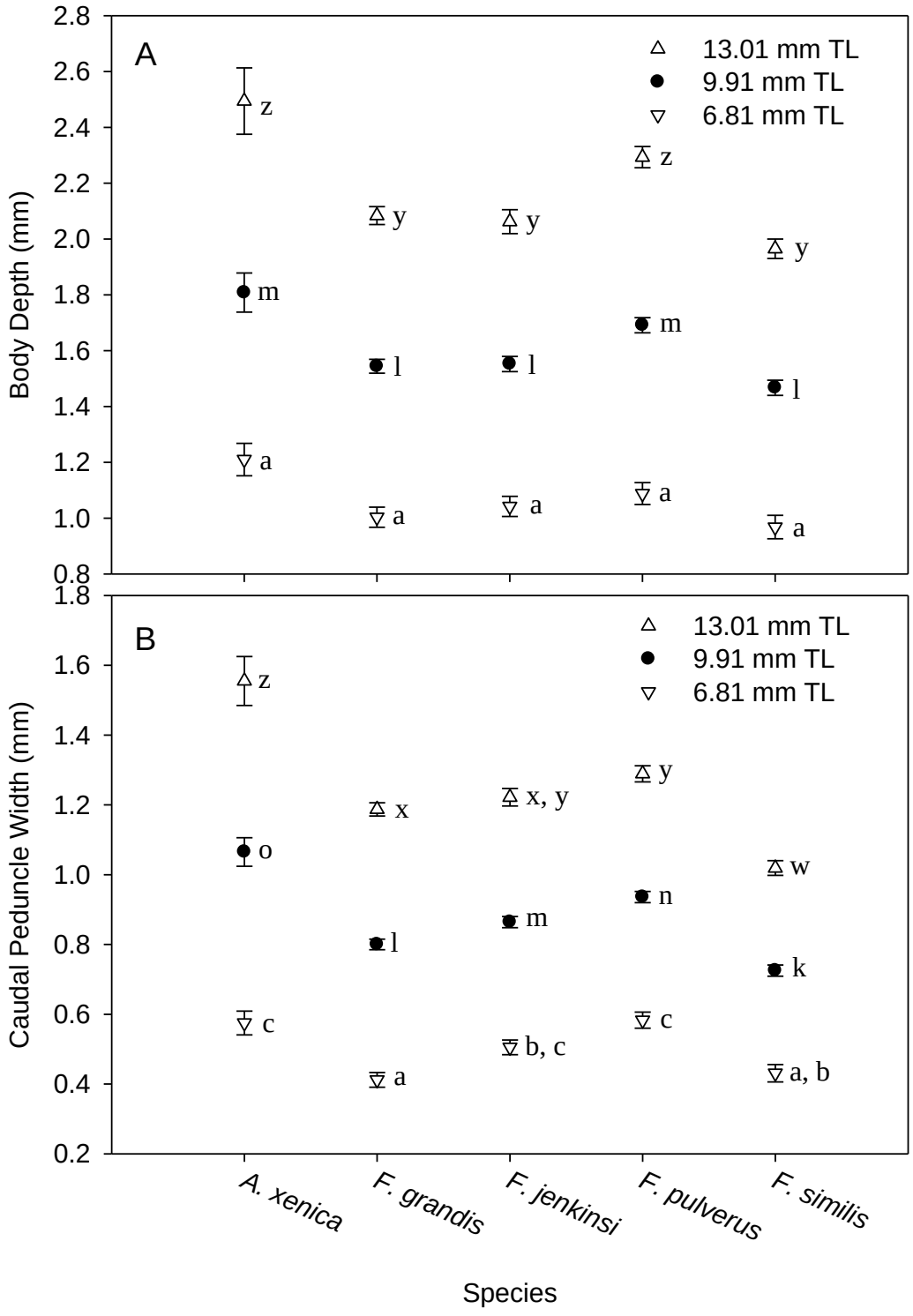

Fig. 9 Plot of body depth (mm, A) and caudal peduncle width (mm, B) ($\bar{x} \pm 1$ SE) adjusted for a low, mean, and high value of total length (TL, mm) by species. Data points within the TL_{low}, TL_{mean}, or TL_{high} values are significantly different ($p \leq 0.05$) if letter labels are not the same, and not significantly different ($p > 0.05$) if the letter labels are the same.

Neither SNL ($F_{4, 88} = 0.648$, $p = 0.630$) nor ED ($F_{4, 88} = 1.917$, $p = 0.115$) adjusted for HL exhibited an interaction term, and thus a direct comparison could be made. Both SNL ($F_{4, 88} = 3.466$, $p = 0.011$) and ED ($F_{4, 88} = 3.128$, $p = 0.019$) were significantly different among species. The SNL of *F. similis* was significantly larger (Sidak, $p < 0.05$) than *F. jenkinsi*, but no other SNL comparisons were different (Fig. 10A). The HL-adjusted ED of *F. similis* was significantly smaller than *F. grandis* (Sidak, $p < 0.05$), whereas all other ED comparisons were not different (Sidak, $p > 0.05$) (Fig. 10B).

The most noticeable character of fundulid larvae is their large size at hatch in comparison to some of the pelagic spawners in the salt marshes of the northern nGOM (Thresher 1984). It is common for demersal spawning fishes to have much larger hatchlings than pelagic spawners (Thresher 1984). The large fundulid larvae come from large eggs (~2 mm) that require copious amounts of energy from the female, and because the female invests so much energy into producing large, well-developed offspring, their survival rate is relatively high (Taylor and DiMichele 1980; Greeley et al. 1988; Berg and Finstad 2008). The hatching of fundulid larvae in a post-flexion state is a reflection of their opportunistic reproductive strategy (Blaxter 1988; Winemiller and Rose 1992). Morphometric and meristic differences in body shape and fin ray counts allowed for diagnostics that were easily discernable. Head scale patterns have been proposed as a possible characteristic that may distinguish some fundulids (Cooke 1965; Wiley and Hall 1975), but the lack of scalation or difficulty seeing the scales in early development led to only using pigment patterns on the dorsal side of the fish for identification.

