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RESEARCH ARTICLE

Dynamic Cohort Analysis Reveals Fluctuating Patterns of Selection Within a Hybrid Zone Between the Killifish *Fundulus heteroclitus* and *F. grandis*

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Abstract

Hybrid zones provide excellent opportunities to study speciation processes and ecological interactions between recently diverged taxa. Historically, hybrid zones have been divided into those in which fitness of hybrids is independent of the environment, and those in which environmental factors influence the fitness of different genotypes. The present study investigated the temporal genetic patterns at a location within a hybrid zone between the killifish *Fundulus heteroclitus* and *F. grandis*, in an effort to determine the extent and directionality of hybridization and the fitness of different genotypes. Fishes collected over the course of three years were placed into two age classes and genotyped at three nuclear loci and one mitochondrial locus that are highly differentiated between the species, allowing for comparison of genetic patterns between different age classes of the same cohorts. Individuals of hybrid descent were prevalent at the study site, the majority of which were likely advanced generation hybrids or backcrosses to one of the parental taxa. The cohort analyses revealed decreased abundance of both single and dilocus hybrid genotypes, and directional changes in allele frequency with increased age in some, but not all cohorts. These fluctuating patterns of selection across the course of the study suggest that fitness is likely strongly influenced by environmental factors.

Keywords Hybridization · *Fundulus* · Exogenous selection · Hybrid fitness · Hybrid zones

Introduction

Hybrid zones are areas where a pair of strongly differentiated taxa overlap in range and interbreed, forming offspring of mixed descent (Harrison, 1993). These areas are often dynamic locations that can be effectively used to measure the strength of reproductive barriers, differences in fitness among genotypes, and the direction and strength of gene flow between the species (Arnold, 1997). Therefore, hybrid zone studies have been essential for the understanding of speciation and the ecology of species interactions and ranges.

Hybrid zones have historically been described as either environmentally independent tension zones, where hybrids have low fitness due to negative epistatic interactions between the genomes of the two species, or environmentally dependent zones where the fitness of hybrid and parental genotypes differ across habitats (Barton & Hewitt, 1985; Curry, 2015). Traditional environmentally dependent hybrid zones can be mosaic, with each species having higher fitness in different and patchily distributed habitats while hybrids have lower or intermediate fitness in those habitats (Harrison, 1986, 1990; Howard, 1986), or areas where hybrids have higher fitness than parental genotypes in intermediate habitats (bounded hybrid superiority)(Moore, 1977). Still, many hybrid zones fail to fit neatly into one of these categories and many studies have suggested that some zones fit multiple models (Arntzen & Wallis, 1991; Bert & Arnold, 1995). Furthermore, hybrid zones are often located in ecotones whether they experience environmentally dependent (exogenous) or environmentally independent (endogenous) selection (Arnold, 1997). Therefore, it is essential to understand the patterns of selection within hybrid zones in order to understand the importance of ecological differences at maintaining species ranges and barriers, and to know

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whether the location of the hybrid zone is likely to shift during environmental perturbations.

Previous work by Gonzalez et al. (2009) revealed a potential hybrid zone between two species of killifish, *Fundulus heteroclitus* and *F. grandis*, in Northeastern Florida. *Fundulus heteroclitus* occupies the bulk of the Atlantic coast of the United States, ranging from Southwestern Newfoundland in Canada to Northeastern Florida in the United States (Able & Felley, 1986). On the other hand, *F. grandis* is the more southern ranging of the two species occupying much of the coast of the Gulf of Mexico and the Atlantic coast of Florida to the area near Daytona (Lee et al., 1980). These two species overlap in range across approximately 40 km in the Atlantic Intracoastal Waterway of Florida and evidence of hybridization was found at a site near Flagler Beach, Florida, where allele frequencies at a pair of highly differentiated loci showed a strong mix of both parental species and putative hybrid genotypes (Gonzalez et al., 2009). This location also lies within an ecotone between *Spartina spp.* dominated salt marsh habitats to the North and mangrove dominated swamps to the South.

Laboratory studies by Barbas and Gilg (2018) verified the possibility of hybridization and quantified the strength of reproductive barriers between the two species. Barbas and Gilg (2018) showed the presence of both pre- and post-zygotic barriers to reproduction with asymmetrical overall isolation; *F. heteroclitus* females were found to be less likely to successfully produce hybrid offspring than were *F. grandis* females. The presence of post-zygotic barriers verified the presence of endogenous selection, but the observation was limited to low hatching success in crosses of *F. grandis* males with *F. heteroclitus* females. Reciprocal crosses did not suffer low hatching success and some F1 hybrids successfully backcrossed showing that at least some hybrids are fertile. No studies have yet focused on naturally hybridizing populations of these *Fundulus spp.* to determine whether the reproductive patterns observed in the laboratory studies are replicated or if exogenous selection also exists.

Therefore, the goal of the present study was to investigate the temporal genetic patterns of the hybrid populations of *Fundulus spp.* near Flagler Beach, Florida in an effort to address five questions. First, how prevalent are hybrid genotypes and do both first and second (multi) generation hybrids exist? Second, how strong are reproductive barriers between these species in the field? Third, do hybrids have low fitness in comparison to parental genotypes? Fourth, do natural hybrid populations of *F. heteroclitus* and *F. grandis* show the same asymmetry in reproductive isolation as predicted by laboratory crosses conducted by Barbas and Gilg (2018)? Lastly, is there evidence for environmentally based selection, or does fitness appear to be independent of the environment? These questions were investigated through the use of a dynamic cohort analysis, whereby fish were divided into age classes and members of the same cohort were compared across years. Given the small home ranges and dispersal tendencies of these species (Skinner et al., 2005; Sweeney et al., 1998; Williams et al., 2008), any consistent genetic changes observed across time are likely to be due to selection as opposed to immigration from distinct populations nearby.

Methods

Marker Development

Previous studies of this population had utilized a single nuclear marker (the 5' UTR of Lactate dehydrogenase B (LDHB)) and one mitochondrial marker (Cytochrome b (Cyt-b)) to distinguish members of each species. Since studies of hybrid zones require the use of multiple nuclear markers to reliably distinguish parental and recombinant genotypes, the first requirement of the present study was to generate additional markers that could reliably distinguish between the parental taxa. The National Center for Biotechnology Information nucleotide databases (GenBank) were searched to identify nuclear loci that had been previously sequenced in both *Fundulus heteroclitus* and *F. grandis*. The findings included recombination activating gene 1 (Rag-1) and glycosyltransferase (Gylt), both of which were sequenced in one or both species of *Fundulus* by Li et al. (2007) and Whitehead (2010). Utilizing the protocols and primers described in Whitehead (2010) we generated additional sequences of each locus for each species utilizing DNA samples from Gonzalez et al. (2009) that had been collected from reference populations; Cedar Key, Florida for *F. grandis* and the St. Mary's River, Georgia for *F. heteroclitus*. Each locus was amplified by PCR and then sequenced in both directions utilizing the same primer combinations used by Whitehead (2010). In total, 11 additional sequences of Rag-1 were obtained of which four were from *F. grandis* and 7 from *F. heteroclitus* (Accession numbers: KU055453-KU055463). We also added 11 new sequences of Gylt, of which six were from *F. grandis* and five from *F. heteroclitus* (Accession numbers: KU055464-KU055474).

