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

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Running title: Seasonal polyphenism and species recognition

## Abstract

Understanding how phenotypic plasticityevolves and in turn affects thecourse 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 howthe seasonal color shift impacts species recognition with the sympatric congener *H. 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 color manipulations of tethered damselflies, we show that the seasonal changes in interspecific interactions are caused not only by the seasonal color shift but also by shifts in discriminatory behavior in both species. We also experimentally tested and rejected the hypothesis that learning underlies the behavioral 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 evolutionof interspecific interactions.


Key words: phenotypic plasticity, mate recognition, competitor recognition, reproductive interference, interspecific aggression, Odonata

## 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, i.e., behavioral discrimination between conspecifics and heterospecifics (Mendelson & Shaw 2012), is often based on specific traits, such as color patches, cuticular hydrocarbons, orvocalizations (Higgie & Blows 2008; Pfennig & Pfennig, 2012).Such traits maydiverge between species through selection resulting from

interspecific interactions in reproductive (i.e., reinforcement/reproductive character displacement, Brown & Wilson, 1956; Pfennig & Pfennig, 2012)or agonistic/territorial contexts (i.e., agonistic character displacement, Lorenz 1962; Grether et al., 2009, 2013), or as a byproduct 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 favors treating heterospecifics as though they were conspecifics in certain contexts, such as when hybridization is advantageous (Pfennig, 2007; Mendelson & Shaw 2012; Willis, 2013)or resource competition favors 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 byproduct 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 experimentsto 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 peakbreeding season to a dark-phase wing phenotype later in the season (Figs. 1, 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 darkvariants 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 color 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 behavioral observations at a site in Veracruz Mexico where *H. titia* is found in sympatry with *H. occisa*. Both sexes of *H. occisa* more closely resemble the early (light) phenotype than the late (dark) phenotype of *H. titia* (Figs. 1, 2, 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 tetheredconspecifics 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 color 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 fore- and hindwings (Fig. 1a-c), and females have amber colored 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, U.S.A, 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 since we vigilantly captured and marked any new individuals along the transect during the entire length of the visit. To quantify the relative proportion of black pigment on the wings of mature male *H. titia*, we photographed the left forewing and hindwing of individuals using a digital camera (Canon 10D or 20D) with a 100mm macro lens and a dual flash (Canon MT-24EX).We used NIH Image to manually measure the black area and total area of each wing (for sample sizes, see Table S1).

To quantify the color variation inmature 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), oriented at 45 degrees relative to the wing surface to eliminate glare, and measured percent reflectance at each wavelength in relation to a Labsphere certified reflectance standard using Ocean Optics' OOIBase32 software. When taking measurements, we placed the wings flatagainst 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 percent reflectance at 2 nm intervals from 300 to 700 nm.We measured the reflectance of a sample of adult females captured without regard for their wing phenotypes near the beginning (*n* = 30, 3/25-4/12/2011) and end (*n* = 26, 5/30-6/10/2011) of our visit.

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, using the "Color Balance" pluginfrom the MBF package. We then used the polygon tool and the "Measure RGB" plugin to analyze the RGB profile of each wing, which we used to calculate an average weighted

grayscale measurement to serve as a photographic index of female wing lightness. This photographic

measure of lightnesscorrelates well withthe 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, 2a) are moresimilar to those of the congener, *H. occisa*, than the dark-phase

phenotypes more abundant later in the season (Figs. 1b, 2b,Fig. 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*individuals along a river transect

(~200-300 m) where both species were present inLa 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 color 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 3/23-5/5/2011 and 4/2-4/26/2012), most*H. titia* were light-phase forms, whereas

in the late season (5/30-6/15/2011 and 5/17-6/11/2012), most*H. titia* individuals were dark-phase forms

(Figs. 1d,e,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 seasonto avoid pseudoreplicationand to maintain a clearer

separation between the early- and late-season cohorts.

*Observationsof naturally occurring fights*

To determine which males were territorial and to record the frequency of naturally occurring

intra- and interspecific fights, we conducted behavioral 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-forthor "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 behavioral censuses.Prior to analysis, multiple recorded bouts of fighting between the same two males on the same day were reduced to a single fight. To avoid over-counting fights involving unmarked (or unidentified) males, those that occurred at the same location (± 5m) 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 intraspecificfightsbetween seasons, wegeneratedexpected 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 behavioral responses, is referred to as the competitor recognition function (Grether *et al.*, 2009; Grether, 2011).

