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.

60   Introduction

61

62   An animal's survival and reproductive success depend on its ability to either operate in

63   variable environments or relocate when conditions become suboptimal. The decision to stay or

64   leave is ultimately based on which option maximizes the animal's fitness (Nguyen et al., 2013;

65   McManus et al., 2014). If the benefits of relocating outweigh the costs, then the animal should

66   disperse (Caughley, 1994). Costs of dispersal include use of energy, risk of injury or death, and

67   outbreeding depression (Bonte et al., 2012), while benefits include escaping unfavorable

68   conditions, obtaining new resources, and decreased chances of inbreeding (Caughley, 1994).

69   However, for an animal to gauge the magnitude of benefits that it might receive from moving, it

70   must have information about alternative habitats, which can be obtained by exploring new areas,

71   contingent upon the aforementioned costs (Nguyen et al., 2013).

72

73   Coastal ecosystems are characterized by diverse microhabitats that are relatively close in

74   proximity, making it possible for mobile aquatic organisms to gather information about

75   surrounding habitats. Aquatic species often have a particular range of salinities that they can

76   tolerate, but also a salinity in which their fitness is highest (Boeuf and Payan, 2001). When the

77   ability to disperse is limited, the habitats in which animals settle can significantly constrain fitness.

78   For example, growth and survivorship of the barnacle *Balanus amphitrite* are negatively impacted

79   when the animal occupies salinities greater than or equal to 10 parts per thousand (ppt) (Qiu and

80   Qian, 1999). Because barnacles remain attached to a substrate during adulthood and cannot

81   readily escape unfavorable environmental conditions, they must endure these consequences in

82   the event of salinity fluctuations. Fishes, on the other hand, have the ability to disperse

83   throughout their lifetime, which allows them to move to areas with more favorable salinities, if

84   available (Bonte et al., 2012). Salinity preference thus plays an important role in habitat selection

85   for aquatic organisms living in brackish environments, which can vary in salinity both spatially and

86   temporally, creating distinct microhabitats (Surge and Lohmann, 2002). Many organisms that

87   inhabit variable environments tend to have wide tolerance ranges (Gabriel, 2005; Schultz, and

88   McCormick 2012). For example, the killifish, *Fundulus heteroclitus*, is able to tolerate shifts in

89   temperature, pH, salinity, and oxygenation; each of these factors vary significantly within their salt

90   marsh habitat (Schulte, 2014). In any case of habitat selection, there are most likely a set of

91   environmental conditions in which the animals experience highest fitness, and a preference for

92   these habitats should be selected for (Kearney and Porter, 2004).

93

94   When organisms occupy a particular habitat, it could be because the conditions in that habitat

95   confer highest fitness, or could reflect biotic and abiotic factors (e.g., competition, predation,

96   salinity, and temperature) that limit occupancy of optimal habitats (Svärdson, 1949; Kearney and

97 Porter, 2004). To determine where an animal achieves highest absolute fitness in a
98 multidimensional environment, all factors other than the one of interest must be controlled for. In
99 addition, animals should be exposed to the full, ecologically relevant range of the abiotic factors
100 (i.e., its fundamental niche) (Pearman et al., 2008). When other things that limit dispersal are
101 present (e.g., competition, predators), the fundamental niche narrows to the realized niche
102 (Morse, 1974), which is typically where organisms are found in their natural environments.
103 Although individuals of a given species are often found within a given niche, among-individual
104 variation around that average niche space can exist, and this variation is then subject to
105 selection. Preference studies can provide insights into whether habitat selection is constrained by
106 other factors, and reveal whether, in the wild, the animals occupy their realized or fundamental
107 niche. In the laboratory, extraneous variables can be controlled while examining preferences,
108 which should provide information about the conditions under which the animal might experience
109 highest absolute fitness. Furthermore, laboratory settings also allow for assessment of
110 repeatability of that preference, which can be difficult in field-based studies on organisms that
111 show low site fidelity. Quantifying repeatability, the proportion of total phenotypic variation that is
112 due to among-individual differences (Falconer and Mackay, 1996; Lessells and Boag, 1987;
113 Boake, 1989), is essential for understanding the potential for selection to drive the evolution of
114 salinity preferences (Brodie and Russell, 1999; Boake, 1989; Arnold, 1994).
115
116 Mangrove rivulus fish, *Kryptolebias marmoratus* (hereafter, 'rivulus')*,* are small, self-fertilizing
117 hermaphroditic vertebrates found in a wide range of microhabitats within mangrove ecosystems
118 of Florida, the Bahamas, parts of the Caribbean and Central America (Huber, 1992). Field data
119 have shown that they exist in a broad range of salinities. Based on data from 274 different field
120 sites, we have collected mangrove rivulus in salinities ranging from 0-65 ppt, with an average
121 salinity of 26 ppt (SE $\pm$ 0.44) (see also Taylor, 2012). However, very few rivulus eggs have been
122 collected in the field so, habitat preferences for egg-laying remain unknown (Taylor, 1990).
123 Rivulus can exist in a wide range of salinities in the field, even over small spatial scales (Sutton et
124 al., 2018) despite the apparent costs that the animals might accrue at both low and high salinities.
125 For example, Lin and Dunson (1999) showed that exposure to different salinities early in life
126 significantly affected adult mass; treatment animals raised at 12 and 40 ppt had significantly
127 higher final masses than those raised at 1 ppt. Mortality rates of mangrove rivulus living at 12 and
128 40 ppt were also significantly greater than those living at the lower salinity (Lin and Dunson,
129 1999). Overall, mangrove rivulus raised in the lowest salinity (1 ppt) matured at a slower rate,
130 grew to a smaller size, and produced fewer eggs than those reared in higher salinities (Lin and
131 Dunson, 1995). When salinities deviate from the isosmotic point animals can incur significant
132 costs, however this is not always the case and it is not always straightforward to predict which
133 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

