Seasonal polyphenism in wing coloration affects species recognition in rubyspot damselflies (Hetaerina spp.)

J. P. DRURY*†, C. N. ANDERSON† & G. F. GREther*

*Department of Ecology and Evolutionary Biology, University of California, Los Angeles, CA, USA
†Dominican University, River Forest, IL, USA

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mate recognition;
Odonata;
phenotypic plasticity;
reproductive interference.

Abstract
Understanding how phenotypic plasticity evolves and in turn affects the course of evolution is a major challenge in modern biology. By definition, biological species are reproductively isolated, but many animals fail to distinguish between conspecifics and closely related heterospecifics. In some cases, phenotypic plasticity may interfere with species recognition. 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 classify different forms of H. titia as separate species. We further show how the seasonal colour shift impacts species recognition with the sympatric congener Hetaerina occisa. Interspecific aggression (territorial fights) and reproductive interference (mating attempts) are much more frequent early in the year, when H. titia more closely resembles H. occisa, compared to later in the year when the dark phase of H. titia predominates. Using wing colour manipulations of tethered damselflies, we show that the seasonal changes in interspecific interactions are caused not only by the seasonal colour shift but also by shifts in discriminatory behaviour in both species. We also experimentally tested and rejected the hypothesis that learning underlies the behavioural shifts in H. occisa. An alternative hypothesis, which remains to be tested, is that the seasonal polyphenism in H. titia wing coloration has resulted in the evolution of a corresponding seasonal polyphenism in species recognition in H. occisa. This study illustrates one of the many possible ways that plasticity in species recognition cues may influence the evolution of interspecific interactions.

Introduction
Biologists have long argued that the ability of organisms to distinguish between members of their own species and others is paramount for species coexistence (Wallace, 1889; Fisher, 1930; Dobzhansky, 1955; Lorenz, 1962; Mayr, 1963; Groning & Hochkirch, 2008). Species recognition, that is behavioural discrimination between conspecifics and heterospecifics (Mendelson & Shaw, 2012), is often based on specific traits, such as colour patches, cuticular hydrocarbons or vocalizations (Higgie & Blows, 2008; Pfennig & Pfennig, 2012a,b). Such traits may diverge between species through selection resulting from interspecific interactions in reproductive (i.e. reinforcement/reproductive character displacement; Brown & Wilson, 1956; Pfennig & Pfennig, 2012a,b) or agonistic/territorial contexts (i.e. agonistic character displacement; Lorenz, 1962; Grether et al., 2009, 2013), or as a by-product of genetic drift or selection in other contexts (Mayr, 1963; West-Eberhard, 1979, 1983; Mendelson & Shaw, 2012).

Evidence that animals distinguish between conspecifics and closely related heterospecifics is surprisingly mixed (Ord & Stamps, 2009; Ord et al., 2011). A likely explanation in some cases is that selection favours treating heterospecifics as though they were conspecif-
ics in certain contexts, such as when hybridization is advantageous (Pfennig, 2007; Mendelson & Shaw, 2012; Willis, 2013) or resource competition favours interspecific territoriality (Cody, 1969, 1973; Grether et al., 2009, 2013; Willis, 2013). In other cases, however, lack of discrimination may be a maladaptive by-product of interspecific overlap in species recognition cues (Murray, 1971; Grether et al., 2009).

Seasonal polyphenisms, cases of phenotypic plasticity in which different phenotypes predominate at different times of year (Shapiro, 1976) provide natural experiments to examine the effects of variation in the degree of interspecific overlap in species recognition cues. Here, we document and investigate the effects of a seasonal polyphenism in the wing pigmentation of smoky rubyspot damselflies (Hetaerina titia). Both sexes shift from a light-phase wing phenotype early in the peak breeding season to a dark-phase wing phenotype later in the season (Figs 1 and 2). The early-season phenotype of H. titia more closely resembles sympatric congeners and is remarkably distinct from the late-season phenotype, leading early researchers to classify light and dark variants of H. titia as separate species (Appendix S1). Previous studies on sympatric Hetaerina species have linked species differences in male wing coloration to interspecific fighting (Anderson & Grether, 2010b, 2011; Drury & Grether, 2014; Grether et al., 2015) and species differences in female wing coloration to reproductive interference (i.e. heterospecific clasping; Drury et al., 2015). That is, species that resemble each other more closely interfere with each other at higher rates, and wing colour manipulations have shown that these relationships are causal. In the case of species pairs that include H. titia, species differences in wing coloration change seasonally, and thus, we predicted that rates of interspecific fighting and reproductive interference would also change seasonally.

