

# Lack of innate preference for morph and species identity in mate-searching *Enallagma* damselflies

Ola M. Fincke · Amélie Fargevielle · Tom D. Schultz

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**Abstract** Insect mate recognition is often viewed as stereotypic, innate, and species-specific. However, male damselflies can learn to identify female-specific color morphs as potential mates. A suite of male mimicry hypotheses assume that heteromorphic females, which differ from males in color pattern, are more easily recognized as “female” and thus lack the inherent, anti-harassment advantage that the more male-like signal provides for andromorphs. Using two measures of male preference, we investigated whether naïve males have a preexisting sensory bias for a given morph color in *Enallagma civile*, a species that appeared to exhibit extreme plasticity in morph expression across generations within a breeding season. *E. civile* males raised in the absence of females exhibited no preference for either morph, whereas males raised with one female type exhibited a learned sensory bias for that morph. Male *Enallagma* also lacked a bias toward conspecific females over a congeneric sister species. In a naturally naïve population of *Enallagma ebrium*, males reacted sexually to both morphs of *Enallagma hageni* as often as they did to conspecific females, whose thoracic spectra were nearly identical with those of *E. hageni*. Moreover, despite the similar thoracic spectra of males and andromorphs, both of which reflected UV, males rarely reacted sexually to other males. Our results falsified

implicit assumptions of male mimicry hypotheses, supported learned mate recognition, and suggested a scenario for speciation via sexual conflict.

**Keywords** Color polymorphism · Harassment · Sexual conflict · Learned mate choice · Pre-existing sensory bias · Reflectance spectra

## Introduction

Learned mate recognition has been documented in several vertebrate taxa, where it has been shown to be important in speciation (e.g., Irwin and Price 1999). Sexual imprinting in young birds can lead to preference as adults for hetero-specific mates (Slagsvold et al. 2002). Learned mate recognition via sexual imprinting has also been demonstrated in guppies, which lack parental care (Magurran and Ramnarine 2004). In insects, learning has been documented, but primarily with respect to foraging behavior (e.g., Papaj and Prokopy 1989; Dukas and Bernays 2000; Weiss and Papaj 2003) and host location (e.g., Godfray and Waage 1988; Cunningham et al. 2001). Perhaps because copulation is so fundamental to fitness, reproductive behavior among insects often involves stereotypic responses elicited by species-specific cues, such as unique pheromone mixes or auditory calls (e.g., Jacobson 1972; Charlton et al. 1993; Phelan 1997; Greenfield 2002; Gemeno and Schal 2004). Nevertheless, studies on mate-searching odonates (Fincke 1994; Miller and Fincke 1999; Van Gossum et al. 2001a,b; Miller and Fincke 2004), courting male flies (Dukas 2005), and female choice in wolf spiders (Hebets 2003), suggest that learning may play more of a role in invertebrate reproductive behavior than has been appreciated previously.

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O. M. Fincke (✉) · A. Fargevielle  
Department of Zoology, University of Oklahoma,  
Norman, OK 73019, USA  
e-mail: fincke@ou.edu

T. D. Schultz  
Department of Biology, Denison University,  
Granville, OH 43023, USA

In both Anisoptera (dragonflies) and Zygoptera (damselflies), female-specific color polymorphisms characterize several large families whose males search for mates rather than defend specific territories (Fincke 2004; Fincke et al. 2005). Usually, the polymorphism consists of one or more “heteromorphs,” whose appearance differs distinctly from males, and a more male-like “andromorph” (Johnson 1975). In *Ischnura* (Johnson 1964, 1966; Cordero 1990; Sánchez-Guillén et al. 2005), and *Ceriagrion* (Andrés and Cordero 1999) inheritance of the color pattern is controlled by a single autosomal locus with a dominance hierarchy of multiple alleles. Relative to monomorphic species whose females are either all andromorphic or all heteromorphic, polymorphic species are more likely to be common than rare, tentatively supporting the hypothesis that the polymorphism functions to reduce sexual harassment by males (Fincke 2004). The cost of sexual harassment is expected to be reduced fecundity, as has been demonstrated in other insects (e.g., Watson et al. 1998; Holland and Rice 1999; McLain and Pratt 1999). In a lab experiment, Sirot and Brockman (2001) found such a cost for andromorphic female *Ischnura ramburii*, but not for heteromorphs. An alternative possibility, that the polymorphism reduces competition among females, lacks support (Sirot et al. 2003).

According to the learned mate recognition (LMR) hypothesis (Fincke 1994; Miller and Fincke 1999; Fincke 2004), when male harassment toward females imposes a fitness cost, polymorphic females, whose color variation makes it more difficult for a mate-searching male to cue to any given female type, should enjoy reduced per capita harassment relative to monomorphic females. In response to this sexual foil, males should increase their search efficiency by cueing to the morph most frequently detected (i.e., correctly recognized as a potential mate). Such males should exert positive, detection-dependent harassment toward female morphs, maintaining multiple female variants by negative frequency-dependent selection acting on each distinguishable type.

At equilibrium, morph fitness should be equal, whereas morph frequency is expected to vary depending on morph-environment interactions affecting the relative fitness cost of morph coloration for females (Fincke 2004). The model is analogous to the maintenance of color polymorphisms by apostatic selection of predators (e.g., Oxford and Gillespie 1998; Bond and Kamil 2002). The LMR hypothesis enjoys support from a negative frequency-dependent population genetics model and frequency-dependent fecundity (Svensson et al. 2005), in addition to the evidence for male learning in two genera (Miller and Fincke 1999; Van Gossum et al. 2001a) and frequency-dependent harassment (Van Gossum et al. 2001b).

