

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.

Keywords Sexual conflict · Frequency-dependent selection · Insectary experiment · Fluorescent dust · Odonata

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),

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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 (Bateman 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, Hinnikint 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; Hammer and Van Gossum 2008; Ting et 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 Sánchez-Guillén 2007; Gosden and Svensson 2007; 2009; Hammer 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 *E. 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 *Enallagma boreale*, which emerged about 2 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., 1.8×3.6×1.8 m) 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° 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 20 cm 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 six ($\bar{x} = 4.23$, $SD = 1.46$), 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 1 day; it took 4 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 Brakefield 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,

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} /15 min (mean±SD)	HG _{OBS} /15 min (mean±SD)	HB _{DUST} /day (mean±SD)	HG _{DUST} /day (mean±SD)
0	0±0	0.5	30	4	–	0.24±0.09	–	0.57±0.23
		2	30	4	–	0.76±0.17	–	0.39±0.11
0.2	0.21±0.06	0.5	30	2	0.49±0.21	0.25±0.07	0.50±0.00	0.88±0.38
		2	30	2	0.39±0.08	0.52±0.08	0.75±0.35	0.43±0.20
0.5	0.52±0.06	0.5	30	2	0.39±0.27	0.43±0.25	0.59±0.02	0.97±0.75
		2	30	3	0.46±0.14	0.35±0.32	0.62±0.13	0.47±0.50
0.8	0.77±0.04	0.5	30	2	0.23±0.004	0.17±0.16	0.75±0.35	1.00±0.00
		2	30	2	0.82±0.29	0.71±0.88	0.77±0.08	0.75±0.35
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 “Materials and methods” section); 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 for blue or green females, respectively, by fluorescent dust

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 from the observation data. 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 sexual 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 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).

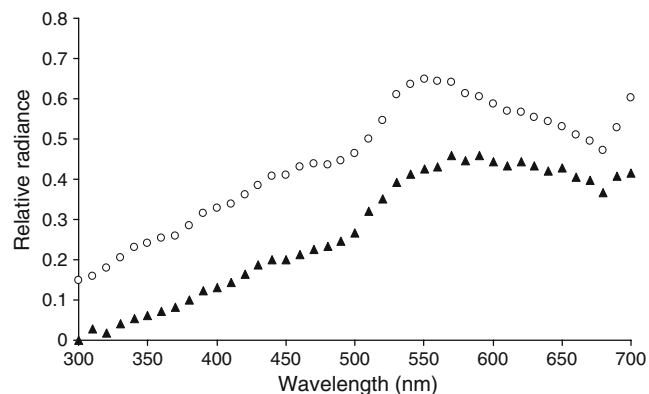


Fig. 1 Relative ambient radiance at shoreline outside the insectaries (open circles), and inside the insectaries (triangles)

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

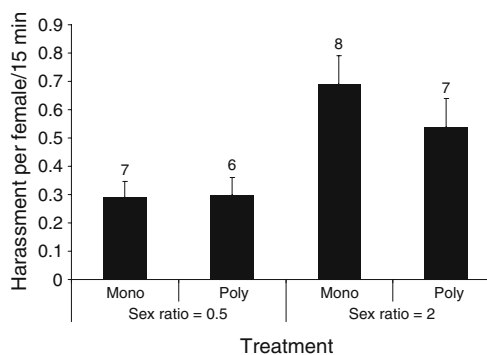


Fig. 2 Mean per capita harassment rate \pm standard error for females. *Mono* monomorphic treatments, *Poly* polymorphic treatments. Sample sizes are above error bars

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

(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.

