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### Authors

Grether, Gregory F  
Drury, Jonathan P  
Berlin, Erin  
et al.

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## RESEARCH PAPER

# The Role of Wing Coloration in Sex Recognition and Competitor Recognition in Rubyspot Damselflies (*Hetaerina* spp.)

Gregory F. Grether\*, Jonathan P. Drury\*<sup>1</sup>, Erin Berlin\* & Christopher N. Anderson†

\* Department of Ecology and Evolutionary Biology, University of California, Los Angeles, CA, USA

† Department of Biological Sciences, Dominican University, River Forest, IL, USA

**Correspondence**

Gregory F. Grether, Department of Ecology and Evolutionary Biology, 621 Charles E. Young Drive South, University of California, Los Angeles, CA 90095-1606, USA.  
E-mail: ggrether@ucla.edu

<sup>1</sup>Current address: Département de Biologie, École Normale Supérieure, 46 Rue d'Ulm, 75005 Paris, France

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**Abstract**

The decision rules that animals use for distinguishing between conspecifics of different age and sex classes are relevant for understanding how closely related species interact in sympatry. In rubyspot damselflies (*Hetaerina* spp.), the red wing coloration of mature males is hypothesized to be a key trait for sex recognition and competitor recognition within species and the proximate trigger for interspecific male–male aggression. We tested this hypothesis by manipulating the wing coloration of tethered conspecific intruders and measuring the responses of territory holders of three species in the field. As predicted, covering the red spots of mature males with black ink nearly eliminated territorial responses, and in some cases, territorial holders clasped the blackened males as if they were females. Adding red spots to female wings triggered territorial responses and nearly eliminated sexual responses. Immature males with artificial red spots were attacked at the same rate as mature male intruders, and much more frequently than were immature male controls. The results varied somewhat by species. In *H. titia*, the only species of *Hetaerina* with substantial black wing pigmentation, the effects of blackening the red spots of intruders varied both geographically and seasonally. But even when blackening the red spots of male intruders did not reduce the aggressive response of *H. titia* territory holders, adding artificial red spots to female wings elicited aggressive responses and nearly eliminated sexual responses. The results of this study further strengthen the evidence that interspecific aggression in *Hetaerina* results from overlap in territorial signals and that the derived black wing pigmentation of *H. titia* reduces interspecific aggression.

**Introduction**

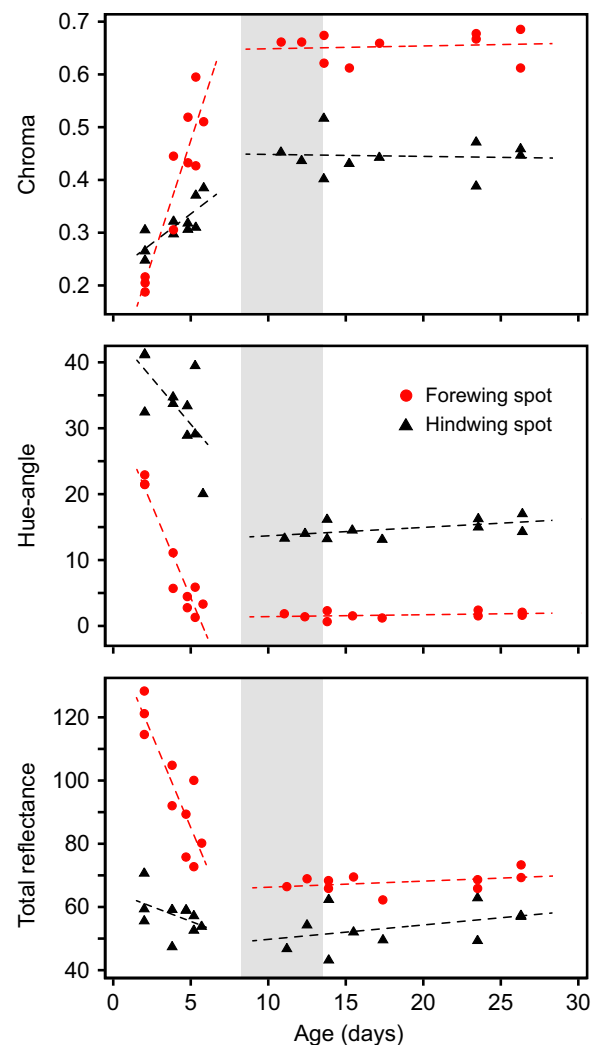
To be effective and efficient, territorial signals must be able to be localized accurately by receivers, to have sufficiently large active space to be perceived at or just beyond the boundaries of a territory, and to differ conspicuously between true competitors and non-competitors (Bradbury & Vehrencamp 1998). Depending on the type of territory being defended, non-competitors may include conspecifics of other age and sex classes, as well as most if not all heterospecifics. For example, in the case of mating territories, territorial behavior is usually restricted to

sexually mature males, and traits that are specific to mature males, and easily detected and localized, such as conspicuous color patches, scent marks, and long-range vocalizations, are most likely to serve as territorial signals. In principle territorial signals could be purely arbitrary traits that serve no other purpose (Bradbury & Vehrencamp 1998), but they are likely to acquire other signaling functions as well (Berglund et al. 1996). If they differ between the sexes, territorial signals may be used for sex recognition at a distance. In species in which females actively seek mating partners, territorial signals are also likely to serve as mate attraction signals. Given their design