170   exhibit salinity preferences, including measurements of strength of preference, number of
171   transitions between salinities, latency to begin exploring the salinity gradient, and the covariance
172   among these traits. Then (2) establish if these behavioral traits are repeatable, (3) determine
173   whether their preference in the laboratory corroborates field collection data, as well as, (4)
174   examine whether mangrove rivulus have a salinity preference for oviposition sites, and (5)
175   determine the effects of developmental salinity on hatching success. We hypothesized that
176   mangrove rivulus would have a salinity preference and that their preference would be repeatable.
177   A preference was inferred if the fish spent significantly more time in one salinity compared to
178   others. It was predicted that the fish would show a preference for 25 ppt because this is the
179   salinity in which rivulus are found most commonly in the wild and at which they are raised in our
180   laboratory colony. Previous work suggests that the isotonic point for mangrove rivulus is nearer to
181   15 ppt (Frick and Wright 2002; Bielmyer et al. 2012), leading to an alternative prediction that the
182   fish would prefer salinities lower than 25 ppt. Additionally, we hypothesized that they would have
183   a preferred salinity in which to lay eggs and that the salinity experienced during development
184   would influence hatching success. We predicted that rivulus would prefer to lay eggs in 25 ppt
185   and that eggs laid in 25 ppt would have the highest hatching success compared to eggs laid in
186   any other salinity. Alternatively, rivulus might choose to lay their eggs, and they eggs might fare
187   better, at salinities closer to the isosmotic point of 15 ppt.
188
189   Materials and Methods
190
191   Housing Conditions
192
193   When not being tested, all fish were housed in ventilated, 1.2 L Rubbermaid® containers filled
194   with 25 ppt salt water (Instant Ocean® salt and aged tap water). All individuals were kept under a
195   12L:12D photoperiod, a temperature of 25.42 ± 0.0043 °C (mean ± SEM), and were fed 2 ml of
196   live brine shrimp (*Artemia*) nauplii reconstituted in water six days per week. All fish were adult
197   hermaphrodites, aged between 133-379 days old when entering their first treatment (Mean ±
198   SEM age: 252 ± 8.62 days old). The University of Alabama Institutional Animal Care and Use
199   Committee approved all procedures described herein (IACUC #15-10-0111).
200
201   Salinity Preference in a Lateral Gradient
202
203   *Genotype selection*. Rivulus are self-fertilizing hermaphrodites and are able to produce isogenic
204   lineages, wherein all individuals share the same genotype. The genotypes used in this study were
205   derived from a broad geographical range, including Belize, the Bahamas, the Florida Keys, East
206   Florida, and West Florida. Sixty-three genotypes whose field-caught progenitor (F0 generation)

207 was homozygous at a minimum of 31 out of 32 microsatellite loci (Avise and Tatarenkov, 2015;
208 Tatarenkov et al., 2012) were selected for salinity preference trials. All fish used in this
209 experiment were one (F1) or two (F2) generations removed from wild-caught progenitors. A
210 sample of 16 genotypes from across the geographical range had animals represented in the
211 salinity preference trials and the control trials (see details below). Separate individuals were used
212 in the control and experimental group. The total sample size for salinity preference trials was thus
213 79 individual fish from 63 genotypes. Animals were selected in this way so as to maximize
214 genetic diversity in the study. We did not have replicates of the same genotype within a
215 treatment, thus the study focused on among-individual variation (repeatability) rather than
216 variation among specific genotypes (heritability).
217
218 *Experimental design.* Lateral salinity gradients were built according to Staaland (1969) with
219 modifications as outlined by McManus et al. (2014) using 74 L aquaria (Fig. 1). The aquarium
220 was divided length-wise using black corrugated plastic to create two gradients per tank, each
221 measuring 74.6 x 14.6 x 29.8 cm, hence forth referred to as half-tank. All dividers were
222 constructed from 6.35 mm black corrugated plastic and secured using marine aquarium silicone,
223 such that each side of the tank was completely separated from the other. Each half-tank salinity
224 gradient consisted of five U-shaped chambers (13.8 x 14.6 x 8.8 cm) containing the experimental
225 salinities - 5, 15, 25, 35, and 45 ppt. The salinity gradient remained stable for at least 7 days, as
226 indicated by a low coefficient of variation for salinity measurements across an 8-day trial, even
227 with a fish allowed to swim freely through the gradient (Table S1). The outside of the aquaria was
228 covered with light green paper to minimize disturbance and to easily visualize fish on videos.
229 Webcams (Logitech, Suzhou) were suspended above the aquaria to monitor the fish's location
230 throughout the salinity preference trials. Two days before each trial, salinity concentrations of 5,
231 15, 25, 35, and 45 ppt were prepared using aged tap water and Instant Ocean® Aquarium Sea
232 Salt (Spectrum Brands, Blacksburg). Salinity concentrations were then checked for accuracy
233 using a handheld refractometer. An air stone was placed in each salinity reservoir to aid in the
234 removal of chlorine from tap water. A pump was placed in the 45 ppt reservoir to prevent the salt
235 from settling at the bottom; agitation from the air stones was sufficient to prevent settling in the
236 other salinities. The temperature of the water was recorded before each trial and maintained an
237 average of 26.8 ± 0.35 ºC.
238
239 Before beginning a trial, all salinity mixtures were checked with a handheld refractometer again
240 for accuracy. Rubber barriers were placed on top of the dividers that were connected to the
241 bottom of the half-tank (Fig. 1a, McManus et al., 2014). To randomize the direction of the gradient
242 each time it was set up, a coin was flipped (heads right, tails left) to determine if the left or right
243 side of the half-tank would contain the lowest salinity concentration. For example, if the left side

