Salinity Preference in the Estuarine Teleost Fish Mummichog (*Fundulus heteroclitus*): Halocline Behavior

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ABSTRACT

Mummichogs prefer seawater (SW) but have wide ability to acclimate to extreme temperatures and salinities. In the field, minnow trapping revealed that mummichogs move progressively into low-salinity warmer water during early spring after ice melt and show significant aversion to colder temperatures and high salinity. First appearance in estuarine shallows occurred above 10°C, and catch increased to 21°C over 4 wk. Three-spine stickle-backs (*Gasterosteus aculeatus*) also preferred warmer low-salinity locations but preferred slowing streams, whereas mummichogs preferred tidal ponds. In the laboratory, artificial haloclines tested isothermal salinity preference, between 28‰ full-strength SW (below) and 10% SW (3.0‰; above). Mummichogs of both sexes acclimated to 5°C in SW strongly preferred SW. Freshwater (0% SW)-acclimated mummichogs at 21°C also preferred SW, but of sexually mature fish acclimated to 21°C SW, only the males preferred SW; the females showed no significant preference for SW, meaning they freely entered low salinity. SW preference was manifested by a stereotypic passive aversion to the dilute upper layer at the halocline. We conclude that the overall movement of mummichogs into summer breeding grounds of low salinity is driven by maturation of females and their preference for warmer water regardless of salinity.

Introduction

In nature, "wedge" estuaries often form haloclines with cooler high-salinity tidal seawater (SW), forming a stable lower-level wedge underneath warmer low-salinity freshwater (FW) above, thus creating a natural salinity preference regime vertically over only a meter or so depth. Little is known of fish behavior around haloclines, yet they are important common features of estuaries. Free-swimming marine cod in a tower tank generally preferred a lower-salinity (26‰) upper layer of the tank, and, henceforth, high salinities were avoided (Claireaux et al. 1995). In reef fishes, some teleost fish (members of Blennidae and Lethrinidae) can respond to halocline position and move deeper as the halocline moves deeper (Irisson et al. 2010). Thus, there seems to be a tendency for these nominally stenohaline marine fish to select lower salinity nearer the isosmotic zone that would minimize their energy expenditure in ion regulation.

By contrast, responses of fish to thermoclines are well known; for instance, the cod and haddock larval population on the Grand Banks was concentrated in the thermocline, and the stronger the stratification was, the more larvae were concentrated at this depth zone (Lough and Potter 1993). Overall, aquatic animals appear to distribute themselves vertically in a thermo-cline according to an ideal free distribution with multiple cost components such as to optimize their fitness, as supported by zooplankton in tower tank experiments (Lampert 2005).

Estuarine teleost fish are generally hardy and can survive wide variation in temperature and salinity, and they often select microhabitats within estuaries to breed. Because of the high metabolic cost of ion regulation in salinity extremes, the general assumption is that fish ought to be able to minimize this cost by behaviorally selecting intermediate salinities near the isosmotic/isoionic point. The mummichog (*Fundulus hete-roclitus* L.) is a model species in physiology and genomics that has been used extensively to study osmoregulation, ion transport, acid/base balance, calcium metabolism, and the hormonal and osmotic control of these processes (Burnett et al. 2007; Patrick et al. 1997b; Taylor 1999; Wood and Marshall 1994). In the northern extreme of the distribution of this species, estuaries freeze in winter, forcing the resident mummichog to retreat to high-salinity depths in winter. In spring, as the water warms up after the ice has melted, the fish return to shallow ponds, where they breed. The fish could be following the temperature and/or salinity gradients, as the shallow, warmer, low-salinity water occupies the breeding ponds. Breeding occurs during spring tides in dilute brackish shallow water, and fertilized embryos stick to vegetation; devel-opment is thus aerial, and hatching occurs after approximately 14 d on the next spring tides (Scott and Scott 1988; Taylor 1999).

Killifish are known to prefer warmer temperatures. The Aus-tralian killifish *Austrofundulus limnaeus* is capable of accurate behavioral thermoregulation, has a thermal preference 26°C, and has a daily cycle of temperature (Podrabsky et al. 2008). In ther-mal preference tests using northern and southern subspecies

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of *F. heteroclitus*, the northern subspecies had a thermal pre-ferendum of 30.6°C, higher than that of the southern subspecies, at 29.0°C (Fangue et al. 2009). Thus, fish could redistribute in the estuary primarily using thermal cues to detect warmer FW suitable for spawning.