We conducted behavioural observations at a site in Veracruz, Mexico, where H. titia is found in sympatry with Hetaerina occisa. Both sexes of H. occisa more

![Fig. 1](image)

**Fig. 1** Seasonal variation in the wing coloration of male *Hetaerina titia*. (a–c) Photographs of males’ wings with different amounts of black pigmentation (forewings on top, hindwings on bottom). (d–f) The proportion of male wings covered with black pigment, plotted against measurement date, from extended visits to sites in Veracruz (D = La Palma 2011, E = La Palma 2012) and Texas (F = Castroville 2013; see Table S2). Red circles represent hindwings, blue triangles represent forewings, and lines are GLM predictions for the proportion of black pigmentation on hindwing (red) and forewing (blue) surfaces. (g,h) The average proportion of black pigmentation on hindwing (circles), forewing (triangles) and total wing (squares) surfaces from individuals sampled on short visits to Armeria (g) and Castroville (h). Error bars (± SE) smaller than the corresponding symbols are not shown. Points connected by solid lines differ significantly, and points connected by dashed lines do not differ significantly, in Tukey's post hoc tests. From left to right, the dates of the short visits were as follows: Armeria: 27 April to 3 May 2008, 30 May to 5 June 2007, 10–14 July 2005 and 23–27 July 2007; Castroville 17–24 April 2008, 21–28 June 2012 and 19–26 August 2008.
closely resemble the early (light) phenotype than the late (dark) phenotype of *H. titia* (Figs 1, 2 and S2). Thus, we predicted that species recognition would be weaker, and hence, rates of interference would be greater, in the early season than in the late season. In addition to recording naturally occurring interactions, we measured the responses of male territory holders of both species to tethered conspecifics and heterospecifics of both sexes in both seasons. To isolate the effects of wing coloration on such responses, and to test for seasonal shifts in the ability of males to discriminate between conspecific and heterospecific wing coloration, we used wing colour manipulations. Finally, to determine whether seasonal shifts in species recognition are due in part to learning, we carried out field experiments in which the opportunity to interact with heterospecifics was manipulated.

**Materials and methods**

**Seasonal polyphenism**

*Hetaerina titia* males have basal red spots on their forewings with varying amounts of black pigmentation on their forewings and hindwings (Fig. 1a–c), and females have amber-coloured wings that vary in opacity (Fig. 2a,b). As with other species of *Hetaerina* (Grether, 1996), the wing phenotypes of adult *H. titia* are fixed upon maturation (Fig. S1).

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, USA; and in Veracruz and Colima, Mexico; see Table S1). At each site, we set up a transect along the river with numbered markers at 1-m intervals and captured, marked and released all adult *Hetaerina* 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 study the influence of maturation date at a finer scale. For these sites, we used the date an individual was photographed as a proxy for the date of its maturation, which is a good approximation as 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 mature 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; Canon U.S.A., Inc., Melville, NY, USA). We used NIH Image to manually measure the black area and total area of each wing (for sample sizes, see Table S1) (Schneider et al., 2012).

![Seasonal variation in the wing coloration of female *Hetaerina titia*.](image)
Veracruz, Mexico (Table S1). We used an Ocean Optics USB-2000 spectrometer equipped with a pulsed xenon light source (Ocean Optics PX-2; Ocean Optics, Inc., Dunedin, FL, USA), oriented at 45° relative to the wing surface to eliminate glare, and measured per cent reflectance at each wavelength in relation to a Lab-sphere-certified reflectance standard using Ocean Optics OOIBase32 software. When taking measurements, we placed the wings flat against the reflectance standard. The readings therefore include 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 per cent 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 \((n = 30, \text{March 25 to 12 April 2011})\) and end \((n = 26, \text{May 30 to 10 June 2011})\) of our visit.

For a finer scale resolution of the influence of date on female wing lightness, we also measured female wing lightness using digital photographs taken as described above. We standardized the white balance relative to the white background of the scale paper included in each photograph in ImageJ (Schneider et al., 2012), using the ‘Color Balance’ plug-in from the 

ImageJ package. We then used the polygon tool and the ‘Measure RGB’ plug-in to analyse the RGB profile of each wing, which we used to calculate an average weighted greyscale measurement to serve as a photographic index of female wing lightness. This photographic measure of lightness correlates well with the spectrometric measure of lightness (Drury et al., 2015).

