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

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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; Gröning & 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-

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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; Drury *et al.*, 2015). 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

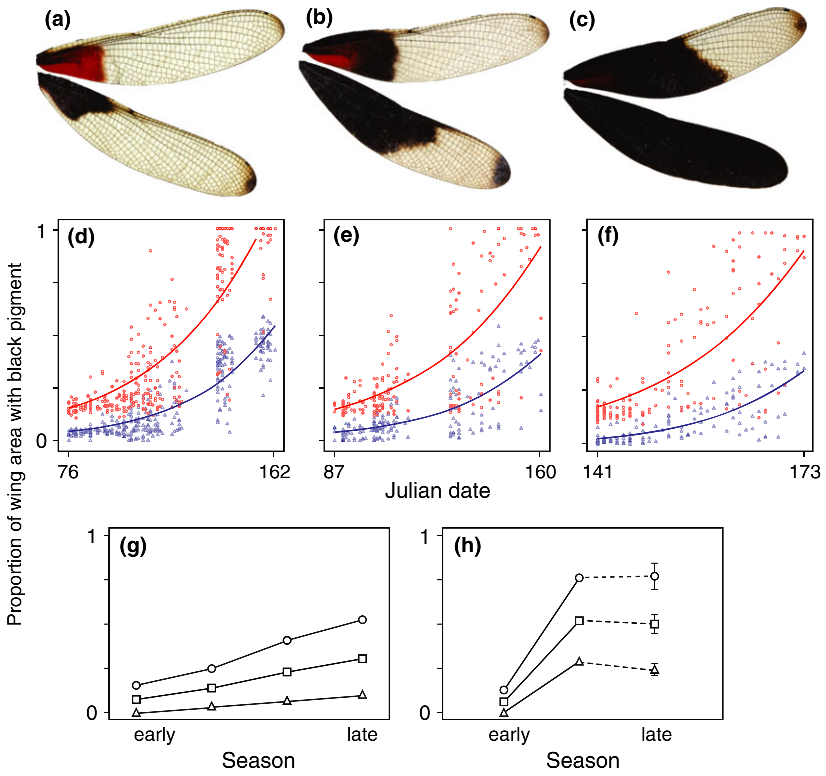

Fig. 1 Seasonal variation in the wing coloration of male *Hetaerina titia*. (a–c) Photographs of males' wings with different amounts of black pigmentation (forewings on top, hindwings on bottom). (d–f) The proportion of male wings covered with black pigment, plotted against measurement date, from extended 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.

