Choice consequences: Salinity preferences and hatchling survival in the mangrove rivulus fish (*Kryptolebias marmoratus)*

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Abstract

In heterogeneous environments, mobile species should occupy habitats in which their fitness is maximized. Mangrove rivulus fish inhabit mangrove ecosystems where salinities range from 0–65 ppt but are most often collected at ~25 ppt. We examined rivulus' salinity preference in a lateral salinity gradient, in the absence of predators and competitors. Fish could swim freely for 8 hours throughout the gradient with chambers containing salinities from 5–45 ppt (or 25 ppt throughout, control). We defined preference as the salinity in which the fish spent most of their time, and also measured preference strength, latency to begin exploring the arena, and number of transitions between chambers. To determine whether these traits were repeatable, each fish experienced three trials. Rivulus spent a greater proportion of time in salinities lower (5-15 ppt) than they occupy in the wild. Significant among-individual variation in the (multivariate) behavioral phenotype emerged when animals experienced the gradient, indicating strong potential for selection to drive behavioral evolution in areas with diverse salinity microhabitats. We also showed that rivulus had a significantly greater probability of laying eggs in low salinities compared to control or high salinities. Eggs laid in lower salinities also had higher hatching success compared to those laid in higher salinities. Thus, although rivulus can tolerate a wide range of salinities, they prefer low salinities. These results raise questions about factors that prevent rivulus from occupying lower salinities in the wild, whether higher salinities impose energetic costs, and whether fitness changes as a function of salinity.

Keywords

Salinity; preference; repeatability; gradient; *Kryptolebias marmoratus*; hatching survival

Introduction

An animal's survival and reproductive success depend on its ability to either operate in variable environments or relocate when conditions become suboptimal. The decision to stay or leave is ultimately based on which option maximizes the animal's fitness (Nguyen et al., 2013; McManus et al., 2014). If the benefits of relocating outweigh the costs, then the animal should disperse (Caughley, 1994). Costs of dispersal include use of energy, risk of injury or death, and outbreeding depression (Bonte et al., 2012), while benefits include escaping unfavorable conditions, obtaining new resources, and decreased chances of inbreeding (Caughley, 1994). However, for an animal to gauge the magnitude of benefits that it might receive from moving, it must have information about alternative habitats, which can be obtained by exploring new areas, contingent upon the aforementioned costs (Nguyen et al., 2013).

Coastal ecosystems are characterized by diverse microhabitats that are relatively close in proximity, making it possible for mobile aquatic organisms to gather information about surrounding habitats. Aquatic species often have a particular range of salinities that they can tolerate, but also a salinity in which their fitness is highest (Boeuf and Payan, 2001). When the ability to disperse is limited, the habitats in which animals settle can significantly constrain fitness. For example, growth and survivorship of the barnacle *Balanus amphitrite* are negatively impacted when the animal occupies salinities greater than or equal to 10 parts per thousand (ppt) (Qiu and Qian, 1999). Because barnacles remain attached to a substrate during adulthood and cannot readily escape unfavorable environmental conditions, they must endure these consequences in the event of salinity fluctuations. Fishes, on the other hand, have the ability to disperse throughout their lifetime, which allows them to move to areas with more favorable salinities, if available (Bonte et al., 2012). Salinity preference thus plays an important role in habitat selection for aquatic organisms living in brackish environments, which can vary in salinity both spatially and temporally, creating distinct microhabitats (Surge and Lohmann, 2002). Many organisms that inhabit variable environments tend to have wide tolerance ranges (Gabriel, 2005; Schultz, and McCormick 2012). For example, the killifish, *Fundulus heteroclitus*, is able to tolerate shifts in temperature, pH, salinity, and oxygenation; each of these factors vary significantly within their salt marsh habitat (Schulte, 2014). In any case of habitat selection, there are most likely a set of environmental conditions in which the animals experience highest fitness, and a preference for these habitats should be selected for (Kearney and Porter, 2004).

When organisms occupy a particular habitat, it could be because the conditions in that habitat confer highest fitness, or could reflect biotic and abiotic factors (e.g., competition, predation, salinity, and temperature) that limit occupancy of optimal habitats (Svärdson, 1949; Kearney and

Porter, 2004). To determine where an animal achieves highest absolute fitness in a multidimensional environment, all factors other than the one of interest must be controlled for. In addition, animals should be exposed to the full, ecologically relevant range of the abiotic factors (i.e., its fundamental niche) (Pearman et al., 2008). When other things that limit dispersal are present (e.g., competition, predators), the fundamental niche narrows to the realized niche (Morse, 1974), which is typically where organisms are found in their natural environments. Although individuals of a given species are often found within a given niche, among-individual variation around that average niche space can exist, and this variation is then subject to selection. Preference studies can provide insights into whether habitat selection is constrained by other factors, and reveal whether, in the wild, the animals occupy their realized or fundamental niche. In the laboratory, extraneous variables can be controlled while examining preferences, which should provide information about the conditions under which the animal might experience highest absolute fitness. Furthermore, laboratory settings also allow for assessment of repeatability of that preference, which can be difficult in field-based studies on organisms that show low site fidelity. Quantifying repeatability, the proportion of total phenotypic variation that is due to among-individual differences (Falconer and Mackay, 1996; Lessells and Boag, 1987; Boake, 1989), is essential for understanding the potential for selection to drive the evolution of salinity preferences (Brodie and Russell, 1999; Boake, 1989; Arnold, 1994).

Mangrove rivulus fish, *Kryptolebias marmoratus* (hereafter, 'rivulus'), are small, self-fertilizing hermaphroditic vertebrates found in a wide range of microhabitats within mangrove ecosystems of Florida, the Bahamas, parts of the Caribbean and Central America (Huber, 1992). Field data have shown that they exist in a broad range of salinities. Based on data from 274 different field sites, we have collected mangrove rivulus in salinities ranging from 0-65 ppt, with an average salinity of 26 ppt (SE ± 0.44) (see also Taylor, 2012). However, very few rivulus eggs have been collected in the field so, habitat preferences for egg-laying remain unknown (Taylor, 1990). Rivulus can exist in a wide range of salinities in the field, even over small spatial scales (Sutton et al., 2018) despite the apparent costs that the animals might accrue at both low and high salinities. For example, Lin and Dunson (1999) showed that exposure to different salinities early in life significantly affected adult mass; treatment animals raised at 12 and 40 ppt had significantly higher final masses than those raised at 1 ppt. Mortality rates of mangrove rivulus living at 12 and 40 ppt were also significantly greater than those living at the lower salinity (Lin and Dunson, 1999). Overall, mangrove rivulus raised in the lowest salinity (1 ppt) matured at a slower rate, grew to a smaller size, and produced fewer eggs than those reared in higher salinities (Lin and Dunson, 1995). When salinities deviate from the isosmotic point animals can incur significant costs, however this is not always the case and it is not always straightforward to predict which salinities are associated with elevated physiological costs (e.g., Ern et al. 2014). While many

freshwater fishes do best (e.g., have lower metabolic rates) in freshwater or have metabolic rates indistinguishable from those at an isotonic salinity, some saltwater fishes do best at the isosmotic point and others show no increase in physiological costs at higher salinities (Ern et al. 2014). It appears that the most pronounced costs are experienced when salinities differ from those the fish was reared in (Ern et al. 2014) but very low or very high salinities might require that the animal dedicate more energy towards osmoregulation, perhaps at the expense of growth, resulting in a smaller fish. Indeed, Sutton et al. (2018) showed, in rivulus, that as salinity concentrations increase, metabolic rates and activity levels increase substantially. Different salinities significantly affect growth rate in a variety of other fish species, both freshwater and marine, which may be due to the relative amounts of energy being devoted to osmoregulation versus somatic processes (Boeuf and Payan, 2001).

Because salinity seems to have such a large impact on the growth, mortality, and reproduction in rivulus, it seems reasonable that selection might have acted to shape relatively narrow salinity tolerances and strong salinity preferences in this species. This motivated a controlled laboratory study to identify the preferred salinity, which is likely the salinity at which the lowest costs would be incurred. While previous studies have demonstrated the ability for rivulus to tolerate various salinities, and the effects that those salinities can have on reproduction and survival (Lin and Dunson, 1995; Lin and Dunson, 1999; Frick and Wright, 2002; Taylor, 2012; Sutton et al., 2018), none have attempted to determine if the species has a salinity preference and if there is among-individual variation around the species-level average preference.

