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Agonistic character displacement of genetically based male colour patterns across darters

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Agonistic character displacement (ACD) occurs when selection to avoid maladaptive interspecific aggression leads to the evolution of agonistic signals and/or associated behavioural biases in sympatry. Here, we test for a pattern consistent with ACD in male colour pattern in darters (Percidae: *Etheostoma*). Male colour 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 behavioural biases. We use a common garden approach to show that differences in male colour pattern among four closely related darter species are genetically based. Additionally, we demonstrate that some aspects of male colour pattern exhibit enhanced differences in sympatric compared to allopatric populations of two darter species, consistent with ACD. However, other male colour traits are more similar between species in sympatry compared with allopatry, indicating that not all signal components are under strong divergent selection in sympatry. This study provides evidence that interspecific male–male 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.

1. 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 [1–3]. 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) [1,4]. 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 [6–11]. 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) [12–16]. Similar to RCD, ACD occurs when selection to avoid interspecific fighting results in the evolution of competitive traits (signals and/or aggression biases) [17,18]. 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 [19,20]. 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 behavioural 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 [21,22], darters (Percidae: *Etheostoma*) represent the only documented example of cascade ACD [16].

This study tests for divergent ACD in a male colour 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: (i) that the signal functions in competitive interactions (rather than male–female mating interactions); (ii) that the signal is genetically based and not due to environmental differences between sympatry and allopatry; and (iii) that a geographical pattern of enhanced signal divergence between species in sympatry compared to allopatry is present [5,17,18]. Several recent studies have shown that male colour pattern functions in male–male competition in darters. Within species, aspects of male colour pattern predict a male's ability to guard a female from rival males and consequently correlate with reproductive success [23]. Male colour pattern also functions in male discrimination between conspecific versus heterospecific male competitors [24–26]. 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 §2a below). Here, we use a common garden approach to ask whether differences in male colour pattern present among four closely related species of darters are genetically based. We then compare multivariate measurements of male colour pattern in sympatric and allopatric population pairs in two darter species to test whether colour pattern divergence is enhanced in sympatry compared to allopatry. This study provides important insight into the evolution of an elaborate sexually dimorphic colour 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 colour pattern divergence between and within species and has implications for RCD, ACD, cascade RCD and cascade ACD in darters.

2. Methods

(a) 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 colour pattern among populations in different drainages [27,28]. 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 F_1 hybrid sex ratios and high levels of backcross hybrid inviability [29]. 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 [26]. Such preferences are absent in orangethroat and rainbow darters when they occur in allopatry with respect to one another [16]. 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 [16]. This suggests that RCD and ACD between orangethroat and rainbow darters have incidentally led to trait evolution and behavioural isolation among lineages within the orangethroat clade (i.e. cascade RCD and cascade ACD).

(b) Common garden study

Our goal here was to test whether colour 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 colour 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 1a) [26]. 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 electronic supplementary material, table S1). 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 3–5 cm of naturally coloured 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 one 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 1 year of age, fish were transferred to 37.9 l tanks, and at 2 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 3 years of age, the laboratory-raised offspring from each of the four species had reached adult size and males had developed an adult breeding coloration. At this time, males from each family were photographed with a Nikon Coolpix D3300 digital camera (mean ± s.e. males per family = 5.5 ± 0.6). Photographs were taken under fluorescent lighting with the camera's factory setting for fluorescent light. Prior to
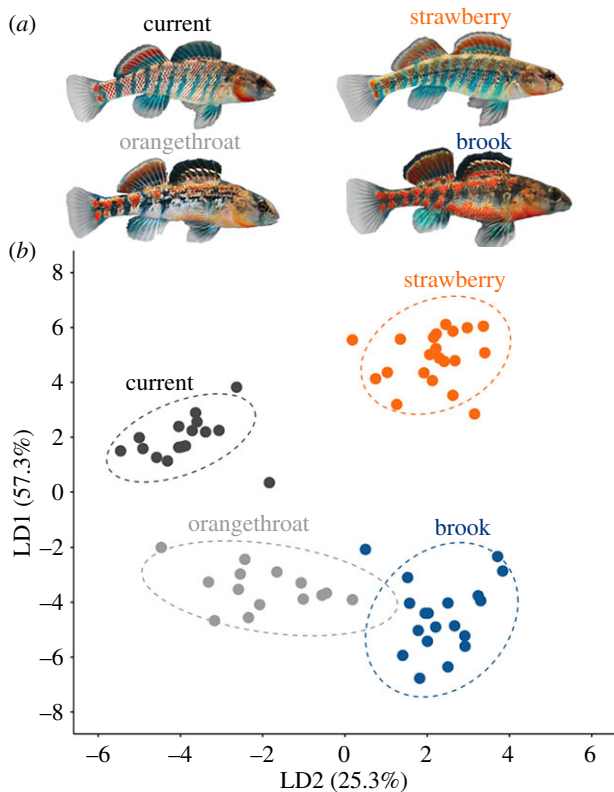
Figure 1. (*a*) Representative example of male colour pattern in strawberry, current, brook and orangethroat darters. (*b*) Biplot of the first two LDs obtained from the LDA on male colour pattern in fish from the common garden study. Ellipses represent 95% CI.

