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2   ## Variation in self-compatibility among genotypes and across ontogeny in a self-fertilizing
3   vertebrate
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10

11   Abstract
12

13   Mixed mating strategies offer the benefits of both self-fertilizing one's own eggs (selfing) and

14   outcrossing, while limiting the costs of both methods. The economics of mixed mating is further

15   determined by individual self-compatibility. In gynodioecious (hermaphrodites, females) and

16   androdioecious (hermaphrodites, males) species, the level of self-compatibility of the

17   hermaphrodites also acts as a selection pressure on the fitness of the other sex. Mangrove rivulus

18   fish populations are comprised of selfing hermaphrodites and males that result from

19   hermaphrodites changing sex. Although hermaphrodites overwhelmingly reproduce through

20   internal selfing, they occasionally oviposit unfertilized eggs. Males can externally fertilize these

21   eggs. Here, we reveal that fecundity and self-compatibility varies within individuals across

22   ontogeny until about 365 days post hatch, and among individuals derived from lineages that vary

23   in their propensity to change sex. Hermaphrodites from high sex changing lineages were

24   significantly less fecund and self-compatible than hermaphrodites from low sex changing

25   lineages. These differences in self-compatibility and fecundity have the potential to drive

26   evolutionary changes on mating strategy and the fitness of males in populations of the mangrove

27   rivulus. This study also illustrates the importance of including lineage variation when estimating

28   the costs and benefits of mixed mating strategies.

29   Introduction

30   As a reproductive strategy, self-fertilization has many benefits. It overcomes the "cost of

31 males," wherein only one half of the population can produce offspring compared to the entire

32 population in asexual or self-fertilizing species (Maynard Smith 1971, 1978). Self-fertilization

33 also assures reproduction when pollinators and/or mates are scarce (Darwin 1876; Baker 1955;

34 Stebbins 1957; Pannell et al. 2015), eliminates physical damage associated with mating and/or

35 sexually transmitted parasites or pathogens (Fowler and Partridge 1989; Clutton-Brock and

36 Parker 1995), and maintains well-adapted genotypes in stable environments (Allard et al. 1972;

37 Dolgin et al. 2007). However, selection for selfing is often counteracted by selection for

38 outcrossing. Benefits of outcrossing include combatting inbreeding depression in two ways: i)

39 preventing accumulation of deleterious alleles in single genotypes, and ii) maintaining

40 heterozygotes that may be more fit than homozygous individuals (Muller 1932). Ultimately,

41 outcrossing represents a bet hedging strategy because it increases the variability of offspring

42 fitness against a changing abiotic environment, or against coevolving parasites, predators and

43 competitors (Muller 1932; Bell 1988; Lively 2010; Morran et al. 2011; Hartfield and Keightley

44 2012).

45   Many plant and invertebrate species have evolved a mixed mating strategy of selfing and

46 outcrossing. Mixed mating allows individuals and populations to reap the rewards of both

47 strategies as biotic and abiotic factors change the fitness landscape across space and time.

48 Because the fitness landscape is variable, it would be expected that self-compatibility also would

49 vary both within and among populations due to changes in the magnitude and/or type of

50 selection acting for and against selfing and outcrossing (Escobar et al. 2011). Plant populations

51 that are often mate- and/or pollinator-limited at the range edge or in ephemeral habitats have

52   increased self-compatibility compared to other populations, as reported for the small angiosperm

53   *Leavenworthia alabamica* (Busch 2005). Populations found near the center of the range and in

54   more permanent habitats are much more likely to be self-incompatible (Busch 2005). When

55   among-individual variation in self-compatibility is heritable, it provides the genetic and

56   phenotypic background on which selection can drive evolutionary change in mating strategies.

57   There are multiple examples of heritable variation in the self-compatibility of plants, including in

58   the creeping bellflower, *Campanula rapunculoide* (Stephenson et al. 2000), and the Carolina

59   horsenettle *Solanum carolinense* (Travers et al. 2004). Genetic and phenotypic variation in self-

60   compatibility, along with environmental variability, can thus drive variation in mating strategy,

61   as they alter the economics of selfing and outcrossing.