Early- and late-season species recognition at a sympatric site

The light-phase phenotypes that \(H. \text{titia}\) males and females exhibit early in the peak emergence season (e.g. Figs 1a and 2a) are more similar to those of the congener, \(H. \text{occisa}\), than the dark-phase phenotypes more abundant later in the season (Figs 1b, 2b and S2). Thus, if between-species similarity in phenotypes results in increased rates of interspecific interactions, the magnitude of interspecific aggression and reproductive interference (sexual interactions between species; Gröning & Hochkirch, 2008) should be reduced in the late season, relative to the early season. To test these predictions, we carried out observations and experiments on \(H. \text{occisa}\) and \(H. \text{titia}\) along a river transect (~200–300 m) where both species were present in La Palma in Veracruz, Mexico, between March and June 2011 and 2012 (Table S1). We captured nearly all individuals in the study area with aerial nets and marked them on the abdomen with unique IDs using DecoColor or colour paints (Anderson et al., 2011).

To compare the responses of \(H. \text{occisa}\) males to light-phase and dark-phase \(H. \text{titia}\) of both sexes, we conducted our experiments in two time periods, referred to as ‘early’ and ‘late’ hereafter. In the early season (corresponding to 23 March to 5 May 2011, and 2 to 26 April 2012), most \(H. \text{titia}\) were light-phase forms, whereas in the late season (30 May to 15 June 2011, and 17 May to 11 June 2012), most \(H. \text{titia}\) individuals were dark-phase forms (Figs 1d,e and 2e,f).

\(Hetaerina\) have an average adult lifespan of approximately 2 weeks (Grether, 1996). For the relatively few individuals that were tested during both periods (< 3 males per species), we only included responses measured in the early season to avoid pseudoreplication and to maintain a clearer separation between the early- and late-season cohorts.

Observations of naturally occurring fights

To determine which males were territorial and to record the frequency of naturally occurring intra- and interspecific fights, we conducted behavioural censuses. During each census, an observer recorded the location of each male along the transect to the nearest 0.1 m. When a fight was observed, the location, species involved, ID of individuals (if marked) and the intensity of the 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 (i.e. \(H. \text{occisa}\) vs. \(H. \text{occisa}\), \(H. \text{occisa}\) vs. \(H. \text{titia}\), \(H. \text{titia}\) vs. \(H. \text{titia}\)) from the behavioural censuses. Prior to analysis, multiple recorded bouts of fighting between the same two males on the same day were reduced to a single fight. To avoid overcounting fights involving unmarked (or unidentified) males, those that occurred at the same location (± 5 m) on the same day were also reduced to a single fight.

If the seasonal shift in \(H. \text{titia}\) wing coloration reduces interspecific aggression, interspecific fights should be relatively more common in the early season than in the late season. To test for a shift in the relative number of interspecific and intraspecific fights between seasons, we generated expected values for the number of fights of each type (i.e. intraspecific, interspecific) from a binomial expansion of the proportion of males of each species marked during each time period (Anderson & Grether, 2011). With these data, we 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 \(\chi^2\) tests.

Competitor recognition experiments

The term ‘competitor recognition’ is analogous to ‘mate recognition’ (Grether et al., 2009; Mendelson, 2015). Here, it specifically refers to males discriminating between mature conspecific and heterospecific males.
Analogous to the way ‘preference function’ and ‘mate recognition function’ are used in the mate choice literature (Ritchie, 1996), the internal mechanism that results in discrimination, and which can be inferred from behavioural responses, is referred to as the competitor recognition function (Grether et al., 2009; Grether, 2011).

We predicted that aggressive responses to intruding heterospecifics would be diminished in the late season, when the species are distinct in wing coloration, compared to the early season. To test this prediction, we measured responses of territory holders to conspecific and heterospecific intruders in both seasons. We considered males to be holding a territory when they consistently perched along the river for two consecutive days within a 1.5 m radius (Anderson & Grether, 2010b). Males used as intruders were captured outside the study transect, tethered with transparent thread and flown within the territories using a modified fishing pole. Each trial lasted two minutes, with at least a 5-min intertrial interval (Anderson & Grether, 2010b). During the trials, we recorded the behaviour of the territorial males, including the amount of time spent chasing the tethered male and the number of physical attacks. We counted as physical attacks instances in which the territory holder charged directly towards or grabbed onto the tethered male using his legs (Anderson & Grether, 2010a). The presentation order of conspecific and heterospecific males was varied systematically 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 (indicating that the male was not defending the site), were excluded from the analysis (when possible, we tested such males on a subsequent day). Hetaerina titia males’ responses to tethered intruders were measured in 2012, and H. occisa males’ responses were measured in both 2011 and 2012.

