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Field tests of multiple sensory cues in sex recognition and harassment of a colour polymorphic damselfly

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The use of multiple sensory modalities in mating decisions has prompted a reassessment of sexual selection in many species. Odonate males have long been assumed to use only visual cues in mate recognition. Using only airborne cues in the laboratory, a previous study of *Ischnura elegans* found that males discriminate between the sexes and exhibit an odour preference for male-like female colour morphs. In a field experiment that required free-flying males to detect and recognize potential mates, we scored nonsexual and sexual reactions of free-flying males to live conspecifics (andromorphic females, which mimic male body colour and pattern; heteromorphic females, which differ from males in body colour and pattern; and males) and empty control dowels positioned at ponds. 'Nonvisual' treatments concealed under a muslin bag offered only olfactory cues, whereas the unbagged 'visual' treatments offered visual plus odour cues. Live conspecifics in the nonvisual treatments did not elicit more sexual reactions than control dowels. In contrast, live individuals in the visual treatment elicited more sexual responses than did controls, suggesting that odour alone was insufficient for detection of conspecifics. However, even with visual cues, males reacted sexually towards other males as often as they did towards either female morph, indicating a failure to discriminate between sex or morph. A second, more realistic visual treatment away from water, where 77% of the solitary mature individuals were males, produced similar results. Thus, we measured natural harassment rates of marked, free-flying females. Both female colour types used similar behaviours to evade males. We found no difference in harassment or mating rates between colour morphs. Our results suggest that visual cues of female *I. elegans* act similarly to the context-dependent signal apparency of *Enallagma* colour morphs, and emphasize the need for laboratory results to be validated by comparison of sensory abilities under natural conditions.

Understanding the sensory cues that organisms use in assessing conspecifics is critical to understanding mating decisions, reproductive isolating mechanisms, and hence, speciation (Coyne & Orr, 2004). Multimodal signalling, used in myriad contexts in animal decision making (reviewed by Partan & Marler, 2005), is common in mating decisions and male competitive interactions (e.g. Bretman, Westmancoat, Gage, & Chapman, 2011; Griffith & Ejima, 2009; Maguire, Lizé, & Price, 2015; Westerman & Monteiro, 2013). This has led to a reassessment of some male and female mating behaviours. For example, the discovery that fish scales and bird feathers often reflect in the ultraviolet (UV) visual range led to a re-examination of the cues used by females to assess mate quality (e.g. Cummings, Rosenthal, & Ryan, 2003; Hausmann, Arnold, Marshall, & Owens, 2002; Ingolf, Riarda, & Bakker, 2006; Pern, Bennett, & Cuthill, 2001). Insects use UV reflectance of flowers in pollinator decisions and sex recognition (reviewed by Silberglied, 1979). Increasing attention has been paid to the potential for such cues to interact with other sensory modalities in female choice (e.g. Papke, Kemp, & Rutowski, 2007) or morphology in determining the outcome of male competitive interactions (e.g. Xu & Fincke, 2015). Olfactory cues in the form of cuticular hydrocarbons and other volatiles are typically species specific in insects (reviewed by Howard & Blomquist, 2005; Symonds & Elgar, 2008). These cues may provide more nuanced mate choice in crickets where acoustical cues have long been thought to be the primary mode of female choice. For example, Maroja et al. (2014) suggested that courting

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males discriminate against hybrid females on the basis of cuticular hydrocarbons, biasing their calling effort towards conspecific females, which favour vigorously calling males (see also Simmons, Thomas, Gray, & Zuk, 2014).

The order Odonata comprises dragonflies and damselflies, which, as adults, possess the most highly acute vision among insects (Futahashi et al., 2015). It has long been assumed that the adults use visual cues to locate and identify prey and potential mates (reviewed by Corbet, 1999). Behavioural support for the latter assumption comes from studies in which the manipulation of visual cues led to changes in behaviour (e.g. Gorb, 1998; Schultz & Fincke, 2009; Xu, Cerreta, Schultz, & Fincke, 2014). Relative to the long antennae of many moths and butterflies, which produce volatile pheromones and rely heavily on chemical cues in decision making (reviewed by Wyatt, 2003), adult odonates possess relatively small antennae and are not known to produce pheromones as adults, although chemical cues are used in foraging and antipredatory behaviour in larval *Enallagma* damselflies (Mortensen & Richardson, 2008). Neuronal responses to chemical cues in adults were only recently documented, first in the antennae of the dragonfly *Libellula depressa* (Rebora, Salerno, Piersanti, Dell'Otto, & Gaino, 2012), and then in the damselfly *Ischnura elegans* (Piersanti, Frati, Conti, Rebora, & Salerno, 2014). Nonvisual airborne cues in an olfactometer were sufficient for male *I. elegans* to discriminate between the sexes (Frati, Piersanti, Conti, Rebora, & Salerno, 2015). Males could also distinguish between 'andromorphic' females, which mimic males in body coloration and pattern, and 'heteromorphic' females, which differ from male colour patterns (reviewed by Van Gossum, Sherratt, & Cordero-Rivera, 2008). Intriguingly, males exhibited a higher positive response to the odours of andromorphs than to odours of heteromorphs. Hence, the authors suggested that the ability to detect nonvisual cues might play a heretofore unrecognized role in the sexual behaviour of males towards the two female colour morphs, specifically suggesting that olfactory cues might function in conjunction with visual ones.

