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Interspecific aggression, not interspecific mating, drives character displacement in the wing coloration of male rubyspot damselflies (*Hetaerina*)

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Traits that mediate intraspecific social interactions may overlap in closely related sympatric species, resulting in costly between-species interactions. Such interactions have principally interested investigators studying the evolution of reproductive isolation via reproductive character displacement (RCD) or reinforcement, yet in addition to reproductive interference, interspecific trait overlap can lead to costly between-species aggression. Previous research on rubyspot damselflies (*Hetaerina* spp.) demonstrated that sympatric shifts in male wing colour patterns and competitor recognition reduce interspecific aggression, supporting the hypothesis that agonistic character displacement (ACD) drove trait shifts. However, a recent theoretical model shows that RCD overshadows ACD if the same male trait is used for both female mate recognition and male competitor recognition. To determine whether female mate recognition is based on male wing coloration in *Hetaerina*, we conducted a phenotype manipulation experiment. Compared to control males, male *H. americana* with wings manipulated to resemble a sympatric congener (*H. titia*) suffered no reduction in mating success. Thus, female mate recognition is not based on species differences in male wing coloration. Experimental males did, however, experience higher interspecific fighting rates and reduced survival compared to controls. These results greatly strengthen the case for ACD and highlight the mechanistic distinction between ACD and RCD.

1. Introduction

When closely related species come into secondary contact, they may overlap in traits used as intraspecific social signals, resulting in costly interactions between species. Evolutionary biologists have focused primarily on the ways in which selection acts to reduce the occurrence of costly reproductive interactions between heterospecific males and females in the context of reproductive character displacement (RCD) and reinforcement [1–7]. Interference competition between species, which in animals usually takes the form of aggressive interactions, is also very common [8], yet agonistic character displacement (ACD), a process whereby natural selection acts on traits that mediate the occurrence or outcome of interspecific aggression, remains relatively understudied [9,10]. While RCD and ACD can result in the same geographical patterns, the processes are conceptually distinct, because interspecific interference competition need not be related to competition for mates [10], and the dynamics of trait evolution can proceed quite differently [9,10]. As such, studies of selection on traits that mediate interspecific social interactions should distinguish between these two processes when drawing conclusions about the evolutionary history of such traits.

Many phenotypic traits function as signals in both mating and competitive contexts [11] (see table 1 in [12]), and, in some cases, the same character displacement patterns (e.g. sympatric shifts in phenotypes) have been attributed to

both ACD and RCD. In the best-known example, male pied flycatchers (*Ficedula hypoleuca*) have black dorsal plumage in allopatry, but in sympatry with the dominant collared flycatcher (*Ficedula albicollis*), most male pied flycatchers have dull, brown plumage, which resembles female collared flycatchers [13] and reduces territorial aggression from male collared flycatchers [14–16]. The same plumage shift also reduces the rate of cross-species mating and hybridization because female pied flycatchers prefer males with brown plumage in sympatry, which represents a reversal of the preference for black males in allopatry [17]. In another well-studied example, male *Calopteryx splendens* damselflies have blue-black wing spots that are larger in allopatry than in sympatry with *C. virgo*, which have fully blue–black wings [18,19]. Moreover, *C. virgo* males are more aggressive to *C. splendens* males with relatively larger wing spots in sympatry, which consequently affects male fitness [18,20,21], yet female mate recognition is also influenced by male wing coloration and shifts in sympatry in a manner consistent with RCD [22,23].