Seasonal shifts in competitor recognition could result from seasonal changes in wing coloration or from seasonal changes in competitor recognition functions, or both. To determine whether competitor recognition functions shift seasonally, it was critical to present territory holders with the same intruder stimuli in both seasons. As light-phase H. titia are only common in the early season, and dark-phase H. titia are only available in the late season, we used colour-manipulated H. occisa intruders in these experiments. H. occisa territory holders were presented with conspecific intruders of the following three treatments: (i) hindwings fully blackened to resemble those of dark-phase H. titia males (black ink [Prismacolor PM-98] from the distal edge of the wingspot to the wing tip), (ii) hindwings half-blackened to resemble intermediate-phase H. titia males (black ink from the distal edge of the wingspot, halfway to the wing tip, and clear ink [Prismacolor PM-121] to the wing tip), and (iii) clear control (clear ink from the distal edge of the wingspot to the wing tip, as a sham control). Hetaerina titia territory holders were presented with males of the following four treatments: (i) H. occisa with fully blackened hindwings (black ink, as above), (ii) H. occisa control (clear ink, as above), (iii) H. titia with fully blackened hindwings and (iv) H. titia control (clear ink). The latter two intruder treatments were not required to test for a shift in the competitor recognition function but enabled a direct comparison of H. titia responses to conspecifics and heterospecifics with and without fully blackened wings. In the late season, when dark-phase H. titia are more prevalent, we used tethered H. titia males with similar extents of wing pigmentation for the control and darkened treatments in tests with a given territory holder. In all cases, tethered males were assigned to treatments at random with respect to their own phenotypes.

Mate recognition experiments

Previous research demonstrated that reproductive interference is most common in species pairs in which females have similar wing colour phenotypes (Drury et al., 2015). Thus, we predicted that the seasonal polyphenism in H. titia female wing coloration influences between-species reproductive interactions. To determine whether the seasonal shift in H. titia female wing phenotypes affects male mate recognition, we measured males’ responses to tethered conspecific and heterospecific females. The females used in these experiments were unmarked, mature females captured outside the study transect. We systematically 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. Hetaerina matings begin when a male clasps a female’s intersternite with his superior and inferior abdominal appendages (i.e. the cerci and paraprocts; Garrison, 1990), and proceed without any courtship ritual. If the focal male clasped the female during her first presentation, we ended the trial; if not, we presented her for another 5 s. We considered cases in which a male pursued a female with his abdomen curled into the clasping position to be sexual responses, whether or not the male actually clasped the female. In our analyses, we only included data from males who responded sexually to at least one tethered female. Both species were tested both years.

Seasonal shifts in male mate recognition could result from changes in female wing coloration or from changes in male mate recognition functions, or both. To determine whether mate recognition functions changed from the early to late season, we presented territorial males in each season with unmanipulated H. occisa females and H. occisa females with wings experimentally darkened to resemble dark-phase H. titia females. We darkened the wings by colouring the hindwing from the base to the tip with a grey marker.
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 and lme4 packages (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, but in a few instances where mixed-effect models did not converge because of complete or near complete separation (i.e. males never responded to a particular tethered treatment), we modelled clasping using the bayesglm function in the package arm (Gelman & Su, 2014).

For experiments conducted in both 2011 and 2012, we determined that there was no effect of year on species recognition unless specifically mentioned (see Appendix S2) and pooled results across years to increase statistical power. After determining that there was no effect on the responses of *H. occisa* territory holders of removing *H. titia* males from the experimental transect in 2012 (see Mechanism of Seasonal Shifts in Species Recognition), we pooled data across transects for subsequent analyses.

**Results**

**Seasonal polyphenism**

Mature *H. titia* males emerging later in the period of peak emergence have more black pigmentation on their wings than mature males emerging earlier (Fig. 1a–c). This was true both within prolonged visits (Fig. 1d–f, Table S4) and across shorter visits to the same study sites (Fig. 1g,h, Table S2). Likewise, mature females emerging later in the year have darker wings than mature females emerging earlier (Fig. 2, Tables S3 and S4).

