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The interplay between agonistic character displacement and reproductive interference in rubyspot damselflies (Hetaerina spp.)

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The interplay between agonistic character displacement and reproductive interference in rubyspot damselflies (*Hetaerina* spp.)

A dissertation submitted in partial satisfaction of the
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in Biology

by

Jonathan Phillip Drury

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ABSTRACT OF THE DISSERTATION

The interplay between agonistic character displacement and reproductive interference in rubyspot damselflies (*Hetaerina* spp.)

by

Jonathan Phillip Drury

Doctor of Philosophy in Biology

University of California, Los Angeles, 2014

Professor Gregory F. Grether, Chair

Aggressive interactions between species are common despite being relatively understudied. Agonistic character displacement (ACD) theory makes predictions about how selection should act on traits that mediate the occurrence of interspecific aggressive interactions. Previous research on rubyspot damselflies (*Hetaerina* spp.) documented several cases of divergent agonistic character displacement acting on wing coloration and competitor recognition to diminish wasteful interspecific aggression. However, these and other studies of the evolutionary consequences of interspecific aggression have largely ignored how interactions between males and females of different species affect interspecific interactions between males. In chapter 1, we present a theoretical model that demonstrates that when individuals engage in local mate competition, selection may actually favor interspecific aggression. We then test this model in rubyspot damselflies (*Hetaerina* spp.) and show that in a comparison of several species pairs, levels of reproductive interference correlate positively with levels of interspecific aggression. In chapter 2, we document a previously undescribed seasonal polyphenism in the wing coloration of male and female smoky rubyspot damselflies (*Hetaerina titia*). We show that this polyphenism—an increasing amount of dark pigmentation on the wings of both sexes over the breeding season—impacts species recognition between *H. titia* and a sympatric congener (*H. occisa*). Additionally, we find that, in accord with comparisons across populations, seasonally decreasing levels of reproductive interference within a population correspond to a decreasing degree of interspecific aggression. In chapter 3, we present a phenotype manipulation experiment carried out on *H. americana*, whose results support the hypothesis that sympatric shifts in male traits resulted from ACD and lead us to reject the alternative hypothesis that reproductive character displacement in female mate recognition is responsible for these shifts. Finally, in chapter 4, we present a new statistical method to correct for phylogenetic non-independence in pairwise species comparisons, and we use this method in conjunction with a new phylogeny we constructed of nine *Hetaerina* species to show that interspecific differences in female wing coloration are correlated with the degree of reproductive interference. We also apply this phylogenetic correction to an analysis of predictors of interspecific territoriality in New World wood warblers (Parulidae).

The dissertation of Jonathan Phillip Drury is approved.

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VITA

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Selected Publications

Drury, J. & Grether, G. *submitted*. Interspecific aggression, not interspecific mating, drives character displacement in the wing colouration of male rubyspot damselflies (*Hetaerina*). *Proceedings of the Royal Society: B*.

Grether, G., Anderson, C., **Drury, J.,** Kirschel, A., Losin, N., Okamoto, K., & Peiman, K. 2013. The evolutionary consequences of interspecific aggression. *Annals of the NY Academy of Sciences.* doi: 10.1111/nyas.12082

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Anderson, C., Córdoba-Aguilar, A., **Drury, J.,** & Grether, G. 2011. An assessment of marking techniques for odonates in the family Calopterygidae. *Entomologia Experimentalis et Applicata* 141:258-261. doi: 10.1111/j.1570-7458.2011.01185.x

Drury, J. & Gowaty, P. 2010. Social Selection, Sexual Selection, and Sexual Conflict. In: *Encyclopedia of Animal Behavior* (Eds. M Breed and J. Moore) Academic Press, San Diego.

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Chapter 1. Reproductive interference explains persistence of aggression between species

Abstract

Interspecific territoriality, which occurs when heterospecific individuals fight over space, may arise spontaneously when populations of closely related territorial species first come into contact. But defense of space is costly, and unless the benefits of excluding heterospecifics exceed the costs, natural selection should favor divergence in competitor recognition until the species no longer interact aggressively. Ordinarily males of different species do not compete for mates, but they may do so when males cannot distinguish sympatric females. With a theoretical model, we demonstrate that reproductive interference caused by undiscriminating males can prevent interspecific divergence, or cause convergence, in traits used to recognize competitors. We then test the model in a genus of visually orienting insects and show that species differences in female coloration are strongly predictive of current levels of reproductive and aggressive interference. Reproductive interference is common and may account for the persistence of interspecific aggression in many taxonomic groups.

Introduction

Interspecific territoriality (Simmons 1951) is expected to be evolutionarily stable under a narrower range of conditions than intraspecific territoriality, for two principal reasons. First, resource competition is generally weaker between than within species, because of past niche divergence and competitive exclusion (Brown Jr. & Wilson 1956; Lorenz 1962; Dhondt 2012). Second, attracting and maintaining priority of access to mates is one of the primary benefits of intraspecific territoriality (Payne & Groschupf 1984), and members of different species generally do not compete for mates (Orians & Willson 1964). Interspecific territoriality may initially arise as a byproduct of intraspecific territoriality when species that still share a common competitor recognition system first come into contact (Orians & Willson 1964; Murray 1971; Grether et al. 2009). But defense of space is costly, and unless the benefits of excluding individuals of other species exceed the costs, selection should favor divergence in competitor recognition until interspecific aggression is eliminated (Lorenz 1962; Orians & Willson 1964; Murray 1971; Brown & Alcock 1990; Grether et al. 2009). Orians and Willson (Orians & Willson 1964) concluded that interspecific territoriality ought to persist only between species that compete for resources that cannot be partitioned and otherwise should only be seen in cases of very recent sympatry caused by range shifts or where gene flow from allopatry prevents local adaptation in sympatry. The data available on birds 50 years ago appeared to support these predictions, but a taxonomically broader view shows that the theory is incomplete. In insects, fishes, frogs and lizards it is common for males of closely related species to compete over mating territories with no common resources at stake (Schwartz & Wells 1985; Singer 1989, 1990; Gerhardt et al. 1994; Nomakuchi & Higashi 1996; Jones et al. 1998; Shimoyama 1999; McLain & Pratt 1999; Tynkkynen et al. 2004, 2006, 2008; Svensson et al. 2007; Outomuro 2009; Anderson & Grether 2010a, 2011; Peiman & Robinson 2010; Dijkstra & Groothuis 2011; Ord et al. 2011; Lailvaux et al. 2012; Iyengar et al. 2014). This is often interpreted as a maladaptive byproduct of intraspecific territoriality and overlap between species in territorial signals (Murray 1971; Singer 1989; Schultz & Switzer 2001; Ord et al. 2011), but an alternative hypothesis is that these are cases in which males of different species actually are in competition for mates (Payne 1980; Nishikawa 1987; Singer 1989).

In fact, interspecifically territorial species, including birds, often interfere with each other reproductively, i.e., males court, attempt to mate, or actually mate with heterospecific females (for examples, see Table 1-1). In hybridizing taxa, mating with heterospecific females might be a "best-of-a-bad-job" tactic for males, but in non-hybridizing taxa, reproductive interference is most likely to occur when males cannot easily distinguish between conspecific and heterospecific females. Although females would benefit from being discriminable in a mating context, ecological factors may prevent reproductive character displacement in female traits. For example, selection for crypsis caused by visually orienting predators (Stamps & Gonn 1983) or prey (Grether & Grey 1996) may constrain divergence in female coloration because mutations that enhance discriminability tend to reduce crypsis (Endler 1991). Regardless of the reasons, reproductive interference between species is quite common (Gröning & Hochkirch 2008).

Species that interfere with each other reproductively effectively compete for mates (Reitz & Trumble 2002). Interspecific territoriality may therefore be profitable even when no other resources are defended (Payne 1980; Nishikawa 1987; Singer 1989). To formally evaluate this hypothesis, we used an individual-based model of agonistic character displacement (Okamoto & Grether 2013) to simulate the evolutionary effects of secondary contact between two species in which males compete for mating territories. Reproductive interference was incorporated into the model as the fractional reduction (d) in a male's expected mating success caused by sharing a territory with one heterospecific male relative to sharing a territory with one conspecific male. The evolvable traits in the model are the central location (μ) and width (σ) of the male competitor recognition template and the male trait (z) upon which competitor recognition is based (for further details, see Okamoto & Grether 2013). In simulations carried out over 10^4 generations, we varied d and the initial values of μ and z. The results show that moderate levels of reproductive interference are sufficient to allow interspecific territoriality to be maintained or even evolve *de novo*.

We tested the model in *Hetaerina*, a damselfly (Zygoptera) genus in which the level of interspecific aggression varies across species pairs included in our study (Table 1-2). Males compete for small mating territories (1-2 m^2) in fast flowing sections of rivers where females oviposit in submerged vegetation. Females usually oviposit outside the territories of their mates and feeding occurs elsewhere (Grether 1996). There is no *a priori* reason to expect interspecific territoriality in *Hetaerina*, and yet it occurs in most sympatric species pairs (Anderson & Grether 2011). In some cases, interspecific fighting is reduced by divergence in male competitor recognition (Anderson & Grether 2010b, 2011) or by species differences in microhabitat use (Anderson & Grether 2011), but in most cases, territory holders are equally aggressive to conspecific and heterospecific male intruders (Table 1-3) and interspecific fights often occur just as frequently as intraspecific fights (Tables 1-4 and 1-5). Evolutionary time lags or gene flow from allopatric populations may explain the failure of particular species pairs to diverge in competitor recognition, but the finding that most sympatric species have not diverged argues for an adaptive explanation. Besides the unexplained variation in interspecific aggression, there are other reasons to think the reproductive interference hypothesis applies to *Hetaerina.* Males have conspicuous, species-specific coloration, but females are cryptic and variable in coloration and can be difficult to identify to the species level (Garrison 1990). To examine whether the male damselflies can distinguish between conspecific and heterospecific females, we presented territory holders at eight sympatric sites with live, flying, tethered females. This is a realistic test of male mate recognition because natural mating sequences begin with the male clasping the female (i.e., no pre-clasping courtship) and males usually clasp tethered conspecific females.

The results of this study provide striking support for our model: variation in the level of reproductive interference, caused by variation in the ability of males to distinguish between conspecific and heterospecific females, explains the variability in the level of aggressive interference between species. Hence, we conclude that both divergent and convergent agonistic character displacement processes have occurred in this genus, depending on the degree to which the interacting species are reproductively isolated.

Materials and Methods

Model. For full details on the basic ACD model (without reproductive interference), we refer readers to Okamoto and Grether (2013). Here we describe only the main features of the model. The model is individual-based (DeAngelis & Mooij 2005) and the loci underlying the evolvable traits are tracked explicitly. We model a sexually reproducing diploid population without overlapping generations. All traits are assumed to be quantitative traits whose breeding values are determined by the additive effects of five autosomal, unlinked loci subject to mutation, and allelic values can take on any real number. During the breeding season (90 days), the model proceeds on a daily time step. On each simulated day, mature males either occupy or do not occupy territories. Males without territories attempt to occupy individual territories that may or may not be occupied by other males. If the territory is occupied, three outcomes are possible: mutual recognition as competitors, one-sided recognition as a competitor, and mutual-non-recognition as competitors. Which of these outcomes is realized is a probabilistic function of the individual values of z, μ and σ of the males encountering each other (Okamoto & Grether 2013). The former two scenarios result in fights, in which males must expend finite energetic reserves. The winner of the fight occupies the territory and the loser is ejected. If there is mutual non-recognition, the resident and intruding males now share the territory. Following the assignment of territories to males, mating occurs. The probability that a given male mates with a given female depends on: (1) whether the male occupies a territory or not, (2) whether the male and the female are conspecifics, and (3) the number of other males with which the male shares a territory who could potentially interfere with his ability to mate with the female. Following mating, females oviposit. The larval stages of the life cycle, during which density-dependent population regulation is assumed to occur, are modeled implicitly.

We ran the model for 10000 generations following secondary contact, after a 1000-generation allopatric burn-in period. At the start of each simulation, the mean values of μ and z were set to equal each other within species, which means that males initially recognize most conspecific males as competitors. The model is based on a damselfly-like system in which intraspecific territoriality is adaptive (Okamoto & Grether 2013). However, it is possible for μ and z to diverge from each other within species, resulting in a loss of intraspecific territoriality. The initial magnitude ∂ of divergence between species in μ and z, which determines whether males of the two species initially respond aggressively to each other, was set at 0, 1.5, or 3 standard deviation units. A ∂ value of 1.5 corresponds to probability of approximately 0.33 that encounters between males of the two species will result in heterospecific recognition (one-sided or two-sided), while a ∂ value of 3 corresponds to a heterospecific recognition probability of about 0.01. We varied the level of reproductive interference between species (d) across simulations ($d = 0.1, 0.21, 0.27, 0.30, 0.33$, or 0.45). A d value of 0.5 would mean that sharing a territory with one heterospecific male is just as costly, in terms of lost mating opportunities, as sharing a territory with a one conspecific male. We ran 15 replicates for each combination of ∂ and d values.

Study sites. We conducted the fieldwork from March to August in the years 2005-2013 at eleven locations in North America, most with two well-represented species of *Hetaerina* damselflies (Table 1-2). We treat one of the locations as two separate sites (PA1 and PA2) because the wing coloration of female *H. titia* undergoes a dramatic seasonal shift from the spring (PA1) to summer (PA2) months. The seasonal color shift affects the predictions of our model because males of the sympatric congener (*H. occisa*) only distinguish between females of the two species after the color shift (PA2, see Table 1-3). Pooling data from PA1 and PA2 did not change the overall results, however (Fig. 1-6).

Behavioral observations. At each site, we captured most of the adult *Hetaerina* along a 100-200 m river transect with aerial nets and marked individuals with unique IDs using a previously described method (Anderson et al. 2011). We conducted behavioral observations (1) to determine which males were defending territories and thus eligible for subsequent experimentation (see below), and (2) to record the frequency of naturally occurring conspecific and heterospecific fights. Observers recorded the location of each male to the nearest 0.1 m by reference to numbered flags. We considered males to be territory holders if they were observed perched near the bank of the river at the same location (within a 3 m radius) for two or more consecutive days (Anderson & Grether 2010b). When fights occurred, we recorded the location, species involved, ID of individuals (if marked), and the level of escalation (1, one-way chase; 2, two-way back-and-forth chase; 3, escalated “circle” fight between two males; and 4, escalated fight involving three or more males). Prior to analysis, multiple recorded bouts of fighting between the same two males on the same day were reduced to a single fight. For fights involving unmarked or unidentified individuals, we only recorded one fight within a 5 m radius per day.

To determine whether interspecific fights occur less often than expected by chance, following Anderson & Grether (2011) we generated the chance expectations from binomial expansions of the relative frequencies of males of each species and conducted a χ^2 goodness-of-fit test on the observed number of fights.

Interspecific aggression. To measure interspecific aggression relative to intraspecific aggression, we followed the protocol of Anderson & Grether (2010b): territory holders were presented with live male intruders that were tethered with a transparent thread and literally flown into the territory with a fishing pole. Each territory holder was presented with one conspecific intruder and one heterospecific intruder, with the order of presentation trials balanced across males. During each trial, a field assistant recorded the behavior of the territory holder, including the amount of time spent chasing the tethered male and the number of slams (defined as attempts to ram the tethered male, whether successful or not) and grabs (defined as extended physical contact with the tethered male) on a continuously running voice recorder. It was not possible for field assistants to be blind to the treatments, but they had no knowledge of our theoretical model or the prediction being tested. Trials were 2 minutes in duration with at least a 5-minute inter-trial interval. Cases in which we were only able to carry out one of the two trials or in which the territory holder did not chase either tethered intruder for at least 60 s were excluded from the analysis (the latter were interpreted as cases in which the male was not actively defending the site; if possible, these males were retested on a subsequent day).

We tested for differences in the attack rate (slams and grabs divided by the duration of the trial) directed at heterospecific versus conspecific males using paired *t*-tests when $\log(x) + 0.01$ transformed data met the assumptions of normality and homoscedasticity. Paired

Wilcoxon paired signed rank tests were used when the data did not meet parametric assumptions. Sample sizes are given in Table 1-3.

To obtain a relative measure of aggression toward heterospecific males, for each species at each site, we divided the mean attack rate toward heterospecific tethered males by the mean attack rate toward conspecific tethered males.

Male mate recognition. We measured male mate recognition by presenting territorial males with tethered females of both sympatric species at a distance of 0.5 m from the male's perch. The presentation order of conspecific and heterospecific females was balanced. Presentations lasted 5 s each, or until the focal male returned to his perch, whichever came last. If the female was clasped during her first presentation, we ended the trial; otherwise we presented her for another 5 s. There is no courtship display in *Hetaerina*. A mating sequence begins with the male clasping the female, usually in midair. Just prior to clasping, the male flies toward the female, curls his abdomen forward, and grasps the intersternite region of the female's thorax with his claspers. We considered a male to have responded sexually if he either clasped or attempted to clasp the female—that is, if he pursued her with his abdomen curled. In most recorded clasping attempts, the male's claspers made contact with the female's intersternite (96.7%) and, in a majority of such cases (63.6%), the male succeeded in clasping the female at least momentarily. Cases in which the male did not respond sexually to either female or we were unable to complete the set of trials were excluded from the analysis.

To test for discrimination between females of different species, we used Fisher's exact tests (for sample sizes, see Table 1-3). To obtain a relative measure of sexual responses from heterospecific males, we divided the proportion of tethered females that elicited sexual responses in trials with heterospecific males by the proportion of tethered females that elicited sexual responses in trials with conspecific males.

Female wing coloration measurements. The wings of female *Hetaerina* vary from nearly clear to nearly black (Fig. 1-4*A*-*E*). To quantify this variation, we measured wing reflectance spectra using an Ocean Optics spectroradiometer (USB 2000) equipped with a reflectance probe (Ocean Optics R200-7-UV-VIS) and a pulsed xenon light source (Ocean Optics PX-2), with reference to a Labsphere certified reflectance standard using Ocean Optics' OOIBase32 software. We placed the reflectance standard behind the wings when taking readings, and the light path was oriented 45 degrees relative to the wing surface to eliminate glare. The resulting measurements include both light reflected off the wings and light transmitted through the wings. We took three repeat measurements at three positions (base, middle, and tip) on the forewings and hindwings and averaged the repeats. From the average spectra, we calculated "lightness" (L) as the sum of percent reflectance at 2 nm intervals from 300 to 700 nm (divided by 10^3 for presentation). To account for the proportionally larger mid-wing area, a weighted measure of lightness was obtained with the formula: $L_{\text{total}} = 0.1L_{\text{base}} + 0.8L_{\text{middle}} + 0.1L_{\text{tip}}$.

It was not practical to scan the wings of all of the females used in the male mate recognition experiments with a spectroradiometer, so we instead took measurements from digital wing photographs. Photographs were taken with the wings flattened against a white background using a Canon 20D equipped with a Canon 100 mm macro lens and Canon MT-24 macro flash. In ImageJ, we used the "Color Balance" plugin in the MBF package to standardize the white balance in each photo relative to the white background of the scale paper included in each photograph. We then used the polygon tool and the "Measure RGB" plugin to analyze the RGB profile of each wing. The average, weighted grayscale (0.299R+ 0.587G+ 0.114B) provided a photographic measure of wing lightness that correlated well with the spectroradiometric measure of wing lightness (Pearson's product-moment correlation $r = 0.78$, $n = 49$, $P < 0.001$).

Female wing color manipulation. To determine whether wing color *per se* influenced male mate recognition, we presented territorial males of *H. occisa* and *H. americana* at several sites (CT, CV, ES, LM, PA2) with (1) unmanipulated conspecific females and (2) conspecific females with wings experimentally darkened to resemble dark *H. titia* females' wings. Females were assigned to treatments at random with respect to their natural wing coloration in an alternating order so as to maintain a balanced design. The same females were presented to *H. titia* territory holders at PA2 and CV. The darkening treatment involved coloring the hindwings from the base to the tip with a gray marker (Warm Gray 90%, Prismacolor PM-107) and the forewings from base to the nodus with a gray marker and from the nodus to the tip with a sepia marker (Prismacolor PM-62). We chose these marker colors because their reflectance spectra best approximated the late season wing coloration of female *H. titia*. We used the same tethering protocol and criteria for male sexual responses and inclusion in analyses as above (for sample sizes, see Fig. 1-4*G*).

Statistical approach. We obtained two measures of interspecific aggression and reproductive interference at each study site, one for each species, but only one measure of the species difference in female wing coloration. To test for correlations between these variables, while circumventing potential non-independence caused by the data structure, we used the following randomization approach: one of the two species at each site was dropped at random and a Spearman correlation was calculated using the remaining data points (in STATA 12.1; Statacorp, Texas). This procedure was repeated 10000 times to yield a distribution of Spearman correlations, the mean ± SD of which are presented in the text. We also performed phylogenetic correction on these data (see Chapter 4).

Results

Model results. With low levels of reproductive interference ($d < 0.28$), the species diverged in their mean values of μ and z until interspecific aggression was eliminated (Fig. 1-1*A*-*C*, Fig. 1-2). By contrast, in the presence of moderate levels of reproductive interference ($d \geq 0.28$), the species converged in their respective values of μ and z until interspecific territoriality was established (Fig. 1-1*D*-*F*, Fig. 1-2). The initial level of divergence (∂) between species had no qualitative effect on the final outcome if $d > 0.1$. With $\partial = 0$ and $d \leq 0.1$, intraspecific territoriality was lost in about one third of the simulation runs (i.e., μ and z diverged within species; Fig. 1-3), but $\partial = 0$ is biologically unrealistic.

Empirical results. We found that males discriminate between heterospecific and conspecific females in the same two species pairs in which they discriminate between heterospecific and conspecific males (i.e., *H. occisa*–*H. titia*, *H. americana*–*H. titia*), and not in the other four species pairs tested (Table 1-3). In the species pairs in which males discriminate between conspecific and heterospecific females, females that are more similar to heterospecific females in wing coloration are more likely to be clasped by heterospecific males (Fig. 1-4*F*), and experimental manipulations confirmed that female wing coloration directly affects male sexual responses (Fig. 1-4*G*).

Rates of reproductive interference and aggressive interference are strongly, positively correlated across sites and negatively correlated with the species differences in female wing lightness (Fig. 1-5). The correlations between the species differences in female wing lightness and both reproductive interference and aggressive interference remain statistically significant after phylogenetic correction (Chapter 4).

Discussion

It is well known that costly interspecific interactions, such as resource competition and hybridization, can drive divergence between species over evolutionary time (Brown & Wilson 1956; Pfennig & Pfennig 2012). It is less intuitive that mutually costly interactions can cause evolutionary convergence. We show, with a mathematical model, that reproductive interference resulting from indiscriminate male mating behavior can cause species to converge in territorial signals. We then test the model's predictions in the field and find that it explains a variable pattern of interspecies fighting in *Hetaerina* damselflies. Recent reviews have highlighted the prevalence of aggressive and reproductive interference (Gröning & Hochkirch 2008; Grether et al. 2009, 2013; Peiman & Robinson 2010; Ord et al. 2011). Our model formally links these two costly interspecific interactions and provides a mechanism through which aggression between species can be maintained by natural selection.

The hypothesis that reproductive interference accounts for interspecific aggression and territoriality was first proposed by Payne (1980) for parasitic *Vidua* finches, which, like the damselflies, only defend mating sites. The hypothesis has also been applied to hybridizing species that defend multi-purpose territories, on the basis that excluding heterospecific males is advantageous at the pair formation stage (Sedlacek et al. 2006) and prevents interspecific extra-pair paternity (Baker 1991; Sedlacek et al. 2006), but very few researchers have made the link between interspecific aggression and reproductive interference, and ours is the first formal model of the phenomenon. While interspecifically territorial species do not always interfere with each other reproductively, not all species that compete for common resources are interspecifically territorial either (Dhondt 2012). Even when resource defense is the primary function of territoriality, reproductive interference might tip the balance in favor of excluding heterospecifics. Extending our model to species that defend resources other than mates would be straightforward.

Whether character displacement is common or rare remains controversial (Pfennig & Pfennig 2012; Gerhardt 2013; Stuart & Losos 2013), but researchers can probably agree that current theory does a poor job of predicting whether species will diverge from each other in sympatry. Indeed, a recent large-scale phylogenetic study of song variation in ovenbirds (Furnariidae) revealed a striking pattern of character convergence between sympatric lineages (Tobias et al. 2013). Our model shows that evolutionary convergence (or stasis maintained by selection) can result, paradoxically, from species being too similar phenotypically to be fully reproductively isolated. This finding defies conventional thinking on the evolutionary effects of cross-species mating, but it appears to account for the variable patterns of character displacement in *Hetaerina* damselflies. If, as our results suggest, selection can favor divergence between some sympatric species and convergence between others within a single genus, broad phylogenetic studies might severely underestimate the influence of species interactions on character evolution. No doubt our model and empirical results will inspire further research on the links between reproductive interference and aggression between species.