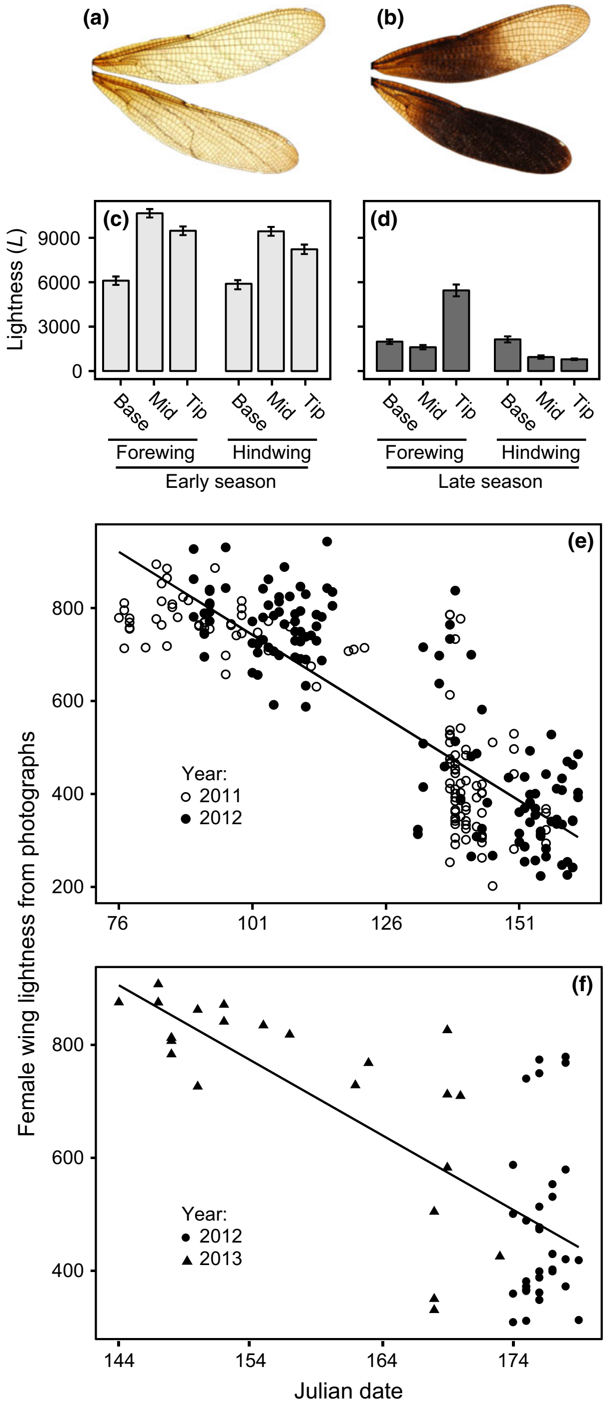

Fig. 2 Seasonal variation in the wing coloration of female *Hetaerina titia*. (a,b) Photographs of representative light-phase (a) and dark-phase (b) female's wings. (c,d) Lightness of female *H. titia* wings (means ± SE) measured with reflectance spectrometry at three positions (base, middle and tip) in the early and late seasons at La Palma. (e,f) Lightness of female *H. titia* wings measured with photography at La Palma in Veracruz (e) and Castroville in Texas (f) vs. capture date. Lines are linear model predictions of wing lightness.

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).

To quantify the colour variation in mature female *H. titia* wings, we measured reflectance spectra with a spectrometer during the 2011 visit to La Palma, in

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 Labsphere-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 ($n = 30$, March 25 to 12 April 2011) and end ($n = 26$, 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 MBF 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. 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. 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. occisa* and *H. 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 colour paints (Anderson *et al.*, 2011).

To compare the responses of *H. occisa* males to light-phase and dark-phase *H. 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. 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. 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. occisa* vs. *H. occisa*, *H. occisa* vs. *H. titia*, *H. titia* vs. *H. 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. 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 χ^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 on the outside surface of the hindwing), (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

(Prismacolor PM-107) and the forewing from base to the nodus with a grey marker (PM-107), and from the nodus to the tip with a sepia marker (PM-62). We chose these colours because their reflectance spectra best approximated dark-phase *H. titia* females (Fig. S3). We used the same criteria for male sexual responses and inclusion in analyses as described above. We limited the wing colour manipulation experiment to *H. occisa* females because dark-phase *H. titia* females are already dark and cannot be manipulated to have *H. occisa*-like wing coloration.

Learning experiments

The competitor recognition experiments (described above) showed that the competitor recognition function of *H. occisa* shifts seasonally (see Results). Specifically, *H. occisa* males discriminated more strongly between blackened and control conspecific intruders in the late season compared to the early season. To test the hypothesis that this shift requires interacting with dark-phase *H. titia* in the late season, we prevented some *H. occisa* males from doing so by removing all male *H. titia* from one study transect for 8 days prior to testing and throughout the testing period in the late season of 2012. Adult maturation takes approximately 10 days from the time of emergence (Grether, 1996), and the first 2–3 days are spent in a fragile teneral form away from the areas where males hold territories. Thus, 8 days was sufficient to ensure that males reaching maturity on the study transect had no territorial interactions with *H. titia*. During the testing period, we presented *H. occisa* territory holders with tethered conspecifics and heterospecifics, using the same treatments and protocols as described above (see Competitor Recognition Experiments and Mate Recognition Experiments). Identical tests were carried out, simultaneously, in a control transect where *H. titia* were not removed (~140 m downstream from the experimental transect).

