BEHAVIORAL AND GENOMIC PATTERNS OF EVOLUTION DURING SPECIATION VIA REPRODUCTIVE AND AGONISTIC CHARACTER DISPLACEMENT

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Ecology, Evolution, and Conservation Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

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ABSTRACT

Interspecific reproductive interactions, such as hybridization or fighting over access to mates, are surprisingly common given that they frequently result in negative fitness consequences. Selection to avoid hybridizing with another species can result in mating trait evolution via reproductive character displacement (RCD). Similarly, selection to avoid aggressive interactions with another species can result in agonistic character displacement (ACD). Both RCD and ACD can lead to a pattern of enhanced preferences for mating or fighting with conspecifics over heterospecifics in areas of sympatry compared to allopatry. Behavioral isolation can potentially evolve among populations within a species as a correlated effect of RCD or ACD, termed cascade RCD (CRCD) or cascade ACD (CACD). My dissertation research integrated behavioral and genomic approaches to investigate the role of character displacement in promoting speciation in a diverse clade of stream fishes called darters (Percidae: Etheostominae). I focused on two groups in the subgenus *Oligocephalus*: the orangethroat darters clade (*Etheostoma: Ceasia*) and the rainbow darter (*Etheostoma caeruleum*). I found behavioral evidence for male-driven RCD and ACD between multiple orangethroat species and their respective sympatric rainbow darter populations. When orangethroat and rainbow darters occur sympatrically, males prefer conspecific over heterospecific females and bias their aggression towards conspecific over heterospecific males. Such preferences are absent when these species occur allopatrically. I also found that RCD and ACD between orangethroat and rainbow darters has secondarily caused mating and agonistic traits to diverge among orangethroat lineages. When orangethroat species occur in sympatry with rainbow darters, orangethroat males preferentially mate and fight with conspecifics over another closely related orangethroat species. However, when orangethroat species are allopatric with respect to rainbow

darters, orangethroat males do not differentiate between conspecific versus heterospecific orangethroat males and females. This is consistent with both CRCD and CACD within the orangethroat darter clade and represents the only known example of CACD to date.

Notably, females do not exhibit preferences for variable components of male coloration between or within species, despite the presence of sexual dimorphism and traditional sex roles. Instead, color pattern functions in male competition. I found that male color pattern is more divergent between orangethroat and rainbow darters in sympatry versus allopatry. Consequently, males bias aggression towards conspecific over heterospecific males in sympatry but not in allopatry. This shows that male competition alone can lead to color pattern divergence between and within species via ACD and CACD. The failure to detect female mate preferences in this system may be due to females facing a high cost to choosiness, as I observed that egg viability decreases rapidly with time since ovulation. Together, these results contradict the classic paradigm that female preference promotes the evolution of behavioral isolation in species where males exhibit elaborate secondary sex traits.

Selection to avoid hybridization has likely promoted the evolution of RCD and ACD between orangethroat and rainbow darters. I used genomic data to show that hybridization is ongoing and that backcross hybrids suffer high mortality. To investigate the genetic mechanisms underlying postzygotic isolation between these species, I assembled the orangethroat darter genome and transcriptome, resulting in the first annotated darter draft genome. I also generated linkage maps for orangethroat and rainbow darters. Using these tools, I identified several putative chromosomal translocations that may be implicated in genetic incompatibilities. Analyses of restriction-site associated DNA sequencing (RADseq) data in laboratory-generated backcross hybrids revealed strong selection against recombinant individuals. This represents one

of the few studies to use fine-scale ancestry mapping in hybrids to characterize genome-wide patterns corresponding to genetic incompatibilities in non-model species. My results indicate that a large proportion of the genome is involved in postzygotic isolation, which in turn (1) directly promotes RCD/ACD in sympatry between orangethroat and rainbow darters and (2) indirectly promotes CRCD/CACD among orangethroat darter lineages. Overall, my dissertation research has significantly changed our understanding of speciation in darters and has provided novel insight into the genomic architecture of hybrid incompatibilities that promote character displacement. The genomic resources that I generated for my dissertation research will undoubtedly serve as valuable tools for future studies on speciation, sexual selection, phylogenetics, mating system evolution, and conservation in darters.

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CHAPTER 1

INTRODUCTION

Speciation is the process by which one species splits into two reproductively isolated groups. Reproductive barriers are traits that prevent species from exchanging genetic material and are classified as either prezygotic or postzygotic (Coyne and Orr 2004). Prezygotic barriers act prior to fertilization and often involve differences in courtship behavior between species. Postzygotic barriers act after fertilization and include intrinsic genetic incompatibles that can result in hybrid sterility and/or inviability. Postzygotic barriers also include extrinsic incompatibilities that cause hybrids with intermediate phenotypes to have reduced fitness in their environment compared to either parental species. A major goal in evolutionary biology is to understand how reproductive barriers evolve, especially in cases where speciation has proceeded in the face of gene flow (Turelli et al. 2001; Feder et al. 2012). When two diverging lineages exchange genetic material via hybridization, co-adapted gene complexes within each group are broken up by recombination. For this reason, hybridization has traditionally been thought of as a homogenizing force that prevents speciation (Mayr 1954; Mallet 2005; Seehausen et al. 2008).

Recent theoretical and empirical work have demonstrated that under some circumstances, hybridization can actually promote speciation (Kelly and Noor 1996; Noor 1999; Kirkpatrick and Ravigne 2002; Servedio and Noor 2003; Yukilevich and True 2006). Selection to avoid maladaptive hybridization can promote divergence in mating traits used in species recognition (Butlin 1987; Howard 1993). This is termed reproductive character displacement (RCD) (Coyne and Orr 2004). Recent work indicates that interspecific male competitive interactions can also

influence trait divergence in sympatry via agonistic character displacement (ACD) (Qvarnström et al. 2012; Drury and Grether 2014). Similar to RCD, ACD occurs when selection to avoid interspecific fighting results in the divergence of competitive traits (signals and/or aggression biases) (Grether et al. 2009). Both RCD and ACD can result in a pattern of enhanced trait divergence between species in sympatry compared to allopatry.

Selection to avoid costly reproductive and aggressive interactions with heterospecifics can also have macroevolutionary consequences. When gene flow among populations within a species is low, RCD and ACD can incidentally cause mismatches among populations within a species in traits associated with mate/competitor evaluation (Comeault and Matute 2016; Yukilevich and Aoki 2016). The evolution of trait divergence among populations as a correlated effect of character displacement with a second species is termed "cascade" character displacement (Figure 1.1). Cascade RCD (CRCD) can cause increased behavioral isolation among populations within species. Cascade ACD (CACD) can alter the likelihood of competitive interactions in the event of secondary contact.

My dissertation research integrated behavioral and genomic approaches to investigate whether character displacement contributes to speciation in a group of stream fishes called darters. Darters are an ideal study system to investigate the evolutionary effects of interspecific reproductive and aggressive interactions as they often occur in diverse communities with multiple congeners (Page 1983) and hybridization is common (Keck and Near 2009). The darter genus *Etheostoma* includes nearly 160 species, representing the most diverse vertebrate genus in North America (Page and Burr 2011). The orangethroat darter clade (*Etheostoma*: *Ceasia*) consists of 15 recently diverged allopatric species (Distler 1968; Ceas and Page 1997; Bossu et al. 2013), 13 of which occur sympatrically with the more distantly related rainbow darter

(*Etheostoma caeruleum*) (Figure 1.2). Orangethroat and rainbow darters exhibit similar male color patterns, mating behavior, and ecology, and hybridization is ongoing in natural populations (Bossu and Near 2013; Moran et al. 2017, 2018b). The overarching goal of my dissertation research was to elucidate (1) which reproductive barriers maintain species boundaries between orangethroat and rainbow darters, and (2) which evolutionary forces promoted the remarkable diversification observed within orangethroat darters.

In chapter 2, I conducted a behavioral experiment that simulated secondary contact in the laboratory between multiple species within the orangethroat darter clade. The four orangethroat darter species examined were all allopatric to one another but sympatric with rainbow darters. I measured behavioral isolation between pairs of orangethroat darters species and between each orangethroat darter species and their respective sympatric population of rainbow darters. This allowed me to compare measurements of behavioral isolation between the more distantly related orangethroat and rainbow darter pairs to behavioral isolation between more recently diverged orangethroat species. In both types of comparisons, I investigated the relative roles of female mate choice, male mate choice, and male competition in behavioral isolation. As color pattern varies subtly among orangethroat darter species (and more obviously between orangethroat and rainbow darters), I also asked whether the magnitude of male color pattern differences between species predicts behavioral isolation while controlling for genetic distance. I observed high levels of behavioral isolation between sympatric orangethroat and rainbow darter populations and surprisingly high levels of behavioral isolation between more recently diverged species within the orangethroat darter clade (Moran et al. 2017). Notably, male but not female mate preferences and male aggressive biases contribute to behavioral isolation between species. Males exhibited strong preferences for mating with conspecific females and chose to engage in aggressive

interactions with conspecific males over individuals from another orangethroat species or rainbow darters. Furthermore, I found that male color pattern differences between species predicted male aggressive biases. This demonstrated that males play a key role in maintaining species boundaries in this system.

I next asked whether RCD and ACD contribute to speciation between orangethroat and rainbow darters. This predicts heightened behavior isolation in sympatry compared to allopatry. In chapter 3, I measured behavioral isolation between orangethroat and rainbow darters that occur allopatrically from one another and compared the results with my measurements of behavioral isolation between sympatric orangethroat and rainbow population pairings from chapter 2. I found strong behavioral evidence for RCD and ACD between orangethroat and rainbow darters. When orangethroat and rainbow darters occur sympatrically, males preferred to mate with conspecific over heterospecific females and biased their aggression towards conspecific over heterospecific males, but such preferences were absent in allopatry (Moran and Fuller 2018b). Previous studies of RCD have largely focused on the evolution of female preferences for male traits (Pfennig and Pfennig 2012), but my results demonstrate that male preferences for female traits can also evolve via RCD.

I then asked whether RCD and ACD between orangethroat and rainbow darters has incidentally caused the heightened behavioral isolation among orangethroat darters species that occur in sympatry with rainbow darters (i.e. due to CRCD and CACD). To test this hypothesis, I examined behavioral isolation between two species of orangethroat darters that both occur allopatrically from rainbow darters (and thus do not experience selection associated with RCD/ACD) (chapter 3). Considered together with data from chapter 2, my results from chapter 3 demonstrated that orangethroat males preferentially mate and fight with conspecifics over

another closely related orangethroat species only when they naturally occur in sympatry with rainbow darters (Moran and Fuller 2018b). This is consistent with both CRCD and CACD and represents the only known example of CACD to date. My results suggest that the recent diversification of the orangethroat clade has been fueled by RCD and ACD with rainbow darters, demonstrating that increased local biodiversity promotes further biodiversity.

Another unique aspect of the orangethroat-rainbow darter system is that female preference for variable aspects of male coloration is absent, despite the presence of striking sexual dimorphism and traditional sex roles (Pyron 1995; Fuller 2003; Zhou et al. 2015; Moran et al. 2017; Moran and Fuller 2018b). The apparent lack of female mate preferences in this system may be explained by a high cost to females being choosy. In chapter 4, I supported this hypothesis by showing that clutch mortality increases rapidly with increasing time between ovulation and spawning in rainbow darters (Moran et al. 2018a). Considered together, my results from chapters 2-4 suggest that male color pattern functions in male contests over access to females and in competitor recognition rather than female mate preferences. In chapter 5, I demonstrated that differences in male color pattern between orangethroat and rainbow darters are enhanced in sympatry compared to allopatry. I also presented a common garden study showing that color variation among populations/species is genetically based (Moran and Fuller 2018a). This implies that interspecific male competition alone can lead to color pattern signal divergence between and within species via ACD and CACD. Together, these findings contradict the classic paradigm that female preferences promote the evolution of behavioral isolation in species where males exhibit elaborate secondary sex traits.

In chapter 6, I asked what the selective forces are that promote the evolution of RCD and ACD in this system. I found evidence suggesting that selection to avoid hybridization has

favored the evolution of RCD and ACD between orangethroat and rainbow darters. I used genomic and morphological data to show that hybridization is ongoing and is associated with decreased fitness (Moran et al. 2018b). F1 hybrid crosses result in male-skewed sex ratios and backcrosses suffer from dramatically reduced viability compared to parental clutches. My results suggest that genetic incompatibilities with large negative fitness effects act as postzygotic barriers between orangethroat and rainbow darters.

My next goal was to investigate the genomic architecture of postzygotic isolation in this system. To this end, I assembled the first darter reference genome and transcriptome for the orangethroat darter, which is presented in chapter 7. I also generated RADseq-based linkage maps for both orangethroat and rainbow darters. Using the linkage maps and the orangethroat darter genome assembly, I analyzed genomic synteny between orangethroat and rainbow darters. This allowed me to determine whether chromosomal rearrangements contribute to postzygotic isolation between these species. I identified several putative chromosomal translocations that may potentially cause genetic incompatibilities. To further investigate the genomic architecture and distribution of genetic incompatibilities between orangethroat and rainbow darters, I used RADseq to examine genome-wide patterns of local ancestry and linkage disequilibrium in lab-generated backcross hybrids. I observed an enrichment of non-recombinant genotypes across all chromosomes in backcrosses to orangethroat darters and across ten chromosomes in backcrosses to rainbow darters. Furthermore, all chromosomes implicated in putative translocation events also exhibited a lack of recombination and/or deviations from Mendelian segregation in backcross hybrids. However, many chromosomes that lacked obvious translocations also exhibited a lack of recombination. My results show that there is likely strong selection acting against recombinant hybrids and a large proportion of the genome underlies postzygotic isolation

between orangethroat and rainbow darters. This research represents one of the few studies to characterize fine-scale, genome-wide patterns of hybrid incompatibilities between two non-model species and provides novel insight into the mechanism underlying postzygotic isolation in darters.

In summary, my dissertation research has drastically changed how we think about speciation in darters, one of the most diverse groups of vertebrates in North America. I have shown that selection to avoid hybridization can promote the evolution of behavioral biases and can simultaneously promote speciation in sympatry (via RCD/ACD) and in allopatry (via CRCD/CACD). Notably, male behavior alone is driving behavioral isolation between orangethroat and rainbow darters and among species within the orangethroat darter clade. Lastly, my dissertation has pioneered the development of genomic resources for darters. The annotated darter refence genome, linkage maps, and RADseq data that I have generated for my dissertation research will provide valuable tools for future studies on the genomics of speciation in these fishes and will greatly assist conservation efforts.

FIGURES

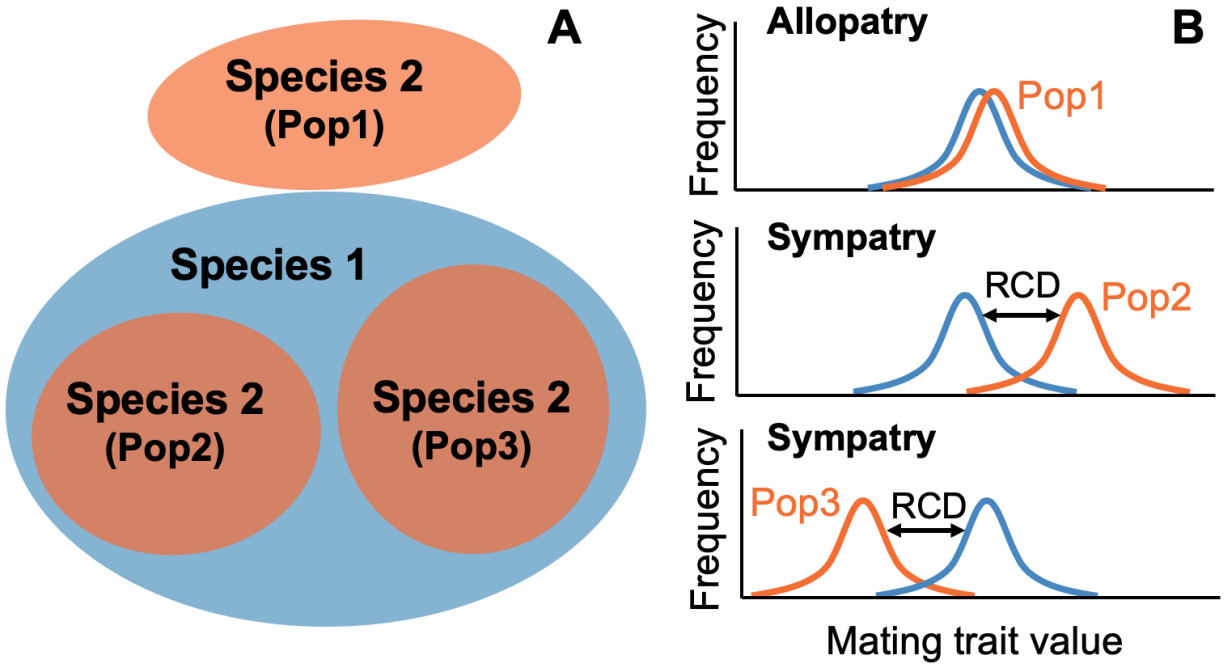

Figure 1.1 Schematic depicting RCD and CRCD. (A) Hypothetical ranges for Species 1 and Species 2. Three populations are shown for Species 2. (B) Mating trait divergence is enhanced in sympatry between Species 1 and 2 due to RCD, resulting in heightened behavioral isolation in sympatry compared to allopatry. Sympatric populations of Species 2 respond to RCD with Species 1 in different ways, which can potentially result in behavioral isolation among populations within Species 2 (i.e. CRCD).

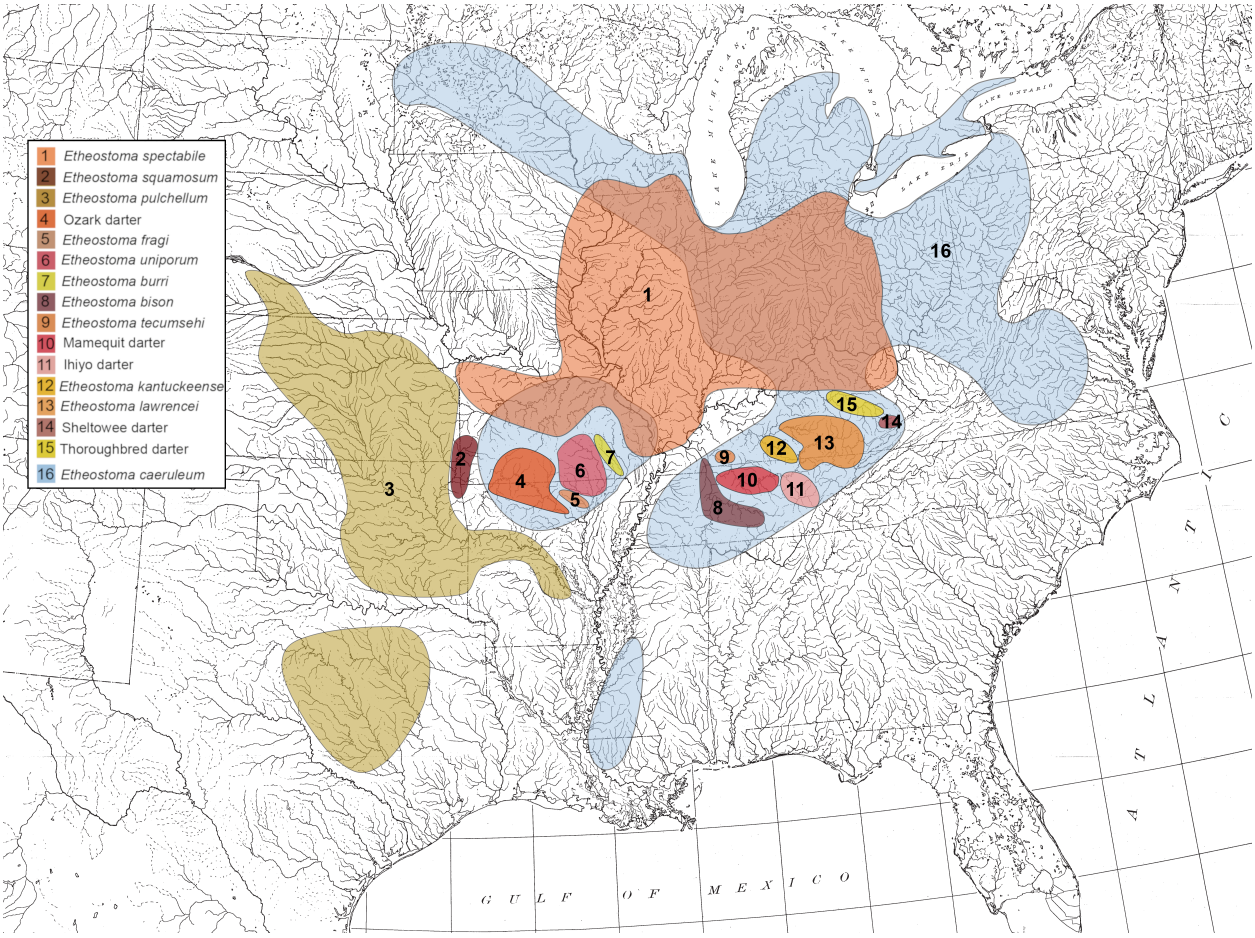

Figure 1.2 Ranges for species within the orangethroat darter clade (# 1-15) and for rainbow darters (#16).

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CHAPTER 2

MALE AND FEMALE CONTRIBUTIONS TO BEHAVIORAL ISOLATION IN DARTERS AS A FUNCTION OF GENETIC DISTANCE AND COLOR DISTANCE[1]

ABSTRACT

Determining which reproductive isolating barriers arise first between geographically isolated lineages is critical to understanding allopatric speciation. We examined behavioral isolation among four recently diverged allopatric species in the orangethroat darter clade (*Etheostoma*: *Ceasia*). We also examined behavioral isolation between each *Ceasia* species and the sympatric rainbow darter *Etheostoma caeruleum*. We asked (1) is behavioral isolation present between allopatric *Ceasia* species, and how does this compare to behavioral isolation with *E. caeruleum,* (2) does male color distance and/or genetic distance predict behavioral isolation between species, and (3) what are the relative contributions of female choice, male choice, and male competition to behavioral isolation? We found that behavioral isolation, genetic differentiation, and male color pattern differentiation were present between allopatric *Ceasia* species. Males, but not females, discerned between conspecific and heterospecific mates. Males also directed more aggression towards conspecific rival males. The high levels of behavioral isolation among *Ceasia* species showed no obvious pattern with genetic distance or male color

[1]Chapter 2 was published in its entirety by Evolution. Moran, R. L., Zhou, M., Catchen, J. M., and R. C. Fuller. 2017. Male and female contributions to behavioral isolation in darters as a function of genetic distance and color distance. Evolution 71(10):2428-2444. This article is reprinted with permission of the publisher and is available from http://onlinelibrary.wiley.com using doi:10.1111/evo.13321

distance. However, when the *E. caeruleum* was included in the analysis, an association between male aggression and male color distance was apparent. We discuss the possibility that reinforcement between *Ceasia* and *E. caeruleum* is driving behavioral isolation among allopatric *Ceasia* species.

INTRODUCTION

Speciation requires the evolution of reproductive isolating barriers between taxa (Mayr 1995). A long-standing goal in speciation research has been to identify the traits/behaviors contributing to reproductive isolation between taxa and the evolutionary forces giving rise to them. Comparative studies of speciation have considered the roles of time, sympatry versus allopatry, divergent ecological selection, and divergent sexual selection due to female choice (reviewed in Coyne and Orr 2004). The emerging consensus is that (a) reproductive isolating barriers increase across evolutionary time separating taxa (e.g., Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002; Fitzpatrick 2002; Russell 2003; Moyle et al. 2004), (b) differences in habitat/ecology are often associated with increased levels of reproductive isolation (e.g., Ryan 1990; Schluter and Price 1993; Boughman 2002; Fuller et al. 2005; Seehausen et al. 2008), (c) sympatric species pairs often have heightened reproductive isolation, presumably due to reinforcement (Coyne and Orr 1989, 1997), and (d) female mating preferences and prezygotic isolation often evolve early, particularly when species are sympatric (Gleason and Ritchie 1998; Turelli et al. 2001; Ritchie 2007). Hence, time since divergence, differences in ecology, reinforcement, and pronounced sexual selection via female mating preferences all favor enhanced reproductive isolation. Here, we consider the other side of the coin and ask how

reproductive isolation evolves in recently diverged allopatric taxa that occupy similar environmental niches, and that (as of yet) lack evidence of female mating preferences. We ask whether discernible levels of reproductive isolation are present, which traits/behaviors predict reproductive isolation, and whether there is evidence that genetic distance (a surrogate for time since divergence) and/or sexual selection can account for the levels of reproductive isolation seen among allopatric taxa.

There are multiple reasons to expect that reproductive isolation should be low or absent among recently diverged allopatric taxa. First, recently diverged allopatric taxa may not have measurable reproductive isolation despite the fact that they differ in traits and/or genetic sequence. This is exemplified by the fact that hybrid swarms often occur when one species is introduced into the range of a close, allopatric relative (e.g., Wilde and Echelle 1992; Huxel 1999; Allendorf et al. 2001; Fitzpatrick et al. 2010). Second, species pairs that occur in similar habitats likely experience little divergent ecological selection, which should lower the likelihood of evolving isolating barriers (Martin and Mendelson 2012). Third, mating systems that are dominated by male-male competition and where sneakers frequently join spawning pairs may offer few opportunities for the evolution of male or female mate choice (Jones et al. 2001; Reichard et al. 2005). Hence, while sexual selection may be intense in such a system, there may be little reason to expect population divergence in preferences and target traits.

Here, we examined (a) whether behavioral isolation was present among four species of allopatric, recently diverged darters, (b) the relative roles of male and female behavior on behavioral isolation, and (c) whether genetic distance and/or color distance predicted behavioral isolation. Behavioral isolation occurs when mismatches in mating traits (signals and/or preferences) prevent mating between two species/populations. To deal with the problem of

animals potentially mating indiscriminately in the laboratory, we also assayed behavioral isolation between each of the four species and a more distantly related sympatric darter species. Previous work on this system has shown behavioral isolation is almost complete between sympatric darter congeners (Zhou and Fuller 2014). The fact that these species are maintained in nature coupled with the fact that sympatric species are reluctant to hybridize in the laboratory provides some reassurance that animals are behaving as they would in a natural setting.

Darters are a highly diverse group of North American benthic stream fishes (Page 1983). Darter speciation appears to occur in allopatry, as the most closely related sister species do not co-occur (Near and Benard 2004; Near et al. 2011). Within a given clade, darters often occupy similar environmental niches, suggesting that early divergence is not due to ecological selection (Schmidt 2009; Martin and Mendelson 2012, 2014). Instead, sexual selection is thought to play a pivotal role in darter speciation. Males of many species exhibit bright coloration or egg mimicry (Page 1983; Page and Burr 2011), and behavioral isolation evolves before larval F1 hybrid inviability (Mendelson 2003). Although many have assumed that male nuptial coloration is the target of female mating preferences (Mendelson 2003; Williams and Mendelson 2010, 2011; Williams et al. 2013), emerging evidence suggests that male coloration may function in aggressive signaling among males (Zhou et al. 2015; Zhou and Fuller 2016; Martin and Mendelson 2016).

The orangethroat darter clade (*Ceasia*) is well suited for studying the early stages of allopatric speciation. *Ceasia* consists of 15 recently diverged species that are all allopatric from one another (Ceas and Page 1997; Page and Burr 2011). A recent study by Bossu et al. (2013) reconstructed palaeodrainage connections in the eastern United States and built a time-calibrated phylogenic tree to investigate the historical biogeography of the *Ceasia* clade. The *Ceasia* clade

is estimated to have originated between 6.6-6.9 mya and to have diversified allopatrically (Bossu et al. 2013). Members of *Ceasia* were raised from the subspecies to species level due to differences in morphology and male coloration (Ceas and Page 1997), and a subsequent study has shown that there is genetic divergence between species (Bossu et al. 2013). However, prior to the present study, behavioral isolation had not been examined between any *Ceasia* species. Here we examined the evolution of behavioral isolation among four allopatric *Ceasia* species. We also compared levels of behavioral isolation among allopatric *Ceasia* species to levels of behavioral isolation between *Ceasia* and a more distantly related sympatric congener, *Etheostoma caeruleum* (rainbow darter). We examined the relationship between male color pattern divergence, genetic divergence, and three components of behavioral isolation: female choice among males, male choice among females, and male recognition of other males as competitors for females.

METHODS

Study Species, Collection, and Maintenance

For our study, we used four allopatric species in the *Ceasia* clade: *Etheostoma fragi* (strawberry darter), *Etheostoma uniporum* (current darter), *Etheostoma burri* (brook darter), and *Etheostoma spectabile* (orangethroat darter), and a more distantly related, sympatric species, *E. caeruleum* (Fig. 2.1; Fig. A.1). We originally used data from previous studies to choose pairs of *Ceasia* species that differed to varying degrees from one another in male color pattern and genetic sequence (i.e., low: *E. fragi* and *E. uniporum*; intermediate: *E. fragi* and *E. burri*; high: *E. fragi* and *E. spectabile*). We used the mitochondrial and nuclear gene phylogeny of Bossu et

al. (2013) to initially select *Ceasia* species that varied in degree of relatedness, but we also measured genetic distance independently using Restriction site-Associated DNA sequencing (RADseq) (see below). Likewise, we used images from field guides (Page 1983; Page and Burr 2011) and our own images to select *Ceasia* species that varied from one another in degree of color pattern similarity, but we also measured color distance between species with digital photography (see below).

Two populations of *E. caeruleum* were used, one from the Ozarks region and the other from Illinois (Table A.1). The three *Ceasia* species from the Ozarks region were tested with the Ozarks *E. caeruleum*, and the *Ceasia* species from Illinois was tested with the Illinois *E. caeruleum*.

Adult fish were collected by kick-seine in March 2015 (localities in Table A.1). Both *Ceasia* and *E. caeruleum* were encountered at each site. Fish were transported back to the laboratory in aerated coolers. They were maintained in 38-liter aquaria separated by species and sex at 20° C with a 13:11 light:dark cycle, and fed frozen bloodworms daily. Behavioral assays were performed prior to feeding on a given day.

Experimental Design for Behavioral Assays

Our behavioral assays aimed to measure behavioral isolation between allopatric *Ceasia-Ceasia* species pairs and between sympatric *Ceasia-E. caeruleum* species pairs, and to determine the relative contributions of males and females to behavioral isolation. Behavioral assays were conducted from March through May 2015. Each trial took place in a 38 L aquarium with gravel substrate. To minimize disturbance, three sides of the observational tank were covered in black plastic. Each trial involved three fish: a *Ceasia* focal male, a *Ceasia* focal female, and a rival

male (Fig. 2.2). Before each trial began, the focal male was placed in the observational tank and allowed to acclimate for 10 min. A conspecific focal female and a rival male were then placed into the tank with the focal male. When darters are first placed into a new tank, they typically respond by freezing and clamping their fins close to their bodies. We did not start a trial until all fish were freely swimming around the observational tank, indicating that they were acclimated. All darters acclimated quickly after being moved to an observational tank, and no fish took longer than 2 min to acclimate. After all three fish were acclimated, they were observed for 30 min. Each 30 min trial was divided into 30 s blocks. A focal male and focal female pair were observed together in three consecutive treatments that varied in the identity of the rival male. Rival males were either a conspecific *Ceasia* male, a heterospecific allopatric *Ceasia* male, or a heterospecific sympatric *E. caeruleum* male (Table 2.1, Fig. 2.2). Unique rival males were used, and the order of the three rival male treatments was randomized for each focal pair. We used rival males that were within 5 mm of the focal male's standard length. All focal females were gravid, discernible by distended abdomens.

Our behavioral assays were organized into three "sets", each using *E. fragi* and one of the three other *Ceasia* species and *E. caeruleum* (Table 2.1). For each set, we performed behavioral assays where each *Ceasia* species (*E. fragi, E. uniporum, E. burri,* and *E. spectabile*) served as the focal male and female. We refer to these as the forward and reverse species sets (Table 2.1). In trials with *E. caeruleum, E. caeruleum* served as a rival male but was never a focal species. A total of 8 replicates were conducted for each combination of species set, species set direction, and rival male treatment (3 species sets x 2 directions x 3 treatments x 8 replicates = 144 behavioral trials).

Male mate choice was measured for the rival males as male pursuit of the female. Male pursuit was measured as the proportion of 30 s blocks in which the rival male was within one body length of the female for at least five consecutive seconds (Zhou et al. 2015), divided by the total number of 30 s blocks in which either male was within one body length of the female for at least five consecutive seconds. Thus, we conducted no-choice tests of male mate preference. Male aggression was measured as the number of fin flares and attacks performed by both the rival and focal male towards the other male during a trial (Zhou et al. 2015).

Female mate choice was measured as the relative proportion of nosedigs and headwags performed within one body length of the rival male. Nosedigs occur when a female jabs her snout into the substrate while searching for a suitable spawning location. Nosedigs are frequently used as a measure of female mating preference (Fuller 2003; Williams and Mendelson 2011). Females perform headwags when actively pursued by a male. Headwags signal receptivity to male courtship (Kozlowski 1979). We recorded the identity of the male(s) present within one body length for all nosedigs and headwags.

A trial was excluded from the analysis of headwags or nosedigs if a female did not perform the behavior in that trial. No trials were excluded from analyses of male behaviors, since at least one male in each trial performed female pursuit and aggressive behaviors. Table A.2 lists sample sizes for each behavior. All raw behavioral data are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.61n4k).

Statistical Analyses of Behavioral Assays

For each of the three species sets, we used generalized linear models with a negative binomial distribution and log link function to analyze two measures of male aggression (i.e.,

number of fin flares and attacks) performed by the focal male and directed towards the rival male. Focal male species identity, rival male species identity (conspecific, heterospecific *Ceasia*, or E. caeruleum), and their interaction were the independent variables. This allowed us to examine whether focal males were more aggressive towards conspecific versus heterospecific rivals, and whether these effects were symmetrical for the forward and reverse trials. All statistical analyses were performed in R (version 3.2.1). Negative binomial generalized linear models were conducted using the glm.nb function in the package MASS (Venables and Ripley 2002). To examine pairwise differences among the rival male treatments, we performed post-hoc tests using Tukey's multiple comparisons with the glht function in the package MULTCOMP (Hothorn et al. 2008). To consider the aggressive behavior of the rival male towards the focal male, we conducted two additional analyses following the same method used to analyze focal male aggressive behavior, but with rival male fin flares and rival male attacks serving as the dependent variables.

For male mate choice, we performed a two-way ANOVA with focal species identity, rival male identity, and the interaction terms as the independent variables. The dependent variable was the amount of time that the rival male pursued the focal female. This allowed us to test the prediction that rival *Ceasia* males would prefer to pursue conspecific over heterospecific females (Zhou et al. 2015). Likewise, *E. caeruleum* should have low levels of pursuit of *Ceasia* females. We conducted post-hoc Bonferroni-adjusted pairwise t-tests to make pairwise comparisons among rival male treatments levels. We did not perform these analyses with focal *Ceasia* males as they were always with conspecific focal females.

Finally, we used ANCOVAs to ask whether females were more likely to respond to

conspecific males compared to allopatric heterospecific *Ceasia* or sympatric *E. caeruleum* males. Previous work has shown that females spawn with the males that guard them (Zhou et al. 2015). Thus, we included male pursuit of female as a covariate in the analysis of nosedigs and in the analysis of headwags. For each of the three species sets, the full model included focal species, rival male identity, the interaction between focal species and rival male identity, and the proportion of time the focal female was guarded by the rival male versus the focal male.

Behavioral Isolation Indices

Behavioral data were used to estimate behavioral isolation indices following Martin and Mendelson (2016). Each index has a value between -1 to 1, where a positive value indicates more conspecific than heterospecific interactions were observed, a negative value indicates more heterospecific than conspecific interactions were observed, and a value of 0 indicates an equal number of conspecific and heterospecific interactions were observed (Stalker 1942; Mendelson 2003; Martin and Mendelson 2016). We calculated indices for female mate choice, male mate choice, and male aggression. Indices were calculated for each replicate within a set and then averaged across each species pair in a set.

To control for differences in the amount of time males spent pursuing females, the female choice index was calculated as the ratio of female nosedigs to the number of times a male attempted to pursue a female. The female mate choice index (FC) was calculated as:

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

where f_c and f_h represent the number of nosedigs females performed near conspecific and heterospecific males, respectively. p_c and p_h represent the number of 30 s time blocks conspecific and heterospecific males spent in pursuit of the female during a trial, respectively.

The male mate choice index (MC) was calculated as:

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

where m_c and m_h represent the proportion of time conspecific and heterospecific males spent pursuing the female during each trial.

The male-male aggression index (MA) was calculated as:

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

where a_c and a_h represent the number of aggressive behaviors (i.e., chases and fin flares) performed between conspecific and heterospecific males.

Color Analysis

We used digital photography to quantify male coloration. We focused on components of male color pattern used in qualitative species diagnoses (Ceas and Page 1997). After each trial, we lightly anesthetized animals (0.01 g/L MS-222 for three min). We then took photographs using a Nikon Coolpix D3300 digital camera under florescent lighting with the camera's factory setting for photography in florescent lighting. Each photograph contained a lateral view of an

individual fish on a background of white 1 mm grid paper next to an X-rite ColorChecker Mini Chart (Grand Rapids, MI). Inclusion of the color checker allows us to color correct digital images in Adobe Photoshop CS4 Extended using the inCamera 4.5 plug-in (PictoColor Software, version 4.0.1), as described by Bergman and Beehner (2008).

For each species, digital photographs of 10 males were used in color analyses. Color analyses were conducted following the methodology outlined in Zhou et al. (2014). For each photograph, we took RGB measurements in Adobe Photoshop CS4 Extended using the Color Sampler Tool. For each fish, we took RGB measurements on both the red and the blue portions of the first dorsal fin, second dorsal fin, anal fin, and lateral bars. We also took RGB measurements on the throat and belly (which were always one solid color). Each RGB measurement gave separate values for R, G, and B. Average R, G, and B values were calculated from three replicate RGB measurements on the same photograph for each location on each fish. Thus, we obtained average R, G, and B values for 10 locations on each fish, for a total of 30 RGB variables.

We also measured the proportion of red and blue color on the first dorsal fin, second dorsal fin, anal fin, anterior body, and posterior body, for a total of 10 color proportion variables. Following Zhou et al. (2014), red and blue color proportions were measured in ImageJ (version 1.50c4) in CIE L*a*b* color space. The perimeter of each body section was traced using the polygon selections tool in ImageJ, and the total number of pixels within each traced area was measured using the histogram tool. Red and blue proportions of each body area were calculated using the Threshold_Color ImageJ plugin (version 1.16, G. Landini; see Zhou et al. 2014 for full details).

Forty color variables (30 RGB and 10 color proportions) were collected from each male. We used the Mahalanobis distance to measure color distance between each species pair (Mahalanobis 1936). The Mahalanobis distance measures trait distances among groups by accounting for the variance and covariance within each group (Mahalanobis 1936; Arnegard et al. 2010; Martin and Mendelson 2014). The multivariate Mahalanobis distance is analogous to the univariate z-score in that it removes the correlation between variables and standard. We calculated the squared Mahalanobis distance between each species pair with the pairwise.mahalanobis function of the HDMD package in R (version 3.2.1). We then took the square root of these values to calculate the interspecific Mahalanobis distance, referred to hereafter as male color distance.

Genetic Distance

We used double digest RADseq to measure genetic distance among the five species. Nuclear DNA was extracted from 12 individuals from each species. Table S3 shows collection locations for individuals used in genetic analyses. Illumina libraries were prepared following Parchman et al. (2012). Nuclear DNA samples were digested with two restriction enzymes (EcoRI and Mse1) and barcoded for identification of individual samples. Samples were then pooled and amplified using 30 cycles of PCR. To obtain DNA fragments of a uniform size, the pooled PCR product was electrophoresed on a 2.5% agarose gel. Bands within the 500 – 600 bp range were excised and purified using a QIAquick Gel Extraction Kit (Qiagen). The pooled libraries were sequenced as 100 bp single-end reads using an Illumina Hi-Seq 2500 platform. We ran one lane of sequencing with 60 individuals total, which resulted in a mean coverage depth of

20X. Sequence reads have been deposited into the NCBI Sequence Read Archive (SRA) and are available under accession number SRP113339.

The Stacks software package (Catchen et al. 2011, 2013) was used to analyze the patterns of genetic structure. The program *process_radtags* was used to de-multiplex samples and remove low quality reads (see Table A.4). We used *ustacks* to build loci and call SNPs *de novo* for each individual, *cstacks* to compile a catalog of loci for each population, and *sstacks* to match each individual against the catalog. A minimum of three identical reads were required to infer a putative allele. We allowed a maximum of three mismatches when merging alleles into loci within an individual, and a maximum of two mismatches between loci when compiling the catalog of all RAD loci. These parameters resulted in a total catalog of 684,956 loci. We used the program *populations* to apply additional filters to the data set and to conduct genetic analyses. Each locus was required to be present in every population and in at least 75% of the individuals within a population to be retained. Minor alleles present at lower than 0.04% were removed to control for false SNPs (i.e., sequencing errors). This filtering retained 18,295 loci. Of these, 17,162 were polymorphic and contained a total of 44,971 SNPs.

We used variant SNPs to calculate Nei's genetic distance (D_{ST}; Nei 1972, 1978) and to conduct STRUCTURE and K-means clustering analyses. The software packages used to conduct these analyses assume independence among SNPs. However, each locus in the catalog has the potential to contain multiple SNPs, which would be linked together on the same 100 bp RAD tag. To ensure only the first SNP was analyzed from each locus, we ran *populations* again with the same parameters as specified above but with the --*write_single_snp* option added. We also ran *populations* while excluding the outgroup, *E. caeruleum*, to obtain a *Ceasia*-specific set of loci that would potentially allow for the detection of finer scale genetic differences among these

species. When all five species were included, populations retained 16,968 variant loci. Excluding *E. caeruleum* resulted in *populations* retaining 19,896 variant loci.

We generated a GenePop (Rousset 2008) file in *populations* using the variant SNPs for all five species. We then imported the file into GenoDive (Version 2.0b27, Meirmans and van Tienderen 2004) and calculated Nei's standard genetic distance (D_{ST}) between each species. We also performed a K-means clustering analysis in GenoDive to obtain an estimate of the number of distinct genetic clusters (K). K was set to range from 1 through 8. We performed 20 repeats of the simulated annealing algorithm with 100,000 Markov Chain Monte Carlo (MCMC) steps. The optimal number of clusters was inferred from the K with the highest value for the pseudo-*F* statistic (Caliński and Harabasz 1974; Meirmans 2012).

We also used STRUCTURE to determine the most likely value of K. We obtained two STRUCTURE (version 2.3.3, Pritchard et al. 2000) formatted output files from *populations* for the two data sets (with and without *E. caeruleum* included). Early STRUCTURE analyses revealed an F1 hybrid *E. caeruleum* x *E. uniporum* individual. This individual was excluded from all analyses. For all STRUCTURE analyses, we used 50,000 burn-in steps with 150,000 MCMC steps. Ranges for K were set to 1 through 8 when all five species were included, and 1 through 7 when *E. caeruleum* was excluded. Analyses for each potential value of K were run 50 times. The true number of genetic clusters present for each data set was determined using the Delta K method (Evanno et al. 2005). Delta K values were calculated using Structure Harvester (Earl and vonHoldt 2012).

Relationship between Behavioral Isolation, Color Distance, and Genetic Distance

To examine the relationship between behavioral isolation and genetic distance, we plotted the three behavioral isolation indices (male choice, male aggression, and female choice) with 95% confidence intervals versus pairwise D_{ST} values (Fig. 2.3). We also examined the relationship between behavioral isolation and male color distance. To control for the potential influence of genetic distance on these variables, each of the three indices of behavioral isolation and male color distance were regressed onto D_{ST}. We then plotted the residuals of these analyses against one another (Fig. 2.4). We visually examined the plots of behavioral isolation versus D_{ST} (Fig. 2.3) and behavioral isolation versus male color distance (Fig. 2.4) to determine whether any trends existed among the three *Ceasia-Ceasia* species comparisons and among the four *Ceasia-E. caeruleum* comparisons. Phylogenetically independent contrasts (Felsenstein 1985) were not feasible due to the number of independent species pairs examined.

RESULTS

Do males discern conspecific from heterospecific male rivals?

Focal male *Ceasia* were more aggressive towards conspecific than heterospecific rivals, indicating that they could discriminate males of closely related species (Table 2). Aggression was lowest towards the more distantly related *E. caeruleum,* and was intermediate towards heterospecific allopatric *Ceasia* males. The results were most striking for fin flares. Across all three species sets, focal males performed 15X more fin flares towards conspecific males compared to *E. caeruleum* males (Figs. A.2-A.4). In one of the three species sets (*E. fragi - E. uniporum - E. caeruleum*), focal males performed significantly more fin flares towards

conspecific than heterospecific *Ceasia*. The same general pattern was observed for attacks, but focal males performed significantly more attacks towards conspecific than heterospecific *Ceasia* only in the *E. fragi* - *E. burri* - *E. caeruleum* species set. This same set was notable because the two focal species differed in aggression. Focal male *E. burri* performed 5x more attacks on both conspecific *Ceasia* and allopatric heterospecific *Ceasia* rivals compared to focal male *E. fragi* (Table 2.2, Fig. A.3).

We observed similar patterns of increased aggression towards conspecifics over heterospecific males in rival males. Conspecific rival males were most aggressive, *E. caeruleum* rival males were least aggressive, and heterospecific *Ceasia* rival males were intermediate (Figs. 2.5, A.5, and A.6). Hence, there were high levels of species discrimination between heterospecific *Ceasia* males even though they are allopatric. Across all three species sets, the numbers of fin flares and the number of attacks directed at the focal male differed as a function of rival male identity (Table 2.3).

Do males discern between conspecific and heterospecific females?

Rival males clearly altered their pursuit behavior depending on whether females were conspecific or heterospecific. Conspecific *Ceasia* rival males spent the most time pursuing the focal female; heterospecific *Ceasia* rivals were intermediate in focal female pursuit. Sympatric *E. caeruleum* rival males spent little time pursuing the focal female (Figs. 2.5c,f, A.5c,f, and A.6c,f). On average, the amount of time spent in pursuit of the focal *Ceasia* female was 5x greater for conspecific *Ceasia* rival males compared to heterospecific *E. caeruleum* rival males (Figs. 2.5c,f, A.5c,f and A.6c,f). These differences between conspecific *Ceasia* versus heterospecific *E. caeruleum* were significant in all three trial sets (Table 1.4). Across all trial

sets, conspecific *Ceasia* rival males spent 2X more time spent pursuing the *Ceasia* focal females compared to heterospecific *Ceasia* rival males. In two of the three species sets, these differences were statistically significant (Table 2.4b,c).

Do females discern between conspecific and heterospecific males?

We found no evidence for female mate preference for conspecifics over heterospecifics. The number of nosedigs and headwags performed towards males did not differ among rival males when rival male pursuit was included as a covariate in the analysis (Figs. A.7-9, Table A.5). Hence, there is no evidence that females adjusted their willingness to spawn due to the identity of the male that was guarding her.

Behavioral Isolation Indices

Behavioral isolation was high for male mate choice and for male aggression, but was low for female mate choice (Table 2.5). For all *Ceasia* species pairs, indices of male choice and male aggression were positive and greater than zero (male choice: $t = 6.50$, $df = 6$, $p<0.001$; male aggression: $t = 7.27$, $df = 6$, $p<0.001$), indicating a behavioral preference for responding to conspecifics over heterospecifics. Male choice and male aggression indices were twice as high for *Ceasia* - *E. caeruleum* pairings compared to heterospecific *Ceasia* pairings. Female choice indices did not differ significantly from zero ($t = -0.69$, $df = 6$, $p = 0.51$), indicating females show little preference for conspecific over heterospecific males.

Among Species Patterns in Genetic Distance

As with our behavioral isolation assays, our genetic analysis indicates that all five species

were distinct evolutionary units; all four *Ceasia* species differed significantly from one another, and *E. caeruleum* was an obvious genetic outgroup to *Ceasia*. One clear F1 hybrid between *E. uniporum* and *E. caeruleum* was detected, but this individual was excluded from the analysis. Table 2.6 shows the population genetic statistics for the total loci retained (both variant and invariant) and the variant loci alone. As expected, *E. caeruleum* had the largest number of private alleles. In general, *E. caeruleum* also harbored greater genetic variation than the *Ceasia* species; the observed heterozygosity, nucleotide diversity, and percent polymorphic loci were highest in *E. caeruleum.* Although these indices of genetic variation were nearly as high in *E. uniporum* as they were in *E. caeruleum*, the observed heterozygosity, nucleotide diversity, and percent polymorphic loci across all loci in *E. caeruleum* were between 1.5-3X higher than that present in *E. fragi*, *E. burri*, and *E. spectabile*.

Pairwise D_{ST} values for *Ceasia*-*Ceasia* and *Ceasia*-*E. caeruleum* species pairs differed significantly from one another (Table 2.5; $t = -6.31$, $df = 2.42$, $p<0.05$). The highest D_{ST} value was 0.348 between *E. spectabile* and *E. caeruleum* and the lowest was 0.206 between *E. fragi* and *E. uniporum*. The D_{ST} values for *Ceasia*-*Ceasia* species pairs ranged from 0.206 to 0.260. The D_{ST} values for *Ceasia*-*E. caeruleum* species pairs ranged from 0.326 to 0.348. All D_{ST} values differed from zero ($t = 13.30$, $df = 6$, $p<0.0001$).

STRUCTURE identified two main clusters when *E. caeruleum* was included in the analysis. One cluster corresponded to *E. caeruleum*, a second to the four *Ceasia* species (Tables A.6-A.7; Fig. A.10a). When *E. caeruleum* was excluded, STRUCTURE identified two main clusters within *Ceasia*. *E. burri* and *E. spectabile* were grouped together into one cluster, and *E. fragi* and *E. uniporum* were grouped together into a second cluster (Tables A.8-A.9; Fig. A.10b).

While STRUCTURE did not detect the four *Ceasia* species as distinct groups, these species were recovered via K-means clustering. When all five species were included, K-means clustering identified each species as a distinct cluster, with an optimal K of 5 (Table A.10).

Among Species Patterns in Color Distance

Analyses of male color distance also revealed significant differences between species. All five species differed from one another in male color pattern (Table 2.5; male color distance > 0 for all species pairs; $t = 4.30$, $df = 6$, $p<0.01$). Differences in male color distance were larger for *Ceasia-E. caeruleum* than for *Ceasia-Ceasia* species pairs ($t = 14.22$, $df = 4.93$, $p< 0.0001$). Within *Ceasia*, genetic distance was not related to male color distance. *E. fragi* and *E. spectabile* had the lowest male color distance, despite having the largest pairwise genetic distance within *Ceasia*. Conversely, *E. fragi* and *E. uniporum* had the lowest pairwise genetic distance within *Ceasia*, yet they exhibited an intermediate male color distance.

Do Genetic Differences and/or Color Differences Predict Behavioral Isolation?

Male components of behavioral isolation were higher among the *Ceasia-E. caeruleum* comparisons than in the *Ceasia-Ceasia* comparisons and these patterns coincide with large differences in genetic distance (Fig. 2.3) and male color distance (Fig. 2.4). Although there were high levels of behavioral isolation (i.e., male choice and male aggression) between *Ceasia* species, there were no obvious correlations with genetic distance (Fig. 2.3a,b). Behavioral isolation values did not vary among the three *Ceasia-Ceasia* comparisons or among the four *Ceasia-E. caeruleum* comparisons, as evidenced by their 95% confidence intervals.

We did not have enough phylogenetically independent species pairs to utilize a phylogenetically controlled regression of behavioral isolation on male color distance. We performed a regression on the raw data and calculated the residuals of male color distance as a function of genetic distance and the residuals of each component of behavioral isolation as a function of genetic distance. We subsequently regressed the behavioral isolation residuals onto the male color distance residuals. This analysis showed that male color distance residuals predicted male aggression residuals ($R^2 = 0.87$, $F_{1,5}=33.65$, $p= 0.002$; Fig 2.4a). This indicates that species pairs with greater differences in coloration were less likely to fight, since a larger male aggression index value represents a larger preference for fighting with conspecifics over heterospecifics. Male color distance did not predict male choice residuals ($R^2 = 0.32$, $F_{1,5} = 2.38$, $p = 0.18$; Fig. 2.4b) or female choice residuals ($R^2 = 0.18$, $F_{1,5} = 1.07$, $p = 0.35$; Fig. 2.4c).

DISCUSSION

Three main results emerged from this study. First, behavioral isolation among taxa was created by male preferences for conspecific over heterospecific females, whereas female mating preferences for conspecific males were absent. Second, males also discerned between conspecific and heterospecific males, preferentially directing aggression towards conspecifics. Additionally, male color distance was associated with the ability of males to discern conspecific (versus heterospecific) male rivals. Third, we showed high levels of behavioral isolation among recently diverged, allopatric *Ceasia* species, yet we were unable to explain how this behavioral isolation evolved; no patterns within *Ceasia* emerged between behavioral isolation, genetic distance, and male color distance. We discuss the implications of these results below.

The Relative Importance of Male versus Female Behavior on Reproductive Isolation

Male darters often show bright, conspicuous coloration that varies among species. This pattern has led to the hypothesis that these colors are important to female mating preferences and reproductive isolation (Williams and Mendelson 2010, 2011; Williams et al. 2013). Yet, here we showed that male mate choice plays a critical role in behavioral isolation. Males of all four species of *Ceasia* discriminated against heterospecific *Ceasia* and *E. caeruleum* females. Hence, males can distinguish between conspecific and heterospecific mates, even at relatively early stages of allopatric divergence. Conversely, female *Ceasia* did not express mate preferences for conspecifics. The lack of female discrimination against heterospecific males is in keeping with numerous other studies on this system that have consistently found no evidence for female mate choice at either the within or among species levels (Pyron 1995; Fuller 2003; Zhou et al. 2015). Instead, there is strong evidence for male mate choice among females (Zhou et al. 2015).

Theoretical and empirical studies of speciation via sexual selection have focused largely on the evolution of female mating preferences (reviewed in Panhuis et al. 2001), with less attention given to the roles of males. The assumption is that females have a larger cost associated with reproduction and experience strong selection to choose high-quality mates (Bateman 1948; Trivers 1972). However, males can also have a significant cost associated with mating which may favor male choice (reviewed in Edward and Chapman 2011; Qvarnström et al. 2012). Male choice need not be limited to systems with reversed sex roles or male parental care. Investment in secondary sex traits (either to attract mates or compete with rivals) can increase male mating costs via increased mortality rates (Kokko and Monaghan 2001). In darters, males engage in frequent, prolonged bouts of competition over access to females, decrease their foraging rates on

the spawning grounds, and can potentially become injured while fighting. In addition, choosiness may be beneficial in darters because mistakenly mating with more distantly related sympatric heterospecifics can result in reduced hybrid viability (Zhou 2014; unpubl. data). The cost of male choice coupled with the benefit of choosiness may favor male discrimination between conspecific and heterospecific females in darters.

