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SEXUAL SIGNALING IN CONFLICTS AND THEIR RESOLUTIONS IN ODONATES

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DEPARTMENT OF BIOLOGY

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Dedicated to all women in science

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ABSTRACT

Nature abounds with conflicts and searching for the best solution to conflicts is of interest to all animals. Among various means to resolve conflicts, signaling may be superior because it can potentially settle conflicts without risk of injury and cost of time and energy associated with physical fights or wars of attritions. In this dissertation, I seek answers to the question, can signaling help animals resolve conflicts, in two different systems.

In nature, the relationship between the two sexes is often far from harmonious. The damselfly *Enallagma hageni* is a species whose males frequently harass females. Males of this species are bright blue, whereas females can be either male-like blue or green. In Chapter 1, I tested for the first time, the hypothesis that female-specific color polymorphism in odonates can resolve sexual conflict by lowering male harassment towards females. Under conditions controlling for sex ratio, population density and morph frequency, I also tested two major hypotheses for the maintenance of the polymorphism. Contrary to the prediction, per capita harassment rate for the female-monomorphic treatments did not differ from that of the female-polymorphic treatments. At a male biased sex ratio, per capita harassment rate towards blue, but not green females increased with morph frequency, providing partial support for frequency-dependent selection that maintains female polymorphism.

Paradoxically, male *E. hageni* were obeserved to sexually harass not only females, but also other males. In Chapter 2, using *E. hageni* as a model system, I tested hypotheses on the causes of male-male harassment and investigated the effect of male-male harassment on male-female mating opportunity in two experiments in outdoor

enclosures. Results from both experiments suggested that male mating harassment towards males had mixed causes: under male-biased sex ratios, male-male clasps may be the result of generalization from the search image of blue females to males, but under equal or female-biased sex ratios, male-male clasps may be mistakes in sexual recognition. The latter type was rare and did not incur short-term cost in male-female mating opportunities.

Discriminating between the sexes is one of the most fundamental tasks in an animal's life, yet such discrimination can be challenging if one sex resembles the other. In Chapter 3, I investigated how male *E. hageni* distinguish sex when the blue females mimic male coloration. I hypothesized that males should minimize confusion by using non-mimetic cues that differ between the models (i.e., males) and the mimics (i.e., the blue females). Both female morphs share an abdominal pattern that differs from the males'. In an experiment where I modified abdominal patterns of males and females of two color morphs, I found that males selectively use both color and pattern in sex recognition: they use pattern only when they encountered blue, but nor green individuals. I then derived and tested potential male decision rules for sex recognition by exposing focal males to females painted with novel orange and pink colors. Males reacted sexually to orange- and pink-painted individuals regardless of their abdominal pattern. Collectively, the results supported a male discrimination rule of 'if not blue, then female', providing insights into the origin of phenotypic novelty in color polymorphic species.

In nature, females are often the limiting sex in reproduction, giving rise to intrasexual conflicts among males over reproductive resources. In the next two chapters, in a Neotropical giant damselfly *Megaloprepus caerulaus*, whose males defend water-filled tree holes to attract mates, I investigated whether signaling can help males resolve territorial conflicts. In Chapter 4, I investigated whether males use information from UV reflectance of a male-specific, white wing band to resolve territorial contests. I staged contests between size- and age-matched, control and UV-reduced males at natural territories. Results showed that males whose UV reflectance of the white wing bands was reduced were more likely to lose contests during early dry season.

However, a challenge to the use of signaling in resolving conflicts is that contestants may signal dishonestly. In Chapter 5, I conducted a split-family rearing experiment to investigate the honesty of the male white wing band and mechanisms maintaining honesty. I proposed that honest signals should show three features of heightened condition dependence: heightened sensitivity to nutrition, steeper isometric slope, and greater variation compared to control traits. Additionally, I hypothesized that the honesty of the male white band is maintained by negative condition-dependent cost of cheating. The male white wing band indeed showed all three features of honest signals. Further, although males in poor condition had disproportionally smaller and less bright white bands, they invested a greater proportion of their total fat to develop the signal. As a result, as predicted, cost of cheating was negative condition dependent because the deduction in fat reserve in order to exaggerate the signal would represent a greater physiological cost for males in poor condition, constraining males in poor condition from cheating.

Chapter 1: Tests of the harassment-reduction function and frequency-dependent maintenance of a female-specific color polymorphism in a damselfly

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ABSTRACT

Color polymorphisms have provided classical examples of how frequency-dependent selection maintains genetic variation in natural populations. Here we tested for the first time, the hypothesized adaptive function of a female-specific color polymorphism in odonates to lower male harassment towards females generally. Under conditions controlling for sex ratio, population density and morph frequency, we also tested two major frequency-dependent selection hypotheses for the maintenance of the polymorphism. Using groups of captive *Enallagma hageni*, whose females are either green or a male-like blue, we varied morph frequency at two sex ratios. We quantified sexual harassment towards females by visual observations, and by the presence of dust on females that was transferred from dusted males. Per capita harassment rate for the female-monomorphic treatments did not differ from that of the female-polymorphic treatments. At a male biased sex ratio, per capita harassment rate towards blue, but not green females increased with morph frequency, providing partial support for frequency-dependent selection resulting from male learning of female morphs. Even at high frequency, green females were not harassed more than blue, contrary to the prediction that males should always recognize green females as mates. Moreover, frequency-dependent harassment towards blue females was not detectable using harassment measured with dust evidence, which greatly underestimated the incidence of sexual harassment. Our findings identified problems with the use of insectaries and the dusting technique to quantify male sexual harassment towards females, as well as with a past insectary

experiment on *Ischnura elegans* that failed to demonstrate frequency-dependent harassment.

INTRODUCTION

Color polymorphisms have a long history of providing evolutionary insights into how negative frequency-dependent selection maintains genetic variation in natural populations (reviewed by Allen 1988; Cook 2003; see also Gigord et al. 2001). Sex-specific color polymorphisms, in which genetically determined morphs co-exist within only one sex, is less common, particularly for females. Female-specific color polymorphisms are known from some reptiles, birds and insects, and, at least in odonates, the polymorphism has been clearly distinguished from neutral variation maintained solely by genetic drift (reviewed by Van Gossum et al. 2008). To understand why females co-exist as two or more color morphs, whereas males of the same species have only a single color type, two fundamental questions need to be answered: 1) why did the polymorphism arise, and 2) how are the morphs currently maintained. Various selective pressures that might favor the evolution of female-specific polymorphisms have been proposed: interspecific mating (Johnson 1975), sex-biased predation (Ohsaki 1995), male harassment of females (Fincke 2004), protection from solar radiation (Cooper 2010), and male mate preference (reviewed by Kunte 2009). In female-specific color polymorphic damselflies, where one female morph (heteromorph) is distinct from males in its coloration and the other morph (andromorph) is similar to the male coloration (Johnson 1975), most of the research to date has been based on the implicit but rarely stated assumption that the polymorphism evolved as an adaptive, harassment-reduction mechanism.

Different evolutionary interests of the two sexes can result in sexual conflict over mating (Batemen 1948; Parker 1979), and sexual harassment often incurs substantial costs for females (e.g., Holland and Rice 1999; Schlupp et al. 2001; Sirot and Brockmann 2001). The male harassment reduction hypothesis predicts that the polymorphism reduces overall per capita harassment towards females, and that the females of current polymorphic species experience equal or higher per capita harassment rates than those of monomorphic species (Fincke 2004). Indirect support for the latter prediction comes from the finding that across species, female monomorphic species experience lower densities than polymorphic ones (Fincke 2004; Fincke unpublished data). However, although it is fundamental to all the polymorphism maintenance hypotheses based on harassment reduction, the prediction that the polymorphism reduces overall per capita harassment towards females generally has not been tested. The ideal test of this prediction would be to compare per capita harassment rate from populations of current polymorphic species with those of their ancestral, monomorphic populations. It is expected that the harassment rate was higher in the ancestral populations, prior to the evolution of the polymorphism. But it is impossible to obtain harassment rates from ancestral populations, and the ancestral state of many current polymorphic species is unclear (Fincke et al. 2005). Thus, we expect that, using males from a current female-polymorphic species, overall per capita harassment towards females in an experimentally created female-monomorphic population should be higher than that of a female-polymorphic population.

Among the various hypotheses for the maintenance of female-specific color polymorphisms in Odonata, two that are based on harassment reduction have received the most attention. A signal detection model of the original male mimicry hypothesis (hereafter MM) by Robertson (1985) assumes that males invariably recognize the heteromorph as a potential mate (see also Johnson 1975, Hinnekint 1987), but predicts that male learning and harassment of andromorph increases as a function of the mimic-to-model ratio (i.e., blue female / male ratio, Sherratt 2001). Alternatively, the learned mate recognition hypothesis (hereafter LMR) proposes that the presence of two or more female types reduces male harassment to all female types by making it difficult for males to form a single, reliable search image. In response, males readily learn to recognize the most common morph as a potential mate, such that per capita harassment rate towards any morph increases with its frequency (Miller and Fincke 1999). Contrary to common misunderstandings (e.g., Cordero and Sánchez-Guillén 2007; Hammers and Van Gossum 2008; Ting el al. 2009), LMR acknowledges signal similarity between blue females and males by predicting different detection functions for a case with two female morphs (Fincke 2004). Indeed, LMR is consistent with harassment protection that females may gain from their similarity with males or other distractor signals, as well as from crypsis with background vegetation (Fincke et al. 2005; Fincke et al. 2007). Because both MM and LMR are detection-dependent hypotheses, inherent in both lies the assumption that male detection of potential mates, as measured by total sexual attention towards females (i.e., sexual attention that is beneficial to females plus unwanted attention), should be correlated with male harassment. However, only when a male's sexual attention is unwanted (i.e., harassment) and costly would it act as a selective pressure on females to confuse males, and thus play a role in the maintenance of the color polymorphism.

Although tests of the above hypotheses have been conducted repeatedly in various species, many suffered from one or more common problems. First, the working definition of 'harassment' varies. Researchers working with the genus *Ischnura* have often measured male harassment towards females in terms of the number of matings by females (e.g., Cordero and Sánchez-Guillén 2007), arguing that the long copulations in that genus (e.g., 180 min, Robertson 1985) are costly for females. The subsequent prediction that the male-like females should mate less often under field conditions has enjoyed considerable support (Robertson 1985; Cordero and Perez 1998; Cordero et al. 1998; Sirot et al. 2003; Cordero and Sanchez-Guillen 2007; Gosden and Svensson 2007; 2009; Hammers and Van Gossum 2008; but see Cordero 1992). However, considering matings as a measure of harassment assumes that females have little control over mating, which is unlikely because females actively resist male mating attempts (reviewed by Fincke 1997; Corbet 1999). Hence, the interpretation of the results from the above studies becomes difficult because some proportion of the matings are likely cooperative, whereas those indicative of harassment would depend on how much control females have over mating (reviewed by Kunte 2009). Moreover, differences in mating frequency could result from different reproductive strategies that are irrelevant to harassment reduction per se. For example, if, for reasons other than harassment, green females produce more egg clutches over their lifespan than do blue females, one might expect green females to mate more often than blue ones. Higher survivorship of blue morph larvae could provide the selective balance that maintains the two morphs in the population. Perhaps most importantly, using mating frequency as the measure of harassment ignores many antagonistic interactions initiated by males, such as escape flights, face-offs, interruption of foraging, and time required for a tandem separation (Xu and Fincke unpublished data; see also Robertson 1985), some of which are likely costly in terms of energy or predation risk.

A second problem in identifying meaningful patterns from data on different species and genera stems from uncontrolled variables in field populations, such as population density, operational sex ratio and frequency of solo females, which can vary even within populations, depending on where the measurements are taken (Fincke unpublished data). Compared with field studies, experiments in insectaries permit better control over the sex ratio and density of captive groups, and allow morph frequency to be experimentally manipulated away from the frequency of the field population, which may be at equilibrium (Fincke 1994). Despite this advantage, the only test to date for negative frequency-dependent harassment of polymorphic females that controlled for density, sex ratio, and morph frequency (Van Gossum et al. 2001a) used an inappropriate dependent variable (i.e., harassment per morph) in the analyses. Van Gossum et al. (2005) subsequently reanalyzed the data set using the appropriate dependent variable of per capita harassment rate, but did not present p values for the relevant regressions. As we show herein, those data failed to support negative frequency-dependent harassment. Thus, to date, the primary support for negative frequency-dependent harassment are studies demonstrating that males learn to recognize females (e.g., Miller and Fincke 1999; Van Gossum et al. 2001b; Fincke et al. 2007; Takahashi and Watanabe 2008).

Here, using captive *Enallagma hageni* in insectaries, we test the fundamental prediction that the female-specific color polymorphism reduces overall per capita harassment towards females, as well as the two major hypotheses (MM and LMR) for the maintenance of the polymorphism. We measured sexual harassment as mating attempts by males towards females that did not result in copulations, as evidenced by female refusals of males both before, and after tandem formation. In contrast, copulations were treated as minimally cooperative sexual interactions because females must raise their abdomen to the males' secondary genitalia in order to mate, and fast, coercive copulations are not a characteristic of this species (reviewed by Fincke 1997). We varied morph frequency at two sex ratios, while keeping population density high and constant. We recorded male harassment in two ways: by visual observation and by the presence of fluorescent dust on females that resulted from interactions with dusted males. Finally, we evaluated the efficiency and adequacy of fluorescent dust as a method to estimate harassment, and compared our results with comparable data from an experiment by Van Gossum et al. (2001a, 2005).

MATERIALS AND METHODS

Study species

The damselfly *Enallagma hageni* is a common species that breeds in calm areas of lakes. Males are bright blue on the thorax and the abdominal stripes whereas females are dimorphic in color. The reflectance patterns of blue females are similar to those of the UV-reflective males but not as bright and usually less saturated. In contrast, green females exhibit low reflectance in the UV and blue range, with a peak around 540 nm (Fincke et al. 2007). In 2009, the year of our experiments at the study site at Chase Osborn Preserve on Sugar Island, MI, USA (46.4°N, 84.2°W), the frequency of blue females was 33.8% (n = 1,288). The only coexisting *Enallagma* species was *E. boreale*, which emerged about two weeks before *E. hageni* and whose population declined dramatically before *E. hageni* became sexually mature. Therefore, for nearly the entire duration of the experiment, *E. hageni* was the sole *Enallagma* species at our study site.

Males search for females in the vegetation that borders breeding site, and in forest light gaps or open fields as far as 150 m away from water (Fincke 1982; 1985; Fincke unpublished data). When a male detects a potential mate, he attempts to take her in tandem by engaging his claspers (cerci) with the mesostigmal plate on her thorax. Once in tandem, a receptive female will then raise her abdomen to the male genitalia on the second segment of his abdomen to form the “wheel” position of copula (Corbet 1999). In *E. hageni*, uninterrupted copulations typically take about 20 min to complete (Fincke 1982).

Insectary conditions and damselfly collection

Six outdoor insectaries (Bioquip Inc., 1.8m × 3.6m × 1.8m) were set up adjacent to Sweet Gale Lake, a small water body contiguous with St. Marys River. All insectaries faced east and were 1 m from the water's edge. About half of the eastern portion of an insectary was covered with wet ground whereas the western portion had natural shrub and fern vegetation, along with an entrance. To block polarized light from the water and to minimize males hanging on that side of the insectary, a white cloth sheet was hung on the side facing the water. To determine the extent to which the tan netting affected light quality inside an insectary, relative irradiance (as compared to solar irradiance, which is the percentage solar radiation radiated in ambient light from a 180 degree solid angle) was measured inside the insectaries and at the shoreline outside with an OceanOptics SD2000 spectrometer (OceanOptics Inc.). Radiance measurements were taken parallel to the ground at midday with a clear sky. Temperature inside the insectaries was taken at 1000 and 1400. At those times, relative humidity was also measured 20cm above ground. Ad lib mosquitoes and small flies were released in the insectaries as food for the damselflies.

Sexually mature individuals of *E. hageni* were collected in late afternoon at the shore and the clasper morphology of males was checked with a field lens to ensure species identity. Collected individuals were put in small plasticine envelopes and stored in a refrigerator overnight. In the morning, we uniquely numbered each individual on its left forewing using an indelible marker. Males were randomly assigned to one of the five morph frequency treatments (i.e., blue morph frequency of 0, 0.2, 0.5, 0.8, or 1) at either a sex ratio of 0.5 or 2 (male:female). Population density was kept at 30 / insectary (Table 1), or 4.6 individuals / m2, similar to densities in the grassy areas where males search for females around lakes (Fincke unpublished data).

Visual observation

Observations were made between 22 June and 2 August 2009, on sunny days from 0930 to 1530. Each replicate was observed for a maximum of 6 ($\bar{x}$ = 4.23, SD = 1.46), 15-min observation periods except if the weather was temporarily overcast. Then, observations were suspended until the sun came out. The observation sequence of different treatments on the same day was randomly assigned. Each replicate was observed for one day; it took four days to complete a full set of two sex ratio, and five morph frequency treatments.

Observations were conducted by a single observer inside an insectary because we could not distinguish an individual's number or female color from outside an insectary. To minimize a possible carry-over effect of a mating that could last for more than one 15 min observation period, at the beginning of each observation period, pairs in copula were gently separated without disrupting other individuals. All intersexual interactions observed during a 15 min period were recorded by individual number, rather than the morph of a female. A male's sexual behavior was categorized as: chase (the male chases a female) and/or face off (the male and the female fly in a rotational manner while facing each other); grab (the male's legs touch the female's prothorax); tandem attempt (the male touches the thorax but fails to engage the female's mesostigmal plate with his clasper), and tandem (formation of a tandem pair). The above male behaviors form an escalating hierarchy, and only the final behavior of the interaction sequence was scored as an independent observation (e.g., if a male chased a female and subsequently clasped her in tandem, only tandem was scored). Females' responses towards males (i.e., acceptance or resistance) were recorded. Whereas a copula was considered female acceptance of a male's sexual attention, chases, grabs, and tandem attempts by males that did not proceed to copulation, as well as face-offs and pre-copulation tandem separations by females, were considered indicative of female resistance to mating. Because we define male harassment as unwanted sexual attention (sensu Joron and Barkefield 2003), all of the above actions were scored as harassment events.

Color dusting

Fluorescent dust used in pollen dispersal and mark-recapture studies of animals (e.g., Stockhouse 1976; Corbet and Rosenheim 1996) has also been used to study male harassment in natural damselfly populations (Gosden and Svensson 2007; 2009). Hence, we also used this method to quantify male harassment, and compared results with those from visual observations. Males in each replicate were randomly assigned to one of five fluorescent colors (orange, yellow, green, pink, blue). Using a grass stem, dust was gently applied to a male's penis on his second abdominal segment and to the claspers (i.e., cerci) at the end of his abdomen. Each color was used for an equal number of males. The males were dusted early in the morning before being released into the insectaries. When properly applied, the dust does not affect longevity or flight ability of insects (Naranjo 1990).

In late afternoon, after observations ended, we collected the females, transferred them to the lab in plasticine envelopes and checked them for dust under UV light, using a dissecting microscope. Any dust on a female's thorax or mesostigmal plate indicated a mating attempt; if there was the same colored dust on her abdominal tip, the interaction was scored as a copulation, otherwise, it was scored as male harassment. Although females found dead were checked for dust, some females missing at the end of a day could not be checked.

Comparison of the two methods and evaluation of fluorescent dusting

Because visual observation and dust are two independent means of estimating harassment, we tested if the two measurements are correlated. However, because visual observation detects harassment categories that cause dust transfer (i.e., tandem attempt and tandem separation) as well as those that do not (i.e., chase, face off, grab), and multiple harassments by males with the same color of dust are counted as a single harassment, we predicted that visual observations would provide a higher estimate of per capita harassment. Hence, we tested this prediction by paired-t test.

When using fluorescent dust as evidence of harassment, it is also important to know how the amount of dust on a male decreases over time and how many harassment events can occur before one can no longer detect harassment with the dust method. Hence, we dusted two focal males with the normal amount of orange or green dust on the penis and claspers, and put each in a separate insectary with ad lib females. Sexual interactions of a focal male with females were recorded by a sole observer from inside each insectary. All females that were in tandem were collected and all interactions between the focal male and females were noted. The experiment ended when the focal male did not sexually interact with any female for 30 min. Females and males were then checked for dust. Photos of the female mesostigmal plates were taken under UV light using a dissecting microscope for further examination of dust.

Data analyses

Per capita harassment rate (i.e., harassment per female/15 min) for each morph was calculated. A total of seven, 15-min observation periods were excluded from data analyses because no harassment occurred towards females. Including data from these observation periods did not change any of the conclusions. Because harassment rates were not normally distributed (Shapiro-Wilk test, $P<0.001$), the log-transformed harassment rate (Shapiro-Wilk test, $P=0.49$) was used for analysis of variance (ANOVA) and correlation analyses. Some males and females died during the day. Thus, the mean number of males and females was calculated by averaging the number of individuals alive before and after the observations of that day. 'Mean morph frequency' refers to mean number of morph females divided by mean number of total females, whereas 'designed morph frequency' refers to morph frequency at the beginning of the day (Table 1).

To test the assumption that the color polymorphism reduces male mating harassment, per capita harassment rates from polymorphic treatments (i.e., mixed morph treatments) and monomorphic treatments (i.e., all green, and all blue female treatments) were compared by analysis of covariance (ANCOVA) controlling for mean number of males. The effect of morph frequency on per capita harassment rate was examined by ANOVA with sex ratio, designed morph frequency and morph type as fixed factors. Independent-sample t tests were used post-hoc to examine the effect of sex ratio on harassment rate. Although MM predicts that harassment varies as a function of the mimic-to-model ratio, the latter is mathematically interchangeable with blue morph frequency by multiplying by the sex ratio. Therefore, we tested MM by female morph frequency at each sex ratio. Both MM and LMR predict that the frequency of blue females affects the harassment rates towards blue and green morphs, but in different ways, and hence, a significant morph by morph frequency interaction would be expected. The relationship between per capita harassment rate and morph frequency was examined using correlation and regression analyses. Mean female morph frequencies were used as independent variables to control for the effect of female mortality. MM predicts that per capita harassment rate of the green morph does not vary with green morph frequency, but per capita harassment rate of the blue morph increases with blue morph frequency. It also predicts that per capita harassment rate of the green morph should be higher than that of the blue morph when the green morph is the majority. In contrast, LMR predicts per capita harassment towards both blue and green females to be positively frequency dependent, although not necessarily equally so. Finally, to test whether monomorphism makes it easier for males to locate females and distinguish their sex, and whether total male attention is frequency-dependent as expected, we repeated the above analyses using total male sexual attention (i.e., harassment plus tandems and copulas) as the variable of interest. We chose a significance level (α) of 0.05 for all statistical tests, and performed all analyses in SPSS Statistics 18.0 (SPSS Inc.).

RESULTS

Physical conditions in the insectaries, mortality, mean sex ratio and morph frequency

The mean temperature inside the insectaries during the experiment was 26.8°C (SD = 3.4). Relative humidity ranged from 34% to 50%. Compared to ambient light spectrum at shoreline outside the insectaries, the relative radiance inside the insectaries was decreased by about 17% overall. The decrease in the radiance was reduced at long wavelengths, causing the spectrum inside the insectaries to be slightly red-biased. Noticeably, the relative radiance decreased slightly more at around 460 nm and 550 nm, which are in the blue and green range respectively (Fig. 1). However, since differences were minor, we assumed that spectral conditions inside the insectaries did not bias a male's ability to detect morph colors.

Females had a higher mortality rate (proportion that died / day) than males (♀: $\bar{x} = 0.18$, SD = 0.12, ♂: $\bar{x} = 0.09$, SD = 0.08; Fisher's exact test, p = 0.002), suggesting that the actual sex ratios were more male biased towards the end of the day. The mortality rates for the two female morphs did not differ (blue: $\bar{x} = 0.17$, SD = 0.13, green: $\bar{x} = 0.17$, SD = 0.15; Fisher's exact test, p = 0.90). The mean sex ratio (mean number of males / mean number of females) for the experimentally designed sex ratio of 0.5 was 0.65 (SD = 0.16), and for designed sex ratio of 2 was 2.13 (SD = 0.26). Mean morph frequencies deviated slightly from the designed morph frequencies (Table 1).

Harassment rate of monomorphic and polymorphic groups

For monomorphic treatments (i.e., all green and all blue female treatments), per capita harassment per 15 min period as measured by observation ($\bar{x} = 0.50$, SD = 0.30) was not significantly higher than that of pooled polymorphic treatments ($\bar{x} = 0.43$, SD = 0.24; $F_{1,24} = 1.06$, p = 0.31) after controlling for the mean number of males. Nor did per capita harassment rate in the monomorphic blue treatment ($\bar{x} = 0.50$, SD = 0.32) differ from that in the monomorphic green treatment ($\bar{x} = 0.50$, SD = 0.31, t = 0.001, df = 13, p = 1.00). Within each sex ratio, per capita harassment rates did not differ at sex ratio of 0.5 (monomorphic: $\bar{x} = 0.29$, SD = 0.15, polymorphic: $\bar{x} = 0.30$, SD = 0.15, t = 0.19, df = 11, p = 0.85), nor at sex ratio of 2 (Fig 2, monomorphic: $\bar{x} = 0.69$, SD = 0.28, polymorphic: $\bar{x} = 0.54$, SD = 0.26, t = 1.04, df = 13, p = 0.32).

Harassment by observation

Per capita harassment rate increased with sex ratio (sex ratio of 0.5: $\bar{x} = 0.29$, SD = 0.14, sex ratio of 2: $\bar{x} = 0.62$, SD = 0.27; t = 4.05, df = 22.0, p = 0.001). As shown in Table 2, there was no significant morph or morph frequency effect on per capita harassment rate, nor a significant morph by frequency interaction. However, in support of both MM and LMR, using only the polymorphic groups, per capita harassment rate of blue females increased with the mean frequency of the blue morph at sex ratio of 2 (r = 0.83, N = 7, p = 0.02). A linear regression revealed similar results: per capita harassment rate of blue females increased with mean blue morph frequency at sex ratio of 2 (LogHar = -0.63 + 0.66B%; $r^2 = 0.69$, $F_{1,5} = 11.07$, p = 0.02), but not at sex ratio of 0.5 ($r^2 = 0.38$, $F_{1,4} = 2.40$, p = 0.20, Fig 3a). However, including the monomorphic treatments would make the correlation non-significant. Contrary to predictions of LMR, per capita harassment rate of green females did not vary with mean green morph frequency at either sex ratio (Fig 3b, sex ratio of 0.5: $r^2 = 0.13$, $F_{1,4} = 0.58$, p = 0.49; sex ratio of 2: $r^2 = 0.04$, $F_{1,5} = 0.20$, p = 0.67). Furthermore, contrary to predictions of MM, even when the blue morph was the minority (i.e., blue morph frequency = 0.2), per capita harassment rate towards blue females was not lower than it was towards green females (Fig 3b, blue: $\bar{x}$ =

0.44, SD = 0.14, green: $\bar{x}$ = 0.39, SD = 0.17, t = 0.48, df = 6, p = 0.65). Total male sexual attention and observed harassment were highly correlated for both blue females (r = 0.91, n = 20, $p < 0.001$) and green females (r = 0.93, n = 21, $p < 0.001$); testing frequency dependence using male sexual attention as the dependent variable did not change any of our conclusions based on male harassment.

Female resistance after tandem formation

We frequently observed tandem females actively resisting a male's mating attempt. A female could separate from the tandem position by shaking her body at a high frequency along the right-left axis, while crawling to a position where the male's abdomen formed a wide angle with the female's thorax, and in some cases, causing him to turn upside down. Of a total of 19 such cases for which the outcome was known, 18 pairs (95%) separated before copula. Tandem females could also resist by holding firmly on vegetation, preventing males from lifting them and flying elsewhere. In nine such cases, five pairs separated before mating. Finally, of four females that kept their abdomens stiff while males were lifting them into the "wheel" position of copula, two separated prior to copula.

Male activity throughout a day

Independent of treatment, multiple observations in the same insectary revealed that male activity level fluctuated throughout the day. There were unpredictable activity 'peaks' when males were observed frequently interacting with females; between the activity peaks there were usually ‘valleys’ when males exhibited little or no activity (e.g., Fig. 4). The numbers of harassment peaks towards the blue morph ($\overline{x}$ = 1.40, SD = 0.60) and the green morph ($\overline{x}$ = 1.38, SD = 0.59) did not differ significantly (t = 0.10, df = 39, p = 0.92).

Harassment as scored by fluorescent dust

Using harassment as scored by dust evidence (Table 1), ANOVA did not reveal any significant main effect of sex ratio, morph, morph frequency, or their interactions (Table 3). There was no correlation between log-transformed per capita harassment rate and mean morph frequency when data from different sex ratios were pooled (blue: r = 0.31, n = 13, p = 0.30, green: r = -0.31, n = 12, p = 0.32) or when analyzed by sex ratio (Fig 5, sex ratio of 0.5: blue: r = 0.50, n = 6, p = 0.31, green: r = -0.27, n = 6, p = 0.61; sex ratio of 2: blue: r = 0.16, n = 7, p = 0.74, green: r = -0.39, n = 6, p = 0.45).

Using data from dust evidence, male sexual attention was not correlated with harassment for either the blue morph (r = 0.41, n = 20, p = 0.08) or the green morph (r = 0.21, n = 21, p = 0.35). Analyzing data by log-transformed male sexual attention using the same model as in Table 3 revealed a significant sex ratio effect ($F_{1,25}$ = 9.10, p = 0.01), a morph type effect ($F_{1,25}$ = 5.57, p = 0.03), and an interaction of morph type, morph frequency and sex ratio ($F_{2,25}$ = 3.71, p = 0.04), while the main effect of morph frequency and all other interactions remained non-significant. Nor were any of the regressions between log-transformed male sexual attention and morph frequency significant.

Comparison of harassment measures

Fluorescent dust and visual observations provided two independent estimates of sexual harassment by males, but in the time units of a day and 15 min, respectively. If the two measurements are unbiased, they should give similar estimates of harassment rate after correcting for the time interval. But even if we conservatively assume that sexual interactions occurred over only 2 hours each day (i.e., 8 observation periods of 15 min), visual observations still captured a much higher harassment rate ($\bar{x}$ = 3.76, SD = 2.17) than did fluorescent dust ($\bar{x}$ = 0.64, SD = 0.27, paired t-test, t = 2.38, df = 27, p =0.03). Chases, face offs, and grabs, which were events not detected from dust, made up 30% of total harassment observed. Harassment rates from fluorescent dust and visual observation were correlated at sex ratio of 0.5 (r = 0.71, n = 13, p = 0.01) but not at sex ratio of 2 (r = 0.10, n = 15, p = 0.72). The per capita harassment rate from visual observation for the two categories that transfer dust (i.e., tandem attempt and tandem separation) was correlated with harassment rate from dust at sex ratio of 0.5 (r = 0.76, n = 13, p = 0.002), but not at sex ratio of 2 (r = 0.08, n = 15, p = 0.78). Of the total 172 females that were not scored from dust as being harassed, 88 (51.2%) were observed to have been harassed; of the 163 females that were not observed to be harassed, 51 (31.3%) had dust evidence of harassment. Including the harassment scored by dust of these 51 females with the observation data did not change the major conclusions: per capita harassment rate of blue females increased with mean blue morph frequency at sex ratio of 2 ($r^2 = 0.59$, $F_{1,5} = 7.13$, $p = 0.04$), but not at sex ratio of 0.5 ($r^2 = 0.42$, $F_{1,4} = 2.89$, $p = 0.16$), and per capita harassment rate of green females did not increase with mean green morph frequency at either sex ratio of 0.5 ($r^2 = 0.07$, $F_{1,4} = 0.29$, $p = 0.61$) or sex ratio of 2 ($r^2 = 0.04$, $F_{1,5} = 0.23$, $p = 0.65$).

Because harassment scored by dust is only a proportion of the total harassment, it is important to understand whether this proportion differed among the two morphs, five morph frequencies and two sex ratios. For example, if any difference in the cuticle of the two female morphs makes the dust stick to one morph more easily, dust may capture a higher proportion of harassment for one morph, invalidating a comparison between the harassment rates of the two morphs. Likewise, because dust can record a maximum of only five harassment events per female (i.e., five different colors of dust), if the actual per capita harassment exceeds five and differs significantly between treatments, then dust would be an inappropriate method to use. Hence, we calculated the proportion of the total observed harassment that was scored by dust. This proportion could be larger than 1 because harassment rate from dust reflects harassment per day, but harassment rate from visual observation reflects harassment per observation period of 15 min. If the measurement of harassment by dust is unbiased, we expect that this proportion will be the same among two morphs, five morph frequencies and two sex ratios. ANCOVA with the proportion of harassment scored by dust as the dependent variable and mean number of males as a covariate revealed no significant morph frequency effect ($F_{4,21} = 0.73$, $p = 0.58$), indicating that the dusting method was not biased towards any morph frequency. Similarly, controlling for the mean number of males, the proportion of harassment scored by dust did not differ between two female morphs ($F_{1,37} = 1.22$, $p = 0.28$). However, this proportion was higher at sex ratio of 0.5 ($\bar{x} = 2.72$, SD = 0.89) than at sex ratio of 2 ($\bar{x} = 1.04$, SD = 0.70; $t = 5.60$, df = 26, $p < 0.001$), indicating that dust was an unreliable method of measuring harassment at the male-biased sex ratio.

