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RESEARCH ARTICLE

Evidence of prezygotic isolation, but not assortative mating, between locally adapted populations of *Fundulus heteroclitus* across a salinity gradient

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Abstract

Selection along environmental gradients can drive reproductive isolation and speciation. Among fishes, salinity is a major factor limiting species distributions, and despite its importance in generating species diversity, speciation events between marine and freshwater are rare. Here, we tested for mechanisms of reproductive isolation between locally adapted freshwater and brackish water-native populations of killifish, *Fundulus heteroclitus*, from either side of a hybrid zone along a salinity gradient. There was evidence for pre-zygotic endogenous reproductive isolation with reduced fertilization success between crosses of freshwater-native males and brackish water-native females. Exogenous pre-zygotic isolation was also present where females had highest fertilization in their native salinity. We used a replicated mass spawning design to test for mate choice in both brackish and fresh water. After genotyping 187 parents and 2523 offspring at 2347 SNPs across the genome, 85% of offspring were successfully assign to their parents. However, no reinforcing mate choice was observed. These results therefore demonstrate emerging, yet limited, reproductive isolation and incipient speciation across a marine to freshwater salinity gradient and suggest that both endogenous and exogenous mechanisms, but not assortative mating, contribute to divergence.

KEYWORDS
adaptation, fishes, reproductive isolation, salinity

1 | INTRODUCTION

Natural selection in divergent environments can result in local adaptation and ultimately drive speciation if barriers to gene flow emerge. During this process, ecological speciation (Nosil, 2012; Schluter, 2000), selection results in the fixation of alleles that are beneficial under one environment but not the other (Schluter, 2009). This in turn can drive reproductive isolation when it limits migration or when hybrids have reduced fitness in either environment. Thus, an essential aspect in understanding ecological speciation is how selection and genetic divergence facilitate reproductive isolation and speciation between populations in distinct environments.

Hybrid zones, where distinct populations or species meet, reproduce, and produce offspring (Barton & Hewitt, 1985), are particularly useful for studying the mechanisms that enable ecological speciation. When a locally adapted population transitions to another along an environmental gradient, gene flow can be limited. Dispersal of individuals along the gradient may be constrained

by selection against hybrids due to endogenous (intrinsic) genetic incompatibilities (Barton & Hewitt, 1989), thereby forming a 'tension zone'. Conversely, hybrid zones can be maintained by environmental or exogenous (extrinsic) mechanisms, where locally adapted types have reduced fitness in the alternate environment (Hewitt, 1988).

To distinguish the mechanisms that limit gene flow between adjacent populations and potentially promote ecological speciation, it is necessary to understand the relative influence of exogenous and endogenous mechanisms. These can each take the form of prezygotic or postzygotic barriers. Prezygotic barriers include mechanisms, such as mate choice, temporal isolation, and gametic competition, whereas postzygotic mechanisms may include hybrid inviability and reduced hybrid fitness (Coyne, 1992; Jiggins & Mallet, 2000). More specifically, endogenous prezygotic mechanisms are those that are independent of the environment and occur before a zygote can be formed, such as mate choice. Prezygotic isolation can also be exogenous, for example, if fertilization depends on specific environmental conditions. Similarly, postzygotic isolation may be endogenous such as when hybrids are always less fit than parents, but exogenous when hybrid fitness depends on the environment.

Environmental salinity provides a strong barrier to migration for aquatic species, where communities in adjacent fresh and salty habitats are almost entirely unique. That is, it is extremely rare for individuals from the same species to occupy both salty and fresh habitats, and this is true across the tree of life, including in fishes (Lee & Bell, 1999; Schultz & McCormick, 2012). Therefore, typically for a taxon to span marine and freshwater environments, adaptive differentiation and speciation is required. However, although speciation events from marine to freshwater have been important in generating species diversity across fishes, these events are relatively rare (Betancur-R et al., 2015; Rabosky, 2020; Vega & Wiens, 2012). A few micro-evolutionary model systems have provided insight into the mechanisms that enable differentiation across the marine-freshwater boundary. For example, in threespine stickleback (*Gasterosteus aculeatus*), selection in freshwater populations has driven divergence in armour plating (Bell, 2001), behaviour (DiPoi et al., 2014), body shape (Walker & Bell, 2000), salinity tolerance (Heuts, 1946), and diet (Ishikawa et al., 2019), among other traits (McKinnon & Rundle, 2002), compared with their marine ancestors. In most cases, freshwater divergence is accompanied by reproductive isolation (McKinnon et al., 2004). Similarly, populations of the killifish *Lucania parva* are locally adapted to different osmotic environments (Kozak et al., 2014) and reproductive isolation has emerged across environmental salinity gradients (Fuller, 2008). Similar mechanisms likely maintain reproductive isolation between sister species *L. parva* (brackish/marine) and *L. goodei* (freshwater) (Kozak et al., 2015). Additional species, such as European flounder (Momigliano et al., 2017), pike (Sunde et al., 2018), sockeye salmon (Wood & Foote, 1996), and others (Schluter, 1996) show patterns of divergence into low salinity and suggest that when a population is able to establish in freshwater, reproductive isolation and divergence can rapidly emerge.