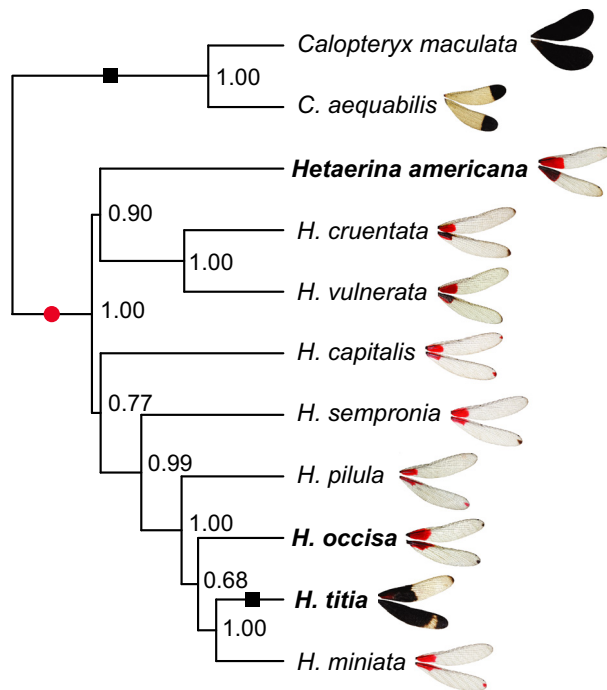
constraints and additional functions, territorial signals may evolve less rapidly than traits that function as signals in more circumscribed contexts, such as short-range communication between the sexes (e.g., courtship signals, genitalic characters). If so, it may often be the case that allopatric populations evolve pre-zygotic barriers to reproduction before diverging appreciably in territorial signals. Indeed, in territory intrusion experiments, closely related species often respond aggressively to each other's territorial signals (Peiman & Robinson 2010; Ord et al. 2011).

Overlap in territorial signals has been hypothesized to be the proximate cause of interspecific fighting in rubyspot damselflies (Odonata: Zygoptera: Calopterygidae: *Hetaerina*; (Anderson & Grether 2010a,b, 2011; Drury & Grether 2014). Mature males of all species in this New World genus display conspicuous red patches at the base of their wings. Females exhibit variable amounts of amber or brown wing coloration but lack red coloration altogether. Sex differences in wing coloration are visible shortly after adult emergence, but males have an extended period of color maturation. For example, in *Hetaerina americana*, the red wing spots of males do not reach a terminal size and color until 10–13 days post-emergence, coincident with the age range in which most males begin fighting for mating territories (Fig. 1; Grether 1995). Similar age-related variation has been observed in other *Hetaerina* species (Guillermo-Ferreira & Del-Claro 2011). Hence, wing coloration alone contains sufficient information to distinguish between the sexes and also to distinguish between mature and immature males. Our main objective in this study was to determine whether male rubyspot damselflies use wing coloration to distinguish between the sexes and age classes, and if so, to evaluate whether species differences in wing coloration correspond to species differences in sex and competitor recognition mechanisms. Some additional background information is required to understand our specific predictions.

In most sympatric *Hetaerina* species pairs that have been studied, territory holders respond aggressively to mature male heterospecifics, and the territories of the two species do not overlap (Anderson & Grether 2011; Drury et al. 2015). The only known exceptions are species pairs that include the smoky rubyspot, *H. titia*. Mature male *H. titia* have extensive black wing coloration, which is a derived character state, given the position of *H. titia* in the phylogeny (Fig. 2; Drury et al. 2015; Svensson & Waller 2013). The hindwings of male *H. titia* can be completely black, while the forewings can be up to approximately two-thirds black, and the basal red spots of some males are



**Fig. 1:** Ontogeny of wing spot color in male *H. americana* in relation to territorial behavior. Specimens chosen to represent a range of ages ( $n = 20$ ) were collected at Bear Creek, California (39.02058, -122.39055) and scanned with a spectroradiometer (LI-COR, Inc., Lincoln NB USA) in John Endler's laboratory at the University of California, Santa Barbara on 7 June 1993. The gray shaded area covers the mean  $\pm$  SD of the age at which males first obtained territories at this site in 1991 (mean  $\pm$  SD 11.0  $\pm$  2.6 days; min. 6.8 days, max. 17.7 days,  $n = 58$ ; for detailed methods see Grether 1995, 1996). Chroma and hue angle were calculated using the segment classification method (Endler 1990) with four 86 nm segments between 350 and 700 nm. Chroma is bounded by 0 and 1 and hue angle is a circular statistic that can take on values between 0 and 360 (redder hues have lower hue angles). Total reflectance is the sum of the proportion of light reflected relative to barium sulfate over 201 2-nm wavelength intervals. Ages were known to 1 day or estimated based on multiple traits other than wing coloration, using a cross-validated regression equation, as described in Grether (1996a). As a visual aid, separate least squares regression lines (dashed lines) were fit to the data for ages 2–7 and 11–26. By chance, there were no males in the sample between ages 7 and 10. The data shown here were included in Grether's (1995) dissertation but were not published elsewhere previously.



**Fig. 2:** Ultrametric phylogenetic tree for nine species of *Hetaerina*, with *Calopteryx maculata* and *C. aqueabilis* as out-groups. Black squares identify branches with extensive black wing coloration; red circles identify branches and species with red wing coloration. Species featured in the text are in bold text. This phylogenetic reconstruction is based on 32 *Hetaerina* specimens, four mitochondrial genes, four nuclear genes, and 89 morphological characters (Drury et al. 2015).

completely masked by black pigment (Calvert 1908). The extent of black wing coloration varies both geographically (Anderson & Grether 2010a) and seasonally within sites (Johnson 1963). The basic seasonal pattern is that males emerging in the spring have little or no black wing coloration while males emerging in the summer months have considerable amounts. The seasonal increase in the amount of black on *H. titia* wings corresponds with a seasonal reduction in interspecific fighting (J.P. Drury et al. unpublished data), and experimental wing color manipulations have demonstrated that black coloration reduces aggression from *H. americana* and *H. occisa* territory holders (Anderson & Grether 2010b, 2011). Whether the black wing coloration of *H. titia* evolved as an adaptation for reducing interspecific fighting is unknown, but it clearly has that proximate effect. The basal red wing coloration on *H. titia* can also vary seasonally and geographically (sometimes, but not always, in an inverse relationship to the extent of black coloration; Anderson & Grether 2010a). Wing coloration also varies geographically and seasonally in other species of *Hetaerina* (Cordoba-Aguilar et al. 2009a; Anderson &

Grether 2010a), but not to the same extent as in *H. titia*.