Males with dust on their claspers were observed grooming their abdomen tips, which should decrease the amount of dust on the claspers. The two males enclosed with ad lib females formed 8 and 9 tandem pairs respectively within 3 hours. Photos revealed that the first 5 females had dust on most of their thorax and part of head, but the dust was considerably less on later tandems. After a male took 7 females in tandem, the dust transferred to females was restricted to a small area around the mesostigmal plate and most dust grains were attached to the hairs instead of cuticle. For the ninth female that was in tandem with the male, the dust consisted of only a few grains, which were difficult to detect under a dissecting microscope (Fig 6).

DISCUSSION

Our study tested for the first time, the hypothesis that the adaptive function of female-specific color polymorphism in damselflies is harassment reduction. At a male-biased sex ratio, per capita harassment rates in experimentally created, female-monomorphic groups were not significantly higher than that of female-polymorphic groups, although the trend was in the expected direction. Our results contradicted field data suggesting that polymorphic species experience significantly higher population densities than monomorphic ones, and presumably, greater sexual harassment (Fincke 2004; Fincke unpublished data).

At a male-biased sex ratio, per capita harassment rate towards blue females increased with their frequency, offering for the first time, direct support for a frequency-dependent mechanism that could maintain the polymorphism. Because the males in our experiments were collected from a natural population where the green morph was the majority, our result suggested that males learned to recognize blue females, and, importantly, did so within the time frame of a single day.

Nevertheless, support for a frequency-dependent mechanism was only partial, as our results were not completely consistent with either of the two major hypotheses (i.e., MM and LMR) for polymorphism maintenance. Per capita harassment rate towards the green females did not increase with green morph frequency, consistent with MM but failing to support LMR. But even when blue morph frequency was 20%, per capita harassment rate towards green females was not higher than that towards blue females, contrary to the MM's assumption that males always recognize the heteromorph as potential mates. Although the conclusions of frequency-dependent harassment might seem dubious, given that about a third of the females that were not observed to be harassed had dust evidence of harassment, the latter is not surprising because visual observations were only intermittent samples of at most, a total of 150 min, whereas dust recorded harassment for the entire study day. More importantly, including harassment scored by dust of these females did not change any of our conclusions based on visual observations alone.

At sex ratio of 0.5, per capita harassment rate did not vary with morph frequency for either morph. Because there were twice as many females at sex ratio of 0.5 than at sex ratio of 2, it should be easier for males to form search images, but because of low male-male competition to acquire a mate, there may be no need for males to quickly recognize potential mates. Indeed, when there is more time available, males may use additional cues, such as the dorsal pattern of a female *Enallagma*'s abdomen, to distinguish sex (e.g. Miller and Fincke 1999; Xu unpublished data). Hence, weak selective pressure for forming a search image and the possibility of using additional cues at low sex ratio, may account for the lack of correlation between per capita harassment rate and morph frequency at female-biased sex ratios.

Across treatments, fluorescent dust recorded, on average, less than one harassment event per female per day, which is low compared to the harassment rates recorded by visual observations. The cost of harassment to females at the low rate detected by dust, if it were real, is likely to be very small. More importantly, at sex ratio of 2, the only treatment at which we detected frequency-dependent harassment by visual observation, the dust data failed to detect this effect. The experiment with a single dusted male enclosed with ad lib females indicated that after 8 interactions with a female, a dusted male no longer left detectable evidence of his tandem attempts. Dust also underestimated contacts made repeatedly by the same individual, or by different individuals with the same color of dust. Furthermore, given the limited number of distinguishable dust colors available for use, the maximum per capita harassment one could score was five. However, fluorescent orange and pink, as well as yellow and green were difficult to distinguish when they co-existed, and one color was represented by only a few grains. Hence, the effective maximum number of detectable harassment events towards a single female was likely even lower than five, a fairly low ceiling effect. These constraints, coupled with the insufficiency of dust to score harassment events other than clasping attempts and tandem separations, raise questions about the effects of male sexual attention on female fecundity drawn from recent field studies using only evidence from dusting to detect harassment (Gosden and Svensson 2007; 2009).

Our data suffered from high sample variance that likely arose from four major sources. First, the fluctuation of male activity level during the course of the day may induce inconsistency because a group could have been observed when male activity was at its peak, at the valley or anywhere between a peak and a valley. Second, even though mortality was equal for the two female morphs, individuals may have died at different times of day, causing actual morph frequency to oscillate throughout time. Third, because most observed interactions were against the screen, any harassment protection that green females may gain from crypsis against background vegetation was likely reduced. Finally, even in an insectary, a male's actual encounter rate with females of each morph may deviate from morph frequency depending on individual experience; hence, although males may indeed learn to recognize females, that learning may not translate into negative frequency-dependent harassment in insectaries or in the field.

Population models using data from *Ischnura elegans* have indicated that female color morphs in damselflies can be maintained by negative frequency-dependent selection (Svensson et al. 2005). Although those authors concluded that female fecundity was negatively frequency-dependent, the evidence presented was indirect, and no proximate mechanism (e.g., frequency-dependent male harassment) was identified for the reported frequency-dependent fecundity. Hence their arguments rely primarily on support from the insectary experiment of Van Gossum et al. (2001a), coupled with evidence for male learning (e.g., Miller and Fincke 1999; Van Gossum et al. 2001b; Fincke 2007). In contrast with our current results, Van Gossum et al. (2001a), using visual observation of harassment, found a significant morph by morph frequency interaction, which supported LMR in I. elegans. Unfortunately, in addition to various problems in the experimental design, an inappropriate variable, harassment rate per morph, rather than harassment rate per female (i.e., per capita rate), was used in their mixed model regression (Table 2, Van Gossum et al. 2001a). The null model would predict that harassment towards a given morph will increase with morph frequency because as frequency increases, the number of females of a given morph available for harassment increases. Hence, a morph by morph frequency interaction could simply be an effect of the number of females present. Indeed, using Van Gossum et al.'s original data, we found that female number had a significant effect of on harassment rate per morph (F5,10 =7.60, p = 0.003). Reanalysis of their data using the same model as in our Table 2 revealed higher per capita harassment at the higher sex ratio, but no significant relationship between morph frequency and per capita harassment for blue (LogHar = 0.02 + 0.22B%; r^2 = 0.07, $F_{1,6}$ = 0.42, p = 0.54) or for green females (LogHar = 0.11 + 0.10G%; r^2 = 0.04, $F_{1,6}$ = 0.26, p = 0.63, Fig. 7) or a significant interaction. Van Gossum et al. (2005) later reanalyzed their original data using per capita harassment rate. They found that mating attempts increased with increasing andromorph to gynomorph (i.e., heteromorph) ratio for the andromorph, but decreased for the gynomorph, but unfortunately, provided no statistics. When we repeated the same analyses using their data, we found that the regression was not significant for either the andromorph [log10 (mating attempts) = 0.010 + 0.052 A/G ratio, r = 0.33, $F_{1,22}$ = 2.62, p = 0.12] or the gynomorph [log10 (mating attempts) = 0.180 - 0.017 A/G ratio, r = 0.17, $F_{1,22}$ = 0.65, p = 0.43]. Therefore, that earlier insectary study actually failed to demonstrate frequency-dependent harassment, and hence supported neither LMR nor MM, despite its frequent citation as evidence for frequency-dependent selection (e.g., Sirot et al. 2003; Fincke 2004; Svensson et al. 2005; Bleay et al. 2007; Fincke et al. 2007; Gosden and Svensson 2007, 2009; Ting et al. 2009; Takahashi and Watanabe 2010).

Although our results here failed to support the harassment-reduction hypothesis, alternative functions for the female-specific color polymorphism seem equally unpromising for both *Enallagma* and *Ischnura*. Across species in both genera, the two female morphs do not seem to differ in their microhabitat use, survivorship, or fecundity (reviewed in Fincke 2004; Fincke unpublished data), with evidence for differential predation absent or at best, weak (e.g., Van Gossum et al. 2004). Similarly, female morphs have not been shown to differ in parasite and pathogen resistance (Joop et al. 2006), or thermoregulation (Bots et al. 2008). Recent work indicated that solar radiation protection may be a relevant function of the color polymorphism in a stream-dwelling species (Cooper 2010), but because those males are territorial, solo females are less likely to be harassed, unlike those of mate-searching coenagrionids. We lack information on potential morph differences at the larval stage (Cordero 1992), as well as knowledge of the relative costs of male harassment and female resistance, and hence, whether convenience polyandry (see Arnqvist and Rowe 2005) is relevant. Thus, perhaps we should not be surprised that after thirty-five years of research since Johnson (1975) proposed the first hypothesis for the maintenance of female-specific polymorphisms in odonates, we are still searching for evidence that builds a parsimonious explanatory story, either within or across genera of species whose males search for mates.

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Table 1. Experimental design and per capita harassment rates of female morphs as measured by visual observation and by fluorescent dust

DBF	MBF (mean ± SD)	Sex ratio	Density	n	HB_{OBS}/15min (mean ± SD)	HG_{OBS}/15min (mean ± SD)	HB_{DUST}/day (mean ±SD)
0	0±0	0.5	30	4	-	0.24 ± 0.09	-
		2	30	4	-	0.76 ± 0.17	-
0.2	0.21±0.06	0.5	30	2	0.49 ± 0.21	0.25 ± 0.07	0.50 ± 0.00
		2	30	2	0.39 ± 0.08	0.52 ± 0.08	0.75 ± 0.35
0.5	0.52±0.06	0.5	30	2	0.39 ± 0.27	0.43 ± 0.25	0.59 ± 0.02
		2	30	3	0.46 ± 0.14	0.35 ± 0.32	0.62 ± 0.13
0.8	0.77±0.04	0.5	30	2	0.23 ± 0.004	0.17 ± 0.16	0.75 ± 0.35
		2	30	2	0.82 ± 0.29	0.71 ± 0.88	0.77 ± 0.08
1	1±0	0.5	30	3	0.35 ± 0.21	-	0.96 ± 0.29
		2	30	4	0.61 ± 0.36	-	0.57 ± 0.31

DBF: designed blue morph frequency; MBF: mean blue morph frequency (see Methods); HB_{OBS}/15 min, HG_{OBS}/15 min: harassment per female/15 min for blue or green females, respectively, by visual observation; HB_{DUST}/day, HG_{DUST}/day: harassment per female/day

Table 2. Analysis of variance using log-transformed harassment rate from visual observation as dependent variable, sex ratio (SR), morph and designed blue frequency (DBF) as fixed effects

Source	F	*df*	*P*
SR	7.23	1,25	0.01
Morph	1.88	1,25	0.18
DBF	0.53	4,25	0.72
Morph*DBF	0.42	2,25	0.66
Morph*SR	0.00	1,25	0.99
DBF*SR	1.29	4,25	0.30
MOR*DBF*SR	0.84	2,25	0.44

Table 3. Analysis of variance using log-transformed harassment rate scored by dust as dependent variable, sex ratio (SR), morph and designed blue frequency (DBF) as fixed effects

Source	F	*df*	*P*
SR	3.23	1,24	0.09
Morph	0.33	1,24	0.57
DBF	1.91	4,24	0.14
Morph*DBF	0.13	2,24	0.88
Morph*SR	3.11	1,24	0.09
DBF*SR	0.84	4,24	0.51
MOR*DBF*SR	0.50	2,24	0.61

FIGURE LEGENDS

Figure 1. Relative ambient radiance at shoreline outside the insectaries (open circles), and inside the insectaries (triangles)

Figure 2. Mean per capita harassment rate ± standard error for females. Mono: monomorphic treatments. Poly: polymorphic treatments. Sample sizes are above error bars

Figure 3. Per capita harassment rates from visual observations, towards **a)** blue females as a function of mean blue morph frequency and **b)** green females as a function of mean green morph frequency. Open circles indicate sex ratio of 0.5, triangles indicate sex ratio of 2 (males : females)

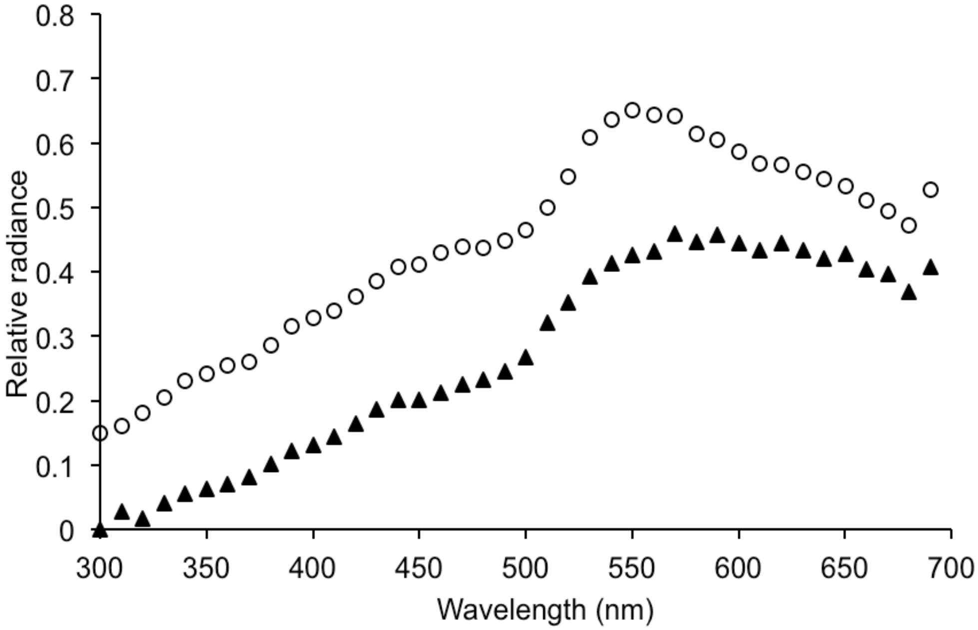
Figure 4. Examples of variation in male activity across observational periods on a given day (observational data): **a)** a replicate of 20% blue females at sex ratio of 2; **b)** a replicate of 80% blue females at sex ratio of 2

Figure 5. Per capita harassment rates scored by fluorescent dust, towards **a)** blue females as a function of mean blue morph frequency and **b)** green females as a function of mean green morph frequency. Open circles indicate sex ratio of 0.5, triangles indicate sex ratio of 2

Figure 6. Photos of females with dust on their thoraxes under dissection microscope and UV light. **a)** the first female to be taken in tandem by a focal male with green dust on his claspers; **b)** the fifth female in tandem with the same male; **c)** the eighth female in tandem with the same male; **d)** the ninth female to be taken in tandem by a male with orange dust on claspers

Figure 7. Mean per capita harassment rate over three days from Van Gossum et al. (2001a) towards **a)** blue females as a function of mean blue morph frequency and **b)** green females as a function of mean green morph frequency. One group with sex ratio of 0.6 (insectary 5) was included in the sex ratio of 0.5 treatment. Open circles indicate sex ratio of 0.5, open squares indicate sex ratio of 1, closed triangles indicate sex ratio of 2

Figure 1



Relative radiance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
300
350
400
450
500
550
600
650
700
Wavelength (nm)

Figure 2

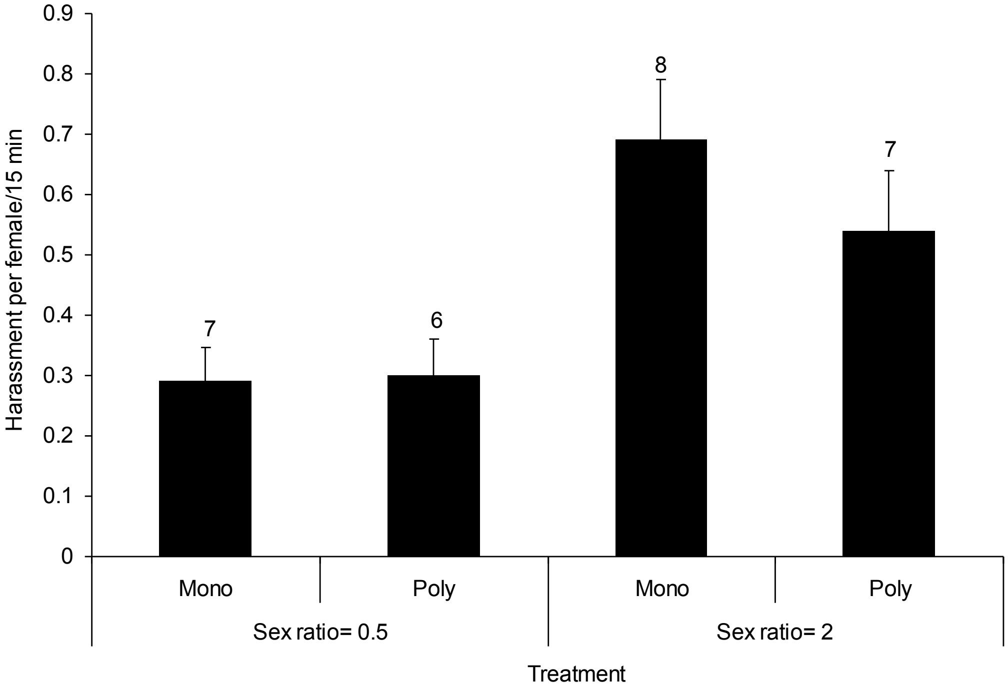

Harassment per female/15 min
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
7
6
8
7
Mono
Poly
Mono
Poly
Sex ratio= 0.5
Sex ratio= 2
Treatment

Figure 3a

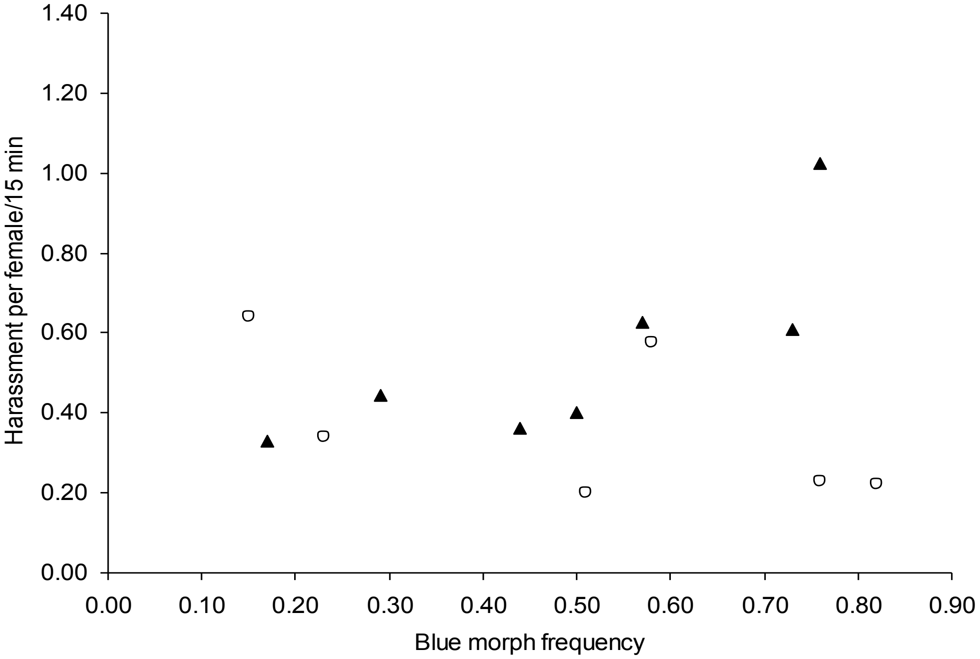

1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Harassment per female/15 min
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
Blue morph frequency

Figure 3b

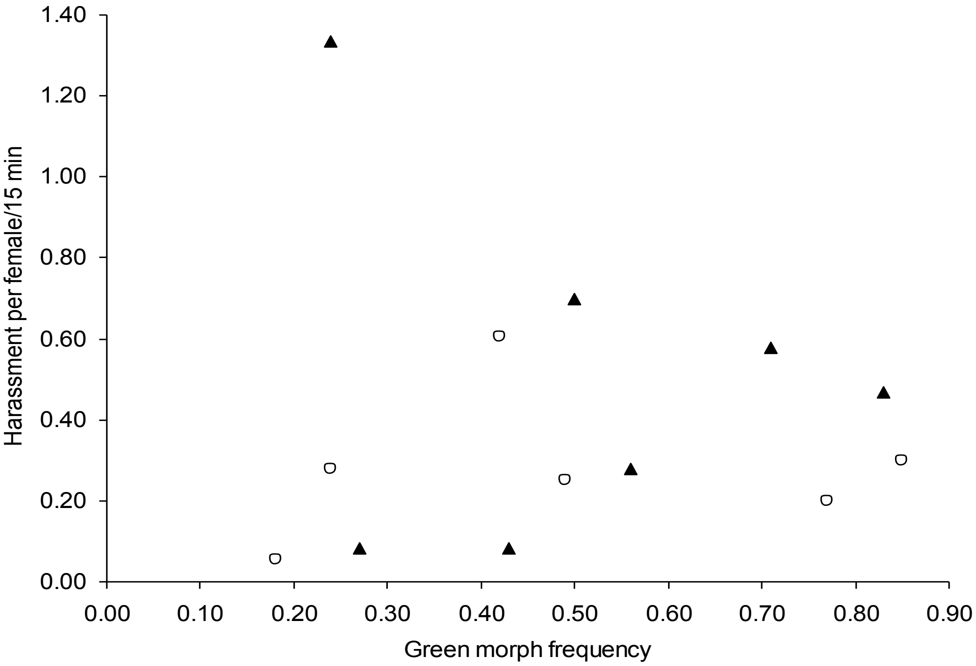

1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Harassment per female/15 min
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
Green morph frequency

Figure 4a

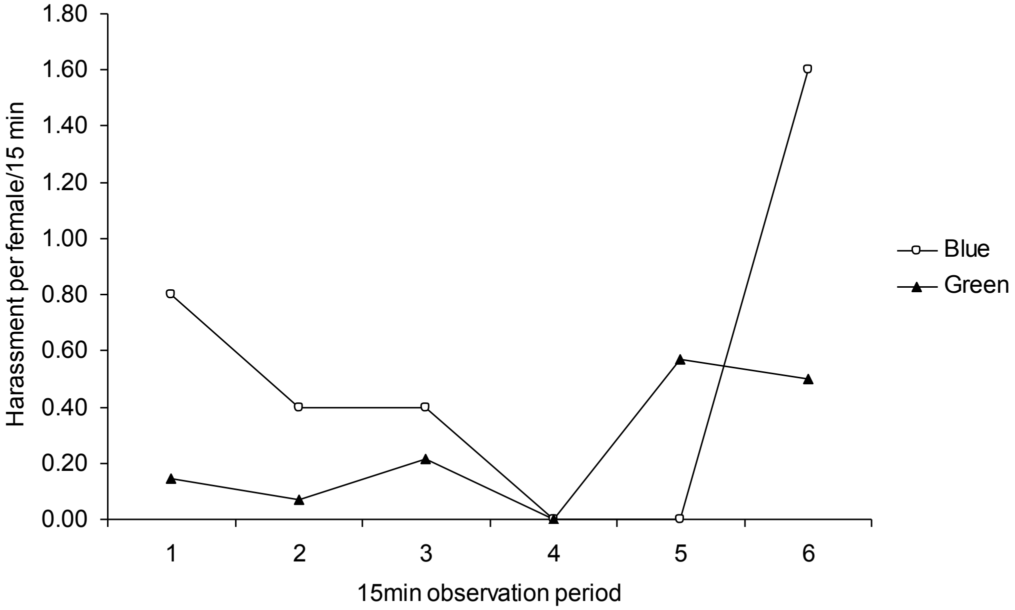
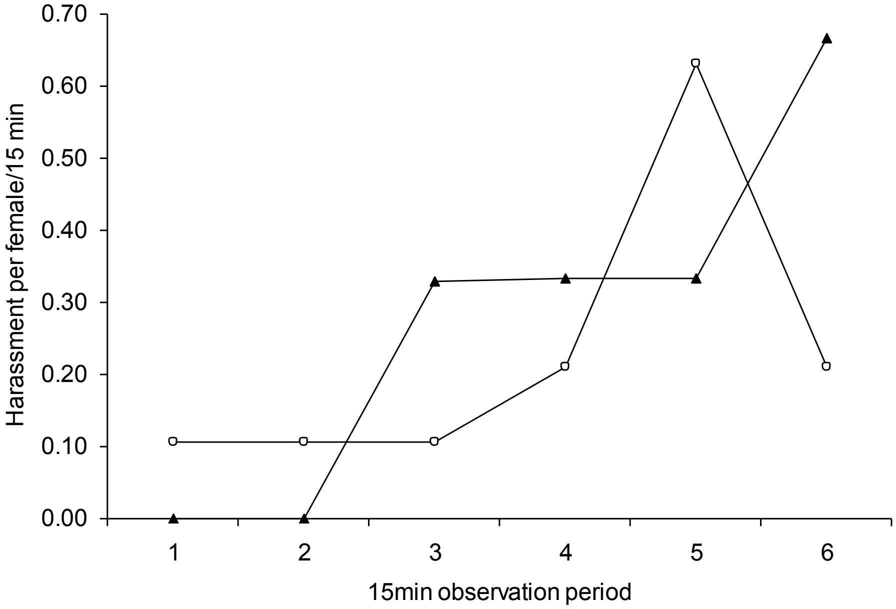

Harassment per female/15 min
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1
2
3
4
5
6
15min observation period
Blue
Green

Figure 4b



0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Harassment per female/15 min
1
2
3
4
5
6
15min observation period

Figure 5a

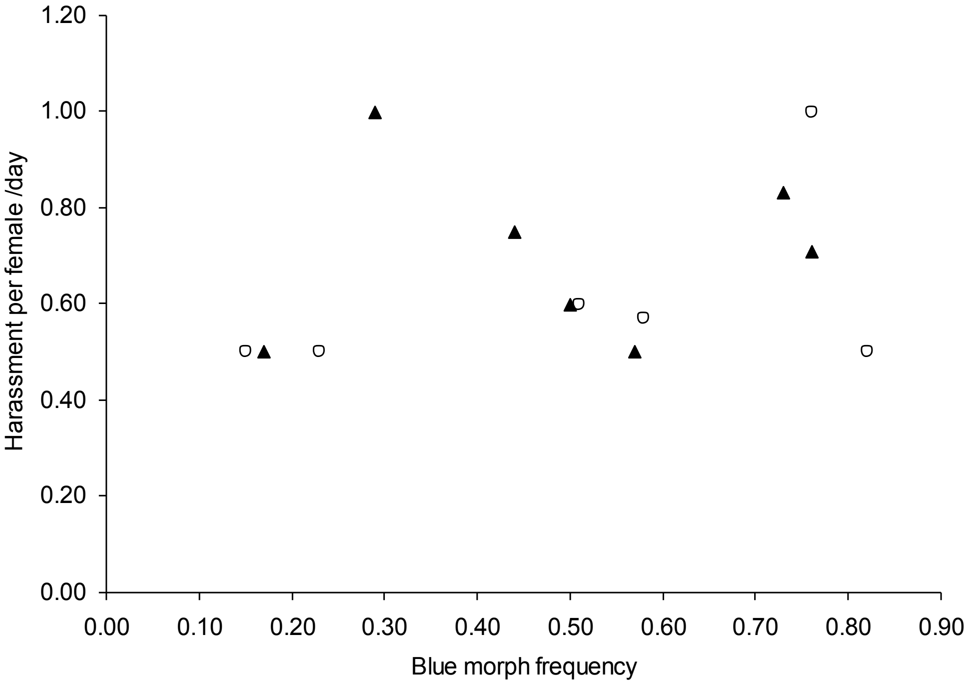

1.20
1.00
0.80
0.60
0.40
0.20
0.00
Harassment per female /day
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
Blue morph frequency

Figure 5b

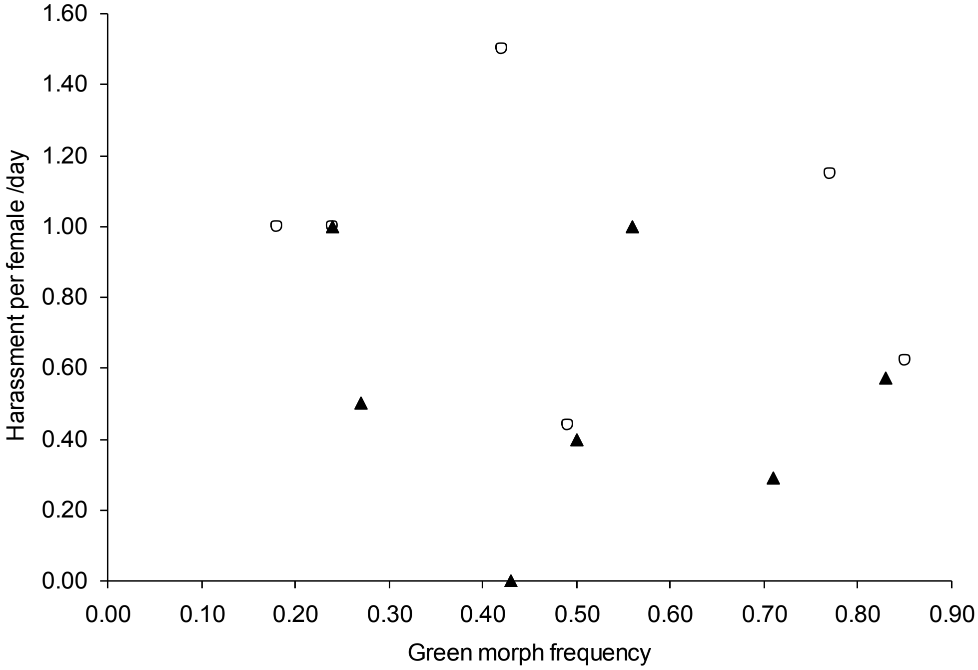

1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Harassment per female /day
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
Green morph frequency

Figure 6

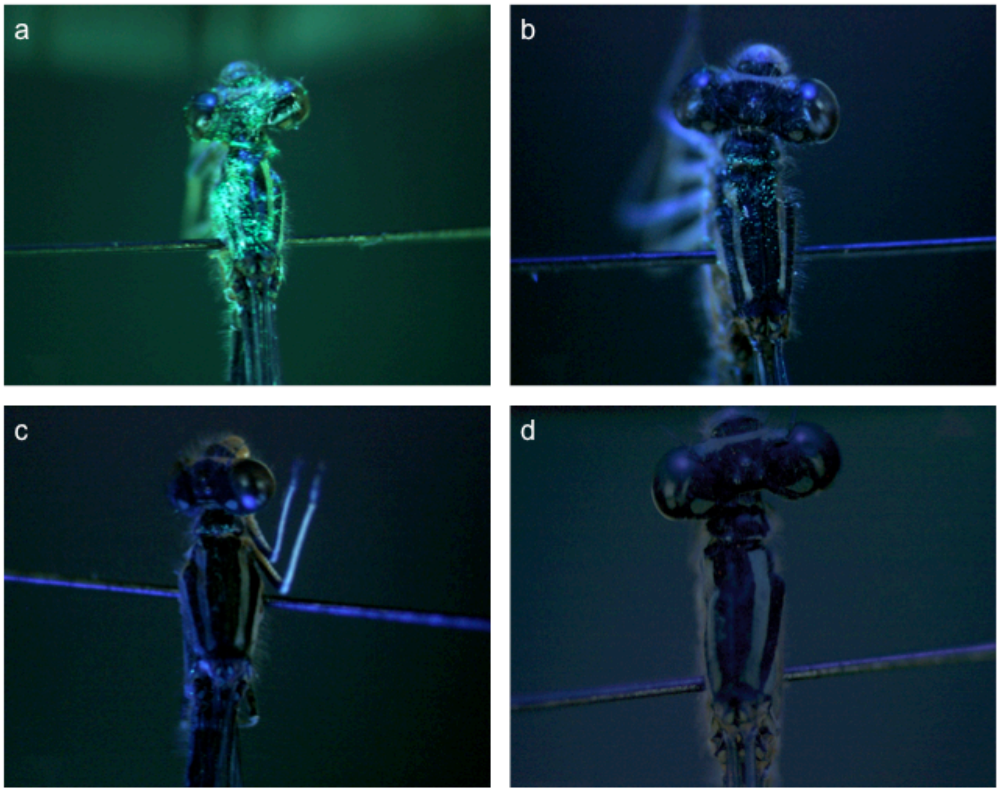


Figure 7a

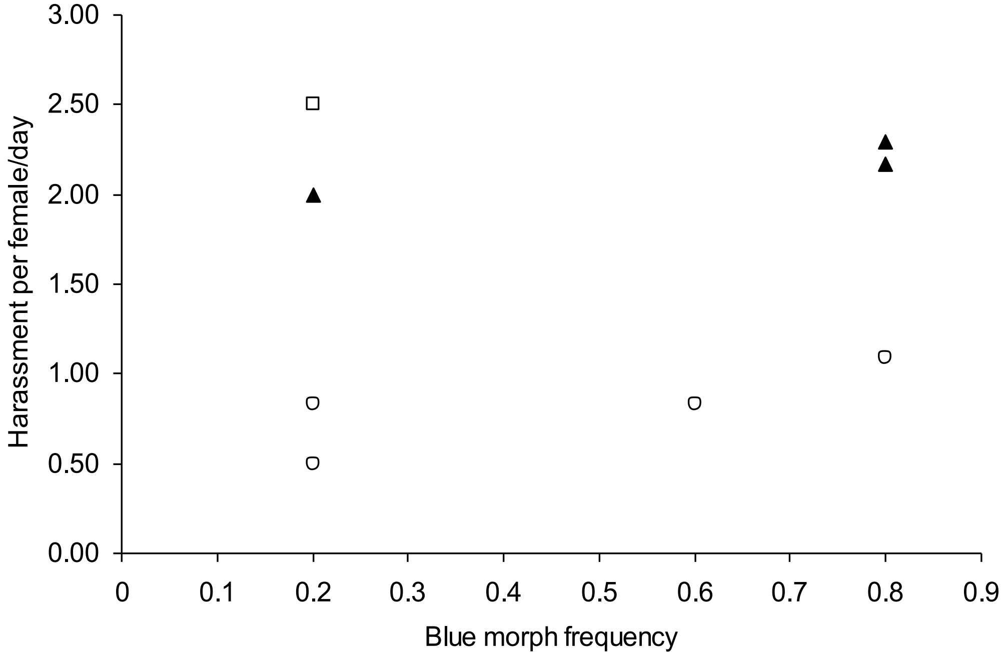
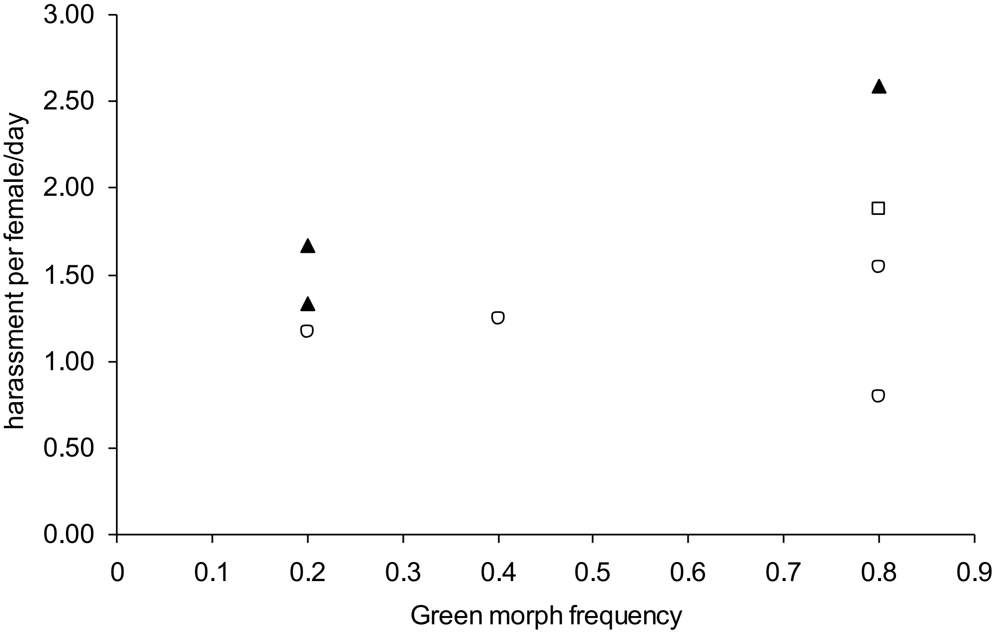

3.00
2.50
2.00
1.50
1.00
0.50
0.00
Harassment per female/day
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Blue morph frequency

Figure 7b


3.00
2.50
2.00
1.50
1.00
0.50
0.00
harassment per female/day
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Green morph frequency

Chapter 2: Male-male harassment has mixed causes and no mating opportunity cost in a sexually mimetic, female-polymorphic damselfly

[At the time of dissertation submission, this chapter was in review at a journal as Xu, M. Male-male harassment has mixed causes and no mating opportunity cost in a sexually mimetic, female-polymorphic damselfly]

ABSTRACT

Male mating harassment towards males is not uncommon, yet it is puzzling why such behavior persists in nature because it is unlikely to result in offspring. In a female-specific color polymorphic damselfly *Enallagma hageni*, males are all blue but females can be either male-like blue (the andromorph) or green (the heteromorph). I tested three hypotheses on the causes of male-male harassment and investigated the effect of male-male harassment on male-female mating opportunity in two experiments in outdoor enclosures. I measured male mating harassment towards other males as male-male clasps. Results from both experiments suggested that male mating harassment towards males had mixed causes: under male-biased sex ratios, male-male clasps may be the result of generalization from the search image of andromorphic females to males, but under equal or female-biased sex ratios, male-male clasps may be mistakes in sexual recognition. The latter type was rare and did not correlate with male-female mating opportunities, measured as male-female clasps. This result failed to support the hypothesis that male-male harassment is 'spillover' of high mating motivation from the heterosexual context to the homosexual context. This study provided insights into the connection between the causes of male-male harassment and male-male competition level, which in turn, can be influenced by the operational sex ratio or local male density.