The lack of female mating preferences in *Ceasia* is notable given that males are so colorful, and that coloration varies among males even within populations (Zhou et al. 2014). We suspect that female mating preferences are costly in darters for three reasons. First, prolonged female choice that delays spawning may reduce egg viability. In many externally fertilizing fish, egg viability decreases with time since ovulation (McEvoy 1984; Formacion et al. 1993; Bromage et al. 1994; de Gaudemar and Beall 1998), and preliminary data indicate that this is the case in darters (in prep.). The optimal strategy for females may be to spawn quickly after ovulation. Second, females may lack the ability to exert mating preferences. Males congregate on gravel riffles where spawning occurs. When ready to spawn, females move to the riffles and are quickly pursued by many males. Females bury themselves in the gravel and wait for a male to initiate spawning. The female cannot see which male has initiated spawning as she is buried in the gravel. Instead, the female spawns with the first male to initiate spawning (Pyron 1995). Third, spawning pairs are often joined by other males acting as sneakers, precluding female choice (Fuller 1999). These three properties – a rapid decline in egg viability following ovulation, an inability to identify the male that initiates spawning, and high levels of sneaker mating – may make female choice costly relative to its benefits. Similar dynamics occur in other external fertilizers (Warner and Robertson 1978; Warner 1987).

Darter species have traditionally been diagnosed using differences in male nuptial ornamentation. Yet our behavioral results suggest that species-diagnostic, female traits are present and that the levels of diversity rival those observed in male sex traits. We doubt that these are visual cues (but see Williams and Mendelson 2010, 2011; Ciccotto et al. 2013). Many darters lack distinguishing female coloration or morphological traits, especially at the within-subgenus level (Page and Burr 2011). In addition, males that come across a heterospecific female already buried in the gravel (and thus with any potential visual cues hidden) often fail to spawn with the female (pers. obs.). This suggests that males use olfactory cues. Several species of darters, including *Ceasia* and *E. caeruleum*, respond to chemical alarm cues from conspecifics and some heterospecifics (Smith 1979; Commens and Mathis 1999; Haney et al. 2001). There is also pronounced variation in olfactory system morphology among darters (Ceas and Page 1997; Page and Burr 2011). Hence, darters may potentially join the ranks of taxa demonstrating large effects of olfaction on species recognition (reviewed in Ache and Young 2005).

Finally, we note that the mating dynamics in *Ceasia* and *E. caeruleum* stand in contrast to those in snubnose darters. Studies examining female mate choice in snubnose darters and its allies have found mixed support for female mate choice depending on whether comparisons were made between sympatric or allopatric species. Female snubnose darters discriminate against sympatric males (Williams and Mendelson 2010, 2011), but do not discriminate against males from closely related allopatric species (Martin and Mendelson 2016). Instead, like our findings in *Ceasia*, male snubnose darters discriminate against allopatric heterospecific females and males (Martin and Mendelson 2016).

The Role of Male Competition and Male Color Pattern

There is strong evidence that male coloration is used by male darters to signal both species identity and competitive ability (Zhou et al. 2015; Zhou and Fuller 2016; Martin and Mendelson 2016). Previous work has shown that within species, male color pattern predicts male reproductive success via ability to guard a female from other males and secure spawnings (Zhou et al. 2015). Furthermore, altering the lighting environment impairs the ability of males to see the red components of the color pattern and decreases aggressive response towards conspecific males (Zhou and Fuller 2016).

We found that male *Ceasia* discerned conspecific male rivals from closely related *Ceasia* males and from *E. caeruleum* males. Additionally, the residuals of male color distance (corrected for genetic distance) predicted behavioral isolation via male aggression residuals (corrected for genetic distance). Hence, species pairs that had higher than expected differences in male color pattern were less likely to engage in male-male competition. The same effects were not found for male mate choice or female mate choice. In some systems (anoles and cichlids), male color pattern is under selection from female mate choice in addition to male competition (Macedonia and Stamps 1994; Seehausen and Schluter 2004; Pauers et al. 2008). Darters are unique in that an elaborate male signal has evolved due to male-male competition without functioning in the context of female choice, and is utilized by males in species recognition.

The Drivers of Reproductive Isolation

We observed surprisingly high levels of behavioral isolation among newly diverged, allopatric species of *Ceasia*. These species were originally described based on qualitative descriptions of variation in male coloration (Ceas and Page 1997). Bossu et al. (2013)

subsequently created a phylogeny using two mitochondrial genes and ten nuclear genes. Here, we used RADseq and digital photography and showed that there is, indeed, significant variation in male coloration and genetic distance among species. The patterns of relatedness that we observed largely reflect those shown previously; *E. fragi* is more closely related to *E. uniporum* than it is to either *E. burri* or *E. spectabile*, and *Ceasia* species are more closely related to one another than they are to *E. caeruleum.* Furthermore, the lower levels of allelic variation present within *Ceasia* compared to *E. caeruleum* reflect the biology of this system. *Ceasia* species are typically restricted to small headwater streams, resulting in low levels of gene flow among populations (Echelle et al. 1975, 1976). In contrast, *E. caeruleum* can be found in larger order streams and rivers, allowing for higher levels of gene flow among populations.

The high levels of male-driven behavioral isolation observed among *Ceasia* species was unexpected. Many closely related, allopatric species will readily hybridize upon secondary contact – whether it be in nature or in the laboratory (e.g., Pinceel et al. 2005; Gay et al. 2007; Harper and Hart 2007). Furthermore, males were presented with a no-choice situation in which they could only choose whether or not to pursue the female. No-choice mating assays are thought to under-estimate levels of behavioral isolation (Foote and Larkin 1988; Verrell 1990; Coyne 1993; Hatfield and Schluter 1996). How these high levels of behavioral isolation evolved among recently diverged, allopatric taxa is unclear. There is no support for the idea that genetic distance or male color pattern distance accounts for behavioral isolation within *Ceasia.* One possibility is that the *Ceasia* species pairs we examined were too similar in genetic distance to detect a meaningful signature. Additionally, the number of within-*Ceasia* species pairings analyzed here is admittedly low.

Clearly, behavioral isolation is higher between *Ceasia* and *E. caeruleum* than it is within *Ceasia,* but these two groups differ in multiple aspects. Both genetic distance and male color pattern distance is higher in *Ceasia*-*E. caeruleum* species pairs compared to *Ceasia*-*Ceasia* species pairs. Perhaps more important is the fact that *Ceasia* and *E. caeruleum* occur in sympatry and likely experience reinforcement. Previous work shows a pattern consistent with reproductive character displacement (RCD) between *Ceasia* and *E. caeruleum*, with preferences for conspecifics heightened in sympatry (Zhou and Fuller 2014). Hybridization occurs between *Ceasia* and *E. caeruleum* in nature (Ray et al. 2008; Keck and Near 2009; Bossu and Near 2009; this study), and postzygotic isolation is present (Zhou 2014; unpubl. data). These observations are consistent with reinforcement (Servedio and Noor 2003; Coyne and Orr 2004).

The presence of reinforcement in this system may also explain why males bias their aggression towards conspecific males. Increased male discrimination against heterospecific females in sympatry via reinforcement may incidentally increase the costs associated with heterospecific male fighting. This can potentially favor increased male discrimination against heterospecific males in sympatry, i.e. agonistic character displacement (ACD; Grether et al. 2009; Qvarnström et al. 2012). Our working hypothesis is that (a) male-male aggression is very costly and (b) males are more likely to escalate aggression when fighting over conspecific females. This creates a positive feedback where selection further favors increased levels of recognition for both conspecific (versus heterospecific) females and conspecific (versus heterospecific) males. These high levels of discrimination may, ironically, allow for *Ceasia* and *E. caeruleum* to occur in very close sympatry (i.e., on the same riffles), increasing their potential to hybridize, and further fueling reinforcement (Vallin et al. 2012).

Another untested hypothesis is that cascade reinforcement has caused heightened behavioral isolation among these allopatric species (reviewed in Ortiz-Barrientos et al. 2009), leading to a pattern of cascade RCD and cascade ACD within *Ceasia*. Cascade reinforcement could occur if reinforcement between *E. caeruleum* and *Ceasia* results in either heightened preferences for conspecifics or radically altered target traits such that allopatric *Ceasia* no longer recognize one another as potential mates. Theoretical studies of cascade reinforcement suggest that it is particularly likely to occur in species with low gene flow (reviewed in Comeault and Matute 2016), such as these headwater species of darters. Obviously, the data presented here do not allow us to test this hypothesis as all of the *Ceasia* species were sympatric with *E. caeruleum*. The critical test is whether *Ceasia* that are allopatric to *E. caeruleum* have lower behavioral isolation than *Ceasia* that are sympatric with *E. caeruleum.* Preliminary evidence indicates that this may be the case (in review).

In conclusion, this study found that recently diverged allopatric *Ceasia* have surprisingly high levels of behavioral isolation that is created by male mate choice and male recognition of rival males. Female mate choice was absent. Neither genetic distance nor male color pattern distance account for the levels of behavioral isolation among allopatric taxa. Reinforcement between *Ceasia* and *E. caeruleum* has likely occurred, and may have resulted in heightened levels of behavioral isolation among lineages of *Ceasia* that are allopatric to one another but are sympatric with *E. caeruleum.*

ACKNOWLEDGEMENTS

The treatment of fish in this project was approved by the University of Illinois Institutional Animal Care and Use Committee (protocol #14097). We would like to thank Phred

Benham for assistance with RADseq library preparation, and the Roy J. Carver Biotechnology Center at the University of Illinois for sequencing work. Grant Czadzeck assisted with collecting fish. Makayla Wycoff and Rachel Siller assisted with behavioral and color data collection. Lance Merry provided the images of darters. This research was supported by funding from the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture (project number ILLU 875-952), the National Science Foundation (DGE 1069157, DEB 1210743, DEB 0953716), and the University of Illinois at Urbana-Champaign.

TABLES

Table 2.1. Each of the three species sets used in behavioral assays in Forward (F) and Reverse (R) direction.

Species Set and Direction	*n*	*Ceasia* Focal Pair	Rival Males		
			Conspecific *Ceasia*	Allopatric *Ceasia*	Sympatric *E. caeruleum*
1F	8	*E. fragi*	*E. fragi*	*E. uniporum*	*E. caeruleum*
1R	8	*E. uniporum*	*E. uniporum*	*E. fragi*	*E. caeruleum*
2F	8	*E. fragi*	*E. fragi*	*E. burri*	*E. caeruleum*
2R	8	*E. burri*	*E. burri*	*E. fragi*	*E. caeruleum*
3F	8	*E. fragi*	*E. fragi*	*E. spectabile*	*E. caeruleum*
3R	8	*E. spectabile*	*E. spectabile*	*E. fragi*	*E. caeruleum**

*Eastern clade *E. caeruleum*. *E. caeruleum* in all other trial sets are from the Mississippi River Corridor clade.

Table 2.2. ANOVA on focal male behavior towards rival males. Post-hoc Bonferroni-adjusted pairwise t-tests are shown for significant effects of rival male identity. The table headings (A-C) list the two *Ceasia* species in the species set (*E. fragi* and a heterospecific allopatric *Ceasia* species) followed by the sympatric, distantly related *E. caeruleum*.

A. *E. fragi – E. uniporum – E. caeruleum* (1F and 1R)

Variable: Focal male fin flares	**df**	**Test statistic**	**p**
Rival male identity	2,42	12.526	**<0.00001**
Conspecific vs. allopatric *Ceasia*	45	-3.4811	**0.0034**
Conspecific vs. sympatric *E. caeruleum*	45	-5.7400	**<0.00001**
Focal male identity	1,42	1.2609	0.2679
Rival male identity *focal male identity	2,42	1.2922	0.2854
Variable: Focal male attacks	**df**	**Test statistic**	**p**
Rival male identity	2,42	1.3671	0.2660
Focal male identity	1,42	1.5545	0.2194
Rival male identity *focal male identity	2,42	0.4307	0.6529

B. *E. fragi – E. burri – E. caeruleum* (2F and 2R)

Variable: Focal male fin flares	**df**	**Test statistic**	**p**
Rival male identity	2,42	8.8088	**<0.001**
Conspecific vs. allopatric *Ceasia*	45	-2.9395	**0.016**
Conspecific vs. sympatric *E. caeruleum*	45	-5.6863	**<0.00001**
Focal male identity	1,42	0.3958	0.5327
Rival male identity *focal male identity	2,42	0.2164	0.8063
Variable: Focal male attacks	**df**	**Test statistic**	**p**
Rival male identity	2,42	26.787	**<0.00001**
Conspecific vs. allopatric *Ceasia*	45	-3.2990	**<0.01**
Conspecific vs. sympatric *E. caeruleum*	45	-4.1934	**<0.001**
Focal male identity	1,42	38.870	**<0.00001**
Rival male identity *focal male identity	2,42	9.2328	**<0.001**

C. *E. fragi – E. spectabile – E. caeruleum* (3F and 3R)

Variable: Focal male fin flares	**df**	**Test statistic**	**p**
Rival male identity	2,42	5.1977	**<0.01**
Conspecific vs. allopatric *Ceasia*	45	-1.5213	0.4055
Conspecific vs. sympatric *E. caeruleum*	45	-5.1874	**<0.0001**
Focal male identity	1,42	0.5725	0.4539
Rival male identity *focal male identity	2,42	0.2062	0.8145
Variable: Focal male attacks	**df**	**Test statistic**	**p**
Rival male identity	2,42	2.3781	0.1051
Focal male identity	1,42	0.3329	0.5608
Rival male identity *focal male identity	2,42	0.3329	0.7187

Table 2.3. ANOVA on rival male behavior towards focal male. Post-hoc Bonferroni-adjusted pairwise t-tests are shown for significant effects of rival male identity. The table headings (A-C) list the two *Ceasia* species in the species set (*E. fragi* and a heterospecific allopatric *Ceasia* species) followed by the sympatric, distantly related *E. caeruleum.*

A. *E. fragi – E. uniporum – E. caeruleum* (1F and 1R)

Variable: Rival male fin flares	**df**	**Test statistic**	**p**
Rival male identity	2,42	10.2619	**<0.001**
Conspecific vs. allopatric *Ceasia*	45	-4.8563	**<0.0001**
Conspecific vs. sympatric *E. caeruleum*	45	-7.1910	**<0.0001**
Focal male identity	1,42	1.3630	0.2496
Rival male identity *focal male identity	2,42	0.4606	0.6341
Variable: Rival male attacks	**df**	**Test statistic**	**p**
Rival male identity	2,42	0.9800	0.3834
Focal male identity	1,42	8.4753	**0.0057**
Rival male identity *focal male identity	2,42	2.8879	0.0668

B. *E. fragi – E. burri – E. caeruleum* (2F and 2R)

Variable: Rival male fin flares	**df**	**Test statistic**	**p**
Rival male identity	2,42	13.4599	**<0.0001**
Conspecific vs. allopatric *Ceasia*	45	-4.2851	**<0.001**
Conspecific vs. sympatric *E. caeruleum*	45	-6.8614	**<0.00001**
Focal male identity	1,42	0.6849	0.4126
Rival male identity *focal male identity	2,42	0.3535	0.7043
Variable: Rival male attacks	**df**	**Test statistic**	**p**
Rival male identity	2,42	13.296	**<0.00001**
Conspecific vs. allopatric *Ceasia*	45	-3.2653	**<0.01**
Conspecific vs. sympatric *E. caeruleum*	45	-4.6727	**<0.00001**
Focal male identity	1,42	9.0481	**<0.01**
Rival male identity *focal male identity	2,42	2.2411	0.1189

C. *E. fragi – E. spectabile – E. caeruleum* (3F and 3R)

Variable: Rival male fin flares	**df**	**Test statistic**	**p**
Rival male identity	2,42	10.845	**<0.001**
Conspecific vs. allopatric *Ceasia*	45	-3.4841	**<0.01**
Conspecific vs. sympatric *E. caeruleum*	45	-5.9064	**<0.000001**
Focal male identity	1,42	1.4469	0.2358
Rival male identity *focal male identity	2,42	0.2704	0.7644
Variable: Rival male attacks	**df**	**Test statistic**	**p**
Rival male identity	2,42	9.7455	**<0.001**
Conspecific vs. allopatric *Ceasia*	45	-4.3483	**<0.001**
Conspecific vs. sympatric *E. caeruleum*	45	-5.7662	**<0.00001**
Focal male identity	1,42	0.4254	0.5178
Rival male identity *focal male identity	2,42	0.1418	0.8682

Table 2.4. ANOVA on rival male behavior towards focal female. Post-hoc Bonferroni-adjusted pairwise t-tests are shown for significant effects of rival male identity. The table headings (A-C) list the two *Ceasia* species in the species set (*E. fragi* and another a heterospecific allopatric *Ceasia* species) followed by the sympatric, distantly related *E. caeruleum.*

A. *E. fragi – E. uniporum – E. caeruleum* (1F and 1R)

Variable: Rival male pursuit of focal female	**df**	**Test statistic**	**p**
Rival male identity	2,42	10.054	**<0.001**
Conspecific vs. allopatric *Ceasia*	45	-1.5139	0.4112
Conspecific vs. sympatric *E. caeruleum*	45	-5.9158	**<0.00001**
Focal pair identity	1,42	0.0153	0.9020
Rival male identity*focal pair identity	2,42	0.6469	0.5288

B. *E. fragi – E. burri – E. caeruleum* (2F and 2R)

Variable: Rival male pursuit of focal female	**df**	**Test statistic**	**p**
Rival male identity	2,42	13.606	**<0.00001**
Conspecific vs. allopatric *Ceasia*	45	-3.2371	**<0.01**
Conspecific vs. sympatric *E. caeruleum*	45	-8.6079	**<0.00001**
Focal pair identity	1,42	2.8817	0.0970
Rival male identity*focal pair identity	2,42	1.1867	0.3153

C. *E. fragi – E. spectabile – E. caeruleum* (3F and 3R)

Variable: Rival male pursuit of focal female	**df**	**Test statistic**	**p**
Rival male identity	2,42	5.3156	**<0.01**
Conspecific vs. allopatric *Ceasia*	45	-2.6836	**<0.01**
Conspecific vs. sympatric *E. caeruleum*	45	-5.1759	**<0.000001**
Focal pair identity	1,42	0.5853	0.4485
Rival male identity*focal pair identity	2,42	0.5790	0.5649

Table 2.5. Behavioral isolation indices for male choice (MC), male aggression (MA), and female choice (FC), male color distance (MCD), and Nei's standard genetic distance (D_{ST}). For each species pair the *Ceasia* species that was used as the focal pair is listed first, followed by the species that was used for the rival male (heterospecific *Ceasia* or *E. caeruleum*). Behavioral isolation indices are shown as mean ± standard error.

Species Pair	MC	MA	FC	MCD	D_{ST}
E. fragi - E. uniporum	0.31±0.07	0.38±0.08	0.01±0.01	457.628	0.206
E. fragi - E. burri	0.30±0.07	0.50±0.06	0.02±0.01	547.442	0.242
E. fragi - E. spectabile	0.34±0.10	0.35±0.06	0.01±0.02	341.987	0.260
E. fragi - E. caeruleum	0.76±0.06	0.80±0.05	0.01±0.04	1685.93	0.345
E. uniporum - E. caeruleum	0.70±0.09	0.82±0.06	-0.11±0.13	1937.85	0.346
E. burri - E. caeruleum	0.66±0.08	0.92±0.03	-0.05±0.05	2086.53	0.326
E. spectabile - E. caeruleum	0.78±0.08	0.86±0.04	0.01±0.02	1884.18	0.348

Table 2.6. Population genetic statistics for the four allopatric *Ceasia* species (*E. fragi*, *E uniporum*, *E. burri*, and *E. spectabile*) and the sympatric *E. caeruleum*. Statistics are shown for the 18,295 fixed and variant loci (All Loci) and for the 17,162 variant loci. Statistics were calculated in Stacks (Catchen et al. 2011, 2013). % Poly = percent polymorphic loci, P = average major allele frequency, H_{obs} = observed heterozygosity, π = nucleotide diversity.

Species	***Private Alleles***	***% Poly***	***All Loci P***	***Variant Loci P***	***All Loci H_{obs}***	***Variant Loci H_{obs}***	***All Loci π***	***Variant Loci π***
E. fragi	7,352	0.2167	0.9994	0.9778	0.0008	0.0298	0.0008	0.0308
E. uniporum	8,178	0.2936	0.9991	0.9686	0.0011	0.0401	0.0012	0.0432
E. burri	4,531	0.2334	0.9993	0.9750	0.0009	0.0339	0.0009	0.0338
E. spectabile	4,417	0.1139	0.9997	0.9891	0.0004	0.0147	0.0004	0.0151
E. caeruleum	12,392	0.3396	0.9991	0.9667	0.0011	0.0417	0.0013	0.0463

FIGURES

Figure 2.1. Males from each of the five species examined in this study: (a) *Etheostoma fragi*, (b) *E. uniporum*, (c) *E. burri*, (d) *E. spectabile,* and (e) *E. caeruleum.*

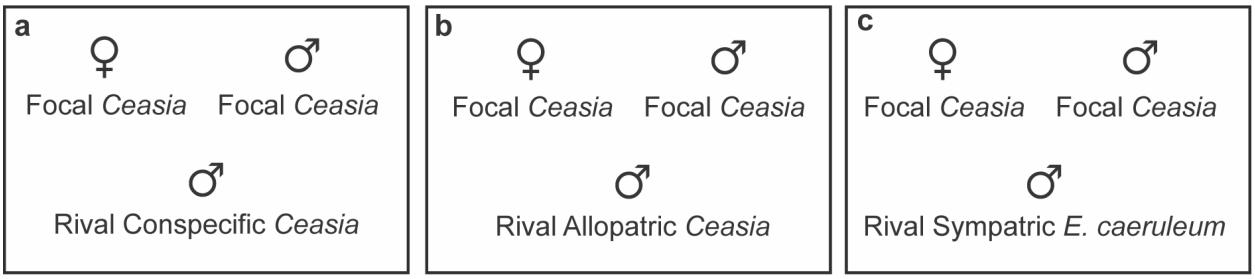

Figure 2.2. Experimental design for behavioral assays. A male and female *Ceasia* focal pair were used in three consecutive trial treatments in which the focal male was either (a) a conspecific *Ceasia*, (b) a heterospecific allopatric *Ceasia*, or (c) a sympatric *E. caeruleum*.

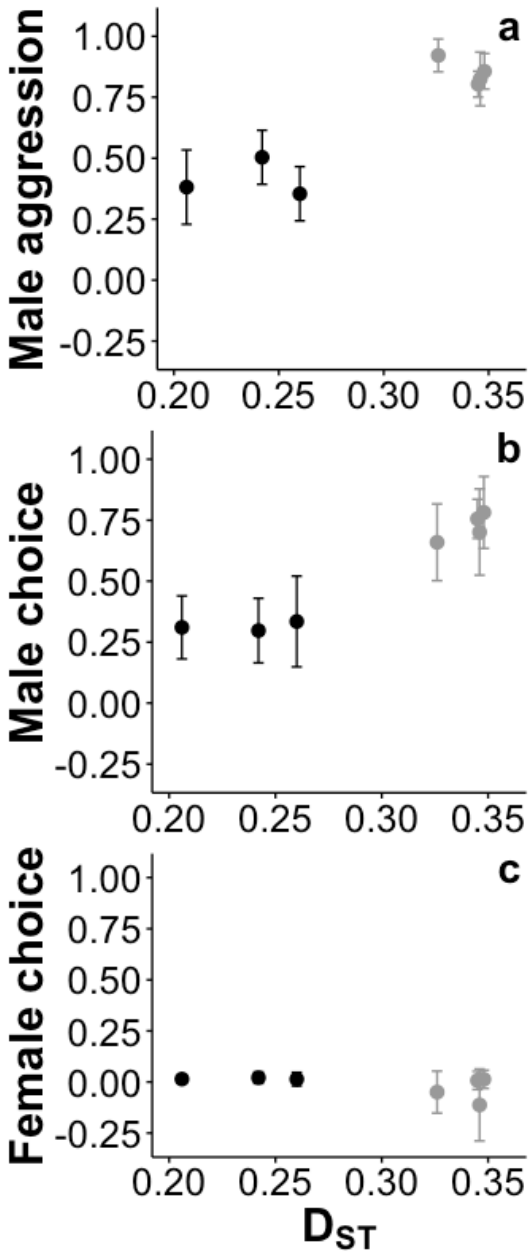

Figure 2.3. Behavioral isolation indices with 95% confidence intervals for (a) male aggression, (b) male choice, and (c) female choice versus Nei's genetic distance (D_{ST}). Each point represents an individual pairwise species comparison. *Ceasia*-*Ceasia* comparisons are shown in black and *Ceasia*-*E. caeruleum* comparisons are shown in gray.

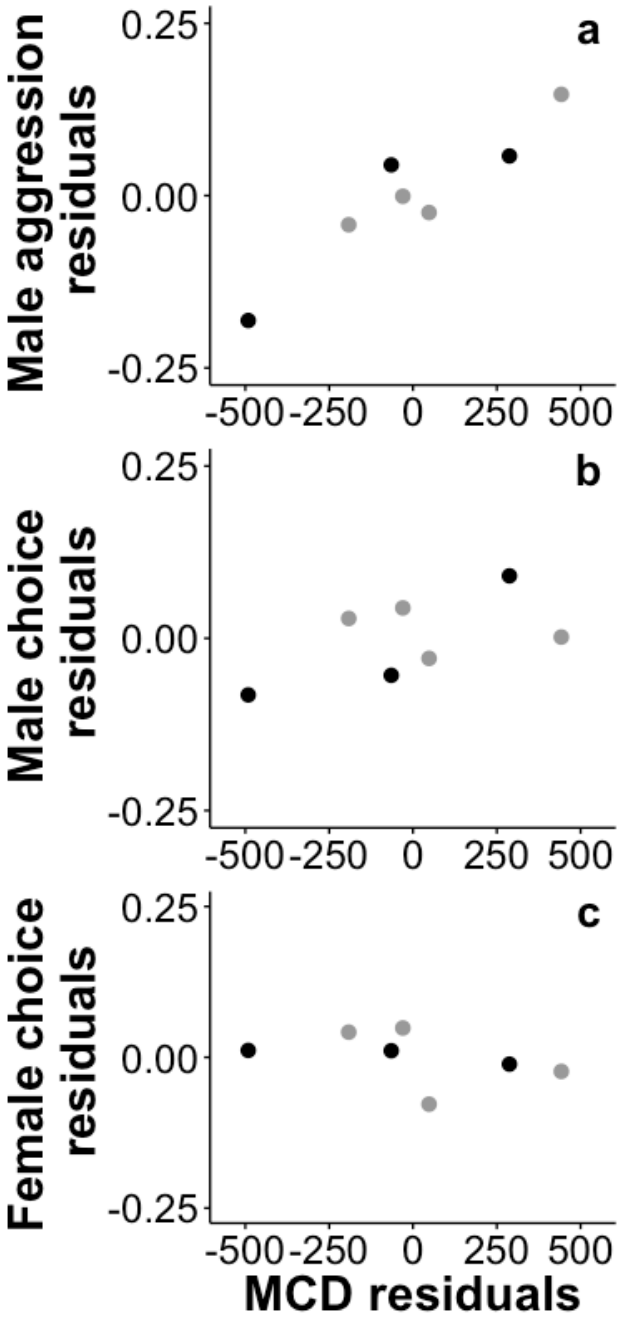

Figure 2.4. Behavioral isolation indices for (a) male aggression, (b) male choice, and (c) female choice versus male color distance (MCD). Each point represents an individual pairwise species comparison. *Ceasia*-*Ceasia* comparisons are shown in black and *Ceasia*-*E. caeruleum* comparisons are shown in gray.

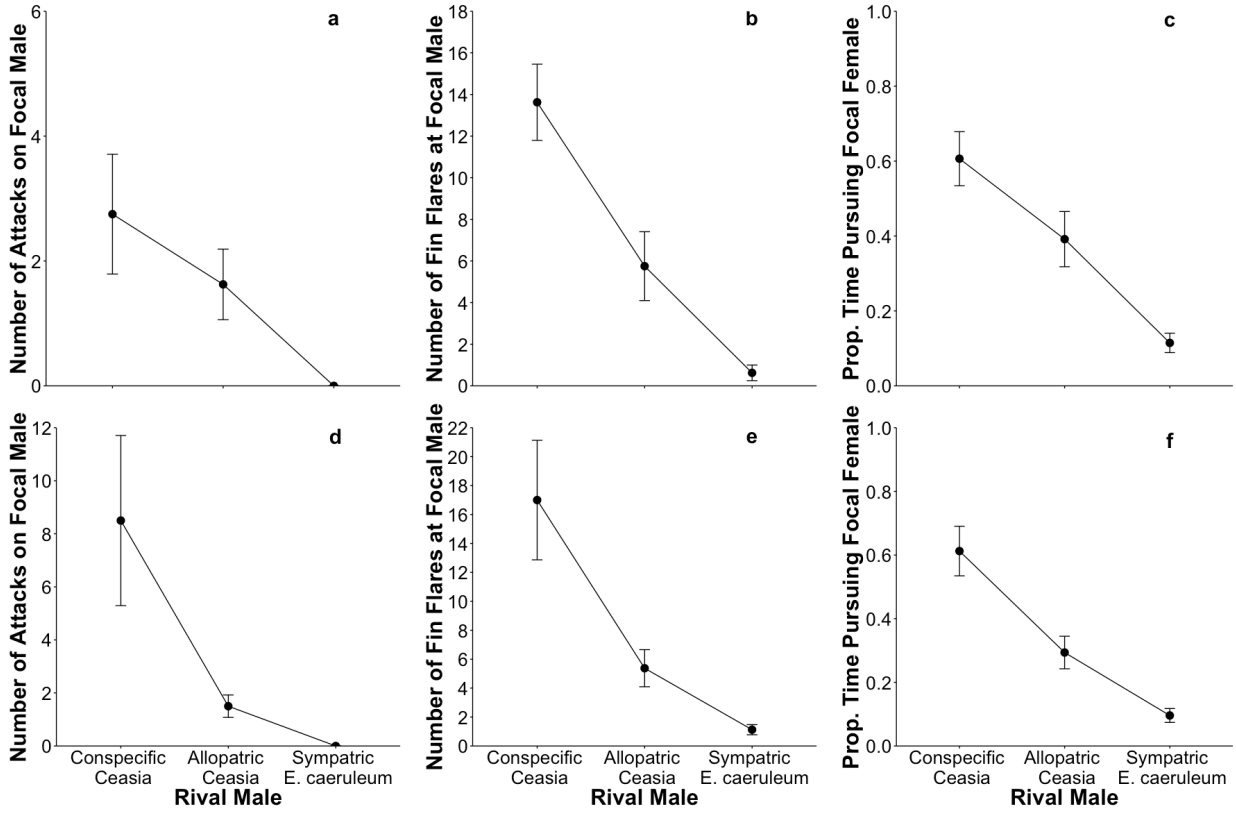

Figure 2.5. Rival male behavior towards focal males and focal females. (a-c) Species set 1F with *E. fragi* as the focal pair and conspecific *Ceasia* rival male, and *E. uniporum* as the allopatric *Ceasia* rival male. (d-f) Species set 1R with *E. uniporum* as the focal pair and conspecific *Ceasia* rival male, and *E. fragi* as the allopatric *Ceasia* rival male. (a,d) Rival male attacks on focal male. (b,e) Rival male fin flares at focal male. (c,f) Rival male pursuit of focal female.

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CHAPTER 3

MALE-DRIVEN REPRODUCTIVE AND AGONISTIC CHARACTER DISPLACEMENT IN DARTERS AND ITS IMPLICATIONS FOR SPECIATION IN ALLOPATRY[2]

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 and cascade ACD). Here, we demonstrate patterns consistent with male-driven RCD and ACD in two 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 cascade RCD 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

[2]Chapter 3 was published in its entirety by Current Zoology. Moran, R. L., and R. C. Fuller. 2018. Male-driven reproductive and agonistic character displacement in darters and its implications for speciation in allopatry. Curr. Zool. 64(1):101–113. This article is reprinted with permission of the publisher and is available from http://academic.oup.com using doi:10.1093/cz/zox069.

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.

INTRODUCTION

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 two species in sympatry compared to 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. Cascade RCD 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 two species are less likely to engage in contests when they occur in sympatry

compared to allopatry. Both RCD and ACD may contribute to trait divergence 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 to test for patterns consistent with RCD, ACD, CRCD, and CACD.

This study focuses on two 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 two *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* (Fig. 3.1). 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 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; R. Moran unpubl. data), providing the potential for RCD to occur via reinforcement (Brown and Wilson 1956; Coyne and Orr 2004). Second, in pairings between four species of *Ceasia* and sympatric *E. caeruleum*, males preferentially mate and fight with conspecifics, suggesting RCD and ACD (Fig. 3.1; Table 3.1; 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 two 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 *E. pulchellum* and *E. caeruleum* that occur in allopatry with respect to one another (Fig. 3.1; Table 3.1). 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 to 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 four *Ceasia* species examined by Moran et al. (2017; Fig. 3.1; Table 3.1), which all occur in sympatry with respect to *E. caeruleum*, prefer conspecific over heterospecific *Ceasia* females and bias their aggression preferentially towards 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 to that previously observed between pairs of *Ceasia* species that occur in sympatry with respect to *E. caeruleum* (Moran et al. 2017).

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 one 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* (Fig. 3.1; Table

3.1). We also tested for a pattern consistent with RCD and ACD in pairings between allopatric *E. pulchellum* and allopatric *E. caeruleum* (Fig. 3.1; Table 3.1). Finally, we tested for a pattern consistent with CRCD and CACD among *Ceasia* species by pairing allopatric *E. spectabile* with allopatric *E. pulchellum* (Fig. 3.1; Table 3.1).

We used two types of behavioral assays ("dichotomous male choice 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; Fig. 3.1; Table 3.1).

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 19º C and fluorescent lighting was provided to mimic the natural photoperiod.

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

Dichotomous male choice assay

We first used a dichotomous male choice 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 (Fig. 3.2a). 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 three 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 three 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) 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 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 (Fig. 3.2b), and once with a rival male that was an *E. caeruleum* (Fig. 3.2c). 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 towards the other male. We calculated four behavioral variables to quantify male aggressive bias towards conspecific males: *focal male fin flare bias*, *focal male attack bias*, *rival male fin flare bias*, and *rival male attack bias* (see Table 3.2). We

asked whether these behavioral variables differed in sympatric versus allopatric pairings. To examine focal male *Ceasia* aggressive behavior, we conducted two 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 two-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 male choice trials), and counted the number of 30-s blocks in which each male pursued the female. Unlike the dichotomous male choice 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 3.2. 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 two-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 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 3.2) 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 for conspecific rival males versus *E. caeruleum* rival males. The number of nosedigs the focal female directed towards 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 male choice assay

To test for patterns consistent with CRCD within *Ceasia*, we paired allopatric *E. spectabile* with allopatric *E. pulchellum* in a dichotomous male choice 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* (Fig. 3.2d). 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 3.2). 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; Fig. 3.2e). 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 3.2. 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 male choice and male competition assays. Repeating all analyses using generalized linear models with a quasibinomial error function and logit link function yielded qualitatively identical results.

Behavioral isolation indices

We used the male aggression, 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 three 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 male aggression (MA) indices for each species pair as:

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

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 male choice (MC) indices as:

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

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), female choice (FC) indices controlled for male pursuit of the female. We calculated the FC indices as:

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

where f_c and f_h represent the number of nosedigs females performed towards conspecific males and towards 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 two sets of comparisons among the three types of behavioral isolation indices (i.e., MA, MC, and FC). First, we tested for differences between *Ceasia-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 towards conspecifics over heterospecifics.

Second, we tested for differences between *Ceasia-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 towards 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 behavioral data are available in the Dryad Digital Repository (http://doi.org/10.5061/dryad.g8d1v).

RESULTS

RCD between Ceasia *and* E. caeruleum

The dichotomous male choice trials revealed a pattern consistent with RCD in focal *Ceasia* male mate preference. RCD predicts that male choice for conspecifics should be heightened in *Ceasia* populations/species that are sympatric with respect to *E. caeruleum. Focal male mate choice* was 2X higher in sympatric *E. spectabile* compared to allopatric *E. spectabile* and allopatric *E. pulchellum*, but did not differ between allopatric *E. spectabile* and allopatric *E.*

pulchellum (Table 3.3; Fig. B.1a). 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 (Fig. B.1b,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 to 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* (Table B.1). 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 to the sympatric *E. caeruleum* rival males (Fig. B.2a). 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 (Fig. B.2b,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 3.4). Females did not exert preference for conspecific males over *E. caeruleum* males, regardless of sympatry with respect to *E. caeruleum* (Table B.2).

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 towards 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 to allopatric *E. spectabile* and allopatric *E. pulchellum* (Table 3.5). Sympatric *E. spectabile* focal males directed 9X more fin flares towards conspecific (versus *E. caeruleum*) rival males (Fig. 3.3d). Similarly, sympatric *E. spectabile* focal males attacked conspecific rival males 6X more than they attacked sympatric *E. caeruleum* rival males (Fig. B.1g). On average, both allopatric *E. spectabile* and allopatric *E. pulchellum* focal males directed an equal number of fin flares (Fig. B.1e,f) and attacks (Fig. B.1h,i) towards 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 towards focal male *Ceasia* compared to allopatric *E. caeruleum* rival males. *Rival male fin flare bias* showed a pattern like that found with focal *Ceasia* males (Table B.3). Sympatric *E. caeruleum* rival males were much less likely to flare their fins towards *E. spectabile* focal males compared to allopatric *E. caeruleum* rival males (Fig. B.2d-f).

Conversely, *rival male attack bias* did not differ between sympatric and allopatric *E. caeruleum* (Table B.3). Both sympatric and allopatric *E. caeruleum* rival males directed a low

number of attacks towards the focal *Ceasia* males (Fig. B.2g-i). Thus, while allopatric *E. spectabile* and allopatric *E. pulchellum* focal males did not bias their aggression more towards 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.

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 two *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* and *E. pulchellum*) that are allopatric with respect to *E. caeruleum* do not prefer conspecific over heterospecific *Ceasia* females. In dichotomous male choice trials, *focal male mate choice* did not differ between allopatric *E. spectabile* and allopatric *E. pulchellum* ($F_{1,22} = 0.29$; $p = 0.60$; Fig. B.3a,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* ($F_{1,22} = 0.12$; $p = 0.73$; Fig. B.4).

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 (Table B.4). There was no significant difference in the proportion of female nosedigs towards rival males as function of their identity (conspecific or heterospecific) when we controlled for the proportion of time each male pursued the female (Table B.5).

CACD between Ceasia *species*

CACD predicts that *Ceasia* males that are sympatric with respect to *E. caeruleum* should bias their aggression towards conspecific over heterospecific *Ceasia* males, despite the fact that the two *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 towards 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* ($F_{1,22} = 1.79$; $p = 0.19$; Fig. B.3c,d), nor did *focal male attack bias* ($F_{1,22} = 0.84$; $p = 0.37$; Fig. B.3e,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 towards focal males (Fig. B.4d). However, in trials where allopatric *E. spectabile* served as focal males, the allopatric *E. pulchellum* rival males directed more fin flares towards the focal males compared to the conspecific *E. spectabile* rival males (Fig. B.3c). This resulted in a significant difference in *rival*

male fin flare bias between allopatric *E. spectabile* and allopatric *E. pulchellum* ($F_{1,22} = 5.79$; $p = 0.025$; Fig. B.4), 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 ($F_{1,22} = 0.10$; $p = 0.75$; Fig. B.4).

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; Figs. 3.3 and 3.4). The pattern in male mating preference was consistent with RCD between *Ceasia* - *E. caeruleum* species pairs and CRCD between *Ceasia* - *Ceasia* species pairs. MC indices were consistently higher between sympatric species pairs compared to 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 to allopatric species pairs ($F_{1,82} = 56.35$, $p < 0.0001$; Fig. 3.3). 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 ($F_{1,70} = 6.64$, $p = 0.01$; Fig. 3.4). The difference in MC indices in sympatry versus allopatry was greater in *Ceasia* - *E. caeruleum* pairings than in *Ceasia*-*Ceasia* pairings (Table 3.6).

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 3.6; Figs. 3.3 and 3.4). This was due to females not exerting any detectable mating preferences for conspecific males.

We observed a pattern consistent with divergent ACD between *Ceasia* - *E. caeruleum* species pairs and CACD between *Ceasia* - *Ceasia* species pairs. MA indices were consistently higher between sympatric species pairs compared to 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 ($F_{1,166} = 136.30$, $p < 0.0001$; Fig. 3.3; indicating ACD) and within the *Ceasia* - *Ceasia* comparisons ($F_{1,142} = 34.17$, $p < 0.0001$; Fig. 3.4; indicating CACD). MA was higher between sympatric *Ceasia*-*E. caeruleum* pairs than it was in sympatric *Ceasia*-*Ceasia* pairs (Table 3.6).

DISCUSSION

Striking patterns of RCD and ACD driven by male behavior are present at two taxonomic levels within *Ceasia*. First, we found evidence for both RCD and ACD among populations within species (Figs. 3 and 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 3.3, 3.4, and B.2). We also found evidence of divergent ACD among populations within *E. spectabile* and *E. caeruleum*. Males preferentially biased their aggression towards conspecific males to a greater extent in sympatric population pairings (Table 3.5). Second, we found evidence for ACD and RCD among closely related species in the *Ceasia* species complex. Males showed no preference for mating (Table 3.3) or fighting (Table 3.5) 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 (Fig. B.2; Table B.1) and ACD in male aggression bias in *E. caeruleum* (Fig. B.2; Tables B.3), 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 towards *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 male aggression bias spreading beyond the zone of sympatry. Indeed, population genetic analyses of four species of *Ceasia* and *E. caeruleum* found increased heterozygosity and higher

levels of nucleotide diversity present in *E. caeruleum* compared to *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; Fig. 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 towards conspecific over heterospecific *Ceasia* males (Figs. B.3 and B.4). 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 male aggression 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 two 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). Female choice 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; R. Moran unpubl. 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 two 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.

ACKNOWLEDGEMENTS

We thank Alycia Lackey, Michael Martin, and Robin Tinghitella for inviting us to submit an article for this special column. We also thank Michelle St. John, Lisa Mitchem, Michael Martin and three anonymous reviewers for providing feedback that improved the quality of the manuscript. We are grateful to Grant Czadzeck for assistance with collecting fish. Images of ranges for each species (shown in Figure 3.1) were modified from SVG files obtained from Wikimedia Commons (Etheostoma_caeruleum_distribution.svg, Etheostoma_spectabile_distribution.svg, Etheostoma_pulchellum_distribution.svg, Etheostoma_fragi_distribution.svg, Etheostoma_uniporum_distribution.svg, Etheostoma_burri_distribution.svg, by Ninjatacoshell and licensed under CC BY-SA 3.0), which were based on ranges depcited in Bossu and Near (2009). This work was supported by the Cooperative State Research, Education, and Extension Service, US Department of Agriculture, under project number ILLU 875-952, the National Science Foundation (DEB 0953716 and IOS 1701676), and the University of Illinois. The treatment of animals was approved by the Institutional Animal Care and Use Committee under protocol #14097.

TABLES

Table 3.1. Collection locations for populations of each species examined in behavioral trials in the present study as well as in Moran et al. (2017). 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 on Figure 3.1.

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	Current study
2	Sympatric	*E. spectabile*	40.054447, -88.089887	Unnamed tributary, Salt Fork of Vermillion River, Champaign County, IL	Current study and Moran et al. (2017)
3	Sympatric	*E. caeruleum*	(Same as above)	(Same as above)	Current study and Moran et al. (2017)
4	Allopatric	*E. spectabile*	40.027663, -88.577180	Unnamed tributary, Sangamon River, Piatt County, IL	Current study
5	Allopatric	*E. pulchellum*	38.952839, -95.517654	Deer Creek, Kansas River, Shawnee County, KS	Current study
6	Sympatric	*E. fragi*	36.304214, -91.927684	Rose Branch tributary of Strawberry River, Fulton County, AR	Moran et al. (2017)

Table 3.1. Continued Collection locations for populations of each species examined in behavioral trials in the present study as well as in Moran et al. (2017). 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 on Figure 3.1.

7	Sympatric	*E. uniporum*	36.250560, -91.359318	Unnamed tributary of Spring River, Sharp County, AR	Moran et al. (2017)
8	Sympatric	*E. caeruleum**	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)

**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).

Table 3.2. Definition of the behavioral variables measured in the dichotomous male choice assay and the male competition assay. 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.

Variable	Definition	RCD	ACD	CRCD	CACD
Dichotomous Male Choice 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 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
Focal Female Mate Choice	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
Focal Male Fin Flare Bias	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

Table 3.2. Continued Definition of the behavioral variables measured in the dichotomous male choice assay and the male competition assay. 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.

Focal Male Attack Bias	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
Rival Male Fin Flare Bias	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
Rival Male Attack Bias	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

+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 3.3. Results of ANOVA testing for RCD in *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* males in three study populations: sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*. Pairwise post-hoc t-test results are also shown for the analysis. P <0.05 indicated in bold.

Focal male mate choice	**df**	**Test Statistic**	**p**
Focal *Ceasia* population identity	2,33	45.21	**<0.00001**
Sympatric *E. spectabile* vs. allopatric *E. spectabile*	22	11.38	**<0.00001**
Sympatric *E. spectabile* vs. allopatric *E. pulchellum*	22	8.10	**<0.00001**
Allopatric *E. spectabile* vs. allopatric *E. pulchellum*	220	-0.38	0.71

Table 3.4. Results ANCOVA testing for RCD in *focal female mate choice* between conspecific rival males and *E. caeruleum* rival males in male competition trials. We asked whether *focal female mate choice* differed among focal *Ceasia* females in three 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.

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

Table 3.5. Results of ANOVA testing for ACD in focal *Ceasia* male aggression bias in male competition trials. We asked whether *focal male fin flare bias* and *focal male attack bia*s differed among focal *Ceasia* males in three study populations: sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum*. Pairwise post-hoc t-test results are also shown for both analyses. P <0.05 indicated in bold.

Focal male fin flare bias	**df**	**Test Statistic**	**p**
Focal *Ceasia* population identity	2,33	8.34	**0.0012**
Sympatric *E. spectabile* vs. allopatric *E. spectabile*	22	5.28	**<0.0001**
Sympatric *E. spectabile* vs. allopatric *E. pulchellum*	22	2.85	**0.0093**
Allopatric *E. spectabile* vs. 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* vs. allopatric *E. spectabile*	22	4.53	**0.0002**
Sympatric *E. spectabile* vs. allopatric *E. pulchellum*	22	3.82	**<0.001**
Allopatric *E. spectabile* vs. allopatric *E. pulchellum*	22	-0.65	0.52

Table 3.6. Behavioral isolation indices (mean ± standard error) for male aggression (MA), male choice (MC), and female choice (FC), calculated from male competition assays that paired two *Ceasia* species or paired *Ceasia* with *E. caeruleum*. 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 two *Ceasia* species, or RCD/ACD in pairings between *Ceasia* and *E. caeruleum*) are listed.

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**	CRCD/CACD	16	0.38±0.08	0.31±0.07	0.01±0.01
Sympatric	*Ceasia - Ceasia*	*E. fragi - E. burri**	CRCD/CACD	16	0.50±0.06	0.30±0.07	0.02±0.01
Sympatric	*Ceasia - Ceasia*	*E. fragi - E. spectabile**	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**	RCD/ACD	48	0.80±0.05	0.76±0.06	0.01±0.04
Sympatric	*Ceasia - E. caeruleum*	*E. uniporum - E. caeruleum**	RCD/ACD	16	0.82±0.06	0.70±0.09	-0.11±0.13
Sympatric	*Ceasia - E. caeruleum*	*E. burri - E. caeruleum**	RCD/ACD	16	0.92±0.03	0.66±0.08	-0.05±0.05
Sympatric	*Ceasia - E. caeruleum*	*E. spectabile - E. caeruleum***	RCD/ACD	32	0.85±0.05	0.84±0.06	0.03±0.02

*Data from Moran et al. (2017).

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

FIGURES

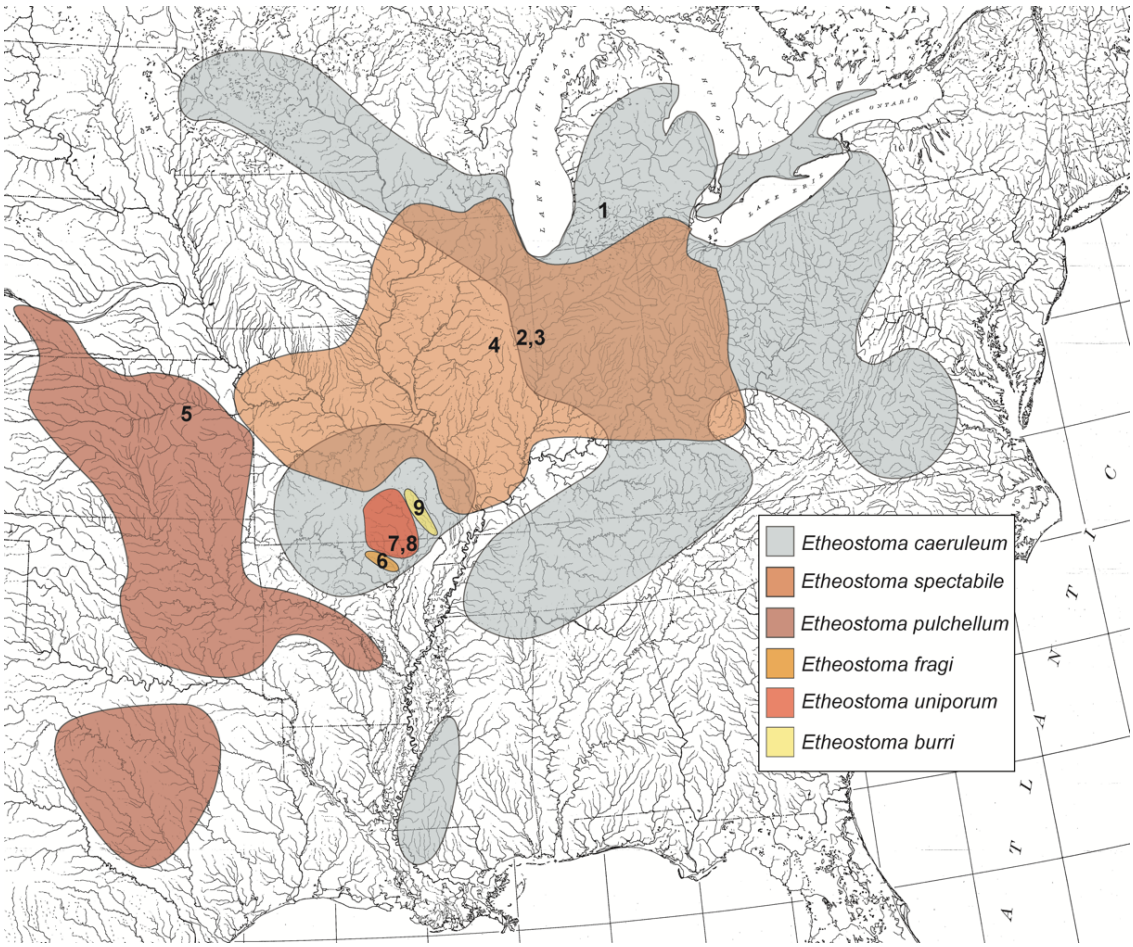

Figure 3.1. Ranges for *Etheostoma caeruleum* and five *Ceasia* species (*Etheostoma spectabile*, *Etheostoma pulchellum*, *Etheostoma fragi*, *Etheostoma uniporum*, and *Etheostoma burri*) used in behavioral assays in the current study and in Moran et al. (2017). Numbers on the map represent approximate collection locations for study populations (see Table 1 for details).

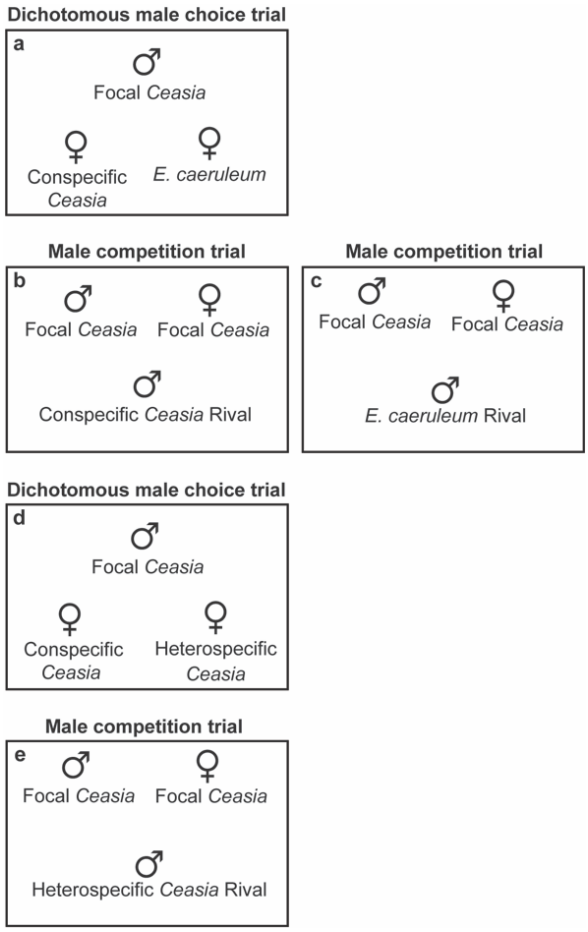

Figure 3.2. (a-c) Trials testing for RCD and ACD. In these trials, sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* served as focal *Ceasia* in turn. Note that in (a) and (c), allopatric *E. caeruleum* were paired with allopatric focal *Ceasia*, and sympatric *E. caeruleum* were paired with sympatric focal *Ceasia*. (a) Experimental set up for dichotomous male choice 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 male choice 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 heterospecific *Ceasia* rival 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).