Environmental conditions in the area where eggs are laid can have significant impacts on offspring survival and phenotype. Specifically, the salinities that aquatic species are exposed to during early life can have considerable effects on the phenotype, which are driven largely by the increased energy demands of osmoregulation (Urbina and Glover, 2015). Some salinities result in reduced hatching success and larval survival, as well as decreased size at hatching and growth rate (Berlinsky et al., 2004; Mihelakakis and Yoshimatsu, 1998; Zhang et al., 2010; Ramee and Allen, 2016). Because osmoregulation comes at a cost, brackish water species can conserve energy by inhabiting areas in which they are isotonic (Boeuf and Payan, 2001); it would then be expected that oviposition sites should also be selected for in this manner to reduce the potential energy cost sustained by offspring during development. During any given egg-laying bout, rivulus lay very few eggs (often 1) (Harrington, 1963; Lomax et al., 2017).

In this study, we controlled for extraneous environmental variables such as water and air temperature, light, food availability, competition and predation to determine how rivulus would distribute along a salinity gradient when salinity was the only difference among available microhabitats. The objectives of this study were to (1) determine whether mangrove rivulus

exhibit salinity preferences, including measurements of strength of preference, number of transitions between salinities, latency to begin exploring the salinity gradient, and the covariance among these traits. Then (2) establish if these behavioral traits are repeatable, (3) determine whether their preference in the laboratory corroborates field collection data, as well as, (4) examine whether mangrove rivulus have a salinity preference for oviposition sites, and (5) determine the effects of developmental salinity on hatching success. We hypothesized that mangrove rivulus would have a salinity preference and that their preference would be repeatable. A preference was inferred if the fish spent significantly more time in one salinity compared to others. It was predicted that the fish would show a preference for 25 ppt because this is the salinity in which rivulus are found most commonly in the wild and at which they are raised in our laboratory colony. Previous work suggests that the isotonic point for mangrove rivulus is nearer to 15 ppt (Frick and Wright 2002; Bielmyer et al. 2012), leading to an alternative prediction that the fish would prefer salinities lower than 25 ppt. Additionally, we hypothesized that they would have a preferred salinity in which to lay eggs and that the salinity experienced during development would influence hatching success. We predicted that rivulus would prefer to lay eggs in 25 ppt and that eggs laid in 25 ppt would have the highest hatching success compared to eggs laid in any other salinity. Alternatively, rivulus might choose to lay their eggs, and they eggs might fare better, at salinities closer to the isosmotic point of 15 ppt.

Materials and Methods

Housing Conditions

When not being tested, all fish were housed in ventilated, 1.2 L Rubbermaid® containers filled with 25 ppt salt water (Instant Ocean® salt and aged tap water). All individuals were kept under a 12L:12D photoperiod, a temperature of 25.42 ± 0.0043 °C (mean ± SEM), and were fed 2 ml of live brine shrimp (*Artemia*) nauplii reconstituted in water six days per week. All fish were adult hermaphrodites, aged between 133-379 days old when entering their first treatment (Mean ± SEM age: 252 ± 8.62 days old). The University of Alabama Institutional Animal Care and Use Committee approved all procedures described herein (IACUC #15-10-0111).

Salinity Preference in a Lateral Gradient

Genotype selection. Rivulus are self-fertilizing hermaphrodites and are able to produce isogenic lineages, wherein all individuals share the same genotype. The genotypes used in this study were derived from a broad geographical range, including Belize, the Bahamas, the Florida Keys, East Florida, and West Florida. Sixty-three genotypes whose field-caught progenitor (F0 generation)

was homozygous at a minimum of 31 out of 32 microsatellite loci (Avise and Tatarenkov, 2015; Tatarenkov et al., 2012) were selected for salinity preference trials. All fish used in this experiment were one (F1) or two (F2) generations removed from wild-caught progenitors. A sample of 16 genotypes from across the geographical range had animals represented in the salinity preference trials and the control trials (see details below). Separate individuals were used in the control and experimental group. The total sample size for salinity preference trials was thus 79 individual fish from 63 genotypes. Animals were selected in this way so as to maximize genetic diversity in the study. We did not have replicates of the same genotype within a treatment, thus the study focused on among-individual variation (repeatability) rather than variation among specific genotypes (heritability).

Experimental design. Lateral salinity gradients were built according to Staaland (1969) with modifications as outlined by McManus et al. (2014) using 74 L aquaria (Fig. 1). The aquarium was divided length-wise using black corrugated plastic to create two gradients per tank, each measuring 74.6 x 14.6 x 29.8 cm, hence forth referred to as half-tank. All dividers were constructed from 6.35 mm black corrugated plastic and secured using marine aquarium silicone, such that each side of the tank was completely separated from the other. Each half-tank salinity gradient consisted of five U-shaped chambers (13.8 x 14.6 x 8.8 cm) containing the experimental salinities - 5, 15, 25, 35, and 45 ppt. The salinity gradient remained stable for at least 7 days, as indicated by a low coefficient of variation for salinity measurements across an 8-day trial, even with a fish allowed to swim freely through the gradient (Table S1). The outside of the aquaria was covered with light green paper to minimize disturbance and to easily visualize fish on videos. Webcams (Logitech, Suzhou) were suspended above the aquaria to monitor the fish's location throughout the salinity preference trials. Two days before each trial, salinity concentrations of 5, 15, 25, 35, and 45 ppt were prepared using aged tap water and Instant Ocean® Aquarium Sea Salt (Spectrum Brands, Blacksburg). Salinity concentrations were then checked for accuracy using a handheld refractometer. An air stone was placed in each salinity reservoir to aid in the removal of chlorine from tap water. A pump was placed in the 45 ppt reservoir to prevent the salt from settling at the bottom; agitation from the air stones was sufficient to prevent settling in the other salinities. The temperature of the water was recorded before each trial and maintained an average of 26.8 ± 0.35 ºC.

Before beginning a trial, all salinity mixtures were checked with a handheld refractometer again for accuracy. Rubber barriers were placed on top of the dividers that were connected to the bottom of the half-tank (Fig. 1a, McManus et al., 2014). To randomize the direction of the gradient each time it was set up, a coin was flipped (heads right, tails left) to determine if the left or right side of the half-tank would contain the lowest salinity concentration. For example, if the left side

had the lowest salinity, then the chambers would increase left to right in the following fashion: 5, 15, 25, 35, 45 ppt. Then, each chamber was filled with 1.8 L of the premixed salinity. For the control group, the half-tank was filled with 25 ppt water in each chamber to evaluate chamber preference independent of salinity concentration. After all of the chambers were filled, the fish was gently placed in the center chamber (25 ppt) with a small fish net and allowed 30 minutes to acclimate to the half-tank. Following acclimation, video cameras were turned on and rubber barriers were removed. After 8 hours, cameras were turned off, and the fish was removed from the half-tank with a small net and returned to their original housing. The half-tanks were then emptied using a siphon and rinsed with fresh water. Videos were then analyzed using JWatcher 1.0 (Blumstein et al., 2006) to determine the amount of time spent per chamber and the number of transitions between chambers. All salinity preference fish were tested 3 times with 21 days between trials to minimize learning or habituation effects (the direction of the gradient was determined randomly for each trial).

Salinity preference trails were run between September and December 2015. Fish were then fed 4 ml of brine shrimp per day and monitored for egg laying. Once a fish had laid eggs they were then used for egg laying preference trials from January through June 2016, as described below. Additional fish were added to the egg laying experiment to account for those from the salinity preference experiment that never laid eggs.