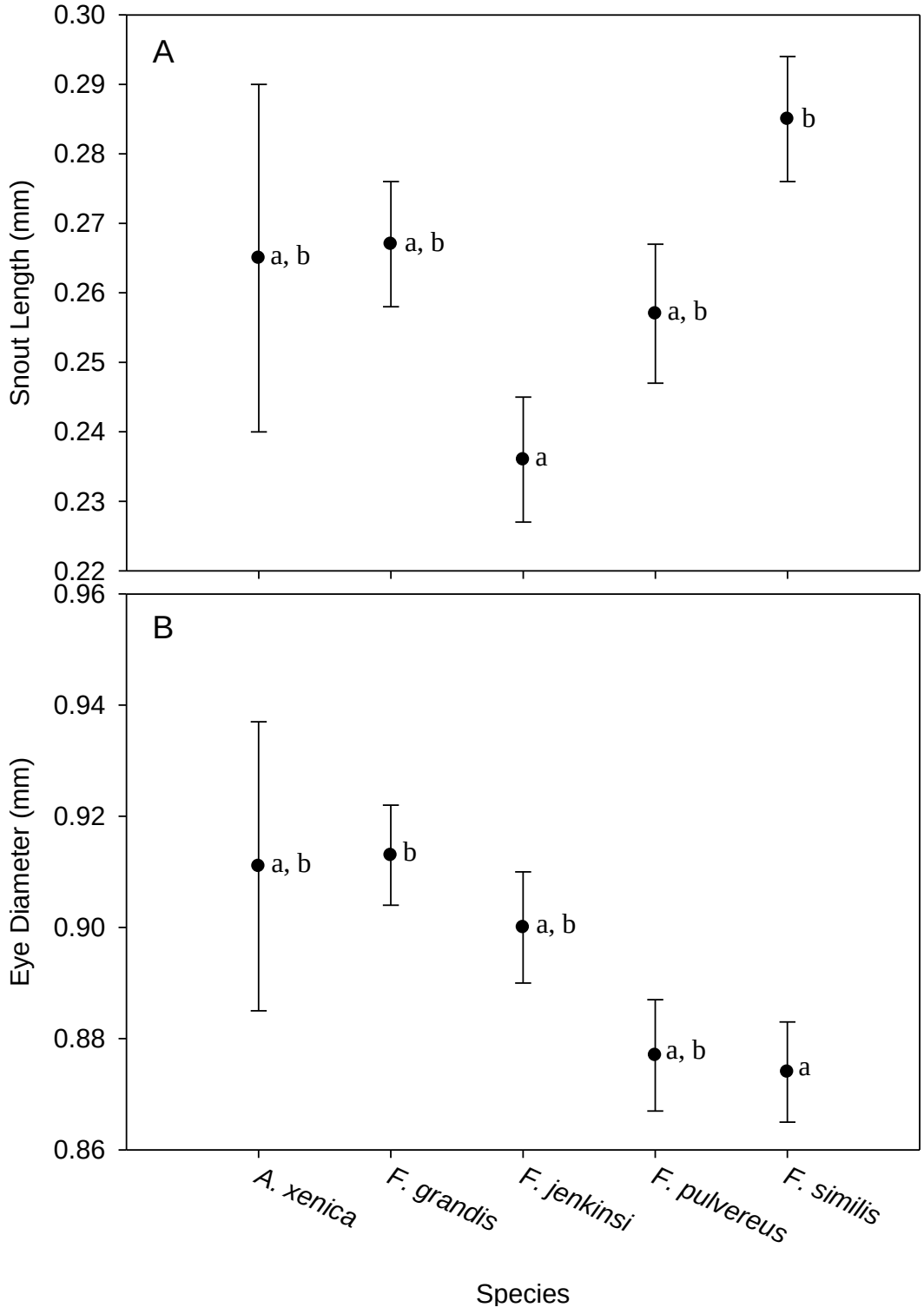

Fig. 10 Plot of snout length (mm, A) and eye diameter (mm, B) ($\bar{x} \pm 1$ SE) adjusted for head length by species. Data points are significantly different ($p \leq 0.05$) if letter labels are not the same, and not significantly different ($p > 0.05$) if the letter labels are the same.

Although species level identification is difficult in the first size class, branchiostegal ray counts can be used to separate the five fundulid species into two groups, but branchiostegal rays were extremely hard to see without being stained. The five species of fundulids can be split into two distinct groups that possess either five (*A. xenica*, *F. grandis*, and *F. pulvereus*) or six (*F. jenkinsi* and *F. similis*) branchiostegal rays (Table 5). Richards and McBean (1966) displayed the same difference in branchiostegals between *F. heteroclitus* and *F. majalis* on the east coast. *Fundulus heteroclitus*, which resembles *F. grandis*, had five branchiostegal rays, whereas *F. majalis*, which resembles *F. similis*, had six branchiostegal rays (Richards and McBean 1966; Hardy 1978). In contrast, most of the anal and dorsal ray counts overlapped between species and size classes, but in size class three and four *F. jenkinsi* had a smaller dorsal fin ray count compared to all species examined. In the second size class, *F. grandis* and *F. similis* had either 0 or 7-9 anal or dorsal rays, but in a very small window of growth could have counts in between 0 and 7. Only two *F. grandis* and *F. similis* combined had less than 7 dorsal or anal rays in size class two. The lack of fully developed anal and dorsal rays until 8.5 mm TL in *F. similis* and *F. grandis* as opposed to *A. xenica*, *F. jenkinsi*, and *F. pulvereus* forming these fin elements at 7 mm TL, reflects that *F. grandis* and *F. similis* are at least 1 mm larger as hatchlings than *F. jenkinsi*, *F. pulvereus*, and *A. xenica*. In some cases, the large size at hatch may hinder survival. Evolutionarily, larger hatchlings may be more motile, but they would also be more visible to avian or marine predators (Koenig and Livingston 1976; Hardy 1978; DeMartini 1999).

From 7 to 9.99 mm TL, morphometrics become more diagnostically useful. *Adinia xenica* and *F. pulvereus* are characterized by having a relatively deep body and wide caudal peduncle compared their TL, but *F. grandis* was slightly slimmer compared to its TL.

Table 5 Descriptive statistics of morphometric and meristic measurement ($\bar{x}$ ± 1 standard deviation) of fundulid species by size class