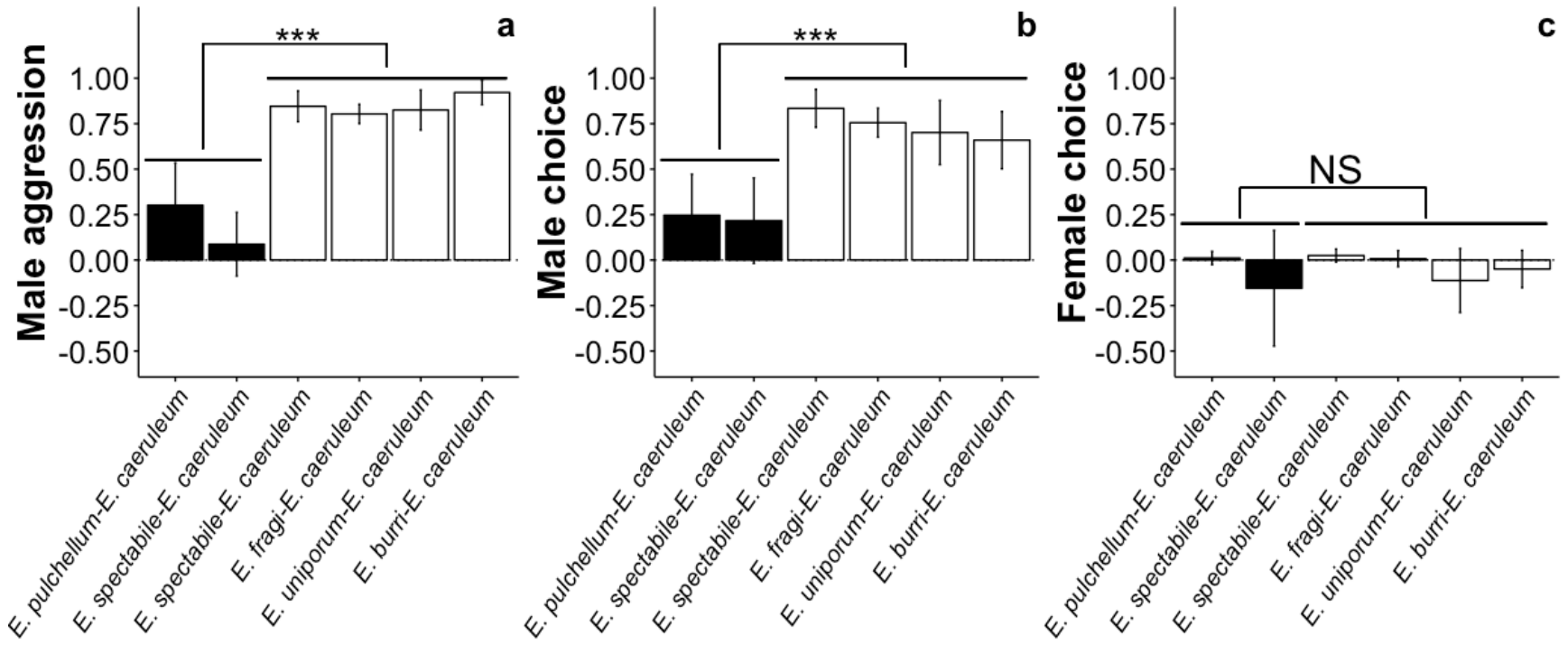

Figure 3.3. Patterns of RCD and ACD between *Ceasia* and *E. caeruleum*. Behavioral isolation indices (with 95% confidence intervals) for (a) male aggression, (b) male choice, and (c) female choice for comparisons between *Ceasia* species and *E. caeruleum.* Allopatric comparisons (i.e., those including *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.

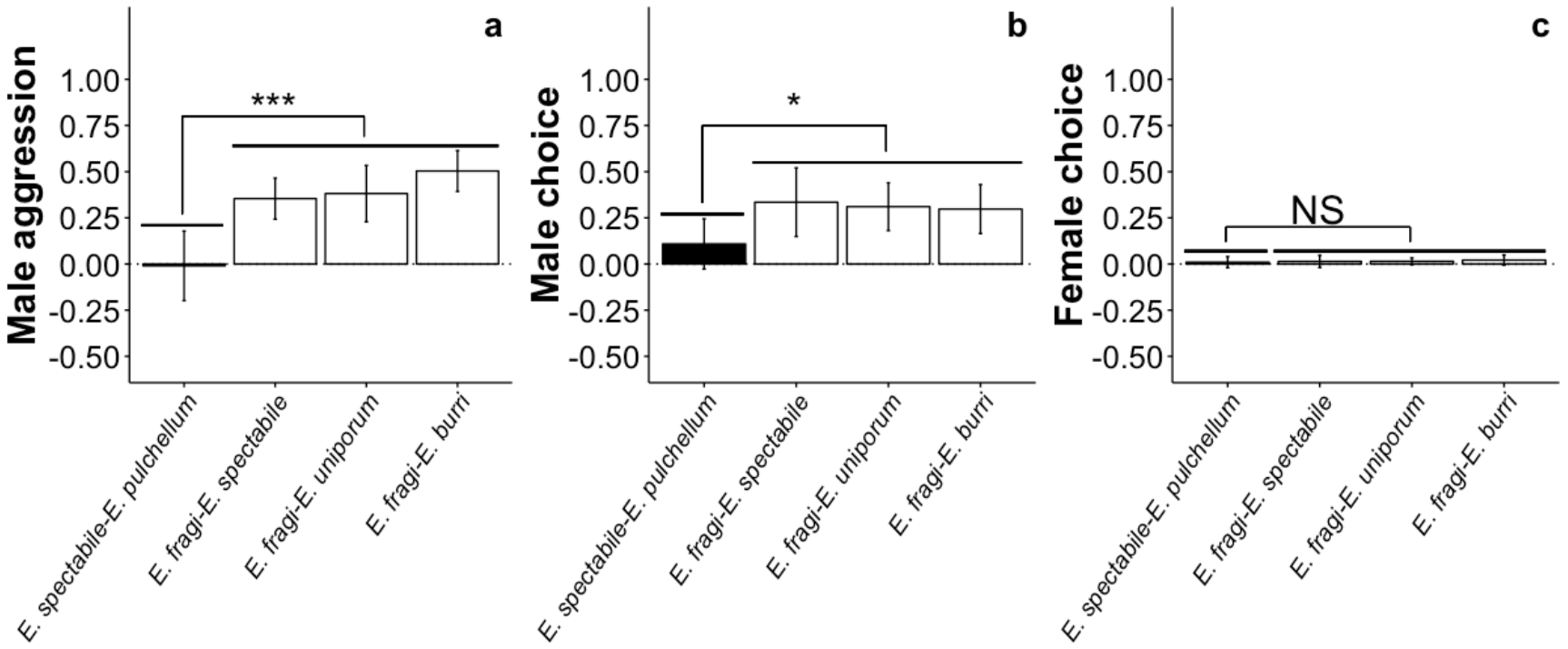

Figure 3.4. Patterns of CRCD and CACD between *Ceasia* species. Behavioral isolation indices (with 95% confidence intervals) for (a) male aggression, (b) male choice, and (c) female choice between pairs of *Ceasia* species. Allopatric comparisons (i.e., comparisons including *Ceasia* species that both 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.

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CHAPTER 4

EGG VIABILITY DECREASES RAPIDLY WITH TIME SINCE OVULATION IN THE RAINBOW DARTER: IMPLICATIONS FOR THE COSTS OF CHOOSINESS[3]

ABSTRACT

Egg viability in the rainbow darter *Etheostoma caeruleum*, a fish apparently lacking female mate choice, was found to decline rapidly after ovulation. It was observed that the majority of a female's clutch may fail to hatch if she is prevented from mating for as little as six hours. These data suggest that exercising female mate preferences may be selectively disfavoured in *E. caeruleum* due to the high cost of delaying mating.

INTRODUCTION

The degree of discrimination exhibited by female animals when choosing a mate ranges from none to extreme. This diversity exists because female mate choice is shaped by a number of variables, including the subset of males made available to her by environmental factors and male-male interactions (Beehler and Foster 1988; Jennions and Petrie 1997; Wong and Candolin 2005), and the costs of mate choice behaviour (Janetos 1980; Real 1990; Kokko et al. 2003). If the costs of choosing outweigh the benefits, the most favoured strategy would be to mate with

[3] Chapter 4 was published in its entirety by Journal of Fish Biology. Moran, R. L., R. M. Soukup, M. Zhou, and R. C. Fuller. 2018. Egg viability decreases rapidly with time since ovulation in the rainbow darter *Etheostoma caeruleum*: implications for the costs of choosiness. Journal of Fish Biology. 92(2):532–536. This article is reprinted with permission of the publisher and is available from http://onlinelibrary.wiley.com using doi:10.1111/jfb.13523.

the first male the female encounters, i.e. random mating. Several costs to female mate choice (e.g. time and energy expenditure, predator exposure) have been investigated across a variety of taxa (reviewed in Reynolds and Gross 1990; Jennions and Petrie 1997), but one has thus far received little attention: decline in gametic quality over time. In fishes, the phenomenon of decreasing egg viability post-ovulation, termed egg overripening, has been reported across a range of species including Atlantic salmon *Salmo salar* (de Gaudemar and Beall 1998), goldfish *Carassius auratus* (Formacion et al. 1993), Atlantic halibut *Hippoglossus hippoglossus* (Bromage et al. 1994), and turbot *Scophthalmus maximus* (McEvoy 1984). Having eggs susceptible to overripening would pressure females to spawn quickly, as waiting could incur a substantial fitness cost. Such selection for rapid mate acquisition may consequently lead to a decrease in female choosiness and/or influence the expression of female mating preferences.

The rainbow darter *Etheostoma caeruleum* is a small benthic fish common in freshwater streams across the eastern United States (Page 1983). During the breeding season from late March to early June, vividly coloured males attempt to guard gravid females from rival males. Females signal their readiness to spawn by performing nosedigs, wherein she pushes her head into the gravel. Spawning involves the female burying herself shallowly in gravel with an arched posture; once a male takes position above her, both vibrate and release eggs and sperm. No parental care is practiced (Winn 1958; Fuller 2003).

Although there is evidence that female *E. caeruleum* favour some types of males in dichotomous trials, such preferences have little apparent effect on the outcome of mating due to male-male competition (Fuller 2003). In any case, female *E. caeruleum* demonstrate no overt choice when allowed to interact freely with males: having assumed the spawning position, the female always spawns with the first male to arrive regardless of his characteristics (R. C. Fuller

and M. Zhou, pers obs.), suggesting that exercising choice may be disadvantageous. Good reasons exist to suspect that mate choice in female *E. caeruleum* may harbour a cost in prolonging the time between ovulation and spawning: in previous experiments, females have been observed expelling and subsequently eating unfertilized eggs when held in isolation (Zhou and Fuller 2014). Furthermore, isolated females subsequently allowed to spawn with males often produce entirely inviable clutches (R. L. Moran, pers obs). This study aimed to formally test the hypothesis that delaying spawning is costly for female *E. caeruleum*, by quantifying change in egg viability as a function of time since ovulation.

METHODS

Etheostoma caeruleum were collected by kick seine from Mill Pond Outlet (Kalamazoo Co., Michigan) in April and May 1998 (year 1) and from an unnamed tributary of the Saline Branch Drainage Ditch (Champaign Co., Illinois) in March 2017 (year 2). Fish from year 1 were maintained at the Kellogg Biological Station. Fish from year 2 were maintained at the University of Illinois at Urbana-Champaign. In both years, fish were housed in male-female pairs in 38 litre aquariums with gravel substrate, maintained at external ambient temperature and light:dark cycle. The fish were fed frozen bloodworms (chironomid larvae) and live tubifex worms twice per day.

Fish were monitored during daylight hours over four to five days post-capture. A female was assumed to have recently ovulated when she performed a nosedig. After a female performed a nosedig, she was moved to an empty tank. Females were kept in isolation for various lengths of time: 0 hours (n=10), 6 hours (n=6), 12 hours (n=7), 24 hours (n=5). Following the isolation

period, a male was introduced. Unique males were used for each female. All males were clearly in breeding condition as evidenced by their nuptial coloration. Males were visually size matched and care was taken to avoid smaller males that might have low sperm production. After the introduction of the male, the pair were left together for 4 hours or until the female finished spawning her clutch. The resulting eggs were collected with a siphon and placed in small tubs filled with water; dilute methylene blue was added to inhibit fungal growth. Hatching success was recorded as the number of eggs yielding fry out of total number of eggs collected. A quasibinomial regression with a logit link function was used to test for a relationship between hatching success and female isolation time. To determine whether there was an effect of collection year and location on hatching success, year was included as a covariate in the model. The quasibinomial error distribution was used to account for overdispersion in the response variable (i.e., hatching success). Statistical analysis was performed in R (version 3.4.0).

RESULTS

All females spawned following the introduction of a male. The number of eggs collected ranged from 17 to 110 (Table 4.1), and was uncorrelated with isolation time or year. Hatching success declined strongly as a function of increasing female isolation time ($F_{1,25} = 5.91$, $p < 0.05$; Fig. 4.1). There was no effect of year on hatching success ($F_{1,25} = 3.30$, $p = 0.08$). Although hatching success varied at each female holding time (Fig. 4.1), these data suggest that on average, greater than 50% of a female's clutch is likely to become non-viable if retained for as little as six hours after ovulation.

DISCUSSION

Given that hatching success declines precipitously over time, gravid female *E. caeruleum* appear to be under strong pressure to fully spawn a clutch of eggs in less than 24 hours. Females who fail to do so risk substantial egg mortality. The time constraint for spawning may be exacerbated by the fact that female *E. caeruleum* release only a small fraction of ovulated eggs per spawning bout and thus must spawn multiple times to fully expel an entire clutch (Fuller 1998). Under these conditions, the cost for a female *E. caeruleum* to reject a male may be unacceptably high.

Female mate choice in *E. caeruleum* may be further disfavoured by strong male-male competition. Male *E. caeruleum* fight vigorously for access to gravid females, attempting to monopolize spawning and prevent the participation of "sneaky" males (Winn 1958; Fuller 2003). Hence, the choice of males within a single patch is likely limited to those that are competitively superior. Furthermore, there may be little additional benefit for the female to choose following male-male competition if male competitive ability predicts fitness benefits to females and her offspring (Wong and Candolin 2005).

Darters are a highly speciose clade that have received increasing attention from evolutionary biologists over the past decade. Spectacular and diverse male colouration in darters has been suggested to act as an agent of speciation by sexual selection, with the most commonly posited mechanism being divergent female mate choice (Mendelson 2003; Williams and Mendelson 2010; Williams et al. 2013). However, evidence is mounting that female choice is limited in at least some darter species (Pyron 1995; Fuller 2003; Zhou et al. 2015; Moran et al. 2013, 2017). This study suggests that female preference in darters is likely costly due to the need

to spawn shortly after ovulation while egg viability remains high. Considering that egg overripening seems to be common across a variety of fish species, often occurring over time frames comparable to or shorter than in *E. caeruleum* (Kjørsvik et al. 1990), its role in shaping female mate choice may be underappreciated.

ACKNOWLEDGEMENTS

This work was supported by the Cooperative State Research, Education, and Extension Service, US Department of Agriculture, under project number ILLU 875-952, the National Science Foundation (DEB 0953716 and IOS 1701676), and the University of Illinois. The treatment of animals was approved by the Institutional Animal Care and Use Committee under protocol #17031.

TABLE

Table 4.1. Isolation time (hrs held after ovulation), total number of eggs collected, and total number of eggs hatched for each female.

Female	Isolation time (hrs)	Year	Eggs collected	Eggs hatched
1	0	1998	83	83
2	0	1998	22	10
3	0	1998	30	25
4	0	1998	110	86
5	0	2017	56	10
6	0	2017	47	31
7	0	2017	27	26
8	0	2017	30	20
9	0	2017	25	16
10	0	2017	28	27
11	6	1998	47	10
12	6	2017	17	11
13	6	2017	20	12
14	6	2017	46	0
15	6	2017	63	59
16	6	2017	21	5
17	12	1998	53	3
18	12	2017	17	6
19	12	2017	21	6
20	12	2017	23	17
21	12	2017	20	11
22	12	2017	45	16
23	12	2017	37	22
24	24	1998	71	15
25	24	1998	92	0
26	24	2017	66	31
27	24	2017	60	12
28	24	2017	54	40

FIGURE

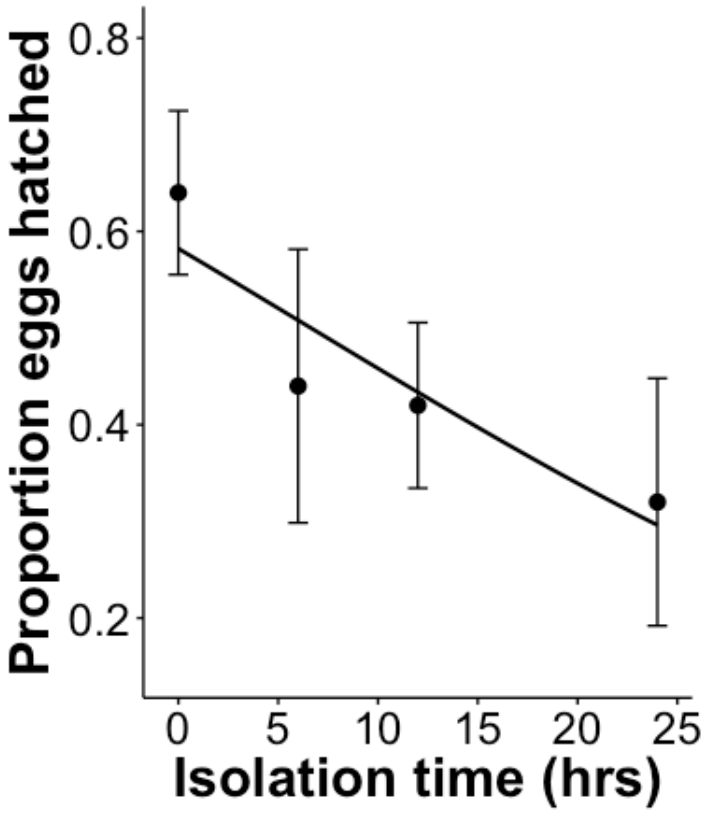

Figure 4.1. The proportion of eggs that hatched from a clutch (mean ± standard error) decreased with increasing time that a female was held in isolation (i.e. prevented from spawning) after ovulation occurred.

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CHAPTER 5

AGONISTIC CHARACTER DISPLACEMENT OF GENETICALLY BASED MALE COLOR PATTERNS ACROSS DARTERS[4]

ABSTRACT

Agonistic character displacement (ACD) occurs when selection to avoid maladaptive interspecific aggression leads to the evolution of agonistic signals and/or associated behavioral biases in sympatry. Here we test for a pattern consistent with ACD in male color pattern in darters (Percidae: *Etheostoma*). Male color pattern has been shown to function in male-male competition rather than female mating preferences in several darter species. Additionally, males bias their aggression towards conspecific over heterospecific males in sympatry but not in allopatry, consistent with divergent ACD in male behavioral biases. We use a common garden approach to show that differences in male color pattern among four closely related darter species are genetically based. Additionally, we demonstrate that some aspects of male color pattern exhibit enhanced differences in sympatric compared to allopatric populations of two darter species, consistent with ACD. However, other male color traits are more similar between species in sympatry compared to allopatry, indicating that not all signal components are under strong divergent selection in sympatry. This study provides evidence that interspecific male-male

[4]Chapter 5 was published in its entirety by Proceedings of the Royal Society B: Biological Sciences. Moran, R. L., and R. C. Fuller. 2018a. Agonistic character displacement of genetically based male colour patterns across darters. Proc. R. Soc. B Biol. Sci. 285:20181248. This article is reprinted with permission of the publisher and is available from http://royalsocietypublishing.org using doi:10.1098/rspb.2018.1248.

aggressive interactions alone can promote elaborate male signal evolution both between and within species. We discuss the implications this has for male-driven ACD and cascade ACD.

INTRODUCTION

Evolutionary biologists have long been interested in secondary contact events between previously allopatric lineages because they provide valuable insight into the process of speciation. Secondary contact can result in a variety of outcomes depending on the degree of reproductive isolation that has accrued (Harrison 1993; Liou and Price 1994; Coyne and Orr 2004). For example, exploitative competition over shared resources might cause one lineage to go locally extinct. Another possibility is that the two lineages freely hybridize upon secondary contact and collapse into a hybrid swarm. Alternatively, selection against maladaptive hybridization between lineages can promote the evolution of reproductive character displacement (RCD), thereby finalizing the speciation process in sympatry. RCD occurs when selection to avoid interspecific mating results in the evolution of mating traits (signals and/or preferences) (Brown and Wilson 1956; Coyne and Orr 2004). Studies of RCD have focused largely on the evolution of female mating preferences and associated male traits (reviewed in 5). However, male mating preferences for female traits can also promote speciation via RCD (Wiernasz 1995; Gabor and Ryan 2001; Albert and Schluter 2004; Shine et al. 2004; Servedio 2007; Kozak et al. 2015). Furthermore, a growing number of studies indicate that interspecific male-male competitive interactions can influence trait divergence and speciation in sympatry via agonistic character displacement (ACD) (Qvarnström et al. 2012; Vallin et al. 2012; Okamoto and Grether 2013; Drury and Grether 2014; Moran and Fuller 2018). Similar to RCD, ACD occurs when

selection to avoid interspecific fighting results in the evolution of competitive traits (signals and/or aggression biases) (Grether et al. 2009, 2017). Both RCD and ACD can result in a pattern of enhanced trait divergence between species in sympatry compared to allopatry.

When gene flow among populations within a species is low, RCD and ACD can incidentally cause mismatches among populations within a species in traits associated with mate/competitor evaluation (Comeault and Matute 2016; Yukilevich and Aoki 2016). The evolution of trait divergence among allopatric populations as a correlated effect of character displacement between sympatric species is termed "cascade" character displacement. Cascade RCD can cause increased behavioral isolation among populations within species. Cascade ACD can alter the likelihood of competitive interactions in secondary contact. Although cascade RCD has been demonstrated in a variety of taxa (Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010), darters (Percidae: *Etheostoma*) represent the only documented example of cascade ACD (Moran and Fuller 2018).

This study tests for divergent ACD in a male color pattern in darters, a diverse group of North American stream fishes. Verifying that the evolution of a given signal trait is a product of divergent ACD requires demonstrating: (1) that the signal functions in competitive interactions (rather than male-female mating interactions), (2) that the signal is genetically based and not due to environmental differences between sympatry and allopatry, and (3) that a geographic pattern of enhanced signal divergence between species in sympatry compared to allopatry is present (Grether et al. 2009, 2017; Pfennig and Pfennig 2012). Several recent studies have shown that male color pattern functions in male-male competition in darters. Within species, aspects of male color pattern predict a male's ability to guard a female from rival males and consequently correlate with reproductive success (Zhou and Fuller 2016). Male color pattern also functions in

male discrimination between conspecific versus heterospecific male competitors (Zhou et al. 2015; Martin and Mendelson 2016a; Moran et al. 2017). Furthermore, there is evidence for cascade RCD and cascade ACD because RCD and ACD between rainbow darters and species in the orangethroat clade leads to heightened isolation between allopatric orangethroat species (see Study System below). Here, we use a common garden approach to ask whether differences in male color pattern present among four closely related species of darters are genetically based. We then compare multivariate measurements of male color pattern in sympatric and allopatric population pairs in two darter species to test whether color pattern divergence is enhanced in sympatry compared to allopatry. This study provides important insight into the evolution of an elaborate sexually dimorphic color trait in a highly diverse group of vertebrates with traditional sex roles but no apparent female mating preferences. Our results demonstrate how interspecific male-male competition can lead to color pattern divergence between and within species and has implications for RCD, ACD, cascade RCD, and cascade ACD in darters.

METHODS

Study System

This study focuses on two groups of darters: the orangethroat darter clade (*Etheostoma*: *Ceasia*) and the rainbow darter (*Etheostoma caeruleum*). The orangethroat darter clade includes 15 recently-diverged allopatric species. These new species have been diagnosed over the past several decades based largely on qualitative differences in male color pattern among populations in different drainages (Distler 1968; Ceas and Page 1997). Recent research suggests that the dramatic diversification within the orangethroat darter clade may be driven by selection against

reproductive and agonistic interactions with the rainbow darter. Thirteen out of the 15 species within the orangethroat clade occur in sympatry with the rainbow darter. These species hybridize at low levels in sympatry, and a substantial amount of postzygotic isolation is present in the form of male-skewed F1 hybrid sex ratios and high levels of backcross hybrid inviability (Moran et al. 2018). When orangethroat and rainbow darters co-occur with one another, males exert strong preferences for mating with conspecific over heterospecific females and bias their aggression towards conspecific over heterospecific males (Moran et al. 2017). Such preferences are absent in orangethroat and rainbow darters when they occur in allopatry with respect to one another (Moran and Fuller 2018). Thus, it appears that selection to avoid costly interspecific interactions has led to male-driven RCD and ACD in sympatry between orangethroat and rainbow darters. Furthermore, orangethroat darter males show enhanced preferences for mating and fighting with conspecifics over individuals from other closely related species within the orangethroat clade only when they co-occur in sympatry with rainbow darters (Moran and Fuller 2018). This suggests that RCD and ACD between orangethroat and rainbow darters has incidentally led to trait evolution and behavioral isolation among lineages within the orangethroat clade (i.e., cascade RCD and cascade ACD).

Common Garden Study

Our goal here was to test whether color pattern differences present among species within the orangethroat clade are genetically based. We chose to focus on four species in the orangethroat clade that were recently shown to differ quantitatively from one another in the color pattern of wild-caught males: the orangethroat darter (*Etheostoma spectabile*), the strawberry darter (*E. fragi*), the current darter (*E. uniporum*), and the brook darter (*E. burri*) (Figure 5.1A)

(Moran et al. 2017). In March 2015, adult male and female fish from one population of each of the four species were collected using a kick seine (locations shown in Table C.1). Fish were transported in aerated buckets back to the University of Illinois at Urbana-Champaign, sorted by sex and species, and maintained in 75.7 L stock tanks. For each species, we set up 37.9 L breeding tanks that contained a conspecific pair of one male and one female. We created three to four replicate crosses (i.e., families) for each of the four species. Breeding tanks were filled with three to five cm of naturally colored aquarium gravel. All stock and breeding tanks contained a sponge filter and tap water treated with dechlorinator. Tanks were maintained in the same room at 19° C under fluorescent lighting set to mimic the natural photoperiod. Fish were fed frozen bloodworms daily *ad libitum*.

Eggs were collected from breeding tanks using a gravel siphon every 1-3 days for a period of one month. All collected eggs were placed in 0.5 L plastic tubs filled with water treated with methylene blue to prevent fungal growth. Offspring from the same family were kept together. After hatching, fry were transferred to a 1 L plastic tub and fed live brine shrimp nauplii every other day. At approximately 1 month of age, fry were large enough for frozen daphnia to be incorporated into their diet. At approximately three months of age, we transitioned to feeding the fry daily with frozen bloodworms. At this time, all families were transferred to 2.5 L tanks. At one year of age, fish were transferred to 37.9 L tanks, and at two years of age they were transferred to 75.7 L tanks. Offspring from all families were housed in the same room at 19° C under fluorescent lighting that mimicked the natural photoperiod.

At approximately three years of age, the lab-raised offspring from each of the four species had reached adult size and males had developed adult breeding coloration. At this time, males from each family were photographed with a Nikon Coolpix D3300 digital camera (mean ±

SE males per family = 5.5 ± 0.6). Photographs were taken under fluorescent lighting with the camera's factory setting for fluorescent light. Prior to photographing, fish were lightly anesthetized using 0.03g/L of MS-222 and were then placed in a petri dish filled with treated water. An X-rite ColorChecker Mini Chart (Grand Rapids, MI) was in each photograph for color correction and standardization with the inCamera 4.5 plug-in for Adobe Photoshop CC (Adobe Systems Inc., San Jose, CA). We also included a ruler in each photograph, which we used to measure the standard length of each fish (i.e., tip of snout to end of caudle peduncle) to the nearest mm in ImageJ (version 1.50c4) (Rasband 2011).

Males from all species within the orangethroat clade exhibit red and blue banding on the lateral side of the body and on the two dorsal fins (Figure 5.1A). To quantify any differences in male color pattern that were present at the species level, we focused our analyses on aspects of male color pattern that have been shown previously to contribute to variation among these species (Moran et al. 2017). We measured RGB values for both the red and blue coloration on the body, as well as the proportion of red and blue coloration present on the body and fins. Darters possess a two-cone visual system, with middle-wavelength-sensitive (green/blue) and long-wavelength-sensitive (red) pigments that overlap with the reflectance spectra of the blue and red elements of male color pattern (Zhou et al. 2015). Hence, our measurements of blue and red coloration are biologically meaningful and capture the components of male color pattern that these fish are able to perceive.

The dropper tool in Adobe Photoshop was used to measure RGB values, which vary 0-255 for each of the three color channels (i.e., red, green, and blue). An RGB value of 0,0,0 represents black and 255,255,255 represents white. We recorded the three values associated with RGB in both the red and blue portion of the color pattern (resulting in six RGB variables total)

on the posterior half of the lateral side of each fish, near the caudal peduncle. The dropper tool was set to sample a 3x3 pixel area within a given color patch. Each location was measured three times, and the average of these measurements was used for each fish in the multivariate analysis. We used ImageJ to measure the proportion of red and blue on the body and fins as described in Moran et al. (Moran et al. 2017, 2018). Briefly, the perimeter of each fin and the body were traced separately using the polygon selection tool and the areas for each part of the fish were calculated with the histogram function. We then isolated the red and blue pixels using the Threshold Color Plugin with the color channel set to CIE *Lab*. Once the red or blue pixels were isolated, we made the image binary and counted the number of black pixels in the regions corresponding to the fins and the body. We measured the proportion of red and the proportion of blue present on the lateral side of the body and the two dorsal fins, for a total of six color proportion variables per fish. The same color pattern measurements described here were also obtained from a previous study that examined color pattern variation among wild-caught fish from each of the four orangethroat species used in the common garden study (Moran et al. 2017). This allowed us to determine whether the components of male color pattern measured in the common garden fish are similar to those present in nature. All raw color pattern measurement data have been deposited in the Dryad Digital Repository (http://doi.org/10.5061/dryad.4vr063d).

All statistical analyses were performed in R (version 3.4.4). We first conducted a two-factor nested MANOVA to examine whether color pattern differed significantly among species and among families (i.e., replicate crosses within a species). Each of the 12 color pattern variables served as dependent variables in this analysis, with species and family (nested within species) included as factors. We also conducted two-factor nested ANOVAs for each dependent

variable to determine whether significant differences existed among families and species. Because size (i.e., standard length in mm) varied among families ($F_{12,58} = 11.72$, $P < 0.00001$), it was included as covariate in the MANOVA and ANOVAs. However, preliminary analyses indicated no effect of size on color pattern differences among individuals. We therefore excluded size from subsequent analyses. We next used Linear Discriminate Analysis (LDA) to reduce the dimensionality of the color data set and to identify which color variables contribute most to differences among species. We used the lda function of the MASS package (Ripley et al. 2017). The 12 color pattern measurements served as dependent variables, and species served as the categorical predictor variable. We then used the Anova function of the car package (Fox 2007) to conduct nested two-factor ANOVAs with species and family (nested within species) as factors and individual LD scores as the dependent variable. We conducted separate ANOVAs for both of the first two LDs. Post-hoc pairwise comparisons were conducted among species using Tukey's tests with the glht function in the multcomp package (Hothorn et al. 2017). Finally, we conducted LDA using color measurements from the common garden study males in addition to previously published color measurement data obtained from 10 wild-caught males from each of these four species (Moran et al. 2017). We conducted ANOVA on the first two LDs with species and rearing environment (i.e., lab-raised or wild-caught, nested within species) as factors and individual LD scores as the dependent variable.

ACD Study

Here our goal was to quantify male color pattern variation in wild-caught sympatric and allopatric populations of the orangethroat darter and the rainbow darter to test for a pattern consistent with divergent ACD. The orangethroat darter is the only species within the

orangethroat clade to occur both in sympatry and in allopatry with respect to the rainbow darter. Previous studies have shown that aspects of male color pattern differ quantitatively between sympatric orangethroat and rainbow darters (Moran et al. 2017, 2018), and that color pattern is variable across populations within species (Zhou et al. 2014). Divergent ACD in male color pattern predicts: (1) enhanced differentiation between species in sympatry compared to allopatry, and (2) differentiation between sympatric and allopatric populations within species.

Adult orangethroat and rainbow darter males were collected with a kick seine in March 2016 from one sympatric and one allopatric population of each species (for a total of four "groups") (Figure 5.2A, Table C.2). We took digital photographs of 10 males from each group, for a total of 40 fish, as described above for the common garden study. Size did not vary among the four groups (ANOVA: $F_{3,36} = 1.88$, $P = 0.15$).

Orangethroat and rainbow darters are both characterized by a male nuptial color pattern that consists of red and blue banding on the lateral sides and dorsal fins. Despite their superficial similarities, their color patterns differ in a few consistent ways (Figure 5.2A). Orangethroat darters lack red coloration on their anal fins, caudal fins, and pectoral fins, but rainbow darters do not. There are also apparent differences in the amount of red and blue banding across the lateral portion of the fish. To quantify variation in male color pattern between and within species, we followed the methods described above for the common garden study. In addition, we measured the proportion of red coloration present on the caudal fin and the proportion of red and proportion of blue coloration present on the anal fin for each fish.

All analyses were conducted in R using the packages described above. We first conducted a two-factor nested MANOVA to examine whether species and geography (i.e., sympatric or allopatric) contributed to differences in male color pattern among groups. Each of

the 16 color pattern variables served as dependent variables in this analysis, with species and geography (nested within species) included as factors. We also conducted two-factor nested ANOVAs for each dependent variable with species and geography as factors. We then used LDA to facilitate pairwise comparisons and to identify which variables contribute most to differences among groups. Here, group (i.e., sympatric rainbow, allopatric rainbow, sympatric orangethroat, or allopatric orangethroat) served as the categorical predictor variable and the color measurements served as dependent variables. Finally, to ask whether individuals' scores for the first two LDs differed among groups, we used nested two-factor ANOVAs. We included the score for the first and second LDs as the dependent variable (in two separate analyses, one for each LD). Species and geography (nested within species) were included as factors. We made post-hoc pairwise comparisons among groups using Tukey's tests.

RESULTS

Common Garden Study

The MANOVA on color pattern measurements in the four lab-raised orangethroat clade species revealed that species identity and family (i.e., replicate cross within a species) both significantly contributed to differences in male color pattern (Table 5.1). There was no effect of size (standard length in mm) on differences in color pattern among individuals (Table 5.1). ANOVAs indicated that the values for nearly every variable differed significantly as a function of species identity or due to an interaction between family and species (Table C.3). The one exception was the proportion of blue present on the second dorsal fin, which varied among families within species but not among species.

The LDA on color pattern measurements in lab-raised fish reduced the dimensionality of the data into three LDs, with the first two LDs explaining 86.6% cumulative variation among groups (LD1: 57.3%, LD2: 25.3%, LD3: 17.4%). Figure 5.1B shows a biplot comparing the scores for LD1 versus LD2 for each individual, grouped by species. The color pattern proportion measurements had higher loadings (i.e., associations) with all three LDs compared to the RGB data, suggesting that differences in the proportion of red and blue coloration on the body and fins is a good predictor of species. The proportion of red on the first and second dorsal fins and the proportion of blue on the first dorsal fin had the highest loadings for LD1. LD2 was associated with the proportion of red on both dorsal fins in addition to the proportion of red present on the body. ANOVAs on LD1 and LD2 revealed significant effects of species (LD1: $F_{3,63} = 35.70$, $P < 0.00001$; LD2: $F_{3,63} = 15.60$, $P < 0.00001$) but not family (nested within species) (LD1: $F_{1,63} = 2.90$, $P = 0.09$; LD2: $F_{1,63} = 1.14$, $P = 0.29$). There was no interaction between species and family for either analysis (LD1: $F_{3,63} = 0.73$, $P = 0.54$; LD2: $F_{3,63} = 0.53$, $P = 0.66$). Post-hoc Tukey's tests indicated that all species differed significantly from one another in scores for LD1 and/or LD2 (i.e., no pair of species overlapped in scores for both LD1 and LD2) (Tables C.4).

The LDA that included color pattern measurements from both lab-raised and wild-caught fish from the four orangethroat clade species resulted in three LDs. The first two LDs accounted for 87.4% of the variation among groups (LD1: 48.9%, LD2: 38.6%, LD3: 12.6%). ANOVAs on LD1 and LD2 revealed a significant effect of species but not rearing environment (i.e., lab-raised versus wild-caught) on male color pattern, and no interaction between species and rearing environment (Table C.5).

ACD Study

Our MANOVA on variation in male color pattern among groups (i.e., allopatric rainbow, allopatric orangethroat, sympatric rainbow, and sympatric orangethroat) revealed an interaction between species identity (orangethroat or rainbow) and geography (sympatric or allopatric, nested within species), but not size (Table 5.2). ANOVAs indicated that the values for nearly every variable differed significantly between sympatric and allopatric populations within species geography or due to an interaction between geography and species (Table C.6). The red (R) value for the blue coloration, the proportion of blue coloration on the body, and the proportion of red coloration on the body and anal fin varied between species but was not associated with geography.

LDA identified three LDs that predicted differences among groups, with the first two LDs explaining 87.9% cumulative variation among groups (LD1: 60.1%; LD2: 27.8%; LD3: 12.1%). Figure 5.2B shows a biplot comparing the scores for LD1 versus LD2 for each individual. The color pattern proportion measurements had higher loadings (i.e., associations) with all three LDs compared to the RGB data, suggesting that differences in the proportion of red and blue coloration on the body and fins is a good predictor of species and geographic relationship between groups.

Contrasting patterns were present in scores for the first two LDs across groups. A pattern consistent with divergent character displacement was evident from LD1 (Figure 5.2B,C). Scores for LD1 showed a closer association between allopatric fish compared to sympatric fish of both species. This pattern was mainly driven by differences between sympatric and allopatric populations of orangethroat darters. LD1 was most strongly associated with the proportion of red coloration present on the anal fin, caudal fin, and body. Conversely, sympatric males of both

species were grouped more closely along LD2 compared to allopatric males of both species (Figures 5.2B, C.1). LD2 was most closely associated with the proportion of blue coloration on the first and second dorsal fin. This suggest that traits corresponding with LD2 may be associated with sharing a common environment and/or introgression.

ANOVAs for both LD1 and LD2 indicated an interaction between species and geography (nested within species) (LD1: $F_{1,36} = 127.78$, $P < 0.0001$; LD2: $F_{1,36} = 178.55$, $P < 0.0001$). Post-hoc pairwise comparisons with Tukey's tests revealed significant differences among all groups in scores for LD1 (Table C.7A). Only one pairing did not differ significantly from one another in scores for LD2: allopatric rainbow darters and sympatric orangethroat darters (Table C.7B).

DISCUSSION

A growing body of literature suggests that interspecific reproductive and aggressive interactions play a surprisingly large role in speciation (Grether et al. 2009, 2017). Interspecific interactions can have broad implications for speciation by directly promoting enhanced behavioral isolation in sympatry and indirectly promoting the evolution of trait divergence and behavioral isolation among allopatric lineages (Ortiz-Barrientos et al. 2009; Hoskin and Higgie 2010). In this study we demonstrated that color pattern differences present in nature among recently diverged allopatric lineages within the orangethroat clade are maintained in a common garden rearing environment, indicating that these differences are genetically based. Additionally, we observed a pattern of enhanced divergence in male agonistic coloration in sympatry (compared to allopatry) between populations of the orangethroat darter and the more distantly related rainbow darters, consistent with divergent ACD. These results have significant

implications for our understanding of speciation and diversification in one of the most diverse groups of vertebrates in North America. More generally, this study provides important insight into the evolution of ACD and cascade ACD in male agonistic signals and response to those signals.

A unique aspect of this study system is that evolution of elaborate male nuptial coloration appears to be driven entirely by male-male interactions between and within species, despite the presence of traditional sex roles. Previous studies on orangethroat and rainbow darters have demonstrated that male coloration functions in male-male competition over access to females within species (Zhou and Fuller 2016), and that male aggressive response towards heterospecific males increases with increasing color pattern similarity between species (Moran et al. 2017). Conversely, studies have consistently failed to detect female preferences associated with variable aspects of male color pattern within or between species (Pyron 1995; Fuller 2003; Zhou et al. 2015; Moran et al. 2017; Moran and Fuller 2018). Here we demonstrated that some male color traits show a clear pattern of divergent character displacement between sympatric orangethroat and rainbow darter populations. We acknowledge that a lack of replication is a limitation of this study. To address this concern, we have re-analyzed data from a previously published survey of color pattern variation within and between populations in the orangethroat darter (*E. spectabile*) across six drainages (Zhou et al. 2014). At least within this species, the pattern of color divergence between populations that are sympatric versus allopatric with rainbow darters appears to be robust.

Male color traits that showed a pattern consistent with divergent ACD between orangethroat and rainbow darters (i.e., those associated with LD1 in the ACD study; Figure 5.2C) included the proportion of red coloration on the anal fin, caudal fin, and body. We suspect

that these traits show the strongest pattern of divergent ACD for two reasons. First, the presence/absence of red coloration on the anal fin and caudal fin are the most obvious differences in color pattern between orangethroat and rainbow darters (Figure 5.2A). Thus, these color traits likely play a large role in visual discrimination between species. Second, a previous study on orangethroat and rainbow darters showed that when lighting filters were used to reduce the ability of males to perceive red coloration, males exhibited reduced aggression towards conspecifics (Zhou et al. 2015). This supports the hypothesis that red coloration is important in male recognition of conspecific rivals.

We also found that some aspects of male color pattern (i.e., those associated with LD2 in the ACD study: proportion of blue coloration on the first and second dorsal fin; Figures 5.2B,C.1) appear to be more strongly correlated with a common environment and/or introgression, and do not show a pattern consist with divergent ACD between orangethroat and rainbow darters. Theoretically, the greater similarity in sympatry compared to allopatry in some color traits may be due to three different phenomena: introgression due to hybridization, local adaptation to a common environment, or phenotypic plasticity due to sharing a common environment. We doubt that phenotypic plasticity accounts for the convergence in color proportion traits on the dorsal fins. Clearly, there are some types of color traits that are plastic. Red coloration in darters is carotenoid based (Zhou et al. 2014), which suggests it may be linked to diet (Kodric-Brown 1989; Hill and McGraw 2006). In rainbow darters, spectral properties of red coloration are associated with parasite load (Ciccotto et al. 2014). Additionally, blue and black coloration present on the side of the body and head can vary rapidly in these species when males escalate aggression (R. Moran pers. obs.). However, these phenomena should affect the red and blue hues and their associated RGB values. The present study has demonstrated that

variation in RBG values account for little of the total variation present between sympatric and allopatric populations/species. Instead, the proportion of red and blue coloration present on the body and fins strongly predict both species identity and geographic relationship between species. The results of our common garden study in combination with another recent study examining male color pattern in orangethroat darters, rainbow darters, and their hybrids (Moran et al. 2018) provide strong evidence that variation in these color elements present among populations and species are largely genetic in nature.

The other two phenomena that can potentially account for the convergence in some color traits are hybridization and local adaptation. Of these two possibilities, we suspect that hybridization is more likely for two reasons. First, hybridization is ongoing in at least three different contact zones. Moran et al. (Moran et al. 2017, 2018) and Bossu and Near (Bossu and Near 2013) have shown that F1 hybrids between rainbow darters and three different orangethroat clade species are present in natural populations. In addition, the traits that are most strongly implicated in species-specific differences between orangethroat and rainbow darters (i.e., the proportion of red and blue coloration on the body, anal fin, and caudal fin) have intermediate values in F1 hybrid males (Moran et al. 2018), suggesting that introgression can cause increased trait similarity between species. Second, although large-scale transitions between genera and sub-genera are associated with ecological divergence in darters (Bossu and Near 2015; Ciccotto and Mendelson 2016), there is strong evidence that differences in male color pattern among more closely related species are primarily driven by intrasexual selection rather than ecological differences (Martin and Mendelson 2014, 2016b; Zhou et al. 2015; Moran et al. 2017).

Importantly, the findings of this study drastically change how we think about the evolution of male color pattern and speciation in darters. Sexual selection in the form of female

mating preference for male color traits was long thought to be the primary catalyst of speciation in these fish. Here we demonstrated that divergence in male color pattern both between species and among populations within species is promoted by sympatry between congeners. We previously found no relationship between the magnitude of male color pattern difference and pairwise genetic distance in multiple comparisons between orangethroat and rainbow darters (Moran et al. 2017). Additionally, within the orangethroat clade, divergence time estimates overlap for species that are sympatric versus allopatric with respect to rainbow darters (Bossu et al. 2013). Together these results suggest that the geographic relationship between orangethroat and rainbow darters (i.e., whether they are sympatric or allopatric) has a greater impact on color pattern divergence than the amount of evolutionary divergence between species.

The pattern of ACD in male color pattern presented here also reflects previously documented behavioral patterns of ACD between orangethroat and rainbow darters and cascade ACD among species in the orangethroat clade. Divergence in male color traits between closely related species within the orangethroat clade that occur in sympatry with rainbow darters (and thus undergo ACD) has resulted in enhanced male competitor bias between species (Moran et al. 2017; Moran and Fuller 2018). This is consistent with cascade ACD in both male agonistic signals and behavioral response to those signals (sensu "convergent sympatry effects" of character displacement) (Comeault and Matute 2016). It remains to be tested whether the divergence in male color pattern traits that we observed between populations within the orangethroat darter (and/or within the rainbow darter) also confer behavioral biases among populations within species (which would indicate "sympatry-allopatry effects" of character displacement) (Comeault and Matute 2016).

Lastly, our findings have implications for the evolution of behavioral isolation via RCD and cascade RCD in this system. Our current hypothesis is that strong selection to avoid maladaptive hybridization after secondary contact (i.e., reinforcement) leads to RCD in male mating preferences and strong behavioral isolation between species (Moran et al. 2018). As a result, females of both species are not a shared resource among males of both species in sympatry, which could cause interspecific male-male aggression over females to be maladaptive. This should promote ACD in male aggressive biases (to avoid needless interspecific aggression), allowing these species to co-occur in close proximity on the breeding grounds and in turn increasing the potential for hybridization. In this manner RCD and ACD may act in a positive feedback loop, mutually strengthening divergence in both mating and fighting traits in males.

To conclude, the results of the present study demonstrate that interspecific interactions in sympatry may play a larger role than previously thought in promoting the evolution of male secondary sex trait diversification both between and within species. Evidence is now growing that female mating preferences are absent or lower compared to male mate preferences in many species of darters. Instead, it appears that male mating and fighting preferences drive trait evolution between and within species, despite the presence of elaborate male secondary sex traits and traditional sex roles.

ACKNOWLEDGEMENTS

The treatment of animals used in this study was in compliance with the University of Illinois Institutional Animal Care and Use Committee (IACUC) under protocols #14097 and #17031. Collection of wild fish was approved by the Illinois Department of Natural Resources

under Scientific Collecting Permits A15.4035 and A16.4035, the Arkansas Game and Fish Commission under Scientific Collection Permit #032020141, the Missouri Department of Conservation under Wildlife Collector's Permit #16392, and the Michigan Department of Natural Resources under a Scientific Collector’s Permit issued to RLM and RCF. We thank Lance Merry for photos of darters that appear in Figure 1A. This research was supported by funding from the National Science Foundation under DGE 1069157 and IOS 1701676 awarded to RLM and DEB 0953716 awarded to RCF. RLM was supported by funding from the United States Department of Agriculture (Cooperative State Research, Education, and Extension Service project number ILLU 875-952).

TABLES

Table 5.1. Results of two-factor nested MANOVA on male color pattern in fish from the common garden study. Species and family (nested within species) were included as factors and size (standard length in mm) was included as a covariate.

Variable	Df	Pillai	approx F	num Df	den Df	P
Species	1, 62	0.61	6.54	12	51	<0.00001
Family	3, 62	2.60	28.86	36	159	<0.00001
Size	1, 62	0.31	1.92	12	51	0.054
Family*Species	3, 62	1.10	2.54	36	159	<0.00001

Table 5.2. Results of two-factor nested MANOVA on male color pattern in fish from ACD study. Species and geography (nested within species) were included as factors.

Variable	Df	Pillai	approx F	num Df	den Df	P
Species	1, 36	0.93	20.73	15	22	<0.00001
Geography	1, 36	0.79	5.62	15	22	<0.001
Species*Geography	1, 36	0.90	13.03	15	22	<0.00001

FIGURES

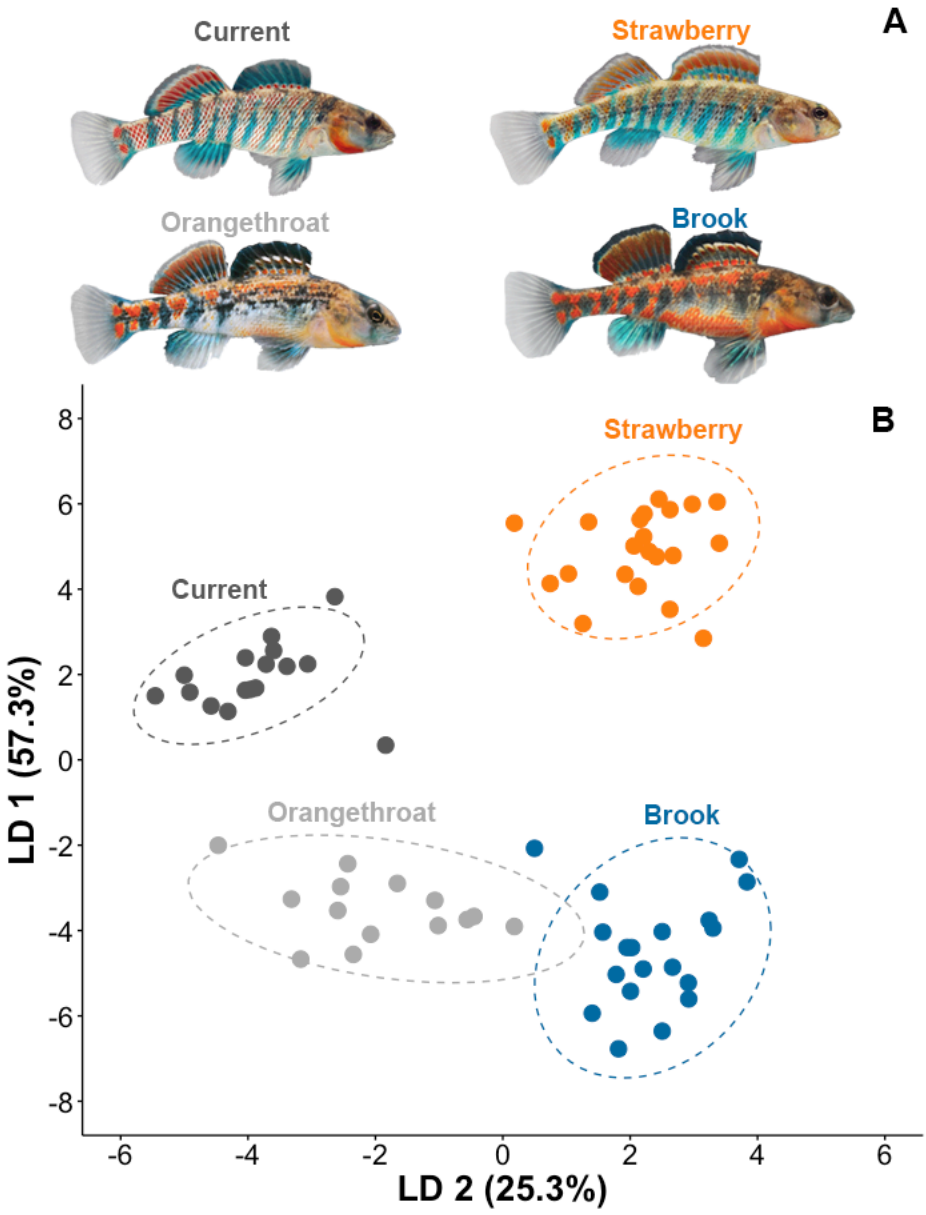

Figure 5.1. (A) Representative example of male color pattern in strawberry, current, brook, and orangethroat darters. (B) Biplot of the first two LDs obtained from the LDA on male color pattern in fish from the common garden study. Ellipses represent 95% CI.

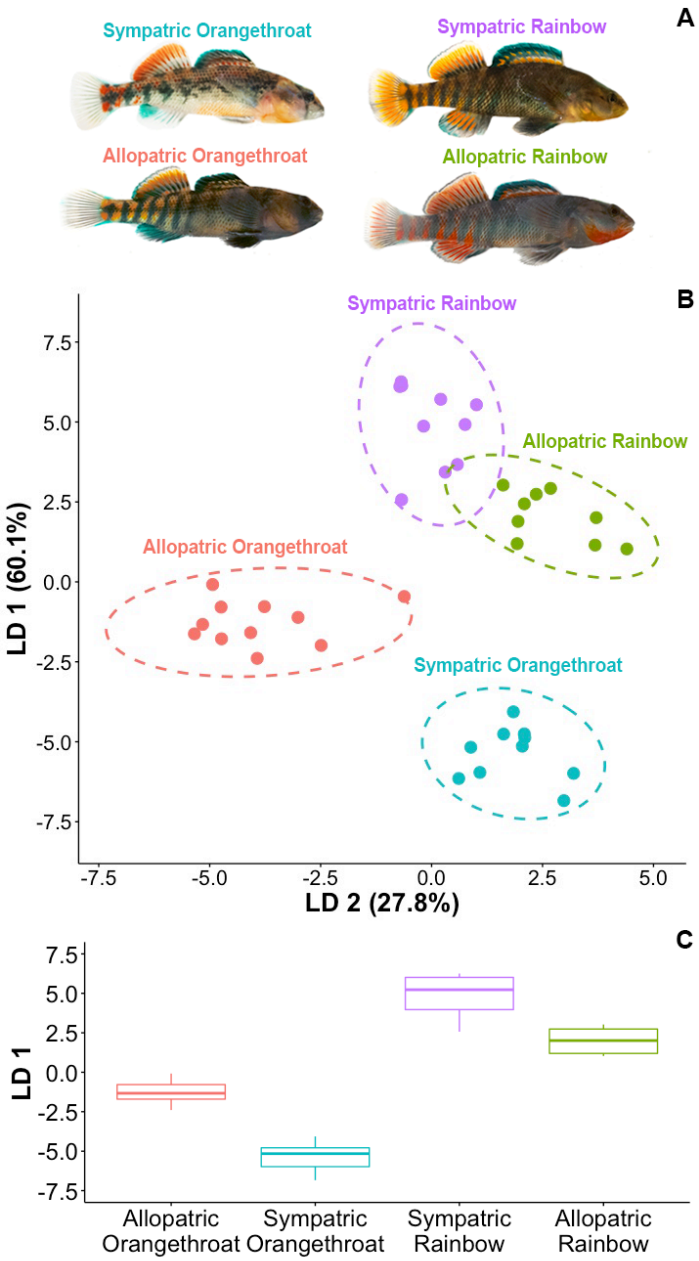

Figure 5.2. (A) Representative example of male color pattern in sympatric orangethroat, sympatric rainbow, allopatric orangethroat, and allopatric rainbow darters. (B) Biplot of the first two LDs obtained from the LDA on male color pattern in fish from the ACD study. Ellipses represent 95% CI. (C) Boxplots of LD2 scores from the LDA on male color pattern in fish from the ACD study.

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CHAPTER 6

HYBRIDIZATION AND POSTZYGOTIC ISOLATION PROMOTE REINFORCEMENT OF MALE MATING PREFERENCES IN A DIVERSE GROUP OF FISHES WITH TRADITIONAL SEX ROLES[5]

ABSTRACT

Behavioral isolation is thought to arise early in speciation due to differential sexual and/or natural selection favoring different preferences and traits in different lineages. Alternatively, behavioral isolation can arise due to reinforcement favoring traits and preferences that prevent maladaptive hybridization. In darters, female preference for male coloration has been hypothesized to drive speciation, because behavioral isolation evolves before F1 inviability. However, as with many long-lived organisms, the fitness of second generation hybrids has not been assessed because raising animals to adulthood in the lab is challenging. Recently, reinforcement of male preferences has been implicated in darters because male preference for conspecific females is high in sympatry but absent in allopatry in multiple species pairs. The hypothesis that reinforcement accounts for behavioral isolation in sympatry assumes that hybridization and postzygotic isolation are present. Here, we used genomic and morphological data to demonstrate that hybridization is ongoing between orangethroat and rainbow darters and used hybrids collected from nature to measure postzygotic barriers across two hybrid

[5]Chapter 6 was published in its entirety by Ecology and Evolution. Moran, R. L., M. Zhou, J. M. Catchen, and R. C. Fuller. 2018. Hybridization and postzygotic isolation promote reinforcement of male mating preferences in a diverse group of fishes with traditional sex roles. Ecol. Evol. 8:9282–9294. This article is reprinted with permission of the publisher and is available from http://onlinelibrary.wiley.com using doi:10.1002/ece3.4434.

generations. We observed sex ratio distortion in adult F1s and a dramatic reduction in backcross survival. Our findings indicate that selection to avoid hybridization promotes the evolution of male-driven behavioral isolation via reinforcement in this system.

