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**THE EVOLUTION AND MAINTENANCE OF FEMALE-LIMITED
POLYMORPHISM IN A DAMSELFLY**

by

Janice J Ting

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of

the requirements for the degree of

Masters of Science

Department of Biology

Carleton University

Ottawa, Ontario, Canada

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Abstract

Many theories have been proposed to explain female polymorphism in damselflies (Odonata). Here I evaluated two contrasting theories - the learned mate recognition (LMR) and the male mimicry (MM) hypotheses, both of which suggest the phenomenon is generated by a form of frequency-dependent male harassment. My study species, *Nehalennia irene*, has two female morphs (andromorph and gynomorph), with morph frequencies varying from 5-95% andromorphs. To discriminate between the theories I evaluated male sexual response towards the two female morphs in populations but also the male response towards other males. My findings support the LMR predictions with regard to male-female interactions when specimens were presented on sticks; however my results fail to support either hypothesis when specimens were presented in cages. Overall, I show the potential for frequency-dependent selection in *N. irene* but propose a more pluralistic approach needs to be applied in explaining the polymorphism in general.

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This thesis would not have been possible without the help, support and friendships from numerous individuals. First and foremost I would like to thank Dr. Tom Sherratt for being an extraordinary supervisor. I am always in awe of how he manages to dedicate so much time and attention to all of his students while juggling three very lively children and always making time for his rugby matches. I feel very fortunate to have had the chance to work with a knowledgeable and passionate scientist like Tom. His pure excitement and joy for science truly inspires me.

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Statement of Contributions

Chapter 2:

Ting JJ, Bots J, Perez-Jvostov F, Van Gossum H, Sherratt TN. Effects of extreme variation in female morph frequencies on the mating behaviour of male damselflies. Behavioral Ecology and Sociobiology (*Accepted*)

Chapter 3:

Ting JJ, Iserbyt A, Bots J, Perez-Jvostov F, Van Gossum H, Sherratt TN. Caged male-mating behaviour experiments in populations varying dramatically in female morph frequencies in a damselfly.

In the work presented in chapters 2 and 3 my contributions to both chapters were that I was integral in the conception and design of the experiments, aided in the collection of the data, was responsible for collating and analysing the data and additionally I wrote the first draft of both chapters. Both chapters are presented in the format of a scientific paper and as such, further details are found in future chapters.

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Chapter 1

General Introduction

Visible polymorphisms

Within single interbreeding populations two or more genetically inherited types can sometimes co-exist with one another. When the occurrence of these types are phenotypically distinct but cannot be explained by recurrent mutation, then such populations are said to exhibit visible polymorphism (Ford 1957). Permanent (balanced) visible polymorphisms occur when the multiple morphs have an equal average fitness at equilibrium. Permanent polymorphisms often imply frequency-dependent selection where morphs experience a selective advantage when rare but also suffer some disadvantages when common (Fisher 1930). Other selective forces that account for permanent polymorphism include heterosis (heterozygotes have a greater fitness than homozygotes) and variability in selection across space or time (Maynard Smith 1998).

Polymorphisms provide an excellent opportunity to explore how natural selection promote and maintain genetic variation in nature. One of the best known examples of visual polymorphism is that of industrial melanism in the peppered moth *Biston betularia* where two morphs (white non-melanic form and black *carbonaria* form; Fig. 1-1a) coexist but differ in frequency in response to changes in the frequency of appropriate light and dark environments (e.g. Majerus 2005). Selection on Batesian mimics is often frequency dependent, where mimics experience the greatest increase in their survivorship when they are rare in comparison to the models (Ruxton et al. 2004). As such, polymorphisms have been frequently observed in Batesian mimics, such as the non-

aggressive social wasp *Mischocyttarus mastigophorus*. This species has two morphs (dark and pale morph) that are thought to resemble *Agelaia xanthopus* and *Agelaia yepocapa*, both aggressive swarm-founding wasps (O'Donnell 1999).

Polymorphisms are also present in plants such as the orchid *Dactylorhiza sambucina* where it has a yellow- and a purple-flower morph – given the lack of the reward, pollinators prefer the rare colour morph and there is therefore negative-frequency selection maintaining the polymorphism (Gigord et al. 2001). Examples of phenotypic polymorphism have been reported in other taxa, such as fish (Franck et al. 2001), birds (Galeotti et al. 2003) and mammals (Ritland et al. 2001). Many instances of polymorphism have been found to be based on variation at a single locus, or several tightly linked loci, and the trait is often generated by genes with pleiotropic effects. For example, in side-blotched polymorphic lizards (*Uta stansburiana*) the characteristic throat color that marks the polymorphism (Fig. 1-1b) has been found to correlate with heritable immune response (Svensson et al. 2001), male mating strategies (Sinervo and Lively 1996), and specific life history strategies (Sinervo et al. 2001).

Figure 1-1. Examples of polymorphism in nature. Figure 1-1a is of the peppered moth (*Biston betularia*) which is polymorphic with a melanic and a *carbonaria* form (Majerus 2009). The side-blotched lizard (*Uta stansburiana*) exhibits genetically based throat-colour polymorphism (orange, blue, or yellow: Fig. 1-1b; Sinervo and Lively 1996).

Sex-limited polymorphism

Phenotypic variation *between* the sexes is known as sexual dimorphism and is common across many taxa (see Andersson 1994). However phenotypic variation can also arise *within* one of the sexes. Sex-limited polymorphism arises where multiple phenotypic variants within a sex are found to co-occur within a population. Sex-limited polymorphisms can generally (but not always) be explained in the context of sexual selection. (Gross 1996). For example, marine isopods, *Paracerceis sculpta*, have polymorphic males (Fig. 1-2) with three coexisting genetically discrete male morphs (Shuster 1989; Shuster and Wade 1991). Large ornamented α males (territorial) guard and defend harems, smaller β males are female mimics (behaviour and morphology) and the tiny γ males (sneakers) resembling juveniles are non-ornamented and invade harems guarded by α males (Shuster 1987). Average mating success is equal among the three male morphs (Shuster and Wade 1991).

Figure 1-2. The four adult morphs in *Paracerceis sculpta*; females are monomorphic and males are polymorphic: α -males are territorial, β -males are female mimics, and γ -males are smallest in size and sneak matings (top photo is © SM Shuster, bottom illustrations are from Shuster 1992).

Female-limited polymorphism is not as clearly understood as that of male-limited polymorphism. Female polymorphism is generally thought to have evolved as a consequence of selection to avoid excessive male harassment (Alexander et al. 1997). However, it is not always clear how the multiple female morphs co-existing within a population are maintained. Female-limited polymorphism has been observed in many taxa such as birds (Galeotti et al. 2003), lizards (Vercken et al. 2007), and arthropods (Reinhardt et al. 2007). For example, females of several species of diving beetles, *Dytiscidae*, vary in the morphology (smooth or textured) of their pronotum and elytra (Bergsten et al. 2001). There is conflict between the sexes and these female morphs occur in different frequencies in response to the number of adhesive discs on male tarsi (Bergsten et al. 2001) which are used to hold onto females during post-mating struggles (Aiken 1992; Miller 2003). A textured ('granulate') female morphology is more effective in dislodging from a males grasp with his many adhesive discs (Bergsten et al. 2001; Fig. 1-3a). Another example of female-limited polymorphism occurs in the African mocker swallowtail butterfly (*Papilio dardanus*). The females occur as at least 14 distinct morphs (Nijhout 2003; Fig. 1-3b). Within a given population, as many as six female morphs coexist (Ford 1936) where the females are Batesian mimics of several unpalatable species of Danaidae and Acraeidae (Poulton 1924; Ford 1936). Many other examples of female-limited polymorphism that are again thought to have evolved in response to male harassment are found in odonates. Indeed, female-limited polymorphism occurs particularly frequently in this taxa as there are 130 reported species where females are polymorphic (Fincke et al. 2005).

Figure 1-3. Examples of female-limited polymorphism. On the top (Fig. 1-3a) an example of the polymorphism in the water beetle (*Dysticidae*) where females differ in their surface texture of their elytra to decrease the effectiveness of adhesive discs on male tarsi (shown to the right of each beetle; Bergsten et al. 2001). On the bottom (Fig. 1-3b) the 14 different homozygous female forms of *Papilio dardanus* along with the monomorphic male (Nijhout 2001).

Female-limited polymorphism in damselflies

Female-limited polymorphism in damselflies appears as discrete variation in body coloration, patterning, and in some cases behaviour. There are generally two female morphs (see Fig. 1-4), one which resembles the male in colour and pattern (from here on referred to as an andromorph) while the other morph(s) looks dissimilar (from here on referred to as a gynomorph). Phenotypic ratios of adult offspring following crosses suggest that the polymorphism is inherited at a single autosomal locus with sex limited expression, where the number of alleles involved equals the number of female morphs (Johnson 1964; Johnson 1966; Cordero 1990b; Andrés and Cordero 1999; Sánchez-Guillén et al. 2005).

Similar to the diving beetle example previously introduced, the most common adaptive explanation for the existence and maintenance of the polymorphism in damselflies is based on sexual selection, specifically sexual conflict where the sexes differ in their optimal number of matings. For some species, female damselflies are able to fertilize their limited number of eggs produced with one mating (Fincke 1987; Cordero 1990a) and thus any subsequent matings are unnecessary. Males on the other hand are able to replenish their sperm and can increase their fitness with the number of matings they achieve. Consequently, males excessively pursue and harass females that are unwilling to mate, thus generally imposing a fitness cost upon the females. Females respond to excessive male harassment with counter-adaptations to reduce these male imposed costs, and in the case of damselflies the counter-adaptation is hypothesized to be expressed in body colour variation.

The evolution of female-limited polymorphism is generally agreed to be attributed to male harassment, where a main assumption is that females suffer a fitness cost induced by male harassment. Unnecessary matings are assumed to be costly to females as matings in damselflies can be quite long in duration (*Nehalennia irene* >18min: Forbes et al. 1997; *Ischnura ramburi* average 3 h: Robertson 1985) thus reducing foraging time, but it may also be physically damaging. Additionally, when paired with a male in a tandem (see Fig. 2-2) it may increase conspicuousness and thus susceptibility to predation for the female, however there is little evidence in support (Fincke 1994). Fitness cost of male harassment was studied in the lab using the damselfly, *Ischnura ramburi*, where male to female ratios were manipulated in small cages (Sirot and Brockmann 2001). With an increase in male to female ratio, andromorphs were shown to have lower lifetime fecundity and reduced foraging efficiency (Sirot and Brockmann 2001). In gynomorphs, no costs were detected with an increase in male to female ratio where the lifetime fecundity of gynomorphs were not influenced by a decrease in lifespan and an increase in egg laying rate (Sirot and Brockmann 2001). Manipulating ratios of males to females as well varying densities and morph frequencies in large outdoor insectaries, female morphs of *Ischnura elegans* (see Fig. 1-4) were found to vary in survivorship and lifetime matings and ovipositions due to the intensity of male harassment (Van Gossum et al. 2005b). More convincing work has been recently conducted in the field where male harassment was quantified by marking male *I. elegans* with various colours of fluorescent dust and counting the number of mating attempts and copulations on females during the following day (Gosden and Svensson 2007). They were able to show that andromorphs are more tolerant in terms of their fecundity to increased harassment and mating, and

gynomorphs (infuscans form; Fig. 1-4) have higher resistance to matings compared to the other morphs where the cost of excessive male harassment was measured in female fecundity (Gosden and Svensson 2007).

The exact mechanism that maintains the polymorphism in damselflies is still unresolved. However two broad theories that have garnered the most attention include the male mimicry hypothesis (abbreviated as MM; Robertson 1985) and the learned mate recognition hypothesis (abbreviated as LMR; Miller and Fincke 1999). These two general theories propose different methods to avoid male harassment. Specifically, the MM hypothesis proposes that it would benefit a female to resemble something unprofitable (i.e. a conspecific or heterospecific male); the LMR hypothesis proposes that it would benefit a female to simply look dissimilar from the common morph. In the MM, the andromorph is considered as a functional male mimic where as the gynomorphs are readily recognized as females (Robertson 1985). In the original LMR hypothesis, males readily recognize conspecific males (Miller and Fincke 1999; Van Gossum et al. 1999). Both theories suggest that female polymorphism in damselflies is maintained through negative frequency dependent selection. The source of the negative frequency dependent selection of the MM hypothesis is the ratio of mimics to models (andromorphs / males) while the source of the LMR is the andromorph frequency (andromorphs / [andromorphs + gynomorphs]).

Figure 1-4. Examples of polymorphic females in damselflies. *Ischnura elegans* (photos © David Kitching) has 3 female morphs while my study species *Nehalennia irene* has two morphs (illustration by Lam 2004; photo © Hans Van Gossum).

Thesis objectives

Previous studies have been poorly equipped to simultaneously address the predictions of both the MM and LMR theories, as all have failed to include testing male-male interactions over a range of morph frequencies (required to test MM). No studies to my knowledge have used multiple natural populations varying dramatically in morph frequencies in order to quantify the extent of negative frequency dependent selection. In chapter 2, I test these two hypotheses using the sedge sprite damselfly, *Nehalennia irene* (Fig. 1-4), in five populations distributed in Ontario and British Columbia, Canada that vary maximally in female morph frequencies (from 5-95% andromorphs).

