Variation in self-compatibility among genotypes and across ontogeny in a self-fertilizing vertebrate

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Abstract

Mixed mating strategies offer the benefits of both self-fertilizing one's own eggs (selfing) and outcrossing, while limiting the costs of both methods. The economics of mixed mating is further determined by individual self-compatibility. In gynodioecious (hermaphrodites, females) and androdioecious (hermaphrodites, males) species, the level of self-compatibility of the hermaphrodites also acts as a selection pressure on the fitness of the other sex. Mangrove rivulus fish populations are comprised of selfing hermaphrodites and males that result from hermaphrodites changing sex. Although hermaphrodites overwhelmingly reproduce through internal selfing, they occasionally oviposit unfertilized eggs. Males can externally fertilize these eggs. Here, we reveal that fecundity and self-compatibility varies within individuals across ontogeny until about 365 days post hatch, and among individuals derived from lineages that vary in their propensity to change sex. Hermaphrodites from high sex changing lineages were significantly less fecund and self-compatible than hermaphrodites from low sex changing lineages. These differences in self-compatibility and fecundity have the potential to drive evolutionary changes on mating strategy and the fitness of males in populations of the mangrove rivulus. This study also illustrates the importance of including lineage variation when estimating the costs and benefits of mixed mating strategies.

Introduction

As a reproductive strategy, self-fertilization has many benefits. It overcomes the "cost of males," wherein only one half of the population can produce offspring compared to the entire population in asexual or self-fertilizing species (Maynard Smith 1971, 1978). Self-fertilization also assures reproduction when pollinators and/or mates are scarce (Darwin 1876; Baker 1955; Stebbins 1957; Pannell et al. 2015), eliminates physical damage associated with mating and/or sexually transmitted parasites or pathogens (Fowler and Partridge 1989; Clutton-Brock and Parker 1995), and maintains well-adapted genotypes in stable environments (Allard et al. 1972; Dolgin et al. 2007). However, selection for selfing is often counteracted by selection for outcrossing. Benefits of outcrossing include combatting inbreeding depression in two ways: i) preventing accumulation of deleterious alleles in single genotypes, and ii) maintaining heterozygotes that may be more fit than homozygous individuals (Muller 1932). Ultimately, outcrossing represents a bet hedging strategy because it increases the variability of offspring fitness against a changing abiotic environment, or against coevolving parasites, predators and competitors (Muller 1932; Bell 1988; Lively 2010; Morran et al. 2011; Hartfield and Keightley 2012).

Many plant and invertebrate species have evolved a mixed mating strategy of selfing and outcrossing. Mixed mating allows individuals and populations to reap the rewards of both strategies as biotic and abiotic factors change the fitness landscape across space and time. Because the fitness landscape is variable, it would be expected that self-compatibility also would vary both within and among populations due to changes in the magnitude and/or type of selection acting for and against selfing and outcrossing (Escobar et al. 2011). Plant populations that are often mate- and/or pollinator-limited at the range edge or in ephemeral habitats have

increased self-compatibility compared to other populations, as reported for the small angiosperm *Leavenworthia alabamica* (Busch 2005). Populations found near the center of the range and in more permanent habitats are much more likely to be self-incompatible (Busch 2005). When among-individual variation in self-compatibility is heritable, it provides the genetic and phenotypic background on which selection can drive evolutionary change in mating strategies. There are multiple examples of heritable variation in the self-compatibility of plants, including in the creeping bellflower, *Campanula rapunculoide* (Stephenson et al. 2000), and the Carolina horsenettle *Solanum carolinense* (Travers et al. 2004). Genetic and phenotypic variation in self-compatibility, along with environmental variability, can thus drive variation in mating strategy, as they alter the economics of selfing and outcrossing.

In mixed mating populations, self-compatibility can also vary across ontogeny, perhaps as a plastic response to environmental conditions. Individuals in populations that have higher rates of outcrossing often become more self-compatible with increasing age. This is often the result of selection favoring a "waiting" strategy for outcrossing opportunities when individuals are young, presumably to derive the benefits of variable and more fit progeny but also to assure some reproduction even if mates and/or pollinators are limited. This has been documented in plants (*Campanula rapunculoide*; Stephenson, Good, and Vogler 2000, *Solanum carolinense;* Travers, Mena-Ali, and Stephenson 2004), and snails (*Physa acute*; Tsitrone, Jarne, and David 2003), but few other self-fertilizing species. Populations can also evolve different mating strategies based on age structure. If younger individuals are more populous, and mating opportunities are plentiful enough to make selfing unnecessary, age-dependent changes in self-compatibility may be lost so that little or no selfing occurs. Alternatively, if populations consist

77 predominantly of older individuals, selfing may continue to be an evolutionary stable component
78 of a mixed mating strategy.