INTRODUCTION

The increasing availability of genomic sequence data for non-model organisms has revealed that hybridization is surprisingly common between species (Mallet 2005; Abbott et al. 2013). As hybridization has traditionally been thought of as a homogenizing force, a major question in evolutionary biology is how speciation can proceed in the face of gene flow (Felsenstein 1981; Bolnick and Fitzpatrick 2007; Feder et al. 2012; Harrison and Larson 2014). Despite a contentious history, it is now recognized that hybridization can actually promote speciation through reinforcement, the process by which enhanced prezygotic isolation is favored in sympatry in response to postzygotic isolation (Dobzhansky 1937; Servedio and Noor 2003; Coyne and Orr 2004). Reinforcement causes reproductive character displacement (RCD), whereby behavioral isolation between two species is heightened in sympatry compared to allopatry. Although multiple different evolutionary forces can lead to such a pattern (reviewed in Hoskin and Higgie 2010), it is considered reinforcement when the mechanism underlying RCD is selection against hybridization (Pfennig and Pfennig 2012). Empirical and theoretical research has indicated that reinforcement may be more common than previously thought (Yukilevich 2012; Hudson and Price 2014), and can both directly finalize speciation in sympatry and indirectly initiate speciation in allopatry (via cascade reinforcement; Ortiz-Barrientos et al. 2009).

Our goal here was to use genomic data to investigate a putative hybrid zone between two species of darters and to examine the strength of multiple postzygotic barriers between these species to test the hypothesis that reinforcement contributes to speciation in this system. The two focal species exhibit a pattern of behavioral isolation consistent with reinforcement of male mating preferences (i.e., male preference for conspecific females is high in allopatry) (Moran and Fuller 2018). Whether or not postzygotic isolation is present is unknown. Previous studies have shown a lack of postzygotic isolation through the F1 larval stage (Hubbs and Strawn 1957). However, the total strength of postzygotic isolation is frequently underestimated by using F1 hybrid inviability as the sole measurement of postzygotic isolation (Wiley et al. 2009; Lemmon and Lemmon 2010). This is particularly problematic because genetic incompatibilities can be masked in F1s due to effects of dominance (Coyne and Orr 2004; Mallet 2006), and maternal provisioning can reduce F1 inviability (Schrader and Travis 2008). Accurate estimates of postzygotic isolation therefore require quantifying postzygotic barriers in F1 adults and in later generation hybrids, but this can be quite challenging in long lived and/or non-model organisms. Measuring the total strength of postzygotic isolation typically necessitates generating multiple generations of hybrid crosses and raising the offspring in the laboratory through the adult life stage. This can be logistically challenging. The current study solves this problem by identifying F1 hybrids in nature and using them to generate second generation hybrids and measure postzygotic isolation.

Darters are a diverse group of stream fishes that have been characterized as a model system for the evolution of speciation via sexual selection. Behavioral isolation evolves before F1 larval inviability in darters (Mendelson 2003; Mendelson et al. 2006, 2007; Williams and Mendelson 2014; Martin and Mendelson 2016b), and there are no known cases of complete F1

inviability through the fertilization and larval hatching stage, even between very distantly related species. The apparent rapid evolution of prezygotic isolation relative to postzygotic isolation in these fish has been attributed to female mate choice on species-specific male color traits (Williams and Mendelson 2010, 2011, 2013). However, recent research in a number of darter species has found that strong conspecific mate preferences are exhibited by males but such preferences are weak (or sometimes absent) in females, and that male coloration functions primarily in male-male competition rather than female mate choice (Zhou et al. 2015; Martin and Mendelson 2016a; Zhou and Fuller 2016; Moran et al. 2017; Mendelson et al. 2018; Moran and Fuller 2018). Thus, males may actually play a stronger role than females in maintaining species boundaries, despite the presence of traditional sex roles and extreme sexual dimorphism.

The present study focuses on the rainbow darter *Etheostoma caeruleum* and the orangethroat darter *Etheostoma spectabile*. The orangethroat darter is a member of the *Ceasia* clade (also referred to as the orangethroat darter clade), which consists of 15 allopatrically distributed species. Time-calibrated gene phylogenies estimate that species within the orangethroat clade last shared a common ancestor 6-7 million years ago (mya) (Bossu et al. 2013). The orangethroat darter clade and rainbow darters are classified together in the subgenus *Oligocephalus.* Divergence time between rainbow and orangethroat darters has been estimated at 22 mya (Near et al. 2011), but these species have very similar male color patterns, ecology, and mating behavior. Thirteen of the orangethroat clade species occur sympatrically with rainbow darters, and ancient hybridization events are evident from the presence of introgressed rainbow darter mitochondrial haplotypes in four orangethroat species (i.e., orangethroat darter *E. spectabile,* current darter *E. uniporum*, brooks darter *E. burri*, and buffalo darter *E. bison*; Ray et al. 2008; Bossu and Near 2009). Molecular evidence also suggests that hybridization is ongoing

between the rainbow darter and two species in the orangethroat darter clade (i.e., the buffalo darter and the current darter), as early-generation hybrids have been documented in nature (Bossu and Near 2013; Moran et al. 2017). However, the evolutionary consequences of hybridization in darters remains unexplored.

Recent studies have suggested that selection against interspecific interactions (i.e., mating and fighting) contribute to behavioral isolation between orangethroat and rainbow darters. In sympatric pairings between rainbow darters and five different orangethroat darter clade species, males have been shown to exert strong preferences for mating with conspecific females and fighting with conspecific males (Moran et al. 2017). Such preferences are absent in allopatric pairings of rainbow and orangethroat darters with similar divergence times to the sympatric pairings (Moran and Fuller 2018). This pattern is consistent with both RCD in male mating preferences and divergent agonistic character displacement (ACD) in male fighting preferences. Divergent ACD occurs when selection against interspecific aggressive interactions leads to the evolution of enhanced bias against fighting with heterospecifics in sympatry (Grether et al. 2009). Additionally, behavioral experiments simulating secondary contact between multiple allopatric orangethroat darter clade species revealed that males also prefer to mate and fight with conspecifics over other orangethroat species, but only when they occur sympatrically with rainbow darters (Moran and Fuller 2018). This suggests that RCD and ACD in sympatry between orangethroat and rainbow darters may have cascading effects by incidentally initiating trait evolution and male-driven behavioral isolation among lineages within the orangethroat darter clade. Surprisingly, studies have consistently failed to detect female preferences in orangethroat and rainbow darters for varying components of male color pattern within or between species (Pyron 1995; Fuller 2003; Zhou et al. 2015; Moran et al. 2017).

Whether reinforcement is causing the pattern of RCD in male mating preferences in orangethroat and rainbow darters remains uncertain. Previous investigations into postzygotic barriers between orangethroat and rainbow darters have been limited to examining F1 larval survival, and have found no evidence of hybrid inviability through this life stage (Hubbs and Strawn 1957; Linder 1958; Hubbs 1967; Bossu 2012; Bossu and Near 2013). Here, we use phenotypic and genomic data to confirm that hybridization is ongoing between the orangethroat darter and the rainbow darter, and then investigate postzygotic isolation between these species using both lab-generated and wild-caught hybrids. We test for inviability, sex ratio distortion, sterility, and mating behavioral abnormalities in F1 hybrids, and inviability in backcross hybrids. This represents the most thorough investigation to date into postzygotic isolation in darters. By utilizing natural hybrids, we were able to reveal that postzygotic isolation is much higher than previously thought. We present evidence that hybridization is ongoing and that it is maladaptive, providing critical support for the hypothesis that male-driven behavioral isolation has evolved via reinforcement (and cascade reinforcement) in these species. More generally, these results contribute to our understanding of the evolution of concurrent RCD and ACD in male mating preferences and fighting biases.

METHODS

Laboratory F1 hybrid cross viability

We first created F1 hybrids in the lab. Adult orangethroat and rainbow darters were collected from two adjacent tributaries of the Vermillion River (Champaign Co., Illinois; Table D.1) using a kick seine in April and May 2012. Fish were transported back to the University of

Illinois at Urbana-Champaign. Crosses were performed by hand-stripping eggs from a single female into a petri dish filled with water from their native stream and subsequently hand-stripping sperm from a single male onto the eggs. Afterward, the water in the petri dish was gently swirled for 1 min to mix the eggs and sperm. Each clutch of eggs was transferred to a separate plastic tub filled with water that was treated with methylene blue (to prevent fungal growth) and stored in an incubator set to 11º C and a 11:14 h light:dark cycle.

Unique male-female pairs were used as parents in each replicate cross. We performed F1 crosses in both direction and "purebred" control crosses with both parental species, with 10–14 replicates per cross type (Table 6.1). The eggs from each replicate were checked daily for development. As fry hatched, they were transferred to a larger tub in the incubator and fed live brine shrimp nauplii every other day. Fry were transferred out of the incubator and into 19 L and 38 L aquaria at approximately three weeks post hatching. Aquaria were maintained at 19º C and the photoperiod was set to mimic natural daylight hours. After transfer to the aquaria, fish were fed daily *ad libitum* with frozen daphnia and frozen bloodworms.

We measured fertilization success (proportion of eggs that developed pigmented eyes), hatching success (proportion fertilized eggs that yielded free-swimming fry), and larval survival (proportion of hatched eggs that survived to 10 months) of each family. Additionally, to determine whether the mean sex ratio of each cross type deviated from the expected 1:1, we measured the sex ratio of each family after 22 months. By this time, all fish exhibited sexually dimorphic coloration.

All statistical analyses were conducted in R (version 3.4.0). We asked whether each viability metric (fertilization success, hatching success, and larval survival) varied among cross types at the family level using generalized linear models (GLMs), with the viability metric as the

independent variable and cross type as the dependent variable. We conducted these analyses using the *glm* function of the *stats* package and specified a quasibinomial distribution with logit link function to account for overdispersion in the data. We used the *Anova* function of the *car* package (Fox 2007) to generate type II analysis of deviance tables and F-tests. We also used one-sample Student's *t*-tests using the *t.test* function of the *stats* package to test whether the proportion of male offspring in a clutch differed from the expected 0.50 in each cross type.

Backcross viability using wild-caught F1 hybrids

Here, we backcrossed wild-caught F1 hybrid males to orangethroat and rainbow darter females. We used F1 individuals collected from a natural hybrid zone as parents in backcrosses rather than using lab-generated F1s because at two years of age most of our lab-raised orangethroat darters and F1 hybrids failed to engage in mating behavior and females were not gravid. This was not completely unexpected, as orangethroat and rainbow darters can take up to three years to reach sexual maturity in the lab (R. Moran pers. obs.). However, it is also possible that the artificial lab rearing environment lacked a critical cue to trigger the onset of spawning. We therefore only used wild-caught fish for backcrosses.

We collected adult male and female orangethroat and rainbow darters and F1 hybrid males from three tributaries of the Vermillion River (Champaign Co., Illinois; Table D.1) in April 2016. We chose to use F1 hybrid males (rather than females) to measure backcross viability because preliminary analyses of our F1 laboratory crosses revealed that: (1) hybrid males are diagnosable due to their color pattern intermediacy between the parental species (see below) (Fig. 6.1), and (2) the sex ratio of F1 hybrid clutches is dramatically skewed towards males, which suggests that F1 females may be quite rare in natural populations (see below). We

confirmed our initial classification of wild-caught fish as orangethroats, rainbows, or hybrids using multivariate phenotypic analyses and genetic sequencing (see below).

We conducted four cross types with six replicates each. We conducted backcrosses in both directions between the wild-caught F1 males and parental species females and conducted "purebred" control crosses with both parental species (Table 6.2). Crosses were conducted in breeding aquaria filled with 5-7 cm of naturally colored aquarium gravel, and fluorescent lighting was provided that mimicked the natural photoperiod. Fish were fed frozen bloodworms *ad libitum* each day.

To generate backcrosses, a hybrid male was rotated daily between two 37.9 L breeding aquaria, one of which contained an orangethroat darter female and the other a rainbow darter female. We used a small dip net to rotate hybrid males from one backcross breeding aquarium to the other every day at noon for 14 consecutive days, so that hybrid males spent seven days with each of the two parental females. Eggs were collected from each breeding aquarium immediately after the hybrid male was transferred to the other parental female's breeding aquarium. Eggs were collected each day for seven days from each purebred parental pair. Purebred parental males were also moved from their breeding aquaria to a separate holding tank for 10 min once a day. During this time, the eggs were collected from the breeding aquaria. Eggs collected from each breeding aquarium were kept together in a 1 L container and maintained as described in the previous section. For each cross, we measured offspring viability at three developmental stages: the proportion of eggs that were fertilized, the proportion of fertilized eggs that survived to hatching, and the proportion of hatched fry that survived to the larval feeding stage (approximately three days post-hatching).

We first asked whether the three measures of viability varied as a function of cross following the same methodology as described above for the F1 crosses. The *glht* function of the *multcomp* R package (Hothorn et al. 2017) was used to make post-hoc pairwise comparisons between cross types. We also asked whether females used in backcrosses were as likely to produce eggs as those used in the purebred parental crosses. We conducted two separate Mann-Whitney U tests to determine whether the total number of eggs produced by orangethroat and rainbow darter females differed depending on the identity of the male that they were paired with (i.e., hybrid male or purebred conspecific male). Female standard length did not differ between species (mean ± SE: orangethroat = 61.45 ± 1.43 mm, n = 12; rainbow = 57.97 ± 1.66 mm, n = 12; two-sample *t*-test: $t_{21.52} = 1.52$, p = 0.14), and male standard length did not differ among groups (mean ± SE: hybrids = 65.4 ± 3.2 mm, n = 6; orangethroat = 68.6 ± 3.0 mm, n = 6; rainbow = 68.0 ± 1.6 mm, n = 6; ANOVA: $F_{2,15} = 0.34$, p = 0.72).

Wild-caught F1 hybrid male mating and competitive behavior

Both orangethroat and rainbow darters congregate in shallow, gravel riffles of headwater streams during the spring breeding season. Males attempt to guard females by chasing off male competitors and flaring their fins in threat displays. Once a female is ready to spawn, she will perform a nosedig into the gravel and bury herself in the substrate. If multiple males are near the female at this time, male fighting will escalate. One to several males will then attempt to spawn with the female (Winn 1958; Fuller 2003).

We conducted two types of behavioral trials to examine mating behavior of wild-caught F1 hybrid males: dichotomous male choice trials and male competition trials. These behavioral trials used the same wild-caught F1 hybrid males as the backcross experiment described above

but used different orangethroat and rainbow darter individuals from the same drainage (i.e., Vermillion River, Champaign Co., Illinois). Previous behavioral studies have shown that orangethroat and rainbow darter males from this drainage exhibit strong preferences for mating and fighting with members of their own species over the other (Zhou and Fuller 2014; Moran et al. 2017; Moran and Fuller 2018). Here, our goal was to ask whether hybrid males show any preference for mating or fighting with members of either parental species. Each behavioral trial involved three fish in a 37.9 L test aquarium positioned under a fluorescent light and filled with 5-7 cm of naturally colored gravel.

For the dichotomous male mate choice trials, a hybrid male was joined by a female orangethroat and a female rainbow darter (n=6). This allowed us to observe whether hybrid males would choose to pursue either female, and if so, whether they exhibited a preference for females of either species. We split each trial into 60 30-s blocks. We scored the number of 30-s blocks in which the male was within one body length of each female for a minimum consecutive time of 5-s (Zhou et al. 2015; Moran and Fuller 2018). We used one-sample Student's *t*-tests with the *t.test* function of the *stats* package in R to test whether the proportion of blocks that the male spent pursuing the orangethroat darter female (versus the total number of blocks spent pursuing either female) differed from the expected 0.50 in each trial.

For the male competition trials, a hybrid male was joined by a male-female pair that were either both orangethroat or both rainbow darters. The goal of these trials was to measure male-male aggressive behavior, but a female was included to elicit male competitive behavior. Each hybrid male participated in two consecutive competition trials, one in which he was joined by an orangethroat darter pair (n=6) and one in which he was joined by a rainbow darter pair (n=6). Thus, each hybrid male was involved in a total of three behavioral trials: one dichotomous male

choice trial and two male competition trials. Hybrid males experienced these trial types in random order. Unique purebred fish were used in each trial. We measured hybrid male aggressive behavior by counting the number of attacks (chasing and biting) and fin flares (male threat displays) that the hybrid male performed towards the purebred male in each trial (Zhou et al. 2015; Moran et al. 2017). We asked whether the number of attacks and fin flares that hybrid males directed towards males of the two purebred species differed. We performed GLMs with a negative binomial distribution and logit link function using the *glm.nb* function of the *MASS* package in R (Ripley et al. 2017). We performed separate GLM analyses that included the number of male aggressive behaviors (fin flares or attacks) performed in each trial as the dependent variable, and the identity of the purebred species pair in the trial (orangethroat or rainbow) as the independent variable. Raw data from the behavioral assays are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad. qf45rf2).

Morphological and histological analyses of testes

To further investigate potential F1 hybrid male sterility, we examined the testes of the six hybrid males and the 12 parental males (six orangethroat and six rainbow darters) that were used in the backcross experiment. Males were euthanized with an overdose of buffered MS-222. We performed gross and histological analyses to compare the testes of the hybrid and purebred males. Testes from each male were fixed in 10% buffered formalin, embedded in paraffin wax, and sectioned. Four μm sections were stained with hematoxylin and eosin and were visually inspected for signs of normal spermatogenesis.

Color analyses

We used digital photographs to perform multivariate phenotypic analyses of wild-caught orangethroat and rainbow darter males, wild-caught putative F1 hybrid males, and laboratory-generated F1 hybrid males. Our aim was to quantify differences in male color pattern in purebred males and hybrid males, and to statistically verify that hybrid color pattern is distinct and intermediate between purebred species. Such a finding would support our classification of wild-caught F1 hybrid males used in backcross experiments.

We chose to focus on components of male color pattern that differ between the parental species. Superficially, the red and blue banding pattern of orangethroat and rainbow darters looks quite similar, but these species differ in several key ways. Figure 6.1A and 6.1B illustrate the differences in male color pattern characteristics between orangethroat and rainbow darters, the most obvious of which are lateral side banding pattern and coloration, anal fin coloration, and caudal fin coloration. Our observations of laboratory-generated and wild-caught F1 hybrid males indicate that hybrids appear to exhibit combinations of both purebred species' color patterns (Fig. 6.1C,D).

We measured 36 male color pattern variables (i.e., 27 RGB variables and 9 color proportion variables) in the wild-caught hybrid males, orangethroat males, and rainbow males (n=6 each) used in backcross experiments, and in 6 lab-generated F1 hybrid males (which each came from unique families; 3 from ♀ rainbow x ♂ orangethroat crosses, 3 from ♀ orangethroat x ♂ rainbow crosses). We photographed laboratory-raised F1 hybrid males once they reached 22 months of age, at which point they exhibited male nuptial coloration. We also photographed the wild-caught putative F1 hybrid males and wild-caught *E. spectabile* and *E. caeruleum* males and females that were used in the backcross and behavioral experiments within 24 hours of their

collection. Fish were lightly anesthetized using MS-222 prior to photographing, and then placed on their sides in a petri dish that was filled with water and on a white background. Photographs were taken under fluorescent lighting using a Nikon Coolpix D3300 digital camera with the factory setting for photography in fluorescent lighting. An X-rite ColorChecker Mini Chart (Grand Rapids, MI) was included in each photo to allow for color correction and standardization across photographs. After photographing, fish were transferred to a bucket of aerated water to recover. Raw files of the digital photographs were color corrected and standardized using the inCamera 4.5 plug-in for Adobe Photoshop CC.

We used two categories of color measurements to quantify male color pattern: RGB values of the red and blue coloration on the body and fins, and proportion of red and blue coloration on the body and fins. We used the Color Sampler Tool in Adobe Photoshop CC to measure the RGB value in both the red and the blue areas (if present) on the lateral side of the body, the first dorsal fin, the second dorsal fin, the anal fin. We also measured the RGB value in the red areas on the caudal fin if present. This resulted in a total of 9 separate locations measured on each fish. Each RGB measurement gives a separate value for R, G, and B that range from 0-255 (where a 0, 0, 0 represents pure black and 255, 255, 255 represents pure white). Thus, three values were recorded for each of the 9 color locations, resulting in 27 RGB variables per fish. If red or blue coloration was absent on a given part of a fish (i.e., no anal fin red coloration), the R, G, and B values for that body part-color combination were recoded as not available. The Color Sampler Tool was set to measure 3x3 pixel samples, and each RGB color sample was taken three times at the same location on the same photograph for each fish. The average of these values was then used in color analyses (Zhou et al. 2014; Moran et al. 2017).

We used ImageJ (version 1.50c4) to measure the proportion of red and blue coloration present on each males' lateral side of the body, first dorsal fin, second dorsal fin, and anal fin. We also measured the proportion of red coloration present on the caudal fin. This resulted in 9 separate color proportion measurements for each fish. ImageJ's Threshold Colour plug-in was set to *L*a*b** color space and used to adjust the color threshold of the photographs (Zhou et al. 2014; Moran et al. 2017). We used *L*a*b** rather than RGB color space, as it allowed for better isolation of the red and blue components of male color pattern. We first used the Polygon Selections tool to trace an outline around the perimeter of the body and each fin one at a time. The Histogram tool was used to obtain a count of the total number of pixels in each of the outlined areas. To isolate only the blue coloration on the fins and body, the Threshold Colour plug-in was set to stop (i.e., exclude) pixels with *L** values above 200 and *b** values above 140. We then converted the image to binary, causing all of the isolated blue pixels to be changed to black. We then used the Polygon Selections tool to trace around each of the fins and the body, and used the Histogram tool to produce a count of the total number of black pixels in each region; this number was divided by the original total number of pixels counted in each region (before the blue coloration was isolated) to obtain the proportion of blue coloration on each fin and the body. The same process was followed to isolate and measure red coloration, but the Threshold Colour plug-in was set to stop pixels with *L** values above 200 and *a** values below 130. If a color was absent on a given part of a fish, the color proportion was recorded as 0.

We performed Linear Discriminate Analysis (LDA) on the male color pattern data with group (i.e., orangethroat*,* rainbow, wild-caught hybrid, or laboratory-generated hybrid) as the predictor variable using the *lda* function of the *MASS* package in R (Ripley et al. 2017). LDA identifies combinations of independent variables that maximize separation between dependent

variables (Mika et al. 1999). Thus, groups with more disparate loadings for a given Linear Discriminant (LD) can be inferred to be more distinct from one another in multivariate signal space. To ask whether male color pattern differs significantly between groups, we conducted multivariate analysis of variance (MANOVA) using the *manova* function of the *stats* package in R. Color measurements served as the independent variables and group served as the dependent variable. Raw data from the color analyses are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad. qf45rf2).

Genotyping wild-caught purebred and hybrid fish

To further verify the purebred or hybrid classification of all fish used in the backcross experiment (42 fish total), we performed single-digest Restriction-site Associated DNA sequencing (RADseq). DNA was isolated from skin and muscle tissue using a modified Puregene protocol. Samples were normalized to a concertation of 15 ng/uL in 50 uL 1x TE. RADseq library preparation with the restriction enzyme *Sbf*I was performed by Floragenex (Eugene, OR, USA), following the methods of Baird et al. (2008). The resulting RADseq library was sequenced as single-end 100 bp reads on two lanes on an Illumina HiSeq 4000 machine. Raw sequence data is available in the NCBI Sequence Read Archive (Accession # SRP152572).

Sequencing resulted in a total of 37,007,596 reads across the 42 individuals, with a mean ± SE of 881,133 ± 197,553 reads per individual. We used the Stacks (v2.0Beta9; Catchen et al. 2011, 2013) *process_radtags* program to demultiplex samples, remove barcodes, and remove reads of low quality or with ambiguous barcodes. This resulted in a total of 36,232,000 retained reads, which were then supplied to the *denovo_map* pipeline in Stacks to construct a catalog of loci and call SNPs. A minimum of three identical reads were required for each locus (-m 3), with

a maximum of three mismatches between loci in each individual (-M 3), and a maximum of two mismatches between loci to be added to the catalog (-n 2). This resulted in a catalog of 63,891 variant sites across 123,901 loci, representing a total of 11,308,200 sites across the genome. The mean ± SE depth of coverage was 23 ± 3X per individual.

The *populations* program in Stacks was used to generate population genetic statistics and to filter loci for analysis of genetic ancestry in Structure (Pritchard et al. 2000). We used *populations* to select loci that were present in all three groups (i.e., orangethroats, rainbows, and putative hybrids) (-p 3) and in at least 50% of the individuals within a group (-r 0.5), with a minimum minor allele frequency of 3%. This filtering resulted in 1,897 SNPs across 1,351 loci (representing a total of 123,472 sites across the genome) for the set of 42 total individuals (6 hybrids, 18 rainbows, 18 orangethroats). To make comparisons between hybrids and parental species, we used *populations* to calculate statistics of genetic differentiation between groups, including SNP-based AMOVA F_{ST} (Weir 1996) and haplotype-based Φ_{ST} (analogous to F_{ST} ; Excoffier et al. 1992) and D_{EST} (Jost 2008). Unlike F_{ST} and Φ_{ST}, D_{EST} is not sensitive to the level of heterozygosity within groups. To obtain an absolute measure of pairwise divergence, we used DnaSP (v6.10.03) (Rozas et al. 2003) to calculate the average number of nucleotide differences between groups (Dxy). To measure the level of genetic diversity within groups, we obtained estimates of nucleotide diversity (π), heterozygosity, the percent of polymorphic sites, and the number of private alleles from *populations*.

In the event that more than one SNP was present at a given RAD locus, we only used first SNP for Structure analyses by supplying the --write_single_snp flag to *populations*. This resulted in 1,073 unlinked SNPs that were output in Structure file format. To infer the number of distinct genetic clusters present in the data, we ran Structure with the ancestry model that allowed for

admixture and a burnin length of 50,000 followed by 150,000 MCMC repetitions. We performed 20 runs for values of K (i.e., genetic clusters) from 1 to 5 and inferred the optimal value of K using the Evanno method (Evanno et al. 2005) in Structure Harvester (Earl and vonHoldt 2012). Preliminary analyses confirmed the presence of two distinct genetic clusters in the data set, one corresponding to orangethroat darters and the other to rainbow darters (see Results).

To infer the proportion of ancestry associated with orangethroat versus rainbow darters in each hybrid male, we also calculated the hybrid index in GenoDive (v2.0b27) (Meirmans and Van Tienderen 2004) following the method of Buerkle (2005). The hybrid index is a maximum-likelihood estimate of the proportion of alleles in a hybrid individual that originated from one parental species versus the other. We imported the Structure file containing genotype data for 1,073 SNPs across all 42 individuals into GenoDive. For a given hybrid individual, a hybrid index closer to 1 would indicate allele frequencies more similar to that of orangethroat darters, a hybrid index closer to 0 would indicate allele frequencies more similar to that of rainbow darters.

RESULTS

Laboratory F1 hybrid cross viability

Fertilization success, hatching success, and larval survival did not differ between F1 hybrid clutches and the "purebred" parental species clutches (Fertilization Success: $F_{3,39} = 0.51$, $p = 0.68$; Hatching Success: $F_{3,39} = 0.04$, $p = 0.99$; Fry Survival: $F_{3,32} = 0.31$, $p = 0.82$; Table 6.1, Fig. D.1). Fertilization success varied greatly across replicate clutches but averaged less than 50% for all cross types. There were five clutches in which none of the eggs developed, possibly due to them being unripe or overly ripe (Moran et al. 2018). Excluding these five crosses from

the analysis did not qualitatively change the results. On average, over 50% of fertilized eggs hatched. Mortality was minimal between 10 and 22 months. Eight hybrids and three purebred fish died during this period, but most deaths could be attributed to husbandry issues (e.g., tank filter failure).

In both F1 hybrid crosses, the sex ratio of the offspring was significantly skewed towards males (♀ orangethroat x ♂ rainbow: mean ± SE proportion male = 0.844 ± 0.104, $t_5 = 3.30$, p = 0.02; ♀ rainbow x ♂ orangethroat: mean ± SE = 0.948 ± 0.037, $t_6 = 12.26$, p < 0.00001) (Fig. 6.2). Only 4 of the 13 F1 hybrid families included females at 22 months. A total of 6 out of 65 F1 hybrids were female. The sex ratio did not differ from the expected 1:1 frequency in purebred crosses (♀ orangethroat x ♂ orangethroat: mean ± SE = 0.594 ± 0.045, $t_5 = 2.13$, p = 0.09; ♀ rainbow x ♂ rainbow: mean ± SE = 0.450 ± 0.121, $t_8 = -0.41$, p = 0.69) (Fig. 6.2). Eleven out of 15 purebred families contained offspring of both sexes at 22 months of age. The total number of offspring per clutch at 22 months did not differ between hybrid and purebred crosses ($F_{1,26} = 0.18$, p = 0.68).

Backcross viability using wild-caught F1 hybrids

Backcrosses suffered higher levels of inviability compared to "purebred" orangethroat and rainbow darter crosses across all three measures of offspring viability (proportion of eggs collected that were fertilized: $F_{3,20} = 19.02$, p < 0.00001; proportion of fertilized eggs that hatched: $F_{3,20} = 3.47$, p < 0.05; proportion of hatched eggs that survived to the feeding larval stage: $F_{3,20} = 6.95$, p < 0.01; Table 6.2, Fig. 6.3). Fertilized eggs were collected in 10 out of 12 (83%) of the hybrid male crosses; one hybrid male x orangethroat darter female backcross

replicate and one hybrid male x rainbow darter female backcross replicate yielded no fertilized eggs. All purebred crosses produced fertilized eggs. Cumulative survival across all developmental stages was 10X higher in purebred crosses than backcrosses (Table 6.2). We did not observe any asymmetry in backcross viability: backcross to both parental species showed equally low levels of viability at each of the three developmental stages measured (Fig. 6.3). Parental crosses also did not differ from one another in viability at any stage (Fig. 6.3).

Orangethroat and rainbow darter females used in the crosses produced a similar number of eggs regardless of whether they were paired with a hybrid or a purebred conspecific male (Table 6.2; orangethroat backcross versus purebred cross: Mann-Whitney U test: U = 18, n = 12, p = 1.00; rainbow backcross versus purebred cross: Mann-Whitney U test: U = 8, n = 12, p = 0.13). In general, rainbow darter females laid fewer, larger eggs compared to orangethroat females, which laid a larger number of smaller eggs (R. Moran pers. obs.). We observed that female orangethroat darters laid two to three times more eggs than female rainbow darters of equivalent size during the duration of this experiment. However, the proportion of offspring surviving through each developmental stage did not differ between species (Fig. 6.3; Table 6.2).

Wild-caught F1 hybrid male mating and competitive behavior

Previous behavioral studies in orangethroat and rainbow darters have shown that males of both species exhibit strong preferences for pursuing females of their own species and preferentially direct aggressive behaviors towards males of their own species (Moran et al. 2017; Moran and Fuller 2018). In contrast, we observed no indication of assortative mating preferences in the wild-caught F1 hybrid males in our dichotomous male choice trials. Hybrid males did not preferentially pursue one purebred species of female over the other (Fig. D.2A; $t_5 = -0.12$, n = 6,

p = 0.91). Similarly, hybrid males did not preferentially bias their aggression towards orangethroat or rainbow darter males in the male competition trials. Hybrid males performed a similar number of fin flares ($X^2 = 0.51$, n = 6, p = 0.48; Fig. D.2B) and attacks ($X^2 = 0.13$, n = 6, p = 0.72; Fig. D.2C) towards males of both parental species. Additionally, all orangethroat and rainbow darter males engaged in aggressive interactions with the hybrid males.

Morphological and histological analyses of testes

Gross examination determined that all hybrid males possessed normally developed testes, compared to the purebred orangethroat and rainbow darter males. Comparative histological analysis of the hybrid and purebred male testes revealed that the testes of all males examined contained mature spermatids, and no obvious irregularities in spermatogenesis were observed. Figure D.3 shows representative images of testes histology for an orangethroat darter male, a rainbow darter male, and two wild-caught F1 hybrid males.

Color analyses

The LDA of male color pattern for orangethroat, rainbow, and F1 hybrid males simplified the multivariate color data set of 27 RGB variables and 9 color proportion variables into three LDs. The first two LDs explained a combined total of nearly 87% of the variance in coloration between groups. We visualized the differences in male color pattern among groups in two-dimensional signal space by plotting scores for LD 1 versus LD 2 for each individual (Fig. 6.4). Orangethroat, rainbow, and F1 hybrid individuals formed tight and well-separated clusters. There was almost complete overlap between the clusters containing the lab-raised and wild-caught F1

hybrid males. Furthermore, hybrid individuals occupied a signal space intermediate between both purebred species along the axis corresponding to LD 1 (Figs. 6.4, D.4).

The color proportion measurements had larger LD coefficients compared to the RGB measurements across all three LDs, indicating that the proportion of red and blue coloration on the body and fins are better predictors of group membership than RGB values (Table D.2). We therefore used the color proportion measurements for subsequent analyses. There was a significant difference in male color pattern between orangethroat, rainbow, and hybrid males (MANOVA: Pillai's Trace = 2.33, $F_{3,20} = 5.40$, $p < 0.000001$). Male color pattern did not differ between the lab-generated and wild-caught F1 hybrid males (MANOVA: Pillai's Trace = 0.95, $F_{1,10} = 4.22$, $p = 0.21$).

Genotyping wild-caught purebred and hybrid fish

As expected, notably higher levels of genetic diversity were observed within the hybrid group compared to either parental species (Table 6.3). Nucleotide diversity (π) and heterozygosity were generally low in both parental species, but higher in rainbow darters compared to orangethroat darters. In the hybrid fish, π was 7.6X higher compared to orangethroat darters and 6.1X higher compared to rainbow darters. Similarly, heterozygosity was 9.8X higher in hybrids compared to orangethroat darters and 5.9X higher in hybrids compared to rainbow darters. The number of private alleles were also an order of magnitude lower in the hybrid group compared to either parental species (Table 6.3), which is to be expected in F1 hybrids that share half of their alleles with each parental species.

Patterns of genetic differentiation between groups also supported our classification of hybrid individuals. The SNP-based F_{ST} was lower compared to the haplotype-based Φ_{ST} and

D_{EST}, but all three measurements of genetic differentiation between groups indicated a high degree of differentiation between orangethroat and rainbow darters, with estimates ranging between 0.689-0.808 (Table 6.4). As expected, comparisons between hybrids and orangethroat darters and between hybrids and rainbow darters revealed lower levels of differentiation. The average number of nucleotide substitutions per site (Dxy) was 0.01 between orangethroat and rainbow darters. Dxy between hybrids and each of the two parental species was 0.005, exactly half of that between the parental species.

The Structure analysis of 1,073 SNPs present in the set of 42 individuals used in the backcross experiment revealed an optimal K of 2 according to the Evanno method implemented in Structure Harvester (Table D.3). As with the color analyses, the genetic analyses confirmed our original diagnosis of the wild-caught orangethroat darters, rainbow darters, and F1 hybrid males that were used in the backcross experiment (Fig. 6.5). With K set to 2, the 18 orangethroat darter individuals were assigned 98% membership to cluster 1, and the 18 rainbow darter individuals were assigned 99% membership to cluster 2. The assignments of the six hybrid males were split between clusters and averaged 53% membership to the orangethroat cluster and 47% membership to the rainbow cluster. The hybrid index scores calculated for the hybrids yielded qualitatively similar results; the maximum likelihood estimate for the proportion of orangethroat darter ancestry in each hybrid male ranged from 0.501-0.566 (Table D.4).

DISCUSSION

Here we tested the hypothesis that reinforcement promotes the previously documented pattern of enhanced male mating preferences for conspecific over heterospecific females in

sympatry compared to allopatry (i.e. RCD) in orangethroat and rainbow darters (Moran and Fuller 2018). Reinforcement occurs when selection to avoid maladaptive hybridization favors divergence in mating signals and/or associated preferences in sympatry between two species (Servedio and Noor 2003; Coyne and Orr 2004). We used morphological and genomic data to show that hybridization is ongoing between orangethroat and rainbow darters. We then used natural and lab-generated hybrids to measure multiple components of postzygotic isolation. Our results suggest that there is a fitness consequence associated with hybridization in these species. This drastically changes how we think about speciation in darters, one of the most diverse groups of vertebrates in North America. Below we discuss the unexpectedly high degree of postzygotic isolation that we observed between orangethroat and rainbow darters and its implications for male-driven speciation via reinforcement and cascade reinforcement.

Patterns of F1 and backcross hybrid inviability

We found high levels of postzygotic isolation between orangethroat and rainbow darters in the form of multiple isolating barriers spanning across hybrid life stages and generations. This system was previously thought to lack substantial postzygotic isolation among species due to high survival of F1 larvae compared to purebred crosses (Hubbs and Strawn 1957; Hubbs 1959). Our results corroborated these previous findings. Clutches resulting from F1 crosses did not exhibit reduced fertilization, hatchability, or survival through adulthood compared to purebred crosses. However, we did observe dramatically distorted sex ratios in F1 crosses. Heterospecific crosses in both directions were heavily skewed towards males. Clutches from purebred crosses did not deviate from a 1:1 sex ratio, and most natural darter populations have also been shown to maintain 1:1 sex ratios in adults (Page 1983). Whether the male-skewed sex ratio in F1 hybrids

creates selection favoring assortative mating and behavioral isolation in areas of sympatry is unclear. Such a scenario may be present in *Neochromis* cichlids, which appear to have evolved assortative mating among incipient species in response to sex ratio distortion in hybrid clutches (Seehausen et al. 1999). The mechanisms underlying the lack of adult F1 females is also unknown. Investigation into the genetics of sex determination in darters would add insight into why female hybrids are missing from F1 hybrid clutches.

We also documented substantial postzygotic isolation between orangethroat and rainbow darters in the backcross generation. When wild-caught F1 males were crossed to females of both parental species, backcross clutches in both directions had dramatically reduced fertilization success, hatching success, and larval survival compared to clutches resulting from purebred parental crosses. The dramatic reduction in fertilization success observed in the backcross clutches is likely attributable to genetic incompatibilities being unmasked in backcross progeny, rather than F1 hybrid male sterility. Wild-caught F1 hybrid males did not exhibit any morphological or histological defects of the testes, and fertilized eggs in 10 out of 12 backcrosses. Many of the backcrosses also produced embryos with obvious developmental abnormalities that died before hatching (R. Moran pers. obs.). A small number of progeny resulting from backcrosses to both parental species were able to survive until the free feeding larval stage, indicating that although intrinsic postzygotic isolation between orangethroat and rainbow darters is very high, it is not complete. This has implications for the evolution of mating preferences in this system, which previous studies have shown to be consistent with reinforcement (see below) (Moran et al. 2017; Moran and Fuller 2018).

Implications for reinforcement

Genome-wide sequence data indicated high genetic differentiation and a 1% nucleotide divergence between orangethroat and rainbow darters. Heterozygosity and nucleotide diversity were generally low in both species, but higher in rainbow darters. This observation is consistent with previous analyses of genetic diversity in these species (Moran et al. 2017) and may reflect higher levels of population connectivity in rainbow darters compared to orangethroat darters (Page 1983).

Notably, our results indicate that F1 hybrids form in nature, and that we can accurately diagnose hybrid males based on color pattern attributes that are intermediate between the two purebred parental species. Molecular markers have also been used to document the presence of naturally occurring F1s, F2s, and backcrosses in both directions between rainbow darters and the orangethroat darter clade species *E. bison* (the buffalo darter) (Bossu and Near 2013), and F1 hybrids between rainbow darters and the orangethroat darter clade species *E. uniporum* (the current darter) (Moran et al. 2017).

The evidence for contemporaneous hybridization between orangethroat and rainbow darters together with the high levels of postzygotic isolation observed provide critical support for previous claims that reinforcement is responsible for driving the patterns of RCD (and potentially ACD) documented in this system (Zhou and Fuller 2014; Moran et al. 2017; Moran and Fuller 2018). Orangethroat and rainbow darter males from sympatric populations consistently show strong biases for mating with conspecific females and fighting with conspecific males (when given a choice between orangethroat or rainbow darters). Such biases are not present in orangethroat and rainbow darters that occur in allopatry with respect to one another (Moran and Fuller 2018). The presence of strong postzygotic isolation and ongoing

hybridization between these species has likely created selection favoring the high levels of behavioral isolation observed in sympatry compared to allopatry. Selection to avoid interspecific male-male aggressive interactions in sympatric populations (i.e., ACD) presumably acts to facilitate the co-occurrence of these species in such close proximity to one another in riffle microhabitats during the spawning season. In turn, the fact that orangethroat and rainbow darters occur syntopically on the same spawning grounds increases the potential for hybridization, which can then further fuel RCD via reinforcement. In this manner RCD and ACD may act in a positive feedback loop to strengthen male behavioral biases against heterospecific females and males (Vallin et al. 2012; Moran and Fuller 2018).

The lack of behavioral biases in wild-caught F1 males stands in contrast to the strong biases that were previously documented for sympatric male orangethroat and rainbow darters from the same drainage (Moran et al. 2017; Moran and Fuller 2018). Wild-caught F1 hybrid males pursued females of both parental species equally and engaged in a comparable amount of aggressive interactions with males of both parental species. Similarly, females and males of both parental species did not show any mating or fighting biases against hybrid males. These observations suggest that F1 males are behaviorally intermediate between the two parental species, similar to the pattern we observed in male color pattern. Furthermore, it has previously been argued that in sympatry, selection favors males who fight with conspecific males (over access to conspecific females) and ignore heterospecific males, in order to avoid costly, unnecessary aggression (Moran et al. 2017; Moran and Fuller 2018). The fact that F1 males engage in contests with males of both parental species suggests that they may pay the costs associated with increased fighting by engaging males of both species.

Evidence from the present study also supports the hypothesis that cascade reinforcement is responsible for the surprisingly high levels of male-driven behavioral isolation present between species within the orangethroat clade (Moran and Fuller 2018). By promoting the evolution of mating traits, reinforcement between two species can incidentally cause behavioral isolation among populations within a single species, termed cascade reinforcement (reviewed in Comeault and Matute 2016). Overtime, cascade reinforcement can cause isolated populations within one species that is experiencing reinforcement with a close relative to diverge to such an extent that they are considered distinct species. We hypothesize that such a phenomenon is occurring in orangethroat darters as a correlated effect of reinforcement with rainbow darters. Males from orangethroat clade species that do not co-occur with one another but do occur sympatrically with rainbow darters exert strong preferences for conspecific over heterospecific orangethroat darter females (Moran et al. 2017). It is possible that the parallel occurrence of reinforcement selecting for increased behavioral isolation between sympatric rainbow darters and multiple species within the orangethroat clade has incidentally led to mismatches in mating preferences and behavioral isolation between species within the orangethroat clade. The alternative hypothesis that sexual selection within species is responsible for this pattern is unlikely, as populations of orangethroat darters that are allopatric from other species in the orangethroat darter clade and from rainbow darters have no detectable levels of behavioral isolation (Moran and Fuller 2018).

Conclusions

We used genomic data to demonstrate that hybridization is ongoing between orangethroat and rainbow darters. These species were previously thought to lack substantial postzygotic

isolation, but we observed dramatically skewed sex ratios in F1s and a high degree of inviability in backcrosses. The results of this study demonstrate that selection to avoid hybridization may be more important than previously thought in darters. Our findings also inform our understanding of how speciation occurs in a highly diverse vertebrate group with traditional sex roles and dimorphism but no apparent female mate preferences. Darters provide a unique example of how male preferences alone can promote mating and fighting trait evolution concurrently between sympatric and allopatric lineages. The extensive amount of postzygotic isolation present between orangethroat and rainbow darters suggests that reinforcement promotes the previously documented patterns of RCD in male mating preferences between these species (Moran and Fuller 2018), which may incidentally favor the evolution of ACD in male aggressive biases. Furthermore, this implies that cascade effects of reinforcement may be responsible for the evolution of male-driven behavioral isolation between recently diverged lineages within the orangethroat darter clade that occur sympatrically with rainbow darters (Moran et al. 2017). Darters provide an intriguing study system for future investigations into the genetics/genomics of hybridization, reinforcement, and speciation.

ACKNOWLEDGEMENTS

The treatment of animals used in this study was in compliance with the University of Illinois Institutional Animal Care and Use Committee (IACUC) under protocols #12055 and #17031. Collection of wild fish was approved by the Illinois Department of Natural Resources under Scientific Collecting Permits A12.4035 and A16.4035. We thank Adam Stern (Veterinary Diagnostic Laboratory, University of Illinois) for assistance with gamete histology, and Jason

Boone (Floragenex) for logistic support with RAD library preparation and sequencing. This research was supported by funding from the National Science Foundation (DEB 0953716, DEB 1210743, DGE 1069157, and IOS 1701676), the United States Department of Agriculture (Cooperative State Research, Education, and Extension Service project number ILLU 875-952), and the University of Illinois at Urbana-Champaign.

TABLES

Table 6.1. Mean (± standard error) number of total eggs stripped, eggs fertilized, eggs hatched, and fry that survived to 10 months of age in the purebred crosses and F1 hybrid crosses.

Cross	# Eggs stripped	# Eggs fertilized	# Eggs hatched	# Fry survived 10 months
♀ O x ♂ O	67.4±9.2 (n=14)	30.6±12.3 (n=11)	24.6±13.0 (n=10)	6.5±0.6 (n=6)
♀ R x ♂ R	76.0±14.2 (n=12)	25.7±9.9 (n=11)	22.4±10.7 (n=9)	5.8±2.2 (n=8)
♀ O x ♂ R	92.8±16.0 (n=10)	23.9±9.7 (n=10)	14.5±5.3 (n=8)	3.8±0.7 (n=5)
♀ R x ♂ O	80.2±12.1 (n=12)	30.6±7.5 (n=11)	22.1±6.8 (n=9)	6.0±1.1 (n=9)

O = orangethroat darter, R = rainbow darter.

Table 6.2. Mean (± standard error) number of total eggs collected, eggs fertilized, eggs hatched, and fry that survived to the independently feeding stage in purebred crosses and backcrosses (n = 6 each).

Cross	# Eggs collected	# Eggs fertilized	# Eggs hatched	# Fry survived to feeding
♂ O x ♀ O	92.33 ± 14.83	82.00 ± 12.41	61.67 ± 9.90	56.17 ± 8.18
♂ R x ♀ R	35.50 ± 5.23	31.17 ± 3.61	21.67 ± 3.19	21.00 ± 3.36
♂ H x ♀ O	88.00 ± 30.46	19.33 ± 14.81	10.33 ± 10.14	6.00 ± 5.80
♂ H x ♀ R	23.17 ± 5.76	8.33 ± 2.39	2.50 ± 1.77	2.17 ± 1.60

O = orangethroat darter, R = rainbow darter, H = F1 hybrid.

Table 6.3. Measurements of genetic diversity within groups (i.e., hybrids, rainbow darters, and orangethroat darters) for all 123,472 sites across 1,351 loci.

Group	**n**	**% Poly**	**# Priv**	**π**	**H_{OBS}**	**H_{EXP}**
Hybrids	6	1.2636	12	0.0061	0.0059	0.0052
Rainbow	18	0.5387	212	0.0010	0.0010	0.0012
Orangethroat	18	0.3694	103	0.0008	0.0006	0.0009

% Poly = percent polymorphic sites, # Priv = number of private alleles, π = nucleotide diversity, H_{OBS} = observed heterozygosity, H_{EXP} = expected heterozygosity.

Table 6.4. Measurements of genetic differentiation and divergence between groups (i.e., hybrids, rainbow darters, and orangethroat darters). The SNP-based fixation statistic (F_{ST}) was calculated using 1,897 variant sites (SNPs). The haplotype-based fixation statistics (Φ_{ST}, D_{EST}) and the average number of nucleotide substitutions per site (D_{XY}) were calculated using 123,472 sites across 1,351 loci.

Comparison	**F_{ST}**	**Φ_{ST}**	**D_{EST}**	**D_{XY}**
Hybrids - Rainbows	0.327	0.495	0.305	0.005
Hybrids - Orangethroats	0.315	0.454	0.271	0.005
Rainbows - Orangethroats	0.689	0.792	0.808	0.010

FIGURES

Figure 6.1. (A) Orangethroat darter and (B) rainbow darter males showing color pattern typical of these species. Orangethroat darters lack the red coloration that is present on the caudal and anal fin in rainbow darters. (C) Wild-caught orangethroat x rainbow darter F1 hybrid male and (D) lab-generated orangethroat x rainbow darter F1 hybrid male showing color pattern characteristics that are combinations of both parental species.

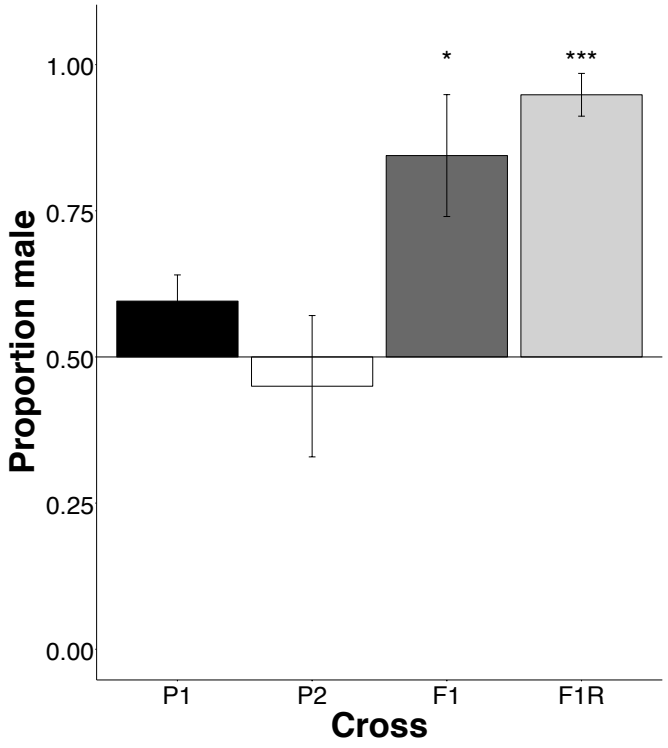

Figure 6.2. Mean proportion (± standard error) of male offspring in the parental crosses and F1 hybrid crosses at 10 months of age. The deviation from a mean of 0.50 male offspring (i.e., a 1:1 male:female sex ratio) is depicted for each cross type (* = $p < 0.05$, *** = $p < 0.001$). P1 = ♀ orangethroat x ♂ orangethroat (n=6), P2 = ♀ rainbow x ♂ rainbow (n=8), F1 = ♀ orangethroat x ♂ rainbow (n=5), F1R = ♀ rainbow x ♂ orangethroat (n=9).

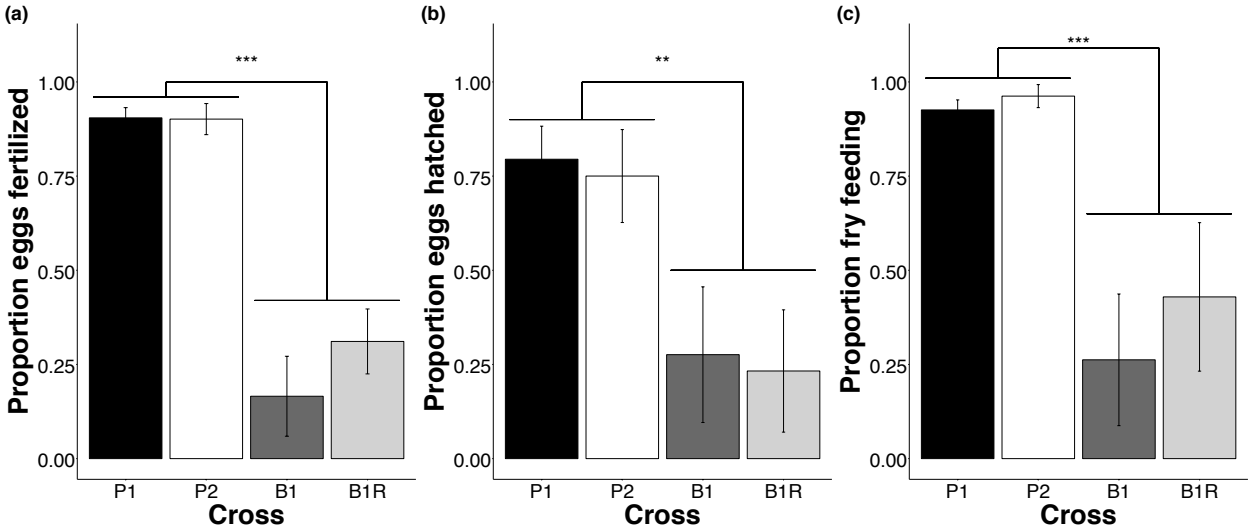

Figure 6.3. Mean proportion (± standard error) of (A) eggs collected that were fertilized, (B) fertilized eggs that hatched, and (C) hatched fry that survived to the independently feeding stage (approximately three days post-hatching) in the parental crosses and backcrosses (n=6 each). Significance levels are indicated for post-hoc comparisons of purebred crosses and backcrosses (** = $p < 0.01$, *** = $p < 0.001$). P1 = ♀ orangethroat x ♂ orangethroat, P2 = ♀ rainbow x ♂ rainbow, B1 = ♀ orangethroat x ♂ F1 hybrid, B1R = ♀ rainbow x ♂ F1 hybrid.