Statistical analysis. The time that it took the fish to first transition out of the 25 ppt central chamber (latency to emerge) was removed for each trial to avoid biasing data by an individual's motivation. We generated an average preference score by multiplying the number of seconds spent in a chamber by the assigned chamber number (centered at 25 ppt with 1 unit difference between chambers, i.e. 5 ppt = -2, 15 ppt = -1, 25 ppt = 0, 35 ppt = 1, and 45 ppt = 2) and then dividing the result by the total number of seconds. A more strongly negative score thus indicates a preference for lower salinities, while a more strongly positive score indicates a preference for higher salinities. This score is then defined as an individual's preference within a given trial. Variance for an individual's preference scores was also calculated, which describes the strength of preference (where low variance indicates high strength of preference for a given salinity). To avoid confusion in the graphics, the negative of the variance was plotted such that higher values indicate stronger preference. The number of transitions between chambers was used calculate the transition probabilities for each individual's trial using Python 2.7 (Python Software Foundation) with code developed by one of the authors (HL, Code S2). To determine the effect of treatment on latency to emerge, preference, strength of preference, and number of transitions we used lme4 package in R (Bates et al., 2015) to conduct general linear mixed models for each variable. For each response variable in turn, we ran models with and without the fixed effect of

281 treatment (note that a random effect of Fish ID is retained in all models, equation shown for
282 preference only):
283
284 $$preference = intercept + \beta_1 \cdot treatment + Fish\ ID + e, \quad (1)$$
285 $$preference = intercept + Fish\ ID + e, \quad (2)$$
286
287 We examined the among-individual covariance structure between preference, strength of
288 preference, number of transitions and emergence latency separately for control and experimental
289 treatments using multivariate mixed models in the ASREML package in R (Butler, 2009). For
290 each treatment, two multivariate models were compared that differed in the among-individual
291 covariance structure. Both models fitted an intercept for each trait, and a trait-specific fixed effect
292 of the round of trials. Each model also included an unstructured covariance matrix for the residual
293 (co)variation between the four traits. The first multivariate model used an unstructured covariance
294 matrix (indicated in equation 3 below as *us:FishID*), enabling the partitioning of all among-
295 individual variances and covariances between the four response traits. The second multivariate
296 model was constrained such that there was no among-individual covariance (indicated in
297 equation 4 as *idh:FishID*) as follows:
298
299 $$preference, strength, transitions, emergence = intercept + \beta_1 \cdot round + us: Fish\ ID + us: e, \quad (3)$$
300 $$preference, strength, transitions, emergence = intercept + \beta_1 \cdot round + idh: Fish\ ID + us: e, \quad (4)$$
301
302 The models were then compared using log-likelihood test to determine if there was any evidence
303 for among-individual correlation structure. This was done following Houslay and Wilson (2017),
304 where a chi-square value was calculated as -2*(Log Likelihood of Model 1 - Log Likelihood of
305 Model 2) to determine whether among-individual correlation structure existed. To determine
306 significance of pairwise correlations, we calculated a z score (estimate/SE), where z scores >
307 |1.96| were considered significant. However, we were unable to estimate the among-individual
308 correlations in the control treatment as only one trait (number of transitions) had any measurable
309 among-individual variation.
310
311 To determine repeatability, we then used the ASREML package in R (Butler, 2009) to run general
312 linear mixed models (GLMM) with treatment (gradient vs. control [just 25 ppt]) and round (first,
313 second, or third trial) as fixed effects and Fish ID as a random effect. A separate model was run
314 for each dependent variable - preference, strength of preference, latency to emerge from the
315 acclimation chamber, and transitions between chambers. Both latency to emerge and transition
316 variables were log transformed to achieve normality of model residuals. To determine if any of the
317 behavioral responses were repeatable, two models were run for each response in each treatment

condition separately to parse the variance into total variance, among-individual variance, and within-individual (residual) variance as follows (shown for preference only):

$$preference = intercept + \beta_1 \cdot round + e, \quad (5)$$

$$preference = intercept + \beta_1 \cdot round + Fish\ ID + e, \quad (6).$$

The variance components from each model were then compared to determine whether behavioral responses were repeatable. This was done following Houslay and Wilson (2017), where a chi-square value was calculated as -2*(Log Likelihood of Model 1 - Log Likelihood of Model 2) to determine whether among-individual variance (random effect of Fish ID) was significant. The pin function in the nadiv package (Wolak et al., 2012) was then used to calculate the adjusted repeatability by dividing among-individual variance by the sum of among-individual and residual variance; this function also provided the standard error for the repeatability estimate.

Egg Laying Preference

Genotype selection. A group of 67 individuals were selected as experimental fish, and an additional 33 were selected as control fish. These animals were derived from fifty-three genotypes, all of which were also represented in the Salinity Preference study. Field-caught progenitors were homozygous at a minimum of 31 out of 32 microsatellite loci (Avise and Tatarenkov, 2015; Tatarenkov et al., 2012). All fish used in this experiment were F1 or F2 generation and laid viable, fertilized eggs (i.e., perivitelline space present) prior to the trial to ensure that they were reproductively active and capable of effective self-fertilization.

Experimental design. We modified the lateral salinity gradient for egg laying by adding to each chamber Poly-Fil fiber situated at the air-water interface as an egg laying substrate. Fish were placed in the gradient for two weeks to lay eggs. After one week, the fish was removed from the gradient and placed into a 1.2 L Rubbermaid® container of the same salinity as the chamber they were located in at the time of capture; this allowed us to check the chambers and Poly-Fil for eggs. The gradient was then emptied and refilled. Once the gradient was re-established, the fish was returned to the chamber in which it was located prior to the egg check. The location and number of eggs per chamber were recorded. At the end of the second week, fish were returned to their original housing area and the gradient and Poly-Fil were checked again for eggs. While in the gradient, fish were fed by adding 2 ml of brine shrimp to each chamber daily (so as to avoid chamber preferences associated with food). All eggs were stored in containers with the same salinity as they were found in until hatching. To determine hatchling success, eggs were checked

daily to record the date of hatching and received weekly water changes with the same salinity in which they were laid.

Statistical analysis. The presence or absence of an egg in each chamber was used for our analysis so as to not bias a particular salinity if a fish laid multiple eggs in a single chamber. This is important because the number of eggs laid by each individual was highly variable, ranging from 0 to 20 eggs across the two week period. Using the lme4 package in R (Bates et al., 2015), we ran a GLMM to test egg laying preference in a salinity gradient. In the following model, treatment refers to either the salinity gradient or the control where all chambers were filled with 25 ppt, while chamber refers to the location the egg was laid. To account for a possible edge effect (Fig. 4b) we included whether a given chamber was an 'edge' in our model. Additionally, because the gradient remained stable for only one week, there were two egg checks for each fish during the two-week period. Thus, we included 'time' as a fixed effect to account for any variance in egg-laying between the two egg-checking periods but, because 'time' itself was not central to the hypotheses that we were testing, we did not include its interactions with other fixed effects.

$$eggs = intercept + \beta_1 \cdot treatment + \beta_2 \cdot chamber + \beta_3 \cdot treatment\ x\ chamber + \beta_4 \cdot edge + \beta_5 \cdot treatment\ x\ edge + \beta_4 \cdot time + Fish\ ID + e, \quad (7).$$

To determine the effects of salinity on hatching (yes or no), a generalized linear mixed model with a binomial distribution and logit link function was used as follows with parent ID as a random effect:

$$hatched = intercept + \beta_1 \cdot salinity + Parent\ ID + e, \quad (8).$$

Comparisons between the salinities were then made by least squares means independent contrasts.

Results

Salinity preference. When in a salinity gradient, mangrove rivulus showed a significant preference for lower salinities (Table 1, Fig. 2a, b). In the control group, where there was no salinity gradient, individuals spent more time in chambers at each edge. Strength of preference was significantly higher in the salinity gradient than in the control (Table 1, Fig. 2c). There was no difference between experimental and control groups in the total number of transitions between chambers (Table 1, Fig. 3) or in latency to emerge from the central chamber at the start of the trial (Table 1). For the experimental group, strength of preference, number of transitions, and latency to emerge

were significantly repeatable, with between 38-53% of the total behavioral variance being attributed to among-individual differences (Table 2). Preference was not repeatable in the experimental group (Table 2). In the control group, only the number of transitions between chambers was repeatable, with ~40% of the behavioral variance being attributed to among-individual differences (Table 2). Multivariate model comparisons of among-individual correlation structures showed strong among-individual correlation structure in experimental treatment (χ^2_6 = 26.698, p = 0.0001; Table 3) but not control (χ^2_6 = 0.021, p = 0.999).

