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Article

Male-driven reproductive and agonistic character displacement in darters and its implications for speciation in allopatry

Rachel L. Moran* and Rebecca C. Fuller

School of Integrative Biology, University of Illinois, Champaign, IL 61820, USA

*Address correspondence to Rachel L. Moran. E-mail: rmoran9@illinois.edu.

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Abstract

Selection against hybridization can cause mating traits to diverge between species in sympatry via reproductive character displacement (RCD). Additionally, selection against interspecific fighting can cause aggressive traits to diverge between sympatric species via agonistic character displacement (ACD). By directly affecting conspecific recognition traits, RCD and ACD between species can also incidentally cause divergence in mating and fighting traits among populations within a species [termed cascade RCD (CRCD) and cascade ACD]. Here, we demonstrate patterns consistent with male-driven RCD and ACD in 2 groups of darters (orangethroat darter clade *Ceasia* and rainbow darter *Etheostoma caeruleum*). In both groups, males that occur in sympatry (between *Ceasia* and *E. caeruleum*) have higher levels of preference for mating and fighting with conspecifics over heterospecifics than do males from allopatry. This is consistent with RCD and ACD. We also found patterns consistent with CRCD and cascade ACD among species of *Ceasia*. *Ceasia* males that are sympatric to *E. caeruleum* (but allopatric to one another) also have heightened preferences for mating and fighting with conspecific versus heterospecific *Ceasia.* In contrast, *Ceasia* males that are allopatric to *E. caeruleum* readily mate and fight with heterospecific *Ceasia.* We suggest that RCD and ACD between *Ceasia* and *E. caeruleum* has incidentally led to divergence in mating and fighting traits among *Ceasia* species. This study is unique in that male preferences evolve via both RCD (male preference for conspecific females) and ACD (male preference to fight conspecific males) which leads to subsequent divergence among allopatric lineages.

Key words: agonistic character displacement, behavioral isolation, cascade reinforcement, reinforcement, reproductive character displacement, speciation.

Reproductive interference between species can cause mating traits (signals and/or preferences) to diverge via reproductive character displacement (RCD; Howard 1993; Servedio and Noor 2003). RCD is often confirmed by a pattern of enhanced behavioral isolation between 2 species in sympatry compared with allopatry. Recent research suggests that secondary effects of RCD in sympatry can also initiate divergence between allopatric lineages (Pfennig and Pfennig 2009; Hoskin and Higgie 2010). Cascade RCD (hereafter CRCD; Ortiz-Barrientos et al. 2009) occurs when behavioral isolation evolves among populations within a species as a correlated effect of RCD. CRCD has been documented in a variety of taxa (e.g., Nosil et al. 2003; Hoskin et al. 2005; Higgie and Blows 2007, 2008; Lemmon 2009; Porretta and Urbanelli 2012; Bewick and Dyer 2014; Pfennig and Rice 2014; Kozak et al. 2015).

Selection against interspecific aggression can also lead to the evolution of traits involved in species recognition. Maladaptive interspecific fighting over resources (such as mates) can cause shifts in aggressive signals and behavior via agonistic character displacement (ACD; Grether et al. 2009; Okamoto and Grether 2013). A pattern of divergent ACD is said to be present when 2 species are less likely to engage in contests when they occur in sympatry compared with allopatry. Both RCD and ACD may contribute to trait divergence

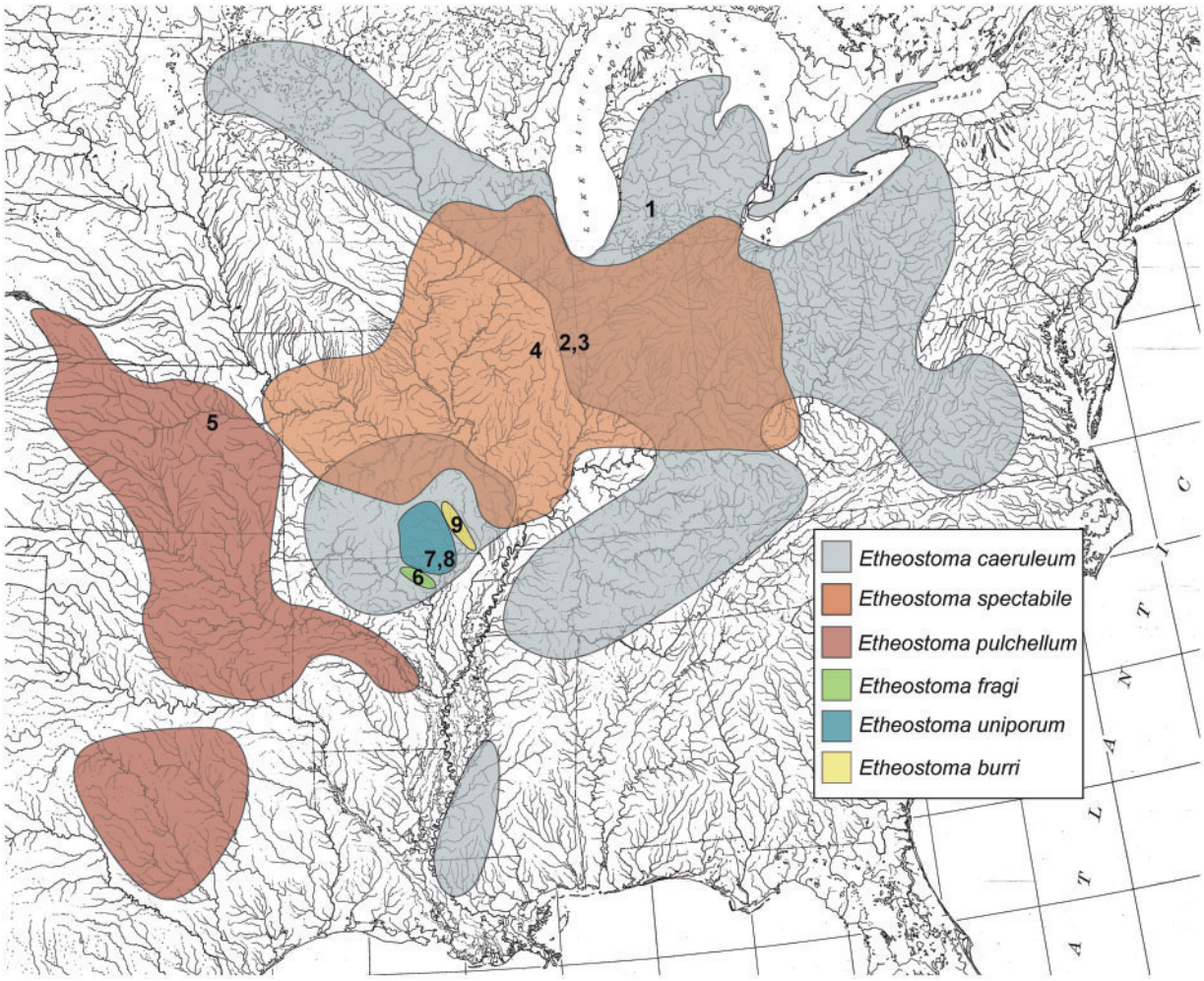

Figure 1. Ranges for *Etheostoma caeruleum* and 5 *Ceasia* species (*Etheostoma spectabile, Etheostoma pulchellum, Etheostoma fragi, Etheostoma uniporum,* and *Etheostoma burri*) used in behavioral assays in the current study and in Moran et al. (2017). Numbers on the map represent approximate collection locations for study populations (see Table 1 for details).

between species that results in decreased heterospecific interactions in sympatry. Although numerous studies have shown that RCD can incidentally lead to divergence in mating traits among populations within species via CRCD, whether selection against interspecific aggression can also cause divergence in agonistic traits among populations within species (i.e., cascade ACD, hereafter CACD) has yet to be determined.

Distinguishing between RCD and ACD is essential to determining the underlying selective pressure (i.e., heterospecific mating or fighting) and relative contribution of male–female and male–male interactions in driving speciation. However, disentangling the importance of RCD versus ACD to speciation can be difficult because many sexually selected traits are used in both female mate choice and male–male competition over mates (Alatalo et al. 1994; Berglund 1996; Sætre et al. 1997; Dijkstra et al. 2007; Saether et al. 2007; Lackey and Boughman 2013; Tinghitella et al. 2015). Here, we examine female mating preferences, male mating preferences, and male–male aggression (MA) to test for patterns consistent with RCD, ACD, CRCD, and CACD.