The method I used to measure male mate response behaviour in chapter 2 was a stick presentation where a live specimen type (andromorph, gynomorph, or male) was adhered to a grass stem and presented to a male freely perched in the field. The benefits of using stick presentations are that the focal male is free to behave spontaneously in his natural environment and the introduction of the specimen type is controlled (movement, distance and orientation). However male responses are largely based on the appearance of the specimen and not the behaviour of the specimen, as being adhered to a grass stem greatly limits the ability of females to attract males or perform refusal behaviours. Previous studies have suggested that males and female morphs differ in their behaviours following approach by males (Robertson 1985; Forbes et al. 1997). Female *N. irene* unwilling to mate seemingly communicate this message to interested males by shaking (rapid body shake), abdomen bending (Forbes 1991), or wing raising (Bick and Bick 1978). In field observations, *N. irene* andromorphs were found to chase away males more

frequently than gynomorphs, whereas gynomorphs performed refusal displays towards males more frequently (wing warning or abdomen bends: Forbes et al. 1997).

Aware of the behavioural limitations in the methodology in chapter 2, I conducted complementary field experiments, again across sites varying dramatically in female morph frequency, examining male mating response behaviour using a cage method. Within each cage four live individuals (two males, one andromorph, and one gynomorph) were placed together allowing for males to interact with the two female morphs as well as a conspecific male (chapter 3). The cage method allowed more response behaviours of the three specimen types used (andromorph, gynomorph, male) to be observed compared to that of the stick presentations (chapter 2); however individuals were unable to hide or flee the vicinity of the harasser due to the environment within the cage. The increase in the ability for a specimen to respond to its harasser may influence how the male responds sexually to the specimen type. Additionally, the recent mating status of females used were known (identified as captured single or in a tandem) which allowed me to examine the behaviours of receptive and unreceptive females. I comment on which of the two methods I used (sticks or cages) more closely relates to what occurs in nature. In chapter 4 I place my findings back into a broader context and suggest future work in the field of polymorphism in damselflies.

Overall, I believe this work expands our knowledge and understanding of female-limited polymorphism in damselflies by addressing methodological limitations that have prevented previous works from directly comparing between the LMR and MM hypotheses. This work provides one of the clearest indications to date that frequency-dependent selection is operating to maintain the polymorphism.

Chapter 2

Effects of extreme variation in female morph frequencies on the mating behaviour of male damselflies

Female-limited polymorphism is often attributed to selection to avoid excessive male mating attempts. It is encountered in various taxonomic groups, but is particularly common in damselflies, where one female morph (andromorph) typically resembles the conspecific male in colour pattern, while the other(s) (gynomorph(s)) do not. Two sets of theories have been proposed to explain the phenomenon in damselflies, which can be broadly classified as the learned mate recognition (LMR) and male mimicry (MM) hypotheses. To test predictions of these hypotheses, I evaluated the rate of male sexual response towards female morphs and conspecific males in the damselfly *Nehalennia irene*. The LMR hypothesis predicts that males should respond sexually to andromorphs at greater rates in populations containing a higher relative frequency of andromorphs. The MM hypothesis makes a similar (yet additional) prediction, namely that males respond more often sexually to *both* andromorphs and males as the ratio of andromorphs to males increases. While LMR predicts that the rate of mating attempts towards gynomorphs should vary with female morph frequency, the MM predicts that it should be relatively fixed. On experimentally presenting live specimens to focal males in five different populations with extreme variation in female morph frequencies, I observed that as the andromorph frequency and ratio of andromorphs to males increased, the proportion of male mating attempts increased on both andromorphs and males, whereas it decreased on gynomorphs. Overall my results are consistent with the LMR hypothesis with regard to male-female interactions, and allow me to reject the MM hypothesis.

INTRODUCTION

It has long been recognised that distinct, genetically inherited phenotypes of a single species can coexist in natural populations. Such polymorphic systems not only represent visually attractive examples of within-species diversity, but provide an opportunity to test broader theories as to how and why genetic diversity is maintained (Gray and McKinnon 2007). Particularly puzzling are polymorphisms limited to only one sex (male- or female-limited polymorphism). Such sex-limited polymorphism is often explained in the context of sexual selection (Gross 1996; Shuster and Wade 2003; Svensson et al. 2009), although there are examples of sex-limited polymorphisms in Batesian mimics, arising out of a combination of differential selection on the sexes and frequency-dependent predation (Stamps and Gon 1983; Joron and Mallet 1998; Ruxton et al. 2004). In particular where sexual selection has led to male-limited polymorphism, it is often thought to have evolved as a consequence of intense male-male competition over access to mates (e.g. damselflies: Plaistow and Tsubaki 2000; e.g. beetles: Simmons et al. 2007). Likewise, female polymorphism is generally – but not always (e.g. Sinervo et al. 2000; Vercken et al. 2007) - thought to have evolved as a counter adaptation to reduce fitness costs imposed by mate-searching males (e.g. diving beetles: Härdling and Bergsten 2006; African bat bugs: Reinhardt et al. 2007). Indeed, empirical studies under both experimental and natural conditions indicate that sexual interactions with males can be costly to female morphs, in that male harassment can upset female time and energy budgets and may ultimately decrease female fecundity (e.g. damselflies: Sirot and Brockmann 2001; Gosden and Svensson 2007) .

The coexistence of multiple female morphs in natural populations has been reported in several animal taxa (Svensson et al. 2009), but it is especially common and most intensively studied in damselflies (Insecta: Odonata) (Fincke et al. 2005; Van Gossum et al. 2008). Where such polymorphism occurs in damselflies, one female morph (andromorph) typically resembles the conspecific male in body colour and pattern, and often also in behaviour, while the other morph(s) are dissimilar (gynomorph(s)) (e.g. Corbet 1999; Van Gossum et al. 2001b). Female-limited polymorphism has been shown to be inherited genetically in several damselfly species, with phenotypic ratios consistent with the trait being controlled at a single autosomal locus with sex limited expression and the number of alleles involved equalling the number of female morphs in the particular species (Johnson 1964; Johnson 1966; Cordero 1990b; Andrés and Cordero 1999; Sánchez-Guillén et al. 2005).

Two broad categories of theory have been proposed to explain the coexistence of the morphs in damselflies. The main premise of both theories is that female polymorphism arose to avoid excessive attention by mate searching males and thereby reduce fitness costs due to male harassment. However, the two sets of theories differ on the precise mechanisms generating the polymorphism. On the one hand, it has been suggested that male mating behaviour is influenced by direct experience, such that males have an increased tendency to respond sexually to the morph they encounter most frequently, typically the more common morph (learned mate recognition hypothesis or LMR; Miller and Fincke 1999). Of course, if a female seeks to avoid male attention, then it might pay to resemble something that is unprofitable to attack, rather than something which simply looks different. Therefore, it has also been suggested that the similarity of

andromorphs to males may not be incidental, such that the andromorphs benefit from male mimicry (male-mimicry hypothesis or MM; Robertson 1985). There are several different specific theories as to how male mimics (andromorphs) may ultimately pay a cost for their mimicry (such as increased vulnerability to predators, or increased conspicuousness to searching conspecific or heterospecific males) thereby ultimately ensuring a balanced polymorphism in these instances (Johnson 1975; Robertson 1985; Hinnekint 1987; Cordero 1992; Sherratt 2001).

Both LMR and MM assume that female polymorphism is maintained by negative frequency-dependent selection, with the primary selective force being costly male sexual harassment (Fig. 2-1a-e). The LMR hypothesis (Miller and Fincke 1999), with its emphasis on learning to recognise female forms, predicts an increase in male mating attempts towards andromorphs, and a concomitant decrease in mating attempts towards gynomorphs, when the andromorph frequency in the female population increases. This is because males are thought to learn to recognise the most frequently encountered morph as a potential mate (Fig. 2-1a,b). While males may need to learn to distinguish males from females, no morph-specific mimicry of conspecific males is assumed in the original formulation of the LMR (Miller and Fincke 1999; Van Gossum et al. 1999), and thus one would expect that the rate of male sexual response towards other males would not change consistently as andromorph frequency changes. However, I refrain from treating this as an explicit prediction of the LMR model, because a more recent formulation of the LMR involving multiple “distractors” (including, but not restricted to, conspecific males) suggests that the relationship could be positive, negative or neutral (Fincke 2004, see Discussion).

Although Batesian mimicry often involves the need of receivers to learn what types are profitable and unprofitable (e.g. see Ruxton et al. 2004), in the simplest case of MM, the primary underlying source of frequency-dependence is the ratio of mimics to models (i.e. the ratio of andromorphs to males). Therefore, assuming that males maximise their payoff per encounter, the MM hypothesis predicts an increase in the male sexual response rate towards andromorphs on encounter, and a concomitant increase in the response rate of males to conspecific males on encounter, as the ratio of andromorphs to males (i.e. the proportion of male-like individuals that are andromorphs) increases (Fig. 2-1c, e). Increases in attack rates on both mimics and models as the ratio of mimics to models increases have been repeatedly observed in the empirical literature on Batesian mimicry (Ruxton et al. 2004, section 10.3.4) and this outcome is widely predicted by mimicry models (Ruxton et al. 2004, Appendix B). Conversely, if gynomorphs are readily recognised as female, then, assuming no opportunity costs or time constraints from the male's response to andromorphs, the rate of male mating attempts towards gynomorphs should not change consistently with andromorph to male ratio, or indeed andromorph frequency (Fig. 2-1d)

It is clear from the above arguments that one way to distinguish between the MM and LMR hypothesis is to compare the male propensity to attempt to mate with andromorphs, gynomorphs and conspecific males in populations where andromorphs are both common and rare (Fig. 2-1a-e). Van Gossum et al. (1999) assessed male mate preference of the damselfly *Ischnura elegans* for female morphs using pairwise choice tests, conducted in five natural populations with distinct female morph frequencies. In contrast to the authors' conclusion (Van Gossum et al. 1999), none of the leading

hypotheses could be firmly rejected because in the one study population where andromorphs were the majority morph (70%), males did not show a significant preference for either female morph, which is in line with both LMR and MM (see above). Moreover, male mate preference was only evaluated against the andromorph frequency, but not against the ratio of andromorphs to males. Most importantly, no attempts have been made to document the frequency of misdirected sexual attention of males towards conspecific males in a range of populations that vary in andromorph to male ratio and/or female morph frequency.

In this study, I assessed variation in male mating response towards conspecific andromorphs, gynomorphs and males in five natural populations of the Sedge Sprite damselfly *Nehalennia irene*. I aimed to study populations that differed maximally in female morph frequencies, and therefore selected populations (based on Van Gossum et al. 2007a) where females represent more than 90% andromorphs as well as populations with more than 90% gynomorphs. Using this approach, this study for the first time has tested the outlined predictions of both LMR and MM under an extremely wide range of natural variation in morph frequency.

Figure 2-1. Simplified predictions of the learned mate recognition hypothesis (LMR: 2-1a to 2-1b) and the male mimicry hypothesis (MM: 2-1c to 2-1e) as to how the *per capita* rates of male harassment on andromorphs, gynomorphs, and males on encounter should vary with an increase in andromorph frequency and the ratio of mimics to models, respectively. I do not expect the relationships to be entirely linear and the aim is simply to depict general trends.

METHODS

Study species and populations

The damselfly *Nehalennia irene* is widely distributed across Canada, from Newfoundland in the east to British Columbia in the west, but it is not present in the far north of the country (Walker 1953). It is a small, non-territorial damselfly (body length 25-28mm; Cannings and Stuart 1977) that inhabits marshy or boggy areas with relatively dense vegetation. The species mostly stays close to the waters edge and rarely ventures out over open water (Walker 1953; Cannings and Stuart 1977).

N. irene exhibits a clear colour polymorphism restricted to the female sex, with morphs classified into andromorphs and gynomorphs based on their body coloration. Andromorphs resemble conspecific males as they have a similar blue-green body colour and black patterning on the dorsum of abdominal segment nine. In contrast, gynomorphs (also sometimes referred to as “heteromorphs”: Johnson 1975) are different from males since their body colour is green-yellow and they lack the distinctive triangular patch which andromorphs and males show on segment eight (see Forbes et al. 1995 for more detailed description). The genetic basis of the colour polymorphism has yet to be established for *N. irene*; however the polymorphism has been confirmed not to be age related (Forbes et al. 1995).