Table 1-1. Examples of interspecifically territorial species pairs in which reproductive interference occurs.

Taxon	Species	Location	References
Insects	Speckled wood butterflies (*Pararge xiphia* & *P. aegeria*)	Madeira	Jones et al. 1998
	Seed-eating bugs (*Neacoryphus bicrucis* & *Margus obscurator*)	United States	McLain & Pratt 1999
	Whiteface dragonflies (*Leucorrhinia* spp.)	United States	Singer 1989, 1990
	Mnais damselflies (*Mnais nawai* & *M. pruinosa*)	Japan	Nomakuchi & Higashi 1996
	Beautiful & western demoiselles (*Calopteryx virgo* & *C. xanthostoma*)	Spain	Outomuro 2009
	Beautiful & banded demoiselles (*Calopteryx virgo* & *C. splendens*)	Finland	Tynkkynen et al. 2004, 2006, 2008; Svensson et al. 2007
	River & ebony jewelwings (*Calopteryx aequabilis* & *C. maculata*)	United States	Iyengar et al. 2014
Crustaceans	European & signal crayfish (*Astacus astacus* & *Pacifastacus leniusculus*)	Sweden	Söderbäck 1995)
	Fiddler crabs (*Uca* spp.)	United States	Aspey 1971; Hyatt & Salmon 1977; Booksmythe et al. 2011
Amphibians	Red-cheeked & Northern slimy salamanders (*Plethodon jordani* & *P. glutinosus*)	United States	Nishikawa 1987
	Cope's gray tree & gray tree frogs (*Hyla chrysoscelis* & *H. versicolor*)	United States	Gerhardt et al. 1994; Reichert & Gerhardt 2014
	Daruma pond & dark-spotted frogs (*Pelophylax brevipoda* & *P. nigromaculata*)	Japan	Shimoyama 1999
Birds	Barred & spotted owls (*Strix varia* & *S. occidentalis*)	United States	Gutiérrez et al. 2006
	Indigo & lazuli buntings (*Passerina cyanea* & *P. amoena*)	United States	Baker 1991
	Chiffchaffs & willow warblers (*Phylloscopus collybita* & *P. trochilus*)	Norway	Saether 1983
	Redstarts & black redstarts (*Phoenicurus phoenicurus* & *P. ochruros*)	Czech Republic	Sedlacek et al. 2006
	Eastern & western meadowlarks(*Sturnella magna* & *S. neglecta*)	United States	Lanyon 1979
	Seaside sparrow & short-tailed sparrow (*Ammodramus maritimus* & *A.* sp.)	United States	Post & Greenlaw 1975
	Dusky indigobirds & paradise whydahs (*Vidua purpurascens* & *V. paradisaea*)	Zambia	Payne 1980
	Vinaceous & ring-necked doves (*Streptopelia vinacea* & *S. capicola*)	Uganda	den Hartog et al. 2007
	Hermit & Townsend's warblers (*Setophaga occidentalis* & *S. townsendii*)	United States	Krosby & Rohwer 2010
	Melodious & Icterine warblers (*Hippolais polyglotta* & *H. icterina*)	N. Europe	Secondi et al. 2011
	Pied & collared flycatchers (*Ficedula hypoleuca* & *F. albicollis*)	Sweden	Vallin et al. 2012
	Reed warblers (*Acrocephalus* spp.)	Europe	Catchpole & Leisler 1986; Leisler 1988; Hoi et al. 1991
	Common & thrush nightingales (*Luscinia megarhynchos* & *L. luscinia*)	Europe	Sorjonen 1986
Mammals	Wolves & coyotes (*Canis lupus* & *C. latrans*)	United States	Benson & Patterson 2013
	Lar & pileated gibbons (*Hylobates lar* & *H. pileatus*)	Thailand	Suwanvecho & Brockelman 2012

Table 1-2. Study site locations (in decimal degrees) and *Hetaerina* species present.

Site name	Species 1	Species 2	Latitude	Longitude
Armeria (AR)	*H. americana*	*H. titia*	18.95001	-103.93351
Bonita Creek (BC)	*H. americana*	*H. vulnerata*	32.91627	-109.49282
Castroville (CV)	*H. americana*	*H. titia*	29.33350	-98.86690
Cuetzalapan (CT)	*H. cruentata*	*H. occisa*	18.37100	-95.00148
El Limon (EL)	*H. americana*	*H. cruentata*	21.36673	-104.61673
Laguna Escondida (ES)	*H. sempronia*	*H. occisa*	18.59245	-95.08390
Lampasas (LM)	*H. americana*	—	31.08271	-98.01973
La Palma (PA)	*H. occisa*	*H. titia*	18.55010	-95.06671
Otapa (OT)	*H. occisa*	*H. titia*	18.68339	-96.38350
Pixquiac (PX)	*H. vulnerata*	*H. cruentata*	19.46679	-96.95018
Upper Cuetzalapan (UC)	*H. sempronia*	*H. capitalis*	18.36733	-94.96500

Table 1-3. Comparisons of territorial males' responses to tethered conspecifics and heterospecifics of both sexes.

			Female tethering		Male tethering		
Focal species	Sympatric congener	Site	*n*	*P**	*n*	Statistic†	*P*
H. americana	*H. titia*	AR	—	—	16	$t = 3.611$‡	0.002
H. americana	*H. vulnerata*	BC	18	0.69	16	$t = -0.051$	0.96
H. americana	*H. titia*	CV	24	0.0065	33	$t = 7.78$‡	<0.0001
H. americana	*H. cruentata*	EL	—	—	17	$t = 0.02$‡	0.98
H. cruentata	*H. occisa*	CT	17	0.28	15	$t = 0.71$‡	0.49
H. cruentata	*H. americana*	EL	—	—	10	$t = 0.85$‡	0.42
H. cruentata	*H. vulnerata*	PX	14	0.22	17	$t = 0.68$	0.50
H. occisa	*H. cruentata*	CT	20	1	16	$t = 0.14$‡	0.89
H. occisa	*H. sempronia*	ES	20	0.80	19	$t = 1.32$	0.20
H. occisa	*H. titia*	OT	7	0.0006	39	$t = 7.33$‡	<0.0001
H. occisa	*H. titia*	PA1	64	0.09	54	$V = 1144.5$	<0.0001
H. occisa	*H. titia*	PA2	42	<0.0001	68	$V = 1653$	<0.0001
H. sempronia	*H. occisa*	ES	10	0.37	14	$t = 3.98$	0.002§
H. sempronia	*H. capitalis*	UC	—	—	16	$V = 59$	0.6685
H. titia	*H. americana*	AR	—	—	14	$t = 5.91$‡	<0.0001
H. titia	*H. americana*	CV	22	<0.0001	30	$t = 8.26$‡	<0.0001
H. titia	*H. occisa*	OT	17	<0.0001	23	$t = 6.56$‡	<0.0001
H. titia	*H. occisa*	PA1	38	0.037	19	$V = 4$	0.0004
H. titia	*H. occisa*	PA2	24	<0.0001	22	$V = 8$	0.006
H. vulnerata	*H. americana*	BC	18	0.15	16	$t = 2.42$	0.03§
H. vulnerata	*H. cruentata*	PX	11	1	10	$t = 0.04$	0.97

Sample sizes are the number of males tested with tethered individuals of both species. Dashes indicate where, for logistical reasons, responses to females were not measured.

*Fisher's exact tests; in all cases where $P < 0.05$, males responded more strongly to conspecific females than to heterospecific females.

†Paired t-tests (t) or Wilcoxon paired sign rank tests (V); if $P < 0.05$, males responded more strongly to conspecific males than to heterospecific males, except where noted otherwise.

‡Previously published data (Anderson & Grether 2010).

§Cases in which males responded more strongly to heterospecific males than to conspecific males.

Table 1-4. Variation in the level of interspecific territoriality relative to intraspecific territoriality, inferred from behavioral observations, including all observed fights.

		Number of fights, observed (expected)*				
Site	Year	Species 1	Interspecific	Species 2	χ^2	*P*†
AR	2005‡	10(8)	20(36)	58(44)	12.74	0.002
AR	2008‡	6(2)	8(18)	42(35)	12.63	0.002
BC	2012	31(28)	12(16)	4(2)	2.57	0.28
CT	2006‡	9(7)	2(9)	8(3)	15.78	<0.001
CV	2008 April‡	6(2)	8(18)	42(35)	9.4	0.009
CV	2008 August‡	24(19)	18(30)	18(12)	23.9	<0.001
CV	2012	15(5)	7(25)	39(31)	34.27	<0.001
EL	2008‡	37(30)	7(19)	8(3)	17.9	<0.001
ES	2013	26(17)	12(26)	16(10)	15.61	<0.001
OT	2006‡	13(11)	14(59)	120(77)	58.55	<0.001
OT	2007‡	8(8)	4(25)	41(19)	42.00	<0.001
OT	2010	13(4)	10(60)	244(203)	66.67	<0.001
PA2	2007‡	13(19)	6(24)	32(8)	88.18	<0.001
PA1	2011	87(133)	120(136)	97(35)	126.44	<0.001
PA2	2011	20(22)	21(35)	29(14)	23.06	<0.001
PA1	2012	16(16)	11(21)	17(7)	17.46	<0.001
PA2	2012	30(19)	14(41)	38(22)	35.55	<0.001
PX	2010	38(40)	23(24)	7(3)	4.46	0.10
PX	2011	25(22)	13(15)	2(3)	0.64	0.76

Species numbers follow Table 1-2.

*Expected number of fights generated through binomial expansion of the relative proportiosn of each species at the site. When expected values were < 5, we calculated *P* values using Monte Carlo simulations.

†In all cases with *P* < 0.05, the rate of interspecific fighting was reduced relative to intraspecific fighting.

‡Previously published data (Anderson & Grether 2011).

Table 1-5. Variation in the level of interspecific territoriality relative to intraspecific territoriality, inferred from behavioral observations, including only escalated fights.

		Number of fights, observed (expected)*				
Site	Year	Species 1	Interspecific	Species 2	χ^2	*P*†
BC	2012	19(18)	8(10)	2(1)	0.74	0.69
CV	2012	8(2)	2(11)	17(14)	22.78	< 0.001
ES	2013	23(14)	9(21)	11(8)	13.93	<0.001
OT	2010	6(2)	0(33)	142(113)	44.83	<0.001
PA1	2011	69(101)	90(102)	70(26)	82.64	<0.001
PA2	2011	15(17)	15(28)	26(11)	27.32	<0.001
PA1	2012	11(9)	4(13)	11(4)	16.33	<0.001
PA2	2012	26(14)	9(29)	23(15)	29.11	<0.001
PX	2010	15(16)	7(9)	4(1)	6.43	0.041
PX	2011	13(11)	6(8)	1(1)	0.62	0.78

Species numbers follow Table 1-2.

*Expected number of fights generated through binomial expansion of the relative proportion of each species at the site. When expected values were < 5, we calculated *P* values using Monte Carlo simulations.

†In all cases with $P < 0.05$, the rate of interspecific fighting was reduced relative to intraspecific fighting.

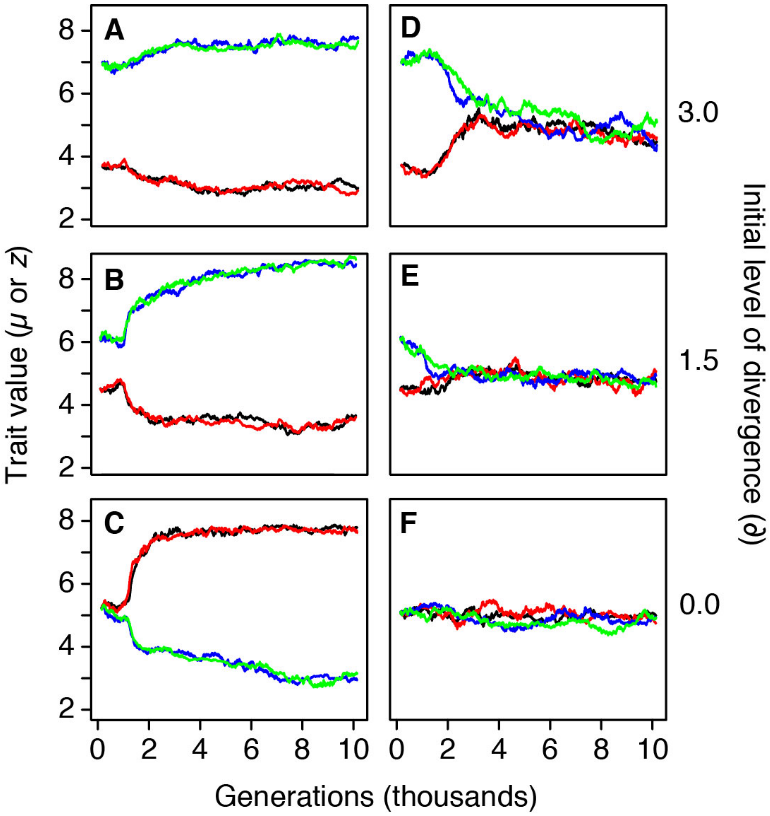

Figure 1-1. Simulations showing the effects of reproductive interference on the evolution of interspecific aggression. (*A*-*C*) illustrate the usual outcome of secondary contact between species with low levels of reproductive interference while (*D*-*F*) represent cases with higher levels of reproductive interference. Plotted values: mean of the male trait z (black, species 1; blue, species 2) and mean of the competitor recognition template μ (red, species 1; green, species 2). Generation 0 is the time of secondary contact. In the examples shown here, $d = 0.1$ (*A*-*C*) and $d = 0.33$ (*D*-*F*).

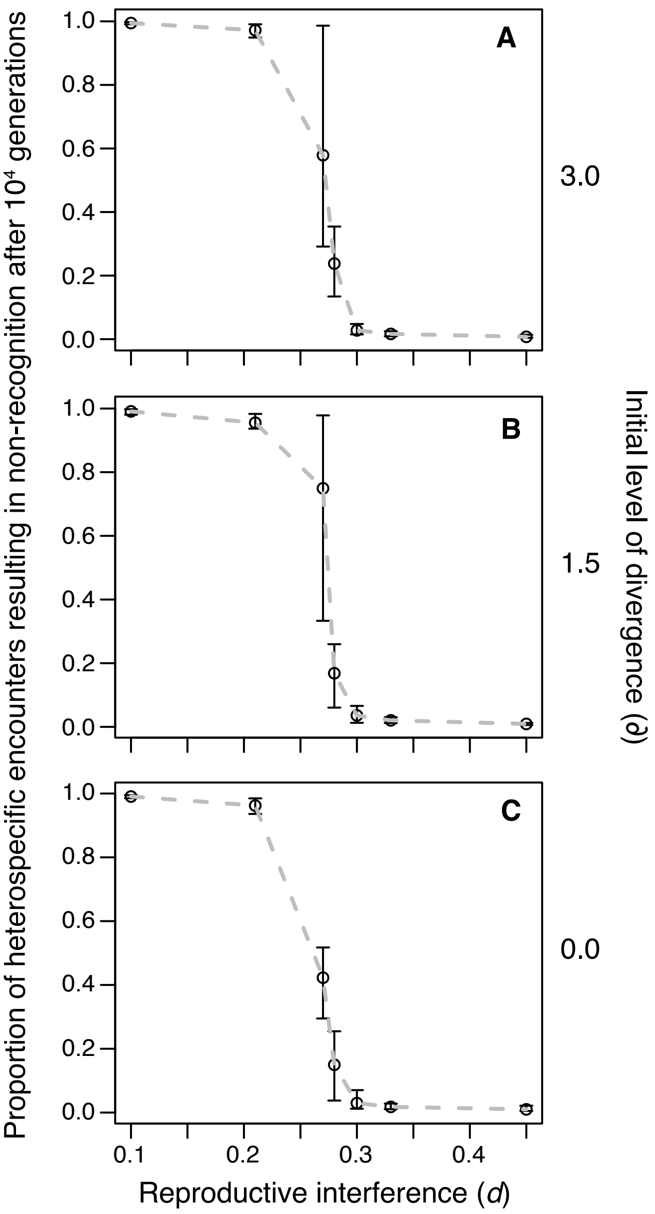

Figure 1-2. (*A*-*C*) illustrate the proportion of heterospecific encounters resulting in mutual non-recognition 10000 generations after secondary contact begins as a function of the intensity *d* of reproductive interference. Except when $d = 0.1$ and $\partial = 0$, open circles represent the average of 15 simulation runs, and the ends of the error bars represent the 5th and 95th percentiles for each set of runs. When $d = 0.1$ and $\partial = 0$, simulations resulting in the loss of territoriality were excluded.

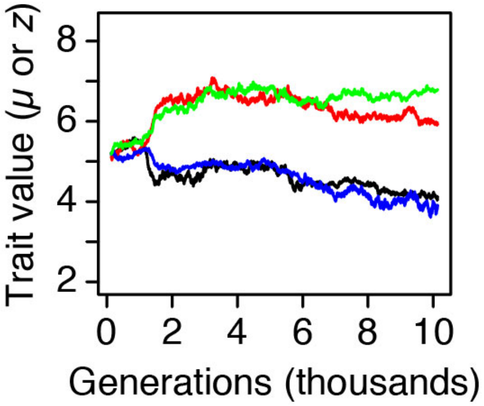

Figure 1-3. Example of a simulation in which intraspecific territoriality was lost as the species diverged from each other. In this and all other cases in which territoriality was lost, the species had the same mean values of z and μ at the time of secondary contact ($\partial = 0$) and reproductive interference was minimal ($d = 0.1$). Instead of tracking each other within species, the male trait z and central location of the recognition parameter μ diverged from each other within (as well as between) species. Territoriality was lost in 5 of 15 simulations with $\partial = 0$ and $d = 0.1$, but 0 of 255 simulations with $\partial > 0$ or $d > 0.1$. The color scheme matches Fig. 1-1.

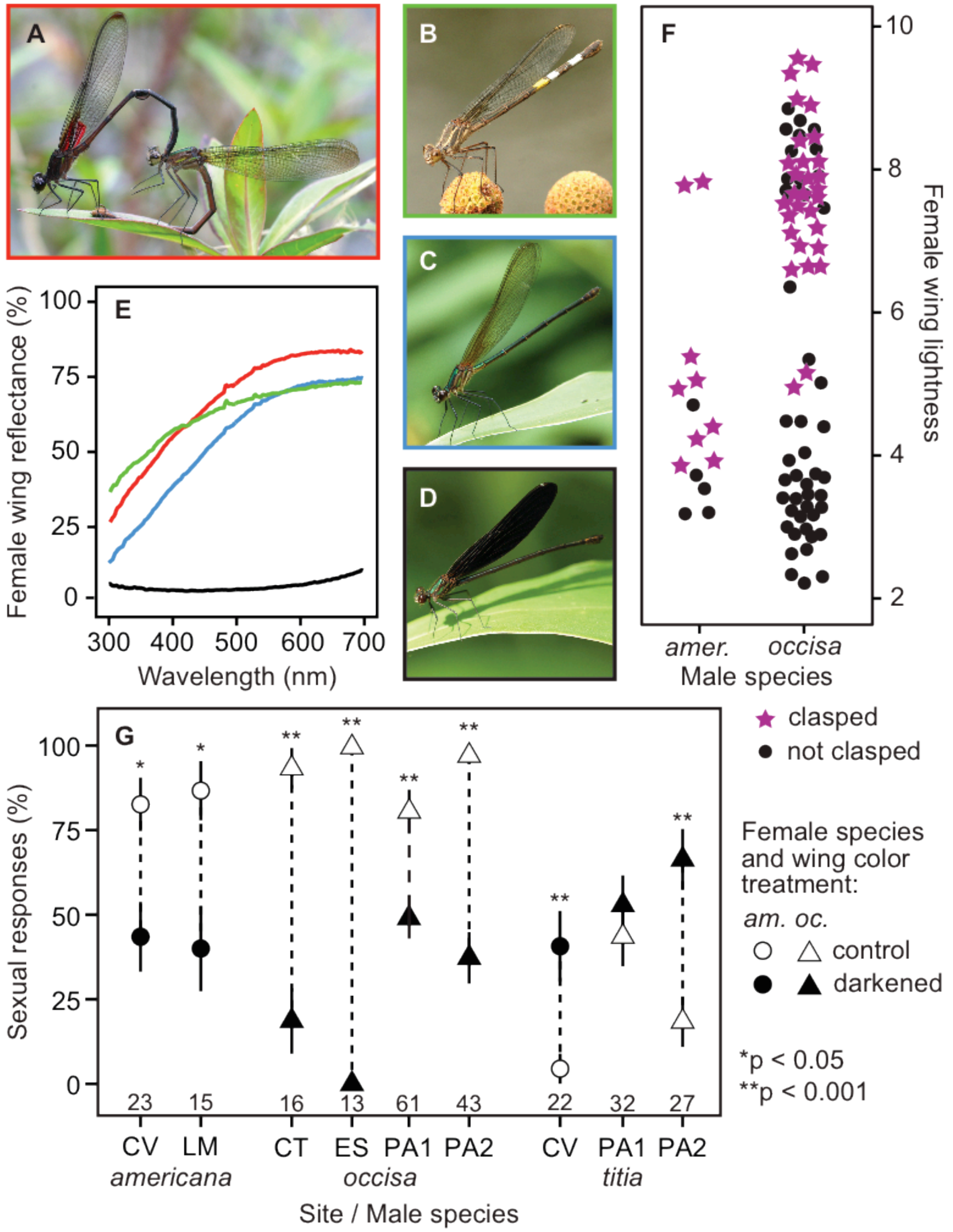

Figure 1-4. Female wing coloration and male sexual responses. Photographs females of four *Hetaerina* species: (*A*) *H. cruentata* (mating), (*B*) *H. americana* (marked for identification), (*C*) *H. occisa,* (*D*) *H. titia*. Sample reflectance spectra of female wings (*E*), with line colors matching the frames of the respective species' photographs (*A*-*D*). Wing lightness (*F*) affects whether *H. titia* females elicit a sexual response (stars) or not (circles) from *H. americana* (two-sided Mann-Whitney test, $n = 14$, $P = 0.01$) and *H. occisa* males ($n = 77$, $P < 0.0001$). Female *H. americana* and *H. occisa* with experimentally darkened wings (*G*) elicit fewer sexual responses from conspecific males and more sexual responses from *H. titia* males than controls (some error bars [SEM] are smaller than the symbols; sample sizes of males tested are given above site labels; significance levels are from Fisher's exact tests). For study site locations, see Table 1-2.

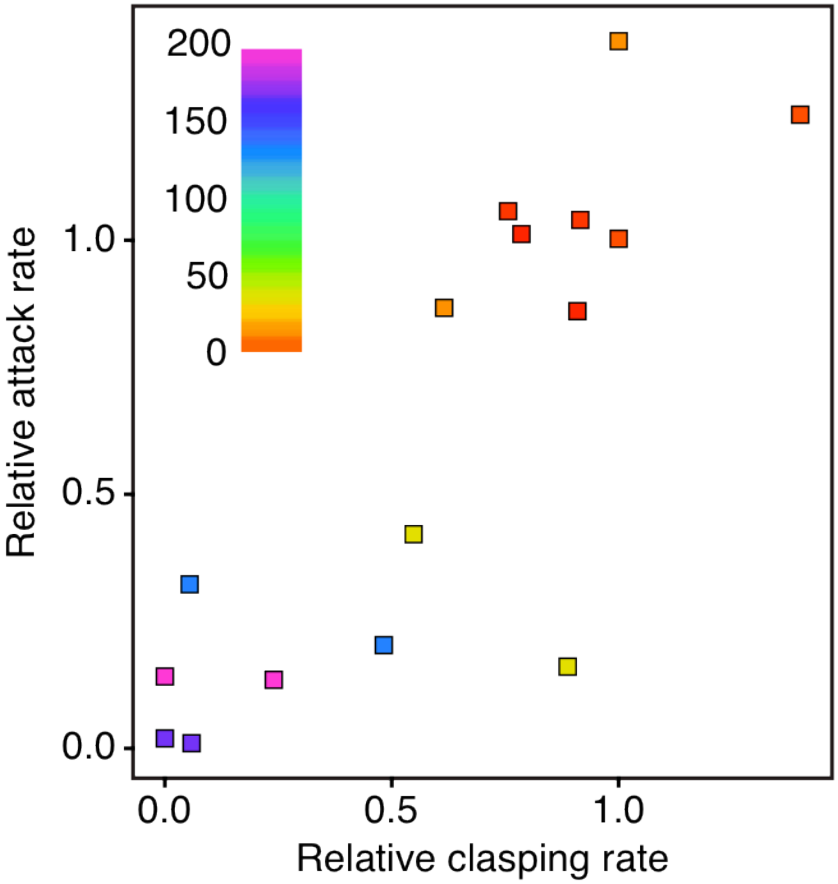

Figure 1-5. Evidence for a link between reproductive interference and interspecific aggression in *Hetaerina* damselflies. Relative attack rate (Y axis): the number of attacks elicited by heterospecific male intruders divided by the number of attacks elicited by conspecific male intruders. Relative clasping rate (X axis): the proportion of tethered females that elicited sexual responses in trials with heterospecific males divided by the proportion of tethered females that elicited sexual responses in trials with conspecific males. Color scale (Z axis): species differences in female wing lightness, as measured by reflectance spectrometry. Each point represents a population at a sympatric site. All three axes are strongly correlated: X vs. Y: mean ± SD Spearman $\rho = 0.84 \pm 0.11$, $P < 0.01$; X vs. Z: $\rho = -0.77 \pm 0.09$, $P < 0.01$; Y vs. Z: $\rho = -0.80 \pm 0.07$, $P < 0.01$.

