

Frequency-Dependent Selection on Female Morphs Driven by Premating Interactions with Males

Jessica Bots,^{1,*} Arne Iserbyt,^{1,2,*†} Hans Van Gossum,¹ Martijn Hammers,^{3,‡}
and Thomas N. Sherratt⁴

1. Evolutionary Ecology Group, University of Antwerp, Antwerp, Belgium; 2. Ethology Group, University of Antwerp, Antwerp, Belgium; 3. Behavioural Ecology and Self-organization, Center for Ecological and Evolutionary Studies, University of Groningen, Groningen, The Netherlands; 4. Department of Biology, Carleton University, Ottawa, Canada

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ABSTRACT: Species showing color polymorphisms—the presence of two or more genetically determined color morphs within a single population—are excellent systems for studying the selective forces driving the maintenance of genetic diversity. Despite a shortage of empirical evidence, it is often suggested that negative frequency-dependent mate preference by males (or diet choice by predators) results in fitness benefits for the rare female morph (or prey type). Moreover, most studies have focused on the male (or predator) behavior in these systems and largely overlooked the importance of female (or prey) resistance behavior. Here, we provide the first explicit test of the role of frequency-dependent and frequency-independent intersexual interactions in female polymorphic damselflies. We identify the stage of the mating sequence when frequency-dependent selection is likely to act by comparing indexes of male mate preference when the female has little (females presented on sticks), moderate (females in cages), and high (females free to fly in the field) ability to avoid male mating attempts. Frequency-dependent male preferences were found only in those experiments where females had little ability to resist male harassment, indicating that premating interactions most likely drive negative frequency-dependent selection in this system. In addition, by separating frequency-dependent male mating preference from the baseline frequency-independent component, we reconcile the seemingly contradictory results of previous studies and highlight the roles of both forms of selection in maintaining the polymorphism at a given equilibrium. We conclude that considering interactions among all players—here, males and females—is crucial to fully understanding the mechanisms underlying the maintenance of genetic polymorphisms in the wild.

Keywords: genetic diversity, *Ichnura elegans*, *Nehalennia irene*, negative frequency-dependent selection, Odonata, phenotypic variation, polymorphism, sexual conflict, social interactions.

Introduction

Negative frequency-dependent selection (NFDS) is considered one of the most important mechanisms maintaining genetic trait variation (Ayala and Campbell 1974). It favors alleles when rare and disfavors them when common, thereby generating balanced genetic polymorphisms within populations (Maynard Smith 1998). However, empirical evidence for the maintenance of genetic polymorphisms through NFDS is rare and generally limited to laboratory or artificial settings and computer simulations (e.g., Allen 1972; Bond and Kamil 1998). Only a small number of studies have succeeded in demonstrating NFDS in the wild (e.g., Shuster and Wade 1991; Chaboudez and Burdon 1995; Sinervo and Lively 1996; Gigord et al. 2001; Hughes et al. 2013). For example, in guppies, rare male phenotypes not only have a mating advantage (Hughes et al. 2013) but also higher survival chances compared to common ones, most likely as a consequence of frequency-dependent predation (Olendorf et al. 2006). Another example comes from female polymorphic damselflies, among which rare morphs gain a fecundity advantage over more common morphs, most likely as a consequence of reduced male harassment (Svensson et al. 2005; Takahashi et al. 2010; Iserbyt et al. 2013). Although these studies on guppies and damselflies have been able to relate morph-specific fitness correlates to morph frequency under natural conditions, the frequency dependence generated by the selective agent (predator or male) per se was not examined directly and requires further investigation. A detailed understanding of the selective agent that drives NFDS is likely to be a complex task, since the behavior of two players (e.g., predator and prey, male and female, host and parasite) may combine to shape the strength of selection. In the garter snake *Thamnophis ordinoides*, for example, specific combinations of antipredator behavior and color patterns result in successful evasion of attacks, and thus both predator and prey behaviors may

* Jessica Bots and Arne Iserbyt contributed equally to this article.

† Corresponding author; e-mail: arne.iserbyt@uantwerpen.be.

‡ Present address: Animal Ecology Group, Alterra Wageningen UR, Wageningen, The Netherlands.

shape the fitness landscape (Brodie 1992). Similarly, if intersexual interactions fuel NFDS in female polymorphic systems, it is likely that not only male mating behavior and harassment but also female response behavior play an important role (Fincke 1997). Together, to increase our general knowledge of NFDS, we need more empirical evidence of the behavior of the selective agent while considering the behavior of all relevant players (Holland and Rice 1998; Maklakov and Arnqvist 2009).