Edited sequence data for sequences obtained in the present study, as well as sequences downloaded from GenBank were uploaded into Sequencher (V. 4.9) to generate maps of all available restriction enzyme cleavage sites. Maps of the cleavage sites were compared between species to identify restriction enzymes that would discriminate between alleles of the two species. This provided a simple and relatively inexpensive method of genetically distinguishing alleles typical of the two species. In the case of Gylt, sequences isolated from *F. heteroclitus* were predicted to be cleaved by the restriction enzyme Eco47-III, while sequences from

F. grandis remain uncleaved. For Rag-1, sequences from *F. heteroclitus* were expected to be cleaved by Mlu-I, while sequences from *F. grandis* were not. Assays involving the presence/absence of a single restriction site such as these can be problematic if DNA quality is low since you don't have the internal control provided by every allele being cut at least once. That said, DNA quality is more likely to cause problems with amplification and once the sample gets amplified the PCR products should be of sufficient quality for a presence/absence assay to work consistently.

In order to generate smaller and more consistently amplified PCR products containing these restriction sites, new primers were designed to flank the restriction site of each locus using Primer3Plus and the assays were then tested on a larger set of samples from reference sites. This included *F. grandis* samples from Cedar Key (N = 71) and Port St. Joe (PSJ; N = 27), Florida, and *F. heteroclitus* samples from St. Marys, Georgia (N = 81) from the Gonzalez et al. (2009) study. We also obtained *F. heteroclitus* samples (N = 54) from Georgetown, South Carolina from staff at the Baruch Marine Laboratory of the University of South Carolina.

A portion of Rag-1 was amplified from each DNA sample utilizing primers RagFa (5′-CAGAGCGAAATGCAATGAAA) and RagRa (5′-CCCGATTTCATCCTGAAAGA) and the PCR protocol of 35 cycles of 95 °C for 45 s., 60 °C for 1 min., 72 °C for 1 min.. These primers generated a 410 bp fragment that was easily amplified from both *F. heterclitus* and *F. grandis*. A 480 bp region of Gylt was amplified from each DNA sample using primers Gylt1F (5′-TAGCCCAGGAGTTCCAAATG) and Gylt1R (5′-GCTGGCTTACTCTTCATGCC) and the PCR protocol of 35 cycles of 95 °C for 45 s., 59 °C for 1 min., and 72 °C for 1 min.. Successful amplification for each was verified using a 1% agarose gel, after which a restriction enzyme was added to the PCR products for genotyping. PCR products of Rag-1 were digested with Mlu-I while PCR products of Gylt were digested with Eco47-III. In both cases manufacturer's instructions were followed for incubation temperature but the volume of restriction enzyme was increased by 50% and incubation time was increased by 20% to ensure complete digestion. All samples were electrophoresed on a 2.5% agarose gel following the incubation period and the samples were scored as homozygous cleaved (two bands), homozygous uncleaved (one band) or heterozygous (three bands) in each case (See Fig. 1 for example gel). All gels were run with a heterozygous positive control to ensure the enzyme was functional and to make for easy comparison of the banding patterns of the samples. While not completely diagnostic, both loci showed highly differentiated allele and genotype frequencies between the two species (Supplemental Tables 1 and 2) and were added to the two previous loci for analysis of samples from the Flagler Beach study site. Gylt was the most differentiated of the nuclear loci with an overall frequency of "cleaved" alleles of 0.964 in *F. heteroclitus* reference populations but only 0.017 in *F. grandis* reference populations. On the other hand, Rag1 was the least differentiated locus with a frequency of "cleaved" alleles of 0.818 in reference populations of *F. heteroclitus* but only 0.008 in reference populations of *F. grandis*.

Fish Collection and Size Structure

Fishes were collected from a marsh in the Gamble Rogers State Park near Flagler Beach, Florida from May–September of 2015, 2016 and 2017 (under FL DEP Scientific Collecting Permit 09061613). Minnow traps baited with dog food were placed in small rivulets and along the edges of marsh grasses (e.g.: *Spartina alterniflora*) and mangroves during falling or rising tides. Similar sampling effort occurred near both vegetation types, much occurring in areas of mixed vegetation. Collected specimens were preserved in 95% ethanol for later genetic analysis. Total length (TL) of each individual was assessed to the nearest 0.1 mm utilizing calipers and fishes were then allocated to 5 mm size classes.

To determine age class membership, we constructed a simple, length-based model that assigns individual fish to an age class as a function of fish length. Specifically, the model estimates the probability of a fish of length l belonging to age class a by fitting a predicted length frequency distribution to observed length data (Johnson & Swenarton, 2016). The predicted length-frequencies were generated by assuming the observed lengths of each age a in each year y

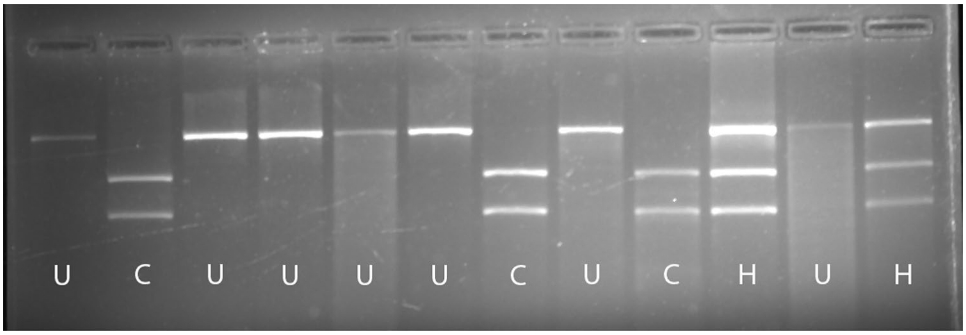

Fig. 1 An example of Gylt PCR products after digestion by Eco47 III. Labels at the bottom of the gel represent uncleaved homozygotes (U), cleaved homozygotes (C) and heterozygous genotypes respectively

followed a normal distribution with mean length ($\bar{l}_{a,y}$) and variance ($\sigma^2_{a,y}$). A two-age class model was most consistent with the known biology of this species (Fritz & Garside, 1975; Kneib & Stiven, 1978; Vastano et al., 2017) and overwhelmingly supported by the data for all years (AIC ω_i = 0.99). The probability of age class membership ($P_{a,y}$) for each size class was then used to assign ages to individual fish so that they could be assigned to a cohort (Table 1). This allowed for comparison of genetic data of members of the same cohort across time. For example, the youngest age class (< 1 year old) collected in 2015 should be members of the same cohort as the 1–2 year old individuals from 2016.

Genetic Analysis

DNA was extracted from a clip of the caudal fin using a Phenol–Chloroform extraction procedure (Sambrook & Russell, 2001). Following extraction, each individual was genotyped at three nuclear loci (LDHB, Rag1 and Gylt) and one mitochondrial locus (Cyt-b). Protocols for Rag1 and Gylt are as described above, while LDHB and Cyt-b assays both followed the protocols outlined in Gonzalez et al. (2009). Briefly, LDHB and Cyt-b were amplified by PCR (using only the internal primers described in Gonzalez et al., 2009) and then digested with PstI and NsiI, respectively. Alleles of *F. heteroclitus* for LDHB are typically cleaved by PstI while alleles of *F. grandis* for Cyt-b are cleaved by NsiI. Genotypes at all nuclear loci were analyzed by NewHybrids v. 1.1 (Anderson & Thompson, 2002) in order to determine whether each individual was likely a member of genotypic classes representing *F. heteroclitus*, *F. grandis*, F1, F2, or backcrosses to either parent species.