We carried out a series of field experiments to investigate the role of wing coloration in sex recognition and competitor recognition in three species of *Hetaerina*, including *H. titia* and two congeners that lack black wing coloration (*H. occisa*, *H. americana*). We predicted that red wing coloration is necessary for males of the latter two species to distinguish males from females and to distinguish mature males from immature males. Specifically, we predicted that mature male intruders with blackened red spots would not be recognized as mature males, while immature males and females with artificial red spots would be attacked as if they were mature males, by conspecific male territory holders. In the case of *H. titia*, our objective was to determine whether red wing coloration has retained its putative ancestral roles in sex recognition and competitor recognition or instead has been functionally replaced by black wing coloration.

## Methods

### Study Sites

We carried out the field experiments described below at La Palma (PA), Veracruz (18.56187, -95.06134) in 2011, Lampasas (LM), Texas (31.08271, -98.01973) in 2012, Bonita Creek (BC), Arizona (32.91627, -109.49282) in 2012, and Castroville (CV), Texas (29.34079, -98.88156) in 2012 and 2013. The *Hetaerina* species present at these sites were *H. occisa* and *H. titia* (PA), *H. americana* and *H. titia* (CV), *H. americana* (LM), and *H. americana* and *H. vulnerata* (BC). The study periods at PA in 2011 and CV in 2012 were after the seasonal color shift in *H. titia* had begun and most individuals in the population were of the dark-phase form. At CV in 2013, some experiments were carried out before the seasonal color shift, when most individuals in the population were of the light-phase form.

### General Procedures

At each site, we captured most of the adult *Hetaerina* along a 100–300 m river transect with aerial nets and gave each individual a unique color code, consisting of four small paint marks on the abdomen, which can easily be read at a distance using binoculars (Anderson et al. 2011). When ambient conditions are suitable for mating, mature males fight to secure small (1–2 m<sup>2</sup>) mating territories in areas with flowing

water and submerged vegetation, where females oviposit (Johnson 1962; Grether 1996; Cordoba-Aguilar et al. 2009b; Guillermo-Ferreira & Del-Claro 2011). To determine which males were defending territories, 2–5 observers walked along the transect recording the location of males by reference to numbered flags ( $\pm 0.1$  m), during the hours that males were active in territory defense (typically, 10:00–18:00 h). We considered a male to be holding a territory if he consistently perched near the bank of the river at the same location ( $\pm 1.5$  m) for two or more consecutive days (Anderson & Grether 2010a).

We carried out several types of territory intrusion experiments (see below). In each experiment, territory holders were presented with conspecific intruders representing two or more treatment groups. In all experiments, we used a repeated measures design. If in a particular experiment, there were  $k$  intruder treatments, then each territory holder was tested  $k$  times, once with each type of intruder, and a complete set of  $k$  tests would constitute one experimental trial. Hence, the number of trials is both the number of territory holders included in the experiment and the sample size for making pairwise comparisons between treatments.

Unmarked damselflies of the species, sex and age class needed as intruders for a particular experiment were captured outside the study transect, assigned to control and experimental treatment groups at random, and held in a mesh container in the shade until the experimental trial was carried out. During a territory intrusion test, the intruder was tethered with ca. 0.3 m of fine transparent thread and flown within 0.5 m of the territory holder's perch using a modified fishing pole, while an observer recorded the behavior of the territory holder on a continuously running audio recorder. From the recordings, we obtained the following response variables: (i) the amount of time the territory holder chased the intruder; (ii) the number of physical attacks; and (iii) whether or not the territory holder responded sexually to the intruder. We counted as physical attacks instances in which the territory holder attempted to ram into the intruder, whether successful or not (Anderson & Grether 2010b). A mating sequence begins with the male clasping the female; just prior to clasping, the male curls his abdomen forward to put his claspers into position. We considered a male to have responded sexually if he curled his abdomen forward and attempted to clasp the intruder, whether successful or not (Drury et al. 2015). Tests within an experimental trial with the same territory holder were separated by at least

5 min. The order of treatments was varied between trials, such that, within a given experiment, no presentation order was repeated before all other presentation orders were used. If a tethered intruder stopped flying before a presentation was completed, we restarted the test with a new tethered individual. Trials that could not be completed, usually because the territory holder left the territory, were excluded from the analysis (there was no more than one incomplete trial per experiment). Two-minute presentations were used in experiments in which all of the intruders were mature males. Naturally occurring fights can last hours, but 2 min is sufficient to elicit the full repertoire of aggressive responses (Anderson & Grether 2010b). It would be unrealistic for females and immature males to fly for such an extended period, and thus, shorter presentations (30–60 s) were used in experiments in which the intruders included females or immature males. Within an experiment, we used presentations of the same length for all treatments. Tethered individuals were retired if they stopped flying, and none were used in more than four tests; afterward, they were photographed and released outside the study transect. In every experiment, multiple exemplars of each tethered intruder treatment were used.

### Red Spot Blackening Experiments

In these experiments, we blackened the red spots of mature conspecific male intruders using black ink (PrismaColor PM-98, Sanford L.P., Oak Brook, IL, USA). Because ink imparts an unnatural sheen to the wings, the red spots of male intruders in the control group were covered with clear ink (PM-121). Blackening the red spots did not always clarify whether the response of the territory holders was affected by the removal of red or the addition of black, so a third treatment was used in most experiments: In addition to blackening the red spots, we added artificial red spots of approximately the same area as, and just distal to, the original red spots, using red ink (PM-4). We refer to the three treatments as black, clear, and black/red. At PA, we used just the black and clear treatments with *H. occisa* and *H. titia* territory holders on May 24–25, 2011 (10 trials and 4–5 exemplars of each tethered intruder treatment per species). At LM, we used all three treatments with *H. americana* on July 3–4, 2012 (10 trials, 3 exemplars per treatment). At CV, we used all three treatments with *H. americana* and *H. titia* on June 24–26, 2012 (*H. americana*: 13 trials, 8–9

exemplars per treatment; *H. titia*: 7 trials, 4–7 exemplars per treatment), and with *H. titia* on May 22–23, 2013 (8 trials, 6–7 exemplars per treatment). During the experiment with *H. titia* at CV in 2012, it became apparent that the black treatment was not reducing aggression but was interfering with sex recognition (i.e., some males in the black treatment were both attacked and clasped). Because, in this case, it was not relevant to ask whether aggression could be ‘restored’ with the red/black treatment, we replaced the black/red treatment with a black/sepia treatment (14 trials, 10–11 exemplars per treatment), to examine whether adding female coloration to the wings of blackened males would decrease aggression or increase sexual responses. Males in this treatment group had black ink covering their red spots and sepia ink (PM-62) on the clear parts of their wings. We used 2-min presentations at PA and CV and 1-min presentations at LM.