Since most large fish clades are exclusively marine or exclusively freshwater (Betancur-R et al., 2015), micro-evolutionary model systems for studying the early/intermediate stages of differentiation along salinity gradients are rare. In addition to a few other models (e.g., sticklebacks, *Lucania* killifish), the Atlantic killifish *Fundulus heteroclitus* provides a model system to study this process. *F. heteroclitus* is a euryhaline species distributed along the eastern coast of North America, from Florida to Nova Scotia, and across the entire salinity continuum from marine to freshwater (Hildebrand & Schroeder, 1928). At least two distinct hybrid zones related to Pleistocene refugia (<20 000 years old) delineate adaptive differentiation across two different environmental gradients across the range (Duvernell et al., 2008). The first hybrid zone is found in coastal New Jersey, and distinguishes northern and southern coastal populations (sub-species) (McKenzie et al., 2016; Ropson et al., 1990), where sub-species differ in genetics, morphology (Able & Felley, 1986), and thermal physiology (DiMichele & Powers, 1982; Fangue et al., 2006). Individuals from either side of the hybrid zone (New Hampshire and North Carolina) showed evidence of endogenous isolation in the form of reduced hatching success as well as assortative mating (McKenzie et al., 2017).

In addition to the coastal zone, another hybrid zone along a salinity gradient is found within the Chesapeake Bay (Whitehead et al., 2011). Although ancestrally marine, *F. heteroclitus* has broad osmoregulatory flexibility (plasticity) and can tolerate a wide range of salinities from dilute freshwater to four-times the salinity of seawater (Griffith, 1974; Whitehead, 2010). Individuals native to freshwater (FW-native) are genetically distinct from their downstream brackish counterparts (BW-native; $F_{ST} = 0.08$; (Brennan et al., 2018; Whitehead et al., 2011)) with a genetic cline centred on the tidal freshwater boundary (~1/32nd the salinity of seawater; ~1 parts per thousand, ppt) (Duvernell et al., 2008; Whitehead et al., 2011). This 1 ppt salinity is also physiologically important. In *F. heteroclitus*, acclimation to salinities across 1 ppt (above or below) requires extensive remodelling of transport epithelia (e.g., gills) (Whitehead et al., 2012) which consumes ~10% of their energy budget (Kidder 3rd et al., 2006a, 2006b). Populations native to each salinity diverge in their salinity-specific performance, for example, showing differences in transcriptional and osmoregulatory plasticity (Brennan et al., 2015; Whitehead et al., 2011), divergence in salinity-specific swimming performance (Brennan et al., 2016), and adaptive genetic divergence due to selection (Brennan et al., 2018). Thus, fresh- and brackish-native populations are locally adapted to their respective osmotic environments and remain genetically distinct despite the presence of a narrow hybrid zone populated by highly admixed individuals (Brennan et al., 2018).

Although there is much evidence that the FW-native and BW-native populations are adaptively diverging across this salinity gradient, the mechanisms that are preventing the homogenization of the populations and maintaining the hybrid zone are not known. Previous work has suggested that hybrids may have reduced low-salinity tolerance relative to the parental populations (Brennan et al., 2018), but no studies have yet attempted to identify the

mechanisms contributing to reproductive isolation between brackish and freshwater populations.

Here, we test for the presence of reproductive isolation that may serve to constrain gene flow between FW-native and BW-native populations of *F. heteroclitus* across a salinity gradient. We ask whether endogenous and exogenous prezygotic and postzygotic isolation exist, which may limit gene flow between the populations. Given the coincidence of the hybrid zone with a physiologically stressful environmental salinity, we predicted that environmental salinity would affect reproductive success between the populations. To test this, we quantified reproductive incompatibilities between FW-native and BW-native parents, and mate choice, in both fresh water and brackish water. First, manual crosses were used to determine if populations could successfully interbreed and produce viable offspring. Second, mate choice was assessed using a mass spawning design where individuals were free to interbreed over the course of a month. We genotyped 2523 offspring and 187 parents at 2347 single-nucleotide polymorphisms (SNPs) across the genome using a RAD-capture approach (Rapture; Ali et al., 2016), and assigned parentage to >2000 of these offspring to determine the mate choice of all parents. These two experiments were conducted in both fresh and brackish water.

2 | METHODS

Adult *F. heteroclitus* were collected in September 2014 and March 2016. FW-native fish were sampled from the Potomac River at Piscataway Park, near Accokeek, Maryland, USA (38°41'42.18''N, 77°3'10.38''W); salinity has not been higher than 0.12 ppt since monitoring began in 1986 (Figure S1; eyesonthebay.dnr.maryland.gov). BW-native fish were from Point Lookout State Park, Scotland, Maryland, USA (38°3'10.90''N, 76°19'34.38''W), where the average salinity between 1984 and 2022 has been 14.8 ppt (Figure S1). These sites are approximately 95 km apart by river distance (Whitehead et al., 2011) and experience similar temperature regimes (Figure S2). Fish were shipped overnight to the University of California-Davis and held in 30-gallon tanks at the salinity close to their collection salinity in 850-gallon recirculating systems equipped with UV, mechanical, and biological filters. Fish were kept at a density less than one per gallon, fed Aquaxcel 4512 daily (Cargill, Minnesota, USA). The light cycle was 10 h light and 14 h dark with 21°C water temperature; previous work has shown that *F. heteroclitus* spawns across a broad range of temperatures from 10.6–27.8°C (Petersen et al., 2010) and a nearby population from the same latitude readily reproduces above 20°C (Taylor et al., 1979). Dechlorinated tap water was used for freshwater (0.2 ppt) and reverse osmosis water mixed with Instant Ocean Sea Salt (Instant Ocean, Spectrum Brands, VA, USA) was used to achieve brackish salinity (15 ppt). Fish were randomly transferred to their experimental salinity and allowed to acclimate for at least month. To distinguish the populations when combined in a single tank, all fish were marked with a fluorescent elastomer tag (Northwest Marine Technology, WA, USA).