79 In androdioecious species, in which populations consist of hermaphrodites and males,
80 variation in self-compatibility should also result in variation in the proportion of males within a
81 population. Populations consisting of individuals with lower self-compatibility should maintain a
82 higher proportion of males, as the "cost of males" is decreased by more outcrossing
83 opportunities. Hypotheses concerning the evolution and variation of self-compatibility and
84 mixed mating have been examined in many plant and invertebrate systems (Busch 2005; Winn et
85 al. 2011; Wright et al. 2013), but not in a vertebrate species. Here we present evidence for
86 individual variation in self-compatibility in one of only two vertebrates known to utilize a mixed
87 mating strategy, the mangrove rivulus fish (*Kryptolebias marmoratus*).

88 The mangrove rivulus (hereafter "rivulus"), is a small euryhaline killifish that inhabits
89 high elevation mangrove forests of Florida, the Bahamas, and Central America. Populations
90 consist primarily of self-fertilizing hermaphrodites with varying proportions of males (no
91 females). Rates of outcrossing and selfing also vary among populations and correlate with the
92 proportion of males in the population; outcrossing rates increase as the proportion of males
93 increase (Mackiewicz et al. 2006*b*; Tatarenkov et al. 2009). While self-fertilization happens
94 internally, males can only achieve reproductive success if/when hermaphrodites lay unfertilized
95 eggs, and there is no evidence that hermaphrodites outcross with each other (Furness et al. 2015).
96 Rivulus males result from sex change, after which selfing hermaphrodites that make the sexual
97 transition become obligate outcrossers. There also appears to be genetic variation for the
98 propensity to change sex (Gresham et al. 2020, Turner 2006). Hermaphrodites fertilize the vast
99 majority of their own eggs (> 94%, Harrington 1971, personal observations), giving males very

limited opportunities for reproductive success. Previously, it was reported that while individuals that change sex to male are significantly more likely to survive environmental challenges than those that remain hermaphrodite, they are much less likely to lay any eggs before sex change occurs (only 2 out of 180, Gresham et al. 2020). This presents the problem of how individuals that change sex might successfully pass their sex-changing alleles on to the next generation.

We hypothesized that the proportion of fertilized eggs (a measure of self-compatibility) would change with age and/or the propensity of a given lineage to change sex. First, we predicted that self-compatibility would increase with age. Cole and Noakes (1997) reported histological evidence that ovarian tissue matures before spermatogenic tissue. We predicted that there would be a period immediately after sexual maturity where oviposition of unfertilized eggs would be more likely. Second, we predicted that overall fecundity and/or self-compatibility would be greater in lineages that are more likely to change sex. This prediction is based on two competing ideas about how sex-changing alleles are successfully maintained in rivulus populations: i) selection should favor individuals in high sex changing lineages to be very fecund and self-compatible prior to changing sex, so as to compensate for any lost reproductive success after the transition, and/or ii) selection should favor individuals derived from lineages that change sex frequently to be highly fecund and self-compatible so that the siblings of sex changers recoup the lost reproductive success of their "brothers."

Methods

We designed and executed an experiment to measure the fecundity and self-compatibility of 213 individual fish representing 100 genetically distinct lineages (determined via microsatellite genotyping using 32 loci; (Mackiewicz et al. 2006*a*; Tatarenkov et al. 2010). We

calculated the propensity to change sex for each of the 100 lineages as the proportion of individuals in each lineage that transition from hermaphrodite to male under common garden conditions (Gresham et al. 2020), which ranged from 0 - 68.6%. All experimental fish were raised from hatching in individual plastic containers (Rubbermaid® Take-a-long Deep Squares) filled with approximately 200 mL 25‰ synthetic saltwater (prepared with Instant Ocean® sea salt and aged tap water) until 30 days post hatching (dph), at which time water volume was increased to 600 mL (25‰). At 60 dph, we suspended a large marble-sized ball of egg-laying substrate (Poly-fil®) from one top corner of the container. Beginning at 67 dph, each container was checked weekly for eggs in the substrate, around the top edge of the container, and on the bottom of the container. Fish were checked weekly for eggs until they changed sex to male or were 365-371 dph, ensuring that each remaining hermaphrodite was at least 365 dph and had been checked weekly 43 times.