62   In mixed mating populations, self-compatibility can also vary across ontogeny, perhaps

63   as a plastic response to environmental conditions. Individuals in populations that have higher

64   rates of outcrossing often become more self-compatible with increasing age. This is often the

65   result of selection favoring a "waiting" strategy for outcrossing opportunities when individuals

66   are young, presumably to derive the benefits of variable and more fit progeny but also to assure

67   some reproduction even if mates and/or pollinators are limited. This has been documented in

68   plants (*Campanula rapunculoide*; Stephenson, Good, and Vogler 2000, *Solanum carolinense;*

69   Travers, Mena-Ali, and Stephenson 2004), and snails (*Physa acute*; Tsitrone, Jarne, and David

70   2003), but few other self-fertilizing species. Populations can also evolve different mating

71   strategies based on age structure. If younger individuals are more populous, and mating

72   opportunities are plentiful enough to make selfing unnecessary, age-dependent changes in self-

73   compatibility may be lost so that little or no selfing occurs. Alternatively, if populations consist

74   predominantly of older individuals, selfing may continue to be an evolutionary stable component

75   of a mixed mating strategy.

76   In androdioecious species, in which populations consist of hermaphrodites and males,

77   variation in self-compatibility should also result in variation in the proportion of males within a

78   population. Populations consisting of individuals with lower self-compatibility should maintain a

79   higher proportion of males, as the "cost of males" is decreased by more outcrossing

80   opportunities. Hypotheses concerning the evolution and variation of self-compatibility and

81   mixed mating have been examined in many plant and invertebrate systems (Busch 2005; Winn et

82   al. 2011; Wright et al. 2013), but not in a vertebrate species. Here we present evidence for

83   individual variation in self-compatibility in one of only two vertebrates known to utilize a mixed

84   mating strategy, the mangrove rivulus fish (*Kryptolebias marmoratus*).

85   The mangrove rivulus (hereafter "rivulus"), is a small euryhaline killifish that inhabits

86   high elevation mangrove forests of Florida, the Bahamas, and Central America. Populations

87   consist primarily of self-fertilizing hermaphrodites with varying proportions of males (no

88   females). Rates of outcrossing and selfing also vary among populations and correlate with the

89   proportion of males in the population; outcrossing rates increase as the proportion of males

90   increase (Mackiewicz et al. 2006*b*; Tatarenkov et al. 2009). While self-fertilization happens

91   internally, males can only achieve reproductive success if/when hermaphrodites lay unfertilized

92   eggs, and there is no evidence that hermaphrodites outcross with each other (Furness et al. 2015).

93   Rivulus males result from sex change, after which selfing hermaphrodites that make the sexual

94   transition become obligate outcrossers. There also appears to be genetic variation for the

95   propensity to change sex (Gresham et al. 2020, Turner 2006). Hermaphrodites fertilize the vast

96   majority of their own eggs (> 94%, Harrington 1971, personal observations), giving males very

97   limited opportunities for reproductive success. Previously, it was reported that while individuals

98   that change sex to male are significantly more likely to survive environmental challenges than

99   those that remain hermaphrodite, they are much less likely to lay any eggs before sex change

100   occurs (only 2 out of 180, Gresham et al. 2020). This presents the problem of how individuals

101   that change sex might successfully pass their sex-changing alleles on to the next generation.

102   We hypothesized that the proportion of fertilized eggs (a measure of self-compatibility)

103   would change with age and/or the propensity of a given lineage to change sex. First, we

104   predicted that self-compatibility would increase with age. Cole and Noakes (1997) reported

105   histological evidence that ovarian tissue matures before spermatogenic tissue. We predicted that

106   there would be a period immediately after sexual maturity where oviposition of unfertilized eggs

107   would be more likely. Second, we predicted that overall fecundity and/or self-compatibility

108   would be greater in lineages that are more likely to change sex. This prediction is based on two

109   competing ideas about how sex-changing alleles are successfully maintained in rivulus

110   populations: i) selection should favor individuals in high sex changing lineages to be very fecund

111   and self-compatible prior to changing sex, so as to compensate for any lost reproductive success

112   after the transition, and/or ii) selection should favor individuals derived from lineages that

113   change sex frequently to be highly fecund and self-compatible so that the siblings of sex

114   changers recoup the lost reproductive success of their "brothers."

