



Sexual conflict does not maintain female colour polymorphism in a territorial damselfly

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Female-limited dimorphism is commonly hypothesized to be an adaptation resulting from male harassment or sexual conflict over female mating rate. We examined whether males discriminate between female colour morphs of the beautiful Hawaiian damselfly, *Megalagrion calliphya*, in order to evaluate whether male harassment could explain the existence and/or maintenance of this dimorphism. Previous studies of this species suggest that spatially varying ecological selection maintains the dimorphism, but these hypotheses are not mutually exclusive. Here, we used a common method of measuring male behaviour towards secured females at mating sites under naturally occurring conditions, using five populations that range in male-like female morph frequency from 0 to 0.86. We found very low rates of interaction in a total of 64 one-hour trials, and male behaviour towards females did not differ significantly between colour morphs. By comparing the populations that vary in female morph frequency, we found no evidence of frequency-dependent sexual selection on colour, suggesting that this polymorphism is maintained by selective forces other than sexual conflict.

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Polymorphic species have long served as model systems for understanding how variation is maintained within populations (Gray & McKinnon, 2007). The coexistence of distinct heritable morphs within a population can be maintained by gene flow between populations with divergent selection or by balancing selective forces such as negative frequency-dependent selection (Enderler, 1977; Fisher, 1930; Ford, 1945). In this study, we evaluated whether frequency-dependent selection could contribute to the maintenance of female-limited colour dimorphism in the Hawaiian damselfly, *Megalagrion calliphya*.

Sex-limited polymorphism, in which only one sex displays multiple morphs, is commonly attributed to sexual selection (Hammers & Van Gossum, 2008). Male-limited polymorphism in many taxa is associated with alternative mating strategies that have evolved due to intrasexual competition over access to mates (Dominey, 1981; Forsyth & Alcock, 1990; Gross, 1991; Sætre & Slagsvold, 1996), while female polymorphism is generally thought

to be the result of intersexual conflict over mating rate (Wellenreuther, Svensson, & Hansson, 2014). High mating rates may increase male fitness, but because females of many species can fertilize their clutch with a single copulation, additional mating or harassment may have a fitness cost (Bateman, 1948; Sirot & Brockmann, 2001; Trivers, 1972). Female-limited dimorphism has been studied most commonly in damselflies, where it has been described in more than 100 species (Fincke, Jödicke, Paulson, & Schultz, 2005). In these species, one female morph (called the andromorph) is similar to the male in coloration, while one or more other morphs (gynomorphs) are distinct from the male coloration.

Two prominent sexual conflict hypotheses explain how selection from male mating harassment drives the evolution of such polymorphisms. The male mimicry hypothesis states that andromorphs escape harassment by mimicking the appearance of males (Cordero, 1992; Hinnikint & Dumont, 1989; Robertson, 1985). This strategy is expected to be successful when andromorphs are rare, but as mimics become more common, the benefits of mimicry become reduced as males increase harassment of all male-like individuals (Fincke et al., 2005; Ting, Bots, Jvostov, van Gossum, & Sherratt, 2009; Xu & Fincke, 2011). The learned mate recognition hypothesis predicts that harassment of both morphs will be

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frequency dependent, as males learn to recognize mates based on which female morph is most common in their population (Fincke, 2004; Miller & Fincke, 1999). Both hypotheses assume that harassment occurs and carries a fitness cost to the female.

In addition to negative frequency-dependent selection, female polymorphisms might be maintained by variation in selection both within and between populations (Endler, 1977; Galeotti, Rubolini, Dunn, & Fasola, 2003; Huxley, 1955). If morphs exploit different ecological niches within populations, both could be maintained (Van Gossum, Sherratt, & Cordero-Rivera, 2008). If selection varies between populations, with each morph having a selective advantage in some areas, gene flow could lead to a range of population morph frequencies (Endler, 1977). In some damselfly species, morph frequencies vary along environmental clines, but the role of ecological selection is often unclear (Hammers & Van Gossum, 2008; Iserbyt, Van Gossum, & Stoks, 2012; Sánchez-Guillén, Hansson, Wellenreuther, Svensson, & Cordero-Rivera, 2011; Takahashi, Morita, Yoshimura, & Watanabe, 2011). There is evidence that female polymorphism is under divergent ecological selection along an elevational cline in the Hawaiian damselfly *M. calliphya* (Cooper, 2010; Cooper, Brown, & Getty, 2016), but sexual selection on this polymorphism has not previously been measured. Since the hypotheses are not mutually exclusive, it is important to test whether both mechanisms may operate on colour in this system (Svensson, 2017).