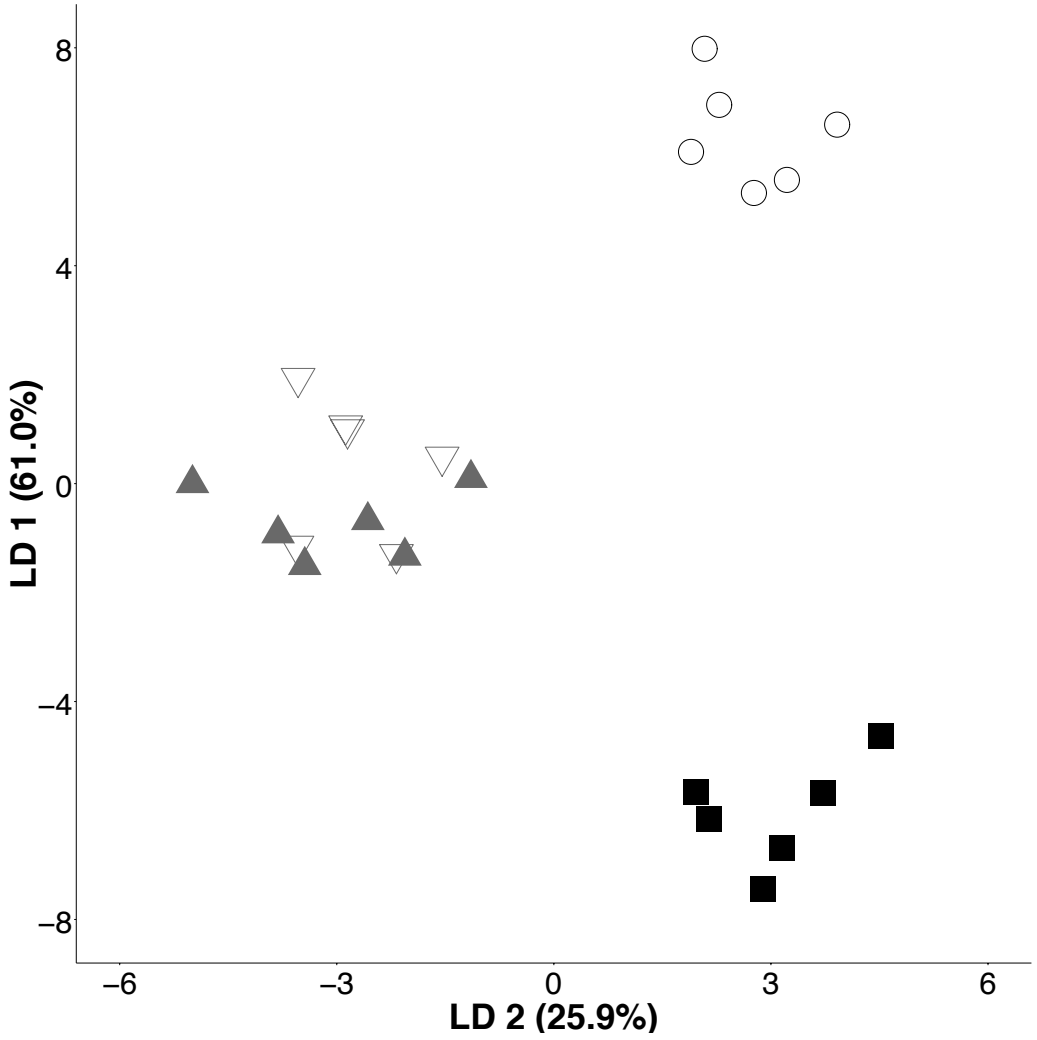

Figure 6.4. Biplot of the first two Linear Discriminant (LD) axes from the male color pattern LDA of wild-caught orangethroat (■), wild-caught rainbow (○), wild-caught F1 hybrid males (▽), and lab-generated F1 hybrid males (▲).

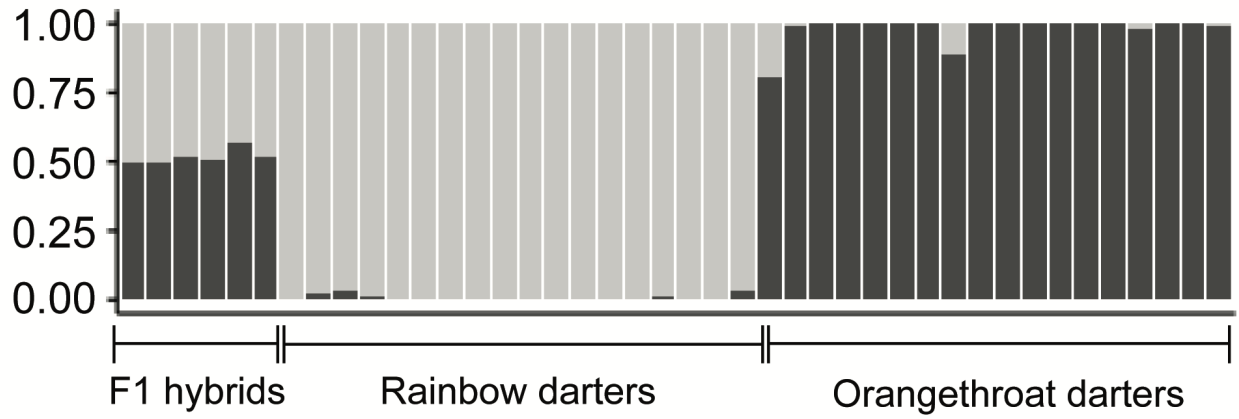

Figure 6.5. Probability of membership to the rainbow darter cluster (light gray) or orangethroat darter cluster (dark gray) for each individual used in the backcross experiment. Structure analysis was conducted using 1,073 SNPs with the number of clusters (K) set to 2.

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CHAPTER 7

THE ORANGETHROAT DARTER GENOME ASSEMBLY FACILITATES AN INVESTIGATION INTO THE GENOMIC ARCHITECTURE OF POSTZYGOTIC ISOLATION BETWEEN TWO HYBRIDIZING DARTER SPECIES

ABSTRACT

Comparative genomic approaches are increasingly being used to study the evolution of reproductive isolating barriers in non-model species. Darters (Percidae) represent the most diverse group of vertebrates in North America and provide a remarkable example of male-driven speciation via intrasexual selection and character displacement. Although numerous studies have examined the behavioral basis of prezygotic isolation in these fishes, investigations into postzygotic barriers have remained rare due to long generation times and a lack of genomic resources. To investigate the genomic architecture of postzygotic isolation between orangethroat and rainbow darters, we used Illumina and PacBio sequencing to generate a chromosome-level, annotated assembly of the orangethroat darter genome and high-density linkage maps for both orangethroat and rainbow darters. The linkage maps were used to identify chromosomal rearrangements that contribute to genetic incompatibilities between species. To investigate whether negative epistatic interactions contribute to postzygotic isolation between orangethroat and rainbow darters, we analyzed genome-wide RADseq data obtained from wild-caught adults of both species and from families of laboratory-generated backcross hybrids. We observed several putative chromosomal translocations between orangethroat and rainbow darters,

suggesting that chromosomal rearrangements may contribute to postzygotic isolation in these species. We also observed large regions in backcross hybrid genomes that showed evidence of selection against recombinant haplotypes and transmission ratio distortion, indicating additional candidate regions underlying genetic incompatibilities. These findings mark significant contributions to our understanding of how genomic architecture facilitates speciation in darters. Additionally, the annotated darter reference genome and linkage maps presented here provide valuable resources for future studies on the genomics of speciation in these fishes and will greatly assist conservation efforts.

INTRODUCTION

Identifying reproductive barriers that prevent gene exchange between taxa remains a central goal of speciation research (Coyne and Orr 2004; Butlin et al. 2012). Understanding the genetic basis of such barriers presents a particular challenge in non-model organisms that are not easily crossed in the laboratory (Orr and Presgraves 2000). However, advances in second- and third-generation sequencing technology have made it recently possible to take a comparative genomic approach to identify barriers to gene flow, even in cases where traditional quantitative genetic approaches are not feasible (Butlin 2010; Wolf et al. 2010; Ellegren 2014). Whether certain genetic mechanisms are more likely to facilitate speciation and maintain reproductive isolation in the face of gene flow remains a major unanswered question.

Genetic incompatibilities underlying postzygotic barriers have been identified across a wide range of taxa (e.g. *Mimulus*: Martin and Willis 2010; *Arabidopsis*: Kradolfer et al. 2013; *Drosophila*: Coyne 1984; Moehring et al. 2006; grasshoppers: Virdee and Hewitt 1992; lake

whitefish: Rogers and Bernatchez 2006; *Ficedula* flycatchers: Sætre et al. 2003; mice: Good et al. 2008). Two mechanisms that appear to be commonly implicated in the evolution of genetic incompatibilities and postzygotic isolation are chromosomal rearrangements and negative epistatic interactions. Chromosomal rearrangements include inversions, fusions, and translocations in a species relative to the ancestral state. If two lineages diverge in gene order collinearity (i.e. synteny) along homologous chromosomes due to such rearrangements, it can cause problems with chromosome pairing and crossing over during meiosis (thus suppressing recombination; Lai et al. 2005; McGaugh and Noor 2012) and can result in complete inviability or other fitness decreases (e.g. hybrid offspring only inheriting one copy of certain genes from their parents in regions associated with rearrangements) (Noor et al. 2001; Rieseberg 2001). Negative epistatic interactions can occur when new alleles arise in each of two diverging lineages and cause no negative impact on fitness within each lineage, but result in decreased fitness when brought together due to hybridization (Orr and Presgraves 2000; Turelli and Orr 2000; Coyne and Orr 2004). Investigations into the mechanism of genetic incompatibilities and the genome-wide frequency and distribution of loci involved in postzygotic barriers between species remain limited and have largely been restricted to model species (sunflowers: Rieseberg et al. 1999; Gardner et al. 2000; mice: Teeter et al. 2010; threespine stickleback: Hohenlohe et al. 2012; swordtail fishes: Schumer et al. 2014; *Drosophila*: Pool 2015; *Populus*: Christe et al. 2016). Here we generate the first genome and linkage maps for darters (Percidae: *Etheostoma*) and use these tools in conjunction with genome-wide sequence data from laboratory-generated backcross hybrids to investigate the genomic architecture and genetic basis of postzygotic isolation in two naturally hybridizing darter species.

The darter genus *Etheostoma* contains nearly 160 species, representing the most speciose vertebrate genus in North America (Page and Burr 2011; Froese and Pauly 2019). Investigating the evolutionary forces that have caused darters to undergo such dramatic diversification has been a large focus of research on speciation and sexual selection over the last six decades (Hubbs and Strawn 1957; Winn 1958; Hubbs 1958; Moerchen 1973; Page and Swofford 1984; Pyron 1995; Fuller 2003; Mendelson 2003; Near and Benard 2004; Martin and Mendelson 2012, 2016b; Bossu and Near 2013; Williams et al. 2013; Zhou and Fuller 2016; Moran et al. 2017; Moran and Fuller 2018b). Female preferences for male nuptial ornamentation were originally credited with driving trait diversification and speciation in darters (Reeves 1907; Winn 1958; Page 1983; Mendelson 2003). However, recent research suggests that male mating and fighting biases and selection to avoid interspecific interactions play an important role in both initiating divergence in allopatry and finalizing speciation in sympatry (Zhou et al. 2015; Martin and Mendelson 2016a; Moran et al. 2017; Mendelson et al. 2018; Moran and Fuller 2018b). Questions remain regarding which barriers contribute most to speciation in darters, in large part due to their long generation time (making crosses in the laboratory a challenge) and a lack of genomic resources.

We focused on two groups of darters in the subgenus *Oligocephalus*. These species were chosen for sequencing because they have become emerging models for the study of speciation via reproductive character displacement (RCD) and agonistic character displacement (ACD) (Zhou and Fuller 2014; Moran et al. 2017; Moran and Fuller 2018b,a). The orangethroat darter (*Etheostoma spectabile*) was recently split into a clade of 15 allopatric species (*Ceasia*), 13 of which occur in sympatry with the rainbow darter (*Etheostoma caeruleum*) (Ceas and Page 1997; Bossu and Near 2009). Although orangethroat and rainbow darters are not sister taxa to one

another and are estimated to have diverged 22 Mya (Near et al. 2011), they share similar ecology, mating behavior, and male color pattern (Page and Burr 2011). There is evidence that historical bouts of introgressive hybridization have occurred between these species. Rainbow darter mitochondrial haplotypes are present in several orangethroat clade species, one of which exhibits complete fixation for the rainbow darter mitochondrial genome (Ray et al. 2008; Bossu and Near 2009). Hybridization is also presently ongoing between orangethroat and rainbow darters. F1, F2, and backcross hybrids have been identified in several zones of sympatry using molecular data (Bossu and Near 2013; Moran et al. 2017, 2018). Recent evidence suggests that hybrids suffer from decreased fitness, as F1 hybrid crosses result in dramatically male-skewed sex ratios and backcrosses exhibit severely reduced viability relative to parental crosses (Moran et al. 2018). Selection to avoid costly hybridization appears to have promoted increased male mating preferences for conspecific over heterospecific females in sympatry compared to allopatry between orangethroat and rainbow darters, consistent with RCD (Moran et al. 2017; Moran and Fuller 2018b). Notably, the signal(s) that males use to discriminate conspecific from heterospecific females remain unknown. Interspecific fighting over access to females has also been documented between male orangethroat and rainbow darters and is likely costly. In turn, selection to avoid interspecific male contests has led to increased male bias for fighting with conspecific over heterospecific males in sympatry compared to allopatry, consistent with ACD (Moran et al. 2017; Moran and Fuller 2018b).

By altering traits used in mate and competitor recognition in sympatry, RCD and ACD between orangethroat and rainbow darters has incidentally caused behavioral isolation to evolve among species within the orangethroat clade. Orangethroat darter males prefer to mate with conspecific females and bias aggression towards conspecific males over individuals from another

closely related orangethroat species only when they naturally co-occur in sympatry with rainbow darters (Moran et al. 2017; Moran and Fuller 2018b). Thus, RCD and ACD between orangethroat and rainbow darters has had "cascade" effects within orangethroat darters, likely promoting the diversification of this clade. Although cascade RCD (CRCD) has now been documented in a number of taxa, this represents the only known example to date of cascade ACD (CACD).

Another unique aspect of this system is that female preference for variable aspects of male color pattern is absent between and within species (Pyron 1995; Fuller 2003; Zhou et al. 2015; Moran et al. 2017). Instead, male color pattern functions in male contests over access to females and in male competitor recognition (Zhou and Fuller 2016; Moran and Fuller 2018b). Males bias their aggression more towards individuals with a color pattern more similar to their own (Moran et al. 2017). Furthermore, differences in male color pattern between orangethroat and rainbow darters is genetically based, and color pattern is more divergent between these species in sympatric compared to allopatric populations (Moran and Fuller 2018a). This demonstrates that interspecific male competition can lead to color pattern signal divergence between and within species via ACD and CACD. Together, these findings contradict the widely-accepted paradigm that female preferences promote the evolution of behavioral isolation in species where males exhibit elaborate secondary sex traits.

The genetic basis of postzygotic isolation between orangethroat and rainbow darters remains unknown. As postzygotic isolation is a necessary prerequisite for RCD (and potentially ACD) in this system, understanding the mechanism underlying genetic incompatibilities between these species will help inform our understanding of how RCD, ACD, CRCD, and CACD evolve. Recent research has shown that lethal genetic incompatibilities are uncovered in the backcross

hybrid generation (Moran et al. 2018). Such incompatibilities could stem from negative epistatic interactions and/or chromosomal rearrangements. There is good reason to suspect that chromosomal rearrangements may play a role in postzygotic isolation. Although all species of darters appear to possess 24 pairs chromosomes, karyotype (e.g. number of metacentric versus acrocentric chromosomes) can vary within and among species (Moerchen 1973; Ross 1973; Danzmann 1979). Variation in chromomere morphology is so pervasive in darters that one previous study suggested karyological diversity may reflect species diversity in these fishes (Ross 1973).

Here we take the first steps towards elucidating the genomic architecture of postzygotic isolation and hybrid incompatibly in darters by evaluating the presence of chromosomal rearrangements and/or negative epistatic interactions between the genomes of orangethroat and rainbow darters. We used both long-read Pacific Biosciences (PacBio) and short-read Illumina sequencing to assemble a genome, transcriptome, and high-density linkage map for the orangethroat darter *E. spectabile*, with the goal of producing a high-quality, annotated draft genome. To test the hypothesis that chromosomal rearrangements are present between orangethroat and rainbow darters, we also constructed a high-density linkage map for the rainbow darter. This allowed us to compare synteny and homology between rainbow darter linkage groups and orangethroat darter chromosomes. To further assist in identifying regions of the genome implicated in postzygotic isolation, we conducted fine-scale SNP mapping in wild-caught adults from both parental species and in laboratory-generated backcross individuals. This allowed us to examine genome-wide patterns of selection against recombinant genotypes, transmission ratio distortion, and linkage disequilibrium in backcross hybrids. This research provides unprecedented insight into the genetic mechanisms promoting the evolution of

RCD/ACD and CRCD/CACD. The genomic resources generated by this study will be instrumental for future investigations spanning the fields of speciation, population genomics, conservation, and systematics in darters and other percids.

METHODS

Linkage Map Sequencing

Orangethroat darter

We used a single male and female pair of orangethroat darters to create an F1 mapping cross. Both orangethroat darter parents were collected via kick seine from the Salt Fork of the Vermillion River Drainage (Champaign County, Illinois) in May 2016. The male parent used in this cross was the same individual whose DNA was used to sequence all genomic libraries described below (i.e., Illumina shotgun, Illumina mate-pair, and PacBio). The pair were housed in the laboratory in a 37 L aquarium with gravel substrate and allowed to spawn. After 24 hours, the gravel was siphoned and a total of 176 eggs were collected. Eggs were maintained as described in Moran et al. (2018) until two months post-hatching. A total of 145 fry survived to this age. At this time, the two parents and 145 offspring were euthanized with an overdose of buffered MS-222, placed in 95% ethanol, and stored at -80 C.

We isolated DNA from the white muscle tissue of both parents and the entire body of each fry. DNA was extracted using a modified Puregene protocol and treated with RNase A. Samples were checked for purity on a Nanodrop 1000 machine and quantified on a Qubit fluorometer. RADseq libraries were constructed at Floragenex (Portland, OR) following the methodology of Baird et al. (2008). The restriction enzyme *SbfI* was used to digest 750 ng of

DNA from each of the progeny. To ensure that diploid genotypes were accurately called at each RAD locus for the two parents, 1.5 ug of DNA was included for both parents in the RADseq libraries. Libraries were sequenced as 1x100 bp reads on two lanes on an Illumina HiSeq4000 machine at the University of Oregon (Eugene, OR).

Rainbow darter

To allow for an investigation of genomic synteny between orangethroat and rainbow darters, we also created an F1 mapping cross with a single rainbow darter pair. Both parents were collected via kick seine from the Salt Fork of the Vermillion River Drainage (Champaign County, Illinois) in April 2018. The pair were allowed to spawn in the laboratory and eggs were collected as described above. Out of 106 total eggs collected, 85 survived to two months post-hatching. At this time, the parents and fry were euthanized and DNA was extracted as described above. A RAD library was constructed using 1000 ng of DNA from each of the parents and offspring. We used the restriction enzyme *SbfI* for RAD library construction at the University of Illinois at Urbana-Champaign (UIUC), following the methodology of Baird et al. (2008). We again ensured a higher depth of coverage for the parents by including 2X the amount of DNA for both parents compared to the fry in the RADseq library. This library was sequenced as 1x100 bp reads on two rapid-run lanes on an Illumina HiSeq2500 machine at the Biotechnology Center at UIUC.

Linkage Map Construction

We used the Stacks (v1.48; Catchen et al. 2011, 2013) program *process_radtags* to demultiplex the raw sequences resulting from the linkage map RADseq libraries (235,042,086

orangethroat darter raw reads; 185,333,523 rainbow darter raw reads) and to remove barcodes and low-quality reads. Eight rainbow darter fry were removed from all further analyses due to a low number of reads, which left 77 rainbow darter fry total. The reads that were retained after the initial quality filtering step (232,255,220 orangethroat darter retained reads; 171,768,983 rainbow darters retained reads) were supplied to the Stacks *denovo_map* pipeline for RAD loci assembly and genotyping for each of the two linkage map families. A minimum of three identical reads (-m 3) were required to form a "stack" (i.e., a putative allele) in each individual. We allowed for a maximum of five differences between stacks to form a locus (-M 5) and a maximum of three differences when merging loci from different individuals to form a catalog (-n 3).

Constructing a linkage map from a single F1 cross requires an identification of polymorphisms that are present in the parents. SNP markers that are informative for mapping in this context include those that are heterozygous in the male parent and homozygous in the female parent (*lm* x *ll*, segregating 1:1), heterozygous in the female parent and homozygous in the male parent (*nn* x *np*, segregating 1:1), heterozygous in both parents with two shared alleles (*hk* x *hk*, segregating 1:2:1), or heterozygous in both parents with one shared allele and one allele specific to each parent (*ef* x *eg*, segregating 1:1:1:1) (Amores et al. 2011). Stacks was used to genotype our linkage map parents and their progeny at each RAD locus. This resulted in 3,478 genotyped loci in the orangethroat darter family and 4,665 genotyped loci in the rainbow darter family that were informative for mapping. For both the orangethroat and the rainbow families, we used the *genotypes* program in Stacks to filter loci for quality and export the resulting data in Cross Pollinator (CP) format. We specified that a locus had to be present in at least 30 out of the 145 total orangethroat darter offspring and in at least 45 out of the 77 total rainbow darter offspring

to be included in the exported files. This resulted in 2,247 orangethroat darter loci and 3,230 rainbow darter loci that were then imported into JoinMap v5 (Van Ooijen 2006). For each species, linkage maps were constructed separately for both parents. Linkage groups with more than two loci shared between the male and female parent were inferred to be homologous and were combined to form a consensus map. Loci with segregation frequencies that differed significantly from Hardy-Weinberg Equilibrium ($p<0.001$) were excluded. Markers were assigned to linkage groups using an LOD of 4.0 for the orangethroat family and 5.0 for the rainbow family. Ungrouped loci were iteratively added to linkage groups by using the Strongest Crosslinked Loci (SCL) option in JoinMap with an LOD cutoff of 4.0. Marker order for each linkage group was calculated using the Kosambi mapping function, which converts recombination frequencies between pairs of markers into genetic distance in centimorgans (cM).

Genome Sequencing

Illumina Paired-End and Mate-Pair Short-Read Libraries

Using standard ethanol precipitation methods, we isolated 19 μg of high molecular weight DNA from a single wild-caught male orangethroat darer (location details described above). This same individual was used as the male parent in the orangethroat darter linkage map cross. Two genomic shotgun libraries with insert sizes of 450 bp and 800 bp, and three mate-pair libraries (3-5 kb, 5-7 kb, 8-12 kb) were prepared and sequenced at the Biotechnology Center at UIUC. Shotgun libraries were prepared with the Hyper Library construction kit from Kapa Biosystems. Mate-pair libraries were constructed with the Nextera Mate Pair library Sample Prep kit (Illumina, CA), followed by the TruSeq DNA Sample Prep kit. Libraries were quantitated with qPCR prior to sequencing.

We sequenced the 450 bp shotgun library together with the RNAseq library (see below for details) on two lanes on a HiSeq 2500 machine in a proportion of 3:1 (favoring coverage for the shotgun library) to produce paired-end 250 bp reads. Fragment sizes ranged from 200 bp to 530 bp with an average of 450bp. We sequenced the 800 bp library and the mate-pair libraries together on one lane for 161 cycles from each end of the fragments on a HiSeq 2500 machine, resulting in 150 bp paired-end reads. Fragment sizes ranged from 600 bp to 900 bp with an average of 800bp.

Sequencing resulted in a total of 391,068,018 overlapping raw reads from the 450 bp insert library, 63,746,270 raw reads from the 800 bp insert library, 97,973,478 raw reads from the 3-5 kb mate-pair library, 91,585,478 raw reads from the 5-7 kb mate-pair library, and 90,833,126 raw reads from the 8-12 kb mate-pair library. For each library, fastq files were generated and demultiplexed with the bcl2fastq v2.17.1.14 Conversion Software (Illumina).

PacBio Long-Read Library

We isolated a total of 40 μg of high molecular weight DNA from the same male orangethroat darter used in the Illumina shotgun, mate-pair, and RAD libraries. A PacBio long-insert (>31kb) library was constructed following standard protocol. Sequencing was conducted on four SMRT cells on a PacBio Sequel machine. Library construction and sequencing were both carried out at the University of Minnesota Genomics Center (St. Paul, MN). A total of 30 Gb of raw sequence data (30X genome coverage) was produced, with a mean ± SE longest sub-read length of 8.4 ± 0.3 kb and a mean ± SE longest subread N50 of 14.6 ± 0.4 kb.

Genome Assembly

We used the program *process_shortreads* in Stacks to remove adaptors and poor-quality reads from the Illumina shotgun libraries. NxTrim was used to remove biotin adaptors and poor-quality reads from the three Illumina mate-pair libraries. After this quality filtering, we retained a total of 388,991,066 overlapping paired-end reads from the 450 bp insert library, 63,200,545 non-overlapping paired-end reads from the 800 bp insert library, 79,139,172 3-5 kb mate-pairs, 76,273,856 5-7 kb mate-pairs, and 78,571,066 8-12 kb mate-pairs. This represents 142X coverage of the approximately 1 Gb orangethroat darter genome. We used Meraculous2 (Chapman 2016) to carry out four *de novo* genome assemblies with kmer length of 49, 59, 69, and 79. We obtained assembly statistics from Meraculous2 v2.2.2.5 (Chapman et al. 2016) and QUAST v4.4 (Gurevich et al. 2013) (Table E.1) and examined the number of Actinopterygii-specific Benchmarking Universal Single-Copy Orthologs (BUSCOs) identified in each assembly with BUSCO v3.0.2 (Simao et al. 2015) (Table E.2). A kmer size of 59 yielded the best assembly based on quality and completeness.

We took two different approaches to improve the Illumina-based assembly obtained from Meraculous2 with PacBio data. First, we supplied the 30 GB of raw PacBio reads to two different long read assemblers: Canu v1.7 (Koren et al. 2017) and to wtdbg2 v2.2 (Ruan and Li 2019). Canu error-corrects raw reads prior to assembly whereas wtdbg2 assembles raw reads and then corrects the assembly based on consensus. Canu was run with an error correction rate of 8.5% (correctedErrorRate=0.085). The corrected and trimmed reads used in the Canu assembly represented 16X coverage of the genome. For the wtdbg2 assembly, reads shorter than 5,000 bp in length were discarded (-L 5000), resulting in 22X coverage of the genome. Each of the two PacBio-only assemblies were polished with Pilon v1.21 (Walker et al. 2014) using 97X Illumina

paired-end reads. Second, we used the PBSuite v15.8.24 (English et al. 2012) program PBJelly2 to conduct scaffolding and gap-filling of the Illumina assembly with the raw PacBio reads. The PBJelly2 assembly was then polished in Pilon with 97X Illumina paired-end reads.

We used two rounds of quickmerge (Alhakami et al. 2017) to merge the PBJelly, Canu, and wtdbg2 assemblies. First, we merged the Canu assembly with the wtdbg2 assembly using the Canu assembly as the reference. Second, we merged the PBjelly assembly with the Canu-wtdbg2 merged assembly using the Canu-wtdbg2 assembly as the reference. For both rounds of assembly merging, contigs were merged using a minimum alignment length of 7 kb (-ml 7000) and a 3.5 Mb length cutoff (-l 3500000) for anchor contigs. Lastly, we performed another round of polishing with pilon, resulting in the final assembly.

Transcriptome Sequencing

To assist in genome annotation, we sequenced and assembled a transcriptome for the orangethroat darter. We isolated RNA from one adult male and one adult female collected from the same location as the male used for genome sequencing. Additionally, we isolated RNA from a one-week old fry. The adults and fry were euthanized using an overdose of buffered MS-222 and placed on ice. Tissue from the eye, gonads, muscle, liver, fins, and brain from each adult and the entire fry were isolated and homogenized with a mortar and pestle. A Qiagen RNeasy extraction kit was used to isolate RNA from each tissue sample. We pooled equal amounts of RNA from the fry and each of the adult tissues to obtain a total of 1 μg RNA.

An RNAseq library was prepared and sequenced at the Biotechnology Center at UIUC. A TruSeq Stranded mRNAseq Sample Prep kit (Illumina) was used to prepare the RNAseq library, but modified so that fragmentation was done at 80° C for 2 min. Resulting cDNA fragments

ranged from 100 bp to 900 bp, with an average of 400 bp. The RNAseq library was sequenced together with the 450 bp shotgun library (see above) to obtain paired-end 250 bp reads. The library was quantitated by qPCR. Sequencing was done on two lanes for 266 cycles from each end of the fragments on a HiSeq2500 machine using a HiSeq Rapid SBS sequencing kit (version 2). This resulted in a total of 128,023,978 reads (64,011,989 forward and 64,011,989 reverse reads).

Transcriptome Assembly

We used the program *process_shortreads* in Stacks to remove adaptors and poor-quality reads present in the RNAseq library. After quality filtering, 127,489,576 paired reads were retained and used to create a *de novo* transcriptome assembly with Trinity v2.5.1 (Grabherr et al. 2013). To determine the percent of the RNAseq reads that were represented in the Trinity assembly, we used Bowtie2 (v2.3.3.1) (Langmead and Salzberg 2012) to align the RNAseq reads back to the assembled transcripts. We also evaluated the number of Actinopterygii-specific BUSCOs present in the transcriptome assembly.

Genome Annotation

We executed three iterative rounds of the Maker v2.31.9 (Cantarel et al. 2008) genome annotation pipeline to predict protein-coding genes. We supplied the following evidence to Maker for the first round of annotation: the orangethroat darter genome assembly, the orangethroat darter transcriptome, protein sequences obtained from five other teleost species (large yellow croaker *Larimichthys crocea* NCBI ASM74293v1, threespine stickleback *Gasterosteus aculeatus* Ensembl BROAD S1, zebrafish *Danio rerio* Ensembl GRCz11, medaka

Oryzias latipes Ensembl HdrR, and tilapia *Oreochromis niloticus* Ensembl Orenil1.0), and the entire set of UniProt Swiss-Prot proteins (http://www.uniprot.org/) (Small et al. 2016). Maker used a list of known transposable elements and a RepBase library to soft mask repetitive elements prior to the initial annotation. We also provided Maker with an orangethroat darter-specific repeat library that was generated with RepeatModeler.

We used the gene predictions produced by the first round of annotation in Maker to train SNAP and Augustus for use in subsequent rounds of gene prediction. When training SNAP, we included gene models with a maximum annotation edit distance (AED) of 0.25 and a minimum length of 50 amino acids. We then conducted the second round of Maker with *ab initio* gene prediction by supplying the transcript, protein and repeat alignments generated in the first round of annotation, and also running the trained SNAP and Augustus gene prediction programs. After completion of the second round of Maker, we used the resulting transcripts to re-train SNAP and Augustus and then performed a third round of Maker with *ab intio* gene predictions. We analyzed the quality of the transcripts produced by the third round of Maker with BUSCO, using the orangethroat darter-specific Augustus HMM. To conduct functional annotation of proteins, we used BLASTP (NCBI) to identify putative matches between the orangethroat darter proteins and those present in the UniProt Swiss-Prot database.

Integrating the Orangethroat Linkage Map and Genome Scaffolds

We used Chromonomer v1.08 (http://catchenlab.life.illinois.edu/chromonomer/) to join and orient scaffolds from the orangethroat darter genome assembly into chromosomes. We aligned the 1,112 linkage map markers (i.e. 100 bp RAD tags) to the assembled orangethroat darter scaffolds with GSNAP (Wu et al. 2016). The resulting SAM file was provided to

Chromonomer, along with AGP and FASTA files for the assembly and a file with the marker names and locations (in cM) for each of the 24 linkage groups.

Synteny and Homology Analyses

To test for the presence of chromosomal rearrangements putatively contributing to postzygotic isolation, we investigated synteny and homology between the orangethroat darter chromosomes and the rainbow darter linkage groups. We aligned rainbow darter linkage map markers to the orangethroat darter assembly using BLASTN with a stringent e-value cutoff of 1^{-10}. To visualize synteny and homology, we constructed a synteny plot with the RCircos package (Zhang et al. 2013) in R v3.4.4. To scale rainbow darter linkage groups for comparisons with orangethroat darter chromosomes, we multiplied the position of each marker in cM x 320,000.

We also examined synteny between the orangethroat darter genome and the closest relative for which a genome is available, the Eurasian perch (*Perca fluviatilis*) (Ozerov et al. 2018). Darters and perch are both in the family Percidae and are estimated to have last shared a common ancestor 58-66 Mya (Stepien and Haponski 2015). We downloaded the Eurasian perch assembly from NCBI's GenBank (BioProject PRJNA450919, version QFAT00000000.1) Although the Eurasian perch genome is not assembled into chromosomes, many scaffolds are at or approaching chromosome length. We used D-Genies (Cabanettes and Klopp 2018), which implements the aligner Minimap2 (Li 2018), to produce a genomic alignment dot plot comparing the orangethroat darter and Eurasian perch scaffolds.

Identifying Genetic Incompatibilities using Backcross Genomes

Genomic regions associated with postzygotic barriers can be identified by quantifying patterns of linkage disequilibrium (LD) and introgression in hybrid genomes. Theory predicts that hybrid genomes will be more likely to exhibit non-admixed haplotypes at areas of the genome associated with genetic incompatibilities, which can include both chromosomal rearrangements and negative epistatic interactions (Barton and Hewitt 1985; Barton and Bengtsson 1986; Rieseberg et al. 1999; Turelli et al. 2001). To identify genomic regions potentially underlying postzygotic isolation between orangethroat and rainbow darters, we examined patterns of local ancestry, LD, and deviations from Mendelian segregation (also called transmission ratio distortion, hereafter TRD)) in backcross hybrid genomes. We then asked whether regions of the genome showing evidence of LD and TRD overlap with: (1) regions of high genetic divergence between parental species, and/or (2) regions showing evidence of chromosomal rearrangements between parental species.

We previously measured backcross viability in the laboratory by crossing wild-caught F1 hybrid males to parental females of both species and comparing their survival to parental control crosses (Moran et al. 2018). To generate the experimental backcrosses, six F1 hybrid males were used in two cross types. Each F1 hybrid male was crossed with a female rainbow darter and with a female orangethroat darter. Backcross clutches suffered from significantly higher mortality rates compared to both parental and F1 hybrid clutches. On average, only 7% of fry per backcross clutch survived at least one week post-hatching. Backcrosses to orangethroat darters resulted in a total of 36 fry from two families that survived to one-week post hatching. Backcrosses to rainbow darters resulted in a total of 13 fry from three families that survived to

one-week post hatching (see Moran et al. 2018 for details). Notably, one F1 hybrid male sired 35 of the surviving orangethroat-backcross fry and 10 of the surviving rainbow-backcross fry.

We generated and sequenced RADseq data for the 49 total backcross offspring following the methods outlined above (see Linkage Map Sequencing – Orangethroat darter). We used the Stacks (v2.0; Catchen et al. 2011, 2013) program *process_radtags* to demultiplex the raw sequences resulting from the linkage map RADseq libraries (116,867,198 orangethroat-backcross raw reads; 27,581,894 rainbow-backcross raw reads), and to remove barcodes and low-quality reads. After quality filtering, we retained 116,424,777 orangethroat-backcross reads and 27,380,460 rainbow-backcross reads. To quantify patterns of genomic divergence between parental species and to facilitate identification of introgressed genomic regions in backcross offspring, we also obtained previously published RADseq data for 18 orangethroat and 18 rainbow darter individuals from NCBI's Sequence Read Archive (SRP152572) (Moran et al. 2018). For our analyses, we only used individuals with < 20% missing data and an ancestry fraction (Q) of > 0.95 (see ADMIXTURE analysis below), indicating non-introgressed individuals. This filtering resulted in a total of 10 orangethroat darters (5 females and 5 males) and 14 rainbow darters (9 females and 5 males). Reads from backcross fry and adult orangethroat and rainbow darters were aligned to the orangethroat darter genome with GSNAP and then supplied to the Stacks *ref_map* pipeline for RAD locus catalog construction and genotyping. The *ref_map* pipeline built and genotyped a total of 81,615 loci (i.e. 100 bp RAD tags) containing 117,524 SNPs with a mean ± SD coverage of 73.9X ± 121.6 per individual across all loci.

We used the *populations* program in Stacks to further filter RAD loci for quality. We specified that a given RAD locus was only to be retained if present in all four populations (i.e. both sets of backcrosses and both parental species) and in a minimum of 50% of the individuals

within each population. To filter out rare loci potentially originating from sequencing errors, we also excluded loci with a minor allele frequency (MAF) across all populations of less than 0.05. This resulted in a set of 29,064 SNPs across 19,772 loci (1.98 million sites) that were shared across both sets of backcross fry and both parental species.

We used the software ADMIXTURE (v1.3.0) (Alexander et al. 2009) to infer genome-wide ancestry proportions from each individual. Because ADMIXTURE assumes independence among SNPs, we kept only the first SNP in each RAD locus (resulting in 19,772 SNPs) for this analysis. We ran ADMIXTURE with 10,000 rounds of bootstrap resampling and specified two ancestral populations.

Mapping local ancestry across backcross hybrid genomes

We used the total set of 29,064 shared SNPs obtained from Stacks to infer local ancestry along backcross hybrid chromosomes with ELAI (Efficient Local Ancestry Inference) (Guan 2014). ELAI uses unphased genotype data to train and implement a two-level Hidden Markov model (HMM) to identify introgressed tracts in the genome. We trained the HMM using the non-admixed individuals from both parental species and then predicted allele dosage along each chromosome for each backcross hybrid individual. We used a mean ± SE of 1,108 ± 56.17 SNPs per chromosome (1 SNP/27 kb) in model training and hybrid ancestry predictions. We expect that this density of SNPs should be sufficient to detect the majority of ancestry switches (i.e. junctions) across the genome for two reasons. First, we used 2nd generation hybrids, which are predicted to have relatively large admixed haplotype blocks, as opposed to the smaller haplotype blocks that are typically observed in more advanced generations of hybrids. Our results are in agreement with this prediction. Second, preliminary analyses of LD decay in across the

orangethroat darter genome indicated that linkage between sites falls to r^2 <0.5 by 100 kb (see below). Thus, we expect that most ancestry blocks should be covered by multiple SNPs at a density of 1 SNP per 27 kb.

Mendelian segregation with at least one crossover event per chromosome predicts that for a given chromosome, backcross offspring will be 50% recombinant, 25% non-recombinant parental (i.e. inherit two copies of a given chromosome from the same parental species), and 25% non-recombinant F1 (i.e. inherit one chromosome from each parental species) (Figure 7.1). We used chi square tests to ask whether backcross offspring deviated from these expected frequencies across all 24 chromosomes. We then used binomial tests to ask whether individual chromosomes deviated from the expected frequencies. All statistical analyses were conducted in R (v3.4.4).

Linkage disequilibrium decay and inter-chromosomal linkage disequilibrium

Selection against genetic incompatibilities underlying reproductive barriers between species can lead to a pattern of LD between non-physically linked genomic regions (Felsenstein 1965; Karlin and Feldman 1970; Slatkin 2008). If alleles found on two different linkage groups are associated with genetic interactions (e.g. due to a chromosomal rearrangement or a negative epistatic interaction), we can expect to see associations in local ancestry between loci on different linkage groups. Specifically, selection against recombined haplotypes due to negative genetic interactions predicts LD between the two implicated genomic regions due to an enrichment of parental haplotypes. We used genotype data from the 10 non-introgressed orangethroat darter individuals to estimate the average rate of LD decay between the set of 29,064 SNPs across the orangethroat darter genome in PopLDdecay (Zhang et al. 2018). Linkage

between sites was measured as the squared Pearson coefficient of correlation, r^2, which ranges from 0 to 1. Two sites are said to be in complete LD if they are tightly linked (i.e. not broken up by recombination), indicated by an r^2 value of 1. Conversely, an r^2 value of 0 indicates complete linkage equilibrium (i.e. no association) between sites.

We then measured inter-chromosomal LD between physically unlinked sites across the genome. To prevent redundancy in our analysis, we thinned the number of SNPs included by only using the first SNP in each 100 bp RAD tag, resulting in 19,772 independent SNPs. To ensure that SNPs were not tightly physically linked, SNPs were only included in the analysis if they were a minimum of 100 kb apart. We used VCFtools to calculate inter-chromosomal r^2 for a total of 31,619,804 pairwise SNP comparisons in the backcrosses to orangethroat darters and 30,409,794 pairwise SNP comparisons in the backcrosses to rainbow darters. We used chi-square tests to calculate p-values for r^2 values between sites (Lewontin 1988; Devlin and Risch 1995). The chi-square test statistic was calculated as $\chi^2 = r^2*N$, where N is equal to the number of chromosomes in the sample. We compared the χ^2 test statistic against a critical value for 1 degree of freedom to calculate p-values. We assessed significance of r^2 values using a strict cutoff of $p<0.001$.

Deviations from Mendelian segregation in backcrosses

Genomic loci that deviate from the expected Mendelian segregation ratios in hybrids may contain genes associated with hybrid lethality (Hall and Willis 2005). TRD in hybrids has been linked to chromosomal rearrangements and negative epistatic interactions (Rieseberg et al. 1995; De Villena and Sapienza 2001; Leppälä et al. 2013). To identify loci showing TRD in backcross hybrid genomes, we first filtered the set of 29,064 shared SNPs to include only SNPs that were

differentially fixed between the parental species. This resulted in a set of 17,611 fixed SNPs across 8,662 loci. We further filtered this SNP set to only include those that mapped to one of the 24 chromosomes, resulting in 16,585 SNPs across 8,177 loci. Because SNPs occurring together on the same 100 bp RAD tag are physically linked, we excluded all but the first SNP from each locus, resulting in 8,177 independent SNPs. Mendelian segregation predicts an MAF of 0.25 across all loci in a backcross population. We tested for a deviation from the expected MAF in both sets of backcross individuals (i.e. the 36 orangethroat-backcross fry and the 13 rainbow-backcross fry). We used Plink v1.9 to calculate allele frequencies at each SNP in both backcross groups. We then used a chi-square test with 1 degree of freedom to test for deviations from the expected MAF of 0.25.

Patterns of Recombination Rate Variation Across Parental Genomes

Examining recombination rate across the genome can help identify certain aspects of genomic architecture, such as the location of centromeres or chromosomal rearrangements. Centromeres and chromosomal inversions are predicted to show a reduction in recombination rate relative to the background level. Conversely, regions of markedly increased recombination rate may indicate a mis-assembly or translocation event. To estimate the population-level recombination rate, rho ($\rho = 4N_e r$), across the genome for both orangethroat and rainbow darters, we provided the set of 29,064 SNPs shared across populations to the Interval program in LDhat (McVean and Auton 2007). Interval uses a reversible-jump MCMC algorithm to estimate recombination rates from population data. We specified a burn-in of 100,000 iterations followed by 2,000,000 iterations with sampling every 5,000 iterations and a block penalty of 1. This allowed us to obtain an estimate of ρ across each chromosome for both species. As the

population-level recombination rate assumes that individuals included in the analysis are unrelated, we were unable to calculate genome-wide estimates of ρ for the backcross offspring.

Genetic Differentiation Between Species

Regions of the genome showing elevated levels of genetic divergence between hybridizing species may indicate reproductive barriers that are resistant to gene flow (Noor and Bennett 2010; Nosil and Feder 2012). Conversely, regions with low genetic divergence can indicate regions that have high permeability to gene flow and/or regions that are identical by descent and have not been under strong divergent selection between species. Our goal was to identify whether regions of the genome exhibiting high levels of TRD in backcross hybrids and chromosomal rearrangements between species also show high levels of genetic divergence between species. We used the refence-aligned RADseq data from adult, wild-caught orangethroat darters (n=10) and rainbow darters (n= 14) described above to calculate genome-wide population genomic statistics in Stacks. We used the Stacks v1.48 *populations* program to select loci that were present in at least 50% of the individuals within each population (i.e. orangethroat darters and rainbow darters). We also specified a MAF cutoff of 0.05. This resulted in a set of 43,503 SNPs across 16,950 RAD loci, with 39,518 SNPs mapping to one of the 24 chromosomes. To conduct genome scans for regions of elevated genetic differentiation between orangethroat and rainbow darters, we used *populations* to calculate the smoothed AMOVA F_{ST} in 500 kb sliding windows across each chromosome. P-values were assigned to each SNP using 10,000 rounds of bootstrap resampling. To assess levels of genetic diversity across the genome within each species, we also obtained the nucleotide diversity (π) at each variant site from *populations*.

RESULTS

Genome Assembly

We estimate the total length of the orangethroat darter genome to be approximately 1 Gb, based on a C-value of 1.06 for another species of darter (the logperch, *Percina caprodes*) (Hardie and Hebert 2004). The Illumina-based Meraculous2 assembly is thus estimated to have a coverage of 142X (Table E.3). Using an optimal kmer size of 59, the Meraculous2 assembly resulted in 4,629 scaffolds > 1 kb, a total assembly length of 719.8 Mb with 10.7% gaps, and an N50 of 2.2 Mb. Additional scaffolding and gap filling of the Illumina assembly with 30X coverage PacBio reads provided substantial reduction in gap sizes and increased continuity of the assembly (see Table 7.1 for intermediate assembly statistics), resulting in 3,345 scaffolds >1 kb, a total assembly length of 855.1 Mb with 0.47% gaps, and an N50 of 8.1 Mb. Analysis with BUSCO indicated that 4,314 out of 4,584 total (94.1%) Actinopterygii orthologs were identified as complete in the assembly. Repetitive elements made up 30.9% (264.2 Mb) of the genome and the GC content was 40.9% of the genome, which is similar to other perciform genomes (e.g. Eurasian perch; Ozerov et al. 2018).

Linkage Maps

We constructed linkage maps using RADseq data from a single orangethroat darter family and a single rainbow darter family. RAD sequencing resulted in a mean ± SE depth of coverage per individual of 44 ± 4.33 for the orangethroat linkage map family (two parents and 145 fry) and 43 ± 1.45 for the rainbow linkage map family (two parents and 77 fry). Markers

clustered into 24 linkage groups in both species. This is in agreement with the number of chromosomes identified in darters previously by karyotyping (Ross 1973; Danzmann 1979). In both orangethroat and rainbow darters, the male parent linkage maps contained fewer loci than the female parent linkage maps. The final female orangethroat darter linkage map contained 930 markers and the final male orangethroat darter linkage map contained 301 markers. The final female rainbow darter linkage map contained 991 markers and the final male rainbow darter linkage map contained 744 markers. Summary statistics for the sex-specific and consensus linkage maps can be found in Table 7.2. A total of 1,112 markers were incorporated into the final orangethroat darter consensus map (Figure E.1) and 1,622 markers were incorporated into the final rainbow darter consensus map (Figure E.2).

Integrating the Orangethroat Linkage Map and Genome Scaffolds

Out of the 1,112 total markers in orangethroat darter linkage map, 988 had primary alignments to the assembly and were used by Chromonomer to join and orient scaffolds into chromosomes. Chromonomer joined 164 of the 3,345 assembly scaffolds into 24 chromosomes. This resulted in a final assembly with 3,204 scaffolds totaling 855.1 Mb in length, 706.7 Mb (83%) of which was integrated into chromosomes.

Transcriptome Assembly

The orangethroat darter transcriptome assembled by Trinity contained a total of 366,416 transcripts for 181,974 genes. The total transcriptome assembly length was 507.2 Mb, with an N50 of 3 kb and a mean contig length of 1.4 kb. The GC content of the transcriptome assembly was 45.4%. Over 93% of the raw RNA sequences aligned to the assembled transcripts. Analysis

with BUSCO indicated that 4,282 out of 4,584 total (93.4%) Actinopterygii orthologs were identified as complete in the transcriptome assembly (Table E.4).

Genome Annotation

Maker identified a total of 18,867 protein-coding genes, with a mean gene length of 13,747.6 bp. Based on homology with proteins in the UniProt Swiss-Prot database, we were able to assign a putative functional annotation to 18,532 (98.2%) of the orangethroat darter proteins.

Synteny and Homology Analyses

Of the 1,622 total markers included in the final rainbow darter linkage map, 1,287 aligned to the orangethroat darter genome assembly using a strict BLASTN e-value cutoff of 1^{-10}. A total of 1,192 of these markers mapped to one of the 24 orangethroat darter chromosomes. Previous karyotypic analyses have shown that all species in the darter family Percidae have a haploid chromosome number of 24. However, chromosome morphology (e.g. number of metacentric versus acrocentric chromosomes) typically varies among darter species and even among populations within species, indicating that chromosol rearrangements may be common in these fishes (Ross 1973; Danzmann 1979). Our synteny analysis revealed a 1:1 homology for most of the 24 orangethroat darter chromosomes and 24 rainbow darter linkage groups (Figure 7.2). We observed five notable deviations from 1:1 homology consistent with putative chromosomal translocations between these species. First, orangethroat darter chromosome 4 mapped to rainbow darter linkage groups 4 and 19 (Figure E.3A). Second, orangethroat darter chromosome 7 mapped to rainbow darter linkage groups 7 and 19 (Figure E.3B). Third, orangethroat darter chromosome 9 mapped to rainbow darter linkage groups 9 and 20 (Figure

E.3C). Fourth, orangethroat darter chromosome 16 mapped to rainbow darter linkage groups 16 and 2 (Figure E.3D). Fifth, orangethroat darter chromosome 24 mapped to rainbow darter linkage groups 24 and 7 (Figure E.3E).

Comparing sequence alignment between the orangethroat darter assembly and the Eurasian perch assembly revealed large stretches of syntenic sequence. Although the Eurasian perch genome is not assembled into chromosomes, some scaffolds appear to be at or approaching chromosome length. Most of the larger Eurasian perch scaffolds exhibited 1:1 homology with the orangethroat darter genome. As the two genomes were assembled independently, the widespread homology we observed with the Eurasian perch assembly provides a second line of support for the orangethroat darter assembly. We also observed several putative rearrangements between the orangethroat darter and Eurasian perch genomes (Figure 7.3). Most putative inversions occurred at the ends of chromosomes, but Eurasian perch scaffold 3 appeared to have multiple inverse regions compared to the homologous orangethroat darter chromosome 3.

Identifying Putative Genetic Incompatibilities using Backcross Genomes

Estimates of genome-wide ancestry proportions from ADMIXTURE were in accordance with our expectation for backcross individuals. The mean ± SE proportion for orangethroat darter ancestry in backcrosses to orangethroat darters was 0.75 ± 0.01 (n=36). The mean ± SE proportion for rainbow darter ancestry in backcrosses to rainbow darters was 0.73 ± 0.02 (n=13) (Figure E.4).

Mapping local ancestry across backcross hybrid genomes

We used a two-level Hidden Markov Model to infer switches in local ancestry (i.e. recombination breakpoints) from homozygous (non-introgressed, non-recombinant) to heterozygous (introgressed, recombinant) regions across backcross hybrid genomes (see Figure 7.4 for representative examples of recombinant and non-recombinant chromosomes). We first asked whether backcross offspring deviated from the expected 50% recombinant, 25% parental (both chromosomes inherited from the same parental species), and 25% F1 (one chromosome inherited from each parental species) across the entire genome (i.e. all 24 chromosomes). We observed a lower than expected number of recombinant haplotypes in backcrosses to orangethroat darters ($\chi^2 = 157.22$, d.f. = 23, n = 36 backcross offspring; $p < 0.0001$) but not in backcrosses to rainbow darters ($\chi^2 = 31.39$, d.f. = 23, n = 13 backcross offspring; $p = 0.11$). Similarly, F1 haplotypes were lower than expected in backcrosses to orangethroat darters ($\chi^2 = 914.67$, d.f. = 23, n = 36 backcross offspring; $p < 0.0001$) but not in backcrosses to rainbow darters ($\chi^2 = 30.31$, d.f. = 23, n = 13 backcross offspring; $p = 0.19$). We observed an enrichment of parental haplotypes in both backcrosses to orangethroat darters ($\chi^2 = 174.22$, d.f. = 23, n = 36 backcross offspring; $p < 0.0001$) and in backcrosses to rainbow darters ($\chi^2 = 93.69$, d.f. = 23, n = 13 backcross offspring; $p < 0.0001$).

On average across all 24 chromosomes, 2.8 % (± 0.7 SE) of offspring from backcrosses to orangethroat darters looked genetically like F1 hybrids (indicating the offspring received a rainbow darter chromosome from the F1 hybrid sire) and 74.8 % (± 2.7 SE) looked genetically like “purebred” orangethroat darters (indicating the offspring received a orangethroat darter chromosome from the F1 hybrid sire). The remaining 22.4% showed evidence of at least one recombination event. On average across all 24 chromosomes, 14.7 % (± 2.4 SE) of offspring

from backcrosses to rainbow darters looked genetically like F1 hybrids (indicating the offspring received an orangethroat darter chromosome from the F1 hybrid sire) and 48.1 % (± 3.1 SE) looked genetically like "purebred" rainbow darters (indicating the offspring received an rainbow darter chromosome from the F1 hybrid sire). The remaining 37.2% showed evidence of at least one recombination event. The average number of crossover events per individual across all 24 chromosomes was 0.67 for backcrosses to orangethroat darters and 1.05 for backcrosses to rainbow darters.

We next asked whether individual chromosomes deviated from the 50% recombinant, 25% parental, and 25% F1 offspring expected from independent assortment. The proportion of chromosomes that were parental, recombinant, or F1 in each backcross direction are shown in Figure 7.5. We observed a dramatic overrepresentation of parental offspring and underrepresentation of recombinant and F1 offspring in backcrosses to orangethroat darters (Table E.5). We observed a similar pattern of enrichment for parental offspring and a lack of recombinant and F1 offspring in backcrosses to rainbow darters, but with fewer chromosomes deviating from the expected frequencies compared to backcrosses to orangethroat darters (Table E.6).

Linkage disequilibrium decay and inter-chromosomal linkage disequilibrium

Our analysis with PopLDdecay indicated that across the orangethroat darter genome, the average genome-wide linkage between physically linked sites (i.e. SNPs occurring on the same chromosome) decayed to $r^2 < 0.5$ by 100 kb and decayed to the mean background level of $r^2 < 0.25$ by 700 kb (Figure E.5). The average r^2 between inter-chromosomal pairs of SNPs in backcrosses to orangethroat darters was 0.039. The average r^2 between inter-chromosomal pairs

of SNPs in backcrosses to rainbow darters was 0.136. Using a strict significance cutoff of $p<0.001$, 477,364 (1.5%) pairs of SNPs were in significant LD in backcrosses to orangethroat darters and 519,481 (1.7%) pairs of SNPs were in significant LD in backcrosses to rainbow darters.

Deviations from Mendelian segregation in backcrosses

We quantified patterns of deviation from the expected MAF of 0.25 in backcross offspring. The mean ± SE MAF in backcrosses to orangethroat darters was 0.254 ± 0.001. The mean ± SE MAF in backcrosses to rainbow darters was 0.258 ± 0.001. The magnitude of transmission ratio distortion varied between the two backcross directions, with backcrosses to orangethroat darters exhibiting a nearly than 2-fold increase in loci showing signs of transmission ratio distortion relative to backcrosses to rainbow darters. We observed that 716 (9%) out of 8,177 SNPs total deviated significantly ($p<0.05$) from expected frequencies in the fry resulting from backcrosses between F1 hybrid males and female orangethroat darters (Figure E.6). Only 358 (4%) out of 8,177 SNPs total deviated significantly from expected frequencies in the fry resulting from backcrosses between F1 hybrid males and female rainbow darters (Figure E.7). However, part of this discrepancy may be attributable to the fact that we had greater power to detect TRD in the backcrosses to orangethroat darters compared to the backcrosses to rainbow darters.

The proportion of loci deviating from the expected MAF of 0.25 varied across chromosomes in both backcross directions (Figure E.8). In backcrosses to orangethroat darters, 56% of all loci deviating from the expected MAF were found on chromosomes 1, 9, 11, 13, and 24. Chromosomes 4, 18, 20, and 22 had the lowest proportion of loci deviating from the expected

MAF in backcrosses to orangethroat darters. In backcrosses to rainbow darters, 58% of all loci deviating from the expected MAF were found on chromosomes 1, 2, 7, 10, 12, 13, 15, and 24. Chromosomes 11, 17, 18, 21, and 22 had the lowest proportion of loci deviating from the expected MAF in backcrosses to rainbow darters. Also of note, in backcrosses to orangethroat darters 48% of loci on chromosome 11 deviated from the expected MAF (with most distorted loci showing an excess of rainbow darter ancestry), whereas in backcrosses to rainbow darters 2% (only 5 out of 236 loci total) deviated from the expected MAF on chromosome 11.