244   had the lowest salinity, then the chambers would increase left to right in the following fashion: 5,
245   15, 25, 35, 45 ppt. Then, each chamber was filled with 1.8 L of the premixed salinity. For the
246   control group, the half-tank was filled with 25 ppt water in each chamber to evaluate chamber
247   preference independent of salinity concentration. After all of the chambers were filled, the fish
248   was gently placed in the center chamber (25 ppt) with a small fish net and allowed 30 minutes to
249   acclimate to the half-tank. Following acclimation, video cameras were turned on and rubber
250   barriers were removed. After 8 hours, cameras were turned off, and the fish was removed from
251   the half-tank with a small net and returned to their original housing. The half-tanks were then
252   emptied using a siphon and rinsed with fresh water. Videos were then analyzed using JWatcher
253   1.0 (Blumstein et al., 2006) to determine the amount of time spent per chamber and the number
254   of transitions between chambers. All salinity preference fish were tested 3 times with 21 days
255   between trials to minimize learning or habituation effects (the direction of the gradient was
256   determined randomly for each trial).
257
258   Salinity preference trails were run between September and December 2015. Fish were then fed 4
259   ml of brine shrimp per day and monitored for egg laying. Once a fish had laid eggs they were then
260   used for egg laying preference trials from January through June 2016, as described below.
261   Additional fish were added to the egg laying experiment to account for those from the salinity
262   preference experiment that never laid eggs.
263
264   *Statistical analysis.* The time that it took the fish to first transition out of the 25 ppt central
265   chamber (latency to emerge) was removed for each trial to avoid biasing data by an individual's
266   motivation. We generated an average preference score by multiplying the number of seconds
267   spent in a chamber by the assigned chamber number (centered at 25 ppt with 1 unit difference
268   between chambers, i.e. 5 ppt = -2, 15 ppt = -1, 25 ppt = 0, 35 ppt = 1, and 45 ppt = 2) and then
269   dividing the result by the total number of seconds. A more strongly negative score thus indicates
270   a preference for lower salinities, while a more strongly positive score indicates a preference for
271   higher salinities. This score is then defined as an individual's preference within a given trial.
272   Variance for an individual's preference scores was also calculated, which describes the strength
273   of preference (where low variance indicates high strength of preference for a given salinity). To
274   avoid confusion in the graphics, the negative of the variance was plotted such that higher values
275   indicate stronger preference. The number of transitions between chambers was used calculate
276   the transition probabilities for each individual's trial using Python 2.7 (Python Software
277   Foundation) with code developed by one of the authors (HL, Code S2). To determine the effect of
278   treatment on latency to emerge, preference, strength of preference, and number of transitions we
279   used lme4 package in R (Bates et al., 2015) to conduct general linear mixed models for each
280   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 $preference = intercept + \beta_1 \cdot treatment + Fish\ ID + e,$ (1)
285 $preference = intercept + Fish\ ID + e,$ (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 $preference, strength, transitions, emergence = intercept + \beta_1 \cdot round + us: Fish\ ID + us: e,$ (3)
300 $preference, strength, transitions, emergence = intercept + \beta_1 \cdot round + idh: Fish\ ID + us: e,$ (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

9

318   condition separately to parse the variance into total variance, among-individual variance, and

319   within-individual (residual) variance as follows (shown for preference only):

320

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

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

323

324   The variance components from each model were then compared to determine whether behavioral

325   responses were repeatable. This was done following Houslay and Wilson (2017), where a chi-

326   square value was calculated as -2*(Log Likelihood of Model 1 - Log Likelihood of Model 2) to

327   determine whether among-individual variance (random effect of Fish ID) was significant. The pin

328   function in the nadiv package (Wolak et al., 2012) was then used to calculate the adjusted

329   repeatability by dividing among-individual variance by the sum of among-individual and residual

330   variance; this function also provided the standard error for the repeatability estimate.

331

332   Egg Laying Preference

333

334   *Genotype selection.* A group of 67 individuals were selected as experimental fish, and an

335   additional 33 were selected as control fish. These animals were derived from fifty-three

336   genotypes, all of which were also represented in the Salinity Preference study. Field-caught

337   progenitors were homozygous at a minimum of 31 out of 32 microsatellite loci (Avise and

338   Tatarenkov, 2015; Tatarenkov et al., 2012). All fish used in this experiment were F1 or F2

339   generation and laid viable, fertilized eggs (i.e., perivitelline space present) prior to the trial to

340   ensure that they were reproductively active and capable of effective self-fertilization.

341

342   *Experimental design.* We modified the lateral salinity gradient for egg laying by adding to each

343   chamber Poly-Fil fiber situated at the air-water interface as an egg laying substrate. Fish were

344   placed in the gradient for two weeks to lay eggs. After one week, the fish was removed from the

345   gradient and placed into a 1.2 L Rubbermaid® container of the same salinity as the chamber they

346   were located in at the time of capture; this allowed us to check the chambers and Poly-Fil for

347   eggs. The gradient was then emptied and refilled. Once the gradient was re-established, the fish

348   was returned to the chamber in which it was located prior to the egg check. The location and

349   number of eggs per chamber were recorded. At the end of the second week, fish were returned to

350   their original housing area and the gradient and Poly-Fil were checked again for eggs. While in

351   the gradient, fish were fed by adding 2 ml of brine shrimp to each chamber daily (so as to avoid

352   chamber preferences associated with food). All eggs were stored in containers with the same

353   salinity as they were found in until hatching. To determine hatchling success, eggs were checked

354 daily to record the date of hatching and received weekly water changes with the same salinity in

355 which they were laid.