In a recent theoretical study, Okamoto & Grether [12] set out to understand whether ACD and RCD can act synergistically to drive evolutionary divergence, or whether one process has priority over the other. They constructed an individual-based theoretical model based on territorial damselflies to explore how RCD and ACD interact when female mate recognition and male competitor recognition are based on the same male trait. The male trait closely tracked the evolution of the mate recognition function, regardless of the relative strength of selection against interspecific mating and interspecific fighting. Even in the absence of selection against cross-species mating, a trait on which female mate recognition is based cannot diverge through ACD in this model. The basic reason is that mutations that reduce interspecific aggression by causing a male's phenotype to deviate from the mean of the other species also reduce his ability to attract conspecific females, and thus have a net negative effect on fitness. Okamoto & Grether's [12] model also showed that sympatric shifts in competitor recognition, which previously were thought to constitute *de facto* evidence for ACD, can arise as a byproduct of trait divergence caused by RCD. This is because males still need to recognize conspecific males as competitors, as the trait diverges through RCD. In short, RCD completely dominates ACD in this model. Therefore, to conclude that ACD is responsible for an observed character displacement pattern, RCD needs to be ruled out as an alternative explanation.

Previous research on two species pairs of rubyspot damselflies (*Hetaerina* spp.) showed that male competitor recognition is based on wing coloration [24,25] and that competitor recognition and male wing coloration in these species pairs diverge in sympatric populations [24,26]. These results are consistent with the hypothesis that ACD has acted in these species pairs. Based on Okamoto & Grether's [12] findings, however, these trait shifts cannot be taken as compelling evidence for ACD unless females do not use male wing coloration for species recognition. While attempts to detect female mate choice based on male coloration within species of *Hetaerina* have yielded no such evidence [27,28], whether females use male coloration for species discrimination is unknown.

Here we test for effects of male wing coloration on female mate recognition in *H. americana* in a population sympatric with *H. titia*, which is one of the species pairs in which sympatric divergence in male coloration and competitor recognition has been detected. Male *H. americana* have large basal red

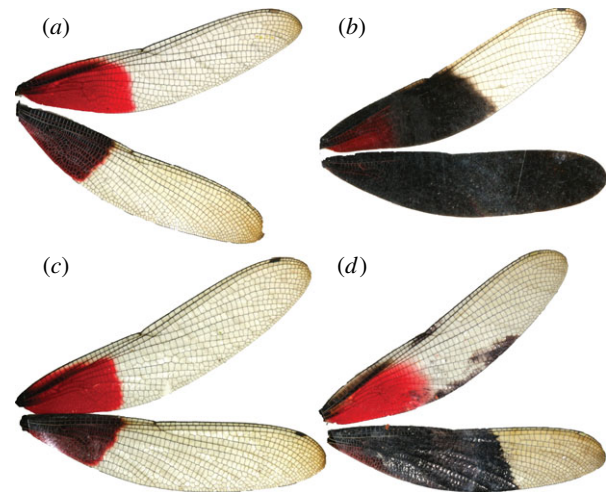


Figure 1. Photographs of a representative (a) unmanipulated *Hetaerina americana* male, (b) a *H. titia* male and *H. americana* males with (c) clear ink and (d) black ink on their hindwings. All males shown here were photographed during the course of the experiment. (Online version in colour.)

wing spots and otherwise clear wings (figure 1a), while male *H. titia* have smaller basal red wing spots and variable amounts of black wing pigmentation (figure 1b; electronic supplementary material, figure S1). To test whether female *H. americana* use the species difference in male wing coloration to avoid mating with heterospecific males, we conducted a field experiment in which a portion of *H. americana* males in the study area were manipulated to resemble *H. titia* males with black ink. We then tracked naturally occurring mating events, territorial fights, changes in territory ownership and survival on a continuous basis for five weeks.

2. Material and methods

(a) Study site and species

We carried out this study on two transects (approx. 100 m each) marked at 1 m intervals along the Medina River in Castroville, TX, USA (29.371797°, –98.896444°; 29.374733°, –98.896769°) from 23 May to 23 June 2013. To minimize dispersal, the study transects were located such that long pools (more than 100 m), which are not suitable as breeding habitat, were located both up and downstream. Every individual American rubyspot (*H. americana*) and smoky rubyspot (*H. titia*) damselfly encountered along these transects was captured with an aerial net and marked on its abdomen with a unique combination of DecoColor paint pens [24,29]. *Hetaerina* perch with their wings folded above their bodies, so abdomen marks are usually clearly visible to observers.