Eggs were collected into 59 mL plastic cups (Fabri-Kal® Greenware GXL250PC) filled with approximately 6 mL 25‰ synthetic saltwater. Eggs were immediately counted and viewed under a stereomicroscope. Eggs were photographed and scored as either fertilized, unfertilized, or inviable. Fertilized and unfertilized eggs were identified by the presence or absence of a perivitelline space, respectively. Inviable eggs were identified by their marked discoloration and opaque appearance (Figure 1). Unfertilized and inviable eggs were removed from the cup and discarded. Fertilized eggs were left in the cups, and water was replaced every 7 days. Cups were checked every day for hatchlings. Hatchlings from the experimental animals were then scored as alive or dead, with live hatchlings being euthanized in 4ºC water. Experimental fish were also observed for signs of sex change each week when the tubs were checked for eggs, as described in (Scarsella et al. 2018); orange freckles or orange skin is the most reliable external character of

sex change. Individuals that changed sex (35 of the 213) were separated from the others and the date and age recorded. All experimental animals were isolated from the general colony for the duration of the experiment, kept on a 12h light: 12 h dark photoperiod, and fed a 4 mL suspension of brine shrimp (*Artemia*) nauplii (~2000 shrimp) six days per week. Room temperature was also maintained at 26.45 ± 1.6 °C (mean ± SEM). The University of Alabama Institutional Animal Care and Use Committee approved all procedures (IACUC Protocol #18-10-1644).

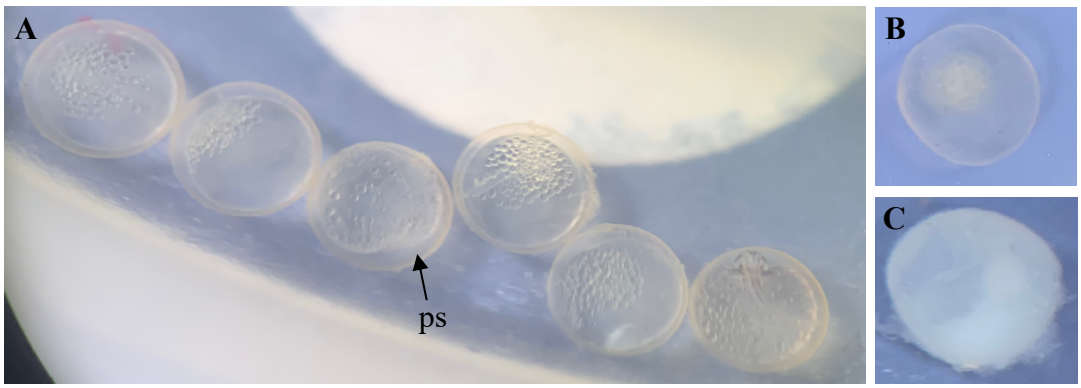

Figure 1. Scoring of eggs. Eggs were scored as A) fertilized, B) unfertilized, or C) inviable. Fertilized eggs were identified by the presence of the perivitelline space (ps). Unfertilized eggs lack the perivitelline space. Inviable eggs were identified by their opaque or discolored appearance.

Fecundity and Self-compatibility Statistical Analysis

We examined the relationship between fecundity (number of eggs laid) and both the propensity for a given lineage to change sex, and the individual's age. To determine whether fecundity varied as a function of the lineages' propensities to change sex, we first ran a nominal logistic model with 'laid eggs' as a categorical dependent variable (yes = 1, no = 0) and lineage sex change propensity as the independent variable. We used a general linear model to examine the relationship between total number of eggs laid (continuous, dependent) and lineage sex change propensity (independent). Residuals of the total number of eggs were left skewed, so we

transformed the number of eggs with square root (x+1). To determine whether fecundity (total number of eggs laid) varied as a function of age, we used a generalized regression model with a zero-inflated Poisson distribution and parent ID as a random effect to account for the fact that each parent was checked for eggs multiple times. An initial plot of the raw data suggested a possible non-linear relationship between age and fecundity so, we constructed linear, quadratic and cubic models and compared model fits using Akaike's Information Criterion (AIC). The linear, quadratic and cubic age terms were scaled by mean and standard deviation (i.e., z-score) prior to running the models. The cubic model fit best (Table 3.1), so we report the results of this model.

We modeled self-compatibility in two ways: by the proportion of eggs that were fertilized and by hatch success of fertilized eggs. To determine whether the proportion of fertilized eggs varied as a function of the lineages' propensity to change sex, we ran a general linear model. To determine whether the proportion of fertilized eggs varied as a function of the parents' age when the eggs were collected, we ran a general linear model with parent ID as a random effect. To determine whether the proportion of fertilized eggs varied as a function of the total number of eggs laid by each parent, we ran a general linear model. We calculated hatch success as the proportion of fertilized eggs that resulted in a live hatchling. To determine whether hatch success varied as a function of the lineages' propensity to change sex, we ran a general linear model. Models were run in JMP Pro version 15.0.0 (JMP®, Version 15 Pro 2019) and in RStudio using the glmmTMB package (RStudioTeam 2016; Brooks et al. 2017; R Core Team 2018). Data files will be deposited on GitHub upon publication acceptance.