In this study, we have focused on intersexual interactions in two species with polymorphic females and attempted to elucidate the roles of both males and females in shaping the pattern of NFDS. In many systems, males pursue a higher mating rate than females, which ultimately results in excessive male mating attempts toward females (Arnqvist and Rowe 2005; Córdoba-Aguilar 2009; Takahashi and Watanabe 2010a). In general, female polymorphisms are thought to have evolved as a counteradaptation to reduce the costs of this harassment (butterflies: Cook et al. 1994; diving beetles: Härdling and Bergsten 2006; African bat bugs: Reinhardt et al. 2007; damselflies: Van Gossum et al. 2008). As an evolutionary response to these confusing multiple morphs, males may adopt a reversible preference to mate with (and thus harass) the more common female type in the population (Van Gossum et al. 2001b). Despite several lines of evidence for frequency-dependent male mating preferences in polymorphic butterflies (Cook et al. 1994; Kunte 2009) and damselflies (Van Gossum et al. 1999; Ting et al. 2009), there remains a key discrepancy between results of male mate preference experiments and field observations. Specifically, when male mate preference behavior in damselflies has been evaluated using live glued or caged female morphs, these studies have generally reported strong frequency-dependent variation in preference among populations (e.g., Van Gossum et al. 1999; Ting et al. 2009). By contrast, other studies that have compared mating probabilities of female morphs in the field to morph frequencies in the population as a whole (e.g., Cordero et al. 1998; Rivera and Sánchez-Guillén 2007; Hammers and Van Gossum 2008) have generally not found evidence of a frequency-dependent change in male preference among populations (reviewed in Van Gossum et al. 2008). The differences in results using these contrasting methodologies have rarely been acknowledged, although it is clear that mating rates in the field are the combined outcome of the behavior of both players, that is, male sexual interest and female response to male mating attempts. Female response behavior to male harassment is ubiquitous (e.g., water striders: Arnqvist and Rowe 2002; fruit flies: Wigby and Chapman 2004; and fowl: Løvlie and Pizzari 2007) and of clear adaptive significance (Rankin et al. 2011). In damselflies, for example, females may actively avoid male harassment by using a particular microhabitat

or by showing refusal behaviors once encountered by a male (e.g., Van Gossum et al. 2001a; Sirot et al. 2003; Xu and Fincke 2011). Here, we attempt to elucidate the role of female behavior in mediating the response to male mating attempts by comparing results of different methods that allow various amounts of female resistance behavior. This has, to the best of our knowledge, never been done before.

We chose to study female polymorphic damselflies since these have proven to be attractive model systems in evolutionary ecology (Van Gossum et al. 2008) and are excellent for examining how intersexual interactions drive patterns of NFDS. Several alternative mechanisms such as morph-specific predation (Van Gossum et al. 2004), parasite susceptibility (Sánchez-Guillén et al. 2013), environmental effects (Cooper 2010), and even stochasticity (Iserbyt et al. 2010, 2012) have been hypothesized to play a role in the maintenance of this polymorphism or at least affect where the equilibrium might be. However, currently, empirical evidence indicates that frequency-dependent male harassment is the predominant balancing selective force (summarized in Iserbyt 2012). One way to avoid harassment by males is to look like a male. Indeed, in many polymorphic damselfly species, one female morph (andromorph) typically resembles the conspecific male in body coloration (Van Gossum et al. 2011), morphology (Abbott and Gosden 2009; Iserbyt et al. 2011), and behavior (Forbes et al. 1997; Van Gossum et al. 2001a), whereas other females (gynomorphs) are clearly distinct. The benefit of such intraspecific mimicry is likely to depend on the ratio of andromorphs to males (Robertson 1985; Sherratt 2001), which naturally predicts a greater advantage to andromorphs when they are rare. Whatever the involved specific mechanism is, rare female morphs have been found to experience less attention from mate-searching males (Van Gossum et al. 2001a; Gosden and Svensson 2009; Ting et al. 2009). Moreover, this frequency-dependent male harassment has been implicated in generating the observed rare morph fecundity advantage in the field (Svensson et al. 2005; Takahashi et al. 2010; Iserbyt et al. 2013). Despite these assertions, the link between the harassment patterns reported and the rare morph fecundity advantage remains largely circumstantial, and to date there has been no attempt to understand why frequency-dependent mate preference tends to be seen with some methods and not with others.