(b) Experimental wing manipulation

When we captured mature *H. americana* males, we assigned them to one of three treatments: (i) unmanipulated control (figure 1a), (ii) clear control: clear ink on the outer surface of the hindwings from the base to halfway between the nodus and the tip using a Prismacolor marker (clear, PM-121, figure 1c) or (iii) blackened: black ink on the same part of the hindwings (black, PM-98, figure 1d), following the protocol of Anderson & Grether [25] (see [30] for a similar approach to phenotype manipulation). To maximize the statistical power to detect effects of the experimental treatment, half of the males were assigned to the blackened treatment and 25% were assigned to each of the control groups. We restricted the experimental blackening to hindwings to prevent males' wings from sticking together. Although some *H. titia* males have extensive black pigmentation on their forewings,

many do not ([31,32]; see also the electronic supplementary material, figure S1), so our experimental manipulation was biologically realistic. Throughout the study, mature *H. americana* males were marked and assigned to a treatment group as they appeared or reached maturity on the transects (mature males have brilliant red forewing spots while immature males have pink to light red forewing spots [33]).

(c) Behavioural observations

During each day of the study, three to five observers, typically four, continuously walked along the transects from approximately 9.00 to 18.00 h, systematically recording the location to the nearest 0.1 m and ID of each individual encountered, with priority given to recording tandem (mating) pairs and fighting individuals. We strived to record all matings, which is quite feasible because tandem pairs are conspicuous and stay together for several minutes.

Hetaerina mating sequences do not include courtship, instead they begin when a male clasps a female's prothorax (intersternite), at which point the pair is in tandem [34]. From here, the tandem pair either breaks up without proceeding, which we considered a failed mating attempt, or continues on to form the characteristic copulatory wheel of odonates. In *H. americana*, after the copulatory wheel breaks, the tandem pair exhibits a jerking motion before the female submerges to oviposit in underwater vegetation [35]. When we encountered a mating pair, we recorded the IDs of both individuals and followed them until the mating was either successful (i.e. we saw a copulatory wheel, jerking or submersion) or the tandem broke. When possible, we recorded the entire length of time the pair was in the copulatory wheel. We also recorded instances where we observed a male pursue and fail to grasp a female and considered these to be failed mating attempts.

When an observer witnessed a fight, the location, species involved, ID of individuals (if marked) and escalation level were recorded; we considered two-way circle chases or back-and-forth chases [27,32] to be 'escalated' in subsequent analyses.

(d) Female mating analyses

Females may make post-copulatory decisions that bias paternity, since subsequent mates can remove previous mates' sperm from females' sperm storage organs [36,37]. To test for this possibility, we analysed (i) the treatments of females' first and last mates during each day and (ii) whether males' treatments influenced whether females remated or the treatment of subsequent mates. Nearly all females' mating bouts (N consecutive days observed in a mated pair) lasted for 3 days or fewer, so to test for the possibility that sperm removal influenced male mating success, we analysed female remating (i) within each day and (ii) across a 3-day window.

(e) Data analyses

In several analyses, we partitioned the reproductive career of individual males into territorial and non-territorial episodes in order to distinguish between the effects of male–male interactions and male–female interactions [27]. The territorial status of a given male on a given day was assessed based only on the male's resighting and fighting record and without knowledge of his treatment group or mating success. We considered males to be holding a territory if they were resighted consistently on a low perch near the bank of the river within a 3 m radius for at least 2 consecutive days [24]. Additionally, we took fighting and resights in the same area near the stream over a period of several hours to be evidence that a male was holding a territory.