We also found a seasonal shift in male mate recognition in *H. occisa* (see Results). To test the hypothesis that this shift results from males associating the dark coloration of late-season *H. titia* females with rejection, we assigned newly emerged *H. occisa* males (< 3 days post-emergence) to either control or clasper removal treatments. In the clasper removal treatment, we removed the males' abdominal appendages (cerci and paraprocts) with iris scissors, which disables them from successfully clasping females. Once the males matured and began defending territories, we presented them with tethered females, as described above (see Mate Recognition Experiments).

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 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 S2) 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.

Table 1 Comparisons of the counts of all naturally occurring conspecific and heterospecific fights across both seasons for each year of the study. Expected values (in parentheses) were calculated from a binomial expansion of the proportion of males of each species present during each time period. χ^2 tests compare the deviations between expected and observed values between seasons.

	Number of fights observed (expected)			Observed/ expected
	Intraspecific *Hetaerina occisa*	Interspecific	Intraspecific *H. titia*	Interspecific fights
2011				
Early	87 (128)	115 (131)	90 (33)	0.88
Late	20 (22)	21 (35)	29 (14)	0.61
χ^2 = 19.01, d.f. = 2, P < 0.001				
2012				
Early	15 (14)	10 (19)	15 (7)	0.52
Late	30 (17)	14 (35)	27 (19)	0.39
χ^2 = 7.31, d.f. = 2, P = 0.026				

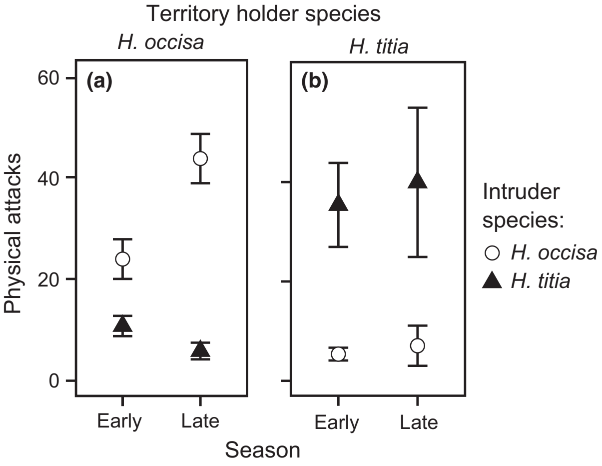

Fig. 3 Responses of males to conspecific and heterospecific tethered male intruders. (a) *Hetaerina occisa* territory holders attacked conspecific intruders at higher rates and heterospecific (*Hetaerina titia*) intruders at lower rates in the late season as compared to the early season. (b) Responses of *H. titia* territory holders to conspecific and heterospecific intruders did not change seasonally. Plotted values: means ± SE.

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

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

Species	Model	*n*	Model term	Estimate	SE	*z*	*P*-value
Hetaerina occisa	Early season	53	Species	−0.89	0.17	−5.26	<0.001
	Late season	50	Species	−2.76	0.35	−7.86	<0.001
	Both seasons	103	Species	−0.90	0.21	−4.30	<0.001
			Season	1.03	0.34	3.02	0.0025
			Species*season	−2.00	0.33	−6.11	<0.001
Hetaerina titia	Early season*	18	Species	−1.90	0.32	−5.95	<0.001
	Late season*	8	Species	−1.88	0.38	−5.0	<0.001
	Both seasons*	26	Species	−1.87	0.34	−5.33	<0.001
			Season	0.34	0.55	0.62	0.54
			Species*season	−0.29	0.76	−0.38	0.70

*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.

