

1 **Male-driven reinforcement and cascade reinforcement in darters**

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## 9 **Abstract**

10 Reinforcement can act in response to maladaptive hybridization by selecting for increased  
11 discrimination against heterospecifics mates in sympatry compared to allopatry (i.e.,  
12 reproductive character displacement - RCD). Additionally, reinforcement can select for  
13 increased discrimination against heterospecifics in a fighting context in sympatry compared to  
14 allopatry (i.e., agonistic character displacement - ACD). Because it directly affects conspecific  
15 recognition traits (signals and/or preferences), reinforcement between two species in sympatry  
16 can incidentally cause behavioral isolation among populations within a species, termed cascade  
17 reinforcement. Here we demonstrate that a pattern consistent with male-driven RCD and ACD is  
18 present between two groups of darters (orangethroat darter clade *Ceasia* and rainbow darter  
19 *Etheostoma caeruleum*). Increased male discrimination against heterospecific females as mates  
20 and increased male discrimination against heterospecific males as competitors is present in  
21 sympatry. Furthermore, there is a pattern consistent with male-driven cascade RCD and  
22 cascade ACD within *Ceasia*. We found low levels of discrimination between two species of  
23 *Ceasia* that occur in allopatry from one another and in allopatry with *E. caeruleum*. This result  
24 contrasts that of a recent study which observed high levels of behavioral isolation between  
25 *Ceasia* species that occurred in sympatry with *E. caeruleum*. We suggest reinforcement  
26 between *Ceasia* and *E. caeruleum* in sympatry has led to the evolution of behavioral isolation  
27 between lineages within *Ceasia* that occur in sympatry with *E. caeruleum* but in allopatry with  
28 respect to one another (i.e., cascade reinforcement). This study demonstrates the ability of male  
29 behavior to simultaneously drive sympatric and allopatric speciation via reinforcement.

## 30 **Introduction**

31 Reinforcement is unique among speciation mechanisms in that it can directly select for  
32 increased behavioral isolation between two species in response to the production of unfit  
33 hybrids in areas of sympatry (Dobzhansky 1940; Butlin 1987; Kelly and Noor 1996).  
34 Consequently, reinforcement causes mating traits (signals and/or preferences) to diverge  
35 between species in sympatry (but not allopatry), leading to a pattern of reproductive character  
36 displacement (RCD; Brown and Wilson 1956; Howard 1993; Coyne and Orr 2004). RCD is the  
37 classic signature used to detect reinforcement, and is evidenced by increased discrimination  
38 against heterospecific mates in sympatry compared to allopatry (Servedio and Noor 2003).

39  
40 Although the most recognized result of reinforcement is increased behavioral isolation between  
41 two species in sympatry, recent research has suggested that reinforcement can also initiate  
42 allopatric speciation (Ortiz-Barrientos et al. 2009; Pfennig and Pfennig 2009; Hoskin and Higgie  
43 2010). By directly affecting traits associated with behavioral isolation with a closely related  
44 sympatric species, reinforcement may alter behavioral isolation among populations within a  
45 species. Heightened behavioral isolation among populations of species that also experience  
46 reinforcement with a close relative has been documented in numerous empirical examples  
47 across a variety of taxa (e.g., Nosil et al. 2003; Hoskin et al. 2005; Higgie and Blows 2007,  
48 2008; Lemmon 2009; Porretta and Urbanelli 2012; Bewick and Dyer 2014; Pfennig and Rice  
49 2014; Kozak et al. 2015). When reinforcement occurs independently in isolated populations  
50 throughout a species range, stochastic processes may cause different mating traits underlying  
51 behavioral isolation to diverge in different populations (reviewed in Comeault and Matute 2016).  
52 This can cause behavioral isolation to evolve among allopatric populations within a species,  
53 termed cascade reinforcement (Ortiz-Barrientos et al. 2009).

54

55 To illustrate this concept, we show a hypothetical range map with one wide-ranging species (A)  
56 and several populations/newly formed species (B1-B4) (Fig. 1). Populations B1-B4 are all  
57 allopatric to one another. Populations B1 and B2 are sympatric with respect to A, but  
58 populations B3 and B4 are allopatric with respect to species A. In this scenario, cascade  
59 reinforcement predicts that there is reinforcement between A and B1 and between A and B2,  
60 independently. Behavioral isolation is thus heightened between A and B1 and between A and  
61 B2. However, because slightly different traits have evolved in B1 and B2 in response to  
62 reinforcing selection, behavioral isolation is also heightened between B1 and B2 as a *by-product*  
63 of reinforcement. In contrast, there is no reinforcement between A and B3 or A and B4, and  
64 behavioral isolation is low in these pairs of taxa. Likewise, behavioral isolation is low between  
65 B3 and B4. The critical test for cascade reinforcement is whether allopatric populations that  
66 experience reinforcement with a more distant relative (i.e., B1 and B2) have higher behavioral  
67 isolation than allopatric populations that do not experience reinforcement (i.e., B3 and B4).

68  
69 The term reinforcement has primarily been used to describe selection acting against  
70 maladaptive heterospecific mating, but reinforcement can also act via selection against  
71 maladaptive heterospecific fighting over resources (such as mates). This form of reinforcement  
72 can cause aggressive signals and/or recognition of competitor's signals to diverge between  
73 species in sympatry, resulting in a pattern of agonistic character displacement (ACD; Grether et  
74 al. 2009; Okamoto and Grether 2013). A pattern of ACD is said to be present when males from  
75 two different species are less likely to engage in contests when they occur in sympatry  
76 compared to allopatry with one another. Both reinforcement leading to RCD and reinforcement  
77 leading to ACD may contribute to speciation. Although numerous studies have shown that RCD  
78 can occur among populations within species due to cascade reinforcement (e.g., Nosil et al.  
79 2003; Hoskin et al. 2005; Lemmon 2009; Rice and Pfennig 2010; Pfennig and Rice 2014; i.e.,

80 cascade RCD, hereafter CRCD), whether reinforcement can also secondarily lead to ACD  
81 among populations within species (i.e., cascade ACD, hereafter CACD) has yet to be  
82 determined.

83

84 Distinguishing between RCD and ACD is essential to determining the underlying selective  
85 pressure (i.e., selection against heterospecific mating or heterospecific fighting) and relative  
86 contribution of male-female and male-male interactions in driving speciation. However,  
87 disentangling the relative contributions of RCD and ACD to speciation can be difficult because  
88 many sexually selected traits are important in both intersexual contexts (such as female mate  
89 choice) and intrasexual contexts (such as male-male competition over mates) (Alatalo et al.  
90 1994; Berglund 1996; Sætre et al. 1997; Dijkstra et al. 2007; Saether et al. 2007; Lackey and  
91 Boughman 2013; Tinghitella et al. 2015). Here we examine both RCD and ACD using a system  
92 where males discriminate against both heterospecific female mates and heterospecific male  
93 rivals, but female mate choice appears to be absent.

94

95 This study focusses on two groups of darters in the in the subgenus *Oligocephalus*: the  
96 orangethroat darter clade *Ceasia* and the rainbow darter *Etheostoma caeruleum*. The clade  
97 *Ceasia* consists of 15 recently diverged species, which all occur in allopatry from one another.  
98 Twelve of the 15 *Ceasia* species occur in sympatry with *E. caeruleum* throughout their range  
99 (*Ceas* and Page 1997; Page and Burr 2011). One wide-ranging *Ceasia* species (orangethroat  
100 darter *Etheostoma spectabile*), occurs both in sympatry and in allopatry with *E. caeruleum*. The  
101 reaming two *Ceasia* species (plains darter *E. pulchellum* and plateau darter *E. squamosum*) are  
102 completely allopatric with respect to *E. caeruleum*. *Ceasia* and *E. caeruleum* have similar male  
103 nuptial coloration, ecology, and mating behavior (Page 1983; Page and Burr 2011). There is  
104 little evidence that male coloration in *Ceasia* and *E. caeruleum* is under selection by females in

105 intersexual mate choice, as females appear to lack preferences within or among species (Pyron  
106 1995; Fuller 2003; Zhou et al. 2015; Moran et al. in press). Instead, male coloration is under  
107 intrasexual selection, functioning as an aggressive signal in male-male competition over access  
108 to females (Zhou and Fuller 2016; Moran et al. in press).

109

110 Evidence from several recent studies suggests that reinforcement is likely occurring in this  
111 system. First, hybridization occurs at low rates between *Ceasia* and *E. caeruleum* in nature  
112 (Zhou and Fuller 2014; Moran et al. in press). Second, postzygotic isolation is present between  
113 at least one *Ceasia* species (*E. spectabile*) and *E. caeruleum*. F1 hybrid clutches between these  
114 species have a male-skewed sex ratios (Zhou 2014), and backcross hybrids suffer from  
115 dramatically reduced viability (R. Moran unpubl. data). Third, a recent study found high levels of  
116 male-driven behavioral isolation between four species of *Ceasia* (strawberry darter *E. fragi*,  
117 current darter *E. uniporum*, brook darter *E. burri*, and *E. spectabile*) and their respective  
118 sympatric populations of *E. caeruleum* (Moran et al. in press). Fourth, a pattern consistent with  
119 RCD was observed in a no-choice mating experiment between *E. spectabile* and *E. caeruleum*  
120 (Zhou and Fuller 2014). Zhou and Fuller (2014) found that pairings of allopatric female *E.*  
121 *spectabile* and allopatric male *E. caeruleum* yielded more eggs than pairings of sympatric  
122 female *E. spectabile* and sympatric male *E. caeruleum*. Together, the results of these previous  
123 studies strongly suggest that reinforcement may be occurring between *Ceasia* and *E.*  
124 *caeruleum*. However, the no-choice assay performed previously by Zhou and Fuller (2014) was  
125 not able to measure the contribution of each sex to behavioral isolation in sympatry, and did not  
126 consider the role of male competition (an important component of behavioral isolation in darters;  
127 Zhou et al. 2015; Martin and Mendelson 2016; Moran et al. in press). Here we provide a direct  
128 measure of mating behavior in both sexes to test for reinforcement. We examine whether

129 behavioral isolation is lower (or absent) between *Ceasia* and *E. caeruleum* when they occur in  
130 allopatry from one another.