Egg laying preference. When given the opportunity to lay eggs along a salinity gradient, individuals laid eggs with greater frequency in lower salinities (Fig. 4). There was no significant overall effect of treatment (χ^2 = 0.07, P = 0.79, df = 1) or time (χ^2 = 0.20, P = 0.66, df = 1), but there was a significant main effect of chamber (χ^2 = 16.6, P = 0.002, df = 4), which was treatment-dependent (treatment x chamber: χ^2 = 9.45, P = 0.05, df = 4). In the control group, individuals were more likely to lay eggs in the edge chambers compared to the central chambers as indicated by a significant chamber effect (χ^2 = 12.82, P = 0.012, df = 4) and *a priori* contrasts (Table 4); these results correspond to the edge effect that was observed in the salinity preference experiment, conducted on a separate set of individuals. Individuals in the experimental group were more likely to lay eggs in 5 ppt than in any other salinity, with a significant salinity effect (χ^2 = 13.26, P = 0.01, df = 4), and significant contrasts between 5 ppt and all other salinities (Table 4). Additionally, there was a significant effect of salinity on hatching (χ^2 = 13.99, P = 0.0013, df = 3, Fig. 5). Eggs laid in the lowest salinity had a significantly higher probability of hatching that those laid at higher salinities (5 ppt vs 15 ppt: χ^2 = 3.59, P = 0.058, df = 3; 5 ppt vs 25 ppt: χ^2 = 4.14, P = 0.04, df = 3; 5 ppt vs 35 ppt: χ^2 = 7.60, P = 0.005, df = 3 ,and 5 ppt vs all other salinities: χ^2 = 10.40, P = 0.001, df = 3).

Discussion

The ability to exist and be phenotypically flexible in a variable environment can come with significant costs (Piersma and Drent, 2003). In aquatic habitats, that cost is often the energy devoted to osmoregulation (Boeuf and Payan, 2001). By investigating salinity preferences and repeatability of those preferences, we can gain insight into whether these traits might evolve in response to natural selection (Boake, 1989). In addition, salinity preferences provide clues into the habitats in which individuals' fitness might be highest. We initially hypothesized that rivulus would prefer to occupy salinities of 25 ppt and that their preference would be both repeatable and would align with field observations (as they are most often found at 25 ppt). Additionally, we hypothesized that rivulus would prefer to lay eggs in 25 ppt and that hatching success would be highest in 25 ppt. Our findings support the hypothesis that rivulus exhibit salinity preferences,

both in terms of where they spend their time and where they lay their eggs. However, in a laboratory environment, free of anything that might constrain their movement (e.g., predators, competitors, physical factors such as temperature), rivulus preferred to occupy salinities below 25 ppt and laid eggs with greatest frequency in 5 ppt. Hatching success also was highest at 5 ppt and decreased precipitously as salinity increased. Moreover, salinity preference was not repeatable, but the strength of preference and latency to emerge were repeatable in the salinity gradient.

We investigated repeatability because the opportunity for selection to drive the evolution of behavior in environments with salinity microhabitats hinges on there being considerable variation among individuals (Boake, 1989; Wolak et al. 2012). Given that the repeatability of a trait is, arguably, the upper bound of its heritability (Boake, 1989; Falconer and Mackay, 1996; but see Dohm, 2002), it is likely that strength of preference and latency to emerge, both of which showed high repeatabilities in the experimental group, could evolve in response to selection. This might be especially true in highly heterogeneous habitats, which can reveal consistent among-individual differences in behavior. These findings are notable because environments with microhabitat options (experimental group) exposed behavioral variation among individuals that was not present in environments with only one option (control group). Given that mangrove environments are replete with microhabitat variation, we expect that such variation would be available in wild rivulus populations for natural selection to act upon. If among-individual differences are underlain by genetic variation, strong selection on exploratory behavior might drive phenotypic divergence between populations with different degrees of microhabitat variation. Due to changes in the influx of both freshwater and saltwater to coastal systems owing to climate change, which is likely to alter microhabitat structure, mangrove forests might provide a unique opportunity to catalog the evolution of behavioral and physiological responses to changing salinity niches (Brennan et al., 2015). For example, when high spatiotemporal variation in salinity occurs, individuals that tend not to explore and have a strong preference for a specific salinity could have reduced fitness compared to those that quickly seek new microhabitats and show a relatively weak preference for a particular salinity.

Rivulus were equally active in control and experimental groups; in each treatment, individuals explored the full experimental apparatus and transitioned between chambers a similar number of times. The number of transitions was repeatable in each group, indicating consistent among-individual differences in activity levels and/or willingness to explore an unfamiliar area. These findings are consistent with Edenbrow and Croft (2012) who showed exploration within a maze to be repeatable in rivulus. However, the pattern of movement was different between control and experimental groups; in the latter, more transitions were made in the direction of lower salinity

chambers. While individuals in the experimental group varied in their activity levels, movement was concentrated in the lower salinities (25 ppt and below, Fig. 3). This significantly reduced among-individual variance in the number of chambers visited during a given trial and resulted in low repeatability for salinity preference in animals exposed to the salinity gradient. The control group also showed very low repeatability for chamber preference in the absence of salinity variation but likely for a different reason; in that group, the vast majority of the variance was within rather than among individuals (Table 2, Fig. 3), indicating that individuals are inconsistent in the chambers they visit most often from trial to trial.

Edenbrow and Croft (2011) also showed significant variation in the expression of behavior among ages and genotypes, indicating some context dependence. It has been previously documented that both abiotic and biotic factors (e.g. temperature, food availability, predation) can influence behavior and behavioral consistency (Nussey et al., 2007; Bell et al., 2009; Edenbrow and Croft, 2013), which was observed in our repeatability analysis for latency to emerge. The time it took rivulus to emerge from the central chamber was not repeatable in the control but was highly repeatable in the salinity gradient treatment (see also Kluen and Brommer, 2013). Strong repeatability for latency to emerge in the experimental group was due to the fact that, when faced with a salinity gradient, some fish sampled their environment quickly and others more slowly. Without the gradient (i.e., controls, all chambers 25 ppt), consistent among-individual differences in latency to emerge disappeared.

Within the experimental group, strength of preference differed consistently among individuals, which could reflect variation in the ability to flexibly adjust physiology along a salinity gradient. Some fish spent the majority of their time in one of the few low salinity chambers, while others transitioned between chambers with salinities ranging from 5 to 25 ppt. Such differences might depend on the individuals' physiology and capacity to respond to the challenges of osmoregulation in fluctuating salinity conditions. Fish rely on multiple structures (gills, gut, kidney; Edwards and Marshall, 2012) for ion and water exchange with their environment. Some individuals could be more efficient at regulating changes in chloride cell function within the gills, aquaporin expression in the intestine, or glomerular filtration rates (Edwards and Marshall, 2012; Cutler and Cramb, 2002). This opens the possibility to explore empirically how individuals with low versus high strength of preference cope with living in different salinities, and whether among-individual differences in physiological flexibility are underlain by genetic variation.

Adult rivulus are most often found at 25 ppt in the wild but we found strong preferences for lower salinities under controlled laboratory conditions. It is also relatively rare to find eggs or hatchlings in the wild (Taylor, 1990; Taylor, 2012). While adult rivulus can clearly tolerate salinities ≥ 25 ppt,

their preference for lower salinities indicates that they are found most often in salinities that are suboptimal, i.e., where they are likely to incur physiological costs of osmoregulation. Based on the findings of this study, our inability to find eggs in the wild is likely due to individuals selecting areas of lower salinity for egg laying and then returning to areas of higher salinity to possibly avoid predators and competitors. Rivulus will actively navigate their microhabitat options via swimming, but also have additional means of exploring their environment. Rivulus can traverse land by terrestrial tail-flip jumping and can survive out of water, as long as it is moist, for 66 days (Taylor, 1990; Pronko et al., 2013; Styga et al., 2017). With a greater ability to explore their environment via terrestrial tail-flip jumping, and having a broad tolerance to many factors that make the mangrove ecosystem a hostile environment for many fish, rivulus is able to take advantage of the many variable microhabitats available in this system (Taylor, 2012). We observed rivulus navigating to low salinities to lay eggs and these eggs had the highest hatching success at 5 ppt (Figs. 4 and 5). The developmental environment can not only affect survival but also the resulting phenotype due to the increased energy demands of osmoregulation (Brown et al., 2012). Independent of energy requirements, developmental plasticity in response to salinity might also change the phenotype in adaptive or non-adaptive ways (West-Eberhard, 2003; Albecker and McCoy 2019). An important area of future research might thus be to examine the extent to which exposure to different salinities early in life might drive physiological, behavioral, and morphological variation.