We measured male behaviour towards secured *M. calliphya* gynomorphs, andromorphs and males at mating sites under naturally occurring conditions, similar to tests of male harassment in other studies (e.g. Andrés & Sánchez-Guillén, 2002; Cordero, Carbone, & Utzeri, 1998; Miller & Fincke, 1999; Ting et al., 2009). We observed male behaviour in five populations that range in andromorph frequency from 0 to 0.86. The sexual selection hypotheses predict that harassment of females occurs and that male behaviour towards females differs between the morphs, with greater attention either to gynomorphs (expected under the male mimicry hypothesis) or the more common morph in population (the learned mate recognition hypothesis). Previous research on this system found no difference in the frequency at which gynomorphs and andromorphs are found in tandem and in copulo in the field, regardless of their frequency in the population (Cooper, 2010). Harassment may be low in this species because *M. calliphya* males are territorial. Territorial behaviour has not been measured in this species, but males display male–male aggression and expulsion behaviours, similar to other *Megalagrion* species described as territorial (Moore, 1983). Females may avoid attention from males by leaving the mating site, and therefore this type of mating system seems unlikely to contain the high levels of harassment seen in damselfly species with mate-searching behaviour by males (Fincke, Fargevieille, & Schultz, 2007; Xu & Fincke, 2011). The previous measures of sexual behaviour in this species (Cooper, 2010), however, included only tandem and in copulo behaviours, not other types of interactions that might lower female fitness. Additionally, that study did not manipulate female presence at the mating

habitat. If females escape unwanted interactions by leaving the area, observational studies can underestimate harassment and its costs (Bots, Iserbyt, Van Gossum, Hammers, & Sherratt, 2015; Fincke et al., 2007; Van Gossum, Stoks, & De Bruyn, 2001). This study, therefore, was designed to measure the levels of harassment experienced by female morphs at the mating habitat.

METHODS

Natural History

The endemic Hawaiian damselfly *M. calliphya* lives near intermittent streams and upland bogs. Males defend territories around open water where females oviposit, while females spend most of their time away from the water and are usually found near the water only when mating and ovipositing. Males initiate mating by clasping the female by the thorax using abdominal appendages. The pair may stay in this position, called tandem formation, for some time before the female chooses to copulate by moving the tip of her abdomen forward to make contact with the accessory genitalia of the male. The pair will then perch just above open water and the female will oviposit in submerged vegetation. Other males may approach the pair and attempt to drive off the male.

In *M. calliphya*, males and andromorphs are red, and gynomorphs are green (Cooper, 2010). The genetic basis of this variation is unknown, but in other damselfly species female-limited colour polymorphisms are determined by one or two autosomal loci (Cordero, 1990; Cordero Rivera & Andrés, 1999; Johnson, 1964, 1966; Sánchez-Guillén et al., 2011; Sanmartín-Villar & Cordero-Rivera, 2016; Takahashi et al., 2011). In *M. calliphya*, morph is fixed; colour does not change between red and green over the life span of individual adults (Cook, Brown, & Cooper, n.d.). A previous study found that the frequency of andromorphs in populations on Hawaii Island ranged from almost 0% to 100% (Cooper, 2010).

Sex and Morph Ratios

We conducted all observations of *M. calliphya* populations on the windward slope of Mauna Loa on Hawaii Island. We measured sex and morph ratios for each of the five populations between 18 June and 8 August 2015 (Table 1). Unmarked adults were netted in multiple passes through the mating habitat between 0800 and 1400 hours on days that it was not raining. Damselflies are rarely encountered outside of the mating habitat, so our measurements of sex and morph ratios are limited to that area. Captured individuals were identified as male, andromorphic female, or gynomorphic female and marked on the wing with a unique number in indelible ink to prevent recounting the same individuals.