Past research with *N. irene* documented considerable variation in female morph frequencies across Canada (Van Gossum et al. 2007a), with andromorphs being nearly absent in the east (New Brunswick) while being by far the majority female morph in the west (British Columbia). Based on estimates made during previous fieldwork (Van Gossum et al. 2007a), five study sites with populations at contrasting female morph

frequencies were selected: two sites (Nukko Lake and Summit Lake) with high, two sites (Jack's Marsh and Barb's Marsh) with low and one site (Otter Marsh) with intermediate andromorph frequencies (see Table 2-1 for details). Comparing data on andromorph frequency collected at the same sites in 2004 (Van Gossum et al. 2007a) suggests that andromorph frequency has been relatively stable at the majority of sites surveyed, in that the BC sites consistently reported > 90% andromorphs, while the low andromorph frequency Ontario sites remained relatively low (see also Iserbyt et al. 2009). Before experimentally evaluating male mating response, the female morph frequencies, male densities and operational sex ratios were estimated at each site, based on sample counts of individuals captured with an insect net in a standardised manner (see Table 2-1). This 2007 survey adopted identical methods to those described in Van Gossum et al. (2007a), and involved the collection of damselflies within 5m of the water's edge, where mating of *N. irene* typically occurs (Forbes et al. 1995). Thus, while walking slowly, I swept the vegetation with an insect net using a "figure-eight" motion and recorded the time taken to complete a sample. The sex, maturity (adult or teneral [i.e. newly metamorphosed individuals that still show glistening body and wings]) and morph of each individual *N. irene* captured was recorded and all individuals were marked prior to their release to preclude recounting individuals that had already been censused. Multiple sweeps were conducted per site to reach a sample size that included a minimum of 30 adult females (see also Van Gossum et al. 2007a).

The arithmetic mean andromorph frequency per sweep was estimated by dividing the number of mature andromorphs caught by the total number of mature females caught in each of the sweeps. I estimated the arithmetic mean ratio of mature andromorphs to

males (putative mimics to models) in the same way (Table 2-1). The ratio of andromorphs to males is simply another way of presenting the relative frequency of andromorphs (this time relative to males) and the main purpose of these two calculations was to directly address the LMR and MM hypotheses. Although one might expect the frequency of andromorphs in the female population to co-vary with the ratio of andromorphs to males, they are demonstrably not equivalent (e.g. see Fig. 2-4). An estimate of the density of males and female morphs was also calculated, based on the total number of individuals caught per minute in individual sweeps (Table 2-1). Immature individuals were excluded from all of these counts as this study focuses on reproductive interactions, which arise among sexually mature individuals only. Even when immature individuals were included in the calculations for andromorph frequency and andromorph to male ratio, the parameter estimates were very similar to those presented in Table 2-1.

Table 2-1 Population details and frequency estimates (± 1 SE) for the five sites studied during the reproductive season of 2007. The first three sites are in Ontario, and the second two sites are in British Columbia. A refers to andromorphs, G to gynomorphs, and M to males. The expected standard errors of andromorph frequencies as well as the ratio of andromorphs to males were calculated from the multiple sweeps taken from each survey. Sites differed significantly in all key parameters investigated (non-parametric Kruskal Wallis test, A/M ratio $\chi^2=13.66$, $df=4$, $P=0.008$; andromorph frequency $\chi^2=18.75$, $df=4$, $P=0.001$; male density $\chi^2=13.35$, $df=4$, $P=0.01$).

Site	Latitude, longitude	Experiment dates	Experiment sample size (A/G/M)	Survey date	Surveying sample size (A/G/M)	Andromorph frequency A/(A+G)	Mimic to model A/M	Overall density of individuals A/G/M (total/min)
Jack's marsh	44°34'00", -76°21'00"	June 27–July 5	14/15/14	July 12	2/45/132	0.04 $\pm$ 0.03	0.02 $\pm$ 0.01	0.14/3.19/9.36
Barb's marsh	44°31'30", -76°22'30"	June 11–15	17/17/17	June 12	2/31/76	0.06 $\pm$ 0.04	0.03 $\pm$ 0.02	0.13/1.95/4.79
Otter marsh	44°32'30", -76°22'00"	July 12–16	21/21/21	July 11	16/20/115	0.44 $\pm$ 0.08	0.14 $\pm$ 0.03	1.31/1.63/9.40
Summit lake	54°18'00", -122°43'30"	June 26–July 3	15/15/15	June 26	77/2/82	0.97 $\pm$ 0.02	0.94 $\pm$ 0.11	3.85/0.10/4.10
Nukko lake	54°04'30", -122°59'30"	June 17–25	15/15/15	June 21	49/1/93	0.98 $\pm$ 0.02	0.53 $\pm$ 0.08	7.35/0.15/13.95

Experimental design

Focal male mating behaviour was examined within 5m of the water's edge by presenting mature live specimen types (andromorphs, gynomorphs and males) to a randomly selected mature focal male (for similar methodology see Forbes et al. 1997; Fincke et al. 2007; Van Gossum et al. 2007b) during the reproductive season of 2007 (mid June to late July). All experiments were conducted between 9am and 4pm local time, on days with suitable weather conditions (sunny without precipitation) when *N. irene* was reproductively active. The specimen types were obtained by gluing a live mature individual (andromorph, gynomorph or male) with the ventral side of the thorax and its mouth parts to the end of a long grass stem (~45cm long) with UHU® Power Glue (see Fig. 2-2). Subsequently, specimen types were presented to sexually mature focal males by holding the grass stem at a 45-65° angle and slowly moving the specimen lateral towards (<1 body length from) the male. The specimens were slowly presented to the focal males to avoid eliciting a startle response. These focal males were found undisturbed, and they were typically perched freely in the surrounding vegetation. To avoid any form of observer bias the same human observer always presented the three different specimen types of a trial on any given occasion.

The qualitative responses of focal males towards the specimen types were observed (see below) and also their latency to respond to the nearest half second up to a maximum of 120 seconds. This latency was recorded, because I expected that males would respond more rapidly to a presented specimen when they perceive it a suitable partner. Presentations where the proposed focal male was startled either by the presenter or by how the specimen was presented (movement was too quick) were not recorded and

a new focal male was used. The behavioural responses of the focal male were categorised as follows: (1) the male did not respond and the observation was stopped after 120s ('no response'), (2) the male showed no interest and flew away (sometimes after inspecting but without touching the specimen) ('fly away'), (3) the male approached and made contact with the presented specimen by landing on its head, thorax, wings or abdomen (but then flew away) ('contact'), or (4) after making contact with the specimen the male attempted to grasp the specimen with his anal appendages ('tandem attempt'). A tandem attempt was considered successful when the focal male was able to securely attach his anal appendages to the female's pronotum (see Fig. 2-2) and unsuccessful if it could not. Attempting the tandem formation is a clear indication of sexual interest by the male (Corbet 1999) and the type of response that is of most interest when evaluating hypotheses such as MM and LMR. After each presentation, the focal male was captured (whenever possible) and marked with a permanent pen (Staedtler® Lumocolor Special) to avoid multiple testing.

The three specimen types (andromorph, gynomorph and male) were alternately presented to a different (unique) focal male to reduce possible bias in male response due to any subtle temporal variation in male reproductive activity. A single trial was completed when each of the three specimen types had been presented, in a systemised order, to five different focal males. Thus for a single trial a total of 15 unique focal males were used (3 specimen types presented to 5 different males). The component of the study conducted at Jack's marsh had an uneven number of specimen types presented because an extra gynomorph was presented out of sequence (Table 2-1). Also there were seven cases where an individual specimen was not presented to five different focal males

because the specimen died, became damaged or was lost during the trial. A minimum of 14 trials and a maximum of 21 trials were carried out at each study population resulting in a maximum of 315 presentations per site (i.e. 21 trials x 3 specimen types x 5 presentations; see Table 2-1 for the number of trials per site). All specimens used were stored in ethanol immediately after the presentations and employed in parallel studies (unpublished results) measuring morphological characteristics and fecundity, as well as a population genetic study.

Figure 2-2. An illustration of the experimental set up where a specimen (andromorph, gynomorph or male) is fixed to a grass stem with glue, and is presented to a focal male in the field, who in this case has successfully achieved tandem with the female specimen.

Statistical analyses

For all trials, the average proportion of each quantified response ('no response', 'contact', 'fly away' or 'tandem attempt') of the focal male was calculated for each specimen type (andromorph, gynomorph or male) based on the five presentations of the specimen to different males. Following this approach, the specimen is the replicate rather than the focal male, thereby avoiding pseudo-replication. The behavioural responses 'no response' and 'contact' occurred at low proportions of presentations ($\bar{X} \pm \text{SE}$: 0.002 ± 0.001 and 0.045 ± 0.006 respectively), leaving 'tandem attempts' (successful and unsuccessful) and 'fly away' as the primary observational endpoints. The overall proportion of presentations resulting in 'tandem attempts' was 0.451 ± 0.018 ($\bar{X} \pm \text{SE}$) while the proportion of 'fly away' was 0.503 ± 0.018 ($\bar{X} \pm \text{SE}$). As might be expected with two mutually exclusive endpoints, there was an extremely strong negative relationship between the mean proportion of 'tandem attempts' and 'fly away' per trial over all trials (Pearson Correlation: $r = -0.95$, $df = 247$, $P < 0.001$). Therefore, I will only present results on 'tandem attempts' since data for 'fly away' gave similar but opposite results.

All analyses were conducted using SPSS® v.15.0. The primary method of analysis involved fitting an overall GLM (general linear model) to the proportion of tandem attempts (following confirmation of approximate normality of residuals and homogeneity of variance). In fitting the GLM, specimen type (andromorph, gynomorph, male) was always treated as a fixed factor while site was treated as a random factor. I also fitted GLMs to each site separately and applied a *post-hoc* Tukey HSD test based on the estimate of the common variance. Due to the binary nature of the distribution of the key response variable ('tandem attempt' or no 'tandem attempt'), I also fitted generalized

linear models (GLZ), with logit link to a binomial distribution employing the number of mating attempts (x) on a specimen out of n presentations (generally $n = 5$) as the response variable. These analyses generated qualitatively similar results and are therefore only reported in abbreviated form.

I took the same set of statistical approaches as above to test for differences in ‘successful tandem attempts’ (a subset of ‘tandem attempts’) among specimen type and sites. Despite 404 total presentations of specimen males to other males and 145 total attempts in forming a tandem, a male never successfully formed a tandem with another male. Additionally no males in the field have been observed or caught in tandem with a conspecific male (pres. obs.) suggesting that there may be some physical constraints of genital morphology preventing successful tandems between two males. Given the clear difference in the rate of successful tandem attempts on males and females, I excluded males and concentrated simply on testing whether males could more readily form tandem with one female morph over the other. This allowed me to detect any sort of differential success that males may have with andromorphs and gynomorphs (i.e. ease of latching his anal appendages onto one morph over the other). To analyse for differences in mean reaction time among specimen types and sites, I also fitted a GLM, using the mean reaction time per presented specimen as the response variable, once again with specimen type as a fixed factor and site as a random factor.

To test whether differences in male mating behaviour towards the different specimen types is mediated in some way by female morph frequency in the local populations, I fitted separate linear regression models to the proportion of tandem attempts on each of the specimen types against both the population’s female morph

frequency ($A/[A+G]$) and the population's andromorph to male ratio (A/M). To help evaluate if and how the regression lines representing the relationships between the proportion of tandem attempts on males vs. A/M ratio and the proportion of tandem attempts on andromorphs vs. A/M ratio differed, I fitted an ANCOVA with an additional covariate*fixed factor interaction term (A/M ratio * specimen type) to allow for unequal gradients. Finally, male density has been shown to occasionally correlate with andromorph frequency in field populations on *N. irene* (Van Gossum et al. 2007a), so I fitted separate linear regression models using the proportion of tandem attempts on each of the specimen types as the dependent variables and male density in each of the populations as the predictor. Additionally Gosden and Svensson (2009) showed that the rate of male harassment towards the gynomorphs increased with the density of these morphs, but the same was not true for andromorphs. Although, not part of my main objectives, my data allows me to address density dependent male mating attempts in populations with a greater range of morph densities (see Table 2-1) compared to that of Gosden and Svensson (2009). I therefore fitted separate linear regression models using the proportion of tandem attempts on each of female morph as the dependent variables and morph density in each site as the predictor.

RESULTS

Variation in male mating behaviour among study sites

The GLM fitted to the proportion of presentations to focal males resulting in tandem attempts, indicated that male response did not vary significantly between specimen type (andromorph, gynomorph, male; $F_{2,8.034}=2.972$, $P=0.108$) or site ($F_{4,8}=1.075$, $P=0.429$)

per se, but that there was a highly significant interactive effect ($F_{8,232}=6.618$, $P<0.001$). The fitted GLZ confirmed this significant interaction ($\chi^2=62.464$, $df=8$, $P<0.001$), but also indicated significant main effects (specimen type $\chi^2=46.997$, $df=2$, $P<0.001$; site $\chi^2=36.197$, $df=4$, $P<0.001$) although here site was necessarily treated a fixed factor rather than a random factor. The consistent interaction term highlighted by both analyses clearly indicates that focal males tend to show a different sexual response to the three specimen types, yet the nature of this response differs among sites (Fig. 2-3).