115

116   Methods

117   We designed and executed an experiment to measure the fecundity and self-compatibility

118   of 213 individual fish representing 100 genetically distinct lineages (determined via

119   microsatellite genotyping using 32 loci; (Mackiewicz et al. 2006*a*; Tatarenkov et al. 2010). We

120   calculated the propensity to change sex for each of the 100 lineages as the proportion of

121   individuals in each lineage that transition from hermaphrodite to male under common garden

122   conditions (Gresham et al. 2020), which ranged from 0 - 68.6%. All experimental fish were

123   raised from hatching in individual plastic containers (Rubbermaid® Take-a-long Deep Squares)

124   filled with approximately 200 mL 25‰ synthetic saltwater (prepared with Instant Ocean® sea

125   salt and aged tap water) until 30 days post hatching (dph), at which time water volume was

126   increased to 600 mL (25‰). At 60 dph, we suspended a large marble-sized ball of egg-laying

127   substrate (Poly-fil®) from one top corner of the container. Beginning at 67 dph, each container

128   was checked weekly for eggs in the substrate, around the top edge of the container, and on the

129   bottom of the container. Fish were checked weekly for eggs until they changed sex to male or

130   were 365-371 dph, ensuring that each remaining hermaphrodite was at least 365 dph and had

131   been checked weekly 43 times.

132   Eggs were collected into 59 mL plastic cups (Fabri-Kal® Greenware GXL250PC) filled

133   with approximately 6 mL 25‰ synthetic saltwater. Eggs were immediately counted and viewed

134   under a stereomicroscope. Eggs were photographed and scored as either fertilized, unfertilized,

135   or inviable. Fertilized and unfertilized eggs were identified by the presence or absence of a

136   perivitelline space, respectively. Inviable eggs were identified by their marked discoloration and

137   opaque appearance (Figure 1). Unfertilized and inviable eggs were removed from the cup and

138   discarded. Fertilized eggs were left in the cups, and water was replaced every 7 days. Cups were

139   checked every day for hatchlings. Hatchlings from the experimental animals were then scored as

140   alive or dead, with live hatchlings being euthanized in 4ºC water. Experimental fish were also

141   observed for signs of sex change each week when the tubs were checked for eggs, as described in

142   (Scarsella et al. 2018); orange freckles or orange skin is the most reliable external character of

143   sex change. Individuals that changed sex (35 of the 213) were separated from the others and the

144   date and age recorded. All experimental animals were isolated from the general colony for the

145   duration of the experiment, kept on a 12h light: 12 h dark photoperiod, and fed a 4 mL

146   suspension of brine shrimp (*Artemia*) nauplii (~2000 shrimp) six days per week. Room

147   temperature was also maintained at 26.45 ± 1.6 °C (mean ± SEM). The University of

148   Alabama Institutional Animal Care and Use Committee approved all procedures (IACUC
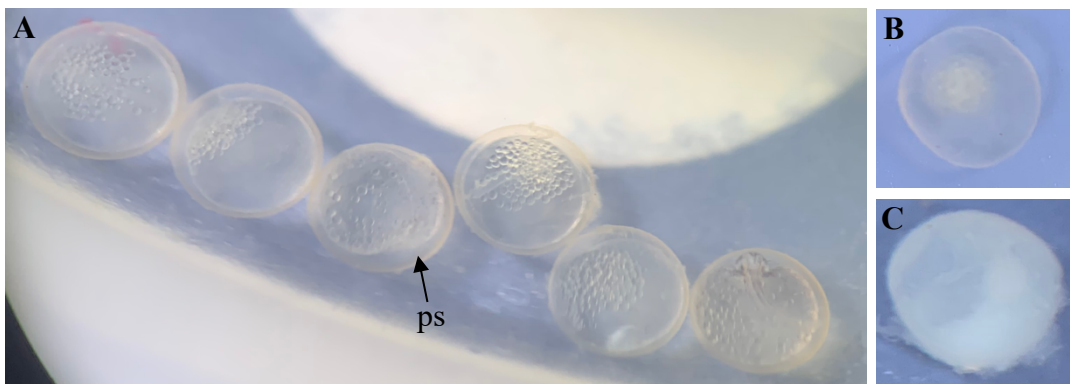
149   Protocol #18-10-1644).

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.