131

132 There is also reason to suspect that cascade reinforcement may be present within *Ceasia*.  
133 Moran et al. (in press) found that surprisingly high levels of behavioral isolation are present  
134 among recently diverged allopatric species of *Ceasia*. Male *Ceasia* discriminate against  
135 heterospecific *Ceasia* female mates and against heterospecific *Ceasia* male rivals (Moran et al.  
136 in press). The behavioral isolation among *Ceasia* species is not associated with differences in  
137 male color pattern or genetic distance. Notably, the *Ceasia* species examined by Moran et al. (in  
138 press) all occur sympatrically with *E. caeruleum*. Therefore, the high levels of behavioral  
139 isolation observed among *Ceasia* may be due to reinforcement between sympatric *Ceasia* and  
140 *E. caeruleum* incidentally causing cascade reinforcement within *Ceasia*. Here we test this  
141 hypothesis by examining whether behavioral isolation is higher among *Ceasia* species that are  
142 sympatric versus those that are allopatric with *E. caeruleum*.

143

144 Our first aim was to determine whether a pattern consistent with RCD and/or ACD is present  
145 between *Ceasia* and *E. caeruleum*. To do this, we compared discrimination against  
146 heterospecifics in the context of male mate choice, female mate choice, and male aggression  
147 among *Ceasia* and *E. caeruleum* populations/species that were sympatric versus allopatric with  
148 respect to one another. RCD predicts higher levels of male and/or female discrimination against  
149 heterospecific mates in sympatry compared to allopatry. If RCD is present due to alterations in  
150 male preferences in sympatry, then males from sympatric populations should only pursue  
151 conspecific females, whereas males from allopatric populations should pursue both conspecifics  
152 and heterospecifics. Likewise, if RCD is present due to changes in female preferences in  
153 sympatry, then females from sympatric populations should only perform nosedigs (a behavior

154 that directly proceeds spawning) when they are being guarded by conspecific males. Allopatric  
155 females should not show such discrimination. ACD predicts higher levels of male discrimination  
156 against heterospecific male rivals in sympatry compared to allopatry. If ACD is present due to  
157 alterations in male competitor recognition in sympatry, then males from sympatric populations  
158 should engage in more aggressive interactions with conspecific males compared to  
159 heterospecific males, whereas males from allopatric populations should not bias their  
160 aggression towards conspecifics.

161  
162 Our second aim was to determine whether a pattern consistent with CRCD and/or CACD is  
163 present within *Ceasia*. To do this, we measured behavioral isolation between *Ceasia* species  
164 that occur allopatrically from one another and allopatrically from *E. caeruleum*. We then  
165 compared these estimates to previous measures of behavioral isolation among *Ceasia* species  
166 that occur sympatrically with *E. caeruleum* (Moran et al in press). CRCD predicts higher levels  
167 of mate discrimination against heterospecific *Ceasia* in populations/species that occur in  
168 sympatry with *E. caeruleum*. CACD predicts higher levels of male discrimination against  
169 heterospecific *Ceasia* male rivals in populations/species that occur in sympatry with *E.*  
170 *caeruleum*.

171

172

## 173 **Methods**

### 174 ***Mating system details***

175 During the spring spawning season, both *Ceasia* and *E. caeruleum* travel to shallow gravel  
176 riffles in headwater streams (Hubbs and Strawn 1957; Hubbs 1985; Heins et al. 1996). Females  
177 look for a suitable place to lay eggs by performing “nosedigs” in which they jab their snout into  
178 the gravel. One to several males will swim in tandem along a female as she searches for a



179 spawning location. Males fight aggressively to ward off rival males, either by actively chasing  
180 them off or by flaring their dorsal and anal fins in a threat display. When the female is ready to  
181 spawn, she will dive into the substrate and position herself so that only her head and caudal fin  
182 are fully visible. Fighting amongst males escalates at this point, as they attempt to secure  
183 access to the female. During spawning, a male positions himself above the female and they  
184 simultaneously release sperm and eggs into the substrate. Spawning often involves multiple  
185 males mating simultaneously with one female, and males sometimes exhibit sneaking behavior.  
186 Females will ovulate clutches of up to 200 eggs periodically throughout the spawning season,  
187 but only release a few eggs per spawning bout (Heins et al. 1996; Fuller 1998). Hence, the  
188 female must spawn multiple times to fertilize all the eggs from a given clutch.

189

#### 190 ***Study species/populations and collection locations***

191 We conducted behavioral trials focusing on two wide-spread species of *Ceasia* (*E. spectabile*  
192 and *E. pulchellum*), and *E. caeruleum*. *E. spectabile* occurs in sympatry with *E. caeruleum* in its  
193 eastern range, but it occurs in allopatry with respect to *E. caeruleum* in the western part of its  
194 range. *E. pulchellum* is allopatric to *E. caeruleum* throughout its range. All species of *Ceasia*  
195 occur in allopatry from one another. Hereafter, when we describe populations of *Ceasia* or *E.*  
196 *caeruleum* as being sympatric or allopatric, we are referring to the geographic relationship  
197 between *Ceasia* and *E. caeruleum* (not between *Ceasia* species). We used three *Ceasia* study  
198 populations: sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*. We  
199 also used a sympatric *E. caeruleum* population (for comparisons with the sympatric *E.*  
200 *spectabile* population) and an allopatric *E. caeruleum* population (for comparisons with the  
201 allopatric *E. spectabile* and the allopatric *E. pulchellum* populations). Table S1 shows collection  
202 locations for each of the five darter populations used in this study.

203

204 In 2016, we conducted dichotomous male choice assays and male competition assays (detailed  
205 below) to measure behavioral isolation in sympatric and allopatric pairings of *Ceasia* and *E.*  
206 *caeruleum* (Table 1). In 2017, we conducted dichotomous male choice assays and male  
207 competition assays to measure behavioral isolation between the two allopatric *Ceasia* species  
208 (Table 2). We then examined the results of these experiments in combination with data from a  
209 previous study (Moran et al. in press) to look for patterns of RCD and ACD between *Ceasia* and  
210 *E. caeruleum*, and patterns of CRCD and CACD within *Ceasia*.

211  
212 Fish were collected with a kick seine in March 2016 and April 2017 and transported back to the  
213 laboratory at the University of Illinois at Urbana-Champaign in aerated coolers. Fish were  
214 separated into stock aquaria according to population and sex, and were fed daily *ad libitum* with  
215 frozen bloodworms. Stock aquaria were maintained at 19° C and fluorescent lighting was  
216 provided to mimic the natural photoperiod.

217

## 218 ***Comparisons between Ceasia and E. caeruleum***

### 219 **Dichotomous male mate choice assays**

220 In 2016, we first used dichotomous male choice assays to test for a pattern of RCD between  
221 *Ceasia* and *E. caeruleum*. In these trials, we placed a single focal male in a test aquarium with a  
222 conspecific female and a heterospecific female - *E. caeruleum* (Fig. 2a). This allowed us to  
223 determine whether males differ in how they respond to conspecific females versus *E. caeruleum*  
224 females. We measured male mate choice for females in three *Ceasia* populations: sympatric *E.*  
225 *spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* (Table 1A). Our prediction was  
226 that male mate choice for conspecifics should be high for *E. spectabile* from the sympatric  
227 population, and that male mate choice for conspecifics should be low for *E. spectabile* from the  
228 allopatric population and for allopatric *E. pulchellum*.

229

230 Behavioral assays occurred in 38 L test aquaria that were filled with 5 cm of naturally colored  
231 aquarium gravel. To minimize disturbance to the fish, test aquaria were covered with black  
232 opaque plastic on three sides. Each trial began by placing the three fish being tested into a test  
233 aquarium and allowing them to acclimatize for 5 min. The trial then began and lasted 30 min. To  
234 avoid exposing fish to chemical cues from fish used in other trials, fish were placed into an  
235 aquarium with freshly changed water for each trial. Each 30 min trial was broken up into 60 30-s  
236 blocks (Zhou et al. 2015; Moran et al. in press).

237

238 We examined male mate choice by measuring focal male pursuit of each female. Previous  
239 studies have shown that male pursuit of a female is highly predictive of spawning in *Ceasia* and  
240 *E. caeruleum* (Zhou et al. 2015; Moran et al. in press). A male was scored as having pursued a  
241 female in a given 30-s block of the trial if he spent a minimum consecutive time of 5-s within one  
242 body length of the female. We calculated focal male mate choice as described in Table 3.

243

244 For both the dichotomous male choice assays and the male competition assays (see below),  
245 analyses were performed using proportional data (i.e., the behavioral variables described in  
246 Table 3) that varied from 0 to 1. A score of 1 would indicate only conspecific interactions  
247 occurred, 0.5 would indicate an equal number of interactions between conspecifics and  
248 heterospecifics, and 0 would indicate only heterospecific interactions occurred. However, for  
249 ease of interpretation, we graphed the raw number of behaviors observed.