A suite of alternative explanations for the maintenance of female-specific-color polymorphisms consider andromorphs as male mimics, which, *because of their greater signal similarity to males*, benefit from reduced sexual harassment relative to heteromorphs. Signal characteristics include both color pattern as well as any morph-specific behavior (e.g., Robertson 1985; Van Gossum et al. 2001b; Sirot et al. 2003). A male’s reaction to either or both can be tested, depending on the experimental design (e.g., Miller and Fincke 1999; and Van Gossum et al. 2001a, respectively). Regardless of the mode of selection proposed, these “mimicry hypotheses” all assume that, relative to heteromorphs, andromorphs are inherently more difficult for a male to recognize as “female,” either by heterospecific (Johnson 1975) or conspecific males (Robertson 1985; Hinnekint 1987; Sherratt 2001). Implicit in that assumption is that males have a preexisting sensory bias (sensu Ryan 1998) toward heteromorphic females. If that were not the case, then even in the two mimicry models that evoke frequency-dependent detection of andromorphs, there would be no need for any *frequency-independent disadvantage for the andromorph*, such as “reduced life expectancy due to predation” (Robertson 1985) or a greater probability of being encountered by males (Sherratt 2001). In those models, the mimicry advantage decreases as andromorph frequency rises because then, males are predicted to learn their sexual identity (Sherratt 2001), whereas the detection of heteromorphs remains *frequency-independent*. Indeed, Sherratt (2001) noted that a key test of his mimicry model is that the “attack rate of males on andromorphs should be higher the higher the perceived ratio of andromorphs to males, while the attack rate of males on heteromorphs should be invariable.”

In contrast, the LMR hypothesis requires no preexisting sensory bias toward either morph type. Moreover, if males learn to recognize potential mates, then signal similarity with multiple distractor types, *not only males*, should be a source of confusion to mate-searching males. For example, among sympatric species, high signal similarity among congeneric females of both morph types should add to a male’s confusion. If males lack an innate ability to recognize conspecific females, then females of either morph may suffer harassment not only by conspecific males, but by syntopic congeneric ones of similar size and reflectance spectra.

Here, we first document an extreme form of plasticity in morph expression in our study population of *Enallagma civile* to illustrate why learning to recognize female signals would be adaptive for mate searching males. We then ask, for the first time, whether experimentally naïve male *Enallagma* males have an innate sensory bias for one female morph over the other, and whether naturally naïve males have an innate preference for conspecific females

compared with congeneric females with similar thoracic spectra. In so doing, we test two general predictions, which distinguish the LMR hypothesis from the suite of mimicry hypotheses.

## Materials and methods

### Study species

Male damselflies in the genus *Enallagma* search for mates in grassy areas around breeding sites on ponds or small lakes (e.g., Bick and Bick 1963; Bick and Hornuff 1966). Males exhibit scramble mate competition, and perform no courtship before taking a female in tandem, which can occur far from the water (Fincke 1982, 1986). Odonates are able to detect UV and a range of colors that include violet, green, and red (Labhart and Nilsson 1995; Yang and Osorio 1996). Potential mates appear to be detected by males using visual cues; use of sexual pheromones has not been documented in any odonate species (Corbet 1999). To achieve tandem, the prerequisite for copula, a male uses anal claspers (cerci) to engage a female's mesostigmal plates on her thorax. Once in tandem, damselfly females can detect a heterospecific male via tactile receptors (Robertson and Paterson 1982). Although females can refuse to copulate with such males, their release from tandem appears to be under a male's control; males often release resisting females within minutes (reviewed by Fincke 1997).

To a human observer, both andromorphs and heteromorphs of many *Enallagma* are nearly indistinguishable from congeneric morphs (see Dunkle 1990; Lam 2004). This is particularly true of female *E. hageni* and *E. ebrium*, two sister species whose phylogeography suggests recent hybridization (Turgeon et al. 2005). Slight differences in the female mesostigmal plates of the two species are discernable with magnification (Lam 2004), whereas males of the two species are readily distinguishable thanks to species-specific anal appendages, a characteristic of the genus (Walker 1953).

In contrast with the characteristic blue and black abdomen of males, female *Enallagma* of both morphs have a dark abdominal dorsum (Walker 1953), which helps cue their sexual identity to males (Miller and Fincke 1999; see also Gorb 1998). In sexually mature females of polymorphic species, the thorax and the sides of the abdomen are blue in the andromorph and shades of green to tan in the heteromorph. In addition to these two primary morph types, *Enallagma civile* in our study population exhibited a third "intermediate" female variant whose thorax is green and abdominal sides, blue (see photo in Miller and Fincke 2004).

To verify that the three sexually mature female variants of *E. civile* do not change morph designation with age, recently emerged teneral were uniquely marked with a number written in indelible ink, and maintained in an outdoor insectary. After gaining mature coloration, females were checked over the next 2 weeks for changes in the initial morph designation. For all study species, the frequency of color morphs were measured in the field during the same period as the male preference trials. In addition, morph frequencies of *E. civile* were measured between June and July 2000, 2003, and 2004, and at the end of the breeding season, between October 12–24, 2000 and 2004. Females and males were captured with a net, either in tandem or alone, as far as 50 m from shore, and marked on the wing with indelible ink to prevent recounts of the same individual. For a subset of the individuals, forewing length, which is highly correlated with abdominal length, tarsus length, and body weight (Fincke 1982), was measured as an indication of body size.

### Reaction of naïve *Enallagma civile* males to conspecific female morphs

*E. civile* was studied at the University of Oklahoma Aquatic Research Facility (ARF) in Norman, Cleveland County, OK. There, adults emerged from artificial ponds in early May and reproduced until mid-October. *Enallagma basidens*, a much smaller species, was the only other *Enallagma* present.