#### Female Color Manipulation Experiments

In these experiments, territory holders were presented with (i) a mature conspecific female with artificial red spots, mimicking mature male wing spots in color, size, and position, (ii) a mature conspecific female with clear ink on same portion of the wings, and (iii) a mature conspecific male. We carried out this experiment with *H. americana* at LM on July 3–4, 2012 (14 trials, 5–9 exemplars per treatment) and BC on July 20–21, 2012 (14 trials, 5–6 exemplars per treatment), and with *H. titia* at CV on June 17–22, 2013 (13 trials, 5–6 exemplars per treatment). We used 1-min presentations at LM and BC and 30-s presentations at CV.

#### Immature Male Experiments

The first of these experiments, at CV on June 28–29, 2012, served to show whether *H. americana* territory holders can distinguish between immature and mature conspecific male intruders (21 trials, 6 exemplars per treatment). In the second experiment, which was carried out at CV between May 23, 2013 and June 5, 2013, *H. americana* territory holders were presented with (i) an immature conspecific male with artificial red spots, mimicking mature male wing spots in color, size, and position; (ii) an immature conspecific male with clear ink on the same portion of the wings; and (iii) a mature conspecific male with clear ink over the red spots (18 trials, 8–9 exemplars per treatment). We used 30-s presentations in these experiments.

#### Statistical Analyses

STATA 12.1 (StataCorp, College Station, Texas) was used for the statistical analyses. To analyze effects of the experimental treatments on the amount of time the territory holder chased the intruder, we used multilevel mixed-effects linear regression (xtmixed), with individual territory holder encoded as a random-effects grouping variable. The arc-sine-square-root transformation of the proportion of time spent chasing, which, based on residual plots, best fit the models’ assumptions, was used as the dependent variable. When possible, we used random-effects negative binomial regression models (xtnbreg) to analyze variation in the rate of physical attacks, with individual territory holder encoded as a random-effects grouping variable and the duration of the presentation as an exposure term. For both types of regression models, the Sidak correction was used for pairwise comparisons between treatment groups. When the regression models were inestimable (as occurred, for example, when there were zero attacks in the black treatment), we analyzed the data using nonparametric Friedman tests and Wilcoxon signed ranks (WSR) tests, with p-values obtained by permutation. For pairwise comparisons between treatment groups using WSR tests, we used Holm’s (1979) method to obtain an adjusted critical p-value at  $\alpha = 0.05$ . To analyze effects of the treatments on clasping frequency, we used two- or three-way contingency tests, depending on the experiment. All reported p-values are two-tailed.

## Results

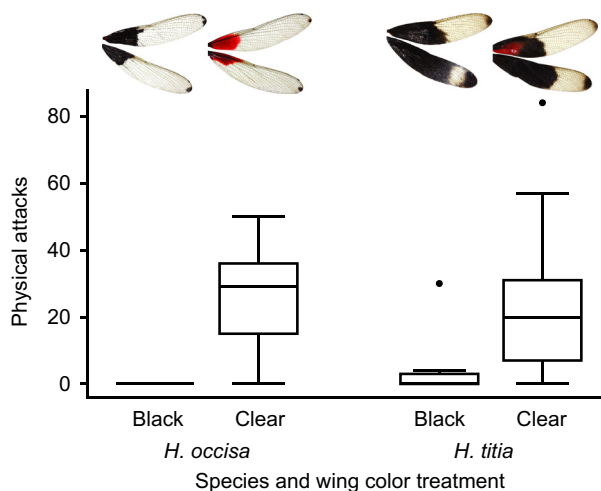
#### Red Spot Blackening Experiments

In most experiments, blackening the red spots of mature male conspecific intruders sharply reduced the aggressive response of territory holders. In some experiments, this color manipulation also interfered with sexual recognition.

At PA in Veracruz, *H. occisa* and *H. titia* territory holders chased intruders with blackened red spots much less than they chased intruders with clear ink on their red spots (xtmixed regression:  $\chi^2 = 134$ ,  $df = 1$ ,  $p < 0.001$ ). The species did not differ in their responses to clear intruders ( $\chi^2 = 0.27$ ,  $df = 1$ ,  $p = 0.60$ ), but *H. titia* males chased black intruders significantly longer than did *H. occisa* territory holders ( $\chi^2 = 7.6$ ,  $df = 1$ ,  $p = 0.006$ ), resulting in a treatment by species interaction ( $\chi^2 = 7.5$ ,  $df = 1$ ,  $p = 0.006$ ). Nine of 10 male *H. occisa* physically attacked the clear intruder (up to 50 times), but none attacked the black

intruder (WSR test on attack count:  $z = 2.76$ , permutation  $p = 0.004$ ). *H. titia* territory holders also attacked black intruders less than clear intruders (Fig. 3; WSR test:  $z = 2.55$ ,  $p = 0.008$ ), but the responses of *H. titia* to black intruders were more variable than those of *H. occisa*. Four of 10 *H. occisa* males and 3 of 10 *H. titia* males clasped the black intruder and none clasped or attempted to clasp the clear control. Across species, blackening the red spots significantly increased the probability of a sexual response (Mantel-Haenszel 3-way contingency test:  $\chi^2 = 8.1$ ,  $df = 1$ ,  $p = 0.004$ ).