**Species recognition**

*Naturally occurring interspecific fights*

Interspecific fights were less frequent than null expectations in both seasons, but the magnitude of the reduction in interspecific fights was greater in the late season in both years, whether the analysis included all fights or only escalated fights (Tables 1 and S5), supporting the hypothesis that between-species similarity in male wing phenotypes results in increased interspecific aggression.
Competitor recognition

As with naturally occurring fights, the competitor recognition experiments on *H. occisa* males supported the hypothesis that similarity in male wing phenotypes increases males’ aggression towards heterospecifics. Specifically, *H. occisa* males discriminated more strongly between conspecifics and heterospecifics in the late season, when dark-phase *H. titia* phenotypes were more abundant, than in the early season, as measured by both attack rate (Fig. 3a, Table 2) and chase duration (Table S6). *Hetaerina titia* males, however, were more aggressive towards conspecific males than towards *H. occisa* males in both seasons, and there was no seasonal shift in the magnitude of relative heterospecific aggression as measured by attack rate (Fig. 3b, Table 2) or chase duration (Table S6).

Manipulations of conspecific wing pigmentation demonstrated that dark wing pigmentation, *per se*, reduces *H. occisa* aggressive responses in the late season more than in the early season. *H. occisa* males shifted from responding equally to conspecific male intruders of all treatment groups in the early season (before the shift in *H. titia* wing coloration) to responding less aggressively towards blackened males than towards control males in the late season (after the *H. titia* colour shift; Table 3). The behavioural shift was less evident in 2012 than in 2011 (Appendix S2, Table S7, Fig. 4a,b). In 2011, territorial *H. occisa* males directed relatively fewer attacks towards experimentally manipulated males with completely blackened wings than towards control males in the late season than in the early season (Table S7, Fig. 4a). In 2012, there was a nonsignificant trend in the same direction, but males were more aggressive overall in the late season in 2012 (Table S7, Fig. 4b). The proportion of time males spent chasing tethered intruders was affected by neither the tethered male treatment nor the season (Table S8).

*Hetaerina titia* males were more aggressive towards *H. occisa* males with experimentally blackened wings compared to *H. occisa* control males, but, consistent with the lack of a shift in conspecific vs. heterospecific discrimination, there was no significant seasonal shift in relative aggression towards manipulated *H. occisa* intruders, as measured by attack rate (Fig. 4c, Table 3) or chase duration (Table S9). Similarly, adding black ink to *H. titia* males resulted in territorial males chasing tethered individuals longer in both seasons (Table S9) and no significant treatment effect on attack rate in either season (Fig. 4c, Table 3).

Mate recognition

The seasonal shift in *H. titia* female wing coloration corresponded with a shift in the ability of males to differentiate between conspecific and heterospecific females. Male *H. occisa* shifted from responding sexually to both heterospecific and conspecific females in the early season to only responding sexually to conspecific females in the late season (Table 4, Fig. 5a). Likewise, *H. titia* males shifted from responding equally to *H. titia* and *H. occisa* females in the early season to largely responding sexually only to conspecific females in the late season (Table 4, Fig. 5b).

Female wing pigmentation, *per se*, influenced the likelihood of male sexual responses to a greater extent in the late season compared to the early season. Male *H. occisa* responded sexually more often to unmanipulated conspecific females than to conspecific females
with experimentally darkened wings in both seasons, but discrimination was more pronounced in the late season (Table 4, Fig. 5c). *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 (Table 4, Fig. 5d).

**Mechanism of seasonal shifts in species recognition**

Male *H. occisa* on the control transect clearly interacted with male *H. titia* because they were observed in interspecific fights (Table 1, 2012 late season), whereas males on the experimental (*H. titia* removal) transect had no opportunity to interact with male *H. titia*. Nevertheless, preventing *H. occisa* males from interacting with *H. titia* during the late season did not affect the development of competitor recognition. Males that matured and established territories on the experimental transect, where *H. titia* had been removed, discriminated between male *H. occisa* and *H. titia* intruders just as strongly as did males on the control transect, where *H. titia* were not removed (Tables 5 and S10). On both transects, *H. occisa* territory holders were more aggressive towards conspecifics than towards heterospecifics, and there was no significant transect-by-intruder species interaction (Tables 5 and S10). The removal of *H. titia* also did not affect how *H. occisa* males responded to tethered conspecific males with and without blackened wings (Table S10).

The *H. titia* removal had no effect on the development of male mate recognition. *H. occisa* males in both control and removal transects responded sexually more often to unmanipulated conspecific females than to *H. titia* females, and there was no significant female species-by-transect interaction (Table 5). Likewise, *H. occisa* males in both transects responded sexually more often to unmanipulated conspecific females than to experimentally darkened conspecifics, and there was no treatment-by-transect interaction (Table 5).