Results

The experiment included 213 individual fish from 100 different lineages. Thirty-five of those fish changed sex to male (16.4%) before the experiment ended at 365 - 371 dph. Of those 35, three individuals laid eggs first (9% of males, 1% of the total cohort). We collected 14,310 eggs, had 10,267 hatchlings (71.7% of eggs hatched), and 10,059 live hatchlings (98% of hatchlings were alive). The three individuals that changed sex to male laid a total of 69 eggs by 197 dph (the oldest age at which a fish was identified as male), which equated to, on average, 23 ± 13 (SEM) eggs per individual among the three, but 1.97 ± 1.4 eggs per capita for all individuals that changed sex to male. The remaining 178 hermaphrodites laid a total of 7,341 eggs before they collectively aged to 197 dph (41.2 ± 2.2 eggs per individual). We started checking for eggs from each fish at 67 dph and collected a single egg from one individual on the first egg check; the egg was fertilized. Overall, the proportion of eggs that were fertilized was 93.4%. The proportion of eggs that were unfertilized was 2.5%, and the proportion identified as "inviable" was 4.1%.

Our results indicate that fecundity initially increases with age until about 175 dph, then decreases until it plateaus at about 350 dph (Table 1 Figure 2A). The proportion of fertilized eggs also increased significantly with age, but not the total fecundity of the parent (Table 1, Figure 2B). Because we did not record the hatch success of each individual collection (i.e., clutch) of eggs, we were not able to analyze whether hatch success changed with age. The lineages' propensity to change sex was significantly related to fecundity and self-compatibility. When individuals that changed sex to male were included in the model, the odds of laying any eggs decreased significantly as the propensity to change sex increased (Table 2). However, this relationship became non-significant when individuals that changed sex to male were excluded

(Table 2). Conversely, the number of eggs laid decreased significantly as the propensity to change sex increased, whether or not males were included in the model (Table 2 Figure 3A). Both the proportion of fertilized eggs and the hatch success of those eggs also decreased significantly with increasing propensity to change sex (Table 2, Figure 3B, C).

Table 1. Summary of fecundity and self-compatibility models with age of parent fish as the dependent variable. AIC = Akaike's Information Criterion; ZI = zero inflation; F = F-ratio; P = P-value; Z = Z-value; df = degrees of freedom.

Fecundity						
Models	AICc	ΔAICc	ZI parameter estimate (p-value)	Estimate	Z	P
# eggs ~_age + age^2 + age^3	33140.0	0.0	-0.98 (<0.0001)	age: 3.06	10.73	<0.0001
				age^2: -5.76	9.85	<0.0001
				age^3: 2.52	7.94	<0.0001
# eggs ~_age + age^2	33198.5	58.5	-0.92 (<0.0001)	age: 0.85	13.01	<0.0001
				age^2: -1.14	-17.67	<0.0001
# eggs ~_age	33527.0	387.0	-0.83 (<0.0001)	age: -0.29	-29.58	<0.0001

Proportion of fertilized eggs				
	Estimate	F	df	P
Parent Age	0.000069	4.08	1	0.04
Number of Eggs Laid	0.000093	0.63	1	0.43

Table 2. Summary of fecundity, self-compatibility, and hatch success models with lineage sex change propensity as the dependent variable. Fecundity was square root (x+1) transformed to ensure normality. The models for proportion of fertilized eggs and hatch success already excluded the males that did not lay any eggs, thus separate models were not needed. Significant effects are shown in bold. F = F-ratio, P = P-value, df = degrees of freedom.

Probability of Laying Eggs	*Odds Ratio*	*χ2*	*df*	*P*
Lineage sex change propensity, **including** males	0.003	23.85	1	**<0.0001**
Lineage sex change propensity, **excluding** males	0.006	2.62	1	0.11
Fecundity (# of eggs laid)	*Estimate*	*F*	*df*	*P*
Lineage sex change propensity, **including** males	-4.17	36.29	1	**<0.0001**
Lineage sex change propensity, **excluding** males	-4.25	9.09	1	**0.003**
Proportion of fertilized eggs	*Estimate*	*F*	*df*	*P*
Lineage sex change propensity, **including** males	-0.10	4.86	1	**0.029**
Hatch Success	*Estimate*	*F*	*df*	*P*
Lineage sex change propensity, **including** males	-0.36	33.85	1	**<0.0001**