Teleost and elasmobranch fish also possess salinity receptors (Nearing et al. 2002) and could therefore sense and prefer salinities that could minimize their energy expenditure in osmo- and ionoregulation. Immature mummichogs prefer higher salinity than the closely related species *Fundulus diaphanous*, offered as evidence for the habitat preference that helps keep the populations separated (Fritz and Garside 1974). Mummichogs also prefer SW, with a salinity preferendum of approximately 22‰, a response that is affected by the ionic content of their diet (Bucking et al. 2012). Given the reports of spawning in dilute parts of estuaries, the SW preference may not be universal, and living in dilute solutions could introduce an added metabolic cost of FW osmoregulation.

We wished to test whether mummichogs preferred high salinity regardless of their acclimation salinity or temperature, sex, or maturity. Because the fish spawn and forage in dilute salinity, clearly there are some situations where the SW preference is overridden. In the cold, at temperatures mimicking winter under-ice conditions, we predicted that the cold-acclimated fish should prefer SW, as dilute zones would be threatened with freezing. In warm temperatures mimicking the active breeding season, we predicted that the salinity preference of SW-acclimated fish would switch to low salinity because these animals breed in low-salinity shallow waters during high spring tides. We also tested fish acclimated to FW, a situation mimicking fish in nature that may have been stranded in FW ponds, predicting that while mummichogs can survive and tolerate dilute environments (Fritz and Garside 1974; Griffith 1974; Patrick et al. 1997*a*), this is likely not their preferred salinity. In the field, we set minnow traps at multiple loci in the marsh during the month the waters were warming and the fish were moving into their summer breeding grounds. In the laboratory, we constructed a halocline tank and presented fed fish with a stable choice between 10% SW and full-strength SW at the acclimation temperature and monitored the time spent in each of the two layers. We found that mature males and immature fish of both sexes show a stereotypic aversion to dilute media and thus prefer SW, whereas mature females have diminished aversion for FW and no strong preference for SW. Thus, mature females initiate movement onto the low-salinity spawning grounds. In the field, mummichogs prefer warmer water of lower salinity, but given the laboratory results, thermal preference apparently is the more important driving force.

Material and Methods

Field Experiment

Ten trap sites (table 1) were selected in the Antigonish estuary of northern Nova Scotia (Canada) in locations where mummichogs are known to breed. Five sites were placed in slow-moving streams (<5 cm/s current velocity at the surface), and five sites were in ponds with no apparent current flow. All

Table 1: Locations and descriptions of trap sites

Latitude (N)	Longitude (W)	River/pond description
45°37′55.85″	61°57′34.76″	West River
45°37′57.06″	61°57′31.27″	West River
45°37′57.74″	61°57′31.60″	Antigonish Landing Pond
45°42′38.33″	61°54′31.26″	Ogden's Pond
45°42′39.50″	61°54′26.33″	Ogden's Pond
45°42′40.26″	61°54′19.73″	Ogden's Pond
45°42′40.92″	61°54′16.27″	Ogden's Pond
45°43′08.83″	61°53′59.86″	Ogden's Brook
45°43′10.28″	61°53′59.61″	Ogden's Brook
45°43′11.31″	61°53′58.94″	Ogden's Brook

Note. Source: Google Earth.

sites were 1.5 m depth or less, often near the minimum depth for trap function (30 cm). Traps were set every other day for 1 h (±4 min); pulled; and the catch identified, counted, and released unharmed on site. Salinity and temperature were recorded by salinometer (YSI model 63 salinometer; ±0.1°C, 1.0 ppt) at each trap locus each day. Tide level was noted, but traps were set at the same time (approx. 10 a.m.) each trap day, so tidal level varied.

Animals

Adult mummichogs were trapped at Ogden's Pond, Antigonish County, using double-cone minnow traps baited with Cheese Nachos, and transferred to aerated coolers, to the St. Francis Xavier Animal Care Facility, and to full-strength SW. Fish were fed twice daily commercial fish flake food plus freeze-dried Tubifex worms and live mealworms in alternating afternoon feedings. Experimental procedures were approved by the Animal Care Committee and followed guidelines administered by the Canadian Council on Animal Care (protocol 12-001-r2). All fish were euthanized by anesthetizing in 1.0 g/L MS-222 adjusted to pH 7.0 in 0.9% saline, followed by decapitation.