Experiments with *E. civile* were conducted between 15 May and 23 July 2003, and 1 May to 15 July 2004. Multiple, newly emerged *E. civile* males were placed in one of three outdoor insectaries (1.8×1.8×1.8 m, ©BioQuip) with: 1) no females, 2) only green females, or 3) only blue females. In the two latter cases, the sex ratio (males to females) was  $\geq 1.0$ . Mature females were added to replace any of 8–10 initial ones that died. No more than 20 individuals were present in an insectary at any time. In this experiment and those that follow, intermediate-type females, which were rare early in the season, were not used. The insectaries were regularly stocked with *Drosophila melanogaster* as prey. Due to logistical limitations, males were not reared in isolation in individual cages. Hence, our design did not eliminate possible bias resulting from males reared with other males.

At sexual maturity, judged by fully developed coloration (i.e., >5 days post emergence) males were uniquely numbered on the wing and tested for their preference of female morph types. We compared two different methods used in past studies. In "presentation trials" (see Miller and Fincke 1999), the legs of a live individual were glued to the horizontal end of a dowel to

mimic a naturally perching damselfly (hereafter referred to as a “damsel on a stick”). The glued individual was presented to a focal male by slowly moving the dowel 5 to 10 cm from, and slightly below, him. The male’s subsequent reaction was recorded. An individual green female, blue female, and mature male, were presented sequentially to the same marked male, alternating the order of the presentation. Male reactions were scored as: 1) approach—male moved toward the individual but did not touch it, 2) grab, 3) tandem formation, or 4) no reaction if the male failed to react within 1 min. Because males may not always be ready to mate, a male that did not react to any of the three types (i.e., two morphs and control male) on a given day, was retested within 3 days. Only if a male then failed to react to any of the three types was he scored as not reacting.

To reduce the variation in male response that might arise due to variation among glued individuals within types, the same set of damsels on sticks were presented to all mature males tested on a given day (i.e., a maximum of four focal males/treatment). Reacting tandem males were gently removed from the glued individuals, which remained alive and undamaged during the trials (<60 min). Fresh individuals were field caught daily; in total, 52 unique females (26 of each morph) and 26 males were presented.

Our second measure of male preference was a “binary choice trial” (see Van Gossum et al. 2001a), in which the same focal males as above were allowed to choose between two free-flying females in a small cage (30×30×30 cm; ©BioQuip). In our experiment, the mature focal male was released into the cage into which had been placed two dowels for perches, a sexually mature blue female, and a mature green one. In the initial trials, none of ten naïve males reacted to either female within 2 min. Consequently, the procedure was modified by first holding a focal male in a black film canister for 1 min (see Van Gossum et al. 1999, 2005a), which greatly increased the number of reacting males. In three cases, a male touched a female but was rebuffed by her abdomen curl and wing flutter, and the male then grabbed the second female. These were scored as a sexual reaction to both female types; scoring only the male’s first reaction did not change any conclusions drawn from the results. In total, 54 unique females (27 of each morph) were used. This design precluded measuring a male’s reaction toward another male whose presence would have introduced even more uncontrolled variation caused by interactions of the non-focal male with the two females. For analyses, grabs and tandems were considered sexual reactions. Two-tailed binomial tests were used to determine whether the proportion of males’ sexual reactions toward each morph differed from an expected 0.5.

Reactions of naïve *E. ebrium* males to *E. hageni* individuals in the field

This experiment was conducted on a naturally naïve population of *E. ebrium* at Stoney Creek, Cheboygan County, Michigan from 19 July to 6 August 2004, and from 19–21 July 2005. The breeding site was a shallow wetland created by a small dam built across Stoney Creek. *E. ebrium* was the only *Enallagma* species at this site. Hence, males were naturally naïve in that they lacked any previous experience with congeneric female *Enallagma*. Another coenagrionid damselfly, *Ischnura verticalis*, was present at this site.

The reactions of *E. ebrium* males to conspecific controls and to *E. hageni* individuals were measured between 1250 and 1700 h, when males actively searched for females in a 30-m-wide grassy area that bordered the shore. In control trials, we used *E. ebrium* males and females collected in tandem to ensure sexual maturity of the females. Dowels with a damsel on a stick were inserted into the ground, 5 to 10 m from the shoreline. Male reactions toward the individual were noted during a 15-min period. A blue female, a green female, and a male were placed sequentially in the same spot, rotating the order of presentation (i.e., 45 min total observation time for the three types).

*E. ebrium* males were scored for grabs or tandems as above. In addition, a “fly by” was scored if a male flew within 10 cm of the damsel on a stick without reacting, or hovered briefly without touching the glued individual. Whenever possible, the reacting male was hand-caught by quickly clasping its wings if in tandem, or with a net after an interaction. These males were collected and their species identity later confirmed by examination of the anal appendages with a hand lens. If a male was not captured because it would have disrupted other males in the immediate area, the event was still counted as an independent observation. In other similar experiments in populations whose males were previously marked, no marked male returned to an experimental individual within 15 min (Miller and Fincke 1999; OM Fincke unpubl. data).