Species	Experiment	Model	*n*	Model term	Estimate	SE	*z*	*P*-value
Hetaerina titia	*Hetaerina occisa* male intruders (C vs. B)	Early season	18	Treatment	−1.13	0.32	−3.56	<0.001
		Late season	8	Treatment	−0.51	0.87	−0.59	0.55
		Both seasons	26	Treatment	−1.12	0.40	−2.78	0.005
				Season	−0.33	0.51	−0.66	0.51
				Treatment*season	0.61	0.73	0.84	0.40
	H. titia male intruders (C v. B)	Early season	18	Treatment	0.30	0.21	1.42	0.16
		Late season	8	Treatment	0.34	0.51	0.67	0.51
H. occisa	*H. occisa* male intruders (C v. HB, C v. B)	Early season	53	HB treatment	−0.04	0.12	−0.33	0.74
				B treatment	−0.02	0.12	−0.15	0.88
		Late season	50	HB treatment	−0.17	0.1	−1.71	0.087
				B treatment	−0.35	0.1	−3.57	<0.001
		Both seasons	103	HB treatment	−0.038	0.11	−0.34	0.73
				B treatment	−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

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).

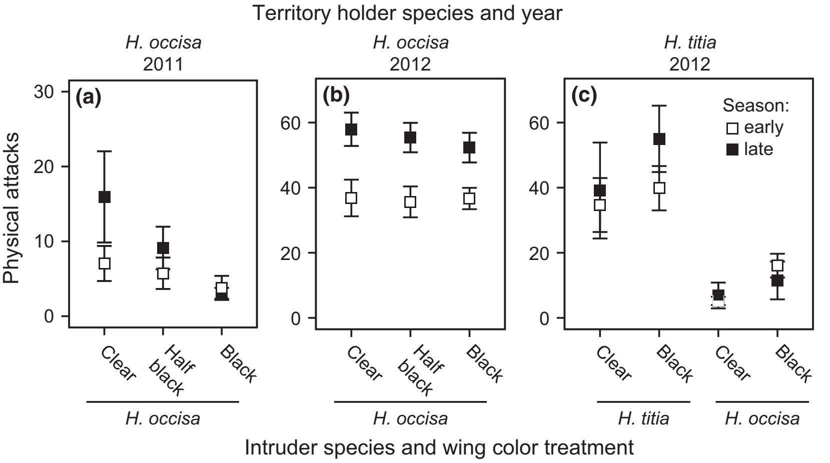

Fig. 4 Responses of males to experimentally manipulated tethered male intruders. (a,b) *Hetaerina occisa* territory holders tended to physically attack blackened conspecific males less in the late season compared to the early season in 2011 (a) and 2012 (b), indicating a shift in competitor recognition may accompany the shift in *Hetaerina titia* male phenotypes. (c) *H. titia* males attacked blackened *H. occisa* males at higher rates than clear *H. occisa* males, but they did not attack blackened conspecific males more than control males or exhibit a seasonal shift in competitor recognition. Plotted values: means ± SE.

Table 4 Results of repeated-measure analyses (mixed-effect logistic regression) of sexual responses of territorial holders of both species towards tethered (a) conspecific and heterospecific females and (b) unmanipulated and experimentally darkened *Hetaerina occisa* females.

Male species	Experiment	Model	*n*	Model term	Estimate	SE	*z*	*P*-value
Hetaerina titia	Conspecific vs. heterospecific females	Early season	38	Female sp.	0.87	0.47	1.83	0.067
		Late season	24	Female sp.	4.23	1.13	3.76	<0.001
		Both seasons	62	Female sp.	0.87	0.47	1.83	0.067
				Season	−0.89	0.57	−1.55	0.12
				Female sp.*season	3.37	1.22	2.76	0.0058
H. occisa		Early season	63	Female sp.	−0.928	0.40	−2.30	0.022
		Late season	42	Female sp.	−7.43	1.43	−5.19	<0.001
		Both seasons	105	Female sp.	−0.93	0.40	−2.30	0.022
				Season	2.37	1.06	2.23	0.025
				Female sp.*season	−6.50	1.49	−4.37	<0.001
H. titia	Darkened vs. control *H. occisa* females	Early season	32	Female treatment	−0.13	0.50	−0.25	0.8
		Late season	26	Female treatment	−2.07	0.65	−3.21	0.0014
		Both seasons	58	Female treatment	−0.13	0.50	−0.25	0.8
				Season	0.64	0.54	1.17	0.24
				Treatment*season	−1.95	0.82	−2.38	0.017
H. occisa		Early season	61	Female treatment	1.61	0.42	3.84	<0.001
		Late season	43	Female treatment	3.54	0.79	4.49	<0.001
		Both seasons	104	Female treatment	1.61	0.42	3.84	<0.001
				Season	−0.42	0.41	−1.04	0.30
				Treatment*season	1.93	0.89	2.16	0.03