356

357 *Statistical analysis.* The presence or absence of an egg in each chamber was used for our

358 analysis so as to not bias a particular salinity if a fish laid multiple eggs in a single chamber. This

359 is important because the number of eggs laid by each individual was highly variable, ranging from

360 0 to 20 eggs across the two week period. Using the lme4 package in R (Bates et al., 2015), we

361 ran a GLMM to test egg laying preference in a salinity gradient. In the following model, treatment

362 refers to either the salinity gradient or the control where all chambers were filled with 25 ppt, while

363 chamber refers to the location the egg was laid. To account for a possible edge effect (Fig. 4b)

364 we included whether a given chamber was an 'edge' in our model. Additionally, because the

365 gradient remained stable for only one week, there were two egg checks for each fish during the

366 two-week period. Thus, we included 'time' as a fixed effect to account for any variance in egg-

367 laying between the two egg-checking periods but, because 'time' itself was not central to the

368 hypotheses that we were testing, we did not include its interactions with other fixed effects.

369

370 $eggs = intercept + \beta_1 \cdot treatment + \beta_2 \cdot chamber + \beta_3 \cdot treatment \; x \; chamber + \beta_4 \cdot edge + \beta_5 \cdot$

371 $treatment \; x \; edge + \beta_4 \cdot time \; + Fish \; ID \; + e$, (7).

372

373 To determine the effects of salinity on hatching (yes or no), a generalized linear mixed model with

374 a binomial distribution and logit link function was used as follows with parent ID as a random

375 effect:

376

377 $hatched = intercept + \beta_1 \cdot salinity + Parent \; ID \; + e$, (8).

378

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

380 contrasts.

381

382 Results

383

384 *Salinity preference.* When in a salinity gradient, mangrove rivulus showed a significant preference

385 for lower salinities (Table 1, Fig. 2a, b). In the control group, where there was no salinity gradient,

386 individuals spent more time in chambers at each edge. Strength of preference was significantly

387 higher in the salinity gradient than in the control (Table 1, Fig. 2c). There was no difference

388 between experimental and control groups in the total number of transitions between chambers

389 (Table 1, Fig. 3) or in latency to emerge from the central chamber at the start of the trial (Table 1).

390 For the experimental group, strength of preference, number of transitions, and latency to emerge

391   were significantly repeatable, with between 38-53% of the total behavioral variance being

392   attributed to among-individual differences (Table 2). Preference was not repeatable in the

393   experimental group (Table 2). In the control group, only the number of transitions between

394   chambers was repeatable, with ~40% of the behavioral variance being attributed to among-

395   individual differences (Table 2). Multivariate model comparisons of among-individual correlation

396   structures showed strong among-individual correlation structure in experimental treatment (χ^2_6 =

397   26.698, p = 0.0001; Table 3) but not control (χ^2_6 = 0.021, p = 0.999).

398

399   *Egg laying preference.* When given the opportunity to lay eggs along a salinity gradient,

400   individuals laid eggs with greater frequency in lower salinities (Fig. 4). There was no significant

401   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

402   there was a significant main effect of chamber (χ^2 = 16.6, P = 0.002, df = 4), which was treatment-

403   dependent (treatment x chamber: χ^2 = 9.45, P = 0.05, df = 4). In the control group, individuals

404   were more likely to lay eggs in the edge chambers compared to the central chambers as

405   indicated by a significant chamber effect (χ^2 = 12.82, P = 0.012, df = 4) and *a priori* contrasts

406   (Table 4); these results correspond to the edge effect that was observed in the salinity preference

407   experiment, conducted on a separate set of individuals. Individuals in the experimental group

408   were more likely to lay eggs in 5 ppt than in any other salinity, with a significant salinity effect (χ^2 =

409   13.26, P = 0.01, df = 4), and significant contrasts between 5 ppt and all other salinities (Table 4).

410   Additionally, there was a significant effect of salinity on hatching (χ^2 = 13.99, P = 0.0013, df = 3,

411   Fig. 5). Eggs laid in the lowest salinity had a significantly higher probability of hatching that those

412   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 =

413   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 =

414   10.40, P = 0.001, df = 3).

415

416   Discussion

417

418   The ability to exist and be phenotypically flexible in a variable environment can come with

419   significant costs (Piersma and Drent, 2003). In aquatic habitats, that cost is often the energy

420   devoted to osmoregulation (Boeuf and Payan, 2001). By investigating salinity preferences and

421   repeatability of those preferences, we can gain insight into whether these traits might evolve in

422   response to natural selection (Boake, 1989). In addition, salinity preferences provide clues into

423   the habitats in which individuals' fitness might be highest. We initially hypothesized that rivulus

424   would prefer to occupy salinities of 25 ppt and that their preference would be both repeatable and

425   would align with field observations (as they are most often found at 25 ppt). Additionally, we

426   hypothesized that rivulus would prefer to lay eggs in 25 ppt and that hatching success would be

427   highest in 25 ppt. Our findings support the hypothesis that rivulus exhibit salinity preferences,