To test the reaction of the naturally naïve Stoney Creek *E. ebrium* males toward *E. hageni*, the latter species was collected from a site about 60 km away at Lake Kathleen, near Pellston, in Emmet County, MI, where *E. hageni* co-occurred with *E. ebrium*. To be certain of species identity, all *E. hageni* females used in this experiment were field-collected by netting tandem pairs; the male’s appendages identified him to species. Individuals were separated by sex and transported in small cages to Stoney Creek. An *E. hageni* damsel on a stick was placed 5–10 m from the water’s edge, and reactions by *E. ebrium* males were recorded during a 15-min period as above except that “tandem attempts” were noted as well as tandems. This was

because although many males tried to clasp a female's thorax with their anal appendages, few male *E. ebrium* achieved tandem with female *E. hageni*. Reacting male *E. ebrium* were collected whenever possible to confirm species identify; later examination of the mesostigmal plates of all Lake Kathleen females used in the experiment confirmed that all were *E. hageni*. Throughout, Fisher exact tests are two-tailed; means are presented  $\pm$  S.E.

To determine the similarity of congeneric morph signals and their similarity with male signals, the spectral reflectance of the thorax of live males and female morphs of *E. hageni* and *E. ebrium* was obtained using an OceanOptics SD2000 spectrometer with a PS-2 xenon light source. The mean reflectance spectra for males and female morphs of each species were calculated. To compare the brightness in color, we calculated the mean radiance in  $\mu\text{mol m}^{-2} \text{s}^{-1} \text{nm}^{-1}$  of each morph under ambient light conditions at midday. Differences in hue and chroma between species and morphs were determined by first standardizing the spectra to the same brightness and then comparing spectral shape with the Euclidean distance measure,  $D_s$  (Endler 1993). The scale for this measure is between 1.0 and 0.0, with the latter representing a perfect match in spectral shape.

## Results

### Morph frequency and adult size

None of the original morph designations changed for any of the 100 marked female *E. civile* that survived in the insectary from 3–14 days. The maximum time female variants were observed was 14 days for andromorphs, 13 days for heteromorphs, and 10 days for phenotypically intermediate females. Wing length differed among all three study species (Table 1). Female *Enallagma* were significantly larger than conspecific males (*E. civile*:  $t=2.05$ ,  $P=0.05$ ; *E. ebrium*:  $t=3.3$ ,  $P=0.001$ ; *E. hageni*:  $t=9.8$ ,  $P<0.001$ ), but morph type had no effect on female wing length ( $F_{1, 138}=1.07$ ,  $P=0.30$ ).

As shown in Table 2, during June and July, the morph frequency of the *E. civile* population at the ARF, OK site did not differ significantly between years 2003 and 2004 ( $n=246$ ,  $P=0.38$ , Fisher exact test). However, in 2004, the frequency of intermediate females was only 10% in the first half of the season, whereas by October, intermediate type females represented 90% of the total ( $n=175$ ,  $P<0.0001$ , Fisher exact test). Periodic abundance of exuvia and teneral individuals suggested that the ARF study population that emerged in May–June produced a second generation that emerged in late Aug to Sept, flying until early October. The frequency of andromorphs in the ARF population in June–

**Table 1** Wing size (mm) of female morphs and males of the study species: *E. civile* (OK), *E. ebrium* (MI), and *E. hageni* (MI)

Species	Female:		Male
	Blue	Green	
<i>E. civile</i> <sup>a</sup>	19.43 $\pm$ 0.16 (13)	19.78 $\pm$ 0.28 (10)	18.75 $\pm$ 0.17* (23)
<i>E. ebrium</i> <sup>b</sup>	18.21 $\pm$ 0.33 (10)	18.08 $\pm$ 0.13 (46)	17.73 $\pm$ 0.09** (75)
<i>E. hageni</i> <sup>c</sup>	18.96 $\pm$ 0.10 (35)	19.17 $\pm$ 0.08 (75)	18.19 $\pm$ 0.06** (101)

Superscripts indicate species differences in wing length (Bonferonni tests,  $P<0.05$ ). Sample sizes are in parentheses. Difference between males and pooled females: \* $P<0.05$ , \*\* $P<0.0001$ .

July differed significantly ( $n=241$ ,  $P<0.0001$ , Fisher exact test) from those of *E. civile* during June–July, 1993 in another population 160 km away (see Miller and Fincke 2004).

### Reactions of naïve *Enallagma civile* males to conspecifics

The mean duration that a teneral male was held before being tested for morph preference was 7.9 $\pm$ 0.22 days (range = 6–13 days), which did not differ among the three treatments ( $F_{2, 63}=0.04$ ,  $P=0.96$ ). Results of the naïve male experiment are presented in Table 3. In the presentation trials, 30% of the naïve but sexually mature males that had never experienced a female conspecific ( $n=43$ ) failed to react to any conspecific, significantly more than the 5.1% of non-reacting males of similar age that were reared with females ( $n=39$ ,  $P=0.01$ , Fisher exact test). In contrast, in the binary trials, there was no difference in the proportion

**Table 2** Frequency of female types between months and sites

Site	Months	Year	<i>n</i>	Morph frequency		
				Green	Intermediate	Blue
ARF <sup>a</sup>	July	2000	26	0.31	0.46	0.23
ARF <sup>b</sup>	October	2000	20	0.10	0.90	0
ARF <sup>c</sup>	June–July	2003	131	0.39	0.15	0.46
ARF <sup>c</sup>	June–July	2004	115	0.50	0.10	0.40
ARF <sup>b</sup>	October	2004	60	0.08	0.90	0.02
Looney Pond <sup>d</sup>	June–July	1993 <sup>c</sup>	126	0.26	0.45	0.29

Aquatic Research Facility (ARF) at OU consists of several artificial ponds in close proximity where *E. civile* breed. Looney Pond is approximately 160 km away. Superscripts refer to populations in which morph frequencies differ significantly (Fisher exact,  $P<0.05$ )

<sup>c</sup>Data from Miller and Fincke (2004)

**Table 3** Responses towards conspecifics, by sexually mature *E. civile* males (*n*) reared under three different treatments in outdoor insectaries