To test for postzygotic isolation, we used five replicate manual crosses for each of the four cross-types: two within-population (BW-native x BW-native, FW-native x FW-native) and two between-population (BW-native♂ x FW-native♀, FW-native♂ x BW-native♀). These 20 crosses were repeated in two salinities: fresh water (0.2 ppt) and brackish water (15 ppt) (40 total crosses). For each replicate, three males and three females were combined in 30-gallon tanks with one 10.2 cm diameter spawning basket and allowed to freely spawn for 30 days. These baskets were covered with 9.5 mm mesh through which spawned embryos could easily fall but adults could not gain access. This is necessary as adults will consume eggs and embryos. Preliminary trials showed that adults recognize these baskets as the primary spawning substrate in the tanks. Baskets were checked for eggs three times per week. All eggs were transferred to 100 × 15 mm plastic petri dishes and checked for fertilization under a stereomicroscope. Fertilization was determined by the appearance of the perivitelline space, which develops minutes after fertilization, or the neural keel, which is visible ~40 h post fertilization (Armstrong & Child, 1965). Fertilized eggs were incubated in 3 ppm methylene blue (Kordon) to prevent fungal growth and water was changed daily. The number of total eggs, fertilized eggs, and hatched fry were recorded. Typical time to hatching is 14 days (Armstrong & Child, 1965), and embryos that did not hatch after 21 days were considered inviable. The brackish water trial was conducted from May 14 to June 12, 2016 and the freshwater trial was conducted from August 14 to Sept 7, 2016.

Fertilization and hatching success were analysed using logistic regressions in R version 4.1.2 (R Core Team, 2022). The response variable was either hatching success counts or fertilization success counts, and main effects included salinity environment (hereafter referred to as 'salinity) and cross-type (hereafter referred to as 'cross'), and their interaction. We tested for the significance of main effects with wald tests using Aod version 1.1 (Lesnoff & Lancelot, 2012), and post hoc differences were calculated with lsmeans version 2.30 (Lenth, 2016).

Mate choice was investigated using a 'choice test' in a free spawning design. In each of four replicate, 30-gallon tanks at both freshwater (0.2 ppt) and five replicate tanks at brackish water (15 ppt) (9 total tanks), five males and five females from both the FW-native and BW-native populations (20 individuals per tank) were combined. Two spawning baskets were placed on either end of each tank to collect eggs. Three replicates of within-population controls were used per population to ensure that both populations were reproductively active. These controls contained three males and three females from one population and one spawning basket. Eggs were collected every 2 days, checked for fertilization, and incubated as described in the no-choice test. For all trials, dead fish were replaced with an individual of the same sex and population (6 total deaths). Upon hatching, fry were preserved for genotyping to assign parentage and identify the cross-type from which they were derived. All samples were

stored at −80°C until processing. Unhatched eggs were again considered non-viable after 21 days of development.

2.1 | Genotyping

Genomic DNA was extracted from whole fry or fin clips with a custom method as in Brennan et al. (2018). Briefly, an overnight digestion at 56°C in 4.2 mg/ml Proteinase K was followed by an Ampure XP bead cleanup and elution in low TE (10 mM Tris– HCl, pH 7.5, 0.1 mM EDTA). Between-population crosses resulted in unequal numbers of hatched fry and replicates with >350 hatched individuals, fry were subsampled down to 350 samples. This included BW replicate 1, 3, and 4, which were reduced to 41%, 45%, and 75% of the total individuals, respectively.

A restriction-associated DNA sequencing (RAD-seq) capture approach (Rapture) was used for genotyping as described by Ali et al. (2016). The strength of this approach is that it allows high-throughput and low-cost genotyping of thousands of samples at hundreds to thousands of loci. 250 ng DNA from each sample was digested with the restriction enzyme *Sbf*I. In sets of 96, individuals were barcoded by adding 2 μl indexed SbfI biotinylated RAD adapter (50 nM), each with a unique 8-bp barcodes. DNA was sheared to 200–500 bp using a Bioruptor NGS sonicator, RAD-tagged DNA fragments were isolated, and the final DNA was prepared for Illumina sequencing using a NEBNext Ultra DNA Library Prep Kit for Illumina. A unique barcode was applied to each set of 96 individuals, enabling multiplexing of thousands of individually indexed individuals.

We designed 866 capture baits based on previously collected *Sbf*I RAD data (Brennan et al., 2018; NCBI BioProject PRJNA428529), specifically targeting loci that contained known polymorphisms between the two populations at intermediate frequencies. These baits were also chosen based on their unique location in the reference genome (on different scaffolds;Reid et al., 2017), unique sequence composition, and optimal GC content (see Data availability for more details). Capture baits (MYbaits) were ordered from MYcroarray (currently Arbor Biosciences, MI, USA). This baitset captured 103 920 bases, plus flanking regions. Equal amounts of all libraries were pooled to a final concentration of 30 ng/μl. This pool was split evenly between three independent capture reactions. Sequence capture proceeded according to the manufacturer's protocol except for the primers used in the final amplification step. Here we substituted universal primers of the sequence AATGATACGGCGACCAC CGAGATCTACACTCTTTCCCTACACGAC*G and CAAGCAGAAGA CGGCATACG*A, where * is a phosphorothioated DNA base.