With *H. americana* at sites LM and CV in Texas, the wing color manipulations strongly affected the length of time intruders were chased (xtmixed regression: LM  $\chi^2 = 52$ ,  $df = 2$ ,  $p < 0.001$ ; CV  $\chi^2 = 1497$ ,  $df = 2$ ,  $p < 0.001$ ) and the rate at which they were physically attacked (Figs. 4a, b; LM, xtnbreg regression:  $\chi^2 = 16$ ,  $df = 2$ ,  $p < 0.001$ ; CV, Friedman test:  $Q = 16$ ,  $p = 0.003$ ). Black intruders were chased much less than either clear intruders (pairwise comparisons with the Sidak p-value adjustment: LM  $z = 6.8$ ,  $p < 0.001$ ; CV  $z = 33.5$ ,  $p < 0.001$ ) or red/black intruders (LM  $z = 5.6$ ,  $p < 0.001$ ; CV  $z = 33.5$ ,  $p < 0.001$ ), and there was no significant difference in the amount of time territory holders spent chasing clear and red/black intruders (LM  $z = -1.14$ ,  $p = 0.59$ ; CV  $z = -0.05$ ,  $p = 1.0$ ). At LM, black intruders were

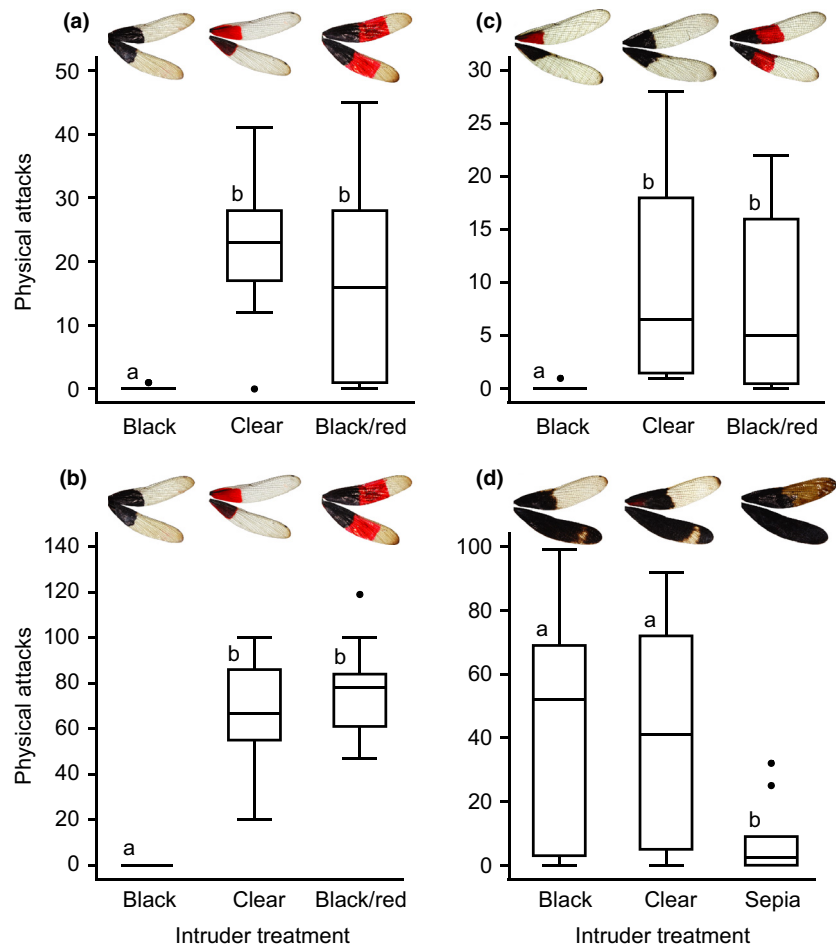


**Fig. 3:** Responses of *H. occisa* and *H. titia* territory holders to mature male conspecific intruders at PA, Veracruz in the late season. Intruder treatments are named below and shown in photographs above the box plots to which they correspond. Box plots depict the median (horizontal line within the box), interquartile range (box), lower and upper adjacent values (whiskers), and outside values (dots). See text for further details and statistical analysis.

physically attacked an average of  $0.20 \pm 0.42$  (mean  $\pm$  SD) times vs.  $21.9 \pm 11.1$  times for clear intruders and  $17.2 \pm 15.45$  times for red/black intruders, during 1-min presentations (Fig. 4a; pairwise comparisons with the Sidak p-value adjustment: black vs. clear  $z = 3.96$ ,  $p < 0.001$ ; black vs. black/red:  $z = 3.3$ ,  $p = 0.003$ ; clear vs. black/red,  $z = 1.19$ ,  $p = 0.55$ ). At CV, black intruders were never physically attacked while clear and red/black intruders were attacked an average of  $65.4 \pm 23.9$  and  $76.8 \pm 20.5$  times, respectively, during 2-min presentations (Fig. 4b; WSR pairwise comparisons with Holm critical  $p = 0.05$ : black vs. clear  $z = 3.18$ ,  $p < 0.001$ ; black vs. black/red  $z = 3.2$ ,  $p < 0.001$ ; clear vs. black/red  $z = 1.68$ ,  $p = 0.10$ ). Thus, adding artificial red spots to the wings of intruders with blackened red spots fully restored the aggressive responses of *H. americana* territory holders. No male–male clasping occurred in these *H. americana* experiments.

In the 2013 study period at CV in Texas, which was before the seasonal shift in *H. titia* wing coloration, the *H. titia* results were essentially the same as the *H. americana* results. The wing color manipulations affected the length of time intruders were chased (xtmixed regression:  $\chi^2 = 12.4$ ,  $df = 2$ ,  $p = 0.002$ ) and the rate at which they were physically attacked (Fig. 4c; xtnbreg regression:  $\chi^2 = 11.6$ ,  $df = 2$ ,  $p = 0.003$ ). Black intruders were chased and attacked less than both clear intruders (pairwise comparisons with the Sidak p-value adjustment: chase duration,  $z = 2.86$ ,  $p = 0.013$ ; attack rate,  $z = 3.4$ ,  $p = 0.002$ ) and red/black intruders (chase duration,  $z = 3.2$ ,  $p = 0.004$ ; attack rate,  $z = 3.0$ ,  $p = 0.008$ ), and there was no difference between clear and red/black intruders in either measure of territorial aggression (chase duration,  $z = 0.36$ ,  $p = 0.98$ ; attack rate,  $z = -0.94$ ,  $p = 0.72$ ). No male–male clasping occurred in this *H. titia* experiment.