photographing, fish were lightly anaesthetized using 0.03 g l^{-1} 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 colour 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 millimetre in IMAGEJ (v. 1.50c4) [30].

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 1a). To quantify any differences in male colour pattern that were present at the species level, we focused our analyses on aspects of male colour pattern that have been shown previously to contribute to variation among these species [26]. 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 the male colour pattern [24]. Hence, our measurements of blue and red coloration are biologically meaningful and capture the components of male colour pattern that these fish are able to perceive.

The dropper tool in Adobe Photoshop was used to measure RGB values, which vary between 0 and 255 for each of the three colour 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 portions of the colour 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 3 × 3 pixel area within a given colour 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 by Moran *et al.* [26,29]. Briefly, the perimeters 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 Colour Plugin with the colour 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 colour proportion variables per fish. The same colour pattern measurements described here were also obtained from a previous study that examined colour pattern variation among wild-caught fish from each of the four orangethroat species used in the common garden study [26]. This allowed us to determine whether the components of male colour pattern measured in the common garden fish are similar to those present in nature.

All statistical analyses were performed in R (v. 3.4.4). We first conducted a two-factor nested MANOVA to examine whether colour pattern differed significantly among species and among families (i.e. replicate crosses within a species). Each of the 12 colour 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 millimetres) 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 colour pattern differences among individuals. We therefore excluded size from subsequent analyses. We next used linear discriminant analysis (LDA) to reduce the dimensionality of the colour dataset and to identify which colour variables contribute most to differences among species. We used the lda function of the MASS package [31]. The 12 colour pattern measurements served as dependent variables, and species served as the categorical predictor variable. We then used the Anova function of the car package [32] to conduct nested two-factor ANOVAs with species and family (nested within species) as factors and individual linear discriminant (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 [33]. Finally, we conducted LDA using colour measurements from the common garden study males in addition to previously published colour measurement data obtained from 10 wild-caught males from each of these four species [26]. We conducted ANOVA on the first two LDs, with species and rearing environment (i.e. laboratory-raised or wild-caught, nested within species) as factors and individual LD scores as the dependent variable.