Libraries were sequenced using 150-bp paired-end reads on an Illumina Hiseq 4000 at the UC Davis Genome Center. Reads were demultiplexed first by Illumina barcode and then by RAD barcode. For RAD barcodes, perfect barcode matches with a partial restriction site were required. Reads were aligned to the reference genome version GCF_000826765.1 (Reid et al., 2017) using BWA-MEM version 0.7.12 (Li, 2013) and PCR duplicates were removed using SAMBLASTER (Faust & Hall, 2014). We removed 117 offspring with mean coverage <5x. Next, variants were filtered to include sites with minor allele frequency greater than 0.05, only biallelic sites, depth between 10 and 110x, minimum quality of 20, and data in at least 90% of individuals. Finally, this set was filtered to include only high confidence SNPs in the parents (to ensure accurate parentage assignment). Across all parental individuals, minor allele frequency was required to be at least 0.01 with at least 85% of individuals with non-missing data. From this, 2347 SNPs were obtained.

To visualize population structure between parental individuals, a principal component analysis was run on the parent samples only using PLINK2 (Chang et al., 2015) with variants pruned for linkage disequilibrium (LD; 1606 variants remained). Weir and Cockerham's Fst (Weir & Cockerham, 1984) was estimated using VCFtools version 0.1.15 (Danecek et al., 2011).

Parentage assignment was conducted using Colony v. 2.0.6.6 (Jones & Wang, 2010). Within each replicate, SNPs were LD pruned and parentage was assigned to the known list of candidate parents using the pairwise likelihood approach. Offspring were filtered to only those that had two parents assigned with 95% confidence. With these data, we were unable to determine the actual number of independent mating events because multiple matings between the same two individuals would be undetectable. Therefore, we considered the mate choice as the proportion of within-population versus between-population mate pairings for each individual, as inferred from parentage assignment of offspring. For example, if one male parent from the BW population produced offspring with one BW female and three FW females, the proportion of like-type matings would be 25%. To test for mate choice, data were analysed with a logistic regression. The count of like versus unlike mate pairings was considered the response variable, whereas main effects included treatment salinity (environment), source population, and sex (and their interactions). Individual length was treated as a covariate, and tank was treated as a random effect. Neither length or tank improved model fit and were dropped from the final models. Models were run using the *glm* function in lme4 (Bates et al., 2014). *Wald tests* were used to test for a statistically significant influence of main effects, with post hoc tests using *lsmeans* (Lenth, 2016), and the *effects* package was used to visualize model predictions (Fox, 2003; Fox & Weisberg, 2018a, 2018b).

3 | RESULTS

3.1 | Population structure

Principal components analysis (PCA) of Rapture SNP variation among 186 adult fish showed that the parental populations native to different osmotic environments were genetically distinct (Figure 1). Weir and Cockerham's weighted F_{ST} between populations was 0.077 which is consistent with other estimates (RAD-seq SNPs: 0.082 (Brennan et al., 2018); Microsatellites: 0.081 (Whitehead et al., 2011)). Although assumptions of ADMIXTURE and DAPC

analyses were violated because of relatedness and family structure for our samples, estimates of individual ancestry with these tools also support the distinctiveness of the parental populations and demonstrate that all offspring generally follow the expected ancestry given their parental assignment (Figure S3; details of parentage analysis below) (Alexander et al., 2009; Jombart et al., 2010).

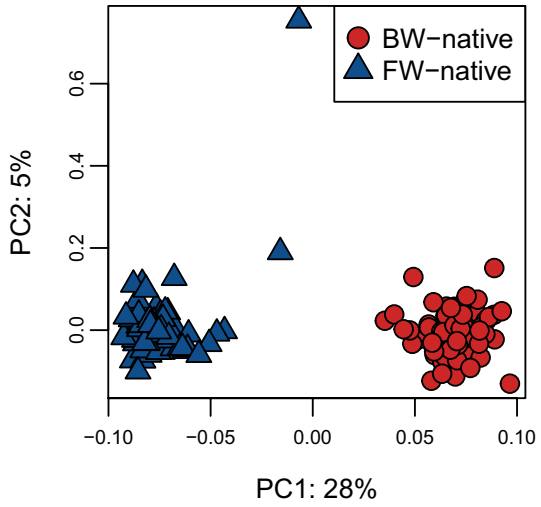

FIGURE 1 Principal components analysis of 1606 linkage disequilibrium pruned SNPs for all BW-native (red circles) and FW-native (blue triangles) parents used in the mate choice experiment. Axis labels show the percent of the total variance explained by principal components one and two.