Males reared with:	<i>N</i>	Male responses								
		Sexual response toward:				Approach only				No response
		B	G	B&G	Male	B	G	B&G	Male	
No females	43	7	3	7	0	5	2	5	1	13
No females <sup>b</sup>	41	10	8	3	–	2	1	0	0	17
B only	23	13	1*	4	0	0	3	1	0	1
B only <sup>b</sup>	24	7	4	0	–	1	1	2	0	9
G only	16	0	12*	1	0	2	0	0	0	1
G only <sup>b</sup>	16	0	9*	0	–	0	0	1	0	6

For each treatment, the response in the presentation trials precedes the response in the binary test trials (<sup>b</sup>, see text). Sexual response includes grabbing a female and tandem formation. Approaches are shown only for males that never reacted sexually to any individual. Categories are unique and mutually exclusive.

B:Blue andromorph, G:green heteromorph, B&G: response to both blue and green females, Male:focal male reacted *only* to a male;

\* $P < 0.05$ , binomial test.

of naïve (41.5%) and female-experienced males (37.5%) that failed to react ( $n=81$ ,  $P=0.82$ , Fisher exact test). Collectively, 18% of the males failed to react in pooled preference trials, whereas 39.5% of the same group of males failed to react in the binary choice trials ( $n=163$ ,  $P < 0.01$ , Fisher exact test).

Despite that difference, the reaction of males in the three treatments differed significantly in both the presentation trials ( $n=82$ ,  $P < 0.0001$ , Fisher exact test) and the binary trials ( $n=81$ ,  $P < 0.001$ , Fisher exact test). In total, only three males (two raised without females, one raised with blue females) reacted sexually toward a male. Among naïve males, those that reacted sexually to only one female morph exhibited no preference for heteromorphs or andromorphs (presentation trials:  $n=10$ ,  $P=0.34$ ; binary trials:  $n=18$ ,  $P=0.81$ , binomial tests). In contrast, when raised with females of a given morph type, males that reacted sexually to only one female type were more likely to react to the morph type they had experienced (i.e., blue females:  $n=14$ ,  $P=0.002$ ; green females:  $n=12$ ,  $P < 0.001$ , binomial test). In the binary trials, the results were similar for

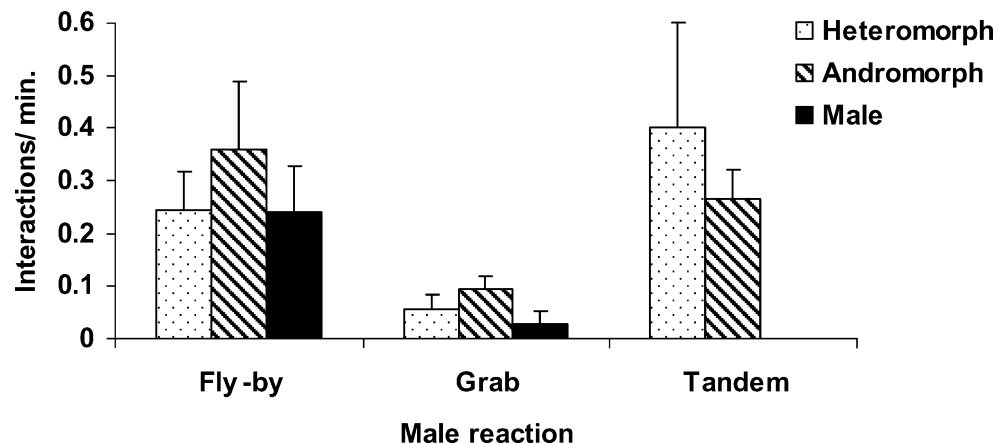
males raised with green females ( $n=9$ ,  $P=0.004$ ), but those raised with blue females did not exhibit a preference for either morph type ( $n=11$ ,  $P=0.55$ , binomial test). In two cases, females approached the focal male before he had reacted, possibly biasing the outcome.

Reactions of naïve *E. ebrium* to conspecifics and to *E. hageni* individuals

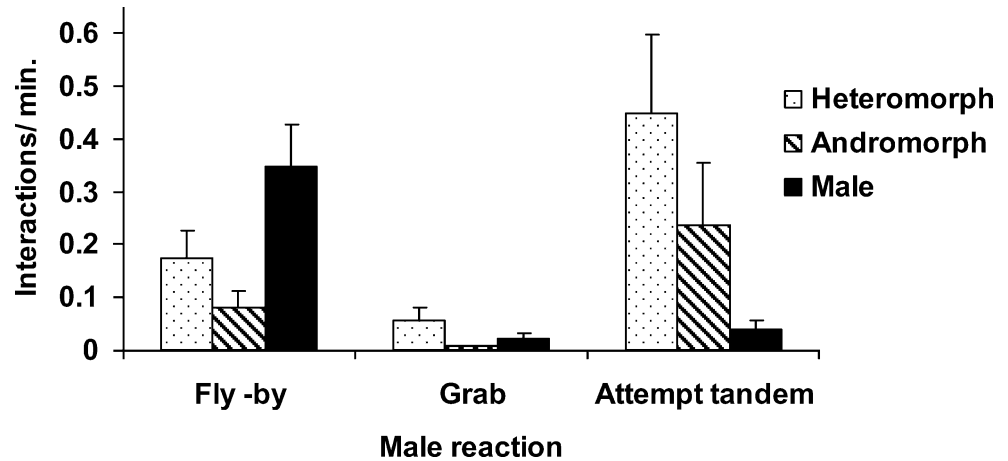
Over the two summers, of the 241 male *Enallagma* caught and marked at Stoney Creek, all were *E. ebrium*. Andromorphs represented 10.5% of the female *E. ebrium* caught ( $n=105$ ). Males searching for mates regularly inspected free-flying males in the area. Both female *E. ebrium* (Fig. 1) and *E. hageni* (Fig. 2) elicited high rates of sexual attention from male *E. ebrium*. In both species and for both morphs, a male usually grabbed a female within the first minute of her placement in the field; sexual interactions by unique males were as high as 5 per minute.