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*,

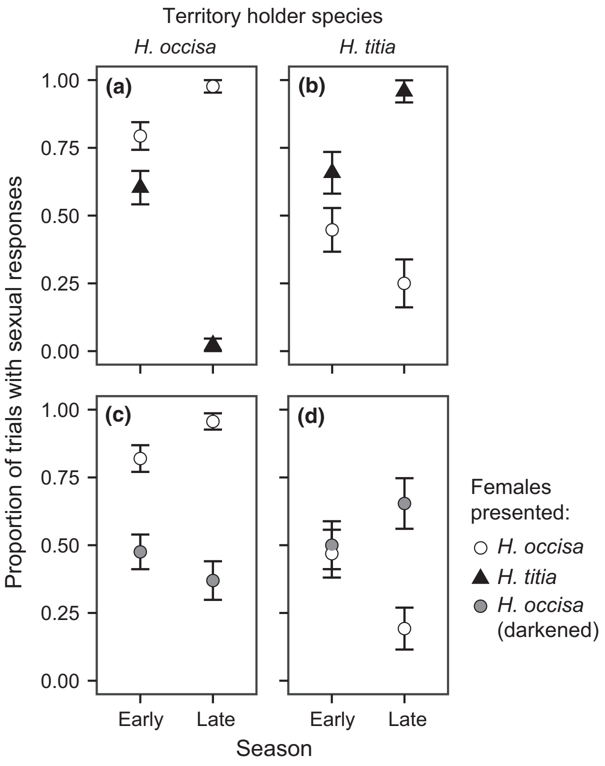

Fig. 5 Responses of males to tethered females. Both *Hetaerina occisa* and *Hetaerina titia* male species recognition and mate recognition shift across the flight season. (a) *H. occisa* and (b) *H. titia* male sexual responses to tethered, unmanipulated females. (c) *H. occisa* and (d) *H. titia* responses to unmanipulated and experimentally darkened *H. occisa* females. All panels show proportions ± SE.

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 reproductively 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 *H. titia* male intruders 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 heterospecifics 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*

Table 5 Analyses testing for an effect of learning on species recognition. Experiments on territory holders after *Hetaerina titia* removal experiment towards (a) conspecific and heterospecific tethered intruders, (b) conspecific and heterospecific tethered females, and (c) unmanipulated and experimentally darkened *Hetaerina occisa* females show no effect of *H. titia* removal on species recognition. Similarly, analyses of abdominal appendage removal experiment comparing sexual responses of territory males towards unmanipulated and experimentally darkened *H. occisa* females show no effect of clasper removal on male responses.

Experiment	Analysis	n	Model term	Estimate	SE	z	P-value
H. titia removal	*H. occisa* vs. *H. titia* males, attack count*	33	Removal transect	−0.67	0.25	−2.66	0.0079
			Tethered male sp.	−1.41	0.27	−5.16	<0.001
			Transect*species	−0.76	0.44	−1.75	0.08
	H. occisa vs. *H. titia* females, clasping†	33	Removal transect	−0.90	0.76	−1.19	0.23
			Tethered female sp.	−4.86	1.11	−4.36	<0.001
			Transect*species	1.92	1.47	1.31	0.19
	Unmanipulated vs. darkened *H. occisa* females, clasping	34	Removal transect	−13.33	6.15	−2.17	0.03
			Tethered female treatment	22.08	4.74	4.66	<0.001
			Transect*treatment	−8.37	5.56	−1.51	0.13
Abdominal appendage removal	Unmanipulated vs. darkened *H. occisa* females, clasping	21	Clasper treatment	0.54	1.49	0.36	0.72
			Tethered female treatment	4.97	1.47	3.38	<0.001
			Clasper*female treatment	10.06	245.1	0.04	0.97

*Negative binomial zero-inflation mixed-effect model.
†bayesglm logistic regression.