By contrast, in the 2012 study period at CV in Texas, which was after the seasonal shift in *H. titia* wing coloration, the red spot blackening treatment had no effect on the duration of chasing (xtmixed regression: black vs. clear,  $z = 1.23$ ,  $p = 0.52$ ) or the attack rate (Fig. 4d; xtnbreg regression: black vs. clear,  $z = 0.28$ ,  $p = 0.99$ ). Nevertheless, in 4 of 21 trials, the territory holder clasped the black intruder. No territory holders responded sexually to clear or red/black intruders. Adding female coloration to the wings of intruders with blackened red spots had no effect on the duration of chasing (pairwise comparisons with the Sidak p-value adjustment: black/sepia vs. clear,  $z = 2.14$ ,



**Fig. 4:** Responses of territory holders of two species to mature male conspecific intruders. (a) *H. americana* at LM, Texas, (b) *H. americana* at CV, Texas, (c) *H. titia* at CV, Texas in the early season, (d) *H. titia* at CV, Texas in the late season. Intruder treatments are named below and shown in photographs above the box plots to which they correspond. Within a panel, treatments with different letters elicited significantly different responses. See Figure 3 for box plot definition and text for further details and statistical analysis.

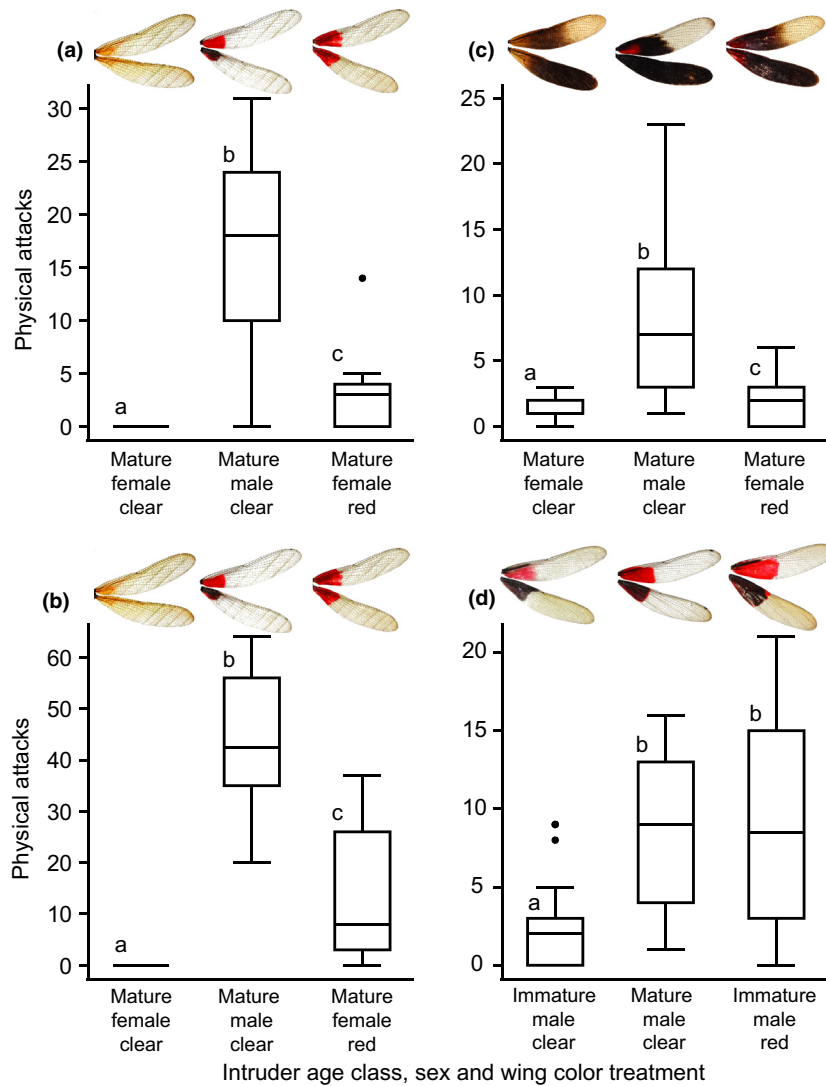
$p = 0.09$ ; black/sepia vs. black,  $z = 1.33$ ,  $p = 0.46$ ) but reduced the attack rate (black/sepia vs. clear,  $z = 4.2$ ,  $p < 0.001$ ; black/sepia vs. black,  $z = 4.3$ ,  $p < 0.001$ ). Black/sepia intruders were clasped in 3 of 14 trials. With data from 2012 and 2013 combined, and restricting the analysis to the black and clear treatments, there were significant season by treatment interactions for both the duration of chasing (xtmixed regression:  $\chi^2 = 16$ ,  $df = 1$ ,  $p = 0.001$ ) and the attack rate (xtnbreg regression:  $\chi^2 = 9.2$ ,  $df = 1$ ,  $p = 0.002$ ). Responses to clear intruders did not differ between seasons (chase duration,  $\chi^2 = 1.13$ ,  $df = 1$ ,  $p = 0.29$ ; attack rate,  $\chi^2 = 0.21$ ,  $df = 1$ ,  $p = 0.65$ ), but territory holders were more aggressive to black intruders in the late season in 2012 than the early season in 2013 (chase duration,  $\chi^2 = 13.8$ ,  $df = 1$ ,  $p < 0.001$ ; attack rate,  $\chi^2 = 10.9$ ,  $df = 1$ ,  $p < 0.001$ ). Across years, blackening the red spots increased the probability of clasping by male *H. titia* at CV (Mantel-Haenszel 3-way contingency test:  $\chi^2 = 4.32$ ,  $df = 1$ ,  $p = 0.038$ ).

#### Female Color Manipulation Experiments

Putting red spots on females invariably caused them to be chased and attacked by male territory holders, and this manipulation also substantially reduced the frequency of sexual responses. Territory holders were not as aggressive to red females as they were to mature male intruders.