In this study, we have attempted to disentangle male and female behavioral components of intersexual interactions by comparing results from direct presentation experiments and mate choice experiments in insect cages with data on female morph mating probabilities under natural conditions. Direct presentation experiments determine male mate preference for a particular female morph since nearly all female resistance behavior is excluded. Male mate choice experiments in small insect cages allow more fe-

male refusal behavior than presentation experiments but still generate a predominate measure of male mate preference. Finally, field observations on the proportion of mated female morphs compared to their relative availability can be considered to be the full outcome of male and female behavioral tactics. We have compared results among these different methods of measuring mate preference in a large number of populations for the polymorphic damselfly *Nehalennia irene*. This species exhibits large spatial variation in female morph frequencies (Iserbyt et al. 2009), which provides a rare opportunity for studying NFDS in the wild. Mating behavior under natural conditions was also assessed for the polymorphic damselfly *Ischnura elegans*, which allowed testing for consistency across species. Using the statistical model of preference developed by Manly (1973), we determine whether frequency-dependent and frequency-independent male mate preference exists. If NFDS is present at all stages of the interaction sequence, then the rare female morph will be disproportionately less preferred by mate-searching males in experiments and found less often engaged in matings in natural populations. However, when female resistance behavior plays an important role in determining the outcome of intersexual interactions, results can be expected to differ between experiments and field observations. Furthermore, a frequency-independent component may be present when a particular morph is consistently less preferred, which may be due to a range of factors including morph conspicuousness. In summary, this study provides the first explicit evaluation of the role of frequency-dependent and frequency-independent interactions between two players in maintaining genetic polymorphisms in the wild.

Material and Methods

Study Species

The sedge sprite (*Nehalennia irene*) is distributed across most of Canada and the Northern parts of the United States, where it inhabits marshy and boggy areas with dense vegetation (Walker 1953). The species exhibits a monomorphic male and two female morphs, which are unambiguously classified as andromorph or gynomorph based on differences in body coloration. Andromorphs closely resemble conspecific males by having a similar blue thorax color and abdominal melanin pattern. Gynomorphs, in contrast, have a green-yellow body coloration and a distinctive abdominal pattern compared to andromorphs and males (for a more detailed description, see Forbes et al. 1995). Genetic inheritance experiments with six coenagrionid damselfly species have generated phenotypic ratios consistent with the hypothesis that the female polymorphism is controlled by a single autosomal locus with sex-

limited expression (reviewed in Sánchez-Guillén et al. 2005). The genetic basis of the polymorphism remains to be examined for *N. irene*. However, considering the species' close phylogenetic relationships with the other six coenagrionid damselfly species (Carle et al. 2008), its inheritance of color polymorphism is expected to be similar. A detailed description of our second study species, *Ischnura elegans*, is given in the appendix, available online. This species has three female morphs, one andromorph and two gynomorphs.

Before mating can occur in damselflies, a mate-searching male needs to grasp a female and form a tandem position (male's anal appendages attached to a female's pronotum). Then the female may bend her abdomen to form a copulation wheel (male's anal appendages attached to a female's pronotum and female's genitalia connected to the male's secondary genitalia on the second abdominal segment; Corbet 1999). After aquatic larvae emerge into flying adults, damselfly populations typically have a male-biased sex ratio (Stoks 2001). This fact, together with the different optimal mating rates between the sexes (Arnqvist and Rowe 2005), causes female damselflies to encounter many more approaches and tandem attempts by mate-searching males than desired. Furthermore, besides such sexual harassment during premating stages, female damselflies may also suffer significant costs from being grasped in a tandem position or forced in an unwanted copulation wheel since these can last up to several hours (Corbet 1999), affecting time and energy budgets.

Female Morph Frequencies

Before conducting experiments and making field observations, each study population was surveyed in a standardized manner to obtain information on the population frequency of adult female morphs. This was done by walking slowly through shoreline vegetation, where reproduction typically occurs, while sweeping figure eights through the vegetation with an insect net (Van Gossum et al. 2007a). All captured individuals were marked on the wing with a permanent marker (Staedtler Lumocolor Special) prior to their release to avoid multiple counts. The total numbers of captured adult males and female morphs were recorded (independent of mating status). Several sets of sweeps were made per population until at least 30 mature females had been counted. In total, 15 populations throughout Canada were included in this study, of which 9 were visited in multiple years (two or three times) and 5 were used in previous experiments (Ting et al. 2009; Iserbyt et al. 2012). The sample size criterion of 30 was reached (or exceeded) in all populations (table A1; tables A1–A3 are available online). For each study population and in each sample year (if applicable), the morph frequency was estimated as the

number of a particular female morph divided by the total number of females observed. These estimates of female morph frequency were used to investigate the frequency-dependent and frequency-independent component of intersexual interactions. Morph frequencies showed limited annual variation (0%–20%; Iserbyt et al. 2009) but varied widely among the populations included in this study, from 2% to 98% of females being andromorph (table A1).