Halocline Aquarium

The halocline aquarium was a Plexiglas box, 40 cm × 40 cm × 35 cm (L × W × H), equipped with a 15 × 15 × 15-cm antechamber with a sliding door to introduce the fish to the halocline chamber. Full-strength SW was added at 0.50 L/min to the lower extreme of the aquarium, and 10% SW was added at 0.50 L/min at the midpoint height, layering on top of the SW. The drain, with the total level controlled by a standpipe, removed fluid from the midpoint such that the halocline became narrower with time as the upper and lower fluids were added. Diagonal stripes on the back of the aquarium accentuated the refractive index difference, allowing the experimenter to visualize exactly where the halocline was located and how wide it was. Typically, the transition zone was narrow, 3–7 cm, confirmed by salinometer readings.

Halocline Behavior Trials

Fish were acclimated to room-temperature SW (21°C, 27‰) or cold SW (5°–6°C, 27‰) for at least 4 wk before salinity preference

trials. Cold-acclimated fish were transferred into a separate aquarium and acclimated to a temperature of 5°C by lowering the temperature by 2°C every other day for a period of 2 wk. A second batch of fish was transferred to dechlorinated Antigonish FW (69 μS/cm, pH 6.6–6.9, 1.0 mM Na$^+$, 1.0 mM Cl$^-$, 100 μM Ca^{2+}) for at least 4 wk before behavioral trials. A third batch of fish including smaller immature fish was also acclimated to room-temperature SW, from which the immature test fish were drawn.

The halocline chamber was drained and cleaned (70% ethanol spray), dried, and refilled between each trial to prevent odorant/pheromone being carried forward from the previous trial. All trials were performed in a controlled environmental chamber under low-light conditions (0.48 μmol/s/m^2 red light), with black plastic on the top and bottom of the aquarium (so light entered only through the transparent sides) to reduce phototactic response. The trial commenced with a 15-min settling period in which the fish was placed in the antechamber where the bottom 3 cm was SW and the majority of the chamber was 10% SW. After the settling period, during which time the fish had been exposed to both salinities, the door opened to allow the fish to enter the main aquarium with the midpoint halocline, and fish movements were video recorded for 30 min. The fish was then returned to its home aquarium, separated from the others so that one fish performed exactly one trial. Time spent above the halocline was summed at times 10, 20, and 30 min (to see whether there was a trend over time) and the total time summed.

Statistics

Catch per unit effort, salinity, and temperature were recorded in Graphpad Prism, version 6.0. Distribution toward temperature and salinity was determined relative to the mean temperature or salinity on the trap day, and catch above and below the average was tested for nonrandom distribution using a one-sample χ^2 test. Trap efficiency was assumed not to vary but was unknown. Similarly, traps that were set in flow (river) locations versus static (pond) locations were tested for nonrandom distribution using a one-sample χ^2 test.

Total time above the halocline in behavioral trials, body mass, and gonadosomatic index (GSI = gonad mass/total body mass × 100%) were expressed as histograms and tested by one-way ANOVA followed by multiple a posteriori tests (Tukey) with $\alpha = 0.05$ in a two-tailed test. If necessary, where sample variance clearly rejected the Bartlett's test ($P < 0.001$), statistics were performed on log-transformed data.

Results

Field Study

The baited minnow traps were effective in trapping mummichogs and three-spine sticklebacks primarily, with few instances of other species (Silverside; *Menidia menidia*, 2; nine-spine stickleback, *Pungitius pungitius*, 6; four-spine stickleback, *Apeltes quadacus*, <5). Initially, catches were zero at all locations, and no fish were observed at any of the locations. Over the sampling period, water temperature warmed from 8°C to above 20°C, and catch increased

with a pause after the midmonth spring tides on the new moon, when catches declined to 2 and 3 before increasing to 220 when water temperatures reached 20°C (fig. 1).

Plotting the catch in relation to average daily temperature (fig. 2) revealed that catch was larger than expected in traps above the average temperature and below the average salinity (observed catch, 452; expected catch, 214.4; $\chi^2 = 264.20$, $P < 0.0001$). Meanwhile, there was a significantly lower than expected catch in traps below the daily average temperature and salinity (observed, 23; expected, 152.1; $\chi^2 = 110.56$, $P < 0.0001$) and a significantly lower than expected catch in low temperature, high salinity (observed, 166; expected, 245; $\chi^2 = 25.60$, $P < 0.001$). There was no significant trend in traps that were above the average for salinity and temperature (observed, 117; expected, 145.5; $\chi^2 = 5.39$, nonsignificant [NS]). For sticklebacks, similar to mummichogs, there was a higher catch than expected in warmer temperature, low salinity (observed, 342; expected, 137; $\chi^2 = 305.86$, $P < 0.0001$) and lower than expected catch in low temperature, low salinity (observed, 22; expected, 157; $\chi^2 = 115.86$, $P < 0.0001$) but unlike mummichogs significantly lower catch in warm temperature, high salinity (observed, 50; expected, 93; $\chi^2 = 19.94$, $P < 0.001$), and (again unlike mummichogs) there was no significant difference in low temperature, high salinity (observed, 71; expected, 98; $\chi^2 = 7.43$, NS).