This study focuses on 2 groups of darters in the subgenus *Oligocephalus*: the orangethroat darter clade *Ceasia* and the rainbow darter *Etheostoma caeruleum*. *Ceasia* and *E. caeruleum* diverged approximately 22 million years ago (Near et al. 2011). Time calibrated gene trees indicate that *Ceasia* subsequently diversified 6–7 million years ago (Bossu et al. 2013). The *Ceasia* clade consists of 15 species, all of which are allopatric with respect to one another (Ceas and Page 1997; Bossu and Near 2009). Phylogenetic and palaeogeographical analyses support allopatric divergence of this clade (Bossu et al. 2013). Twelve *Ceasia* species occur in sympatry with respect to *E. caeruleum* throughout their range, and 2 *Ceasia* species occur in allopatry with respect to *E. caeruleum* throughout their range (see Bossu and Near 2009; Page and Burr 2011). The one remaining *Ceasia* species (orangethroat darter *Etheostoma spectabile*) occurs in both sympatry and allopatry with respect to *E. caeruleum* (Figure 1). Within *Ceasia*, time since divergence does not differ significantly between lineages that occur in sympatry versus allopatry with respect to *E. caeruleum* (Bossu et al. 2013). *Ceasia* and *E. caeruleum* have similar male coloration, mating behavior, and ecology. There is little evidence that male coloration in either *Ceasia* or *E. caeruleum* is the target of female mate choice; females lack preferences for either male size or color pattern within species, and *Ceasia* females lack preferences for conspecific over heterospecific *Ceasia* and *E. caeruleum* males (Pyron 1995; Fuller 2003; Zhou et al. 2015; Moran et al. 2017). Instead, there is

Table 1. Collection locations for populations of each species examined in behavioral trials in the present study as well as in Moran et al. (2017)

Range map population number	Geography	Species	Collection location	Drainage information	Source of behavioral data
1	Allopatric	*E. caeruleum*	42.426825, −85.428370	Prairieville Creek, Kalamazoo River, Barry County, MI	Present study
2	Sympatric	*E. spectabile*	40.054447, −88.089887	Unnamed tributary, Salt Fork of Vermillion River, Champaign County, IL	Present study and Moran et al. (2017)
3	Sympatric	*E. caeruleum*	(Same as above)	(Same as above)	Present study and Moran et al. (2017)
4	Allopatric	*E. spectabile*	40.027663, −88.577180	Unnamed tributary, Sangamon River, Piatt County, IL	Present study
5	Allopatric	*E. pulchellum*	38.952839, −95.517654	Deer Creek, Kansas River, Shawnee County, KS	Present study
6	Sympatric	*E. fragi*	36.304214, −91.927684	Rose Branch tributary of Strawberry River, Fulton County, AR	Moran et al. (2017)
7	Sympatric	*E. uniporum*	36.250560, −91.359318	Unnamed tributary of Spring River, Sharp County, AR	Moran et al. (2017)
8	Sympatric	*E. caeruleum*[a]	36.065396, −91.610420	Mill Creek tributary of Strawberry River, Sharp County, AR	Moran et al. (2017)
9	Sympatric	*E. burri*	37.146415, −90.907459	North Fork Webb Creek, Black River Drainage, Wayne County, MO	Moran et al. (2017)

Notes: Sympatry and allopatry refer to the geographic relationship between *Ceasia* and *E. caeruleum* (all species of *Ceasia* are allopatric from one another). Range map population number refers to numbers shown in Figure 1.

[a]*Etheostoma caeruleum* study population used in sympatric comparisons with *Ceasia* species from the Ozarks regions (i.e., *E. fragi*, *E. uniporum*, and E. *burri*) in Moran et al. (2017).

strong evidence that male coloration is under intrasexual selection and functions as an aggressive signal in male–male competition over access to females (Zhou and Fuller 2016; Moran et al. 2017).

Several recent studies have indicated that RCD and ACD are likely occurring in this system. First, hybridization occurs between *Ceasia* and *E. caeruleum* in nature (Bossu and Near 2009; Moran et al. 2017), and their hybrids have reduced fitness (Zhou 2014; Moran R, unpublished data), providing the potential for RCD to occur via reinforcement (Brown and Wilson 1956; Coyne and Orr 2004). Second, in pairings between 4 species of *Ceasia* and sympatric *E. caeruleum*, males preferentially mate and fight with conspecifics, suggesting RCD and ACD (Figure 1 and Table 1; Moran et al. 2017). Third, a pattern consistent with RCD was observed in a no-choice mating experiment which found that allopatric pairings of female *E. spectabile* and male *E. caeruleum* yielded more eggs than sympatric pairings (Zhou and Fuller 2014). Zhou and Fuller (2014) is the only study to date to compare sympatric and allopatric pairings between a *Ceasia* species and *E. caeruleum*, but the no-choice assay they used was not able to measure the contribution of each sex to behavioral isolation in sympatry. Furthermore, Zhou and Fuller (2014) did not consider male competition, and could not test for ACD.

A unique aspect of this study system is that it allows us to test for patterns consistent with RCD and ACD at 2 taxonomic levels within *Ceasia*: populations within a species, and closely related species within a recently diverged clade. We first tested for RCD and ACD between populations of a single species of *Ceasia* as a function of sympatry with *E. caeruleum*. We next asked whether RCD and ACD are present between species of *Ceasia* as a function of sympatry with *E. caeruleum*. Most studies involving RCD and ACD have considered differences in mating traits between populations within a pair of species as a function of sympatry versus allopatry. However, RCD can also influence species diversification at a macroevolutionary scale (Pfennig and Pfennig 2012; Grether et al. 2017). Over time, CRCD and CACD can cause isolated populations within a species to diverge from one another to such an extent that they merit classification as distinct, allopatric species. The outcome of this process can result in a complex of closely related, allopatric species that exhibit enhanced mating trait divergence with one another (via CRCD/CACD), and with a more distantly related sympatric species (via RCD/ACD). In this manner, CRCD and CACD can fuel hierarchical "speciation cascades" among allopatric lineages at multiple taxonomic levels simultaneously (Pfennig and Ryan 2006). We hypothesize that this scenario is ongoing in the *Ceasia*–*E. caeruleum* system.

To test for RCD and ACD, we measured preferences for mating and fighting with conspecifics in pairings between *E. spectabile* and *E. caeruleum* that occur in sympatry versus allopatry with respect to one another. This allowed us to examine whether patterns consistent with RCD and ACD are present at the population level within *E. spectabile* and *E. caeruleum*. Additionally, we measured preferences for mating and fighting with conspecifics in pairings between *Etheostoma pulchellum* and *E. caeruleum* that occur in allopatry with respect to one another (Figure 1 and Table 1). Because *E. pulchellum* and *E. caeruleum* do not co-occur, these species should show a reduced level of bias against mating and fighting with one another compared with species of *Ceasia* and *E. caeruleum* that do co-occur. Measuring mating and fighting biases in allopatric pairings of *Ceasia* and *E. caeruleum* thus serves as a critical test against which we can compare levels of behavioral preferences in sympatric pairings of *Ceasia* and *E. caeruleum* that were previously reported by Moran et al. (2017).

We also investigated whether patterns consistent with CRCD and CACD are present among *Ceasia* species. Males within the

4 *Ceasia* species examined by Moran et al. (2017; Figure 1 and Table 1), which all occur in sympatry with respect to *E. caeruleum*, prefer conspecific over heterospecific *Ceasia* females and bias their aggression preferentially toward conspecific over heterospecific *Ceasia* males. This divergence in male mating and fighting traits among *Ceasia* species is not associated with differences in male color pattern or genetic distance. Therefore, RCD and ACD between *Ceasia* and *E. caeruleum* may have incidentally contributed to species divergence within the *Ceasia* clade via CRCD and CACD. To test this hypothesis, we examine preferences for mating and fighting with conspecifics (over a heterospecific member of the *Ceasia* clade) in pairings between *E. spectabile* and *E. pulchellum* that occur in allopatry with respect to *E. caeruleum*. We then ask whether *E. spectabile* and *E. pulchellum* have lower levels of preference for mating and fighting with conspecifics compared with that previously observed between pairs of *Ceasia* species that occur in sympatry with respect to *E. caeruleum* (Moran et al. 2017).

Materials and Methods

Mating system details

During the spring spawning season, *Ceasia* and *E. caeruleum* travel to shallow gravel riffles in headwater streams (Hubbs and Strawn 1957; Hubbs 1985). Females look for a suitable place to lay eggs by performing "nosedigs" in which they jab their snout into the gravel. One to several males swim in tandem with a female as she searches for a spawning location. Males fight aggressively to ward off rival males by actively chasing them off and/or by flaring their dorsal and anal fins in a threat display. When the female is ready to spawn, she dives into the substrate, leaving only her head and caudal fin fully visible. Spawning initiates when a male positions himself above the female, and they release sperm and eggs into the substrate. Spawning often involves multiple males mating simultaneously with 1 female, and males sometimes exhibit sneaking behavior. Females will ovulate clutches of up to 200 eggs throughout the spawning season, but only release a few eggs per spawning bout (Heins et al. 1996; Fuller 1998). Hence, the female must spawn multiple times to fertilize all the eggs from a given clutch.

Study species/populations and collection locations

All *Ceasia* species occur in allopatry with respect to one another. Throughout the rest of this paper, the terms "allopatric" and "sympatric" refer to the geographic relationship between *Ceasia* and *E. caeruleum* (not between *Ceasia* species). To test for RCD and ACD between *E. spectabile* and *E. caeruleum*, we examined preferences for mating and fighting with conspecifics over heterospecifics in pairings between allopatric *E. spectabile* and allopatric *E. caeruleum* versus pairings between sympatric *E. spectabile* and sympatric *E. caeruleum* (Figure 1 and Table 1). We also tested for a pattern consistent with RCD and ACD in pairings between allopatric *E. pulchellum* and allopatric *E. caeruleum* (Figure 1 and Table 1). Finally, we tested for a pattern consistent with CRCD and CACD among *Ceasia* species by pairing allopatric *E. spectabile* with allopatric *E. pulchellum* (Figure 1 and Table 1).

We used 2 types of behavioral assays ["dichotomous male choice (MC) assay" and "male competition assay," detailed below] to compare preferences for engaging in mating and fighting with conspecifics versus heterospecifics. We then compared these behavioral measurements to those documented in pairings between sympatric *Ceasia* and sympatric *E. caeruleum*, and pairings between sympatric *Ceasia* species, in Moran et al. (2017; Figure 1 and Table 1).

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

Testing for RCD and ACD between *Ceasia* and *E. caeruleum*

Dichotomous MC assay

We first used a dichotomous MC assay to test for RCD in male mate choice. Each trial included a focal male *E. spectabile* or *E. pulchellum* with a conspecific female and a heterospecific (*E. caeruleum*) female (Figure 2A). This assay allowed males to choose between (1) sympatric *E. spectabile* and sympatric *E. caeruleum*, (2) allopatric *E. spectabile* and allopatric *E. caeruleum*, and (3) allopatric *E. pulchellum* and allopatric *E. caeruleum* females ($n = 12$ each). RCD predicts that preferences for conspecific mates should be higher in sympatric *E. spectabile* focal males than both allopatric *E. spectabile* and allopatric *E. pulchellum* focal males.