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### Table 2 Results of repeated-measure analyses (mixed-effect negative binomial models) comparing the number of attacks by territory holders directed towards tethered conspecific and heterospecific males.

<table>
<thead>
<tr>
<th>Species</th>
<th>Species</th>
<th>Season</th>
<th>n</th>
<th>Treatment</th>
<th>Estimate</th>
<th>SE</th>
<th>z</th>
<th>P-value</th>
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<td><em>Hetaerina occisa</em></td>
<td>Early season</td>
<td>53</td>
<td>Species</td>
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<td>0.17</td>
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<td>Both seasons</td>
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<td>Species</td>
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<td>–4.30</td>
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<td>Season</td>
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<td></td>
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<td><em>Hetaerina titia</em></td>
<td>Early season*</td>
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<td>Species</td>
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<td>0.32</td>
<td>–5.95</td>
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<tr>
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<td>Late season*</td>
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<td>Species</td>
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<td>Both seasons*</td>
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<td>Species</td>
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<td>0.76</td>
<td>–0.38</td>
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</table>

*Negative binomial zero-inflation mixed-effect model.

### Table 3 Results of repeated-measure analyses (mixed-effect negative binomial models) comparing the number of attacks by territory holders towards experimentally manipulated tethered intruders (*C* = clear, *HB* = half-blackened, *B* = blackened), pooled across years.

<table>
<thead>
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<th>Species</th>
<th>Experiment</th>
<th>Model</th>
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<th>Model term</th>
<th>Estimate</th>
<th>SE</th>
<th>z</th>
<th>P-value</th>
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<tbody>
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<td><em>Hetaerina occisa</em> male intruders (<em>C vs. B</em>)</td>
<td>Early season</td>
<td>18</td>
<td>Treatment</td>
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<td>Late season</td>
<td>8</td>
<td>Treatment</td>
<td>–0.51</td>
<td>0.87</td>
<td>–0.59</td>
<td>0.55</td>
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<td></td>
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<td>Both seasons</td>
<td>26</td>
<td>Treatment</td>
<td>–1.12</td>
<td>0.40</td>
<td>–2.78</td>
<td>0.005</td>
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<td></td>
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<td></td>
<td>Season</td>
<td>–0.33</td>
<td>0.51</td>
<td>–0.66</td>
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<td>Treatment*season</td>
<td>0.61</td>
<td>0.73</td>
<td>0.84</td>
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<td><em>H. titia</em> male intruders (<em>C v. B</em>)</td>
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<td>18</td>
<td>Treatment</td>
<td>0.30</td>
<td>0.21</td>
<td>1.42</td>
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<td>Late season</td>
<td>8</td>
<td>Treatment</td>
<td>0.34</td>
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<td><em>H. occisa</em></td>
<td><em>H. occisa</em> male intruders (<em>C v. HB, C v. B</em>)</td>
<td>Early season</td>
<td>53</td>
<td>HB treatment</td>
<td>–0.04</td>
<td>0.12</td>
<td>–0.33</td>
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<td>B treatment</td>
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<td>–0.15</td>
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<td>50</td>
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<td>0.15</td>
<td>–2.29</td>
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Removing the claspers of *H. occisa* males did not prevent them from developing the ability to discriminate between females on the basis of wing coloration. Males in both the clasper removal and control groups attempted to clasp unmanipulated *H. occisa* females much more often than darkened *H. occisa* females, and there was no significant male treatment-by-female treatment interaction (Table 5).

**Discussion**

**Seasonal polyphenism**

Across a broad geographic area, smoky rubyspots (*H. titia*) of both sexes undergo a striking seasonal shift in wing coloration, from a light-phase phenotype that resembles other sympatric congeners, such as *H. occisa*,...
to a dark-phase phenotype that is distinct from any sympatric species. Whereas such a pattern could potentially be caused by a genetic polymorphism in which dark- and light-phase morphs were reproducively isolated by differences in emergence time, the continuous variation in wing coloration phenotypes and especially the presence of some light-phase individuals during the late season (Figs 1 and 2) argue against this hypothesis. In addition to documenting this seasonal polyphenism, we show that it coincides with, and appears to contribute to, seasonal changes in the levels of reproductive and aggressive interference between species. *Hetaerina occisa* territory holders were far more aggressive to tethered heterospecific males early in the breeding season compared to later in the breeding season. In the late season, blackening the wings of conspecific intruders led to a reduction in *H. occisa* territorial aggression compared to controls, but no such reduction in aggression was found in the early season. Thus, the seasonal shift in species recognition is a product of both seasonal changes in the wing coloration of *H. titia* and seasonal changes in competitor recognition in *H. occisa*. Males of both species were more likely to respond sexually to tethered heterospecific females in the early season compared to the late season. Manipulations of female wing coloration showed that male mate recognition also changes seasonally in both species. Hence, reproductive interference is reduced both by the seasonal shift in female wing coloration in *H. titia* and by seasonal shifts in male mate recognition in both species. From the standpoint of interspecific interference, it is surprising that *H. titia* does not develop the dark-phase phenotype throughout the year. Presumably, the seasonal polyphenism is adaptive in some other context (e.g. parasite resistance, visual predation, thermoregulation) or reflects an unknown developmental constraint.