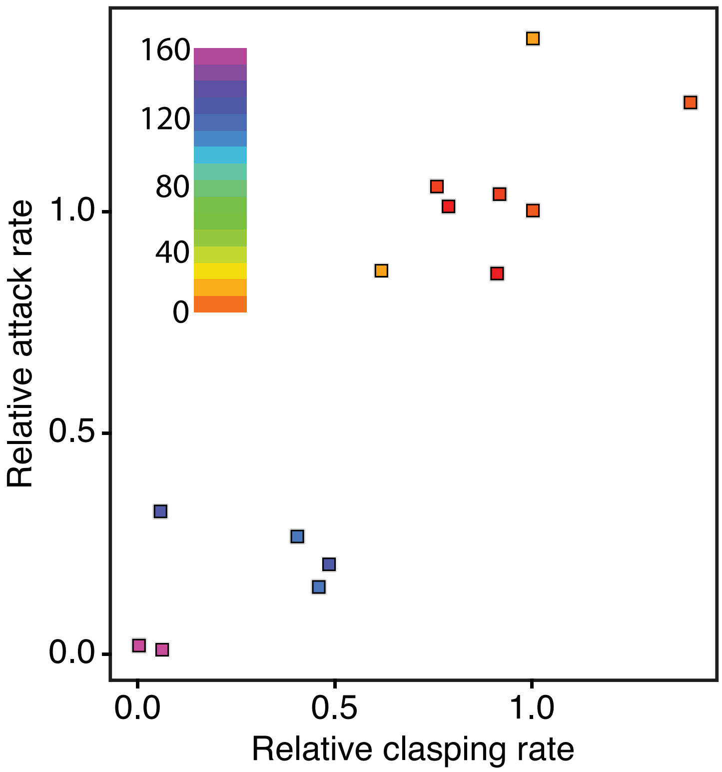

Figure 1-6. Evidence for a link between reproductive interference and interspecific aggression in *Hetaerina* damselflies. This alternative version of Fig. 1-5 shows that the results remain qualitatively unchanged if data from the early (PA1) and late (PA2) season at the La Palma site are pooled (relative clasping rate vs. relative attack rate, mean ± s.d. Spearman $\rho = 0.87 \pm 0.07$, $P < 0.01$; relative clasping rate vs. lightness difference, mean ± s.d. $\rho = -0.75 \pm 0.10$, $P < 0.01$

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Chapter 2. Phenotypic plasticity in wing coloration affects species recognition in rubyspot damselflies (*Hetaerina* spp.)

Abstract

The assumption that individuals must be able to distinguish conspecifics from heterospecifics is foundational for many classical evolutionary theories, which posit that the inability to do so would impede coexistence or speciation. Existing empirical studies of species recognition largely treat phenotypes as species recognition cues that are fixed, yet in many taxa, these signals exhibit substantial phenotypic plasticity. Here, we document a seasonal polyphenism in the degree of dark wing pigmentation in smoky rubyspot damselflies (*Hetaerina. titia*)—a shift so pronounced that it led early researchers to treat different forms of *H. titia* as separate species. We then demonstrate that this seasonal polyphenism impacts species recognition at a site with *H. titia* and a sympatric congener (*H. occisa*). When light-phase *H. titia* forms, which resemble *H. occisa* individuals, are more abundant, reproductive interference and interspecific fighting are common. Shifts in species recognition are due to both the shift in *H. titia* wing coloration and a shift in mate recognition in both species and competitor recognition in *H. occisa*. This research highlights the importance of understanding how plasticity in signal phenotypes influences the evolutionary dynamics of interspecific behavioral interactions and suggests that phenotypic plasticity may, in some cases, impede interspecific divergence.

Introduction

Biologists have long argued that the ability of species to distinguish between members of their own species and heterospecifics in reproductive and/or agonistic contexts is paramount to species coexistence (Wallace 1889; Fisher 1930; Dobzhansky 1955; Lorenz 1962; Mayr 1963; Gröning and Hochkirch 2008). Species recognition may evolve because selection favors trait variants that facilitate species recognition (Fisher 1930; Dobzhansky 1937; Brown and Wilson 1956) or as a byproduct of divergence from selection acting in other contexts (Mayr 1963; West-Eberhard 1979, 1983). In spite of the perceived importance of species recognition, evidence that species do distinguish between conspecifics and heterospecifics is surprisingly mixed, and variation in the efficacy of species recognition mechanisms remains largely unexplained (Ord and Stamps 2009; Ord et al. 2011).

Many studies of species recognition treat the signal phenotypes that individuals use as species recognition cues as static entities, but this assumption may not be warranted. Indeed, many traits known to influence between-species interactions exhibit varying degrees of phenotypic plasticity (Table 2-1). Moreover, given that traits targeted by sexual selection are often involved in between-species discrimination (Ryan and Rand 1993; Andersson 1994; Price 1998) and that some classical theories of sexual selection posit that selection should favor phenotypic plasticity in these traits (e.g., as condition dependent handicap traits, Zahavi 1975), it is likely that there are many other examples of signal plasticity as yet unstudied in the context of species recognition.

The evolutionary consequences of plasticity in species recognition traits have largely been ignored, in spite of recent interest in understanding the role of plasticity in the context of speciation and/or reproductive isolation (Ingleby et al. 2010; Pfennig et al. 2010; Rodríguez et al. 2013). These investigators have generally focused on the influence of plasticity on intraspecific rather than interspecific interactions (but see Pfennig and Pfennig 2012). Furthermore, although the majority of researchers interested in between-species interactions have focused on reproductive isolation, species that come into secondary contact may also overlap in traits that mediate interference competition, resulting in wasteful between-species aggression if those species do not compete for resources (Grether et al. 2009, 2013). Given the role of species recognition in classical evolutionary theoretical frameworks, understanding how plasticity in signaling traits affects species recognition in both reproductive and agonistic contexts is an important and currently unaddressed problem in the field.

In this study, we document a seasonal polyphenism, a type of phenotypic plasticity whereby traits vary within years due to changing environmental inputs, in a trait previously shown to influence species recognition in *Hetaerina titia* damselflies (Anderson and Grether 2010a, 2011). *H. titia* males and females vary in the amount of dark pigmentation in their wings, historically leading investigators (e.g. Kellicott 1899; Calvert 1908) to refer to light-phase and dark-phase forms as separate species (*H. bicolor* [Burmeister] and *H. titia* [Drury], respectively) until they were synonymized in 1912 (Williamson 1912).

We then present a series of experiments conducted on males of *H. titia* and a sympatric congener (*H. occisa*) to test the hypothesis that the documented seasonal polyphenism affects species recognition. *H. occisa* males, like other *Hetaerina* spp., have clear wings with a red basal wingspot, and *H. occisa* females have light amber wings. As light-phase forms of both sexes of *H. titia* look similar to *H. occisa*, we predicted species recognition to be weaker in tests with light-phase forms compared to dark-phase forms. We first used tethered presentations of unmanipulated conspecifics and heterospecifics to investigate species recognition. Next, we experimentally confirmed the role of wing pigmentation by using phenotype manipulation. Finally, to test the hypothesis that shifts in species recognition are due to *H. occisa* males learning to distinguish between heterospecifics and conspecifics when dark-phase *H. titia* individuals are abundant, we compared the responses to heterospecifics of control males and males experimentally deprived of the opportunity to learn species recognition.

Methods

Seasonal polyphenism of H. titia *wings*

Hetaerina titia males have basal red spots on their forewings with varying amounts of black pigmentation on their fore- and hindwings (Fig 2-2a-c), and females have amber colored wings that vary in opacity (Fig 2-3a,b). Thus, analyses of the proportion of wing surfaces with black pigment in photographs best measure variation in male wing phenotypes (cf. (Anderson and Grether 2010b)), whereas variation in female wing coloration is best characterized using reflectance spectroscopy. As with other species of *Hetaerina* (Grether 1996), the wing phenotypes of adult *H. titia* are fixed upon maturation (Fig. 2-1).

We collected data on *H. titia* male wing coloration on several different visits from 2005 to 2012 to three sites during the peak emergence period (in Texas, U.S.A, and in Veracruz and Colima, Mexico, see Table 2-2). At each site, we set up a transect marked at 1 m intervals and collected all adult individuals found along these transects. Most visits lasted for a week or less, and we treat the entire visit as a categorical variable for these data in subsequent analyses. However, on three occasions we spent more than 30 days at a site, which permitted us to address maturation date at a finer scale. For these sites, we used the date an individual was photographed as a proxy for the date of their maturation, which is a good approximation since we vigilantly captured and marked any new individuals along the transect during the entire length of the visit. To quantify the relative proportion of black pigment on the wings of male *H. titia*, we photographed the left forewing and hindwing of individuals using a digital camera (Canon 10D or 20D) with a 100 mm macro lens and a dual flash (Canon MT-24EX). We used NIH Image to manually measure the proportion of each wing with black pigmentation on all marked individuals at each study site including data from images of tethered when available (see Table 2-2).

To quantify the coloration of female *H. titia* wings, we measured reflectance spectra of individuals' wings during the 2011 visit to La Palma, in Veracruz, Mexico (Table 2-2). We used an Ocean Optics USB-2000 spectrometer equipped with a pulsed xenon light source (Ocean Optics PX-2) oriented at 45 degrees relative to the wing surface to eliminate glare and measured percent reflectance at each wavelength in relation to a Labsphere certified reflectance standard using Ocean Optics' OOIBase32 software. When taking readings, we placed the wings on the reflectance standard behind the wings. Wing color measurements include both light reflected off the wings and light transmitted through the wings. We averaged three measurements each for the base, middle, and tip of forewings and hindwings. From these averaged spectra, we calculated lightness (*L*) as the sum of percent reflectance at 2 nm intervals from 300 to 700 nm. We measured the reflectance of a sample of adult females captured without regard for their wing phenotypes near the beginning (*n* = 30, 3/25-4/12/2011) and end (*n* = 26, 5/30-6/10/2011) of our visit.

Species recognition at a sympatric H. occisa *and* H. titia *site*

The light-phase phenotype that *H. titia* males and females exhibit early in the peak emergence season (e.g., Figs. 2-2a, 2-3b) is similar to that of a heterospecific congener, *H. occisa,* with which they are sympatric at a river near La Palma in Veracruz, Mexico. Both sexes of *H. occisa* appear more similar to light-phase *H. titia* forms than to dark-phase forms. To assess the impact of the seasonal polyphenism in *H. titia* wing on interspecific behavioral interactions, we carried out observations and experiments on *H. occisa* and *H. titia* at a river transect (200-300 m) in La Palma (abbreviated PA, hereafter) between March and June 2011 and 2012 (Table 2-2). We captured nearly all individuals in the study area with aerial nets and marked individuals with unique IDs using DecoColor color paints (Anderson et al. 2011).

To compare the responses of *H. occisa* males to light-phase and dark-phase *H. titia* individuals, we conducted our experiments in two time periods, referred to as “early” and “late” hereafter. In the early season (corresponding to 3/23-5/5/2011 and 4/2-4/26/2012), most *H. titia* were light-phase morphs, whereas in the late season (5/30-6/15/2011 and 5/17-6/11/2012), most *H. titia* individuals were dark-phase forms (Figs. 2-2d,e, 2-3c). *Hetaerina* have an average adult lifespan of approximately 2 weeks (Grether 1996), and we only included the responses measured in the early season from the relatively few individuals that we did observe in both periods (< 3 males per species).

Behavioral observations and naturally occurring fight frequencies

We conducted behavioral censuses to determine which males were territorial and to record the frequency of naturally occurring intra- and interspecific fights. During each behavioral census, an observer recorded the location of each male along the transect to the nearest 0.1 m. When an observer witnessed a fight, the location, species involved, ID of individuals (if marked), and the intensity of a fight were recorded. For analyses, we considered escalated fights to be those exhibiting prolonged two-way back-and-forth or “circle” fights. We identified unique fights of each type (*H. occisa* vs. *H. occisa*, *H. occisa* vs. *H. titia*, *H. titia* vs. *H. titia*) from the behavioral censuses. Prior to analysis, multiple recorded bouts of fighting between the same two males on the same day were reduced to a single fight. For fights involving unmarked or unidentified individuals, we only recorded one fight within a 5 m radius per day.

To determine if there was a shift in the relative number of interspecific fights as a result of changing abundance of light-phase and dark-phase *H. titia* forms, we first generated expected values for the number of fights of each type from a binomial expansion of the proportion of males of each species based on the marking record (Anderson and Grether 2011). We then tested whether the deviations between observed and expected values were more extreme in the late season (i.e., reflecting a decrease in interspecific aggressive interference) using Pearson's χ^2 test.

Competitor recognition

To determine if the seasonal polyphenism in *H. titia* male wing phenotypes affects species recognition in an agonistic context, we conducted territory intrusion experiments to measure responses to conspecific and heterospecific intruders. The subjects of the experiment were established territory holders. We considered males to be holding a territory when they were observed two consecutive days within a 3 m radius, perched near the bank of the river (Anderson and Grether 2010a).

We presented territory holders with live conspecific and heterospecific males tethered to a transparent thread to measure aggression. Each trial lasted two minutes, with at least a five-minute inter-trial interval (Anderson and Grether 2010a). During the experimental trials, we recorded the behavior of the territorial males, including the amount of time spent chasing the tethered male and the number of slams (defined as charges directed toward the tethered male) and grabs (defined as prolonged physical contact with the tethered male). We systematically varied the presentation order of conspecific and heterospecific males in a balanced manner. Cases in which we were unable to present all of the treatments or in which the territory holder did not chase either tethered intruder for at least 60 s were excluded from the analysis, as we considered these males to not be defending the site. When possible, we aimed to test these males on a subsequent day within the season. *H. titia* males' responses to tethered intruders were measured in 2012, and *H. occisa* males' responses were measured in both 2011 and 2012.

Shifts in species recognition can result from changes in signal phenotypes themselves (i.e., resulting from the seasonal polyphenism) and/or from changes in the underlying neural template that individuals use to distinguish heterospecifics from conspecifics (Grether et al. 2009; Grether 2011). To determine whether *H. occisa* male competitor recognition changes due to the relative frequency of light-phase and dark-phase *H. titia* forms, we measured *H. occisa* males' responses in each season to tethered conspecific male intruders with (1) wings blackened to resemble those of *H. titia* males (black ink [Prismacolor PM-98] above the wingspot on the outside surface of the hindwing), (2) wings half-blackened (black ink immediately above the wingspot, halfway to the tip, and clear ink [Prismacolor PM-121] to the tip), and (3) control males (clear ink above their wingspots). Similarly, we tested *H. titia* males' responses in each season to (1) conspecific males with blackened wings (as above), (2) control, conspecific males (clear ink, as above), (3) *H. occisa* males with blackened wings, and (4) control *H. occisa* males. In all cases, tethered males were assigned to treatments at random with respect to their own phenotypes. In the late season, when dark-phase *H. titia* forms are more prevalent, we aimed to select males with similar extents of wing pigmentation for the control and darkened treatments.

Mate recognition

To determine if the seasonal shift in *H. titia* female wing phenotypes affects male mate recognition, we conducted experiments to measure males' responses to conspecific and heterospecific tethered females. *Hetaerina* matings begin when a male clasps a female's intersternite (Garrison 1990), and proceed without any courtship ritual. We considered an abdomen curl or attempted clasp (defined as a male approaching a female with his abdomen moving toward her intersternite), or successful clasp to be a sexual response. We varied the presentation order of conspecific and heterospecific females. Each presentation lasted 5 s, or until the male returned to his perch, whichever came last. If the focal male clasped the female during her first presentation, we ended the trial; if not, we presented her for another 5 s. In our analyses, we only included data from females presented to males who responded positively to at least one tethered female. Both species were tested both years.

To determine whether a shift in reproductive interference is solely the result of the shift in *H. titia* female wing coloration or if there is also a shift in male mate recognition, we presented territorial males in each season with unmanipulated *H. occisa* females and with *H. occisa* females with wings experimentally darkened to resemble *H. titia* females of the darker, late season form. We darkened experimental females' wings by coloring the hindwing from the base to the tip with a gray marker (Prismacolor PM-107), and the forewing from base to the nodus with a gray marker and from the nodus to the tip with a sepia marker (Prismacolor PM-62). We chose these colors because their reflectance spectra best approximated the late season wing coloration of female *H. titia* among the markers available. We used the same criteria for male sexual responses and inclusion in analyses as above.

Mechanisms of seasonal shift in species recognition

A shift in competitor recognition could result from learning; male *H. occisa* may learn to discriminate between competitors and non-competitors when *H. titia* wings appear sufficiently distinct from those of conspecifics, resulting in aggression toward *H. titia* in the early season and not in the late season when *H. titia* males are mostly dark-phase forms. Likewise, a shift in mate recognition may result from a shared developmental mechanism of responses (e.g., via pleiotropy) to heterospecific males. To investigate these possibilities, we conducted a removal experiment of *H. titia* males in the late season to deprive *H. occisa* males of the opportunity to learn to distinguish conspecifics and heterospecifics (e.g., by associating black wings with heterospecific fighting style, lack of mate competition, etc.). We removed all *H. titia* males that we found along a transect located ~140 meters above the transect used for other experiments and translocated them to a site away from both the experimental and the control transects. We conducted the same male and female tethering tests described above on territory holders in the experimental transect.

Similarly, if trial-and-error learning shapes male mate recognition (e.g., if *H. occisa* males learn that dark winged females' intersternite morphology does not correspond to conspecifics, or if *H. occisa* males associate dark wings with a high probability of rejection), *H. occisa* males that cannot clasp females should not gain sufficient experience to discriminate between conspecific and heterospecifics when *H. titia* females exhibit the dark-phase phenotype. To test this hypothesis, we assigned immature males (i.e., males with brownish wing spots, ≤ 3 days post-emergence) to either control or removal treatments, wherein we removed their cerci and paraprocts (thereby disabling them from successfully clasping females). We then conducted female-tethering tests as described above once these males matured and began to defend territories.

Statistical analyses

We conducted all statistical analyses in R (R Core Team 2013). We ran repeated-measure analyses of responses to tethered individuals by including a random intercept term for the ID of the territorial male. To model the count of attacks and proportion of time spent chasing tethered males, we used the glmmADMB package (Fournier et al. 2012; Skaug et al. 2012) to run generalized mixed effect linear regressions with negative binomial structures for models of attack counts and Gaussian structures for models of proportion of time chasing. In some instances where males did not respond to a particular treatment, we used zero inflation models when they had higher likelihoods than standard negative binomial models. To model the response of males to tethered females, we performed mixed effect logistic regression analyses using glmmADMB, but in a few instances where mixed effect models did not converge because of complete separation (i.e., males never responded to a particular tethered treatment), we modeled clasping using the bayesglm() function in the package arm (Gelman and Su 2014).

For experiments conducted in both 2011 and 2012, if we determined that there was no effect of year on species recognition, we pooled results across both years to increase statistical power. In analyses from 2012, when we determined that there were no effects on *H. occisa* territory holders of the experiment conducted on the experimental transect (see *Mechanism of seasonal shift in species recognition*), we grouped data from both transects for subsequent analyses of competitor and mate recognition.

Results

Seasonal polyphenism of H. titia *wings*

Adult *H. titia* males emerging later in the period of peak emergence have more black pigmentation on their wings than adults emerging earlier (Fig. 2-2a-c). This was true both within prolonged visits (Fig. 2-2d-f, effect of date in GLMs of proportion of black pigment, all models with log links, PA 2011 hindwing *d.f.* = 337, $t = 28.23$, $p < 0.001$, forewing *d.f.* = 335, $t = 25.6$, $p < 0.001$, total proportion *d.f.* = 331, $t = 27.61$, $p < 0.001$; PA 2012 hindwing *d.f.* = 179, $t = 13.74$,

$p < 0.001$, forewing *d.f.* = 179, $t = 12.67$, $p < 0.001$, total proportion *d.f.* = 178, $t = 13.58$, $p < 0.001$; CV 2013 hindwing *d.f.* = 146, $t = 9.41$, $p < 0.001$, forewing *d.f.* = 145, $t = 12.55$, $p < 0.001$, total proportion *d.f.* = 145, $t = 12.17$, $p < 0.001$) and across shorter visits to the same study sites (Fig. 2-2g,h, effect of visit in one-way ANOVAs of proportion of wing with black pigmentation, AR hindwing *d.f.* = 3, 268, $F = 272.5$, $p < 0.001$, forewing *d.f.* = 3, 265, $F = 42.91$, $p < 0.001$, total proportion *d.f.* = 3, 265, $F = 216.3$, $p < 0.001$, CV hindwing *d.f.* = 2, 204, $F = 87.4$, $p < 0.001$, forewing *d.f.* = 2, 203, $F = 69.72$, $p < 0.001$, total proportion *d.f.* = 2, 202, $F = 85.22$, $p < 0.001$).

Likewise, females emerging later in the year had darker wings than females emerging earlier (Fig. 2-3a-c, main effect of season in a repeated-measures ANOVA of total wing lightness with season and wing patch d.f. = 1, $F = 407$, $p < 0.001$).

Behavioral observations

In both years, the ratio of the observed:expected number of naturally occurring interspecific fights decreased from the early to the late season, whether including all fights or restricting the analysis to only escalated fights (Table 2-3). Additionally, the relative proportion of naturally occurring conspecific and heterospecific fights shifted between seasons each year such that a larger proportion of fights were intraspecific in the late season (Table 2-3).

Competitor recognition

(a) H. occisa *males*

In the competitor recognition experiments, *H. occisa* males discriminated more strongly between conspecifics and heterospecifics in the late season, when dark-phase *H. titia* forms were more abundant, than in the early season (Fig. 2-4a, mixed effect negative binomial model of attack count [slams + grabs], early season *n* males = 53, $z = -5.26$, $p < 0.001$, late season *n* males =50, $z = -7.86$, $p < 0.001$, species*season interaction, *n* males = 103, $z = -6.11$, $p < 0.001$, main effect of season $z = 3.02$, $p = 0.0025$; mixed effect linear model of the proportion of time spent chasing tethered males, early season *n* males = 53, $z = -4.91$, $p < 0.001$, late season *n* males = 50, z = -18.7, $p < 0.001$, species*season interaction, *n* males = 103, $z = -7.13$, $p < 0.001$, main effect of season $z = 1.61$, $p = 0.11$). There was no difference between years in these relationships (all three-way species*season*year interaction terms NS).

H. occisa males shifted from responding equally to all conspecific males, regardless of their color treatment, in the early season to responding less aggressively toward blackened males in the late season after the shift in *H. titia* wing phenotypes (Table 2-4). The behavioral shift was less evident in 2012 than in 2011 (Table 2-5, Fig. 2-5a,b; treatment*year interaction in model of attacks, *n* males = 103, $z = 3.45$, $p < 0.001$). In 2011, territorial *H. occisa* males directed relatively fewer attacks toward experimentally manipulated males with completely blackened wings than toward control males in the late season than in the early season (Table 2-5, Fig. 2-5a). In 2012, this only appeared as a nonsignificant trend in the data (Table 2-5, Fig. 2-5b). The proportion of time males spent chasing tethered intruders was affected by neither the tethered male treatment nor the season (Table 2-6).