Halocline Behavior

The reflex pattern we observed frequently as the fish crossed the halocline was an immediate cessation of fin movements when the head entered the dilute water layer, which resulted in the fish passively sinking back into the lower layer. We infer that this is an aversive behavior to dilute salinity dominated by
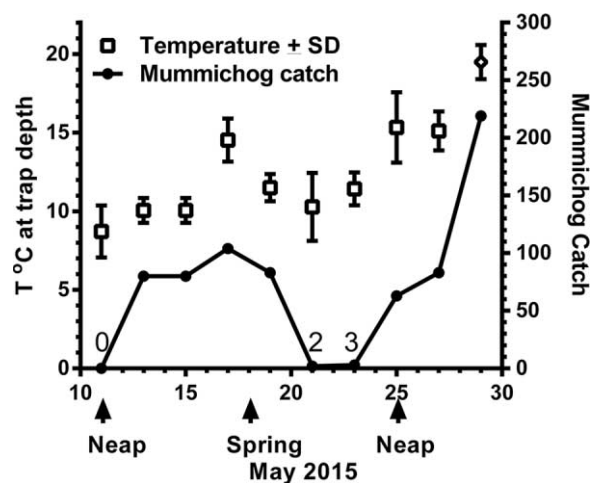

Figure 1. Catch of *Fundulus heteroclitus* (mummichog) in 10 sites in Nova Scotia estuaries in May 2015 as the fish move into their summer breeding locations. Total mummichog catch per unit effort (trap-hours) is plotted over time (d) with mean ± range of water temperature (T) at trap depth. Temperature and catch increased over the trapping period (stickleback catch not shown).
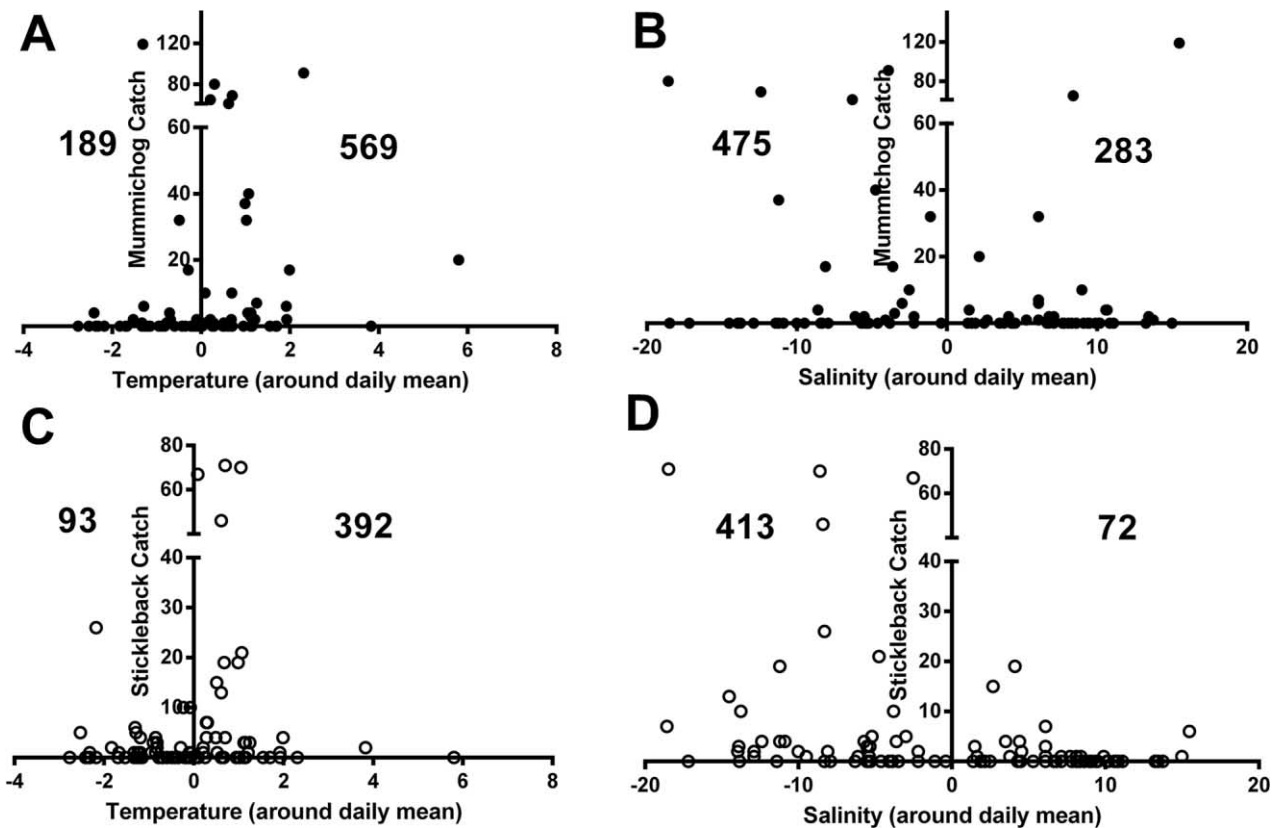
Figure 2. Mummichog catch per unit effort plotted against mean daily temperature (*A*) and mean daily salinity (*B*) for minnow traps set in 10 estuarine sites during spring 2015. *Gasterosteus aculeatus* (three-spine stickleback) catch is plotted against mean daily temperature in *C* and salinity in *D*. See text for statistical analysis.