At the low andromorph frequency sites Jack's Marsh (percent andromorph: 4.3% A) and Barb's Marsh (6.1% A) in Ontario males responded overall more sexually towards gynomorphs > andromorphs > males. Post-hoc tests following separate fit of the GLMs to data from each of these sites revealed that differences at these particular sites were driven by the significant differences in the propensity of males to attempt tandem with gynomorphs compared to males, while the response towards andromorphs was intermediate, not differing significantly from either gynomorphs or males (see Fig. 2-3). At Otter Marsh (44.4% A) in Ontario there was no significant variation in male mating behaviour towards any of the specimen types. In contrast, at the high andromorph frequency sites I found significant variation in the rate of mating attempts of focal males towards specimen types at both Summit Lake (97.5%) and Nukko Lake (98.0%) in British Columbia, with a hierarchy of andromorphs > males > gynomorphs. Post-hoc tests revealed that these results were driven by the differences between andromorphs and males, and andromorphs and gynomorphs, but not males and gynomorphs (see Fig. 2-3). When I conducted the same sets of analyses (GLZ and GLM) as above, this time using the proportion of presentations resulting in successful tandems (rather than the proportion

of presentations resulting in tandem attempts of any kind) as the dependent variable, similar statistically significant patterns to those listed above were found (excluding comparisons with males).

No significant interaction between specimen type and site were found with regards to reaction time of focal males (GLM: $F_{8,232}=1.341$, $P=0.224$). A significant effect of specimen type was not detected either ($F_{2,8.17}=1.49$, $P=0.280$), but there was a significant difference in reaction time of focal males between study populations ($F_{8,323}=7.09$, $P=0.010$) in that males on average took longer to react to presented specimens at Jack's Marsh compared to all other sites (possibly as a result of higher winds during the presentation period, pers. obs.). The mean proportions of those tandem attempts (as opposed to the proportion of total presentations) ending in success per specimen were 0, 0.66 and 0.63, for males, andromorphs and gynomorphs respectively. The rate of successful tandem formation from tandem attempts did not differ significantly between the two female morphs, even when accounting for site and a morph*site interaction (all of which proved non-significant, not shown).

Figure 2-3. The mean proportion of tandem attempts (± 1 SE), which focal males made towards the three specimen types (scored out of a maximum number of 5 presentations, each to different males) at each site (the first 3 sites are in Ontario, the last 2 sites are in British Columbia): gynomorph (G), andromorph (A), male (M). The sites are arranged in increasing andromorph frequency from left to right. Lowercase letters indicate significant statistical differences ($P < 0.05$) in the mean proportion of tandem attempts among specimen types based on Tukey HSD for within site analyses.

Evaluating LMR and MM hypotheses

While I cannot rule out a potential role for other geographically-varying factors, the highly significant site*specimen type interaction in the analysis of presentations resulting in tandem attempts in the above analyses (Fig. 2-3), may well be mediated by the variation in morph frequency and/or mimic to model ratio, which vary significantly among my sample populations (see Table 2-1 legend). As predicted by both LMR (Fig. 2-1a) and MM (Fig. 2-1c) and, the proportion of tandem attempts on presented andromorphs were found to increase as the proportion of females that were andromorphs in a population increased ($r^2=0.27$; regression ANOVA $F_{1,80}=28.79$, $P<0.001$; Fig. 2-4a) and as the ratio of andromorphs to males increased ($r^2=0.26$; $F_{1,80}=28.31$, $P<0.001$; Fig. 2-4c). As predicted by the LMR (Fig. 2-1b) hypothesis (but contrary to the MM hypothesis; Fig. 2-1d), the proportion of tandem attempts on presented gynomorphs declined significantly with andromorph frequency ($r^2=0.13$; $F_{1,81}=12.51$; $P=0.001$; Fig. 2-4b) and a similar effect was seen when I regressed the proportion of tandem attempts on gynomorphs with andromorph to male ratio ($r^2=0.14$; $F_{1,81}=12.94$, $P=0.001$; Fig. 2-4d). However, as predicted by the MM (Fig. 2-1e) hypothesis, the proportion of tandem attempts on males was also found to be positively correlated with the ratio of mimic to male ($r^2=0.09$; $F_{1,80}=7.49$, $P=0.008$; Fig. 2-4e).

An ANCOVA allowing for unequal gradients revealed that there was no significant interaction between morph frequency (covariate) and specimen type (male or andromorph) ($F_{4,154}=1.26$, $P=0.288$), so that the slopes of the regression lines of male sexual responses to andromorphs and to males did not differ significantly between males and andromorphs (Fig. 2-4). In effect, this means that males consistently attempted to

mate more often with andromorphs than with males when the andromorph frequency increased and that the rate of increase of male responsiveness was similar for both specimen types.

Finally, although not part of my primary hypotheses I set out to test, I note that despite significant among population variation in densities (see Table 2-1), there were no statistically significant relationships between the proportion of tandem attempts on andromorphs, gynomorphs and males and male density over my 5 populations (response to andromorphs vs. male density: $r^2=0.0002$; $F_{1,81}=0.017$, $P=0.898$; gynomorphs vs. male density: $r^2=0.006$; $F_{1,81}=0.46$, $P=0.499$; males vs. male density: $r^2=0.005$; $F_{1,81}=0.0423$, $P=0.517$). However, when examining the relationship between morph density and proportion of tandem attempts towards that morph, I found that as morph density increased, the proportion of tandem attempts also increased (andromorphs: $r^2=0.139$; $F_{1,80}=12.893$, $P=0.001$; gynomorphs: $r^2=0.142$; $F_{1,81}=13.410$, $P<0.001$).

Figure 2-4. The mean proportion of tandem attempts (± 1 SE) on andromorphs (orange circles), gynomorphs (green triangles) and males (blue squares) and how these responses vary with andromorph frequency (± 1 SE) (2-4a and 2-4b) and the ratio of mimics to models (± 1 SE) (2-4c to 2-4e). Linear regression lines were fitted through the raw data (mean data points shown) which indicate changes in the proportion of tandem attempts on andromorphs (orange solid line), gynomorphs (green broken line), and males (blue dotted line). The upper case letters identify the sites (J) Jack's marsh, (B) Barb's marsh, (O) Otter marsh, (S) Summit lake, and (N) Nukko lake.

DISCUSSION

Frequency-dependent selection is one of the best known mechanisms driving the evolution of visible polymorphisms, and many types of interaction are known capable of creating such outcomes, ranging from social interactions (e.g. Sinervo et al. 2000) to predators that focus on common types of prey (Ruxton et al. 2004). Here I have attempted to test two related theories that have been proposed to explain the evolution of female-limited polymorphisms in damselflies. Both of these theories assume that the polymorphism is driven by some form of frequency-dependent harassment of females, but the precise nature of this frequency-dependence differs between theories. In particular, my current results show that male damselflies more readily attempt to mate with specific female phenotypes over others, but that the phenotype of preference differ among sites. Such an outcome is not surprising given that the sites differ significantly in the proportion of females that are andromorph and the ratio of andromorphs to males - both MM and LMR hypotheses explicitly predict that these factors should mediate male mating behaviour. Clearly, if males were simply attempting tandems with any individual that was in close proximity, then my results would have shown no variation in male sexual response for any specimen type independent of andromorph frequency. Moreover, the fact that specimen types were glued onto sticks clearly indicates that the decision of focal males to attack specimens was strongly influenced by the visual appearance of the specimen (although the specimens were still able to exhibit a range of behaviours, see below).

These results indicate for the first time that as andromorph frequency increases between populations, the propensity of males to attempt to mate with conspecific males tends to increase. Indeed, the readiness of males to respond sexually towards andromorphs increased at an approximately similar rate to that of the sexual response of males towards males. Such results are suggestive of a system in which males do not get any better at discriminating males from andromorphs as andromorph frequency rises, but rather their threshold for attempting to mate with male-like forms increases. This outcome is readily understood from the perspective of signal detection theory which is now widely applied to understand the evolution of mimicry (e.g. see Oaten et al. 1975; Getty 1985; Holen and Johnstone 2004; McGuire et al. 2006). Thus, if appearance distributions for males and andromorphs do not change, but the frequency of male-like individuals that are andromorph increases, then males should be more prepared to attack male-like individuals (whether they ultimately turn out to be males or andromorphs), because the underlying chances of a correct classification are higher.

In contrast to the above observations which are consistent with the MM hypothesis, I note that when gynomorphs were extremely rare, males showed extremely low propensity to attempt to mate with them, supporting one of the predictions of the LMR but not the MM hypothesis (see also Van Gossum et al. 2005a). Of the studies examining male mating behaviour in species of damselflies with polymorphic females, relatively few studies have been conducted in populations where gynomorphs are the minority female morph (Svensson and Abbott 2005; Svensson et al. 2009). Comparisons of the frequency of female morphs of *Ischnura elegans* mating with those found single in Louro, Spain (approximately 90% andromorphs), found that gynomorphs were either

approximately equally represented in the mating population (2004) or significantly over-represented (2004) – see Cordero Rivera and Sánchez-Guillén (2007). Earlier pairwise presentations of live specimens in the same population (2001, approximately 85% andromorphs) found that the percentage of males that sexually responded to the two main morphs did not differ (Cordero Rivera and Sánchez-Guillén 2007). Likewise, in separate pairwise presentation tests of female morphs of *I. elegans* to males, andromorphs were not significantly preferred over gynomorphs despite representing approximately 70% of the population (Van Gossum et al. 1999). Finally, Conrad and Pritchard (1989) could not reject the null hypothesis that males exhibited equal preference for andromorphs and gynomorphs in experimental choice tests involving female morphs of the damselfly *Argia vivida*, despite the fact that females consisted of approximately 66% andromorphs. This study therefore represents the strongest evidence to date that gynomorphs can indeed be overlooked by males when this female morph is extremely rare.

Intriguingly, in my study when andromorphs were extremely rare, they were only marginally less attractive to males than gynomorphs, indicating a strong degree of asymmetry in the relationship. Several earlier studies have found that gynomorphs were preferred over andromorphs under these conditions (e.g. Robertson 1985; Fincke 1994; Cordero et al. 1998; Miller and Fincke 1999; Andrés et al. 2002; McKee et al. 2004). The asymmetry observed in this current study may have several possible sources. In particular, andromorphs may constitute a somewhat more conspicuous target when presented to focal males than gynomorphs (Fincke et al. 2007), making them somewhat more noticeable in my experimental approach, despite their potential similarity to males.

Although I had anticipated that males might differ in their latency to attempt to mate with the three different types of specimen, I did not find any significant difference in mean reaction times. This may have arisen for a number of reasons. By definition I was restricting myself to specimens that males eventually found sexually attractive. Perhaps more importantly, environmental conditions such as wind may play an important role in influencing the times taken to sexually respond (as evidenced by a significant site effect), and the high variance in reaction times may have considerably reduced the statistical power.

As male density increases, the rate at which males encounter females increases, and the intensity of male harassment selection maintaining female polymorphism may therefore also change. Male density has previously been found to positively correlate with andromorph frequency (Van Gossum et al. 2007a), yet here I have shown that male density does not directly influence the nature of male mating attempts in *N. irene*, indicating that density-dependent changes in male behaviour are not driving the detected relationships. Nevertheless as morph density increased, male tandem attempts towards that morph were found to increase. The results from this study differ from those of Gosden and Svensson (2009) where they detected a positive relationship in *Ischnura elegans* between the rate of male tandem attempts and morph density, but only for non-andromorphs (*infuscans* and *rufescens-obsoleta*). A possible reason as to why I was able to detect a change in both morphs is that in this study the range of morph densities was greater than that of Gosden and Svensson (2009). However, while the results are indicative of some form of density-dependent response caution should be applied –

andromorph density and gynomorph density are negatively correlated and they also correlate with andromorph frequency, so it is very difficult to isolate their effects.

One might argue that offering model specimens on sticks to males is unnatural in that males do not need to search for a potential mate in my experiment. However, signal detection theory – which provides a formal basis for both the MM and LMR models (Sherratt 2001; Fincke 2004) applies to discriminative decisions post encounter, so my current design provides an entirely appropriate test of these arguments, even if the presentations were rather close range (see also Forbes et al. 1997; Fincke et al. 2007 for additional examples of direct “damsel on a stick” presentations to males). More importantly, I note that although females in my experiment were capable of some refusal behaviour, such as wing spreading and abdomen curling, they could not fly away or chase males. Such female behaviour may also play an important role in the outcome of male mate attempts. Indeed, Forbes et al. (1997) indicated that andromorphs of *N. irene* were more likely to refuse matings by chasing the male away, while gynomorphs were more likely to refuse matings with wing raises or by flying away (see also Van Gossum et al. 2001a; Sirot et al. 2003 for similar accounts for other damselfly species). Recent work has evaluated male mate preference in *N. irene* in British Columbia and Ontario using cage presentations (which allows for more flexibility in female behaviour) and indicates a similar between-population effect of andromorph frequency on male mating attempts as reported here (chapter 3).