Coordinated behavioral responses to environmental cues were evident in this study. Some among-individual correlations were expected. For example, if some individuals consistently took longer to emerge than others, they then had less time to explore the apparatus, leading to a negative among-individual correlation between latency to emerge and the number of transitions. There was also a negative among-individual correlation between number of transitions and strength of preference (negative of the variance, such that high values indicate higher strength of preference; Figure 2); individuals that had strong preferences made fewer transitions. The positive but not statistically significant among-individual correlation between strength of preference and latency to emerge indicates that more 'cautious' individuals (those that take longer to emerge) also tend to find their preferred salinity and stay there. There was only among-individual correlation structure in the experimental treatment, and this structure remained consistent over time, perhaps representing a behavioral syndrome (Sih et al., 2004). In this context, the syndrome reflects a gradient of exploratory phenotypes. On one end are individuals that are quick to explore novel environments, actively move through the area, and exhibit weak preferences. On the other end are individuals that take a more restrained approach to novel environments, move around less, and exhibit strong preferences. We used genotypes from across rivulus' expansive geographic range, leaving two primary explanations for the behavioral

variation that we observed: i) individuals were derived from populations under divergent selection, (e.g., those with and without significant microhabitat variation) and we used a representative sample of genotypes from these areas; and/or ii) mangroves exhibit considerable spatiotemporal variation in microhabitat characteristics and/or stability such that fluctuating selection maintains behavioral variation within populations.

Some facets of our experimental treatment (whether the exposure to a salinity gradient or increased microhabitat heterogeneity) revealed consistent differences among individuals that were not present in a uniform environment (control). When phenotypic variation emerges as a result of microhabitat heterogeneity, it suggests variation among individuals in phenotypic flexibility across environments (i.e., slope of the reaction norm). If this emergent variation is heritable, there should be increased opportunity for selection to drive evolutionary change in heterogeneous environments.

In addition to present-day conditions, the evolutionary history of a species can impact how they will respond to future selection (Crowley et al., 2019). Species with a previous history of inhabiting variable environments should be able to respond to the changing environment appropriately given that habitat selection can have significant fitness consequences. When only considering osmoregulation demands, aquatic species that inhabit brackish environments should select microhabitats in which they are isotonic and where the metabolic cost of osmoregulation is minimal (Boeuf and Payan, 2001; Sutton et al. 2018). Based on our field observations indicating that rivulus are most frequently observed at salinities close to 25 ppt, and the fact that rivulus' isotonicity point is nearer to 15 ppt (Frick and Wright, 2002; and Bielmyer et al. 2012), it does not appear that rivulus are occupying ideal habitats in the wild. This could be because rivulus are excluded from lower salinity microhabitats by competitors or predators. Other factors also could impact their ability to osmoregulate efficiently (e.g., diet, temperature, dissolved oxygen) such that inhabiting an area of higher salinity may result in higher fitness (Hammerschlag, 2006). More work is needed to: i) identify the possible abiotic and biotic factors that exclude rivulus from their preferred salinity in the wild; ii) understand the physiological costs associated with occupying higher salinities and; iii) understand whether physiological differences among genotypes might explain among-individual variation in strength of preference.

Funding:
This study was supported by a Grants-in-Aid of Research from the Society of Integrative and Comparative Biology.

Acknowledgements:

We would like to thank Sarah Blackwell, Brady Hudson, Marly Lowery, Mack Padgett, Judson Russell, and Erin Yepsen for their assistance.

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Table 1. Summary of general linear mixed models for salinity preference, strength of preference (negative of the variance), transitions between chambers, and latency to emerge from the central chamber. Statistically significant P-values are indicated in bold.

	Preference			**Strength**			**Transitions**			**Latency to Emerge**		
	β ± SE	X^2_1	*P*	*β ± SE*	X^2_1	*P*	*β ± SE*	X^2_1	*P*	*β ± SE*	X^2_1	*P*
Treatment effect	-0.721 ± 0.161	18.115	**0.000**	0.306 ± 0.083	12.677	**0.000**	-0.138 ± 0.199	0.499	0.480	0.251 ± 0.345	0.540	0.462
Round	-0.007 ± 0.076	0.009	0.927	-0.123 ± 0.030	16.376	**0.000**	-0.133 ± 0.060	4.838	**0.028**	0.074 ± 0.090	0.681	0.409
	x̄ ± SE			*x̄ ± SE*			*x̄ ± SE*			*x̄ ± SE*		
Experimental	-0.392 ± 0.072 (score) ~21.05 ± 3.866 (ppt)			-0.982 ± 0.031			30.296 ± 1.796			46.196 ± 7.346		
Control	0.329 ± 0.139			-1.291 ± 0.070			37.896 ± 4.538			19.051 ± 3.264		
	Median			Median			Median			Median		
Experimental	-0.478 (score) 20.194 (ppt)			-1.028			26			771.5		
Control	0.391 (score)			-1.445			32.5			809.5		

Table 2. General linear mixed model by residual maximum likelihood for repeatability of preference, strength of preference, transitions between chambers, and latency to emerge from the central chamber. Repeatability estimates reported as 0.000±0 were very small, e.g., all $R < 7.132 \times 10^{-7}$. Statistically significant P-values are indicated in bold.

	Preference			**Strength**			**Transitions**			**Latency to Emerge**		
	V ± SE			*V ± SE*			*V ± SE*			*V ± SE*		
$V_{individual}$ Experimental	0.057 ± 0.074			0.062 ± 0.0182			0.379 ± 0.105			1.374 ± 0.327		
$V_{residual}$ Experimental	0.843 ± 0.112			0.099 ± 0.0131			0.534 ± 0.070			1.226 ± 0.157		
	R ± SE	X^2_1	*P*	*R ± SE*	X^2_1	*P*	*R ± SE*	X^2_1	*P*	*R ± SE*	X^2_1	*P*
Repeatability Experimental	0.064 ± 0.08	0.643	0.211	0.383 ± 0.08	21.377	**0.000**	0.415 ± 0.08	27.551	**0.000**	0.529 ± 0.07	47.724	**0.000**
	V ± SE			*V ± SE*			*V ± SE*			*V ± SE*		
$V_{individual}$ Control	0.000* ± 0.000*			0.000* ± 0.000*			0.348 ± 0.197			0.000* ± 0.000*		
$V_{residual}$ Control	0.959 ± 0.202			0.203 ± 0.043			0.532 ± 0.137			1.381 ± 0.291		
	R ± SE	X^2_1	*P*	*R ± SE*	X^2_1	*P*	*R ± SE*	X^2_1	*P*	*R ± SE*	X^2_1	*P*
Repeatability Control	0.000*	0.000	0.500	0.000*	0.000	0.500	0.395 ± 0.16	6.351	**0.006**	0.000*	0.000	0.500

* Estimates reported at 0.000 denote instances where the among-individual variance estimate was bound at the edge of allowable parameter space and, as a consequence, no SE is estimated when using ASReml.

Table 3. Multivariate among individual correlation structure across all rounds in lateral salinity gradient for experimental treatment.

Variable	By Variable	*r*	*SE*	*Z score*
Transitions	Preference	-0.800	0.350	**-2.287**
Emerge	Preference	1.061	0.384	**2.763**
Emerge	Transitions	-0.496	0.132	**-3.748**
Strength	Preference	0.210	0.361	0.582
Strength	Transitions	-0.616	0.127	**-4.846**
Strength	Emerge	0.308	0.193	1.597

Z scores >|1.96| are significant and indicated in bold.