(b) H. titia *males*

H. titia males were more aggressive toward conspecific males than toward *H. occisa* males in both seasons and there was no seasonal shift in the magnitude of heterospecific aggression (Fig. 2-4b, mixed effect negative binomial zero inflation model of attack count [slams + grabs], early season *n* males = 18, $z = -5.95$, $p < 0.001$, late season *n* males = 8, $z = -5.0$, $p <$

0.001, species*season interaction, n males = 26, $z = -0.38$, $p = 0.70$, main effect of season $z = 0.62$, $p = 0.54$; mixed effect linear model of the proportion of time spent chasing tethered males, early season n males = 18, $z = -6.61$, $p < 0.001$, late season n males = 8, $z = -2.37$, $p = 0.018$, species*season interaction n males = 26, $z = 0.22$, $p = 0.83$, main effect of season $z = -0.27$, $p = 0.79$).

H. titia males were more aggressive toward *H. occisa* males with experimentally blackened wings than toward control heterospecific males, a difference that remained consistent in both the early and late season although they were more aggressive overall in the late season (Fig. 2-5c, mixed effect negative binomial model of attack count toward heterospecifics, early season treatment effect, n males = 18, $z = -3.56$, $p <0.001$; late season treatment effect n males = 8, $z = -0.59$, $p = 0.55$; season*treatment interaction n males = 26, $z = 0.84$ $p = 0.40$, main effect of season $z = -0.66$, $p = 0.51$; linear model of the proportion of time chasing tethered males, early season treatment effect, n males = 18, $z = -4.49$, $p < 0.001$; late season treatment effect, n males = 8, $z = -0.23$, $p = 0.82$; season*treatment interaction n males = 26, $z = 1.81$, $p = 0.07$, main effect of season $z = -2.34$, $p = 0.019$). *H. titia* males did not direct more attacks toward conspecific males with experimentally blackened wings than toward control conspecific males, although they did chase blackened males for longer, which also did not vary across seasons (Fig. 2-5c, negative binomial model of attack count toward tethered conspecifics, early season treatment effect n males = 18, $z = 1.42$, $p = 0.16$; late season treatment effect n males = 8, $z = 0.67$, $p = 0.51$; season*treatment interaction n males = 26, $z = 0.68$, $p = 0.50$, main effect of season $z = 0.17$, $p = 0.87$; linear model of the proportion of time chasing tethered males, early season treatment effect n males = 18, $z = 2.98$, $p = 0.0029$; late season treatment effect n males =

8, $z = 2.52$, $p = 0.012$; season*treatment interaction $n = 26$, $z = 0.25$, $p = 0.81$, main effect of season $z = -0.31$, $p = 0.75$).

Mate recognition

(a) H. occisa *males*

In our mate recognition experiment, *H. occisa* and *H. titia* males shifted from responding sexually (i.e., clasping or attempting to clasp) to heterospecific and conspecific females in the early season to only responding sexually to conspecific females in the late season (*H. occisa*: Fig. 2-6a mixed effect logistic regression of clasp [yes = 1, no = 0], early season main effect of species n males = 63, $z = -2.30$, $p = 0.022$, late season main effect of species n males = 42, $z = -5.19$, $p < 0.001$, species*season interaction n males = 105, $z = -4.37$, $p < 0.001$, main effect of season $z = 2.23$, $p = 0.025$). There was no effect of the year on the outcome of the analyses (species*season*year interaction NS).

Male *H. occisa* responded sexually more often to unmanipulated conspecific females than to conspecific females with darkened wings in both seasons, but discrimination was more pronounced in the late season (Fig. 2-6c, mixed effect logistic regression of clasp [yes = 1, no = 0], early season main effect of treatment n males = 61, $z = 3.84$, $p < 0.001$, late season main effect of treatment n males = 43, $z = 4.49$, $p < 0.001$, treatment*season interaction n males = 104, $z = 2.16$, $p = 0.03$, main effect of season $z = -1.04$, $p = 0.30$). There was no effect of year on the response of *H. occisa* males to the female wing darkening experiment (year*season*treatment interaction NS).

(b) H. titia *males*

Similarly, *H. titia* males shifted from responding equally to *H. titia* and *H. occisa* females in the early season to only responding sexually to conspecific females in the late season. (Fig. 2-6b, mixed effect logistic regression of clasp [yes = 1, no = 0], early season main effect of species, n males = 38, $z = 1.83$, $p = 0.067$, late season main effect of species n males = 24, $z = 3.76$, $p < 0.001$, species*season interaction, n males = 62, $z = 2.76$, $p = 0.0058$, main effect of season $z = -1.55$, $p = 0.12$). This relationship was independent of the year in which measurements were taken analyses (all species*season*year interactions NS).

H. titia males did not discriminate between experimentally blackened *H. occisa* females and unmanipulated *H. occisa* females in the early season, but they clasped blackened females more often than unmanipulated females in the late season (Fig. 2-6d, pooled years: mixed effect logistic regression of clasp [yes = 1, no = 0], early season main effect of treatment n males = 32 , $z = -0.25$, $p = 0.8$, late season main effect of treatment n males = 26, $z = -3.21$, $p = 0.0014$, treatment*season interaction n males = 58, $z = -2.38$, $p = 0.017$, main effect season $z = 1.17$, $p = 0.242$). The same trends were seen in both years but the strength of the relationship differed between years (season*year interaction n males = 58, $z = -1.99$, $p = 0.047$; 2011: early season main effect of treatment n males = 9, $z = -0.48$, $p = 0.63$, late season main effect of treatment n males = 16, $z = -2.94$, $p = 0.0032$, treatment*season interaction n males = 27, $z = -1.92$, $p = 0.055$, main effect season $z = 1.34$, $p = 0.18$; 2012: early season main effect of treatment n males = 23, $z = 0.0$, $p = 1.0$, late season main effect of treatment [bayesglm] *d.f.* = 15, $z = -0.679$, $p = 0.50$, treatment*season interaction n males = 31, $z = -0.04$, $p = 0.97$, main effect season $z = -1.47$, $p = 0.14$).

Mechanisms of shifts in H. occisa *species recognition*

Removing *H. titia* males in the late season did not affect the response of *H. occisa* territory holders to tethered *H. titia* male intruders: while the removal did lower overall attack rates, *H. occisa* males were more aggressive toward conspecifics than toward heterospecifics in both transects (negative binomial zero inflation model of attack count [slams + grabs], n males = 33, species*transect interaction $z = -1.75$, $p = 0.0796$; main effect of male species $z = -5.16$, $p < 0.001$; main effect of transect $z = -2.66$, $p = 0.0079$**;** linear model of the proportion of time chasing tethered males, n males = 33, male species*transect interaction $z = -0.59$, $p = 0.55$, main effect of male species $z = -10.45$, $p < 0.001$; main effect of transect $z = -0.22$, $p = 0.83$). Similarly, the removal did not affect the response of *H. occisa* to tethered conspecific males (see Table 2-7).

Likewise, the late season *H. titia* removal experiment had no effect on *H. occisa* male mate recognition; *H. occisa* males in both the control and removal transects responded sexually more often to unmanipulated conspecific females than to experimentally darkened conspecifics or *H. titia* females and there was no significant female treatment by transect interaction (*H. occisa* unmanipulated female vs. darkened females, logistic regression of clasping [yes = 1, no = 0], treatment*transect interaction n males = 46, $z = 0.06$, $p = 0.95$, main effect of transect $z = -0.36$, $p = 0.72$, main effect of treatment $z = 2.54$, $p = 0.011$; unmanipulated *H. occisa* vs. *H. titia* female, female treatment*transect interaction n males = 45, $z = -0.61$, $p = 0.54$, main effect of experimental transect $z = 0.0$, $p = 1.0$, main effect of female treatment $z = -2.82$, $p = 0.0047$).

There was no difference between the response of control males and males whose claspers were removed; males in both treatment groups attempted to clasp unmanipulated *H. occisa* females much more often than darkened *H. occisa* females (logistic regression of clasping [yes = 1, no = 0], clasper treatment*female treatment interaction n males = 21, $z = 0.04$, $p = 0.97$, main effect of female treatment $z = 3.38$, $p < 0.001$, main effect of clasper treatment $z = 0.36$, $p = 0.72$).

Discussion

Across a broad geographic area, smoky rubyspots (*H. titia*) of both sexes undergo a striking seasonal shift in wing coloration, from a light phase form that resembles other sympatric congeners, such as *H. occisa*, to a dark phase form that is distinctly different from any sympatric species. In addition to documenting this seasonal polyphenism in wing coloration, we show that it coincides with and is causally related to seasonal changes in the levels of reproductive and aggressive interference between *H. titia* and *H. occisa*. *H. occisa* territory holders were far more aggressive to tethered *H. titia* male intruders before compared to after the color shift. How *H. occisa* males respond to conspecific intruders with experimentally blackened wings also changed seasonally, however, which suggests that the reduction in interspecific aggression that occurs from the early to the late season is a product of both the *H. titia* color shift and a shift in *H. occisa* competitor recognition (no shift in competitor recognition was detected in *H. titia*). Males of both species were more likely to clasp tethered heterospecific females before compared to after the color shift. Manipulations of female wing coloration showed that male mate recognition also changes seasonally in both species. Hence, reproductive interference between these species is reduced both by the seasonal shift in female wing coloration in *H. titia* and by shifts in male mate recognition in both species.

Our field experiments provided no evidence that the seasonal shifts in competitor recognition and mate recognition are a product of learning. Removing all *H. titia* males from one study transect did not affect the development of competitor or mate recognition in male *H.*

occisa, compared to a control transect where *H. titia* were not removed. Likewise, preventing male *H. occisa* from clasping females (by removing their claspers) did not prevent them from discriminating between conspecific females with experimentally darkened wings and control females. Thus, it appears that prior fighting experience is not required for males to discriminate between conspecific and heterospecific males during the late season and that prior sexual experience is not required for males to discriminate between females based on wing coloration.

The direction of the seasonal polyphenism documented here accords with previous research (Johnson 1962): *H. titia* males and females shift from light-phase forms early in the peak emergence season to dark-phase forms in the late season. Also in agreement with Johnson (1962), interspecific aggression was more intense between *H. occisa* and light-phase *H. titia* individuals than between *H. occisa* and dark-phase *H. titia* individuals—in his study, interspecific territoriality was more common between light-phase forms and a clear-winged congener (*H. americana*). Previous research documenting a character displacement pattern in the influence of *H. titia* wing coloration on competitor recognition (Anderson and Grether 2010a,b, 2011) is unchallenged by the documented seasonal polyphenism, as competitor recognition of individuals in allopatric populations of *H. occisa* was unaffected by the extent of black on male wings (Anderson and Grether 2010a). The current study raises the possibility that documented character displacement patterns in both male wing patterns and competitor recognition may have evolved via selection acting on the reaction norms of both the seasonal polyphenism and the development of species recognition. Finally, a recent study demonstrates that in many species, including *Hetaerina* damselflies, interspecific territoriality may be an adaptive response to between-species competition for access to mates (Drury et al., *submitted*). The results of the current study show that the shift in *H. occisa* competitor recognition follows a sharp decline in the reproductive interference inflicted by *H. titia*, consistent with this hypothesis and recapitulating at a population level the phenomenon described between several species pairs.

The role of learning in shaping mate recognition has been described previously (e.g., Irwin and Price 1999; Verzijden et al. 2012), and has even been documented in species with seasonal polyphenisms (e.g., Westerman et al. 2014). In this study, however, in spite of finding evidence for shifts in mate recognition accompanying changes in the relative abundance of dark-phase *H. titia* forms, we did not find that learning played a role in this shift. This could be because there is a genetic polymorphism at the loci that contribute to wing pigmentation, with dark and light-phase forms being temporally isolated, but the continuous variation in wing phenotypes and the strong relationship with time (Fig. 2-2, 2-3), in spite of the fact that *Hetaerina* spp. spend most of their lives as larvae (Zloty et al. 1993), point to an environmental mechanism behind the shift (e.g., developmental shift cued by photoperiod). Alternatively, other forms of learning or developmental mechanisms could result in the shift in species recognition templates.

In spite of the number of cases where environmental variation influences the expression of traits used for species recognition (Table 2-1), few studies (empirical or theoretical) have explicitly explored the impact such phenotypic plasticity may have on the evolutionary dynamics of signal phenotypes. Pfennig & Pfennig (2012) argue that phenotypic plasticity may be an important first step of character displacement. However, they define plasticity in the context of character displacement as arising because of heterospecific competition, rather than in other contexts (as in a polyphenism) that in turn influence the outcome of between species competitive interactions. Moreover, in most examples that they give, and in their “plasticity-first” hypothesis, they posit that plasticity is initially adaptive (i.e., in the direction of divergence). They do, however, acknowledge that in some cases, plasticity could actually impede or prevent character displacement. We have shown that plasticity in *H. titia* wing coloration actually increases wasteful between species interactions in the early part of the year. We do not currently know whether the documented seasonal polyphenism is adaptive in other contexts, or if it simply reflects some sort of developmental constraint.

This study raises several questions about the evolutionary consequences of plasticity in traits used for species recognition:

(1) How does genetic accommodation (West-Eberhard 2003) target the developmental mechanisms behind signal trait production and species recognition templates? For example, character displacement could proceed via selection operating to decrease the degree of phenotypic plasticity (e.g., genetic assimilation, Waddington 1942, 1956) in traits mediating species recognition. Alternatively, selection could favor increased plasticity, if shifts in traits facilitate coexistence and eventually, character displacement (Pfennig & Pfennig 2012). Selection could also act on the timing of the expression of plasticity, for example if mate recognition is learned in some parts of the year and not in others (e.g., Westerman et al. 2014).

(2) Given that phenotypic plasticity may move populations away from local adaptive peaks (Ghalambor et al. 2007), how often and under what circumstances does phenotypic plasticity preclude, rather than facilitate, species coexistence?

(3) Our results indicate that the presence of phenotypic plasticity can in some cases result in wasteful between-species reproductive and agonistic interactions, and beg the question of why selection should favor the maintenance of plasticity in spite of putatively maladaptive consequences. In the case of *H. titia*, for example, why are males and females not dark year-

round? Although between-species interference occurs in sympatry, has selection adjusted development of signals or recognition relative to allopatric populations?

Understanding how variable phenotypic expression can both change the dynamics of evolution and itself become the target of selection is a major challenge in current evolutionary biology (West-Eberhard 2003; Pigliucci 2010), and we hope that our results extend this ongoing synthesis to studies of traits involved in mediating between-species social interactions.

Table 2-1. Cases where traits used to distinguish between conspecifics (CS) and heterospecifics (HS) exhibit plasticity.

Species	**Trait**	**Evidence for plasticity**	**Evidence for role in species recognition**	**Citations**
Invertebrates				
Calopteryx splendens	male wing spot	seasonal polyphenism in wing spot allometry	allopatric shift in affect on female mate recognition	(Hardersen 2010; Svensson et al. 2010)
Chorthippus biguttulus	male song	songs altered in presence of road noise	females prefer songs similar to those of conspecifics	(Safi et al. 2006; Lampe et al. 2014)
Gryllus crickets	male song	change in pulse rate	females differentiate between species	(Walker 2000; Gray 2004)
Hetaerina americana	male wing spot	seasonal change in relative wing spot size	allopatric shift in affect on interspecific aggression	(Córdoba-Aguilar et al. 2009; Anderson and Grether 2010a)
Hetaerina titia	male wing melanization	seasonal polyphenism in wing melanization	allopatric shift in affect on interspecific aggression	(Anderson and Grether 2010a), this study
Photinus greeni	male light pulse (interpulse interval)	temperature dependent	used to discriminate CS and HS mates	(Lloyd 1966, 1969; Michaelidis et al. 2006)
Pieris occidentalis	male wing melanization	seasonal polyphenism in wing melanization	used to discriminate CS and HS mates	(Kingsolver and Wiernasz 1991; Wiernasz and Kingsolver 1992)
Pieris rapae	female wing phenotype	seasonal polyphenism in wing melanization	used to discriminate CS and HS mates	(Ohguchi and Hidaka 1988; Stoehr and Goux 2008)
Pieris protodice	male wing melanization	seasonal polyphenism in wing melanization	used to discriminate CS and HS mates	(Shapiro 1969; Wiernasz and Kingsolver 1992)
Uca capricornus	female carapace coloration	changes within 20 minutes, reversible	used to discriminate neighbors; other exoskeleton coloration shown to influence spp. rec.	(Detto et al. 2006, 2008; Umbers et al. 2014)
Vertebrates				
Gasterostereus aculeatus spp.	olfactory cues	influenced by diet	mediate female mate recognition (cs/hs trials)	(Ward et al. 2004; Rafferty and Boughman 2006)
Plethodon cinereus	olfactory cues	olfactory cues influenced by tail loss, diet	individuals avoid HS congener scent	(Walls et al. 1989; Sullivan et al. 2003; Wise et al. 2004)
Podarcis hispanicus spp.	male olfactory cues	influenced by immunocompetence	males distinguish between scents of different populations of species complex	(López and Martín 2005; Gabirot et al. 2012)

Table 2-2. Study locations, visit dates, and number of *H. titia* males' wings analyzed.

Site	Latitude, longitude (dec. degrees)	Dates of visit	*n* males
Armeria	18.950, -103.934	7/10-7/14/2005	89
		5/30-6/5/2007	85
		7/23-7/27/2007	24
		4/27-5/3/2008	74
Castroville	29.333, -98.867	4/17-4/24/2008	32
		8/19-8/26/2008	11
		6/21-6/28/2012	165
		5/21-6/22/2013	147
La Palma	18.550, -95.067	3/17-6/11/2011	342
		3/28-6/9/2012	181

Table 2-3. Comparisons of the counts of naturally occurring conspecific and heterospecific fights across both seasons for each year of the study. Expected values were calculated from a binomial expansion of the proportion of males of each species present on during the time period. Chi-squared analyses test for the independence of deviations of expected and observed values from the early to late season.

		count of fights, observed (expected)			proportion of *H. occisa* fights interspecific	proportion of *H. titia* fights interspecific	observed/expected interspecific fights
		H. occisa	interspecific fights	*H. titia*			
all fights							
2011	early	87 (128)	115 (131)	90 (33)	0.57	0.56	0.88
	late	20 (22)	21 (35)	29 (14)	0.51	0.42	0.61
							$\chi^2 = 19.01$, *d.f.* = 2, $p < 0.001$
2012	early	15 (14)	10 (19)	15 (7)	0.4	0.4	0.52
	late	30 (17)	14 (35)	27 (19)	0.32	0.34	0.39
							$\chi^2 = 7.31$, *d.f.* = 2, $p = 0.025$
escalated fights							
2011	early	69 (100)	90 (102)	70 (26)	0.57	0.56	0.88
	late	15 (17)	15 (28)	26 (11)	0.5	0.37	0.54
							$\chi^2 = 14.49$, *d.f.* = 2, $p < 0.001$
2012	early	11 (9)	4 (13)	11 (4)	0.27	0.27	0.32
	late	26 (14)	9 (29)	23 (15)	0.26	0.28	0.31
							$\chi^2 = 6.99$, *d.f.* = 2, $p = 0.03$

Table 2-4. Results of repeated-measure analyses (mixed effect negative binomial models) comparing responses of *H. occisa* territory holders to experimentally manipulated tethered intruders, pooled across both years. Models compare the count of attacks (slams + grabs) directed toward control males with responses toward males with half-blackened (HB) and blackened (B) wings in the early and late seasons.

dataset	**model term**	**estimate**	**std. error**	***z***	***p*-value**
early season	HB	-0.04	0.12	-0.33	0.74
(*n* males = 53)	B	-0.02	0.12	-0.15	0.88
late season	HB	-0.17	0.1	-1.71	0.087
(*n* males = 50)	B	-0.35	0.1	-3.57	< 0.001
both seasons	HB	-0.038	0.11	-0.34	0.73
(*n* males = 103)	B	-0.015	0.11	-0.14	0.89
	season	0.99	0.33	3.02	0.0025
	HB*season	-0.13	0.15	-0.89	0.38
	B*season	-0.35	0.15	-2.29	0.022

Table 2-5. Results of models comparing responses of *H. occisa* territory holders to experimentally manipulated tethered intruders, presented separately for each year. Models compare the count of attacks (slams + grabs) directed toward control males with responses toward males with experimentally half-blackened (HB) and blackened (B) wings.

year	**dataset**	**model term**	**estimate**	**std. error**	***z***	***p*-value**
2011	early season	HB	-0.30	0.29	-1.03	0.31
	(*n* males = 23)	B	-0.72	0.31	-2.34	0.019
	late season	HB	-0.51	0.18	-2.86	0.0043
	(*n* males = 17)	B	-1.51	0.22	-6.91	< 0.001
	both seasons	HB	-0.27	0.24	-1.13	0.26
	(*n* males = 40)	B	-0.70	0.26	-2.74	0.0062
		season	1.03	0.55	1.87	0.06
		HB*season	-0.24	0.33	-0.72	0.47
		B*season	-0.77	0.37	-2.09	0.037
2012	early season	HB	0.02	0.12	0.19	0.85
	(*n* males = 30)	B	0.15	0.12	1.26	0.21
	late season	HB	-0.05	0.09	-0.51	0.61
	(*n* males = 33)	B	-0.11	0.09	-1.21	0.23
	both seasons	HB	0.02	0.11	0.16	0.87
	(*n* males = 63)	B	0.14	0.11	1.28	0.20
		season	0.62	0.18	3.47	0.00052
		HB*season	-0.07	0.15	-0.45	0.65
		B*season	-0.25	0.15	-1.73	0.084

Table 2-6. Results of models comparing responses of *H. occisa* territory holders to experimentally manipulated tethered intruders. Models compare the proportion of time spent chasing control males with responses toward males with half-blackened (HB) and blackened (B) wings in the early and late seasons in 2011, 2012, and pooled across both years.

year	**dataset**	**model term**	**estimate**	**std. error**	*z*	***p*-value**
2011	early season	HB	-0.10	0.06	-1.67	0.095
	(*n* males = 23)	B	-0.06	0.06	-0.97	0.33
	late season	HB	-0.03	0.06	-0.45	0.65
	(*n* males = 17)	B	-0.08	0.06	-1.39	0.16
	both seasons	HB	-0.10	0.06	-1.77	0.076
	(*n* males = 40)	B	-0.06	0.06	-1.03	0.3
		season	0.07	0.08	0.86	0.39
		HB*season	0.07	0.09	0.85	0.4
		B*season	-0.03	0.09	-0.29	0.77
2012	early season	HB	0.06	0.05	1.16	0.25
	(*n* males = 30)	B	0.10	0.05	2.03	0.043
	late season	HB	0.02	0.03	0.69	0.49
	(*n* males = 33)	B	0.01	0.03	0.16	0.87
	both seasons	HB	0.06	0.04	1.39	0.17
	(*n* males = 63)	B	0.10	0.04	2.43	0.015
		season	0.08	0.05	1.72	0.09
		HB*season	-0.04	0.06	-0.64	0.52
		B*season	-0.10	0.06	-1.67	0.09
pooled	early season	HB	-0.01	0.04	-0.28	0.78
	(*n* males = 53)	B	0.03	0.04	0.80	0.42
	late season	HB	0.005	0.03	0.16	0.87
	(*n* males = 50)	B	-0.25	0.03	-0.87	0.39
	both seasons	HB	-0.01	0.03	-0.32	0.75
	(*n* males = 103)	B	0.03	0.03	0.92	0.36
		season	0.08	0.04	1.74	0.08
		HB*season	0.02	0.05	0.32	0.75
		B*season	-0.06	0.05	-1.15	0.25

Table 2-7. Results of models comparing responses of *H. occisa* territory holders to experimentally manipulated tethered intruders, comparing control males with males on a transect where all *H. titia* males were removed. Models compare the count of attacks (slams + grabs) directed toward control males with responses toward males with half-blackened (HB) and blackened (B) wings.

response variable	model term	estimate	std. error	*z*	*p*-value
attack count	HB	-0.02	0.11	-0.18	0.85
(*n* males = 33)	B	-0.09	0.11	-0.83	0.41
	transect	-0.67	0.18	-3.79	0.00015
	HB*transect	-0.08	0.19	-0.44	0.66
	B*transect	-0.05	0.19	-0.26	0.79
proportion of time chasing	HB	0.05	0.04	1.24	0.22
(*n* males = 33)	B	0.02	0.04	0.58	0.56
	transect	-0.02	0.06	-0.29	0.77
	HB*transect	-0.07	0.06	-1.14	0.26
	B*season	-0.05	0.06	-0.75	0.45

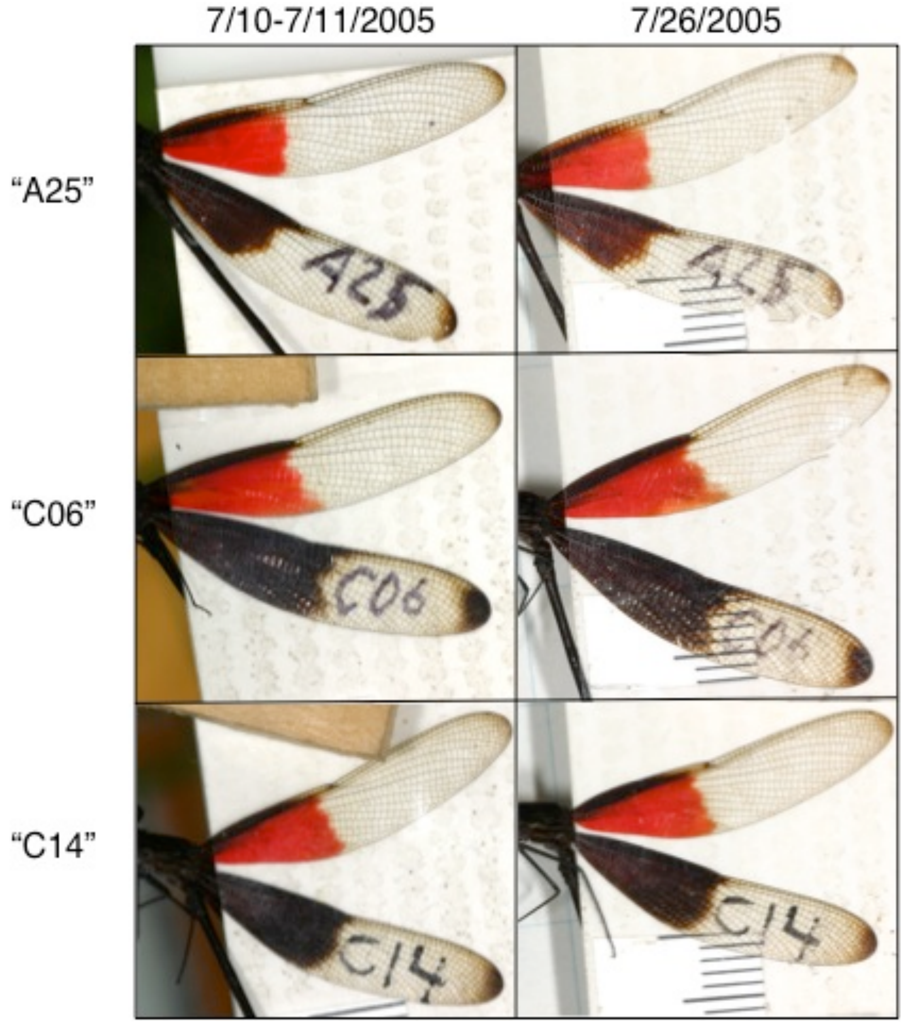

Figure 2-1. A sample of males that were photographed at two points separated by two weeks, showing that the extent of black pigment on the wings of individual males does not change after maturation.