Quantification of Intersexual Interactions

We disentangled male and female behavioral components of intersexual interactions by comparing indexes of male preference mediated by female resistance using three different methods: (1) direct presentation experiments that excluded nearly all female mating resistance behaviors, with the exception of abdomen curling and wing raising; (2) mate choice experiments in insect cages that permitted female mating resistance, including the ability to fly short distances; and (3) field observations under natural conditions where male and female mating behavior was unrestricted. The direct presentation and cage experiments are commonly used to study Odonate mate preference (Corbet 1999) and are generally considered to limit female refusal behavior.

Male Mate Preference Determined from Direct Presentation Experiments. Data on the preference by males for female morphs was obtained from previously published records ($n = 6$ populations; Van Gossum et al. 2007b; Ting et al. 2009; Iserbyt and Van Gossum 2011). Additional data were collected in two populations during the reproductive season of 2011 (for sample size details, see table A2). In short, live adult andromorphs and gynomorphs were glued (UHU Power Glue) on a grass stem and alternately presented to randomly selected sexually active males in the field (Forbes et al. 1997; Van Gossum et al. 2007b; Ting et al. 2009). Behavioral responses of the focal free-living males toward the glued individuals were observed. To this end, we scored whether a tandem attempt—a measure of male sexual interest—occurred. A tandem attempt was defined as a male's attempt to grasp a female model's pronotum with his anal appendages to form the tandem position required for mating (Corbet 1999). This included both successful and unsuccessful attempts. Females glued to grass stems were able to raise their wings and curl their abdomen, which are typical refusal behaviors of female damselflies (Corbet 1999). However, such behaviors were previously found not to influence the outcome of a presentation (Iserbyt and Van Gossum 2011). A single trial was completed when each of the models had been presented to three (Van Gossum et al. 2007b), five (Ting et al. 2009; Iserbyt and Van Gossum 2011), or six (Van Gossum et al. 2007b; A. Iserbyt, un-

published data) different focal males. In total, a minimum of 3 and a maximum of 35 trials were carried out in each study population (for sampling details, see table A2).

Male Mate Preference Determined from Choice Experiments in Cages. Preference experiments were conducted in the field in small insect cages (30 cm × 30 cm × 30 cm, BugDorm-4030 Insect Rearing Cage) in which the sexual interactions between four mature live models (one andromorph, one gynomorph, and two males) were observed. In these cages, female resistance behavior was limited since they could not use microhabitat to make themselves less or more conspicuous. The observation was terminated when a successful tandem with a female was formed or after 20 min if no successful tandem occurred. We scored whether a tandem attempt occurred. Male-male mating attempts in cages were generally rare, and simultaneous mating attempts by both males were never observed. A single trial was completed when a pair of females (andromorph and gynomorph) was presented to three independent pairs of males. A minimum of 19 trials and a maximum of 24 trials were carried out for each of the six study populations (for sampling details, see table A2). In these six study populations, both cage and presentation experiments were performed (table A1).

Field Survey of Mated Female Morphs. In the field, the probabilities of females mating are the outcome of male preference behavior and female resistance behavior. For instance, unreceptive females can attempt to hide in dense vegetation, flee, or confront and chase away mate-searching males (Forbes et al. 1997; Van Gossum et al. 2001a; Sirot et al. 2003). The relative frequencies of mating andromorphs and gynomorphs were determined while walking slowly through the area where reproduction takes place and involved capturing all mating pairs (tandem and copula) one by one. This was done the day after determining morph frequencies in undisturbed conditions. To avoid multiple counts, mated individuals were marked with a small black spot on one of the forewings using a permanent pen (Staedtler Lumocolor Special). The procedure was continued until a minimum of 20 mating pairs had been counted in each study population. Mating pairs included males and females in tandem position or copulation wheel. Since it is possible that in *N. irene* transitions from copula to tandem occurred upon encounter, we cannot distinguish between females in tandem and copula position and therefore refer to these collectively as mated females. Data were collected in 13 populations during the reproductive seasons between 2008 and 2010. Our sampling criterion (minimum 20 mating pairs) was fulfilled in all populations (see table A3 for details). Presentation and cage experiments were conducted in

7 and 6 of the above 13 populations, respectively (table A1). A field survey as described above was also performed for *I. elegans* (appendix).