Table 4. Differences in the probability of laying eggs between chambers in control and salinities in experimental groups. All analyses have df=1. Significant *a priori* contrasts (P<0.05) are shown in bold; contrast that approach significance (P<0.07) are in italics.

Control			**Experimental**		
Chamber Comparison	χ^2	*P*	Salinity (ppt) Comparison	χ^2	*P*
1 vs. 2	2.32	0.127	5 vs. 15	*3.74*	*0.053*
1 vs. 3	**8.74**	**0.003**	5 vs. 25	**5.50**	**0.019**
1 vs. 4	*3.37*	*0.066*	5 vs. 35	**7.74**	**0.005**
1 vs. 5	0.05	0.827	5 vs. 45	**10.59**	**0.001**
2 vs. 3	2.13	0.144	15 vs. 25	0.15	0.698
2 vs. 4	0.04	0.843	15 vs. 35	0.72	0.721
2 vs. 5	2.33	0.127	15 vs. 45	1.81	0.179
3 vs. 4	1.30	0.253	25 vs. 35	0.17	0.676
3 vs. 5	**8.75**	**0.003**	25 vs. 45	0.86	0.353
4 vs. 5	*3.37*	*0.066*	35 vs. 45	0.23	0.634

Figure 1. Side view of salinity gradient tank with (A) and without (B) barriers (gray squares) in place. Rubber barriers were used when filling the tank to prevent mixing.

Figure 2. Average time spent in each salinity (a), preference (b), and strength of preference (c) in control and experimental treatments. For preference, the scores have been converted to ppt for ease of interpretation and strength of preference is graphed as the negative of the variance such that higher scores for strength (= 1/lower variance) indicates a stronger preference.

Figure 3. Probability of transitioning from one chamber to the next, probabilities derived from the number of transitions between each chamber by each fish. Wider arrows indicate greater likelihoods of transitioning between adjacent chambers, and the arrowhead indicates the direction of transition. Actual transition probabilities are associated with their respective arrows.

Figure 4. The number of fish that laid eggs in each chamber for the A) control and B) experimental groups; some fish laid eggs in multiple chambers. In the control group each chamber contained 25 ppt. 32 of 67 experimental fish laid eggs and 18 of 33 control fish laid eggs while in the gradient.

Figure 5. Hatching success at each salinity in the experimental group, where fish had the option of laying eggs in any of the five salinities.

Figure 1.

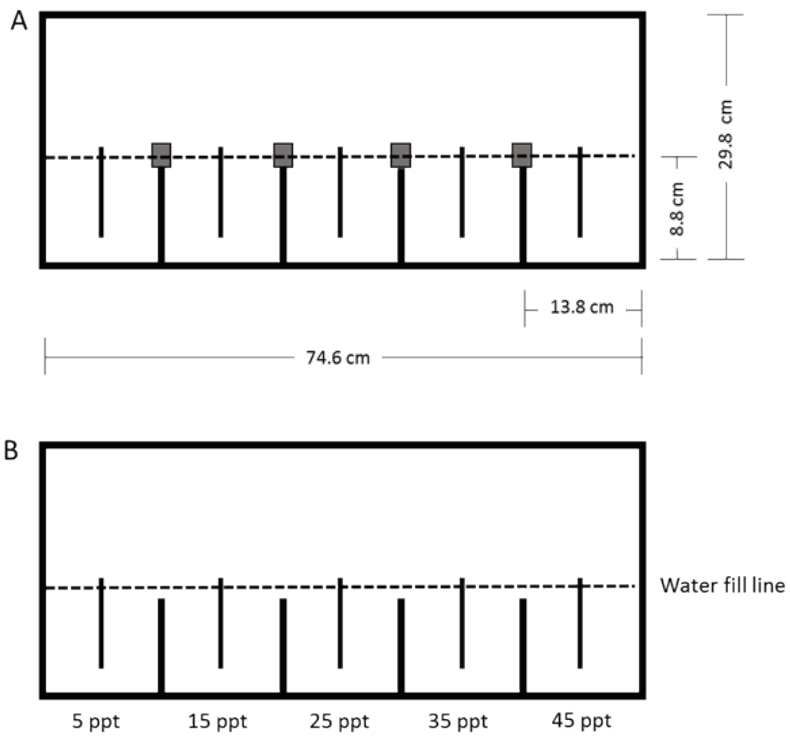

A
29.8 cm
8.8 cm
13.8 cm
74.6 cm
B
Water fill line
5 ppt
15 ppt
25 ppt
35 ppt
45 ppt

Figure 2.

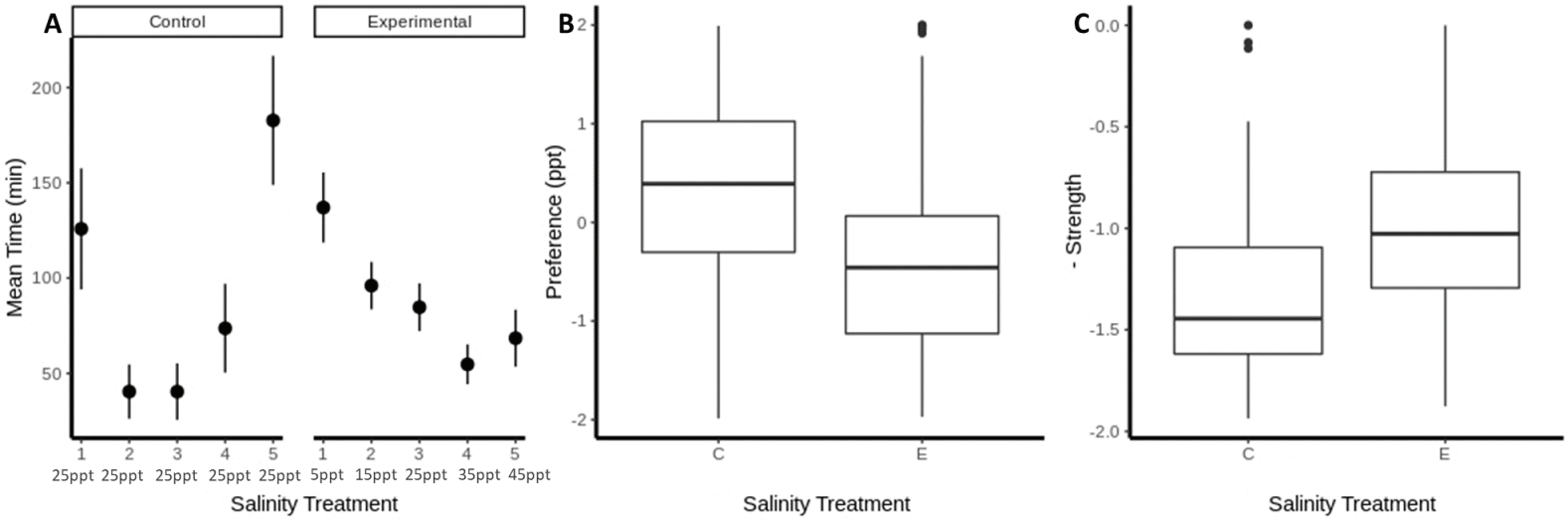

A
Control
Experimental
Mean Time (min)
200
150
100
50
1 2 3 4 5
25ppt 25ppt 25ppt 25ppt 25ppt
1 2 3 4 5
5ppt 15ppt 25ppt 35ppt 45ppt
Salinity Treatment
B
Preference (ppt)
2
1
0
-1
-2
C
E
Salinity Treatment
C
- Strength
0.0
-0.5
-1.0
-1.5
-2.0
C
E
Salinity Treatment

Figure 3.