To analyse fighting rates, we took three approaches: (i) treating all recorded fights between the same two males as a single fight (as in [25]), (ii) treating fights between the same two males on N different days as N different fights (i.e. one fight recorded per dyad per

day) and (iii) treating all fights as unique whether they were between the same or different males (i.e. all fights recorded per dyad per day). *Hetaerina titia* male densities were not consistent along the entire length of the transect. Because the wing blackening treatment was only expected to affect males that interacted with *H. titia* males, we restricted some analyses to males that were observed within close proximity (less than or equal to 4 m), of a *H. titia* territory holder. The 4 m criterion was chosen, *a priori*, based on the observation that the reaction distance of territory holders to conspecific male intruders is less than or equal to 2 m and that adjacent territories are typically less than or equal to 2 m apart, as reported previously [25].

Because the opportunity for males to fight and mate depended on the number of days they were present in the study, we analysed the data using count models with exposure terms of the logarithm of the number of days that a male was resighted. For analyses partitioned into territorial and non-territorial episodes, the exposure term was the number of days males held or did not hold territories during the study.

To include repeated measurements on individuals when available, we used mixed-effect models with random intercepts for individual IDs. We used R [38] to conduct all statistical analyses, using the packages MASS [39] for negative binomial regression, survival [40,41] for survival analyses, glmmADMB [42,43] and lme4 [44] for mixed-effect GLMs, pscl for zero-inflation models [45,46] and ggplot2 [47] for figures.

3. Results

(a) Sample sizes and preliminary results

We marked and included 146 *H. americana* males in the experiment, recorded 444 unique *H. americana* mating events involving marked males (82 failed mating attempts; 362 successful matings; mean number of successful matings per male = 3.26, s.d. = 4.74) and made 1207 observations of fights involving at least one *H. americana* male. We resighted 111 males, or 76.03% of the number marked, at least 1 day after marking, and resighted males' locations were recorded an average of 12.1 times per day. Among these resighted males, the median number of days resighted was 6, and most were resighted every day prior to their final disappearance (mean proportion of days on which males were resighted = 0.93). We witnessed five failed mating attempts of *H. americana* males with *H. titia* females; a tandem was successfully formed in three of these cases but broke prior to copulation. In no cases did the sham (clear) and unmanipulated control groups differ significantly from each other (see the electronic supplementary material), and thus the control groups were pooled for comparison to the experimental (blackened) group.

(b) Female mate recognition

There was no overall effect of the wing blackening treatment on: (i) the proportion of attempted tandems that resulted in a successful mating (figure 2a, mixed-effect binomial model of tandems (success = 1, failure = 0) with a random intercept for male IDs, treatment $n = 444$, $z = -0.14$, $p = 0.89$), (ii) the rate of successful matings (figure 2b, negative binomial model of the count of matings with an offset term for the log of the total number of days resighted, treatment d.f. = 110, $z = -1.02$, $p = 0.31$) or (iii) the duration of copulatory wheels (figure 2c, mixed-effect model of the logarithm of the duration of copulatory wheels with a random intercept for male IDs, treatment $n = 119$, $z = 0.26$, $p = 0.8$).