Behavioral trials occurred in 38 L test aquaria filled with 5 cm of naturally colored aquarium gravel. To minimize disturbance to the fish, test aquaria were covered with black opaque plastic on 3 sides. We used unique fish in each trial, chosen haphazardly from stock tanks. Females in each trial were size matched to within 10% of their total body length. Each trial began by placing the 3 fish being tested into a test aquarium and allowing them to acclimatize for 5 min. The trial then began and lasted 30 min. Each trial was broken up into 60 30-s blocks (Zhou et al. 2015; Moran et al. 2017).

We examined male mate choice by measuring focal male pursuit of each female in each trial. Male pursuit of a female is highly predictive of spawning in *Ceasia* and in *E. caeruleum* (Zhou et al. 2015; Moran et al. 2017). A male was scored as having pursued a female during a 30-s block if he spent a minimum consecutive time of 5-s within one body length of the female. We calculated a *focal male mate choice* behavioral variable from this data as described in Table 2.

We performed analyses using proportional data (i.e., the behavioral variables described in Table 2) that varied from 0 to 1. A score of 1 indicates only conspecific interactions occurred, 0.5 indicates an equal number of interactions between conspecifics and heterospecifics, and 0 indicates only heterospecific interactions occurred. However, for ease of interpretation, we graphed the raw number of behaviors observed.

We used analysis of variance (ANOVA) to test for RCD in male mating preference by asking whether *focal male mate choice* differed among the focal *Ceasia* study populations (i.e., sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*). We included *focal male mate choice* as the dependent variable, and focal male population identity as the independent variable. We then used post hoc *t*-tests to directly compare populations. We also asked whether *focal male mate choice* differed from a null expectation of 0.5 (equal amounts of time spent with each female) in each population using one-sample *t*-tests.

Male competition assay

We conducted a second type of assay in which males could compete with one another to test for RCD and ACD. This assay paired (1)

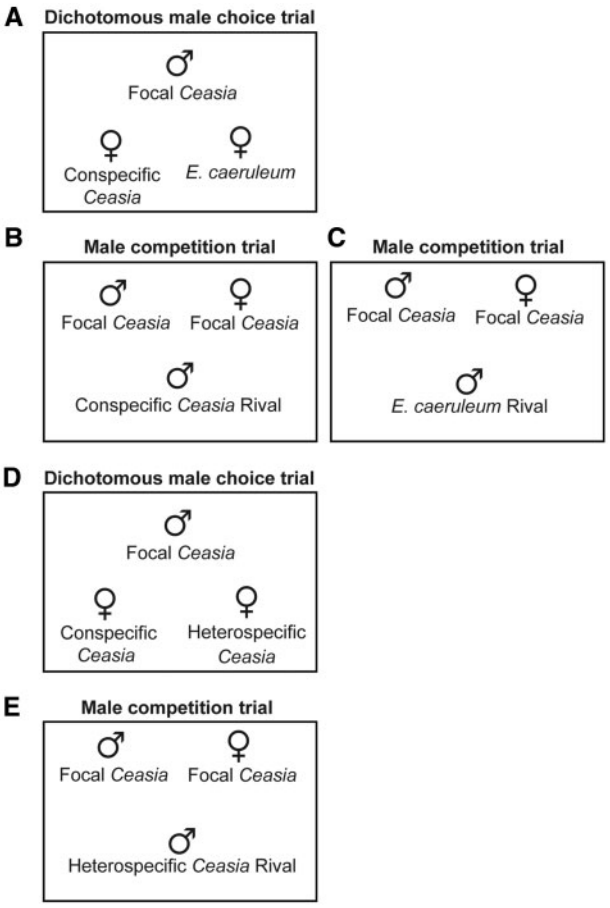

Figure 2. Setup for behavioral experiments. (**A–C**) Trials testing for RCD and ACD. In these trials, sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* served as focal *Ceasia* in turn. Note that in (A) and (C), allopatric *E. caeruleum* were paired with allopatric focal *Ceasia*, and sympatric *E. caeruleum* were paired with sympatric focal *Ceasia*. (A) Experimental setup for dichotomous MC trials that tested for RCD in focal *Ceasia* male mate choice. (B–C) Experimental set up for male competition trials that tested for patterns consistent with RCD in *E. caeruleum* rival male mate preference, RCD in focal *Ceasia* female mate preference, ACD in focal *Ceasia* male aggressive behavior, and ACD in *E. caeruleum* rival male aggressive behavior. (**D–E**) Trials testing for CRCD and CACD. In these trials, allopatric *E. spectabile* and allopatric *E. pulchellum* acted as focal *Ceasia* and as heterospecific *Ceasia* in turn. (D) Experimental set up for dichotomous MC trials that tested for patterns consistent with CRCD in focal *Ceasia* male mate choice. (E) Experimental set up for male competition trials that tested for patterns consistent with CRCD in heterospecific *Ceasia* rival male mate preference, CRCD in focal *Ceasia* female mate preference, and CACD in focal *Ceasia* male and heterospecific *Ceasia* rival male aggressive behavior. 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).

sympatric *E. spectabile* and sympatric *E. caeruleum*, (2) allopatric *E. spectabile* and allopatric *E. caeruleum*, and (3) allopatric *E. pulchellum* and allopatric *E. caeruleum* ($n = 12$ each). Each trial included a focal male and a focal female pair from the same *Ceasia* study population. Each focal *Ceasia* pair was observed once with a rival male that was conspecific to them (Figure 2B), and once with a rival male that was an *E. caeruleum* (Figure 2C). Male color pattern in these species is complex and varies within populations (Zhou et al. 2014), allowing us to distinguish conspecific males. Males in each trial were size matched within 10% of their total body length to control for any larger differences in color pattern and competitive ability associated with body size (Zhou et al. 2014). In each trial, we measured the behavior of the focal female, the focal male, and the rival male. Due to low collection numbers, some allopatric *E. caeruleum* males were used twice, but never more than once on the same day or with the same *Ceasia* study population.

To test for ACD, we recorded the number of aggressive behaviors (i.e., fin flares and attacks) that both males in a trial directed toward the other male. We calculated 4 behavioral variables to quantify male aggressive bias toward conspecific males: *focal male fin flare bias*, *focal male attack bias*, *rival male fin flare bias*, and *rival male attack bias* (see Table 2). We asked whether these behavioral variables differed in sympatric versus allopatric pairings. To examine focal male *Ceasia* aggressive behavior, we conducted 2 separate ANOVAs with *focal male fin flare bias* and *focal male attack bias* as the dependent variables, and focal *Ceasia* male identity (sympatric *E. spectabile*, allopatric *E. spectabile*, or allopatric *E. pulchellum*) as the independent variable in both analyses. Similarly, to examine the aggressive behavior of *E. caeruleum* rival males relative to *Ceasia* rival males, we conducted ANOVAs with *rival male fin flare bias* and *rival male attack bias* as dependent variables, and focal *Ceasia* male identity as the independent variable. Additionally, we made pairwise comparisons among groups using post hoc 2-sample *t*-tests.

To test for RCD in male mate preference, we split each male competition trial into 60 30-s blocks (as in the dichotomous MC trials), and counted the number of 30-s blocks in which each male pursued the female. Unlike the dichotomous MC assay, the male competition assay considers the preference of male *E. caeruleum* for *E. spectabile* and *E. pulchellum* females. We calculated *rival male mate choice* as described in Table 2. As focal males were always paired with conspecific females in the male competition trials, we did not measure focal male mate choice in these trials. The male competition assay presented males with a no-choice situation, where they could choose whether to pursue a female. This assay also examined male mate preference in the presence of a male competitor, which is closer to what a male would experience in nature during the spawning season. We asked whether *rival male mate choice* differed between sympatric and allopatric trial sets. We conducted an ANOVA with *rival male mate choice* as the dependent variable and trial set (i.e., sympatric *E. spectabile*, allopatric *E. spectabile*, or allopatric *E. pulchellum* as the focal pair) as the independent variable, followed by pairwise post hoc 2-sample *t*-tests.

Finally, we tested for RCD in female mating preferences. The setup of the male competition assay was equivalent to a dichotomous female choice (FC) assay. We counted the number of nosedigs a female performed towards the rival male in each trial. Females typically perform nosedigs directly before spawning, and this behavior is often used to measure female mating preferences in darters (Fuller 2003; Williams and Mendelson 2011; Zhou et al. 2015; Zhou and Fuller 2016). We asked whether *focal female mate choice* (Table 2) differed among sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* using ANCOVA. The model included *focal female mate choice* as the dependent variable and focal female identity as the independent variable. We included the proportion of time that conspecific rival males pursued the focal female as a covariate in the analysis, as male pursuit has been shown to predict female nosedigs and spawning (Zhou et al. 2015; Moran et al. 2017). We also used ANCOVA to test for focal female mate preference

Table 2. Definition of the behavioral variables measured in the dichotomous MC assay and the male competition assay

Variable	Definition	RCD	ACD	CRCD	CACD
Dichotomous MC assay (2 females, 1 male)					
Focal Male Mate Choice	Number of time blocks spent pursuing the conspecific divided by the total number of time blocks spent pursuing either female.	Yes	NA	Yes	NA
Male competition assay (2 males, 1 female)					
Rival Male Mate Choice	Proportion of time blocks the focal female was pursued by conspecific versus heterospecific rival males across 2 trials = Number 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
Focal Female Mate Choice	Proportion of nosedigs towards conspecific versus heterospecific rival males across 2 trial = Number of nosedigs toward conspecific rivals/(sum of nosedigs toward conspecific and heterospecific rivals); the analysis of this variable was corrected for male pursuit.	No	NA	No	NA
Focal Male Fin Flare Bias	Proportion of fin flares toward conspecific versus heterospecific rivals across 2 trials = Number of fin flares to conspecific rival/(sum of fin flares to conspecific and heterospecific rivals).	NA	Yes	NA	Yes
Focal Male Attack Bias	Proportion of attacks toward conspecific versus heterospecific rivals across 2 trials = Number of attacks on conspecific rival/(sum of attacks on conspecific and heterospecific rivals).	NA	Yes	NA	Yes
Rival Male Fin Flare Bias	Proportion of fin flares performed by conspecific versus heterospecific rivals across 2 trials = Number of 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
Rival Male Attack Bias	Proportion of attacks performed by conspecific versus heterospecific rivals across 2 trials = Number of attacks by conspecific rival toward the focal male/(sum of attacks by conspecific and heterospecific rivals towards the focal male).	NA	Mixed[a]	NA	Yes

Notes: We indicate whether we observed a pattern consistent with predictions for RCD, ACD, CRCD, and CACD for each behavioral variable, or whether the behavioral variable was not applicable (NA) to testing a given prediction.