inhibitory sensory-motor pathways. It was this pattern that was less obvious in the females that spent roughly equal time below and above the halocline; these animals repeatedly crossed the halocline vigorously and by the end of the 30-min trial had added to the mixing layer so that the halocline was noticeably wider.

Salinity Preference

There were no apparent trends in time spent in the upper layer in the first, second, and third 10-min intervals, so the data were pooled for the entire 30-min trials. A one-way ANOVA of the entire data set ($F = 3.220$, df $= 7, 62$, $P = 0.0056$) was significant, but on the basis that sexual behavior may intervene in mature animals, the data set was divided into male (fig. 3) and female (fig. 4) responses. ANOVA of the male results produced no significant trends in halocline behavior ($F = 1.886$, df $= 3, 34$, $P = 0.1515$). The males from all four treatments spent on average less than 5.1 min above the halocline, and there were no significant differences between the treatments. As expected, the immature fish tested had significantly smaller total body mass (ANOVA, $F = 18.70$, df $= 3, 27$, $P < 0.0001$) and very low GSI (fig. 3*B*, 3*C*). One-way ANOVA of male GSI data ($F = 18.70$, df $= 3, 27$, $P < 0.0001$) indicated that males in SW at 20°C had

significantly higher GSI (fig. 3*C*; Tukey a posteriori test, $P < 0.01$) compared to the immature and cold-acclimated fish, consistent with their maturity, as they also manifested breeding colors.