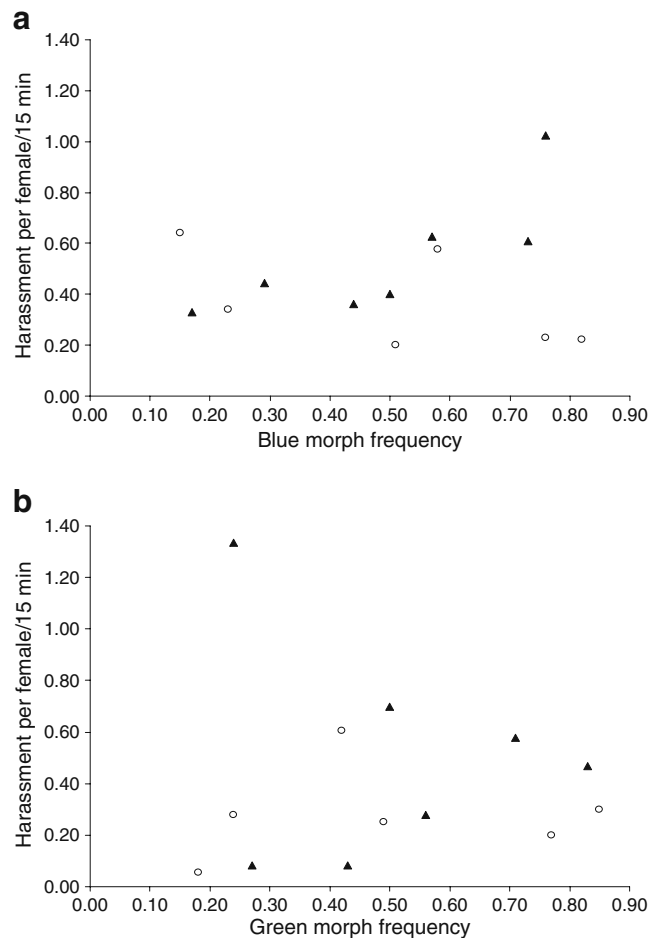
Male activity throughout a day

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 ($\bar{x} = 1.40$, $SD=0.60$) and the green morph ($\bar{x} = 1.38$, $SD=0.59$) 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: $r=-0.39$, $n=6$, $p=0.45$).

**Fig. 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)

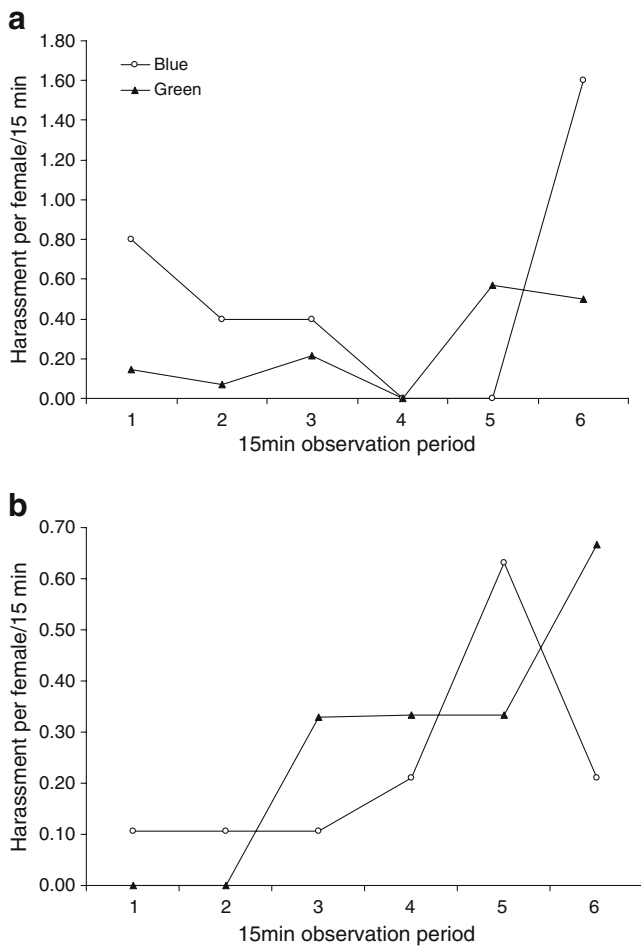


Fig. 4 Examples of variation in male activity across observational periods on a given day (observational data): **a** a replicate of 20% blue females at sex ratio of 2; **b** a replicate of 80% blue females at sex ratio of 2

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 ($F_{1,25}=9.10$, $p=0.01$),