[a]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.

for conspecific rival males versus *E. caeruleum* rival males. The number of nosedigs the focal female directed toward each rival male was the independent variable, the rival male's identity (conspecific or *E. caeruleum*) was the dependent variable, and the proportion of time the rival male spent in pursuit of the female was included as a covariate. We note that although the females' ability to exert mating preferences may be precluded by the outcome of male contests, male competition over females is pervasive in these species, so this assay reflects what females most frequently encounter in nature.

Testing for CRCD and CACD between *Ceasia* species

Dichotomous MC assay

To test for patterns consistent with CRCD within *Ceasia*, we paired allopatric *E. spectabile* with allopatric *E. pulchellum* in a dichotomous MC assay. We conducted this assay in the manner described above to test for RCD, but here the heterospecific female was an allopatric *E. spectabile* or allopatric *E. pulchellum*, in place of an *E. caeruleum* (Figure 2D). We performed trials in which allopatric *E. spectabile* acted as the focal male and conspecific female, with *E. pulchellum* as the heterospecific female, and vice versa ($n = 12$ each). CRCD predicts no significant difference between allopatric *E. spectabile* and allopatric *E. pulchellum* in *focal male mate choice* (Table 2). To compare *focal male mate choice* between these species, we conducted ANOVAs that included *focal male mate choice* as the dependent variable and focal male identity (allopatric *E. spectabile* or allopatric *E. pulchellum*) as the independent variable. We also tested whether *focal male mate choice* for the conspecific female differed from a null expectation of 0.5 (equal amounts of time spent with each female) using one-sample *t*-tests.

Male competition assay

We also conducted a male competition assay between allopatric *E. spectabile* and allopatric *E. pulchellum* to test for patterns consistent with CRCD and CACD. Earlier work showed that *Ceasia* males that are sympatric with *E. caeruleum* prefer to mate and fight with conspecifics over heterospecific *Ceasia* (Moran et al. 2017). Here, we asked whether *Ceasia* males that are allopatric with respect to *E. caeruleum* lacked such preferences. We performed trials in which both allopatric *E. spectabile* and allopatric *E. pulchellum* acted as the focal pair and as the heterospecific rival male in turn ($n = 12$ each; Figure 2E). CRCD and CACD predict that allopatric *E. spectabile* and allopatric *E. pulchellum* should show similarly low levels of preference for mating and fighting with conspecifics over heterospecifics. We measured *rival male mate choice*, and *focal female mate choice*, *focal male fin flare bias*, *focal male attack bias*, *rival male fin flare bias*, and *rival male attack bias* as described in Table 2. We conducted ANOVAs as described above for the male competition trials that tested for RCD and ACD, but with the appropriate species (i.e., *E. spectabile* or *E. pulchellum*) in place of *E. caeruleum* as the heterospecific rival male.

We used ANOVA to test for RCD, ACD, CRCD, and CACD in both sets of dichotomous MC and male competition assays. Repeating all analyses using generalized linear models with a quasi-binomial error function and logit link function yielded qualitatively identical results.

Table 3. Results of ANOVA testing for RCD in focal *Ceasia* male mate choice between conspecific females and *E. caeruleum* females in dichotomous MC male trials

Focal male mate choice	*df*	Test statistic	*P*
Focal *Ceasia* population identity	2, 33	45.21	<0.00001
Sympatric *E. spectabile* versus allopatric *E. spectabile*	22	11.38	<0.00001
Sympatric *E. spectabile* versus allopatric *E. pulchellum*	22	8.10	<0.00001
Allopatric *E. spectabile* versus allopatric *E. pulchellum*	220	−0.38	0.71

Notes: We asked *focal male mate choice* differed among focal *Ceasia* males in 3 study populations: sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*. Pairwise post-hoc *t*-test results are also shown for the analysis.

Behavioral isolation indices

We used the MA, male mate choice, and female mate choice data from both sets of male competition assays (i.e., those testing for RCD and ACD, and those testing for CRCD and CACD) to calculate 3 behavioral isolation indices following Moran et al. (2017). Behavioral isolation indices were calculated individually for each trial and then averaged across all replicates within each species comparison. These indices allowed for a comparison of levels of preference for mating and fighting with conspecifics over heterospecifics at a macroevolutionary scale among *Ceasia–E. caeruleum* and *Ceasia–Ceasia* species pairs. Indices range from −1 (complete preference for heterospecifics) to 1 (complete preference for conspecifics), with 0 indicating no preference for conspecifics versus heterospecifics (Stalker 1942; Martin and Mendelson 2016; Moran et al. 2017).

We calculated MA indices for each species pair as:

$$\mathrm{MA} = \frac{a_c - a_h}{a_c + a_h},$$

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

We calculated MC indices as:

$$\mathrm{MC} = \frac{m_c - m_h}{m_c + m_h},$$

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

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

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

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

We used ANOVA to make 2 sets of comparisons among the 3 types of behavioral isolation indices (i.e., MA, MC, and FC). First, we tested for differences between *Ceasia* and *E. caeruleum* pairs that occur in sympatry versus allopatry with respect to one another. RCD predicts higher MC and FC indices in *Ceasia–E. caeruleum* pairings that occur in sympatry versus allopatry, indicating enhanced mate preference for conspecifics. Similarly, divergent ACD predicts higher MA indices in *Ceasia–E. caeruleum* pairs that occur in sympatry versus allopatry. This would indicate that sympatric males bias their aggression more toward conspecifics over heterospecifics.

Second, we tested for differences between *Ceasia* and *Ceasia* species pairs that occur in sympatry versus allopatry with respect to *E. caeruleum*. CRCD predicts higher MC and FC indices in *Ceasia–Ceasia* pairings that occur in sympatry with respect to *E. caeruleum*, indicating enhanced mate preference for conspecific over heterospecific *Ceasia*. Likewise, CACD predicts higher MA indices in *Ceasia–Ceasia* pairings that occur in sympatry with respect to *E. caeruleum*. This would indicate that *Ceasia* males that occur in sympatry with respect to *E. caeruleum* bias their aggression more toward conspecific males versus heterospecific *Ceasia* males.

For all analyses, we used Type III sums of squares using the "car" package in R (version 3.4.0). Raw data have been deposited in Dryad (doi:10.5061/dryad.g8d1v).

Results

RCD between *Ceasia* and *E. caeruleum*

The dichotomous MC trials revealed a pattern consistent with RCD in focal *Ceasia* male mate preference. RCD predicts that MC for conspecifics should be heightened in *Ceasia* populations/species that are sympatric with respect to *E. caeruleum*. *Focal male mate choice* was 2× higher in sympatric *E. spectabile* compared with allopatric *E. spectabile* and allopatric *E. pulchellum*, but did not differ between allopatric *E. spectabile* and allopatric *E. pulchellum* (Table 3 and Supplementary Figure S1a). In addition, *focal male mate choice* was much greater than the null expectation of 0.5 in trials with sympatric *E. spectabile* serving as the focal male (mean ± SE: 0.97 ± 0.01; one-sample *t*-test: $t_{11} = 51.58$, $P < 0.00001$). Conversely, *focal male mate choice* did not differ from 0.5 in trials where allopatric *E. spectabile* and allopatric *E. pulchellum* served as the focal males (Supplementary Figure S1b, c; allopatric *E. spectabile* mean ± SE: 0.51 ± 0.04; one-sample *t*-test: $t_{11} = 0.17$, $P = 0.87$; *E. pulchellum* mean ± SE: 0.53 ± 0.05; one-sample *t*-test: $t_{11} = 0.60$, $P = 0.56$).

RCD in male mate preference was also indicated in the male competition trials, which compared *E. caeruleum* rival male preference for the focal *Ceasia* female with that of the conspecific *Ceasia* rival male. RCD predicts that sympatric *E. caeruleum* males should be less likely to pursue *Ceasia* females than allopatric *E. caeruleum* males. *Rival male mate choice* differed significantly between sympatric and allopatric *E. caeruleum* (Supplementary Table S1). In trials where sympatric *E. spectabile* served as the focal *Ceasia* pair, conspecific rival males were much more likely to pursue the focal female compared with the sympatric *E. caeruleum* rival males (Supplementary Figure S2a). In both trials where allopatric

E. spectabile and *E. pulchellum* served as the focal *Ceasia* pair, conspecific rival males and allopatric *E. caeruleum* rival males spent roughly the same amount of time pursuing the focal female (Supplementary Figure S2b, c). Hence, allopatric *E. caeruleum* males chose to pursue allopatric *E. spectabile* and allopatric *E. pulchellum* females. Sympatric *E. caeruleum* males largely ignored sympatric *E. spectabile* females.