Behaviour Trials

We measured male behaviour experienced by focal individuals at the five populations during a total of 64 trials between 28 June

Table 1
Elevation and demographic information for the five populations, including the total number (*N*) of gynomorph females (*G*), andromorph females (*A*) and males (*M*) captured

Population	GPS	Elevation (m)	<i>N</i> G, A, M	Andro. freq. A/(G+A)	Male freq. M/(M+A+G)	<i>N</i> trials
Ninole	19°10.58'N, 155°33.61'W	646	11, 0, 101	0.00	0.90	12
Portuguese	19°5.863'N, 155°36.727'W	792	63, 6, 380	0.09	0.85	12
Waiaele	19°06.96'N, 155°36.68'W	853	91, 70, 451	0.43	0.74	16
UMHR	19°8.15'N, 155°36.77'W	1036	50, 28, 426	0.36	0.85	14
Kulani	19°35.69'N, 155°20.73'W	1676	5, 31, 289	0.86	0.89	10

UMHR: Upper Mountain House Road.

and 11 July 2015. Focal individuals (males, andromorphs, gynomorphs) were collected from two mid-elevation populations, Waiaele (19°06.96'N, 155°36.68'W) and Upper Mountain House Road (19°08.15'N, 155°36.77'W), where both morphs are abundant. The collected adults were held for less than 48 h and then brought to the study sites in coolers packed with ice and damp paper towels to reduce the stress of transport. This species often experiences multiple days of cool, rainy weather, which keeps them from flying and hunting, and so this treatment is unlikely to harm the focal individuals; individuals kept in such fashion readily resume normal behaviours, including mating interactions, when their body temperature rises. To ensure that all individuals were treated equally before the trials and to control for any effects on behaviour, we held individuals overnight even if they were used in trials at their population of origin. Within each trial, all focal individuals originated from the same population.

In each trial we spaced one gynomorph, one andromorph and one male approximately 30 cm from each other around the edge of a pool. We secured them by their feet to the vegetation or rocks using Beacon Fabri-Tac® adhesive. This method allowed natural movement of the head, abdomen and wings but not flight or departure from the mating site. The use of females that are secured by tethering or gluing is common in studies of harassment in odonates (e.g. Andrés & Sánchez-Guillén, 2002; Cordero et al., 1998; Miller & Fincke, 1999; Ting et al., 2009). We did not actively present females to males, as in some studies of individual male mate preferences, because our aim was to measure the male attention experienced by females.

A stationary observer at least 1 m away from the pool recorded local male behaviour towards all focal individuals for 1 h during each trial. For each focal individual, we recorded how often local males came within 10 cm (approaches), the incidence and duration of males hovering without contact (inspections), contacts and tandem attempts (both successful and unsuccessful). Copulations were not included because none were observed. Because copulations are initiated by females, this supports the interpretation of male behaviours as unwelcome and potentially harassment. Multiple males were active in the vicinity during all trials, and therefore more than one focal individual could be experiencing male attention simultaneously. We did not control for repeated interactions by local males, as they were not marked individually and could not be identified. This study was not intended to measure the preferences of individual males, but instead aimed to measure the interactions experienced by females at the mating site in natural conditions.

Each day of the study, behavioural trials were initiated once mating pairs were seen at the site, and the trials were run throughout the peak mating period, between 0830 and 1330 hours. Trials were set up only around pools where at least one territorial male had been seen that day, which indicated that the pool was suitable mating habitat and multiple males were active in the area. These conditions prevented artificially low estimates of interaction rates due to sampling during poor weather or in unused territory.

Statistical Analysis

We quantified the intensity of male interactions with focal individuals using the number and duration of approaches, inspections, contacts, tandem attempts and tandems. To account for variation between trials due to weather or male density at that site, we calculated differences in the number and duration of interaction types between morphs (andromorph – gynomorph) and between sexes (female morph average – male) for each trial. The differences were tested against zero using Mann–Whitney *U* tests when symmetrically distributed and sign tests when not. Differences

were compared between sites using Kruskal–Wallis tests. Ties were broken randomly. All statistical analyses were performed in R 3.4.0 (R Core Team, 2017).

RESULTS

Overall, there was little evidence for harassment of either female morph, with many individuals having zeros in both the number and duration of interactions. Of 128 females, 101 had no contact with males over the course of an hour, even though males were active in the area. In 49 of those cases, males approached within 10 cm of the female, but did not attempt to mate with her. There were only 45 successful tandem events in 128 damselfly-hours of trial time, for an average of 0.35 tandems per hour. Most of the 64 total trials, however, contained some interactions with focal individuals. Seven trials contained no interactions with any focal individual, despite the presence of males in the area, and were therefore removed from our comparison of interaction differences within trials. All trials were included in the comparison of interaction amounts between sites, which showed that sites differed in the level of activity (Table 2). Post hoc Nemenyi tests showed that these differences were due to activity at Kulani being higher than that at Ninole (medians: 1.1 more approaches, 0.4 more inspections and contacts, 0.3 more tandems and 7.0 more seconds of interaction time per trial). All further analyses were performed with the 57 trials in which there was at least one interaction.