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

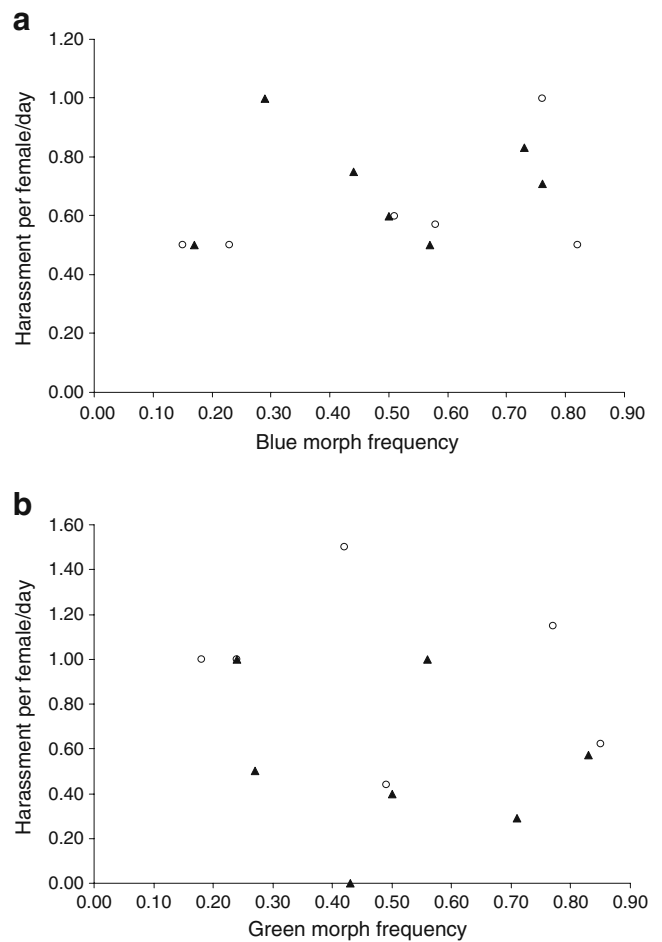


Fig. 5 Per capita harassment rates scored by fluorescent dust, towards **a** blue females as a function of mean blue morph frequency and **b** green females as a function of mean green morph frequency. Open circles indicate sex ratio of 0.5, triangles indicate sex ratio of 2

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 h each day (i.e., eight 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 eight and nine tandem pairs respectively within 3 h. Photos revealed that the first five 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 seven 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).