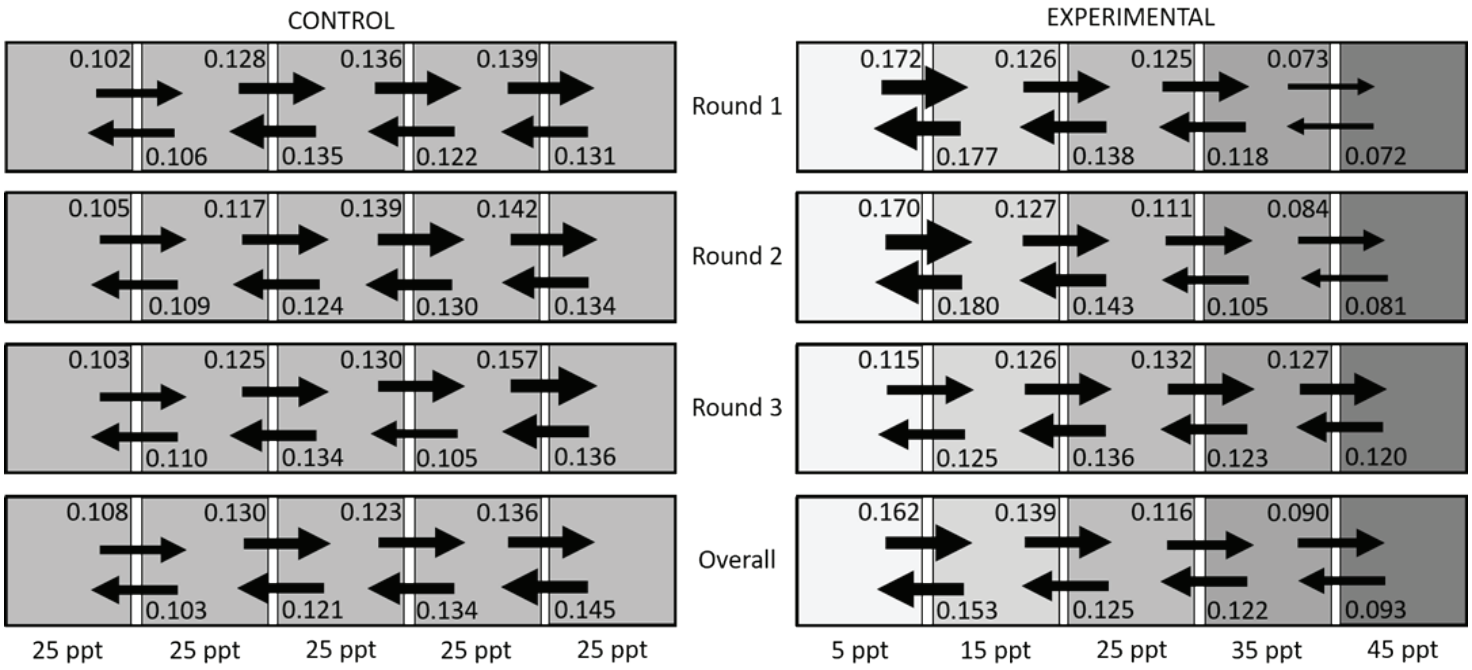

CONTROL
EXPERIMENTAL
0.102
0.128
0.136
0.139
0.106
0.135
0.122
0.131
Round 1
0.172
0.126
0.125
0.073
0.177
0.138
0.118
0.072
0.105
0.117
0.139
0.142
0.109
0.124
0.130
0.134
Round 2
0.170
0.127
0.111
0.084
0.180
0.143
0.105
0.081
0.103
0.125
0.130
0.157
0.110
0.134
0.105
0.136
Round 3
0.115
0.126
0.132
0.127
0.125
0.136
0.123
0.120
0.108
0.130
0.123
0.136
0.103
0.121
0.134
0.145
Overall
0.162
0.139
0.116
0.090
0.153
0.125
0.122
0.093
25 ppt
25 ppt
25 ppt
25 ppt
25 ppt
5 ppt
15 ppt
25 ppt
35 ppt
45 ppt

Figure 4.

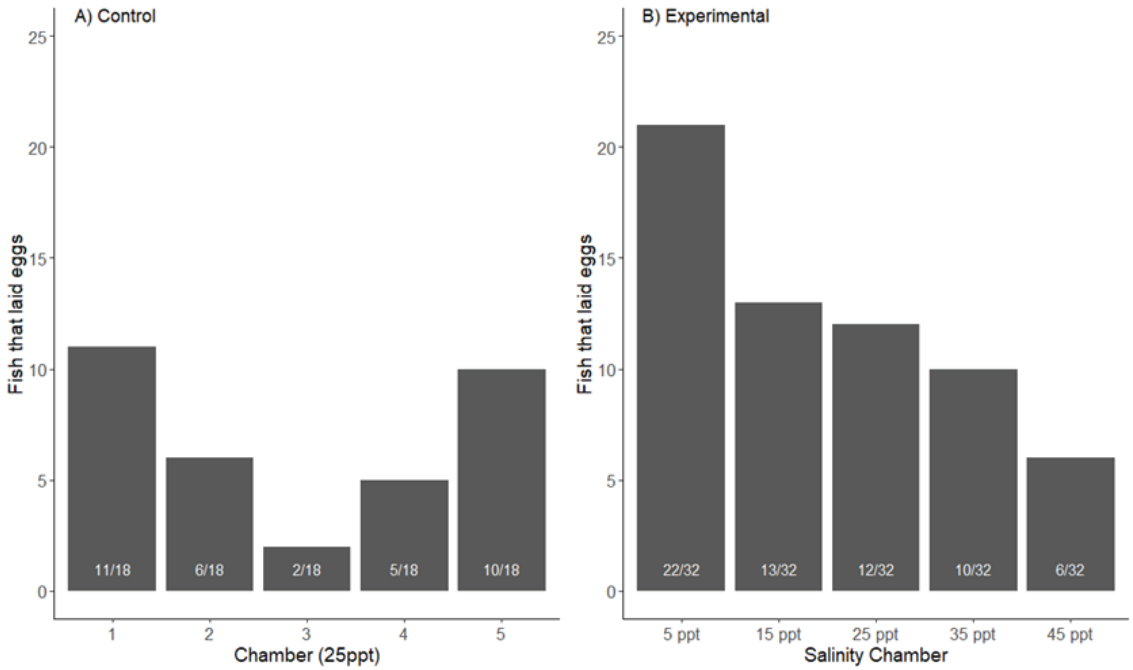
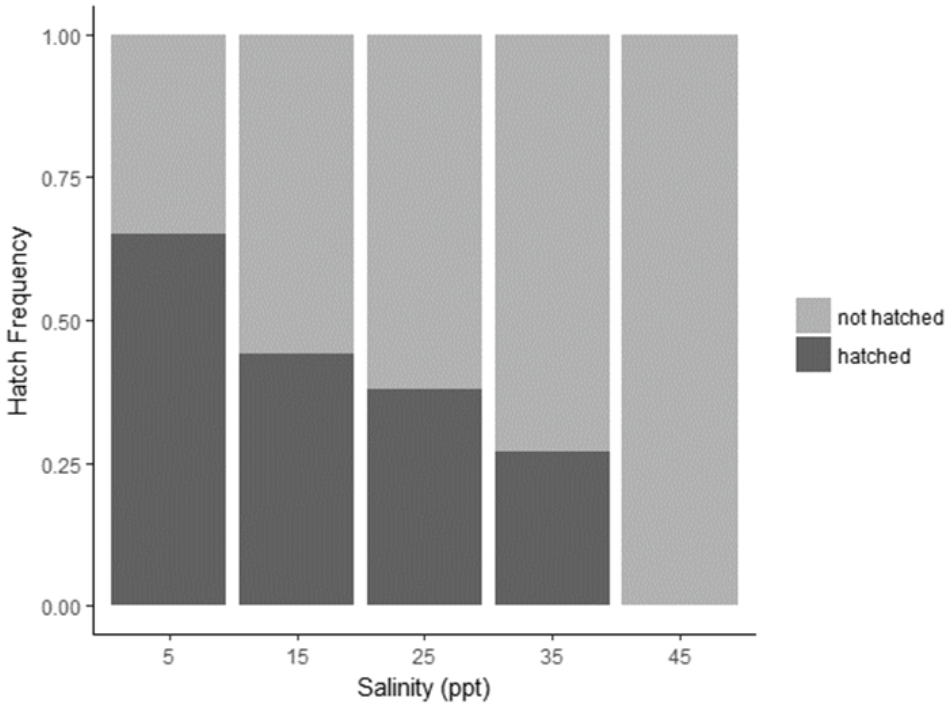

A) Control
Fish that laid eggs
25
20
15
10
5
0
11/18
6/18
2/18
5/18
10/18
1
2
3
4
5
Chamber (25ppt)
B) Experimental
Fish that laid eggs
25
20
15
10
5
0
22/32
13/32
12/32
10/32
6/32
5 ppt
15 ppt
25 ppt
35 ppt
45 ppt
Salinity Chamber

Figure 5.