Female FW-acclimated fish, SW-acclimated fish at 5°C, and SW 20°C immature fish all had, on average, less than 5 min above the halocline (fig. 4*A*), indicating preference for SW. However, one-way ANOVA of the data from all females ($F = 3.027$, df $= 3, 28$, $P = 0.046$) was significant, and mature female SW fish at 20°C averaged 9.72 min above the halocline (fig. 4*A*), significantly greater (Tukey a posteriori test, $P < 0.05$) than the 5°C females and more than twice the average time for the other two groups (not significantly different likely due to small sample size). Of the SW 20°C females, 5/13 spent more than half their time above the halocline, whereas none of the females in any of the other three groups (0/19) spent more than half their time above the halocline. On dissection of the 5°C females, while yolky oocytes were present, there were no apparent ovulated eggs in or around the ovary, but on dissection of the SW 20°C females, 8/13 had ovulated eggs, and the GSI was the highest on average for all four groups (fig. 4*C*), evidence of reproductive readiness. As expected, the immature females had significantly lower total body mass (ANOVA, $F = 4.261$, df $= 3, 26$, $P = 0.0142$) and lowest GSI (ANOVA, $F = 7.183$, df $= 3, 26$, $P = 0.0011$; fig. 4*B*, 4*C*). Somewhat surprisingly, the FW-
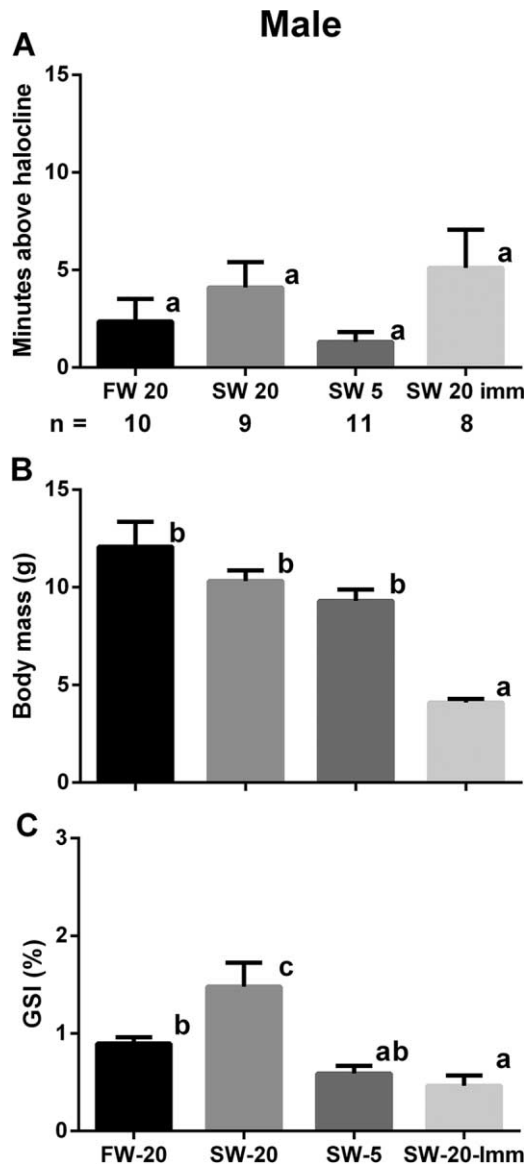
Male

by mummichogs over riverine sites with current. The fish show a significant preference for warmer water of lower salinity and a significant aversion to colder sites while also showing no aversion to warm sites of high salinity. Our results confirm the previous thermal preference experiments (Fangue et al. 2009) that mummichogs have a strong preference for warmer temperatures. Thermal gradients in nature are not steep, and a difference of only 1° appears sufficient to attract these fish. Our results also confirm the segregation of three-spine sticklebacks from mummichogs on the basis of flow, with the sticklebacks preferring riverine areas
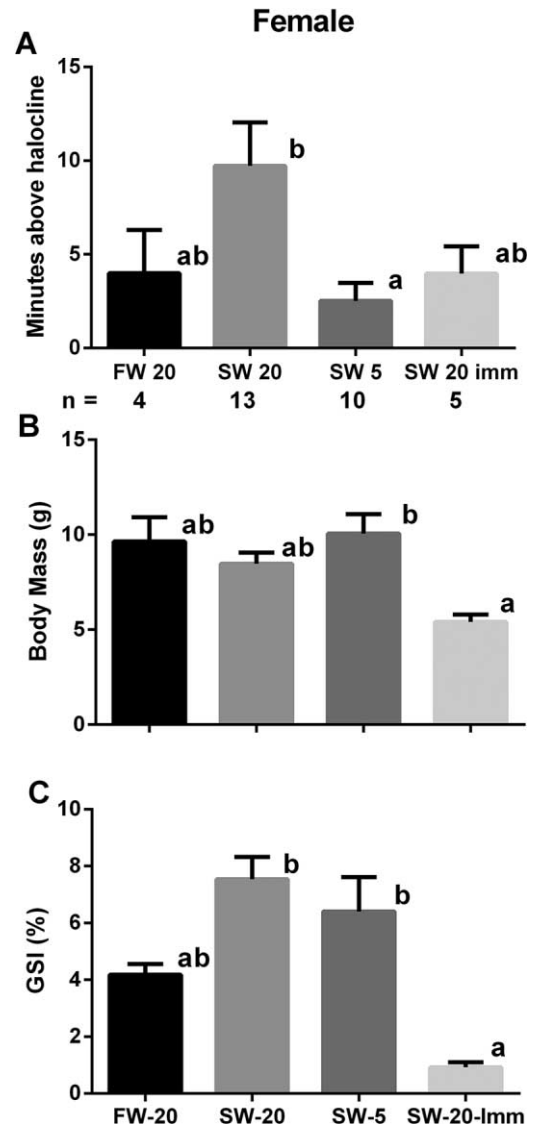
Female