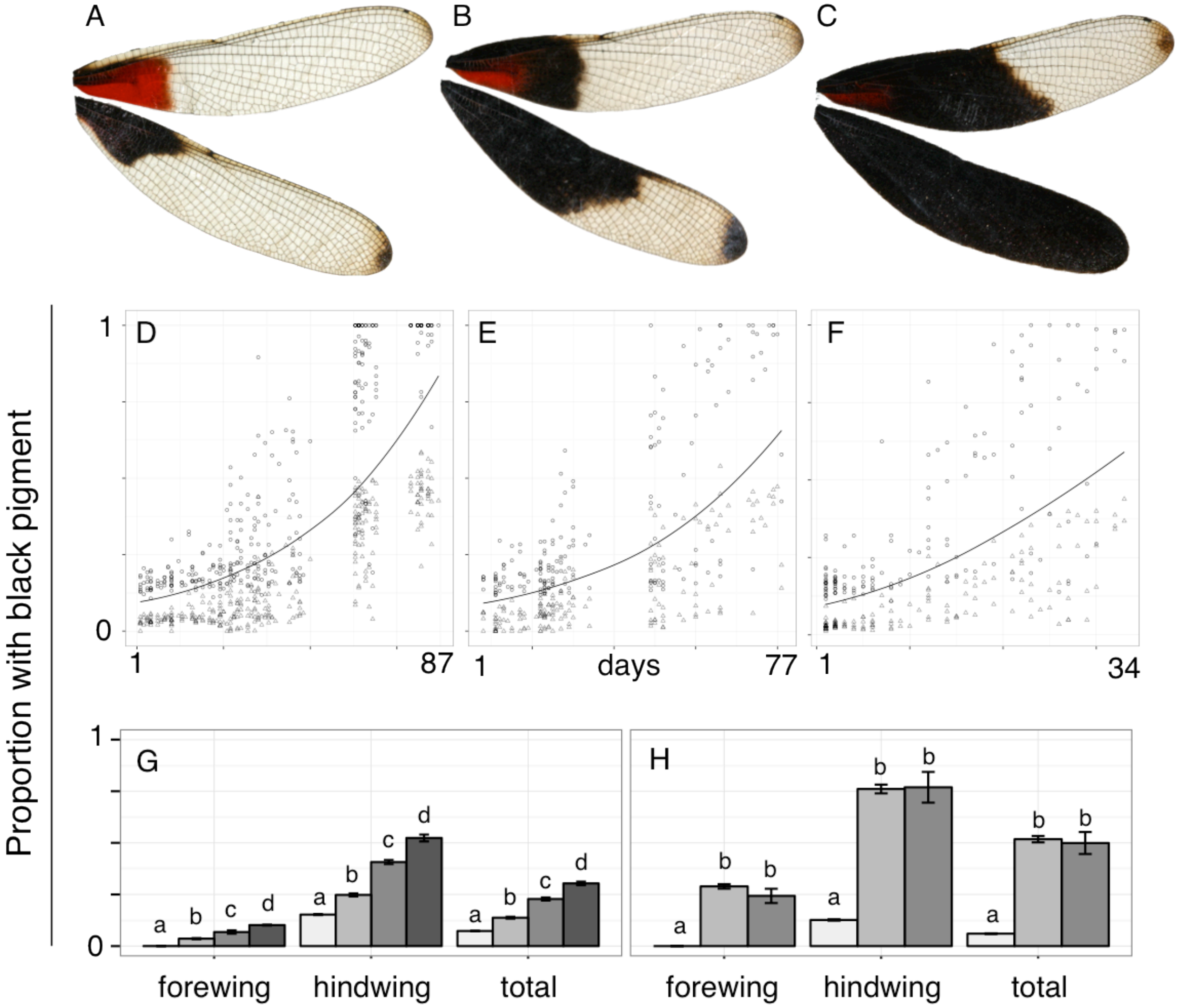

Figure 2-2. Examples of *H. titia* male wings and analyses of the seasonal change in wing pigmentation. A-C. Photographs of males' wings with increasing amounts of black pigmentation (forewings are on top, hindwings on bottom). D-F. The proportion of wing pigmentation on individuals photographed over the course of longer visits to streams (D= La Palma 2011, E= La Palma 2012, F= Castroville 2013, see Table 2-2). Empty circles represent hindwing data, empty triangles represent forewing data, and lines are GLM predictions for the total proportion of black pigmentation on both wing surfaces. The range of x-axes represent the length of visits, with the first day indicated by 1. G & H. The proportion of black pigmentation on both wing surfaces from individuals sampled on shorter visits to Armeria (G) and Castroville (H). Bars (means ± SE) are sorted from left to right on the x-axis by the time of the year of the visit (Armeria 1. 4/27-5/3/2008, 2. 5/30-6/5/2007, 3. 7/10-7/14/2005, 4. 7/23-7/27/2007; Castroville 1. 4/17-4/24/2008, 2. 6/21-6/28/2012, 3. 8/19-8/26/2008). Letters represent significantly different groups from Tukey post-hoc tests.

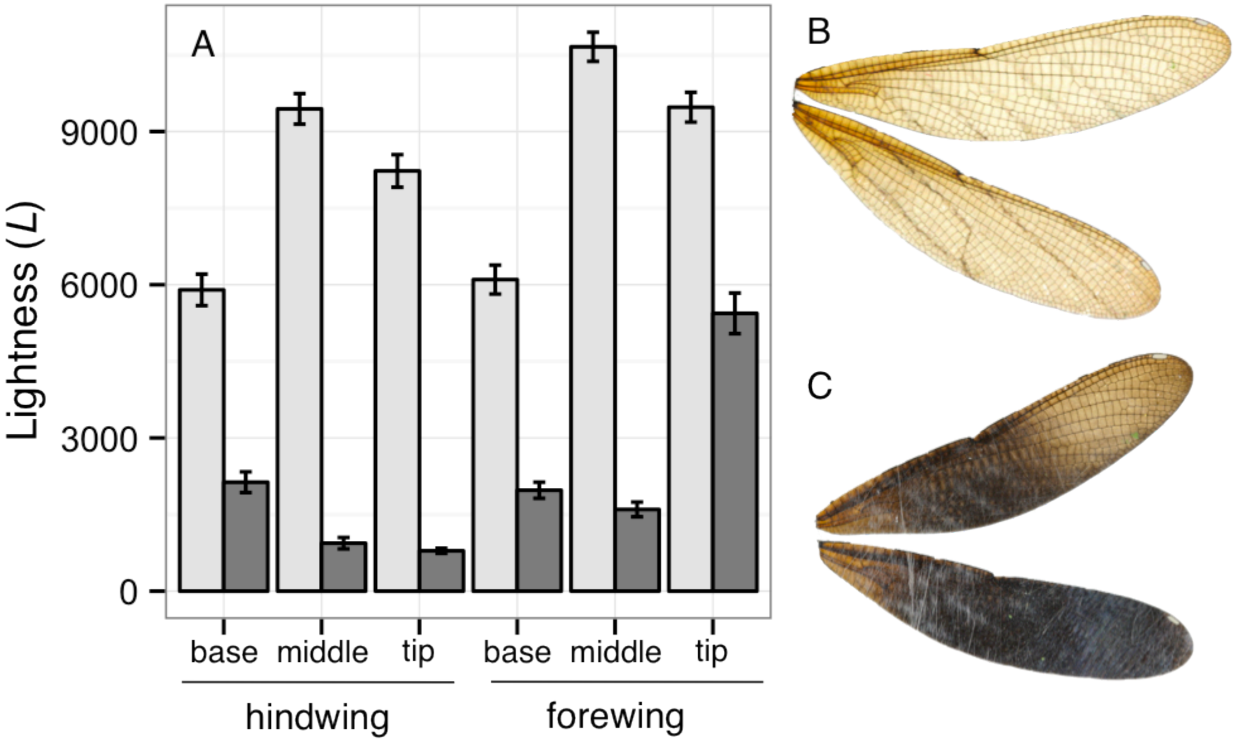

Figure 2-3. Lightness of *H. titia* females of light-phase and dark-phase forms and representative photographs. A. Difference in lightness between the early (light gray bars) and late (dark gray bars) emerging *H. titia* females' wings (means ± SE); lower values are darker. B. The wings of a light-phase form *H. titia* female (forewings are on top, hindwings on bottom). C. The wings of a dark-phase form *H. titia* female.

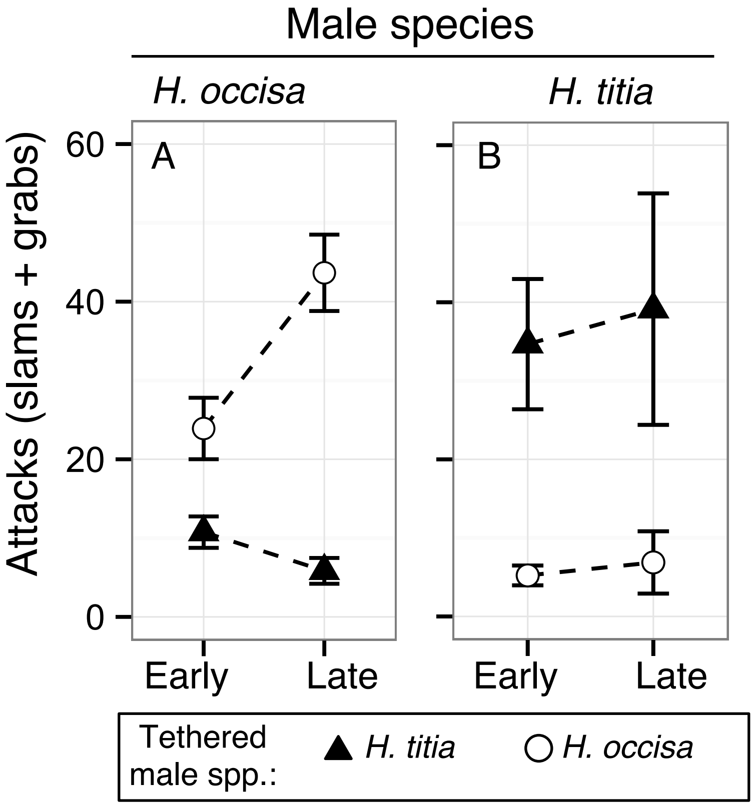

Figure 2-4. Responses of males to conspecific and heterospecific tethered intruders. A. *H. occisa* males direct more attacks (slams + grabs) toward conspecific males more often and heterospecific males less often in the late season (after *H. titia* exhibit darker phenotypes) B. Responses of *H. titia* males to tethered conspecific and heterospecific males do not change in the late season. All panels depict means ± SE.

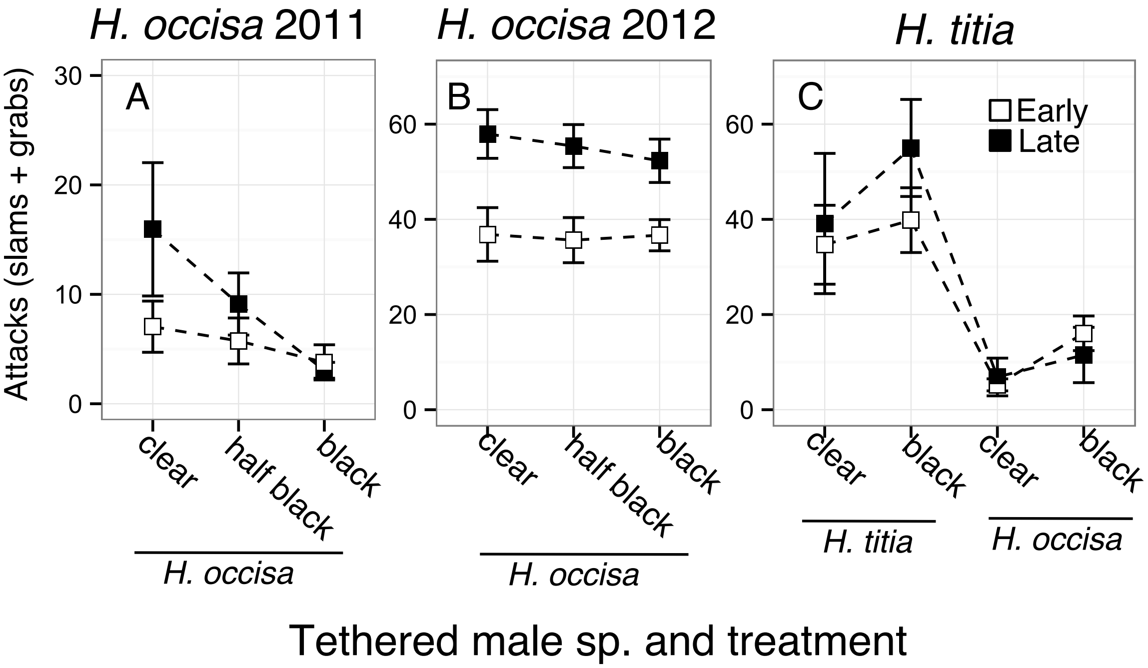

Figure 2-5. Responses of males to conspecific, experimentally manipulated tethered intruders. A & B. There is a weak but consistent tendency for territorial *H. occisa* males to reduce the relative number of attacks (slams +grabs) toward blackened conspecific males in the late season in 2011 (A) and 2012 (B), indicating a shift in competitor recognition accompanying the shift in *H. titia* male phenotypes. C. *H titia* males attack blackened *H. occisa* males more than clear *H. occisa* males, but do not attack blackened conspecific males more than control males and do not exhibit a shift in competitor recognition from the early to late season. All panels depict means ± SE.

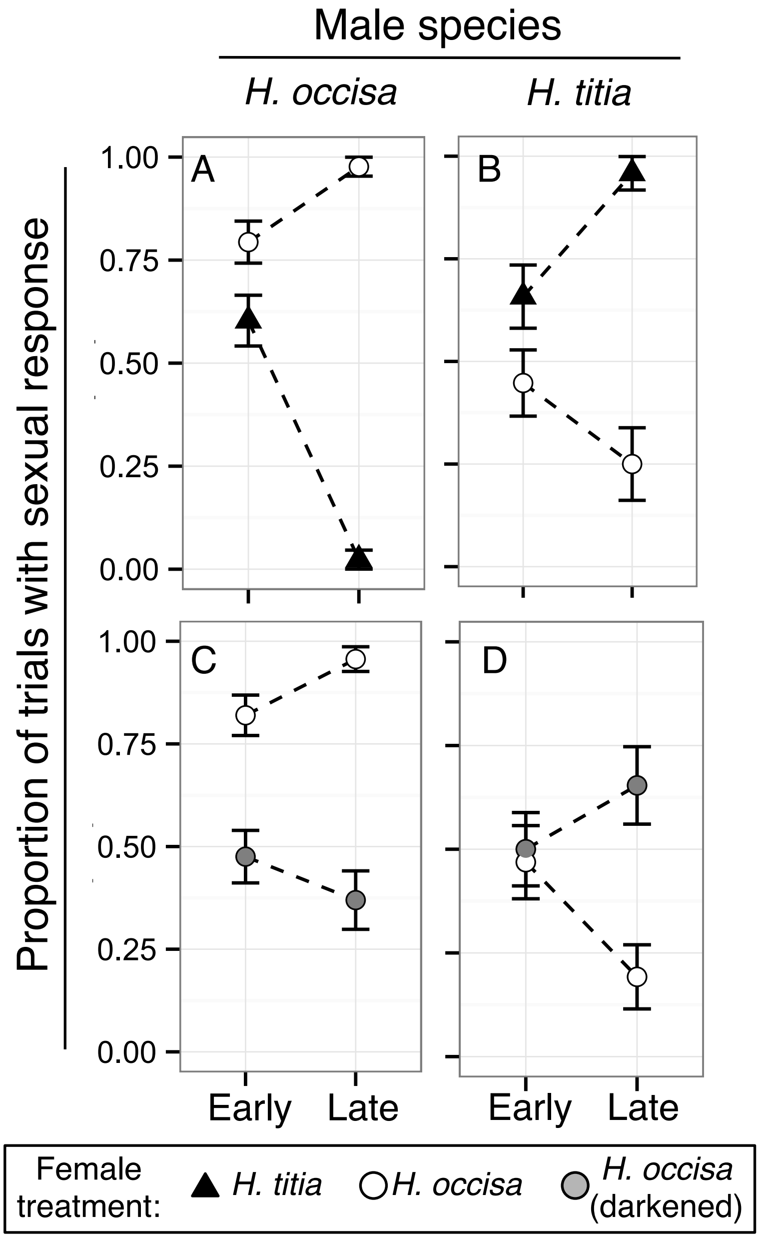

Figure 2-6. Responses of males to tethered females. Both *H. occisa* and *H. titia* male species recognition and mate recognition shift across the flight season. A *H. occisa* and B. *H. titia* male sexual responses to tethered, unmanipulated females. C. *H. occisa* and D. *H. titia* responses to unmanipulated and experimentally darkened *H. occisa* females. All panels show means ± SE.

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Chapter 3. Interspecific aggression, not interspecific mating, drives character displacement in the wing colouration of male rubyspot damselflies (*Hetaerina*)

Abstract

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 if female mate recognition is based on male wing colouration 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 colouration. 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 wasteful interactions between species. Evolutionary biologists have historically 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 [1,3,6–10]. Interference competition between species, which in animals usually takes the form of aggressive interactions, is also very common [11], yet agonistic character displacement (ACD), a process whereby natural selection acts on traits that mediate the occurrence or outcome of interspecific aggression, remains an understudied evolutionary phenomenon [12,13].

Many phenotypic traits function as signals in both mating and competitive contexts [14] (see Table 1 in [15]), 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 [16] and reduces territorial aggression from male collared flycatchers [17–19]. 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 [20]. 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 [21,22]. Moreover, *C. virgo* males are more aggressive to *C. splendens* males with relatively larger wing spots in sympatry, which consequently affects male fitness [21,23,24], yet female mate recognition is also influenced by male wing colouration and shifts in sympatry in a manner consistent with RCD [25,26].

In a recent theoretical study, Okamoto & Grether [15] set out to understand whether ACD and RCD can act synergistically to drive evolutionary divergence, or whether one process is more important than the other. In their study, 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. On the other hand, 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, because as the trait diverges though RCD, males still need to recognize conspecific males as competitors. 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 for trait shifts.

Previous research on two species pairs of rubyspot damselflies (*Hetaerina* spp.) showed that male competitor recognition is based on wing colouration [27,28] and that competitor recognition and male wing colouration both exhibit geographic patterns consistent with ACD [27,29]. Based on Okamoto & Grether's [15] findings, however, these trait shifts cannot be taken as compelling evidence for ACD unless females do not use male wing patterns for species recognition. While attempts to detect female mate choice based on male colouration within species of *Hetaerina* have yielded no such evidence [30,31], whether females use male colouration for species discrimination was previously unknown.

Here we test for effects of male wing colouration 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 colouration and competitor recognition has been detected. Male *H. americana* have large basal red wing spots and otherwise clear wings (Fig. 3-1a) while male *H. titia* have smaller basal red wing spots and variable amounts of black wing pigmentation (Fig. 3-1b). To test whether female *H. americana* use the species difference in male wing colouration 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 5 weeks.

2. Methods

(a) Study site and species

We carried out this study on two transects (~100 m each) marked at 1 m intervals along the Medina River in Castroville, TX (29.371797°, -98.896444°; 29.374733°, -98.896769°) from May 23rd to June 23rd 2013. To minimize dispersal, the study transects were located such that long pools (> 100 m), which are not suitable as breeding habitat, were located both up and downstream. Every individual American rubyspot (*Hetaerina 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 [27,32].

(b) Experimental wing manipulation

When we captured mature *H. americana* males, we assigned them to one of three treatments: 1) unmanipulated control (Fig. 3-1a), 2) 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, Fig. 3-1c), or 3) blackened: black ink on the same part of the hindwings (black, PM-98, Fig. 3-1d). 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. 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, 3-5 observers, typically 4, continuously walked along the transects from ~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 approach 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 [30,36] to be "escalated" in subsequent analyses.

(d) 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 [30]. 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 two consecutive days [27]. 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 analyze fighting rates, we took three approaches: (1) treating all recorded fights between the same two males as a single fight (as in [28]), (2) 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 (3) treating all fights as unique whether they were between the same or different males (i.e., all fights recorded per dyad per day). *H. 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 (4 m, the scale of a male's territory) of a *H. titia* territory holder.

Because the opportunity for males to fight and mate depended on the number of days they were present in the study, we analyzed 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 nonterritorial 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 [37] to conduct all statistical analyses, using the packages MASS [38] for negative binomial regression, survival [39,40] for survival analyses, glmmADMB [41,42] and lme4 [43] for mixed-effect GLMs, pscl for zero-inflation models [44,45], and ggplot2 [46] for figures.

(e) *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 [47,48]. To test for this possibility, we analyzed (1) the treatments of females' first and last mates during each day and (2) 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 three days or fewer, so to test for the possibility that sperm removal influenced male mating success, we analyzed female remating (1) within each day and (2) across a three-day window.