250

251 We used analysis of variance (ANOVA) to determine whether focal male mate choice differed  
252 among the three focal *Ceasia* populations (i.e., sympatric *E. spectabile*, allopatric *E. spectabile*,  
253 and allopatric *E. pulchellum*) in the dichotomous male choice trials. We included focal male

254 mate choice as the dependent variable, and focal *Ceasia* population as the independent  
255 variable. We then used post-hoc t-tests to directly compare populations. We also asked whether  
256 focal male mate choice differed from a null expectation of 0.5 (equal amounts of time spent with  
257 each female) in each focal *Ceasia* population using sample t-tests.

258

### 259 **Male competition assays**

260 We conducted a second type of assay in 2016 in which males could compete with one another.  
261 This assay allowed us to look for patterns of RCD and ACD between *Ceasia* and *E. caeruleum*.  
262 We conducted the 2016 male competition assay using the same *Ceasia* and *E. caeruleum* study  
263 populations as in the 2016 dichotomous male choice assay (Table 2). Male competition trials  
264 included a focal male and focal female *Ceasia* pair, and a rival male that was either (a)  
265 conspecific to the focal *Ceasia* pair or (b) an *E. caeruleum*. Each focal male and focal female  
266 *Ceasia* pair was observed twice: once in a trial where the rival male was a conspecific *Ceasia*  
267 (Fig. 2b; Table 2A), and once in a trial where the rival male was an *E. caeruleum* (Fig. 2c; Table  
268 2B). Due to low collection numbers, some allopatric *E. caeruleum* males were used twice (i.e.,  
269 once as a rival male in a trial with allopatric *E. spectabile* and once as a rival male in a trial with  
270 allopatric *E. pulchellum*), but never more than once on the same day or with the same *Ceasia*  
271 population.

272

273 The male competition assay allowed us ask whether sympatric male *Ceasia* and *E. caeruleum*  
274 were more likely to discriminate against heterospecific males in a competitive context compared  
275 to allopatric male *Ceasia* and *E. caeruleum*. We also asked whether sympatric males showed  
276 higher levels of discrimination against heterospecific females compared to allopatric males  
277 when they could simply choose whether or not to pursue a female (i.e., a no-choice situation).  
278 Additionally, this assay allowed us to examine whether female mate preference for conspecific

279 males versus *E. caeruleum* males was present, and whether it differed among allopatric and  
280 sympatric populations.

281

282 To measure the aggressive response of both males (focal and rival) towards the other male in  
283 each trial, we recorded the number of aggressive behaviors (i.e., fin flares and attacks)  
284 performed by each male. We calculated four behavioral variables to quantify male discrimination  
285 against heterospecific males: focal male fin flare bias, focal male attack bias, rival male fin flare  
286 bias, and rival male attack bias (see Table 3 for variable calculations).

287

288 To measure male mate preference in the male competition trials, trials were split into 60 30-s  
289 blocks (as in the dichotomous male choice trials) and we counted the number of 30-s blocks in  
290 which each male pursued the female in each trial. We calculated rival male mate choice as  
291 described in Table 3. We did not measure focal male mate choice in the male competition trials,  
292 as focal males were always paired with a conspecific female in these trials. Unlike the  
293 dichotomous male choice assay, the male competition assay examines male mate preference in  
294 the presence of a male competitor (which is closer to what a male would most often face in  
295 nature during the spawning season). Additionally, the male competition assay considers the  
296 preference of male *E. caeruleum* for *Ceasia* females, whereas the dichotomous choice trials  
297 only considered the preference of *Ceasia* males for conspecific females versus heterospecific *E.*  
298 *caeruleum* females. As male mate preference has been previously shown to be important in  
299 maintaining species boundaries in these species (Zhou et al. 2015; Moran et al. in press),  
300 examining mate preference from both the perspective of male *Ceasia* and male *E. caeruleum* is  
301 critical to estimating behavioral isolation between species.

302

303 Finally, we measured focal female mate choice by counting the number of nosedigs a female  
304 performed towards the rival male in each trial (see Table 3). Females typically perform nosedigs  
305 directly before spawning, and this behavior is often used to measure female mating preferences  
306 in darters (Fuller 2003; Williams and Mendelson 2011; Zhou et al. 2015; Zhou and Fuller 2016).

307

308 To test for a pattern of ACD between *Ceasia* and *E. caeruleum*, we asked whether the  
309 behavioral variables that we used to measure male aggression differed among sympatric and  
310 allopatric populations. To examine focal male behavior, we conducted two separate ANOVAs  
311 with focal male fin flare bias and focal male attack bias as the dependent variables, and focal  
312 *Ceasia* population as the independent variable in both analyses. Similarly, to examine rival male  
313 behavior, we conducted ANOVAs with rival male fin flare bias and rival male attack bias as  
314 dependent variables, and focal *Ceasia* population as the independent variable. Additionally, we  
315 performed post-hoc analyses using two-sample t-tests to make direct comparisons between  
316 focal *Ceasia* populations.

317

318 To test for a pattern of RCD in male mate preferences, we asked whether male mate choice  
319 differed among sympatric and allopatric focal *Ceasia* populations. We conducted an ANOVA  
320 with male mate choice as the dependent variable, and focal *Ceasia* population as the  
321 independent variable, followed by post-hoc two-sample t-tests between populations.

322

323 To test for a pattern of RCD via increased female discrimination against heterospecific males in  
324 sympatry, we asked whether focal female mate choice differed among the three focal *Ceasia*  
325 populations examined using ANCOVA. The model included focal female mate choice as the  
326 dependent variable and focal *Ceasia* population as the independent variable. We also included  
327 the proportion of time that conspecific rival males pursued the *Ceasia* focal female as a

328 covariate in the analysis, since previous studies have shown that male pursuit predicts female  
329 nosedigs and spawning (Zhou et al. 2015; Moran et al. in press). As the goal of this analysis  
330 was to test for a pattern of increased female preference for conspecific mates in sympatry  
331 compared to allopatry (i.e., RCD in female mate preference), and previous studies have  
332 indicated that females do not exert preferences among males within or among species (Pyron  
333 1995; Fuller 2003; Zhou et al. 2015; Moran et al. in press), we also used ANCOVA to test for  
334 female mate choice between conspecific and *E. caeruleum* rival males within each focal *Ceasia*  
335 population. For these within-population analyses, the number of nosedigs the focal females  
336 directed towards each rival male was the independent variable, the rival male's identity  
337 (conspecific or *E. caeruleum*) was the dependent variable, and the proportion of time the rival  
338 male spent in pursuit of the female was included as a covariate.

339

#### 340 ***Comparisons between Ceasia species***

##### 341 **Dichotomous male choice assays**

342 In 2017, we examined behavioral isolation between allopatric *Ceasia* species. We asked  
343 whether allopatric *Ceasia* males were able to discriminate between conspecific females and  
344 allopatric heterospecific *Ceasia* females. The hypothesis that CRCD is occurring within *Ceasia*  
345 predicts that male discrimination against heterospecific females should be low between the two  
346 allopatric *Ceasia* species (as neither occur in sympatry with *E. caeruleum*, and thus do not  
347 experience reinforcement). To test this, we compared the allopatric *E. spectabile* and allopatric  
348 *E. pulchellum* study populations that were used in the 2016 comparisons between *Ceasia* and  
349 *E. caeruleum*.

350

351 We performed dichotomous male choice assays as described above for the *Ceasia* and *E.*  
352 *caeruleum* comparisons conducted in 2016, but with a heterospecific *Ceasia* female in place of

353 the *E. caeruleum* female (Fig. 2d). We performed trials in which allopatric *E. spectabile* acted as  
354 the focal male and conspecific female, with *E. pulchellum* as the heterospecific *Ceasia* female,  
355 and vice versa (Table 1B). We asked whether male preference for conspecifics differed among  
356 the two allopatric *Ceasia* populations. As this set of trials only compared allopatric populations,  
357 we expected to find no significant difference between the two *Ceasia* focal populations in focal  
358 male mate choice (Table 3). To compare male mate choice between populations, we included  
359 focal male mate choice as the dependent variable, and focal *Ceasia* population (allopatric *E.*  
360 *spectabile* or allopatric *E. pulchellum*) as the independent variable. We also tested whether focal  
361 male mate choice for the conspecific female differed from a null expectation of 0.5 (equal  
362 amounts of time spent with each female).

363

#### 364 **Male competition assays**

365 We also conducted male competition assays between the two allopatric *Ceasia* species in 2017.  
366 A previous study by Moran et al. (in press) found high levels of male-driven behavioral isolation  
367 between *Ceasia* species that occur in sympatry with *E. caeruleum*. Our hypothesis that CRCD is  
368 present in *Ceasia* predicts low levels of mate discrimination against heterospecific *Ceasia* in  
369 species that occur allopatrically from *E. caeruleum*. Additionally, our hypothesis that CACD is  
370 present in *Ceasia* predicts low levels of male competitive discrimination against heterospecific  
371 *Ceasia* males in species that occur allopatrically from *E. caeruleum*. To test these hypotheses,  
372 we performed male competition assays as described above for the trials examining interactions  
373 between *Ceasia* and *E. caeruleum* conducted in 2016, but using a heterospecific *Ceasia* rival  
374 male in place of the *E. caeruleum* rival male (Fig. 2e). In these trials, we paired allopatric *E.*  
375 *spectabile* with allopatric *E. pulchellum* (Table 2C). We performed trials in which each of these  
376 allopatric *Ceasia* species acted as the focal pair and as the heterospecific rival male. We did not



377 repeat trials conducted in 2016 in which each of these *Ceasia* species were paired with a  
378 conspecific rival male.