Species	TL (mm)	TL/HL	TL/BD	TL/CPW	HL (mm)	HL/SNL	HL/ED	Dorsal Fin Rays	Anal Fin Rays	Branchiostegal Rays
Size Class 1 (0-6.99 mm)										
Adinia xenica (*n* = 2)	4.97 ± 0.41	4.59 ± 0.47	6.97 ± 1.00	16.08 ± 1.65	1.09 ± 0.20	11.99 ± 0.39	2.08 ± 0.07	0	0	5
Fundulus grandis (*n* = 4)	6.16 ± 0.76	4.70 ± 0.57	5.99 ± 0.69	16.40 ± 1.12	1.33 ± 0.26	9.35 ± 1.35	2.27 ± 0.16	0	0	5
Fundulus jenkinsi (*n* = 7)	6.10 ± 0.80	4.19 ± 0.29	6.71 ± 0.66	15.32 ± 3.29	1.47 ± 0.29	11.97 ± 1.49	2.34 ± 0.23	0	0	6
Fundulus pulvereus (*n* = 5)	5.91 ± 0.74	4.18 ± 0.14	6.69 ± 0.83	13.15 ± 3.50	1.42 ± 0.20	10.06 ± 1.79	2.31 ± 0.06	0	0	5
Fundulus similis (*n* = 3)	6.46 ± 0.55	4.67 ± 0.60	7.05 ± 1.52	15.30 ± 1.61	1.40 ± 0.22	9.76 ± 3.58	2.31 ± 0.30	0	0	6
Size Class 2 (7-9.99 mm)										
Adinia xenica (*n* = 2)	8.58 ± 1.85	4.56 ± 0.47	5.83 ± 0.18	11.17 ± 0.93	1.88 ± 0.35	10.81 ± 1.63	2.26 ± 0.07	8	8	5
Fundulus grandis (*n* = 9)	8.51 ± 0.92	4.63 ± 0.57	6.99 ± 0.77	14.26 ± 1.03	1.83 ± 0.14	10.64 ± 3.94	2.45 ± 0.08	0-7	0-7	5
Fundulus jenkinsi (*n* = 5)	8.98 ± 1.16	4.17 ± 0.17	6.29 ± 0.62	12.05 ± 1.54	2.15 ± 0.24	10.77 ± 1.17	2.46 ± 0.09	6-7	0	6
Fundulus pulvereus (*n* = 5)	8.49 ± 1.13	3.62 ± 0.30	5.83 ± 0.45	11.20 ± 1.28	2.03 ± 0.22	9.27 ± 2.54	2.33 ± 0.21	7-8	7	5
Fundulus similis (*n* = 7)	8.92 ± 1.21	4.38 ± 0.33	6.90 ± 0.49	14.44 ± 1.08	2.05 ± 0.38	8.33 ± 1.58	2.55 ± 0.22	0-9	0-8	6
Size Class 3 (10-11.99 mm)										
Adinia xenica (*n* = 1)	11.81	4.22	5.2	8.07	2.8	7.78	2.78	8	10	5
Fundulus grandis (*n* = 6)	11.01 ± 0.64	4.82 ± 0.08	6.54 ± 0.14	12.08 ± 0.87	2.28 ± 0.12	8.74 ± 0.89	2.47 ± 0.11	9-11	9-11	5
Fundulus jenkinsi (*n* = 4)	11.48 ± 0.63	4.20 ± 0.22	6.30 ± 0.35	10.82 ± 0.39	2.74 ± 0.17	9.98 ± 1.59	2.57 ± 0.16	7-8	11	6
Fundulus pulvereus (*n* = 4)	10.84 ± 0.65	4.07 ± 0.02	5.86 ± 0.30	10.37 ± 0.59	2.66 ± 0.15	9.17 ± 1.14	2.74 ± 0.02	10	10	5
Fundulus similis (*n* = 6)	10.61 ± 0.51	4.44 ± 0.36	6.37 ± 0.39	14.34 ± 0.87	1.64 ± 0.24	6.88 ± 0.34	2.30 ± 0.15	8-10	8-10	6
Size Class 4 (> 12 mm)										
Fundulus grandis (*n* = 6)	14.85 ± 1.95	4.42 ± 0.41	6.06 ± 0.39	10.41 ± 0.69	3.42 ± 0.77	8.01 ± 1.24	2.66 ± 0.24	11	10-11	5
Fundulus jenkinsi (*n* = 5)	12.94 ± 1.27	4.09 ± 0.18	6.47 ± 0.21	10.87 ± 0.50	3.16 ± 0.29	8.57 ± 0.91	2.77 ± 0.09	7-8	10-11	6
Fundulus pulvereus (*n* = 6)	13.92 ± 0.90	4.14 ± 0.21	5.64 ± 0.29	9.96 ± 0.21	3.37 ± 0.34	8.25 ± 0.43	2.87 ± 0.07	11	10	5
Fundulus similis (*n* = 6)	13.99 ± 2.43	4.25 ± 0.24	6.44 ± 0.50	12.33 ± 0.91	3.29 ± 0.48	7.89 ± 1.09	2.87 ± 0.14	9-12	8-10	6

Adinia xenica differed from *F. grandis* and *F. pulvereus* in being the only fundulid with five branchiostegal rays to have a dorsal fin origin far anterior to its anal fin origin. When *F. grandis* grew above 12 mm TL, its BD became much larger in relation to its TL, but all other morphometric differences listed above also apply for individuals > 10 mm TL. Richards and McBean (1966) found that *F. heteroclitus* exhibited a deeper body even at a smaller size (< 10 mm TL), but the drastic allometry in BD of *F. grandis* may indicate a need for smaller individuals to occupy shallower water. The fundulids with six branchiostegal rays (*F. jenkinsi* and *F. similis*) are mainly distinguished by SNL and CPW. The large SNL of *F. similis* is not only a key diagnostic character of adult forms (Ross 2001; Boschung and Mayden 2004), but also differentiates it from *F. jenkinsi* in larval to post-larval stages. *Fundulus jenkinsi* CPW also is larger than that of *F. similis* in fish 7 to 9.99 mm TL, whereas all other morphometric measurements seemed to not sufficiently separate the two species.

Illustrations and Pigment

A residual yolk sac was present on the newly hatched larvae in some of the fundulid species illustrated. The size or presence of the residual yolk sac may depend on how long it takes for the egg to hatch; if the fundulid develops in the egg fourteen days or longer there will be little to no residual yolk, but some eggs of the larvae illustrated or measured hatched as early as ten days after fertilization and had a large yolk sac. A large residual yolk sac may be due to incubation in water rather than air but most likely due to the lack deferred hatching that coastal fundulids are known for in the wild (Harrington 1958; Koenig and Livingston 1976; Taylor et al. 1977; Waas and Strawn 1983; Greeley et al. 1988).

Adinia xenica is relatively long and slender as a hatchling compared to its deep-bodied adult form (Fig. 11). In the first two size classes, the belly and operculum are highly pigmented, and both have a strong mid-lateral line of pigment that extends from the base of the pectoral fin to the caudal peduncle. On each side of the external mid-lateral pigment there is internal pigment bordering the spine that is not visible to the naked eye in fish > 10 mm TL due to the amount of external pigment covering it. Although the dorsum of *A. xenica* is highly pigmented, it is diffuse and absent of a pattern (Fig. 11).

Fundulus grandis is sparsely pigmented (Fig. 12), with small melanophores scattered on the head, operculum, mid-lateral body, and both dorsal and ventral surfaces of the fish. Internal pigment bordering the spine is visible in all stages of *F. grandis* < 15 mm TL. Most newly hatched larvae have a residual yolk sac that is present until ~6 mm, and dorsal or anal fin rays are not visible until > 8.5 mm TL.

Pigment in *F. jenkinsi* hatchlings is sparse, but specimens > 6 mm TL are moderately pigmented (Fig. 13). The 4.2 mm TL illustrated specimen possessed a yolk sac that would have been absorbed by about 5.5 mm TL, but not every newly hatched *F. jenkinsi* had residual yolk, as the presence of a yolk sac depends on length of gestation. The dorsal pigment of *F. jenkinsi* from the head to the caudal peduncle is relatively concentrated, whereas the interior pigment bordering the spine was visible through all stages < 15 mm TL. In addition, pigment formed an X or sideways hourglass between the anterior half of the eyes on *F. jenkinsi* specimens ranging from hatch to 9.99 mm TL.

The hypaxial muscle of *F. pulvereus* smaller than about 7 mm TL is heavily pigmented internally from the vertebrae to the ventral edge of the fish (Fig. 14). This

pigment becomes less visible when fish reach 6.5 to 7 mm TL, but a strong blanket of external pigment begins to cover the fish as it grows. So much pigment is present on *F. pulvereus* that there is barely a discernable dorsal pattern.