To determine if females bias either their first or last matings toward control males, we first estimated the proportion of each treatment we would expect if females were mating randomly with respect to treatment. Given that females sometimes mated with unmarked, unmanipulated males that had yet to enter the experiment, we calculated an expected proportion of these males from the recorded fights (4.78%) and used this to adjust the null expectations for the proportion of non-experimentally blackened mates (expected proportion of blackened mates = 0.37, control and unmarked mates = 0.63). To calculate the actual proportion of males of each treatment with which females mated, we fit binomial mixed effect models of the mate's treatment (experimental vs. control/unmarked) with random intercepts for female IDs to estimate the proportion of mates ± standard error.

3. Results

(a) *Sample sizes and preliminary results*

We marked and included 146 *H. americana* males in the experiment, recorded 472 unique *H. americana* mating events (including failed attempts), and made 1207 observations of fights involving at least one *H. americana* male. In no cases did the sham (clear) and unmanipulated control groups differ significantly from each other (all statistical tests NS), 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: (1) the proportion of attempted tandems that resulted in a successful mating (Fig. 3-3a, 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**)**, (2) the rate of successful matings (Fig. 3-3b, 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 (3) the duration of copulatory wheels (Fig. 3-3c, 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).

In *H. americana*, holding a territory is not essential for mating but males generally mate more often when they hold a territory [30]. 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 nonterritorial episodes to further examine the effect of the experimental treatment on male mating success [30]. 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; for a model built with the treatment of the first male a female mated with each day, the mean proportion of control/unmarked mates was 0.6424 (95% CI: 0.5696-0.7093), and for a model built with the treatment of females' last mates, the mean proportion of control/unmarked mates was 0.6664 (95% CI: 0.5820902-0.7413). In both cases, the confidence interval contains the expected proportion (0.63), so there is no evidence for discrimination among treatments for either first or last mates. Similarly, the treatment of a female's mates did not influence her probability of remating within one day (Fig. 3-3d, 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 three days (Fig. 3-3d, 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 analyzed within one day (binomial lag model with a lag variable for the subsequent mate treatment used as a predictor with a random intercept for female/1day, *n* = 76, *z* = -0.811, *p* = 0.42) or over a three day period (binomial lag model with a lag variable for the subsequent mate treatment used as a predictor with a random intercept for female/3day, *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 3-1). We found little evidence for an effect of the experimental treatment on intraspecific fighting rates (Table 3-1). 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 3-1, Fig. 3-4).

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, $n = 146$, $z = 2.549$, $p = 0.011$). 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, $n = 111$, $z = 1.694$, $p = 0.09$). Thus, the blackening treatment appeared to affect overall survival and/or dispersal only immediately after males were marked.

Experimentally blackened males were just as likely as control males to perch and defend territories near heterospecifics (mixed-effect model of the logarithm of the distance to the nearest *H. titia* territory holder with a random intercept for male ID, territory holders, $n = 673$, $z = 0.08$, $p = 0.93$; all males, $n = 1045$, $z = 0.42$, $p = 0.68$; probability of being within a 4 m radius of a territorial *H. titia* male in a binomial mixed-effect model with a random intercept for male ID, territory holders, $n = 674$, $z = 0.08$, $p = 0.93$; all males, $n = 673$, $z = -0.24$, $p = 0.81$). 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 (Fig. 3-2, Cox proportional hazard treatment coefficient = 0.643, $n = 62$ $z = 2.154$, $p = 0.031$). 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*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 colouration. 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 (Fig. 3-2a,b). The only hint of an effect of the 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. Post-copulatory means of discrimination are possible in calopterygid damselflies, where there is extensive evidence that males remove stored sperm from females during copulation [48–50]. If the amount of time spent in copula is under female control (but see [51–53]), 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 [47,54,55]. 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 toward particular males, remating more often after mating with a non-preferred male [47,56], or similarly, biasing remating toward a particular treatment, yet none of these indicators of cryptic female choice occurred in our experiment, whether we analyzed single days or three 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 [47,56,57], or manipulating fecundity [58,59].

H. americana females may use traits other than wing colouration 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 [60,61]. 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 [28], 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 a survival cost compared to control males, likely resulting from the increase in fights with heterospecific males.

Together, our results strengthen the hypothesis that previously documented shifts in both competitor recognition and male wing colouration [27–29] have resulted from ACD. One previous study documented a pattern of character displacement in male breeding colouration of benthic and limnetic forms of three-spined sticklebacks (*Gasterosteus aculeatus*) [62] that cannot be explained by a shift in female preferences or colour sensitivity [63,64], effectively ruling out RCD as a potential explanation for the observed shift. Nevertheless, to 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 supports ACD over RCD as the cause of a character displacement pattern. Although some investigators have grouped character displacement influencing interspecific aggression under RCD (e.g. [3]), this study highlights the conceptual distinction between RCD and ACD, and rules out the former as an explanation for the evolution of wing patterns in *Hetaerina*.

Table 3-1. Effects of the experimental treatment on intraspecific and interspecific fighting rates.

data set	**intraspecific fights (*H. americana* v. *H. americana*)**		**interspecific fights (*H. americana* v. *H. titia*)**	
	all fight types	**only escalated fights**	**all fight types**	**only escalated fights**
1) one fight per dyad[1]	$n = 666$, $\chi^2 = 4.17$, $p = 0.041$	$n = 374$, $\chi^2 = 0.22$, $p = 0.64$	$n = 115$, $\chi^2 = 4.69$, $p = 0.00099$	$n = 82$, $\chi^2 = 11.66$, $p = 0.00064$
2) one fight per dyad per day[2]	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[2]	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)

(Statistical tests compare blackened and control males. Data sets 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.)

[1] Chi-squared goodness-of-fit test of count of fights, comparing experimental versus control to a null expectation of fights based on the resighting record

[2] Negative binomial model of the number of interspecific fights, offset by the log of the number of days territorial or fighting (if larger)

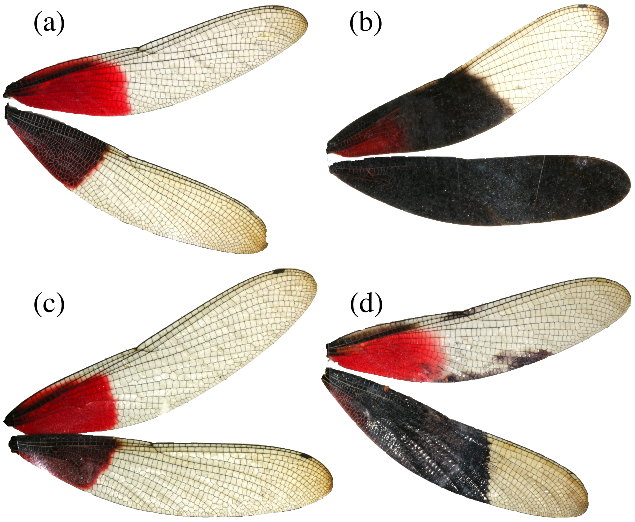

Figure 3-1. Photographs of a representative (a) unmanipulated *Hetaerina americana* male, (b) a *H. titia* male, and *H. americana* males with (c) clear ink and (d) black ink on their hindwings.

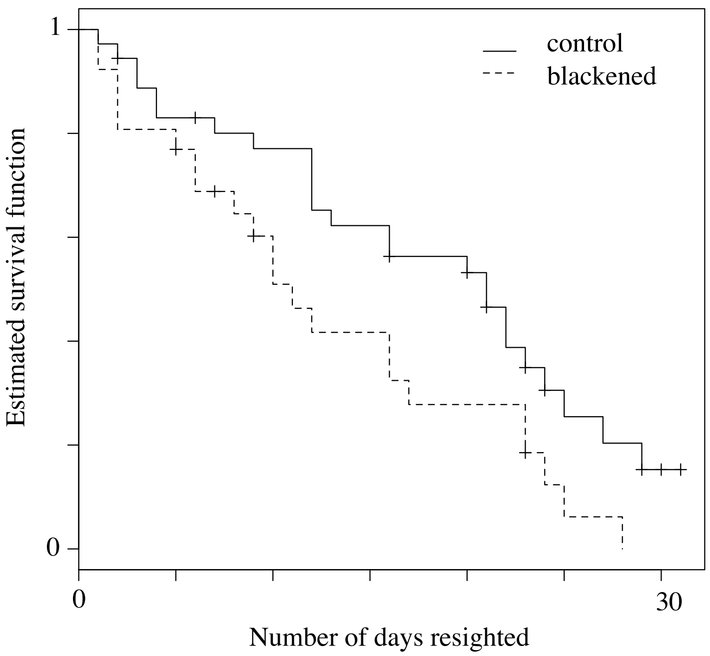

Figure 3-2. Effects of the experimental treatment on survival of males seen in close proximity to *H. titia* males. Kaplan-Meier plot, crosshatches indicate censored data points.

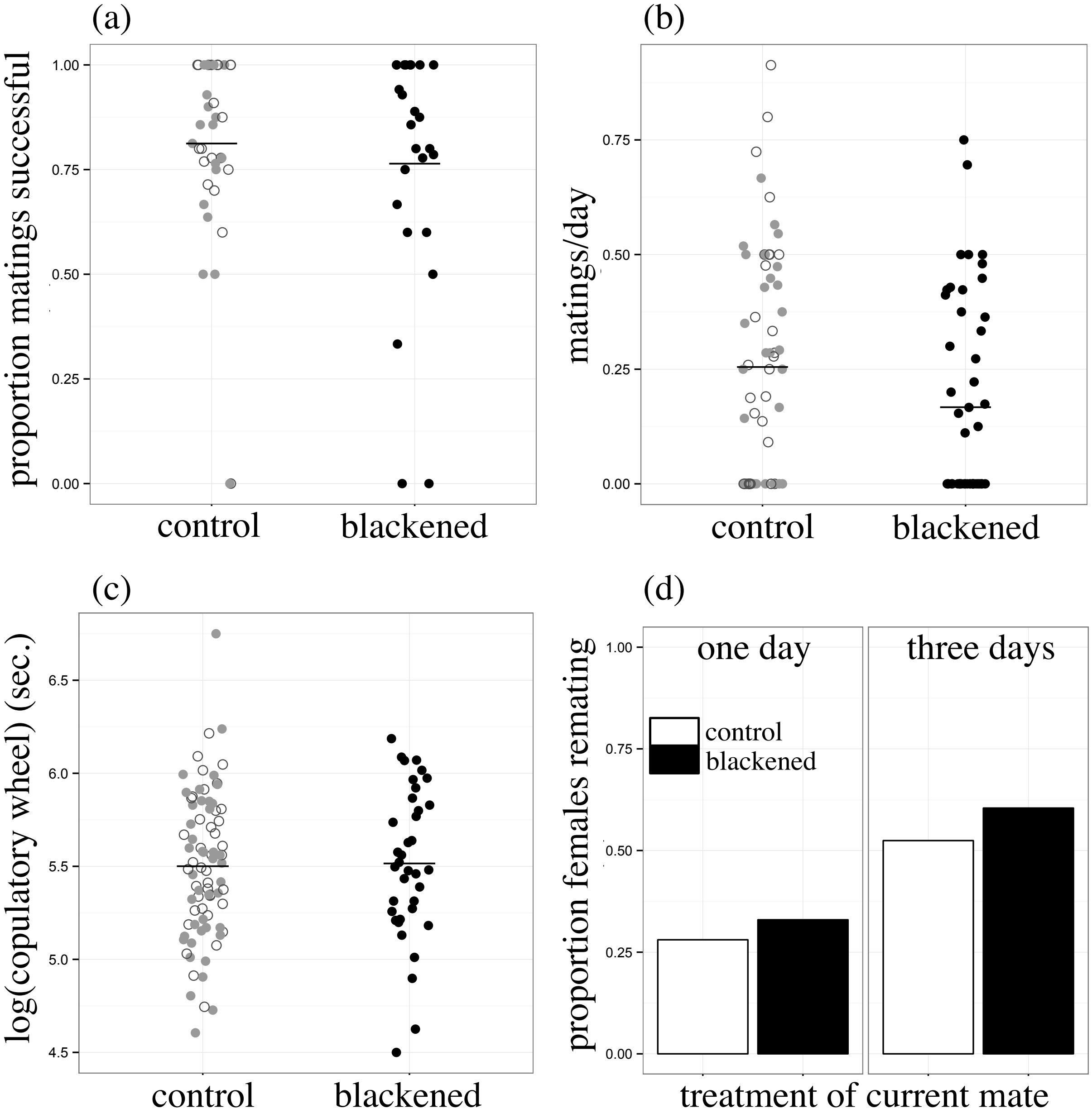

Figure 3-3. Lack of an effect of the experimental treatment on overall male mating success, measured either as (a) the proportion of successful tandems, (b) the mating rates of males, (c) the length of the copulatory wheel, or (d) the probability of a female remating within one or three days. In panels (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.

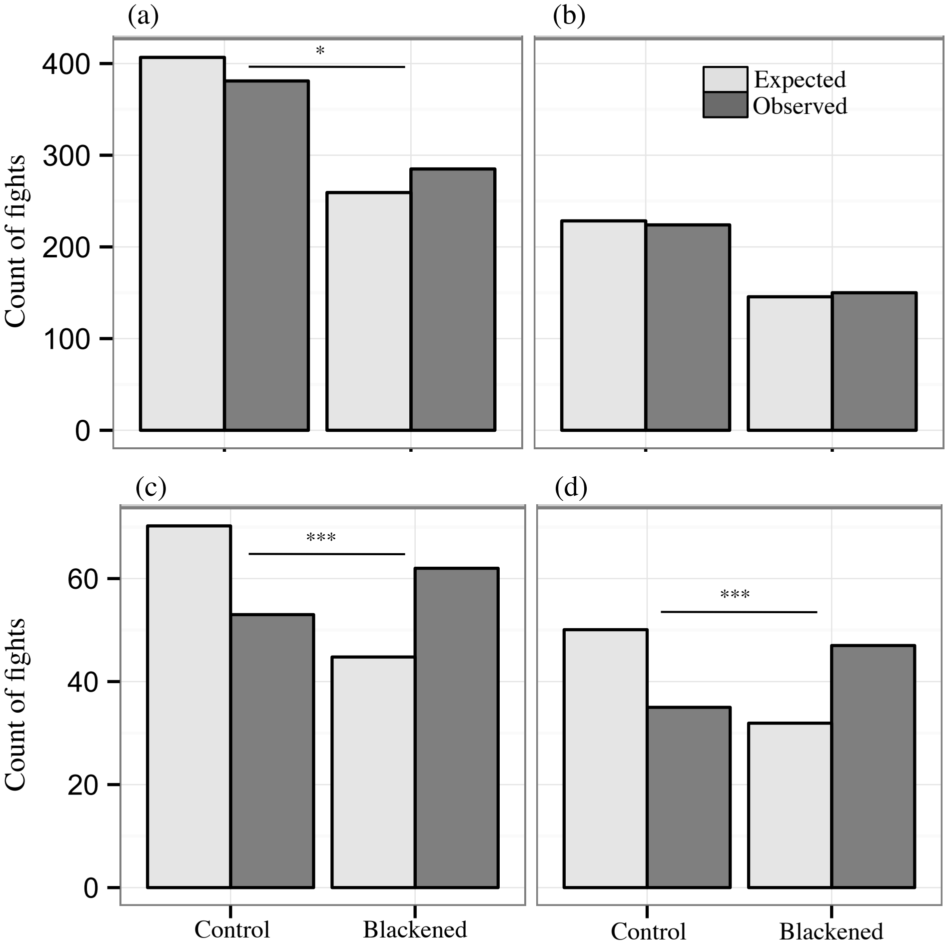

Figure 3-4. Expected and observed values of fights (reduced to one fight per dyad). See Table 3-1 for statistical analyses. Panels (a) & (b) present data for intraspecific fights, and panels (c) & (d) present data for interspecific fights. (b) & (d) show only escalated fights). *$p < 0.05$, *** $p < 0.001$

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Chapter 4. A new method of phylogenetic correction for analyses of pairwise species interactions

Abstract

Many hypotheses in evolutionary biology pertain to between-species interactions, yet few methods exist to account for the phylogenetic non-independence of species in such analyses. Currently, variations of Mantel tests are often used to correct for the phylogeny in statistical analyses of pairwise differences between taxa, but several investigators have recently pointed out that such tests perform poorly. Moreover, these tests are designed for complete cross-species comparison matrices, although some hypotheses are more appropriately tested on a subset of possible species pairs (e.g., sympatric species). Here, we present a simple simulation-based approach to generate a distribution of phylogenetically corrected test statistics against which raw test statistics can be compared. We then apply this method to conduct phylogenetically corrected statistical analyses of (1) predictors of interspecific territoriality in North American wood warblers and (2) the relationship between similarity in female wing coloration and the intensity of between-species reproductive and territorial interactions in rubyspot damselflies (*Hetaerina* spp.). This method can be applied easily to a wide range of questions in ecology and evolution.

Introduction

Interactions between species are a fundamental component of life on earth, and understanding the evolutionary and ecological consequences of such interactions are a central goal of many classical theoretical frameworks in ecology and evolutionary biology. In many statistical analyses of between-species interactions, the variables of interest are pairwise species differences in traits and/or behavioral interactions between species**.** For example, a recent comparative study on guenon monkeys showed that pairwise similarity in facial signals predict range overlap, with species with more similar signals being less likely to coexist (Allen et al. 2014). Another example comes from a recent study on New World wood warblers, which showed that species similarity in songs predicts the occurrence of hybridization (Willis et al. 2013). Recently, another study on warblers showed that genetic distance predicts the occurrence of interspecific territoriality (Losin 2012), although the specific traits associated with aggression were not identified. Understanding both the predictors of interspecific interactions and the consequences of such interactions for diversification and coexistence is an important contemporary research area.

As with other comparative approaches, it is important to account for the phylogenetic relationships among taxa included in the analyses, as these taxa have shared, non-independent evolutionary histories (Felsenstein 2004). Given that the data in analyses like those described above are pairwise comparisons, rather than “tip values” belonging to a single species, standard independent contrasts (Felsenstein 1985) cannot be used for analyzing between-species interactions. To circumvent the non-independence of species comparisons, previous investigators have implemented variations of Mantel tests (Mantel 1967), most notably the phylogenetically permuted partial Mantel test (Lapointe and Garland 2011), to test for relationships between predictor variables and interspecific comparisons. Nevertheless, several investigators have criticized these approaches for having both low power and inflated Type I error rates (e.g., Guillot & Rousset, 2013; Harmon & Glor, 2010). Moreover, for many biologically relevant comparisons, the pairwise species comparison matrices are necessarily incomplete. For example, to understand predictors of hybridization, it only makes sense to include species that are sympatric, and thus able to encounter one another, in analyses. Yet accounts of Mantel and partial Mantel tests only describe procedures for complete matrices (Mantel 1967; Lapointe and Garland 2011).

We employed a phylogenetic simulation method for analyzing incomplete species pair matrices that is similar to simulation methods developed for analyzing tip values (Martins and Garland 1991; Garland et al. 1993). Briefly, we simulated character evolution across phylogenies scaled to empirical data, calculated pairwise comparisons of interests on these simulated datasets, and conducted statistical analyses to generate a phylogenetic null distribution of test statistics against which to compare the test statistic calculated on the raw data (Fig. 1). We then applied this method to two test cases.

Case 1: Interspecific territoriality in North American wood warblers. Previous research on interspecific territoriality (hereafter referred to as IT) in wood warblers demonstrated that interspecific territoriality occurs more often between species with similar body mass and feeding ecology (Losin 2012). However, in an analysis correcting for genetic distance, these variables were no longer significant predictors of IT. This approach did not account for the hierarchical relationships between the species of warblers that result from the branching patterns of evolution. Here, we present results using the phylogenetic approach that we developed. Additionally, since interspecific territoriality may occur when species have overlapping agonistic signals (e.g., visual or audio cues used to distinguish competitors from non-competitors,Grether et al. 2009; Grether 2011), we expanded the analysis to include two other traits that individuals may use to differentiate between competitors and non-competitors: plumage and song.

Case 2: Female wing coloration and interspecific interactions in rubyspot damselflies (Hetaerina spp.). Rubyspot damselflies live in sympatry with congeners throughout much of their ranges, and previous research documented substantial variation in the magnitude of interspecific aggression (Anderson and Grether 2010) and reproductive interference (Chapter 1). Previous experimental research demonstrated that males use female wing coloration for mate recognition and that variation in heterospecific wing coloration predicts male responses to heterospecifics. Furthermore, across several species pairs, between-species interactions are correlated with female phenotypic similarity such that species pairs with more similar looking females exhibit elevated rates of reproductive interference and interspecific aggression (Chapter 1). Yet, whether this relationship is robust to phylogenetic correction or it simply reflects phylogenetic constraint (i.e., closely related species appear more similar to one another and are thus more likely to interfere behaviorally) is unknown. Here, we build a molecular and morphologically based phylogeny of several *Hetaerina* species and apply our phylogenetic correction to test for an evolutionary relationship between similarity in female wing coloration and between-species behavioral interactions.

Methods

(a) Statistical approach

We wrote an R program that employs a simulation approach to calculate phylogenetically corrected test statistics (Fig. 1, after Martins & Garland 1991, Garland et al. 1993, Lapointe & Garland 2001). In the program, character data of predictor variables are simulated *N* times under a specified model of evolution (e.g., Brownian motion [BM], Ornstein-Uhlenbeck [OU]) across a known phylogeny using the fastBM() function in the phytools package for R (Revell 2012). After each simulation, differences between the known interacting tips (e.g., sympatric species) are calculated. In this way, only the relevant species comparisons (sympatric species, in our analyses) are included, and a full species interaction matrix is not necessary. Using these simulated differences, statistical analyses of the relationship between the raw (observed) response variables and the simulated predictor variables are carried out. This yields a distribution of *N* test statistics against which the test statistic from the same analysis conducted with the raw data can be compared. If the test statistic from the analysis conducted on the raw data is more extreme than that of 95% of the simulated datasets, then one can infer that the relationship between the response variable and the predictor variable(s) is not due to shared ancestry alone.

Prior to running the evolutionary simulations, we estimated an evolutionary rate parameter (σ^2) for each continuous character by fitting an evolutionary model (e.g., BM, OU) to the raw data using the fitContinuous() function from the R package geiger (Harmon et al. 2008). We further constrained the evolutionary simulations to match the range and mean of the raw data. However, σ^2 cannot be estimated directly from the raw data for variables that are inherently species pair characteristics (e.g., interspecific territoriality). For such variables, we simulated the evolution of a continuous variable and then transformed the species differences. For binary variables (e.g. overlap in feeding guild), we transformed the species differences using a threshold function, such that the resulting dataset had the same number of species pairs in each binary category as the raw dataset. For continuous variables (e.g., plumage similarity), we transformed the species differences such that the resulting distribution matched the mean and standard deviation of the raw distribution (for an example, see the transformations described in section b.iii, below).

(b) Example 1: Predictors of interspecific territoriality in New World warblers (Aves: Parulidae)

(i) species included and ecological characters

Given that allopatric species do not have the opportunity to exhibit interspecific territoriality, we only included sympatric species pairs in the analysis. More specifically, only parulid species pairs that include one of the species known to be interspecific territorial (see below) at a given site and in a given time period were included in the analysis. Sympatry determinations were based on the Breeding Bird Survey dataset (Sauer et al. 2014). A total of 42 species, and 290 species pairs, were included in the analysis (see Losin 2012 for more details).

Data on interspecific territoriality, feeding guild overlap, and degree of syntopy were taken from Losin (2012). Briefly, IT was defined as the presence of ongoing interspecific aggression that reduces territory overlap between the species (Simmons 1951). Feeding guild overlap is a binary measure of ecological similarity, where a value of “1” indicates that the interacting species share the same food type (e.g., fruit, insects), feeding substrate (e.g., ground, upper canopy), and feeding technique (e.g., gleaning, sallying). Degree of syntopy was calculated as the ratio of the observed:expected number of sites where species overlap (values larger than 1 indicate that species exist in sympatry more often than would be expected if their geographical distributions were random).

(ii) morphological and song data

We obtained mass and bill measurements (specifically, exposed culmen length) for each species by searching the Birds of North America (Poole 2005) and BIOSIS databases. When possible, data included were restricted to measurements of breeding season males, calculated using the weighted average from all studies. In most cases, we were able to find measurements recorded within a polygon delimited by the GPS locations of sites where IT had been recorded.

To quantify the pairwise differences between the plumages of the warblers in our study, we had human observers compare images of species pairs from field guides. Since males are the primary territory defenders (e.g., MacArthur 1958), we scanned two field guides with color illustrations of males of the species in our study (Sibley 2000; Dunn and Alderfer 2006). We compiled the images into 18 different surveys with ~49 comparisons in each survey using QuestionPro (QuestionPro 2012).