Hybrid fitness and the strength of reproductive barriers were both assessed utilizing tests of Hardy–Weinberg and gametic disequilibrium. Specifically, the strength of reproductive barriers between the two species was estimated from single locus heterozygote deficiencies and pairwise gametic disequilibrium coefficients of the smallest size classes in each year. Since these represent the most recently produced offspring, a significant lack of hybrid genotypes would suggest the presence of strong reproductive barriers between the two parental species. This could be either due to infrequent matings with heterospecific individuals or to low survival prior to, or soon after hatching. It is also possible that low hybrid fitness does not manifest immediately, and instead takes the form of lower survival compared to parental genotypes. In this case it would be expected that the deficiency in heterozygotes and dilocus recombinant genotypes should increase with age. Therefore, Wright's F statistic (F_{IS}) and standardized gametic disequilibrium coefficients (D′) were compared across different age classes of each cohort to determine if they showed the increase expected of low hybrid survival. All assessments of Hardy–Weinberg and gametic disequilibrium were performed using GenAlEx v. 6.5 (Peakall & Smouse, 2012) with estimation of D' as described in Lewontin (1964). A Bonferroni correction for multiple tests was utilized to determine statistical significance of any values.

As an additional test for low hybrid fitness we estimated a folded hybrid index (FHI) for each age class of each cohort utilizing the methods of Dowling and Moore (1985). Briefly, a principal component analysis (PCA) was conducted on the three-locus genotypes of samples from the reference populations of *F. heteroclitus* and *F. grandis*. PC1 accounted for 91.8% of the variance with coefficients as follows: Rag1, 0.559; Gylt, 0.590; LDHB, 0.583. A PC1 score then serves as a weighted hybrid index with large positive scores representing *F. grandis*, large negative scores representing *F. heteroclitus* and various hybrids showing scores intermediate to the two extremes. The hybrid index is then "folded" by centering the distribution around zero and calculating the absolute value of PC1 scores for all individuals to obtain a score of relative hybridity. The relative hybridity scores for each individual were then averaged to estimate the average relative hybridity for the small and large size classes for each of the two cohorts. If hybrids are selected against, then mean FHI is expected to increase from one age group to the next within a cohort since that would indicate a decrease in the average hybridity of the population.

To test for potential directional effects of selection, allele frequencies were compared across age classes of the same cohort using a G-test of independence. Low survival of hybrid genotypes or patterns of directional allele frequency changes in later age classes would provide evidence of environmentally based selection occurring well after

Table 1 Probabilities of inclusion of different 5 mm size classes (total length) of fishes in two different age classes (P_0 = < 1 year old, P_1 = 1–2 years old)

Size (mm)	25	30	35	40	45	50	55	60	65	70	75 +
P_0	0.959	0.963	0.954	0.928	0.863	0.704	0.412	0.138	0.028	0.003	~ 0
P_1	0.041	0.037	0.046	0.072	0.137	0.296	0.588	0.862	0.972	0.996	~ 1

Values shown are for fishes pooled across three collection years (2015, 2016 and 2017). All fishes above 75 mm in Total Length had approximately the same probabilities of inclusion development and hatching. Similarly, allele frequencies at each locus were compared between the adults of one cohort and the yearlings of the next, which should be their offspring (i.e.: > 1 year olds of 2016 vs. < 1 year olds of 2016). This was done to investigate the possibility of selection associated with reproduction and, again, involved use of a G-test of independence.

To test for potential asymmetry in hybridization a pair of methods were utilized. First, the genotype at the Cyt-b locus of individuals identified as likely hybrids by NewHybrids was utilized in a series of Chi-square goodness-of-fit tests under the assumption that the maternal lineage was equally likely to be *F. heteroclitus* or *F. grandis*. Separate tests were conducted on: (1) putative F1 hybrids only, (2) each of the backcross directions, and (3) for all of the likely hybrids in the data set. Second, tests of cytonuclear disequilibrium were conducted between all three nuclear genotypes and the cyt-b cytotype. These tests were conducted with the program CNDd (Asmussen & Basten, 1996) utilizing Fisher exact tests to determine if Cyt-b cytotypes were non-randomly associated with nuclear genotypes at each locus.

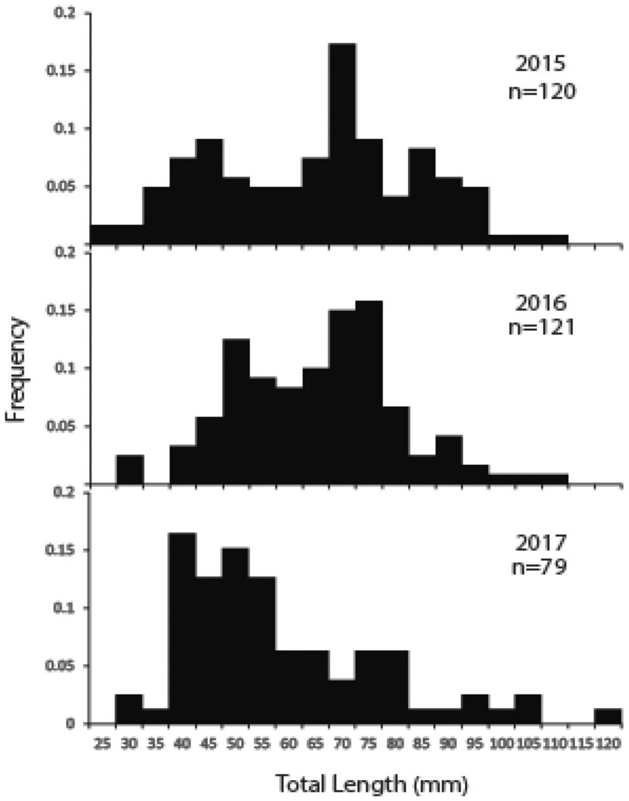

Fig. 3 The frequency of fishes collected for different 5 mm size classes in each of the three collection years

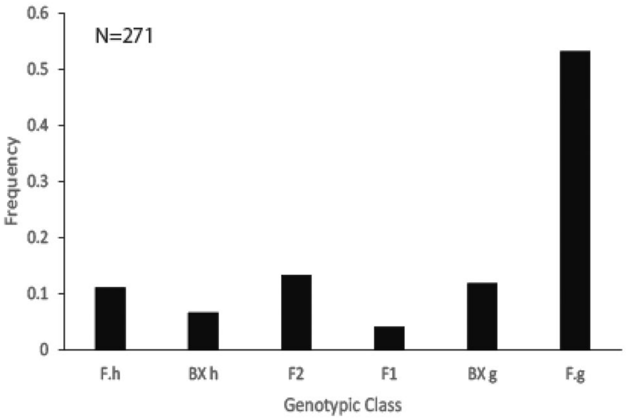

Fig. 2 Proportions of fishes with genotypes conforming to one of six possible classes of parental or hybrid genotypes with a scaled likelihood > 0.5 according to a NewHybrids (Anderson & Thompson, 2002) analysis. F.h and F.g respectively represent *Fundulus heteroclitus* and *F. grandis* parental genotypes. F1 and F2 denote first and second-generation hybrids while BXh and BXg represent backcrosses to *F. heteroclitus* and *F. grandis* respectively

Results

The NewHybrids analysis confirmed that hybrid genotypes exist in substantial numbers at Flagler Beach. While three nuclear markers that are not completely diagnostic are insufficient to statistically confirm which genotypic class the majority of the fish belonged to, many of the genotypes observed had an extremely low probability of being either pure *F. heteroclitus* or *F. grandis* (Fig. 2). A total of 67 individuals genotyped from the study site (n = 321) had posterior probabilities > 0.95 of being a hybrid of some kind. Of those, 57 had a posterior probability > 0.95 of being an F2 hybrid while the other 10 could not be placed into a specific hybrid category with P > 0.95. Even with a posterior probability > 0.95 of being an F2 hybrid it should be noted that this designation is dubious with data from only three genetic markers. Therefore, it would be best to simply consider these as being advanced generation hybrids of some kind.