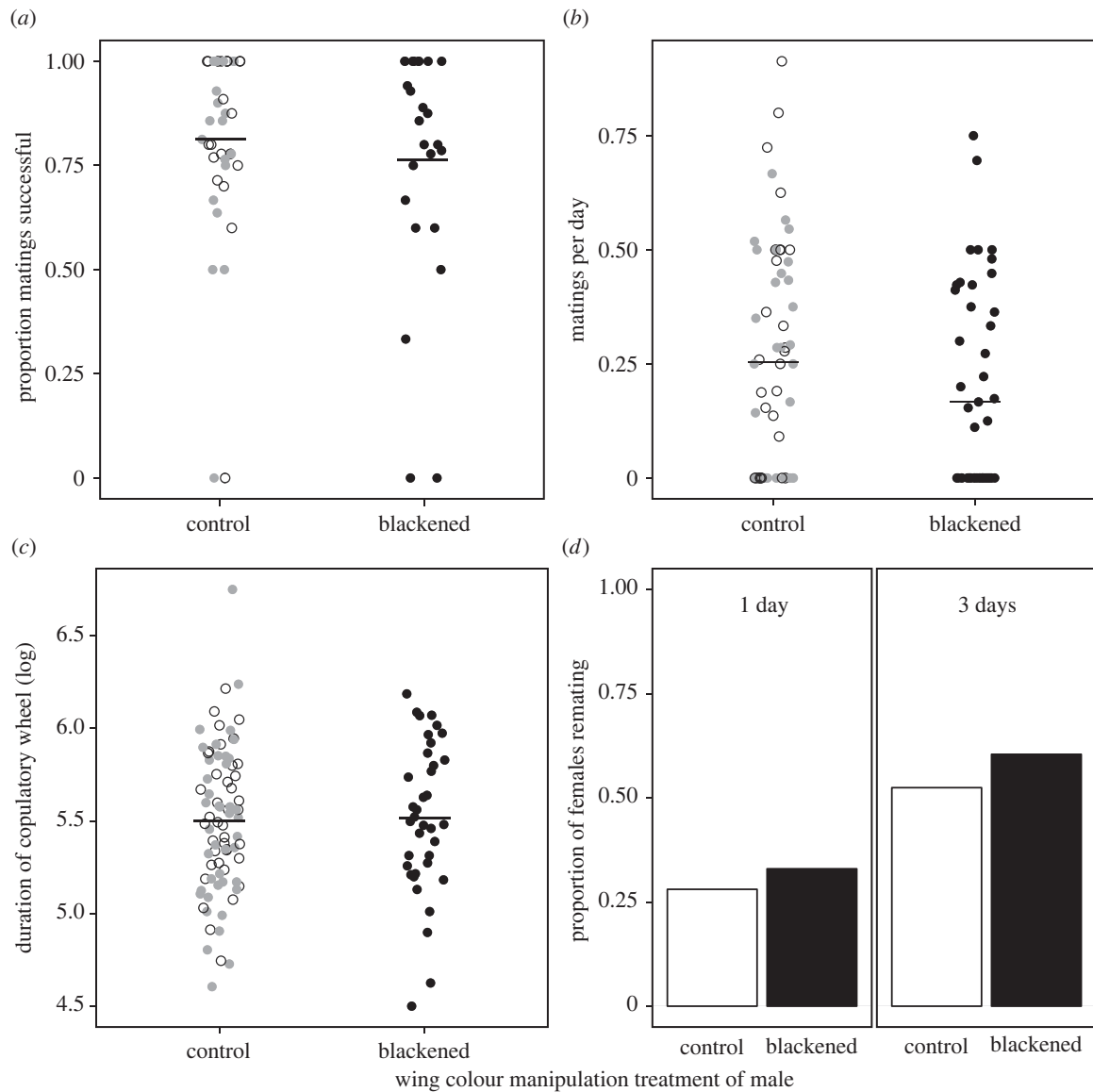


Figure 2. Lack of an effect of the experimental treatment on overall male mating success, measured either as (a) the proportion of successful tandems, (b) the mating rates of males, (c) the length of the copulatory wheel or (d) the probability of a female remating within 1 or 3 days. In panels (a–c), black dots indicate blackened males, grey dots indicate males with clear ink, empty circles indicate unmanipulated males and horizontal lines represent group means. In panel (d), black bars represent blackened males and empty bars represent control males.