With *H. americana* at LM in Texas, female intruders with artificial red spots were physically attacked in 10 trials, mature male intruders were attacked in 13 trials, and female intruders with clear ink on their wings were never attacked, out of 14 trials in total. The difference between red females and clear females in attack frequency was highly significant (Fisher's exact test,  $p < 0.001$ ). Mature males were attacked at higher rates and chased for longer periods than were red females (Fig. 5a; attack rate xtnbreg regression:  $\chi^2 = 52$ ,  $df = 1$ ,  $p < 0.001$ ; chase duration xtmixed regression:  $\chi^2 = 23$ ,  $df = 1$ ,  $p < 0.001$ ). Territory holders clasped or attempted to clasp clear females in 10





**Fig. 5:** Responses of territory holders of two species to conspecific intruders of various age and sex classes and wing color treatments. (a) *H. americana* at LM, Texas, (b) *H. americana* at BC, Arizona, (c) *H. titia* at CV, Texas in the late season, (d) *H. americana* at CV, Texas in the late season. Intruder treatments are named below and shown in photographs above the box plots to which they correspond. Within a panel, treatments with different letters elicited significantly different responses. See Figure 3 for box plot definition and text for further details and statistical analysis.

trials and never attempted to clasp red females or mature males (Fisher's exact test,  $p < 0.001$ ).

With *H. americana* at BC in Arizona, red females and mature males were always attacked and clear females were never attacked, out of 14 trials in total (Fig. 5b). The difference between red females and clear females in attack frequency was highly significant (Fisher's exact test,  $p < 0.001$ ). Mature males were attacked at higher rates and chased for longer periods than were red females (attack rate xtnbreg regression:  $\chi^2 = 29$ ,  $df = 1$ ,  $p < 0.001$ ; chase duration xtmixed regression:  $\chi^2 = 40$ ,  $df = 1$ ,  $p < 0.001$ ). Territory holders responded sexually to clear females in half of the trials (4 clasps and 3 abdomen curls without clasping) and the red female and mature male were each clasped in one trial. The difference between red females and clear females in sexual

response frequency was significant (Fisher's exact test,  $p = 0.033$ ). The territory holder that clasped the mature male also clasped the clear female but not the red female.

With *H. titia* at CV in Texas, in June 2013 after the seasonal shift in wing coloration, red females were attacked in nine trials, mature males were always attacked, and clear females were never attacked, out of 13 trials in total (Fig. 5c). The difference between red females and clear females in attack frequency was highly significant (Fisher's exact test,  $p < 0.001$ ). Mature males were attacked at higher rates and chased for longer periods than were red females (attack rate xtnbreg regression:  $\chi^2 = 11.7$ ,  $df = 1$ ,  $p = 0.0006$ ; chase duration xtmixed regression:  $\chi^2 = 4.93$ ,  $df = 1$ ,  $p = 0.026$ ). Territory holders responded sexually to clear females in 11 trials

(9 clasplings and 2 abdomen curls without claspling) and red females in one trial (1 claspling) and never to mature males. The difference between red females and clear females in claspling frequency was highly significant (Fisher's exact test,  $p < 0.001$ ).

### Immature Male Experiments

*H. americana* territory holders clearly distinguished between unmanipulated immature and mature male intruders. At CV in 2012, mature male intruders were more likely to be physically attacked (21 of 21 trials) than immature male intruders (13 of 21) trials (Fisher's exact test,  $p = 0.005$ ), and mature males were attacked at higher rates and chased for longer periods than were immature males (attack rate xtnbreg regression:  $\chi^2 = 42$ ,  $df = 1$ ,  $p < 0.001$ ; chase duration xtmixed regression:  $\chi^2 = 33$ ,  $df = 1$ ,  $p < 0.001$ ). No male–male claspling attempts occurred in this experiment.

Adding artificial red spots to the wings of immature males caused them to be chased and attacked as if they were mature males (Fig. 5d). At CV in 2013, there was no difference in the responses evoked by red immature males and clear mature males (attack rate xtnbreg regression:  $z = 0.24$ ,  $p = 0.99$ ; chase duration xtmixed regression:  $z = 0.99$ ,  $p = 0.69$ ) and both red immature males and clear mature males were attacked at higher rates and chased for longer periods than were clear immature males (attack rate xtnbreg regression: red immature vs. clear immature,  $z = 5.0$ ,  $p < 0.001$ ; red immature vs. clear mature,  $z = 5.2$ ,  $p < 0.001$ ; chase duration xtmixed regression: red immature vs. clear immature,  $z = 5.61$ ,  $p < 0.0001$ ; clear mature vs. clear immature,  $z = 6.6$ ,  $p < 0.001$ ). In two trials of this experiment, the territory holder responded sexually to the clear immature male but did not actually clasp him; there were no claspling attempts on red immature or clear mature males.

### Discussion

We confirmed that wing coloration plays a key role in competitor recognition in *Hetaerina* damselflies. Covering the red spots of mature male intruders with black ink essentially shut off the aggressive responses of *H. occisa* and *H. americana* territory holders (Figs. 3 and 4). Adding red ink to the wings of males with blackened red spots restored the aggressive responses of *H. americana* territory holders (Fig. 4), which demonstrates that red wing coloration, *per se*, is necessary to elicit territorial aggression. This may explain why,

in a previous study, covering the red spots of male *H. americana* with blue ink reduced their territory holding ability (González-Santoyo et al. 2014).

Our evidence that wing coloration affects sex recognition is equally clear. It is extremely rare for males to clasp each other under natural circumstances, and no male–male clasplings were observed in hundreds of tethered intruder tests carried out with these species in previous studies (Anderson & Grether 2010b; Drury et al. 2015), but in the red spot blackening experiment, several male *H. occisa* and *H. titia* with blackened spots were clasped as if they were females. Furthermore, adding red ink to female wings nearly eliminated sexual responses by *H. americana* and *H. titia* territory holders, and females and immature males with artificial red spots were attacked at much higher rates than controls (Fig. 5).