Statistical Analysis

The full data set obtained from the different experiments and field surveys is deposited in the Dryad Digital Repository (<http://dx.doi.org/10.5061/dryad.86b5m> [Bots et al. 2015]) and is analyzed by fitting the model proposed by Manly (1973). This model allows one to distinguish between overall preference (based on an intercept) from change in preference with estimated population morph frequencies (based on a gradient); see also Greenwood and Elton (1979). Manly's model was originally designed to understand diet choice by visually hunting predators by testing whether predators consistently overeat rare (positive frequency-dependent predation) or common forms of prey (negative frequency-dependent predation). However, Manly's model is not limited to analyzing frequency-dependent predation, and to the best of our knowledge, this is the first study applying it to mating behavior. There are several advantages to using the model of Manly (1973): (1) the underlying statistical properties of preference index $\hat{\beta}$ (see below) are well understood (Manly 1973); (2) the nonindependence of data collected in the same study populations on different sample dates can be taken into account; and (3) variation in sample size can be controlled for by weighting analyses.

The first step in the analysis was to calculate the measure of male mate preference for the different female morphs in each study population (and study year, if appropriate). This measure (Manly $\hat{\beta}$) is calculated according to equation (9) in Manly (1973):

$$\hat{\beta} = \frac{(e_1/A_1)}{(e_1/A_1) + (e_2/A_2)},$$

where e_1 and e_2 are the numbers of each morph selected and A_1 and A_2 are the total numbers (absolute or relative) of each morph available/presented. As a simple proportion, Manly $\hat{\beta}$ can range between 0 and 1, with 0.5 indicating no preference. The above equation is best applied to situations where the chosen or "eaten" morphs are replaced ("type one selection experiment," Manly 1974, pp. 282–285). This is the case in mate-searching male damselflies, because females will be available again for mate-searching males after mating. Manly $\hat{\beta}$ was calculated for all methods (direct presentations, cage trials, field survey). In the equation above, andromorphs were consistently treated as morph 1 and gynomorphs as morph 2. For the presentation and cage experiments, e_1 and e_2 were the numbers of tandem attempts toward each female morph, whereas A_1 and A_2 were used as the total numbers of preference tests (because they were effectively presented at equal frequencies, they cancel out);

see table A2. For the natural survey, e_1 and e_2 represented the numbers of mated andromorphs and gynomorphs observed, while A_1 and A_2 represented the total numbers of each female morph observed in our survey, regardless of their mating status (table A3).

The standard error (SE) of the estimate of $\hat{\beta}$ was calculated according to Manly (1973), equation (10) where $n = e_1 + e_2$:

$$SE(\hat{\beta}) = \frac{A_1\beta}{(e_1A_2 + e_2A_1)} \frac{(ne_2)^{1/2}}{e_1}.$$

This equation allowed weighting all subsequent analyses to take into account variation in sample size between study populations (see tables A1–A3 for sampling details), thereby putting more emphasis on data based on higher sample sizes. These weights were inversely proportional to the expected variance of $\hat{\beta}$ in each population and were calculated as

$$w = \frac{1}{SE(\hat{\beta})^2}.$$

The next step in the analysis was to test for frequency-dependent and frequency-independent mate preference. This was done by evaluating the relationship between Manly $\hat{\beta}$ and the andromorph frequency with a linear regression model in SAS 9.3 (SAS Institute, Cary, NC) using the MIXED procedure. The andromorph frequency was based on the estimates of the population frequency, which had been assessed in each study population prior to conducting the experiments. All data points were weighted using w (see above). Degrees of freedom of the fixed effects were adjusted for statistical dependence using the Kenward-Roger method (Kenward and Roger 1997). In addition, since data collected in the same study population on multiple sample years are not independent, site was added using the repeated statement. The appropriate covariance structure was determined for each model based on the lowest Bayesian information criterion values. Repeating all analyses with averaged data for each study population gave qualitatively and quantitatively similar results, as did conducting unweighted regression analyses (not shown).

Our analysis was conducted in several steps. First, the Manly $\hat{\beta}$'s obtained for the different methods were compared in one model in which method (categorical), andromorph frequency (continuous), and their interaction were included as explanatory variables. The interaction between method and andromorph frequency was significant (see "Results"), indicating frequency-dependent male preference toward female morphs but in a different manner among methods. Therefore, the analyses were also carried out for all methods separately. A significant relationship between andromorph frequency and Manly $\hat{\beta}$ was interpreted as evidence for frequency-dependent mate preference (slope of the relationship statistically different from zero).