Figure 3. Male mummichog halocline trials as time (minutes of a 30-min trial) spent above the halocline in the low-salinity zone (*A*). Both layers (full-strength seawater [SW], 28‰, below, and 10% SW, 3‰ brackish water, above) were held at the acclimation temperature ($\pm 0.1°C$). Column labels indicate acclimation salinity (SW or freshwater [FW]) and acclimation temperature (°C). Data are expressed as mean $\pm$ SEM; dissimilar letters indicate significant differences at $P < 0.05$ or better using the Tukey a posteriori test after one-way ANOVA; sample size is indicated below *A*. Also plotted were body mass (g; *B*) and gonadosomatic index (GSI; *C*) as mean $\pm$ SEM; statistics as per *A*. imm = immature animals.

acclimated females had small GSI, averaging 4.1%, not significantly different from the immature females (fig. 4*C*).

Discussion

Movement to the Spawning Areas

Spring migration to the spawning areas occurs once the temperature rises above 10°C, and pond environments are preferred

Figure 4. Female mummichog halocline trials as time spent above the halocline in 10% seawater (SW; 3‰) rather than below the halocline in full-strength SW (28‰). Column labels and experimental conditions as per figure 3. Data are expressed as mean $\pm$ SEM; dissimilar letters indicate significant differences at $P < 0.05$ or better using the Tukey a posteriori test after one-way ANOVA; sample size is indicated below *A*. Also plotted were body mass (g; *B*) and gonadosomatic index (GSI; *C*) as mean $\pm$ SEM; statistics as per *A*. imm = immature animals.

with current (Scott and Scott 1988) and the mummichogs preferring ponds with negligible current (Allen et al. 2007), so whereas the breeding seasons of the two species overlap, their hatchlings will be in different microhabitats and not be in direct competition. The field data also point to a preference for lower salinity, which runs counter to the reported SW preference of mummichogs (Bucking et al. 2012), but the laboratory experiments were carefully isothermal, while the field data were not. Whereas it seems that these fish favor low salinity, this response could be secondary to the fish seeking warmer water. The haloclines occurring naturally typically have colder SW underneath warmer brackish water, so it is unclear which of salinity or temperature governs the observed movement into the upper layer.

Halocline Behavior

Considerations. The halocline trials were strictly isothermal to show only salinity preference, as the strong preference for warmer water (Fangue et al. 2009) would likely override a salinity preference. Fed fish were used to reduce foraging behavior contributing to movements, as top minnows usually forage at the surface; thus, the fed fish would likely stay in the upper layer only if they preferred that salinity, whereas unfed fish would likely actively forage above and below the halocline. In previous work (Fritz and Garside 1974), unfed (24-h) fish were used in salinity preference trials. The diet was similar to the high-NaCl, high-Ca^{2+} diet used in the previous study (Bucking et al. 2012) that evoked preference for SW (30‰–32‰) in the immature fish (2–6 g) used in that study. The mature fish used in our study (6–18 g) were in reproductive colors (yellowing of male undersides), and the GSI, particularly of females, were in the range for active spawning (GSI 6–9). In the 20°C SW group, the majority of females (8/13) were ovulating. The study coincided also with spawning season in the field, although all fish were kept on a 12L:12D photoperiod rather than ambient at the time (approx. 16L:8D). Whereas depth (geotaxis) is a consideration in vertical selectivity tests, previous experiments with horizontal two-way preference tests confirmed the salinity selection trends established by vertical tests with *Fundulus heteroclitus* (Fritz and Garside 1974). In consideration of the results, the sexual maturity and the fed condition are major factors, and the fish would likely occupy the upper layer only if they were neutral to or preferred the lower salinity.