INTRODUCTION

In nature, the relationship between the two sexes is often far from harmonious. Differences in potential reproductive rate between the two sexes often result in male mating harassment towards females (Bateman 1948; Parker 1979), famously illustrated by examples including water striders (reviewed by Rowe et al. 1994), waterfowl (McKinney et al. 1983; Brennan 2010), and elephant seals (Galimberti et al. 2000). In these and other cases (Holland and Rice 1999; Schlupp et al. 2001; Sirot and Brockmann 2001), male mating harassment towards females incurs costs for females. While much of the interest has been focused on male harassment towards females, male mating harassment towards males has been largely understudied, probably because they were considered rare, and hence, play a far less important role in sexual conflict.

However, recently, reports started to accumulate that male mating harassment towards other conspecific males are not uncommon. For example, male-male courtships has been observed among fruit flies *Drosophila melanogaster* (Dukas 2010) and *D. affinis* (McRobert and Tompkins 1988), hissing cockroach *Gromphadorhina portentosa* (Logue et al. 2009), parasitic wasp *Psyttalia concolor* (Benelli and Canale 2012), guppies *Poecilia reticulata* (Field and Waite 2004), a social parrot *Melopsittacus undulatus* (Abbassi and Burley 2012) and a house finch (McGraw and Hill 1999). Male-male pairings and copulations have been reported among numerous beetles (Serrano et al. 1991, Harari et al. 2000, Switzer et al. 2004, Levan et al. 2009, Stojkovic et al. 2010). Unlike male harassment towards females, male harassment towards other males are not likely to result in offspring, hence, the prevalence and persistence of these behaviors in nature represent an evolutionary puzzle (Bailey and Zuk 2009).

To solve this puzzle, we need to answer three basic questions: how often do male-male mating harassment occur; what are the causes; and what effects do they have on male mating success. Although we have substantial understanding on the above issues in social vertebrates and especially, primates (Chevalier-Skolnikoff 1976; Oi 1990; Abbassi and Burley 2012; Bierbach et al. 2013; also reviewed by Vasey 1995), relatively less is known among invertebrates, and even within invertebrates, our understanding seems to derive from a taxonomically biased survey that primarily included beetles.

To date, there are two major hypotheses for the cause of male-male mating harassment among invertebrates. First, accumulating evidence suggested that many male harassment towards other males are random mistakes in male sex discrimination (e.g., Harari et al. 2000; Bailey and French 2012). These random mistakes are maintained in nature either because the cost of male-male mating harassment is very low, hence, selection on more accurate male sex discrimination has been loosened, or, these mistakes could indeed incur a substantial cost to males, such as time and energy waste as well as opportunistic cost of losing a female, but this cost is outweighed by the cost of evolving more accurate sex discrimination mechanisms. Therefore, under the mistake hypothesis, male-male mating harassment would have negative to neutral effect on male-female matings success. Alternatively, it has been proposed that male-male mating harassment could be caused by ‘spillover’ of high mating motivation from male-female interaction context to the male-male interaction context (Sih and Watters 2005), a phenomenon also termed as the ‘libido’ behavioral syndrome (Logue et al. 2009). Empirical support for the ‘spillover’ hypothesis come from studies in several insect species where some males showed high tendency to mate in both heterosexual and homosexual contexts (Serrano et al. 1991, Castro et al. 1994, Sih and Watters 2005, Logue et al. 2009, Stojkovic et al. 2010). Under this hypothesis, selection has yet to decouple mating motivations in the heterosexual and homosexual contexts, such that male-male harassment is positively correlated with male-female mating success (Logue et al. 2009).

The female-specific color polymorphic damselflies are a system where male mating harassment towards females is common. In these damselfly species, males usually have bright coloration whereas females have two or more color morphs: the andromorph has coloration similar to that of the males, and the heteromorph displays coloration that is distinct from the male coloration (Sherratt and Forbes 2001; Fincke et al. 2007). Except for visual cues, there is no evidence to date suggesting that damselflies use other mode of communication in their sex recognition. In this system, mate-repelling behaviors of females (Fincke 1987; Utzeri 1988), the long duration of the copulations (Robertson 1985; Cordero 1990), and difference in reproductive rates between the two sexes (Fincke 1986) all suggested that male mating harassment towards females should frequently occur. Male mating harassment that imposes costs to females (Sirot and Brockmann 2001) has been proposed to be one, but not the sole, selection force maintaining the female color polymorphism (Robertson 1985, Miller and Fincke 1999, Sherratt 2001; see also Cooper 2010).

In a female-specific color polymorphic damselfly *Enallagma hageni*, there is scramble competition for mates among the males, and in one study, roughly 40% of the males never mated during their lifetimes (Fincke 1982), representing strong selection for a reduced discrimination criterion that minimizes the probability of missing a female. The reduced discrimination criterion, on the other hand, also increases the probability of false alarms, favoring the occurrence of male mating harassment towards males (Thornhill and Alcock 1983). Moreover, in at least two major genera of these damselflies, evidence showed that males are able to learn to recognize females (Miller and Fincke 1999; Van Gossum et al. 2005; Fincke et al. 2007). Because of the similarity between andromorphic females and males, once a male learned to recognize an andromorphic female, he could subsequently generalize the learned image to males (Sherratt 2001), resulting in male harassment towards males. Indeed, recently, evidence showed that male mating harassment towards males does occur among two major genera of the polymorphic damselflies (Van Gossum et al. 2005; Fincke et al. 2007; Ting et al. 2009).

Using a female-specific color polymorphic damselfly *Enallagma hageni*, I tested three hypotheses for the cause of male mating harassment towards males: they are (1) random mistakes in male sex discrimination (the ‘mistake’ hypothesis), (2) the result of generalizing the search image of a mate from andromorph to males (the 'generalization' hypothesis), or (3) the spillover of high mating motivation from the heterosexual context to the homosexual context (the 'spillover' hypothesis). Additionally, I investigated the effect of male-male mating harassment on short-term heterosexual mating opportunity of males. The mistake and the generalization hypothesis both predict that male-male harassment should have neutral or negative effect on male-female matings opportunity, whereas the spillover hypothesis predicts male-female mating opportunity to be positively correlated with male-male harassment. I measured male-male mating harassment as male-male clasps and heterosexual mating opportunity as male-female clasps.

METHODS

Study Species

Enallagma hageni is a common North American damselfly whose males have a blue thorax and black-and-blue striped abdomen. There are two female morphs. Andromorph females are male-like blue and heteromorph females are green on the thorax and lateral side of abdomen. Both female morphs have uniformly black abdomen dorsum. At the study site, the andromorph made up roughly 1/3 of the female population and this ratio was relatively stable across years (Fincke unpubl.). The only locally co-occurring species that is morphologically similar is *E. boreale*, but the reproduction seasons of *E. boreale* and *E. hageni* did not overlap at my study site.

Males search for mates at the water's edge and far from water (Fincke 1985). Once a male detects a female, he hovers behind the female from the top, grabs her thorax, and takes her in tandem by engaging the claspers (cerci) at the tip of his abdomen with the mesostigmal plates on the female's prothorax (Corbet 1999). If a female is receptive, she then raises her abdomen to the male's penis to mate with him; otherwise, she can refuse to mate and use various behavioural tactics to separate from the male (Xu and Fincke 2011). If a male is approached by another male, he lifts wings and lowers his abdomen without curling it. The approaching male usually, but not always, leaves in response to this signal. In cases where male-male tandem pairs are formed, they usually do not last long (Xu personal observation). The study was conducted in the Chase Osborn Preserve, Sugar Island, MI, USA (46.4°N, 84.2°W) in June and July 2011 and 2012.

Experiment 1: the frequency, causes and consequences of male-male clasps

I conducted this experiment in outdoor cages (1.8 × 1.8 × 1.8 m3, Bioquip Inc.) located in a forest gap. Each cage included blueberry bushes, ferns, and maple and pine seedlings. I used white sheets to cover the top of the cages facing direct sunlight to block polarized light that attracts damselflies. The netting and the sheets slightly decreased the radiance inside the cages and shifted the light towards red; however, comparing to the ambient light outside the cages, the change in the light condition was minimal, and should not affect males' ability to detect individuals inside the cages (Xu and Fincke 2011). During the experiment, each cage contained *ad libitum* food (mosquitoes and small flies) for the damselflies. To increase humidity and lower temperature, I placed a shallow pool of water (diameter: 1 m, depth: 10 cm) in each cage. I also sprayed the cages with water twice each day, once before the training session and once before the test session. The experiment was only conducted on sunny days.

I collected females from tandem or mating pairs in late afternoon at the lakeshore. To ensure species identity of the females, I checked the species-specific clasper morphology of the males that were in the tandem or mating pairs. The females were then stored in small envelopes in the refrigerator overnight to be used the next day. Refrigerating females for one night does not affect their ability or willingness to fly and mate (Xu and Fincke 2011). Males were collected in the early mornings before they started to fly (i.e., they had no interaction with other damselflies on that day) and were used on the same day. All individuals used for this experiment were young. I numbered all individuals on the left hind wings with an indelible marker.

Training Sessions

I randomly assigned males to one of the four treatments in a factorial design with two factors (Table 1): female morph (andromorph or heteromorph) and training ratio (♀ / ♂) of 0.25 or 4. Each male was trained with 20 other individuals. The training ratio in the andromorph treatment is equivalent to the mimic-to-model ratio in previous studies (Sherratt 2001, Ting et al. 2009). However, because the term “mimic-to-model ratio” does not apply to the heteromorph treatment, I here use “training ratio” (♀ / ♂) as a universal term for both female morphs. The training sessions began between 1040-1130 h and lasted for 2 h. Evidence showed that *Ischnura* and *Enallagma* males are not likely to retain their learned experience overnight (Miller and Fincke 2004; Takahashi and Watanabe 2009), and *E. hageni* males are able to override experience from the field and learn andromorph inside enclosures over the frame of a few hours (Xu and Fincke 2011). Because males and females tend to stay in tandem pairs for a long time if they cannot find an oviposition site after copulation, I gently separated all post-mating tandem pairs. To increase a male’s chance to interact with other individuals, if a male was in a tandem pair for 15 min but did not proceed to copulate, I separated him from the female.

Test Sessions

Immediately after the training session, I excluded males that did not show any activity during the entire training session, and from the remaining males, I randomly chose three males from each session as focal males for the test session. The three focal males were tested simultaneously in the test session. All test sessions included 10 females of the same morph with which a focal male was trained, and 10 non-focal males (Table 1).

To prevent non-focal males from taking females in tandem or mating pairs, I clipped off the claspers of non-focal males with a fingernail clipper. Clipping the claspers of non-focal males does not interfere with their willingness to mate

(Robertson and Paterson 1982), and as expected, none of the clipped males formed a tandem pair. To detect harassment and mating, I dusted the penises and claspers of the three focal males with either blue, green or pink florescent fingerprinting dust (Gosden and Svensson 2007; 2009; Xu and Fincke 2011). The dust can be transferred to another individual through contacts with dusted claspers or penises (Supplimentary Materials).

The test sessions lasted for 1 h. During the test sessions, an observer blind to the training ratios separated post-mating tandem pairs and tandem pairs that lasted for more than 15 min.

Dust Check

After the test sessions, each individual was placed in its own small plastic bag to be transferred to the lab. An observer blind to the treatments checked for the presence of dust under a dissecting microscope with UV lights in a dark room. Male-female clasps were indicated by the presence of dust on a female's mesostigmal plates, and occasionally, on her prothorax. If the same color dust was also present on the female's ovipositor or the ventral side of the abdomen tip, it was scored as a mating. Otherwise, it was scored as an unsuccessful mating attempt. Male-male clasps were indicated by the presence of dust on a male's mesostigmal plates. The dusting method has been shown efficient in capturing up to 9 heterosexual clasps of a single focal male (Xu and Fincke 2011). It is also efficient in detecting male-male clasps (for more details see Supplimentary Materials).

Experiment 2: Male-male Clasps in a More Competitive Environment

I quantified male-male clasps by similar dusting method in an earlier experiment conducted with the same study population but with more realistic conditions (Xu and Fincke 2011) than the current experiment. That experiment included 14 single morph replicates of a factorial design with two factors: female morph (andromorph or heteromorph), and sex ratio (♂ / ♀ of 0.5 or 2). The density was kept at 30 individuals / large cage (1.8 × 1.8 × 3.6 m^3). In addition, there were 13 mixed morph replicates. In these 13 replicates, I did not directly vary andromorph-to-male ratio, but varied female morph frequency (andromorph females / total females) at a density of 30 individuals / cage and sex ratios of either 0.5 or 2 (for details see Xu and Fincke 2011). After accounting for the effect of mortality, the female morph frequency manipulation in the mixed morph and andromorph-only treatments together resulted in andromorph-to-male ratios ranging from 0.08 to 1.87 (mean ± SD = 0.68 ± 0.56, median = 0.50). Lastly, the experiment included 3 replicates where 30 males were kept with no female in the cage.

Because this experiment included treatments with male-biased sex ratio and all males were able to clasp, the general male-male competition level as indicated by the operational sex ratio was similar to that seen in nature (Fincke 1985). Because this experiment was not originally designed to focus on male-male clasps, there was no independent training and test session; instead, males interacted with the same pool of individuals continuously for one day. Multiple males carried the same color of dust, such that interactions as indicated by the presence of dust could not be attributed to individual males. Hence, I calculated the per capita male-male clasps of a replicate (total male-male clasps / number of males).

Statistical Analyses

In 14 out of 48 test sessions in the current experiment, I did not catch all the 11 males and 10 females at the end. Thus, interactions of the focal males with these missing individuals were undetected. The average number of males caught after the test sessions was 10.6 ± 1.1 (mean ± SD) and the average number of females caught was 9.8 ± 1.1 (mean ± SD). To control for the number of individuals that were checked for dust, I used the number of males or females caught after test sessions as offset variables (Zuur et al. 2009) in the models.

In Experiment 1, I tested the effect of female morph, training ratio, and their interaction on male-male clasps by a generalized linear mixed model (GLMM) with Poisson error distribution and test session as a random effect. Similarly, I used GLMM to examine the effect of morph, training ratio and their interaction on male-female clasps. I also used GLM to investigate the relationship between male-male clasps and male-female clasps. In Experiment 2, I used an independent-sample t test to compare the per capita male-male clasp rates (clasps per male per day) between the two single morph treatments, and an analysis of covariance (ANCOVA) to investigate the relationship between per capita male-male clasps and andromorph-to-male ratio controlling for sex ratio. All statistical tests were two tailed and were performed in R (version 2.15.1, R Core Team, 2012). Throughout the paper, means are shown with standard deviations.

If male-male clasps are entirely random events as in the mistake hypothesis, male-male clasps should occur at an equal frequency among all treatments, hence, none of the variables should have significant effect on male-male clasps in both experiments. If male-male clasps were the results of generalization of the learned blue image from andromorph to males, I expect that male-male clasps should occur more frequently in the andromorph treatment than in the heteromorph treatment, and that a higher training ratio or andromorph-to-male ratio that facilitate learning of a blue search image should also increase the probability of male-male clasps. Both mistake hypothesis and generalization hypothesis predict neutral to slightly negative relationship between male-male clasps and male-female clasps. Lastly, under the spillover hypothesis, male-male clasps and male-female clasps should be positively correlated. I also expect a training ratio effect in Experiment 1 and a sex ratio effect in Experiment 2 because higher level of male-male competition should promote mating motivation of males.

RESULTS

Experiment 1: Male-male Clasps

Of the 145 focal males I trained and tested, 125 males had at least one interaction during the test session (Table 1). Twenty males from 4 treatments (heteromorph high: 5, heteromorph low: 6, andromorph high: 4, andromorph low: 5) did not interact with any individual during the test session and thus, were excluded from data analyses. Of the 125 focal males, 94 males (75.2%) did not make any male-male clasp during the test sessions, 29 males (23.2%) made one male-male clasp, and 2 males (1.6%) made two male-male clasps. All but 1 of the 125 focal males clasped with at least 1 female (range: 1-8, 2.5 ± 1.5) during the test sessions.

There was no significant effect of female color morph, training ratio, or their interaction on male-male clasps after controlling for the number of males caught after the test sessions (Table 2). Controlling for total activity level (sum of male-male and male-female clasps) of focal males did not change the above conclusion. The number of male-male clasps (Table 1) did not differ significantly between the two female morph treatments either with pooled training ratios (Fig. 1, GLMM: $\chi^2 = 1.94$, $df = 1$, $P = 0.16$), or within each training ratio (high: $\chi^2 = 0.88$, $df = 1$, $P = 0.35$, low: $\chi^2 = 0.82$, $df = 1$, $P = 0.36$). Furthermore, training ratio did not affect male-male clasps in either female morph treatment (Table 1, Fig. 1, GLMM: andromorph: $\chi^2 = 0.000$, $df = 1$, $P = 0.99$, heteromorph: $\chi^2 = 0.0003$, $df = 1$, $P = 0.99$). Using a focal male response index (whether the focal male did or did not clasp other males) as a binary dependent variable in the GLMM yielded similar results.

Experiment 1: Male-female Clasps

After controlling for the number of females caught after the test sessions, focal males trained with the higher female-to-male ratio made more male-female clasps than males trained with the lower ratio (Fig. 1, GLMM: $\chi^2 = 3.74$, $df = 1$, $P = 0.05$), but there was no significant effect of female morph on male-female clasps ($\chi^2 = 0.05$, $df = 1$, $P = 0.83$). However, when analyzed within each female morph, the training ratio effect was not significant for either morph. The female morph-by-training ratio interaction was not significant and was dropped from the final model to obtain a higher statistic power.

Experiment 1: Relationship Between Male-male clasps and Male-female Clasps

Male-male and male-female clasps were not correlated across treatments (Fig. 2, GLM: $\chi^2 = 0.005$, $df = 1$, $P = 0.94$). Similarly, the correlation between male-male clasps and male-female clasps was not significant when analyzed within each female morph treatment (andromorph: $\chi^2 = 0.02$, $df = 1$, $P = 0.88$, heteromorph: $\chi^2 = 0.08$, $df = 1$, $P = 0.78$) or within each training ratio (high: $\chi^2 = 1.52$, $df = 1$, $P = 0.22$, low: $\chi^2 = 2.44$, $df = 1$, $P = 0.12$). Although males that clasped other males had higher total sexual activities (sum of male-male and male-female clasps, 3.5 ± 1.8) than males that did not clasp other males (2.4 ± 1.4, GLM: $\chi^2 = 10.23$, $df = 1$, $P = 0.001$), the number of male-female clasps made by focal males that clasped other males (2.5 ± 1.7) did not differ from those made by males that did not clasp other males (2.5 ± 1.4, GLM: $\chi^2 = 0.06$, $df = 1$, $P = 0.80$).

Experiment 2: Male-male Clasps in a more realistic environment

In contrast, in single morph treatments in Experiment 2, when sex ratio treatments (♂ / ♀ = 0.5 and 2) were pooled, mean per capita male-male clasps was higher in the andromorph treatment (0.5 ± 0.2) than in the heteromorph treatment (0.2 ± 0.1, independent two-sample t-test: $t = 3.53$, $df = 12$, $P = 0.004$). However, when analyzed within sex ratio, per capita male-male clasps were higher in the andromorph treatment than in the heteromorph treatment in the high sex ratio (♂ / ♀= 2) treatment ($t = 3.38$, $df = 5$, $P = 0.02$), but not in the low sex ratio (♂ / ♀= 0.5) treatment ($t = 1.70$, $df = 5$, $P = 0.15$). Using data from all but heteromorphy-only treatments, after controlling for sex ratio effect, male-male clasps did not vary with andromorph-to-male ratio (Fig. 3, ANCOVA: $F_{1,16} = 1.45$, $P = 0.24$). Lastly, in the three male-only replicates, a male made an average of 0.4 ± 0.1 male-male clasps per day.

DISCUSSION

In the female-specific color polymorphic damselfly *E. hageni*, given that only roughly 40% of males have a chance to mate during their lifetimes and that male-male scramble competition for mates is fierce (Fincke 1982), the value of a mating is expected to be high to a male. As a result, the cost of missing a potential mate may be much higher than the cost of a false alarm (i.e., pursue a male as a potential mate). The fact that andromorphic females resemble males in coloration only makes male-male harassment more probable. Indeed, evidence showed that males of another female-polymorphic damselfly *Ischnura elegans* were more likely to clasp males than females after being enclosed with only males for 2 days (van Gossum et al. 2005). Field studies in the genus *Ischnura* also reported high incidences of male-male mating harassment (Robertson 1985; Cordero 1989), and one study in *Nehalennia irene* using individuals glued to grass stems reported male-male clasps almost as common as male-female clasps (Ting et al. 2009). However, despite the promises from theoretical and empirical evidence, in Experiment 1 in our study, only about 1 / 4 of the 125 focal males made male-male clasps in comparison with all but one that made male-female clasps. For the males that did clasp both males and females, roughly 90% of their total clasps were with females. These results indicated that under the present experimental conditions, male-male clasps were in fact rare. This finding is congruent with reports from the same species and congeneric species that male-male mating harassment occurs at a similar or even lower frequency in field experiments (Fincke 1994; Fincke et al. 2007). If the difference in male-male harassment frequencies between *Enallagma* and *Ischnura* damselflies is true, we suggest that it may be a result of difference in the benefit and cost of a hit (i.e., correctly identify a female as 'female') versus a false alarm (identify a male as 'female'), as well as the greater degree of similarity between andromorphic female *Ischnura* and their males.

Our findings did not fully support any of the hypotheses for the cause of male-male mating harassment. Results from Experiment 2 showed a higher male-male harassment rate in the andromorph treatment than in the heteromorph treatment under a male-biased sex ratio (♂ / ♀ = 2), supporting the generalization hypothesis and contradicting the hypothesis that male-male harassment are random mistakes. However, this difference disappeared under an even sex ratio during the testing sessions in Experiment 1 and under a female biased sex ratio (♂ / ♀ = 0.5) in Experiment 2, suggesting that the effect of generalization on male-male mating harassment may depends sex ratio. In Experiment 1, female morph did not affect male-male harassment, suggesting that male-male mating harassment may have been random mistakes in sex discrimination under a loosened male-male competition regime.

In both Experiment 1 and 2, andromorph-to-male ratio did not affect male-male clasps, contrary to the generalization hypothesis and contradicting the finding from field studies in *Ischnura elegans* and *Nehalennia irene* that male clasp attempts with other males increased with andromorph-to-male ratio (Hammers and van Gossum 2008, Ting et al. 2009). The positive correlation between male-male tandem attempts and mimic-to-model ratio (i.e., andromorph-to-male ratio) is a key prediction for the male mimicry hypothesis for the maintenance of female color polymorphism in damselflies (Sherratt 2001). Although it was not our focus to test hypothesis for the maintenance of female polymorphism, our results did not support the male mimicry hypothesis. An alternative explanation for the lack of training ratio effect is that 2 h-long trainings were not sufficient to override the effect of interactions between focal males and a majority of heteromorphic females in the field. However, we do not think this was likely the case. Male-female harassment data from Experiment 2 showed that male harassment towards andromorphic females were the highest in andromorph majority and monomorphic andromorph treatments, suggesting that males from a heteromorph-majority population were able to learn andromorph, and most importantly, they did so within a time frame of a few hours (Xu and Fincke 2011). In addition, two lines of evidende showed that males do not store their learned experience over night. First, data from two congeneric species showed that males were more likely to sexually interact with heterospecifics early in the day than late in the day (Miller and Fincke 2004). In addition, male *Ischnura senegalensis* do not prefer either female morph in the morning but they gradually preferred the majority morph later in the day (Takahashi and Watanabe 2009). Because all focal males in both experiments in this study were caught early in the morning before males started to fly, i.e., before they had any interaction with other individuals on that day, I assume they did not have a preference for a particular female morph when the trails began. It was also unlikely that the training effect had diminished before the testing sessions because focal males made more male-female clasps in the test sessions when they were trained with higher female-to-male ratio for both female morphs. If training effect had diminished before the test sessions, we would not expect a training ratio effect on male-female clasps because all test sessions for a morph treatment were universal.

Finally, in Experiment 1, male-male clasps were not correlated with male-female clasps. Hence, I did not find support for the spillover hypothesis. More specifically, in Experiment 1, male-female clasps were common and differed between the two training ratios, but male-male clasps remained relatively rare in all combinations of female morph and training ratios, suggesting that unlike what the spillover hypothesis assumes, selections on mating motivations in the heterosexual and homosexual contexts have been decoupled and are operating independently.

In summary, our results suggested that male-male harassment may have mixed causes under different conditions. When male-male competition is relaxed, male-male clasps may be simply random mistakes in male sex discrimination, and these male-male clasps may represent a baseline level of mistakes. On the other hand, generalization may be operational under a male-biased sex ratio, i.e., a higher male-male competition level. If speed of reaction towards an approaching individual largely determines which male gets access to it, males may not afford to take extra time to discriminate. Instead, they may trade accuracy for speed of reaction to out-compete other males (Chittka and Osorio 2007), a condition favoring generalization. This explanation is supported by several previous studies that found males were more likely to sexually pursue other males under higher sex ratios (McGraw and Hill 1999; Switzer et al. 2004). At the extreme, in all-male treatment in Experiment 2, male-male clasps were common. This finding resonates with the finding from several studies where high rates of male-male sexual interactions were observed when the opposite sex was depleted from the population during a reproductive season (Conover and Hunt 1984; McGraw and Hill 1999; Young et al. 2008). It has been suggested that male-male harassment in an all-male environment may be a result of the extreme sex ratio under which males become hyper-aggressive, a behavioral type known to be linked to high level of male-male harassment (Sih and Watters 2005).

Hence, data from all-male treatment further supported our conclusion on the effect of sex ration on the cause of male-male harassments.

Male-male clasps and male-female clasps were not correlated, suggesting that male-male harassment had no cost on short-term heterosexual mating opportunities for males, perhaps because male-male clasps were usually short. This finding agreed with evidence from several other insects that mating harassment towards other males did not affect a male’s subsequent heterosexual mating success (McRobert and Tompkins 1988; Dukas 2010; Benelli and Canale 2012), and in this study, I found no evidence that suggests male mating harassment towards males may have direct benefits to males, such as mitigating male-male aggression, mating practice, and indirect sperm translocation (Peschke 1985; McRobert and Tompkins1988; Preston-Mafham 2006; Levan et al. 2009). Lack of a short-term mating opportunistic cost, however, could be the result after selection has eliminated those male mating harassment towards other males that incur more substantial costs. Because the main cause of deaths in nature were by spider and dragonfly predation (Fincke 1982), it would be interesting to investigate whether male-male harassment increases predation risk of males since activities involved in harassment should increase the likelihood of getting caught in a spider web and being detected by motion-sensitive dragonflies.

Our results suggested that there may be multiple causes underlying male-male harassment in *Enallagma* damselflies in nature. The current forms and prevalence of male-male harassment observed seem not to have a significant effect on heterosexual mating success of males, indicating that unlike many social vertebrates, male-male sexual behaviors among invertebrates are unlikely to have a direct fitness benefit to males. Yet one question that remains is why male-male harassment is more common in *Enallagma*'s sister genus, *Ischnura*, another female-polymorphic damselflies. I suggest that two important differences may have contributed: *Ischnura* tandem pairs seem more likely to form around shore, areas of high male-male competition, and that the andromorphic females are more similar in appearance to males in *Ischnura* than in *Enallagma*. A cross-genera comparative study on the morphological, behavioral, and ecological differences that may contribute to differences in benefit-cost matrices for the male-male harassment would further deepen our understanding of this paradoxical phenomenon.