We did not find support for RCD in female mating preferences in the male competition trials. When male pursuit was included as a covariate in the analysis, *focal female mate choice* did not differ among the sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* trials (Table 4). Females did not exert preference for conspecific males over *E. caeruleum* males, regardless of sympatry with respect to *E. caeruleum* (Supplementary Table S2).

ACD between *Ceasia* and *E. caeruleum*

The aggressive behavior of focal *Ceasia* males in the male competition trials was consistent with divergent ACD. Divergent ACD predicts that *Ceasia* males that are sympatric with respect to *E. caeruleum* should bias their aggression toward conspecific rival males over *E. caeruleum* rival males. *Focal male fin flare bias* and *focal male attack bias* were higher for sympatric *E. spectabile* compared with allopatric *E. spectabile* and allopatric *E. pulchellum* (Table 5). Sympatric *E. spectabile* focal males directed 9× more fin flares toward conspecific (versus *E. caeruleum*) rival males (Supplementary Figure S1d). Similarly, sympatric *E. spectabile* focal males attacked conspecific rival males 6× more than they attacked sympatric *E. caeruleum* rival males (Supplementary Figure S1g). On average, both allopatric *E. spectabile* and allopatric *E. pulchellum* focal males directed an equal number of fin flares (Supplementary Figure S1e, f) and attacks (Supplementary Figure S1h, i) toward conspecific rival males and allopatric *E. caeruleum* rival males.

We also found a pattern consistent with divergent ACD in *E. caeruleum* male aggressive behavior. Divergent ACD predicts that sympatric *E. caeruleum* rival males should show higher levels of aggression toward focal male *Ceasia* compared with allopatric *E. caeruleum* rival males. *Rival male fin flare bias* showed a pattern like that found with focal *Ceasia* males (Supplementary Table S3). Sympatric *E. caeruleum* rival males were much less likely to flare their fins toward *E. spectabile* focal males compared with allopatric *E. caeruleum* rival males (Supplementary Figure S2d, f).

Conversely, *rival male attack bias* did not differ between sympatric and allopatric *E. caeruleum* (Supplementary Table S3). Both sympatric and allopatric *E. caeruleum* rival males directed a low number of attacks toward the focal *Ceasia* males (Supplementary Figure S2g, i). Thus, while allopatric *E. spectabile* and allopatric *E. pulchellum* focal males did not bias their aggression more toward conspecific rival males (versus allopatric *E. caeruleum* rival males; see previous paragraph), allopatric *E. caeruleum* rival males typically preferred not to attack allopatric *E. spectabile* and allopatric *E. pulchellum* focal males.

Table 4. Results ANCOVA testing for RCD in focal *Ceasia* female mate choice between conspecific rival males and *E. caeruleum* rival males in male competition trials

Focal female mate choice	*df*	Test statistic	*P*
Focal *Ceasia* population identity	2, 32	0.09	0.92
Male pursuit	1, 32	0.74	0.40

Notes: We asked whether *focal female mate choice* differed among focal *Ceasia* females in 3 study populations: sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*. Male pursuit of the female was included as a covariate in the analysis.

CRCD between *Ceasia* species

CRCD predicts that males from *Ceasia* species that are sympatric with respect to *E. caeruleum* should show higher levels of male mate preference for conspecific females over heterospecific *Ceasia* females, despite the fact that the 2 *Ceasia* species are allopatric with respect to one another. Moran et al. (2017) showed that in *Ceasia* species that are sympatric with respect to *E. caeruleum*, male mate preference for conspecific over heterospecific *Ceasia* females was surprisingly high. This study shows that male *Ceasia* (i.e., *E. spectabile*

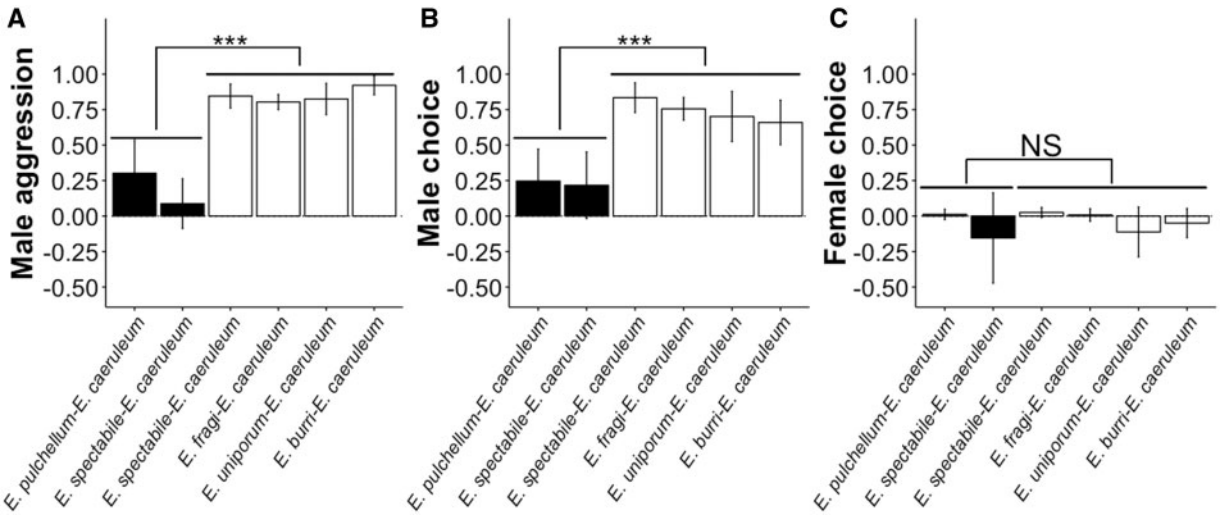

Figure 3. Patterns of RCD and ACD between *Ceasia* and *E. caeruleum*. Behavioral isolation indices (with 95% confidence intervals) for (**A**) MA, (**B**) MC, and (**C**) FC for comparisons between *Ceasia* species and *E. caeruleum*. Allopatric comparisons (i.e., those including *Ceasia* and *E. caeruleum* that occur in allopatry with respect to one another) are shown in black. Sympatric comparisons (i.e., those including *Ceasia* and *E. caeruleum* that occur in sympatry with respect to one another) are shown in white. Grouping bars are also used to indicate allopatric species pairs (left) versus sympatric species pairs (right). Significance levels from ANOVAs comparing allopatric and sympatric species pairs are shown.

Table 5. Results of ANOVA testing for ACD in focal *Ceasia* MA bias in male competition trials

Focal male fin flare bias	*df*	Test statistic	*P*
Focal *Ceasia* population identity	2, 33	8.34	0.0012
Sympatric *E. spectabile* versus allopatric *E. spectabile*	22	5.28	<0.0001
Sympatric *E. spectabile* versus allopatric *E. pulchellum*	22	2.85	0.0093
Allopatric *E. spectabile* versus allopatric *E. pulchellum*	22	−0.84	0.41
Focal male attack bias	*df*	Test statistic	*P*
Focal *Ceasia* population identity	2, 33	9.12	<0.001
Sympatric *E. spectabile* versus allopatric *E. spectabile*	22	4.53	0.0002
Sympatric *E. spectabile* versus allopatric *E. pulchellum*	22	3.82	<0.001
Allopatric *E. spectabile* versus allopatric *E. pulchellum*	22	−0.65	0.52

Notes: We asked whether *focal male fin flare bias* and *focal male attack bia*s differed among focal *Ceasia* males in 3 study populations: sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*. Pairwise post-hoc *t*-test results are also shown for both analyses.

and *E. pulchellum*) that are allopatric with respect to *E. caeruleum* do not prefer conspecific over heterospecific *Ceasia* females. In dichotomous MC trials, *focal male mate choice* did not differ between allopatric *E. spectabile* and allopatric *E. pulchellum* ($F_{1,22} = 0.29$; $P = 0.60$; Supplementary Figure S3a, b). Additionally, *focal male mate choice* did not differ from a null expectation of 0.5 in allopatric *E. spectabile* (mean ± SE: 0.42 ± 0.04; one-sample *t*-test: $t_{11} = -1.94$, $P = 0.08$) or in allopatric *E. pulchellum* (mean ± SE: 0.45 ± 0.04; one-sample *t*-test: $t_{11} = -1.28$, $P = 0.23$). Similarly, in the male competition trials *rival male mate choice* did not differ between allopatric *E. spectabile* and allopatric *E. pulchellum* ($F_{1,22} = 0.12$; $P = 0.73$; Supplementary Figure S4).

In contrast, there was no evidence for CRCD in female mating preference. *Focal female mate choice* did not differ between allopatric *E. spectabile* and allopatric *E. pulchellum*, and these preferences did not differ from 0.5 (Supplementary Table S4). There was no significant difference in the proportion of female nosedigs toward rival males as function of their identity (conspecific or heterospecific) when we controlled for the proportion of time each male pursued the female (Supplementary Table S5).

CACD between *Ceasia* species

CACD predicts that *Ceasia* males that are sympatric with respect to *E. caeruleum* should bias their aggression toward conspecific over heterospecific *Ceasia* males, despite the fact that the 2 *Ceasia* species are allopatric with respect to one another. CACD also predicts that *Ceasia* males that are allopatric with respect to *E. caeruleum* should not bias their aggression more toward conspecific versus heterospecific males. Moran et al. (2017) paired *Ceasia* species that occur in sympatry with respect to *E. caeruleum* and found high levels of male preference for fighting with conspecific over heterospecific *Ceasia* males. Here, we show that *Ceasia* species (i.e., *E. spectabile* and *E. pulchellum*) that are allopatric with respect to *E. caeruleum* show no such male bias in aggressive behavior. *Focal male fin flare bias* did not differ between allopatric *E. spectabile* and allopatric *E. pulchellum* ($F_{1,22} = 1.79$; $P = 0.19$; Supplementary Figure S3c, d), nor did *focal male attack bias* ($F_{1,22} = 0.84$; $P = 0.37$; Supplementary Figure S3e, f).