379

380 We measured male aggression, male mate choice, and female mate choice as described in  
381 Table 3. We conducted ANOVAs as described above for the 2016 male competition trials that  
382 paired *Ceasia* with *E. caeruleum*, but with heterospecific *Ceasia* in place of *E. caeruleum*. As  
383 the analyses for these 2017 trials compared discrimination against heterospecifics in two  
384 allopatric *Ceasia* populations, our prediction for CRCD and CACD was that there would be no  
385 significant differences between these two populations (i.e., both allopatric *Ceasia* populations  
386 should show low levels of discrimination against one another).

387

### 388 ***Behavioral isolation indices***

389 We used the male aggression, male mate choice, and female mate choice data from the male  
390 competition assays to calculate three behavioral isolation indices following Moran et al. (in  
391 press). Behavioral isolation indices were calculated individually for each trial and then averaged  
392 across all replicates within each species comparison. These indices allowed for a comparison  
393 among species pairs (i.e., for each *Ceasia* - *E. caeruleum* and *Ceasia* - *Ceasia* comparison) of  
394 levels of discrimination against heterospecifics. Indices range from -1 (complete preference for  
395 heterospecifics) to 1 (complete preference for conspecifics), with 0 indicating no preference for  
396 conspecifics versus heterospecifics (Stalker 1942; Martin and Mendelson 2016; Moran et al. in  
397 press).

398

399 We calculated male aggression (MA) indices for each species pair as:

400

401

402 
$$MA = \frac{a_c - a_h}{a_c + a_h}$$

403

404

405 where  $a_c$  and  $a_h$  represent the combined number of fin flares and attacks performed between  
406 conspecific males and between heterospecific males, respectively.

407

408 We calculated male choice (MC) indices as:

409

410

411 
$$MC = \frac{m_c - m_h}{m_c + m_h}$$

412

413

414 where  $m_c$  and  $m_h$  represent the proportion of time in each trial that conspecific males and  
415 heterospecific males spent pursuing the *Ceasia* female.

416

417 As previous studies have indicated that male pursuit of a female is highly correlated with female  
418 nosedigs (a measure of female mating preference), female choice (FC) indices controlled for  
419 male pursuit of the female. We calculated the FC indices as:

420

421

422 
$$FC = \frac{f_c}{p_c} - \frac{f_h}{p_h}$$

423

424

425 where  $f_c$  and  $f_h$  represent the number of nosedigs females performed towards conspecific males  
426 and towards heterospecific males, respectively.  $p_c$  and  $p_h$  represent the number of 30-s blocks in  
427 which conspecific males and heterospecific males were scored as having pursued the female  
428 during a trial, respectively.

429

430

## 431 **Results**

### 432 ***Reproductive Character Displacement between Ceasia and E. caeruleum***

433 There was a clear pattern of RCD between *Ceasia* and *E. caeruleum* in the dichotomous male  
434 choice trials, due to increased male mate discrimination against *E. caeruleum* females in  
435 sympatry. Focal male mate choice differed among sympatric and allopatric *Ceasia* populations,  
436 but not between allopatric *Ceasia* populations (Table 4). Sympatric *E. spectabile* focal males  
437 showed 2X higher levels of discrimination against sympatric *E. caeruleum* females than did  
438 allopatric *E. spectabile* males (Fig. 3a). Sympatric *E. spectabile* male mate choice was much  
439 greater than the null expectation of 0.5 (mean  $\pm$  SE:  $0.97 \pm 0.01$ ; one-sample t-test:  $t_{11}=51.58$ ,  
440  $p<0.00001$ ). Conversely, male mate choice did not differ from 0.5 in the allopatric *Ceasia* males  
441 (Fig. 3b,c; allopatric *E. spectabile* mean  $\pm$  SE:  $0.51 \pm 0.04$ ; one-sample t-test:  $t_{11}=0.17$ ,  $p=0.87$ ;  
442 *E. pulchellum* mean  $\pm$  SE:  $0.53 \pm 0.05$ ; one-sample t-test:  $t_{11}=0.60$ ,  $p=0.56$ ).

443

444 RCD in male mate choice (i.e., increased discrimination against heterospecific females in  
445 sympatric populations) was also indicated in the male competition trials that considered male *E.*  
446 *caeruleum* mate choice. Rival male mate choice differed significantly among sympatric and  
447 allopatric *Ceasia* populations, but not between allopatric *Ceasia* populations (Table S2). In  
448 sympatric trials, conspecific rival males were much more likely to pursue the focal female  
449 *Ceasia* compared to *E. caeruleum* rival males (Fig. S1a). In allopatric trials, conspecific and *E.*

450 *caeruleum* rival males and spent roughly the same amount of time pursuing the focal female  
451 *Ceasia*. Hence, sympatric *E. caeruleum* males discriminated against sympatric *E. spectabile*  
452 females, but allopatric *E. caeruleum* males did not discriminate against allopatric *E. spectabile*  
453 or allopatric *E. pulchellum* females.

454  
455 Unlike male *Ceasia*, female *Ceasia* mating preference for conspecific versus *E. caeruleum*  
456 males did not differ among the focal *Ceasia* populations. When male pursuit was included as a  
457 covariate in the analysis, female mate choice did not differ among *Ceasia* populations (Table 5).  
458 This was due to females not exerting any preference for conspecific males over *E. caeruleum*  
459 males across all three populations, regardless of sympatry with *E. caeruleum* (Table S3).

460

#### 461 ***Agonistic Character Displacement between Ceasia and E. caeruleum***

462 The male competition trials revealed a pattern consistent with ACD between *Ceasia* and *E.*  
463 *caeruleum*, due to increased male discrimination against heterospecific competitors in sympatry.  
464 Focal male fin flare bias and focal male attack bias differed significantly among sympatric  
465 versus allopatric *Ceasia* populations, but did not differ between allopatric *Ceasia* populations  
466 (Table 6). Sympatric *E. spectabile* focal males directed 9x more fin flares towards conspecific  
467 rival males compared to *E. caeruleum* rival males (Fig. 3d). Similarly, sympatric *E. spectabile*  
468 focal males attacked conspecific rival males 6x more than they attacked sympatric *E. caeruleum*  
469 rival males (Fig. 3g). On average, both allopatric *E. spectabile* and allopatric *E. pulchellum* focal  
470 males directed an equal number of fin flares (Fig. 3e,f) and attacks (Fig. 3h,i) towards  
471 conspecific rival males and allopatric *E. caeruleum* rival males.

472

473 Rival male fin flare bias also showed a pattern consistent with ACD and was similar to what was  
474 found with focal males (Table S4). Sympatric *E. caeruleum* rival males were much less likely to

475 flare their fins towards *E. spectabile* males than were allopatric *E. caeruleum* rival males (Fig.  
476 S1d-f). Conversely, rival male attack bias did not differ among trials with sympatric versus  
477 allopatric focal *Ceasia* (Table S4). Both sympatric and allopatric *E. caeruleum* directed a low  
478 number of attacks towards focal male *Ceasia* (Fig. S1g-i). Thus, while focal males in allopatric  
479 *Ceasia* populations failed to discriminate against allopatric *E. caeruleum* males, allopatric *E.*  
480 *caeruleum* rival males did discriminate somewhat against allopatric *Ceasia* males.

481

#### 482 **Mate discrimination between allopatric *E. spectabile* and allopatric *E. pulchellum***

483 As expected, both allopatric populations of *Ceasia* failed to discriminate against heterospecific  
484 *Ceasia* mates. In dichotomous male choice trials, focal male mate choice did not differ between  
485 allopatric *E. spectabile* and allopatric *E. pulchellum* ( $F_{1,22} = 0.29$ ;  $p = 0.60$ ; Fig. S2a,b). Allopatric  
486 *E. spectabile* focal male mate choice did not differ from a null expectation of 0.5 (mean  $\pm$  SE:  
487  $0.42 \pm 0.04$ ; one-sample t-test:  $t_{11} = -1.94$ ,  $p = 0.08$ ). The same pattern was observed for  
488 allopatric *E. pulchellum* focal male mate choice (mean  $\pm$  SE:  $0.45 \pm 0.04$ ; one-sample t-test:  $t_{11} =$   
489  $-1.28$ ,  $p = 0.23$ ). Similarly, rival male mate choice did not differ between allopatric *E. spectabile*  
490 and allopatric *E. pulchellum* in the male competition trials ( $F_{1,22} = 0.12$ ;  $p = 0.73$ ; Fig. S3).

491

492 Additionally, focal female mate choice did not differ between allopatric *E. spectabile* and  
493 allopatric *E. pulchellum*, and these preferences did not differ from 0.5 (Table S5). There was no  
494 significant difference in the proportion of female nosedigs towards rival males as function of  
495 their identity (conspecific or heterospecific *Ceasia*) when we controlled for the proportion of time  
496 each male pursued the female (Table S6).

497

#### 498 **Competitor discrimination between allopatric *E. spectabile* and allopatric *E. pulchellum***

499 Consistent with our prediction, neither of the populations of allopatric *Ceasia* discriminated  
500 against heterospecific *Ceasia* male competitors. Focal male fin flare bias did not differ between  
501 allopatric *E. spectabile* and allopatric *E. pulchellum* ( $F_{1,22} = 1.79$ ;  $p = 0.19$ ; Fig. S2c,d), nor did  
502 focal male attack bias ( $F_{1,22} = 0.84$ ;  $p = 0.37$ ; Fig. S2e,f).