I was able to use populations of *N. irene* that varied considerably in andromorph frequency, but the underlying cause for this cross-continental variation in andromorph frequency is still being investigated (see Iserbyt et al. 2009). One possibility is that the

system has a single equilibrium morph frequency maintained by frequency-dependent selection but that frequency-independent environmental factors (such as male density or temperature) influence where that equilibrium lies. For example, the ambient summer temperature is lower in British Columbia (see Iserbyt et al. 2009) which may favour andromorphs for thermoregulatory reasons (c.f. Forsman et al. 2002; but see Bots et al. 2007). A system with two non-trivial stable equilibria is not possible without an intermediate unstable equilibrium (Cockerham et al. 1972). Alternatively, I therefore may have a three equilibrium system (2 stable, 1 unstable) such that populations in the west have evolved to one equilibrium (rare gynomorphs), while populations in the east have evolved to another equilibrium (rare andromorphs). Finally, because of the great distance between my study populations that differed most markedly in morph frequency, I cannot rule out a role for other factors that may also indirectly have influenced male behaviour through mechanisms other than the social environment (i.e. the $A/[A+G]$ or A/M ratio's). In mitigation, I note that one of my Ontario study sites - Otter marsh, with 44% andromorphs – was within 10km of two other Ontario study sites that comprised 4% and 5% andromorphs, yet the response data for Otter marsh tended to fall intermediate between the populations with very low (4-5%) and very high (97-98%) andromorph frequency.

Overall, my findings did not conform to the specific forms of frequency-dependence predicted by the MM, but they are consistent with the LMR theory where it is able to make predictions. Nevertheless, I note that my data do exhibit some properties that are predicted by the MM model that are not explicit predictions of the LMR model, indicating that the best explanation may involve some combination of the two theories.

Previous researchers have likewise suggested that the MM and LMR may not be mutually exclusive (e.g. Sirot et al. 2003). Indeed, there are variants of both LMR and MM theories where the distinction between the theories becomes particularly subtle. For example, the most recent version of the LMR allows for the possibility that female morphs can be confused with conspecific males (and heterospecifics), due to their phenotypic similarity (Fincke 2004 page 836, paragraph 1; see also Fincke et al. 2007 page 1122, penultimate paragraph). Likewise, if one were to include opportunity costs of males mistakenly rejecting a female on encounter into the basic MM model, then one would end up with a form of frequency-dependent selection not dissimilar to that predicted by the LMR model (Staddon and Gendron 1983; Fincke 2004 page 842, paragraph 2). What one chooses to call these types of explanations is a matter of semantics, but they might be thought of as “pluralistic hypotheses” since they effectively combine elements of mimicry with other cognitive constraints. In showing that the rate of male response towards conspecific males increases with andromorph to male ratio, and the rate of male response towards gynomorphs and andromorphs increases as their relative frequency increases, the data presented here provide important support for a more pluralistic perspective. Continued study of male mating behaviour across populations that differ in morph frequencies may help to further characterise the underlying mechanistic and cognitive basis of the significant relationships I have reported. The prevalence of polymorphisms in damselflies coupled with their high abundance makes them excellent subjects for disentangling the various factors that may generate phenotypic diversity in natural populations (Van Gossum et al. 2008). Nevertheless, it is clear that we have some

way to go before a general consensus is reached as to the precise mechanisms that serve to promote and maintain the polymorphisms even in these species.

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Chapter 3

Caged male-mating behaviour experiments in populations varying dramatically in female morph frequencies a damselfly

Many hypotheses have been proposed to explain the coexistence of multiple female morphs in damselflies (Zygoptera: Odonata) which has been attributed to selection to avoid excessive male harassment (unwanted sexual interest). In damselflies, one female morph is similar in colour and pattern to conspecific males (andromorph) while the other female morph(s) looks dissimilar (gynomorphs(s)). In this study I set out to test two sets of theories that can be broadly classified as the learned mate recognition (LMR) and male mimicry (MM) hypotheses. The LMR hypothesis suggests that males learned to recognize the most common morph as a potential mate while the MM hypothesis suggests that andromorphs are functional male-mimics. Both theories propose that the polymorphism is driven by some sort of negative frequency-dependent selection however the exact source differs between the two. Using the sedge sprite, *Nehalennia irene*, I was able to address both theories directly as I evaluated male mating behaviour of males towards female morphs and conspecific males in populations that varied maximally in morph frequencies. Cage experiments were conducted with four damselflies (andromorph, gynomorph, 2 males) whose interactions and behaviours were recorded. Overall my results are inconsistent with both the LMR and the MM hypotheses but I do show evidence of frequency-dependent changes in morphs preference and an influence of female response behaviours on the outcome of mating attempts.

gynomorph. I was able to observe the mating behaviours of males towards the different specimen types to see if their responses varied between specimen types within each site. Including the second male was essential to address the male mimicry (MM) hypothesis (see chapter 2). Allowing specimens to behave relatively freely, I could examine avoidance of repeated sexual interactions by sexually aggressive males. The mating status (captured single or tandem) of females used was also known, thus allowing me to examine if unreceptive females behave in different ways when approached by sexually aggressive males. Once again, I conducted the cage experiments at six populations that varied maximally in female morph frequencies (<5% andromorphs to >95% andromorphs), providing the opportunity to test the predictions of LMR and MM and examining potential differences in response behaviours between specimen types (andromorphs, gynomorphs and males) and mating status (captured single or in a tandem).

METHODS

Study species and populations

The sedge sprite damselfly *Nehalennia irene* is distributed across Canada, in marshy or boggy areas with relatively dense vegetation and they stay close to the water's edge avoiding the open water (Walker 1953; Cannings and Stuart 1977). *N. irene* is a non-territorial damselfly and is small in size (body length 25-28mm; Cannings and Stuart, 1977). This species exhibits female-limited polymorphism with morphs classified by their body coloration and patterning. From here on, females that resemble conspecific males are referred to as andromorphs and females that look dissimilar are referred to as

gynomorphs (see Fig. 1-4). Andromorphs are similar in coloration to males with a blue-green body colour and black patterning on the dorsum of abdominal segment nine. In contrast, gynomorphs are different from males since their body colour is green-yellow and they lack the distinctive triangular patch which andromorphs and males show on segment eight (see Forbes et al. 1995 for more detailed description).

Based on estimates made during previous fieldwork (Van Gossum et al. 2007a; Iserbyt et al. 2009; chapter 2), six study sites with populations at contrasting female morph frequencies were selected (see Table 3-1 for details): two sites with high andromorph frequency (Nukko lake and Airpark marsh, both in BC), two sites with low andromorph frequency (Jack's marsh and Barb's marsh, both in ON) and two sites with intermediate andromorph frequencies (Otter marsh and Quebec marsh, in ON and QU respectively). Similar to my field season in 2007, I surveyed each site to estimate morph frequencies before the start of the mate preference experiments (see chapter 2 for surveying methodology) with the exception of Airpark marsh which was surveyed on the last day of the experiments as the previous estimates taken prior to the experiments did not include adults in the surveying sweeps (June 16, 17, 29: however there were adults found in the field on June 29, 2008 to conduct experiments). All estimates calculated excluded individuals that were not sexually mature, as my study examines mating behaviours. I sampled and conducted my experiments less than five meters from the water's edge where mating is known to occur (Forbes et al. 1995).

Andromorph frequency and ratio of mimics to models (andromorphs to males) were calculated (see chapter 2 for methodology) for each site to allow for direct comparisons of the LMR and MM hypotheses. Male density was also calculated as it was

previously found to occasionally correlate with andromorph frequency (Van Gossum et al. 2007a) and as such I have calculated male density in order to determine if it has an effect on male mating behaviours. Additionally, female morph densities were calculated to address recent findings in support of changes in male mating behaviour with female morph density (Gosden and Svensson 2009; chapter 2).

As in 2007 (chapter 2) I calculated all estimates from an OSR (operational sex ratio) near the water's edge and not from sex ratio at emergence. The cage experiments described below were part of a wider study which includes comparing the proportion of single andromorphs and gynomorphs with the proportion of these female types in tandem or copula ("natural preference experiments", Bots et al. unpublished data) and direct behavioural observations of continuously monitored focal individuals in the field (Iserbyt et al. unpublished data).

Experimental design

The experiments were conducted during the reproductive season of 2008 (mid June to late July). Male mating behaviour experiments were carried out by placing four mature live specimen types (one andromorph, one gynomorph and two males) into a cage (internal dimensions 30 x 30 x 30cm BugDorm-4030 Insect Rearing Cage <http://bugdorm.megaview.com.tw>: Fig. 3-1). The materials of the cage consisted of clear vinyl on the front (for viewing) with an 18cm mesh sleeve opening, a white opaque panel for the top panel and the other four sides were of fine polyester insect netting (150x150 mesh). The cage was orientated in such a manner with the opaque panel on the top to provide some shade from direct sunlight. All mate preference experiments were

conducted between 9am and 4pm local time, on days with suitable weather conditions (sunny without precipitation and strong winds) when *N. irene* was reproductively active.

A pair of females, one andromorph and one gynomorph, were placed into the cage and allowed to acclimate for two minutes. Female pairs were matched for mating status (i.e. single/tandem/copula/unknown status caught in sweep nets) while all males used were found single and patrolling in the vegetation for mates (to ensure as far as possible that males used would be sexually interested in the females in the cage). Each pair of males used were marked with a permanent pen (Staedtler® Lumocolor Special) with either one or two small dots on a single wing for identification purposes. After the females had acclimated to the cage (2 minutes), the pair of marked males were then placed into the cage (total of four individuals at a time). Observations were then made continually up until the point that one of the males formed a successful tandem, or until 20 minutes were over. A single trial was completed when the same pair of females (andromorph and gynomorph) were presented to 3 separate pairs of unique males (a total of six males – each time taken directly from the field before the start of the experiment; see Fig. 3-2). A minimum of 19 full trials and a maximum of 24 full trials were carried out at each study population resulting in a maximum of 72 preference tests per site (i.e. 24 pairs of females x 3 pair of males; see Table 3-1 for the number of presentation trials per site). Trials where at least one cage (pair of males) ended in a successful tandem were included in the analyses (Table 3-1). Using this criterion, an overall total of 14 trials were excluded from the analyses (no successful tandems): Jack's marsh = 2 trials, Barb's marsh = 5 trials, Otter marsh = 1 trial, Quebec marsh, = 1 trial, Airpark marsh = 1 trial, Nukko lake = 4 trials.

Figure 3-1. A schematic drawing of the cage used. The internal dimensions are 30x30x30cm and the external dimensions are 32.5x32.5x32.5cm. The front panel with the mesh sleeve is a clear vinyl, the top panel is an opaque white vinyl, while the other four sides are made of fine insect netting (150x150 mesh).

Figure 3-2. An illustration of the experimental setup where a presentation consists of four damselflies in a cage: one andromorph (orange damselfly) one gynomorph (green damselfly) and two males (blue damselfly: one marked with two dots and the other marked with one dot on its wing). A trial consists of three presentations where the same pair of females are introduced to three different pairs of males. Each trial a different pair of females were used (i.e. in the next trial the females used would be A2 and G2). Note that the illustration is not drawn to scale.

Table 3-1. Population details and frequency estimates (± 1 SE) for the six populations studied during the reproductive season of 2008. The first three sites are in Ontario, the fourth site is in Quebec, and the last two sites are in British Columbia, Canada. A refers to andromorphs, G to gynomorphs, and M to males. The number of cage trials includes those where at least one cage presentation ended in a successful tandem. An arithmetic mean was calculated for andromorph frequency, mimics: models, overall population density, overall specimen density, and the operational sex ratio (OSR) and from the values calculated from the multiple sweeps within a survey, the expected standard errors were calculated.