Patterns of recombination rate variation across parental genomes

The population-level recombination rate, rho ($\rho = 4N_e r$), was generally uniform throughout the genome and rates were similar for both orangethroat and rainbow darters (orangethroat darters: mean ± SE ρ = 0.93 ± 0.01; rainbow darters: mean ± SE ρ = 0.88 ± 0.03) (Figures E.9-E.10). In both species, most chromosomes showed at least one region of suppressed recombination, potentially corresponding to centromere location. Recombination rate varied between 0 to 8 orangethroat darters and 0 to 25 in rainbow darters. In orangethroat darters, extreme recombination rate peaks above 7 were observed on chromosomes 22 and 23. In rainbow darters, extreme recombination rate peaks above 7 were observed on chromosomes 1, 4, 6, 7, 8, 12, 22, and 23. Such recombination rate hotspots may indicate regions harboring chromosomal rearrangements relative to the reference genome or mis-assemblies in the reference genome.

Genetic differentiation between species

Despite the presence of ongoing hybridization, we observed that F_{ST} between orangethroat and rainbow darters is generally high across the genome (F_{ST} = 0.83) (Figure E.11). Consistent with previous studies (Moran et al. 2017, 2018), we observed that nucleotide diversity is nearly 2X higher in rainbow darters (π = 0.076) compared to orangethroat darters (π = 0.033) (Figure E.12). Theory predicts that genomic regions with elevated recombination rate are more likely to have increased F_{ST}. F_{ST} between orangethroat and rainbow darters is correlated with recombination rate, but in opposite directions between the two species. In orangethroat darters, we observed a positive correlation between genome-wide measurements of F_{ST} and the population-level recombination rate (t = 3.73, d.f. = 26,323, p = <0.001; Pearson's correlation ± 95% CI = 0.023 ± 0.012). In rainbow darters, we observed a negative correlation between genome-wide measurements of F_{ST} and the population-level recombination rate (t = -12.8, d.f. = 26,323, p < 0.00001; Pearson's correlation ± 95% CI = -0.079 ± 0.012).

DISCUSSION

Here we presented the first reference genome, transcriptome, and linkage maps for darters, the most diverse group of vertebrates in North America. We used the orangethroat darter genome and rainbow darter linkage map to examine genomic synteny between these species and to identify putative chromosomal rearrangements that are potentially underlying postzygotic isolation. We also used RADseq to genotype (1) non-admixed individuals from natural populations of orangethroat and rainbow darters and (2) individuals produced from laboratory-generated backcrosses between wild-caught F1 hybrid males and females of both parental

species. We conducted fine-scale ancestry mapping in backcross hybrid genomes and identified patterns consistent with wide-spread genetic incompatibility, suggesting that there is likely strong selection against recombinants. Our analyses of the darter genome assembly, linkage maps, and RADseq data provide new insights into the genomic architecture of postzygotic isolation in species undergoing male-driven RCD and ACD.

The first darter genome, transcriptome, and linkage maps

We produced a highly contiguous, chromosome-level annotated assembly of the orangethroat darter genome by combining Illumina and PacBio whole-genome sequencing with a RADseq-based linkage map (Table 7.1). By generating a RADseq-based linkage map for the rainbow darter, we were also able to compare genomic synteny and homology between orangethroat and rainbow darters. We generally observed 1:1 homology between the orangethroat darter genome and rainbow darter linkage groups, as well as extensive homology between the orangethroat darter genome and the Eurasian perch genome. However, several putative chromosomal rearrangements were observed in both comparisons (Figures 7.2-7.3, E.3), which may play a role in conferring hybrid incompatibility (see below).

The genomic resources for darters generated here will undoubtedly facilitate future studies aimed at examining the genomics of speciation, sexual selection, mating system evolution, and ecological adaptation in this highly diverse group of fishes. Furthermore, we anticipate that the darter genome will greatly assist with conservation efforts. Darters are highly sensitive to anthropogenic disturbances (Albritton 1994; Juracek et al. 2017), and nearly half of all species within the Percid family are considered imperiled (Helfman et al. 2009). Having a high-quality genome available will open the door to future studies aimed at quantifying and

preserving genomic variation in populations of conservation concern (Fitzpatrick et al. 2014; Juracek et al. 2017).

Synthesizing patterns associated with genetic incompatibilities

Multiple chromosomes appear to be implicated in genetic incompatibilities between orangethroat and rainbow darters. The genomes of viable backcross fry showed a striking bias for homozygous ancestry from a single parental species (Figure 7.5). We observed evidence of wide-spread deviations from Mendelian segregation in both backcross directions, but the lack of recombinant offspring and increased TRD was enhanced in backcrosses to orangethroat darters relative to backcrosses to rainbow darters (Figures 7.5, E.8). Putative chromosomal translocation events were observed on chromosomes 2, 4, 7, 9, 16, 19, 20, and 24. This observation is in agreement with a previous study of chromosome morphology that identified karyotypic differences between these species (Moerchen 1973). Notably, chromosomes 4 and 7 show evidence of a chromosomal translocation between orangethroat and rainbow darters and also exhibit regions of increased recombination rate in rainbow darters (Table 7.3).

We did not observe any patterns in our data that strongly implicate a single mechanism of genetic incompatibility between our focal species. If structural differences underlie postzygotic isolation, we would expect to see a reduction in recombinant genotypes and TRD at chromosomes exhibiting rearrangements between orangethroat and rainbow darters. Indeed, all eight chromosomes associated with a putative chromosomal translocation event also showed a significant reduction in recombinant offspring in backcrosses to orangethroat darters (Table 7.3). Furthermore, four out of eight chromosomes associated with a putative chromosomal translocation event showed an above average proportion of SNPs in TRD in backcrosses to

orangethroat and/or rainbow darters. Several chromosomes that exhibited no evidence of chromosomal rearrangements in our synteny analysis also exhibited a reduction in recombinant offspring and TRD. For example, chromosomes 11, 17, 18, and 23 had an excess of parental genotypes in both backcross directions but our synteny analysis between the orangethroat darter genome and the rainbow darter linkage map did not indicate any apparent rearrangements on these chromosomes. However, we observed regions of increased population-level recombination rate in both species on chromosome 23, potentially indicating a rearrangement that was not detected by our synteny analysis. Future efforts aimed at sequencing the rainbow darter genome will be necessary to resolve the location of fine-scale rearrangements between orangethroat and rainbow darters. Overall, our results provide evidence that both chromosomal rearrangements and negative epistatic interactions may contribute to postzygotic isolation between orangethroat and rainbow darters.

We observed no clear relationship between regions of the genome showing heightened levels of genetic divergence in parental species and regions of the genome exhibiting putative rearrangements, reduced recombination in backcross offspring, and/or high levels of TRD in backcross offspring. This was likely due to the fact that estimates of genetic differentiation between orangethroat and rainbow darters were generally high throughout the genome (Figure E.10). Our results suggest that even with hybridization ongoing, “islands” of genomic divergence are not localized at a few discrete genomic regions (Turner et al. 2005; Harr 2006; Nosil et al. 2009). Instead, we observed widespread genomic divergence between orangethroat and rainbow darters, implying that numerous regions across the genome act as barriers to gene flow. Similar patterns of genome-wide divergence despite ongoing geneflow have also been observed in

Anopheles mosquitoes (Lawniczak et al. 2010), threespine stickleback (Hohenlohe et al. 2010; Roesti et al. 2012), and *Drosophila* (McGaugh and Noor 2012).

Theory suggests that widespread divergence can evolve rapidly even in the face of gene flow when selection is acting on multiple loci throughout the genome, *sensu* "multifarious selection", in conjunction with genomic hitchhiking (Rice and Hostert 1993; Feder and Nosil 2010). Such a scenario may be most likely to occur with secondary contact (Barton and Hewitt 1985; Barton and Bengtsson 1986). There is good reason to suspect that orangethroat and rainbow darters initially diverged in allopatry followed by a secondary contact event, as orangethroat and rainbow darters are not sister taxa (Near et al. 2011) and speciation appears to be initiated in allopatry in darters (Near and Benard 2004; Hollingsworth and Near 2009). In addition to selection against genetic incompatibilities, strong selection for enhanced prezygotic isolation in sympatry via RCD and ACD has likely also played a large role in promoting genomic divergence between orangethroat and rainbow darters (Moran and Fuller 2018b,a). Examining genomic divergence between multiple sympatric and allopatric population pairs of orangethroat and rainbow darters may help to distinguish regions of the genome under selection due to RCD and ACD versus neutral regions that have accumulated divergence in allopatry.

We note that a limitation of our study was that the majority of backcross offspring in both cross directions were sired by one F1 hybrid male (35 out of 36 offspring in backcrosses to orangethroat darters; 10 out of 13 offspring in backcrosses to rainbow darters). Thus, the reduction in recombinant haplotypes that we observed in backcross offspring may be due to some intrinsic attribute specific to this F1 male that suppressed crossing over during meiosis. However, the overall qualitative patterns appear to hold in the few offspring sired by two other F1 hybrid males. Our low sample size for backcrosses to rainbow darters (13 offspring total) may

have also affected our ability to detect negative genetic interactions. These low sample sizes were unavoidable as the overall survival rate was low in back crosses (7%) compared to parental clutches (65%) (Moran et al. 2018). Whether the reduced number of recombinants we observed is a result of selection acting during gametogenesis or embryogenesis is an intriguing question that clearly requires a larger sample size and the ability to genotype sperm.

Conclusions

The orangethroat darter genome and linkage maps for orangethroat and rainbow darters presented here in conjunction with fine-scale genomic data for backcross hybrids have provided an unprecedented insight into the genomic architecture and distribution of postzygotic barriers in darters. Notably, this study represents one of the only investigations to date to characterize genome-wide patterns of selection related to hybrid incompatibilities in a long-lived, non-model species. The presence of several putative chromosomal rearrangements and an enrichment of parental genotypes and TRD across the genomes of hybrid offspring indicate that there are a large number of genetic incompatibilities contributing to postzygotic isolation in this system. Our results suggest that chromosomal rearrangements and negative epistatic interactions may be contributing to hybrid inviability and thus maintaining strong postzygotic isolation between orangethroat and rainbow darters despite the occurrence of viable, fertile F1 hybrids in natural populations (Moran et al. 2018). The low abundance of hybrids with recombinant chromosomes suggests pervasive genetic incompatibilities throughout the genome fuel selection against hybridization that in turn favors the evolution of strong prezygotic barriers in sympatry between orangethroat and rainbow darters via RCD and ACD (Moran et al. 2017; Moran and Fuller 2018b). The findings of the present study contrast those of several previous studies in this system

that concluded postzygotic isolation is likely an insignificant barrier to gene flow between congeneric darter species (Hubbs and Strawn 1957; Hubbs 1959). Lastly, the genomic tools generated here provide the opportunity to further develop darters into a model system for studying the genomics of speciation via RCD/ACD and CRCD/CACD (Moran et al. 2017, 2018; Moran and Fuller 2018b), and also constitute a valuable resource for conservation efforts focused on darters.

ACKNOWLEDGEMENTS

The treatment of animals used in this study was in compliance with the University of Illinois Institutional Animal Care and Use Committee (IACUC) under protocol #17031. Collection of wild fish was approved by the Illinois Department of Natural Resources under Scientific Collecting Permit A16.4035. We thank Christina Cheng, Angel Rivera-Colón, and Niraj Rayamajhi for assistance with molecular work and RADseq library preparation. We thank Ray Ming for assistance with linkage map construction. Floragenex, the University of Minnesota Genomics Center, and the University of Illinois at Urbana-Champaign Roy J. Carver Biotechnology Center provided valuable consultation. This research was supported by funding from the School of Integrative Biology (SIB) and the Program in Ecology, Evolution and Conservation Biology (PEEC) at the University of Illinois Urbana-Champaign (PEEC Summer Research Grant to RLM, SIB Harley J. van Cleave Award to RLM, SIB Hawkes Award to RCF and JMC), and by a Sigma Xi Grant-in-Aid of Research, a Society for Integrative & Comparative Biology Grant in Aid of Research, an American Society of Naturalists Student Research Award, a Society for the Study of Evolution Rosemary Grant Advanced Award, and a

National Science Foundation Doctoral Dissertation Improvement Grant (IOS 1701676) to RLM. RLM was supported by a National Institute of Health Science Education Partnership Award (R25OD020203-01A1).

TABLES

Table 7.1. Summary statistics for the orangethroat darter genome assemblies.

Assembly program	Input data	# scaffolds	Sequence total (Mb)	% gaps	N50 (Mb)	Complete BUSCOs
Meraculous	Illumina mate pair and shotgun reads	4,629	719.8	10.68	2.2	94.5%
PBJelly	Meraculous assembly and PacBio raw reads	3,554	855.2	1.42	3.8	96.3%
wtdgb2	PacBio raw reads	2,593*	774.4	0	2.9	95.0%
Canu	PacBio raw reads	6,669	776.2	0	0.4	92.4%
Quickmerge	Canu and wtdbg2 assemblies	4,469	778.2	0	4.5	93.1%
Quickmerge	Canu-wtdbg2 merged assembly and PBJelly assembly	3,345	855.1	0.47	8.1	94.1%
Chromonomer	Canu/wtdbg2/PBJelly merged assembly and linkage map	3,204	855.1 (83% in chromosomes)	0.47	30.5	94.1%

***** wtdgb2 discards scaffolds < 5 kb in length.

Table 7.2. Summary statistics for the sex-specific and consensus linkage maps for orangethroat (OT) and rainbow (RB) darters.

	OT Female	OT Male	OT Consensus	RB Female	RB Male	RB Consensus
Mean inter-maker distance (cM)	1.64	5.11	1.57	1.83	2.97	1.45
Total map length	1,488.89	1,414.41	1,770.27	1,804.78	2,137.51	2,304.04
Mean recombination rate (cM/MB)	2.11	2.00	2.50	2.68	3.15	3.42

Table 7.3. Summary of patterns observed for chromosomal rearrangements, deviations from expected proportions of recombinant offspring in backcrosses both species, levels of TRD above the genome wide average in backcrosses to both species, and population-level recombination rate hotspots in both species. BC = backcross, OT = orangethroat darter, RB = rainbow darter.

Chr	Rearrange-ment	Low # Recomb BC-OT	Low # Recomb BCRB	High TRD BCOT	High TRD BCRB	Rec Rate Hotspot OT	Rec Rate Hotspot RB
1		x		x	x		x
2	x	x			x		
3							
4	x	x					x
5		x					
6							x
7	x	x			x		x
8		x					x
9	x	x		x			
10		x			x		
11		x	x	x			
12		x			x		x
13		x		x	x		
14							
15					x		
16	x	x					
17		x	x				
18		x	x				
19	x	x					
20	x	x					
21							
22		x				x	x
23		x	x			x	x
24	x	x		x	x		

FIGURES

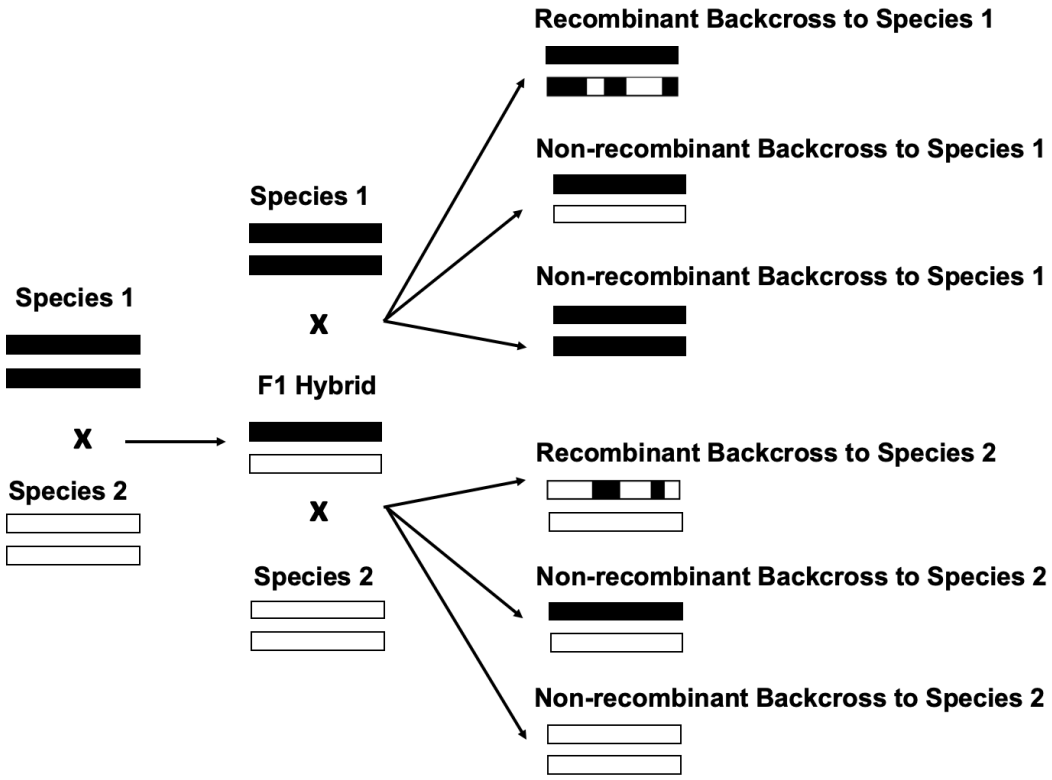

Figure 7.1. Schematic depicting crossing design and expected genetic structure for backcrosses to two parental species (Species 1 and Species 2). Homologous pairs of chromosomes are represented by rectangles. Black indicates Species 1 ancestry and white indicates Species 2 ancestry. F1 hybrids are expected to be heterozygous across the genome, with one set of chromosomes from each parental species. In F1s, crossing over during meiosis results in recombinant gametes. In a given chromosomal tetrad, crossing over only occurs in two of the four DNA strands. This predicts that F1 gametes will be 50% recombinant and 50% non-recombinant (25% Species 1 and 25% Species 2) at each chromosome. Thus, for a given homologous chromosome pair, backcross offspring can have one recombinant and one non-recombinant chromosome, two chromosomes from the same parental species, or one chromosome from each parental species (i.e. appear genetically to be an F1 hybrid).

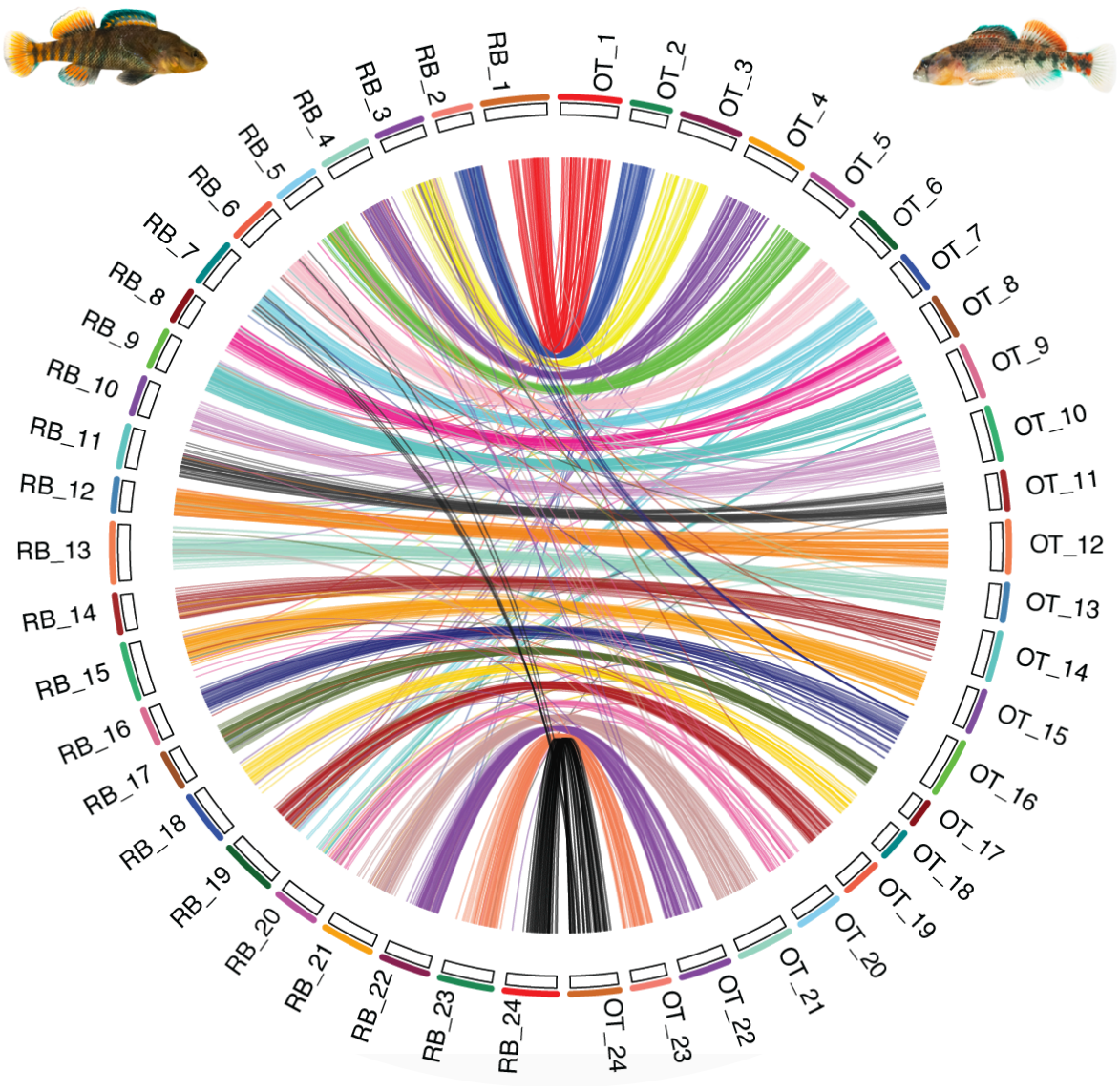

Figure 7.2. Syntenic relationship between orangethroat darter chromosomes (OT 1-24) and rainbow darter linkage groups (RB 1-24). Lines represent alignment position of 1,192 rainbow darter linkage map markers (i.e. 100 bp RAD tags) to the orangethroat darter genome.

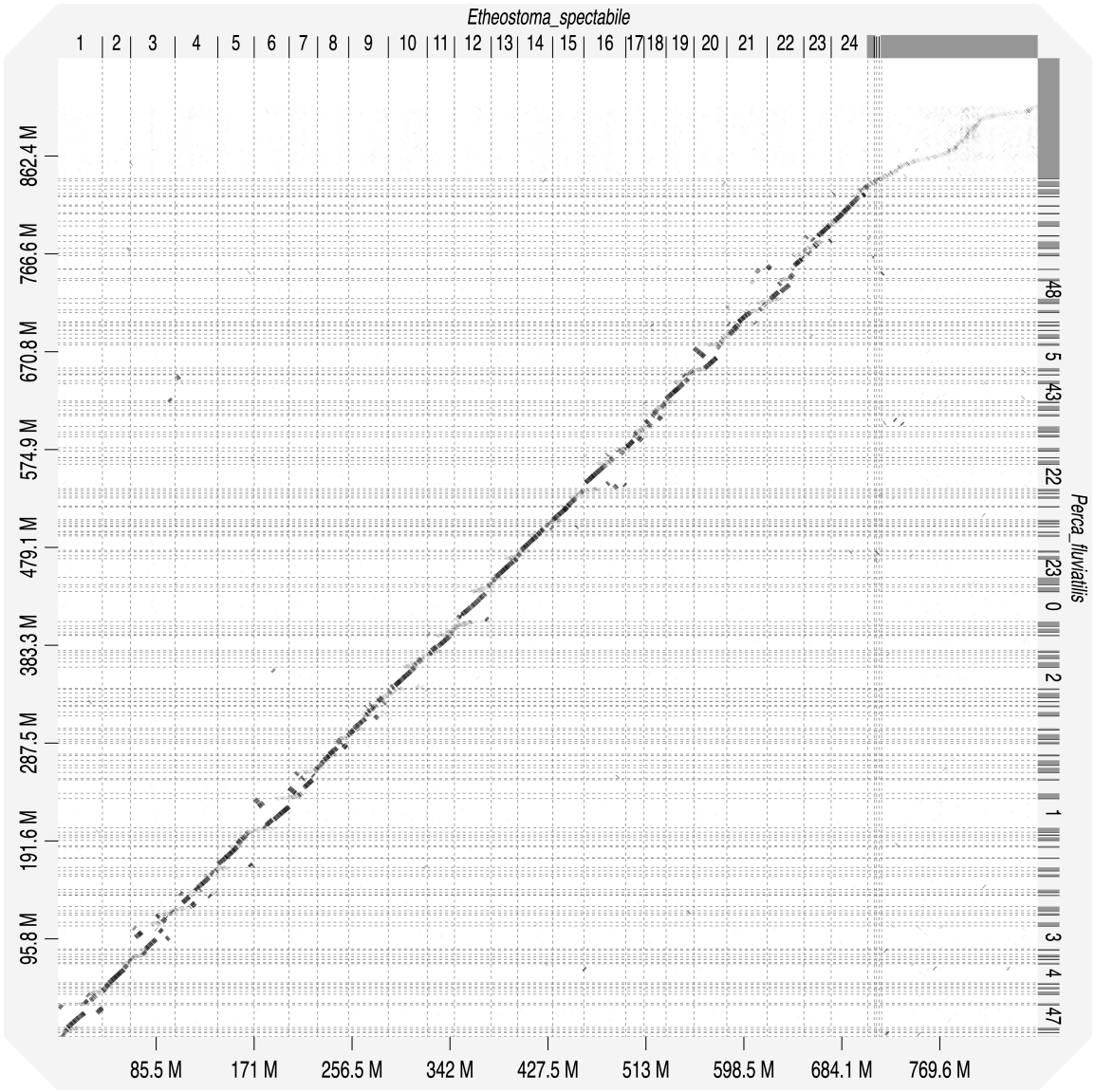

Figure 7.3. Genome alignment dot plot showing synteny and homology between the orangethroat darter (*Etheostoma spectabile*) chromosomes and the Eurasian perch (*Perca fluviatilis*) scaffolds. M = megabase.

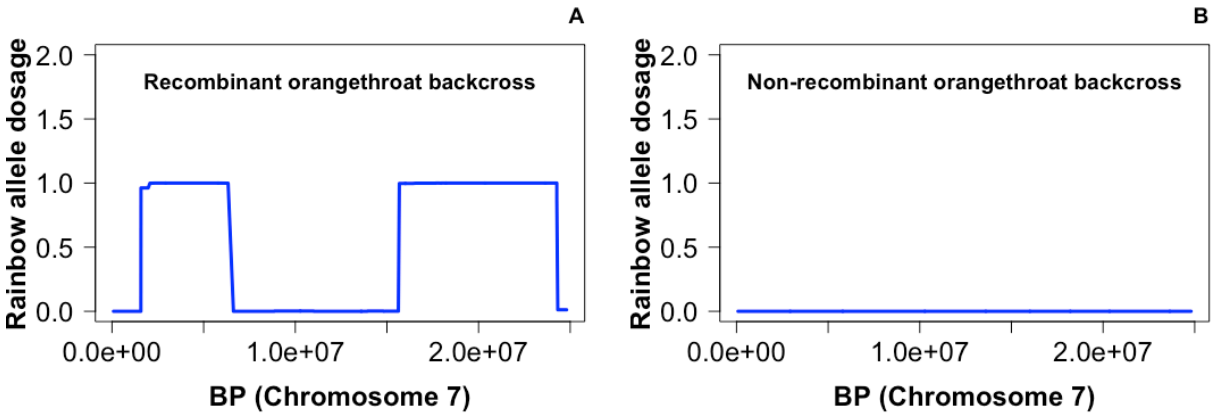

Figure 7.4. Local ancestry along chromosome 7 for (A) a recombinant orangethroat-backcross individual and (B) a non-recombinant orangethroat-backcross individual. Both backcross individuals shown originated from the same family, which was produced by crossing an F1 hybrid male to an orangethroat female. The minor parent (i.e. rainbow darter, "RB") allele dosage is shown on the y axis. An allele dosage of 1 represents admixed regions of the genome with one rainbow allele and one orangethroat allele. An allele dosage of 0 represents non-admixed regions of the genome with zero rainbow darter alleles and two orangethroat darter alleles.

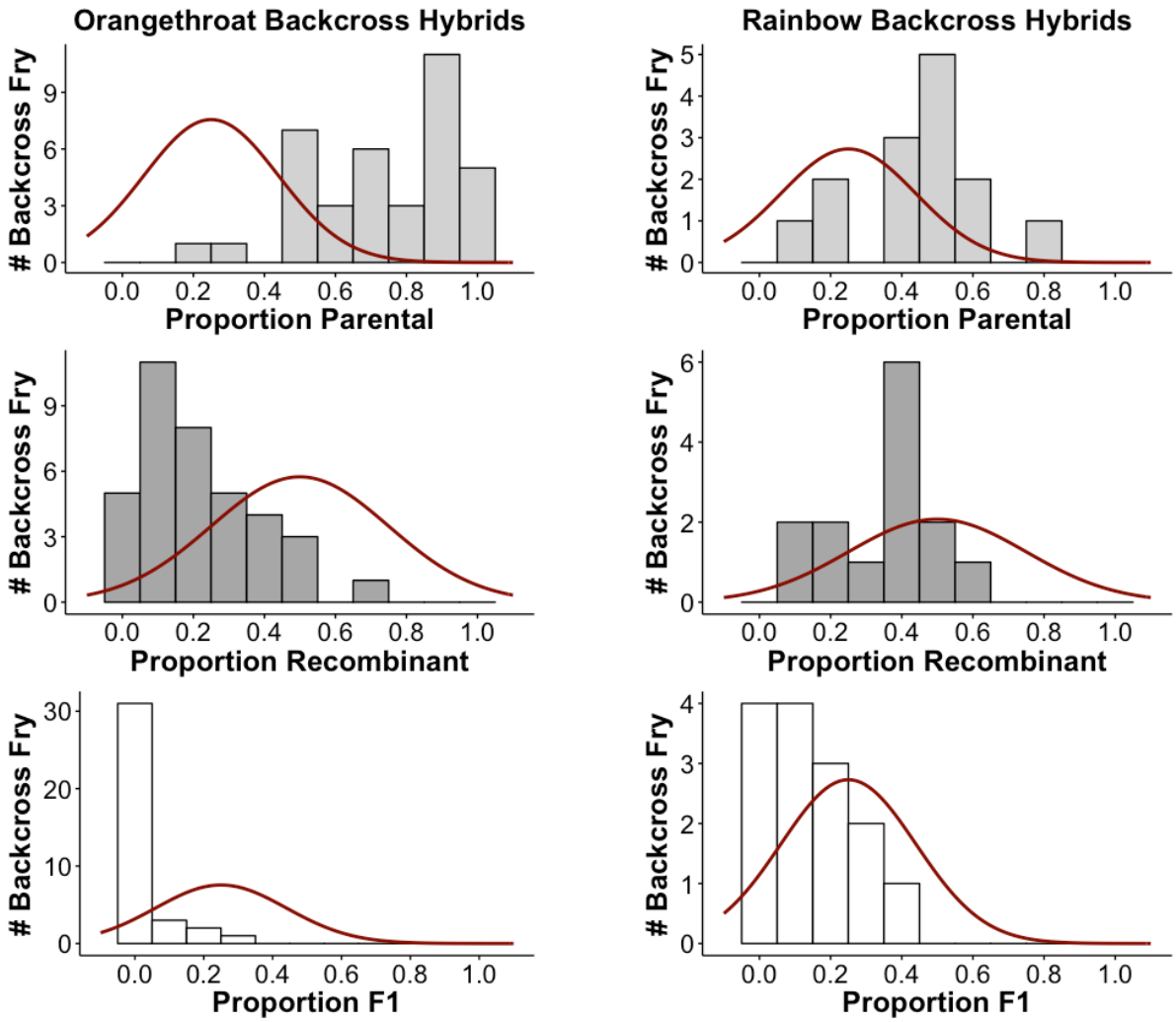

Figure 7.5. Distributions of the proportion of parental, recombinant, and F1 haplotypes observed across all 24 chromosomes in backcrosses to orangethroat darters (left column) and in backcrosses to rainbow darters (right column). The expected normal distribution is overlaid in red.

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APPENDIX A

SUPPLEMENTARY MATERIALS FOR CHAPTER 2

TABLES

Table A.1. Collection site location information for species used in behavioral assays.

Species	Latitude, Longitude	Collection Site Drainage Information
E. fragi (strawberry darter)	36.304214, -91.927684	Rose Branch tributary of Strawberry River, White River Drainage, Salem, AR
E. uniporum (current darter)	36.250560, -91.359318	Unnamed tributary of Spring River, White River Drainage, Williford, AR
E. burri (brook darter)	37.146415, -90.907459	North Fork Webb Creek, Black River Drainage, Logan Township, MO
E. spectabile (orangethroat darter)	40.089035, -88.143440	Unnamed tributary of Salt Fork Vermilion River, Wabash River Drainage, Champaign, IL
E. caeruleum (Mississippi River Corridor clade rainbow darter)*	36.065396, -91.610420	Mill Creek tributary of Strawberry River, White River Drainage, Evening Shade, AR
E. caeruleum (Eastern clade rainbow darter)**	40.055556, -88.091667	Unnamed tributary of Salt Fork Vermilion River, Wabash River Drainage, Champaign, IL

*Used as sympatric rival male in trials where *E. fragi*, *E. uniporum*, and *E. burri* served as focal pair.
** Used as sympatric rival male in trials where *E. spectabile* served as focal pair.

Table A.2. Number of trials included for each behavior analyzed.

Trial Set	Nosedigs	Headwags	Male pursuit	Attacks	Fin flares
1F and 1R	19	38	48	48	48
2F and 2R	17	30	48	48	48
3F and 3R	16	29	48	48	48

Table A.3. Collection site location information for species used in genetic analyses.

Species	Latitude, Longitude	Collection Site Drainage Information
E. fragi (strawberry darter)	36.304214, -91.927684	Rose Branch tributary of Strawberry River, White River Drainage, Salem, AR
E. uniporum (current darter)	37.057146, -91.022982	Pine Valley Creek, Current River, White River Drainage, Van Buren, MO
E. burri (brook darter)	37.146415, -90.907459	North Fork Webb Creek, Black River Drainage, Logan Township, MO
E. spectabile (orangethroat darter)	40.089035, -88.143440	Unnamed tributary of Salt Fork Vermilion River, Wabash River Drainage, Champaign, IL
E. caeruleum (Mississippi River Corridor clade rainbow darter)	37.031917, -91.036867	Pine Valley Creek, Current River, White River Drainage, Van Buren, MO

Table A.4. Information on number of reads discarded and retained by *process_radtags* in Stacks.

Total Number of Reads	Read Length (bp)	Reason Read was Discarded			Reads Retained	Percent of Reads Retained
		Ambiguous Barcodes	Low Quality	Ambiguous RAD-Tag		
251,420,894	100	15,257,833	6,199,861	9,410,201	220,552,999	87.72

Table A.5. Results from ANCOVA analyses examining focal female behavior towards rival males. The table headings (A-C) list the two *Ceasia* species in the species set (*E. fragi* and a heterospecific allopatric *Ceasia* species) followed by the sympatric, distantly related *E. caeruleum.*

A. *E. fragi – E. uniporum – E. caeruleum* (1F and 1R)

Variable: Headwags towards rival male	**df**	**Test statistic**	**p**
Rival male identity	2,31	2.9876	0.0651
Focal pair identity	1,31	1.4044	0.2450
Pursuit by rival male	1,31	47.235	**<0.0001**
Rival male identity * focal pair identity	2,31	1.5383	0.2307
Variable: Nosedigs towards rival male	**df**	**Test statistic**	**p**
Rival male identity	2,12	0.7963	0.4735
Focal pair identity	1,12	0.4070	0.5355
Pursuit by rival male	1,12	23.753	**<0.001**
Rival male identity * focal pair identity	2,12	0.0160	0.9841

B. *E. fragi - E. burri - E. caeruleum* (2F and 2R)

Variable: Headwags towards rival male	**df**	**Test statistic**	**p**
Rival male identity	2,23	0.1731	0.8421
Focal pair identity	1,23	2.0644	0.1642
Pursuit by rival male	1,23	3.7075	0.0666
Rival male identity * focal pair identity	2,23	0.4727	0.6292
Variable: Nosedigs towards rival male	**df**	**Test statistic**	**p**
Rival male identity	2,9	0.3383	0.7217
Focal pair identity	1,9	4.002	0.0765
Pursuit by rival male	1,9	0.1709	0.6890
Rival male identity * focal pair identity	2,9	1.5930	0.2557

C. *E. fragi – E. spectabile – E. caeruleum* (3F and 3R)

Variable: Headwags towards rival male	**df**	**Test statistic**	**p**
Rival male identity	2,22	1.1741	0.3277
Focal pair identity	1,22	3.6363	0.0697
Pursuit by rival male	1,22	16.407	**<0.001**
Rival male identity * focal pair identity	2,22	0.4653	0.6340
Variable: Nosedigs towards rival male	**df**	**Test statistic**	**p**
Rival male identity	2,9	1.4891	0.2763
Focal pair identity	1,9	1.0152	0.3400
Pursuit by rival male	1,9	9.2894	**0.0138**
Rival male identity * focal pair identity	2,9	0.4668	0.6414

Table A.6. Results of the STRUCTURE analysis for the four species of *Ceasia* and *E. caeruleum*. Using the Delta K method for estimating K (Evanno et al. 2005), the optimal value of K is 2. Calculations were performed using Structure Harvester (Earl and vonHoldt 2012).

K	Reps	Mean LnP(K)	SD LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	50	-38618.65	3.69	-	-	-
2	50	-27344.16	907.97	11274.49	14299.14	**15.75**
3	50	-30368.81	67588.97	-3024.65	22201.82	0.33
4	50	-11191.64	2045.29	19177.17	17988.48	8.80
5	50	-10002.95	2559.90	1188.69	798.27	0.31
6	50	-9612.53	2259.66	390.42	620.44	0.27
7	50	-8601.66	2099.72	1010.86	870.17	0.41
8	50	-8460.97	2036.82	140.69	-	-

Table A.7. Proportion of membership of each pre-assigned population in each of the two clusters in STRUCTURE for analysis including all four *Ceasia* species and *Etheostoma caeruleum*.

Species	Cluster 1	Cluster 2	Number of Individuals
Etheostoma burri	1.00	0.00	12
Etheostoma spectabile	1.00	0.00	12
Etheostoma fragi	1.00	0.00	12
Etheostoma uniporum	1.00	0.00	11
Etheostoma caeruleum	0.00	1.00	12

Table A.8. Results of the STRUCTURE analysis for only the four species of *Ceasia,* excluding *E. caeruleum*. Using the Delta K method for estimating K (Evanno et al. 2005), the optimal value of K is 2. Calculations were performed using Structure Harvester (Earl and vonHoldt 2012).

K	Reps	Mean LnP(K)	SD LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	50	-38562.30	3.31	-	-	-
2	50	-22179.45	2537.34	16382.85	142651.47	**56.22**
3	50	-148448.07	959138.00	-126268.62	334482.29	0.35
4	50	-609198.99	2396921.89	-460750.91	902296.72	0.38
5	50	-167653.18	901024.69	441545.80	611030.98	0.68
6	50	-337138.36	1647994.64	-169485.18	298040.65	0.18
7	50	-208582.89	1418176.47	128555.47	-	-

Table A.9. Proportion of membership of each pre-assigned population in each of the two clusters in STRUCTURE for the analysis including all four *Ceasia* species but excluding *Etheostoma caeruleum.*

Species	Cluster 1	Cluster 2	Number of Individuals
Etheostoma burri	0.00	1.00	12
Etheostoma spectabile	0.00	1.00	12
Etheostoma fragi	1.00	0.00	12
Etheostoma uniporum	1.00	0.00	11

Table A.10. K-means clustering analysis results for variant SNP data set including all five species.

K	Pseudo-*F*
1	0.000
2	23.95
3	36.07
4	47.73
5	**59.88**
6	51.44
7	46.13
8	41.06

FIGURES

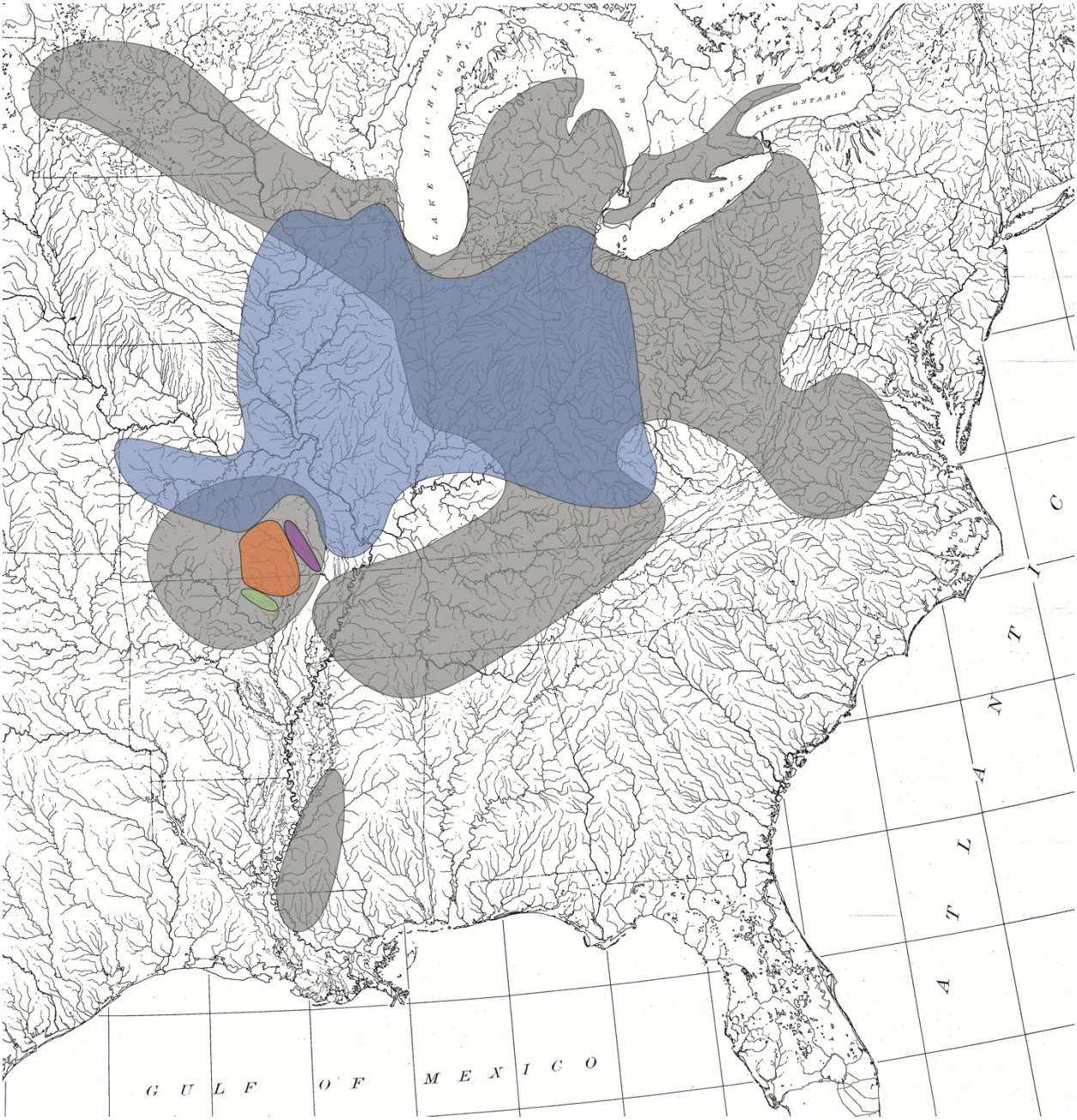

Figure A.1. Range map for study species. Gray = *Etheostoma caeruleum*, blue = *E. spectabile*, green = *E. fragi*, orange = *E. uniporum*, and purple = *E. burri.*

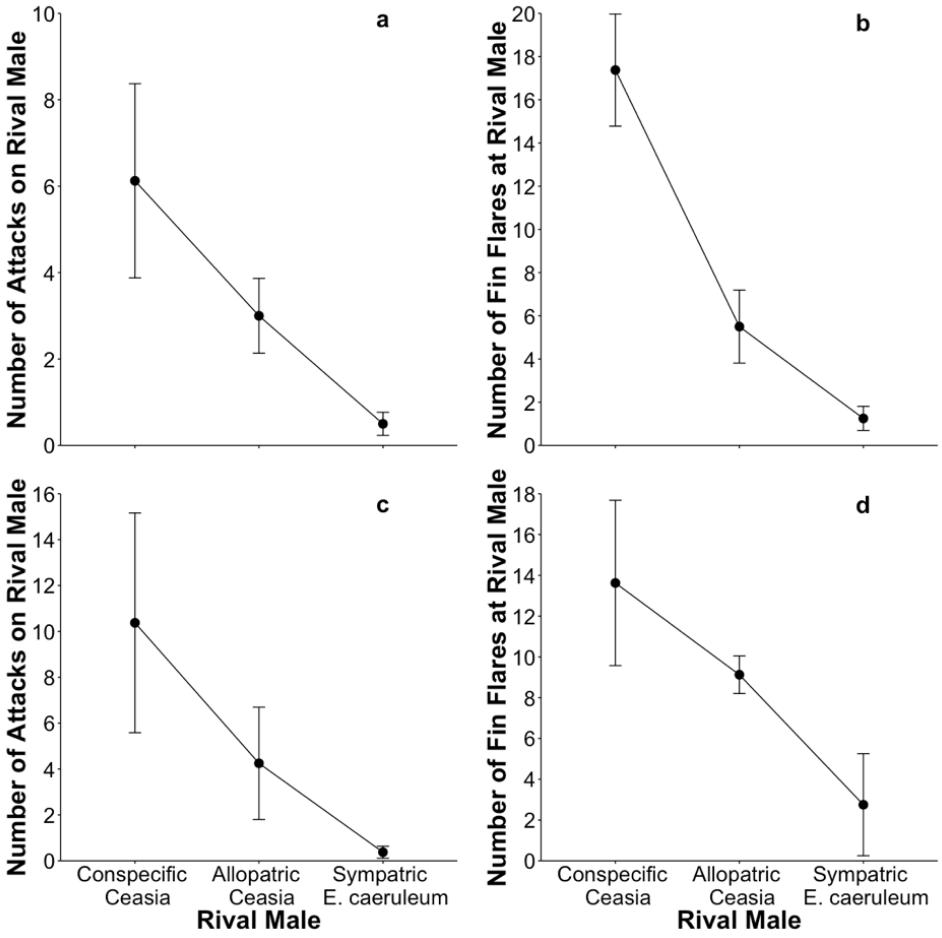

Figure A.2. Focal male behavior towards rival males. (a,b) Species set 1F with *E. fragi* as the focal pair and conspecific *Ceasia* rival male, and *E. uniporum* as the allopatric *Ceasia* rival male. (c,d) Species set 1R with *E. uniporum* as the focal pair and conspecific *Ceasia* rival male, and *E. fragi* as the allopatric *Ceasia* rival male. (a,c) Focal male attacks on rival male across rival male trial types. (b,d) Focal male fin flares at rival male across rival male trial types.

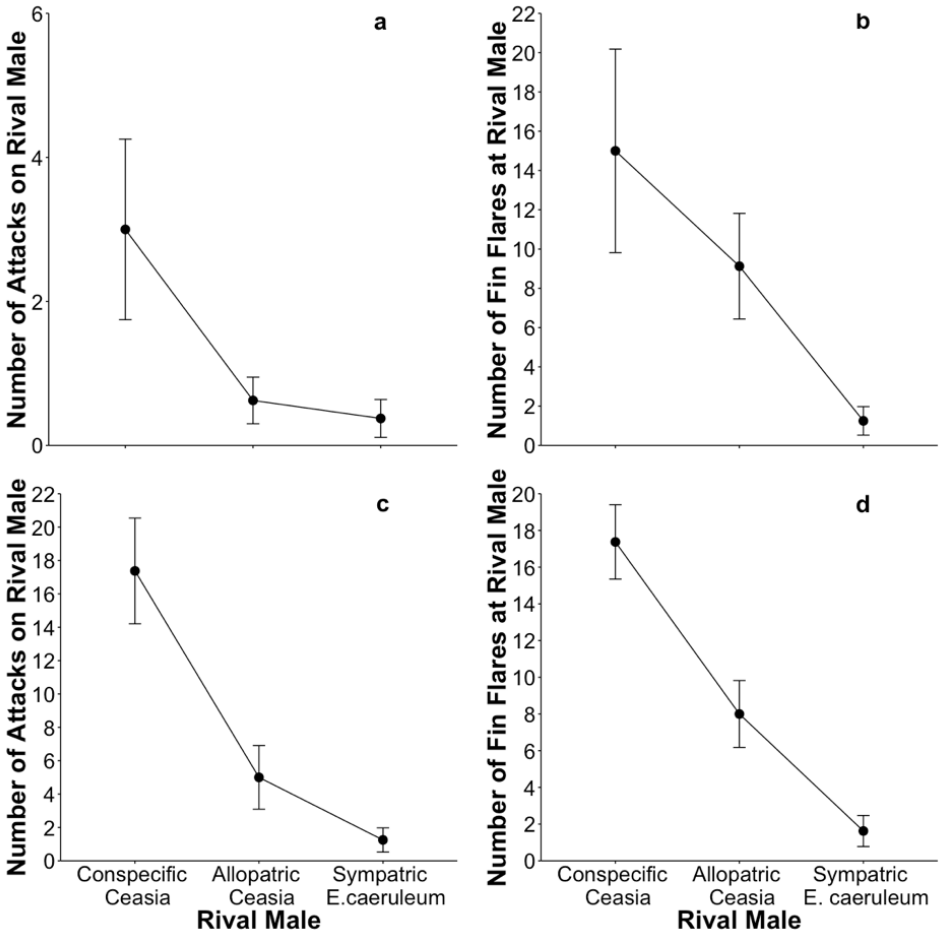

Figure A.3. Focal male behavior towards rival males. (a,b) Species set 2F with *E. fragi* as the focal pair and conspecific *Ceasia* rival male, and *E. burri* as the allopatric *Ceasia* rival male. (c,d) Species set 2R with *E. burri* as the focal pair and conspecific *Ceasia* rival male, and *E. fragi* as the allopatric *Ceasia* rival male. (a,c) Focal male attacks on rival male across rival male trial types. (b,d) Focal male fin flares at rival male across rival male trial types.

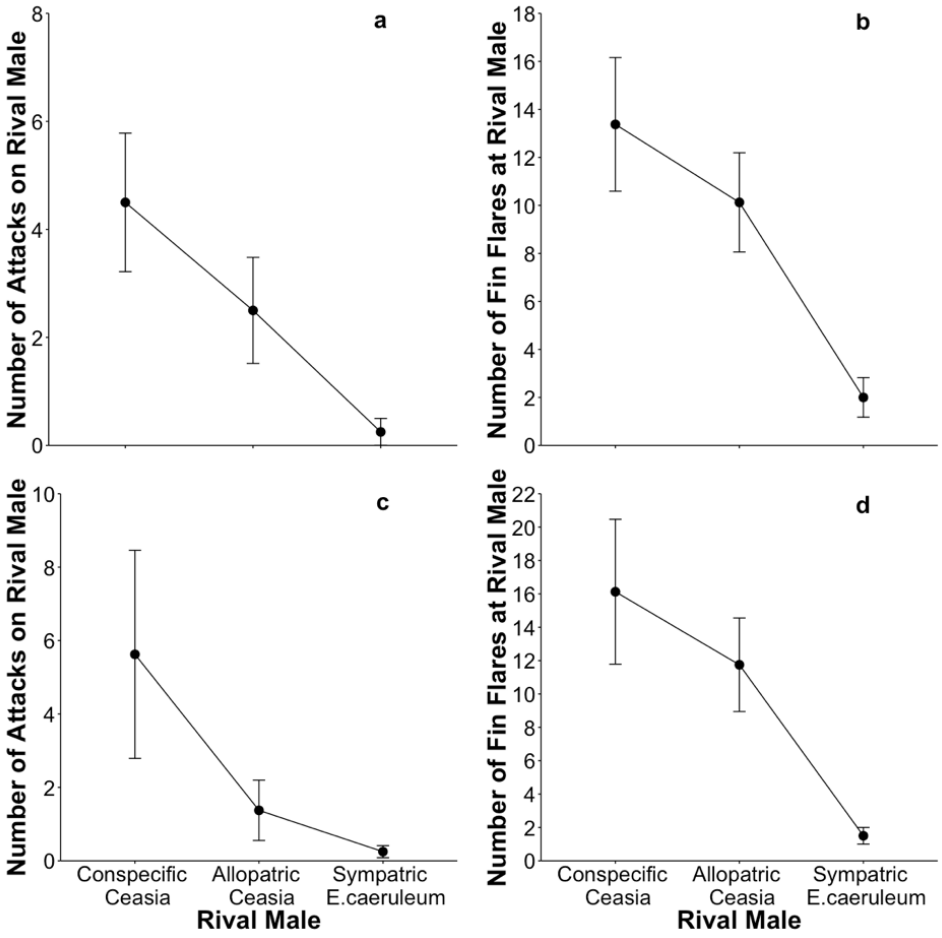

Figure A.4. Focal male behavior towards rival males. (a,b) Species set 3F with *E. fragi* as the focal pair and conspecific *Ceasia* rival male, and *E. spectabile* as the allopatric *Ceasia* rival male. (c,d) Species set 3R with *E. spectabile* as the focal pair and conspecific *Ceasia* rival male, and *E. fragi* as the allopatric *Ceasia* rival male. (a,c) Focal male attacks on rival male across rival male trial types. (b,d) Focal male fin flares at rival male across rival male trial types.