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Table 1. Experimental design, sample sizes and mean (± SD) number of male-male and male-female clasps of the damselfly *Enallagma hageni* in outdoor enclosure in Experiment 1

female morph	training session		test session		n	♂-♂ clasp	♂-♀ clasp
	#♀	#♂	#♀	#♂			
Blue	4	16	10	10	31	0.3 ± 0.5	2.3 ± 1.6
Blue	16	4			30	0.3 ± 0.6	2.9 ± 1.9
Green	4	16	10	10	34	0.2 ± 0.4	2.0 ± 1.0
Green	16	4			30	0.2 ± 0.5	2.7 ± 1.3

Table 2. Summary of results from the generalized linear mixed model of effect of female color morph (MORPH) and training ratio (TR, female / male) on the number of male-male clasps by each focal male *Enallagma hageni* in Experiment 1

source	χ^2	df	p
MORPH	0.92	1	0.34
TR	0.00	1	1.00
MORPH*TR	0.001	1	0.98

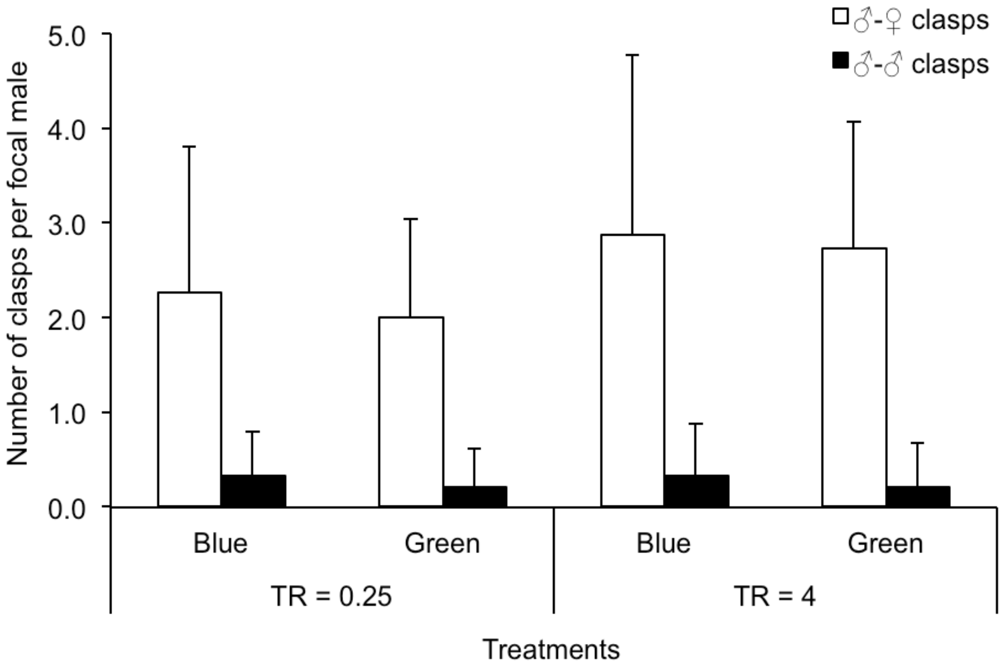
FIGURE LEGENDS

Figure 1. Mean male-female and male-male clasps by focal *E. hageni* males for two female morph treatments at two ♀ / ♂ training ratios from the Experiment 1. Error bars show standard deviation

Figure 2. The number of male-female clasps against the number of male-male clasps in Experiment 1. The sizes of the data points are proportional to the number of focal males

Figure 3. Per capita male-male clasps per day against andromorph-to-male ratio from the mixed-morph and andromorph-only treatments in Experiment 2

Figure 1



□♂-♀ clasps
■♂-♂ clasps
Number of clasps per focal male
5.0
4.0
3.0
2.0
1.0
0.0
Blue
Green
Blue
Green
TR = 0.25
TR = 4
Treatments

Figure 2

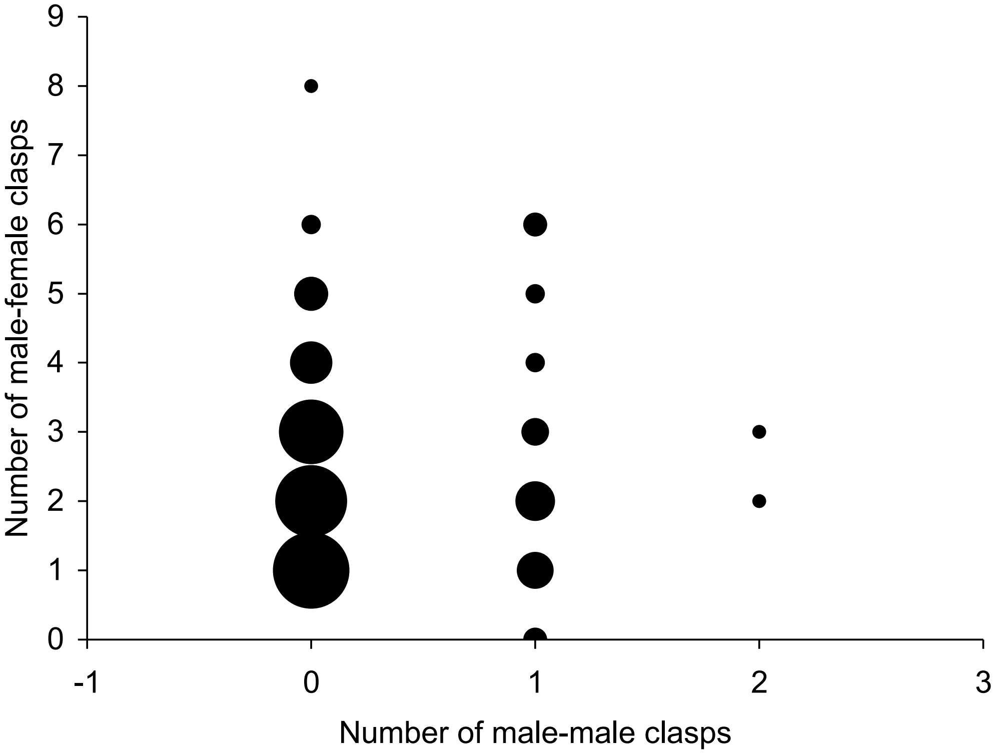
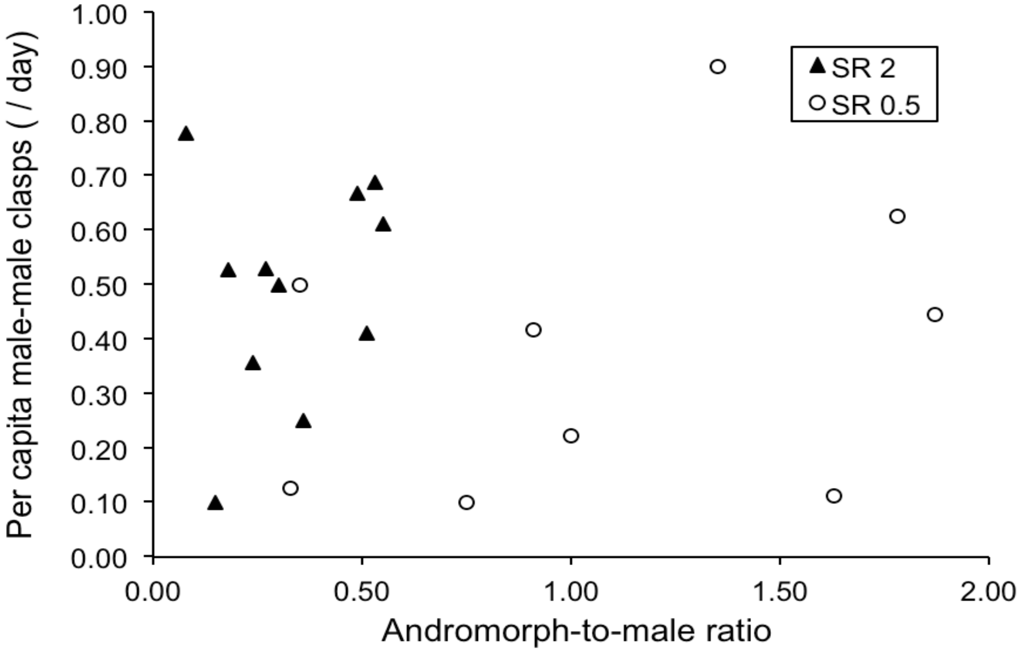

Number of male-female clasps
0
1
2
3
4
5
6
7
8
9
-1
0
1
2
3
Number of male-male clasps

Figure 3


Per capita male-male clasps (/ day)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.00
0.50
1.00
1.50
2.00
Andromorph-to-male ratio
▲ SR 2
○ SR 0.5

Chapter 3: Selective use of multiple cues by males reflects a decision rule for sex discrimination in a sexually mimetic damselfly

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ABSTRACT

Discriminating between the sexes when one sex resembles the members of the other sex may be challenging. When sexual mimicry imposes costs on signal receivers, receivers can minimize confusion by using non-mimetic cues that differ between the models and the mimics. We tested this hypothesis in a female-specific polymorphic damselfly *Enallagma hageni*, whose blue coloration of andromorphic females resembles that of males, whereas the heteromorphic females have a distinctive green colour. Both female morphs share an abdominal pattern that differs from the males'. We predicted that males selectively use both colour (the mimetic cue) and pattern (the non-mimetic cue) in sex recognition: they use the non-mimetic cue only when the encountered individual has the mimetic colour. We modified the abdominal pattern of males, andromorphs and heteromorphs to resemble that of the opposite sex, and recorded males' reactions to pattern-altered and control individuals both in an arena and in the field. Our results supported our hypothesis. We then derived and tested potential male decision rules based on the two visual cues for sex recognition. We presented focal males with unnatural, orange females possessing either a male or female abdominal pattern, and recorded the reactions of mate-searching males to individuals with a novel pink-painted phenotype. Males reacted sexually to orange- and pink-painted individuals regardless of the abdominal pattern. Collectively, our results supported a male discrimination rule of 'if not blue, then female', providing insights into the origin of phenotypic novelty in colour polymorphic species.

INTRODUCTION

An animal’s life is filled with discrimination tasks, such as differentiating between its own and other species, kin and non-kin, palatable and non-palatable prey, as well as mates of various qualities (Duncan & Sheppard, 1965; Walker, 1974; Ryan, 1990; Hepper, 2008). Among such choices, one of the most fundamental is to discriminate between one’s own and the opposite sex. Individuals from a wide range of invertebrates and vertebrates are known to distinguish sex using sex-specific visual (Rutowski, 1977; Sætre & Slagsvold, 1992), acoustic (von Helversen & von Helversen, 1997) or chemical cues (Tregenza & Wedell, 1997; Ferkin & Johnston, 1995). However, the task becomes challenging when one sex of a species resembles the opposite sex or other species. Bluegill sunfish *Lepomis macrochirus* represent an example of intraspecific sexual mimicry where some smaller males may mimic female morphology as an alternative mating strategy while avoiding aggression from other males during spawning (Dominey, 1980; Gross, 1982). Another well-known example is the interspecific sexual deception of orchids that attract male hymenopterans as pollinators by mimicking mating signals of the female hymenopterans (Schiestl, 2005).

Numerous studies have focused on demonstrating how sexually mimetic signals fool the signal receivers (Slagsvold & Sætre, 1991; Oscar, Abby, & Morris, 2010; Gaskett, 2011), yet it is equally important to understand how receivers respond to the mimicry beyond their initial confusion. Sexually mimetic signals can be costly for the receivers (Semple & McComb, 1996). For example, mating between a nesting female and a female-mimicking male pied flycatcher (*Ficedula hypoleuca*) can compromise the fitness of a territorial male that fails to recognize its competitor (Sætre & Slagsvold, 1995). Understanding how receivers perceive and react to sexually mimetic cues is integral to predicting the dynamics between receivers and signalers in a sexual signaling system. However, although the evolutionary responses of receivers have long been studied in the context of predator-prey and brood parasite-host interactions (Lotem et al., 1995; Turner & Speed, 1996), we know relatively little about how receivers recognize sex in the presence of sexual mimicry (but see Wong, Salzmann, & Schiestl, 2004).

One way receivers can lower the cost of mimicry is to adjust their discrimination level in response to external cues (Holen & Johnstone, 2006). For example, superb fairy-wrens have been found to lower their discrimination threshold for egg rejection when brood parasites were present near the nest (Langmore, Cockburn, Russell, & Kilner, 2009). Alternatively, , we propose that receivers can lower the cost of mimicry by increasing attention to non-mimetic cues, an addition to Arnqvist's (2006) hypothesis for receiver resistance to sensory exploitation. Furthermore, if discrimination of non-mimetic cues imposes assessment or other costs to the receivers, we propose that a receiver seeking to optimize its efficiency of discrimination should use non-mimetic cues only when the mimetic cue is insufficient to distinguish sex.

Evidence for such use of non-mimetic cues comes from egg mimicry of a brood parasite. The eggs of a cuckoo finch *Anomalospiza imberbis* and those of its host *Prinia subflava* are similar in colour and pattern proportion, but differ significantly in pattern dispersion. The host uses multiple visual cues to reject parasitic eggs, among which colour (a mimetic cue) and pattern dispersion (a non-mimetic cue) are the two most important cues (Spottiswoode & Stevens, 2010). In the context of sexual mimicry, an experiment on the Augrabies flat lizard *Platysaurus broadleyi* showed that female-mimicking males ('she-males') mimic visual, but not chemical signals of females; accordingly, 'he-males' courted she-males based on visual signals alone at long distance, however, at closer range where chemical signals become detectable, he-males become less likely to court she-males (Whiting, Webb, & Keogh, 2009).

Female-specific colour polymorphic damselflies provide an advantageous system to test the optimal use of non-mimetic cues. In such species, there are usually two female morphs: an andromorph whose coloration is similar to that of the males and a heteromorph whose coloration distinctly differs from that of the males (Johnson, 1975). Both female morphs share traits that differ from those of males, such as body size, abdomen width, wing shape, and the melanin pattern (Gorb, 1998; Abbott & Svensson, 2008). In a female colour polymorphic damselfly *Enallagma ebrium*, the sexually dimorphic abdominal dorsal pattern, in addition to coloration, affects sex recognition of mate-searching males (Miller & Fincke, 1999). Odonates have highly acute vision (Briscoe & Chittka, 2001; Bybee, Johnson, Gering, Whiting, & Crandall, 2012) and, even though the chemoreceptors are present in the antenna of odonates (Slifer & Sekhon, 1972; Rebora, Salerno, Piersanti, Dell'Otto, &

Gaino, 2012), to date there is no evidence that damselflies use modes of communication other than vision in mate-searching and sex recognition (Corbet, 1999).

Using the female-specific polymorphic damselfly, *Enallagma hageni*, we tested the hypothesis that males determine the sex of the blue andromorphic females using the sexually dimorphic abdominal dorsal pattern. Here, 'colour' refers to spectral reflectance properties and 'pattern' refers to the distribution of colour patches across body parts. Throughout the paper, we refer to 'mimicry' in the sense of signal similarity in coloration between andromorphic females and males. We modified the abdominal pattern to resemble that of the opposite sex in individuals of three colour types: andromorph, heteromorph, and male, and presented a control and a pattern-altered individual of the same colour type to focal males (Fig. 1). We had two specific predictions: (1) painting the abdominal pattern of a male or an andromorphic female (i.e., the mimetic female) to resemble that of the opposite sex would change the probability of a male's sexual reaction, and (2) painting the abdominal pattern of a heteromorphic female (i.e., the non-mimetic female) to resemble that of a male would not affect the probability of sexual reactions towards her. Additionally, to further deduce how males make decisions based on information from colour and pattern, we recorded the reactions of males to unnatural, orange-painted females with either a female or male pattern both in the arena and in the field. We also recorded the reactions of mate-searching males in the field to individuals of both sexes that were painted pink (i.e., a completely novel colour in the genus) with their natural abdominal patterns (Fig. 3). Our results suggested differential use of visual cues by males in sex recognition in the presence of female polymorphism with sexual mimicry, and a possible decision rule for sex recognition that could facilitate the origin of novel colour phenotypes in polymorphic systems.

METHODS

Study species and general methods

Male Enallagma damselflies search for mates around breeding sites on the edge of ponds and lakes but also in areas far from shore (Fincke, 1986). There is intense scramble competition for mates among males (Fincke, 1982), although operational sex ratios (male : female) at lakeshores are much higher than in surrounding forests (Fincke unpubl. data).

Like the majority of species in the family Coenagrionidae (Fincke, Jödicke, Paulson, & Schultz, 2005), the common North American bluet damselfly *Enallagma hageni* exhibits female-specific color polymorphism. The andromorph has blue coloration similar to that of the males, except that the andromorph's reflectance peaks at a slightly longer wavelength, and is less bright and saturated, whereas the green coloration of the heteromorph has a spectral reflectance distinct from that of the males and the andromorph (Fincke, Fargevieille, & Schultz, 2007). Whereas both sexes have a striped thorax, both female morphs have a uniformly black abdominal dorsum that is distinct from the male's striped abdominal pattern. In addition to the abdominal dorsal pattern, there are several additional visual cues that differ between the sexes, such as body size and abdomen width (Fincke, 1982, Gorb, 1998). However, the two female morphs do not differ in body size as measured by wing length (Fincke, 1994) or abdomen width (Fincke unpubl. data); nor do andromorphic females mimic males in behaviour (Fincke unpubl. data). The two female morphs do not differ in fecundity (Fincke, 1994) and thus are of equal value to mating males. Here we limited our investigations to the use of colour- and pattern-related traits by males. Hence, heteromorphic females, which do not differ from andromorphic females in other sex cues (e.g. abdominal width, body size, presence of an ovipositor), represented a within-species control for visual cues to sex other than colour and pattern. Andromorphs comprised 1/4-1/3 of the females in our study populations in 2010, 2011 and 2013.

Male *E. hageni* should be able to perceive orange and pink because a long-wavelength photoreceptor appears to be ancestral in damselflies (Bybee et al., 2012). Whereas the genus *Enallagma* includes an orange species, *E. signatum* that co-occurs with *E. hageni* over much of *E. hageni*'s range (Schultz, Anderson, & Symes, 2008; Schultz & Fincke, 2013), pink coloration is a novel trait for North American members of the family Coenagrionidae (i.e., the European *Ischunura elegans rufescens* may have a spattering of pink on the underside of the orange thorax), distinct in reflectance from that of red or violet damselflies (e.g., *Enallagma pictum*, Schultz et al., 2008; *Argia fumipennis violacea*, Schultz, unpubl. data, repectively; see also Romney & Tarow, 2002). Color and pattern manipulation in the experiments were done using the non-toxic acrylic paint colours (DecoArt, Standford, USA)

‘Lamp Black’, ‘Ocean Blue’, ’Mistletoe’ (green), ‘Bright Orange’, or ‘Dragon Fruit’ (pink). In the experiments below, we collected only sexually mature individuals found in tandem pairs as control or manipulated individuals. We glued the tips of legs of a live *E. hageni* to the end of a wooden dowel with either Beacon quick grip adhesive (Beacon Adhesive Inc., Mount Vemon, USA) or Duco cement household glue (Duco Technology Inc., Henderson, USA), such that the damselfly was perched in a natural posture and was able to move its head and wings. All individuals remained alive throughout a test. Except where noted, each individual was used for only one trial, after which it was released by gently separating the tips of legs from the glue; most flew away, although sometimes one or more legs were autotomized at the joint. Occasionally, we found painted individuals in tandem within minutes of being released. In field experiments, individuals on the dowels were placed at the level of the top of surrounding vegetation. Only the most sexually extreme reaction for each reacting male was recorded. In field tests, to prevent reacting males from being counted twice, they were collected, marked on the wing, and held for the duration of the trial. All trials were conducted in full sun.

Experiment 1a, b: use of colour and pattern in sex recognition: arena and field

Presentations of live individuals to focal males were conducted in a white plastic arena placed in a forest gap in the Chase Osborn Preserve, Sugar Island, MI, USA (46.4°N, 84.2°W) between June and July 2010. The arena was cylindrical, 110 cm in diameter, 60 cm in height, and had an observation window of 10 cm diameter near the top. The white wall and top of the arena (a white sheet) provided standard background that eliminated the confounding effect of the conspicuousness of the colour types against vegetation background in the field. A small blueberry bush in the arena served as a perch site for focal males.

Focal males were collected early in the morning before they started to fly and randomly assigned to one of the three colour type treatments (Fig. 1). We presented to a total of 30, 36 and 30 focal males a pair of either males, andromorphs, or heteromorphs, respectively (i.e., 192 total trials). In the male treatment, the focal male was sequentially presented with a control male and a male whose abdominal dorsum was painted uniformly black (i.e., the female pattern). We used natural controls in 19 of those trials and sham controls painted with male coloration in 11 trials. In the andromorph treatment, the focal male was presented with a control female (natural control in 24 trials and black-sham-painted controls in12 trials) and a blue female that was painted with blue paint to create the male blue-and-black striped dorsal pattern (Fig. 2a). The corresponding heteromorph treatment used either a natural (19 trials) or black-sham-painted control female (11 trials) and a female painted with the male abdominal pattern. The presentation sequence of female or male pattern was randomized among focal males. The individual on the dowel was slowly moved towards a perching focal male from behind until the presented individual was in front of, and approximately 3 cm below the focal male's head. Such a presentation position has been shown to be most likely to elicit a focal male's reaction (Gorb, 1998). A focal male's reaction towards a presented individual was noted as: no response within 1 min, switch perch, hover, grab, tandem attempt, or tandem. ‘No response’ and ‘switch perch’ were treated as a male’s lack of interest in mating, and were excluded from the analyses. Hover was regarded as a non-sexual reaction, whereas grab, tandem attempt and tandem were regarded as sexual reactions. The presentation ended if the focal male grabbed, attempted tandem or formed tandem with the presented specimen; otherwise, the presentation was repeated three times and the most extreme sexual reaction was used for analysis.

To determine whether the results from the arena were consistent with male behaviour under more natural conditions of sex ratio, male-male competition for mates, and the presence of males as andromorph distractors, the reactions of free-flying males to the 6 types of individuals on dowels (i.e., male, andromorph, and heteromorph with either male or female dorsal pattern) were measured in the field over three days in July 2010. A total of six replicates were conducted for each type: three in grass close to the lakeshore and three in forest light gaps. We ran trials at these two locations because males search for mates at both sites (Fincke, 1986). Three observers recorded the reactions of the first 10 males (but the first 15 in two trials and the first 12 in one trial) that reacted to the individual on the dowel. In sum, we observed reactions of a total of 391 mate-searching males to 36 focal individuals on the dowels. The observers, blind to the purpose of this experiment, stood 1 m away from the dowels. At this distance, the type of the individual on the dowel was not easily discernible (e.g. a natural male or a blue female painted with male pattern). As in the arena, male reactions were categorized as: hover, grab, tandem attempt, and tandem. Hover was treated as a non-sexual reaction whereas the rest were considered sexual reactions.

Experiments 2a, b: Deduction of male decision rule from orange-painted individuals

In experiment 2a, we individually presented each of 35 focal males with orange-painted, heteromorphic females in the arena in August 2010 and June 2011. Each focal male was randomly assigned to either the female pattern or the male pattern treatment. Females were painted orange on their thoraces and abdomens where the natural coloration was green (Fig. 1). Additionally, in experiment 2b, using the protocol as in field experiment 1b, we recorded the reactions of 80 unique mate-searching males towards orange-painted females with either a female ($N = 4$) or male pattern ($N = 4$) in late July 2011 at Cheboygan Marsh, MI (45.38°N, 84.28°W).

Experiments 3: Deduction of male decision rule from pink-painted individuals

To determine whether a male’s decision rule could be generalized to completely novel phenotypes of both males and females in the field, on 20 sunny days between 26 June and 22 July 2013 at Larks Lake, MI (45.1°N, 84.2°W), we recorded the reactions of mate-searching males to control or pink-painted males and females of both morphs. Manipulated individuals were painted pink where their natural colours were blue or green (Fig. 2b) whereas controls were either natural (for all three colour types) or sham-painted (for andromorphs and heteromorphs only).

The natural abdominal dorsal pattern was retained for all three colour types (Fig. 3). Individuals on dowels were positioned in grass 3-5 m from shore. We used six types of individuals: 17, 23, and 21 pink-painted, and 13, 18, and 19 natural control andromorphs, heteromorphs and males respectively, as well as 9 and 14 sham control andromorphs and heteromorphs (Fig. 3). Each of the six types was positioned one at a time at the same site in a random sequence; sites changed among replicates.

We recorded reactions of passing males for 10 minutes, scoring them as ‘fly-by’ (male passed within 5 cm of the individual on the dowel without subsequent reaction), ‘hover’, ‘grab’, ‘tandem attempt’ or ‘tandem’ as described above. ‘Fly-by’ and ‘hover’ were considered non-sexual reactions, whereas the remaining three were considered sexual reactions. We also recorded the number of non-reacting males passing within a 1 m^2 square around the dowel; the total number of males / m^2 / 10 min was used to control for variation in male density among trials. A few painted individuals were used in two trials; none were used more than twice.

Spectrum analysis of the paints

To assess how closely the paints matched the natural colours of the damselflies, we measured the relative reflectance of 9 males, 12 andromorph females, 11 heteromorph females, as well as 5 samples of each paint with an OceanOptics SD 2000 spectrometer and a PX-2 xenon light source (OceanOptic, Dunedin, USA). We compared the hue (wavelength at the maximum reflectance) and brightness of the blue paint to those of andromorph females and males, and the green paint to those of the heteromorph females in Avicol v.6 (Gomez, 2006). We also calculated colour contrasts in the unit of ‘just noticeable differences’ (JNDs, Vorobyev & Osorio, 1998) with a tetra-chromatic model of odonate colour vision (for details, see Schultz & Fincke, 2013) to examine whether males could detect the difference between the paints and the damselfly colours. A colour contrast value of 1 JND is usually considered detectable by the signal receivers.

Statistical analyses

The presentation sequence (painted individual first or control individual first) and the type of control (i.e., natural control or sham control) had no effect on the reactions of focal males in experiment 1a (generalized linear mixed model, sequence: $\chi^2_1 = 0.001$, $P = 0.97$; control type: $\chi^2_1 = 0.0002$, $P = 0.99$). Hence, data from the two presentation sequences and the two control types were pooled in the analyses below. Using data from all three treatments, we first used a generalized linear mixed model (GLMM) with binomial error distribution to investigate the general effects of colour and pattern on sex recognition by focal males. In this model, focal male reaction (i.e., sexual vs non-sexual) was used as a binary dependent variable; focal male was treated as a random effect, and pattern was nested within focal male. We performed two additional tests to further examine the relationship between colour and pattern cues. First, we used Fisher’s exact test to investigate whether the effect of pattern on sex recognition was contingent on colour. Second, we used a generalized linear model (GLM) to investigate whether the effect of pattern differed in magnitude among the three colour types. In this model, we used 'difference in response' as the dependent variable. This variable was scored '0' if a focal male's reaction towards the pattern-altered individual was the same as towards the control individual, and '1' if a focal male changed his reactions. The dependent variable was colour type (i.e. andromorph, heteromorph, or male). Both tests required excluding two focal males that did not react sexually to control heteromorphic females and a male that reacted sexually to a control male. We interpret the former two cases as a focal male lacking an interest in mating, and the latter as a mistake in male sex recognition. These cases provided no information on how males recognize sex using colour or pattern cues. Within each colour type, we used Wilcoxon-signed rank test to investigate whether the abdominal pattern modification changed the focal males' reactions. In this test, zeros and ties were handled according to Pratt's modification of the original Wilcoxon test (Pratt, 1959), and the exact *P* values were calculated. In field experiment 1b, male reactions towards individuals on the dowel did not differ between the two field locations (grass near water or forest light gap, GLM, $\chi^2_1 = 0.001$, $P = 0.98$). Hence, data from the two locations were pooled in the analyses. We used GLM with binomial error distribution to examine male reactions (i.e., sexual or non-sexual) to the control and the pattern-altered individuals in Experiment 1b.

In the arena presentations of orange females in Experiment 2a, each focal male was only presented one individual, thus, we used Fisher's exact test to investigate the effect of abdominal dorsal pattern on focal males' reaction. We also used a GLM similar to the above to examine the reaction of mate-searching males towards orange-painted individuals. To examine male reactions to the control and the pink-painted individuals in the field in Experiment 3, we used generalized linear models (GLM) with the number of sexual and non-sexual reactions by males as dependent variables, and total number of males in the m^2 area as a covariate. Because males were less likely to react sexually towards andromorphic sham control females than towards natural control females ($\chi^2_1 = 6.68$, $P = 0.01$), we excluded andromorphic sham control females from analyses; including them did not change the results.

We used Bonferroni correction to adjust the α levels for our planned pairwise comparisons. All statistical tests were performed in R (version 2.15.1, R Core Team, 2012). Throughout, means are shown with standard errors.

RESULTS

Experiments 1a, b: Use of color and pattern in sex recognition: arena and field

In the arena, except for two focal males from the heteromorph treatment and one male from the male treatment, the focal males all reacted sexually to control females and non-sexually to control males as expected (Fig. 4). No male reacted non-sexually to the model with the female pattern and sexually to the model with the male pattern. Both colour and pattern had significant effects on focal male reaction (GLMM, colour: $\chi^2_2 = 9.55$, $P = 0.01$; pattern: $\chi^2_1 = 10.39$, $P = 0.001$). Because the pattern by colour interaction was not significant, we dropped it from the final model to recover power. When analyzed within each treatment, focal males were less likely to react sexually to the blue andromorphic females painted with the male-like striped abdominal dorsal pattern than to the control blue females (Wilcoxon-signed rank test, α' = 0.017, $W_{35} = 4.24$, $P < 0.001$, Fig. 4). Similarly, focal males were more likely to react sexually to males painted with the female uniform black abdominal pattern than to control males ($W_{29} = 4.80$, $P < 0.001$). Finally, as expected, there was no difference between the focal males' sexual reactions to green heteromorphic females painted with a green-and-black striped male pattern and their sexual reactions to control females ($W_{29} = 1.41$, $P = 0.50$).

Excluding the three focal males that did not react to control individuals in the expected way, the effect of abdominal dorsal pattern on male sex recognition was contingent on colour (Fisher's exact test, $P = 0.007$). Additionally, the magnitude of the pattern effect depended on colour type (GLM, $\chi^2_2 = 34.51$, $P < 0.001$). The post hoc comparisons revealed a significant gradient in the magnitude of response to the pattern alteration among three treatments with the most drastic change in the male treatment and the least change in the heteromorph treatment (Fig. 4, Tukey's test, andromorph – heteromorph: $P = 0.004$, andromorph – male: $P = 0.045$, heteromorph – male: $P < 0.001$).

Reactions by males in the field experiment 1b were similar to those of males in the arena presentations (Fig. 5). Overall, the probability of male sexual reactions differed among three colour types ($\chi^2_1 = 12.91$, $P = 0.0003$), and between two pattern treatments ($\chi^2_2 = 37.41$, $P < 0.001$), but the pattern by treatment interaction was not significant ($\chi^2_2 = 2.45$, $P = 0.29$). When analyzed within each treatment, males were more likely to react sexually to the control andromorphic females than to andromorphic females painted with the male abdominal pattern (GLM, $\alpha' = 0.017$, $\chi^2_1 = 12.91$, $P = 0.0003$). Similarly, males were more likely to react sexually to males painted with the female abdominal pattern than to control males ($\chi^2_1 = 9.47$, $P = 0.002$). In contrast, the probability of male sexual reactions towards heteromorphic females with female or male patterns did not differ ($\chi^2_1 = 0.27$, $P = 0.60$).

Experiment 2a, b: Deduction of male decision rule from orange-painted individuals

In the arena experiment 2a, 6 out of 19 (32 %) focal males reacted sexually to orange-painted females with the female pattern, whereas 2 out of 16 (13 %) focal males reacted sexually to orange-painted females with the male pattern. There was, however, no difference in male sexual reactions towards the two types of orange females (Fisher's exact test, $P = 0.24$).

The overall sexual response ratios towards orange individuals in the arena were low. Nevertheless, two previously unreacting males did take an orange female with female pattern and an orange female with male pattern in tandem as the females were withdrawn from the arena respectively, even though these could not be included in the analysis. Including these two cases does not change the conclusion above. Focal males were more likely to react sexually to orange-painted individuals with a female pattern than to control males (Fisher's exact test, $\alpha' = 0.025$, $P =$

0.015). However, male sexual responses towards orange-painted females with a male pattern were not significantly different from those towards control males ($P = 0.27$).

In the field experiment 2b, males readily reacted sexually to orange-painted females regardless of the abdominal pattern. The probability of a sexual reaction by a mate-searching male towards an orange individual with a female pattern (0.95 ± 0.03) did not differ from that towards an orange individual with a male pattern (0.85 ± 0.06, GLM, $\chi^2_1 = 2.32$, $P = 0.13$).

Experiment 3: Deduction of male decision rule from novel, pink individuals: field

In the field experiment using pink-painted individuals and the controls, the probability of sexual reactions by males decreased with total number of males in the 1 m^2 area ($\chi^2_1 = 15.47$, $P < 0.001$). However, controlling for total number of males in the area, the total number of reactions (i.e., sexual plus non-sexual reactions) did not differ between controls and pink-painted individuals for any colour type (andromorph: $\chi^2_1 = 1.01$, $P = 0.32$; heteromorph: $\chi^2_1 = 1.81$, $P = 0.18$; males: $\chi^2_1 = 2.44$, $P = 0.12$).

Overall, the probability of sexual reactions by mate-searching males differed among the three colour types (GLM, $\chi^2_2 = 81.15$, $P < 0.001$, Fig. 6); there was no significant effect of the pink paint treatment ($\chi^2_1 = 0.00$, $P = 0.99$) on the probability of sexual reactions, but colour type by pink paint treatment interaction was significant ($\chi^2_2 = 25.36$, $P < 0.001$). More specifically, controlling for total number of males, the probability of a male's sexual reaction did not differ between control andromorphic females and the pink-painted andromorphic female (GLM, $\alpha' = 0.017$, $\chi^2_1 = 0.48$, $P = 0.48$), or between control heteromorphic females and the pink-painted heteromorphic females ($\chi^2_1 = 3.12$, $P = 0.08$). In contrast, males were more likely to react sexually to pink-painted males than to control males ($\chi^2_1 = 28.28$, $P < 0.001$). The probability of sexual reactions did not differ among the three types of pink individuals, two of which had the female pattern and one had the male pattern (GLM, $\alpha' = 0.025$, $\chi^2_2 = 4.45$, $P = 0.11$).