Male *E. ebrium* reacted sexually toward females of both species significantly more than they did to either species of

**Fig. 1** Rate of reactions (mean+S.E.) of *Enallagma ebrium* males toward conspecifics on a stick at Stoney Creek, MI



**Fig. 2** Rate of reactions (mean+S.E.) of naïve *Enallagma ebrium* males toward *E. hageni* individuals at Stoney Creek. “Attempt tandem” also includes successful tandems (see text)



male (*E. ebrium*:  $n=126$ ,  $P<0.001$  Fisher exact test; *E. hageni*:  $n=188$ ,  $P<0.0001$ , Fisher exact test, Table 4). The reactions of male *E. ebrium* to male *E. hageni* did not differ from their reactions to male conspecifics ( $n=72$ ,  $P=0.715$ , Fisher exact test). Male *E. ebrium* reacted sexually more often to the majority green conspecific females than to the minority blue andromorphs ( $n=106$ ,  $P=0.046$ , Fisher exact test, Table 4). In contrast, male *E. ebrium*, which had no prior experience with *E. hageni* females, reacted sexually to *E. hageni* females without discriminating between morphs ( $n=136$ ,  $P=0.35$ , Fisher exact test). Of the 39 reacting males that were caught, all were *E. ebrium*. Whereas all of the five male *E. ebrium* that attempted to take a male *E. hageni* in tandem succeeded in doing so, of the 104 male *E. ebrium* that attempted to form a tandem with an *E. hageni* female, only 16 (15%) succeeded, a significant difference ( $n=109$ ,  $P=0.0002$ , Fisher exact test). An unsuccessful male would fly up, realize the female was not attached, and try again to form tandem. After several such attempts, he flew away. In a successful tandem, the male tried to fly off with the glued female for as long as 15 s before releasing her. Using a dissecting scope, no

obvious differences in the morphology of the male claspers between successful and unsuccessful male *E. ebrium* were observed.

The reflectance curves from the thorax of *E. ebrium* and *E. hageni* exhibited little variation within sex and morph, or between species (Figs. 3 and 4). Males of both species were similar in brightness ( $4.92$  and  $4.49 \mu\text{mol m}^{-2} \text{s}^{-1} \text{nm}^{-1}$ , respectively) and exhibited a broad reflectance peak between 350 and 475 nm. The difference in hue and chroma,  $D_s$ , between the mean radiance of *E. ebrium* and *E. hageni* males was 0.064. Although not as bright as the males (Fig. 3), the andromorphic females of *E. ebrium* reflected the same hue and saturation as males. Andromorphic *E. hageni* were not as bright or saturated as the males (Fig. 4), and exhibited a peak reflectance at 490 nm rather than 400 nm. The pale green heteromorphs of both species were similar to each other in brightness ( $3.52$  and  $4.05 \mu\text{mol m}^{-2} \text{s}^{-1} \text{nm}^{-1}$ ), hue and saturation (Figs. 3 and 4,  $D_s=0.032$ ). In contrast with the males and andromorphs, heteromorphic females of both species exhibited very low reflectance in the ultraviolet range.

**Table 4** Responses during 15-min observation trials in the field of naïve male *E. ebrium* toward conspecifics (damsels on a stick), and *E. hageni*

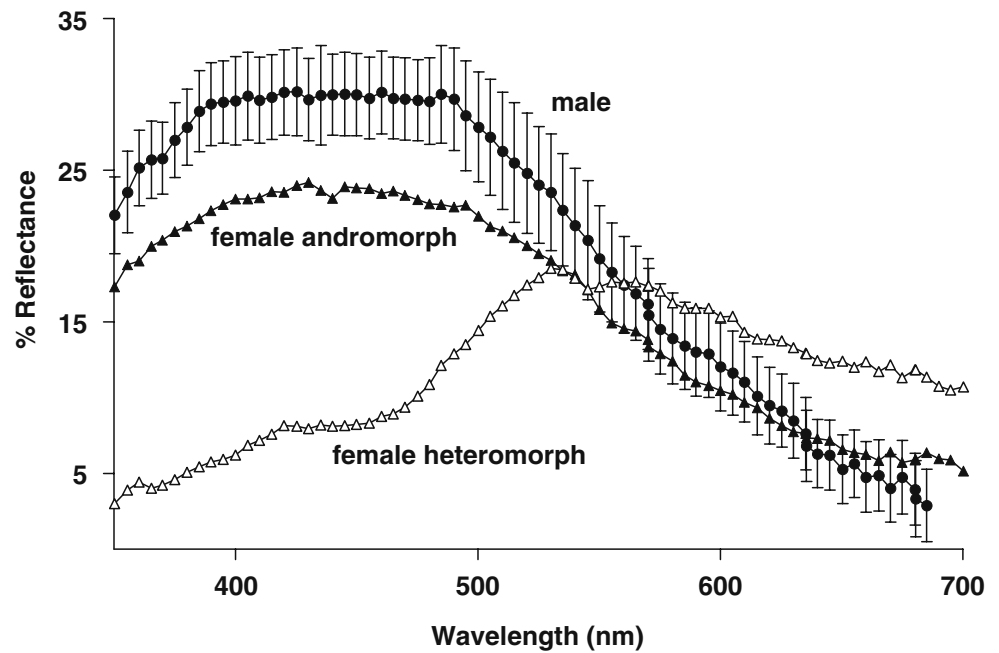
Reaction to	Male responses:			
	Fly-by	Grab	Tandem	Attempt tandem
<i>E. ebrium</i> :				
Green female	22	5	36	–
Blue female	24	5	14	–
Male	18	2	0	–
<i>E. hageni</i> :				
Green female	15	6	10	62
Blue female	10	1	6	26
Male	44	3	5	

All categories are mutually exclusive.