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; Verzijden *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 (Pfennig & Pfennig, 2012a). 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 help to extend this ongoing synthesis to studies of signal phenotypes involved in mediating between-species social interactions.

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Conflict of interest

The authors declare no conflict of interests.

References

Anderson, C.N. & Grether, G.F. 2010a. Character displacement in the fighting colours of *Hetaerina* damselflies. *Proc. R. Soc. B Biol. Sci.* **277**: 3669–3675.

Anderson, C.N. & Grether, G.F. 2010b. Interspecific aggression and character displacement of competitor recognition in *Hetaerina* damselflies. *Proc. R. Soc. B Biol. Sci.* **277**: 549–555.

Anderson, C.N. & Grether, G.F. 2011. Multiple routes to reduced interspecific territorial fighting in *Hetaerina* damselflies. *Behav. Ecol.* **22**: 527–534.

Anderson, C.N., Córdoba-Aguilar, A., Drury, J.P. & Grether, G.F. 2011. An assessment of marking techniques for odonates in the family Calopterygidae. *Entomol. Exp. Appl.* **141**: 258–261.

Brown, W.L. & Wilson, E.O. 1956. Character displacement. *Syst. Zool.* **5**: 49–64.

Cody, M.L. 1969. Convergent characteristics in sympatric species: a possible relation to interspecific competition and aggression. *Condor* **71**: 223–239.

Cody, M.L. 1973. Character convergence. *Annu. Rev. Ecol. Syst.* **4**: 189–211.

Corbet, P.S. 1999. *Dragonflies: Behavior and Ecology of Odonata*. Comstock Pub. Associates, Ithaca, NY.

Dobzhansky, T. 1955. *Evolution, Genetics, and Man*. John Wiley & Sons, Inc., New York & Chapman & Hall, Ltd., London.

Drury, J.P. & Grether, G.F. 2014. Interspecific aggression, not interspecific mating, drives character displacement in the wing coloration of male rubyspot damselflies (*Hetaerina*). *Proc. R. Soc. B Biol. Sci.* **281**: 20141737.

Drury, J.P., Okamoto, K., Anderson, C.N. & Grether, G.F. 2015. Reproductive interference explains persistence of aggression between species. *Proc. R. Soc. B Biol. Sci.* **282**: 20142256.

Fincke, O.M. 1997. Conflict resolution in the Odonata: implications for understanding female mating patterns and female choice. *Biol. J. Linn. Soc.* **60**: 201–220.

Fisher, R.A. 1930. *The Genetical Theory of Natural Selection*. Oxford University Press, London.

Fournier, D., Skaug, H., Ancheta, J., Ianelli, J., Magnusson, A., Maunder, M. *et al.* 2012. AD model builder: using automatic differentiation for statistical inference of highly parameterized complex nonlinear models. *Optim. Methods Softw.* **27**: 233–249.

Garrison, R.W. 1990. A synopsis of the genus *Hetaerina* with descriptions of four new species. *Trans. Am. Entomol. Soc.* **116**: 175–259.

Gelman, A. & Su, Y.-S. 2014. arm: Data analysis using regression and multilevel/hierarchical models. http://cran.r-project.org/package=arm.

Grether, G. 1996. Sexual selection and survival selection on wing coloration and body size in the rubyspot damselfly *Hetaerina americana*. *Evolution* **50**: 1939–1948.

Grether, G.F. 2011. The neuroecology of competitor recognition. *Integr. Comp. Biol.* **51**: 807–818.