Frequency-independent mate preference can be defined as baseline preference for a given morph, which is present irrespective of the frequency of that particular morph in the population. Significance of frequency-independent mate preference was obtained using the same statistical model as above. To be able to distinguish frequency-independent preference from frequency-dependent preference, it was determined whether a particular morph was preferred more or less than can be expected at an andromorph frequency of 0.5. Specifically, we applied a *t*-test to determine whether the corresponding $\hat{\beta}$ at an intercept of 0.5 andromorph frequency differed from 0.5 (Manly $\hat{\beta}$ of 0.5 represents no preference). When Manly $\hat{\beta}$ was significantly less than 0.5 at this point, this was interpreted as evidence of frequency-independent preference for gynomorphs, whereas a Manly $\hat{\beta}$ significantly higher than 0.5 at this point indicated frequency-independent preference for andromorphs.

Frequency-dependent and frequency-independent mate preference in the natural survey were also examined for *I. elegans*, for which the data are deposited in the Dryad Digital Repository: <http://dx.doi:10.5061/dryad.86b5m> (Bots et al. 2015). However, Manly $\hat{\beta}$ was determined for the three morphs separately (see appendix).

Results

Male Preference among Different Methods

The relationship between Manly $\hat{\beta}$ and andromorph frequency differed among the direct presentation experiment, the cage trials, and the natural survey as indicated by the significant “method by andromorph frequency” interaction ($F_{2,36,1} = 3.33$, $P = .047$; see fig. 1). Therefore, this relationship was evaluated for all methods separately.

Patterns of Frequency Dependence

In the direct presentation experiment, Manly $\hat{\beta}$ increased with andromorph frequency ($F_{1,8} = 144.02$, $P < .0001$; fig. 1A). Such a pattern strongly indicates the presence of frequency-dependent mate preference, with male preference for andromorphs increasing as they became relatively more common in the population. For the cage experiment, Manly $\hat{\beta}$ also increased with andromorph frequency, although this relationship was marginally nonsignificant ($F_{1,4} = 5.89$, $P = .072$; fig. 1B). Both experiments indicate that at low andromorph frequency, males had an overall preference for gynomorphs, whereas andromorphs were the preferred female morph at high andromorph frequency (see fig. 1A, 1B). By contrast, Manly $\hat{\beta}$ in the natural survey was not related to andromorph frequency ($F_{1,13} = 0.01$, $P = .92$), which is confirmed by the estimated slope of the relationship being zero (fig. 1C).

Patterns of Frequency Independence

Significant frequency-independent preference was observed in the presentation experiment ($df = 8$, $t = 7.03$, $P = .0001$), with andromorphs being preferred at an andromorph frequency of 0.5 (fig. 1A). However, this pattern does not hold for the cage experiment ($df = 4$, $t = 1.11$, $P = .33$). Furthermore, a frequency-independent preference of marginal significance was found in the natural survey, with gynomorphs being overall more engaged in matings ($df = 13.4$, $t = -2.16$, $P = .05$). Indeed, figure 1C illustrates that Manly $\hat{\beta}$ for the majority of the populations was lower than 0.5, suggesting a baseline preference for gynomorphs.

Discussion

This study presents empirical evidence for negative frequency-dependent selective mechanisms maintaining heritable trait variation in the wild. We use female polymorphic damselflies as a model system and indicate the importance of (1) disentangling the contribution of behavioral components of all players (here, males and females) when studying selective mechanisms and (2) considering not only experimental but also unmanipulated natural conditions to fully understand the complexity of a selective agent driving patterns of NFDS. Specifically, comparison of how our mate preference index (Manly β) varied with andromorph frequency shows that different methods to investigate male preference and mating patterns differ profoundly in terms of whether frequency dependence is evident. These patterns most likely reflect the degree of female mate resistance behavior that each method allowed and thus suggest an important, though often neglected, role of female response in influencing the outcome of intersexual interactions. If male mate preference would simply translate in the number of matings observed under natural conditions, frequency dependence should have been observed in both the male preference experiments and the field data, which is clearly not the case.