Salinity Preference in Males. Mature and immature male mummichogs clearly prefer SW in halocline behavior trials, spending on average 2–5 min (7%–17% of the trial) above the halocline, confirming the findings of Fritz and Garside (1974) and extending their findings to include cold-acclimated and mature fish. There was frequent observation of a stereotypic behavior pattern where the fish approached the halocline head-up at approximately 45°, swimming slowly using mostly pectoral fin motions until the head emerged above the halocline; then, fin motion abruptly ceased, and the fish slowly sank back through the halocline in a head-neutral (horizontal) posture. A possible mechanism would include a sensory-motor pathway where sensing the dilution of the water produces activation of an inhibitory pathway that stops fin movements. In many cases, only three or four such brief forays across the halocline occurred; in other cases, more extensive exploration of the whole tank occurred, but in no instance (males and immature fish of both sexes) did the time above the halocline exceed half (15 min). In shuttle box trials, salinity differences were smaller (5‰ per step), and the final preferred salinity was intermediate (22‰) but still well above the isoionic/isosmotic salinity that would minimize metabolic cost of osmoregulation (Bucking et al. 2012). There is little evidence of a large impact on metabolism by salinity increase, as mummichogs have the same total oxygen consumption in 10‰ as in 30‰ (Kidder et al. 2006), so salinity selection may not occur to minimize the metabolic cost of osmoregulation. In two species of *Fundulus*, swimming performance (highest speed) was significantly better in SW (salinity 31‰–35‰) than in low salinity (15‰–16‰) for male *F. heteroclitus* and for *Fundulus majalis* of both sexes, but there was no difference for female *F. heteroclitus* (Yetsko and Sancho 2015); thus, there may be a survival value for the SW preference based on better predator avoidance through higher burst swimming speed.

Salinity Preference in Females. The most intriguing result was that mature ovulating females in warm water were the most likely to spend more than 50% of their time above the halocline, hence preferring low salinity. The females spent more time (Tukey test, $P < 0.05$) above the halocline than the cold-acclimated female fish and twice the time compared to the males acclimated to 20°C, but this was only a trend (unpaired *t*-test, two tailed, $P = 0.077$). Overall, the mature females were the only group that had individuals that preferred the dilute medium (5/13 spent more than 50% of time above the halocline), and this group stood out as being highly active (searching constantly) and appearing to ignore the halocline. The above-mentioned aversive behavior to dilute media was not observed in these warm-acclimated mature females. In nature, the logical result is that the females, when ready to spawn, could lead the males to shallows, where the vegetation is available for the adherent embryos. Responses to environmental cues (e.g., vegetation, higher temperature) are known in pursuit-type spawning teleost fish that lay eggs on vegetation, such as the goldfish *Carassius aculeatus*, and in this species both sexes respond to pheromones (Kobayashi et al. 2002). Evolutionarily, females being pursued by multiple males constitutes a "cooperative" type of reproductive strategy (Taborsky 2001). Future experiments could correlate endocrine state, ovarian maturity, and ovulation with changes in salinity preference during the reproductive season to clarify the role of hormones in the behavior.

An unusual result was that FW-acclimated fish (males and females) immediately showed a strong preference for SW rather than the 10% SW in the upper layer; thus, the animals selected SW rather than selecting the much smaller salinity step change from FW to 10% SW. There was no outward evidence that the animals were in shock from the transfer, and they were actively swimming all of the time, but future experiments could offer a FW choice. This result confirms previous findings that FW-acclimated mummichogs seem to prefer high salinity (20‰–31‰) in both vertical and horizontal tests (Fritz and Garside 1974). The behavior of the FW fish seems energetically unwise, as the osmoregulatory

structures they develop to cope with FW have to be replaced with SW-type structures at considerable metabolic cost over a week or more, as has been demonstrated in many FW-SW experimental transfers in a variety of fish species (LeBlanc et al. 2010; Sangiao-Alvarellos et al. 2003; Schettino and Lionetto 2003; Wood and Grosell 2008). The SW preference of FW-acclimated fish underscores the fundamentally marine nature of this species in spite of its survival plasticity.

In the cold, preference for SW was also present in males and females even though osmoregulation would be predicted to be a much larger percentage of total metabolic rate and the relief from the large ion gradients and passive ion diffusive gain might be an advantage. Activity at 5°C is reduced, which could account for the larger time below the halocline, but in every case the fish did encounter the dilute solution and did not stay in it. In all cases, in the antechamber all fish experienced, at least briefly, the low-salinity upper layer. It would be interesting to evaluate the effect of slightly warmer water above the halocline on the overall aversion of mummichogs to dilute media, given that in nature the halocline is usually accompanied by warmer surface water.

In conclusion, we confirmed that mummichogs in several salinity- and temperature-acclimation states prefer SW to lower salinity and found a novel reduction in that aversion in actively breeding females. Thus, the apparent series of events surrounding spawning in *F. heteroclitus* is that mature females, lacking the aversion to dilute media, as demonstrated by halocline behavioral trials in laboratory conditions, easily enter warm, shallow, dilute waters followed by (several) males, so spawning occurs in dilute media in spite of the continuing aversion by males to dilute media and preference for SW.

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