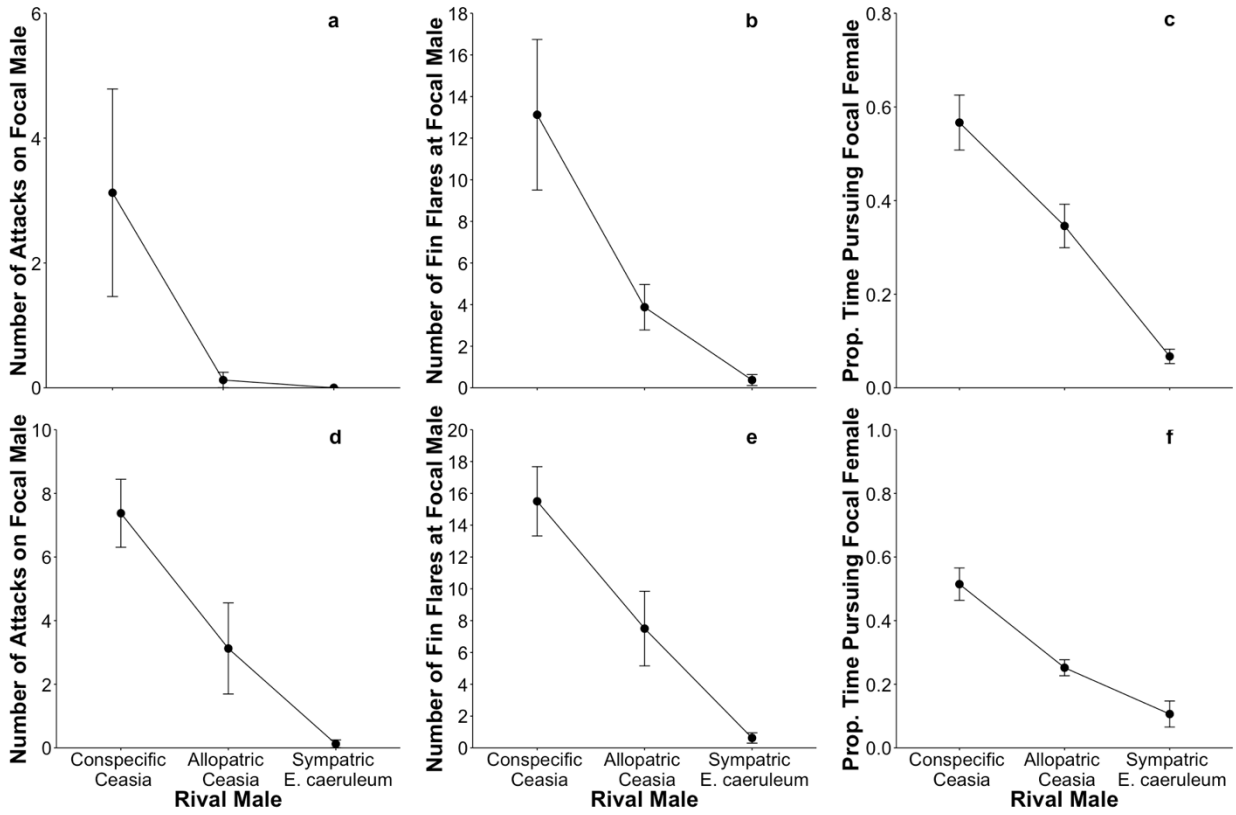

Figure A.5. Rival male behavior towards focal males and focal females. **(**a-c) Species set 2F with *E. fragi* as the focal pair and conspecific *Ceasia* rival male, and *E. burri* as the allopatric *Ceasia* rival male. (d-f) Species set 2R with *E. burri* as the focal pair and conspecific *Ceasia* rival male, and *E. fragi* as the allopatric *Ceasia* rival male. (a,d) Rival male attacks on focal male. (b,e) Rival male fin flares at focal male. (c,f) Rival male pursuit of focal female.

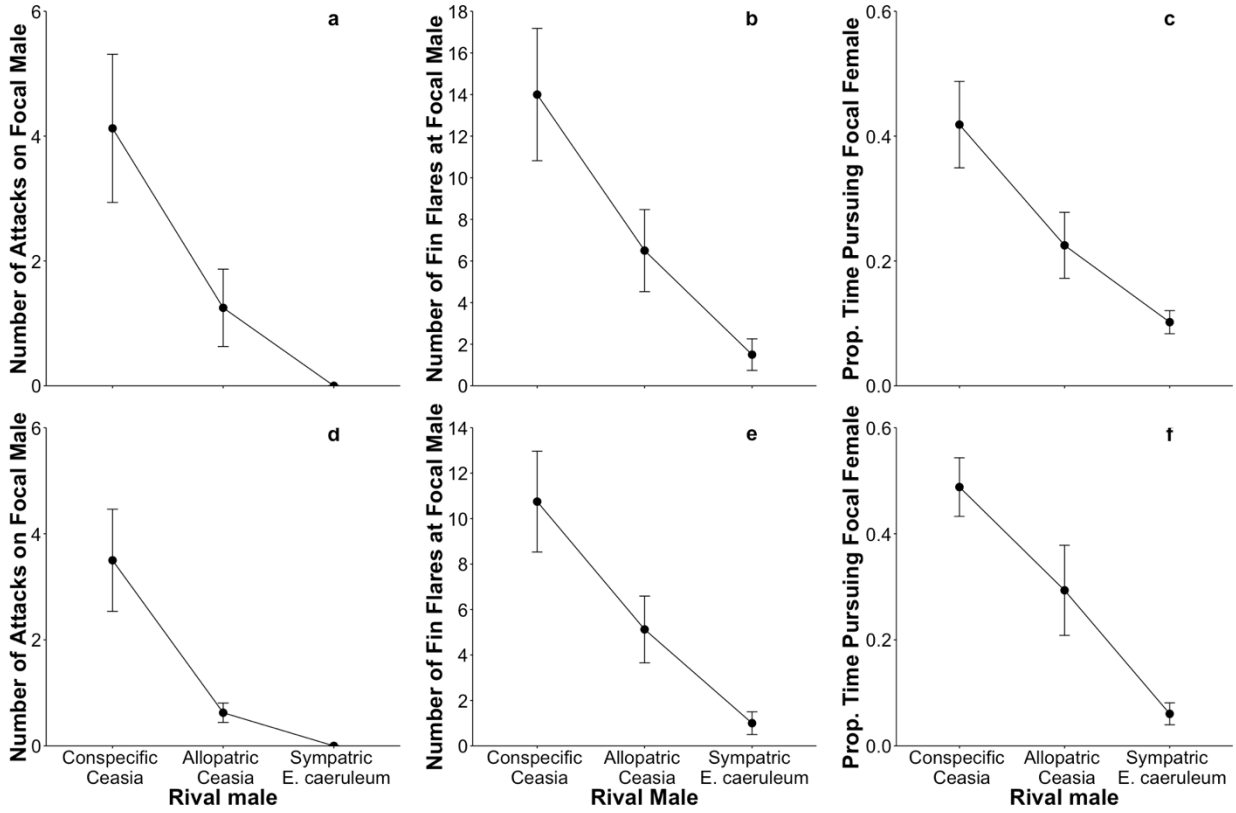

Figure A.6. Rival male behavior towards focal males and focal females. (a-c) Species set 3F with *E. fragi* as the focal pair and conspecific *Ceasia* rival male, and *E. spectabile* as the allopatric *Ceasia* rival male. (d-f) Species set 3R with *E. spectabile* as the focal pair and conspecific *Ceasia* rival male, and *E. fragi* as the allopatric *Ceasia* rival male. (a,d) Rival male attacks on focal male. (b,e) Rival male fin flares at focal male. (c,f) Rival male pursuit of focal female.

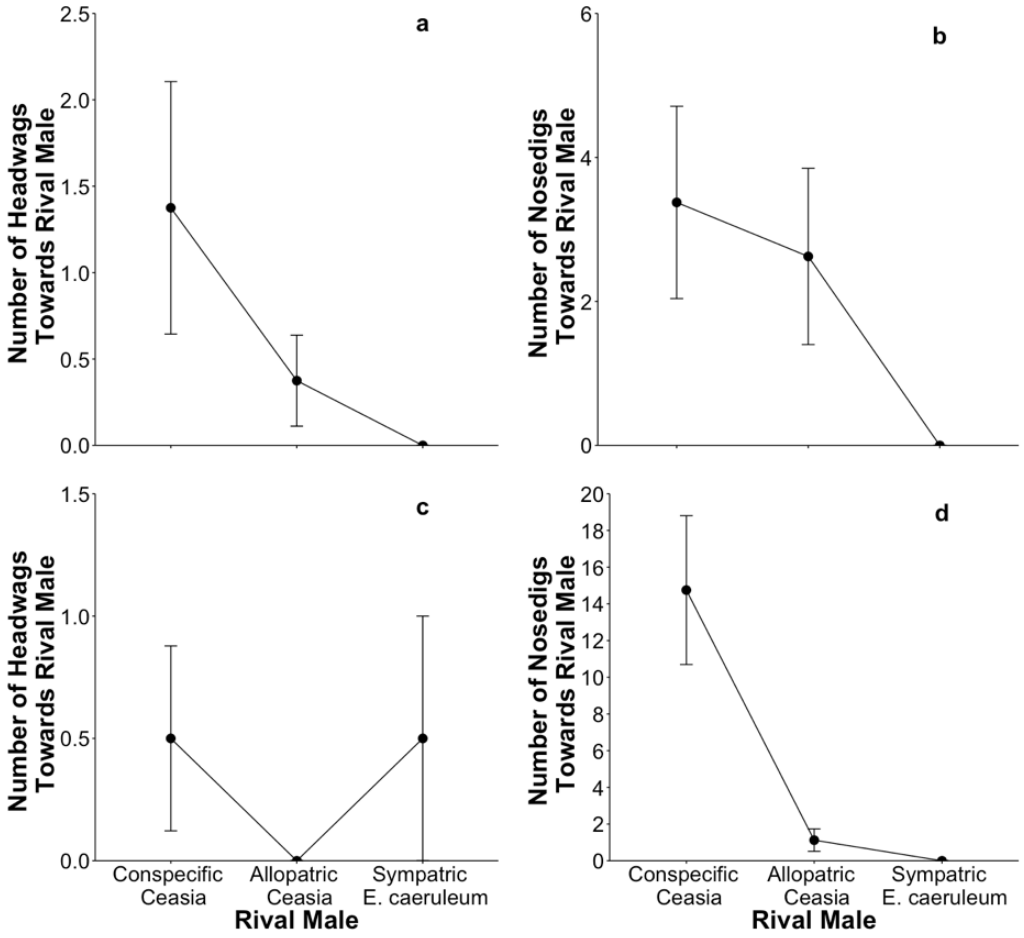

Figure A.7. Focal female behavior towards rival males. (a-b) Species set 1F with *E. fragi* as the focal pair and conspecific *Ceasia* rival male, and *E. uniporum* as the allopatric *Ceasia* rival male. (c-d) Species set 1R with *E. uniporum* as the focal pair and conspecific *Ceasia* rival male, and *E. fragi* as the allopatric *Ceasia* rival male. (a,c) Focal female headwags towards rival male across rival male trial types. (b,d) Focal female nosedigs towards rival male across rival male trial types.

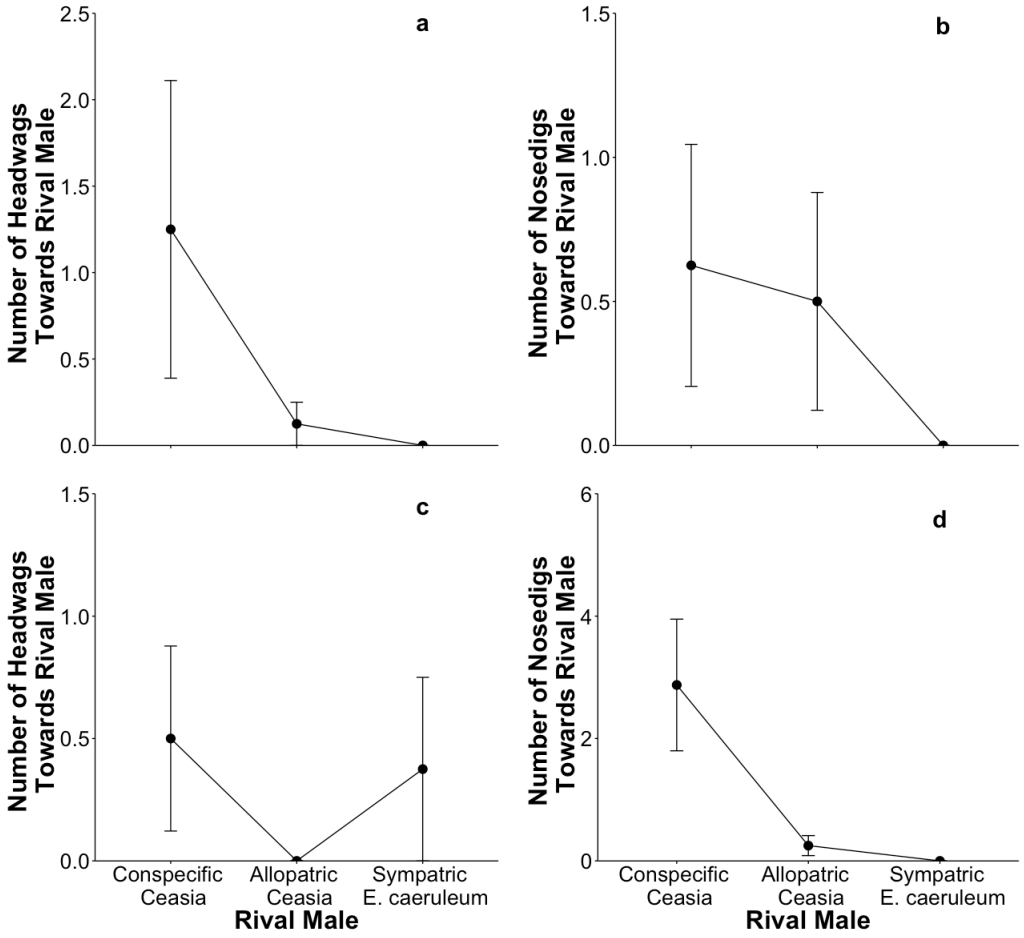

Figure A.8. Focal female behavior towards rival males. (a-b) Species set 2F with *E. fragi* as the focal pair and conspecific *Ceasia* rival male, and *E. burri* as the allopatric *Ceasia* rival male. (c-d) Species set 2R with *E. burri* as the focal pair and conspecific *Ceasia* rival male, and *E. fragi* as the allopatric *Ceasia* rival male. (a,c) Focal female headwags towards rival male across rival male trial types. (b,d) Focal female nosedigs towards rival male across rival male trial types.

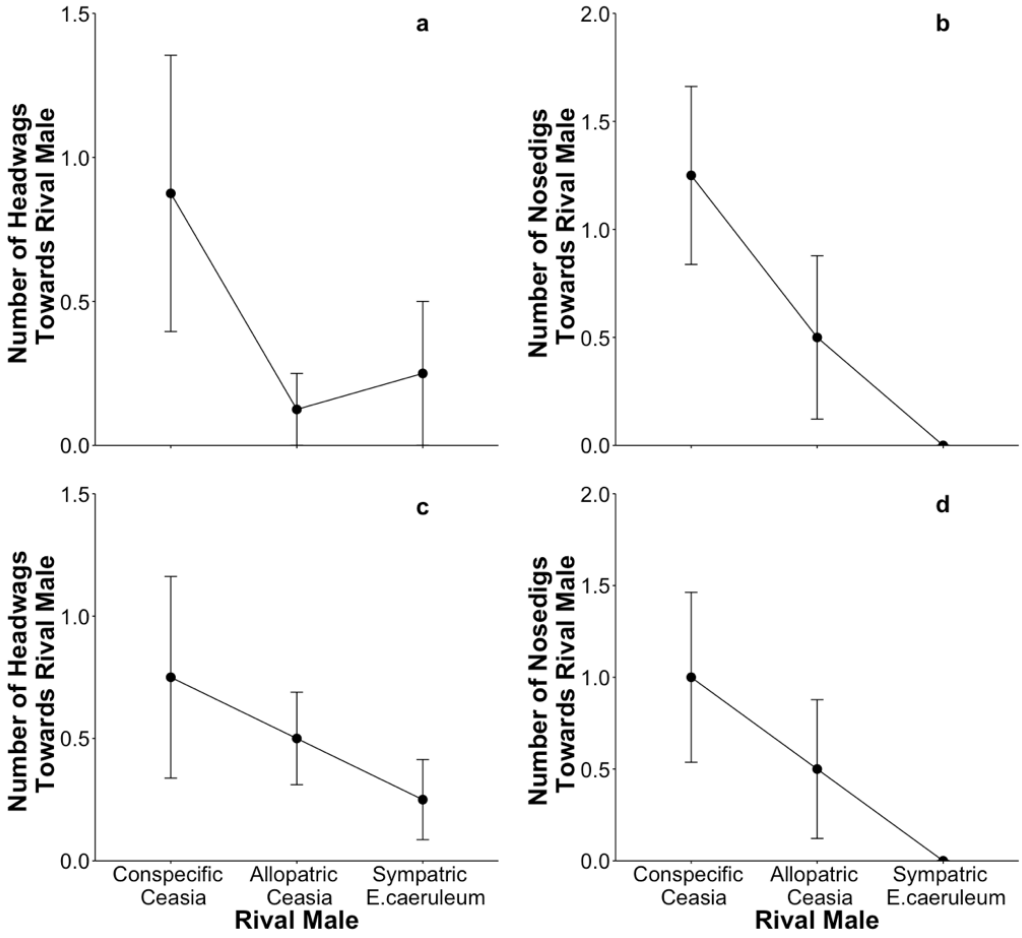

Figure A.9. Focal female behavior towards rival males. (a-b) Species set 3F with *E. fragi* as the focal pair and conspecific *Ceasia* rival male, and *E. spectabile* as the allopatric *Ceasia* rival male. (c-d) Species set 3R with *E. spectabile* as the focal pair and conspecific *Ceasia* rival male, and *E. fragi* as the allopatric *Ceasia* rival male. (a,c) Focal female headwags towards rival male across rival male trial types. (b,d) Focal female nosedigs towards rival male across rival male trial types.

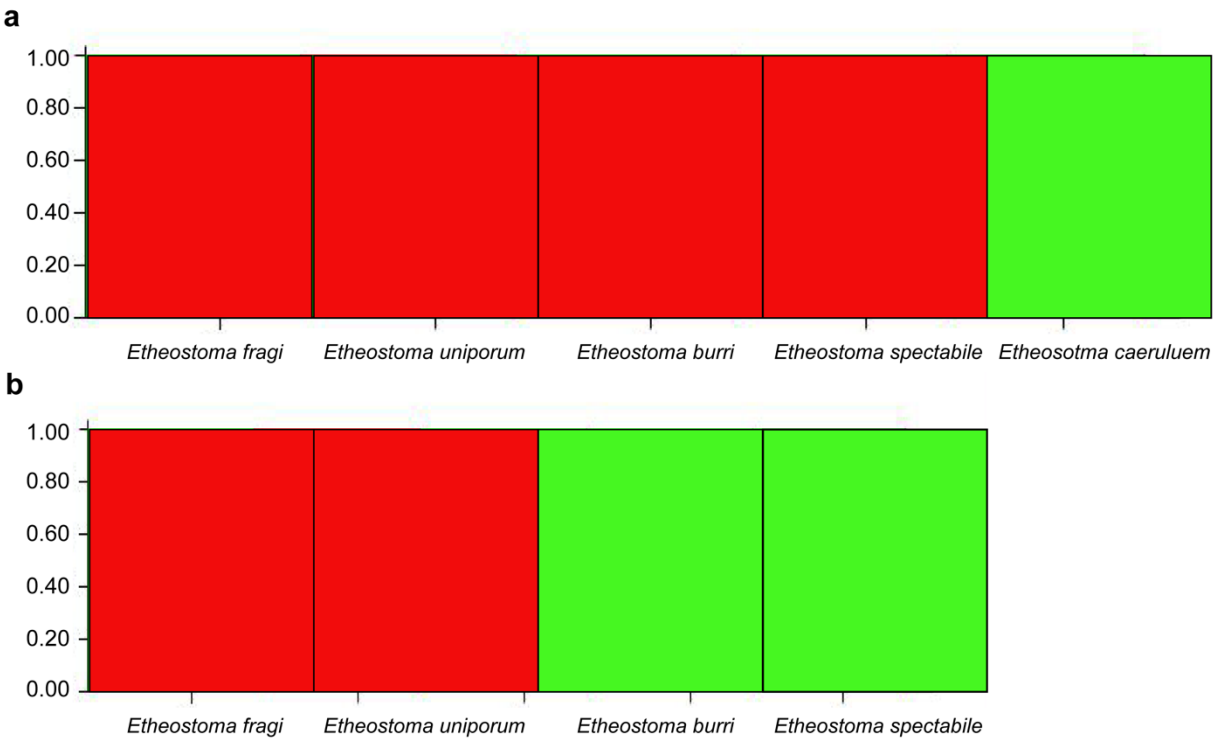

Figure A.10. STRUCTURE bar plot showing the probability for each individual of belonging to a cluster (See Tables A.5-A.8). (a) STRUCTURE analysis including all four *Ceasia* species and the more distantly related *Etheostoma caeruleum*. The optimal number of distinct clusters (K) determined to be two using the Delta K method (Evano et al. 2005). (b) STRUCTURE analysis only including the four *Ceasia* species and excluding *E. caeruleum.* The optimal number of distinct clusters (K) determined to be two using the Delta K method (Evano et al. 2005).

APPENDIX B

SUPPLEMENTARY MATERIALS FOR CHAPTER 3

TABLES

Table B.1. Results of ANOVA testing for patterns consistent with RCD in *E. caeruleum* rival male mate choice in the male competition trials. *Rival male mate choice* compared *E. caeruleum* rival male preference for the focal *Ceasia* female to conspecific *Ceasia* rival male preference for the focal *Ceasia* female. We asked whether *rival male mate choice* differed among trials in which sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* served as the focal *Ceasia* pair (note that allopatric *E. caeruleum* rival males were paired with allopatric focal *Ceasia* pairs, and sympatric *E. caeruleum* rival males were paired with sympatric focal *Ceasia* pairs). Pairwise post-hoc t-test results are also shown for the analysis.

Rival male mate choice	**df**	**Test Statistic**	**p**
Focal *Ceasia* population identity	2,33	17.68	**<0.00001**
Sympatric *E. spectabile* vs. allopatric *E. spectabile*	22	6.35	**<0.00001**
Sympatric *E. spectabile* vs. allopatric *E. pulchellum*	22	5.35	**<0.0001**
Allopatric *E. spectabile* vs. allopatric *E. pulchellum*	22	-0.25	0.80

Table B.2. Results of ANCOVA testing for patterns consistent with RCD in focal *Ceasia* female preference for conspecific *Ceasia* rival males versus *E. caeruleum* rival males. We asked whether the number of nosedigs that focal females performed towards rival males differed as a function of rival male identity (conspecific versus *E. caeruleum*) in trials where the focal *Ceasia* pair were (A) sympatric *E. spectabile*, (B) allopatric *E. spectabile*, and (C) allopatric *E. pulchellum*. Male pursuit was included as a covariate in the analyses.

A. Sympatric *E. spectabile*

Nosedigs	**df**	**F**	**p**
Male identity	1,21	0.29	0.59
Male pursuit	1,21	2.69	0.12

B. Allopatric *E. spectabile*

Nosedigs	**df**	**F**	**p**
Male identity	1,21	0.01	0.93
Male pursuit	1,21	2.42	0.13

C. Allopatric *E. pulchellum*

Nosedigs	**df**	**F**	**p**
Male identity	1,21	0.04	0.85
Male pursuit	1,21	4.17	0.054

Table B.3. Results of ANOVA testing for patterns consistent with ACD in *E. caeruleum* rival male aggression bias in male competition trials. *Rival male fin flare bias* and *rival male attack bias* compared the number of fin flares and attacks that the *E. caeruleum* rival male performed towards the focal *Ceasia* male, relative to number of fin flares and attacks that the conspecific *Ceasia* rival male performed towards the focal *Ceasia* male. We asked *rival male fin flare bias* and *rival male attack bias* differed among trials in which sympatric *E. spectabile*, *allopatric E. spectabile*, and allopatric *E. pulchellum* served as the focal *Ceasia* pair (note that allopatric *E. caeruleum* rival males were paired with allopatric focal *Ceasia* pairs, and sympatric *E. caeruleum* rival males were paired with sympatric focal *Ceasia* pairs). Pairwise post-hoc t-test results are also shown for both analyses.

Rival male fin flare bias	**df**	**Test Statistic**	**p**
Focal *Ceasia* population identity	2,33	6.13	**0.0054**
Sympatric *E. spectabile* vs. allopatric *E. spectabile*	22	3.62	**0.0015**
Sympatric *E. spectabile* vs. allopatric *E. pulchellum*	22	2.04	**0.054**
Allopatric *E. spectabile* vs. allopatric *E. pulchellum*	22	-1.36	0.19

Rival male attack bias	**df**	**Test Statistic**	**p**
Focal *Ceasia* population identity	2,33	1.71	0.20
Sympatric *E. spectabile* vs. allopatric *E. spectabile*	22	1.90	0.071
Sympatric *E. spectabile* vs. allopatric *E. pulchellum*	22	0.87	0.39
Allopatric *E. spectabile* vs. allopatric *E. pulchellum*	220	-0.95	0.36

Table B.4. Results of ANCOVA testing for patterns consistent with CRCD in focal *Ceasia* female mate preference for conspecific over heterospecific *Ceasia* males in male competition trials. We asked whether *focal female mate choice* differed among trials in which allopatric *E. spectabile* and allopatric *E. pulchellum* served as the focal *Ceasia* pair. Male pursuit of the female was included as a covariate in the analysis.

Focal female mate choice	**df**	**Test Statistic**	**p**
Focal *Ceasia* population identity	1,21	0.57	0.46
Male pursuit	1,21	1.27	0.27

Table B.5. Results of ANCOVA testing for patterns consistent with CRCD in focal *Ceasia* female preference for conspecific rival males versus heterospecific *Ceasia* rival males. We asked whether the number of nosedigs that focal females performed towards rival males differed as a function of rival male identity (conspecific versus heterospecific *Ceasia*) in trials in which (A) allopatric *E. spectabile* and (B) allopatric *E. pulchellum* served as the focal *Ceasia* pair. Male pursuit was included as a covariate in the analyses.

A. Allopatric *E. spectabile*

Nosedigs	**df**	**F**	**p**
Male identity	1,21	0.35	0.56
Male pursuit	1,21	2.87	0.11

B. Allopatric *E. pulchellum*

Nosedigs	**df**	**F**	**p**
Male identity	1,21	0.13	0.72
Male pursuit	1,21	7.20	**0.014**

FIGURES

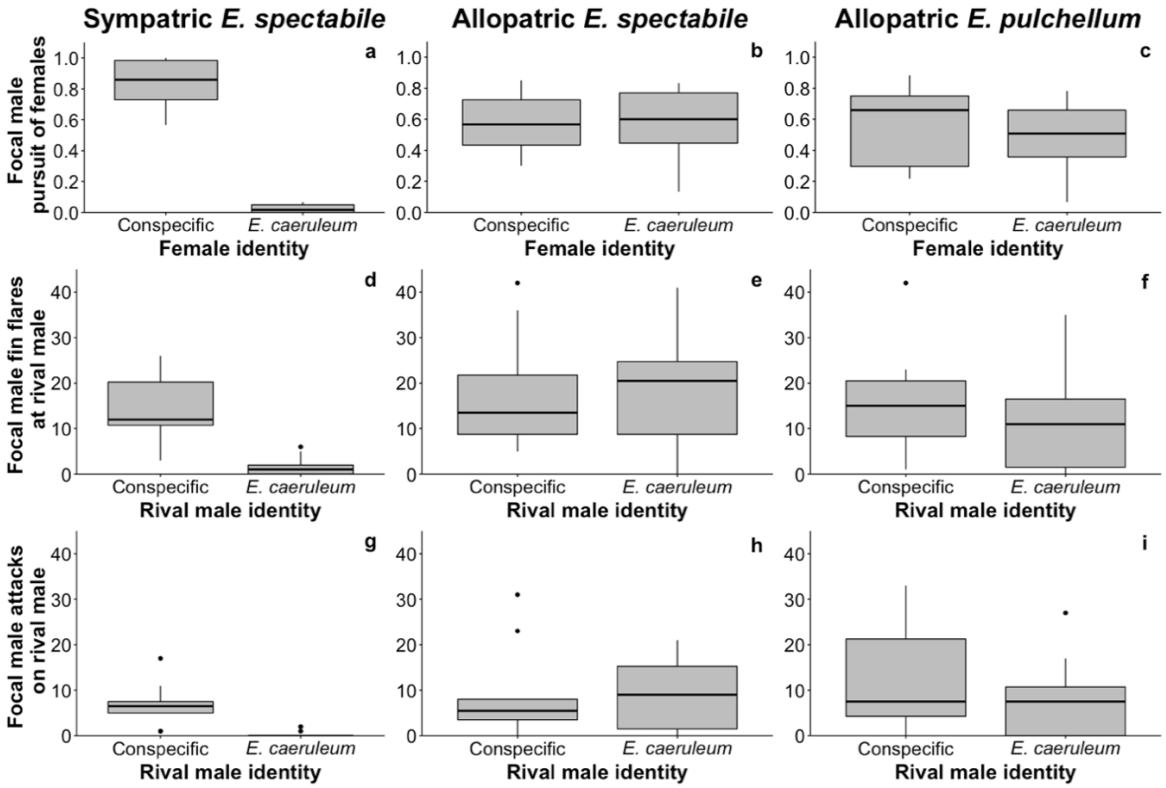

Figure B.1. Focal *Ceasia* male behavior in the dichotomous male choice trials (a-c) and male competition trials (d-i) that tested for RCD and ACD between *Ceasia* and *E. caeruleum*. Columns from left to right show results for trials with sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* as the focal *Ceasia*, respectively. (a-c) Test for RCD in focal *Ceasia* male mate preference for conspecific females. Proportion of time focal males spent in pursuit of conspecific versus *E. caeruleum* female in dichotomous choice trials. (d-f) Test for ACD in focal *Ceasia* male fin flare bias towards conspecific males. Number of focal male fin flares directed at conspecific *Ceasia* rival males versus *E. caeruleum* rival males in male competition trials. (g-i) Test for ACD in focal *Ceasia* male attack bias towards conspecific males. Number of focal male attacks directed at conspecific *Ceasia* rival male versus *E. caeruleum* rival males in male competition trials.

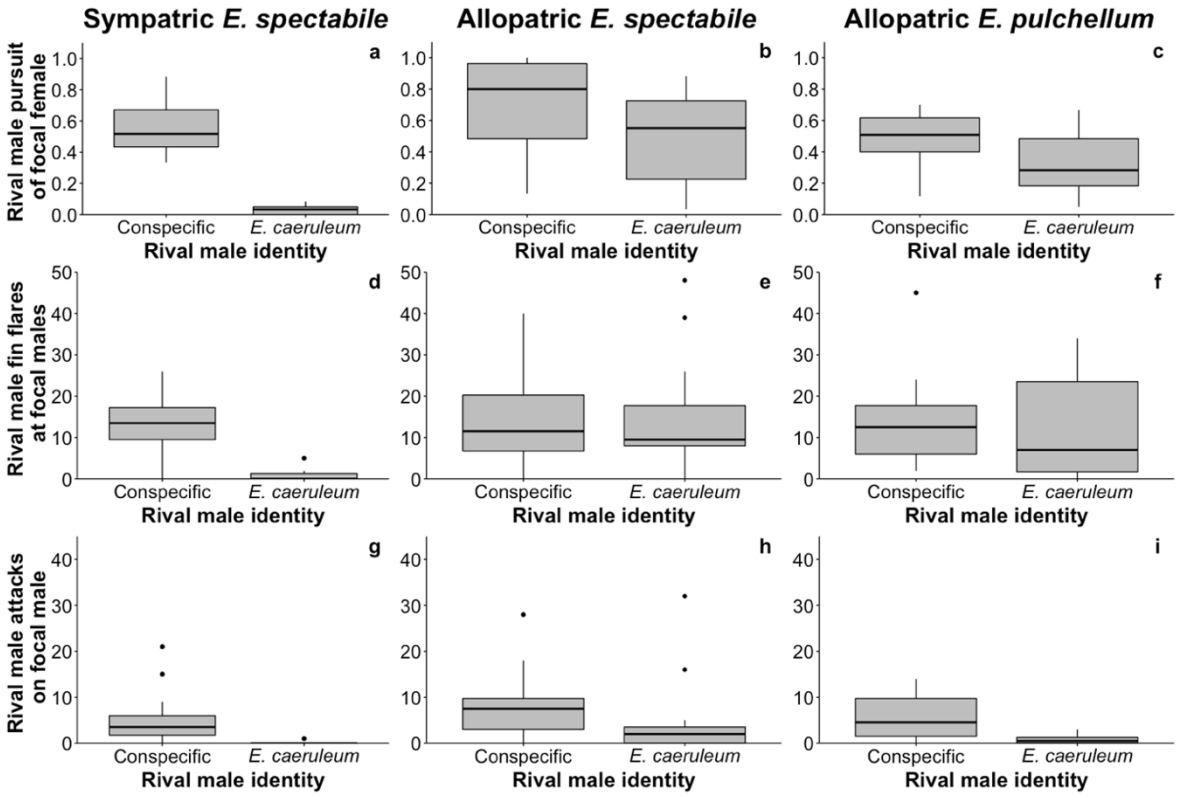

Figure B.2. Rival male behavior in the male competition trials that tested for RCD and ACD between *Ceasia* and *E. caeruleum*. Columns from left to right show results for trials with sympatric *E. spectabile*, allopatric *E. spectabile*, and allopatric *E. pulchellum* as the focal *Ceasia*, respectively. (a-c) Test for RCD in *E. caeruleum* rival male mate preference for conspecific females. Proportion of time the conspecific *Ceasia* rival male versus *E. caeruleum* rival male spent in pursuit of the focal *Ceasia* female. (d-f) Test for ACD in *E. caeruleum* rival male fin flare bias. Number of fin flares performed towards the focal *Ceasia* male by the conspecific *Ceasia* rival male versus the *E. caeruleum* rival male. (g-i) Test for ACD in *E. caeruleum* rival male attack bias. Number of attacks performed towards the focal *Ceasia* male by the conspecific *Ceasia* rival male versus the *E. caeruleum* rival male.

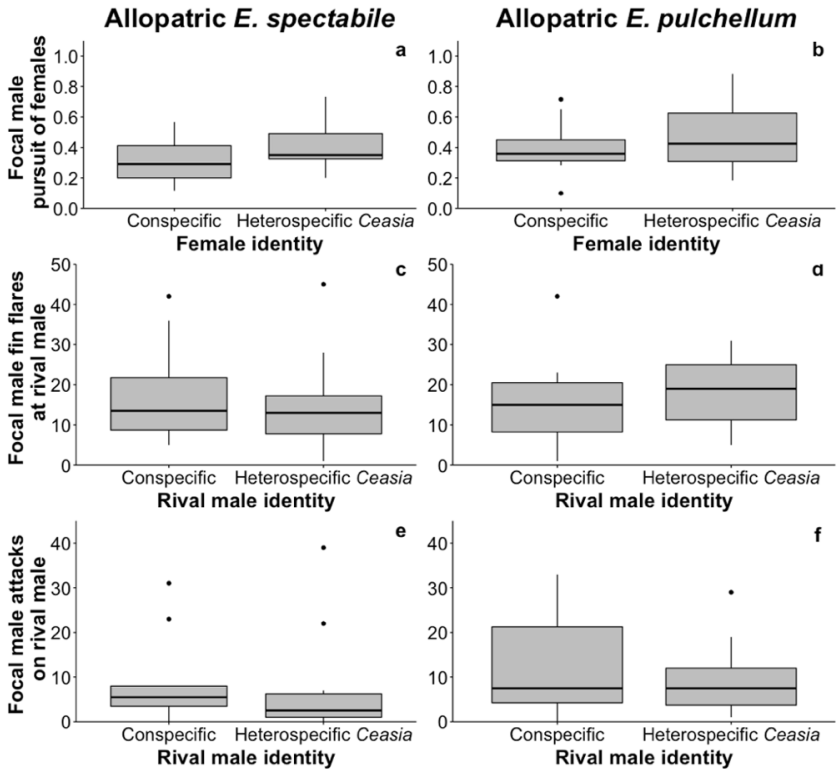

Figure B.3. Focal *Ceasia* male behavior in dichotomous male choice trials (a,b) and male competition trials (c-f) that tested for a pattern consistent with CRCD and CACD in pairings between two *Ceasia* species. The left column shows results for trials where allopatric *E. spectabile* were the focal *Ceasia* and conspecific male rival, and allopatric *E. pulchellum* were the heterospecific *Ceasia*. The right column shows results for trials where allopatric *E. pulchellum* were the focal *Ceasia* and conspecific male rival, and allopatric *E. spectabile* were the heterospecific *Ceasia*. (a,b) Test for CRCD in focal *Ceasia* male mate preference for conspecific females. Proportion of time the focal *Ceasia* male spent in pursuit of the conspecific *Ceasia* female versus heterospecific *Ceasia* female in dichotomous male choice trials. (c,d) Test for CACD in focal *Ceasia* male fin flare. Number of fin flares the focal *Ceasia* male directed at conspecific *Ceasia* rival males versus heterospecific *Ceasia* rival males in male competition trials. (e,f) Test for CACD in focal *Ceasia* male attack bias. Number of attacks the focal *Ceasia* male directed at conspecific *Ceasia* versus heterospecific *Ceasia* rival males in male competition trials. Focal *Ceasia* male repose to conspecific *Ceasia* rival males (c-f) is also presented next to focal *Ceasia* male response to *E. caeruleum* rival males in Figure S1.

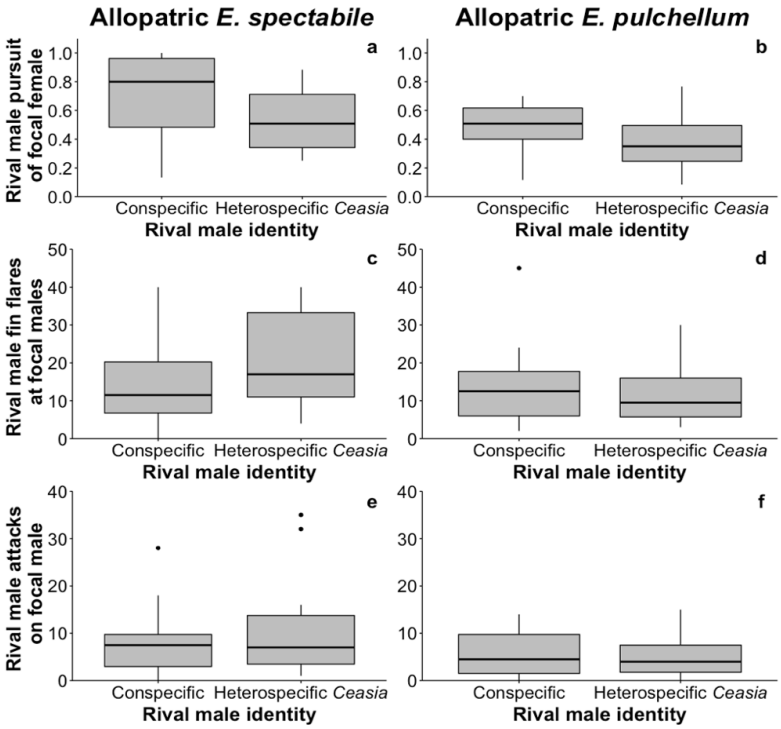

Figure B.4. Rival *Ceasia* male behavior in the male competition trials that tested for a pattern consistent with CRCD and CACD in pairings between two *Ceasia* species. The left column shows results for trials where allopatric *E. spectabile* were the focal *Ceasia* and conspecific male rival, and allopatric *E. pulchellum* were the heterospecific *Ceasia*. The right column shows results for trials where allopatric *E. pulchellum* were the focal *Ceasia* and conspecific male rival, and allopatric *E. spectabile* were the heterospecific *Ceasia*. (a,b) Test for CRCD in heterospecific *Ceasia* rival male mate preference for conspecific females. Proportion of time the conspecific *Ceasia* rival male versus heterospecific *Ceasia* rival male spent in pursuit of the focal *Ceasia* female. (c,d) Test for CACD in heterospecific *Ceasia* rival male fin flare bias. Number of fin flares performed by the conspecific *Ceasia* rival male versus the heterospecific *Ceasia* rival male towards the focal *Ceasia* male. (e,f) Test for CACD in heterospecific *Ceasia* rival male attack bias. Number of attacks performed by the conspecific *Ceasia* rival male versus the heterospecific *Ceasia* rival male towards the focal *Ceasia* male. Conspecific *Ceasia* rival male repose to the focal *Ceasia* male is also presented next to *E. caeruleum* rival male response to focal *Ceasia* male in Figure S2.

APPENDIX C

SUPPLEMENTARY MATERIALS FOR CHAPTER 5

TABLES

Table C.1. Collection locations for the parents used to generate offspring for the common garden study.

Species	Drainage	Latitude	Longitude
Strawberry darter	Strawberry River	36.304214	-91.927684
Current darter	Spring River	36.250560	-91.359318
Brook darter	Black River	37.146415	-90.907459
Orangethroat darter	Vermillion River	40.089035	-88.143440

Table C.2. Collection locations for the male fish used in the ACD color pattern study.

Species	Geography	Drainage	Latitude	Longitude
Orangethroat darter	Allopatric	Sangamon River	40.027663	−88.577180
	Sympatric	Vermillion River	40.089035	-88.143440
Rainbow darter	Sympatric	Vermillion River	40.089035	-88.143440
	Allopatric	Kalamazoo River	42.426825	−85.428370

Table C.3. Common garden study results from of two-factor nested ANOVAs on each color variable with species and family (nested within species) included as factors. R = red coloration, B = blue coloration, r = R value, g = G value, b =B value, Prop = proportion, DF = dorsal fin.

R_r

	Df	F	P
family	1	8.80	<0.01
species	3	60.67	<0.00001
family*species	3	3.38	<0.05
Residuals	63		

R_g

	Df	F	P
family	1	6.66	<0.05
species	3	47.16	<0.00001
family*species	3	3.34	<0.05
Residuals	63		

R_b

	Df	F	P
family	1	5.96	<0.05
species	3	13.83	<0.00001
family*species	3	6.36	<0.001
Residuals	63		

B_r

	Df	F	P
family	1	6.77	<0.05
species	3	7.90	<0.001
family*species	3	1.63	0.19
Residuals	63		

B_g

	Df	F	P
family	1	0.98	0.33
species	3	8.51	<0.0001
family*species	3	1.99	0.12
Residuals	63		

Table C.3. Continued Common garden study results from of two-factor nested ANOVAs on each color variable with species and family (nested within species) included as factors. R = red coloration, B = blue coloration, r = R value, g = G value, b =B value, Prop = proportion, DF = dorsal fin.

B_b

	Df	F	P
family	1	1.30	0.26
species	3	9.59	<0.0001
family*species	3	2.21	0.1
Residuals	63		

DF1_Prop_B

	Df	F	P
family	1	11.45	<0.01
species	3	43.07	<0.0001
family*species	3	1.29	0.29
Residuals	63		

DF1_Prop_R

	Df	F	P
family	1	0.44	0.51
species	3	28.49	<0.0001
family*species	3	0.47	0.7
Residuals	63		

DF2_Prop_B

	Df	F	P
family	1	8.38	<0.01
species	3	1.63	0.19
family*species	3	0.47	0.71
Residuals	63	3.00	

DF2_Prop_R

	Df	F	P
family	1	0.48	0.49
species	3	23.13	<0.0001
family*species	3	3.44	<0.05
Residuals	63		

Table C.3. Continued Common garden study results from of two-factor nested ANOVAs on each color variable with species and family (nested within species) included as factors. R = red coloration, B = blue coloration, r = R value, g = G value, b =B value, Prop = proportion, DF = dorsal fin.

Body_Prop_B

	Df	F	P
family	1	27.49	<0.0001
species	3	22.93	<0.0001
family*species	3	1.62	0.2
Residuals	63		

Body_Prop_R

	Df	F	P
family	1	7.80	<0.01
species	3	24.10	<0.0001
family*species	3	0.82	0.49
Residuals	63		

Table C.4. Pairwise post-hoc Tukey's tests comparing scores for (A) LD1 and (B) LD2 from LDA on color pattern in fish from the common garden study. This analysis included four allopatric species in the orangethroat darter clade (i.e., orangethroat, strawberry, current, and brook darters).

(A) LD1

Species 1	Species 2	Estimate	Std. Error	t-value	P
Strawberry	Brook	8.33	0.95	8.74	<0.001
Orangethroat	Brook	0.21	0.96	0.22	0.996
Current	Brook	5.56	0.98	5.66	<0.001
Orangethroat	Strawberry	-8.11	0.98	-8.25	<0.001
Current	Strawberry	-2.76	1.01	-2.75	0.038
Current	Orangethroat	5.35	1.01	5.29	<0.001

(B) LD2

Species 1	Species 2	Estimate	Std. Error	t-value	P
Strawberry	Brook	0.74	0.93	0.80	0.855
Orangethroat	Brook	-3.18	0.93	-3.41	<0.01
Current	Brook	-5.01	0.95	-5.25	< 0.001
Orangethroat	Strawberry	-3.92	0.96	-4.10	< 0.001
Current	Strawberry	-5.75	0.98	-5.89	< 0.001
Current	Orangethroat	-1.83	0.98	-1.86	0.254

Table C.5. Results of ANOVAs comparing scores for (A) LD1 and (B) LD2 from LDA on male color pattern in wild-caught and lab-reared fish from four allopatric species in the orangethroat darter clade (i.e., orangethroat, strawberry, current, and brook darters). Species and rearing environment (i.e., lab-raised or wild-caught; nested within species) were factors in these analyses.

(A) LD1

Factor	Df	F	P
Species	3, 103	122.96	<0.000001
Environment	1, 103	1.51	0.222
Species* Environment	3, 103	1.62	0.190

(B) LD2

Factor	Df	F	P
Species	3, 103	117.98	<0.000001
Environment	1, 103	0.42	0.520
Species* Environment	3, 103	1.61	0.192

Table C.6. ACD study results from of two-factor nested ANOVAs on each color variable with species and geography (nested within species) included as factors. R = red coloration, B = blue coloration, r = R value, g = G value, b =B value, Prop = proportion, DF = dorsal fin, AF = anal fin, CF = caudal fin.

R_r

	Df	F	P
geography	1	4.19	<0.05
species	1	1.21	0.28
geography*species	1	0.29	0.59
Residuals	36		

R_g

	Df	F	P
geography	1	0.09	0.77
species	1	0.18	0.68
geography*species	1	2.27	<0.01
Residuals	36		

R_b

	Df	F	P
geography	1	0.93	<0.01
species	1	1.38	0.25
geography*species	1	0.16	0.7
Residuals	36		

B_r

	Df	F	P
geography	1	3.55	0.07
species	1	6.30	<0.05
geography*species	1	3.65	0.06
Residuals	36		

B_g

	Df	F	P
geography	1	5.10	0.03
species	1	0.31	0.58
geography*species	1	2.22	0.15
Residuals	36		

Table C.6. Continued ACD study results from of two-factor nested ANOVAs on each color variable with species and geography (nested within species) included as factors. R = red coloration, B = blue coloration, r = R value, g = G value, b =B value, Prop = proportion, DF = dorsal fin, AF = anal fin, CF = caudal fin.

B_b

	Df	F	P
geography	1	3.33	0.08
species	1	0.06	0.8
geography*species	1	4.26	<0.05
Residuals	36		

DF1_Prop_B

	Df	F	P
geography	1	12.19	<0.01
species	1	2.48	0.12
geography*species	1	8.31	<0.01
Residuals	36		

DF1_Prop_R

	Df	F	P
geography	1	0.03	0.87
species	1	7.32	<0.05
geography*species	1	11.67	<0.01
Residuals	36		

DF2_Prop_B

	Df	F	P
geography	1	0.09	0.77
species	1	0.87	0.36
geography*species	1	17.83	<0.001
Residuals	36		

DF2_Prop_R

	Df	F	P
geography	1	0.81	0.37
species	1	4.12	<0.05
geography*species	1	1.70	0.2
Residuals	36		

Table C.6. Continued ACD study results from of two-factor nested ANOVAs on each color variable with species and geography (nested within species) included as factors. R = red coloration, B = blue coloration, r = R value, g = G value, b =B value, Prop = proportion, DF = dorsal fin, AF = anal fin, CF = caudal fin.

AF_Prop_B

	Df	F	P
geography	1	0.15	0.7
species	1	37.55	<0.0001
geography*species	1	5.05	<0.05
Residuals	36		

AF_Prop_R

	Df	F	P
geography	1	0.00	0.99
species	1	173.00	<0.0001
geography*species	1	0.00	0.99
Residuals	36		

Body_Prop_B

	Df	F	P
geography	1	0.01	0.93
species	1	7.49	<0.01
geography*species	1	0.02	0.9
Residuals	36		

Body_Prop_R

	Df	F	P
geography	1	12.98	<0.001
species	1	1.07	0.31
geography*species	1	0.85	0.36
Residuals	36		

CF_Prop_R

	Df	F	P
geography	1	10.55	<0.01
species	1	65.34	<0.00001
geography*species	1	10.55	<0.01
Residuals	36		

Table C.7. Pairwise post-hoc Tukey's tests comparing scores for (A) LD1 and (B) LD2 from ACD color pattern analysis on sympatric (SYM) and allopatric (ALLO) orangethroat (O) and rainbow (R) darters.

(A) LD1

Group 1	Group 2	Estimate	Std. Error	t-value	P
ALLO RB	ALLO OT	3.14	0.45	7.02	< 0.000001
SYM OT	ALLO OT	-4.02	0.45	-9.00	< 0.000001
SYM RB	ALLO OT	6.27	0.45	14.01	< 0.000001
SYM OT	ALLO RB	-7.16	0.45	-16.02	< 0.000001
SYM RB	ALLO RB	3.13	0.45	6.99	< 0.000001
SYM RB	SYM OT	10.29	0.45	23.01	< 0.000001

(B) LD2

Group 1	Group 2	Estimate	Std. Error	t-value	P
ALLO RB	ALLO OT	6.60	0.45	14.75	< 0.001
SYM OT	ALLO OT	6.06	0.45	13.56	< 0.001
SYM RB	ALLO OT	4.21	0.45	9.42	< 0.001
SYM OT	ALLO RB	-0.53	0.45	-1.19	0.64
SYM RB	ALLO RB	-2.39	0.45	-5.34	< 0.001
SYM RB	SYM OT	-1.85	0.45	-4.14	<0.01

FIGURE

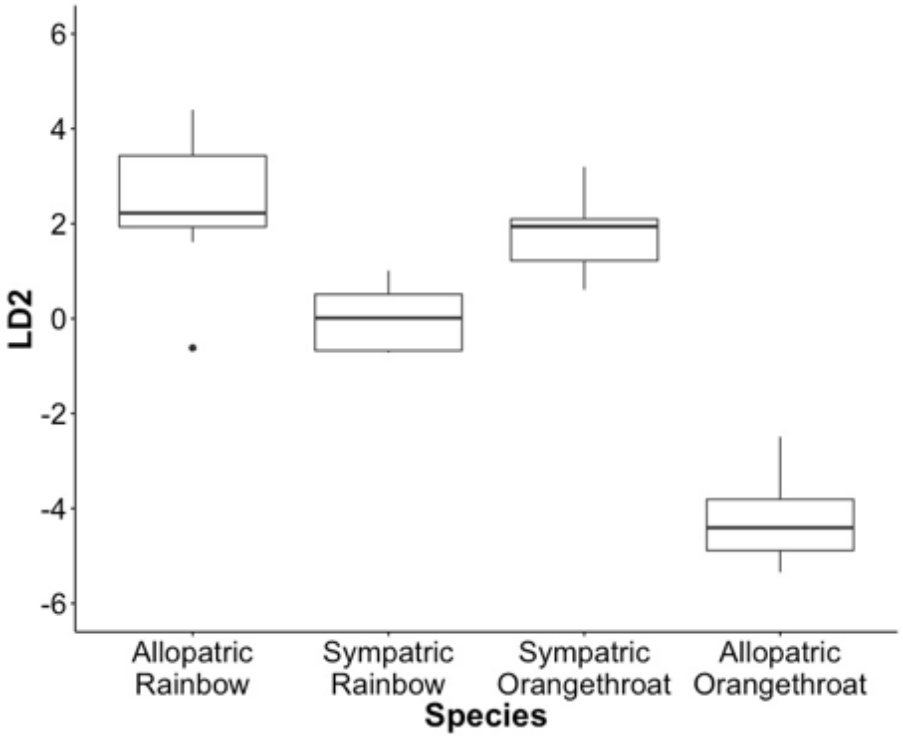

Figure C.1. Boxplots of LD2 scores from the LDA on male color pattern in fish from the ACD experiment.

APPENDIX D

SUPPLEMENTARY MATERIALS FOR CHAPTER 6

TABLES

Table D.1. Collection locations for orangethroat darters, rainbow darters, and F1 hybrids.

Species	Experiment	Latitude	Longitude
Orangethroat	F1 cross, Backcross	40.088394	-88.142504
Rainbow	F1 cross	40.055556	-88.091667
	Backcross	40.123949	-88.209260
F1 hybrid	Backcross	40.116161	-88.204336

Table D.2. Coefficients of linear discriminants (LDs) from male color pattern LDA in orangethroat darters, rainbow darters, and F1 hybrid males. Df = dorsal fin, Af = anal fin, Lat = lateral side, Caud = caudal fin, R = red, B = blue, PR = proportion red, PB = proportion blue.

Measurement type	Variable	LD1	LD2	LD3
RGB value	Df1R_r	0.016	0.002	-0.002
	Df1R_g	0.044	-0.009	0.014
	Df1R_b	-0.008	0.078	0.058
	Df1B_r	-0.003	-0.006	0.007
	Df1B_g	-0.003	0.011	-0.015
	Df1B_b	-0.003	0.009	-0.002
	Df2R_r	0.006	0.020	-0.033
	Df2R_g	-0.004	0.008	-0.026
	Df2R_b	0.015	-0.036	0.044
	Df2B_r	0.009	-0.011	0.007
	Df2B_g	-0.003	-0.001	0.019
	Df2B_b	0.040	-0.018	0.007
	AfR_r	0.007	-0.005	-0.001
	AfR_g	0.010	-0.004	0.001
	AfR_b	-0.003	-0.001	0.003
	AfB_r	-0.030	0.009	0.025
	AfB_g	-0.010	-0.001	0.005
	AfB_b	0.003	-0.007	0.003
	LatR_r	-0.002	0.005	-0.021
	LatR_g	0.012	0.000	-0.006
	LatR_b	0.001	-0.029	0.078
	LatB_r	0.012	-0.014	0.000
	LatB_g	0.013	0.013	-0.007
	LatB_b	-0.015	0.005	-0.010
	CaudR_r	0.005	-0.006	0.005
	CaudR_g	-0.003	0.002	0.005
	CaudR_b	0.032	-0.008	0.003
Color proportion	Df1_PB	1.100	1.248	-1.181
	Df1_PR	3.956	-6.657	3.330
	Df2_PB	7.123	-0.927	2.941
	Df2_PR	-1.403	-4.787	-0.119
	Af_PB	2.121	-1.079	1.291
	Af_PR	4.877	10.087	-5.367

Table D.2. Continued Coefficients of linear discriminants (LDs) from male color pattern LDA in orangethroat darters, rainbow darters, and F1 hybrid males. Df = dorsal fin, Af = anal fin, Lat = lateral side, Caud = caudal fin, R = red, B = blue, PR = proportion red, PB = proportion blue.

Body_PB	5.086	2.154	-2.949
Body_PR	-5.217	3.768	5.815
Caud_PR	7.544	3.553	3.011

Table D.3. Results of Structure (Pritchard et al. 2000) analysis on 1,073 SNPs obtained from the 42 individuals used in the backcross experiment (orangethroat darters: n = 18, rainbow darters: n = 18, F1 hybrids: n = 6). Summary statistics presented for each value of K were generated using Structure Harvester (Earl and vonHoldt 2012). The optimal value of K (i.e., the number of distinct genetic clusters) was inferred to be 2 using the Evanno method (Evanno et al. 2005), which identifies K as the largest value of Delta K.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	20	-34,929.50	0.00	NA	NA	NA
2	20	-11,038.83	0.83	23,890.68	2,5047.61	3,0191.22
3	20	-12,195.76	3,987.59	-1,156.94	611.51	0.15
4	20	-12,741.19	3,376.52	-545.43	2,060.81	0.61
5	20	-11,225.81	524.95	1,515.38	NA	NA

Table D.4. Hybrid indices (h) for each of the six wild-caught F1 hybrid males. The value for h represents the maximum likelihood estimate of the proportion of orangethroat darter (versus rainbow darter) alleles present in each hybrid male. Also shown are the likelihood value and the upper and lower limits of the 95% confidence interval for each individual.