428 both in terms of where they spend their time and where they lay their eggs. However, in a
429 laboratory environment, free of anything that might constrain their movement (e.g., predators,
430 competitors, physical factors such as temperature), rivulus preferred to occupy salinities below 25
431 ppt and laid eggs with greatest frequency in 5 ppt. Hatching success also was highest at 5 ppt
432 and decreased precipitously as salinity increased. Moreover, salinity preference was not
433 repeatable, but the strength of preference and latency to emerge were repeatable in the salinity
434 gradient.
435
436 We investigated repeatability because the opportunity for selection to drive the evolution of
437 behavior in environments with salinity microhabitats hinges on there being considerable variation
438 among individuals (Boake, 1989; Wolak et al. 2012). Given that the repeatability of a trait is,
439 arguably, the upper bound of its heritability (Boake, 1989; Falconer and Mackay, 1996; but see
440 Dohm, 2002), it is likely that strength of preference and latency to emerge, both of which showed
441 high repeatabilities in the experimental group, could evolve in response to selection. This might
442 be especially true in highly heterogeneous habitats, which can reveal consistent among-individual
443 differences in behavior. These findings are notable because environments with microhabitat
444 options (experimental group) exposed behavioral variation among individuals that was not
445 present in environments with only one option (control group). Given that mangrove environments
446 are replete with microhabitat variation, we expect that such variation would be available in wild
447 rivulus populations for natural selection to act upon. If among-individual differences are underlain
448 by genetic variation, strong selection on exploratory behavior might drive phenotypic divergence
449 between populations with different degrees of microhabitat variation. Due to changes in the influx
450 of both freshwater and saltwater to coastal systems owing to climate change, which is likely to
451 alter microhabitat structure, mangrove forests might provide a unique opportunity to catalog the
452 evolution of behavioral and physiological responses to changing salinity niches (Brennan et al.,
453 2015). For example, when high spatiotemporal variation in salinity occurs, individuals that tend
454 not to explore and have a strong preference for a specific salinity could have reduced fitness
455 compared to those that quickly seek new microhabitats and show a relatively weak preference for
456 a particular salinity.
457
458 Rivulus were equally active in control and experimental groups; in each treatment, individuals
459 explored the full experimental apparatus and transitioned between chambers a similar number of
460 times. The number of transitions was repeatable in each group, indicating consistent among-
461 individual differences in activity levels and/or willingness to explore an unfamiliar area. These
462 findings are consistent with Edenbrow and Croft (2012) who showed exploration within a maze to
463 be repeatable in rivulus. However, the pattern of movement was different between control and
464 experimental groups; in the latter, more transitions were made in the direction of lower salinity

465 chambers. While individuals in the experimental group varied in their activity levels, movement
466 was concentrated in the lower salinities (25 ppt and below, Fig. 3). This significantly reduced
467 among-individual variance in the number of chambers visited during a given trial and resulted in
468 low repeatability for salinity preference in animals exposed to the salinity gradient. The control
469 group also showed very low repeatability for chamber preference in the absence of salinity
470 variation but likely for a different reason; in that group, the vast majority of the variance was within
471 rather than among individuals (Table 2, Fig. 3), indicating that individuals are inconsistent in the
472 chambers they visit most often from trial to trial.
473
474 Edenbrow and Croft (2011) also showed significant variation in the expression of behavior among
475 ages and genotypes, indicating some context dependence. It has been previously documented
476 that both abiotic and biotic factors (e.g. temperature, food availability, predation) can influence
477 behavior and behavioral consistency (Nussey et al., 2007; Bell et al., 2009; Edenbrow and Croft,
478 2013), which was observed in our repeatability analysis for latency to emerge. The time it took
479 rivulus to emerge from the central chamber was not repeatable in the control but was highly
480 repeatable in the salinity gradient treatment (see also Kluen and Brommer, 2013). Strong
481 repeatability for latency to emerge in the experimental group was due to the fact that, when faced
482 with a salinity gradient, some fish sampled their environment quickly and others more slowly.
483 Without the gradient (i.e., controls, all chambers 25 ppt), consistent among-individual differences
484 in latency to emerge disappeared.
485
486 Within the experimental group, strength of preference differed consistently among individuals,
487 which could reflect variation in the ability to flexibly adjust physiology along a salinity gradient.
488 Some fish spent the majority of their time in one of the few low salinity chambers, while others
489 transitioned between chambers with salinities ranging from 5 to 25 ppt. Such differences might
490 depend on the individuals' physiology and capacity to respond to the challenges of
491 osmoregulation in fluctuating salinity conditions. Fish rely on multiple structures (gills, gut, kidney;
492 Edwards and Marshall, 2012) for ion and water exchange with their environment. Some
493 individuals could be more efficient at regulating changes in chloride cell function within the gills,
494 aquaporin expression in the intestine, or glomerular filtration rates (Edwards and Marshall, 2012;
495 Cutler and Cramb, 2002). This opens the possibility to explore empirically how individuals with
496 low versus high strength of preference cope with living in different salinities, and whether among-
497 individual differences in physiological flexibility are underlain by genetic variation.
498
499 Adult rivulus are most often found at 25 ppt in the wild but we found strong preferences for lower
500 salinities under controlled laboratory conditions. It is also relatively rare to find eggs or hatchlings
501 in the wild (Taylor, 1990; Taylor, 2012). While adult rivulus can clearly tolerate salinities ≥ 25 ppt,

14

502   their preference for lower salinities indicates that they are found most often in salinities that are

503   suboptimal, i.e., where they are likely to incur physiological costs of osmoregulation. Based on

504   the findings of this study, our inability to find eggs in the wild is likely due to individuals selecting

505   areas of lower salinity for egg laying and then returning to areas of higher salinity to possibly

506   avoid predators and competitors. Rivulus will actively navigate their microhabitat options via

507   swimming, but also have additional means of exploring their environment. Rivulus can traverse

508   land by terrestrial tail-flip jumping and can survive out of water, as long as it is moist, for 66 days

509   (Taylor, 1990; Pronko et al., 2013; Styga et al., 2017). With a greater ability to explore their