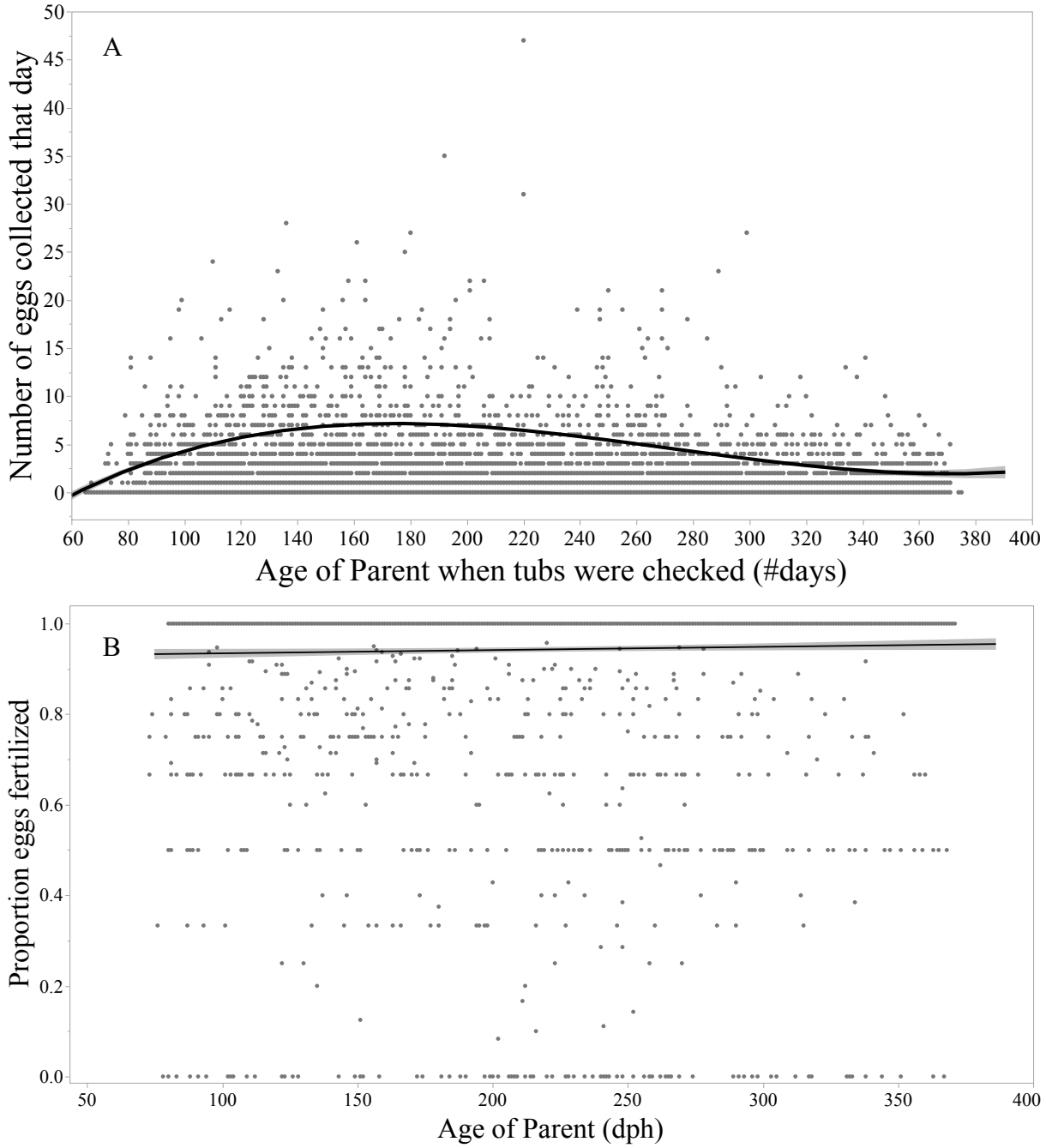

Figure 2. Age effects on the number of eggs collected and the proportion of those eggs that were fertilized. A) Age affected fecundity cubically; the number of eggs oviposited increased initially, then decreased between 170 – 180 dph, then plateaued around 350 dph. B) The proportion of eggs that were fertilized increased significantly with fish age. The additional straight line at the top of the graph is a string of points representing all of the egg collections where 100% of the eggs were fertilized.