For each pairwise comparison, participants were presented with images of a pair of males from the same field guide. We asked them to rate the overall difference between the plumages of the two images using a 0-4 scale (Table 1). The survey software randomized the order of the images for each respondent. To distribute the surveys, we advertised them through social networking platforms and sent out announcements to several birding listservs. Thus, respondents were a mixture of experienced birders, scientists, and laypeople. For each species pair, we calculated the mean difference score across the two field guides.

We also created surveys to quantify the pairwise difference of the songs of the warblers in our study. We downloaded two song exemplars for each species from xeno-canto.org. As with mass and bill measurements, in most cases we found songs recorded within a polygon delimited by the GPS locations of IT sites for a given species. When such recordings were not available, we downloaded the exemplar from the closest site to the polygon. We then used Audacity v. 2.0.3 (Audacity 2012) to edit the songs for the surveys. First, we removed background noise (noise reduction = 22 dB, sensitivity = -7.97 dB, frequency smoothing = 260 Hz, attack/decay time = 0.01 secs). Then we cut out all parts of the recordings except for two bouts of singing, separated by a few seconds of silence. Finally, we normalized the edited song files. Each of the two final exemplars for a species thus included two songs from an individual bird. We compiled the songs into 32 different surveys with ~20 comparisons in each survey (QuestionPro 2012).

For each pairwise comparison, participants were presented with a pair of exemplars. As with the plumage comparison surveys, we asked them to rank the overall similarity of the songs using a 0-4 scale (Table 1). The survey software randomized the order of the pairwise song comparisons for each respondent. We distributed the surveys using the same channels as the plumage comparison surveys.

Since birds may respond to different aspects of songs than humans, we included a measure of song similarity calculated from spectrogram cross-correlation (SPCC) (Clark et al. 1987). Briefly, this method calculates the maximum similarity between two spectrograms by sliding the spectrograms until the peak cross-correlation value is achieved. To calculate SPCC scores for each species pair, we used RavenPro v. 1.4 (Bioacoustics Research Program 2011) with the default settings (biased normalized spectrogram correlation, no filter). Since all of our exemplars consisted of two songs, and the SPCC score is calculated by comparing songs in a pairwise fashion, we split each exemplar into two. Thus, the species-pair SPCC score reflects the mean of the SPCC scores for the two exemplars for each species, which themselves are the mean of the SPCC scores for the two songs contained in each exemplar.

(iii) Warbler phylogeny and simulation

We obtained a well-supported maximum likelihood phylogeny of New World warblers (Lovette et al. 2010, their Fig. 5) built with both mitochondrial and nuclear sequence data. We pruned the tree to contain only species included in our study for subsequent analyses.

To simulate similarity in mass and bill length, we used the approach outlined above for continuous variables by calculating the observed σ^2 value from the data and using this value to generate 5,000 simulated datasets (Table 2). To simulate guild overlap, we used the approach outlined above for binary variables such that the simulated data had the same number of species with overlapping guilds as the raw dataset. For our other variables (human determined plumage and song similarity, and SPCC song similarity), we transformed the simulated difference distributions using a log-transformation.

To do this, we started with the equation:

(Eq. 1) $$\frac{|\log_a(D) + b|^c}{d}$$

Where D is the distribution of pairwise differences of simulated tip data, a is the base of the logarithm, and b-d are positive integers. Specifically, a and c change the shape of the distribution while b and d together change the range of the distribution to scale the simulated data to the raw data.

To minimize the difference between the simulated dataset and the empirical dataset, we wrote a script that sampled 1 million times each from 4 distributions (a = uniform(1.1,40), b = uniform(0,10), c = uniform(1,10), d = uniform(0.1,100)), compared the mean and standard deviation of the empirical distribution with the mean and s.d. of the simulated distribution transformed using these sampled values, and chose the values from the combination of a-d that yielded a transformed distribution closest in mean and standard deviation to the empirical distribution.

For the parameter values used in the simulation for each predictor variable, and statistical tests comparing the simulated and empirical distributions, see Table 2. We did not simulate the evolution of syntopy, but we included raw syntopy variables in the statistical analysis to control for species overlap.

(*iv*) Statistical analysis

We began by building a full logistic regression model of interspecific territoriality, using all of the predictor variables described above, using transformations of variables in order to meet parametric assumptions (Table 2,7). We then used backwards model selection to identify the "best" model (i.e., the model with the lowest AIC). We then re-ran both the full and best models using the 5,000 simulated datasets to generate null distributions for the test statistics (z).

(c) Example 2: Reproductive interference and female wing color in Hetaerina *damselflies*

(i) Reproductive interference, interspecific aggression, and female wing color

To quantify the magnitude of reproductive interference, we conducted female tethering tests on male territory holders at 7 sites, representing 6 different sympatric species pairs (Table 3). Males were individually marked, and we considered males perched in the same location (±3 m) to be territory holders (see Chapter 1). We presented males sequentially with tethered conspecific and heterospecific females and recorded their response, systematically varying the presentation order. *Hetaerina* matings begin when a male clasps a female's intersternite (Garrison 1990), and proceed without any courtship ritual. If a male either approached the female with his abdomen curled, actually attempted to clasp the female at her intersternite, or successfully clasped her, we considered a male to have responded sexually. Each presentation lasted 5 s, or until the male returned to his perch, whichever came last. If the focal male clasped the female during her first presentation, we ended the trial; if not, we presented her for another 5 s. In our analyses, we only included data from females presented to males who responded positively to at least one tethered female. From these experiments, we calculated a heterospecific clasping ratio by dividing the proportion of tethered females that elicited sexual responses in trials with heterospecific males by the proportion of tethered females that elicited sexual responses in trials with conspecific males. For each species, the heterospecific clasping ratio serves as an index of the magnitude of reproductive interference imposed by a sympatric congener.

To measure interspecific aggression relative to intraspecific aggression, we presented territory holders with tethered male intruders (Anderson & Grether 2010). Each territory holder was presented sequentially with a conspecific and heterospecific male, with the order of presentation trials balanced across males (for a detailed explanation, see Chapter 1). Briefly, during each trial, we recorded the behavior of the territory holder, including the time spent chasing the tethered male and the number of slams (defined as attempts to ram the tethered male, whether successful or not) and grabs (defined as extended physical contact with the tethered male). To obtain a relative measure of aggression toward heterospecific males (hereafter referred to as the "aggression ratio"), for each species at each site, we divided the mean attack rate (slams and grabs divided by the duration of the trial) toward heterospecific tethered males by the mean attack rate toward conspecific tethered males.

We measured the reflectance spectra of females' wings at the same sites where we measured responses of territorial males to tethered females. We used an Ocean Optics USB-2000 spectrometer equipped with a pulsed xenon light source (Ocean Optics PX-2) oriented at 45 degrees relative to the wing surface to eliminate glare and measured intensity as the proportion reflectance relative to a Labsphere certified reflectance standard using Ocean Optics' OOIBase32 software. During measurements, we placed the wings on the reflectance standard. Wing color measurements include both light reflected off the wings and light transmitted through the wings. We averaged three measurements each for the base, middle, and tip of forewings and hindwings. For each of these averaged spectra, we calculated lightness (L) from the sum of percent reflectance at 2 nm intervals from 300 to 700 nm. Since the color in the middle of females' wings makes up a relatively larger proportion of the wing, a weighted measure of lightness was obtained for each species with the formula: $L_{\text{total}} = 0.1L_{\text{base}} + 0.8L_{\text{middle}} + 0.1L_{\text{tip}}$ (see Table 3 for species L values and sample sizes).

(ii) Taxon & specimen sampling

To construct the phylogeny, we included 32 specimens from 9 *Hetaerina* species, sampling several individuals from different populations where possible (Table 4). We included individuals of *Calopteryx maculata* and *Calopteryx aqueabilis* as outgroups.

(iii) Morphological and sequence data and alignment preparation

We obtained a matrix of adult female and male morphological characters used in the creation of the key to the genus *Hetaerina* (Table 5, Garrison 1990, R. Garrison, pers. comm.). We also included morphological character data from a published account of *Hetaerina* larvae (see Table 3 in Zloty et al. 1993).

We extracted DNA from wing muscle tissue of ethanol preserved specimens using Qiagen DNEasy kits (Qiagen, Valencia, CA, USA). Several target mitochondrial and nuclear sequences (Table 6) were amplified using the polymerase chain reaction (PCR). In most cases, the PCR amplification thermocycling regime was 15 min at 95°C, followed by 12 cycles of 30s at 94°C, 90s at 60°C and 60s at 72°C, 32 cycles of 30s at 94°C, 90s at 55°C and 60s at 72°C, and a final elongation at 60°C for 30 min. We modified this regime and/or increased the volume of extracted DNA in cases where samples amplified poorly. In some cases, samples were cleaned using an ExoSap (Amersham Biosciences) procedure and cycle-sequenced with BigDye Terminator v.3.1 (Applied Biosystems) prior to sequencing at either the UCLA GenoSeq Core (Los Angeles, CA) or Cornell Genomics Facility (Ithaca, NY). However most PCR products were sent to Beckman Coulter Genomics (Danvers, MA) for sequencing. Resulting forward and reverse chromatograms were aligned in Geneious 4.8.3 (Biomatters, Inc.), checked, and assembled into contigs. Consensus sequences for each locus were aligned using Muscle v.3.8.31 alignment software (Edgar 2004), inspected visually, and altered manually if necessary. For three sequences that yielded very long branch lengths in gene trees from an initial analysis (16s/tRNA-leu/ND-I: *H. pilula* 3 & *H. miniata* 4, tubulin alpha: *H. capitalis* 4), we checked sequences against BLAST and found that BLAST returned taxonomically distant matches. As a result, we excluded these sequences from final multiple alignments. Concatenated sequence files were created using SequenceMatrix (Vaidya et al. 2011). Our final concatenated matrix totaled 3853 nucleotides (Table 6) and 89 morphological characters, which were added to one individual for each species.

(iv) Phylogenetic inference

When it was possible to verify sequence alignments to ensure the accuracy of codon partitioning using BLAST (Altschul et al. 1990), we partitioned loci at the codon level. We then used PartitionFinder v. 1.1.1 (Lanfear et al. 2014) to identify the best-fit partitioning scheme and suitable models of evolution for each partition using BIC model selection and the "greedy" search algorithm. Final phylogenetic inferences were conducted on alignments with the partitions identified in PartitionFinder and three additional partitions for morphological characteristics (females, males, and larvae).

We used MrBayes 3.2.2 for Bayesian reconstruction of the phylogeny (Ronquist and Huelsenbeck 2003). The analysis was run for 20 million generations, sampling every 5000 generations, with four chains (one cold, three heated). Chain convergence was assessed using Tracer 1.6 (Rambaut and Drummond n.d.), 25% of trees were discarded as burnin, and the maximum clade credibility tree was calculated from post-burnin trees using TreeAnnotator v.1.7.4 (Rambaut and Drummond 2012).

We used RAxML 8.0.9 for maximum likelihood (ML) phylogeny reconstruction (Stamatakis 2014) using alignments without morphological data since ambiguity in these characters is not supported by RAxML. Searches were conducted using a random starting tree and with the GTR + gamma model assigned to each partition. Bootstrap support was calculated with 1000 bootstrap replicates.

Both Bayesian and ML analyses were run using the Cipres Web Portal (Miller et al. 2010). To obtain an ultrametric tree for modeling trait evolution, we rendered the maximum clade credibility tree obtained from mrBayes (Fig. 2) ultrametric using the chronos() function in the ape package in R (Paradis et al. 2004) and dropped tips so that the topology had a single tip for each species (Fig. 4).

(v) Statistical analyses

Because the pairwise difference between species in female wing coloration is not independent within sites (i.e., the difference for one species is simply the negative of the difference from the other species' perspective), we calculated Spearman rank correlations between similarity in female wing lightness and interspecific interactions 1,000 times, each time randomly choosing one species' value of either the clasping ratio or aggression ratio for each site. From this distribution of empirically derived ρ values, we calculated the mean ρ for each pair of variables. To obtain a phylogenetically corrected statistical test, we used fitContinuous() as described above to simulate female wing coloration values 1,000 times using both Brownian motion and OU models of evolution. On each simulated dataset, we created a distribution of 1,000 ρ values from simulated female wing lightness and empirical clasping and aggression ratios in the same way and stored the mean value of this distribution. We then compared this distribution of simulated mean ρ values to the empirically calculated mean ρ value using a one-sample t-test.

Hetaerina titia female wings exhibit a seasonal polyphenism in their wing lightness (Chapter 2). In the overall analysis, one site's (La Palma in the early season, Table 3) measurements were taken on light-morph females. To determine if the phylogenetically corrected statistic is robust to changes in how we modeled the evolution of wing color, we ran analyses including a mean value of *H. titia* female wings calculated across all sites and another analysis excluding data from La Palma in the early season, where most females were light-phase morphs.

Results

Predictors of interspecific territoriality in New World wood warblers

Even without a phylogenetic correction, species differences in body mass, bill size, and feeding guild overlap were not predictive of interspecific territoriality after controlling for variation in song similarity, plumage similarity and syntopy (Table 7). In the raw model, degree of syntopy predicted the occurrence of IT, with more syntopic species being more likely to exhibit IT (Table 7), so we included this as a covariate in all phylogenetic models.

Plumage similarity significantly predicted the occurrence of IT in all the raw data models, and the phylogenetically corrected full model (Table 7). Specifically, IT is more likely in species with similar looking plumage, as rated by humans. However, in the phylogenetically corrected best model, plumage similarity was no longer a significant predictor of IT (Table 7). SPCC song similarity was strongly, positively predictive of IT, both before and after phylogenetic correction in all models (Table 7).

Hetaerina *tree construction*

Bayesian and ML reconstructions largely coincided, except for the placement of *H. americana* (Fig. 2, 3), which was placed in a separate clade with *H. vulnerata* and *H. cruentata* in the Bayesian tree.

Reproductive interference, interspecific aggression, and female wing coloration

Phylogenetically simulated species differences in female wing lightness did not differ from empirically measured differences across the sites (including all sites: BM, σ^2 = 5330548216.91, starting mean = 200985.31, bounded by range of female lightness data, OU model, σ^2 = 15341937971.12, starting mean = 197860.50, α = 2.72; removing early season La Palma: BM, σ^2 = 8410441272.12, starting mean = 196303.53, bounded by range of female lightness data, OU model, σ^2 = 24299197168.71, starting mean = 192220.63, α = 2.72).

Species differences in female wing lightness were negatively correlated with the level reproductive interference and this correlation remained highly significant after the phylogenetic correction (Fig. 5a; mean observed ρ = -0.772; BM model of evolution, t = 59.11, *d.f.* = 999, $p <$ 0.001; OU model of evolution, t = 57.78, *d.f.* = 999, $p <$ 0.001; excluding early season La Palma: mean observed ρ = -0.730; BM model of evolution, t = 52.16, *d.f.* = 999, $p <$ 0.001; OU model of evolution, t = 50.74, *d.f.* = 999, $p <$ 0.001).

Likewise, species differences in female wing lightness were negatively correlated with the magnitude of interspecific aggression, and this correlation remained highly significant after the phylogenetic correction (Fig. 5b; mean observed ρ = -0.804; BM model of evolution, t = 55.31, *d.f.* = 999, $p <$ 0.001; OU model of evolution, t = 53.55, *d.f.* = 999, $p <$ 0.001; excluding early season La Palma: mean observed ρ = -0.722; BM model of evolution, t = 51.34, *d.f.* = 999, $p <$ 0.001; OU model of evolution, t = 50.65, *d.f.* = 999, $p <$ 0.001).

Discussion

(a) Agonistic signal similarity and interspecific territoriality in warblers

After controlling for phylogeny, we found strong evidence for a relationship between song similarity (measured through SPCC analysis) and interspecific territoriality—sympatric species with similar territorial songs are more likely to be interspecifically territorial than those with dissimilar songs. Willis et al. (2013) found a similar result for a relationship between SPCC similarity and the occurrence of hybridization. Plumage similarity, scored by humans, was less consistently predictive of IT in our analyses, and depended on the other terms included in models. This may indicate insufficient power in the dataset to draw strong conclusions about the relationship between overlap in visual signals and IT. That plumage similarity is a significant predictor in the raw analysis but less consistently so in phylogenetically corrected analyses may also indicate that closely related species, which appear similar to one another, are constrained by overlap in visual signals used in territorial interactions. Surprisingly, agonistic signals were more predictive of IT than ecological overlap, contrary to many theoretical expectations for adaptive IT (e.g., Orians and Willson 1964; Cody 1969, 1973). This may be because interspecific interference competition is a maladaptive consequence of overlap in agonistic signals (Murray 1981), or may indicate that exploitative competition for access to resources not included in our analyses (including, for example, mating competition, see Chapter 1) explains the maintenance of overlap in such agonistic signals. Future research studying signals such as territorial song variation at the population level could distinguish between these possibilities.

(b) Species similarity in female wing coloration and between-species interactions in rubyspot damselflies

Pairwise species differences in female *Hetaerina* wing lightness were predictive of empirically observed levels of reproductive interference and interspecific aggression, even after correcting for shared ancestry. Phylogenetic corrections were robust to changes in the evolutionary model used to simulate the data. In conjunction with experimental and observational evidence demonstrating that female wing coloration is important for male mate recognition in *Hetaerina* (Chapter 1), this constitutes strong evidence for a relationship between similarity in female wing phenotypes and reproductive interference, which in turn is a strong predictor of interspecific territoriality. Since female rubyspot damselflies are completely submerged while ovipositing, they are vulnerable to both aerial and aquatic predators (Johnson 1961). As such, female wing coloration may be under strong selection for crypsis, thus impeding evolutionary changes that could facilitate species recognition.

(c) Simulation approach to phylogenetic correction of pairwise interactions

The statistical approach outlined here could easily be applied to other instances where phylogenetically permuted partial Mantel tests have been used to study pairwise species interactions in comparative frameworks, such as understanding the causes or consequences of similarity between species in signal morphospace (Martin and Mendelson 2012; Allen et al. 2014) or predictors of between species behavioral interactions (Willis et al. 2013, this study). Our approach may also be a viable alternative to statistical approaches employed by ecologists interested in the influence of trait overlap on community assembly while controlling for phylogenetic relatedness (e.g., Webb et al. 2002; Cavender-Bares et al. 2004). In cases where complete species interaction matrices are biologically reasonable, some investigators have included phylogenies as random effect terms in linear mixed-effect models (e.g., Tobias et al. 2013; Willis et al. 2013). Our method provides an alternative or additional means to statistically correct for the shared ancestry of species in such comparative frameworks. Understanding the relative strengths and weaknesses of these various methods, and/or demonstrating that conclusions are robust to different means of phylogenetic correction where possible, is paramount as the use of phylogenetic statistics continues to increase in ecology and evolutionary biology.

Table 4-1. Rating scales provided to web survey participants for (a) plumage and (b) song comparison surveys.

(a) Plumage comparison

Score	*Description*
0	No difference. The birds are virtually identical in plumage color & pattern.
1	Some difference in color and/or pattern, but the birds' plumages are nearly identical.
2	Moderately different. The birds overlap in color and/or pattern of plumage, but marked differences are present.
3	Somewhat similar in color and/or pattern, but mostly different.
4	Extremely different. The birds are completely different in both plumage color and pattern.

(b) Song comparison

Score	*Description*
0	Songs practically indistinguishable
1	Songs similar in most respects, but recognizably different
2	Songs similar in many respects, but different in others
3	Songs similar in some respects, but quite different
4	Songs do not resemble each other whatsoever

Table 4-2. Parameters used for phylogenetic simulation of warbler IT, transformations of simulated data, and comparison of simulated datasets to empirical difference distributions. See main text for description of variables and transformations. (*) see Eq. 1 in main text.

variable	BM model parameters			transformation
	σ^2	starting mean	bounds	
sqrt(difference in mass)	0.135813	10.69561	6.6, 19.82105	--
sqrt(difference in exposed culmen length)	0.047829	10.3906	8.083226, 14.3	--
plumage difference (from surveys)	0.1	2.5	0, 5	*a = 27.82601, b = 6.175658, c = 2.93477, d = 51.96569
song difference (from surveys)	0.1	2.5	0, 5	*a = 7.244837, b = 7.013548, c = 2.675404, d = 49.68288
sqrt(SPCC song similarity)	0.1	2.5	0, 5	sqrt of values from Eq. 1 (*a = 39.87552, b = 1.321954, c = 4.125054, d = 41.89644)
guild overlap	0.001	1	0, 2	85 most similar pairs assigned to "1"

Table 4-3. (‡) indicates data from Anderson & Grether 2010, all other data from Chapter 1.

site	species 1	species 2	clasping ratio (n males)		aggression ratio (n males)		wing lightness L_{total} (n females)	
			sp. 1	sp. 2	sp. 1	sp. 2	sp. 1	sp. 2
Bonita Creek	*H. americana*	*H. vulnerata*	1 (18)	1.4 (18)	1.003 (16)	1.247 (16)	225004.7 (25)	218297.1 (20)
Castroville‡	*H. americana*	*H. titia*	0.0545(24)	0.482 (22)	0.323 (33)	0.203 (30)	202008.2 (24)	69345.37 (26)
Cuetzalapan	*H. cruentata*	*H. occisa*	0.915 (17)	0.756 (20)	1.04 (15)	1.057 (16)	235271.8 (6)	231365.7 (25)
Laguna Escondida	*H. sempronia*	*H. occisa*	1 (10)	0.615 (20)	1.392 (14)	0.867 (19)	216139.3 (7)	234272.8 (27)
La Palma-early season	*H. occisa*	*H. titia*	0.594 (48)	0.944 (38)	0.349 (56)	0.156 (16)	228482.1 (30)	189813 (30)
La Palma-late season	*H. occisa*	*H. titia*	0.24 (37)	0 (25)	0.135 (16)	0.141 (19)	222894.4 (24)	30624.86 (26)
Otapa‡	*H. occisa*	*H. titia*	0.0588 (7)	0 (17)	0.01 (39)	0.02 (23)	204119.2 (4)	38976.18 (9)
Pixquiac	*H. vulnerata*	*H. cruentata*	0.786 (11)	0.909 (14)	1.012 (10)	0.861 (17)	216954.5 (13)	217889.9 (25)

Table 4-4. Specimens used in the reconstruction of the phylogeny and their GenBank accession numbers. Locus numbers follow codes in Table 4-6 (shaded cells correspond to missing sequences).