3.2 | Reproductive incompatibilities

Fertilization and hatching success were high across all cross-type and salinity environment treatment groups during the tests for reproductive incompatibilities (mean ± standard error; fertilization = 0.90 ± 0.01; hatching = 0.84 ± 0.02). Variation in fertilization success between populations and between environments provided evidence of endogenous and exogenous reproductive isolation (Figure 2a). For endogenous isolation, there was an effect of cross (Wald test, $\chi^2(3) = 32.2$, $p = 4.8\text{e-}07$) where crosses between FW-native♂ and BW-native♀ had lower fertilization success than all other crosses ($p < 0.05$), though the effect size of this was small (maximum reduction: 8.7%; reduction of fertilization success ± std. error: 0.082 ± 0.0126). Though there was no significant effect of salinity ($\chi^2(1) = 2.9$, $p = 0.086$), the marginally significant interaction between salinity and cross ($\chi^2(3) = 7.8$, $p = 0.05$) suggests the possible influence of extrinsic isolation due to salinity environment. This interaction effect was due to the lower fertilization success of BW-native♀ in fresh water (0.831 ± 0.019; 0.912 ± 0.009) relative to brackish (0.875 ± 0.008; 0.931 ± 0.006) as compared with higher success of FW-native♀ in fresh water (0.949 ± 0.01022; 0.910 ± 0.02302) relative to brackish (0.920 ± 0.0129; 0.900 ± 0.0245; Figure 2a). That is, females tended to have higher fertilization success in their matched salinity environment (e.g., BW-native♀ in BW environment, and FW-native♀ in FW environment) compared with their un-matched salinity environment (e.g., BW-native♀ in FW environment, and FW-native♀ in BW environment).

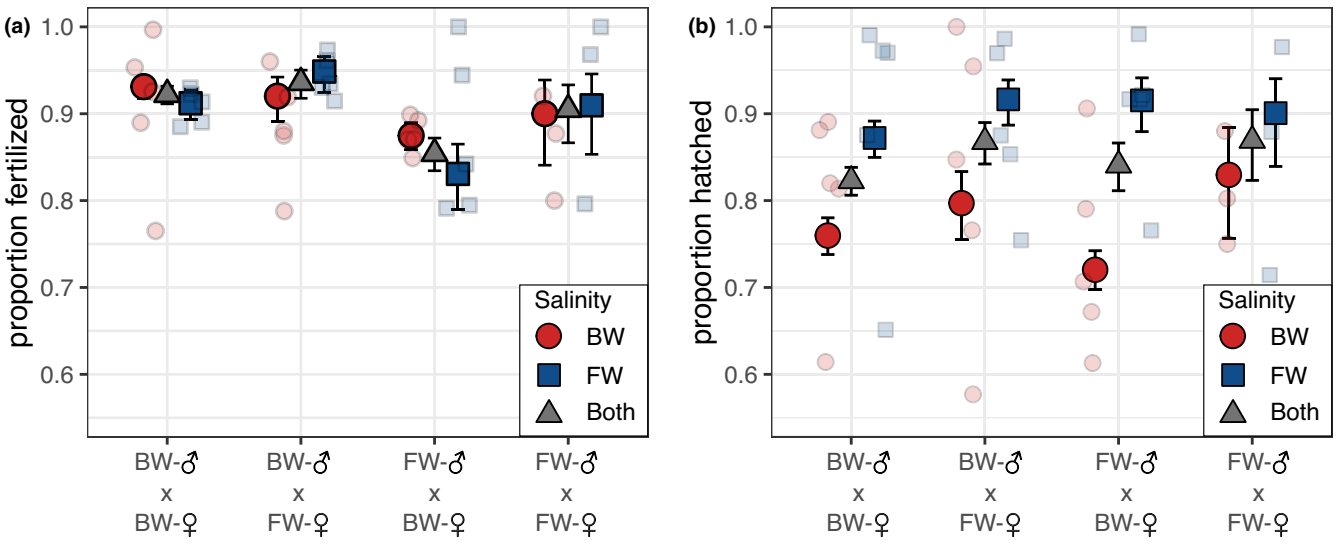

FIGURE 2 Results from the manual crosses for (a) fertilization and (b) hatching success. Small transparent points show individual replicates whereas larger bold coloured points are the model estimates including standard error. Results are separated by salinity where red circles are the brackish treatment and blue squares are the freshwater treatment. The grey triangles are the average of both salinities within the cross-type. (a) The FW-native♂ x BW-native♀ cross had lower fertilization success than other crosses and there was an interaction effect where BW-native♀ in brackish water had higher fertilization than in fresh water. The opposite pattern was detected for FW-native♀ with higher fertilization success in fresh water than in brackish water. (b) Hatching success showed a significant salinity effect and was higher in fresh water. There was also a significant effect of cross where BW-native♂ x BW-native♀ had lower hatching than BW-native♂ x FW-native♀. There was a significant interaction between cross and salinity due to a significant salinity effect in all cross-types except FW-native♂ x FW-native♀ crosses.

For hatching success, we detected a significant effect of salinity ($\chi^2(1) = 46.9$, $p = 7.5e\text{-}12$) with consistently higher hatching in fresh versus brackish water across crosses (difference ± std. error: 0.123 ± 0.0146) (Figure 2b). But in contrast to fertilization, there was no evidence of reduced hatching success for inter-population crosses relative to within-population crosses ($p > 0.05$). There was a significant effect of cross-type ($\chi^2(3) = 17.1$, $p = 0.0007$) where BW-native♂ x BW-native♀ had lower hatching success than BW-native♂ x FW-native♀ (difference ± std. error: 0.045 ± 0.015), though this was a relatively small effect size (5% reduction). Finally, there was a significant interaction between cross and salinity ($\chi^2(3) = 8.8$, $p = 0.032$) that was driven by differences between brackish and freshwater treatments that was apparent for all crosses ($p < 0.0001$) except for FW-native♂ x FW-native♀ ($p = 0.67$).