Grether, G.F., Losin, N., Anderson, C.N. & Okamoto, K. 2009. The role of interspecific interference competition in character displacement and the evolution of competitor recognition. *Biol. Rev.* **84**: 617–635.

Grether, G., Anderson, C., Drury, J.P., Losin, N., Peiman, K. & Okamoto, K. 2013. The evolutionary consequences of interspecific aggression. *Ann. N. Y. Acad. Sci.* **1289**: 48–68.

Grether, G.F., Drury, J.P., Berlin, E. & Anderson, C.N. 2015. The role of wing coloration in sex recognition and competitor recognition in rubyspot damselflies (*Hetaerina* spp.). *Ethology*. doi: 10.1111/eth.12382.

Gröning, J. & Hochkirch, A. 2008. Reproductive interference between animal species. *Q. Rev. Biol.* **83**: 257–282.

Hardersen, S. 2010. Seasonal variation of wing spot allometry in *Calopteryx splendens* (Odonata Calopterygidae). *Ethol. Ecol. Evol.* **22**: 365–373.

Higgie, M. & Blows, M. 2008. The evolution of reproductive character displacement conflicts with how sexual selection operates within a species. *Evolution* **62**: 1192–1203.

Ingleby, F.C., Hunt, J. & Hosken, D.J. 2010. The role of genotype-by-environment interactions in sexual selection. *J. Evol. Biol.* **23**: 2031–2045.

Irwin, D.E. & Price, T. 1999. Short review sexual imprinting, learning and speciation. *Heredity (Edinb)* **82**: 347–354.

Kingsolver, J.G. & Wiernasz, D.C. 1991. Seasonal polyphenism in wing-melanin pattern and thermoregulatory adaptation in *Pieris* butterflies. *Am. Nat.* **137**: 816–830.

Lorenz, K. 1962. The function of colour in coral reef fishes. *Proc. R. Inst. G. B.* **39**: 282–296.

Mayr, E. 1963. *Animal Species and Evolution*. Harvard University Press, London, Oxford University Press.

McPeek, M.A., Symes, L.B., Zong, D.M. & McPeek, C.L. 2011. Species recognition and patterns of population variation in the reproductive structures of a damselfly genus. *Evolution* **65**: 419–428.

Mendelson, T.C. 2015. Distinguishing perceptual and conceptual levels of recognition at group boundaries. *Evol. Ecol.* **29**: 205–215.

Mendelson, T.C. & Shaw, K.L. 2012. The (mis)concept of species recognition. *Tree* **27**: 421–427.

Murray, B.G. Jr 1971. The ecological consequences of interspecific territorial behavior in birds. *Ecology* **52**: 414–423.

Ohguchi, O. & Hidaka, T. 1988. Mate recognition in two sympatric species of butterflies, *Pieris rapae* and *P. melete*. *J. Ethol.* **6**: 49–53.

Ord, T.J. & Stamps, J.A. 2009. Species identity cues in animal communication. *Am. Nat.* **174**: 585–593.

Ord, T.J., King, L. & Young, A.R. 2011. Contrasting theory with the empirical data of species recognition. *Evolution* **65**: 2572–2591.

Payne, R.B. 1980. Behavior and songs of hybrid parasitic finches. *Auk* **97**: 118–134.

Peiman, K.S. & Robinson, B.W. 2010. Ecology and evolution of resource-related heterospecific aggression. *Q. Rev. Biol.* **85**: 133–158.

Pfennig, K.S. 2007. Facultative mate choice drives adaptive hybridization. *Science* **318**: 965–967.

Pfennig, D.W. & Pfennig, K.S. 2012a. Development and evolution of character displacement. *Ann. N. Y. Acad. Sci.* **1256**: 89–107.

Pfennig, D.W. & Pfennig, K.S. 2012b. *Evolution's Wedge. Competition and the Origins of Diversity*. University of California Press, Berkeley, CA.