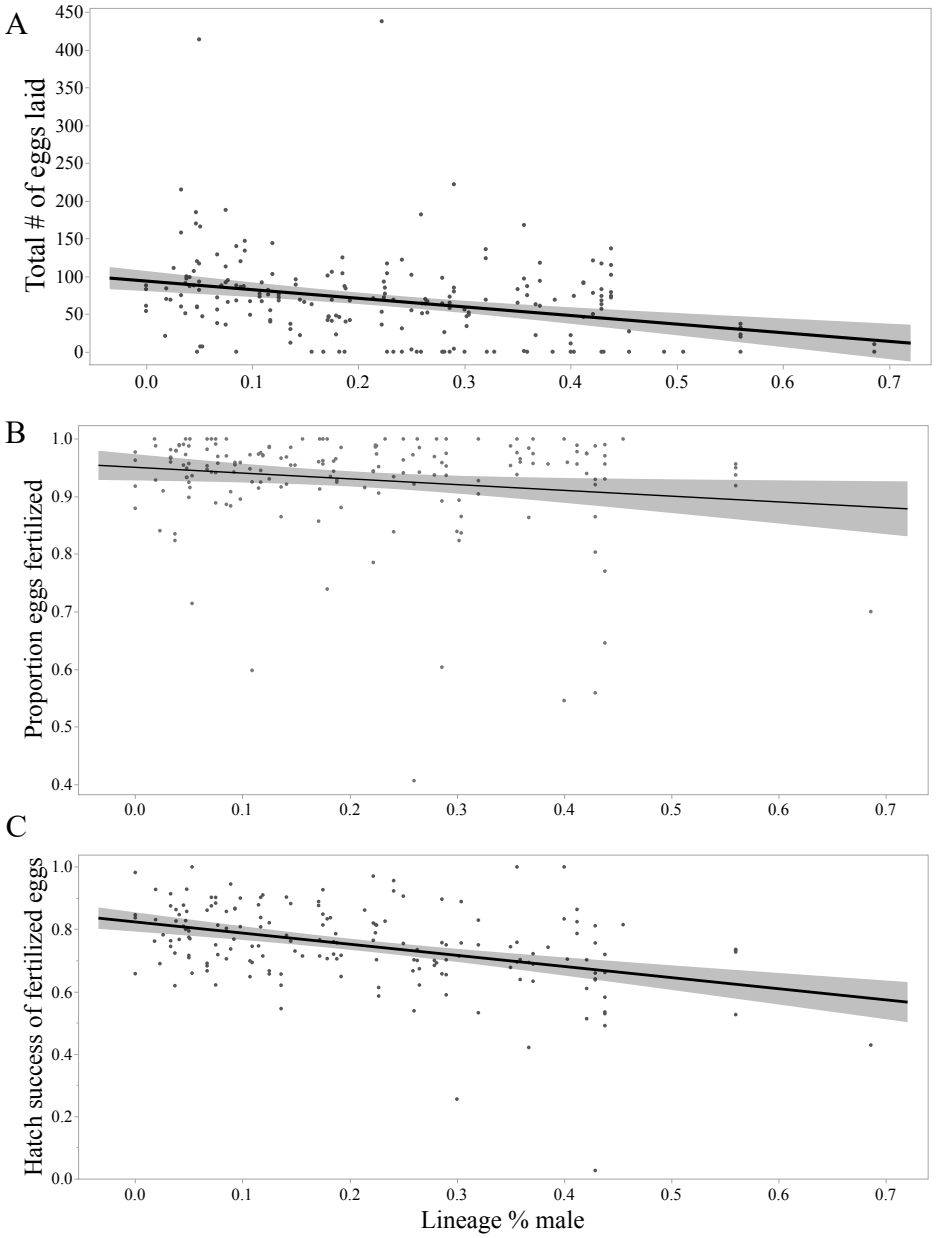

Figure 3. Lineage sex change propensity effects on A) fecundity, B) the proportion of eggs that were fertilized, and C) hatch success of fertilized eggs. Lineage sex change propensity is measured as the proportion of each lineage that change sex in isolation under common garden conditions in our colony (lineage % male).

Discussion

Mixed mating strategies offer the fitness rewards of both outcrossing with conspecifics and fertilizing one's own eggs. The cost-to-benefit ratio of each strategy is expected to vary across space and time as the external and internal environments also change (Maynard Smith 1971; Lively and Lloyd 1990; Lehtonen et al. 2012; Layman et al. 2017; Lynch et al. 2018). There is also variation among individuals in characters that affect mating strategy: fecundity and self-compatibility (Stephenson et al. 2000; Travers et al. 2004; Busch 2005). Variation in self-compatibility, combined with external factors such as pollinator and/or mate availability or parasite pressure, has the potential to drive evolutionary changes in mating strategy. For instance, if self-compatible individuals have greater reproductive fitness (produce more offspring and/or offspring have greater survival rates) and survive just as well as self-incompatible individuals, then the population may evolve to complete or near complete selfing. Comparatively, if there are plenty of mates/pollinators and/or inbreeding depression that increases the benefits to cost ratio of outcrossing, populations will evolve to complete or near complete outcrossing. We tested, in the only self-fertilizing hermaphroditic vertebrate, the hypothesis that self-compatibility and fecundity would vary with age and as a function of the lineages' propensities to change sex.