Variables	Site	Jack's marsh	Barb's marsh	Otter marsh	Quebec marsh	Airpark marsh	Nukko lake
Latitude, longitude		44°34'00", -76°21'00"	44°31'30", -76°22'30"	44°32'30", -76°22'00"	46°46'30", -70°58'00"	54°01'06", -123°03'33"	54°04'30", -122°59'30"
Cages dates (mm/dd)		07/02 –07/07	06/24 –07/01	07/07 –07/12	07/15 –07/19	06/29 –07/13	07/02 –07/12
Cages <i>n</i> (# of trials)		19	18	18	19	19	20
Cages <i>n</i> (# of A/G/M)		19/19/114	18/18/108	18/18/108	19/19/114	19/19/112	20/20/120
Survey date (mm/dd)		07/02	06/24	07/07	07/15	07/13	07/01
Survey <i>n</i> (# of A/G/M)		1/62/168	2/86/245	14/53/197	24/13/94	36/3/83	66/2/79
Andromorph frequency A/(A+G)		0.008 $\pm$ 0.008	0.059 $\pm$ 0.053	0.237 $\pm$ 0.051	0.682 $\pm$ 0.116	0.949 $\pm$ 0.027	0.981 $\pm$ 0.019
Mimics: models A/M		0.004 $\pm$ 0.004	0.019 $\pm$ 0.017	0.070 $\pm$ 0.012	0.302 $\pm$ 0.071	0.424 $\pm$ 0.066	0.838 $\pm$ 0.149
Overall population density (total/min)		23.43 $\pm$ 0.96	13.86 $\pm$ 1.21	13.01 $\pm$ 2.38	6.92 $\pm$ 0.96	10.96 $\pm$ 1.78	17.50 $\pm$ 2.74
Overall specimen density A/G/M (total/min)		0.09 $\pm$ 0.09 6.10 $\pm$ 0.81 17.24 $\pm$ 2.45	0.16 $\pm$ 0.13 3.66 $\pm$ 0.64 10.06 $\pm$ 1.04	0.70 $\pm$ 0.17 2.64 $\pm$ 0.80 9.67 $\pm$ 1.66	1.44 $\pm$ 0.33 0.54 $\pm$ 0.14 4.95 $\pm$ 0.94	3.37 $\pm$ 0.73 0.36 $\pm$ 0.23 7.23 $\pm$ 0.94	7.93 $\pm$ 1.82 0.20 $\pm$ 0.20 9.36 $\pm$ 1.09
OSR (male: female)		2.84 $\pm$ 0.27	3.29 $\pm$ 0.36	4.52 $\pm$ 1.09	2.56 $\pm$ 0.37	2.67 $\pm$ 0.78	1.42 $\pm$ 0.26

Table 3-2. A glossary of common terminologies used along with a sample calculation. Abbreviations of the specimen types used are: andromorph (A), gynomorph (G) and male (M)

Terminology	Definition	Calculation
Andromorph frequency	The proportion females in the population that are andromorphs	Arithmetic mean: $=A/(A+G)$ per sweep; than a single value for each site was calculated as an average was taken from x sweeps
Mimics: models	The ratio of mimics to models in the population	Arithmetic mean: $=A/M$ per sweep; than a single value for each site was calculated as an average was taken from x sweeps
Presentation	Two different males are introduced to an A and a G in a cage for a maximum of 20 min	
Trial	Maximum of three presentations using the same A and G but a different pair of males each time (6 males)	
Successful tandem attempts (Ts)	When a male successfully grasps onto a female's prothorax with his anal appendages. Males cannot successfully form tandems with conspecific males.	Geometric mean: <i>Proportion of successful tandems</i> for A (per trial) = # of Ts with A in a trial / total # of Ts in a trial
Tandem attempts (Ta)	Includes unsuccessful and successful tandem attempts. Males are included in this calculation	Arithmetic mean: <i>Proportion of tandem attempts</i> for A (per trial) = # of Ta with A in a presentation / total # of Ta in a presentation; then a single value for each trial was calculated as an average was taken from the 3 [maximum] presentations
Response behaviour	A behaviour in response to a male (fly away, wing movements, body movements, offensive behaviours)	Geometric mean: <i>Proportion of response behaviour</i> ('wing' movements by A) = # of wing by A in a trial / total # of response behaviours by A in a trial

All interaction behaviours within each cage presentation were recorded, including who instigated and received the behaviour (see below) and the trial was terminated after 20 minutes or when a successful tandem was formed. The behaviours exhibited by the instigator (almost invariably a male) were categorised as follows: approach, contact (brief), sit (long contact), chase, face-off, unsuccessful tandem attempt (the male attempts to grasp the specimen with his anal appendages but fails), successful tandem attempt (the male's anal appendages successfully grasps onto a female's prothorax). Males are only able to form tandems successfully with females and not with other males (see chapter 2). The response(s) of the receiver (male or female) was recorded as follows: no response, wing flutter (wings opening and closing quickly, wing spread (wings held open), abdominal curl, fly away, face off, charge/chase, or shake (body shake). I grouped response behaviours as follows: wing movement = wing flutter/spread, body movement = abdomen curl/body shake, offensive behaviour = face off/chase/charge, and fly away. These combined classifications of responses were created (along *a priori* rational lines) as it turned out that many of the response behaviours were infrequent.

Statistical analysis

For all mate preference trials, a single frequency score of the response *tandem attempt* (see Table 3-2) was calculated (see below for further details) for each specimen type (andromorph, gynomorph, male) based on the combined data from three cage presentations (*trial*; see Table 3-2) which used the same females but a unique pair of males each time. Hence trial was treated as the replicate. All analyses were conducted using SPSS® v.15.0. Following normality and homogeneity tests, I began the analysis on

the *proportion of tandem attempts* (both successful and unsuccessful for all specimen types: andromorph, gynomorph, male) in the cage experiments (see Table 3-1 for a sample calculation). A total of 16 trials had only two presentations as in 15 of the trials one cage had no activity during the 20 minutes of observations (Jacks: 2, Barbs: 6, Otter: 4, Quebec: 3) while a single trial at Airpark marsh excluded the third presentation as the gynomorph died. I began by fitting separate GLMs to the *proportion of tandem attempts* on each specimen type and treated andromorph frequency (and subsequently the ratio of mimics to models [andromorphs/males] and morph density [individuals captured/minute]) as a covariate, to see if the proportion of tandem attempts changed with an increase in andromorph frequency (or the ratio of putative mimics to models or morph density). The proportion of tandem attempts on each specimen type from each site were also analysed to determine if the mean *proportion of tandem attempts* towards each specimen type (andromorph, gynomorph, male) occurred more often than chance alone (a one sample t-test with the population mean test value as 0.333). Clearly the *proportion of tandem attempts* towards the specimen types within a trial are not independent of one another as when a male interacts with another male then both males are occupied and unable to interact with the female morphs.

I then focused on the *proportion of successful tandems* (see Table 3-2 for sample calculation) with each of the female morphs (necessarily excluding males from the analysis as males cannot form successful tandems with other males) and how such metrics vary with population parameters. Here I report only the *proportion of successful tandems* towards andromorphs as the results for gynomorphs would simply be the converse. Thus, a general linear model was applied to test whether the rate of successful

tandems with andromorphs covaried with andromorph frequency. A similar analysis was conducted where the covariate was the ratio of mimics to models (andromorphs to males) and morph density (individuals captured per minutes).

Prior encounters

Males that attempt tandems with conspecific males may exhibit a preference for male-like individuals (andromorphs or males) such that these males are more likely to subsequently form a successful tandem with an andromorph. In order to investigate any influence of prior encounters of a focal male in the experiment I selected males in all presentations (males used in each presentation were different [unique] and thus considered independent for this analysis) which met the following criteria: 1) a successful tandem was formed, 2) the male that formed the successful tandem had >1 interactions and 3) one of the interactions was a tandem attempt. I recorded the first tandem attempt that occurred and categorized it by sex (male or female) and I recorded the female morph (andromorph or gynomorph) that that particular male eventually formed a successful tandem with. With this data a 2x2 contingency table was created and a Fisher's Exact test was applied to test the Null Hypothesis that the female morph a male ended up forming tandem with was independent of the sex the male initially attempted a tandem with.

Response behaviours

Using the data collected from the cage experiments I set out to examine if there were differences in response behaviour of andromorphs, gynomorphs and males (wing movement, body movement, offensive behaviour, and fly away). I did not analyse differences across sites as there were multiple observers and thus the possibility of observer bias confounding the results. For each specimen type (andromorph, gynomorph, male) used in a trial, I calculated a single frequency score for each behaviour in response to a male in each trial (see *proportion of response behaviours* in Table 3-1 for a sample calculation). In order to test if specimen types (andromorphs, gynomorphs, males) differ in the proportion of behaviours performed (fly away, wing movements, body movements, offensive behaviours) in response to a male, I conducted separate GLMs for each response behaviour where specimen type (andromorph, gynomorph, male) was treated as a fixed factor. Additionally a Tukey's *post hoc* test was conducted for response behaviours that were found to differ significantly between specimen types to determine which relationships were driving the results.

Additionally, it has been previously suggested that female response behaviours may indicate non-receptivity (Forbes et al. 1997). I tested to see if mating status of females (captured as single or in a tandem) differed in the *proportion of response behaviours* performed in response to males by conducting a GLM for each response behaviour (dependent variable) and I treated female mating status and female morph as fixed factors. Females captured as copula or with an unknown status were excluded from the analyses as they were rare occurrences (copula: 1 trial; unknown: 4 trials).

Effectiveness of response behaviours

Although not part of my primary questions, I tested the effectiveness of response behaviours (all grouped together) to see if a female responding to a tandem attempt by a male would influence the success of the tandem. For each trial I looked at all tandem attempts and categorized them as successful or not, then within each category I calculated the frequency of the presence or absence of any response behaviour. With this data I created a 2x2 contingency table and applied a Fisher's Exact test to test the Null Hypothesis that a response behaviour from a female will not influence the subsequent outcome of a tandem attempt. This was done for females grouped together and for female morphs (andromorphs and gynomorphs) separated.

RESULTS

Male mate response behaviour

When looking at tandem attempts (successful and unsuccessful) I found that the proportion of tandem attempts towards andromorphs had a slight, non significant, tendency to increase with andromorph frequency (GLM: $F_{1,122}=0.992$, $p=0.321$; Fig. 3-3a) and no change in the proportion of tandem attempts on andromorphs was detected with an increase in the ratio of mimics to models ($F_{1,122}=0.033$, $p=0.856$; Fig. 3-3d). The proportion of tandem attempts towards gynomorphs was found to decrease significantly with increasing andromorph frequency ($F_{1,122}=5.869$, $p=0.017$; Fig. 3-3b) and with the ratio of mimics to models ($F_{1,122}=6.029$, $p=0.015$; Fig. 3-3e). As andromorph frequency increased, the proportion of male tandem attempts towards males did not change significantly but had a tendency to increase ($F_{1,122}=2.396$, $p=0.124$; Fig. 3-3c).

Nevertheless as the ratio of mimics to models increased the propensity for a male to attempt a tandem with a conspecific male was found to significantly increase ($F_{1,122}=6.131, p=0.015$; Fig. 3-3f).

Like chapter 2, I found that the mean proportion of successful tandems that males made with andromorphs was found to increase with an increase in andromorph frequency (GLM: $F_{1,111}=7.216, p=0.008$; Fig. 3-4a), and an increase in the ratio of mimics to males ($F_{1,111}=7.550, p=0.007$; Fig. 3-4b). By definition, the proportion of successful tandem attempts with gynomorphs shows the reverse relationship and is not independent.

When exploring the proportion of tandem attempts at each site separately, there are few discernable patterns of preference across the sites or even within each site (Fig. 3-5). Males at Quebec marsh were found to attempt tandems with males significantly less than 1/3 ($t=-2.449, df=19, p=0.024$). At Airpark marsh, males were found to attempt tandems with gynomorphs less than expected ($t=-2.381, df=19, p=0.028$) and towards andromorphs the rate of interaction tended to be more than expected, ($t=1.831, df=19, p=0.083$). Males at Nukko lake attempted tandems with conspecific males significantly more than expected ($t=2.088, df=19, p=0.048$) and tended to attempt tandems with gynomorphs less than 1/3 ($t=-1.834, df=19, p=0.080$).

Figure 3-3. The relationship between andromorph frequency (3-3a to 3-3c) and the ratio of mimics to models (3-3d to 3-3f) of the mean proportion of tandem attempts (± 1 SE) on andromorphs (orange circles: 3-3a and 3-3d), gynomorphs (green triangles: 3-3b and 3-3e), and males (blue squares: 3-3c and 3-3f). Linear regression lines were fitted to the raw data: andromorphs (orange solid line), gynomorphs (green dashed line), and males (blue dotted line) and p-values indicate level of significance ($p < 0.05$). Upper case letters identify the sites (J) Jack's marsh, (B) Barb's marsh, (O) Otter marsh, (A) Airpark marsh, and (N) Nukko lake.

Figure 3-4. The mean proportion of successful tandems (± 1 SE) with an andromorph plotted against andromorph frequency (3-4a) and the ratio of mimics to models (3-4b). The letters identify the sites (J) Jack's marsh, (B) Barb's marsh, (O) Otter marsh, (Q) Quebec marsh, (A) Airpark marsh, and (N) Nukko lake. A linear regression line was fitted to the raw data (solid line).

Figure 3-5. The mean proportion of tandem attempts (unsuccessful and successful) (± 1 SE), which males made towards the three specimen types (gynomorph (G), andromorph (A), male (M)) at each site. The sites are arranged in increasing andromorph frequency (% A) from left to right. The asterisks (*) indicate a significant difference ($p < 0.05$) of the mean proportion of tandem attempts towards each specimen type differing from the probability of occurring by chance alone ($0.333 [1/3]$), indicated with the dotted line.

Although not part of my primary hypotheses I explored the possible relationship between male density and male mating behaviour. However I found no statistically significant relationships between the proportion of tandem attempts on andromorphs, gynomorphs and males and male density over my 6 populations (response to andromorphs vs. male density : $r^2=0.022$, $F_{1,122}=2.786$, $p=0.098$; gynomorphs vs. male density: $r^2=0.009$, $F_{1,122}=1.151$, $p=0.285$; males vs. male density: $r^2=0.003$, $F_{1,122}=0.327$, $p=0.569$). When examining the relationship between morph density and proportion of tandem attempts towards that morph, I found that as gynomorph density increased, the proportion of tandem attempts towards gynomorphs also increased ($r^2=0.035$, $F_{1,122}=4.454$, $p=0.037$), however this was not found to be true in andromorphs ($r^2=0.000$, $F_{1,122}=0.010$, $p=0.922$).