510   environment via terrestrial tail-flip jumping, and having a broad tolerance to many factors that

511   make the mangrove ecosystem a hostile environment for many fish, rivulus is able to take

512   advantage of the many variable microhabitats available in this system (Taylor, 2012). We

513   observed rivulus navigating to low salinities to lay eggs and these eggs had the highest hatching

514   success at 5 ppt (Figs. 4 and 5). The developmental environment can not only affect survival but

515   also the resulting phenotype due to the increased energy demands of osmoregulation (Brown et

516   al., 2012). Independent of energy requirements, developmental plasticity in response to salinity

517   might also change the phenotype in adaptive or non-adaptive ways (West-Eberhard, 2003;

518   Albecker and McCoy 2019). An important area of future research might thus be to examine the

519   extent to which exposure to different salinities early in life might drive physiological, behavioral,

520   and morphological variation.

521

522   Coordinated behavioral responses to environmental cues were evident in this study. Some

523   among-individual correlations were expected. For example, if some individuals consistently took

524   longer to emerge than others, they then had less time to explore the apparatus, leading to a

525   negative among-individual correlation between latency to emerge and the number of transitions.

526   There was also a negative among-individual correlation between number of transitions and

527   strength of preference (negative of the variance, such that high values indicate higher strength of

528   preference; Figure 2); individuals that had strong preferences made fewer transitions. The

529   positive but not statistically significant among-individual correlation between strength of

530   preference and latency to emerge indicates that more 'cautious' individuals (those that take

531   longer to emerge) also tend to find their preferred salinity and stay there. There was only among-

532   individual correlation structure in the experimental treatment, and this structure remained

533   consistent over time, perhaps representing a behavioral syndrome (Sih et al., 2004). In this

534   context, the syndrome reflects a gradient of exploratory phenotypes. On one end are individuals

535   that are quick to explore novel environments, actively move through the area, and exhibit weak

536   preferences. On the other end are individuals that take a more restrained approach to novel

537   environments, move around less, and exhibit strong preferences. We used genotypes from

538   across rivulus' expansive geographic range, leaving two primary explanations for the behavioral

539   variation that we observed: i) individuals were derived from populations under divergent selection,
540   (e.g., those with and without significant microhabitat variation) and we used a representative
541   sample of genotypes from these areas; and/or ii) mangroves exhibit considerable spatiotemporal
542   variation in microhabitat characteristics and/or stability such that fluctuating selection maintains
543   behavioral variation within populations.
544
545   Some facets of our experimental treatment (whether the exposure to a salinity gradient or
546   increased microhabitat heterogeneity) revealed consistent differences among individuals that
547   were not present in a uniform environment (control). When phenotypic variation emerges as a
548   result of microhabitat heterogeneity, it suggests variation among individuals in phenotypic
549   flexibility across environments (i.e., slope of the reaction norm). If this emergent variation is
550   heritable, there should be increased opportunity for selection to drive evolutionary change in
551   heterogeneous environments.
552
553   In addition to present-day conditions, the evolutionary history of a species can impact how they
554   will respond to future selection (Crowley et al., 2019). Species with a previous history of inhabiting
555   variable environments should be able to respond to the changing environment appropriately given
556   that habitat selection can have significant fitness consequences. When only considering
557   osmoregulation demands, aquatic species that inhabit brackish environments should select
558   microhabitats in which they are isotonic and where the metabolic cost of osmoregulation is
559   minimal (Boeuf and Payan, 2001; Sutton et al. 2018). Based on our field observations indicating
560   that rivulus are most frequently observed at salinities close to 25 ppt, and the fact that rivulus'
561   isotonicity point is nearer to 15 ppt (Frick and Wright, 2002; and Bielmyer et al. 2012), it does not
562   appear that rivulus are occupying ideal habitats in the wild. This could be because rivulus are
563   excluded from lower salinity microhabitats by competitors or predators. Other factors also could
564   impact their ability to osmoregulate efficiently (e.g., diet, temperature, dissolved oxygen) such that
565   inhabiting an area of higher salinity may result in higher fitness (Hammerschlag, 2006). More
566   work is needed to: i) identify the possible abiotic and biotic factors that exclude rivulus from their
567   preferred salinity in the wild; ii) understand the physiological costs associated with occupying
568   higher salinities and; iii) understand whether physiological differences among genotypes might
569   explain among-individual variation in strength of preference.
570

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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		
	$\beta \pm SE$	X^2_1	P	$\beta \pm SE$	X^2_1	P	$\beta \pm SE$	X^2_1	P	$\beta \pm SE$	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
	$\bar{x} \pm SE$			$\bar{x} \pm SE$			$\bar{x} \pm SE$			$\bar{x} \pm SE$		
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 < 7.132x10^{-7}. Statistically significant P-values are indicated in bold.