Rival male behavior showed a similar pattern consistent with CACD. In the trials where allopatric *E. pulchellum* served as focal males, both conspecific *E. pulchellum* rival males and the allopatric *E. spectabile* rival males directed a similar number of fin flares toward focal males (Supplementary Figure S4d). However, in trials where allopatric *E. spectabile* served as focal males, the allopatric *E. pulchellum* rival males directed more fin flares toward the focal males compared with the conspecific *E. spectabile* rival males (Supplementary Figure S3c). This resulted in a significant difference in *rival male fin flare bias* between allopatric *E. spectabile* and allopatric *E. pulchellum* ($F_{1,22} = 5.79$; $P = 0.025$; Supplementary Figure S4), despite the pattern being consistent with the prediction for CACD. *Rival male attack bias* did not differ between trials with allopatric *E. spectabile* versus allopatric *E. pulchellum* serving as the focal male ($F_{1,22} = 0.10$; $P = 0.75$; Supplementary Figure S4).

Behavioral isolation indices

To examine macroevolutionary patterns of RCD and ACD among *Ceasia–E. caeruleum* species pairs, and CRCD and CACD among *Ceasia–Ceasia* species pairs, we compared the behavioral isolation indices calculated in this study with behavioral isolation indices calculated by Moran et al. (2017; Table 6 and Figures 3 and 4). The pattern in male mating preference was consistent with RCD between *Ceasia* and *E. caeruleum* species pairs and CRCD between *Ceasia* and *Ceasia* species pairs. MC indices were consistently higher between sympatric species pairs compared with allopatric species pairs, signifying enhanced preference for mating with conspecifics in sympatry. RCD was indicated in the *Ceasia–E. caeruleum* comparisons as MC was higher for sympatric compared with allopatric species pairs ($F_{1,82} = 56.35$, $P < 0.0001$; Figure 3). CRCD was indicated in the *Ceasia–Ceasia* comparisons as male *Ceasia* that are sympatric with respect to *E. caeruleum* had heightened MC indices, despite the fact that all *Ceasia* are allopatric to one another ($F_{1,70} = 6.64$, $P = 0.01$; Figure 4). The difference in MC indices in sympatry versus allopatry was greater in *Ceasia–E. caeruleum* pairings than in *Ceasia–Ceasia* pairings (Table 6).

Conversely, we did not observe a pattern consistent with RCD or CRCD in female mating preferences. FC indices did not differ as a function of sympatry with respect to *E. caeruleum* in *Ceasia–E. caeruleum* ($F_{1,82} = 0.96$, $P = 0.33$) or *Ceasia–Ceasia* comparisons ($F_{1,70} = 0.18$, $P = 0.67$; Table 6 and Figures 3 and 4). This was due to females not exerting any detectable mating preferences for conspecific males.

We observed a pattern consistent with divergent ACD between *Ceasia* and *E. caeruleum* species pairs and CACD between *Ceasia* and *Ceasia* species pairs. MA indices were consistently higher between sympatric species pairs compared with allopatric species pairs, indicating increased male preference for fighting with conspecific over heterospecific males in sympatry. This pattern was present both within the *Ceasia–E. caeruleum* comparisons ($F_{1,166} = 136.30$, $P < 0.0001$; Figure 3; indicating ACD) and within the *Ceasia–Ceasia* comparisons ($F_{1,142} = 34.17$, $P < 0.0001$; Figure 4; indicating CACD).

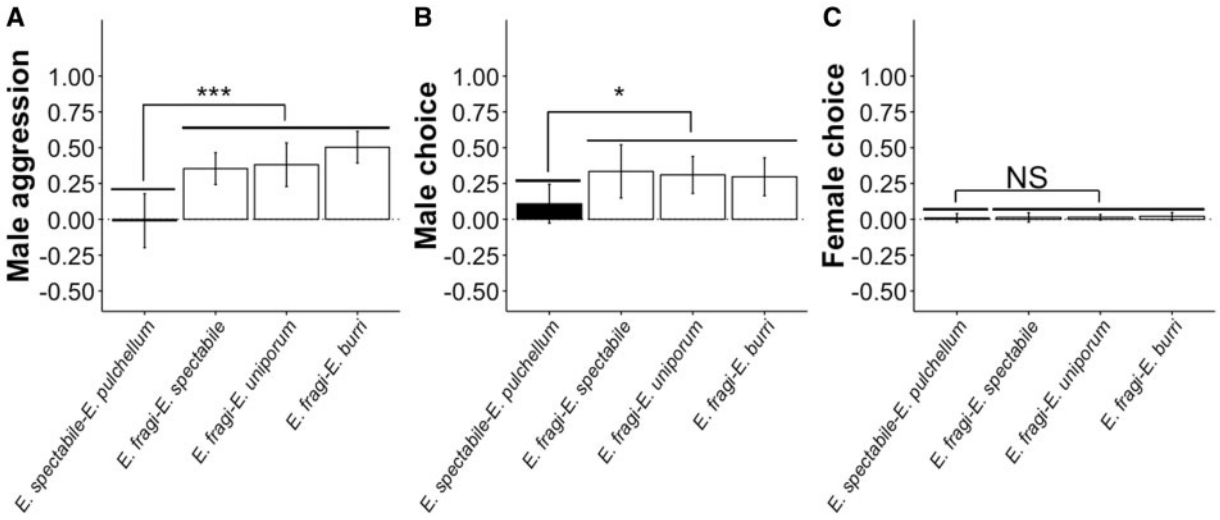

Figure 4. Patterns of CRCD and CACD between *Ceasia* species. Behavioral isolation indices (with 95% confidence intervals) for (**A**) MA, (**B**) MC, and (**C**) FC between pairs of *Ceasia* species. Allopatric comparisons (i.e., comparisons including *Ceasia* species that both occur in allopatry with respect to *E. caeruleum*) are shown in black. Sympatric comparisons (i.e., comparisons including *Ceasia* species that both occur in sympatry with respect to *E. caeruleum*) are shown in white. Grouping bars are also used to indicate allopatric species pairs (left) versus sympatric species pairs (right). Significance levels from ANOVAs comparing allopatric and sympatric species pairs are shown.

Table 6. Behavioral isolation indices (mean ± standard error) for MA, MC, and FC, calculated from male competition assays that paired 2 *Ceasia* species or paired *Ceasia* with *E. caeruleum*

Geography	Pairing	Species	Hypotheses tested	*n*	MA	MC	FC
Allopatric	*Ceasia–Ceasia*	*E. spectabile–E. pulchellum*	CRCD/CACD	24	−0.01±0.07	0.11±0.07	0.01±0.02
Sympatric	*Ceasia–Ceasia*	*E. fragi–E. uniporum*[a]	CRCD/CACD	16	0.38±0.08	0.31±0.07	0.01±0.01
Sympatric	*Ceasia–Ceasia*	*E. fragi–E. burri*[a]	CRCD/CACD	16	0.50±0.06	0.30±0.07	0.02±0.01
Sympatric	*Ceasia–Ceasia*	*E. fragi–E. spectabile*[a]	CRCD/CACD	16	0.35±0.06	0.34±0.10	0.01±0.02
Allopatric	*Ceasia–E. caeruleum*	*E. spectabile–E. caeruleum*	RCD/ACD	24	0.09±0.09	0.22±0.12	−0.16±0.16
Allopatric	*Ceasia–E. caeruleum*	*E. pulchellum–E. caeruleum*	RCD/ACD	24	0.30±0.12	0.25±0.12	0.01±0.02
Sympatric	*Ceasia–E. caeruleum*	*E. fragi–E. caeruleum*[a]	RCD/ACD	48	0.80±0.05	0.76±0.06	0.01±0.04
Sympatric	*Ceasia–E. caeruleum*	*E. uniporum–E. caeruleum*[a]	RCD/ACD	16	0.82±0.06	0.70±0.09	−0.11±0.13
Sympatric	*Ceasia–E. caeruleum*	*E. burri–E. caeruleum*[a]	RCD/ACD	16	0.92±0.03	0.66±0.08	−0.05±0.05
Sympatric	*Ceasia–E. caeruleum*	*E. spectabile–E. caeruleum*[b]	RCD/ACD	32	0.85±0.05	0.84±0.06	0.03±0.02

Notes: As all species of *Ceasia* occur allopatrically with respect to one another, here geography for a given pairing refers to the relationship between *Ceasia* and *E. caeruleum*. For each species pairing, 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*). Sample size (*n*) and hypotheses tested (CRCD/CACD in pairings between 2 *Ceasia* species, or RCD/ACD in pairings between *Ceasia* and *E. caeruleum*) are listed.

[a]Data from Moran et al. (2017).

[b]Calculated using data from the present study combined with data from Moran et al. (2017).

MA was higher between sympatric *Ceasia* and *E. caeruleum* pairs than it was in sympatric *Ceasia* and *Ceasia* pairs (Table 6).

Discussion

Striking patterns of RCD and ACD driven by male behavior are present at 2 taxonomic levels within *Ceasia*. First, we found evidence for both RCD and ACD among populations within species (Figure 3 and Supplementary Figure S1; Table 2). We observed RCD in male mate choice among populations of *E. spectabile* and *E. caeruleum*. Male (but not female) preference for conspecific mates was enhanced in sympatric (versus allopatric) population pairings of these species (Tables 3, 4, and Supplementary Table S2). We also found evidence of divergent ACD among populations within *E. spectabile* and *E. caeruleum*. Males preferentially biased their aggression toward conspecific males to a greater extent in sympatric population pairings (Table 5). Second, we found evidence for ACD and RCD among closely related species in the *Ceasia* species complex. Males showed no preference for mating (Table 3) or fighting (Table 5) with conspecifics over heterospecifics in pairings of allopatric *E. pulchellum* and allopatric *E. caeruleum*. This stands in contrast to the results of Moran et al. (2017), which found high levels of male preference for mating and fighting with conspecifics over heterospecifics in sympatric pairings of *Ceasia* species and *E. caeruleum*. We discuss how the data from the present study and Moran et al. (2017) reveal a pattern consistent with RCD and ACD at a macroevolutionary scale between *Ceasia* species and *E. caeruleum* (see below).