Colour contrasts of damselflies and paint as perceived by an odonate

The green paint matched the heteromorph colour in both hue (green paint: 540.0 ± 0.0 nm, heteromorph: 543.6 ± 2.1 nm, Welch t test: $t_{5.55} = 1.80$, $P = 0.13$, Fig. 7a) and relative brightness (green paint: 10.41 ± 0.53 %, heteromorph: 9.37 ± 0.23 %, $t_{10.0} = 1.70$, $P = 0.12$). Using the visual physiology model, the chromatic (colour) and achromatic (brightness) contrasts between green-painted and natural heteromorphic females were not likely noticeable by a male damselfly in the field (chromatic: 0.63 JND, achromatic: 0.36 JND). The blue paint peaked at a shorter wavelength (459.0 ± 0.4 nm) than the naturally andromorphic blue (486.8 ± 2.5 nm, $t_{11.68} = 10.90$, $P < 0.001$), but this difference was likely not noticeable to males (chromatic contrast = 0.70 JND). However, the relative brightness of the blue paint (19.69 ± 0.42 %) was significantly greater than the natural blue of andromorphic females (11.95 ± 0.47 %, $t_{12.81} = 12.89$, $P < 0.001$, Fig. 7b). This difference may be detectable by males (achromatic contrast =1.23 JND), although the contrast value was close to the borderline value of 1.0 JND. In fact, to a damselfly, the blue paint was probably as bright as males (male brightness = 17.02 ± 0.62 %, achromatic contrast = 0.53 JND). Both orange and pink paints were noticeably different from the natural blue and green colour types in both hue and brightness (Fig. 7a).

DISCUSSION

Our study offers one of the few examples from a sexual mimicry system that demonstrates the selective use of a non-mimetic cue by receivers in sex discrimination. Male *E. hageni* used information from at least two types of visual cues, body coloration (the mimetic cue) and abdominal dorsal pattern (the non-mimetic cue) to distinguish sex. Moreover, results from experiment 1 from both arena and field suggested that the two cues were not used in the same manner. Although the lack of a significant interaction between pattern and colour in the models for data from experiment 1 restricted our ability to draw a definitive conclusion on the hierarchical use of colour and pattern cues, the Fisher's exact test for data from the arena experiment showed that the effect of pattern in male sex recognition depended on colour type. Furthermore, the GLM using the difference in response as a dependent variable revealed a significant difference in the magnitude of effect of the pattern cue among three colour types. Similarly, results from the field component of experiment 1 indicated that male attention to pattern was more important in sex recognition of andromorphs relative to heteromorphs (Fig. 5).

More specifically, Experiment 1 showed two distinct types of response to pattern alteration. Imposing a male abdominal pattern on the green heteromorph did not significantly diminish a male's ability to correctly recognize her sex. However, when the individual was blue, altering its pattern from the natural one significantly increased the probability of a male's mistake in sexual recognition. Moreover, males made more mistakes in response to the blue males with a female abdominal pattern than they did to the blue andromorphic females with a male pattern. This difference in the magnitude of the pattern effect between males and andromorphic females suggested that males did not interpret the information from pattern equally across the two blue colour types. Apparently, males paid more attention to other cues that differed between the sexes (e.g., reflectance of blue coloration, abdominal width, body size, or the presence of an ovipositor or penis) when they assessed andromorphic females, compared to when they assessed other males. A male's reaction to green-painted males with male and female patterns would have helped to infer the importance of a male's colour versus these other sexually dimorphic visual cues. In the pattern-altered heteromorph treatment, we do not know whether males only assessed the green colour cue or whether they assessed the pattern as well but chose to ignore it. Nevertheless, the use of the pattern cue enhanced the accuracy of a male's sex discrimination only when colour alone was insufficient to indicate sexual identity. In this sense, the sexually dimorphic abdominal pattern may act as a 'backup signal' (Johnstone, 1996) for sexual identity and a male's selective attention to it is potentially adaptive for males.

The value of the arena experiment was that it eliminated both types of crypsis that colour morphs enjoy under natural conditions (Schultz & Fincke, 2013). Other males, which normally serve as signal distractors for blue andromorphic females (Fincke unpubl. data) were absent. Similarly, against the white background of the arena, a normally cryptic green heteromorph was readily visible to a focal male. Finally, testing a single focal male at a time eliminated any confounding effect of male-male competition. Importantly, under these conditions, our results demonstrated that a focal male was equally good at distinguishing control andromorphic and heteromorphic females. Thus, in the analogous field experiment, the (non-significant) trend for control heteromorphs on dowels to elicit more sexual interactions relative to andromorphic females on dowels was most likely the result of the presence of male distracters, rather than some innate pre-disposition of males for green females. Learning experiments with naive *Enallagma* males (Fincke et al., 2007) also support this conclusion.

An interesting question is why the use of colour appears to be higher in the decision hierarchy than abdominal pattern. Theory has predicted that differential error rates among multiple signals can affect a receiver's optimal assessment strategy (Fawcett & Johnstone, 2003). In *Enallagma* species, the sexually dimorphic abdominal pattern is a more accurate cue to sex than colour, raising the question why males do not use the pattern cue exclusively. One reason may be due to trade-offs between speed and accuracy (Chittka & Osorio, 2007; Chittka, Skorupski, & Raine, 2009; Abbott & Sherratt, 2013). Colour may be more easily discerned at a greater distance than the details of the abdominal pattern. Being able to distinguish sex from further away should be advantageous because it allows a male to react faster, especially when there is scramble competition for mates among males in this species (Fincke, 1982). Although male *E. hageni* react to blue andromorphic females at greater distances than they do to green females (Schultz & Fincke, 2013), distance at which pattern is detected has yet to be measured.

Consistent with our results, male *E. ebrium* also cue to the sexually dimorphic abdominal pattern, (Miller & Fincke, 1999), which is characteristic of all female *Enallagma*. In that experiment, both andromorphic and heteromorphic females that were painted with a male-like blue tip and stripes on part of the abdomen tended to receive fewer sexual reactions than control females, although the trend was not significant for the andromorph ($P = 0.07$). Similarly, when the abdominal dorsum of *E. ebrium* males was painted completely black, the males received more sexual reactions than control males. The latter finding was also reported for a confamilial polymorphic damselfly *Coenagrion puella* (Gorb, 1998). Additionally, sexually dimorphic wing reflectance patterns have also been found to function in sex recognition in several odonates (Schultz & Fincke, 2009; Guillermo-Ferreira, Therezio, Gehlen, Bispo, & Marletta, 2014, reviewed by Corbet, 1999).

Results from our system were not consistent with a similar discrimination problem for avian predators learning to recognize Batesian mimics with different colours and patterns. Domestic chicks that were trained to distinguish palatable and unpalatable prey did not learn the difference between the two kinds of prey that had the same colour but differed in pattern (Aronsson & Camberale-Stille, 2008, 2012). Whether such differences generally occur between the two types of mimicry systems is unknown.

Our results from novel-coloured individuals provide insights, for the first time, on a decision rule that males seem to use in sex discrimination. Results from experiment 1 suggested that colour seemed to be higher in the hierarchy than pattern in male sex discrimination. Based on that result, we deduced two possible binary decision rules used by males to recognize sex. Males may use the decision rule:

1) 'If green, then female; otherwise, consider pattern', or
2) 'If not blue, then female; otherwise, consider pattern'

Results from Experiment 1 were consistent with both scenarios for *E. hageni*, which has only two naturally existing female colour morphs. The two seemingly similar decision rules can be differentiated by measuring male reactions to novel-coloured individuals. If males consider novel-coloured individuals as conspecifics, Rule 1 predicts that the abdominal pattern of orange and pink individuals should affect a male's reaction. In contrast, Rule 2 predicts that orange or pink-painted individuals should be recognized as 'female' regardless of their abdominal pattern.

Three pieces of evidence from male reactions to both orange- and pink-painted females in the field suggested that mate-searching males use the more general Rule 2 of 'if not blue, then female'. First, males readily reacted sexually to orange females, even to those with a male abdominal pattern. Second, not only did mate-searching males treat pink females with female patterns as 'female', but surprisingly, males were more likely to react sexually to pink-painted males with male abdominal pattern than to control males. Finally, the probability of males reacting sexually towards pink-painted andromorphic and heteromorphic females did not differ from that towards the respective control females, further supporting the use of Rule 2. Indeed, male sexual reactions towards the three kinds of pink-painted individuals, two of which had the female pattern and one of which had the male pattern, did not differ, suggesting that males did not use the abdominal dorsal pattern as a cue when the individual was pink. However, relatively few males reacted sexually to the orange-painted individuals in the arena, and as a result, the sexual reactions towards orange individuals with male pattern were not significantly different from that of control males, contradicting Rule 2. While we cannot give an explanation on this, we do know that males sometimes behave differently in enclosures from in the field (Xu & Fincke, 2011). Additionally, one focal male sexually reacted towards a control male, an event usually much rarer in enclosures (Xu, in review).

In contrast with our study, a previous one on another polymorphic species, *Nehalennia irene*, found that male mating attempts towards control males did not differ from that towards pink or black-painted males (Iserbyt & van Gossum, 2011). These results indicated that males were not using the decision rule, 'if not andromorphic coloration, then female'. However, unlike *E. hageni* males, in that study male *N. irene* often reacted sexually to control males, even more than they did to heteromorphic females, and we think this may have been one reason for the discrepancy in the findings. Compared to *Enallagma* males, males of both *Nehalennia* and many *Ischnura,* which are more similar to andromorphic females in both colour and pattern, more often mistake males for females (e.g., Cordero & Andrés, 1996; Cordero & Sánchez-Guillén, 2007). Hence, in such species, we expect that the male decision rule 'if not andromorph, then consider non-mimetic cues' should still apply, although instead of pattern, the traits would more likely be body size, abdomen width, and/or the presence of an ovipositor.

Although the decision rule for *E. hageni* male sex recognition deduced in our study was based on only two visual cues (i.e., colour and pattern), and hence, may not be a complete one, the rule has important implications for the evolution of polymorphic systems. First, the specific decision rule 'if not blue, then female' differed from Sherratt's (2001) key assumption in his signal detection model for the maintenance of female colour polymorphism, namely, that the heteromorph is always recognized by males as 'female'. Surprisingly in our field experiments, males often reacted to novel-coloured conspecifics as if they were females even without any prior experience with them. Although that result contradicted the prediction that males learn to recognize females as potential mates (Fincke, 2004), we do not know the effect of experience in the formation of a male's decision rule. Moreover, males made lots of mistakes in sexual recognition of pink individuals; our results would still be consistent with male learning if more experience with pink individuals enabled males to form search images and reduce mistakes when pink females increased in the population. Comparative studies that contrast the behaviour of sexually mature, naive and experienced males to novel morphs should offer promising insights into the evolution and development of male decision rules.

Finally and most importantly, our study provides the first empirical evidence that a general rule ‘if not andromorphic colour, then female’ could permit a novel female colour morph to invade a population because the mutant females would still be able to find mates. Hence, such a rule may offer a proximate, behavioural mechanism for the origin of female-specific colour polymorphisms, and perhaps more generally, other types of polymorphisms.

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FIGURE LEGENDS

Figure 1. Designs and predictions of Experiment 1 and 2 for the control and pattern-altered individuals of Enallagma hageni in the arena and field. C: control individuals; M: pattern manipulated individuals. The control individuals had natural coloration (males and andromorphic females were blue, heteromorphic females were green). The black parts show the natural melanin or black-painted abdominal dorsal pattern

Figure 2. **(a)** An andromorphic female E. hageni painted with the striped male abdominal dorsal pattern (above), and a natural male (below) glued to wooden dowels and **(b)** an andromorphic female E. hageni painted with pink paint on its body where the original colour was blue

Figure 3. Design of Experiment 3 for the control and pink-painted individuals of Enallagma hageni in the field. The control individuals had natural coloration. The abdominal dorsal patterns were unaltered in this experiment

Figure 4. Number of responses by focal male Enallagma hageni in paired presentations of control and pattern-altered individuals in the arena. Responses in the female pattern and the male pattern treatments from each colour type on the dowel (i.e., andromorph, heteromorph and male) are from the same focal males. The numbers above the bars show numbers of focal males

Figure 5. Mean (± S.E.) proportion of male Enallagma hageni exhibiting sexual reactions towards 6 control and 6 abdominal dorsal pattern-altered andromorphic, heteromorphic and male Enallagma hageni in the field

Figure 6. Mean number (± S.E.) of sexual and non-sexual reactions per 10 min from mate-searching males towards the control and pink-painted andromorphic, heteromorphic and male Enallagma hageni in the field. The numbers above the bars show numbers of focal males

Figure 7. The relative reflectance of **(a)** heteromorphic female and **(b)** male and andromorphic female Enallagma hageni and the paints used to alter the abdominal dorsal pattern. Damselflies were from the study population at Duck Lake, Sugar Island, MI, USA

Figure 1

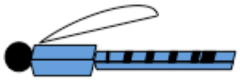
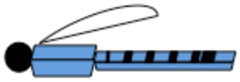
colour type	pattern		predictions: % sexual reaction
	control (C)	manipulated (M)	
male			C < M
andromorph			C > M
heteromorph			C = M
orange			C = M

Figure 2

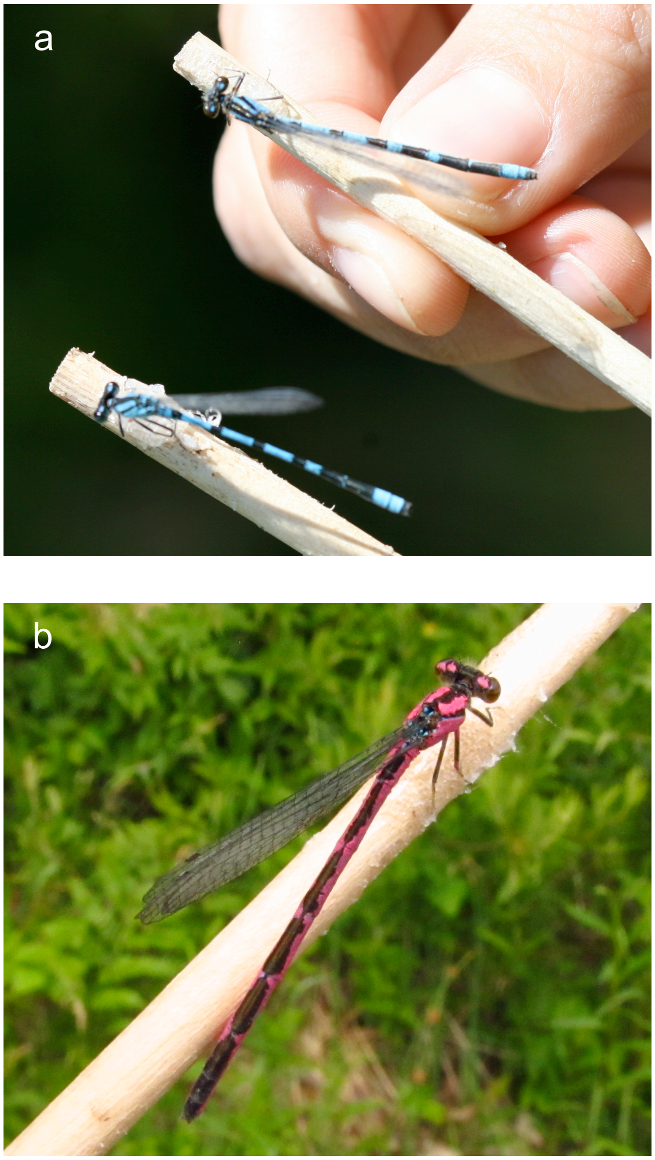


Figure 3

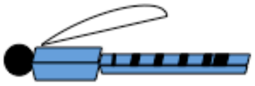
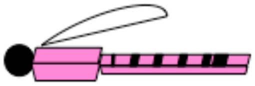
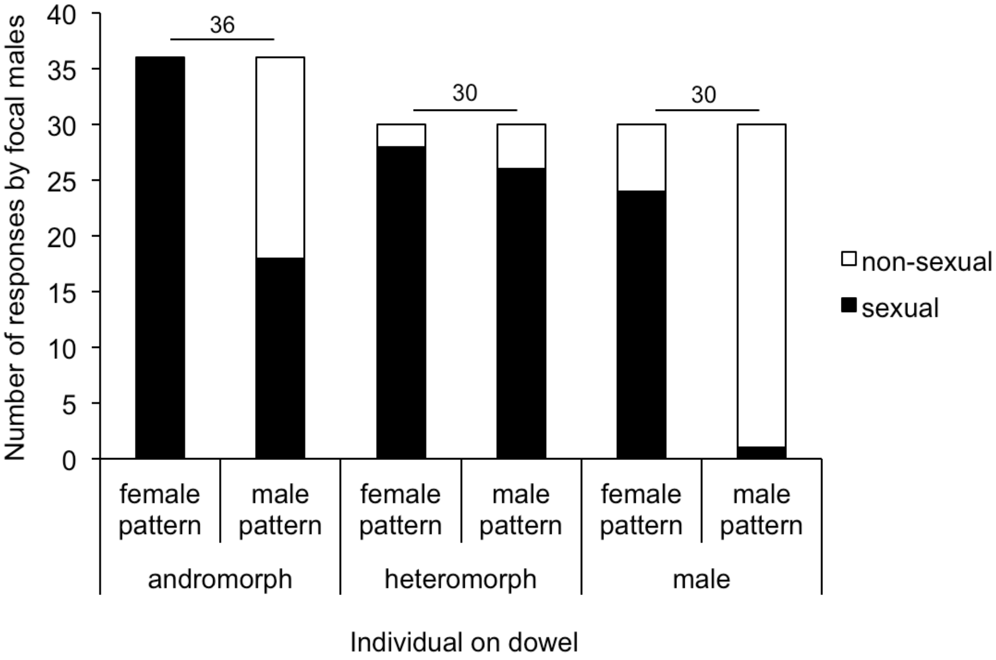
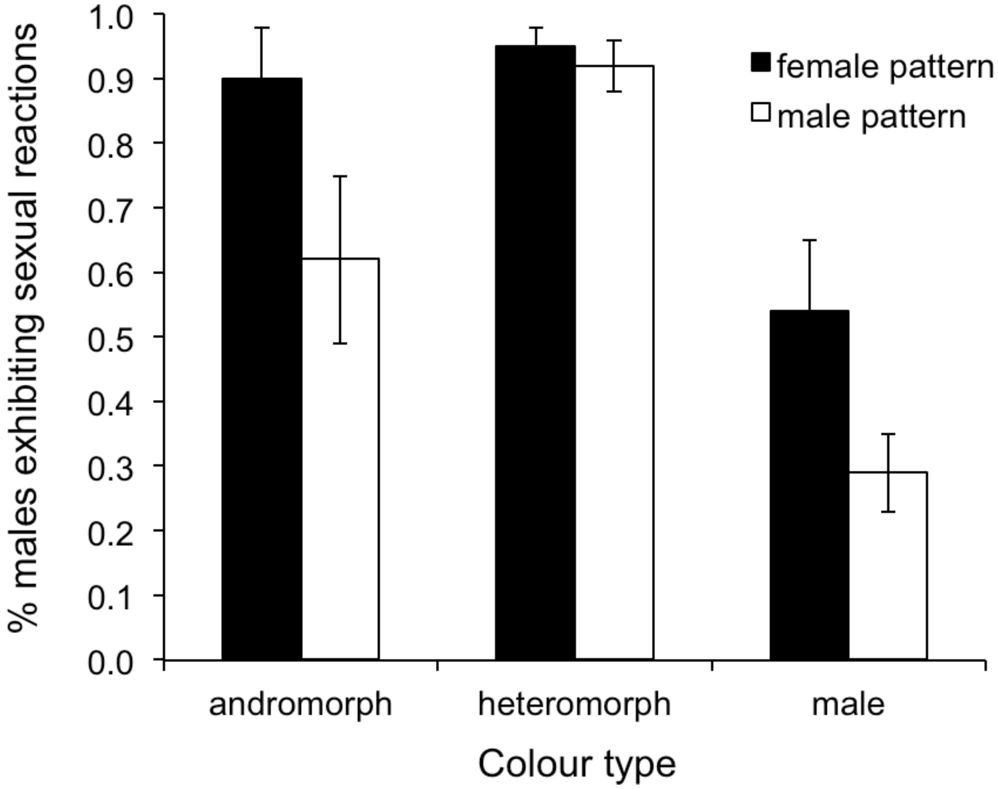
individual type	colour treatment	
	control	pink
male		
andromorph		
heteromorph		

Figure 4



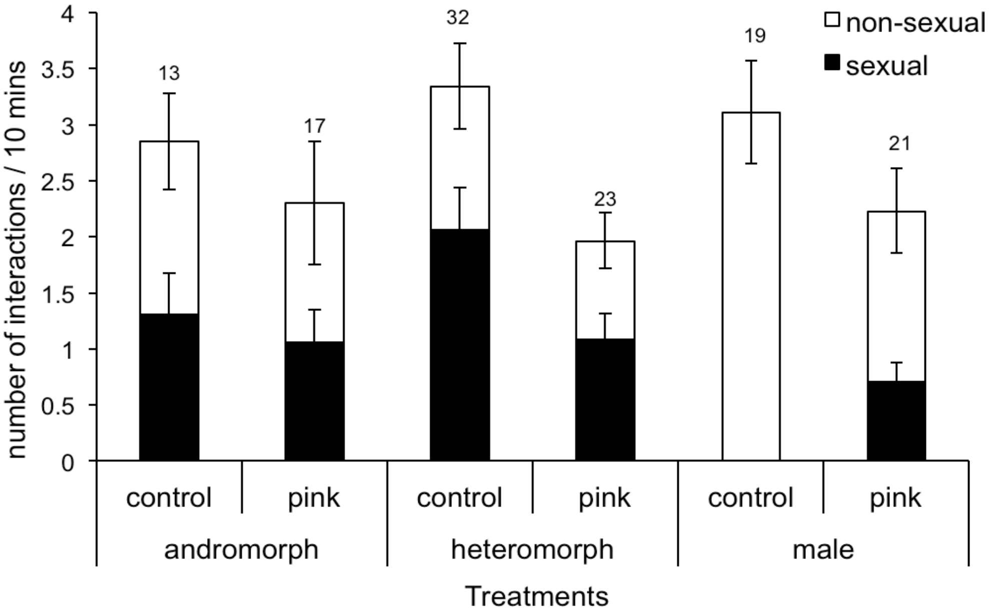
36
30
30
40
35
30
25
20
15
10
5
0
Number of responses by focal males
female pattern
male pattern
female pattern
male pattern
female pattern
male pattern
andromorph
heteromorph
male
Individual on dowel
□ non-sexual
■ sexual

Figure 5



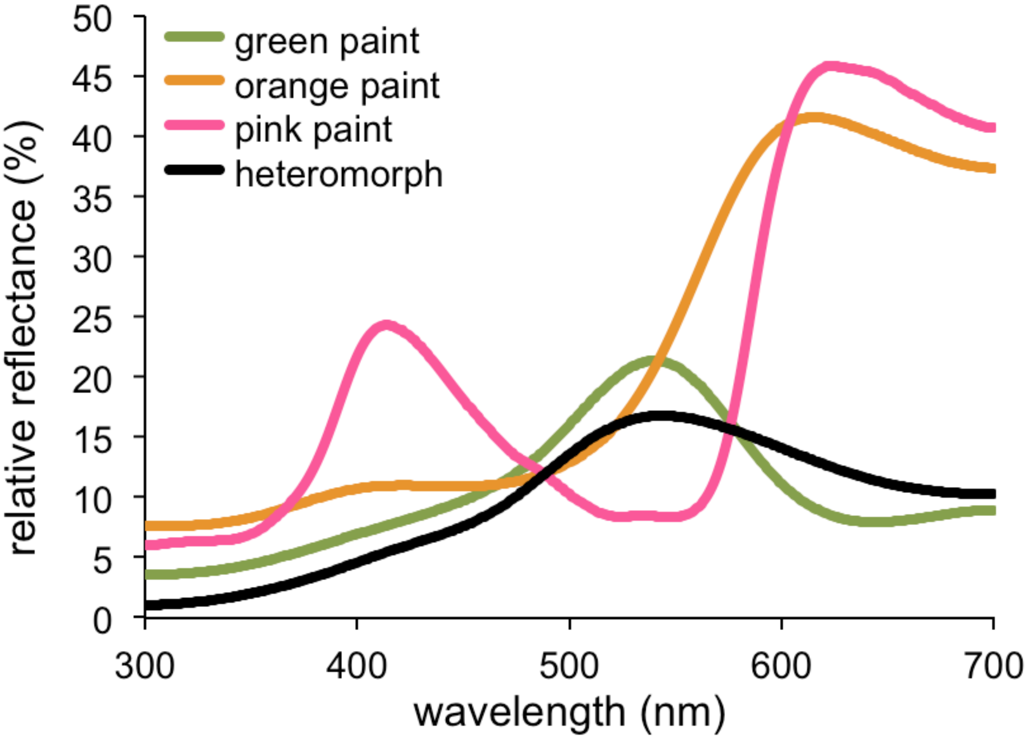
% males exhibiting sexual reactions
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
■ female pattern
□ male pattern
andromorph
heteromorph
male
Colour type

Figure 6



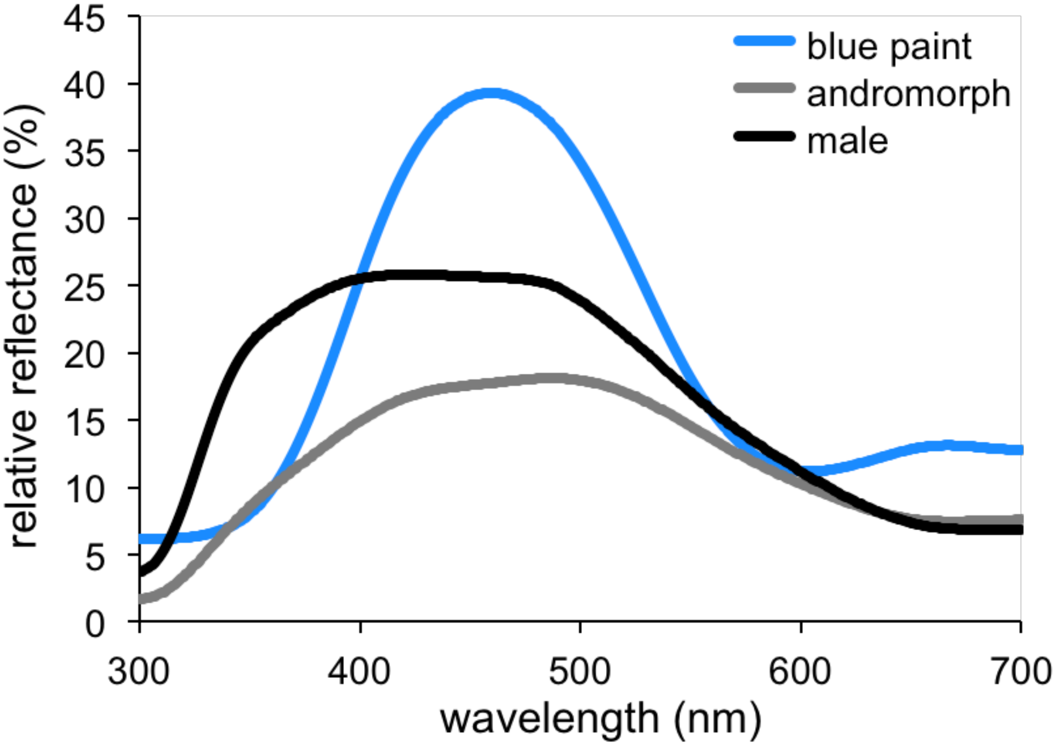
number of interactions / 10 mins
0
0.5
1
1.5
2
2.5
3
3.5
4
13
17
32
23
19
21
☐ non-sexual
■ sexual
control
pink
control
pink
control
pink
andromorph
heteromorph
male
Treatments

Figure 7a



50
45
40
35
30
25
20
15
10
5
0
relative reflectance (%)
green paint
orange paint
pink paint
heteromorph
300
400
500
600
700
wavelength (nm)

Figure 7b


45
40
35
30
25
20
15
10
5
0
relative reflectance (%)
300
400
500
600
700
wavelength (nm)
blue paint
andromorph
male

Chapter 4: Ultraviolet wing signal affects territorial contest outcome in a sexually dimorphic damselfly

[At the time of dissertation submission, this chapter was in review at a journal as Xu, M., Fincke, O. M. A male wing color trait offers honest signal and incurs differential cost of cheating in a territorial damselfly]

ABSTRACT

Ultraviolet (UV) reflectance and UV vision are both common among animals, and are known to function in mate choice and male-male competition among numerous vertebrates. In comparison, examples of functional UV reflectance among invertebrates are scarce. In a territorial damselfly *Megaloprepus caerulatus*, data from natural territorial contests indicated that males assessed the male wing band of rivals. We investigated the functions of (1) UV reflectance of the male-specific white wing band in territorial contests by staging contests between size- and age-matched, control and UV-reduced males, and (2) UV reflectance of the female-specific wing tip in sex recognition by presenting control and UV-reduced females to territorial males. Results showed that males whose UV reflectance of the white wing bands was reduced were more likely to lose contests. This effect dissipated late in the reproductive season, when breeding sites typically decrease in value. UV reflectance of the female wing tips did not affect male sex recognition, nor did it affect the detectability of a female at a male territory. Our study provides the first direct evidence from invertebrates, and one of the few among all animals, that UV reflectance of wing ornamentation affects the outcome of male contests in the field.

INTRODUCTION

Colouration of organisms was recognized by early naturalists as a key target of natural and sexual selection (Darwin, 1859; Bates, 1862). Today, we have a much better understanding of the diverse roles human-visible colour traits play in animal communication systems, such as the cryptic colouration of peppered moths (Cook, 2003) to avoid detection, the warning colour of poisonous frogs to facilitate predator learning (Maan & Cummings, 2012), the nuptial colours of fish to attract mates (Kodric-Brown, 1998), and colouration as signals of individual, sex or species identity (Sætre & Slagsvold, 1992; Couldridge & Alexander, 2002; Tibbetts, 2002). However, unlike many animals, humans do not see ultraviolet (UV) light (wavelengths between 200-400 nm, Bennett, Cuthill, & Norris, 1994). Although UV reflectance was described in insects more than four decades ago (Silberglied & Taylor, 1973; Silberglied, 1979), only recently has it been demonstrated that UV reflection (Eaton & Lanyon, 2003) and sensitivity to UV (Jacobs, 1992; Marshall & Oberwinkler, 1999; Briscoe & Chittka, 2001; Douglas & Jeffery, 2014) are much more widespread in animals than previously thought.

A growing body of work has documented diverse functions of UV reflectance in different signaling systems. Birds and bees are known to use the UV reflectance of fruits and flowers as a guide in forging (Jones & Buchmann, 1974; Wheelwright & Janson, 1985; Chittka, Shmida, Troje, & Menzel, 1994; Siitari, Honkavaara, & Viitala, 1999). UV reflectance can also be used as a private communication channel by prey. For example, male northern swordtails (*Xiphophorus*) use conspicuous UV reflectance to attract females, while remaining cryptic to their major predator, the Mexican tetra (*Astyanax mexicanus*), which cannot detect UV (Cummings, Rosenthal, & Ryan, 2003). On the other hand, predators, such as the web-building spider, *Argiope argentata*, are known to use its UV-reflective dorsal surface to attract flower-seeking pollinator prey (Craig & Ebert, 1994). Finally, UV reflectance is an important sexual signal, used in sex recognition (Lim, Land, & Li, 2007; Ries, Spaethe, Sztatecsny, Strondl, & Hödl, 2008; Guillermo-Ferreira, Therezio, Gehlen, Bispo, & Marletta, 2014) and female mate choice (Bennett, Cuthill, Partridge, & Lunau, 1997; Johnsen, Andersson, Ornborg, & Lifjeld, 1998; Hunt, Cuthill, Bennett, & Griffiths, 1999; White, Partridge, & Church, 2003; Robertson & Monteiro, 2005; Liu, Siefferman, Hill, 2007; Lim, Li, & Li, 2008; Detto & Backwell, 2009; Bajer, Molnar, Torok, Herczeg, 2010; but see Kurvers, Delhey, Robert, & Peters, 2010) among a broad range of taxa.

Less clear is whether UV reflectance affects male-male competition (Stapley & Whiting, 2006). Several examples from vertebrates indicate that UV reflectance influences the escalation and duration of male-male competition in the damselfish *Pomacentrus amboinensis* (Siebeck, 2004), three-spined stickleback *Gasterosteus aculeatus* (Rick & Bakker, 2008), Augrabies flat lizard *Platysaurus broadleyi* (Stapley & Whiting, 2006, Whiting, Stuart-Fox, O'Connor, Firth, Bennett, et al, 2006), blue tit *Cyanistes caeruleus* (Remy, Gregoire, Perret, & Doutrelant, 2010) and eastern bluebird *Sialia sialis* (Siefferman & Hill, 2005). Yet only a handful of studies have shown that UV reflectance affects the outcome of male-male competition in vertebrates, including the European green lizard *Lacerta viridis* (Bajer, Molnar, Torok, & Herczeg, 2011) and blue tit (Vedder, Schut, Magrath, & Komdeur, 2010), and few studies have been conducted in field populations.