Patterns of Frequency Dependence

Male mate preference was frequency dependent in the direct presentation experiment, with preference for a female morph increasing as its frequency rose. Similarly, in the cage trials, a trend for frequency-dependent male preference was detected. The slope of the relationship between Manly $\hat{\beta}$ and andromorph frequency was less steep in the cage trials than in the presentation experiments, indicating that the strength of frequency-dependent male preference was weaker, possibly because more female resistance behavior was allowed in the cages. Conversely, frequency-

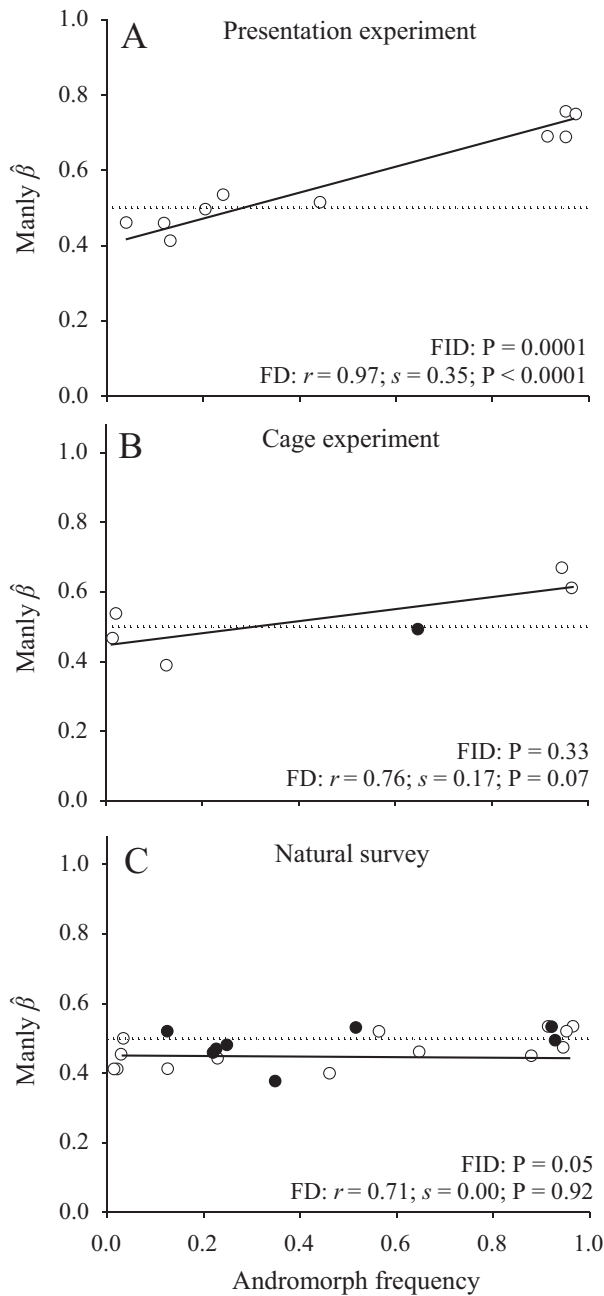


Figure 1: Comparison of the relationship between Manly $\hat{\beta}$ and andromorph frequency in *Nehalennia irene* for the presentation experiment (A), the cage experiment (B), and the natural survey (C). Each circle represents a separate population. Open circles refer to populations from the presentation experiment that were also sampled for the other three methods; filled circles refer to other populations. FID refers to frequency-independent male mate preference. The dotted horizontal line at Manly $\hat{\beta} = 0.5$ indicates absence of overall preference for both female morphs. Data points above or below this line indicate frequency-independent preference for andromorphs or gynomorphs, respectively. FD refers to frequency-dependent mate preference, which is characterized by an association

dependent male preference was absent in the natural survey, which was determined in the field where a full arsenal of female resistance behavior, including microhabitat use, fleeing, and all possible refusal displays could be utilized (e.g., Forbes et al. 1997; Sirot and Brockmann 2001; Van Gossum et al. 2001a; Iserbyt and Van Gossum 2009). A similar analysis in *Ischnura elegans* (see appendix) also confirmed the absence of frequency-dependent preference during natural observations. These results suggest that female behavior mediates frequency-dependent male mating preferences. The estimated slope of the relationship between Manly $\hat{\beta}$ and andromorph frequency was close to zero for the natural survey, which may indicate that if female morphs are willing to mate, they may solicit matings to fulfill their reproductive needs when rare. Conversely, females of the common morph may to an extent use refusal or evasion behavior to avoid male sexual attention when their optimal number of matings is exceeded (Gosden and Svensson 2009).