The red wing spots appear to have retained their putative ancestral function in sex recognition but are no longer necessary to elicit territorial aggression in some *H. titia* populations. We found both geographic and seasonal variation in the responses of this species. Blackening the red spots of male intruders sharply reduced the aggressive responses of *H. titia* territory holders at PA (Veracruz), at a time when most of the *H. titia* in the population were of the late-season phenotype (Fig. 3), and also at CV (Texas) in the early season, prior to the seasonal color shift (Fig. 4c). However, at CV in the late season, blackening the red spots of intruders had no effect on the aggressive responses of *H. titia* territory holders (Fig. 4d). Intruders with blackened red spots were clasped as if they were females in several trials at PA and at CV in the late season but not at CV in the early season. Putting female coloration on the wings of males with blackened red spots reduced the attack rate (Fig. 4d) but did not increase the frequency of sexual responses, at CV in the late season. A possible proximate explanation for the site difference in the effects of the blackening treatment is that the red spots are naturally masked by black (melanin) pigment to a greater degree, and therefore are less visible to the damselflies, at CV than at PA, in the late season (compare *H. titia* photographs in Figs 3 and 4d). Similarly, the seasonal differences in the effects of the red spot blackening treatment on sexual responses may be understood in terms of the seasonal shift in female wing coloration, because males with blackened red spots look more like females in the late season, when most females have dark brown wings, than in the early season, when females have light amber-colored wings. In the late season, *H. titia* may have to learn to distinguish between males and females without

relying on the red spots. Learning is known to play an important role in mate recognition in other species (Bateson 1978; Irwin & Price 1999; Verzijden et al. 2012) including damselflies (Miller & Fincke 1999; Svensson et al. 2010; Verzijden et al. 2012). The ultimate reasons for both the seasonal color shift and the geographical variation in the extent to which melanin masks the red pigment remain to be investigated.

The coloration of immature male *Hetaerina* is analogous to that of subadult male birds in species with delayed plumage maturation, except that the coloration of male *Hetaerina* develops gradually, with no equivalent of a plumage molt (Fig. 1). As in birds, male damselflies are capable of mating before they are competitively mature but rarely do so. The function of delayed plumage maturation in birds has been the subject of considerable research (Berggren et al. 2004; Karubian et al. 2008; Hawkins et al. 2012). The leading hypotheses are that subadult plumage is (i) a form of female mimicry, (ii) a form of crypsis, (iii) the product of a constraint on color development, and (iv) an honest signal of low competitive ability (the status signaling hypothesis) (reviewed in Hawkins et al. 2012). The female mimicry hypothesis can be eliminated as a possible reason for delayed color maturation in *Hetaerina*, because immature males do not resemble females and rarely elicit sexual responses from mature males. The remaining hypotheses are plausible and need not be mutually exclusive. The gray-brown hindwing spots of immature males may confer some degree of crypsis against the typical visual background (rocks, earth, vegetation) of visually orienting predators (e.g., birds, dragonflies, frogs, lizards; Grether 1997) and prey (e.g., mayflies, caddisflies, midges; Grether & Grey 1996) by covering the pink-red forewing spots when the wings are folded at rest. It may take several days for enough red pigment to be synthesized or converted from colorless molecules to reach the vibrant red color of mature wing spots (the red pigment has not been identified but is probably an ommochrome; Stavenga et al. 2013). However, the development of territorial fighting ability itself is developmentally constrained; it takes several days for the cuticle to fully harden and to build wing muscles and fat stores (Corbet 1999). Whether by design or constraint, color maturation in *Hetaerina* coincides closely with when males first begin fighting for territories (Fig. 1; Grether 1995). Thus, the wing spots of immature males may serve as honest signals of low fighting ability, and the responses of territory holders in our experiments support this hypothesis. We found that *H. americana* territory holders show reduced aggression toward immature male intruders and that

putting red ink on the wings of immature males caused them to be attacked at the same rate as fully mature males (Fig. 5d). Under normal circumstances, immature males also behave differently than mature males (e.g., they perch higher and/or in different parts of streams than mature males), but our results indicate that immature wing coloration, *per se*, is sufficient to reduce territorial aggression.

While wing coloration appears to be the dominant trait for sex recognition in *Hetaerina*, red females were not attacked as aggressively as were mature male intruders, and a few red females were clasped, which indicates that other factors besides wing coloration influences male responses. Gorb (1998) found that step-by-step elimination of male color pattern elements from damselfly models progressively increased the probability of male sexual responses in the damselfly *Coenagrion puella*. In *Enallagma hageni*, a polymorphic damselfly in which males and one female morph have blue body coloration, males appear to use the simple decision rule 'if not blue, then female' for distinguishing between males and heteromorphic (green) females, but males rely on other color pattern elements for distinguishing between males and andromorphic (blue) females (Xu et al. 2014).

There are numerous examples of red color patches being used as agonistic signals in vertebrates (Collias 1990; Pryke 2009; Ninnis & Andersson 2014) but relatively few insect examples. A likely reason is that opsins sensitive to long wavelengths are uncommon in insects (Briscoe & Chittka 2001). Our results demonstrate that *Hetaerina* damselflies can distinguish not only between black and red objects but also between different shades of red. Long-wavelength opsins have been detected in other branches of the Odonata phylogeny (Bybee et al. 2012), and given our results, it is likely that long-wavelength opsins will be found in *Hetaerina*.

Previous research has shown that male *H. titia* respond more aggressively, and male *H. occisa* and *H. americana* in populations that are sympatric with *H. titia* respond less aggressively, to intruders with black coloration distal to the red spots (Anderson & Grether 2010a). Thus, it was surprising that adding red to the wings of males with blackened red spots fully restored the aggressive responses of *H. americana* at a sympatric site in Texas (Fig. 4b). Apparently, the relative position of the red and black patches on the wing also affects how males respond.

Red wing coloration appears to play a similar role in sex and competitor recognition in *Mnesarete* (Guillermo-Ferreira et al. 2014), the sister genus to *Hetaerina*

(Dijkstra et al. 2014). In *Mnesarete pudica*, both sexes initially develop black wing coloration but by sexual maturity, males have red wings and females have light brown wings (Guillermo-Ferreira et al. 2014). Adding red to the wings of female *M. pudica* caused them to be attacked by territorial males, while immature males with black wings were not attacked (Guillermo-Ferreira et al. 2014). Black wing coloration appears to have evolved independently in *Mnesarete* and *Hetaerina* and presumably serves different functions, as it occurs only in immature *M. pudica* but throughout adulthood in *H. titia*.

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