153

154   *Fecundity and Self-compatibility Statistical Analysis*

155   We examined the relationship between fecundity (number of eggs laid) and both the

156   propensity for a given lineage to change sex, and the individual's age. To determine whether

157   fecundity varied as a function of the lineages' propensities to change sex, we first ran a nominal

158   logistic model with 'laid eggs' as a categorical dependent variable (yes = 1, no = 0) and lineage

159   sex change propensity as the independent variable. We used a general linear model to examine

160   the relationship between total number of eggs laid (continuous, dependent) and lineage sex

161   change propensity (independent). Residuals of the total number of eggs were left skewed, so we

162   transformed the number of eggs with square root (x+1). To determine whether fecundity (total

163   number of eggs laid) varied as a function of age, we used a generalized regression model with a

164   zero-inflated Poisson distribution and parent ID as a random effect to account for the fact that

165   each parent was checked for eggs multiple times. An initial plot of the raw data suggested a

166   possible non-linear relationship between age and fecundity so, we constructed linear, quadratic

167   and cubic models and compared model fits using Akaike's Information Criterion (AIC). The

168   linear, quadratic and cubic age terms were scaled by mean and standard deviation (i.e., z-score)

169   prior to running the models. The cubic model fit best (Table 3.1), so we report the results of this

170   model.

171   We modeled self-compatibility in two ways: by the proportion of eggs that were fertilized

172   and by hatch success of fertilized eggs. To determine whether the proportion of fertilized eggs

173   varied as a function of the lineages' propensity to change sex, we ran a general linear model. To

174   determine whether the proportion of fertilized eggs varied as a function of the parents' age when

175   the eggs were collected, we ran a general linear model with parent ID as a random effect. To

176   determine whether the proportion of fertilized eggs varied as a function of the total number of

177   eggs laid by each parent, we ran a general linear model. We calculated hatch success as the

178   proportion of fertilized eggs that resulted in a live hatchling. To determine whether hatch success

179   varied as a function of the lineages' propensity to change sex, we ran a general linear model.

180   Models were run in JMP Pro version 15.0.0 (JMP®, Version 15 Pro 2019) and in RStudio using

181   the glmmTMB package (RStudioTeam 2016; Brooks et al. 2017; R Core Team 2018). Data files

182   will be deposited on GitHub upon publication acceptance.

183

184

185   Results

186   The experiment included 213 individual fish from 100 different lineages. Thirty-five of

187   those fish changed sex to male (16.4%) before the experiment ended at 365 - 371 dph. Of those

188   35, three individuals laid eggs first (9% of males, 1% of the total cohort). We collected 14,310

189   eggs, had 10,267 hatchlings (71.7% of eggs hatched), and 10,059 live hatchlings (98% of

190   hatchlings were alive). The three individuals that changed sex to male laid a total of 69 eggs by

191   197 dph (the oldest age at which a fish was identified as male), which equated to, on average, 23

192    $\pm$ 13 (SEM) eggs per individual among the three, but 1.97 $\pm$ 1.4 eggs per capita for all

193   individuals that changed sex to male. The remaining 178 hermaphrodites laid a total of 7,341

194   eggs before they collectively aged to 197 dph (41.2 $\pm$ 2.2 eggs per individual). We started

195   checking for eggs from each fish at 67 dph and collected a single egg from one individual on the

196   first egg check; the egg was fertilized. Overall, the proportion of eggs that were fertilized was

197   93.4%. The proportion of eggs that were unfertilized was 2.5%, and the proportion identified as

198   "inviable" was 4.1%.

199   Our results indicate that fecundity initially increases with age until about 175 dph, then

200   decreases until it plateaus at about 350 dph (Table 1 Figure 2A). The proportion of fertilized

201   eggs also increased significantly with age, but not the total fecundity of the parent (Table 1,

202   Figure 2B). Because we did not record the hatch success of each individual collection (i.e.,

203   clutch) of eggs, we were not able to analyze whether hatch success changed with age. The

204   lineages' propensity to change sex was significantly related to fecundity and self-compatibility.

205   When individuals that changed sex to male were included in the model, the odds of laying any

206   eggs decreased significantly as the propensity to change sex increased (Table 2). However, this

207   relationship became non-significant when individuals that changed sex to male were excluded

208   (Table 2). Conversely, the number of eggs laid decreased significantly as the propensity to

209   change sex increased, whether or not males were included in the model (Table 2 Figure 3A).

210   Both the proportion of fertilized eggs and the hatch success of those eggs also decreased

211   significantly with increasing propensity to change sex (Table 2, Figure 3B, C).