In *H. americana*, holding a territory is not essential for mating but males generally mate more often when they hold a territory [27]. Thus, a male's mating rate is influenced by the proportion of his life spent holding a territory. In this experiment, males mated 2.1 times more frequently when they held a territory than when they did not (zero-inflated negative binomial model of the count of successful matings with a random intercept for male ID, $n = 180$, $z = 5.03$, $p < 0.001$). To separate effects of territory competition from female choice, we partitioned males' careers into territorial and non-territorial episodes to further examine the effect of the experimental treatment on male mating success [27]. In other words, differences in mating success between treatments could be a result of males of one treatment being unable to hold territories, a phenomenon independent of female mate recognition. When males held territories, neither the proportion of attempted tandems that resulted in a successful mating (mixed-effect binomial model of tandems (success = 1, failure = 0) with a random intercept for male IDs, $n = 353$, treatment $z = -0.50$, $p = 0.62$) nor the mating rate (negative binomial model of the

count of matings with an offset term for the log of the total number of days territorial, treatment d.f. = 71, $z = -1.69$, $p = 0.092$) were influenced by the experimental treatment. Likewise, when males did not hold territories, the proportion of successful tandems did not depend on treatment (mixed-effect binomial model of tandems (success = 1, failure = 0) with a random intercept for male IDs, $n = 91$, treatment $z = 0.89$, $p = 0.37$). However, the mating rate of non-territory holding blackened males was 1.74 times higher than that of controls (negative binomial model of the count of matings with an offset term for the log of the total number of days non-territorial, treatment d.f. = 107, $z = -1.992$, $p = 0.046$).

The post-copulatory behaviour of females did not distinguish among males based on their treatments. Neither a female's first nor last mate of the day depended on the male's treatment group (estimated from intercept of a mixed-effect model of first or last male treatment with a random intercept for female ID, both $p > 0.05$, see the electronic supplementary material). Similarly, the treatment of a female's mates did not influence her probability of remating within

Table 1. Effects of the experimental treatment on intraspecific and interspecific fighting rates. Statistical tests compare blackened and control males. Datasets correspond to those described in the main text. Analyses presented in italics restrict males to those seen within 4 m of a territorial *H. titia* male. The ratios of blackened male interspecific fighting rates to control male interspecific fighting rates are presented in parentheses.

dataset	intraspecific fights (<i>H. americana</i> versus <i>H. americana</i>)		interspecific fights (<i>H. americana</i> versus <i>H. titia</i>)	
	all fight types	only escalated fights	all fight types	only escalated fights
(1) one fight per dyad ^a	$n = 666$, $\chi^2 = 4.17$, $p = 0.041$	$n = 374$, $\chi^2 = 0.22$, $p = 0.64$	$n = 115$, $\chi^2 = 4.69$, $p = 0.00099$	$n = 82$, $\chi^2 = 11.66$, $p = 0.00064$
(2) one fight per dyad per day ^b	d.f. = 81, $z = 1.07$, $p = 0.28$	d.f. = 81, $z = 0.18$, $p = 0.86$	d.f. = 81, $z = 2.38$, $p = 0.017$, (1.94) d.f. = 55, $z = 3.01$, $p = 0.0026$, (2.25)	d.f. = 81, $z = 2.92$, $p = 0.0035$, (2.5) d.f. = 55, $z = 3.53$, $p = 0.00043$, (2.96)
(3) all fight observations ^b	d.f. = 81, $z = 0.18$, $p = 0.86$	d.f. = 81, $z = -0.48$, $p = 0.63$	d.f. = 81, $z = 2.82$, $p = 0.0049$, (2.36), d.f. = 55, $z = 3.38$, $p = 0.00072$, (2.75)	d.f. = 81, $z = 3.02$, $p = 0.0026$, (2.76), d.f. = 55, $z = 3.52$, $p = 0.00043$, (3.26)

^aChi-squared goodness-of-fit test of count of fights, comparing experimental versus control to a null expectation of fights based on the resighting record (see the electronic supplementary material).

^bNegative binomial model of the number of interspecific fights, offset by the log of the number of days territorial or fighting (if larger).