3.3 | Mate choice

For the control parental tanks and the experimental tanks, we observed relatively high and even levels of both fertilization (mean ± standard error: FW-Native in FW: 0.80 ± 0.07; FW-Native in BW: 0.86 ± 0.03; BW-Native in FW: 0.84 ± 0.03; BW-Native in BW: 0.86 ± 0.04; Experimental tank in FW: 0.89 ± 0.01; Experimental tank in BW: 0.93 ± 0.02) and hatching success (FW-Native in FW: 0.88 ± 0.05; FW-Native in BW: 0.65 ± 0.04; BW-Native in FW: 0.80 ± 0.10; BW-Native in BW: 0.69 ± 0.03; Experimental tank in FW: 0.74 ± 0.03; Experimental tank in BW: 0.73 ± 0.06). If there were differences in fertilization success or hatching success between the populations, we might have observed apparent differences in mate choice that were actually due to physiological limitations. The successful reproduction of each parental population provides confidence that the observed differences in mate pairings were due to choice rather than physiological limitations. For mate choice, complete data were collected for nine experimental tanks: five replicates in the brackish salinity environment and four replicates in the freshwater salinity environment. One FW environment replicate was lost because BW-native males were accidentally not added to this treatment. An average of 433 ± 84 eggs were produced per experimental replicate (14.4 eggs/day) with a maximum of 1431 and a minimum of 142.

Overall, 2523 offspring with greater than 5x sequence coverage (Figure S4) and 187 parents, were genotyped at 2347 SNPs across the genome. Of the 2523 offspring, 85% (2138) were successfully assigned to two parents each with 95% confidence. Overall, despite removing individuals with coverage <5x, low-parentage assignment was in excess for individuals with low sequencing depth, but it was not restricted to only low depth individuals (Figure S5). All samples and parents were also run in a single Colony analysis and the number of impossible parents (i.e., those not in the same experimental tank) was determined. Only 1% of assignments (25) were impossible (assigned to parents from different tanks), providing confidence that the parentage assignment was robust.

Of the 187 parents, 137 had at least one offspring assigned, representing 63 males and 74 females. The number of offspring assigned to each individual was variable and ranged from 1 to 201 (mean ± standard error: 31.2 ± 3.5) and was similar between males (33.9 ± 5.9) and females (28.9 ± 4.0; two-sided t-test: $p = 0.48$; Figure S6, S7). Overall, family size ranged from 1 to 89 with a mean of 11 ± 1.15. Finally, the number of mates per individual with assigned offspring ranged from 1 to 9 (2.77 ± 0.16) with no difference between males (3.02 ± 0.27) and females (2.57 ± 0.17; two-sided *t*-test: $p = 0.17$) and no relationship with the length of the individuals (ANOVA: $p = 0.99$; Figures S8).

There was limited evidence of mate choice. A random effect of experimental replicate did not improve model fit (Likelihood ratio test: $p = 1$) and was not included in the final model. The mean size of adults was 7.04 ± 0.05 cm and FW-native individuals (7.26 ± 0.072 cm) were significantly larger than BW-native individuals (6.85 ± 0.06 cm; $p = 0.0003$; Figure S9). Despite this, there was no effect of length on mate choice ($p = 0.99$; Figure S10). Overall, the results of the logistic regression did not support the influence of mate choice in these data (Figure 3) as there was no main effect of salinity (Wald test, $\chi^2(1) = 0.004$, $p = 0.98$), cross ($\chi^2(3) = 5.5$, $p = 0.14$), or their interaction ($\chi^2(3) = 5.8$, $p = 0.12$). Consistent with this, all model estimates overlapped and most, with the exception of BW-native females in FW (estimate and 95% CI: 0.68 [0.51, 0.81]), were not different from 0.5 (Figure 3), indicating that mating was largely random throughout the experiment.

4 | DISCUSSION

Fundulus heteroclitus are distributed across steep salinity gradients where there is strong genetic structure, local adaptation to osmotic environments, and reduced performance of hybrids. An apparent barrier to gene flow is localized to a region of the salinity gradient that is physiologically important (~1 ppt), and co-localizes with a zone of admixture (hybrid zone). As such, we hypothesized that exogenous isolating mechanisms were restricting gene flow between populations. Indeed, we detected evidence for prezygotic exogenous isolation, where females tended to have higher fertilization success in their matched salinity environment, compared with when fertilizations were attempted in their unmatched salinity environment (Figure 2a). Simultaneously, we detected prezygotic endogenous isolation, where FW-native♂ x BW-native♀ hybrids had reduced fertilization success regardless of salinity. No evidence of postzygotic isolation was found (Figure 2b). Although the effect sizes were relatively small, these mechanisms could contribute to reduced gene flow near the tidal freshwater boundary that distinguishes populations inhabiting fresh versus salty water.