## Discussion

As is the case for other polymorphic *Enallagma* (e.g., Fincke 1982, 1994), *E. civile* morphs maintained the same morph designation over a 2-week period, longer than the mean natural lifespan of a closely related species (Fincke 1986). Andromorph frequency varied more than 20-fold between generations within a breeding season of our Oklahoma study population, compared with only a twofold difference in mean frequency between populations, and was nearly as great as the maximum known difference in andromorph frequency between populations of any polymorphic coenagrionid (reviewed by Fincke et al. 2005). The nearly monomorphic fall generation, 90% of whose females were intermediates, gave rise to the more distinctly

**Fig. 3** Mean reflectance spectra ( $n=15$ ) of the thorax of males and female morphs of *Enallagma ebrium*. Error bars indicating SD are only shown for males

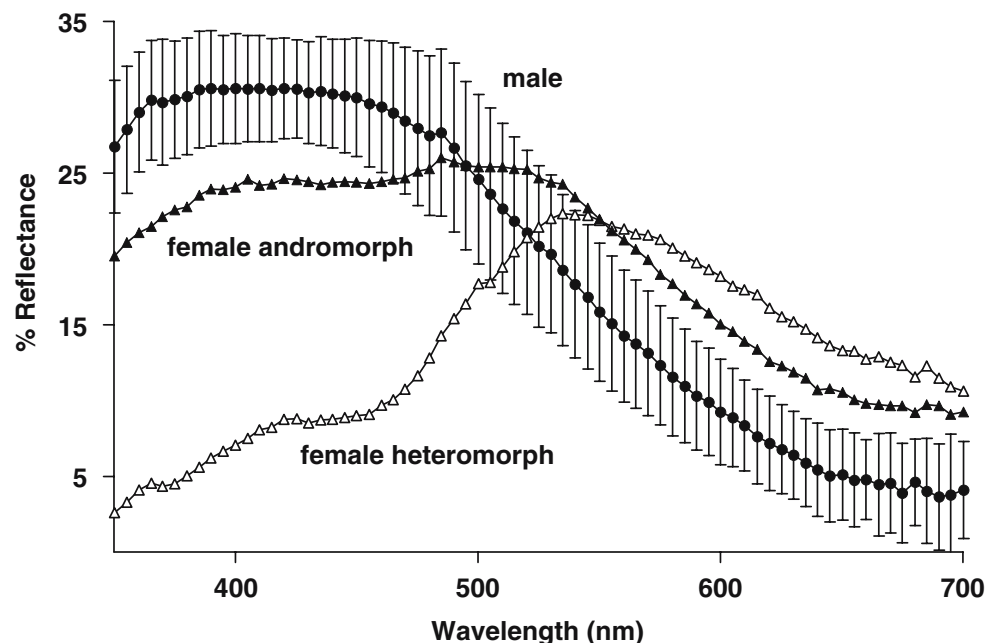


dimorphic spring population, in which intermediates were relatively rare. Selection is unlikely to explain the change between early and late season, which was not only consistent across years (Table 2), but also occurred in several populations in the region (OM Fincke, unpublished data). Although unlikely to be a purely seasonal polyphenism as in some butterflies (e.g., Fric et al. 2004; Wiklund and Tullberg 2004), the change may reflect a gene-environment interaction in the phenotypic expression of the underlying Mendelian genes controlling for color,

possibly mediated by larval density or temperature. Regardless of the cause, this rapid and high fluctuation in female phenotypes should make the learning of sexual signals particularly advantageous for mate-searching males.

Results from the presentation trials on male *E. civile* indicated that naïve males lacked a preexisting sensory bias toward heteromorphs, contrary to the suite of mimicry hypotheses. The latter implicitly assume that, unlike andromorphs, heteromorphs are inherently recognized as “female” (Johnson 1975; Robertson 1985; Hinnekint 1987;

**Fig. 4** Mean reflectance spectra ( $n=15$ ) of the thorax of males and female morphs of *Enallagma hageni*. Error bars indicating SD are only shown for males



Sherratt 2001), a view unfortunately reinforced by referring to heteromorphs as “gynomorphs” (e.g., Van Gossum et al. 2005a,b). Teneral male *E. civile* reared without females were less likely than experienced males of the same age to react sexually to any female, and when they did, they exhibited no preference for either morph, whereas males raised with a given morph exhibited a sexual bias toward that morph. These results strengthen conclusions drawn from an earlier learning experiment on another population of *E. civile*, in which field-experienced males preferred andromorphs after a 2-day exposure to only blue females (Miller and Fincke 1999). Although a 2-day exposure to only green females did not result in a significant preference for green, relatively few males reacted to the individuals presented.

With one exception, our two different methods for measuring a male’s preference provided similar conclusions. Unlike the presentation trials, the binary choice trials did not indicate that being raised with blue females resulted in a learned bias for andromorphs, a result that by itself, is consistent with an innate male preference for heteromorphs. However, the binary trials did not control for actions of free-flying females, and males did not react at all unless first put in a dark film canister, an effect possibly explained by dark-adapted sensitivity, which is greatest in the first minute (Chapman 1971). Moreover, only a third of the males reared with females reacted sexually toward them in the binary trials, fewer than half that did so in similar tests using *Ischnura elegans* (Van Gossum et al. 2001a), in which males formed a temporary sensory bias to both morphs.