1 day (figure 2d, blackened versus control in a binomial mixed-effect model of remating with a random intercept for female ID, $n = 255$, $z = 0.82$, $p = 0.41$) or within 3 days (figure 2d, blackened versus control in a binomial mixed-effect model of remating with a random intercept for female ID, $n = 255$, $z = 1.28$, $p = 0.20$). Furthermore, the treatment of the male with which a female remated was not influenced by the treatment of her previous mate, whether analysed within 1 day (binomial lag model with a lag variable for the subsequent mate treatment used as a predictor with a random intercept for female/1 day, $n = 76$, $z = -0.811$, $p = 0.42$) or over a 3 day period (binomial lag model with a lag variable for the subsequent mate treatment used as a predictor with a random intercept for female/3 day, $n = 141$, $z = -0.784$, $p = 0.43$).

(c) Treatment effects on fighting, disappearance, and territory tenure

Compared to controls, blackened *H. americana* males were more likely to fight with *H. titia* males, with an increasing effect of treatment in escalated fights and for males who were resighted within 4 m of *H. titia* territory holders (table 1). We found little evidence for an effect of the experimental treatment on intraspecific fighting rates (table 1). In the analysis for which we reduced all fights between the same two males to a single observation, there was a marginally significant trend for blackened males to be involved in more intraspecific fights than control males, but this effect disappeared when the analysis was restricted to escalated fights, and there was no such trend in the other two datasets (table 1; electronic supplementary material, figure S2).

The rate at which blackened males disappeared from the study transects was 1.57 times higher than that of controls (Cox proportional hazard treatment coefficient = 0.4541, $n = 146$, $z = 2.549$, $p = 0.011$; limiting analysis to clear and blackened treatments, Cox proportional hazard treatment

coefficient = 0.474, $n = 110$, $z = 2.12$, $p = 0.034$). Among all males that were resighted at least once, however, there was no difference in the disappearance rate of blackened males and controls (Cox proportional hazard treatment coefficient = 0.3531, $n = 111$, $z = 1.694$, $p = 0.09$; limiting analysis to clear and black ink treatments, Cox proportional hazard treatment coefficient = 0.432, $n = 80$, $z = 1.65$, $p = 0.10$).

Experimentally blackened males were just as likely as control males to perch and defend territories near heterospecifics (see the electronic supplementary material). However, blackened males suffered a survival cost from interacting with *H. titia* males; blackened males whose median perch locations were ever within 4 m of *H. titia* males had 1.9 times higher disappearance rates than control males (figure 3, Cox proportional hazard treatment coefficient = 0.643, $n = 62$, $z = 2.154$, $p = 0.031$; limiting analysis to clear and black ink treatments, Cox proportional hazard treatment coefficient = 0.992, $n = 42$, $z = 2.37$, $p = 0.018$). Experimentally blackened males also held territories for fewer days than control males when they were ever within 4 m of *H. titia* males, but experienced no such difference when they were never within 4 m of *H. titia* males (negative binomial model of territorial days with offset term for the log number of total days resighted, treatment \times proximity d.f. = 110, $z = -2.427$, $p = 0.015$).

4. Discussion

Female mate recognition appeared to be unaffected by the species difference in male wing coloration. Manipulating *H. americana* male wings to appear similar to those of *H. titia* males had no discernable effect on mating—females neither rejected experimentally blackened males more often after being clasped nor mated less often with experimentally blackened males (figure 2a,b). The only hint of an effect of the

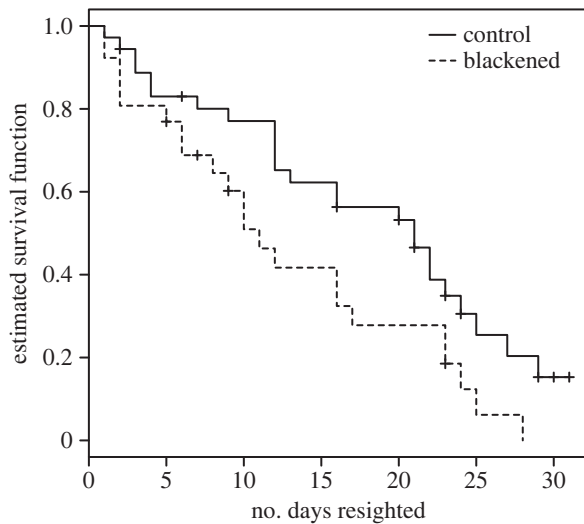


Figure 3. Effects of the experimental treatment on survival of males seen in close proximity to *H. titia* males. Kaplan–Meier plot, crosshatches indicate censored data points.

experimental treatment on female responses was opposite to the predicted direction: among non-territory holders, blackened males mated at a marginally significantly higher rate than controls. Mating rates in this study are similar to those measured previously in *H. americana* [35].