Successful tandems vs. tandem attempts

As noted above, while the proportion of successful tandems with andromorphs increases, the proportion of total tandem attempts towards andromorphs does not change with an increase in andromorph frequency or the ratio of mimics to models. I must address why there is a discrepancy in andromorphs, since successful tandems are a subset of all tandems. Other than failure to reject a false NH, there are two obvious explanations. 1. as andromorph frequency increases it becomes easier to mate with an andromorph or 2. lacking independence (a male attempting tandem with one type is necessarily not attempting tandem with other types), the results are driven indirectly by a change in responses towards gynomorphs and males. My first hypothesis, that fewer attempts are required for a male to successfully form a tandem with andromorphs as andromorph

frequency rises was tested as follows; within a trial for every successful tandem per female morph, the ease of forming a tandem was evaluated by calculating the average number of attempts made by the successful male. There was no female morph effect (GLM: $F_{1,5,016}=0.331, p=0.590$), no site effect ($F_{5,5}=2.153, p=0.210$) or interactive effect ($F_{5,214}=0.674, p=0.643$). So it does not appear that the morphs differ in their ease of forming a successful tandem. To address my second hypothesis, in order to see if the change in gynomorphs influenced the results in andromorphs, I excluded males from the calculations of proportion of tandem attempts (thus altering the denominator) to allow for a direct comparison between andromorphs and gynomorphs. I found that there was no change in the proportion of tandem attempts towards andromorphs or gynomorphs as andromorph frequency (GLM: $F_{1,116}=0.466, p=0.496$) or ratio of mimics to models increased (GLM: $F_{1,116}=1.423, p=0.235$). It does not appear that the results seen in andromorphs are driven by a change in gynomorphs leaving a two other possibility, type II error, where we falsely accept the null hypothesis, or perhaps it can be attributed to the experimental design.

Prior encounters

Of the 375 possible presentations, 111 presentations were excluded as they did not end in a successful tandem and 173 did not have the male attempt a tandem prior to succeeding, leaving 91 presentations to be included in the analyses below. The first specimen which a focal male attempted a tandem with (male or female) did not influence the female morph (andromorph or gynomorph) in which a successful tandem was formed (Pearson Chi-Square: $\chi^2=0.026, df=1, p=0.871$) when all sites were grouped together. A total of 67

focal males were found to have the first tandem attempt be with another male and 32 (expected = 31.7) of those focal males subsequently formed a tandem with a gynomorph and 35 (expected = 35.3) with an andromorph. There were 24 focal males that attempted their first tandem with a female and 11 (expected = 11.3) focal males that successfully formed a tandem with a gynomorph and 13 (expected = 12.7) with an andromorph. I do not present the non-significant results for sites analysed separately as the sample size for each site was quite low (Jack's: $n=21$, Barb's: $n=10$, Otter: $n=16$, Quebec: $n=7$, Airpark: $n=19$, Nukko: $n=18$).

Response Behaviours

Specimen types were found to differ in their proportion of flying away in response to a male (GLM: $F_{2, 306}=4.867$, $p=0.008$; Fig. 3-6). Males were found to fly away significantly more than andromorphs ($p=0.007$), but not gynomorphs ($p=0.099$), while andromorphs and gynomorphs were not found to differ ($p=0.583$). The proportion of interactions with males resulting in wing movements were not found to differ significantly between specimen type ($F_{2, 306}=0.399$, $p=0.671$; Fig. 3-6). Body movements as a response to males was found to have a specimen effect ($F_{2, 306}=3.556$, $p=0.030$; Fig. 3-6). It was revealed with a Tukey's *post-hoc* test (Fig. 3-6) that females were found to respond to conspecific males with body movements more than males (males vs. gynomorph: $p=0.043$, males vs. andromorphs: $p=0.079$), however female morphs were not found to differ ($p=0.971$). Offensive behaviours were not found to differ significantly between specimen type ($F_{2, 306}=0.791$, $p=0.454$; Fig. 3-6).

A total of 80 females were captured as a single while 114 were caught in tandem. The only response behaviour to males found to be influenced by female mating status was body movements (GLM: status: $F_{1, 192}=7.840, p=0.006$, see Fig. 3-7) where single females responded to males with body movements more often than males. A female morph effect and an interactive effect were not detected (morph: $F_{1, 192}=0.064, p=0.800$; interaction: $F_{1, 192}=2.470, p=0.118$). The other behaviours were not found to have a significant female status effect, female morph effect or an interactive effect (Fly away: status: $F_{1, 192}=0.944, p=0.333$; morph: $F_{1, 192}=1.753, p=0.187$; interaction: $F_{1, 192}=0.064, p=0.801$; Wing movements: status: $F_{1, 192}=2.645, p=0.106$; morph: $F_{1, 192}=0.475, p=0.492$; interaction: $F_{1, 192}=0.552, p=0.458$; Offensive behaviours: status: $F_{1, 192}=0.650, p=0.421$; morph: $F_{1, 192}=0.588, p=0.444$; interaction: $F_{1, 192}=2.950, p=0.088$).

Body movements are comprised of abdomen bends and body shakes where a total of 274 abdomen bends and 70 body shakes were recorded for all females. Thus I predict that it is abdomen bends that are driving the significant differences seen between single females and tandem females (see above). In order to confirm the prediction I reanalyzed the body movements data by separating out abdomen bends and body shakes. Similar results were found with body movements (see above) for abdomen bends (status: $F_{1, 190}=6.669, p=0.011$; morph: $F_{1, 190}=0.430, p=0.513$; interaction: $F_{1, 190}=0.174, p=0.677$) but different non-significant results for body shake (status: $F_{1, 190}=0.547, p=0.449$; morph: $F_{1, 190}=0.196, p=0.659$; interaction: $F_{1, 190}=2.777, p=0.097$). These results confirm that single females and tandem females differ in their propensity to respond to males with body movements, specifically abdomen bends.

Response behaviour

Figure 3-6. Error bars with the mean proportion (± 1 SE) of response behaviours (fly away, wing movements, body movements, offensive behaviours) of each specimen type (G=gynomorph, A=andromorph, M=male) in response to a conspecific male across all sites. Lowercase letters indicate significant statistical differences ($p < 0.05$) in the mean proportion of response behaviours among specimen types based on Tukey HSD.

Figure 3-7. Error bars with the mean proportion (± 1 SE) of response behaviours (fly away, wing movements, body movements, offensive behaviours) for females with a mating status of captured either as a single or in a tandem. Lower case letters indicates a significant difference ($p < 0.05$) in the proportion of the behaviour performed in response to a male between the two female mating statuses.

Effectiveness of response behaviours

When examining the effectiveness of refusal behaviours (grouped together) against male harassment, a refusal behaviour exhibited by a female was found to influence the success of a tandem attempt (Pearson Chi-Square: $\chi^2=83.624$, $df=1$, $p<0.001$) when all sites and female types were grouped together (Fig. 3-8). Similar results were seen when looking at gynomorphs separately ($\chi^2=25.622$, $df=1$, $p<0.001$) and andromorphs separately ($\chi^2=60.904$, $df=1$, $p<0.001$). Females that refused a male's tandem attempt were found to increase the likelihood of the tandem being unsuccessful more than expected ($n=64$: gynomorph [G]=25, andromorph [A]=39). While females that did not refuse an attempt but the male still did not succeed in forming a tandem was found to occur lower than expected ($n=14$: G=8, A=6). Females that did refuse a tandem, but the male was still able to successfully form a tandem was found to occur less than expected ($n=656$: G=35, A=30). The occurrence of males succeeding in forming a tandem with females that did not refuse was found occur more than expected ($n=199$: G=91, A=108).

Figure 3-8. The frequency of events where a female responded (R= response, No R = no response) to a tandem attempt by a male, and the subsequent success of the male's attempt (Ts= successful tandem, Ta = unsuccessful tandem). The black bars represent the observed values and the grey bars represent the expected values.

DISCUSSION

This study was conducted to complement my previous work (chapter 2). In both studies I set out to test two proposed theories that attempt to explain the evolution of female-limited polymorphism in damselflies, but using two different experimental methods. In the two theories being tested (LMR and MM), both assume that the polymorphism is driven by male harassment and is maintained through frequency dependent selection where the precise mechanism differs between the two theories. My current results fail to statistically support either of the proposed hypotheses, LMR or MM. That said, I still uncovered significant elements of frequency-dependence. Moreover, there was some evidence that response behaviours differ between specimen types. Interestingly I found that response behaviours from females, regardless of female morph, may be communicating their physiological state to males which I was unable to address in chapter 2 as specimens were physically restricted in the types of response behaviours they could perform.

In chapter 2 I was able to provide empirical evidence in support of the LMR hypothesis where as andromorph frequency increased the propensity of a male to attempt a tandem towards andromorphs increased and towards gynomorphs decreased. Here I was unable to support either the LMR or the MM hypothesis even though I show that males increased their propensity to successfully form a tandem with andromorphs as andromorph frequency increased. The discrepancy that prevented me from supporting either of the proposed theories was the non-significant trend of an increase in a male's propensity to attempt a tandem with andromorphs as andromorph frequency increased (Fig. 3-3a). However, consistent with my findings in chapter 2 is the decrease in tandem

attempts towards gynomorphs as andromorph frequency increased (Fig. 3-3b: supporting LMR) and increase towards males as the ratio of mimics to models increase (Fig. 3-3f: supporting MM). Likewise there is an increase in the proportion of successful tandems with andromorphs as andromorph frequency increased.

Contrary to chapter 2 I do not see a strong distinctive pattern in the propensity of males to attempt tandems on specific specimen types in my six sites (Fig. 3-5). There does appear to be a trend in gynomorphs where they are attacked more than expected in Ontario and Quebec (lower andromorph frequency sites) and less than expected in British Columbia (high andromorph frequency sites). This would corroborate the predictions of the LMR. Nevertheless, I note that as the ratio of andromorphs to males increases, then so too does the proportion of tandem attempts on males. Although LMR makes no direct prediction with respect to male-male interactions, on the basis that males can be confused with any number of “distractors” (Fincke et al. 2005), it stands to reason that an excellent candidate “distractor” in this instance is the andromorph – not only because they look alike, but also because the tendency to attempt to mate with the males increases with the andromorph frequency.

Male density has previously been found to positively correlate with andromorph frequency (Van Gossum et al. 2007a), but I have shown in two separate studies (chapter 2; chapter 3) that there is no relationship between male density and male mating attempts in *N. irene*, such that density has no influence on male mating behaviour. Similar to Gosden and Svensson (2009), but different from chapter 2, I found that as morph density increased, male tandem attempts towards that morph were found to increase in gynomorphs, but not in andromorphs. However, caution must be applied when

interpreting these results as andromorph density and gynomorph density are negatively correlated across my populations and they also correlate with andromorph frequency, so it is very difficult to isolate their effects.

I set out to see if one could predict a male's preference (female morph a male forms a successful tandem with) by the sex of the damselfly he previously attempted his first tandem on. I was unable to reject my NH of no association and therefore unable to predict the female morph in which a male would successfully form a tandem with from a male's first tandem attempt.

Male *N. irene* are non-territorial and thus utilise a scramble mating tactic (Cannings and Stuart 1977), where males rush at females near the water's edge (male-female interactions are observed to occur here; Forbes et al. 1995) and attempt to grasp a female's prothorax with their anal appendages (Robertson 1985; Corbet 1999). Single females captured near the water's edge (mating site) are interpreted to be females that have recently mated/oviposited and flying away from the mating site to replenish her egg supply. While females captured in tandem at mating sites are assumed to be physiologically ready to mate and have yet to copulate. In this study I show that females (independent of morph) captured as a single were more often observed to respond to a male with a body movement (abdomen bend or body shake) compared to females captured in tandem.

Body movements were observed to be more of a female behaviour than a male behaviour (Fig. 3-6), although andromorphs and gynomorphs did not differ significantly in the frequencies of their responses. I suspect that females are signalling, with an abdomen bend, to males their physiological state potentially providing an honest signal of

their lack of mature eggs. When females are receptive and possess numerous mature eggs their abdomens are swollen in comparison to females that have few or lack mature eggs completely (see Gosden and Svensson 2009; *N. irene*: Iserbyt et al. unpublished data). It is thought that females with swollen abdomens are unable to bend their abdomens (Fincke 1987) and thus a female that can perform this behaviour is able to inform males that her current physiological state is not an efficient or effective way to increase a male's fitness. Males were observed more frequently than females to respond to conspecific males by flying away (Fig. 3-6), however the size of the cage prevented the behaviour from being effective in avoiding further attempts. Additionally I was able to show that a response behaviour (of any type: fly away, wing movement, body movement, or offensive behaviour) of females (independent of morph) towards males is effective in preventing a sexually aggressive male from forming a successful tandem (Fig. 3-8) and thus appropriately classified as refusal behaviours.