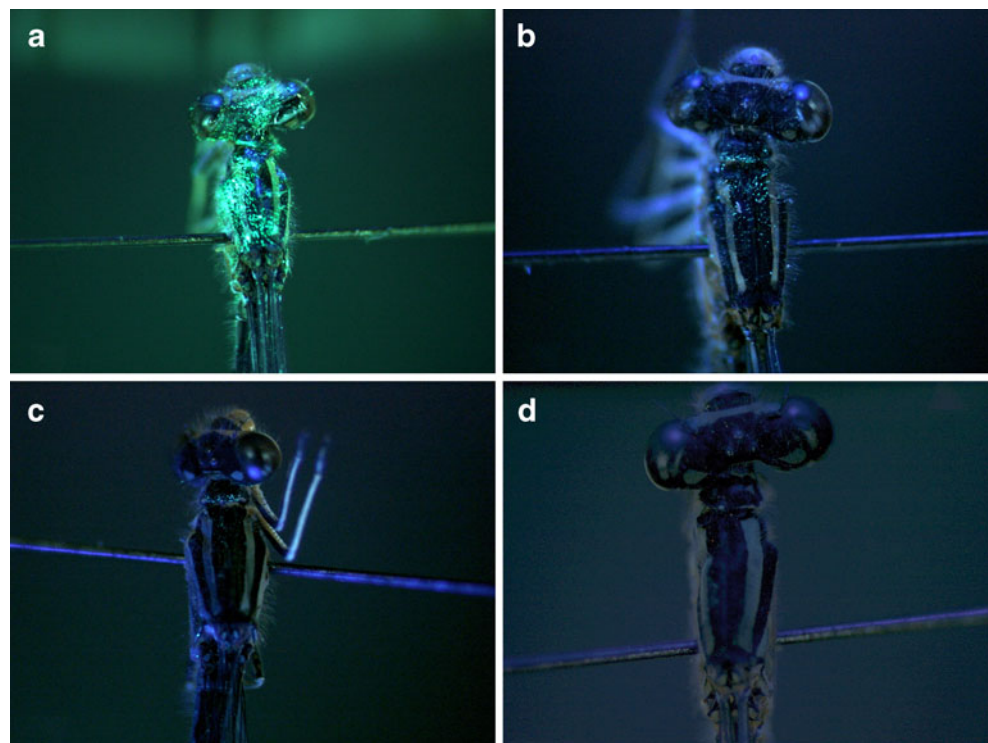
Discussion

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.

Fig. 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



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 eight 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 et al. 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 ($F_{5,10}=7.60$, $p=0.003$). 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 ($\text{LogHar}=0.02+0.22\text{B}\%$; $r^2=0.07$, $F_{1,6}=0.42$, $p=0.54$) or for green females ($\text{LogHar}=0.11+0.10\text{G}\%$; $r^2=0.04$, $F_{1,6}=0.26$, $p=0.63$, 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)

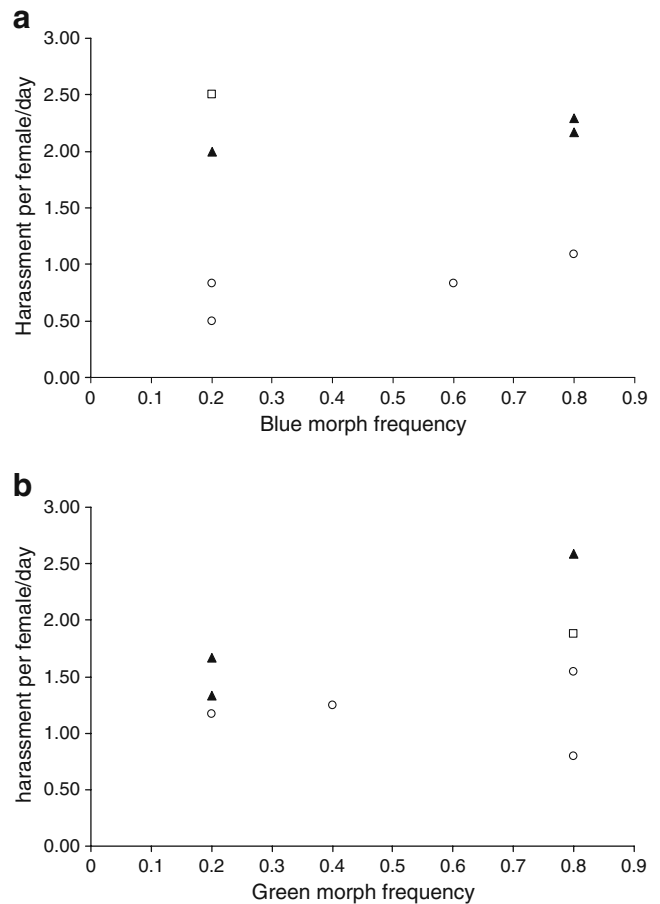


Fig. 7 Mean per capita harassment rate over 3 days from Van Gossum et al. (2001a) towards **a** blue females as a function of mean blue morph frequency and **b** green females as a function of mean green morph frequency. One group with sex ratio of 0.6 (insectary 5) was included in the sex ratio of 0.5 treatment. *Open circles* indicate sex ratio of 0.5, *open squares* indicate sex ratio of 1, *closed triangles* indicate sex ratio of 2

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 [$\log_{10}(\text{mating attempts})=0.010+0.052\text{ A/G ratio}$, $r=0.33$, $F_{1,22}=2.62$, $p=0.12$] or the gynomorph [$\log_{10}(\text{mating attempts})=0.180-0.017\text{ A/G ratio}$, $r=0.17$, $F_{1,22}=0.65$, $p=0.43$]. 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 35 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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