Salinity tolerance of gametes has a major impact on the ability of a population to occupy novel osmotic environments (Green et al., 2020) and mismatch between gametes and external salinity can limit gene flow even if adults can survive. In F. *heteroclitus*,

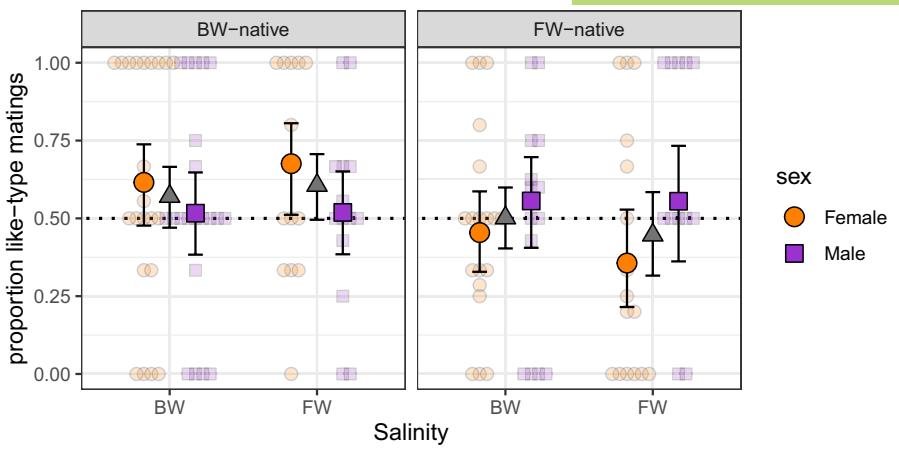

FIGURE 3 Results from the mate choice test for assortative mating. The y-axis is the proportion of pairings with mates from the same population (like-type matings). The left panel shows BW-native individuals and the right panel shows FW-native individuals. Within each panel, points are separated by the environmental salinity, and the colour and shape of each point represents the sex (orange circles for female and purple squares for male). Small transparent points are the results from each individual replicate whereas larger bold-coloured points are the model estimates for the treatment with standard error. The grey triangles are the average across both sexes within a treatment. There were no significant main effects or interactions.

mature eggs are released into the environment and subsequently fertilized. Under normal conditions, sperm fertilizes the egg by entering a single opening in the chorion (the micropyle) (Brummett & Dumont, 1981). Once the sperm enters, the egg is activated and the micropyle is blocked, preventing polyspermy. However, this activation can occur due to salinity alone, thus blocking fertilization and resulting in a non-viable egg (Bush & Weis, 1983). Bush and Weis (1983) showed variation in salinity-induced activation between coastal populations of *F. heteroclitus*, suggesting that local adaptation can impact the effects of salinity-induced activation. Given this, the improved fertilization for females in their native salinity (Figure 2) could be explained by salinity-induced egg activation that blocks successful fertilization. This result fits into the concept of immigrant reproductive dysfunction (IRD), where migration into a different environment can inhibit successful reproduction, limiting gene flow between populations and facilitating ecological speciation (Svensson et al., 2017). For example, sand goby (*Pomatoschistus minutus*) and Atlantic herring (*Clupea harengus*) both show divergence in sperm motility between populations locally adapted to different salinities (Berg et al., 2019; Leder et al., 2021), and salinity-specific gamete performance is common between closely related species that occupy different osmotic environments (Elofsson et al., 2003; Lindström et al., 2021). Our results are consistent with the findings of these previous studies and show that non-native salinity drives a small reduction in fertilization success for females, where this effect likely acts on the egg rather than the sperm (Figure 2). Therefore, IRD may be promoting divergence in this system. However, it should be noted that this is a conservative estimation as there are numerous environmental differences between the two habitats (e.g., pH, predators, nutrients, pathogens, oxygen, diet, symbionts, tidal rhythmicity, etc.) that could influence performance and fitness between the populations and thereby limit gene flow (Austin & Austin, 2007; Lozupone & Knight, 2007; Osborne et al., 2015; Ou et al., 2015).

To test for the influence of assortative mating, we leveraged high-throughput genotyping with a replicated mass spawning experiment across multiple environments. We were able to accurately and rapidly genotype and assign parentage to thousands of offspring. This approach recapitulated known genetic relationships between the parental populations and resulted in high parentage assignment across the experiment. However, we did not detect evidence of assortative mating at either salinity (Figure 3). This is in contrast to other model systems, such as cichlids or swordtails, where assortative mating and sexual selection is the dominant factor limiting homogenization of populations and species (Knight & Turner, 2004; Salzburger et al., 2006; Schumer et al., 2017). Similarly, in the closely related killifish, *Lucania* sp., strong assortative mating was identified between populations and sister species adapted to different salinities (Fuller, 2008; Berdan & Fuller, 2012; Kozak et al., 2015), but in none of these cases did assortative mating differ between salinities.

The lack of mate choice in *F. heteroclitus* suggests that the sexual selection and assortative mating observed in other systems seems to play a more minor role. Reproduction in *F. heteroclitus* occurs daily during the spawning season where individual females can produce eggs in clutches of 10–100 with up to 300 per day during peak spawning season (Taylor, 1986). There is strong sexual dichromatism where males take on nuptial coloration and mating begins with a courtship ritual after which males clasp the anal and dorsal fins of females and external fertilization occurs (Newman, 1907). A variety of spawning modes can occur and group spawns happen in the wild (Petersen et al., 2010). However, males are territorial. They attempt to prevent multi-pair matings (Newman, 1907), and we observed male territorial behaviour during our experiment. Nevertheless, the observed lack of assortative mating in this experiment could be due to multi-male spawning events in the experiment that were due to sneak matings or true lack of preference; additional direct observations would be required to further illuminate these dynamics.