Even when reared only with conspecific males, male *E. civile* rarely reacted sexually toward other males, unlike male *I. elegans*, which, after a 2-day exposure to only males, preferentially formed tandems with other males (Van Gossum et al. 2005a). Nevertheless, male *I. elegans* that experienced females took andromorphs in tandem more often than males, similar to our results with *E. ebrium* under field conditions (Table 4), and in contrast with studies indicating that males do not distinguish between andromorphs and males (Robertson 1985; Cordero 1989).

Although our methods could not resolve whether the aversion of *Enallagma* males to other males is innate or learned, male *E. civile* reared only with males clearly did not exhibit any aversion to andromorphs (Table 3). Hence, even if the purported andromorph advantage of the mimicry hypotheses stems from a learned aversion to males that is transferred to andromorphs, our results still failed to support even the frequency-dependent male mimicry hypotheses (see also Van Gossum et al. 2005b). In fact, the few naïve male *E. civile* that exhibited a morph preference reacted sexually to andromorphs more often than to heteromorphs, although that trend was not significant. Males might learn to associate

blue coloration with conspecifics more generally, or the clade of blue *Enallagma* species may be physiologically more sensitive to blue wavelengths. Either possibility would be contrary to the assumption of an inherent anti-harassment advantage for andromorphs, as were our actual results.

As importantly, both morphs of *E. hageni* were more confusing to male *E. ebrium* at Stoney Creek than were males of either species. Male *E. ebrium* and *E. hageni* were nearly identical in brightness, hue, and reflectance of near ultraviolet wavelengths, comparable to the conspicuously blue signals of other male *Enallagma* (TD Schultz, unpublished data). Although their thoracic spectra indicated that andromorphs were more similar to males than to heteromorphs, consistent with expectations of a male mimic, andromorphic spectra were most similar to their congeneric counterpart (Figs. 3 and 4). The female morphs of *E. hageni* were equally attractive to male *E. ebrium*, indicating that males lacked a species-specific visual bias against either morph, contrary to Johnson’s prediction (1975). It might be that males had an innate preference for conspecifics, but that male density at Stoney Creek was so high that the optimal decision rule was to try to mate with any remotely female-like signal (see Fincke 2004). This alternative seems unlikely because during the same time, males reacted sexually more often to the majority green female *E. ebrium* than to the minority blue morph, as predicted under conditions favoring the learning rule. The greater relative apparency of andromorphs helps explain why male reactions to them were higher than that expected from their frequency alone.

At Stoney Creek, of the many male *E. ebrium* that tried, only 16% formed tandems with female *E. hageni*. Although we were unable to attribute successful tandems to any obvious morphological difference, further analysis using confocal microscopy of the secondary genitalia of both sexes is planned. In a reciprocal experiment, naturally naïve male *E. hageni* similarly did not discriminate against female *E. ebrium*; the few successful tandems were maintained only briefly (OM Fincke, unpublished data). Because incompatibility in the secondary genitalia is known to maintain reproductive isolation in many odonate species (Paulson 1974), such variation in tandem formation is reminiscent of a coevolutionary arms race driven by sexual conflict (e.g., Arnqvist and Rowe 1995). The phylogeography of North American *Enallagma* indicates that the *E. hageni* clade radiated rapidly, within the past 250,000 years. (Turgeon et al. 2005). Those authors hypothesized that speciation might be driven by sexual selection, presumably female mate choice (e.g., Panhuis et al. 2001) or sexual conflict (e.g., Arnqvist et al. 2000; Gavrillets et al. 2001; Gavrillets and Waxman 2002).

Our results favor the latter hypothesis. Once polymorphic, females could further reduce harassment costs

by diverging in their mesostigmal plates such that fewer males could take them in tandem. Thus, sexual harassment could reinforce divergence in secondary genitalia that occurred by drift in isolated glacial refugia. Mapping color states onto phylogenies indicated that the polymorphic state is ancestral in *Enallagma* (Fincke et al. 2005), and within families, polymorphic genera are more speciose than monomorphic ones (OM Fincke, unpublished data), further supporting a scenario of speciation via sexual conflict.

Once reproductive isolation is complete, learning to discriminate against congeneric females should make mate-searching more efficient for males, particularly given the high potential for sexual interactions (Fig. 2) and the wide overlap of co-occurring *Enallagma* species (e.g., Turgeon and McPeck 2002). At Lake Kathleen, where *E. ebrium* males and *E. hageni* are naturally syntopic, interspecific sexual harassment is common (OM Fincke, unpublished data; see also Nielsen and Watt 2000). The spectral similarity of these sister species may prevent males from learning species identity, although a male hovering above a female may be able to distinguish the tan post-ocular spots of female *E. ebrium* from the typically green ones in *E. hageni*. Where *E. civile* were syntopic with *E. aspersum*, males appeared to learn to discriminate against congeneric females over the course of a day (Miller and Fincke 2004), possibly cueing to differences in the UV reflectance of these two species (TD Schultz, unpublished data). Interestingly, in *Calopteryx haemorrhoidalis*, a territorial species whose wing patterns, but not secondary genitalia, vary among congeners, males are able to discriminate visually against congeneric females (Beukema 2004). This is an ability that may also be learned (see Fincke 2004). The extent to which individuals learn species identity is critical to understanding the fitness consequences of interspecific sexual harassment for both sexes.

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