We predicted that aggressive responses to intruding heterospecifics wouldbe 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 heterospecificintruders 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 five-minute inter-trial interval (Anderson & Grether, 2010b). During the trials, we recorded the behavior 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 toward 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). *H. 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 wing coloration or from seasonal changes in competitor recognition functions, or both.To determine whether competitor recognition functions shiftseasonally,it was critical to present territory holders with the same intruder stimuli in both seasons. Since 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 color-manipulated *H. occisa* intruders in these experiments. *H. occisa* territory holders were presented with conspecific intruders of the following three treatments: (1) 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), (2) 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 (3) clearcontrol (clear ink from the distal edge of the wingspot to the wing tip, as a sham-control). *H.*

*titia* territory holders were presented with males of the following four treatments (1) *H. occisa* with fully blackened hindwings (black ink, as above), (2) *H. occisa* control (clear ink, as above), (3) *H. titia* with fully blackened hindwings, (4) *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 color 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 if 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 colorationor from changes inmale mate recognitionfunctions, or both.To determine if mate

recognitionfunctionschanged 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 coloring the hindwing from the base to the tip with a gray marker (Prismacolor PM-107) and the forewing from base to the nodus with a gray marker (PM-107), and from the nodus to the tip with a sepia marker (PM-62).We chose these colors 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 color 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.

*Learningexperiments*

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 periodin 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 modeled 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* malesemerging 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, Table S3, 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 (Table 1, Table 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 toward 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). *H. titia* males, however, were more aggressive toward conspecific males than toward *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 toward blackened males than toward control males in the late season (after the *H. titia* color shift; Table 3).The behavioral 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 toward experimentally manipulated males with completely blackened wings than toward control males in the late season than in the early season (Table S7, Fig. 4a). In 2012, there was a non-significant 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).

*H. titia* males were more aggressive toward *H. occisa* males with experimentally blackened wings compared to *H. occisa* control males, but, consistent with the lack of a shift in conspecific versus heterospecific discrimination, there was no significant seasonal shift in relative aggression toward 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 pronouncedin the late season (Table 4, Fig. 5c).*H. titia* males did not discriminate betweenexperimentally 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), while 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, S10).On both transects *H. occisa* territory holders were more aggressive toward conspecifics than toward heterospecifics, and there was no significant transect by intruder species interaction (Table 5, Table 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).

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. While 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 (Fig. 1, 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. *H. 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 and 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 clasper-less 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 color 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 – e.g., photoperiod.If so, this this could be an example of a seasonal recognition shift in one species (*H. occisa*) evolving in response to a seasonal color 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 matesin 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; Ord et al. 2011l; Peiman & Robinson 2010), should bea priority for further research (Drury et al. 2015).

Reproductive interference between *H. occisa* and *H. titia*in the early seasondoes 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 (Pers. Obs.). 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 pre-zygotic 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 outin 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 (*Calopteryx splendens*), for example, demonstrates that the ability of females to discriminate between conspecific and heterospecific (*C. virgo*) males is largely learned, perhaps as a mechanism of local adaptation in the presence of gene flow (Svensson *et al.*, 2010, 2014). While this and other research showing 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 understanding of the role of plasticity in evolution generally. For example, in 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, 2012). 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.

## Acknowledgements

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**Table 1.** Comparisons of the counts of all naturally occurring conspecific and heterospecific fights across both seasonsfor 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. Chi-squared tests compare the deviations between expected and observed values between seasons.

| | | Number of fights observed (expected) | | | |
|---|---|---|---|---|---|
| | | Intraspecific *H. occisa* | Interspecific | Intraspecific *H. titia* | Observed/expected interspecific fights |
| 2011 | early | 87 (128) | 115 (131) | 90 (33) | 0.88 |
| | late | 20 (22) | 21 (35) | 29 (14) | 0.61 |
| $\chi^2$ = 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 |
| $\chi^2$ = 7.31, *d.f.* = 2, *P* = 0.026 | | | | | |

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

| Species | Model | n | Model term | Estimate | Std. error | z | P-value |
|---------|-------|---|------------|----------|-----------|---|---------|
| *H. 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 |
| *H. titia* | early season[1] | 18 | species | -1.90 | 0.32 | -5.95 | < 0.001 |
| | late season[1] | 8 | species | -1.88 | 0.38 | -5.0 | < 0.001 |
| | both seasons[1] | 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 |