There was no difference between gynomorphs and andromorphs in the rate at which they experienced any male behaviour (Fig. 1a, Table 3), and this was true across all populations (Table 4). Males and females were not approached, inspected or contacted by males at different rates, but females experienced more tandem attempts (Fig. 1b, Table 3). Females experienced a greater total duration of all interactions than males due to the long duration of tandems. This difference varied between sites (Table 4, Fig. 2b); females experienced 50 more seconds of interactions than males at Kulani, but there was no median sex difference at Ninole (Nemenyi: $P = 0.005$). There was no difference between the female morphs in total duration of interactions either overall (Table 3) or between sites (Table 4, Fig. 2a).

DISCUSSION

We found no evidence of selection from male harassment on female colour in this system. Males did not interact with one female morph more than the other for any of the behaviours measured, and this was true across sites with different morph frequencies. Additionally, rates of approaches, inspections and contacts towards females were no higher than those towards males, perhaps because male–male interactions are common territorial behaviours. This attention to focal males is additional evidence that male mimicry would not be an effective way for females to avoid harassment for

Table 2

Differences in the rate of each interaction per trial between sites, using Kruskal–Wallis tests

	χ^2_4	<i>P</i>
Approach	19.757	0.001
Inspection	13.238	0.010
Contact	18.767	0.001
Tandem attempts	13.298	0.001
Total interaction time	13.380	0.009

Significant differences are indicated in bold. Post hoc Nemenyi tests showed that all behaviours were more frequent at the Kulani site than at the Ninole site ($P < 0.05$).

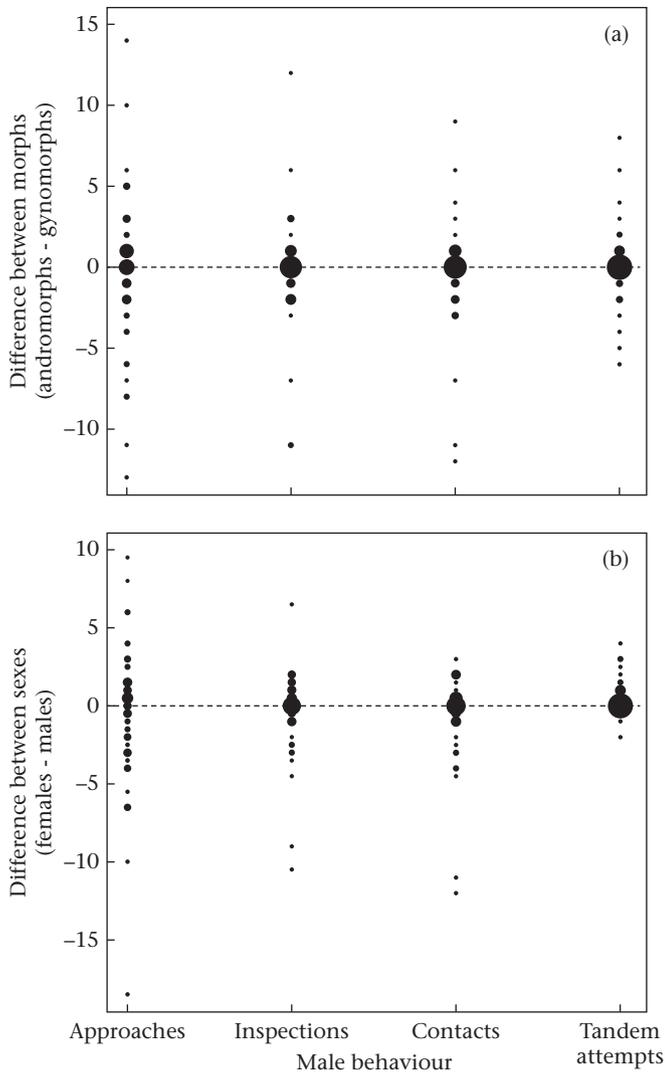


Figure 1. Differences between (a) female morphs and (b) the sexes in their experience of different male behaviours.