The most important finding was that both fecundity and self-compatibility decreased as lineages became more likely to change sex, which is the exact opposite of what we predicted. We predicted that fecundity and/or self-compatibility would increase with the lineages' propensity to change sex for one of two reasons: either individuals that eventually change sex would produce more eggs with greater self-compatibility, or the remaining hermaphrodites from high sex changing lineages would produce at least as many eggs with equal self-compatibility as those from lineages less likely to change sex. Given that neither of these predictions were

227 supported, it remains unclear how sex change from a self-fertilizing hermaphrodite to an obligate
228 outcrossing male is maintained in rivulus populations. Theory suggests that males incur a cost
229 because, when compared to self-fertilizing hermaphrodites, they cannot produce offspring and
230 also reduce the parent's genetic contribution to offspring by one half (Maynard Smith 1971,
231 1978). Males must overcome this cost by producing many extremely fit offspring.

232 Prior experiments have provided evidence of outbreeding depression on fecundity
233 (Gresham et al. 2020) in several ecologically relevant conditions, increasing the cost of males
234 and outcrossing. However, Ellison et al. (2011) provide evidence of potential inbreeding
235 depression under parasitic stress, therefore decreasing the cost of males. Male size also increases
236 with heterozygosity, possibly allowing more heterozygous males to secure more resources and
237 have greater access to unfertilized eggs (Molloy et al. 2011). The results from this experiment
238 suggest that the cost of being male is highly variable among lineages. Only three individuals laid
239 eggs before changing sex to male, indicating that they were not as fecund as self-fertilizing
240 hermaphrodites. The odds of laying any eggs decreased as sex change propensity increased when
241 all individuals were included in the model. However, when we excluded individuals that changed
242 sex, there was not an effect of lineage sex change propensity, demonstrating that the
243 hermaphrodites from high sex changing lineages are just as likely to lay *an* egg as those from
244 low sex changing lineages. In contrast, hermaphrodites from high sex changing lineages laid
245 significantly fewer eggs, even if the males were excluded from the model. Self-compatibility and
246 hatch success also decreased as propensity to change sex increased. These results indicate that
247 individuals from high sex changing lineages that remain hermaphrodite are not very fecund or
248 self-compatible. This exemplifies that the cost of changing sex to male might vary among

genetically distinct lineages. In particular, individuals from low sex changing, highly fecund and self-compatible lineages are likely to experience the highest cost of sex change.

We found that fecundity initially increases with age, then decreases, and finally plateaus as individuals age from about 60 dph to about 370 dph. The only other report of potential age-related changes in fecundity in rivulus comes from Harrington (1971). From 37 fish, he reported significant seasonality in "ovarian function" (i.e., fecundity) over three years when exposed to natural day light cycles, but not a significant change in fecundity with age. The seasonal decline reported by Harrington (1971), which occurred from September through December, aligns with the end of our experiment. The fish in our experiment hatched and then were removed from the experiment over a four-month period between 30 August 2018 and 22 December 2019. It is possible that the association between age and fecundity that we found, particularly the shallow decline in egg production from 180-300 dph represents the same seasonal decline described by Harrington (1971). He allowed his fish to experience natural daylight cycles. Our fish were kept on a constant photoperiod and a relatively constant temperature, and so should have been removed from any external signals of season. To truly understand how fecundity is affected by age and seasonality, we need additional years of records from multiple lineages.

Overall, 93.4% of the eggs we collected were fertilized. This is very close to the proportion of eggs fertilized reported by Harrington (1971). From six fish of one lineage and 1,056 eggs, he identified 94.3% as fertilized. We recorded 2.5% unfertilized and 4.1% inviable, compared to Harrington (1971), who reported 4% unfertilized and 1.7% inviable. It is likely that most of the inviable eggs we recorded were unfertilized when oviposited, but then became inviable and discolored by the time we collected them. Harrington (1971) collected eggs every day and was more likely to record unfertilized eggs before they became inviable. Our first

272 fertilized egg was collected at 67 dph, indicating the egg was oviposited sometime between 60
273 dph and 67 dph. This is the earliest reported oviposition in rivulus.