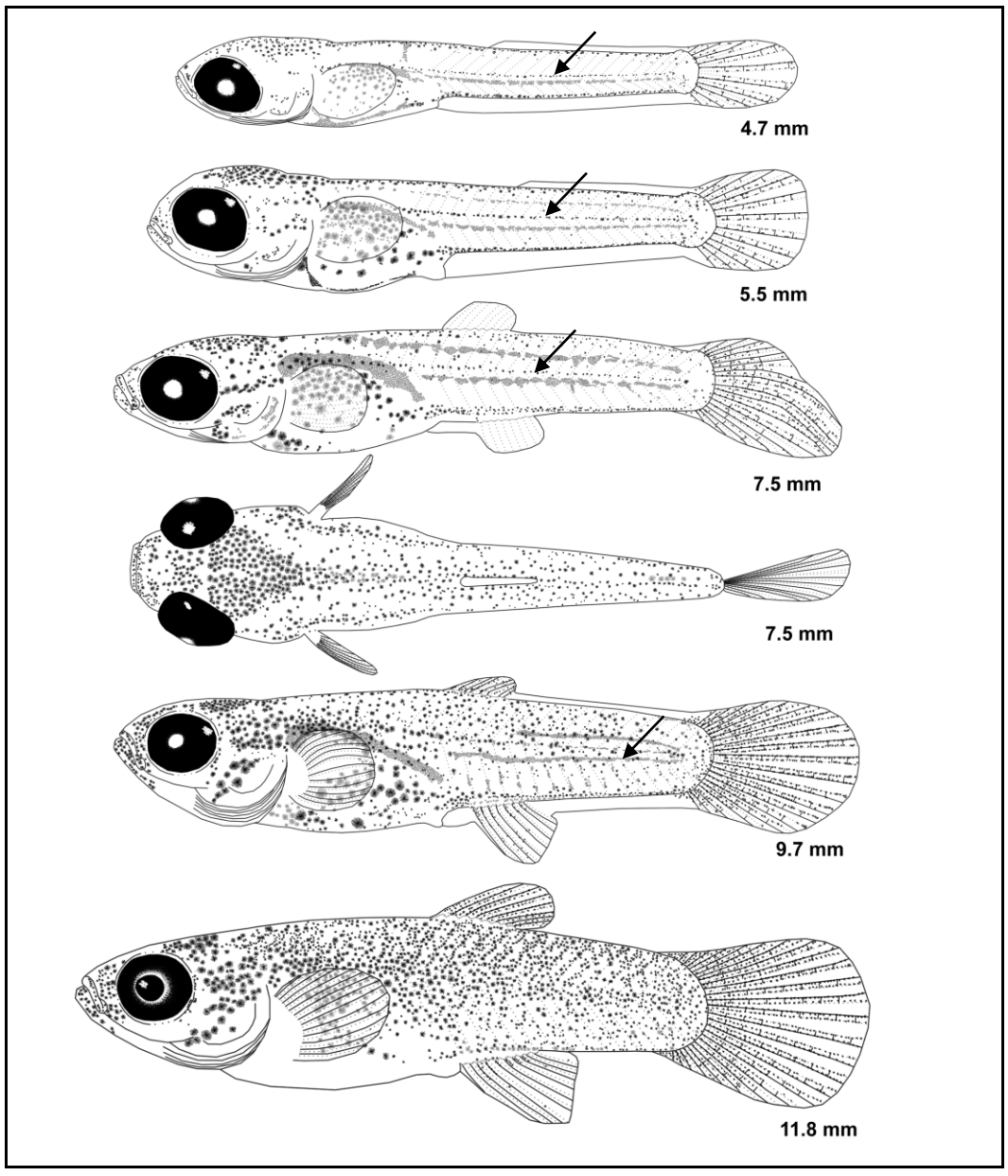

Fig. 11 Various stages (TL) of *Adinia xenica* development. Arrows note mid-lateral pigment and largest size where internal pigment around spine is visible. Size class one (0-6.99 mm TL), size class two (7-9.99 mm), size class three (10-11.99 mm), and size class four (12-15 mm).

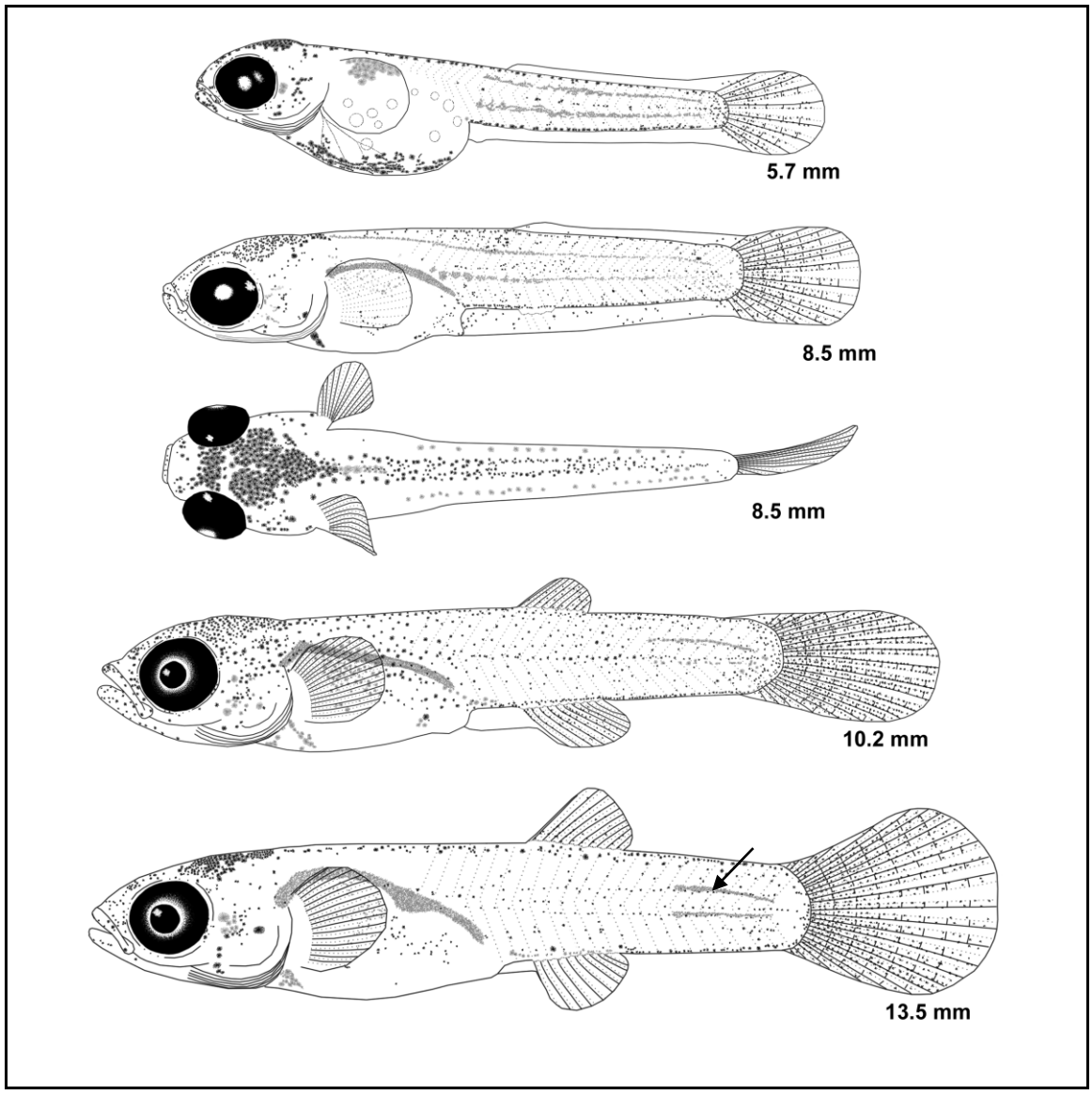

Fig. 12 Various stages (TL) of *Fundulus grandis* development. Arrow indicates largest size where internal pigment around spine is visible. Size class one (0-6.99 mm TL), size class two (7-9.99 mm), size class three (10-11.99 mm), and size class four (12-15 mm).