212

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 $\sim$_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 $\sim$_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 $\sim$_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*	*$\chi 2$*	*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**
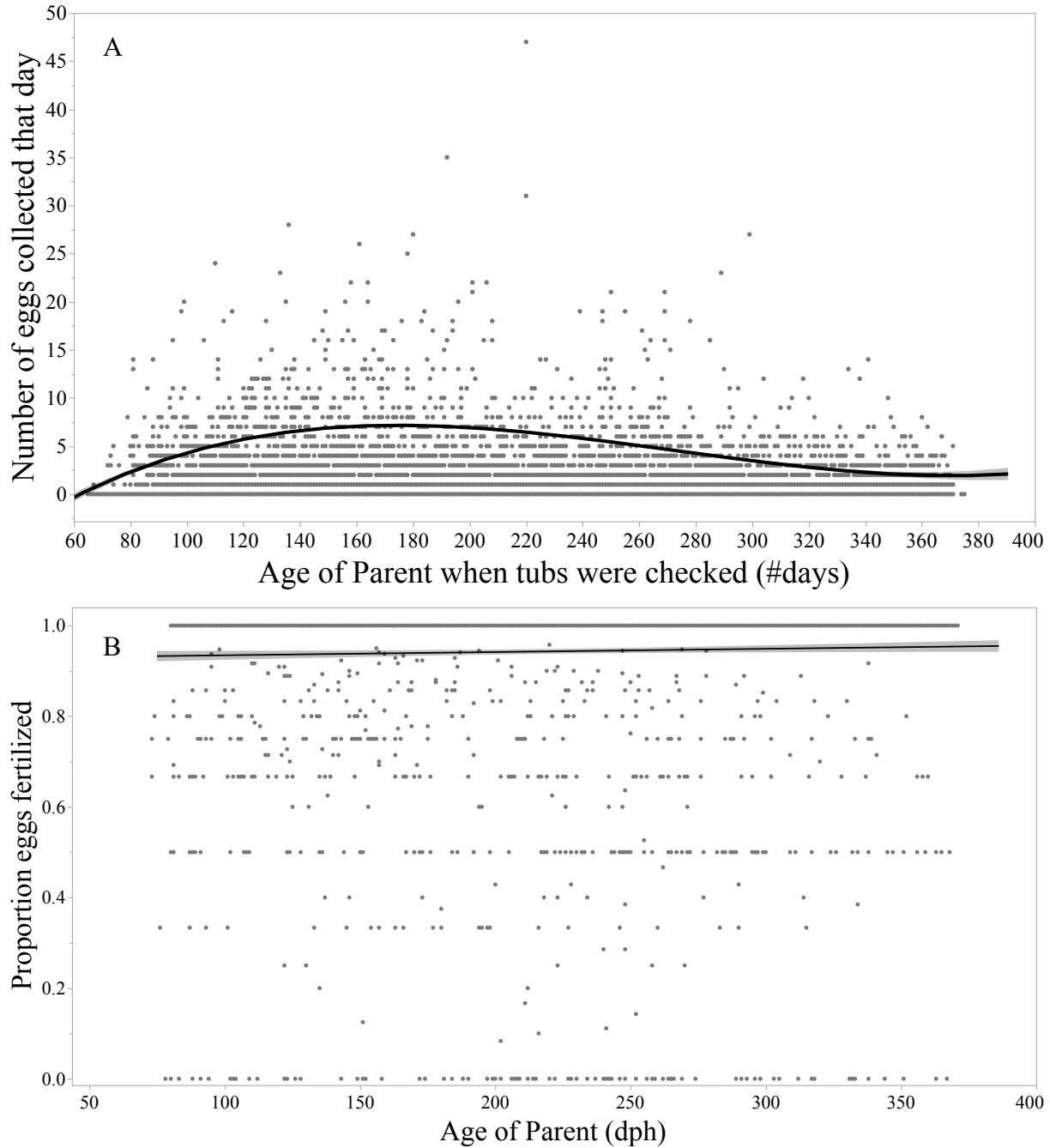
Figure 2. Age effects on the number of eggs collected and the proportion of those eggs that were fertilized. A) Age affected fecundity cubically; the number of eggs oviposited increased