Since the majority of individuals could not be placed in a specific category with > 0.95 confidence, we also utilized the scaled likelihood output of NewHybrids to get a less conservative estimate of the genotypic category of each fish. Using the scaled likelihood output of NewHybrids, an individual that was scored as homozygous for alleles typical of *F. heteroclitus* at all three nuclear loci has a probability of ~ 0.869 of being a pure *F. heteroclitus*. Similarly, an individual that was homozygous for alleles typical of *F. grandis* at all three nuclear loci has a ~ 0.840 probability of being a pure *F. grandis*. Using the larger of these two values (0.87) as a threshold, there were approximately 26.8% of the samples that had a probability > 0.87 of being a hybrid of some kind. So hybrid genotypes likely make up at least 20% of all of the individuals collected (based on the posterior probabilities > 0.95) and possibly 27% or more (based on scaled likelihood).

Of the 321 samples genotyped, the majority belonged to one of the six NewHybrids genotypic classes (*F. heteroclitus*, *F. grandis*, F1, F2, backcross to *F. heteroclitus*, backcross to *F. grandis*) with a scaled likelihood > 0.5 (Fig. 2). Those that did not have a probability of inclusion in a single class > 0.5 were considered ambiguous and not included in Fig. 2. Given that this methodology is not as statistically sound as use of posterior probabilities, the results should be taken as preliminary until the patterns can be confirmed with additional loci. Approximately half of the samples genotyped from Flagler Beach had the highest probability of being pure *F. grandis*, with F2, backcrosses to *F. grandis* and pure *F. heteroclitus* being the next most abundant genotypic classes with frequencies between 0.11 and 0.14. Putative F1 hybrids were the least abundant genotypic class, comprising < 5% of all samples.

Size-frequency distributions of all fishes collected in each year of the study are shown in Fig. 3. The age-class probability analysis revealed that each year's size distribution was best explained with two modes, likely representing a pair of age classes (AIC $\omega_i = 0.99$). Since these species typically live two years in the field a bimodal distribution makes biological sense (Fritz & Garside, 1975; Kneib & Stiven, 1978; Vastano et al., 2017). Therefore, the three-year data set of the present study represents two complete cohorts. Cohort one includes the members of the smallest size classes (smaller mode) in 2015 and the larger size classes (mode) in 2016. Cohort two are the members of the smaller mode in the 2016 collection and the larger mode in year 2017. When combining the data sets from each year the age-class probability analysis shows that fishes < 55 mm in TL have at least a 70% probability of inclusion in the small size class, while fishes > 60 mm in TL have at least an 86% probability of inclusion in the large size class (Table 1). The 55–60 mm size class is ambiguous, and was therefore, not included in any of the analyses.

Analysis of each nuclear locus shows that all three conform to Hardy–Weinberg expectations in the small sizes classes of both 2015 and 2016, with no significant lack of heterozygous genotypes (Table 2). On the other hand, two of the three nuclear loci show a significant lack of heterozygous genotypes in the large size class of 2016 and all three loci have a significant heterozygote deficiency in the large size

Table 2 Comparisons of deviation from Hardy–Weinberg expected heterozygosity (F_{IS}) and gametic disequilibrium (D) and standardized gametic disequilibrium (D′) for two different size and age classes of *Fundulus spp*. in each of three collection years in a hybrid zone

Locus	Test Statistic	Large 2015	Small 2015	Large 2016	Small 2016	Large 2017	Small 2017
			——Cohort 1——		——Cohort 2——		
Rag 1	N	**77**	37	67	27	**29**	37
	F_{IS}	**0.324**	− 0.042	0.202	0.264	**0.513**	0.313
	(P)	**(< 0.001)**	(0.797)	(0.095)	(0.171)	**(0.006)**	(0.057)
Gylt	N	**78**	37	**69**	27	**28**	**36**
	F_{IS}	**0.571**	− 0.057	**0.479**	0.339	**0.659**	**0.468**
	(P)	**(0.005)**	(0.728)	**(< 0.001)**	(0.078)	**(< 0.001)**	**(0.005)**
LDHB	N	**78**	37	**79**	29	**30**	**37**
	F_{IS}	**0.454**	− 0.014	**0.473**	0.215	**0.630**	**0.630**
	(P)	**(< 0.001)**	(0.934)	**(< 0.001)**	(0.248)	**(0.001)**	**(< 0.001)**
Rag 1 / Gylt	N	**77**	37	**63**	**25**	27	34
	D	**0.165**	0.009	**0.203**	**0.088**	0.047	0.068
	(P)	**(< 0.001)**	(0.190)	**(0.003)**	**(0.006)**	(0.316)	(0.107)
	D’	**0.938**	0.231	**0.910**	**1.000**	0.364	0.706
Rag 1 / LDHB	N	**77**	37	**63**	**25**	**27**	**34**
	D	**0.221**	0.001	**0.115**	**0.113**	**0.060**	**0.189**
	(P)	**(< 0.001)**	(0.763)	**(0.003)**	**(< 0.001)**	**0.999**	**(< 0.001)**
	D’	**0.965**	0.077	**0.569**	**0.785**	**(0.018)**	**0.813**
Gylt / LDHB	N	**77**	37	**63**	**25**	27	**34**
	D	**0.189**	0.002	**0.141**	**0.063**	0.038	**0.170**
	(P)	**(< 0.001)**	(0.724)	**(0.001)**	**(0.006)**	(0.256)	**(0.001)**
	D’	**0.995**	0.154	**0.806**	**0.788**	0.562	**0.913**

P values are shown in parentheses below each test statistic with significant values in bold and the number of individuals in the data set provided for each test (N). Columns are color coded for comparisons of samples that represent different age classes of the same cohort. The two complete cohorts (cohort 1 = small 2015 and large 2016; cohort 2 = small 2016 and large 2017) are labeled for ease of comparison class of 2017. This suggests that heterozygous genotypes have been lost with an increase in age in both cohorts. While the large size class of 2015 cannot be directly compared to younger members of the same cohort, the patterns observed were consistent with the large size classes of 2016 and 2017 in that all loci showed a significant heterozygote deficiency. Unlike the small size classes of 2015 and 2016, however, fishes belonging to the small size class of 2017, tended to deviate significantly from Hardy–Weinberg expectations with a significant lack of heterozygotes observed at both Gylt and LDHB, and a non-significant trend in the same direction at Rag1.