1.00
0.75
0.50
0.25
0.00
Hatch Frequency
5
15
25
35
45
Salinity (ppt)
not hatched
hatched

Supplementary Materials

Table S1. Daily salinity gradient measurements with a fish freely swimming through.

	Side A						Side B				
Day	**5 ppt**	**15 ppt**	**25 ppt**	**35 ppt**	**45 ppt**		**5 ppt**	**15 ppt**	**25 ppt**	**35 ppt**	**45 ppt**
1	5	15	25	35	45		5	15	25	35	45
2	5	15	25	35	45		5	15	25	34	45
3	5	13	24	34	46		5	16	24	35	45
4	6	13	23	32	45		5	16	25	35	45
5	6	15	24	34	45		6	15	24	35	46
6	6	15	25	34	46		6	17	26	36	45
7	6	15	25	34	45		6	17	25	35	45
8	6	15	25	36	45		7	18	26	36	44
Average	5.63	14.50	24.50	34.25	45.25		5.625	16.13	25.00	35.13	45.00
Std. Dev.	0.52	0.93	0.76	1.17	0.46		0.74	1.13	0.76	0.64	0.54
CV (%)	9.20	6.39	3.09	3.40	1.02		13.23	6.98	3.02	1.83	1.19
		BOTH Sides		**5 ppt**	**15 ppt**	**25 ppt**	**35 ppt**	**45 ppt**			
		Average		5.63	15.31	24.75	34.69	45.13			
		Std. Dev.		0.62	1.30	0.78	1.01	0.41			
		CV (%)		11.00	8.50	3.13	2.93	0.92			

Supplementary material 2. Representative raw data and Python Code for Transition Probabilities

Representative .dat file from JWatcher for experimental fish in round 1

* bold, italicized text is not part of JWatcher output; they are notes from the authors

FirstLineOfData=25
#---
Name: E24E25 rd 1.mp4.dat
Format: Focal Data File 1.0
Updated: Wed Dec 16 09:29:07 CST 2015
#---
FocalMasterFile=/Users/Dropbox/salinity preference share/Salinity_Preference.fmf
# Observation started: Wed Dec 16 08:46:46 CST 2015
StartTime=1450277206472
# Observation stopped: Wed Dec 16 09:16:46 CST 2015
StopTime=1450279006475

Answer.1=1
Answer.2=BP11
Answer.3=left

#BEGIN DATA ***(formatted as "Time in milliseconds", "Chamber in which the fish was located")***
1705, 3
83631, 2
84881, 1
169944, 2
170421, 3
171293, 4
171573, 5
172381, 4
241859, 3
242476, 2
257203, 3
257587, 4
257939, 5
580470, 4
580998, 3
628708, 2
691299, 1
693667, 2
768785, 1
894686, 2
896822, 3
1045659, 2
1047178, 1
1048603, 2
1151016, 3
1156504, 4
1156825, 5
1160535, 4
1161394, 3
1296230, 2
1296836, 1
1308540, 2
1332107, 3
1333219, 4
1334454, 3
1336963, 2
1338045, 3
1354114, 2
1376527, 1
1478398, 2
1510931, 1
1676727, 2
1703110, 3
1713734, 4

1713896, 5
1718622, 4
1722014, 5
1727679, 4
1727958, 3
1789268, 2
1789852, 1
1792908, 2
1800003, 1
1800003, EOF ***(EOF = end of file)***

Python code for calculating transition probabilities (code generated by Huanda Lu)

```
# -*- coding: utf-8 -*-
#!/usr/bin/python
"""
Created on Wed Feb 3 09:08:59 2017

@author: Huanda Lu
huandalu@gmail.com
"""
import os
import numpy as np

data_base_dir = "./data"
MAX_ROUND = 3
behavior_code_map={
'1':0,
'2':1,
'3':2,
'4':3,
'5':4,
}

behavior_code_list=['1','2','3','4','5']

def get_seq(data_file):
    sample_time_list = []
    behavior_code_seq = []
    f = open(data_file)
    start_line_no = 99999
    line_no = 0
    for line in f:
        line_no = line_no + 1
        if line_no == 1:
            items = line.split("=")
            if len(items) == 2:
                start_line_no = int(items[1])
        else:
            if line_no >= start_line_no:
                line = line.replace('\n','')
                line = line.replace('\r','')
                line = line.replace('\t',',')
                line = line.replace('"','')
                items = line.split(",")
                sample_time = int(items[0])
                behavior_code = items[1]
                behavior_code = behavior_code.replace(' ','')
                if behavior_code != "EOF" and behavior_code != 'e':
                    sample_time_list.append(sample_time)
                    behavior_code_seq.append(behavior_code)
    f.close()
    return sample_time_list,behavior_code_seq


def is_valid_behavior_code(behavior_code_seq):
    for behavior_code in behavior_code_seq:
```

```
        if behavior_code not in behavior_code_map:
            return False
    return True

def gen_prob_matrix(tag,output_dir):
    data_dir = '%s/%s' %(data_base_dir,tag)
    for round_i in range(1,MAX_ROUND + 1):
        round_data_dir = "%s/round %d" %(data_dir,round_i)
        trans_prob_matrix = np.zeros(shape=(len(behavior_code_map),len(behavior_code_map)), dtype=float, order='F')
        prob_matrix = np.zeros(shape=(len(behavior_code_map),1), dtype=float, order='F')

        for file_name in os.listdir(round_data_dir):
            if os.path.splitext(file_name)[1] != '.dat':
                continue
            info = "Processing %s/%s" %(round_data_dir,file_name)
            print info
            data_file = "%s/%s" %(round_data_dir,file_name)
            sample_time_list,behavior_code_seq = get_seq(data_file)
            if is_valid_behavior_code(behavior_code_seq):
                for t in range(0,len(behavior_code_seq)-1):
                    trans_prob_matrix[behavior_code_map[behavior_code_seq[t]],behavior_code_map[behavior_code_seq[t+1]]] =
trans_prob_matrix[behavior_code_map[behavior_code_seq[t]],behavior_code_map[behavior_code_seq[t+1]]] + 1
                for t in range(0,len(behavior_code_seq)):
                    prob_matrix[behavior_code_map[behavior_code_seq[t]],0] = prob_matrix[behavior_code_map[behavior_code_seq[t]],0] + 1


        trans_count_matrix_file =  "%s/trans_count_%s_round_%d.csv" %(output_dir,tag,round_i)
        np.savetxt(trans_count_matrix_file,trans_prob_matrix,delimiter=',',fmt='%d')

        count_matrix_file =  "%s/count_%s_round_%d.csv" %(output_dir,tag,round_i)
        np.savetxt(count_matrix_file,prob_matrix,delimiter=',',fmt='%d')

        total_count = np.sum(prob_matrix)
        for i in range(0,len(behavior_code_map)):
            if np.sum(trans_prob_matrix[i,]) > 0:
                trans_prob_matrix[i,] = trans_prob_matrix[i,]/np.sum(trans_prob_matrix[i,])
            if total_count > 0:
                prob_matrix[i,0] = prob_matrix[i,0]/total_count

        trans_prob_matrix_file = "%s/trans_prob_%s_round_%d.csv" %(output_dir,tag,round_i)
        np.savetxt(trans_prob_matrix_file,trans_prob_matrix,delimiter=',',fmt='%f')
        prob_matrix_file = "%s/prob_%s_round_%d.csv" %(output_dir,tag,round_i)
        np.savetxt(prob_matrix_file,prob_matrix,delimiter=',',fmt='%f')


if __name__ == '__main__':
    result_dir = "./result"
    if not os.path.exists(result_dir):
        os.makedirs(result_dir)

    gen_prob_matrix('control',result_dir)
    gen_prob_matrix('experimental',result_dir)
```