initially, then decreased between 170 – 180 dph, then plateaued around 350 dph. B) The proportion of eggs that were 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.
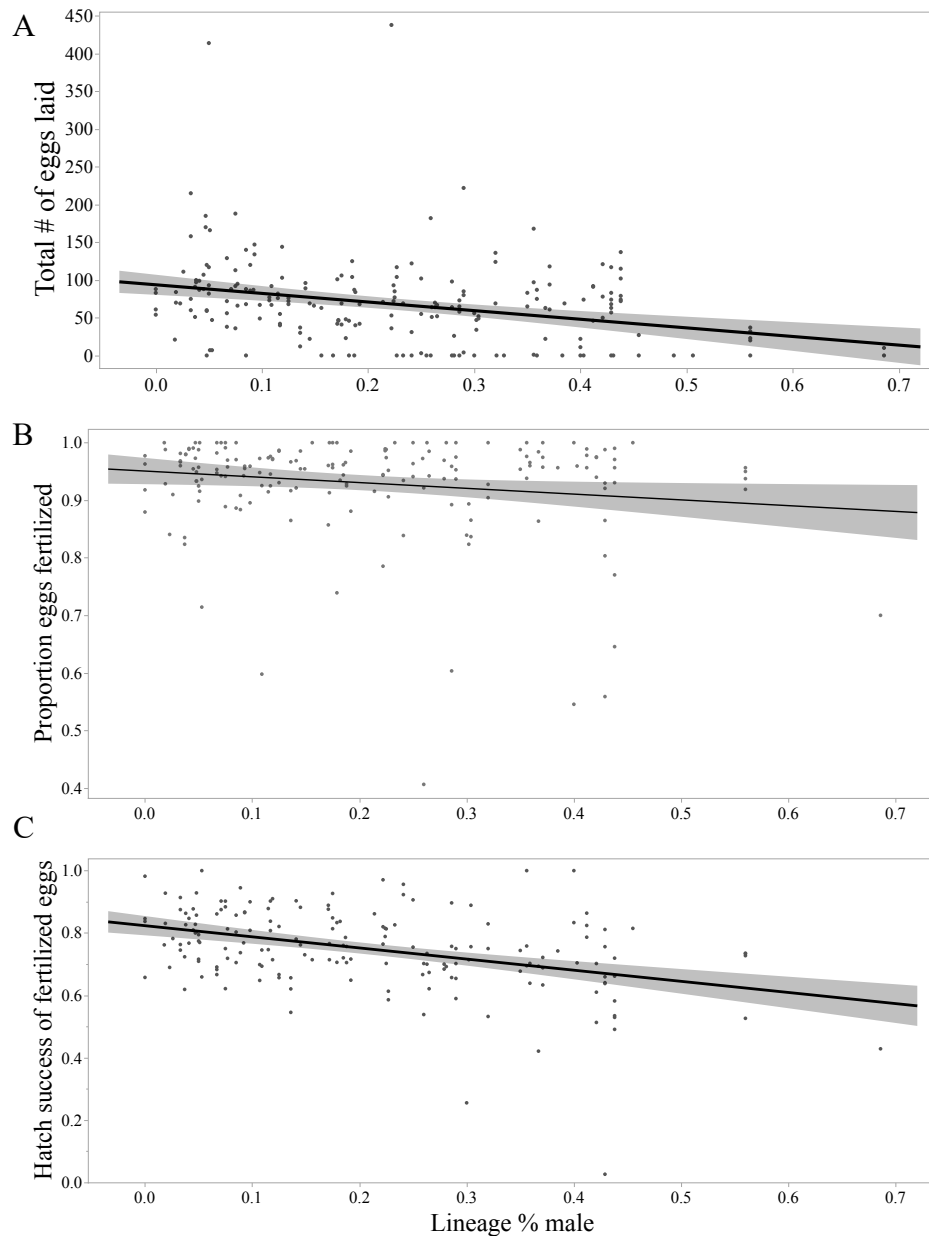
Figure 3. Lineage sex change propensity effects on A) fecundity, B) the proportion of eggs that were fertilized, and C) hatch success of fertilized eggs. Lineage sex change propensity is measured as the proportion of each lineage that change sex in isolation under common garden conditions in our colony (lineage % male).