Tests of gametic disequilibrium show a similar pattern to the single locus comparisons with a few additional discrepancies (Table 2). An apparent loss of recombinant (hybrid) genotypes is observed in the first cohort where none of the pairwise comparisons of loci in the small size class of 2015 deviate significantly from null expectations, but hybrid genotypes are strongly lacking in the large size class of 2016. Data from the second cohort is not as clear since two of the three pairwise comparisons of loci significantly lack recombinant genotypes in the small size class of 2016 and only one pairwise comparison differs from expectations in the large size class of 2017. So instead of values of D′ getting larger in the older individuals of cohort two, a slight reduction was observed in two of the three pairwise comparisons. While the large size class collected in 2015 and the small size class collected in 2017 cannot be directly compared to members of their same cohort, the patterns of gametic disequilibria for both are similar to that seen in the single locus tests. Fishes in the large size class collected in 2015 showed a significant lack of recombinant genotypes for all pairs of loci, as did the small size class collected in 2017.

Analysis of the FHI for each age class in each cohort provided additional evidence for loss of hybrid genotypes with age. The FHI showed a dramatic increase from 0.202 to 0.958 between the small size class and the large size class of the first cohort (for comparison, FHI of the reference samples was 1.549). The second cohort also showed an increase in FHI, but to a much lesser extent with the small and large age classes having scores of 0.574 and 0.623 respectively. Sample sizes can be obtained from Table 2.

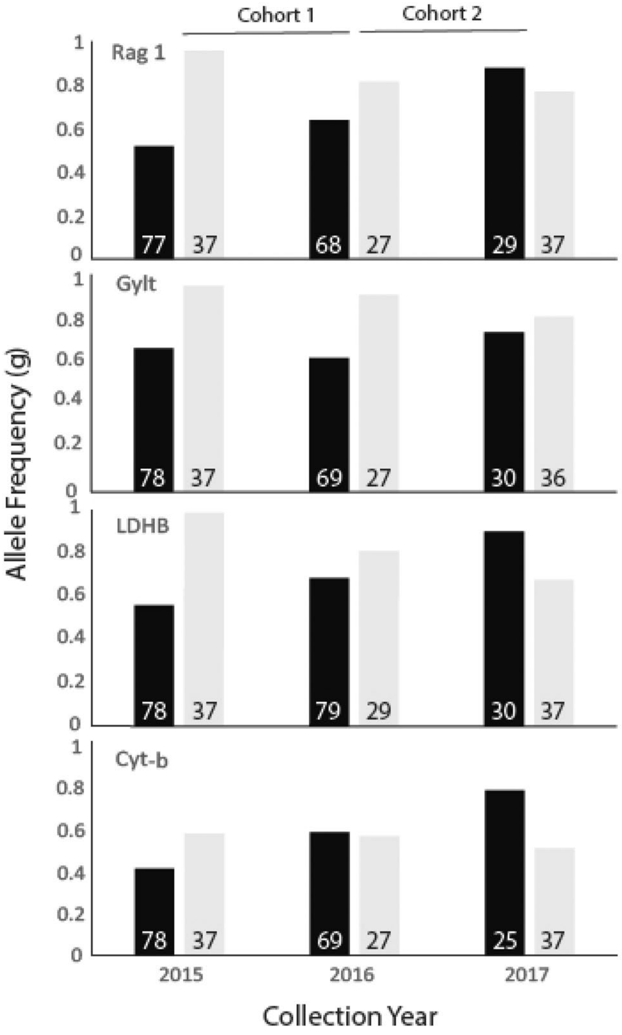

Fig. 4 Frequencies of alleles typical of *Fundulus grandis* (g) at four loci for fishes of different size (age) classes collected in each of the three years of the study. Black bars represent the larger of the two size classes while grey bars identify the smaller of the two size classes. These collections encompass two cohorts, the small fishes collected in 2015 and the large fishes of 2016 representing cohort one, and the small fishes collected in 2016 and the large fishes from 2017 representing cohort two (identified by horizontal bars at the top of the figure). The numbers at the bottom of each bar show the number of individuals in the sample

Allele frequencies in the first two years of collection show remarkably similar patterns with small size classes in both years having significantly higher frequencies of alleles typical of *F. grandis* at all three nuclear loci than do the large size classes (Fig. 4) (2015: Rag1: $G = 53.2$, $P < 0.001$; Gylt: $G = 26.3$, $P < 0.001$; LDHB: $G = 57.9$, $P < 0.001$; 2016: Rag1: $G = 5.87$, $P < 0.05$; Gylt: $G = 18.1$, $P < 0.001$; LDHB: $G = 3.52$, $P < 0.05$; $df = 1$ for all tests). This pattern is also very apparent in cohort one, where the frequency of *F. grandis*-alleles at all nuclear loci drops from 0.94 + in the small size class of 2015 to between 0.6–0.7 in the large size class of 2016 (Rag1: $G = 32.2$, $P < 0.001$; Gylt: $G = 32.0$, $P < 0.001$; LDHB: $G = 36.7$, $P < 0.001$; $df = 1$ for all tests). This drop in the frequency of *F. grandis*-alleles is due to a precipitous drop in the frequency of homozygous genotypes for those alleles at each nuclear locus while both heterozygous, and homozygous *F. heteroclitus* genotypes increase in frequency (Fig. 5). This pattern shifts in the third year of the study (2017), however, where samples in the small size class show no allele frequency differences from the large size class at either Rag1 ($G = 2.9$, $df = 1$, $P > 0.05$) or Gylt

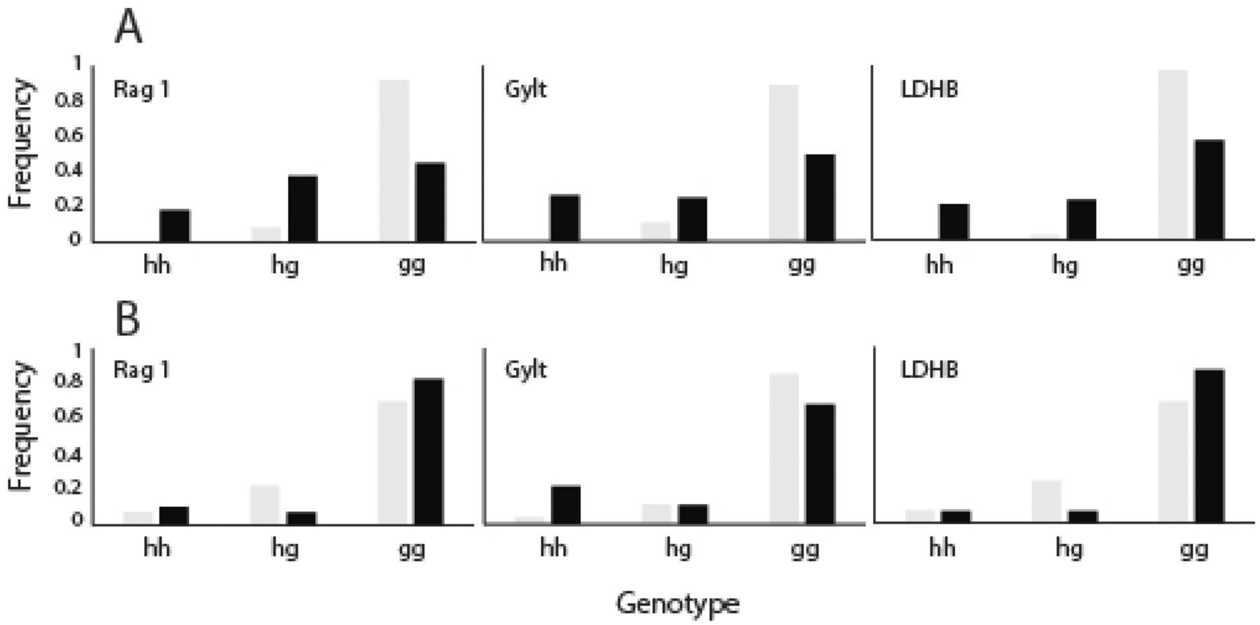

Fig. 5 Genotype frequencies of three nuclear loci for two different cohorts of killifish. Panel A shows cohort 1 (small size of 2015 in grey, large size of 2016 in black) while panel B shows cohort 2 (small size of 2016 in grey, large size of 2017 in black). Alleles typical of *F. heteroclitus* are denoted with "h" while alleles of *F. grandis* are "g."