(c) Agonistic character displacement study

Here our goal was to quantify male colour 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 colour pattern differ quantitatively between sympatric orangethroat and rainbow darters [26,29], and that colour pattern is variable across populations within species [34]. Divergent ACD in male colour pattern predicts: (i) enhanced differentiation between species in
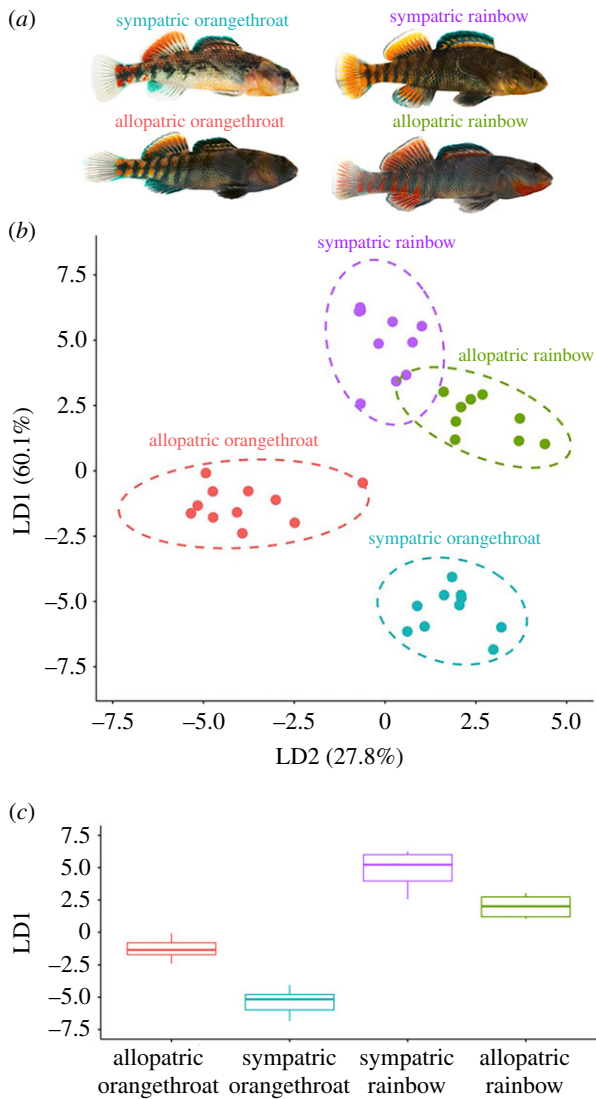
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Figure 2. (*a*) Representative example of male colour pattern in sympatric orangethroat, sympatric rainbow, allopatric orangethroat and allopatric rainbow darters. (*b*) Biplot of the first two LDs obtained from the LDA on male colour pattern in fish from the ACD study. Ellipses represent 95% CI. (*c*) Boxplots of LD2 scores from the LDA on male colour pattern in fish from the ACD study.

sympatry compared to allopatry and (ii) 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 2*a*; electronic supplementary material, table S2). 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: $F_{3,36} = 1.88$, $p = 0.15$).

Both orangethroat and rainbow darters are characterized by a male nuptial colour pattern that consists of red and blue banding on the lateral sides and dorsal fins. Despite their superficial similarities, their colour patterns differ in a few consistent ways (figure 2*a*). 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 colour 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 colour pattern among groups. Each of the 16 colour 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 colour 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 scores 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.

3. Results

(a) Common garden study

The MANOVA on colour pattern measurements in the four laboratory-raised orangethroat clade species revealed that species identity and family (i.e. replicate cross within a species) both significantly contributed to differences in male colour pattern (table 1). There was no effect of size (standard length in mm) on differences in colour pattern among individuals (table 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 (electronic supplementary material, table S3). 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 colour pattern measurements in laboratory-raised fish reduced the dimensionality of the data into three LDs, with the first two LDs explaining 86.6% of the cumulative variation among groups (LD1: 57.3%, LD2: 25.3%, LD3: 17.4%). Figure 1*b* shows a biplot comparing the scores for LD1 versus LD2 for each individual, grouped by species. The colour pattern proportion measurements had higher loadings (i.e. associations) with all three LDs compared with the RGB data, suggesting that differences in the proportion of red and blue coloration on the body and fins are 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

Table 1. Results of two-factor nested MANOVA on male colour pattern in fish from the common garden study. Species and family (nested within species) were included as factors and size (standard length in millimetres) was included as a covariate.

variable	d.f.	Pillai	approx F	num d.f.	den d.f.	p-value
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 2. Results of two-factor nested MANOVA on male colour pattern in fish from ACD study. Species and geography (nested within species) were included as factors.

variable	d.f.	Pillai	approx F	num d.f.	den d.f.	p-value
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

species overlapped in scores for both LD1 and LD2) (electronic supplementary material, tables S4).

The LDA that included colour pattern measurements from both laboratory-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. laboratory-raised versus wild-caught) on male colour pattern and no interaction between species and rearing environment (electronic supplementary material, table S5).

(b) Agonistic character displacement study

Our MANOVA on variation in male colour 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; table 2). ANOVAs indicated that the values for nearly every variable differed significantly between sympatric and allopatric populations within species due to geography or due to an interaction between geography and species (electronic supplementary material, table S6). The red 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% of cumulative variation among groups (LD1: 60.1%; LD2: 27.8%; LD3: 12.1%). Figure 2b shows a biplot comparing the scores for LD1 versus LD2 for each individual. The colour 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 are a good predictor of species and geographical 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 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 with allopatric males of both species (figure 2b; electronic supplementary material, figure S1). LD2 was most closely associated with the proportion of blue coloration on the first and second dorsal fins. This suggests 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 (electronic supplementary material, table S7A). Only one pairing did not differ significantly from one another in scores for LD2: allopatric rainbow darters and sympatric orangethroat darters (electronic supplementary material, table S7B).