Among invertebrates, and insects especially, we know surprisingly little about the role of UV in male-male competition (Lim, & Li, 2006; Detto, & Backwell, 2009; but see Rutowski, 1992; Kemp, & Macedonia, 2006), with no direct evidence that UV reflectance affects contest outcome. Therefore, our knowledge is taxonomically biased towards vertebrates (Detto, & Backwell, 2009). Even among invertebrates, studies have mainly focused on butterflies and jumping spiders. Consequently, the question of whether UV reflectance functions in agonistic interactions among invertebrates remains largely unanswered.

The broad-winged neotropical damselfly, *Megaloprepus caerulatus*, exhibits sexually dimorphic wing colouration and flight kinematics that are well adapted for sexual signaling (Schultz, & Fincke, 2009; Hilfert-Rüppell, & Rüppell, 2013). In Panama, both males and females possess iridescent blue bands on the wings, whereas males have a sex-specific white band proximal to the blue band. In contrast, females have white tips distal to the blue band. The female white tips signal sexual identity (Schultz, & Fincke, 2009), yet it was unclear which colour component has the signaling function. The white bands and tips are structural colours that reflect strongly in the UV (Schultz, & Fincke, 2009). Like most insects, odonates can detect UV (Yang, & Osorio, 1991; Schultz, Anderson, & Symes, 2008; Huang, Chiou, Marshall, & Reinhard, 2014).

Male *M. caerulatus* defend forest light gaps that contain large, water-filled tree holes where females come to mate and lay eggs (Fincke, 1984; 1992a). Females do not exert direct mate choice of male phenotype but by mating only at territories, they indirectly select larger males; body size predicts fight outcome (Fincke, 1992b). The proportion of the wing surface covered by the white band is positively correlated with male body size (Schultz, & Fincke, 2009), suggesting a potential signaling role.

In the present study, we first determine whether rivals assess the white wing band and the type of assessment they use in natural contests (i.e., self-assessment, opponent-assessment, mutual assessment, Arnott, & Elwood, 2009; Elwood, & Arnott, 2012). In terms of receiver psychology, we hypothesize that assessment should be mutual, and that longer contests are more escalated. Here, we define "escalation" as contests that involve more behavioral elements and energetically more expensive behaviors (Enquist, Leimar, Ljungberg, Mallner, & Segerdahl, 1990). We subsequently investigate the potential functions of the UV reflectance of the male white wing bands and female white wing tips in two field experiments. Specifically, we hypothesize that the UV reflectance of the male white wing band signals male fighting ability during the territorial contests. Additionally, we hypothesize that the UV reflectance of the female wing tips is an indispensible component of the sexual identity signal used in sex recognition by territorial males. Further, because UV reflectance is conspicuous against UV-absorptive vegetation background (Schultz, & Fincke, 2009), we hypothesize that the UV reflectance of the female wing tips may also enhance the detectability of a female to a territorial male.

METHODS

Study site

The study was conducted in the lowland moist forest on Barro Colorado Island (hereafter BCI), Panama (9.15° N, 79.85° W), where the dry season begins in December and continues until late April (Windsor, 1990). The 9-month reproductive season of *M. caerulatus* begins in the wet season after tree holes fill with water, and continues through mid dry season. Most holes dry up by the end of March (Fincke, 1992b).

Rival assessment during natural contests

We quantified male agonistic behavior and the duration of 50 natural contests from unique dyads of unmanipulated males between July 1996 and February 1997, July 1997 and February 1998, and between December 2010 and February 2011. We marked each male with a unique number on the left hindwing with a black permanent Sharpie marker pen (Sanford Corporation, Keysborough, USA) and measured left forewing length as a proxy for body size (Fincke, & Hadrys, 2001), width of the white band (details in Schultz, & Fincke, 2009) and territorial status (i.e., resident or intruder). Male behaviors during the contests were noted as ‘chase’, ‘contact’, ‘face-off’ and ‘spiral flight’. During a ‘face-off’ each male beats its wings synchronously while directly facing the opponent. In an upward ‘spiral’ two males circle upward while facing each other. Face-off and spiral flight are more expensive than forward flight (i.e., chase) and contact, and spiral flight is the most energetically expensive (Rüppell, & Fincke, 1989; Dudley, 2000). We assigned each behaviour an escalation score between 1 (low) and 4 (high). We used the criteria that energetically more expensive behaviours are more escalated, and among low-cost behaviours, contact behaviour is more escalated than non-contact behaviour (i.e., chase). This resulted in the following escalation scores: 1 = chases; 2 = contacts; 3 = face-offs; 4 = spiral flight. Total contest duration was the sum of all interaction durations (not including perching time) that occurred before the loser left the gap and did not return for ≥ 30 min. All contests occurred in the absence of females. Male characteristics were measured from only marked males, whereas contest duration and behavioral components of contests were quantified from both marked and unmarked males.

Design of UV manipulation experiments

UV manipulation experiments were conducted over two dry seasons (December 13, 2008-Feburary 27, 2009 and December 25, 2010-Feburary 24, 2011) at one natural tree hole and five artificial tree holes (i.e. plastic basins tied to the trunk of fallen trees in light gaps). Throughout the experiment, water volume in all experimental holes was kept at 4 liters. All individuals used in the experiments were collected in the field, and marked and measured as above. Age category of an individual (i.e., young, medium or old) was scored by the wear on the wings.

For tests of both hypotheses on UV function, we compared a UV-reduced treatment with a control treatment. Individuals in the UV-reduced treatment were gently painted with a thin layer of Neutrogena ultra sheer dry-touch sunscreen (SPF45, Neutrogena, Los Angeles, USA) on the white bands or tips. We had two types of controls: natural controls were individuals whose white bands or tips were intact, and sham controls were individuals that were painted with a thin layer of UV-reflective zinc white gouache paint (Winsor & Newton, Piscataway Township, USA) on the white wing bands or tips. To assess the effectiveness of the sunscreen treatment and how closely the zinc paint matched the natural wing colour, we measured the relative reflectance of 3 individuals of each sex before and after the sunscreen treatment and the zinc paint treatment with an OceanOptics SD 2000 spectrometer and a PX-2 xenon light source (OceanOptic, Dunedin, USA). To specifically compare brightness of sunscreen-treated males and sham controls, we measured relative reflectance of 6 females, each treated with zinc paint on the left hindwing and sunscreen on the right hindwing. We interpolated the spectrum and calculated brightness in the UV and human visible range for each individual in Avicol v.6 (Gomez, 2006). The sunscreen treatment significantly decreased UV reflectance of the white bands and tips comparing to those of the natural controls (Fig. 1a, natural control: 7.58 ± 1.19 %, UV-reduced: 2.99 ± 0.68 %, paired t-test, $t_5 = 16.65$, $p < 0.001$), but it also significantly increased brightness in the human visible range (wavelength 400-700 nm, natural control: 5.74 ± 0.94 %, UV-reduced: 7.13 ± 1.36 %, $t_5 = 2.67$, $p = 0.04$). As expected, the zinc paint did not significantly change reflectance in either the UV range (Fig. 1b, natural control: 7.84 ± 0.64 %, zinc-painted: 8.07 ± 0.56 %, $t_5 = 0.63$, $p = 0.56$) or the human visible range (natural control: 6.12 ± 0.64 %, zinc-painted: 6.69 ± 0.56 %, $t_5 = 1.50$, $p = 0.19$). From 6 females that were treated with both sunscreen and zinc paint, the brightness in the human-visible region did not differ significantly (sunscreen: 8.01 ± 0.48 %, zinc paint: 7.40 ± 0.41 %, $t_5 = 0.85$, $p = 0.44$).

Does UV reflectance predict the outcomes of male territorial contests?

We paired males of similar size and within the same age category into 26 dyads. In each dyad, one male was randomly assigned to the UV-reduced treatment, and the other male was either left intact as a natural control (N = 18) or was sham painted with zinc paint (N = 8). One male in a dyad was the original resident male at the tree hole, and the other was a territorial resident collected from another defended site. To arrange a contest, the non-resident male was released at the experimental hole either while the original resident was out of the gap foraging, or after it was gently chased away from the gap. When the introduced male failed to perch near the hole, a tethered female was allowed to fly near the tree hole until she attracted the male's attention, but before any interaction occurred. A dyad was used in the analysis only if the introduced male exhibited territorial behavior (e.g., fluttered at the water surface and perched directly above the hole) before a fight occurred such that both males had a chance to establish residency. A territorial contest began when the original resident returned to the gap and engaged the new male. Contest duration and outcome was determined as above. All dyads used a unique combination of males. Five males were used in two different dyads, however, they were assigned to different treatments and different territorial status (i.e., the original or the new resident).

Does UV reflectance of females affect male sex recognition?

We presented 17 territorial males with a UV-reduced female and a control female (N = 12 natural controls, N = 5 sham controls) from the same age category. The females were presented sequentially with the sequence randomly assigned. The females were tethered with 8× (0.076 mm diameter) flyfishing line (Black Knight Industries, Inc., Oil City, USA) around the groove between the head and the thorax, anterior to the mesostigmal plates such that it did not interfere with tandem formation. The other end of the line was tied to a twig held by the experimenter. A presentation began by fluttering a female 1 m directly in front of a perched male. Male responses were noted as: hover, grab, tandem attempt and tandem. Hover was regarded as a non-sexual reaction, whereas the rest were regarded as sexual reactions. The presentation ended if the male reacted sexually; otherwise, the presentation was repeated 3 times and the most extreme reaction was used for analysis. Additionally, for a subset of 11 focal males, we recorded reaction time towards females. Male reaction time, a proxy for the detectability of females, was defined as the time elapsed from the beginning of the presentation to when a focal male took off from the perch to fly towards the female. Afterwards, females were released from the tethers with no fishing line attached, and all flew away. No female was injured from tethering.

Statistical analyses

Durations of natural contests were log transformed for all analyses. We examined the correlation between contest duration and the difference between rivals in body size and signal size (i.e., absolute value of each difference). We also investigated the correlation between contest duration and number of behavioural elements and the escalation level. Because body size decreases across the reproductive season (Fincke, & Hadrys, 2001), we controlled for day of season (first day set as May 1) using part (semipartial) correlations.

We examined whether males from the control or the UV-reduced treatment were more likely to win the contests with a binomial test. We also tested whether any possible confounding factors could have affected the contest outcomes. We used a binomial test to investigate whether males of different territorial status (i.e., original or new resident) differed in the probability of winning. Further, we calculated the difference in the body sizes of contestants as the forewing length of the winner minus the forewing length of the loser, and tested if this value was significantly different from zero with a one-sample t-test. The effect of year (2008-2009 or 2010-2011 study period), control type (natural or sham), and number of days since the proximate beginning of the dry season (December 1) on which treatment was more likely to win was examined with generalized linear models (GLM). We used the treatment of the winner (i.e., control or UV-reduced) as a binary dependent variable for the GLMs.

We used a generalized linear mixed model (GLMM) with binomial error distribution to investigate whether UV reflectance of the wing tips signaled a female's sex identity to males. In this model, focal male reaction (i.e., sexual vs non-sexual) was used as a binary dependent variable; focal male was treated as a random effect, and female treatment (control or UV-reduced) was nested within focal male. Additionally, we investigated whether control and UV-reduced females differed in their detectability to territorial males by comparing male reaction times towards the two female treatments. Because male reaction time was not normally distributed even after a log transformation (Shapiro-Wilk test, control: $W = 0.51$, $p < 0.001$, UV-reduced: $W = 0.76$, $p = 0.002$), we used the Wilcoxon signed ranks test for the comparison.

Statistical tests were performed in R (version 2.15.1, R Core Team, 2012) and SAS (version 9.1, SAS Institute Inc, Cary N.C.). Throughout, means are shown with standard errors.

RESULTS

Male assessment of rivals in natural contests

Contest duration decreased as the reproductive season progressed ($r_{48} = -0.44$, $p = 0.001$). Controlling for day of the reproductive season, contest duration decreased with the difference in white band size between the two males (Fig. 2, $r_{48} = -0.29$, $p = 0.04$), but not with difference in body size ($r_{48} = -0.20$, $p = 0.17$). Contest duration was negatively correlated with winner band size ($r_{48} = -0.34$, $p = 0.02$) but not with loser band size ($r_{48} = 0.06$, $p = 0.66$).

Larger males and males with larger white bands were more likely to win contests (binomial test, body size: $p < 0.001$, band size: $p < 0.001$). The resident male in a dyad was more likely to win the contest (binomial test, $p < 0.001$), but was on average 3.2 ± 1.08 mm larger than the intruder rival (one-sample t-test, $t_{49} = 3.17$, $p = 0.003$). Of the 40 residents winning contests, 7 were smaller or the same size as their rival whereas of the 10 intruders that won contests, 3 were smaller than their resident rival. Hence, a resident had no inherent advantage over larger rivals (Fisher's exact test, $p = 0.40$). After controlling for day of reproductive season and white band size difference, the duration of contests won by residents did not differ from those won by intruders (ANCOVA, $F_{1,45} = 1.08$, $p = 0.31$).

Territorial contests were stereotypic and acrobatic. Chases were the most common, occurring in 90% of the fights, followed by contact (55%), spiral flight (31%), and face-offs (27%). The number of behavioural elements increased with contest duration ($r_{71} = 0.32$, $p = 0.006$). The level of escalation attained in a contest also increased with contest duration ($r_{71} = 0.28$, $p = 0.017$). Controlling for the difference in size of white bands, contests with one or more face-offs tended to be shorter than those without any face-offs (ANCOVA, $F_{2,43} = 3.24$, $p = 0.08$).

The function of UV reflectance of male white bands

Year and control type had no effect on which treatment won the contest (GLM, year: $\chi^2_1 = 0.005$, $p = 0.95$; control type: $\chi^2_1 = 0.24$, $p = 0.62$). Therefore, we pooled results from the two years and two control types. Thirteen out of 26 contests were won by the smaller males in the dyads. The body size difference between the winner and the loser (0.26 ± 0.78 mm) was not significantly different from zero (one-sample t-test, $t_{25} = 0.33$, $p = 0.74$), and the larger male in a dyad did not enjoy a higher probability of winning than the smaller male (binomial test, $p = 1.00$). Additionally, original residents and new residents were equally likely to win contests (binomial test, $p = 0.33$).

Among the 26 dyads, control males won in 18 dyads. Overall, control males and UV-reduced males did not differ in their probability of winning the contests (binomial test, $p = 0.08$). However, the number of days since December 1 had a significant effect on which treatment won the contest (GLM, $\chi^2_1 = 6.29$, $p = 0.01$). Thus, we divided each study period into roughly two halves in number of days: early dry season (Dec. 13 – Jan. 24, 13 dyads) and late dry season (Jan. 25 – Feb. 27, 13 dyads). All 8 cases where the UV-reduced males won the contests occurred during the late season, whereas during early season, control males won all contests; this seasonal effect was repeatable for two field seasons. In early dry season, control males were more likely to win the contests (Fig. 3, binomial test, $p < 0.001$). In contrast, in late season, control males and UV-reduced males did not differ in their likelihood of winning the contests (Fig. 3, binomial test, $p = 0.58$). Moving the dividing date for ± 10 days did not change the above conclusions. After size-matching, winners and the losers did not differ significantly in body size in either the early season (absolute size difference: 0.20 ± 1.30 mm, one-sample t-test, $t_{12} = 0.16$, $p = 0.88$) or the late season (absolute size difference: 0.32 ± 0.94 mm, $t_{12} = 0.34$, $p = 0.74$).

The function of UV reflectance of female wing tips

Focal male reactions towards sham control females did not differ from reactions towards natural control females (GLM, $\chi^2_1 = 0$, $p = 1.00$). Therefore, we pooled results from the two types of controls in the following analyses. Among 17 focal males tested, 13 males reacted sexually towards both control and UV-reduced females, 4 males reacted sexually towards control females and non-sexually towards UV-reduced females (Fig. 4). There was no difference in focal male reactions towards the control and UV-reduced females (GLMM, $\chi^2_1 = 0$, $p = 0.997$). Male reaction time towards control females (mean: 1.5 s, median: 1.0 s, interquartile range: 0.0 s) did not differ from that towards UV-reduced females (mean: 2.5 s, median: 1.0 s, interquartile range: 4.0 s, Wilcoxon signed rank test, $W_{10} = 4.5$, $p = 0.25$).

DISCUSSION

Our results indicated that UV reflectance of a male wing colour ornament can affect the outcome of male territorial contests in a damselfly. This is, to our knowledge, the first evidence from an invertebrate that UV reflectance functions in resolving male-male competition. At least two previous studies have investigated the function of UV reflectance *per se* in the display component of male agonistic interactions among invertebrates. In a fiddler crab, results showed that the UV reflectance of the male major claw was used only by females in mate choice, not by male competitors to choose with whom to fight (Detto, & Backwell, 2009). In contrast, in a salticid spider, *Cosmophasis umbratica*, males showed more agonistic elements in their display of body colouration under UV light source than when ambient UV light was absent (Lim, & Li, 2006). These studies offered pioneering insights into the function of UV in the dynamics of male contests, yet because in these studies males could not actually fight because they were either tethered or were kept in separate compartments in the arena, they could not tell us whether UV reflectance can affect contest outcomes. In another study on the effect of UV-green iridescence on *C. umbratica* male contests, the band separation (i.e., the difference in wavelengths between the UV and the green reflectance peaks), instead of the hue (i.e., the wavelength at the reflectance peak) of UV or green colouration correlated with which male initiated the fight and how long a male persisted, but neither band separation nor the hue of UV affected contest outcome (Lim, & Li, 2013). By isolating the UV reflectance from other wavelengths and by staging male contests between size and age matched males in nature, our results provide the first direct evidence from an invertebrate that males use information from UV reflectance in settling their territorial contests. This result is consistent with findings from vertebrates that stronger UV reflectance gives males an advantage in winning male-

male agonistic interactions (e.g., Siefferman, & Hill, 2005; Vedder, Schut, Magrath, & Komdeur, 2010; Bajer, Molnar, Torok, & Herczeg, 2011; but see Stapley, & Whiting, 2006).

Natural contest duration was correlated with the difference in male white band size, but surprisingly, not with difference in male body size measured by wing length, although larger males, independent of territorial status, were more likely to win the contests (see also Fincke, 1992b). Thus, our results strongly suggested that it was the white band that males assessed in settling their territorial conflicts. Because the proportion of the wing covered by the white band is a predictor of body size (Schultz, & Fincke, 2009), the conspicuous white band may serve as a ‘biological billboard’ (sensu “amplifier”, “exaggerator”, Hasson, 1991; Maynard Smith, & Harper, 2003) to facilitate the assessment of body size that could otherwise be difficult to perceive in the scramble of the fight. In contrast with predictions of self or mutual assessment, contest duration did not increase with the white band of losers. Rather, our results (Fig. 2) were most consistent with opponent-only assessment (Arnott, & Elwood, 2009; Elwood, & Arnott, 2012). As expected, the longer a contest lasted, the more escalated it became as the number of behaviours and more costly behaviours increased. The most energetically expensive behaviors, face-offs and spirals, were relatively rare. Such contests are akin to those of cichlid fish, which assess rivals sequentially (Enquist, Leimar, Ljungberg, Mallner, & Segerdahl, 1990). Face-offs, the most obvious wing displays, seemed to decrease contest duration (a marginally significant result after controlling for differences in white band between rivals).

Results from observations of natural contests indicated that it was the size, specifically, of the white wing band males assessed during their territorial contests. The UV manipulation experiment further suggested that males specifically assessed the UV reflectance of the white band during territorial contests, a conclusion consistent with other work that suggested that UV acts as a signal of male quality. In an experiment that varied food availability among full-sibling *M. caerulatus* reared as larvae, UV reflectance of the adult wings had the highest signal variation among all other colour components (i.e., short wavelength: 400-500 nm, medium wavelength: 500-600 nm and long wavelength: 600-700 nm, Xu, 2014). In addition, UV showed the strongest response to food treatment and was the only colour component that was correlated with body size and fat reserves, two major body condition measures.

Interestingly, the effect of UV reflectance on contest outcome depended on the time during the field season: the UV reflectance affected contest outcome but not late in the reproductive season. One possible explanation for this effect is that late-season males do not pay as much attention to the signal as early dry season males do because late-season males are reluctant to fight as holes diminish in value late in the season (i.e. larvae yet to emerge die when holes dry up). Indeed, we found that duration of natural contests decreased as the reproductive season progressed. Eventually, males abandon holes even when they are kept full, because high heat and low humidity of late dry season became intolerable (Fincke, 1992b; Fincke, & Hedström, 2008). Although the sunscreen treatment increased brightness in the human-visible region, we suggest that the lower probability of winning for UV-reduced males was not due to increased brightness because brightness of sham control males was also increased and did not differ from that of sunscreen-treated males, which nevertheless were less likely to win fights.

A contest can be settled by asymmetries in many factors, such as body size or weight, past experience, existence or size of weaponry, physiological status, sensory cues (Arnott & Elwood 2009), as well as territorial status (Jennion, & Backwell, 1996; Kemp, & Wiklund, 2001). Further, the value of the resource is known to affect the cost, duration, and outcome of contests (Enquist, & Leimar, 1987). In this study, we explicitly controlled the effect of body size, age and territorial status of the two males in a dyad; hence not surprisingly, none had a significant effect on the contest outcome. Even in late field season when the UV reflectance did not affect contest outcome, the slight size difference between the contestants after size-matching did not affect contest outcomes. Therefore, contests in late season may have been settled by other asymmetrical qualities or a male's motivation to obtain a territory, if they were not settled completely randomly.

Although the female white wing tips is a signal of sex identity to males (Schultz, & Fincke, 2009), it was unclear which colour is the signaling component. Here, we demonstrated here that reducing the UV reflectance of the wing tip signal had no effect on a male's sex recognition. Therefore, UV is likely not a functional component of the sex identity signal in this species. Our results differ from findings from another territorial damselfly *Mnesarete pudica*, whose males were less likely to react towards mature, UV-reduced females relative to controls (Guillermo-Ferreira, Therezio, Gehlen, Bispo, & Marletta, 2014). In that species, UV reflectance of the wing colouration is likely a signal of sexual maturity because both juvenile males and females have significantly lower UV reflectance than sexually mature adults. Indeed in *M. pudica*, male reactions towards UV-reduced females were identical to those towards juvenile females. In contrast, the UV reflectance of juveniles and sexually mature individuals in *M. caerulatus* does not show such an ‘all-or-none’ qualitative difference (Xu, 2014), and so it was not surprising that we did not find a similar function of the UV reflectance. In addition, although the UV reflectance of the female wing tips are conspicuous against the UV-absorptive forest background, males did not react faster to females with UV blocked wing tips but our sample size was low (n = 11).

Our study provides the first evidence from an invertebrate that UV reflectance of a colour trait is used by males to settle territorial contests, and it provides one of the few examples among animals that UV reflectance affects the outcome of contests in the field. Opponents likely use the size of a male’s white band as an advertisement for overall male size. Our UV reflectance manipulation by applying sunscreen, decreased UV reflectance without decreasing the size of the white color signal, suggesting that males are responding to the decrease in the UV component. However, because sunscreen reduced roughly half of the UV reflectance at the wavelength of the UV receptor, it will be important to quantify natural variation in UV reflectance among males in the field, and investigate whether difference in UV reflectance within the range of the natural variation could also affect the outcome of contests. Finally, our experiment does not exclude the possibility that other wavelengths could play a role in sexual communication (Hunt, Cuthill, Bennett, Church, & Patridge, 2001). Hence, it would be interesting to manipulate other wavelengths and compare the relative effect of different colour components.

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FIGURE LEGENDS

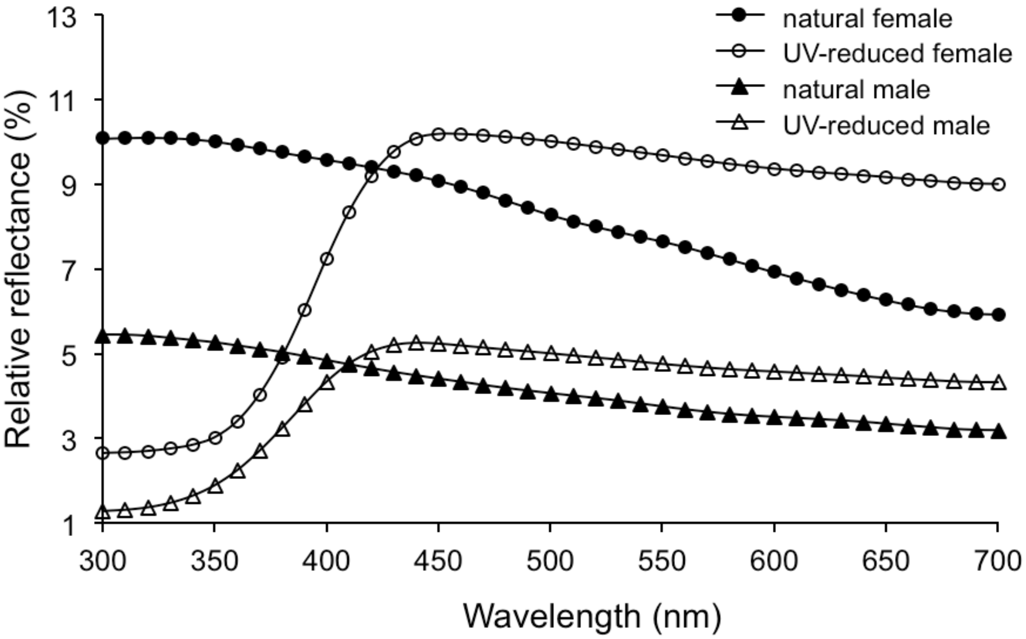
Figure 1. Relative reflectance of *Megaloprepus caerulatus* **(a)** natural and sunscreen-treated male wing bands (N = 3) and female wing tips (N = 3) and **(b)** natural and zinc-painted male wing bands (N = 3) and female wing tips (N = 3).

Figure 2. After controlling for day of reproductive season, relationship between contest duration of 50 natural fights between unmanipulated *Megaloprepus caerulatus* males and **(a)** absolute difference in white band size between contestants, **(b)** winner white band size and **(c)** loser white band size. Standard residuals from the regression between contest duration and day of reproductive season are used as the dependent variable.

Figure 3. Numbers of size- and age-matched dyads of *Megaloprepus caerulatas* won by control or UV-reduced males in early dry season (December 13 – January 20) and late dry season (January 21 – February 27) over two field seasons.

Figure 4. Number of sexual and nonsexual responses by focal male *Megaloprepus caerulatas* in paired presentations of control and UV-reduced females. Responses in the control and the UV-reduced treatments are from the same focal males.

Figure 1a



natural female
UV-reduced female
natural male
UV-reduced male
Relative reflectance (%)
13
11
9
7
5
3
1
300
350
400
450
500
550
600
650
700
Wavelength (nm)

Figure 1b

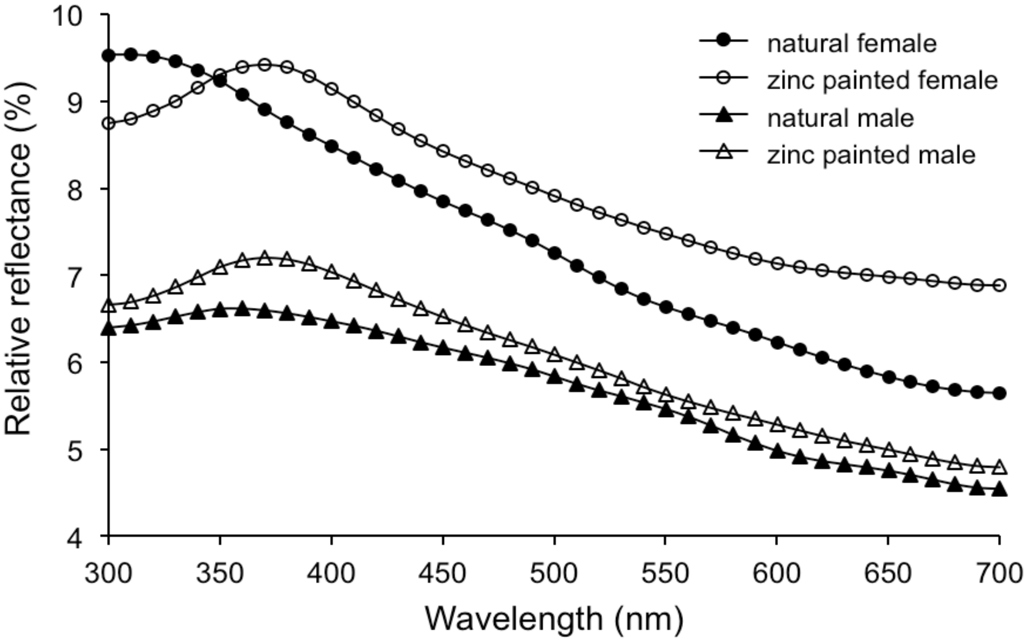

10
9
8
7
6
5
4
Relative reflectance (%)
300
350
400
450
500
550
600
650
700
Wavelength (nm)
natural female
zinc painted female
natural male
zinc painted male

Figure 2a

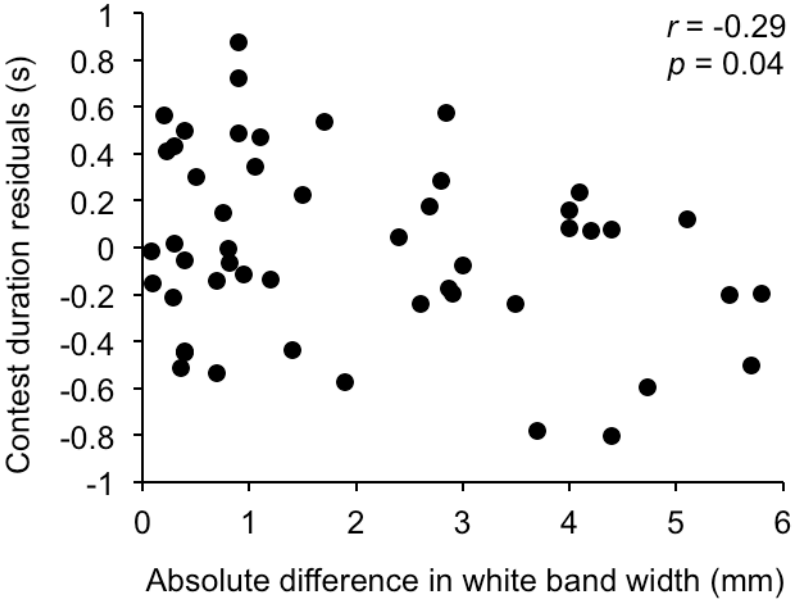

r = -0.29
p = 0.04
Contest duration residuals (s)
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
0
1
2
3
4
5
6
Absolute difference in white band width (mm)

Figure 2b

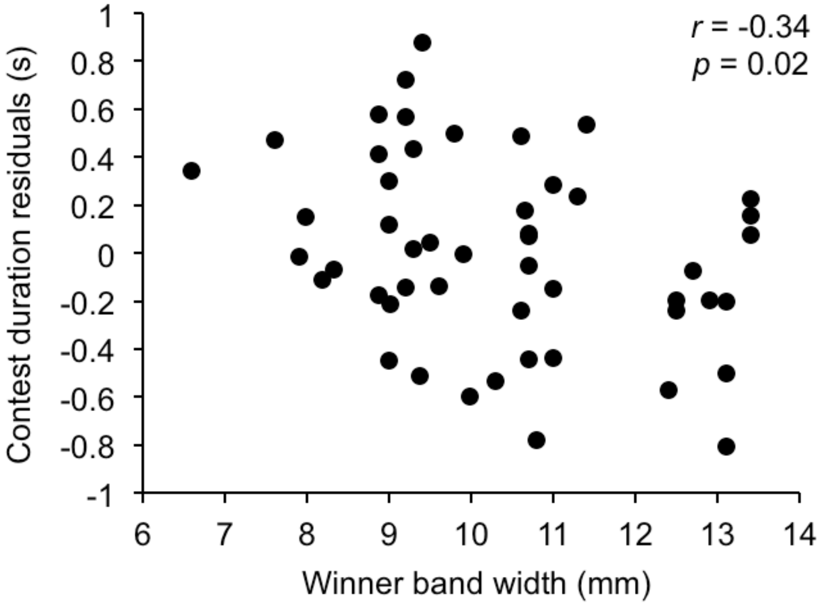

r = -0.34
p = 0.02
Contest duration residuals (s)
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
6
7
8
9
10
11
12
13
14
Winner band width (mm)

Figure 2c

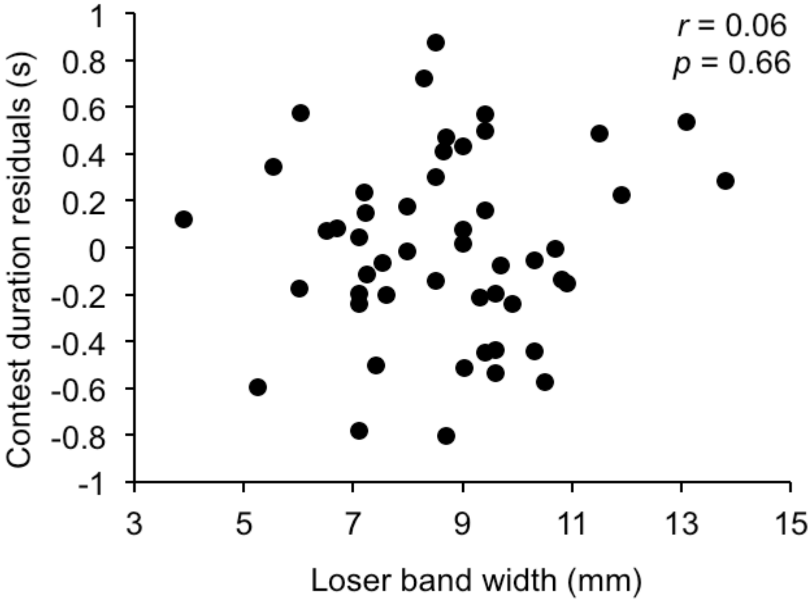

r = 0.06
p = 0.66
Contest duration residuals (s)
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
3
5
7
9
11
13
15
Loser band width (mm)