($G = 0.9$, df = 1, $P > 0.05$) while *F. grandis* allele frequencies are significantly higher in the large size class at locus LDHB ($G = 10.2$, df = 1, $P < 0.01$). Also, allele frequencies in cohort two show no significant difference between the small size class of 2016 and the large size class of 2017 at either Rag1 ($G = 0.9$, df = 1, $P > 0.05$) or LDHB ($G = 1.9$, df = 1, $P > 0.05$) while *F. grandis* alleles at Gylt show a significant decrease in frequency from ~ 0.91–0.73 ($G = 6.1$, df = 1, $P < 0.05$). The Gylt locus did not show a change in the frequency of heterozygotes in cohort two, but homozygous genotypes for *F. heteroclitus* and *F. grandis* alleles increased and decreased respectively (Fig. 5).

Unlike the nuclear markers, allele frequencies at Cyt-b stayed relatively consistent for most of the study (Fig. 4). Allele frequencies did not differ significantly between size classes in either of the first two sample years but had significantly higher frequencies of alleles typical of *F. grandis* in the large size class than the small size class of 2017 (2015: $G = 2.8$, $P > 0.05$; 2016: $G = 0.0$, $P > 0.05$; 2017: $G = 6.0$, $P < 0.05$; df = 1 in all tests). No significant changes in allele frequency were detected between the age classes of either cohort one ($G = 0.2$, df = 1, $P > 0.05$) or cohort two ($G = 3.2$, df = 1, $P > 0.05$) although there was a trend toward an increase in *F. grandis* alleles in the large size class of cohort two.

Hybrid genotypes of each category identified by NewHybrids were produced by females of each of the two species in approximately equal proportions. Putative F1 hybrids were few in number with $n = 4$ individuals produced by a *F. heteroclitus* mother and $n = 7$ produced by a *F. grandis* mother ($X^2 = 0.82$, df = 1, $P > 0.05$). Analysis of all putative hybrids were also nearly equally split between the two maternal lineages with 0.56 having mtDNA from *F. heteroclitus* and 0.44 from *F. grandis* ($X^2 = 1.61$, df = 1, $P > 0.05$). Likewise, the maternal lineage of each class of backcross did not show significant asymmetry, but they did show a trend of the Cyt-b genotype matching the direction of the backcross. Approximately 0.65 of putative backcrosses to *F. grandis* also had Cyt-b from *F. grandis* ($X^2 = 2.94$, df = 1, $P < 0.1$) while approximately 0.72 of putative backcrosses to *F. heteroclitus* had Cyt-b from *F. heteroclitus* ($X^2 = 3.56$, df = 1, $P < 0.1$).

Tests of cytonuclear disequilibrium between Cyt-b and each nuclear locus also showed no evidence of directionality. In each case, strong disequilibrium was shown for both homozygous genotypes ($P < < 0.001$ for both homozygotes at each nuclear locus) with an overabundance of matching cytotypes and nuclear genotypes (i.e.: hh:h and gg:g). No disequilibrium was observed between the heterozygous genotype and mitochondrial cytotype at any of the three loci (Gylt: $P = 0.751$, Rag 1: 0.126, LDHB: 0.288).

Discussion

Hybridization Patterns

Hybrid genotypes between *F. grandis* and *F. heteroclitus* at Flagler Beach were relatively common with evidence of both first and second (or beyond) generation hybrid individuals. The low proportion of putative F_1 genotypes suggests that either heterospecific mating events are relatively rare, or that F_1 offspring have low survival during development. Genotypes indicative of second generation (and potentially beyond) hybrids were considerably more common resulting in a bimodal hybrid index at this site. The overall lack of F_1 hybrids conforms fairly well to the patterns observed in laboratory crosses of *F. heteroclitus* and *F. grandis* by Barbas and Gilg (2018). Under competitive conditions (a female housed with males of both species) very strong reproductive barriers were estimated for females of both species (total reproductive isolation values of 0.56 and 0.90 for *F. grandis* and *F. heteroclitus* females respectively). When females were housed with a single male (either conspecific or heterospecific) estimates of total reproductive isolation were lower but still substantial for females of both species (values of 0.25 and 0.55 for *F. grandis* and *F. heteroclitus* females respectively). That said, samples of the smallest size classes (young-of-the-year) in some years conformed to Hardy–Weinberg expectations and were less likely to be in gametic disequilibrium than were samples from larger size classes. These data suggest that hybrid mating events may be fairly common at times and that the lack of hybrid genotypes may be due to lower survival than parental genotypes (discussed below). In other years, however, young-of-the-year samples showed significant deviation from both Hardy–Weinberg and gametic equilibrium suggesting that mating was often not occurring at random.

While the data from Barbas and Gilg (2018) showed a relative lack of successful heterospecific matings under laboratory conditions when females were exposed to males of both species simultaneously, it is unknown whether females at Flagler Beach are often courted by males of both species at the same time. If they are not, then higher rates of interspecific matings would be expected since the strength of reproductive isolation was considerably lower under non-competitive conditions. It is also possible that heterospecific mating events simply occur less often in the field due to environmental parameters not replicated in the lab, or to reproductive barriers that were not tested by Barbas and Gilg (2018). Differences in reproductive timing or habitat use, for example, could limit heterospecific matings beyond what was observed in the laboratory. The breeding season of the two species partially overlap, with data from allopatric regions suggesting that *F. grandis* breeds between March and August (Greeley & MacGregor, 1983) and *F. heteroclitus* breeds between May and August (Brummett, 1966; Selman, 1986). If these differences also exist when the species are sympatric, it could further reduce heterospecific mating events resulting in limited production of F_1 offspring, but to our knowledge, no data are currently available on the breeding seasons in areas of sympatry. Similarly, differential habitat use for spawning could also factor into the lack of F1 hybrids, but again, the extent to which the two species utilize different habitats for spawning is unknown.

The relative abundance of each species can also have a strong influence on hybridization patterns, resulting in different outcomes in the laboratory and the field. Studies of hybridization in fishes have often showed that in cases of hybridization where the two parental taxa exist in unequal numbers that the rarer of the two species tends to hybridize more (Avise & Saunders, 1984; Dowling et al., 1989). At Flagler Beach, killifish populations tend to be dominated by *F. grandis,* with putatively "pure" *F. grandis* making up more than 53% of our samples while only ~ 11% of our samples were putatively "pure" *F. heteroclitus*. This difference in relative abundance would lead to an expectation of more hybridization by *F. heteroclitus* than expected from laboratory crosses. This is obviously not significantly affecting the production of F1 hybrids, but backcrosses to *F. heteroclitus* are fairly common relative to the proportion of *F. hetreoclitus* at this location. This should lead to greater introgression into *F. heteroclitus* overall.