4. Discussion

A growing body of the literature suggests that interspecific reproductive and aggressive interactions play a surprisingly large role in speciation [17,18]. Interspecific interactions can have broad implications for speciation by directly promoting enhanced behavioural isolation in sympatry and indirectly promoting the evolution of trait divergence and behavioural isolation among allopatric lineages [21,22]. In this study, we demonstrated that colour 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 [23], and that male aggressive response towards heterospecific males increases with increasing colour pattern similarity between species [26]. Conversely, studies have consistently failed to detect female preferences associated with variable aspects of male colour pattern within or between species [16,24,26,35,36]. Here, we demonstrated that some male colour 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-analysed data from a previously published survey of colour pattern variation within and between populations in the orangethroat darter (E. spectabile) across six drainages [34]. At least within this species, the pattern of colour divergence between populations that are sympatric versus allopatric with rainbow darters appears to be robust.

Male colour 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 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 colour pattern between orangethroat and rainbow darters (figure 2a). Thus, these colour 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 [24]. This supports the hypothesis that red coloration is important in male recognition of conspecific rivals.

We also found that some aspects of male colour pattern (i.e. those associated with LD2 in the ACD study: proportion of blue coloration on the first and second dorsal fin; figure 2b; electronic supplementary material, figure S1) 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 with allopatry in some colour 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 colour proportion traits on the dorsal fins. Clearly, there are

some types of colour traits that are plastic. Red coloration in darters is carotenoid based [34], which suggests it may be linked to diet [37,38]. In rainbow darters, spectral properties of red coloration are associated with parasite load [39]. 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 2015, personal observation). However, these phenomena should affect the red and blue hues and their associated RGB values. The present study has demonstrated that variation in RGB values accounts 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 predicts both species identity and geographical relationship between species. The results of our common garden study in combination with another recent study examining male colour pattern in orangethroat darters, rainbow darters and their hybrids [29] provide strong evidence that variation in these colour elements present among populations and species is largely genetic in nature.

The other two phenomena that can potentially account for the convergence in some colour 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. [26,29] and Bossu & Near [40] have shown that F_1 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 F_1 hybrid males [29], suggesting that introgression can cause increased trait similarity between species. Second, although large-scale transitions between genera and subgenera are associated with ecological divergence in darters [41,42], there is strong evidence that differences in male colour pattern among more closely related species are primarily driven by intrasexual selection rather than ecological differences [24,26,43,44].

Importantly, the findings of this study drastically change how we think about the evolution of male colour pattern and speciation in darters. Sexual selection in the form of female mating preference for male colour traits was long thought to be the primary catalyst of speciation in these fish. Here, we demonstrated that divergence in male colour 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 colour pattern difference and pairwise genetic distance in multiple comparisons between orangethroat and rainbow darters [26]. Additionally, within the orangethroat clade, divergence time estimates overlap for species that are sympatric versus allopatric with respect to rainbow darters [45]. Together these results suggest that the geographical relationship between orangethroat and rainbow darters (i.e. whether they are sympatric or allopatric) has a greater impact on colour pattern divergence than the amount of evolutionary divergence between species.

The pattern of ACD in male colour pattern presented here also reflects previously documented behavioural patterns of ACD between orangethroat and rainbow darters and cascade ACD among species in the orangethroat clade. Divergence in

male colour traits between closely related species within the orangethroat clade that occurs in sympatry with rainbow darters (and thus undergo ACD) has resulted in enhanced male competitor bias between species [16,26]. This is consistent with cascade ACD in both male agonistic signals and behavioural response to those signals (*sensu* 'convergent sympatry effects' of character displacement) [20]. It remains to be tested whether the divergence in male colour pattern traits that we observed between populations within the orangethroat darter (and/or within the rainbow darter) also confers behavioural biases among populations within species (which would indicate 'sympatry–allopatry effects' of character displacement) [20].

Lastly, our findings have implications for the evolution of behavioural 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 behavioural isolation between species [29]. 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. The evidence is now growing that female mating preferences are absent or lower compared with 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.

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