[1]Negative binomial zero-inflation mixed-effect model

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

| Species | Experiment | Model | n | Model term | Estimate | Std. error | z | P-value |
|---------|-----------|-------|---|------------|----------|-----------|---|---------|
| *H. titia* | *H. occisa* male | early season | 18 | treatment | -1.13 | 0.32 | -3.56 | < 0.001 |
| | intruders | late season | 8 | treatment | -0.51 | 0.87 | -0.59 | 0.55 |
| | (C vs. B) | 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 | early season | 18 | treatment | 0.30 | 0.21 | 1.42 | 0.16 |
| | intruders | late season | 8 | treatment | 0.34 | 0.51 | 0.67 | 0.51 |
| | (C v. B) | | | | | | | |

| | | | | HB treatment | -0.04 | 0.12 | -0.33 | 0.74 |
|---|---|---|---|---|---|---|---|---|
| *H. occisa* | *H. occisa* male intruders | | early season | 53 | | | | |
| | | | | B treatment | -0.02 | 0.12 | -0.15 | 0.88 |
| | (C v. HB, C v. B) | 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 |

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

| Male species | Experiment | Model | *n* | Model term | Estimate | Std. error | *z* | *P*-value |
|---|---|---|---|---|---|---|---|---|
| *H. titia* | conspecific vs. | early season | 38 | female sp. | 0.87 | 0.47 | 1.83 | 0.067 |
| | heterospecific | late season | 24 | female sp. | 4.23 | 1.13 | 3.76 | < 0.001 |
| | females | 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. | early season | 32 | female treatment | -0.13 | 0.50 | -0.25 | 0.8 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| control *H.* | late season | 26 | female treatment | -2.07 | 0.65 | -3.21 | 0.0014 |
| *occisa* females | 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 |

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

| Experiment | Analysis | *n* | Model term | Estimate | Std. error | *z* | *P*-value |
|---|---|---|---|---|---|---|---|
| *H. titia* removal | *H. occisa* vs. *H. titia* males, attack count[1] | 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[2] | 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 | unmanipulated vs. | 21 | clasper treatment | 0.54 | 1.49 | 0.36 | 0.72 |
| appendage | darkened *H. occisa* | | tethered female treatment | 4.97 | 1.47 | 3.38 | < 0.001 |
| removal | females, clasping | | clasper * female treatment | 10.06 | 245.1 | 0.04 | 0.97 |

[1]Negative binomial zero-inflation mixed-effect model

[2]bayesglm logistic regression

**Figure legends**

**Figure 1.** Seasonal variation in the wing coloration of male *H. 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 post-hoc tests. From left to right, the dates of the short visits were as follows: Armeria 4/27-5/3/2008, 5/30-6/5/2007, 7/10-7/14/2005, 7/23-7/27/2007; Castroville 4/17-4/24/2008, 6/21-6/28/2012, 8/19-8/26/2008.

**Figure 2.**Seasonal variation in the wing coloration of female *H. titia*. A & B: Photographs of representative light-phase (A) and dark-phase (B) female's wings. C & D: Lightness offemale *H. titia* wings (means ± SE) measured with reflectance spectrometry at three positions (base, middle, and tip) in the early and late season 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) versus capture date. Lines are linear model predictions of wing lightness.

**Figure 3.**Responses of males to conspecific and heterospecific tethered male intruders. A. *H. occisa*territory holders attacked conspecificintruders at higher rates and heterospecific (*H. 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 seasonallyPlotted values: means ± SE.

**Figure 4.**Responses of males to experimentally manipulated tethered male intruders. A & B. *H. occisa* territory holders tended to physically attack blackened conspecific males less in the late seasoncompared to the early season in 2011 (A) and 2012 (B), indicating a shift in competitor recognition may accompany the shift in *H. 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 malesor exhibit a seasonal shift in competitor recognition. Plotted values: means ± SE.

**Figure 5.**Responses of males to tethered females. Both*H. occisa* and *H. titia*male species recognition and mate recognition shift across the flight season. A *H. occisa* and B. *H. titia* male sexual responses to tethered, unmanipulated females. C. *H. occisa* and D. *H. titia* responses to unmanipulated and experimentally darkened *H. occisa* females. All panels show proportions ± SE.

Territory holder species

*H. occisa*      *H. titia*

Territory holder species and year

Physical attacks

Intruder species and wing color treatment

Season:
☐ early
■ late

Territory holder species
*H. occisa*          *H. titia*

Proportion of trials with sexual responses

Season

Females presented:
○ *H. occisa*
▲ *H. titia*
◉ *H. occisa* (darkened)