	Preference			Strength			Transitions			Latency to Emerge		
	$V \pm SE$			$V \pm SE$			$V \pm SE$			$V \pm SE$		
$V_{individual}$ 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		
	$R \pm SE$	X^2_1	P	$R \pm SE$	X^2_1	P	$R \pm SE$	X^2_1	P	$R \pm SE$	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**
	$V \pm SE$			$V \pm SE$			$V \pm SE$			$V \pm SE$		
$V_{individual}$ 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		
	$R \pm SE$	X^2_1	P	$R \pm SE$	X^2_1	P	$R \pm SE$	X^2_1	P	$R \pm SE$	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			Salinity (ppt)		
Comparison	χ^2	P	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 <mark>negative of the variance</mark> 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.
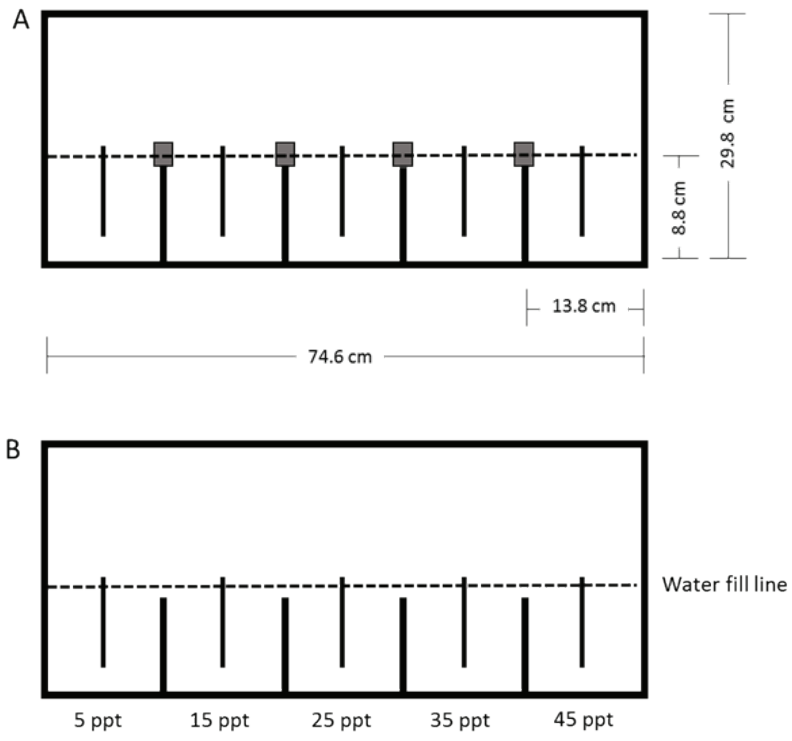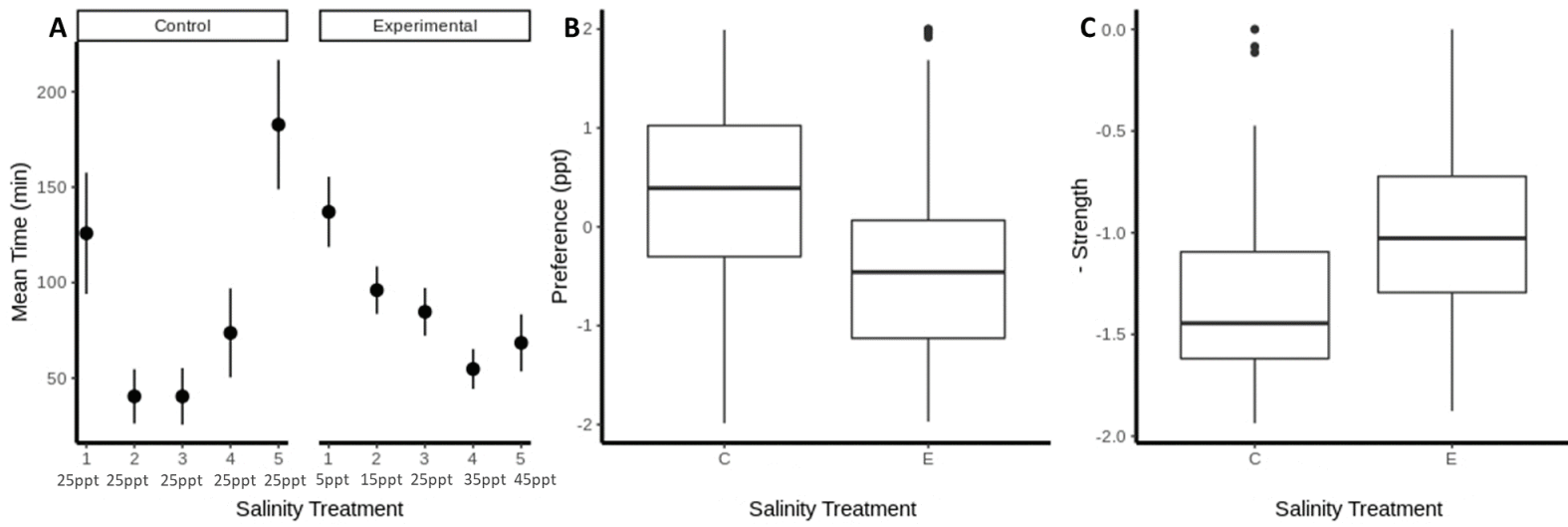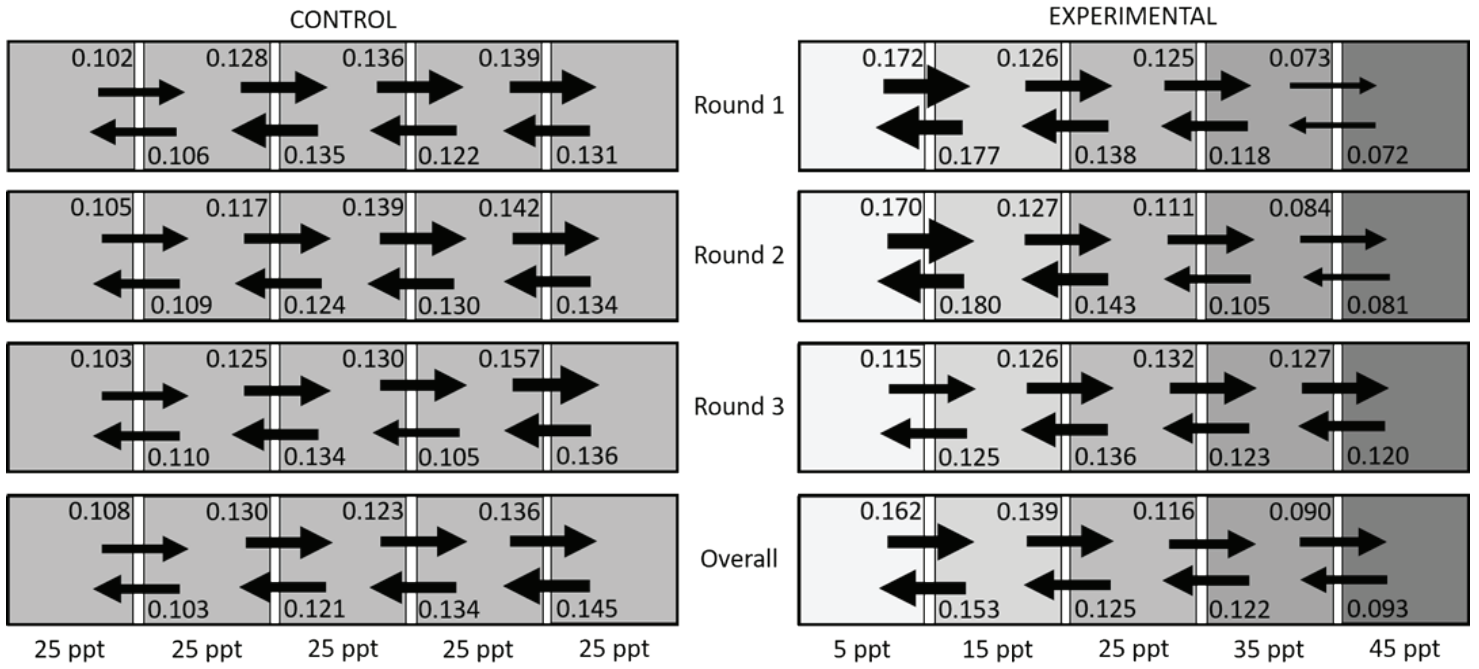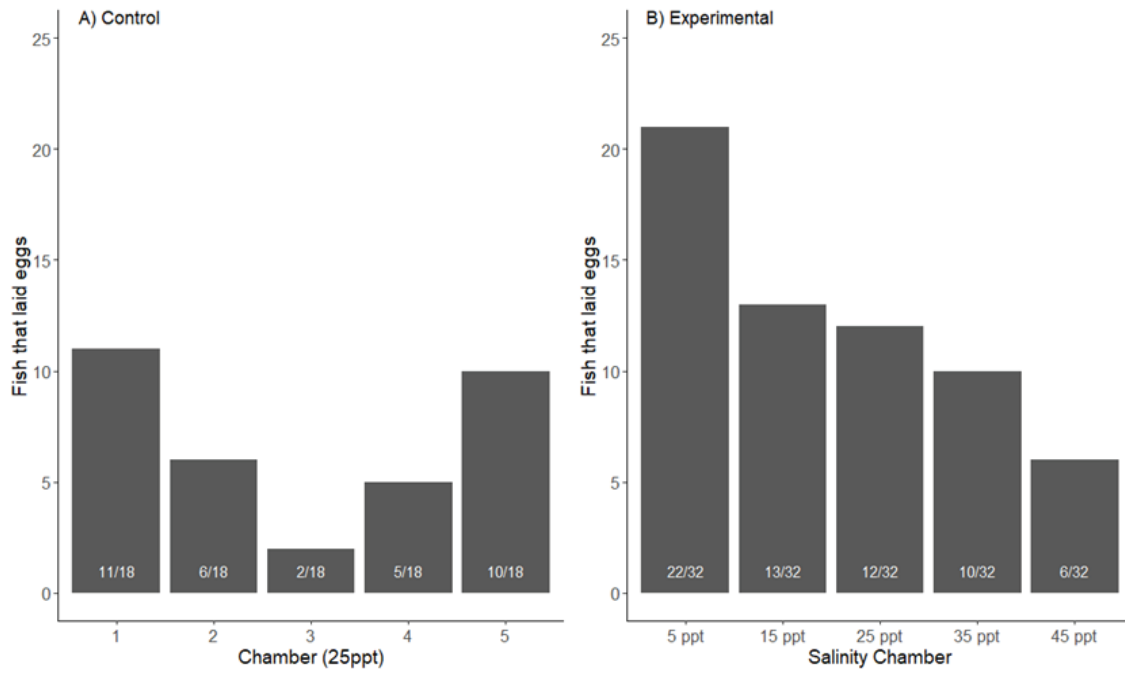
Figure 1.

Figure 2.

Figure 3.

CONTROL

0.102	0.128	0.136	0.139	
0.106	0.135	0.122	0.131	Round 1
0.105	0.117	0.139	0.142	
0.109	0.124	0.130	0.134	Round 2
0.103	0.125	0.130	0.157	
0.110	0.134	0.105	0.136	Round 3
0.108	0.130	0.123	0.136	
0.103	0.121	0.134	0.145	Overall

25 ppt   25 ppt   25 ppt   25 ppt   25 ppt

EXPERIMENTAL

0.172	0.126	0.125	0.073	
0.177	0.138	0.118	0.072	Round 1
0.170	0.127	0.111	0.084	
0.180	0.143	0.105	0.081	Round 2
0.115	0.126	0.132	0.127	
0.125	0.136	0.123	0.120	Round 3
0.162	0.139	0.116	0.090	
0.153	0.125	0.122	0.093	Overall

5 ppt   15 ppt   25 ppt   35 ppt   45 ppt

Figure 4.

Figure 5.