503

504 Allopatric *Ceasia* males also failed to discriminate against heterospecific *Ceasia* males when  
505 they acted as the rival male. In the trials where allopatric *E. pulchellum* were the focal males,  
506 conspecific rival males and allopatric *E. spectabile* (heterospecific *Ceasia*) rival males directed a  
507 similar number of fin flares towards focal males (Fig. S3d). However, in trials where allopatric *E.*  
508 *spectabile* were the focal males, allopatric *E. pulchellum* (heterospecific *Ceasia*) rival males  
509 directed more fin flares towards the focal males compared to the conspecific rival males (Fig.  
510 S3c). This resulted in a significant difference in rival male fin flare bias between the two  
511 allopatric *Ceasia* populations ( $F_{1,22} = 5.79$ ;  $p = 0.025$ ; Fig. S3), despite the pattern being  
512 consistent with our prediction for CACD. Rival male attack bias did not differ between trials with  
513 allopatric *E. spectabile* versus allopatric *E. pulchellum* as the focal *Ceasia* ( $F_{1,22} = 0.10$ ;  $p = 0.75$ ;  
514 Fig. S3).

515

### 516 ***Is there a pattern consistent with RCD and CRCD across Ceasia?***

517 To examine patterns of character displacement across *Ceasia*, we compared the behavioral  
518 isolation indices we calculated in this study with behavioral isolation indices calculated by Moran  
519 et al. (in press) (Tables 7, S7; Fig. 4). Behavioral isolation was measured between the same  
520 populations of sympatric *E. spectabile* and sympatric *E. caeruleum* in the present study and in  
521 Moran et al (in press; Table S7). Calculations of behavioral isolation indices for the pairing of  
522 sympatric *E. spectabile* with sympatric *E. caeruleum* did not differ between this study and Moran  
523 et al (in press) (two-sample t-tests; MA:  $t_{38} = 0.31$ ,  $p = 0.76$ ; MC:  $t_{18} = -1.44$ ,  $p = 0.17$ ; FC:  $t_{18} = -0.98$ ,

524  $p=0.34$ ). Thus, the MA, MC, and FC indices presented here for the pairing of sympatric *E.*  
525 *spectabile* with sympatric *E. caeruleum* (Table 7; Fig. 4) were calculated by pooling the  
526 behavioral isolation data from this study with behavioral isolation data from Moran et al. (in  
527 press).

528  
529 RCD predicts higher levels of discrimination against heterospecific mates (i.e., higher MC and/or  
530 FC indices) in sympatric compared to allopatric *Ceasia - E. caeruleum* pairs. CRCD predicts  
531 increased levels of discrimination against heterospecific mates (i.e., higher MC and/or FC  
532 indices) in sympatric compared to allopatric *Ceasia - Ceasia* pairs. In other words, *Ceasia* that  
533 are sympatric with *E. caeruleum* (but allopatric to one another) should have increased  
534 behavioral isolation.

535  
536 We observed a pattern consistent with RCD and CRCD. MC behavioral isolation indices were  
537 consistently higher between sympatric species pairs compared to allopatric species pairs, both  
538 within the *Ceasia - E. caeruleum* comparisons and within the *Ceasia - Ceasia* comparisons  
539 (Table 7; Fig. 4b). The difference between sympatry and allopatry was more pronounced in  
540 *Ceasia - E. caeruleum* comparisons.

541  
542 There was no pattern among FC indices as a function of sympatry with *E. caeruleum* (Table 7;  
543 Fig. 4c). This was due to females not exerting any detectable mating preferences for conspecific  
544 males.

545  
546 ***Is there a pattern consistent with ACD and CACD across Ceasia?***

547 ACD predicts higher levels of male discrimination against heterospecific rival males (i.e., higher  
548 MA indices) in sympatric compared to allopatric *Ceasia - E. caeruleum* pairs. CACD predicts

549 increased levels of male discrimination against heterospecific rival males (i.e., higher MA  
550 indices) in sympatric compared to allopatric *Ceasia* - *Ceasia* pairs.

551  
552 We observed a pattern consistent with ACD and CACD. MA behavioral isolation indices were  
553 consistently higher between sympatric species pairs compared to allopatric species pairs, both  
554 within the *Ceasia* - *E. caeruleum* comparisons and within the *Ceasia* - *Ceasia* comparisons  
555 (Table 7; Fig. 4a). As with MC, the difference between sympatry and allopatry was more  
556 pronounced in *Ceasia* - *E. caeruleum* comparisons.

557

558

## 559 **Discussion**

560 The results of this study suggest that (1) reinforcement has occurred multiple times between  
561 *Ceasia* and *E. caeruleum* throughout their range of sympatry, and (2) cascading effects of  
562 reinforcement between *Ceasia* and *E. caeruleum* has incidentally contributed to allopatric  
563 speciation within the *Ceasia* clade. Although theory predicts that cascading effects of  
564 reinforcement can lead to allopatric speciation (McPeck and Gavrillets 2006; Pfennig and Ryan  
565 2006), the majority of empirical studies that have examined cascade reinforcement to date have  
566 tested behavioral preferences among closely related populations within species. In addition,  
567 many other studies have compared populations within species where there is behavioral  
568 isolation between populations that are allopatric versus sympatric with another species (Nosil et  
569 al. 2003; Lemmon 2009; Hopkins et al. 2014; Kozak et al. 2015; Comeault and Matute 2016;  
570 Comeault et al. 2016). The implication with these studies is that reinforcement changes  
571 preferences and target traits in such a way that increases behavioral isolation between  
572 sympatric and allopatric populations (i.e., “sympatry-allopatry effects”). Here, there are high  
573 levels of behavioral isolation between *Ceasia* species that have independently undergone



574 reinforcement with *E. caeruleum*. This suggests that different species-specific preferences and  
575 traits have evolved in *Ceasia* species that are sympatric with *E. caeruleum* (i.e., “convergent-  
576 sympatry effects”). This study provides an important example of how cascade reinforcement can  
577 cause high levels of behavioral isolation to evolve between closely related populations and  
578 result in allopatric speciation.

579  
580 We observed a striking pattern of RCD and ACD between *Ceasia* and *E. caeruleum*, primarily  
581 driven by male *Ceasia* behavior. Sympatric *E. spectabile* males strongly discriminated against  
582 *E. caeruleum* female mates and male competitors, but allopatric *E. spectabile* and allopatric *E.*  
583 *pulchellum* males did not discriminate against allopatric *E. caeruleum* of either sex. As  
584 expected, allopatric *E. spectabile* and allopatric *E. pulchellum* also did not discriminate against  
585 one another in a mating or fighting context. Furthermore, our results agree with several previous  
586 studies in this system that have failed to detect female mate preference for conspecific versus  
587 heterospecific males in sympatric or allopatric populations of *Ceasia*. Thus, it appears that  
588 males play an important role in maintaining species boundaries in these species.

589  
590 There is also a pattern consistent with our predictions for RCD and ACD in male *E. caeruleum*,  
591 but the pattern of ACD was less striking in *E. caeruleum* males compared to *Ceasia* males.  
592 Sympatric *E. caeruleum* discriminated against sympatric *E. spectabile* females and males.  
593 However, *E. caeruleum* males did not show as low a level of discrimination against *Ceasia*  
594 males in allopatry. We hypothesize that the discrimination against *Ceasia* males demonstrated  
595 by allopatric *E. caeruleum* males may be due to differences in the level of gene flow  
596 experienced by *Ceasia* versus *E. caeruleum*. Patterns of character displacement due to  
597 reinforcement are more likely to be maintained over time (and to lead to cascading effects  
598 among populations within species) when gene flow is low among populations (Hoskin et al.

599 2005; Lemmon 2009; Kozak et al. 2015; Yukilevich and Aoki 2016). *Ceasia* and *E. caeruleum*  
600 both occur in small headwater streams, but *E. caeruleum* can also inhabit larger order streams  
601 and rivers (Page 1983; Page and Burr 2011), leading to more opportunities for gene flow among  
602 populations (Echelle et al. 1975, 1976). Indeed, population genetic analyses of four species of  
603 *Ceasia* and *E. caeruleum* found increased heterozygosity and higher levels nucleotide diversity  
604 are present within *E. caeruleum* relative to the *Ceasia* species, indicating lower levels of gene  
605 flow in species of *Ceasia*. This key difference in the biology of *Ceasia* and *E. caeruleum* may  
606 explain why *E. caeruleum* has not diversified to the extent that *Ceasia* has, despite being  
607 similarly widespread.

608

609 Our results together with the results of a recent study by Moran et al. (in press) support the  
610 hypothesis that reinforcement has occurred multiple times between *Ceasia* and *E. caeruleum*.  
611 Sympatric *Ceasia* species have consistently shown almost complete levels of behavioral  
612 isolation with their respective sympatric populations of *E. caeruleum*, but allopatric *Ceasia* do  
613 not show any such discrimination (this study; Moran et al. in press; Zhou and Fuller 2014). The  
614 observed pattern of RCD and ACD across *Ceasia* together evidence of high levels of  
615 postzygotic isolation between *Ceasia* and *E. caeruleum* (Zhou 2014; R. Moran unpubl. data)  
616 suggests that reinforcement is responsible for driving behavioral isolation between *Ceasia* and  
617 *E. caeruleum* in sympatry.