Individual	h	ln(likelihood)	Low	Up
1	0.501	-766.435	0.475	0.527
2	0.522	-307.065	0.473	0.571
3	0.514	-711.405	0.485	0.544
4	0.510	-764.757	0.481	0.540
5	0.566	-324.111	0.519	0.613
6	0.538	-823.007	0.511	0.565

FIGURES

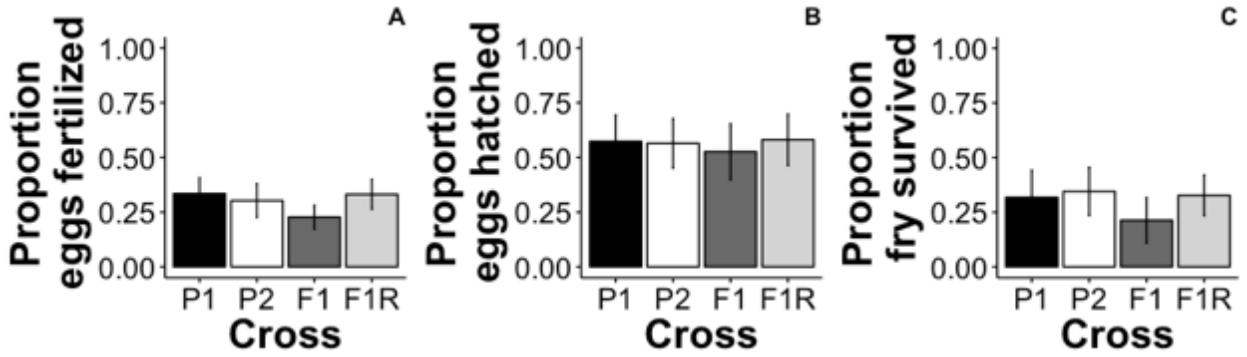

Figure D.1. Mean proportion (± standard error) of (A) hand stripped eggs that were fertilized (n=10-14 each), (B) fertilized eggs that hatched (n=10-11 each), and (C) hatched fry that survived to 10 months of age (n=5-9 each) in the two parental cross types and two hybrid cross types. P1 = ♀ orangethroat x ♂ orangethroat, P2 = ♀ rainbow x ♂ rainbow, F1 = ♀ orangethroat x ♂ rainbow, F1R = ♀ rainbow x ♂ orangethroat.

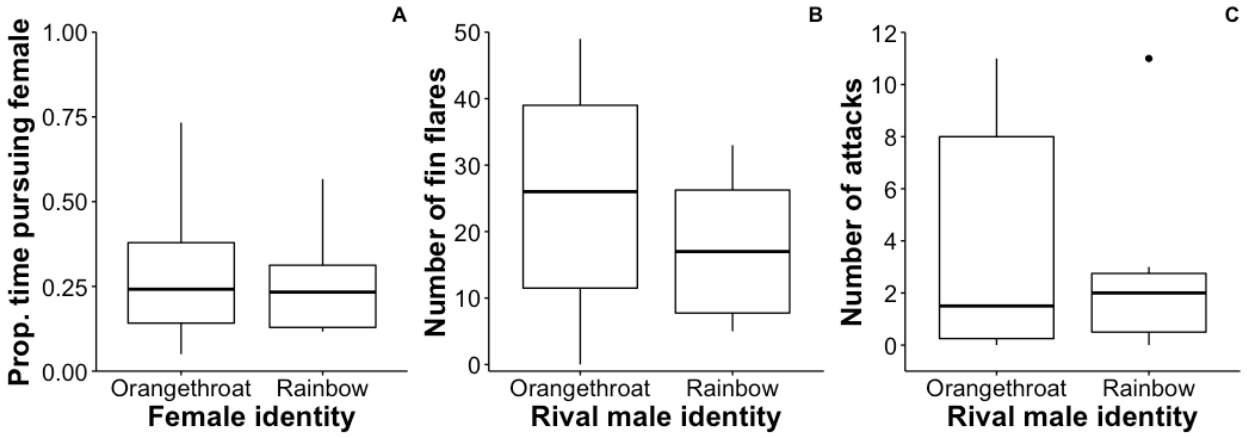

Figure D.2. Wild-caught orangethroat darter x rainbow darter F1 hybrid male mating and aggressive behavior. (A) Proportion of time hybrid males spent pursuing orangethroat versus rainbow darter females in dichotomous choice trials. (B) Number of fin flares performed by hybrid males towards orangethroat versus rainbow darter rival males. (C) Number of attacks performed by hybrid males towards orangethroat versus rainbow darter rival males.

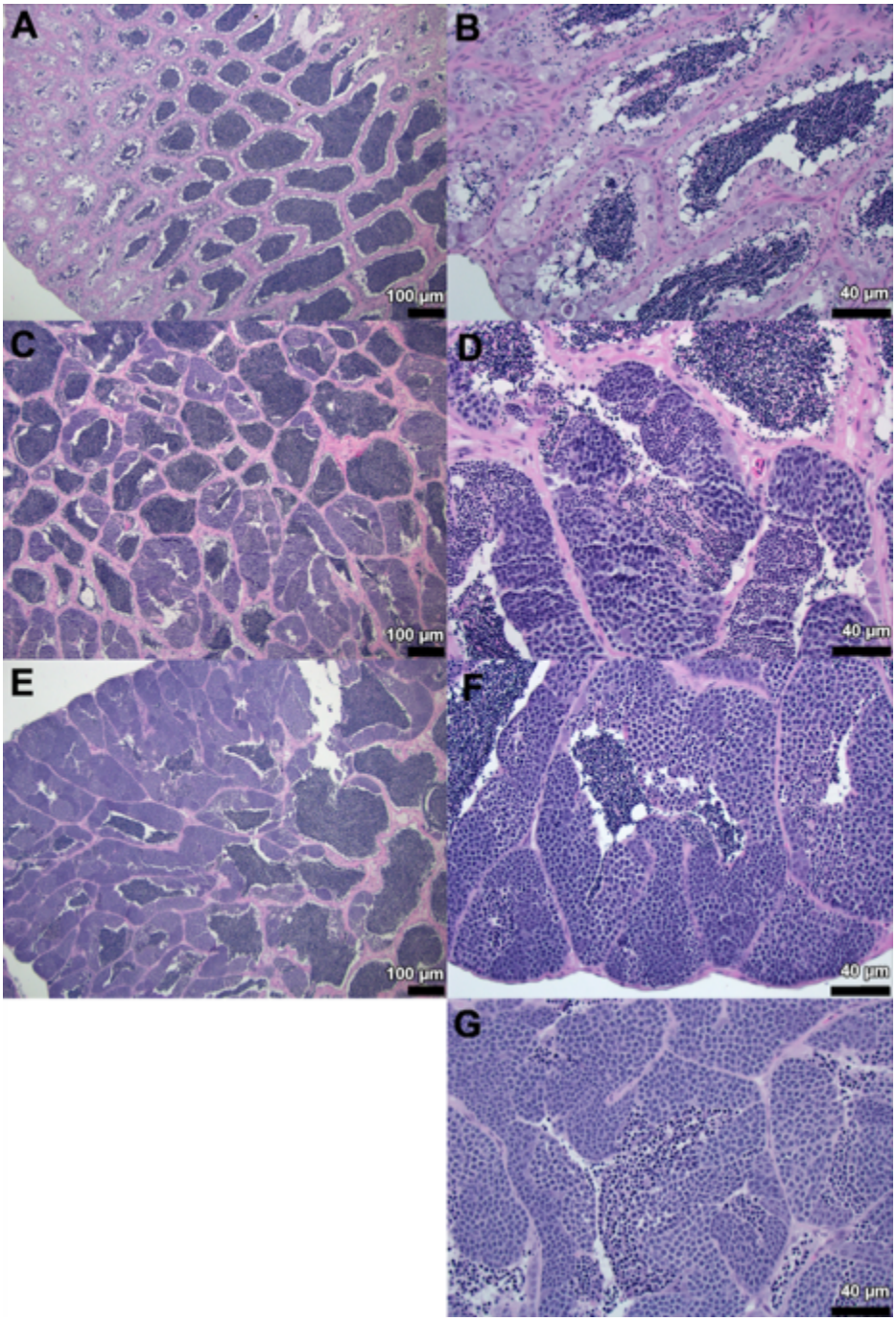

Figure D.3. Cross section of testes from orangethroat darter (A,B), rainbow darter (C,D) and F1 hybrid (E-G) males.

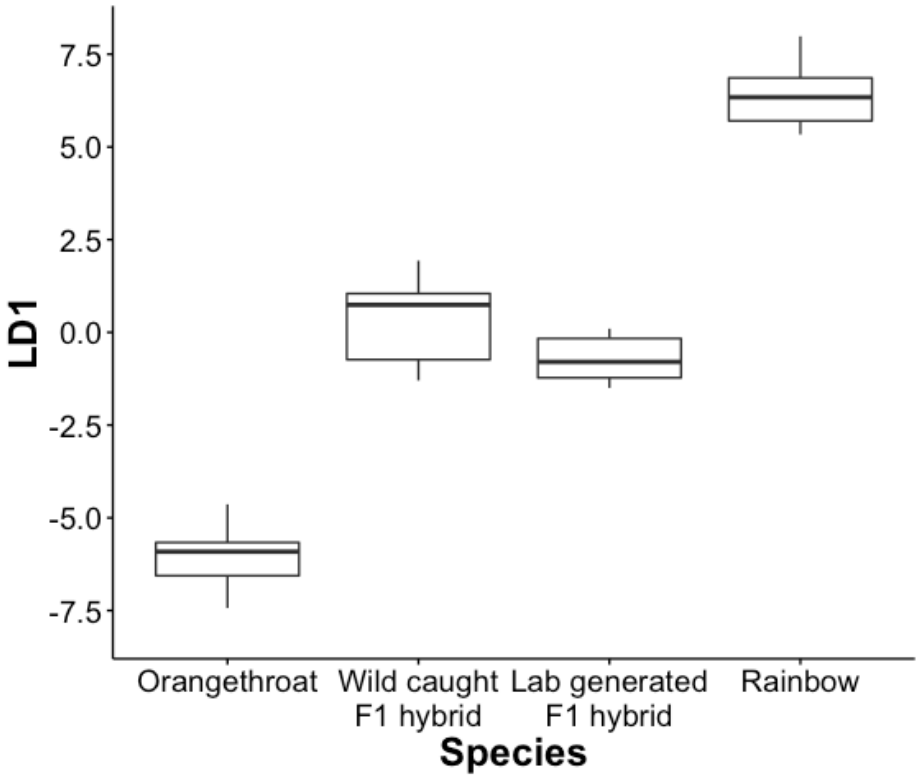

Figure D.4. Scores for LD 1 of the male color pattern LDA for orangethroat darters, wild-caught F1 hybrids, lab-generated F1 hybrids, and rainbow darters.

APPENDIX E

SUPPLEMENTARY MATERIALS FOR CHAPTER 7

TABLES

Table E.1. Statistics for the Meraculous genome assemblies using various kmer lengths produced with Illumina shotgun and mate-pair libraries.

kmer length	# scaffolds 1kb+	scaffold sequence total (Mb)	# contigs	% gaps in assembly	scaffold N50 (Mb)	contig N50 (kb)	# scaffolds > 50 kb
49	4623	717.5	368,313	11.6	2.4	6.2	805
59	4629	719.8	277,301	10.7	2.2	7.8	839
69	4988	716.4	220,375	10.1	2.5	9.3	781
79	5429	714.3	193,878	9.5	2.3	10.1	773

Table E.2. The number of Actinopterygii-specific Benchmarking Universal Single-Copy Orthologs (BUSCOs) present in each of the Meraculous genome assemblies produced using Illumina short-read sequencing.

kmer length	Complete BUSCOs	Complete single-copy BUSCOs	Complete and duplicated BUSCOs	Fragmented BUSCOs	Missing BUSCOs	Total BUSCO groups searched
49	4347 (94.8%)	4247 (92.6%)	100 (2.2%)	86 (1.9%)	151 (3.3%)	4584
59	4334 (94.5%)	4241 (92.5%)	93 (2.0%)	93 (1.9%)	157 (3.5%)	4584
69	4331 (94.4%)	4233 (92.3%)	98 (2.1%)	86 (1.9%)	167 (3.7%)	4584
79	4320 (94.2%)	4219 (92.0%)	101 (2.2%)	91 (2.0%)	173 (3.8%)	4584

Table E.3. Total amount of sequence data retained for each Illumina library after quality filtering.

Library	Read length	# Reads retained	# Total bps	Coverage
450 bp shotgun	250	388,991,066	97,247,766,500	97X
800 bp shotgun	150	63,200,545	10,112,087,200	10X
3-5 kb mate-pair	150	79,139,172	12,662,267,520	13X
5-7 kb mate-pair	150	76,273,856	12,203,816,960	12X
8-12 kb mate-pair	150	78,571,066	12,571,370,560	13X

Table E.4. The number of Actinopterygii-specific Benchmarking Universal Single-Copy Orthologs (BUSCOs) present in the transcriptome assembly.

Complete BUSCOs	Complete single-copy BUSCOs	Complete and duplicated BUSCOs	Fragmented BUSCOs	Missing BUSCOs	Total BUSCO groups searched
4370 (95.3%)	4370 (31.0%)	2949 (64.3%)	150 (3.3%)	64 (1.4%)	4584

Table E.5. Proportion of parental, recombinant, and F1 haplotypes observed at each chromosomes (Chr) in backcrosses to orangethroat darters and P values from binomial tests for deviations from the expected proportions (i.e., 0.25 parental, 0.50 recombinant, and 0.25 F1 for each chromosome). Significant P values (<0.05) are bolded. CI= Confidence interval, Prop = proportion. *Chromosomes involved in rearrangements.

	Parental				Recombinant				F1			
Chr	Prop	CI low	CI high	P value	Prop	CI low	CI high	P value	Prop	CI low	CI high	P value
1	0.75	0.58	0.88	**<0.0001**	0.19	0.08	0.36	**<0.001**	0.06	0.01	0.19	**<0.01**
2*	0.83	0.67	0.94	**<0.0001**	0.14	0.05	0.29	**<0.0001**	0.03	0.00	0.15	**<0.001**
3	0.42	0.26	0.59	**0.03**	0.58	0.41	0.74	0.41	0.00	0.00	0.10	**<0.001**
4*	0.75	0.58	0.88	**<0.0001**	0.25	0.12	0.42	**<0.01**	0.00	0.00	0.10	**<0.001**
5	0.83	0.67	0.94	**<0.0001**	0.14	0.05	0.29	**<0.0001**	0.03	0.00	0.15	**<0.001**
6*	0.61	0.43	0.77	**<0.0001**	0.33	0.19	0.51	0.07	0.06	0.01	0.19	**<0.01**
7*	0.64	0.46	0.79	**<0.0001**	0.31	0.16	0.48	**0.03**	0.06	0.01	0.19	**<0.01**
8	0.81	0.64	0.92	**<0.0001**	0.19	0.08	0.36	**<0.0001**	0.00	0.00	0.10	**<0.001**
9*	0.94	0.81	0.99	**<0.0001**	0.06	0.01	0.19	**<0.0001**	0.00	0.00	0.10	**<0.001**
10	0.75	0.58	0.88	**<0.0001**	0.25	0.12	0.42	**<0.01**	0.00	0.00	0.10	**<0.001**
11	0.72	0.55	0.86	**<0.0001**	0.17	0.06	0.33	**<0.001**	0.11	0.03	0.26	0.06
12	0.81	0.64	0.92	**<0.0001**	0.17	0.06	0.33	**<0.001**	0.03	0.00	0.15	**<0.001**
13	0.81	0.64	0.92	**<0.0001**	0.19	0.08	0.36	**<0.0001**	0.00	0.00	0.10	**<0.001**
14	0.67	0.49	0.81	**<0.0001**	0.33	0.19	0.51	0.07	0.00	0.00	0.10	**<0.001**
15	0.42	0.26	0.59	**0.03**	0.47	0.30	0.65	0.87	0.11	0.03	0.26	0.06
16*	0.69	0.52	0.84	**<0.0001**	0.28	0.14	0.45	**0.011**	0.03	0.00	0.15	**<0.001**
17	0.92	0.78	0.98	**<0.0001**	0.06	0.01	0.19	**<0.0001**	0.03	0.00	0.15	**<0.001**
18	0.75	0.58	0.88	**<0.0001**	0.19	0.08	0.36	**<0.001**	0.06	0.01	0.19	**<0.01**
19*	0.83	0.67	0.94	**<0.0001**	0.17	0.06	0.33	**<0.001**	0.00	0.00	0.10	**<0.001**
20*	0.81	0.64	0.92	**<0.0001**	0.19	0.08	0.36	**<0.001**	0.00	0.00	0.10	**<0.001**
21	0.64	0.46	0.79	**<0.0001**	0.36	0.21	0.54	0.13	0.00	0.00	0.10	**<0.001**

Table E.5. Continued Proportion of parental, recombinant, and F1 haplotypes observed at each chromosomes (Chr) in backcrosses to orangethroat darters and P values from binomial tests for deviations from the expected proportions (i.e., 0.25 parental, 0.50 recombinant, and 0.25 F1 for each chromosome). Significant P values (<0.05) are bolded. CI= Confidence interval, Prop = proportion. *Chromosomes involved in rearrangements.

22	0.86	0.71	0.95	**<0.0001**	0.11	0.03	0.26	**<0.0001**	0.03	0.00	0.15	**<0.001**
23	0.86	0.71	0.95	**<0.0001**	0.11	0.03	0.26	**<0.0001**	0.03	0.00	0.15	**<0.001**
24*	0.83	0.67	0.94	**<0.0001**	0.14	0.05	0.29	**<0.0001**	0.03	0.00	0.15	**<0.001**

Table E.6. Proportion of parental, recombinant, and F1 haplotypes observed at each chromosomes (Chr) in backcrosses to rainbow darters and P values from binomial tests for deviations from the expected proportions (i.e., 0.25 parental, 0.50 recombinant, and 0.25 F1 for each chromosome). Significant P values (<0.05) are bolded. CI= Confidence interval, Prop = proportion. *Chromosomes involved in rearrangements.

	Parental				Recombinant				F1			
Chr	Prop	CI low	CI high	P value	Prop	CI low	CI high	P value	Prop	CI low	CI high	P value
1	0.69	0.39	0.91	**<0.01**	0.23	0.05	0.54	0.09	0.08	0.00	0.36	0.21
2*	0.77	0.46	0.95	**<0.0001**	0.23	0.05	0.54	0.09	0.00	0.00	0.25	**0.048**
3	0.38	0.14	0.68	0.33	0.62	0.32	0.86	0.58	0.00	0.00	0.25	**0.048**
4*	0.38	0.14	0.68	0.33	0.38	0.14	0.68	0.58	0.23	0.05	0.54	1.00
5	0.46	0.19	0.75	0.10	0.31	0.09	0.61	0.27	0.23	0.05	0.54	1.00
6*	0.54	0.25	0.81	**0.02**	0.23	0.05	0.54	0.09	0.23	0.05	0.54	1.00
7*	0.38	0.14	0.68	0.33	0.38	0.14	0.68	0.58	0.23	0.05	0.54	1.00
8	0.46	0.19	0.75	0.10	0.46	0.19	0.75	1.00	0.08	0.00	0.36	0.21
9*	0.69	0.39	0.91	**<0.01**	0.31	0.09	0.61	0.27	0.00	0.00	0.25	**0.048**
10	0.31	0.09	0.61	0.75	0.38	0.14	0.68	0.58	0.31	0.09	0.61	0.75
11	0.69	0.39	0.91	**<0.01**	0.00	0.00	0.25	**<0.001**	0.31	0.09	0.61	0.75
12	0.46	0.19	0.75	0.10	0.46	0.19	0.75	1.00	0.08	0.00	0.36	0.21
13	0.38	0.14	0.68	0.33	0.46	0.19	0.75	1.00	0.15	0.02	0.45	0.54
14	0.31	0.09	0.61	0.75	0.69	0.39	0.91	0.27	0.00	0.00	0.25	**0.048**
15	0.23	0.05	0.54	1.00	0.77	0.46	0.95	0.09	0.00	0.00	0.25	**0.048**
16*	0.38	0.14	0.68	0.33	0.62	0.32	0.86	0.58	0.00	0.00	0.25	**0.048**
17	0.54	0.25	0.81	**0.02**	0.15	0.02	0.45	**0.02**	0.31	0.09	0.61	0.75
18	0.54	0.25	0.81	**0.02**	0.15	0.02	0.45	**0.02**	0.31	0.09	0.61	0.75
19*	0.38	0.14	0.68	0.33	0.31	0.09	0.61	0.27	0.31	0.09	0.61	0.75
20*	0.46	0.19	0.75	0.10	0.46	0.19	0.75	1.00	0.08	0.00	0.36	0.21
21	0.54	0.25	0.81	**0.02**	0.38	0.14	0.68	0.58	0.08	0.00	0.36	0.21

Table E.6. Continued Proportion of parental, recombinant, and F1 haplotypes observed at each chromosomes (Chr) in backcrosses to rainbow darters and P values from binomial tests for deviations from the expected proportions (i.e., 0.25 parental, 0.50 recombinant, and 0.25 F1 for each chromosome). Significant P values (<0.05) are bolded. CI= Confidence interval, Prop = proportion. *Chromosomes involved in rearrangements.

22	0.62	0.32	0.86	**<0.01**	0.23	0.05	0.54	0.09	0.15	0.02	0.45	0.54
23	0.69	0.39	0.91	**<0.01**	0.15	0.02	0.45	**0.02**	0.15	0.02	0.45	0.54
24*	0.31	0.09	0.61	0.75	0.46	0.19	0.75	1.00	0.23	0.05	0.54	1.00

FIGURES

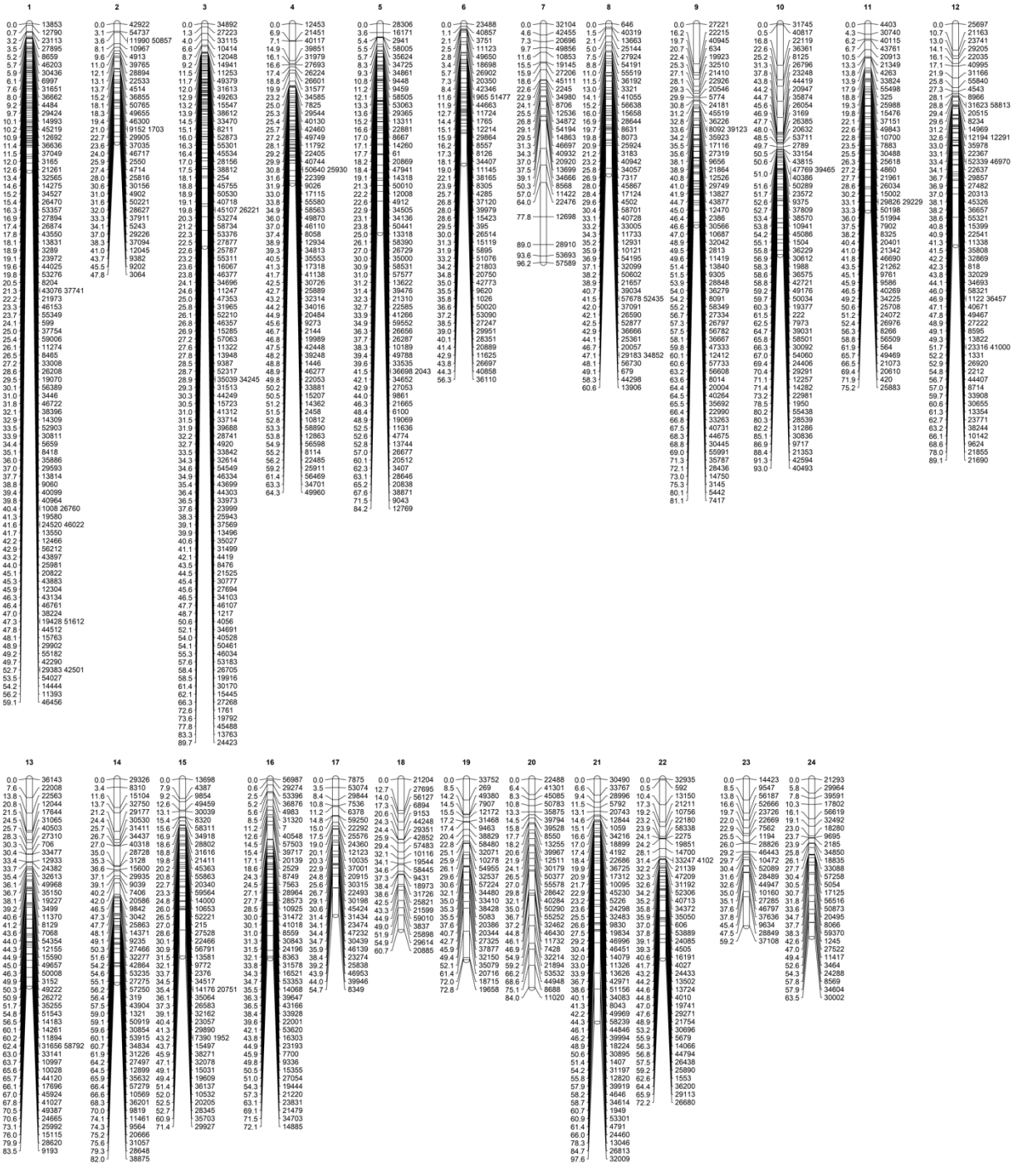

Figure E.1. Positions in cm for the 1,112 markers in the orangethroat darter consensus map.

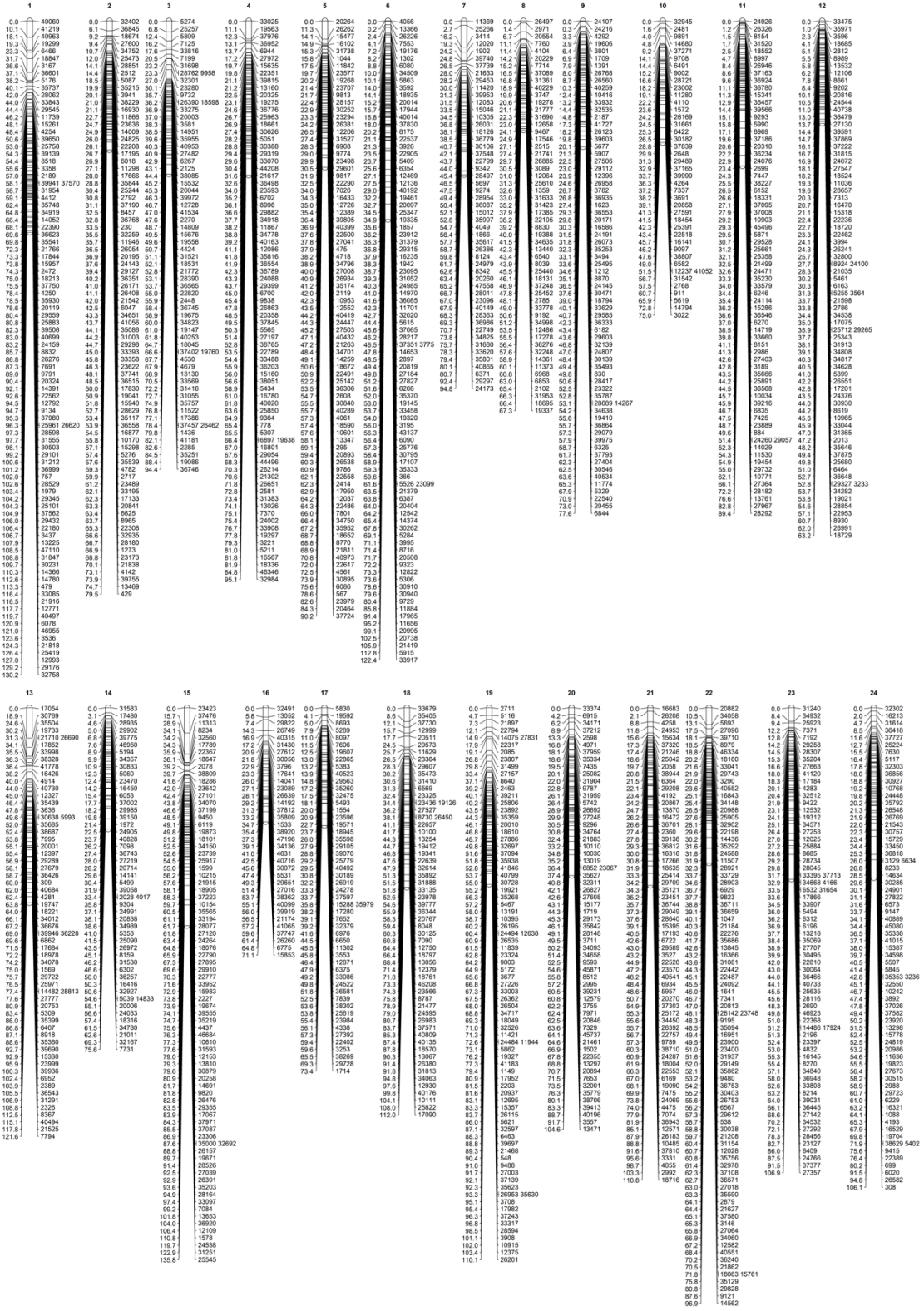

Figure E.2. Positions in cm for the 1,622 markers in the rainbow darter consensus map.

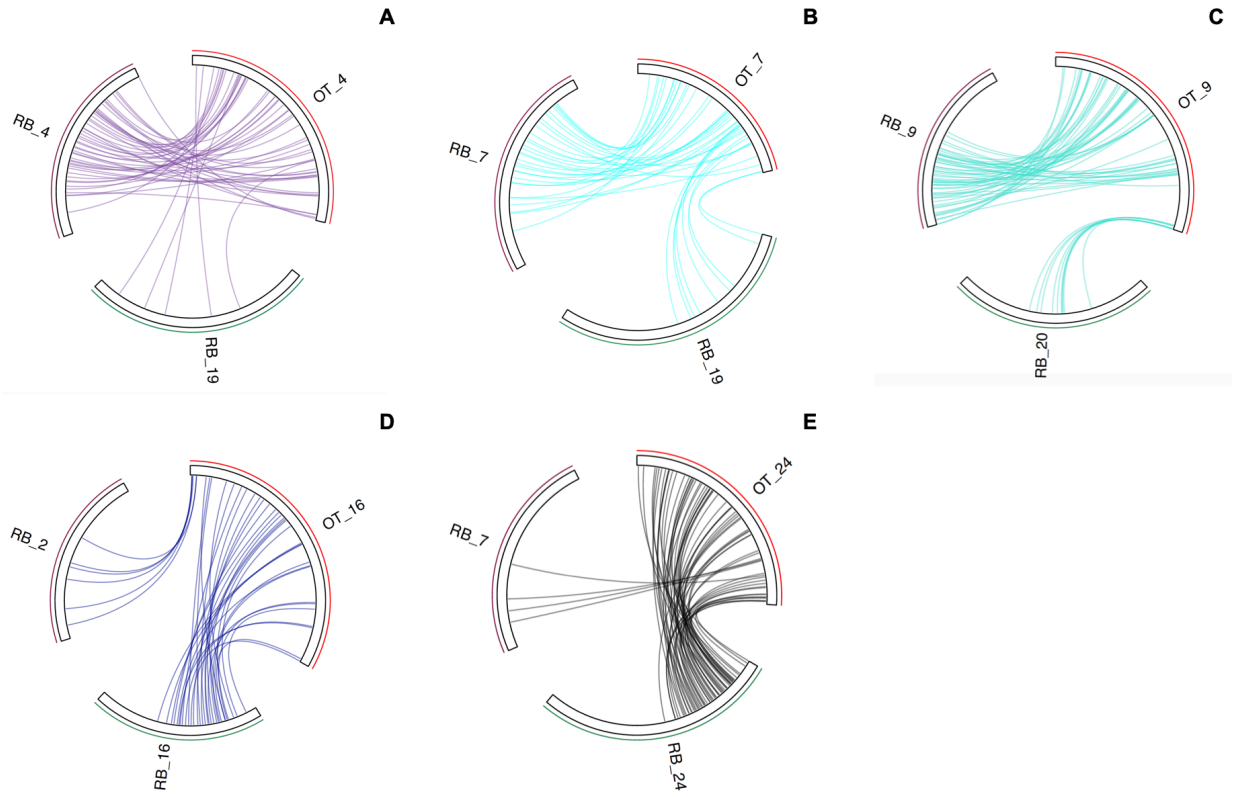

Figure E.3. Alignment of rainbow (RB) darter linkage map markers (i.e. 100 bp RAD tags) to the orangethroat (OT) darter chromosomes. Mapping of five putative translocations are shown. (A) Orangethroat chromosome 4 maps to rainbow linkage groups 4 and 19. (B) Orangethroat chromosome 7 maps to rainbow linkage groups 7 and 19. (C) Orangethroat chromosome 9 maps to rainbow linkage groups 9 and 20. (D) Orangethroat chromosome 16 maps to rainbow linkage groups 16 and 2. (E) Orangethroat chromosome 24 maps to rainbow darter linkage groups 24 and 7.

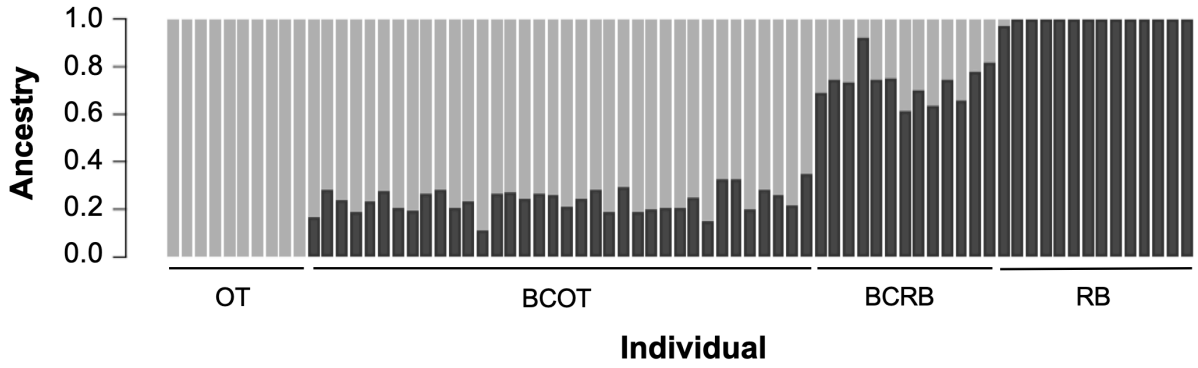

Figure E.4. Individual ancestry proportions for orangethroat darters (OT), backcrosses to orangethroat darters (BCOT), backcrosses to rainbow darters (BCRB), and rainbow darters (RB). Ancestry proportions were obtained from ADMIXTURE by specifying two ancestral populations. Light gray corresponds to orangethroat darter ancestry and dark gray corresponds to rainbow darter ancestry.

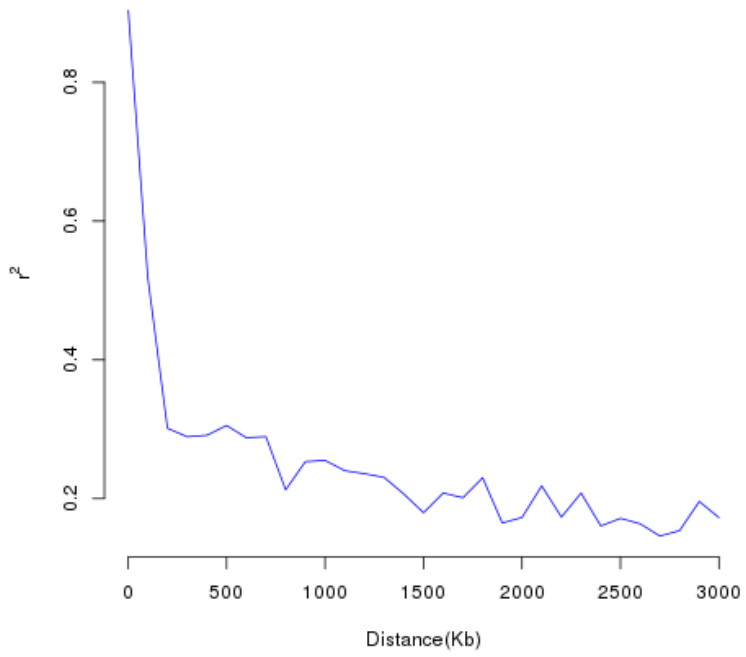

Figure E.5. Linkage disequilibrium decay (r^2) calculated between pairs of SNPs across the orangethroat darter genome.

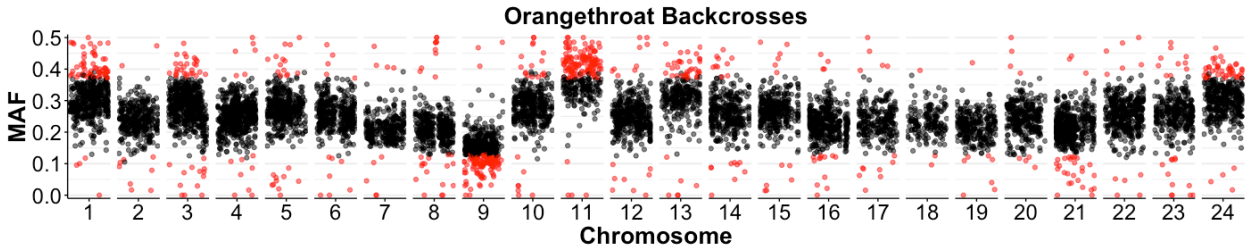

Figure E.6. Minor allele frequency (MAF) for set of 8,177 SNPs in backcrosses to orangethroat darters. SNPs that deviate significantly from the expected MAF of 0.25 are shown in red ($p<0.05$).

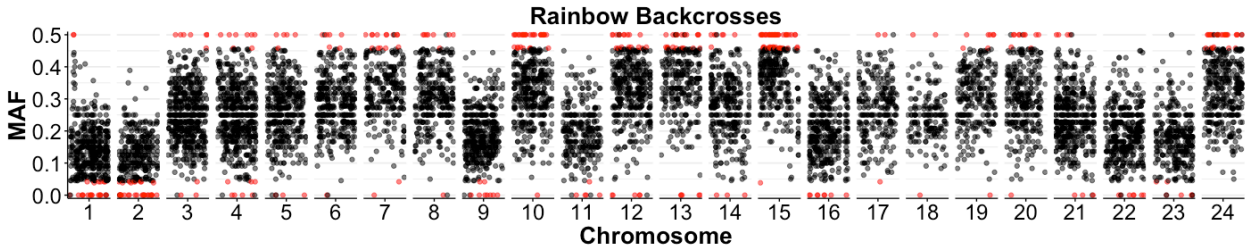

Figure E.7. Minor allele frequency (MAF) for set of 8,177 SNPs in backcrosses to rainbow darters. SNPs that deviate significantly from the expected MAF of 0.25 are shown in red ($p<0.05$).

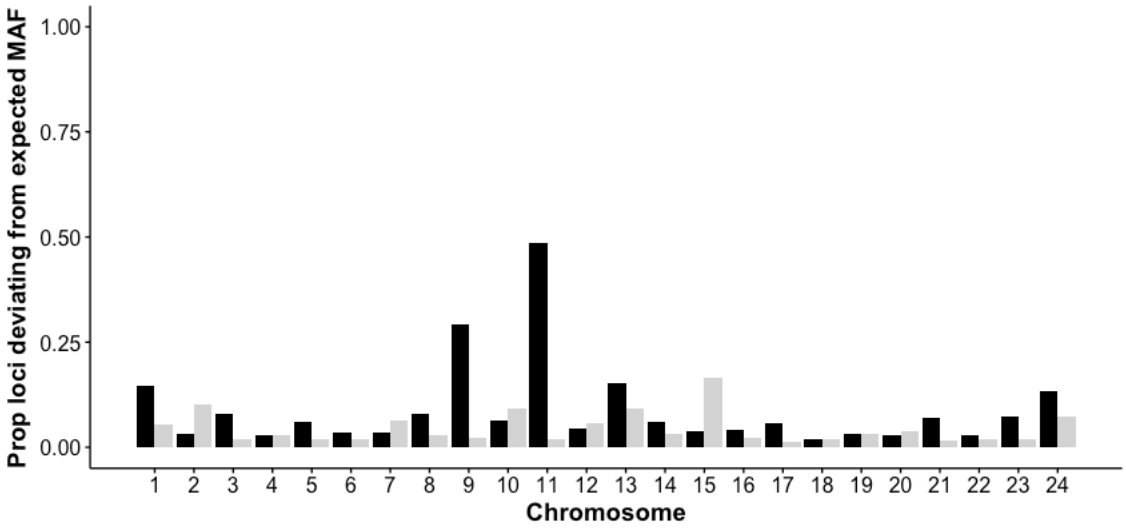

Figure E.8. The proportion of loci deviating from the expected MAF of 0.25 in each of the 24 chromosomes in backcrosses to orangethroat darters (black) and backcrosses to rainbow darters (gray).

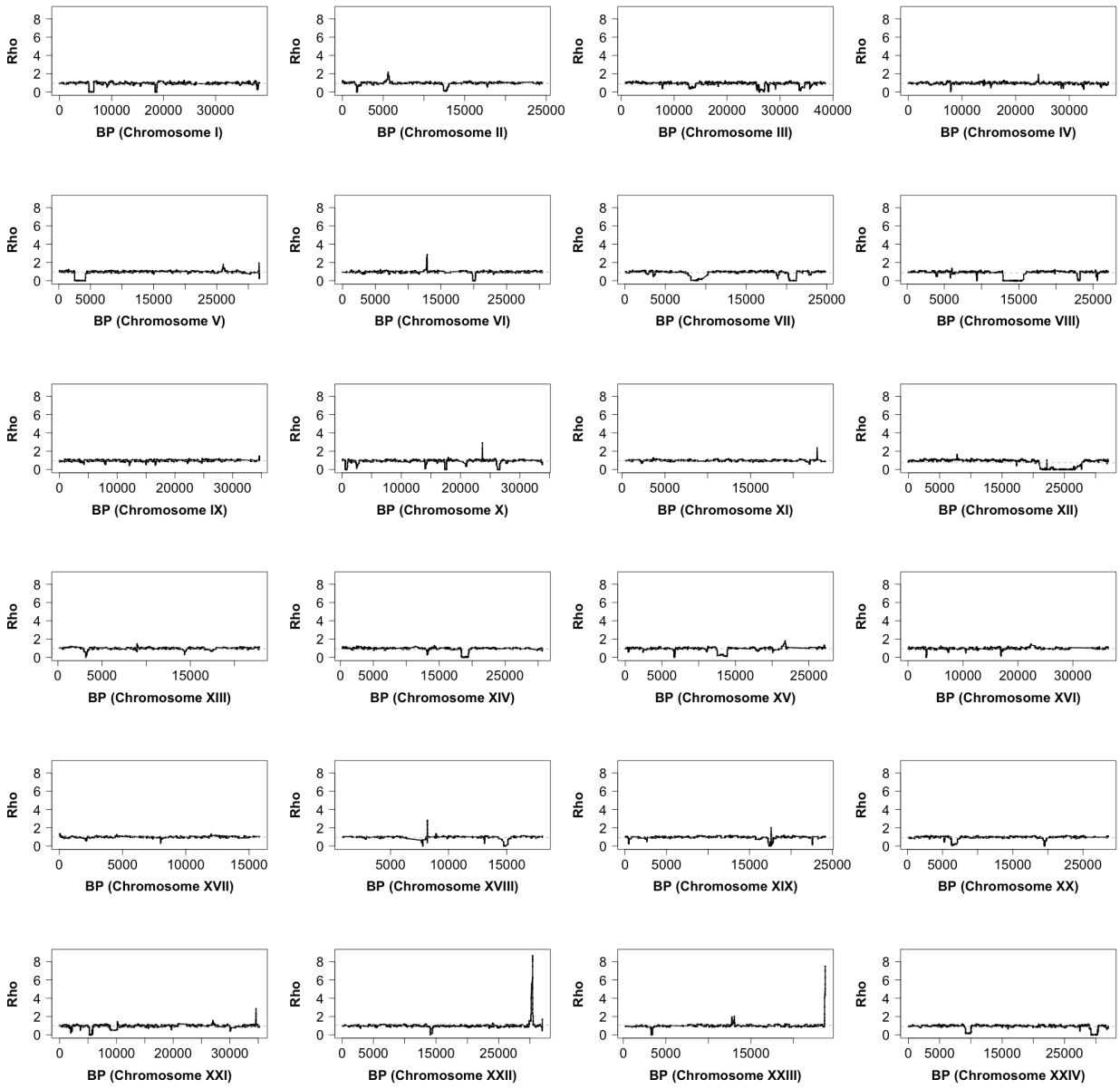

Figure E.9. Genome-wide population level estimates of the recombination rate (rho) for orangethroat darters. Dashed gray lines represented the mean recombination rate for a given chromosome.

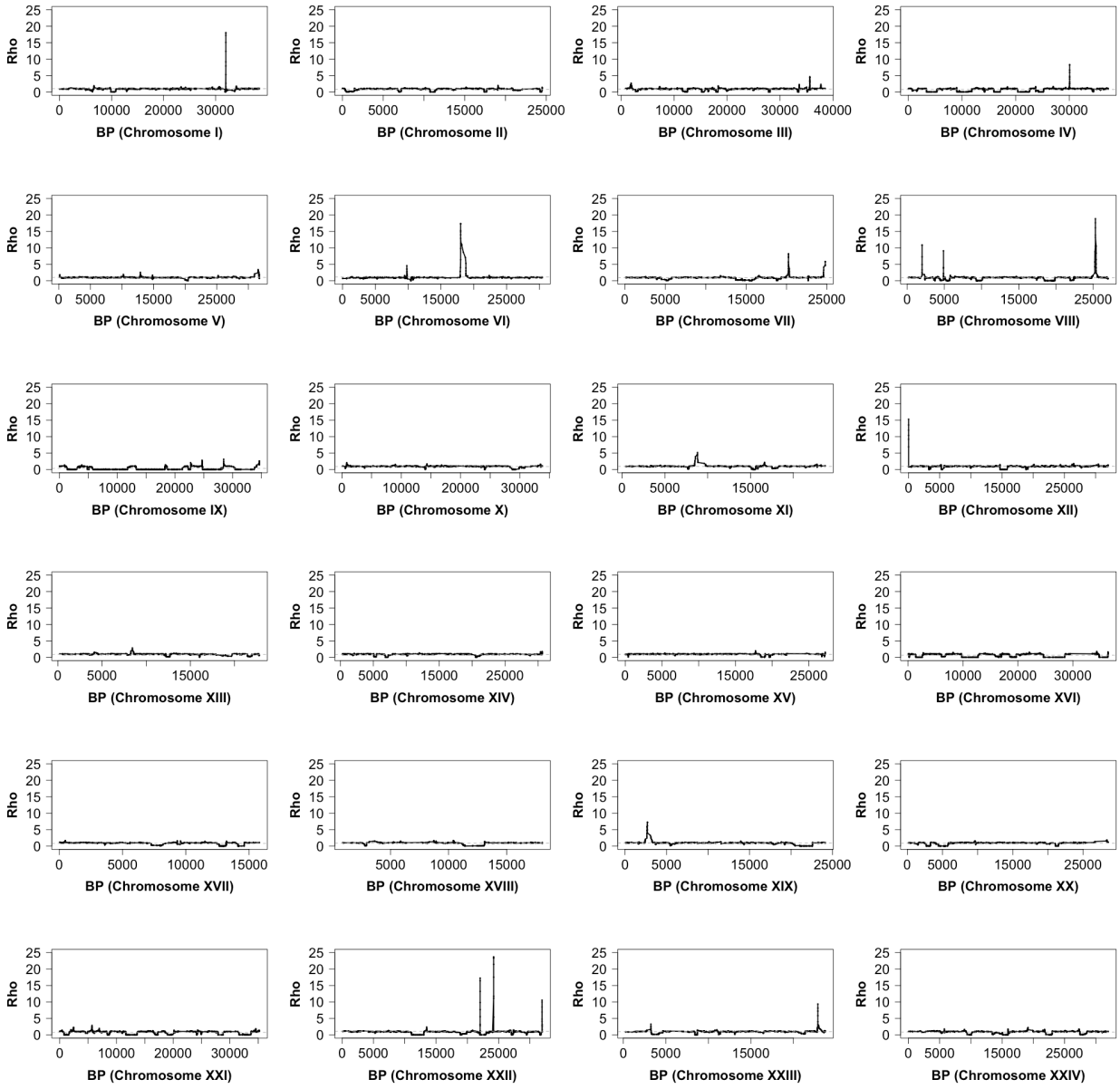

Figure E.10. Genome-wide population level estimates of the recombination rate (rho) for rainbow darters. Dashed gray lines represented the mean recombination rate for a given chromosome.

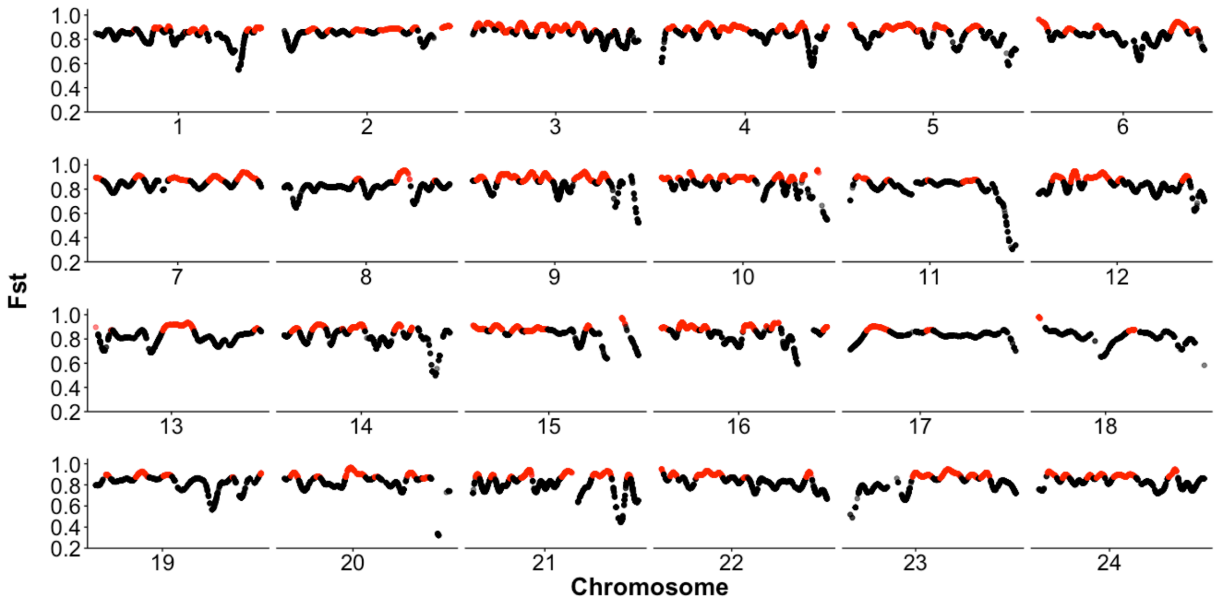

Figure E.11. Smoothed Fst for 39,518 SNPs across the 24 chromosomes. Fst was calculated in non-overlapping 500 kb windows and p-values were calculated using 10,000 rounds of bootstrap resampling (red = $p<0.05$).

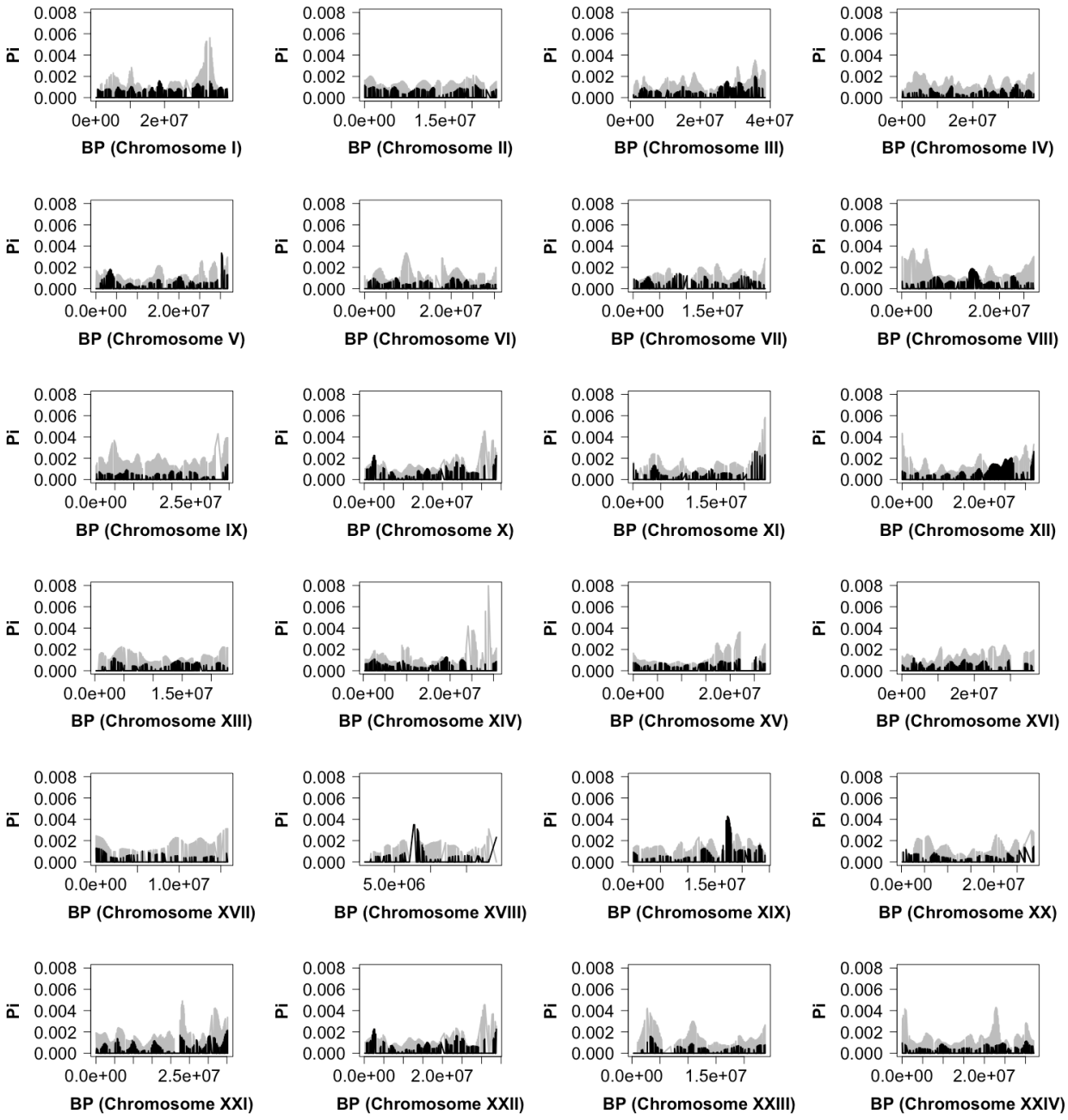

Figure E.12. Smoothed nucleotide diversity, π (Pi), within orangethroat darters (black) and rainbow darters (gray) for 44,688 sites across the 24 chromosomes.