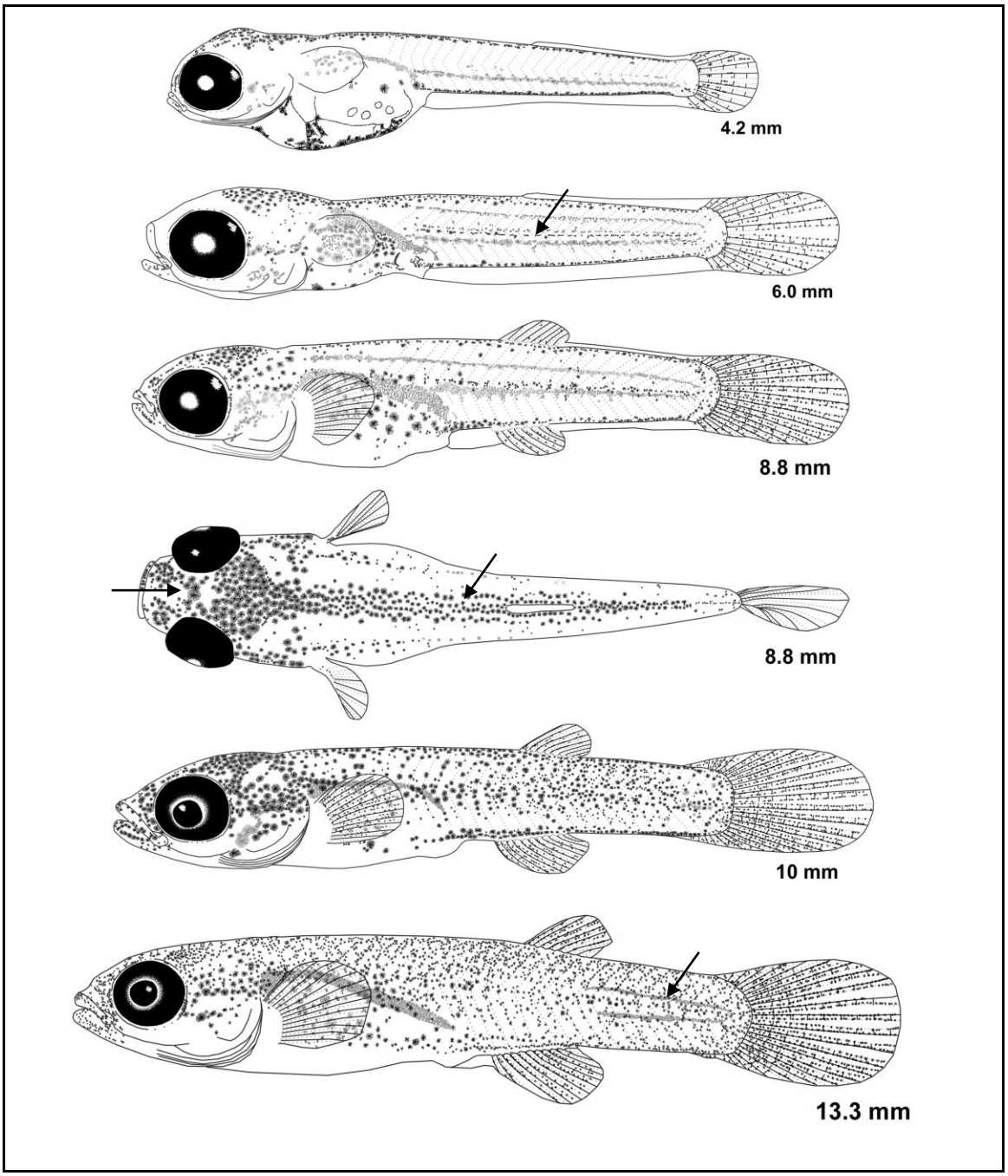

Fig. 13 Various stages (TL) of *Fundulus jenkinsi* development. Arrows indicate cluster of pigment from head to caudal peduncle, pigment forming sideways X or hourglass, and largest size where internal pigment around spine is visible. Size class one (0-6.99 mm TL), size class two (7-9.99 mm), size class three (10-11.99 mm), and size class four (12-15 mm).