618

619 We also observed a pattern consistent with CRCD and CACD across *Ceasia*. Species of *Ceasia*  
620 that occur in sympatry with *E. caeruleum* show surprisingly high levels of male discrimination  
621 against heterospecific *Ceasia* mates and competitors, but no such discrimination is present  
622 among *Ceasia* that occur in allopatry from *E. caeruleum*. This observation together with the  
623 evidence for reinforcement between *Ceasia* and *E. caeruleum* suggest that cascade

624 reinforcement is occurring within *Ceasia*. Cascade reinforcement may occur if slightly different  
625 mating traits (signals and/or preferences) arise in different populations across the range of a  
626 species experiencing reinforcement with a heterospecific. Theory predicts that cascade  
627 reinforcement can readily occur when gene flow between populations within a species is low (as  
628 is the case with organisms that occur in isolated headwater streams, such as darters), and  
629 when populations respond to reinforcing selection on mating traits and their underlying loci in  
630 unique ways due to stochastic processes (i.e., mutation-order selection; Abbott et al. 2013;  
631 Mendelson et al. 2014; Comeault and Matute 2016; Yukilevich and Aoki 2016). Under mutation-  
632 order selection, species divergence may occur despite the presence of similar types of  
633 ecological and sexual selection. In this way, stochastic variation in response to the same  
634 selective pressures (i.e., selection against maladaptive heterospecific interactions in sympatry)  
635 can potentially lead to allopatric divergence among populations within species.

636  
637 This study corroborates the results of several recent studies that have shown that male mate  
638 choice and male competition play an important role in driving sympatric and allopatric speciation  
639 in darters (Ciccotto et al. 2013; Zhou et al. 2015; Zhou and Fuller 2016; Martin and Mendelson  
640 2016; Moran et al. in press). Multiple studies in several different species of darters have also  
641 found little or no female mate preference for conspecific over heterospecific males (Martin and  
642 Mendelson 2013, 2016; Ciccotto et al. 2014; Zhou et al. 2015; but see Williams and Mendelson  
643 2010, 2011). Furthermore, although the presence of elaborate male nuptial coloration is most  
644 commonly attributed to intersexual selection via female mate preferences (Panhuis et al. 2001),  
645 male coloration in darters appears to be under intrasexual selection due to intense male-male  
646 competition (Zhou et al. 2015; Martin and Mendelson 2016; Zhou and Fuller 2016).  
647 Reproductive and agonistic character displacement can lead to shifts in behavioral response to  
648 heterospecifics and the signals used to recognize conspecifics versus heterospecifics in

649 sympatry (Brown and Wilson 1956; Grether et al. 2009). Thus, future studies that examine  
650 whether character displacement in male color pattern corresponds to the observed ACD and  
651 CACD in male aggressive response to heterospecifics would be of interest.

652

653 In conclusion, this study provides empirical evidence of male-driven reinforcement and cascade  
654 reinforcement in darters. As far as we are aware, this is the first documented case  
655 demonstrating that ACD between species can incidentally lead to CACD among populations  
656 within species (or in this case, among closely related species within a clade). Although the clear  
657 majority of reinforcement studies to date have focused on the evolution of female mating  
658 preferences for males, the results of this study demonstrate that male behavior alone can drive  
659 speciation between and within species via reinforcement and cascade reinforcement. This  
660 underscores the necessity of considering the behavior of both sexes when evaluating whether  
661 reinforcement may be at work in a given system. Finally, this study provides important  
662 groundwork for future studies examining the effects of reinforcement on sympatric and allopatric  
663 speciation in darters. Further research is needed to explore the extent to which reinforcement  
664 has been involved in generating the extraordinary diversity of darters.

665

666

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674

675

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821

**Table 1.** Comparisons made in dichotomous choice trials, which included a focal male *Ceasia* and a conspecific female *Ceasia* with (A) an *E. caeruleum* female, or (B) a heterospecific *Ceasia* female.

**A. Conspecific *Ceasia* vs. *E. caeruleum* dichotomous male choice trials**

<b>Focal <i>Ceasia</i> male</b>	<b>Conspecific female</b>	<b>Heterospecific female</b>	<b>n</b>
Allopatric <i>E. spectabile</i>	Allopatric <i>E. spectabile</i>	Allopatric <i>E. caeruleum</i>	12
Allopatric <i>E. pulchellum</i>	Allopatric <i>E. pulchellum</i>	Allopatric <i>E. caeruleum</i>	12
Sympatric <i>E. spectabile</i>	Sympatric <i>E. spectabile</i>	Sympatric <i>E. caeruleum</i>	12

**B. Conspecific *Ceasia* vs. heterospecific *Ceasia* dichotomous male choice trials**

<b>Focal <i>Ceasia</i> male</b>	<b>Conspecific female</b>	<b>Heterospecific female</b>	<b>n</b>
Allopatric <i>E. spectabile</i>	Allopatric <i>E. spectabile</i>	Allopatric <i>E. pulchellum</i>	12
Allopatric <i>E. pulchellum</i>	Allopatric <i>E. pulchellum</i>	Allopatric <i>E. spectabile</i>	12

**Table 2.** Comparisons made in male competition trials, which included a conspecific focal male and focal female *Ceasia* pair together with (A) a conspecific *Ceasia* rival male, (B) an *E. caeruleum* rival male, or (C) a heterospecific *Ceasia* rival male.

**A. Male competition trials with conspecific *Ceasia* rival male**

<b><i>Ceasia</i> focal male</b>	<b><i>Ceasia</i> focal female</b>	<b>Rival male</b>	<b>n</b>
Allopatric <i>E. spectabile</i>	Allopatric <i>E. spectabile</i>	Allopatric <i>E. spectabile</i>	12
Allopatric <i>E. pulchellum</i>	Allopatric <i>E. pulchellum</i>	Allopatric <i>E. pulchellum</i>	12

**B. Male competition trials with *E. caeruleum* rival male**

<b><i>Ceasia</i> focal male</b>	<b><i>Ceasia</i> focal female</b>	<b>Rival male</b>	<b>n</b>
Allopatric <i>E. spectabile</i>	Allopatric <i>E. spectabile</i>	Allopatric <i>E. caeruleum</i>	12
Allopatric <i>E. pulchellum</i>	Allopatric <i>E. pulchellum</i>	Allopatric <i>E. caeruleum</i>	12
Sympatric <i>E. spectabile</i>	Sympatric <i>E. spectabile</i>	Sympatric <i>E. caeruleum</i>	12

**C. Male competition trials with heterospecific *Ceasia* rival male**

<b><i>Ceasia</i> focal male</b>	<b><i>Ceasia</i> focal female</b>	<b>Rival male</b>	<b>n</b>
Allopatric <i>E. spectabile</i>	Allopatric <i>E. spectabile</i>	Allopatric <i>E. pulchellum</i>	12
Allopatric <i>E. pulchellum</i>	Allopatric <i>E. pulchellum</i>	Allopatric <i>E. spectabile</i>	12

**Table 3.** Definition of the behavioral variables measured in dichotomous male mate choice assays and male competition assays. We also indicate whether each behavioral variable showed a pattern that was consistent with our predictions for RCD, ACD, CRCd, and CACD (i.e., lack of discrimination against heterospecifics in allopatric populations, and high discrimination against heterospecifics in sympatric populations). NA denotes that the behavioral variable was not applicable to testing the predictions for that type of character displacement.

Variable	Definition	RCD	ACD	CRCd	CACD
<b>Dichotomous Male Choice Assays (2 females, 1 male)</b>					
<i>Focal Male Mate Choice</i>	Number of time blocks spent pursuing the conspecific divided by the total number of time blocks spent pursuing either female.	yes	NA	yes	NA
<b>Male Competition Assays (2 males, 1 female)</b>					
<i>Rival Male Mate Choice</i>	Proportion of time blocks the focal female was pursued by conspecific versus heterospecific rival males across two trials = # of time blocks conspecific rival male pursued the female / (sum of time blocks the conspecific and heterospecific rivals pursued the female).	yes	NA	yes	NA

<i>Focal Female Mate Choice</i>	Proportion of nosedigs towards conspecific versus heterospecific rival males across two trial = # of nosedigs towards conspecific rivals / (sum of nosedigs towards conspecific and heterospecific rivals); the analysis of this variable was corrected for male pursuit.	no	NA	no	NA
<i>Focal Male Fin Flare Bias</i>	Proportion of fin flares towards conspecific versus heterospecific rivals across two trials = # fin flares to conspecific rival / (sum of fin flares to conspecific and heterospecific rivals).	NA	yes	NA	yes
<i>Focal Male Attack Bias</i>	Proportion of attacks towards conspecific versus heterospecific rivals across two trials = # attacks on conspecific rival / (sum of attacks on conspecific and heterospecific rivals).	NA	yes	NA	yes
<i>Rival Male Fin Flare Bias</i>	Proportion of fin flares performed by conspecific versus heterospecific rivals across two trials = # fin flares by conspecific rival toward the focal male / (sum of fin flares by conspecific and heterospecific rivals toward the focal male).	NA	yes	NA	yes

<i>Rival Male</i>	Proportion of attacks performed by conspecific versus heterospecific rivals across two trials = # attacks by conspecific rival toward the focal male / (sum of attacks by conspecific and heterospecific rivals towards the focal male).	NA	mixed	NA	yes
<i>Attack Bias</i>			+		

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+Allopatric *E. caeruleum* males tended to attack allopatric *E. spectabile* males more than sympatric *E. caeruleum* males attacked sympatric *E. spectabile* males, but no other differences were found.

**Table 4.** Results of ANOVA on focal male mate choice between conspecific females and *E. caeruleum* females in dichotomous male choice male trials. We asked focal male mate choice differed among focal *Ceasia* populations (sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*). Pairwise post-hoc t-test results are also shown for the analysis.