Ischnura damselflies (especially, *I. elegans*) have provided leading examples of models of sexual conflict (Zuk, Garcia-Gonzalez, Herberstein, & Simmons, 2014) and studies designed to elucidate the mechanisms that maintain female-specific colour morphs (reviewed by Van Gossum et al., 2008) and the role of antagonistic selection in speciation (e.g. Takahashi, Nagata, & Kawata, 2014). Female-specific colour polymorphisms are common in coenagrionid damselflies with nonterritorial mating systems whose males must search for mates in areas around the breeding sites (reviewed by Fincke, 2004). There is growing consensus that in nonterritorial species, colour polymorphisms function to reduce sexual harassment of females by mate-searching males. That conclusion, however, has rested on the assumption that males are solely using visual cues of female colour and pattern to find and recognize mates. Mechanical cues are known to be used by a female to reject heterospecific males after a male clasps a female by her mesostigmal plates to form a tandem, prior to copula (Robertson & Paterson, 1982). However, such cues that may be used in post-tandem decisions by females are beyond the scope of our study, which focuses on a male's ability to detect and recognize potential mates among conspecifics.

Field conditions under which female-specific colour polymorphisms evolved are noisy, not only visually (e.g. Endler, 1978; Schultz & Fincke, 2013), but chemically (e.g. Ferry et al., 2007; Riffell et al., 2014). Moreover, many breeding sites on lakes are also windy, making it difficult to detect odour cues other than highly volatile pheromones (Vickers, 2000). A key question is whether the abilities of males demonstrated in the Frati et al. (2015) laboratory study similarly function in mate detection and recognition in nature. In the laboratory experiment, the odour from eight females was sufficient for males to discriminate between the sexes and between female colour morphs. Thus, our goal was to test the hypothesis that, under natural conditions, males can differentiate between the sexes and between female heteromorphs and andromorphs in the absence of visual cues. Even with visual cues, male sexual reactions were low. To rule out a possible anomaly, we ran a more realistic visual test and quantified natural harassment rates towards free-flying females and their subsequent reactions. We discuss the relevance of our findings for assumptions about multimodal sexual attention and sexual conflict more generally.

METHODS

Study Site and Species

Ischnura elegans is a common European coenagrionid damselfly whose males search for females around ponds and calm, protected areas of large lakes. Our study site was the area surrounding a series of artificial ponds at a fish hatchery (Centro Ittiogenico del Trasimeno, Sant'Arcangelo; 43°05′12.8″N, 12°09′05.4″E) on the shores of Lake Trasimeno near Perugia, Italy. Here, *I. elegans* was the only damselfly present, save for relatively few *Ischnura pumilio* and an occasional *Coenagrion* sp. The study was conducted on 15 sunny days between 5 and 22 September 2016, when males and females were abundant at the site. Although *I. elegans* was not found breeding along the exposed lake shore adjacent to the study site, solitary females and males, as well as pairs in copula could be found as far as 40 m from the ponds. This distance appeared to be limited by lack of natural habitat farther away on the south, east and west, and the proximity of the lake to the northern side. *Ischnura elegans* is well known for a complicated genetic colour polymorphism among females (Sánchez-Guillén, Van Gossum, & Cordero-Rivera, 2005). In our population, mated males and females included some types that have been considered sexually immature (Van Gossum et al., 2011). Hence, we limited our experiments to the reactions of blue males, excluding any by subadult green males (Fig. 1a and b, respectively). We used female andromorphs that resembled mature males in both colour and pattern (Fig. 1c, but not 1d) and the heteromorphs *infuscans–obsoleta* and *infuscans* (Fig. 1e and f, respectively), which both differed in colour and pattern from the blue males. We excluded subadult orange *infuscans–obsoleta* heteromorphs (Fig. 1g) and subadult 'violet' females (Fig. 1h).

Pond Experiment Testing Relative Importance of Nonvisual Cues in Mate Searching

This experiment employed the stationary damsel-on-a-dowel technique, designed to reveal the ability of free-flying males to detect and correctly recognize potential mates (Fincke, 2015). Observers quantified reactions by free-flying males that were elicited by live individuals attached to dowels positioned in the ground. Our experiment had two treatments, a 'visual' one that provided both visual and any odour cues and a 'nonvisual', covered treatment. We attached two live *I. elegans* of the same sex and colour morph to a 45 cm dowel (diameter = 4.7 mm) in a perching position using Patafix™ (UHU Bostik, Milan), a white, repositionable, nonvolatile adhesive. To offer wild males the same 'dosage' of odour cues that were sufficient to trigger sex discrimination in the Frati et al. (2015) laboratory study, each of three live 'dowel types' consisted of a total of four dowels with two damselflies per dowel, for a total of eight live individuals of the same sex and morph (andromorph, heteromorph or male). The fourth dowel type was a control that consisted of four dowels each with two strips of Patafix but without damselflies.