Most of our efforts were directed at testing for RCD and ACD in *Ceasia*. However, we also found evidence for RCD in male mate choice (Supplementary Figure S2 and Table S1) and ACD in MA bias in *E. caeruleum* (Supplementary Figure S2 and Tables S3), but the pattern of divergent ACD observed in male *E. caeruleum*

behavior was not as extreme as that observed in *Ceasia*. ACD was indicated in *E. caeruleum* in that sympatric male *E. caeruleum* were less likely to flare their fins at sympatric male *E. spectabile*, but *E. caeruleum* males from both sympatric and allopatric populations did not perform many attacks toward *E. spectabile* or *E. pulchellum* males. We hypothesize that this difference may be related to the level of gene flow present between populations of *Ceasia* species versus *E. caeruleum*. RCD and ACD are more likely to be maintained over time (and to lead to CRCD and CACD) when gene flow is low among populations within species (Yukilevich and Aoki 2016). *Ceasia* and *E. caeruleum* both occur in small headwater streams, but *E. caeruleum* can also inhabit larger order streams and rivers (Page 1983), leading to more opportunities for gene flow among populations (Echelle et al. 1975, 1976). Gene flow from sympatric to allopatric populations of *E. caeruleum* may result in the loci for MA bias spreading beyond the zone of sympatry. Indeed, population genetic analyses of 4 species of *Ceasia* and *E. caeruleum* found increased heterozygosity and higher levels of nucleotide diversity present in *E. caeruleum* compared with *Ceasia* (Moran et al. 2017), indicating lower levels of gene flow in species of *Ceasia*.

We also tested for patterns consistent with CRCD and CACD between species of *Ceasia* (Table 2 and Figure 4). We observed that allopatric *E. spectabile* and allopatric *E. pulchellum* males showed no preference for conspecific over heterospecific *Ceasia* females, nor did they bias their aggression more toward conspecific over heterospecific *Ceasia* males (Supplementary Figures S3 and S4). Our previous work indicated that sympatric *Ceasia* species have a clear preference to mate and fight with conspecific over heterospecific *Ceasia* (Moran et al. 2017). Together, these data reveal a clear pattern of CRCD in male mate choice and CACD in MA among *Ceasia* species (see below).

Relationship to previous studies in darters

Considering our results together with those of a recent study by Moran et al. (2017) reveals 2 macroevolutionary patters: (1) RCD and ACD are present between species of *Ceasia* and *E. caeruleum* and (2) cascading effects of RCD and ACD between *Ceasia* and *E. caeruleum* have incidentally contributed to allopatric divergence among closely related lineages within the *Ceasia* clade (i.e., CRCD and CACD). RCD and ACD are indicated in that *Ceasia* species that occur in sympatry with *E. caeruleum* consistently show almost complete preference for mating and fighting with conspecifics over *E. caeruleum*, but no such preferences exist in *Ceasia* species that occur in allopatry with *E. caeruleum* (this study; Zhou and Fuller 2014). Similarly, CRCD and CACD are indicated in that *Ceasia* species that occur in sympatry with *E. caeruleum* (but allopatry with respect to one another) show surprisingly high levels of male preference for mating with and fighting with conspecifics over heterospecific *Ceasia*, but these preferences are absent in pairings of *Ceasia* that occur in allopatry with respect to *E. caeruleum* (this study; Moran et al. 2017). Future studies should determine whether patterns of CRCD and CACD are also present among populations within individual species of *Ceasia* (as is the case with RCD and ACD within *E. spectabile*).

This study corroborates the results of several recent studies which have shown that male mate choice and male competition play an important role in driving sympatric and allopatric trait divergence in darters (Ciccotto et al. 2013; Zhou et al. 2015; Zhou and Fuller 2016; Martin and Mendelson 2016; Moran et al. 2017). Furthermore, although the presence of elaborate male coloration is typically attributed to intersexual selection via female mate preferences (Panhuis et al. 2001), male coloration in darters appears to be under intrasexual selection due to intense male–male competition. RCD and ACD can lead to shifts in behavioral response to heterospecifics and in the signals used in species recognition (Brown and Wilson 1956; Grether et al. 2009). Thus, examining whether character displacement in male color pattern corresponds to the observed ACD and CACD in male aggressive response to heterospecifics would be of interest.

Our results also uphold previous examinations of female mate choice in this system, which have consistently failed to detect female preferences for conspecific males in sympatric or allopatric pairings of *Ceasia* and *E. caeruleum* (Pyron 1995; Fuller 2003; Zhou et al. 2015; Moran et al. 2017). FC may be prevented by the presence of intense male competition in these species. Further study is needed to determine whether females exhibit any cryptic forms of mate choice (Eberhard 1996), such as adjusting the number of eggs laid when mating with conspecific versus heterospecific males.

Selection underlying RCD and ACD

The presence of hybridization in conjunction with high levels of postzygotic isolation between *Ceasia* and *E. caeruleum* (Zhou 2014; Moran R, unpublished data) suggests that RCD in these species may occur via reinforcement. Selection for males to prefer conspecific mates (to avoid maladaptive hybridization) would establish females as an unshared resource between species, making interspecific fighting over females costly. Theoretical treatments of ACD predict that selection may favor divergence in male aggressive traits between species when males compete for separate resources (i.e., females), which decreases the prevalence of interspecific aggression in sympatry (Okamoto and Grether 2013). In the case of *Ceasia* and *E. caeruleum*, a lowered aggressive response to heterospecific males may also facilitate their co-occurrence within the same habitat in sympatric drainages. The fact that the 2 species can co-occur in sympatry provides further opportunities for interspecific encounters and hybridization, further strengthening selection for divergence in mating traits and behavioral isolation via RCD. In this manner, RCD and ACD may strengthen one another in a positive feedback loop. There is evidence for such a feedback loop scenario between types of character displacement acting in *Ficedula* flycatchers (Qvarnström et al. 2012; Vallin et al. 2012).

Selection underlying CRCD and CACD

Theory predicts that CRCD or CACD can occur when populations stochastically respond to selection on mating and fighting traits in unique ways during RCD and ACD (i.e., mutation-order selection; Abbott et al. 2013; Mendelson et al. 2014; Comeault and Matute 2016). Under mutation-order selection, trait divergence may occur despite the presence of similar types of ecological and sexual selection. In this way, stochastic variation in response to the same selective pressures (i.e., maladaptive heterospecific interactions in sympatry) can potentially lead to allopatric divergence among populations within species.

Although theory predicts that CRCD and CACD can lead to allopatric speciation (McPeek and Gavrilets 2006; Pfennig and Ryan 2006), the majority of empirical studies that have examined CRCD and CACD to date have only tested for differences in behavioral preferences among populations within species. In addition, many studies have tested for CRCD by comparing levels of behavioral isolation between populations within species that are allopatric versus sympatric with respect to another species (Nosil et al. 2003;

Lemmon 2009; Hopkins et al. 2014; Kozak et al. 2015; Comeault et al. 2016). The implication with these studies is that RCD changes mating traits in such a way that increases behavioral isolation between sympatric and allopatric populations within a species (i.e., "sympatry–allopatry effects"). In *Ceasia* and *E. caeruleum*, there are high levels of preferences for mating and fighting with conspecifics in pairings between *Ceasia* species that have independently undergone RCD and ACD with *E. caeruleum*. This suggests that different species-specific traits have evolved in *Ceasia* species that are sympatric with respect to *E. caeruleum* (i.e., "convergent-sympatry effects").

Conclusions

This study provides empirical evidence of male-driven RCD, ACD, CRCD, and CACD in darters. As far as we are aware, this is the first documented case demonstrating that ACD between species can incidentally lead to CACD among populations within species (or in this case, among closely related species within a clade). Although the clear majority of RCD studies to date have focused on the evolution of female mating preferences for males, the results of this study demonstrate that male behavior can drive trait divergence between and within species via RCD and CRCD. This underscores the necessity of considering the behavior of both sexes when evaluating character displacement in a given system. Finally, this study provides important groundwork for future studies examining the extent to which RCD and ACD have been involved in generating the extraordinary species diversity present in darters.

Data Archival Location

Behavioral data are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.g8d1v).

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Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

References

Abbott R, Albach D, Ansell S, Arntzen JW, Baird SJE et al., 2013. Hybridization and speciation. *J Evol Biol* **26**:229–246.

Alatalo RV, Gustafsson L, Lundberg A, 1994. Male coloration and species recognition in sympatric flycatchers. *Proc R Soc B Biol Sci* **256**:113–118.

Berglund A, 1996. Armaments and ornaments: an evolutionary explanation of traits of dual utility. *Biol J Linn Soc* **58**:385–399.

Bewick ER, Dyer KA, 2014. Reinforcement shapes clines in female mate discrimination in *Drosophila subquinaria*. *Evolution* **68**:3082–3094.

Bossu CM, Near TJ, 2009. Gene trees reveal repeated instances of mitochondrial DNA introgression in orangethroat darters (Percidae: *Etheostoma*). *Syst Biol* **58**:114–129.

Bossu CM, Beaulieu JM, Ceas PA, Near TJ, 2013. Explicit tests of palaeodrainage connections of southeastern North America and the historical biogeography of orangethroat darters (Percidae*: Etheostoma: Ceasia*). *Mol Ecol* **22**:5397–5417.

Brown WL, Wilson EO, 1956. Character displacement. *Syst Zool* **5**:49.