Dependent variable: Focal male mate choice	Test		
	df	Statistic	p
Population identity	2,33	45.21	<0.00001
Sympatric <i>E. spectabile</i> vs. allopatric <i>E. spectabile</i>	22	11.38	<0.00001
Sympatric <i>E. spectabile</i> vs. allopatric <i>E. pulchellum</i>	22	8.10	<0.00001
Allopatric <i>E. spectabile</i> vs. allopatric <i>E. pulchellum</i>	220	-0.38	0.71



**Table 5.** Results ANCOVA examining focal female mate choice between conspecific rival males and *E. caeruleum* rival males in male competition trials. We asked whether female mate choice differed among focal *Ceasia* populations (sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*). Male pursuit of the female was included as a covariate in the analysis.

<b>Dependent variable: Focal female mate choice</b>	<b>Test</b>		
	<b>df</b>	<b>Statistic</b>	<b>p</b>
Population identity	2,32	0.09	0.92
Male pursuit	1,32	0.74	0.40

**Table 6.** Results of ANOVA on focal male *Ceasia* fin flares and attacks at rival males in male competition trials. We asked whether focal male fin flare bias and focal male attack bias differed among focal *Ceasia* populations (sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*). Pairwise post-hoc t-test results are also shown for both analyses.

Dependent variable: Focal male fin flare bias	Test		
	df	Statistic	p
Population identity	2,33	8.34	<b>0.0012</b>
Sympatric <i>E. spectabile</i> vs. allopatric <i>E. spectabile</i>	22	5.28	<b>&lt;0.0001</b>
Sympatric <i>E. spectabile</i> vs. allopatric <i>E. pulchellum</i>	22	2.85	<b>0.0093</b>
Allopatric <i>E. spectabile</i> vs. allopatric <i>E. pulchellum</i>	22	-0.84	0.41

Dependent variable: Focal male attack bias	Test		
	df	Statistic	p
Population identity	2,33	9.12	<b>&lt;0.001</b>
Sympatric <i>E. spectabile</i> vs. allopatric <i>E. spectabile</i>	22	4.53	<b>0.0002</b>
Sympatric <i>E. spectabile</i> vs. allopatric <i>E. pulchellum</i>	22	3.82	<b>&lt;0.001</b>
Allopatric <i>E. spectabile</i> vs. allopatric <i>E. pulchellum</i>	22	-0.65	0.52

**Table 7.** Behavioral isolation indices (mean  $\pm$  standard error) for male aggression (MA), male choice (MC), and female choice (FC).

For each species pair, the *Ceasia* species that acted as the focal *Ceasia* in behavioral trials is listed first, followed by the species that it was observed with (a heterospecific *Ceasia* or *E. caeruleum*).

Geography	Pairing	Species	MA	MC	FC
Allopatric	<i>Ceasia</i> - <i>Ceasia</i>	<i>E. spectabile</i> - <i>E. pulchellum</i>	-0.01 $\pm$ 0.07	0.11 $\pm$ 0.07	0.01 $\pm$ 0.02
Sympatric	<i>Ceasia</i> - <i>Ceasia</i>	<i>E. fragi</i> - <i>E. uniporum</i> *	0.38 $\pm$ 0.08	0.31 $\pm$ 0.07	0.01 $\pm$ 0.01
Sympatric	<i>Ceasia</i> - <i>Ceasia</i>	<i>E. fragi</i> - <i>E. burri</i> *	0.50 $\pm$ 0.06	0.30 $\pm$ 0.07	0.02 $\pm$ 0.01
Sympatric	<i>Ceasia</i> - <i>Ceasia</i>	<i>E. fragi</i> - <i>E. spectabile</i> *	0.35 $\pm$ 0.06	0.34 $\pm$ 0.10	0.01 $\pm$ 0.02
Allopatric	<i>Ceasia</i> - <i>E. caeruleum</i>	<i>E. spectabile</i> - <i>E. caeruleum</i>	0.09 $\pm$ 0.09	0.22 $\pm$ 0.12	-0.16 $\pm$ 0.16
Allopatric	<i>Ceasia</i> - <i>E. caeruleum</i>	<i>E. pulchellum</i> - <i>E. caeruleum</i>	0.30 $\pm$ 0.12	0.25 $\pm$ 0.12	0.01 $\pm$ 0.02
Sympatric	<i>Ceasia</i> - <i>E. caeruleum</i>	<i>E. fragi</i> - <i>E. caeruleum</i> *	0.80 $\pm$ 0.05	0.76 $\pm$ 0.06	0.01 $\pm$ 0.04
Sympatric	<i>Ceasia</i> - <i>E. caeruleum</i>	<i>E. uniporum</i> - <i>E. caeruleum</i> *	0.82 $\pm$ 0.06	0.70 $\pm$ 0.09	-0.11 $\pm$ 0.13
Sympatric	<i>Ceasia</i> - <i>E. caeruleum</i>	<i>E. burri</i> - <i>E. caeruleum</i> *	0.92 $\pm$ 0.03	0.66 $\pm$ 0.08	-0.05 $\pm$ 0.05
Sympatric	<i>Ceasia</i> - <i>E. caeruleum</i>	<i>E. spectabile</i> - <i>E. caeruleum</i> **	0.85 $\pm$ 0.05	0.84 $\pm$ 0.06	0.03 $\pm$ 0.02

\*From Moran et al. (in press).

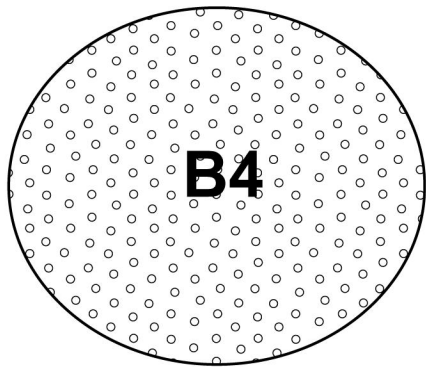
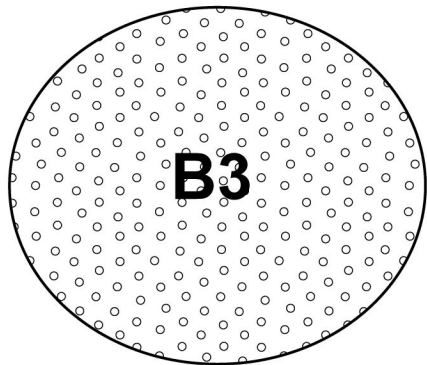
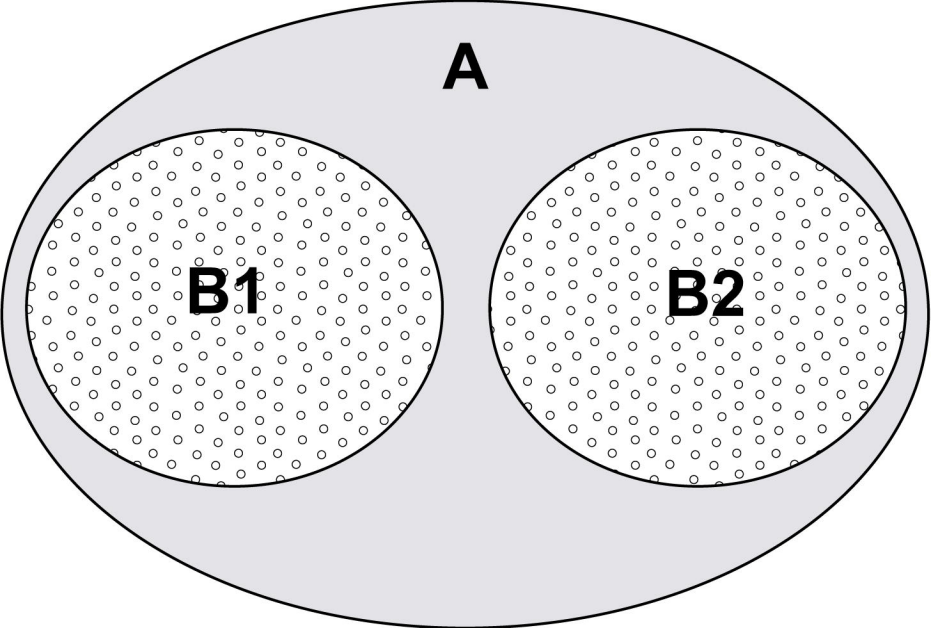
\*\*Calculated using data from the present study combined with data from Moran et al. (in press).

**Figure 1.** Hypothetical ranges for two species, A and B, with four populations shown within B (B1-B4). Populations B1-B4 are allopatric from one another. Populations B1 and B2 are sympatric with species A. Populations B3 and B4 are allopatric from species A. Reinforcement occurring independently between A and B1 and between A and B2 can incidentally cause heightened behavioral isolation between B1 and B2 compared to behavioral isolation present between B3 and B4 (i.e., cascade reinforcement).

**Figure 2.** (a-c) Experimental set up for dichotomous male choice trials and male competition trials conducted between *Ceasia* and *E. caeruleum* in 2016. Sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* acted as focal *Ceasia* in these trials. (d-e) Experimental set up for dichotomous male choice trials and male competition trials conducted between *Ceasia* species in 2017. Allopatric *E. spectabile* and allopatric *E. pulchellum* acted as focal *Ceasia* and as heterospecific *Ceasia* in turn in these trials. In 2017, we did not repeat male competition trials in which a conspecific *Ceasia* acted as the rival male (shown in b). We compared the behavior of individuals in trials with a conspecific *Ceasia* rival male (b) to individuals in trials with an *E. caeruleum* rival male (c). We also compared the behavior of individuals in trials with a conspecific *Ceasia* rival male (b) to individuals in trials with a heterospecific *Ceasia* rival male (e).