The relatively high proportion of second generation and beyond hybrid individuals show that hybrids are at least partially fertile and capable of successfully interbreeding with both of the parental taxa and other hybrids. Barbas and Gilg (2018) showed that hybrid offspring produced in the lab were capable of successfully mating with both of the parent species, but sample sizes were too limited to draw strong conclusions as to how common second-generation hybridization would be expected to be. The overall abundance of advanced generation hybrids and backcrosses from the present study would suggest that at least some of the barriers to reproduction between *F. heteroclitus* and *F. grandis* appear to be reduced when dealing with the reproduction of hybrids, at least at FB. This bimodal pattern of genotypes within hybrid zones is not an uncommon pattern that is more often linked with high levels of assortative mating as opposed to post-zygotic isolation (Reviewed in Jiggins & Mallet, 2000). Other hybrid zones involving species in the genus *Fundulus* that have been investigated also reveal the presence of both first and second generation and beyond hybrids, but most appear to have considerably higher proportions of putative F1 genotypes than the present study (Chavez & Turgeon, 2007; Duvernell et al., 2006; Schaefer et al., 2009, 2016). Therefore, it would appear that hybrids within this genus are often capable of successful reproduction, but the likelihood of successful heterospecific matings depend on the species pair involved.

The results from Barbas and Gilg (2018) also suggested that *F. heteroclitus* females are considerably less likely to produce hybrid offspring than are *F. grandis* females. If this were the case, then the majority of hybrids collected should have Cyt-b genotypes of *F. grandis*. This pattern, however, was not observed and instead all analyses suggested that there was no significant directionality to either the heterospecific mating events, or those involving hybrids. The lack of significant directionality could be due to the presence of previously unquantified reproductive barriers that limit the production of hybrids by *F. grandis* females, resulting in more similar levels of reproductive isolation in females of both species. Since Barbas and Gilg (2018) was a laboratory study, environmentally associated or site-specific barriers were not taken into account and may play a role in the patterns observed in the present study. Alternatively, the apparent lack of directionality may be a result of differences in overall abundance between the two species at this location. Since *F. grandis* is considerably more common at this site, encounters between *F. heteroclitus* females and *F. grandis* males may exceed conspecific encounters in *F. heteroclitus*. In fact, based on the proportions of different genotypic classes from the NewHybrids analysis, we would expect encounters between a *F. heteroclitus* and a *F. grandis* genotype to be approximately five times as likely as an encounter between two *F. heteroclitus* individuals. Therefore, even if reproductive barriers are stronger, the increased number of heterospecific encounters involving *F. heteroclitus* females may effectively erase the expected pattern of directionality.

Fitness Patterns

Evidence for reduced fitness of hybrids was observed throughout portions of the study. This was most clear in the first year of the study (2015), as well as the first cohort (small 2015 vs. large 2016). The young-of-the-year fishes collected in 2015 conformed to Hardy–Weinberg expectations of heterozygosity at the three nuclear loci, and all pairs of loci were in gametic equilibrium. The older fishes in both 2015 and 2016, however, consistently lacked both single locus heterozygotes and recombinant dilocus genotypes suggesting that hybrid genotypes had lower survival than either of the parental species. These differences between age classes are intriguing since it suggests that the barriers to reproduction between the two species are not always strong, but that differential survival often generates a lack of hybrid genotypes. This is also supported by the dramatic increase in FHI between the two age classes of the first cohort, confirming a decrease in hybridity with an increase in age.

While the aforementioned increase in both single locus and dilocus disequilibria with increased age was clear in the first cohort, the second cohort showed a different pattern. In cohort two, there was a substantial reduction in single-locus heterozygotes in the large size class compared to the small size class, again suggesting low survival of hybrid genotypes. The dilocus comparisons, however, were not as clear and in several cases the amount of disequilibrium was greater in the small size class than the large. The small size class in 2017 also showed significant deviations from both Hardy–Weinberg and gametic equilibrium at two of three individual loci and two of three dilocus genotypes. These data show that some cohorts show an initial lack of hybrid genotypes, due either to non-random mating patterns or lower developmental success in hybrids, but do not necessarily show a loss of hybrid genotypes as age increases. Similarly, comparisons of FHI between the two size classes of the second cohort suggested an overall reduction in hybrid genotypes with age, but not nearly as much as was observed in the first cohort.

The inconsistency of these patterns suggest that hybrid fitness varies over time at the study location, indicating that environmentally based (exogenous) selection is likely a factor. Barbas and Gilg (2018) showed the existence of some disruption of development in F_1 hybrids produced by matings of *F. grandis* males with *F. heteroclitus* females, providing evidence for endogenous selection against some hybrids. If hybrid genotypes have intrinsically low fitness, however, the observed patterns should be consistent across time and space. Therefore, it would appear that exogenous selection is also involved in producing the observed patterns.

The inconsistency in fitness patterns is also apparent in the allele frequency changes observed throughout the study. Allele frequencies can be compared among different groups of samples to assess differences in survival or reproduction. For example, changes in allele frequencies between age classes of the same cohort can be used to assess differences in survival among genotypes. Alternatively, differences between the 1–2 year old individuals and the < 1 year old fishes collected in the same year can shed light on potential reproductive advantages since the < 1 year old fishes are likely the offspring of the 1–2 year old fishes. In the present study, there is evidence of directional selection working in opposite directions with regard to survival and reproductive success, and the patterns were not temporally consistent.

The first cohort of the study showed a precipitous drop in the frequency of alleles typical of *F. grandis* between the young-of-the-year in 2015 and the 1–2 year old fishes of 2016 at all three loci. While there was no young-of-the-year size class to compare to the 1–2 year old samples from 2015, they also had a lower frequency of *F. grandis* alleles compared to other samples. These data suggest that, at least early in the study, individuals with alleles typical of *F. heteroclitus* had a survival advantage over those with more *F.*

grandis-like genotypes. This pattern was repeated in the second cohort at the Gylt locus, but the other two nuclear loci did not show significant changes in allele frequency.

In regard to reproductive success, however, the small size classes of both 2015 and 2016 had significantly higher frequencies of alleles typical of *F. grandis* than did the larger size classes of the same years. This suggests that individuals with *F. grandis*-like genotypes tended to dominate the production of offspring in those two years. As was the case with patterns of survival, however, the samples from 2017 showed effectively no change in allele frequency between the large and small size classes of the same year, so the reproductive advantage of *F. grandis*-like genotypes was no longer apparent. Future assessments of the killifish populations at this location will be necessary to determine the stability of the patterns observed in the present study.

The inconsistencies in these patterns across time suggests an environmental component to fitness differences, but what is the most likely environmental component? While abiotic factors such as temperature and salinity can be important factors in determining species ranges and survival in many fishes (Fry, 1947; Sylvester, 1975), these are unlikely to play a direct role in the survival of *F. grandis* and *F. heteroclitus* at the study site. Both species have been shown to be tolerant of salinities nearing 0 ppt to > 100 ppt (Griffith, 1974; Whitehead, 2010). Likewise, temperature tolerance is extremely wide in these two species and far exceeds the temperatures encountered at Flagler Beach, ranging from near freezing to > 40 °C (Bulger, 1984; Fangue et al., 2006; Galleher et al., 2010). That said, it is also possible that while the two species can both tolerate similar temperatures and salinities, they may have different optima. Therefore, this should be investigated more thoroughly in the future.