Discussion

204

205   Mixed mating strategies offer the fitness rewards of both outcrossing with conspecifics

206   and fertilizing one's own eggs. The cost-to-benefit ratio of each strategy is expected to vary

207   across space and time as the external and internal environments also change (Maynard Smith

208   1971; Lively and Lloyd 1990; Lehtonen et al. 2012; Layman et al. 2017; Lynch et al. 2018).

209   There is also variation among individuals in characters that affect mating strategy: fecundity and

210   self-compatibility (Stephenson et al. 2000; Travers et al. 2004; Busch 2005). Variation in self-

211   compatibility, combined with external factors such as pollinator and/or mate availability or

212   parasite pressure, has the potential to drive evolutionary changes in mating strategy. For instance,

213   if self-compatible individuals have greater reproductive fitness (produce more offspring and/or

214   offspring have greater survival rates) and survive just as well as self-incompatible individuals,

215   then the population may evolve to complete or near complete selfing. Comparatively, if there are

216   plenty of mates/pollinators and/or inbreeding depression that increases the benefits to cost ratio

217   of outcrossing, populations will evolve to complete or near complete outcrossing. We tested, in

218   the only self-fertilizing hermaphroditic vertebrate, the hypothesis that self-compatibility and

219   fecundity would vary with age and as a function of the lineages' propensties to change sex.

220   The most important finding was that both fecundity and self-compatibility decreased

221   as lineages became more likely to change sex, which is the exact opposite of what we predicted.

222   We predicted that fecundity and/or self-compatibility would increase with the lineages'

223   propensity to change sex for one of two reasons: either individuals that eventually change sex

224   would produce more eggs with greater self-compatibility, or the remaining hermaphrodites from

225   high sex changing lineages would produce at least as many eggs with equal self-compatibility as

226   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

249   genetically distinct lineages. In particular, individuals from low sex changing, highly fecund and

250   self-compatible lineages are likely to experience the highest cost of sex change.

251   We found that fecundity initially increases with age, then decreases, and finally plateaus

252   as individuals age from about 60 dph to about 370 dph. The only other report of potential age-

253   related changes in fecundity in rivulus comes from Harrington (1971). From 37 fish, he reported

254   significant seasonality in "ovarian function" (i.e., fecundity) over three years when exposed to

255   natural day light cycles, but not a significant change in fecundity with age. The seasonal decline

256   reported by Harrington (1971), which occurred from September through December, aligns with

257   the end of our experiment. The fish in our experiment hatched and then were removed from the

258   experiment over a four-month period between 30 August 2018 and 22 December 2019. It is

259   possible that the association between age and fecundity that we found, particularly the shallow

260   decline in egg production from 180-300 dph represents the same seasonal decline described by

261   Harrington (1971). He allowed his fish to experience natural daylight cycles. Our fish were kept

262   on a constant photoperiod and a relatively constant temperature, and so should have been

263   removed from any external signals of season. To truly understand how fecundity is affected by

264   age and seasonality, we need additional years of records from multiple lineages.

265   Overall, 93.4% of the eggs we collected were fertilized. This is very close to the

266   proportion of eggs fertilized reported by Harrington (1971). From six fish of one lineage and

267   1,056 eggs, he identified 94.3% as fertilized. We recorded 2.5% unfertilized and 4.1% inviable,

268   compared to Harrington (1971), who reported 4% unfertilized and 1.7% inviable. It is likely that

269   most of the inviable eggs we recorded were unfertilized when oviposited, but then became

270   inviable and discolored by the time we collected them. Harrington (1971) collected eggs every

271   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

318   in a population in the propensity to change sex, but the magnitude of variation is population

319   specific (Gresham et al. 2020).

320   Males, and lineages that frequently produce males, may also have better success in

321   populations with many young hermaphrodites. While we did not find evidence that younger

322   hermaphrodites reproduce as "pure females" and only lay unfertilized eggs, younger

323   hermaphrodites did lay more unfertilized eggs. If the fitness of progeny derived from selfing and

324   outcrossing is relatively equal, population age structure can act as a selection pressure for or

325   against lineages that frequently change sex. For instance, populations that lose a greater

326   proportion of juveniles and young adults to predation will have proportionally fewer unfertilized

327   eggs available for males than those populations in which younger individuals thrive; selecting

328   against high sex changing lineages. These results open up fascinating opportunities for continued

329   laboratory studies on lineage variation in traits such as size, fecundity, and sex change across

330   different environments, and continued field studies to track demographic and population genetic

331   characteristics such as selfing and outcrossing rates, the proportion of males, and population size

332   and density.

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