What proximate mechanism underlies the seasonal shifts in competitor recognition and mate recognition? Perhaps the simplest hypothesis is that species recognition involves learning and only males that are present during the late season have the opportunity to learn to discriminate between the late-season wing coloration phenotypes of the two species. Learned mate recognition (Irwin & Price, 1999; Verzijden et al., 2012) has been found in butterflies with seasonal polyphenisms (e.g. Westerman et al., 2014), and in other species of damselflies (Fincke, 1997; Svensson et al., 2010, 2014). To test the learning hypothesis, we manipulated the opportunity for males to gain experience interacting with heterospecífics during the late season. In one field experiment, we removed all *H. titia* from an experimental transect and compared the responses of male *H. occisa* that matured in that transect to those of males that matured in a control transect where *H. titia* was not removed. Although *H. occisa* males in the control transect were observed interacting with *H. titia* males (Table 1, late season 2012) and males in the experimental transect had no opportunity to do so, this had no effect on the propensity of males to distinguish between the late-season wing coloration phenotypes of the two species. In a second field experiment, we prevented male *H. occisa* from obtaining mating experience by removing their claspers before they became sexually active. We found that clasperless males were just as capable as intact males of distinguishing between late-season female wing coloration phenotypes of the two species. Thus, the seasonal shifts in mate recognition and competitor recognition do not appear to be an effect of the seasonal colour shift on learning.

An alternative hypothesis that does not require learning is that the seasonal shifts in recognition are developmentally plastic responses to an environmental cue – presumably the same environmental cue that triggers the shift in *H. titia* wing coloration – for example photoperiod. If so, this could be an example of a seasonal recognition shift in one species (*H. occisa*) evolving in response to a seasonal colour polyphenism in another species (*H. titia*). This character displacement hypothesis makes a clear and testable prediction: allopatric *H. occisa*
populations should not exhibit the seasonal shift in mate recognition and competitor recognition.

Interspecific aggression may be an adaptive response to between-species competition for access to mates in animals generally (Payne, 1980; Drury et al., 2015). Previous research showed that variation in the magnitude of reproductive interference predicts contemporary levels of interspecific aggression across several Hetaerina populations and species pairs (Drury et al., 2015). Our finding that H. occisa discriminate between conspecific and heterospecific males only after the seasonal decline in reproductive interference provides additional support for this hypothesis. Alternatively, the relationship between interspecific aggression and reproductive interference could potentially arise from pleiotropy between mate recognition and competitor recognition. The extent to which variation in the level of reproductive interference accounts for unexplained variation in interspecific aggression in other taxa, such as mammals, birds and reptiles (Ord & Stamps, 2009; Peiman & Robinson, 2010; Ord et al., 2011), should be a priority for further research (Drury et al., 2015).

Reproductive interference between H. occisa and H. titia in the early season does not appear to result in hybridization or gamete wastage. In odonates generally, males cannot force females to copulate (Corbet, 1999), and all interspecific Hetaerina pairs that we have observed have broken up before the copulation stage (J. Drury, C. Anderson & G. Grether, personal observation). As for the mechanism of reproductive isolation, post-clasping mate recognition is probably based on tactile cues. Male cerci and paraprocts, and the structures on females to which they attach (intersternites), vary in shape among species (Garrison, 1990), and cerci morphology has been implicated in prezygotic isolation in Enallagma damselflies (McPeek et al., 2011). In principle, post-clasping mate recognition also could be based on species differences in male wing coloration, but this mechanism has been ruled out in other sympatric Hetaerina species (Drury & Grether, 2014).