Pfennig, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D. & Moczek, A.P. 2010. Phenotypic plasticity's impacts on diversification and speciation. *Trends Ecol. Evol.* **25**: 459–467. Elsevier Ltd.

Pigliucci, M. 2010. Phenotypic plasticity. In: *Evolution: The Extended Synthesis* (M. Pigliucci & G.B. Müller, ed.), pp. 137–174. MIT Press, Cambridge, MA.

R Core Team 2013. *R: A Language and Environment for Statistical Computing*. R Core Team, Vienna, Austria.

Ritchie, M.G. 1996. The shape of female mating preferences. *Proc. Natl. Acad. Sci. USA* **93**: 14628–14631.

Rodríguez, R.L., Rebar, D. & Fowler-Finn, K.D. 2013. The evolution and evolutionary consequences of social plasticity in mate preferences. *Anim. Behav.* **85**: 1041–1047.

Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**: 671–675.

Shapiro, A. 1969. An extreme phenotype of *Pieris protodice* (Pieridae). *J. Lepid. Soc.* **23**: 189–190.

Shapiro, A.M. 1976. Seasonal polyphenism. *Evol. Biol.* **9**: 259–333.

Skaug, H., Fournier, D., Nielsen, A., Magnusson, A. & Bolker, B. 2012. Generalized linear mixed models using AD model builder (R package version 0.7.2.12).

Stoehr, A. & Goux, H. 2008. Seasonal phenotypic plasticity of wing melanisation in the cabbage white butterfly, *Pieris rapae* L. (Lepidoptera: Pieridae). *Ecol. Entomol.* **33**: 137–143.

Svensson, E.I., Karlsson, K., Friberg, M. & Eroukhmanoff, F. 2007. Gender differences in species recognition and the evolution of asymmetric sexual isolation. *Curr. Biol.* **17**: 1943–1947.

Svensson, E.I., Eroukhmanoff, F., Karlsson, K., Runemark, A. & Brodin, A. 2010. A role for learning in population divergence of mate preferences. *Evolution* **64**: 3101–3113.

Svensson, E.I., Runemark, A., Verzijden, M.N. & Wellenreuther, M. 2014. Sex differences in developmental plasticity and canalization shape population divergence in mate preferences. *Proc. R. Soc. B Biol. Sci.* **281**: 20141636.

Tynkkynen, K., Rantala, M.J. & Suhonen, J. 2004. Interspecific aggression and character displacement in the damselfly *Calopteryx splendens*. *J. Evol. Biol.* **17**: 759–767.

Tynkkynen, K., Kotiaho, J.S., Luojumäki, M. & Suhonen, J. 2006. Interspecific territoriality in *Calopteryx* damselflies: the role of secondary sexual characters. *Anim. Behav.* **71**: 299–306.

Verzijden, M.N., ten Cate, C., Servedio, M.R., Kozak, G.M., Boughman, J.W. & Svensson, E.I. 2012. The impact of learning on sexual selection and speciation. *Trends Ecol. Evol.* **27**: 511–519.

Wallace, A.R. 1889. *Darwinism, 2007 Facsi*. Cosimo Inc, New York.

West-Eberhard, M.J. 1979. Sexual selection, social competition, and evolution. *Proc. Am. Philos. Soc.* **123**: 222–234.

West-Eberhard, M.J. 1983. Sexual selection, social competition, and speciation. *Q. Rev. Biol.* **58**: 155–183.

West-Eberhard, M.J. 2003. *Developmental Plasticity and Evolution*. Oxford University Press, USA.

Westerman, E.L., Chirathivat, N., Schyling, E. & Monteiro, A. 2014. Mate preference for a phenotypically plastic trait is learned, and may facilitate preference-phenotype matching. *Evolution* **68**: 1661–1670.

Wiernasz, D.C. & Kingsolver, J.G. 1992. Wing melanin pattern mediates species recognition in *Pieris occidentalis*. *Anim. Behav.* **43**: 89–94.

Willis, P.M. 2013. Why do animals hybridize? *Acta Ethol.* **16**: 127–134.

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.

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