Together, these results indicate that even though males prefer to mate with the most common female morph in the population (Miller and Fincke 1999; Ting et al. 2009; Takahashi and Watanabe 2010b; this study) and can switch their preference following experimental changes in morph frequency (Van Gossum et al. 2001b), such frequency dependence is not present for mated female morphs in the wild. Nevertheless, it has been shown for *Nehalennia irene*, *I. elegans*, and *Ischnura senegalensis* that the most common female morph in a given population consistently oviposits fewer eggs (Svensson et al. 2005; Takahashi et al. 2010; Iserbyt et al. 2013). These fecundity costs likely reflect the outcome of NFDS. By comparing the extent of frequency-dependent male mating preference using different methods, we have established that sexual interactions during premating (and not mating) stages are most likely to drive the observed NFDS in our study system. This is because the frequency-dependent exhibition of male preference (i.e., in terms of tandem attempts) may have a large effect on female morph fitness, even if it does not result in a mating. Indeed, each unsuccessful male mating attempt and associated female resistance behavior may involve fitness costs to single females in terms of time and energy that could be allocated elsewhere (isopods: Jormalainen et al. 2001; beetles: den Hollander and Gwynne 2009; bees: Rossi et al. 2010; damselflies: Córdoba-Aguilar 2009; Takahashi and Watanabe 2010a). Consequently, we suggest that interacting effects of costly premating female

between Manly $\hat{\beta}$ and andromorph frequency. Fitted regressions are represented by the correlation coefficient r and the slope s . Slopes close to zero indicate absence of FD, and the greater the departure from zero, the stronger the estimated FD.

resistance behavior and excessive male mating attempts mainly drive NFDS in this model system.

Patterns of Frequency Independence

According to intraspecific mimicry theory, males learn to avoid unprofitable interactions with other males, including phenotypes that resemble them (Robertson 1985; Sherratt 2001). As a consequence, male-like andromorphs may overall be less readily recognized as a female by mate-searching males, at least when rare compared to males. We therefore tested whether males have an underlying (baseline) preference for a given female morph when morphs are statistically controlled to be at equal frequency in the population. There was no evidence of such frequency-independent male mate preference in the cage experiment. However, in contrast with our predictions, andromorphs were more preferred than can be expected at 0.5 morph frequency in the direct presentation experiment. This method does not allow andromorphs to make use of behavioral mimicry (as suggested by Forbes et al. 1997), and moreover, given their conspicuous body coloration against the background vegetation, andromorphs may be more easily detected when directly presented to mate-searching males (Iserbyt and Van Gossum 2011). However, it is worth emphasizing that the above inference refers to male preference when andromorph frequency is statistically controlled to 0.5. Therefore, an overall preference for gynomorphs can still arise if there is frequency-dependent male mate preference (as observed) and andromorphs are rare.

On the other hand, observations under natural conditions are consistent with expectations based on intraspecific male mimicry. Specifically, andromorphs were overall less often mated than gynomorphs in the natural survey, while there was no frequency-dependent change in the preference index. This largely corresponds with our analysis for *I. elegans* (see appendix), for which the *infuscans* gynomorph was consistently more often observed mated, whereas the reverse was true for andromorphs. In line with our results, previous studies examining mating probabilities in the field also found that andromorphs were less often mated than gynomorphs, which is especially profound in *Ischnura* species (e.g., Sirot et al. 2003; Hammers and Van Gossum 2008; Gosden and Svensson 2009). All these results suggest that the putative male mimic is overall less often mated under natural conditions.

Conclusions

Previous studies related variation in female morph frequencies with female morph fecundity patterns and found evidence for NFDS maintaining female polymorphism in the

wild (Svensson et al. 2005; Takahashi et al. 2010; Iserbyt et al. 2013). However, details on the role of the selective agent per se, and especially the role of the two players involved in NFDS (males and females), were lacking. Here, we have attempted to disentangle male and female behavioral components of intersexual interactions by comparing measures of male mate preference under different conditions. By taking into account the varying degrees of female resistance behavior allowed in three different male preference evaluations, we show that premating intersexual interactions most likely drive the observed NFDS in this model system. This illustrates the importance of dissecting the interactions among all players involved in driving NFDS. We suggest that this approach is not only applicable to female polymorphic systems but may potentially serve as a general framework in predator-prey and host-parasite systems. As such, our findings add to the understanding of the role of social interactions as a driving force for the emergence, evolution, and maintenance of the genetic and phenotypic variation observed in nature (Sinervo et al. 2001; Lyon and Montgomerie 2012).

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