274 Regardless of the cost of changing sex to male, there is still the issue of passing sex
275 changing alleles to the next generation. How do lineages that are less fecund, less self-
276 compatible, and that frequently change sex to male not disappear from populations? It is possible
277 that many of these lineages do go extinct but, some Belizean populations maintain relatively high
278 male proportions (> 20%) and non-trivial rates of outcrossing (Davis 1990; Turner et al. 1992;
279 Tatarenkov et al. 2009, 2015), which suggests that males and outcrossing are under positive
280 selection in some places. Males must be able to successfully find unfertilized eggs, but we are
281 not sure how that happens. There have been multiple attempts to mate males and hermaphrodites
282 in the lab. Mackiewicz et al. (2006*a*) paired multiple nearly-senescent hermaphrodites and males
283 in individual tubs and confirmed two out of 32 offspring were outcrossed progeny. Nakamura et
284 al. (2008) surgically removed eggs from two hermaphrodites for *in vitro* outcrossing, but only
285 one out of thirteen hatchlings were outcrossed instead of self-fertilized. Our laboratory has tried
286 pairing hermaphrodites and males in different ratios with no outcrossing success. In behavior
287 trials, reported interactions between pairs of hermaphrodites and males have ranged from no
288 courtship behaviors (Martin 2007) to significantly more courtship behaviors than same sex pairs
289 (Luke and Bechler 2010) or courtship behaviors by the males with reciprocal attacks by the
290 hermaphrodites (Turner et al. 1992). It is clear that we have not yet reproducibly identified
291 conditions that favor outcrossing.

292 There often are opposing selection pressures for and against biparental sex (outcrossing).
293 Mixed mating individuals can benefit from outcrossing when the costs of biparental sex are low,
294 but self when the costs of biparental sex are high or when mates and/or pollinators are sparse.

295 The relative benefit of selfing or outcrossing among individuals is also dependent on the
296 individual level of self-compatibility, which can have a genetic basis and/or respond to the
297 external environment in a plastic or flexible manner. Individuals and lineages that are not very
298 self-compatible cannot exploit the benefits of selfing as much as individuals that are more self-
299 compatible, even when the costs of outcrossing are high. In the mangrove rivulus fish, we have
300 found significant variation in self-compatibility that correlates with age and with the lineages'
301 propensities to change sex. Individuals from lineages with a higher sex change propensity laid
302 significantly fewer eggs and a significantly lower proportion of the eggs were self-fertilized (i.e.,
303 they were less self-compatible). Also, the proportion of fertilized eggs increased with age.
304 In wild populations, interactions between individuals' level of self-compatibility and
305 external conditions have the potential to drive evolutionary changes. The internal and external
306 environments can alter the age structure of populations and/or the economics of outcrossing and
307 selfing. In populations that have few or no lineages that frequently change sex (such as those
308 recently founded by highly self-compatible individuals), lineages with higher propensities to
309 change sex would likely struggle to become established, if offspring of selfed and outcrossed
310 matings are equally fit. In these populations, individuals that change sex frequently will find an
311 environment with very few unfertilized eggs, and very few opportunities to pass their sex
312 changing alleles on to the next generation. However, once high sex change propensity lineages
313 become established, possibly due to additional migration or outcrossed progeny having a fitness
314 advantage over selfed progeny, these lineages can maintain sex change and males. Indeed,
315 among rivulus populations across the geographic range we find a significant increase in the rate
316 of outcrossing that correlates with an increase in the proportion of sex changing lineages
317 (Mackiewicz et al. 2006*b*; Tatarenkov et al. 2015). We also find variation among lineages within

in a population in the propensity to change sex, but the magnitude of variation is population specific (Gresham et al. 2020).

Males, and lineages that frequently produce males, may also have better success in populations with many young hermaphrodites. While we did not find evidence that younger hermaphrodites reproduce as “pure females” and only lay unfertilized eggs, younger hermaphrodites did lay more unfertilized eggs. If the fitness of progeny derived from selfing and outcrossing is relatively equal, population age structure can act as a selection pressure for or against lineages that frequently change sex. For instance, populations that lose a greater proportion of juveniles and young adults to predation will have proportionally fewer unfertilized eggs available for males than those populations in which younger individuals thrive; selecting against high sex changing lineages. These results open up fascinating opportunities for continued laboratory studies on lineage variation in traits such as size, fecundity, and sex change across different environments, and continued field studies to track demographic and population genetic characteristics such as selfing and outcrossing rates, the proportion of males, and population size and density.

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