If abiotic tolerances are unlikely to determine differences in survival, then the most likely factor for survival would seem to be habitat. Since the hybrid zone roughly coincided with an ecotone between mangrove dominated marsh systems to the south and grass (*Spartina spp.*) dominated salt marshes to the north, Gonzalez et al. (2009) hypothesized that the ranges of the two species were related to differences in vegetation. *Fundulus heteroclitus* is known to occupy salt marsh habitats dominated by *Spartina alterniflora* throughout its range (Kneib, 1986), while *F. grandis* often occupies habitats dominated by species of mangroves like *Avicennia germinans* (Duggins et al., 1989; Ellis & Bell, 2004). That said, *F. grandis* also occupies *Spartina* dominated areas in the more northern portions of its range in the Gulf of Mexico (Rozas & Reed, 1993). At least partially due to a decreased frequency of extreme freeze events in Northeastern Florida, mangroves have been expanding their range northward over at least the last two decades or more (Rodriguez et al., 2016) and in 2014 were found approximately 24 km north of St. Augustine Inlet near Jacksonville, Florida (Williams et al., 2014), a location well inside the known range of *F. heteroclitus* (Gonzalez et al., 2009). The study site near Flagler Beach contains a mixture of these plants and has since at least the Gonzalez et al. (2009) study. No data on relative abundances of mangroves and marsh grasses is available for Flagler Beach over the course of the present study, but it is likely that it is following the current trend toward increasing mangrove coverage found in the region (Cavanaugh et al., 2019; Radabaugh et al., 2017; Rodriguez et al., 2016). Therefore, it is possible that in the first part of the present study that mangrove cover was low enough that individuals with *F. heteroclitus*-like genotypes tended to have higher survival, but as mangrove habitat increased this survival advantage has been mostly lost. Direct tests of this hypothesis will be required to determine its validity.

The apparent advantage in reproduction of *F. grandis*-like genotypes may have four components. First, the overall greater abundance of *F. grandis* genotypes at this location may result in a larger proportion of encounters with *F. grandis*-like mates. Second, *F. grandis* males tend to grow to larger sizes than do *F. heteroclitus* males (Barbas & Gilg, 2018). While the effects of male size on mate choice has not been tested in these species to our knowledge, male body size has been shown to influence mate choice in other Cyprinodontiform fishes (Reynolds & Gross, 1992). Therefore, it is possible that both *F. grandis* and *F. heteroclitus* females preferentially mate with the larger *F. grandis* males leading to increased frequencies of alleles typical of *F. grandis* in the < 1 year old fishes when compared to the 1–2 year old adult population that likely produced them. Third, *F. grandis* has been shown to have an earlier and more extended mating season than does *F. heteroclitus*, at least in allopatric portions of their ranges (Brummett, 1966; Greeley & MacGregor, 1983; Selman, 1986). This could increase the success of *F. grandis* in at least two ways. The extended season could allow for more reproductive events (both conspecific and heterospecific) for *F. grandis* individuals, and, if many *F. grandis*-like individuals are born in the early part of the season they may grow to a size capable of feeding on eggs or larvae produced by *F. heteroclitus*. Piscivory, including cannibalism, has been observed in both the field and the laboratory, at least in *F. heteroclitus* (Able et al., 2007), and both species are known to eat the eggs of conspecifics (Able & Hata, 1984). Fourth, it is also possible that the apparent reproductive advantage of *F. grandis* is related to habitat in some way, but it is not known whether these species show any kind of habitat-specific patterns in areas of sympatry. Future studies should focus on the breeding seasons and habitat use of these species in the sympatric portions of their range and on the effect of male body size on both conspecific and heterospecific reproductive success.

An alternative explanation for the observed changes in allele frequency is that there have been species-specific patterns of movement into and out of the study site. Over the course of the study, the population of killifishes at Flagler Beach tended became more dominated by *F. grandis*-like genotypes, especially in the larger size classes. It is therefore, possible, that the allele frequency changes are not being caused by differences in survival, but by the movement of *F. heteroclitus*-like individuals away from Flagler Beach and the movement of *F. grandis*-like individuals into the location. This hypothesis seems implausible for several reasons. First, it would require not only genotype-specific movement patterns but also age-specific movement patterns with young *F. grandis*-like genotypes moving into the study site along with older *F. heteroclitus*-like genotypes in the first two years. These patterns would also need to change across the temporal span of the study with movement of older *F. grandis*-like genotypes into Flagler Beach by 2017. Second, previous studies of these species suggest that they typically show limited movement and very small home ranges. Mark-recapture studies by Lotrich (1975), Sweeney et al. (1998) and Skinner et al. (2005) all showed movement distances of less than a few hundred meters in natural populations of *F. heteroclitus*. Comparable studies show similarly small home ranges for *F. grandis* (Nelson et al., 2014; Jenson et al., 2019). These home ranges are easily encompassed by the size of the study site and are significantly smaller than the distances to the nearest locations with substantial populations of these species (M.R. Gilg, pers. obs.). Thus, it would appear much more likely that the changes in allele frequency are produced by selection and not migration.

Synopsis

Fundulus heteroclitus and *F. grandis* hybridize to a large extent at Flagler Beach with production of both first and second generation and beyond hybrids. Previous work by Barbas and Gilg (2018) showed the presence of endogenous selection against hybrids in the form of lower developmental success in one direction of the cross. The present study provides evidence of both selection against hybrids and also directional selection affecting both survival and reproduction, but the observed patterns differ across years suggesting that exogenous selection is also likely affecting the population at this location. Therefore, the data would suggest that the hybrid zone between *F. heteroclitus* and *F. grandis* is unlikely to be a true tension zone, but it is currently unknown how allele frequency and fitness vary across space and throughout the rest of the genome. Future work should focus on fine scale spatial dynamics of the hybrid zone utilizing a larger panel of molecular markers to further test whether this hybrid zone best fits an environmentally-based model.

If environmental factors are confirmed to affect fitness of hybrids in this zone, then it is conceivable that the hybrid zone could shift. The data in the present study suggested that *F. heteroclitus* had a survival advantage in the early part of the study but the advantage was not observed in the latter half. This may suggest that the population at Flagler Beach will become more dominated by *F. grandis* in the near future since *F. grandis* appeared to have an advantage in the production of offspring at this site. That said, the allele frequencies estimated in the present study are fairly consistent with those observed in Gonzales et al. (2009). Two of the loci used in the present study were also used in Gonzales et al. (2009), including Cyt-b and LDHB. Frequencies of alleles typical of *F. grandis* were 0.6 at Cyt-b and 0.77 at LDHB in Gonzales et al. (2009). Pooling members of both size classes from each year of the present study results in frequencies of *F. grandis* alleles ranging from 0.46 to 0.63 at Cyt-b and from 0.69 to 0.786 for LDHB. Therefore, very little overall change has been observed in approximately one decade between the two studies, over which span the population has completely turned over approximately five times. This suggests that fluctuations in the direction of selection across relatively short time scales as shown here may result in relatively stable allele frequency patterns over greater spans of time.

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Data Availability The datasets generated during and/or analyzed during the current study are available upon contacting the corresponding author.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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