Ceas PA, Page LM, 1997. Systematic studies of the *Etheostoma spectabile* complex (Percidae; Subgenus *Oligocephalus*), with descriptions of four new species. *Copeia* **1997**:496–522.

Ciccotto PJ, Gumm JM, Mendelson TC, 2013. Male association preference for conspecifics in the redband darter *Etheostoma luteovinctum* (Teleostei: Percidae) based on visual cues. *Copeia* **2013**:154–159.

Comeault AA, Matute DR, 2016. Reinforcement's incidental effects on reproductive isolation between conspecifics. *Curr Zool* **62**:135–143.

Comeault AA, Venkat A, Matute DR, 2016. Correlated evolution of male and female reproductive traits drive a cascading effect of reinforcement in *Drosophila yakuba*. *Proc R Soc B Biol Sci* **283**:20160730.

Coyne J, Orr H, 2004. *Speciation*. Sunderland (MA): Sinauer Associates.

Dijkstra PD, Seehausen O, Pierotti MER, Groothuis TGG, 2007. Male–male competition and speciation: aggression bias towards differently coloured rivals varies between stages of speciation in a Lake Victoria cichlid species complex. *J Evol Biol* **20**:496–502.

Eberhard WG, 1996. *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton (NJ): Princeton University Press.

Echelle A, Echelle A, Smith M, Hill L, 1975. Analysis of genic continuity in a headwater fish *Etheostoma radiosum* (*Percidae*). *Copeia* **1975**: 197–204.

Echelle A, Echelle A, Taber B, 1976. Biochemical evidence for congeneric competition as a factor restricting gene flow between populations of a darter (Percidae: *Etheostoma*). *Syst Biol* **25**:228–235.

Fuller RC, 1998. Fecundity estimates for rainbow darters *Etheostoma caeruleum* in Southwestern Michigan. *Ohio J Sci* **98**:2–5.

Fuller RC, 2003. Disentangling female mate choice and male competition in the rainbow darter *Etheostoma caeruleum*. *Copeia* **2003**:138–148.

Grether GF, Losin N, Anderson CN, Okamoto K, 2009. The role of interspecific interference competition in character displacement and the evolution of competitor recognition. *Biol Rev* **84**:617–635.

Grether GF, Peiman KS, Tobias JA, Robinson BW, 2017. Causes and consequences of behavioral interference between species. *Trends Ecol Evol* **32**: 760–772.

Heins DC, Baker JA, Tylicki DJ, 1996. Reproductive season, clutch size, and egg size of the rainbow darter *Etheostoma caeruleum*, from the Homochitto River, Mississippi, with an evaluation of data from the literature. *Copeia* **1996**:1005–1010.

Higgie M, Blows MW, 2007. Are traits that experience reinforcement also under sexual selection? *Am Nat* **170**:409–420.

Higgie M, Blows M, 2008. The evolution of reproductive character displacement conflicts with how sexual selection operates within a species. *Evolution* **62**:1192–1203.

Hopkins R, Guerrero RF, Rausher MD, Kirkpatrick M, 2014. Strong reinforcing selection in a Texas wildflower. *Curr Biol* **24**:1995–1999.

Hoskin CJ, Higgie M, 2010. Speciation via species interactions: the divergence of mating traits within species. *Ecol Lett* **13**:409–420.

Hoskin CJ, Higgie M, McDonald KR, Moritz C, 2005. Reinforcement drives rapid allopatric speciation. *Nature* **437**:1353–1356.

Howard D, 1993. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. In: Harrison RG, editor. *Hybrid Zones and the Evolutionary Process*. New York (NY): Oxford, 46–69.

Hubbs C, 1985. Darter reproductive seasons. *Copeia* **1985**:56–68.

Hubbs C, Strawn K, 1957. Relative variability of hybrids between the Darters, *Etheostoma spectabile* and *Percina caprodes*. *Evolution* **11**:1–10.

Kozak GM, Roland G, Rankhorn C, Falater A, Berdan EL et al., 2015. Behavioral isolation due to cascade reinforcement in *Lucania* Killifish. *Am Nat* **185**:491–506.

Lackey ACR, Boughman JW, 2013. Divergent sexual selection via male competition: ecology is key. *J Evol Biol* **26**:1611–1624.

Lemmon EM, 2009. Diversification of conspecific signals in sympatry: geographic overlap drives multidimensional reproductive character displacement in frogs. *Evolution* **63**:1155–1170.

Martin MD, Mendelson TC, 2016. Male behaviour predicts trait divergence and the evolution of reproductive isolation in darters (*Percidae*: *Etheostoma*). *Anim Behav* **112**:179–186.

McPeek M, Gavrilets S, 2006. The evolution of female mating preferences: differentiation from species with promiscuous males can promote speciation. *Evolution* **60**:1967–1980.

Mendelson TC, Martin MD, Flaxman SM, 2014. Mutation-order divergence by sexual selection: diversification of sexual signals in similar environments as a first step in speciation. *Ecol Lett* **17**:1053–1066.

Moran RL, Zhou M, Catchen JM, Fuller RC, 2017. Male and female contributions to behavioral isolation in darters as a function of genetic distance and color distance. *Evolution* **71**:2428–2444.

Near TJ, Bossu CM, Bradburd GS, Carlson RL, Harrington RC et al., 2011. Phylogeny and temporal diversification of darters (*Percidae: Etheostomatinae*). *Syst Biol* **60**:565–595.

Nosil P, Crespi BJ, Sandoval CP, 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. *Proc R Soc B Biol Sci* **270**:1911–1918.

Okamoto KW, Grether GF, 2013. The evolution of species recognition in competitive and mating contexts: the relative efficacy of alternative mechanisms of character displacement. *Ecol Lett* **16**:670–678.

Ortiz-Barrientos D, Grealy A, Nosil P, 2009. The genetics and ecology of reinforcement: implications for the evolution of prezygotic isolation in sympatry and beyond. *Ann N Y Acad Sci* **1168**:156–182.

Page LM, 1983. *Handbook of Darters*. Neptune City (NJ): TFH Publications Inc.

Page LM, Burr BM, 2011. *Peterson Field Guide to Freshwater Fishes of North America North of Mexico*. 2nd edn. Boston: Houghton Mifflin Harcourt.

Panhuis TM, Butlin R, Zuk M, Tregenza T, 2001. Sexual selection and speciation. *Trends Ecol Evol* **16**:364–371.

Pfennig KS, Pfennig DW, 2009. Character displacement: ecological and reproductive responses to a common evolutionary problem. *Q Rev Biol* **84**:253–276.

Pfennig D, Pfennig K, 2012. *Evolution's Wedge: Competition and the Origins of Diversity*. Berkeley (CA): Univeristy of California Press.

Pfennig KS, Rice AM, 2014. Reinforcement generates reproductive isolation between neighbouring conspecific populations of spadefoot toads. *Proc R Soc B* **281**:20140949.

Pfennig K, Ryan M, 2006. Reproductive character displacement generates reproductive isolation among conspecific populations: an artificial neural network study. *Proc Biol Sci* **273**:1361–1368.

Porretta D, Urbanelli S, 2012. Evolution of premating reproductive isolation among conspecific populations of the sea rock-pool beetle *Ochthebius urbanelliae* driven by reinforcing natural selection. *Evolution* **66**:1284–1295.

Pyron M, 1995. Mating patterns and a test for female mate choice in *Etheostoma spectabile* (Pisces, Percidae). *Behav Ecol Sociobiol* **36**: 407–412.

Qvarnström A, Vallin N, Rudh A, 2012. The role of male contest competition over mates in speciation. *Curr Zool* **58**:493–509.

Saether SA, Saetre G-P, Borge T, Wiley C, Svedin N et al., 2007. Sex chromosome-linked species recognition and evolution of reproductive isolation in flycatchers. *Science* **318**:95–97.

Sætre G, Moum T, Bures S, Kral M, Adamjan M et al., 1997. A sexually selected character displacement in flycatchers reinforces premating isolation. *Nature* **387**:1995–1998.

Servedio MR, Noor MAF, 2003. The role of reinforcement in speciation: theory and data. *Annu Rev Ecol Evol Syst* **34**:339–364.

Stalker HD, 1942. Sexual isolation studies in the species complex *Drosophila virilis*. *Genetics* **27**:238–257.

Tinghitella RM, Lehto WR, Minter R, 2015. The evolutionary loss of a badge of status alters male competition in three-spine stickleback. *Behav Ecol* **26**: 609–616.

Vallin N, Rice AM, Bailey RI, Husby A, Qvarnström A, 2012. Positive feedback between ecological and reproductive character displacement in a young avian hybrid zone. *Evolution* **66**:1167–1179.

Williams TH, Mendelson TC, 2011. Female preference for male coloration may explain behavioural isolation in sympatric darters. *Anim Behav* **82**: 683–689.

Yukilevich R, Aoki F, 2016. Is cascade reinforcement likely when sympatric and allopatric populations exchange migrants? *Curr Zool* **62**:155–167.

Zhou M, 2014. Speciation and the evolution of male breeding coloration in darters [PhD thesis]. Univeristy of Illinois at Urbana-Champaign.

Zhou M, Johnson AM, Fuller RC, 2014. Patterns of male breeding color variation differ across species, populations, and body size in rainbow and orangethroat darters. *Copeia* **2014**:297–308.

Zhou M, Fuller RC, 2014. Reproductive isolation between two darter species is enhanced and asymmetric in sympatry. *J Fish Biol* **84**:1389–1400.

Zhou M, Fuller RC, 2016. Intrasexual competition underlies sexual selection on male breeding coloration in the orangethroat darter, *Etheostoma spectabile*. *Ecol Evol* **6**:3513–3522.

Zhou M, Loew ER, Fuller RC, 2015. Sexually asymmetric colour-based species discrimination in orangethroat darters. *Anim Behav* **106**:171–179.