Table 3

Comparisons of median differences in male behaviour towards focal individuals to zero, using Mann–Whitney *U* tests with exact *P* values

	Morph difference		Sex difference	
	<i>Z</i>	<i>P</i>	<i>S</i>	<i>P</i>
Approach	−0.0572	0.9564	27	1
Inspection	−0.1334	0.8961	20	0.8714
Contact	0.0334	0.9754	17	0.7428
Tandem attempts	0.3145	0.7557	21	0.0003
Total interaction time	0.1479	0.8838	35	0.0002

For sex differences, one-sample sign tests were used. The statistic *S* is the number of trials in which there was a positive difference (females > males). All median differences were zero, with the exception that total interaction time was 3.5 s longer for females than for males. Significant values are indicated in bold.

this species, as hypothesized in other studies (Cordero, 1992; Hinnekint & Dumont, 1989; Robertson, 1985).

The only majority-andromorph site, Kulani, had higher rates of all interactions than the monomorphic gynomorph site, Ninole. While this result initially appears to support the idea that andromorphs escape from intense harassment, we note that gynomorphs and andromorphs experienced equal interest from males at both

Table 4
Tests of differences between sites, using Kruskal–Wallis sum rank tests

	Morph difference		Sex difference	
	χ^2_4	<i>P</i>	χ^2_4	<i>P</i>
Approach	1.5123	0.8245	1.501	0.8264
Inspection	2.7157	0.6065	7.419	0.1154
Contact	1.4432	0.8367	4.066	0.3972
Tandem attempts	7.0166	0.135	4.444	0.3492
Total interaction time	4.7213	0.3171	12.537	0.0138

sites. We suggest that ecological differences between the sites may have greater effects on rates of sexual interactions than on morph frequency. Kulani is a high-altitude bog where limited surface water is available for territory defence and oviposition despite abundant underground aquatic habitat to support a large population size, which may lead to more interactions between adults. Weather is also highly variable because of a trade wind inversion at this elevation that can trap moisture in clouds and lead to more limited periods of clear weather for mating than at lower elevation. Because we conducted our behavioural trials on clear days only, our

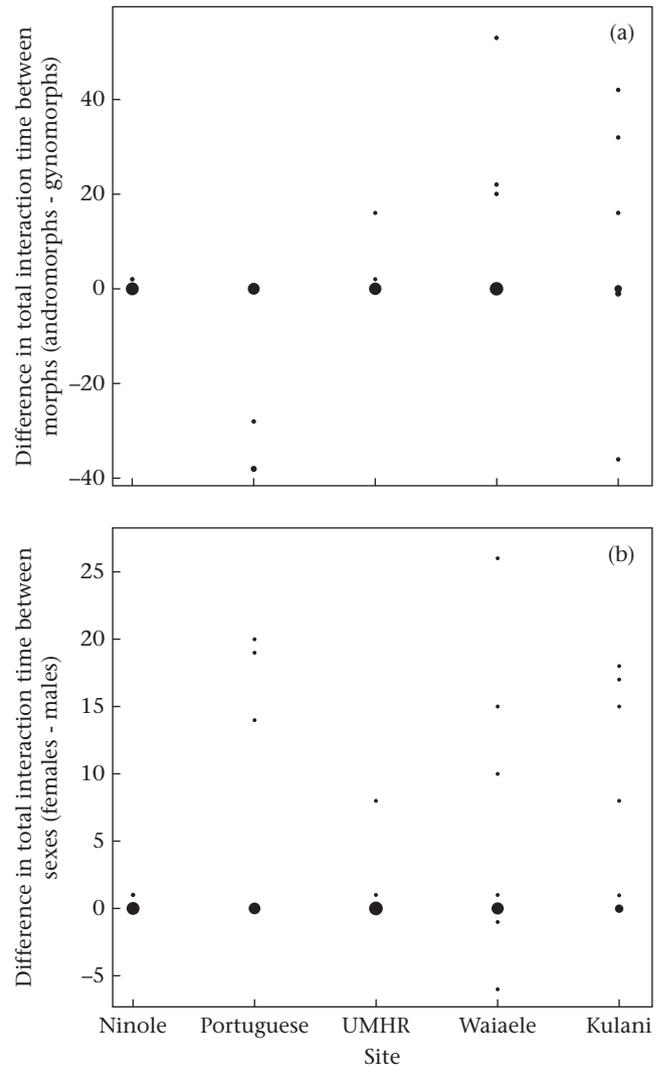


Figure 2. Difference in total interaction time between (a) female morphs and (b) the sexes. Populations are ordered by increasing elevation. UMHR: Upper Mountain House Road.