Figure 3

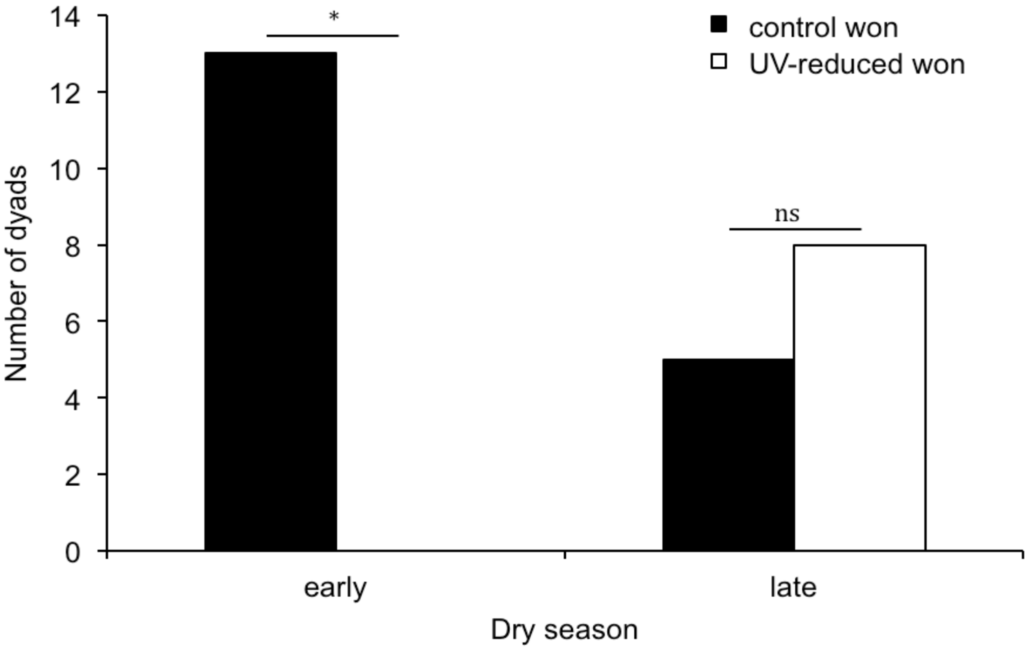
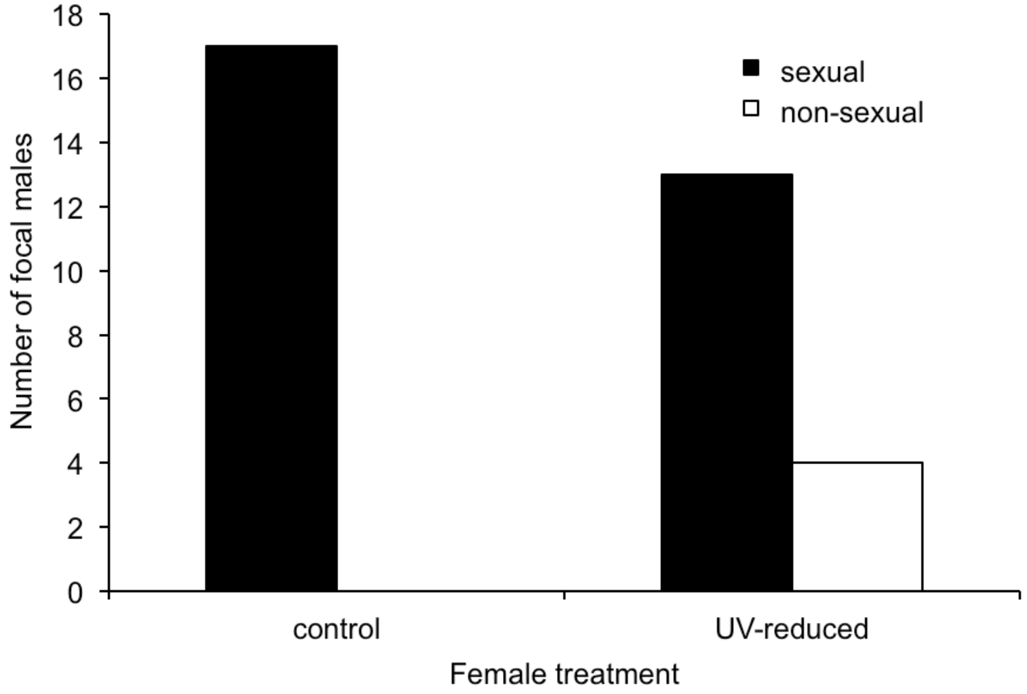

14
12
10
8
6
4
2
0
Number of dyads
*
ns
■ control won
□ UV-reduced won
early
late
Dry season

Figure 4


18
16
14
12
10
8
6
4
2
0
Number of focal males
■ sexual
□ non-sexual
control
UV-reduced
Female treatment

Chapter 5: A male wing color trait offers honest signal of quality and imposes differential potential cost of cheating in a territorial damselfly

[At the time of dissertation submission, this chapter was in review at a journal as Xu, M., Fincke, O. M. A male wing color trait offers honest signal of quality and imposes differential potential cost of cheating in a territorial damselfly]

ABSTRACT

Signals that correlate with the underlying quality of individuals can help animals resolve conflicts without physical fights. Because the stability of a signaling system relies on the signal being honest on average, it is thus important to investigate the mechanisms that can maintain the honesty of the signal. In a territorial damselfly *Megaloprepus caerulatus*, males defend water-filled tree holes to attract mates. Males are known to use information from a white wing band, made of fat deposited on the wings, to resolve their territorial contests. We conducted a split-family rearing experiment to investigate signaling components, information content, honesty and mechanisms maintaining the honesty of this male color signal. We proposed that an honest signal should show three features of heightened condition dependence: heightened sensitivity to nutrition, steeper isometric slope, and greater variation compared to control traits. Additionally, we hypothesized that the honesty of this signal is maintained by a negative condition-dependent cost of cheating. The male white wing band showed all three features of an honest signal. Further, although males in poor condition had disproportionally smaller and less bright white bands, they invested a greater proportion of their total fat to develop the signal. As a result, cost of cheating was negatively condition dependent because the reduction in fat reserves in order to exaggerate the signal would represent a greater physiological cost for males in poor condition.

INTRODUCTION

Nature abounds with conflicts over limited resources, such as food, territory or mates (Huntingford et al. 1987). There are multiple ways to resolve these conflicts; for example, animals can use war of attrition (Maynard Smith 1974; Hammerstein and Parker 1982) or physical fights (e.g., Sandegren 1976). These resolutions either cost time and energy, or inflict risks of injury or even death (Sandegren 1976; Marden and Waage 1980; Parker and Thompson 1980; Bean and Cook 2001). In contrast, signaling might offer a superior way to settle conflicts if contestants each display signals that correlate with their underlying quality, whether physiological condition, genetic quality, or aggressiveness. By assessing the signals of rivals or against a pre-determined criterion, rivals may be able to settle conflict without all-out fights (Rohwer 1975; Gosling and Roberts 2001; Maynard Smith and Harper 2003).

However, rival signals are worth respecting only if contestants advertise their quality honestly (Maynard Smith and Harper 2003). An elemental question is thus, how honest are signals used in antagonistic interactions. Similar to signals used in female mate choice, honest signals used to settle conflicts are expected to show heightened condition-dependent expression compared with non-signaling traits (Cotton et al. 2004a, b). Synthesizing multiple arguments (Cotton et al. 2004a; Shingleton et al. 2007; Emlen et al. 2012; Warren et al. 2013), the following three predictions of the heightened condition dependence hypothesis offer the most stringent test on the honesty of a signal to date: compared to non-signaling traits, signaling traits should: (1) exhibit heightened sensitivity to nutrition, (2) increase disproportionally with body size (or other internal conditions), and (3) show greater variation in trait value. It has been argued that honest signals should show heightened nutrition sensitivity because it more finely advertises any slight difference in the amount of nutrition acquired. Similarly, a steeper isometric slope (or higher allometric exponent) and a greater degree of variation would allow the signaling trait to better capture any slight difference in the animal's condition, often manifested as overall body size, such that it can be perceived more easily. It is important to note that showing these three features does not guarantee signal honesty. In fact, a signal only needs to be honest on average, thus, a low level of cheating is expected and can be evolutionarily stable (Dawkins and Guilford 1991; Johnstone and Grafen 1993). Rather, a signal showing these features simultaneously should be more revealing, and hence, should function as a more effective 'biological billboard' in advertising the animal's underlying quality.

Much evidence for the heightened condition dependence of morphological traits come from body parts as signals (reviewed by Cotton et al. 2004a), such as dung beetle horns (Emlen 1997), stalk-eyed fly eye stalks (Cotton et al. 2004b), and carrion fly antennae (Bonduriansky and Rowe 2005). Recent evidence shows that enhanced sensitivity to insulin or insulin-like growth factor may be a ubiquitous mechanism underlying heightened condition-dependence of such body parts (Shingleton et al. 2008; Emlen et al. 2012). On the other hand, because color traits are a modification added to a body part later in development, it seems unlikely that the same pathway is responsible for the development of color traits. Hence, it remains unclear whether honest color traits follow the same set of criteria for signal honesty that were originally proposed for body parts. Furthermore, although condition dependence of color traits have often been found in birds (reviewed by Cotton et al 2004a), there, such signals are plastic over a rival's lifetime via seasonal molts. In contrast, many body parts and color traits of invertebrates have determined development (i.e., are fixed at the adult stage), raising the question of whether such permanent signals can honestly reflect quality across the adult's life. Finally, unlike body structures whose most important aspect is size, color traits have two distinct features: size and spectral reflectance. Yet little is known about how size and spectral features of a color trait interact to function as a whole (but see McGlothlin et al. 2007), nor do we have a good understanding of whether different components of a color trait can be under different selective pressures (Badyaev et al. 2001).

Signal honesty plays a pivotal role in maintaining the stability of a signaling system because a high frequency of cheating can cause the receivers to abandon using information from the signal, and eventually, lead to collapse of the signaling system (Maynard Smith and Harper 2003). However, pinpointing mechanisms that maintain honesty of signals is often challenging. Some signals, such as the vibration of a spider's web and the pitch of a red deer's roar, are physically or physiologically constrained such that they cannot be exaggerated (e.g., Riechert 1978; Reby and McComb 2003). However, such cases are rare. Animals that have repeated contacts and some ability for individual recognition may use social punishment to curtail cheating (Tibbetts and Dale 2004). But this mechanism would be ineffective for animals that rarely have repeated encounters. Alternatively, cheating could be discouraged by the mechanism of the "handicap principle", an idea whose meaning has been refined significantly since originally proposed four decades ago (Zahavi 1975; 1977). The current, generally accepted version of this principle assumes that signals are costly to make, but it is the potential cost of exaggeration (i.e., cost of positive deviation from equilibrium), rather than the realized cost (i.e., cost at signaling equilibrium) of signal production or efficacy, that maintains the honesty (Számadó 2011). Under these assumptions, the potential marginal cost (i.e., cost per unit of signal incremental) of exaggeration should be relatively higher for individuals in poor condition, therefore, a handicap (Zahavi 1977; Grafen 1990; Getty 2006).

However, empirical evidence for the handicap principle is scarce (Kotiaho 2001). Besides difficulty in identifying the potential costs of cheating, especially for structural colors (Kemp et al. 2012), there may have been a few common misconceptions of the handicap principle. First, evidence of realized cost does not necessarily render honesty (Lachmann et al. 2001; Számadó 2011). Instead, to measure the potential cost of cheating, researchers can manipulate the signal (e.g., enlarge or brighten) to take an individual away from the signaling equilibrium. This approach, considerably more difficult than correlational studies, has not been a popular choice (reviewed by Számadó 2011, but see, e.g., Møller and de Lope 1994; Tibbetts and Dale 2004; McGlothlin et al. 2007; Contreras-Garduño et al. 2008; Bortolotti et al. 2009). Second, demonstrating that there is a cost of cheating is not enough; instead, one must provide evidence that the cost of cheating decreases as an individual's condition increases. To date, numerous studies from a broad range of taxa and various signal types have quantified realized cost associated with a sexual signal (e.g., Leech and Leonard 1996; Salvador et al 1996; Brant 2003; Spencer et al. 2003; also reviewed by Burk 1988), and a handful of studies measured the potential cost of exaggeration (Møller et al. 1995; Candolin 1999; Kotiaho 2000; Tibbetts and Dale 2004; Bortolotti et al. 2009; Laubach et al. 2013). But studies that were able to measure, or merely infer, both a potential cost of cheating and condition dependence of this cost are extremely rare (Møller and de Lope 1994; Candolin 1999).

The broad-winged, neotropical damselfly *Megaloprepus caerulatus* provides a promising model for studying the honesty of a color-based signal in male territorial contests. *M. caerulatus* exhibits sexually dimorphic wing coloration in most of its range. In Panama, both males and females have iridescent blue bands on the wings; males also have a sex-specific white band proximal to the blue band, whereas females have white tips distal to the blue band. All of the above wing color ornamentations reflect ultraviolet light (UV, wavelength 200-400 nm). Interestingly, the white bands and tips are structural colours made of nano-sized wax filaments covering both sides of the wing sheath, which generates the white color by Mie scattering. The female white wing tips are a signal of sex identity used by males to distinguish the sex of conspecifics (Schultz and Fincke 2009).

Male *M. caerulatus* defend forest light gaps that contain large water-filled tree holes where females come to mate and lay eggs (Fincke 1984). Male territorial contests, which usually last between a few seconds to a few minutes, are highly ritualized and acrobatic (Xu and Fincke, in review). The proportion of a male's wing surface covered by the white band is positively correlated with body size (Schultz and Fincke 2009). Larger males are more likely to win contests (Fincke 1992). Interestingly, however, contest duration is negatively correlated with the difference in the white band size between the contestants, but not the difference in wing length. In addition, a male whose UV reflectance of the white bands was experimentally reduced was more likely to lose contests (Xu and Fincke, in review). Females do not exert direct mate choice of male phenotype, but by mating only at territories, they indirectly select larger males (Fincke 1992).

The objective of this study is twofold. First, we aim to investigate the signaling component and information content of the white wing band of male *M. caerulatus*. Specifically, we hypothesized that both size and spectral feature of the white wing band have signaling functions. The information content of this signal may be general body size, flight muscle mass that provides power for the flight, fat reserve inside the body that provides energy for the contest, or some combination of these variables (see Contreras-Garduño et al. 2008).

Second, we aim to investigate the honesty of this signal and the mechanisms maintaining the honesty. We propose that the male white band should show all three features of honest signals: heightened nutrition sensitivity, steeper isometric slope (or higher allometric exponent), and greater variation in trait value compared to control traits. Here, we use "isometry" and "allometry" in the narrow sense (Voje and

Hansen 2012). The former term refers to a relationship of $Y = a + bX$, and the latter refers to $Y = aX^b$, where Y is the trait size and X the body size. We selected two color traits as controls. The female white wing tip is also made of wax filaments, and it functions as a sex identity signal, as opposed to a quality signal. More interestingly, newly emerged adult males all have female-like white tips, which significantly dim or completely disappear after a few days. Thus, the male white wing tip provides a developmental control trait within the same sex.

In terms of mechanisms that maintain honesty, we hypothesized that honesty of the male white band is at least partly maintained by the handicap principle. We propose that the potential cost of cheating is a decrease in a male's fat reserves, a cost that poses a greater challenge for males in poor condition than for males in good condition. We tested the above hypotheses in a split-family rearing experiment.

METHODS

Egg collection and condition manipulation

We mated field caught male and female *M. caerulatus* from the lowland moist forest on Barro Colorado Island (hereafter BCI), Panama (9.15° N, 79.85° W) during the wet season between September 24 and November 17, 2010. Each female mated with a different territorial male. After a mating was completed, we caught both parents and measured various traits as mentioned below. The female was placed in a plastic jar with wet filter paper as oviposition substrate to lay eggs (Fincke and Hadrys 2001). We collected egg clutches from a total of 7 unique pairs, as well as an additional female who was found ovipositing in an undefended tree hole (i.e., its mate was unknown). During multiple, repeated cycles of copula (Fincke 1984), a *M. caerulatus* male removes sperm from previous mates before adding its own sperm. Thus, eggs laid immediately after a mating are likely full siblings sired by the last mate (Fincke and Hadrys 2001; Fincke 2011).

The filter papers with eggs inserted were labeled and kept in plastic containers with 250 ml water and checked daily for hatchlings. Neonates from a family that hatched on the same day were placed in plastic containers in groups of 5 with 250 ml aerated water, dead leaves and *ad lib.* brine shrimp eggs. Brine shrimp hatched readily and over a long period even without salt added, and they served as food for neonates. Once a larva reached 4 mm in length (head to the last abdominal segment), it was randomly assigned to one of the three food treatments. Larvae from the high, medium and low food treatments were fed every one, two, or four days, respectively. One-day old *Physalaemus (Engystomops) pustulosus* tadpoles and mosquito larvae were used as the food source. Very occasionally, larvae of the drone fly *Eristalis tenax* about the same size of mosquito larvae were used when we did not have enough mosquito larvae. The mean dry weight of the tadpoles and the mosquito larvae used was 0.75 ± 0.02 mg (n = 29) and 0.47 ± 0.03 mg (n = 18), respectively. The amount of food per feeding was adjusted for larval size across all three treatments: 4-7 mm larvae received 2 tadpoles or 6 mosquito larvae about 5 mm, 7-10 mm larvae received 4 tadpoles or 8 mosquito larvae, 10-15 mm larvae between received 6 tadpoles or 10 mosquito larvae, and larvae > 15 mm received 8 (i.e., *ad* *lib.*) tadpoles. Small larvae were kept individually in small plastic containers with 200 ml water, whereas those > 15 mm were kept individually in large plastic containers with 1 L water. We changed water and cleaned the containers every time a larva molted (roughly once every 1-2 weeks).

Adult trait measurements

Once a final instar larva with swollen wing pads stopped feeding, signaling imminent emergence, we put its container in a cylindrical emergence cage made of window screening. Two sticks placed in the container with one end touching the cage served as emergence sites. Tenerals were kept in the cage for 36 h until their wings dried, and then they were frozen at -5˚C. We measured adult forewing length as a proxy for body size (Fincke 1982; Fincke and Hadrys 2001). We took a photo of the left hindwing with a ruler in view, and measured the size of the left hindwing and the wing tips for both sexes, as well as the size of the white wing band for males using ImageJ (National Institutes of Health, Bethesda, USA).

The spectral reflectance of the male white band and female white tip of the left hidewing were measured with an OceanOptics SD 2000 spectrometer and a PX-2 xenon light source (OceanOptic, Dunedin, USA). We divided a male's white band into three regions, and measured the maximum relative reflectance from each region on both sides of the hindwing. Similarly, we measured the maximum relative reflectance from the upper and lower regions of a female's white tips. We interpolated all spectra between 300-700 nm, averaged relative reflectance from different regions of an individual, and calculated brightness (mean relative reflectance), hue (wavelength at the reflectance maximum) and UV chroma ($R_{300\text{-}400\ \text{nm}} / R_{300\text{-}700\ \text{nm}}$) for each individual in Avicol v.6 (Gomez, 2006). Like most insects, odonates can detect UV (Yang and Osorio, 1991; Schultz et al. 2008; Huang et al. 2014).

We also measured dry weight of wings, thorax, and the rest of the body for all adults. Body parts were dried in an oven at 65°C until the weight was constant. To estimate adult flight muscle mass, we carefully cut open the oven-dried thorax and submerged it in 0.35 mol / L NaOH solution for 24 h (Marden 1987). We then washed away the muscle tissue with a jet of distilled water, and re-dried the cuticle. The weight difference before and after NaOH treatment was the flight muscle mass. Additionally, we measured the amount of wax on the wings that made up the white bands or tips, and the fat reserves inside the body. We wrapped wings and crushed body parts in Whatman No. 42 quantitative ashless filter paper (GE Healthcare Bio-Sciences, Piscataway, USA), and submerged them in chloroform for 8 h. We subsequently extracted lipids from wings and body with chloroform in a Soxhlet apparatus for 8 h (Marden 1989). Wings and body were dried after the extraction and weighed. Fat content was calculated as the difference in dry weight before and after the lipid extraction. Because we euthanized adult offspring 36 h after emergence when male white tips had almost disappeared, the wax extracted from male wings was mostly used to make male white wing bands.

Statistical analyses

To see if the food treatment worked as designed, we calculated the number of days each larva stayed in a treatment, as well as the total dry weight fed to a larva. Because the total amount of food fed overlapped among the three treatments, we used the total amount of dry weight fed as a random variable in the analyses to increase power. Using treatment as a fixed effect variable did not change major conclusions. We controlled for family effect in all analyses below.

We used analysis of covariance (ANCOVA) to investigate whether the size of male band, male tip and female tip increased with total dry weight fed. Similarly, we used ANCOVA to test if the three color traits increased with wing size. Using linear models (LMs), we tested whether male band size was more sensitive than female tip size to nutrition and had a steeper isometric slope (i.e., b in $Y = a + bX$) or allometric exponent/slope (i.e., b in $Y = aX^b$, or $\log(Y) = \log(a) + b\log(X)$). In these models, color trait size was the dependent variable, trait type was a fixed effect independent variable, family was a random effect variable, and total dry weight fed or wing size was used as covariate. We also included trait type* covariate interactions in the models. Similarly, we compared nutrition sensitivity and isometry/allometry between male band size and male tip size with linear mixed models (LMMs). Variables were similar to those of the linear models above, but male tip and male band were treated as repeated measures. If male band size was more sensitive to nutrition than the size of either control trait, we expect the total dry weight fed * trait type interaction to be significant. Likewise, if the male band size had higher isometry or allometry than either control trait, we expect trait type * wing size interaction to be significant. In addition, we calculated coefficient of variation (CV) of the three color traits of families. In this analysis, we used four families that produced more than three adults of each sex. We then used paired t-test to compare CV of male band size and female tip size, as well as of male band size and male tip size.

To investigate whether the male band size signaled any quality independent of body size, we calculated the band size residual as the standard residual from a regression between male band size and wing size, then performed ANCOVAs with band size residual as a dependent variable, family as a random effect dependent variable and fat reserve or flight muscle mass as the covariate.

We examined whether total fat content of an individual (i.e., fat reserves inside the body + wax deposited on the wings) increased with total food fed and compared total fat between male and female offspring with linear models controlling for family effect. We also calculated the fraction of total fat that was deposited on the wings to make white bands and tips. We then investigated whether this fraction correlated with total fat in both sexes, and compared the slopes between males and females using ANCOVA.

Finally, we investigated whether spectral features of male bands and female tips signal body size, fat reserves or flight muscle mass. Because hue was highly correlated with UV chroma (individuals with low UV chroma had hue at longer wavelengths, $r = -0.77$, $df = 39$, $p < 0.001$), we only used mean brightness and UV chroma in our analyses. A principal components analysis suggested the same two color variables as the first two principle components.

Statistical tests were performed in R (version 2.15.1, R Core Team, 2012) and SPSS (version 20, IBM Corp., Armonk, USA). Throughout, means are shown with standard errors.

RESULTS

Treatment verification, larval development and father-son regression

Food fed to larvae in the medium and low food treatments was almost always completely consumed before the next feeding, however, food fed to larvae in the high food treatment was always *ad lib*. Larval developmental time between hatching and emergence differed significantly among treatments (high: 119.4 ± 1.5 days, medium: 130.5 ± 1.6 days, low: 156.6 ± 2.0 days, $F_{2,101} = 111.47$, $p < 0.001$). The amount of time a larva stayed in treatment before emergence (high: 98.0 ± 1.1 days, medium: 109.9 ± 1.3 days, low: 130.4 ± 1.8 days) decreased with food availability ($F_{2,101} = 140.58$, $p < 0.001$). Although the difference in treatment duration partially diminished the effect size of the designed food treatments, total dry weight fed was still significantly different among treatments (high: 293.77 ± 4.24 mg, medium: 216.20 ± 5.39 mg, low: 184.02 ± 3.79 mg, $F_{2,100} = 138.70$, $p < 0.001$).

The range of wing size of sons (12.44 mm, 57.37 ~ 69.81 mm) was smaller than that of fathers (27.33 mm, 54.49 ~ 81.82 mm, Fig. 1a). Similarly, the range of band size of sons (94.52 mm^2, 74.38 ~ 168.90 mm^2) was roughly half of that of fathers (182.11 mm^2, 60.06 ~ 242.17 mm^2, Fig 1b). Mean son wing size from 7 families with known fathers were not correlated with father wing size (Fig. 1a, LM, $F_{1,5} = 0.16$, $p = 0.71$), nor were mean son wing band size and father wing band size correlated (Fig. 1b, $F_{1,5} = 0.92$, $p = 0.38$).

Nutrition sensitivity

Forewing length of both male and female offspring increased with total dry weight fed (ANCOVA, male: $F_{1,33} = 10.78$, $p = 0.002$, female: $F_{1,50} = 38.28$, $p < 0.001$, Fig. 2a). Male and female forewing length did not differ in their sensitivity to food fed (LM, total dry weight fed * sex interaction: $F_{1,90} = 0.94$, $p = 0.34$). Excluding four families that produced fewer than four adults of either sex from the analysis did not change this conclusion.

The size of both male white band and female white tip increased with total dry weight fed (ANCOVA, male band: $F_{1,31} = 4.89$, $p = 0.03$, female tip: $F_{1,47} = 11.41$, $p = 0.001$, Fig. 2b), and the size of male wing tip marginally significantly increased with total dry weight fed ($F_{1,33} = 3.67$, $p = 0.06$, Fig. 2b). As predicted, male band size was more sensitive to food availability than female tip size (LM, total dry weight fed * sex interaction: $F_{1,85} = 4.38$, $p = 0.04$). Similarly, male band size was marginally more sensitive to food availability than male tip size (LMM, total dry weight fed * trait interaction: $F_{1,30} = 3.75$, $p = 0.06$).

Heightened isometry and variation

The size of the male band, male tip and female tip all increased with forewing size (ANCOVA, male band: $F_{1,33} = 12.74$, $p = 0.001$, male tip: $F_{1,36} = 67.43$, $p < 0.001$, female tip: $F_{1,51} = 71.53$, $p < 0.001$). In the original scale, male band size increased with forewing size at a steeper slope than female tip size (Fig. 3a, LM, wing size * sex interaction: $F_{1,91} = 10.16$, $p < 0.002$). After log-transforming color trait size and wing size, both male band size and female tip size showed significant positive allometry (i.e., common regression slope > 1 in log(trait size) – log(body size) regression, $\beta = 1.05$, $F_{1,91} = 22.59$, $p < 0.001$), although male and female slopes did not differ (Fig. 3b, log(wing size) * sex interaction: $F_{1,91} = 2.46$, $p = 0.12$). Similarly, using male wing tips as a developmental control trait, male band size had steeper isometric slope than male tip size (Fig. 3a, LMM, wing size * male trait interaction: $F_{1,33} = 6.06$, $p = 0.02$), but the allometric slopes did not differ (Fig. 3b, log(wing size) * male trait interaction: $F_{1,33} = 0.15$, $p = 0.70$).

Excluding four families that produced fewer than four adults of either sex, male band size had higher CV than that of female tip size (paired t-test, $t_3 = 5.16$, $p = 0.01$), and this difference remained statistically significant when sizes were log-transformed ($t_3 = 3.79$, $p = 0.03$). However, the CVs did not differ between male band size and male tip size ($t_3 = 2.31$, $p = 0.10$), nor did it differ between log-transformed sizes ($t_3 = 0.74$, $p = 0.51$).

Information content independent of body size

On average, fat reserves and flight muscle mass made up 8.5 ± 0.3 % and 25.8 ± 0.2 % of total dry weight among male offspring, respectively. Using data from male offspring only, the band size residual (residual of band size – forewing size regression) did not explain fat reserve (ANCOVA, $F_{1,33} = 0.07$, $p = 0.79$, Fig. 4a), nor flight muscle mass ($F_{1,33} = 1.38$, $p = 0.25$, Fig. 4b). However, there was one outlier in both models above (Fig. 4): the male that had the largest, negative band size residual (-2.59) also had both the largest fat reserves (6.7 mg) and the greatest flight muscle mass (14.3 mg) among all male offspring. Excluding this outlier, band size residual still did not explain fat reserve ($F_{1,32} = 2.52$, $p = 0.12$), but flight muscle mass increased with band size residual ($F_{1,32} = 4.87$, $p = 0.03$). However, band size residual did not correlate with flight muscle ratio (flight muscle mass / body mass), regardless of whether the outlier was excluded or not (including the outlier: $F_{1,33} = 0.06$, $p = 0.81$, excluding the outlier: $F_{1,32} = 0.06$, $p = 0.81$).

Realized production cost and potential cost of cheating

Using offspring from four families that produced more than three adults of both sexes, total fat increased with total food fed (LM, $F_{1,62} = 22.98$, $p < 0.001$), and males had marginally more total fat than did females (male: 4.32 ± 0.19 mg, female: 3.88 ± 0.16 mg, $F_{1,81} = 3.67$, $p = 0.06$). The two sexes did not differ in the rate of assimilating food into fat (tot dry weight fed * sex interaction, $F_{1,62} = 0.42$, $p = 0.52$), therefore, we dropped the interaction from the model to recover power.

Using data from all adult offspring whose fat had been extracted, males deposited 0.96 ± 0.03 mg wax (23.8 ± 0.8% of total fat) on the wing to make white bands, whereas females deposited 0.89 ± 0.02 mg fat (22.5 ± 0.7% of total fat) to make white tips. Larger color traits need more wax to make (LM, $F_{1,61} = 6.55$, $p = 0.01$), and males deposited more wax on the wings than females ($F_{1,61} = 23.56$, $p < 0.001$). However, the proportion of total fat that was deposited on the wing did not differ between males and females (LM on arcsine transformed proportion of wing fat, $F_{1,91} = 1.00$, $p = 0.32$), despite that female wing tips were considerably smaller.

Both fat reserves and wax on the wings increased with total fat (fat reserve: $F_{1,68} = 1100.58$, $p < 0.001$; fat on the wing: $F_{1,68} = 9.37$, $p = 0.003$). But surprisingly, male offspring with more total fat allocated a smaller proportion of their fat to the wings to make white bands (ANCOVA, $F_{1,39} = 17.3$, $p < 0.001$, Fig. 5a). Similarly, female offspring with more total fat allocated a smaller proportion of fat make white tips ($F_{1,43} = 36.05$, $p < 0.001$, Fig. 5b). Using fat reserves as a covariate that is completely independent of wax on the wings yielded the same conclusions.

Spectral features and their interactions with trait size

Overall, female white tips were brighter than male white bands (relative reflectance: female tip: 6.99 ± 0.16 %, male band: 6.17 ± 0.16%, ANOVA, $F_{1,83} = 17.57$, $p < 0.001$), and they had lower UV chroma (i.e., proportion of reflectance in UV) than male white bands (female tips: 25.67 ± 0.30 %, male band: 30.74 ± 0.28%, $F_{1,83} = 157.40$, $p < 0.001$). The brightness of neither female tips nor male bands were sensitive to total dry weight fed (ANCOVA, female tip: $F_{1,38} = 3.08$, $p = 0.09$, male band: $F_{1,28} = 0.14$, $p = 0.71$). However, UV chroma of both female tips and male bands decreased with total dry weight fed (female tip: $F_{1,38} = 8.64$, $p = 0.006$, male band: $F_{1,28} = 5.40$, $p = 0.03$).

Neither the brightness nor UV chroma of the female tips were correlated with any potential quality measures, whereas male band brightness was positively correlated with body size, fat reserves and flight muscle mass, and UV chroma of the male bands was negatively correlated with fat reserve (Table 1).

Individuals with more total fat deposited a higher density of wax (wax / mm^2) on the wings in both sexes ($F_{1,62} = 11.97$, $p < 0.001$). A higher wax density resulted in greater brightness when sexes were pooled ($F_{1,52} = 7.35$, $p = 0.009$), although when analyzed in each sex, the effect was marginally significant for males ($F_{1,26} = 3.31$, $p = 0.08$) but not for females ($F_{1,21} = 0.14$, $p = 0.71$) .

DISCUSSION

Is the male white wing band an honest signal?

Overall, our data showed all three features of heightened condition dependence: heightened nutrition sensitivity, steeper isometric slope and greater variation compared to control traits, suggesting that the male white wing band is, on average, an honest signal of male quality. Because the three criteria we used represent a stringent test for signal honesty, our study provides stronger evidence in signal honesty than studies that only showed a correlation between the signal property and the underlying quality (e.g., Keyser and Hill 2000; Bortolotti et al. 2006; Forsman and Hagman 2006; Velando et al. 2006), as well as those that showed condition dependence of the signaling traits without proper control (reviewed by Cotton et al. 2004a). Further, a previous study on territorial contests of male *M. caerulatus* in the field provided direct evidence that males use information from the white wing band to settle territorial contests (Xu and Fincke, in review). The current study built upon that previous study to show that the white band is not only used by males, but also is honest. Below, we discuss the implications of each of the three criteria.