Post-copulatory means of discrimination are possible in calopterygid damselflies, where there is extensive evidence that males remove stored sperm from females during copulation [37,48,49]. If the amount of time spent in copula is under female control (but see [50–52]), females may be able to control how much sperm from previous mates is removed by her current mate, the amount of sperm that the male is able to transfer, or the amount of time she spends with the current male at the expense of time for future matings [36,53,54]. Yet, in our study, copulation duration was also unaffected by the experimental treatment. Since females are sometimes clasped by different males after emerging from ovipositing, cryptic female choice may take the form of females biasing either first or last matings towards particular males, remating more often after mating with a non-preferred male [36,55], or similarly, biasing remating towards a particular treatment, yet none of these indicators of cryptic female choice occurred in our experiment, whether we analysed single days or 3 day windows for each female (given the possibility of sperm storage across days of a female's reproductive bout). We did not test the possibility that females discriminated between the treatments via some other cryptic choice mechanism such as biasing paternity sperm storage [36,55,56] or manipulating fecundity [57,58].

Hetaerina americana females may use traits other than wing coloration to differentiate between conspecific and heterospecific males. In *Enallagma* damselflies, the appendages that males use to clasp females (cerci) have evolved in a correlated fashion with the corresponding structures on females—consistent with the hypothesis that these structures

are involved in species recognition [59,60]. Female *Hetaerina* may also use tactile information from male cerci and/or paraprocts (i.e. inferior and superior clasping appendages), as these structures are highly variable and species specific [34].

In agreement with previous research [25], manipulating the wings of *H. americana* males to resemble those of *H. titia* males increased the occurrence of interspecific fighting. We further documented effects of the experimental manipulation on the rate and intensity of interspecific fights and the proportion of a male's life during which he held a territory. Moreover, blackened *H. americana* males in our study close in proximity to *H. titia* males suffered reduced survival compared to control males, probably resulting from the increase in fights with heterospecific males. We also documented an immediate effect of the phenotype manipulation: a reduction in the probability that blackened males were resighted. Whether this early attrition of blackened males reflects mortality or dispersal is not clear, but if weaker/lower quality males were more likely to be lost from the study, this might account for the relatively high non-territorial mating rates of the remaining blackened males.

Together, our results strengthen the hypothesis that previously documented shifts in both competitor recognition and male wing coloration [24–26] have resulted from ACD. One previous study documented a pattern of character displacement in male breeding coloration of benthic and limnetic forms of three-spined sticklebacks (*Gasterosteus aculeatus*) [61] that cannot be explained by a shift in female preferences or colour sensitivity [62,63], effectively ruling out RCD as a potential explanation for the observed shift. Nevertheless, to the best of our knowledge, the current study is the first to experimentally demonstrate that a target of male competitor recognition is not also a target of female mate recognition and thus the first to support ACD over RCD as the cause of a character displacement pattern. Although some investigators have grouped character displacement influencing interspecific aggression under RCD [2], this study highlights the mechanistic distinction between RCD and ACD: our phenotype manipulation experiment confirmed that the species difference *H. americana* male wing coloration influences interspecific aggression but does not influence female mate recognition. Based on these results, we can reject the hypothesis that previously documented sympatric shifts in male traits are by-products of RCD.

Data accessibility. Datasets used in our analyses are available at Dryad (dryad.org): doi:10.5061/dryad.158pj.

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