new tree name	collection location	decimal degrees (lat, long)	Locus							
			1	2	3	4	5	6	7	8
Calopteryx aequabilis	Horse Creek, CA	41.824, -123.000		KM383956	KM383925	KM383900	KM383991	KM383865		KM383796
C. maculata	Burr Ferry, LA	31.076, -93.489	KM383849	KM383957	KM383926		KM383992	KM383864		
Hetaerina americana 1	Arroyo de Piedra	19.456, -96.479	KM383858	KM383984	KM383951		KM384000	KM383870	KM383832	KM383824
H. americana 2	Bonita Creek, AZ	32.916, -109.493	KM383853	KM383982	KM383950	KM383899		KM383872	KM383833	KM383825
H. americana 3	Bonita Creek, AZ	32.916, -109.493	KM383854	KM383983	KM383949	KM383898	KM383999	KM383871	KM383834	KM383826
H. capitalis 1	Los Organos	18.657, -95.151		KM383978	KM383928	KM383914		KM383889		KM383813
H. capitalis 2	Upper Cuetzalapan	18.367, -94.965		KM383979	KM383929	KM383915		KM383890		KM383811
H. capitalis 3	Upper Cuetzalapan	18.367, -94.965		KM383980	KM383930	KM383916		KM383891		KM383812
H. capitalis 4	Río Limón	21.367, -104.617	KM383861	KM383981	KM383927	KM383913	KM384009	KM383888		KM383810
H. cruentata 1	Cuetzalapan	18.371, -95.001		KM383986	KM383954	KM383918	KM383998	KM383893	KM383842	KM383818
H. cruentata 2	Los Organos	18.657, -95.151	KM383850	KM383987	KM383955	KM383919	KM383996	KM383894	KM383841	KM383819
H. cruentata 3	Pixquiac	19.467, -96.95		KM383985	KM383953	KM383917	KM383997	KM383892	KM383840	KM383820
H. miniata 1	Bartola	10.989, -84.334		KM383966	KM383939	KM383922	KM384014	KM383884	KM383831	KM383801
H. miniata 2	Bartola	10.989, -84.334		KM383967	KM383942		KM384015	KM383885	KM383828	KM383802
H. miniata 3	Bartola	10.989, -84.334		KM383968	KM383940	KM383905	KM384016	KM383886	KM383829	
H. miniata 4	Bartola	10.989, -84.334		KM383969	KM383941		KM384017	KM383887	KM383830	KM383803
H. occisa 1	La Palma	18.550, -95.067		KM383972	KM383933	KM383906	KM384001	KM383866	KM383848	
H. occisa 2	Benito Juarez	18.359, -95.000		KM383971	KM383934	KM383904	KM384002	KM383868	KM383846	
H. occisa 3	Benito Juarez	18.359, -95.000		KM383973	KM383935	KM383911	KM384004	KM383869	KM383847	KM383809
H. occisa 4	La Palma	18.550, -95.067	KM383857	KM383970	KM383932	KM383902	KM384003	KM383867	KM383845	KM383808
H. pilula 1	Cuetzalapan	18.371, -95.001		KM383962	KM383945	KM383903	KM384011	KM383881		KM383804
H. pilula 2	Laguna Escondida	18.592, -95.084		KM383964	KM383943		KM384012	KM383883	KM383827	KM383806
H. pilula 3	Laguna Escondida	18.592, -95.084		KM383965	KM383944		KM384010	KM383882		KM383807
H. pilula 4	Balzapote	18.614, -95.073	KM383859	KM383963			KM384013			KM383805
H. sempronia 1	Laguna Escondida	18.592, -95.084	KM383856	KM383975	KM383948	KM383908	KM384005	KM383878		KM383814
H. sempronia 2	Laguna Escondida	18.592, -95.084	KM383855	KM383974	KM383946	KM383901	KM384006	KM383877		KM383815
H. sempronia 3	Los Organos	18.657, -95.151		KM383976	KM383947	KM383909	KM384007	KM383879		KM383816
H. sempronia 4	Upper Cuetzalapan	18.367, -94.965		KM383977		KM383910	KM384008	KM383880		KM383817
H. titia 1	La Palma	18.550, -95.067	KM383863	KM383959	KM383937		KM384019	KM383873	KM383838	KM383800
H. titia 2	Bartola	10.989, -84.334		KM383960		KM383923	KM384021	KM383874	KM383836	KM383799
H. titia 3	Burr Ferry, LA	31.076, -93.489		KM383958	KM383936	KM383907	KM384018	KM383875	KM383837	KM383798
H. titia 4	Armeria	18.950, -103.934	KM383860	KM383961	KM383938	KM383912	KM384020	KM383876	KM383835	KM383797
H. vulnerata 1	Pixquiac	19.467, -96.95	KM383851	KM383989	KM383952	KM383921	KM383993	KM383896	KM383839	KM383822
H. vulnerata 2	Pixquiac	19.467, -96.95	KM383852	KM383990		KM383924	KM383995	KM383897	KM383844	KM383823
H. vulnerata 3	Sierra Vista, AZ	31.480, -110.337	KM383862	KM383988	KM383931	KM383920	KM383994	KM383895	KM383843	KM383821

Table 4-5. Morphological characters for adult males and females used to build the morphological matrix. Figure numbers from Garrison 1990.

females

labrum brilliant metallic blue-green
labrum black with white (or pale) spot on each side)
labrum concolorus (all pale or all dark)
epicranium matte black
epicranium with a metallic blue-green luster
epicranium with a metallic red luster
1st antennal segment pale, contrasting with 2nd segment
without post-occipital tubercules
with post-occipital tuberacles as in Fig. 54
with pronounced postoccipital tuberacles as in Fig. 53
prothorax with well developed horns Fig. 78, 99a
intersternite (inst.) dorsally directed digit Fig. 78-80
inst.widened dorsally Fig. 84,94,103
inst. dorsal and with a v-or v-shaped cleft Fig. 89,103
inst. dorsal end with only an anterior shoulder, cleft obtuse eg Fig. 81
inst. dorsal end opposing bi or trilobate (e.g. Fig. 88)
inst. with isolated sclerite dorsally eg. Fig. 99
inst. with anterior arm shorter than posterior arm
inst. anterior arm subecust toposterior arm Fig. 103a
inst. anterior arm longer than posterior arm (Fig. 103c)
synthx(synthorax) mark color metallic red (Fig. 117, 126)
synthx: upper end of metallic color meeting humoral suture (Fig. 129)
synthx: upper end of metallic color not meeting humeral suture (Fig. 128)
synthx: with metallic color on mesenfraepisternum (Fig. 134,137)
synthx: mesepisternal & meseprimeral stripes connected at upper end (Fig. 125)
without a pterostigma
ab dorsal dark and lateraventral pale areas contrasting (Fig. 140)
abd. segment 10 posteriorly with no dorsolateral spine (Fig. 144-145)
dorsal carina on abd 10: spine (Fig. 141,147)
dorsal carina on abd 10: a keel or nonexistant (Fig. 145)
ab10: with a ventrolateral spine (Fig. 144)

males

labrum brilliant metallic green
labrum black with white (or pale) spot on each side
labrum concolorus (all pale or all dark)
epicranium with a metallic luster
1st antennal segment pale, contrasting with 2nd segment
with occipital tubercles (Fig. 51-52)
some metallic green on metepisternum (e.g. Fig. 66,69)
without a pterostigma
red apical spot in forewing
brown (or slightly smoky) apical spot in forewing
forewing tip hyaline (clear)
red apical spot in hindwing
brown (or slightly smoky) apical spot in hindwing
hindwing tip hyaline (clear)
abd10: posteriorly with a small dorsolateral spine (Fig. 55)
dorsal carina on abd 10 a spine surpassing segment margin Fig. 57
dorsal carina on abd 10 a spine not surpassing segment margin (Fig. 58-60)
dorsal carina on abd 10: a keel or nonexistant at end of segment (Fig. 61)
inf. app.: mesobasal process:long thumblike > 3x longer than wide (Fig. 3b, 29, 30a, 31c,48a)
inf app: distal process: rudimentary (e.g. Fig. 4-6)
inf. app.: distal process 0.24 or less the length of sup. app. (e.g. Fig. 3)
inf. app.: distal process ca .3 to .5 the length of sup. app (e.g. Fig. 7,8, 11-14)
inf. app.: distal process ca .66 or more the length of sup. app. (e.g. Fig. 9,15,22,29)
inf. app.: distal process expanded at, forming a club or racquet (Fig. 9,10)
inf. app.: distal process strongly curved in lateral view (e.g. Fig. 45-46)
inf. app.: distal process tip truncate (excludes recurved tooth) (e.g. Fig. 14,37)
inf. app.: distal process tip attenuate (excl. recurved tooth) (eg. Fig. 26,28)
inf. app.: distal process tip with a single (or with small 2nd) tooth (Fig. 15,22)
inf. app.: distal process tip with 2-4 small recurved teeth (Fig. 16-41)
inf. app.: distal process tip with a single tooth or none (Fig. 27,31)
sup. app: basal 0.33 with strong almost transverse elevated ridge (Fig. 2)
sup. app.: base with rudimentary tubercles or none (e.g. Fig. 20,22)
sup. app.: anterior margin of median lobe at right angle to sup. app (Fig. 13)
sup. app.: median lobe evenly convex (e.g. Fig. 14,20,23,25)
sup. app.: median lobe bimodal or bidentate (eg. Fig. 22,28-32)
sup.app.: median lobe unidentate,triangular (Fig. 26)
sup.app.: with well defined (complete) transverse ridge (e.g. Fig. 17,19)
sup.app.: with well defined (complete) superior ridge (e.g. Fig. 17,19)
sup.app.:sup and transverse ridge meeting at acute angle (e.g. Fig. 17)
sup.app.: sup and transverse ridge meeting at rounding angle (e.g. Fig. 21,24)

inst=intersternite, abd=abdomen, inf. app=inferior appendange (male clasper), sup. app=superior appendage (male clasper)

Table 4-6. Loci and primers used to generate sequence data for the phylogeny

locus	primers (5'→3' forward top, 5'→3' reverse bottom)	total alignment length	min/max length of sequenced product (bp)	reference
<u>*mitochondrial*</u>				
1. cytochrome oxidase I	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	664	611/664	Folmer et al. 1994
2. 16s rRNA^{p}	GCTCCGDTTTGAACTCAGAT AGTTCTCGCCTGTTTATCAAA	534	512/523	Fleck et al. 2008
3. 12s rRNA^{p}, tRNA-valine^{c}, 16s rRNA^{p}	GATCTGATGAAGGTGGATTT TAGCTCTTCTGAAATCGAGA	421	363/411	Fleck et al. 2008
4. 16s rRNA^{p}, tRNA-leucine^{c}, NADH dehydrogenase I^{p}	TTCAAACCGGTGTAAGCCAGG TAGAATTAGAAGATCAACCAG	534	387/532	Rach & deSalle 2008
<u>*nuclear*</u>				
5. elongation factor α^{p}	GCYGARCGYGARCGTGGTATYAC CATGTTGTCGCCGTGCCAAC	459	421/449	Monteiro & Pierce 2001
6. histone 3	ATGGCTCGTACCAAGCAGACVGC ATATCCTTRGGCATRATRGTGAC	328	328/328	Terry & Whiting 2005
7. tubulin alpha	GAAACCRGTKGGRCACCAGTC GARCCCTACAAYTCYATTCT	155*	447/466	Mugleston et al. 2013
8. 18s & 28s rRNA^{p} 5.8s rRNA^{c}	TAGAGGAAGTAAAAGTCG GCTTAAATTCAGCGG	758	460/697	Dumont et al. 2005

^{p}=partial
^{c}=complete
*only one codon was variable

Table 4-7. Results of logistic regression models predicting the occurrence of interspecific territoriality. For descriptions models and the predictor variables, see the main text. Predictors with *z*-values that are more extreme than 95% of the phylogenetically simulated *z*-values are in bold. Asterisks indicate significance in the raw binomial model (* = <0.05, ** = <0.01)

model	variable	estimate	std. error	*z*-value	*p*	phylogenetically simulated *z*-value 5th, 95th quantiles
(a) *full model*	sqrt(difference in mass)	-0.86835	0.65387	-1.328	0.18417	-2.5498552, 0.5589984
	sqrt(difference in exposed culmen length)	-0.40608	0.79545	-0.511	0.60970	-2.502162, 0.565033
	plumage difference (from surveys)	**-1.22551**	**0.52527**	**-2.333**	**0.01964***	**-1.769950, 1.066464**
	guild overlap	0.69477	0.65772	1.056	0.29082	-0.8459854, 2.5154025
	log(syntopy)	1.89284	0.63710	2.971	0.00297**	2.862095, 3.667015
	song difference (from surveys)	0.02768	0.42431	0.065	0.94799	-1.187302, 1.698583
	sqrt(SPCC song similarity)	**12.49932**	**4.81397**	**2.596**	**0.00942****	**-2.327728, 0.963512**
(b) *best model*	sqrt(difference in mass)	-0.9337	0.6180	-1.511	0.13085	-2.6169981, 0.4236344
	plumage difference (from surveys)	-1.2089	0.5213	-2.319	0.02040*	-2.3903001, 0.9442075
	log(syntopy)	1.9425	0.6221	3.123	0.00179**	3.043996, 3.644523
	sqrt(SPCC song similarity)	**11.7653**	**4.5194**	**2.603**	**0.00923****	**-2.3723174, 0.8540829**

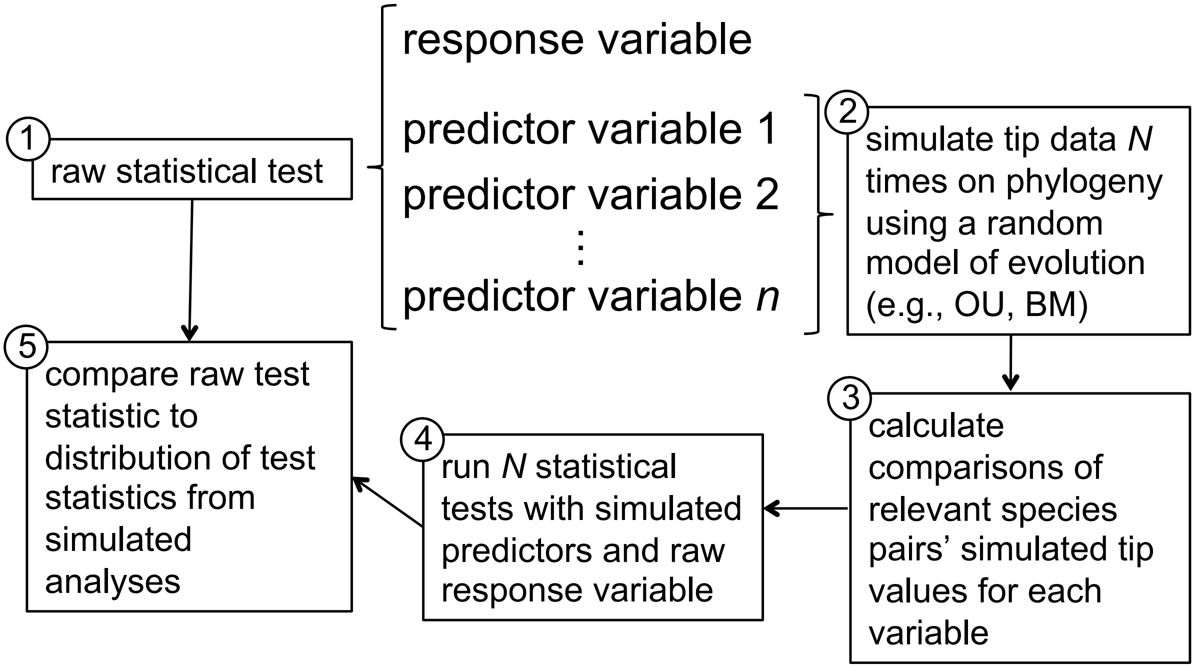

Fig. 4-1. Schematic diagram of the algorithm used to test for a phylogenetically corrected statistical relationship between response variables and predictor variables measured as between-species comparisons.

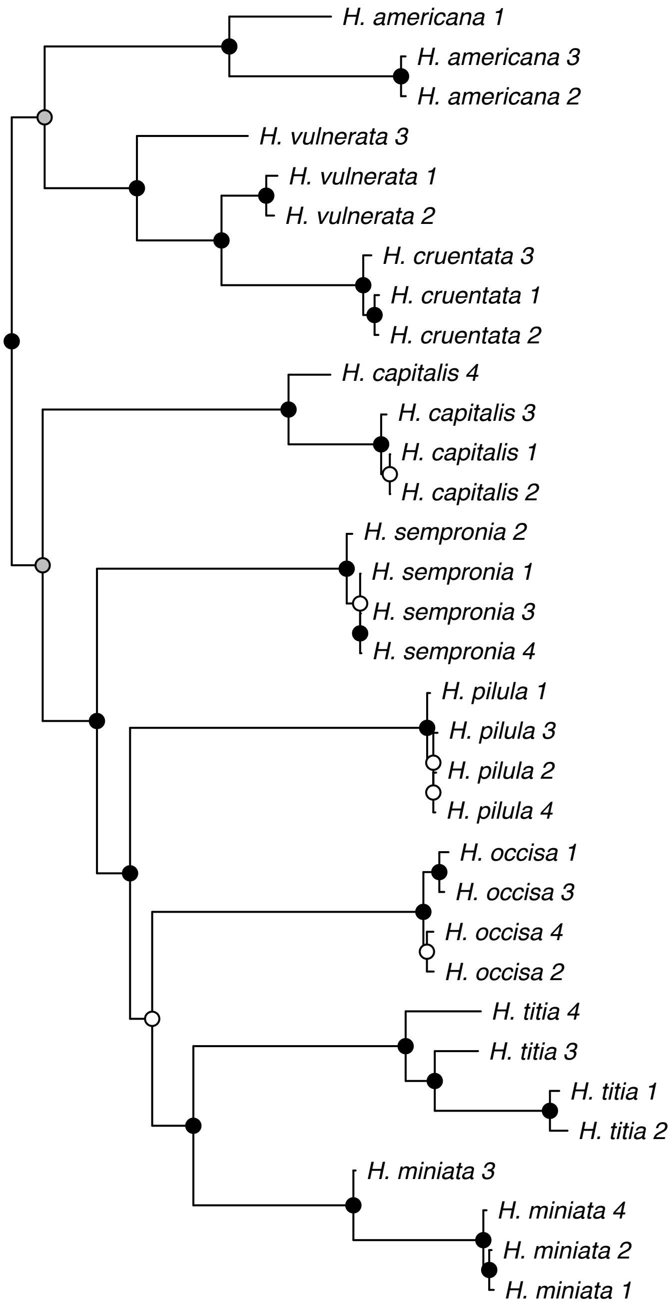

Fig. 4-2. Maximum clade credibility tree calculated from partitioned data set using Bayesian tree inference. Black circles indicate mean posterior probability >0.95, gray circles >0.75 and < 0.95, and empty circles <0.75.

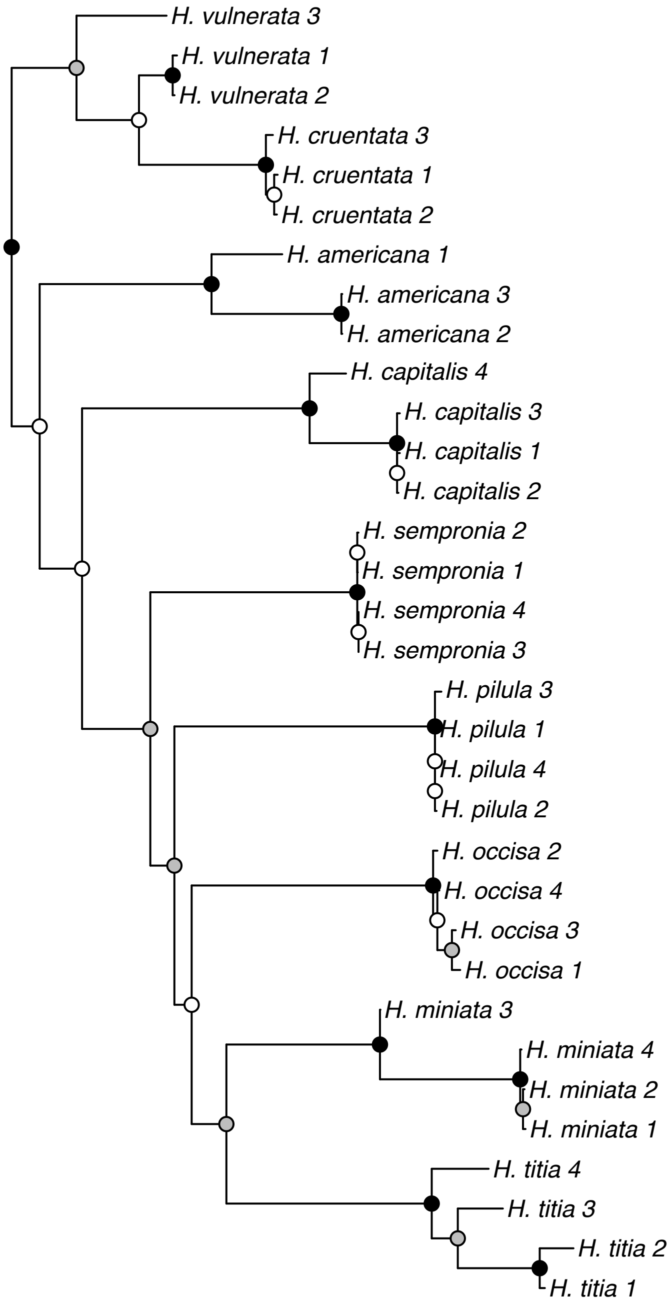

Fig. 4-3. Maximum likelihood tree calculated from partitioned data set using ML tree inference. Black circles indicate bootstrap support >0.95, gray circles >0.75 and < 0.95, and empty circles <0.75.

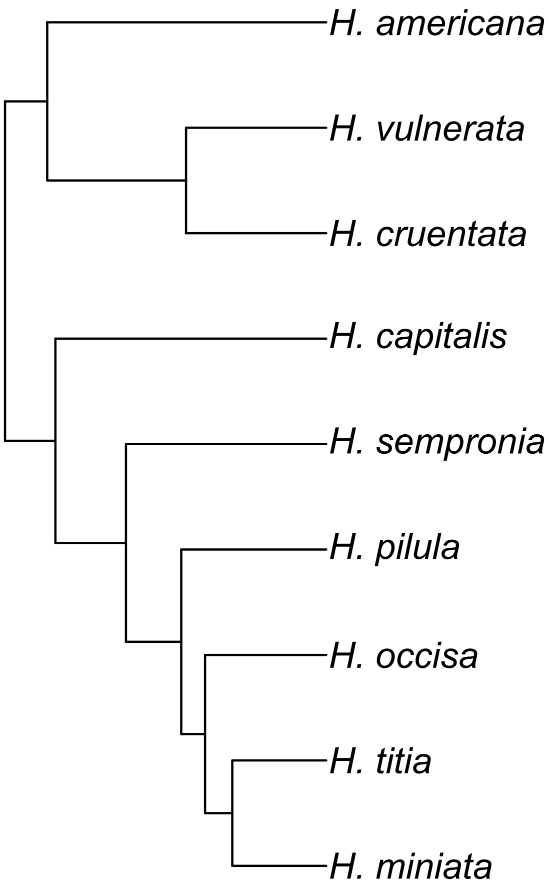

Fig. 4-4. Ultrametric species tree pruned from Bayesian maximum clade credibility tree.

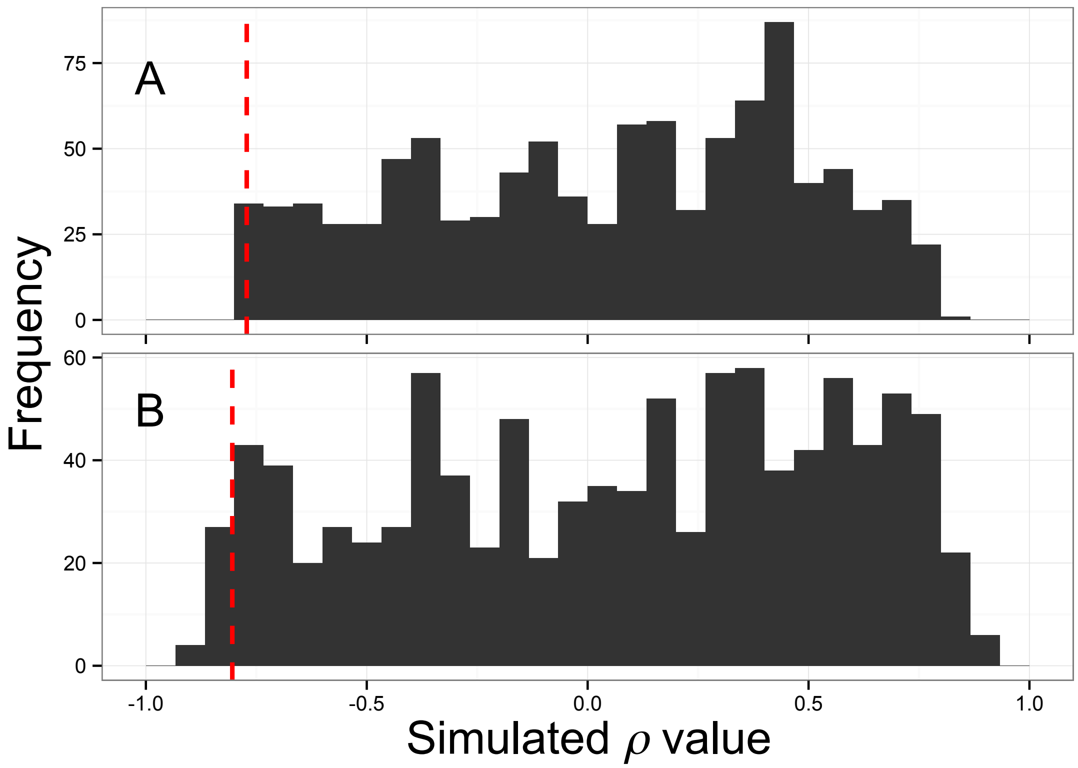

Fig. 4-5. Comparisons of Spearman correlations calculated from *Hetaerina* female wing lightness datasets simulated with Brownian motion across the phylogeny (histograms) to those calculated from empirical datasets (dashed red lines) for (A) correlations between species differences in female wing coloration and the clasping ratio and (B) correlations between species differences in female wing coloration and the aggression ratio. In both cases, empirically derived values are more extreme than expected from Brownian motion simulations (see text for statistical tests).

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