It is likely that our findings are not unique to rubyspot damselflies and that seasonal polyphenisms often affect species recognition. Indeed, several species of garden white butterflies (Pieris spp.) undergo seasonal changes in wing phenotypes (Shapiro, 1969; Kingsolver & Wiernasz, 1991; Stoehr & Goux, 2008), and wing phenotypes in these species influence discrimination between species (Ohguchi & Hidaka, 1988; Wiernasz & Kingsolver, 1992). Similarly, wing pigmentation is known to influence both mate and competitor recognition in Calopteryx damselflies (Tynkkynen et al., 2004, 2006; Svensson et al., 2007, 2010), and Calopteryx splendens males undergo a seasonal shift in the allometry of wing pigmentation (Hardersen, 2010). The influence of these polyphenisms on interspecific interactions is currently unknown. Seasonal polyphenism is just one form of phenotypic plasticity. Other forms of plasticity in the expression of traits used as agonistic or sexual signals mediating interspecific interactions are also likely to influence the efficacy of species recognition.

### Phenotypic plasticity, species recognition and evolution

Several investigators have explored how plasticity in sexual ornaments and/or in mate preferences may influence the dynamics of sexual selection and interactions between species (Ingleby et al., 2010; Pfennig et al., 2010; Verzijsen...
et al., 2012; Rodríguez et al., 2013). Recent research on banded demoiselles (C. splendens), for example, demonstrates that the ability of females to discriminate between conspecific and heterospecific (Calopteryx virgo) males is largely learned, perhaps as a mechanism of local adaptation in the presence of gene flow (Svensson et al., 2010, 2014). Whereas this and other research show how phenotypic plasticity in mating preferences can be important evolutionary processes, phenotypic plasticity in the signal traits that are themselves used to discriminate between heterospecifics and conspecifics likely also has important evolutionary consequences.

Studies of phenotypic plasticity in species recognition traits may also advance the understanding of the role of plasticity in evolution generally. For example, in the light of our results, the previously documented character displacement patterns in male wing coloration and competitor recognition in Hetaerina damselflies (Anderson & Grether, 2010a,b) may have evolved via selection shifting the timing of the switch to dark-phase phenotypes or the degree of black pigmentation in dark-phase variants in sympatric populations. If so, this would be an example of genetic accommodation (West-Eberhard, 2003) underlying character displacement (Pfenng & Pfenng, 2012a). Understanding how variable phenotypic expression can both change the dynamics of evolution and itself become the target of selection is an example of genetic accommodation (West-Eberhard, 2012a). Understanding how variable phenotypic expression can both change the dynamics of evolution and itself become the target of selection is an example of genetic accommodation (West-Eberhard, 2012a).

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Conflict of interest

The authors declare no conflict of interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary methods.
Appendix S2 Statistical tests for the effect of year on behavioral responses.

Figure S1 A sample of males that were photographed at two points separated by 2 weeks, showing that the extent of black pigment on the wings of individual males does not change after maturation.

Figure S2 Representative photographs of several *H. occisa* males (left column) and females (right) from La Palma.

Figure S3 Representative spectra from the forewings (base, middle, and tip) of a darkened *H. occisa* female (red), an unmanipulated *H. occisa* female (green), and an unmanipulated, darkphase form *H. titia* female (blue).

Table S1 Study locations, visit dates, and number of *H. titia* males’ wings analyzed.

Table S2 Results of analyses of the relationship between the capture date and the proportion of black pigmentation on male *H. titia* wings.

Table S3 Repeated-measures ANOVA of total wing lightness from reflectance spectra of *H. titia* females.

Table S4 Photographic measurements of *H. titia* female wing lightness demonstrate that female wings undergo a seasonal shift in coloration (Fig. 2).

Table S5 Comparisons of the counts of naturally occurring, escalated conspecific and heterospecific fights across both seasons for each year of the study.

Table S6 Results of mixed-effect models comparing the chase duration of *H. occisa* and *H. titia* territory holders toward heterospecific and conspecific tethered intruders.

Table S7 Results of mixed-effect negative binomial models comparing responses of *H. occisa* territory holders to experimentally manipulated tethered intruders, presented separately for each year.

Table S8 Results of mixed-effect linear models comparing responses of *H. occisa* territory holders to experimentally manipulated tethered intruders.

Table S9 Results of mixed-effect models comparing the chase duration of *H. titia* territory holders toward experimentally manipulated (a) heterospecific and (b) conspecific tethered intruders with black (B) and clear (C) ink on their wings.

Table S10 Results of mixed-effect models comparing responses of *H. occisa* territory holders to tethered intruders, comparing control males with males on a transect where all *H. titia* males were removed.

Data deposited at Dryad: doi: 10.5061/dryad.7k824

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