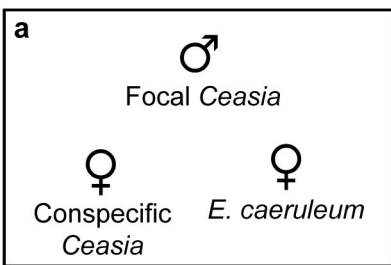
**Figure 3.** Focal male behavior in trials that paired *Ceasia* with *E. caeruleum*. Columns from left to right show results for trials with sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* as the focal *Ceasia*, respectively. (a-c) Proportion of time focal males spent in pursuit of conspecific versus *E. caeruleum* female in dichotomous choice trials. (d-f) Number of focal male fin flares directed at conspecific versus *E. caeruleum* rival males in male competition trials. (g-i) Number of focal male attacks directed at conspecific versus *E. caeruleum* rival males in male competition trials.

**Figure 4.** Behavioral isolation indices (with 95% confidence intervals) for (a) male aggression, (b) male choice, and (c) female choice. Each point represents a comparison between two *Ceasia* species (*Ceasia* - *Ceasia*) or between a *Ceasia* species and *E. caeruleum* (*Ceasia* - *E. caeruleum*). Allopatric comparisons (i.e., comparisons including *Ceasia* that do not co-occur with *E. caeruleum*) are shown in black. Sympatric comparisons (i.e., comparisons including *Ceasia* that do co-occur with *E. caeruleum*) are shown in gray.

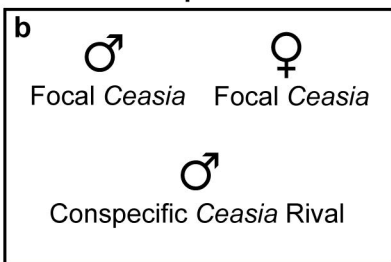




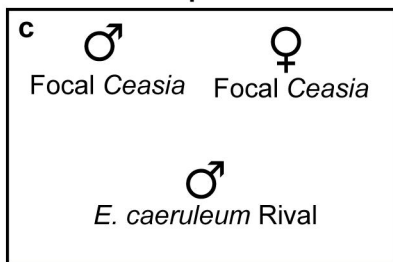
**Dichotomous male choice trial**



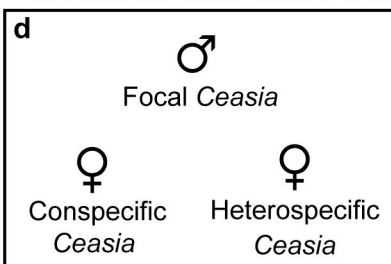
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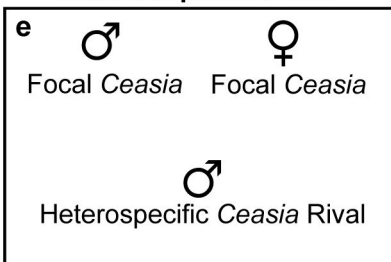
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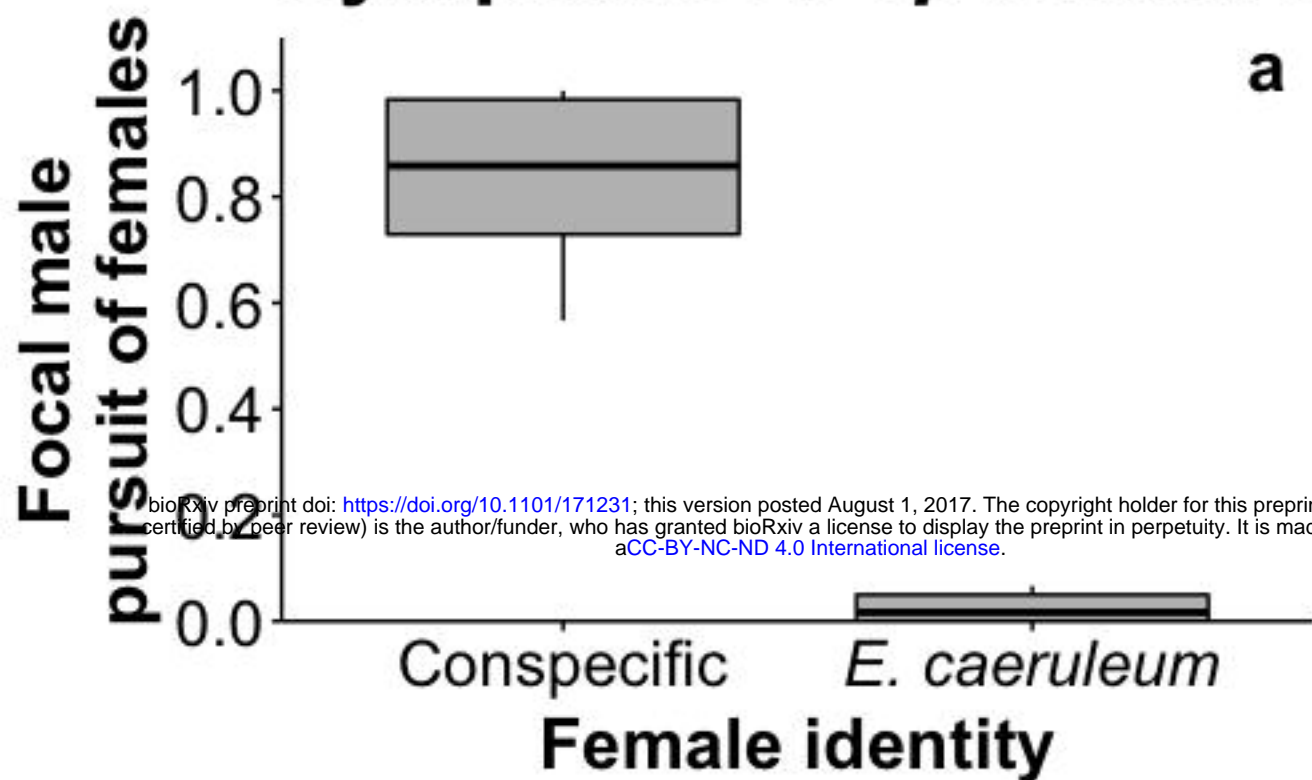
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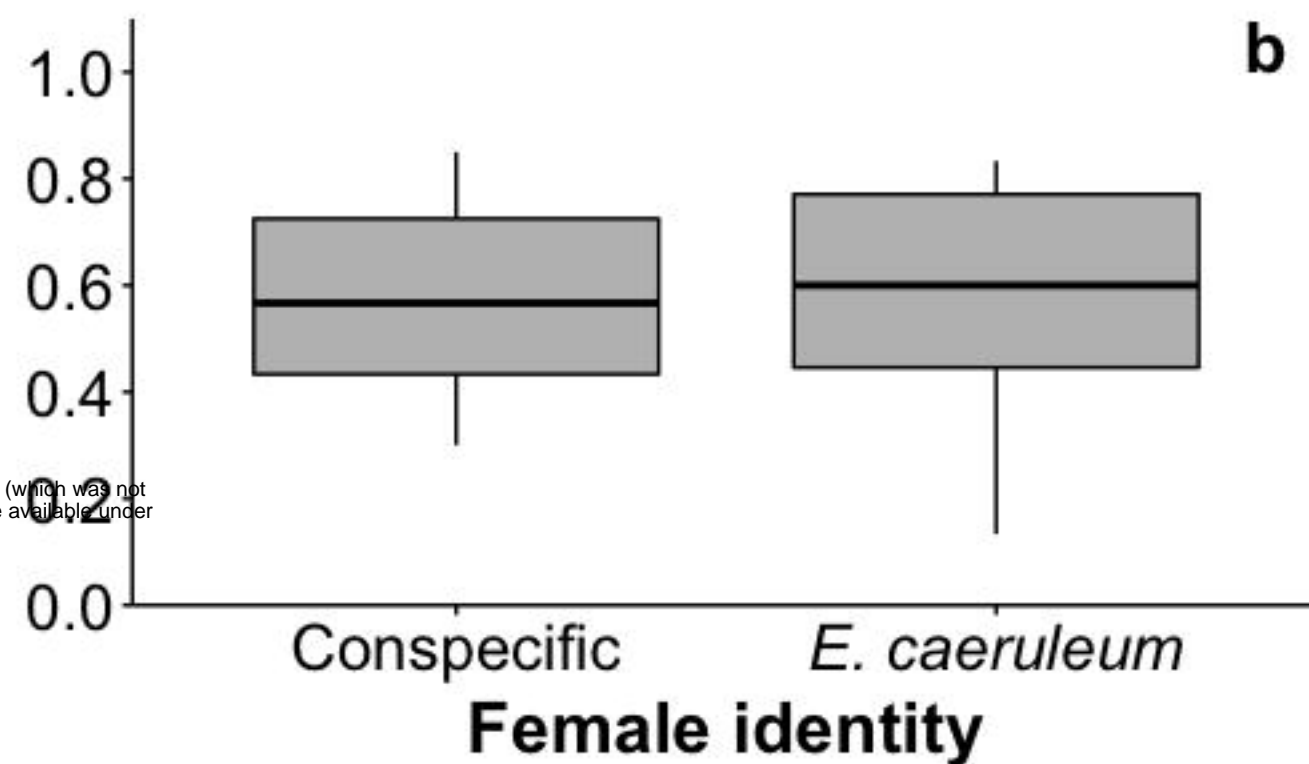
**Male competition trial**



# Sympatric *E. spectabile*



# Allopatric *E. spectabile*



# Allopatric *E. pulchellum*