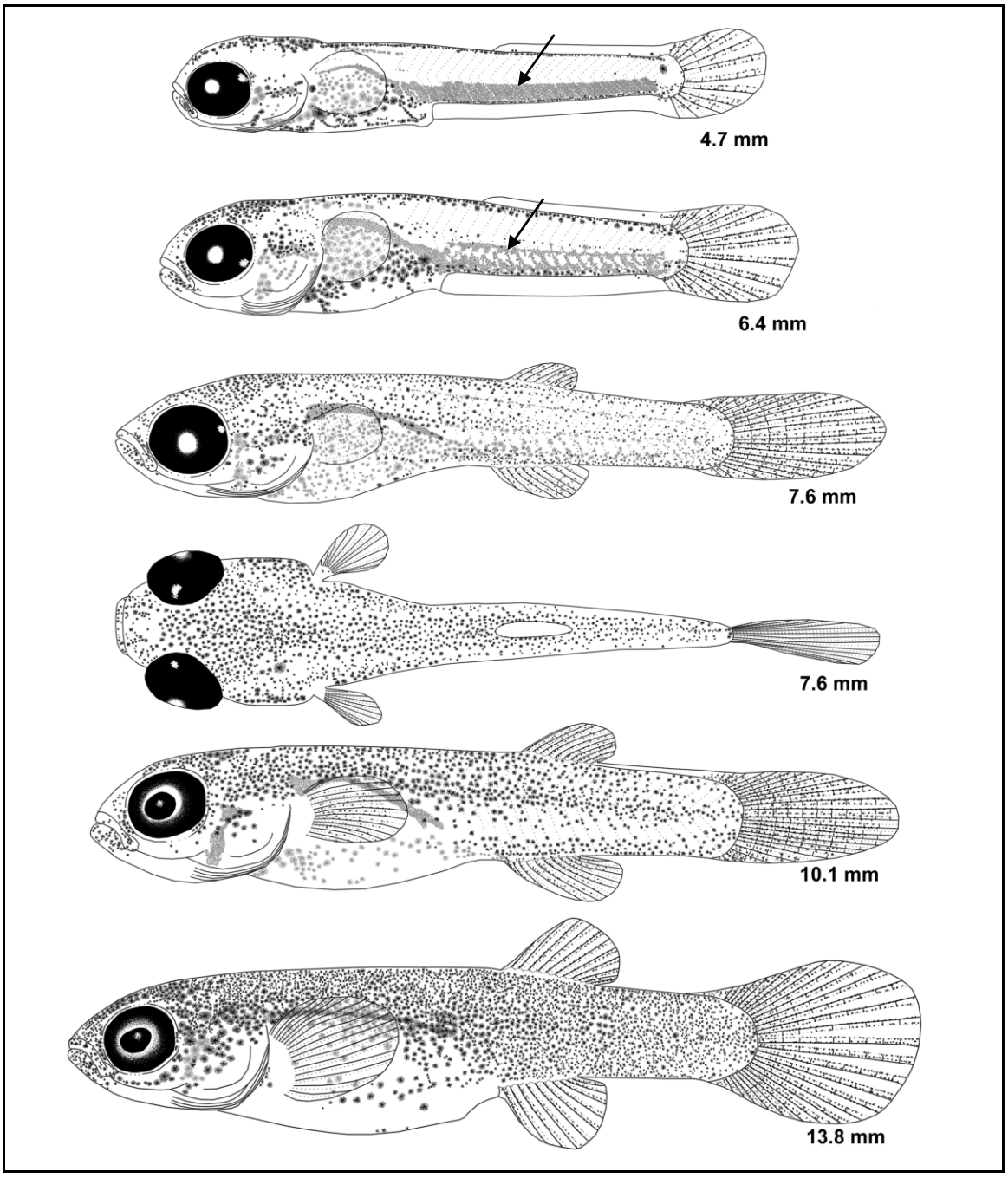

Fig. 14 Various stages (TL) of *Fundulus pulvereus* development. Arrows note interior hypaxial muscle pigment. Size class one (0-6.99 mm TL), size class two (7-9.99 mm), size class three (10-11.99 mm), and size class four (12-15 mm).

Although the illustrated 6.7 mm TL specimen of a newly-hatched *F. similis* possessed a yolk sac, it had been absorbed in many newly-hatched larvae and was never found in fish larger than 7 mm TL (Fig.15). Melanophores of *F. similis* were relatively

stellate and large in diameter, and the dorsal pigment was distinct with three or five rows of large melanophores extending from the head to the origin of the dorsal fin. The vertical bars that are characteristic of adult *F. similis* start to form with internal pigment at 10 mm TL and become external bars by 12 mm TL.

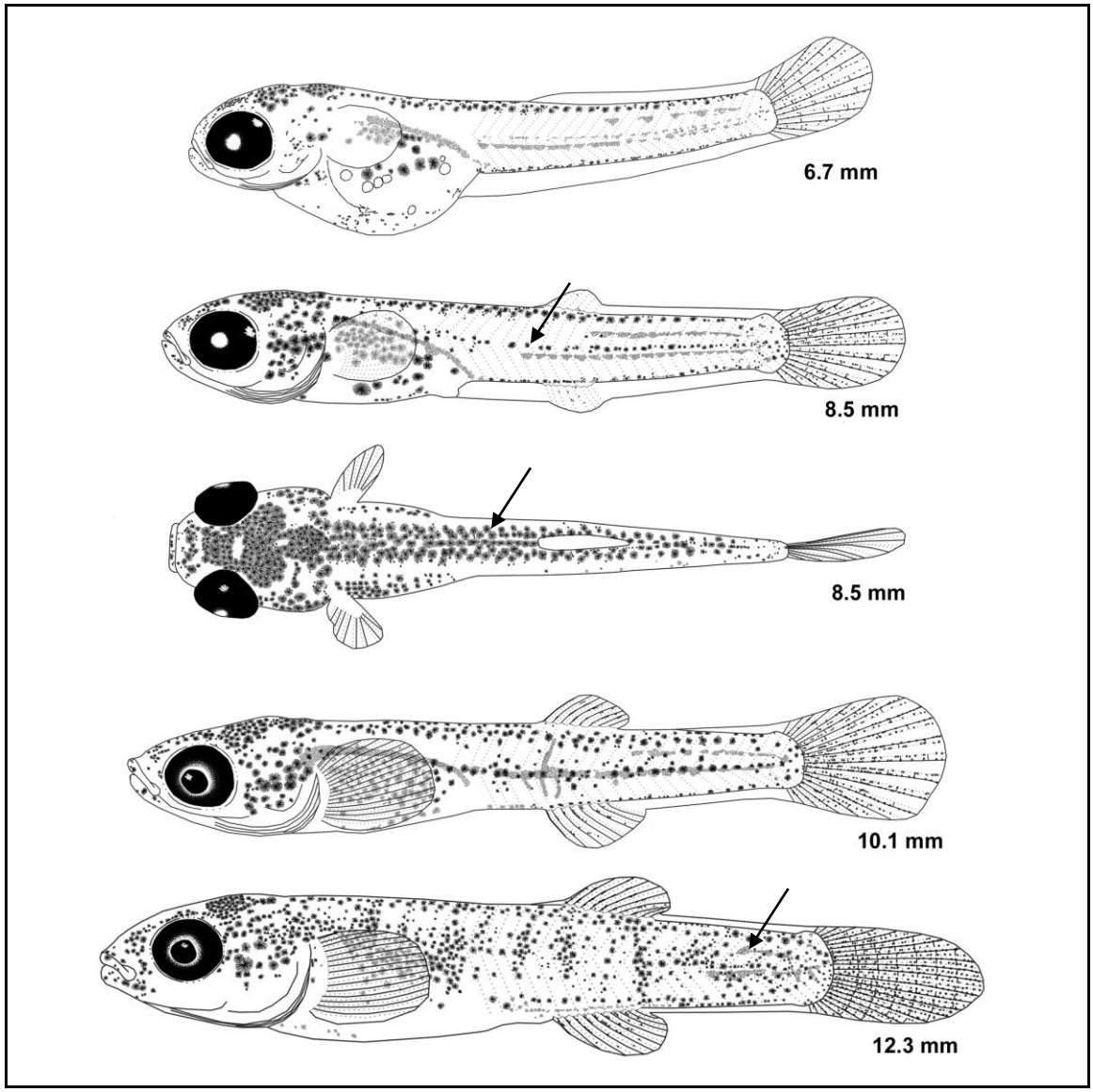

Fig. 15 Various stages (TL) of *Fundulus similis* development. Arrows indicate mid-lateral pigment, 3-5 rows of large pigment from head to origin of dorsal fin, and largest size where internal pigment around spine is visible. Size class one (0-6.99 mm TL), size class two (7-9.99 mm), size class three (10-11.99 mm), and size class four (12-15 mm).