Mate choice may also be impacted by the condition of individuals, where unhealthy individuals will tend to find fewer mates (Neff & Cargnelli, 2004). Thus, under natural conditions, it is possible that failure of a non-native fish to completely acclimate to a mismatched osmotic environment could impair long-term health and performance, thereby leading to mate choice and selection against migrants (Hendry, 2004). Additional studies would be required to further explore this hypothesis.

Although we detected the influence of pre-zygotic mechanisms that may contribute to limiting gene flow across the adaptive transition zone at the tidal freshwater boundary, the effect sizes were not large. Here, we consider caveats to our experimental design, and the potential influence of other mechanisms. There was no evidence of postzygotic isolation at the F1 generation in this experiment. However, F1 individuals are heterozygous and it is common that endogenous postzygotic isolation only emerges when deleterious alleles are in a homozygous state, for example, at F2 or backcross stages (Orr & Presgraves, 2000). The lack of evidence here does not preclude the emergence of postzygotic isolation in more advanced backcrosses. Additionally, we treated developing embryos with fungicide, which is common practice in fish husbandry. However, research in *Lucania* killifish found that hybrids between FW and BW types had reduced survival in fresh water, but that this effect was masked when embryos were treated with fungicide (Kozak et al., 2012). Future experiments with *F. heteroclitus* may consider trials both with and without fungicide treatment. We also observed lower hatching success in brackish water than fresh water. This was unexpected but was consistent across both populations and each part of the experiment. We can think of no obvious reason for this pattern. Although no differences in hatching emerged in these experiments, it is possible that this potential artefact could be masking differences. Additionally, behavioural effects could be present in the wild that may not manifest in captivity. For example, evidence for assortative mating in swordfish under field conditions disappeared when animals were studied in captivity (Schumer et al., 2017). Egg deposition locations differ between coastal populations *F. heteroclitus* (Able, 1984). If mating events occur at different or unsuitable locations in each salinity environment, then reproduction of immigrants could be reduced thereby limiting gene flow. Indeed, potential killifish spawning substrates (aquatic plants) differ between fresh and brackish water habitats (Haramis & Carter, 1983).

Assortative mating is less efficient at maintaining hybrid zones than reductions in hybrid fitness; a small reduction in hybrid fitness will have a much larger effect than even strong mate preference (Irwin, 2020). Given this, it is possible that migrants and hybrids are less fit than native individuals inhabiting their native salinity environment but assortative mating has not emerged. We have previously shown that admixed individuals have reduced physiological tolerance to fresh water relative to either parental population, suggesting impaired hybrid fitness. This could limit gene flow and serve to maintain genetic differentiation between the locally adapted populations inhabiting either side of the tidal freshwater boundary.

Previous work across coastal temperature gradients in *F. heteroclitus* has shown reduced hatching rates and mate choice between northern and southern populations (McKenzie et al., 2017). Though the coastal environmental gradient correlates with temperature and the Chesapeake gradient with salinity, we hypothesized that strong population structure across any physiologically relevant environmental gradient would result in reproductive isolation and mate choice. However, we observed weaker evidence for reproductive isolation and no evidence of mate choice between FW-native and BW-native populations. The differences between the two environmental gradients could be due to lower genetic differentiation between populations that span the Chesapeake salinity transition zone (Fst = 0.08) than between those that span the coastal latitudinal transition zone (Fst = 0.18) (Adams et al., 2006). Greater time since isolation can lead to increased genetic differentiation and accumulation of incompatibilities (Coyne & Orr, 1989). Furthermore, the nature of natural selection and adaptation along the coastal gradient (thermal physiology) and the Chesapeake gradient (osmoregulatory physiology) differs and could therefore have different impacts on reproductive and developmental traits (Lindtke & Buerkle, 2015). Finally, experimental design considerations could play a role. For example, McKenzie et al. used strip spawning to generate a single experimental replicate with 47 individuals, whereas we used natural crosses across nine replicates each with 20 individuals.

The results presented here show the emergence of reproductive isolation between populations of killifish locally adapted to local salinity and indicate the potential initial steps of incipient speciation. Additional work could help disentangle the drivers underlying this emerging isolation. For example, identifying the mechanisms leading to the higher fertilization success of females in their native salinity could provide insight into how osmotic environments can limit gene flow. Further, behavioural assays, including spawning substrate preference, could provide additional nuance into how the populations interact and may give more detailed information as to the mating preferences of each population. Finally, it is necessary to understand the impact of other abiotic and biotic factors that differ between marine and freshwater environments on population performance and ultimately gene flow. By understanding the mechanisms that lead to reproductive isolation and speciation across salinity gradients, we can gain insight into the potential mechanisms that have enabled diversification of fishes between marine and freshwater environments.

AUTHOR CONTRIBUTIONS

Reid Brennan: Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Andrew Whitehead:** Conceptualization (equal); funding acquisition (equal); investigation (equal); methodology (equal); writing – original draft (supporting); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequence reads are available at the National Center for Biotechnology Information (NCBI; Bioproject no. PRJNA872341). Other data including hatching success, fertilization, mate choice, the capture probe fasta, environmental data, and code to run all analyses are available on Zenodo (https://doi.org/10.5281/zenodo.7041408).

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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