measurements of sexual interactions reveal the activity level in those conditions only, not an average level of activity that might better reflect total harassment experienced by females. These conditions at high elevation, therefore, may concentrate the adult interactions in time and space. Ninole, by contrast, is the lowest-elevation site and is composed of many intermittent pools in a wide rocky streambed, with a large number of potential territories and mating sites.

We detected low rates of harassment across populations, morphs and sexes. It is possible that our methods underestimate true rates of interaction, if males are more likely to see females in flight than when stationary. However, this difference should apply to all studies of secured damselflies, some of which have reported much higher rates of interaction. In this study, females were clasped in tandem on average once every 3 h. By contrast, Fincke et al. (2007) found that *Enallagma ebrium* females glued to perches were clasped in tandem approximately once every 3 min, on average. Many of the studies of female polymorphism in damselflies have been conducted in *Enallagma*, *Ischnura* and *Nehalennia* species, all of which have ‘scramble competition’ mating systems. In these species, males actively search for females rather than remaining in place (Fincke, 2004; Fincke et al., 2007; Xu & Fincke, 2011). Males of *M. calliphya*, however, are territorial (Moore, 1983) and therefore females may be able to avoid unwanted interactions much more easily by simply leaving the mating territory. Harassment seems to be a weaker pressure in this species than in other damselflies, due to different mating systems. This could be tested by measuring interaction intensity in other territorial species and examining the effect of variation in mating system on male interaction intensity using comparative methods.

This study found no evidence of differential harassment on female colour morphs in *M. calliphya*, suggesting that this polymorphism is maintained by other selective forces. A phylogenetic analysis suggests that female polymorphism evolved independently in four *Megalagrion* species under spatially varying ecological selection from abiotic factors (Cooper et al., 2016). Colour variation throughout the radiation is correlated with canopy cover: green species and morphs are correlated with shaded habitat and red species and morphs are correlated with exposed habitat (Cooper et al., 2016). In *M. calliphya*, green gynomorphic females are prevalent in low-elevation habitats with dense canopy cover, and red andromorphic females are common at high-elevation populations with sparse vegetation. All males are red and, because they defend territories over open water, occupy exposed habitat throughout their elevational range (Cooper, 2010). The red pigment function may be an antioxidant defence against oxidative damage from ultraviolet damage in both males and females (Cooper, 2010; Park, Hsieh, Cooper, & Brown, n.d.). Green females may have an advantage in shaded habitat due to a physiological cost or a predation risk of the red pigmentation. A spatial pattern of selection, combined with gene flow between populations, could lead to a cline in morph frequencies (Endler, 1977).

Debate over the relative contributions of ecological and sexual selection to the evolution of sexual dimorphism dates back to Darwin and Wallace (Darwin, 1888; Wallace, 1889; also see Andersson, 1994; Kottler, 1980; Punzalan & Hosken, 2010). In *M. calliphya*, both sexual dimorphism and female dimorphism are associated with differences in the habitats occupied by red and green individuals, and appear to be the result of ecological rather than sexual selection. In recent decades, work on female polymorphism has mostly focused on sexual conflict hypotheses, but multiple selective forces may act on polymorphic traits (Iserbyt et al., 2012; Sánchez-Guillén et al., 2011; Svensson, 2017). Historical events may also impact the geographical distribution of morphs, for example in the damselfly *Nehalennia irene* (Iserbyt et al., 2012),

along with temperature regimes (Hammers & Van Gossum, 2008) and temporal variation in selection pressure via seasonal fluctuations in population density (Cordero, 1992). Finally, female polymorphisms are not limited to damselflies and can occur due to ecological selection even when sexual selection has produced sexual dimorphism, as shown in Kunte's (2008) studies of female polymorphism in swallowtail butterflies. Future studies of female polymorphism should test ecological as well as sexual selective forces, as the same patterns of polymorphism may be the result of different processes, especially when the species differ in ecology, natural history or mating system.

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