Pigment common among estuarine fundulids consisted of highly concentrated melanophores on the posterior interorbital region, pigment bordering the fin rays, and internal pigment bordering the spine (Hardy 1978; Able and Fahay 1998). Regardless of the variation of conspecific hatchling development stage, unique pigment patterns were noticed for certain fundulids. From hatch size to 6.99 mm TL, *F. pulvereus* has a unique, fully pigmented hypaxial muscle segment that extends from the belly to the caudle peduncle, which separates it from all other nGOM fundulid species. This hypaxial muscle pigment only lasts until 7 mm TL, but any *F. pulvereus* > 7 mm TL is the most densely pigmented fundulid on the nGOM coast. All *A. xenica* from hatch size to 9.99 mm TL have a mid-lateral row of pigmentation that is more concentrated than *F. grandis*. Koenig and Livingston (1976) noted that the dorsal pigment of *A. xenica* was scattered at the caudal peduncle and became more concentrated at the head of the lab reared fish, but the *A. xenica* that was illustrated here seemed to have less of a discernable pattern other than the posterior interorbital pigment that was present in all fundulids. The lack of replication may have restricted observations with only, at the most, two fish in each size class. *Fundulus grandis* had a distinct line of dorsal pigment that extended from the anterior region of its head to the caudal peduncle, but the rest of the body was very lighty pigmented compared to other sympatric fundulid species. The pigment between the anterior half of *F. jenkinsi* eyes forms an X or sideways hour glass, and *F. similis* has three to five rows of large distinct stellate melanophores that extend from its head to the origin of its dorsal fin. Hardy (1978) noticed that same large dorsal melanophore pattern in the allopatric counterpart of *F. similis*, *F. majalis*, along the western Atlantic. *Fundulus jenkinsi* pigment is abundant and scattered throughout the body, and its dorsal fin origin

is posterior to its anal fin origin. Many of the traits that characterize these fish as adults are shown, in subdued form, in specimens > 10 mm TL, but it was necessary to develop diagnostic characters for the smaller fish that will aid in future identification of fundulids to the species level. The identification of these species at the larval and post-larval stage is necessary to understand habitat preference of young fundulids.

Killifish are known to utilize shallow water and the inundated marsh for feeding and reproduction (Kneib 1984; Kneib 1997; Lopez et al. 2010b), but there is little information on the habitat preference of the pre-juvenile stages. Fundulids are thought to be residents of estuarine marsh habitat, and in consequence are thought to have no ontogenetic shift in habitat use (Beck et al. 2001). Juvenile conspecifics occupy shallow water (Kneib 1984), and areas of high marsh, where water becomes entrained into very shallow pools that may be accessed by the smaller fundulid larval and postlarval stages. It is believed that fundulids use the high marsh for feeding on insects and other small organisms (Lopez et al. 2010b), and tidal pools and depressions seem to be habitat in which small assemblages of insect larvae thrive (Campbell and Denno 1978; Robles and Cubit 1981). Thus, high marsh tidal pools and depressions may act as essential habitat for developmental stages of all killifish. These areas are typically occupied by very young stages of organisms which are often difficult to accurately identify. Therefore, their comparative description will aid research scientists and resource managers in more accurate assessments of habitat use.

Dichotomous Key (fishes ≤ 15 mm TL)

1. 5 Branchiostegal rays (Fig. 16A) 2

   6 Branchiostegal rays (Fig. 16B) 4

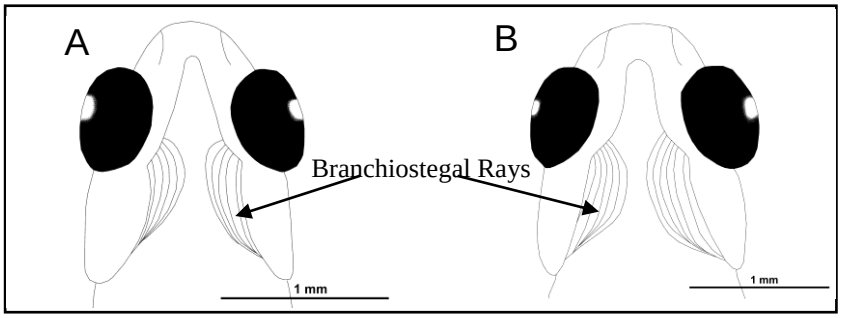

Fig. 16 The five branchiostegal rays of *Fundulus grandis* (A) and the six branchiostegal rays of *Fundulus similis* (B).

2. Hypaxial muscle pigment forms a line laterally under the spine or is sparse, TL 4.3-5.5 times longer than HL (< 7 mm TL); overall body pigment coverage is moderate to sparse, TL 3.9-4.9 times longer than HL (> 7 mm TL) 3

   Internal pigment covers all of hypaxial muscle from belly to caudal peduncle (Fig. 17), TL 4.0-4.3 times longer than HL (< 7 mm TL); pigment is heavily concentrated throughout the body, TL 3.3-4.4 times longer than HL (> 7 mm TL) .....*Fundulus pulvereus*

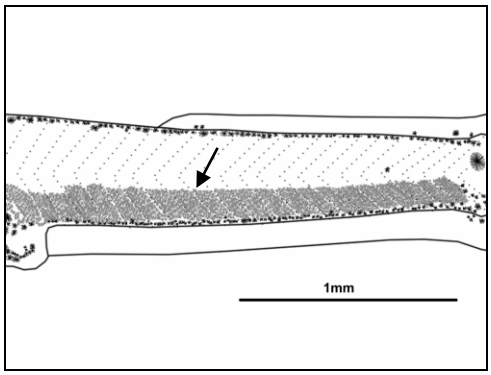

Fig. 17 Internal hypaxial pigment of *Fundulus pulvereus*.

3. Mid-lateral external pigment is more concentrated and extends from the caudal peduncle to the origin of the pectoral fin (Fig. 18), TL 6.3-7.7 times longer than BD, TL 14.9-17.3 times longer than CPW (< 7 mm TL); dorsal fin origin far anterior to anal fin origin, TL 5.2-6.0 times longer than BD, TL 8.0-11.0 times longer than CPW (> 7 mm TL) (Fig. 19)..... *Adinia xenica*

Mid-lateral external pigment is present but sparse (Fig. 18), TL 5.0-6.5 times longer than BD, TL 15.3-18.0 times longer than CPW (< 7 mm TL); anal and dorsal fin origin about vertically aligned (Fig. 19), TL 5.6-7.9 times longer than BD, TL 9.6-16.7 times longer than CPW (> 7 mm TL) ..... *Fundulus grandis*

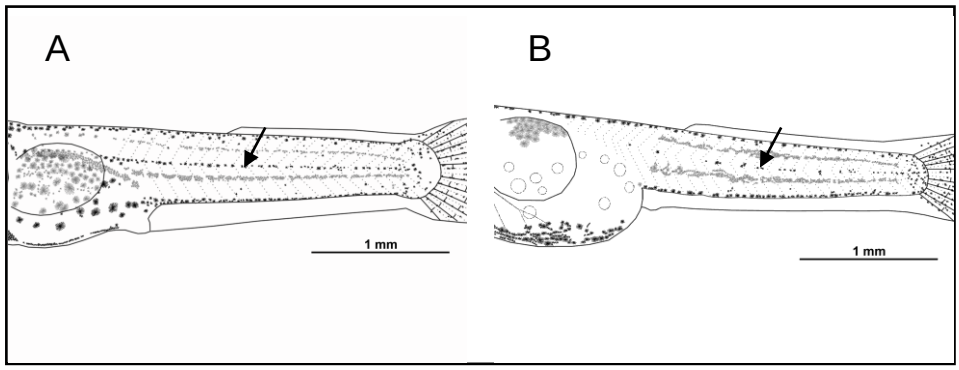

Fig. 18 Mid-lateral external pigment of *Adinia xenica* (A) and *Fundulus grandis* (B) denoted by arrows.