The size of the male white band showed a stronger response to nutrition treatment compared to both female and male white tips. In comparison, although both male and female body size increased with food availability, their sensitivity to nutrition did not differ. This result has important ecological implications. Because both male white bands and female white tips have determined development (i.e., they develop shortly before eclosion and are not replenished over an adult's lifetime), all the fat needed to produce both signals are thus derived from larval feeding. Additionally, the larvae of *Megaloprepus* develop in water-filled tree holes, a detritus-based food web, where nutrients are limited (Kitching 2000). Under natural conditions, *M. caerulatus* larvae feed on mosquito larvae, and when available, tadpoles, or conspecific larvae; cannibalism is size-dependent (Fincke 1994). Thus, given *M. caerulatus*' larval ecology, the male white band size should reflect larval habitat quality and foraging success of a larva. Our results support conclusions from several previous studies (Cotton et al. 2004b; Tibbetts 2010), but differ in one aspect with findings from paper wasps (Tibbetts and Curtis 2007). In the latter study, the control trait, facial pattern brokenness as an individual identity signal, did not respond to supplemental food. However, in our experiment, the size of female wing tips, a signal of sexual identity, increased with food availability. There may be two reasons for this difference in results. First, unlike the size of a trait, facial pattern brokenness would not be expected to scale with general body size. In addition, we suspect that facial pattern brokenness is most often used when two wasps are in close contact, and thus when detectability of the signal is not an issue. In contrast, a damselfly entering a territory should benefit from advertising its sex such that the territorial male could quickly decide to fight or mate with this individual. Hence, the ideal signal design for female white tips should be perceivable at a considerable distance, which may explain why the sexual identity signal in this species also showed condition dependence, although not as strongly as did the male quality signal.

Similar to many other sexually selected traits (reviewed by Bonduriansky 2007), the size of both male white band and female white tip showed positive allometry (i.e., increased with wing size at an exponent greater than 1, Fig. 3b), suggesting relatively heavier investment in signaling traits than other traits. Additionally, as predicted, male band size increased at a steeper slope with wing size than both control traits, augmenting the true difference between the advertised qualities. Male band size did not have steeper allometric slope than either female or male tip size. However, because we are testing a hypothesis on signal honesty, not the allometry-as-constraint hypothesis (Huxley 1932; Gould 1966), a steeper isometric slope should be considered sufficient evidence (Houle et al. 2011).

Finally, male white bands had a larger coefficient of variance compared to female tips, supporting our last prediction for heightened condition dependence of this signaling trait. This result is similar to findings from the stalk-eyed flies (Cotton et al. 2004b). Like a steeper isometric slope, greater variation also allows the signal to closely track variation in the underlying male quality. The male white band size, however, did not show higher CV than the male tip, perhaps because the two male traits are developmentally linked.

Mechanisms for the maintenance of honesty

Our results showed that the male white band imposed a production cost as measured by fat investment, consistent with findings from many previous studies that have shown a realized cost of sexual signals (e.g., Leech and Leonard 1996; Salvador et al 1996; Brant 2003; Spencer et al. 2003; also reviewed by Burk 1988; Kotiaho 2001). However, our novel finding lies in the negative condition-dependent potential cost of cheating. In our experiment, both male body size and the size of the resource pool (total fat) depended on larval feeding. At emergence, the total amount of fat acquired as a larva was allocated to two different places: inside the body as fat reserves, and onto the wings as signals. Fat reserves serve as fuel in male territorial contests as well as normal life functions, such as foraging flight or flight to search for territories. Wax on the wings, on the other hand, serves as a signal to deter rivals. We could easily imagine that an optimal allocation rule exists for males. Too little wax on the wing would put a male at a disadvantage in territorial contests because its true quality would be underestimated by the opponent. On the other hand, too much wax on the wings would result in a suboptimal fat reserves size, and in the case where this seemingly strong individual does not fool the receiver and is involved in an escalated fight, the cost of being beaten up (*sensu* "receiver retaliation", "receiver imposed cost") could easily outweigh the potential benefit of appearing strong (Molles and Vehrencamp 2001). Although overall, fat reserve and wax on the wings both increased with total fat, male fat allocation was negative condition-dependent: males in poor condition invested a larger proportion of their total fat to make white bands than did males in good condition. Nevertheless, males in poor condition still only made disproportionally smaller and less bright signals. Collectively, males showed condition dependence in both resource acquisition and allocation.

Now, let us suppose that a male in poor condition and a male in good condition were to exaggerate their signal by the same area at the same density of wax, both males will relocate the same amount of fat from reserves inside body to the wings. Because the male in poor condition begins with disproportionally smaller fat reserves, the deduction in fat reserve size would inevitably represent a greater physiological cost. Therefore, our study is among the very few that was able to show not only a potential cost to exaggeration, but that the cost is condition dependent. In our study, differential recourse allocation is the proximate mechanism that rendered differential cost of cheating.

The clearest, and perhaps the only direct evidence for the handicap hypothesis to date comes from barn swallows, *Hirundo rustica* (Møller and de Lope 1994). Male swallows whose outermost tail feathers were elongated suffered increase mortality, but the mortality cost was greater for males in poor condition. Similarly, male *Hygrolycosa rubrofasciata* wolf spiders suffered increased mortality when drumming rate was experimentally increased by exposing them to a female, compared with drumming without a female (Kotiaho 2000). However, in our opinion, this latter study measured the cost of using, rather than exaggerating, a sexual signal and hence, is less relevant as a test of the handicap principle. Finally, the close relationship between resource allocation and (dis)honesty of a male nuptial color has been reported in three-spined stickleback *Gasterosteus aculeatus* (Candolin 1998; 1999). In that species, when food deprived or exposed to high predation risk, males in poor condition develop dishonestly larger red spots. Food deprivation can reduce an already poor-conditioned male's residual reproductive value to below a threshold such that it is unlikely to reproduce again; high predation risk can have similar effects. In this case, the assumption of the handicap hypothesis, negative condition-dependent cost of cheating, as measured by reduction in residual reproductive value, was not fulfilled. Accordingly, the signal did not honestly reflect condition, providing contrapositive evidence to the handicap hypothesis.

Interestingly, in our study, we detected one outlier male that had the greatest amount of total fat but also showed the largest negative signal residual (Fig. 3a). Although we are cautious in drawing any conclusion based on one data point, it would be interesting to know whether this male represents the other kind of cheating by underestimating the true quality. Because this male is very likely to win any contest, and by developing smaller wing bands it can save fat to be stored as fat reserves. Such “modest” mutants can invade the badge of status signaling system (Johnstone and Norris 1993, but see Maynard Smith and Harper 2003), yet it is unclear if modest mutants can invade signaling systems of handicaps or indices. We argue that such outliers in future research, especially in field studies, should not be too quickly dismissed.

Signaling components and information content

Our results showed that both size and spectral reflectance of the male white band were correlated with one or more quality measures. In the field, relative size and UV reflectance of the male white band affect duration and outcome of territorial contests, respectively (Xu and Fincke, in review). Hence, the signaling components of the male white band appear to be both size and color. Further, our current data show that the sizes of both male band and female tip were condition dependent. The spectral features of the male band were condition dependent (Table 1), consistent with findings from studies on structural-based, sexual colors in both insects and birds (Keyser and Hill 1999; Kemp and Rutowski 2007). In contrast, the spectral features of the female tip did not correlate with any potential quality or condition (Table 1). In addition, males who made larger-sized bands also deposited higher density of wax on the wings, resulting in brighter bands. This seemingly counterintuitive relationship between size and brightness was caused by males' ability to acquire more resource and, thus, relaxed the constraint on total resource that would otherwise lead to a trade-off between making a large signal and a bright one. Similarly, females deposited higher density of wax on the tips than did males on the bands, resulting in higher brightness.

As for the information content of the signal, it is not surprising that we found the male white band to track variation in general male body size. Independent of body size, the signal residual correlated with flight muscle mass, but not flight muscle ratio or fat reserves. During a male territorial contest, males often perform mechanistically challenging behavioral tournaments, such as straight upward flight and hover, or fast horizontal chase with hits and abrupt turns (Xu and Fincke, in review). These behaviors rely heavily on power and coordination from flight muscle (Dudley 2002). It is known that the maximum lifting force in flight was highly correlated with flight muscle mass in several species of odonates (Marden 1987). Therefore, it is understandable that flight muscle mass should be an important predictor of the contest performance and outcome. However, in the dragonfly *Plathemis lydia*, which had the highest flight muscle ratio among animals tested, it was flight muscle ratio (flight muscle mass / body mass), not flight muscle mass *per se*, that was correlated with mating success, which depends on winning aerial contests of territories (Marden 1989). But due to its unusually high flight muscle ratio, it is unclear whether this finding applies to other flying insects.

An intriguing question concerns the time dimension of the signal and its information content. In *M. caerulatus,* both body size and the white band size does not change after emergence. However, male *M. caerulatus* can double their mass as young adults (Anholt et al 1991) and odonates greatly increase flight muscle mass during maturation (Marden 1989). Although adult male fat reserves can also change before or after sexual maturation (Marden 1989; Fitzstephen and Getty 2000), males cannot replenish wax on the wings, therefore, new fat acquired as an adult does not affect the signal.

Conclusions and perspectives

Data from our experiment showed that the male white wing band in *M. caerulatus* exhibited all three features of honest signals. Both the size and spectral property of the male white band had signaling functions, and the information content of this signal is primarily body size, but also flight muscle mass.

Our data indicated that the cost of exaggerating the wing band was greater for males in poor conditions, and that resource allocation could be an underlying mechanism for the differential cost of cheating. In fact, researchers have realized the tandem relationship between recourse allocation and allometry of sexual traits (see Voje and Hansen 2012), but the concept of resource allocation has only recently been modeled to explain maintenance of signal honesty (Kokko 1998, Lindström et al. 2009), and empirical evidence for this mechanism is scarce (see Candolin 1999; Lindström et al. 2009). It would be a rewarding research direction, in our minds, to examine how ubiquitous this mechanism is in mediating differential cost of cheating for sexual signals.

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Table 1. Correlations between the two spectral features of two color traits and potential underlying qualities in *M. caerulatus*. +: statistically significant positive correlation; –: statistically significant negative correlation; ns: non-significant correlation

trait	spectral feature	quality		
		forewing length	fat reserve	muscle mass
male wing band	brightness	+	+	+
	UV chroma	ns	–	ns
female wing tip	brightness	ns	ns	ns
	UV chroma	ns	ns	ns

FIGURE LEGENDS

Figure 1. Relationship of **(a)** body size measured as forewing length and **(b)** white band size of fathers and sons from seven families of *Megaloprepus caerulatus* (excluding one family where the father was unknown). The mean body size and white band size values of sons from each family were used

Figure 2. Response of **(a)** male and female body size measured as forewing length and **(b)** size of male white wing band, female white wing tip and immature male white wing tip from 8 clutches of *Megaloprepus caerulatus* to the estimated total amount of dry weight fed during larval development

Figure 3. **(a)** Isometric scaling and **(b)** allometric scaling of male white wing band, female white wing tip and immature male white wing tip from 8 clutches of offspring in the rearing experiment. Female white wing tip and immature male white wing tip were used as control traits

Figure 4. Relationship between male white wing band signal residual (i.e., the standardized residual from male wing band size—body size regression) and **(a)** fat reserves and **(b)** flight muscle mass

Figure 5. Allocation of fat, measured as the fraction of fat (wax) that was used to produce the signals on the wings out of total fat, as a function of total amount of fat for **(a)** male offspring and **(b)** female offspring

Figure 1

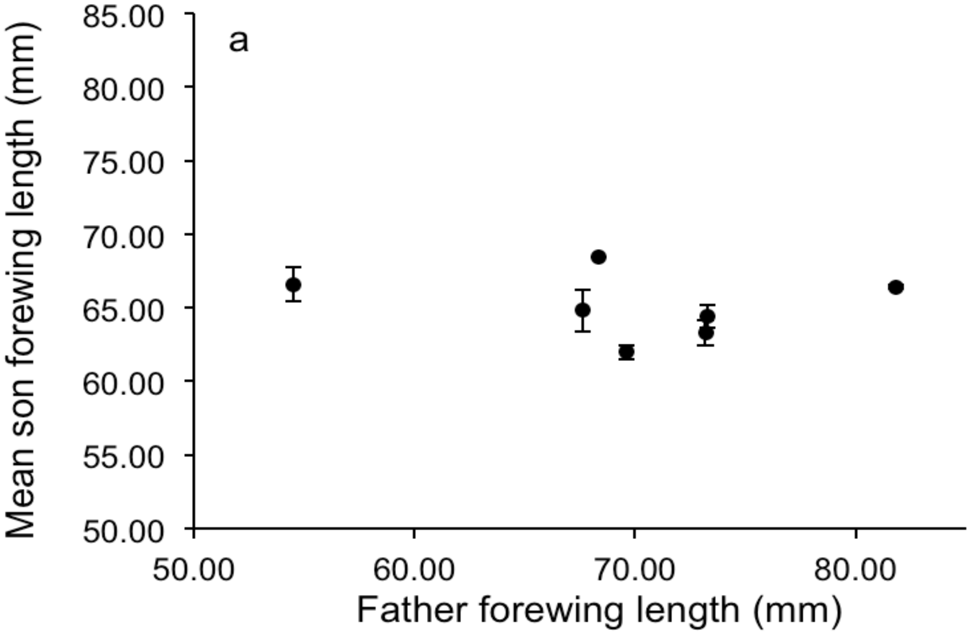

a
Mean son forewing length (mm)
85.00
80.00
75.00
70.00
65.00
60.00
55.00
50.00
50.00
60.00
70.00
80.00
Father forewing length (mm)

Figure 1

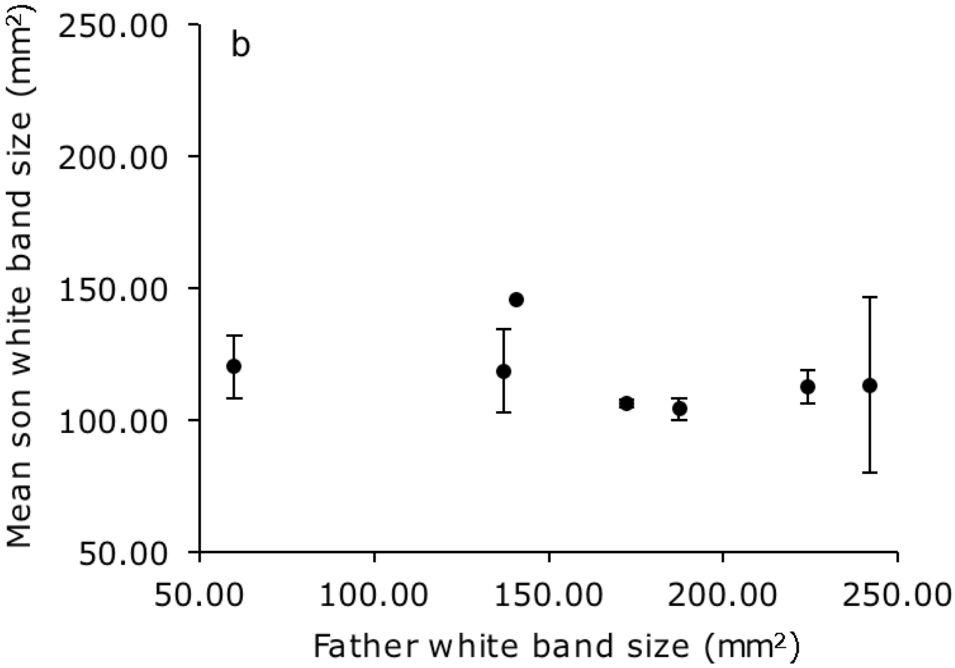

b
Mean son white band size (mm2)
250.00
200.00
150.00
100.00
50.00
50.00
100.00
150.00
200.00
250.00
Father white band size (mm2)

Figure 2

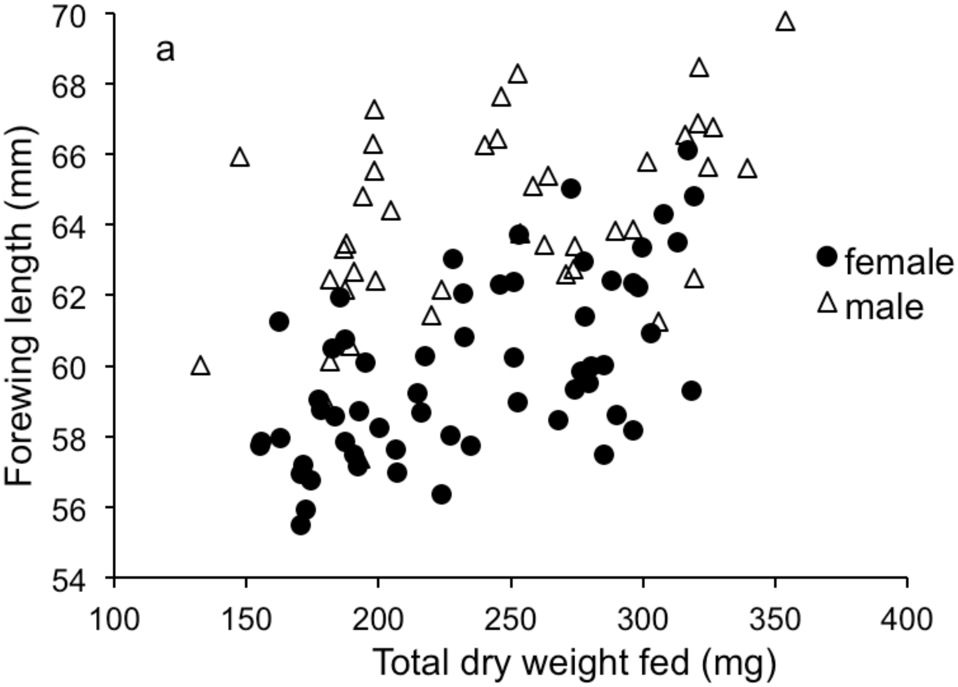

a
70
68
66
64
62
60
58
56
54
Forewing length (mm)
100
150
200
250
300
350
400
Total dry weight fed (mg)
● female
△ male

Figure 2

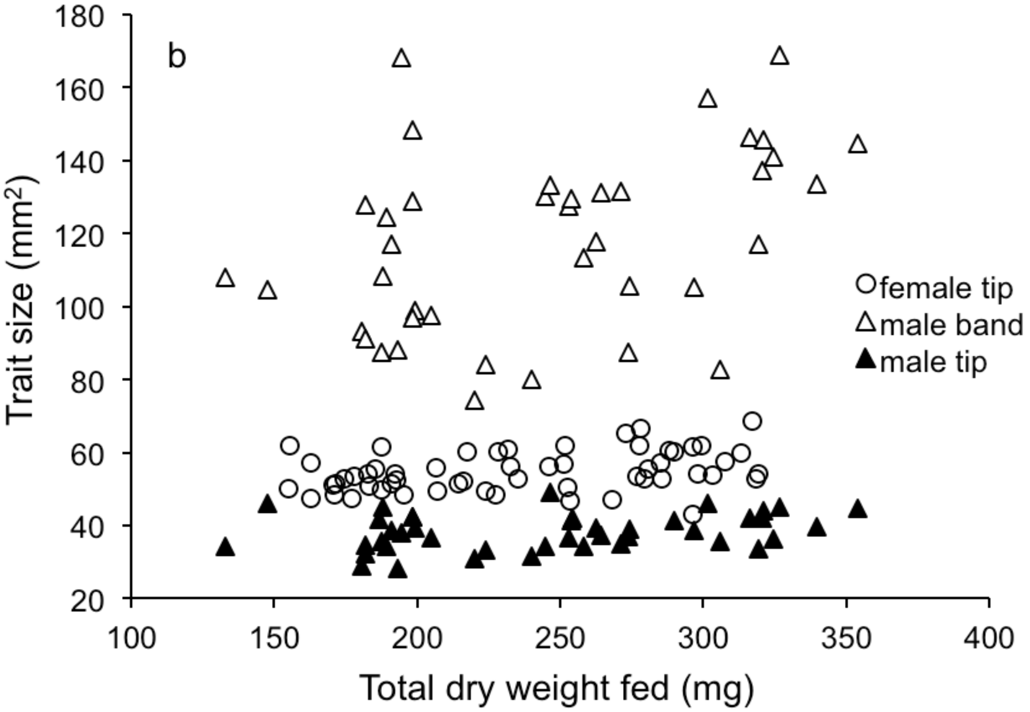

b
Trait size (mm^2)
180
160
140
120
100
80
60
40
20
100
150
200
250
300
350
400
Total dry weight fed (mg)
○ female tip
△ male band
▲ male tip

Figure 3

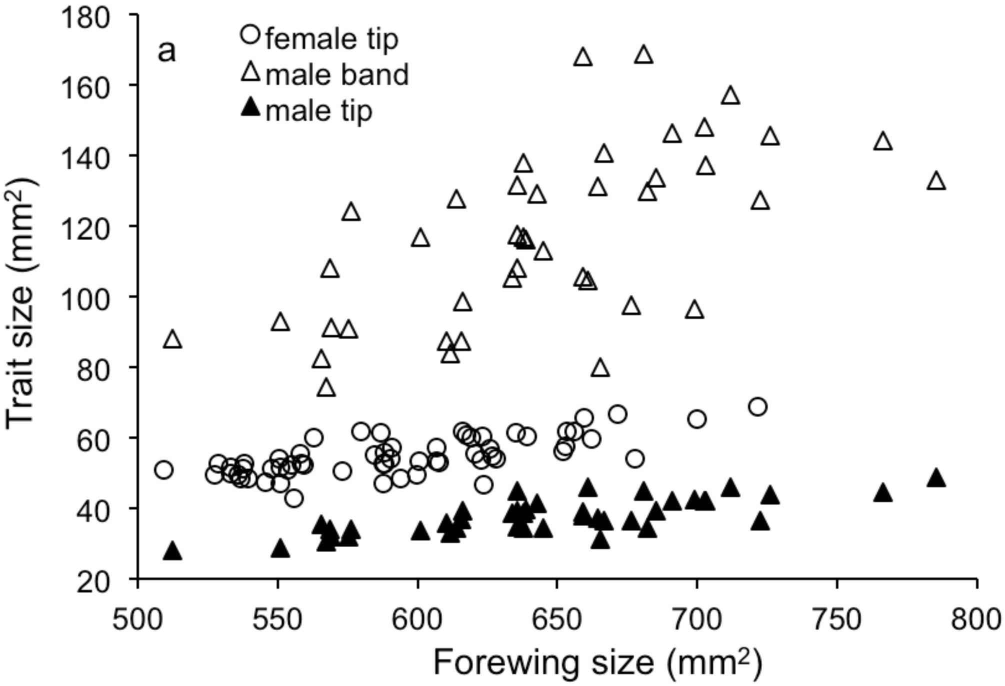

a
○ female tip
△ male band
▲ male tip
Trait size (mm^2)
180
160
140
120
100
80
60
40
20
500
550
600
650
700
750
800
Forewing size (mm^2)

Figure 3

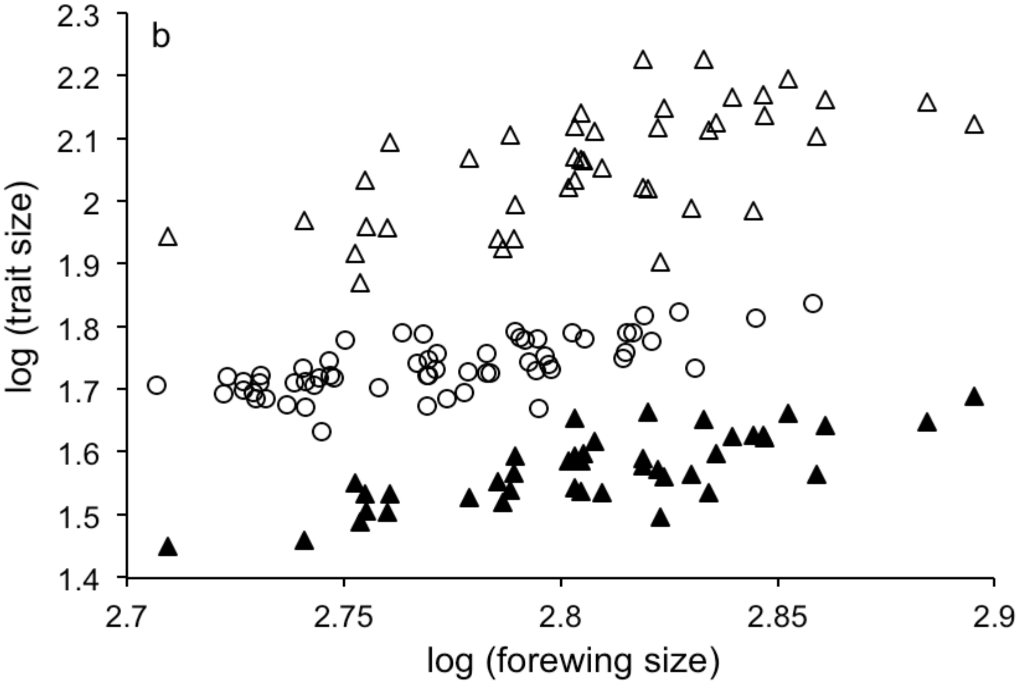

b
log (trait size)
2.3
2.2
2.1
2
1.9
1.8
1.7
1.6
1.5
1.4
2.7
2.75
2.8
2.85
2.9
log (forewing size)

Figure 4

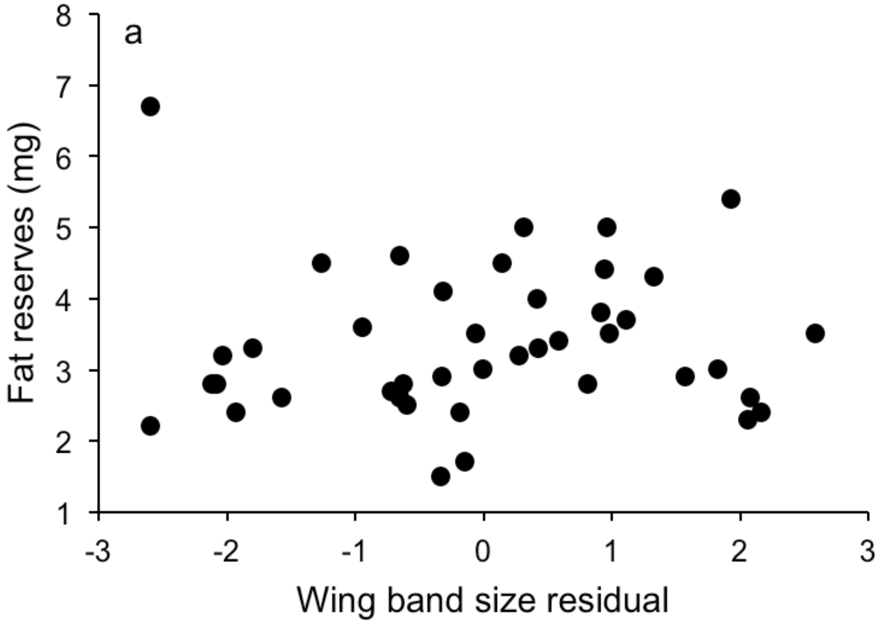


Figure 4

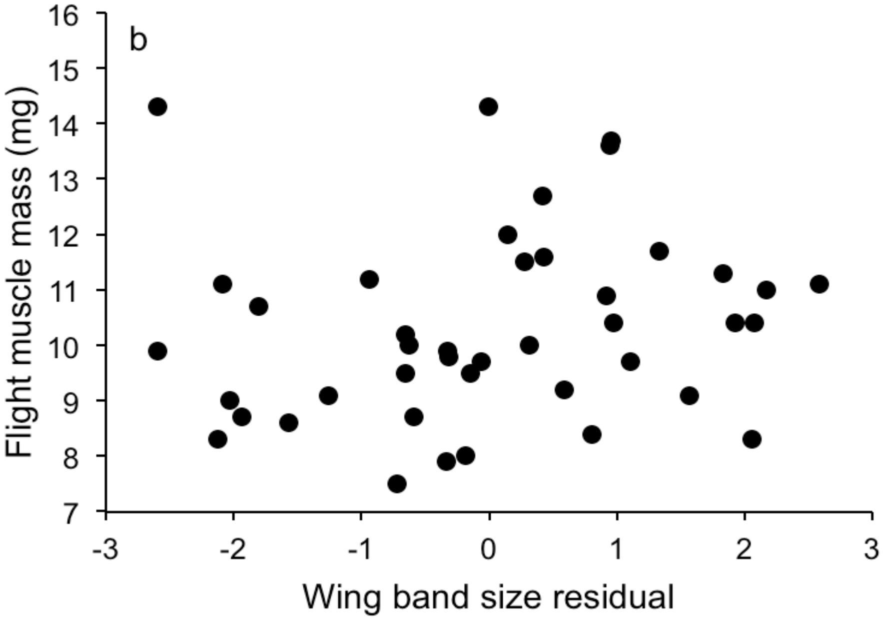

b
Flight muscle mass (mg)
16
15
14
13
12
11
10
9
8
7
-3
-2
-1
0
1
2
3
Wing band size residual

Figure 5

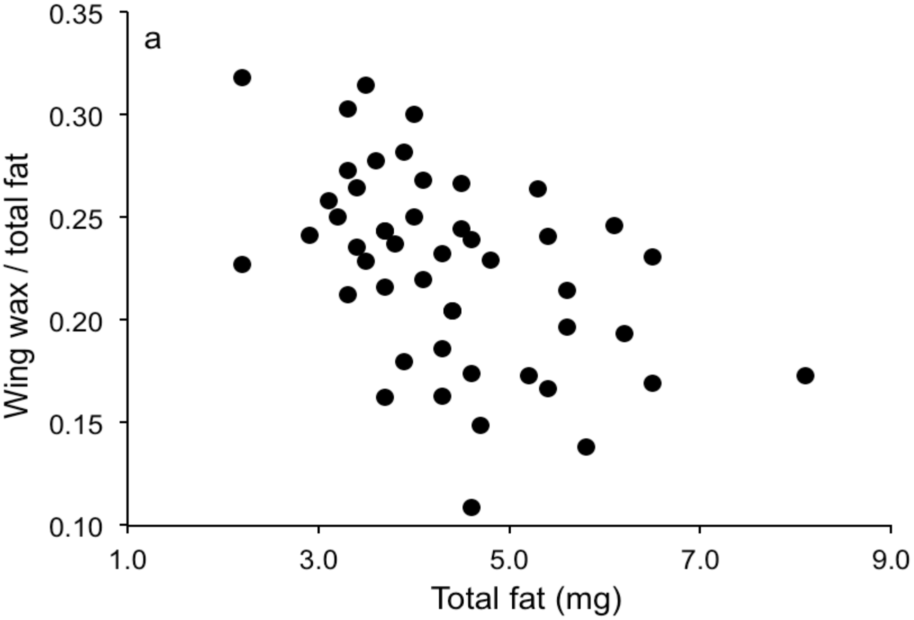
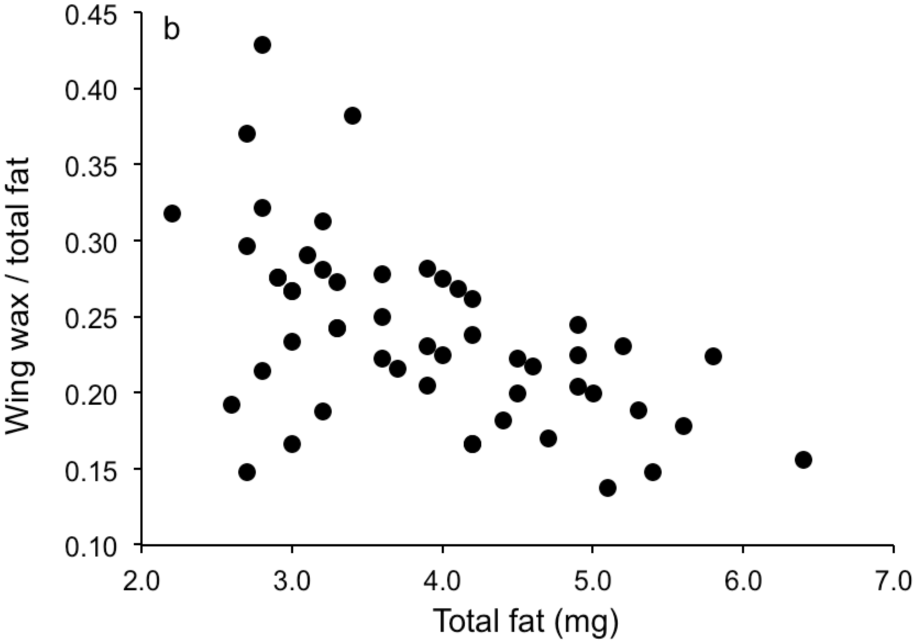

a
Wing wax / total fat
0.35
0.30
0.25
0.20
0.15
0.10
1.0
3.0
5.0
7.0
9.0
Total fat (mg)

Figure 5


b
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
Wing wax / total fat
2.0
3.0
4.0
5.0
6.0
7.0
Total fat (mg)