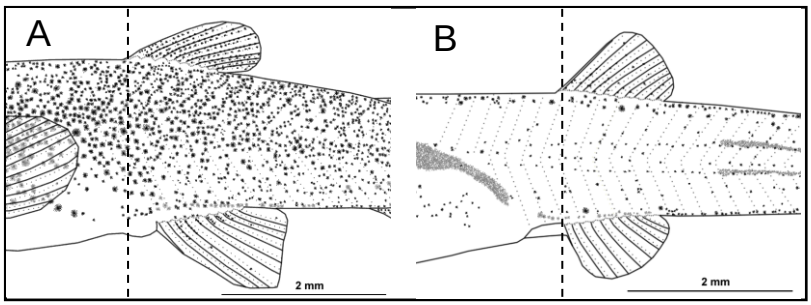

Fig. 19 Dorsal fin origin in reference to anal fin origin for *Adinia xenica* (A) and *Fundulus grandis* (B).

4. Small dorsal pigments cluster to form a line from the head to the caudal peduncle, melanophores form X or sideways hour glass between anterior half of eyes (Fig. 20), TL 10.4-14.6 times longer than SNL (< 7 mm TL); dorsal fin origin starts on the same vertical plane as the third anal ray (Fig. 20), TL 7.3-11.9 times longer than SNL (> 7 mm TL)..... *Fundulus jenkinsi*

Distinct 3-5 mid-dorsal rows of large stellate melanophores extending from head to dorsal fin origin, TL 7.6-13.0 times longer than SNL (< 7 mm TL); dorsal and anal fin about the same origin on a vertical plane (Fig. 21), TL 6.5-9.8 times longer than SNL (> 7 mm TL)..... *Fundulus similis*

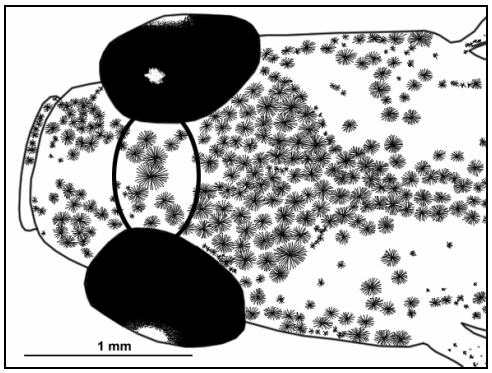

Fig. 20 Melanophores forming an X or sideways hour glass between the anterior half of eyes of *Fundulus jenkinsi* as denoted by oval.

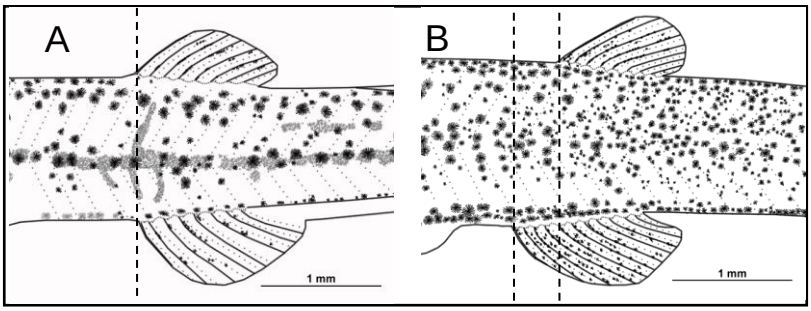

Fig. 21 Dorsal fin origin in reference to anal fin origin for *Fundulus similis* (A) and *Fundulus jenkinsi* (B).

CHAPTER IV

CONCLUSIONS

The reproduction and development data obtained From this work lead to key insights into the life history of *F. jenkinsi*. For example, *F. heteroclitus* has been shown to spawn in a semi-lunar fashion, whereas *F. grandis* from the nGOM coast spawns in succession only on late ascending tides that lead into the spring tide (Taylor and DiMichele 1980; Greeley et al. 1988). In contrast, the spawning of *F. jenkinsi* occurs on late ascending, spring, and early descending tides. The key component of *F. jenkinsi* spawning seems to be inundated saltmarsh, while moon phase or declination seems to have little impact. Secondly, *A. xenica* and *F. heteroclitus* deposit eggs on inundated marsh vegetation and all estuarine fundulids have been shown to strand their large, demersal eggs in the intertidal marsh habitat (Hastings and Yerger 1971; Taylor et al. 1977). Although the substrate where *F. jenkinsi* deposits its eggs is not known, reproductive histology and the size of hatchlings indicated many similarities in reproductive behavior with other fundulids. Therefore, some method of *F. jenkinsi* egg attachment in the intertidal environment would be logical. In addition, *F. heteroclitus* and *F. luciae* juveniles swim with the tide higher into the saltmarsh and generally stay in shallower water than the adults (Taylor 1984). These east coast fundulids have a similar life history pattern and this may indicate that the smaller larval and post-larval stages stay on the leading edge of the tide where the water is shallowest. Because of similarities in habitat preference of nGOM fundulids, it appears that the larval and post-larval stages of each species occupy the same habitat. For this reason, the constructed dichotomous key from known parents and using meristic, morphometric, and pigment characters for five

common sympatric nGOM fundulids will be required to identify fishes to species in the intertidal environment. In closing, the information on the reproductive life history of *F. jenkinsi* and the constructed dichotomous key for the five sympatric nGOM fundulids will be integral to continued conservation of the species and their habitat.

APPENDIX A

ORDER AND TIME OF REAGENTS FOR STAINING TISSUE WITH HEMATOXYLIN 2 AND EOSIN-Y.

Solution	**Time**
Xylene Substitute	3min
Xylene Substitute	3min
Xylene Substitute	3min
Absolute ethanol	10 dips
Absolute ethanol	10 dips
95% ethanol	10 dips
95% ethanol	10 dips
80% ethanol	10 dips
80% ethanol	10 dips
50% ethanol	10 dips
Distilled Water	1 min
Hematoxylin	5 min
Water rinse well	
Acid water	2 dips
Water rinse well	
Blueing water	30 sec
Water rinse well	
95% ethanol rinse well	10 dips
Eosin	1.25 min
Blot Blot Blot	
95% ethanol	10 dips
95% ethanol	10 dips
95% ethanol	10 dips
95% ethanol	10 dips
Absolute ethanol	1 min
Absolute ethanol	1 min
Absolute ethanol	1 min
Absolute ethanol	1 min
Xylene Substitute	1 min
Xylene Substitute	1 min
Xylene Substitute	1 min
Xylene Substitute	1 min

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