The cage method utilised in this study was employed in order to create a slightly more natural setting, compared to the stick presentations of chapter 2, to allow any potential behavioural differences between morphs to surface and potentially influence male mating behaviour in damselflies. I clearly show that andromorphs and gynomorphs do not differ in their refusal behaviour however refusal behaviours are effective in preventing a tandem from succeeding. In figure 3-9 a direct comparison can be made among the four sites that were used in both the sticks (chapter 2) and cage (chapter 3) experiments. It can be seen that the propensity of tandem attempts in the cage experiments were generally similar to that of the stick experiments. An obvious difference is the proportion of tandem attempts towards andromorphs at Nukko lake as it

is much greater in the sticks than in the cages (Fig. 3-9). A more direct comparison to the sticks would have been to introduce a male to one specimen at a time in a cage but with the addition of males interacting with conspecific males.

With regards to which method is more representative of what occurs naturally in these populations, I suspect the cage experiment provides more information about male-mating preference (i.e. successful tandem) while the stick experiments are more informative about male harassment (i.e. tandem attempts). Both methods, males are using short range detections where in the cages males are basing their decisions on both visual cues and behavioural cues which are likely to be at least some, if not all of the cues that they are using in the field to identify potential mates. In the cage experiments, males have a genuine choice among three specimen types (andromorph, gynomorph, male) with the assumption that they are aware of the presence of all other individuals. Whereas in the stick method males are not given a choice of specimen type, their only choice is to attack or not. In the sticks males were given one specimen at a time, which increased the likelihood that the male recognized the presence of the specimen and that his decision to attack was based on the visual cues from that particular specimen type. Although males had a choice in the cages, these choices were not independent while the choice to attack or not in the sticks was independent. Perhaps the lack of effect in the propensity of tandem attempts towards andromorphs was hidden by the non-independent interactions in the cage.

I found that response behaviours, of any type, by females decreases the success of a tandem attempt, however it was not found to differ between female morphs (andromorphs, gynomorphs). Even though the cage method appears to more

representative of natural occurrences, it does not appear to be necessary nor as direct about male harassment in damselflies. I believe that the stick experiments are more informative of male harassment (tandem attempts) as the behaviour of the specimen can be controlled as well as which specimen is being presented as well as eliminating male-male competition. Thus, male harassment can be measured on each specimen type independent of other confounding variables (i.e. which specimen type would a male select if there was no influence from the specimen itself). The stick experiments were less intrusive as the focal males were undisturbed in their natural habitat while in the cage experiments all specimens were introduced into a foreign and unnatural setting.

Figure 3-9. A comparison of the mean proportion of tandem attempts (unsuccessful and successful) (± 1 SE), which males made towards the three specimen types (gynomorph (G), andromorph (A), male (M)) at the four sites used in both the cage and sticks experiments. The sites are arranged in increasing andromorph frequency (% A) from left to right. The top row are results from the cage experiments where the asterisks (*) indicate a significant difference ($p < 0.05$) of the mean proportion of tandem attempts towards each specimen type differing from the probability of occurring by chance alone (0.333 [1/3]), indicated with the dotted line. While the bottom row are results from the stick experiments where lower case letters indicate significant statistical differences ($p < 0.05$) in the mean proportion of tandem attempts among specimen types based on Tukey HSD for within site analyses. Only one site, Nukko lake is located in British Columbia, while the other three sites are located in Ontario.

As mentioned previously, the work included in this thesis is part of a multiyear broad-scale project on *Nehalennia irene*. Colleagues have also conducted natural preference experiments (“natural”) comparing the proportion of single andromorphs and gynomorphs with the proportion of these female types in tandem or copula occurring naturally in the field (Bots et al. unpublished data) and continuous direct behavioural observations of focal individuals (“focal”) in the field (Iserbyt et al. unpublished data). The “natural” and “focal” field observations are methods that are probably more representative of the natural tandem endpoint in the field as specimens were not confined, disturbed or manipulated. Preliminary analyses of the data collected suggest that there is frequency-independent male preference for gynomorphs over andromorphs and this pattern does not change with andromorph frequency in the “natural” (Bots et al. unpublished data). This differs from my results from the sticks (chapter 2) and cage (chapter 3). The “natural” preference data is the outcome of both short and long range encounters but is restricted to detecting females who have eventually agreed to mating (tandem or copula), whereas my presentational work is restricted to short-distance encounters and involves harassment that does not end in copulation.

A previous study on *N. irene* utilising various methods to measure male mate preference conducted similar experiments (sticks, cages, field trials) and also found varying results (see Forbes et al. 19997). It has been suggested that perhaps activity pattern may have an influence which has been confirmed by the “focal” observations where active individuals (greater number of flights) are approached by males more frequently and this is found to be equal for all specimen types (andromorph, gynomorph, male) (Iserbyt et al. unpublished data). Additionally, preliminary results from my “focal”

observations suggest that female morphs do not differ in activity pattern, solitary microhabitat use, or in refusal displays (Iserbyt et al. unpublished data). This is contrary to results in Forbes et al. (1997) where gynomorphs were more likely to respond with wing warnings or abdomen bending than andromorphs while andromorphs were more likely to respond with chases than gynomorphs. Also contrary to the “focal” observations, I found in this chapter that male and females differed in response behaviour as well as females of different mating statuses (single/tandem) and that response behaviours were effective in preventing a successful tandem, suggesting that unreceptive females can avoid repeated interactions with sexually aggressive males.

The cline observed in morph frequency in *N. irene* is currently being studied. It is uncertain as to what factors are mediating the cline, but I cannot rule out the possibility that they may in turn explain some of the variation in male-mating behaviour I observed. I speculate that environmental factors (biotic and abiotic) may help mediate the cline. Indeed, there is preliminary evidence that ambient temperature may be one of these key factors (Iserbyt et al. 2009). Average ambient summer temperatures in BC are lower than that of ON which may also help explain the lower densities out in Western Canada. In this context, it is worth noting that there are unpublished studies on polymorphism in the Hawaiian *Megalagrion* where there is also variation in andromorph frequencies from about 0 to 100% (Cooper et al. unpublished data). There appears to be an altitudinal gradient where predominately gynomorphs are found at low altitude, and predominately andromorphs at high altitude, and intermediate frequencies in between. The variation seen in *Megalagrion* may also be influenced by ambient temperature as well, as temperatures are cooler at higher altitudes compared to lower altitudes. Additionally recent work on

Ischnura elegans found higher andromorph frequencies in populations to the north and west in the Netherlands and Belgium with lower ambient temperatures (Hammers and Van Gossum 2008). In *N. irene*, *Megalagrion*, and *I. elegans* a common trend arises that sites that are expected to be cooler in ambient temperatures are inhabited predominantly by andromorphs; however future work is required to empirically support this correlation.

I believe that cage experiments however more inclusive of natural factors influencing male-mating behaviour, is not as direct as addressing male harassment in damselflies as when using stick presentations. Response behaviours were found to be effective in refusing male sexual behaviours but there was no systematic difference between the morphs. From the cage experiments I reject both the LMR and MM hypotheses but suspect the non-independent interactions may have attributed to the weak relationships compared to the results of the stick presentations. Alternatively with hindsight, both models were a little simplistic and perhaps other variables should be incorporated. For example, I suspect that the frequency dependent selection in male preference towards gynomorphs and males may be complicated by other factors (i.e. geographical difference between BC and ON). Clearly the mating behaviours of polymorphic damselflies are influenced by numerous variables (i.e. andromorph frequency, response behaviour) and more work is required to determine the precise mechanisms maintaining polymorphisms in damselflies.

Ting JJ, Iserbyt A, Bots J, Perez-Jvostov F, Van Gossum H, Sherratt TN. Caged male-mating behaviour experiments in populations varying dramatically in female morph frequencies in a damselfly.

Chapter 4

General Conclusions

Visible polymorphism is a general phenomenon that provides evolutionary biologists a means to study the processes that promote and maintain genetic variation in nature.

Multiple morphs can co-exist when they have equal fitness at equilibrium, and this often arises as a consequence of a fitness advantage when rare (negative frequency-dependent selection). When the polymorphism is restricted to one of the sexes it can be generally explained in the context of sexual selection (although female-limited polymorphisms in Batesian mimics are a notable exception, see chapter 1). Female-limited polymorphism is particularly common in odonates, and the aim of this thesis was to test specific theories as to the selective mechanisms that are involved in the maintenance of this phenomenon in the damselfly *Nehalennia irene* (Odonata: Zygoptera).

In the first chapter I briefly summarised some of the current literature on polymorphisms, focusing on female-limited polymorphism in damselflies. I introduced the various theories that have been proposed to explain the evolution and maintenance of the polymorphism in damselflies and focused on two particular theories that have been part of an ongoing debate – the learned male recognition (LMR) and the male-mimicry (MM) hypothesis. I emphasised two main properties of these models that previous studies had failed to address adequately when testing one or the other theory. One of the shortfalls was the lack of any work comparing the tendency of males to attempt to mate with other males across a range of female morph frequencies, which is crucial in

addressing the MM hypothesis. The other shortfall is evaluating male mating behaviour in natural populations that vary maximally in andromorph frequencies, since the two theories predict marked changes in male preference for all morphs and males under slightly different forms of frequency-dependent selection.

In the second chapter I conducted field experiments quantifying male mate preference of andromorphs, gynomorphs and males in the sedge sprite, *Nehalennia irene*, working in natural populations in Ontario and British Columbia that varied dramatically in andromorph frequency. The experimental design allowed a direct comparison between the two theories as I could address male-male sexual interactions (MM) as well as potential changes in male mating behaviour with increasing andromorph frequencies (LMR) and the ratio of mimics to models (MM). I presented undisturbed and unrestrained males in the field with a specimen (andromorph, gynomorph or male) that had been adhered to a grass stem and recorded mating behaviours. My data was in support of the LMR hypothesis with regard to male-female interactions, as I found as andromorph frequency increased male mating attempts towards andromorphs increased and towards gynomorphs decreased. I was also able to show that as andromorph frequency increased the propensity of mating attempts towards conspecific males significantly increased, a prediction made by the MM but of little or no relevance to the LMR (although later versions of the LMR appear to make this prediction for much the same reason – male mimicry - that MM does). The work from chapter 2 is the first to directly test the outlined predictions of both the LMR and MM theories in populations that covered a wide range of morph frequencies. However my choice of presentation method greatly limited any response behaviours from the specimen which could influence male-mating behaviours

and mating success. This justified a full exploration in my second field season (chapter 3) and provided the opportunity to use another one of the many male-preference methodologies previously used in past studies to see if similar results would be obtained.

In chapter 3 I again looked at male-mating behaviours of *N. irene* across sites with extremely high and low morph frequencies, but instead of stick presentations that were utilised in chapter 2, I used cages to allow specimens to behave more freely in order to create a slightly more natural setting. Within each presentation two males were introduced into a cage with two females (andromorph and gynomorph) which allowed males to interact with both female morphs as well as with a conspecific male. Results from the cage experiments did not strictly conform to either of the predictions of LMR or MM. The proportion of total tandem attempts towards andromorphs was independent of morph frequencies, an outcome that is not consistent with either the LMR or MM models. With this more natural method, I was able to show that response behaviours of the females are an important factor to incorporate into male-mating behaviour experiments, as they influence the outcome of a tandem attempt. Also I suggest that females signal their inability to mate (lacking mature eggs) to males with abdominal bends - although this conclusion was based on the female mating status (single or tandem) at the time of capture, dissections are required to confirm these suspicions.

Current efforts are ongoing to further explore the cline seen in *N. irene* exploring the possible role of ambient temperature (as well as other biotic or abiotic factors) in the change in morph frequencies (see chapter 3 discussion). I suggest in chapter 3 that perhaps the different methods are measuring different types of harassment, so I suggest future work focus on exploring harassment in free-flying damselflies (although such an

approach has its own methodological challenges, as keeping track of individually marked damselflies in the field is not always easy).

With so many theories attributing the evolution of the polymorphism to male harassment it is crucial to understand what behaviour is considered “harassment” and what costs it imposes on damselflies. There are current efforts to quantify harassment and examine its effects; however more work is still required (see Gosden and Svensson 2009). In general, it is of interest to distinguish costs in damselflies that are imposed by tandem attempts that are unsuccessful and tandem attempts that are successful. It may be more costly to refuse copulation than it is to refuse a tandem attempt as the struggle to be released by a male takes more time and energy than dodging a male’s grasp. Additionally when a tandem is successful, how often does it lead to copulation in *N. irene* is currently unknown. It is possible that the proportion of successful tandems is correlated to the proportion of copulations which would suggest that females that are found in tandem are willing to copulate. Or alternatively, they copulate in order to avoid a costly struggle with males.

Additional molecular work would also help in elucidating the specific genes that are involved in the polymorphism and possibly at what life history stage these genes “turn on”. Even with the ever growing literature on female-limited polymorphism in damselflies it appears that the solution may be more complex than once thought. A limiting factor in the use of damselflies to address the maintenance of polymorphisms is their difficulty of maintaining multi-generation populations in the laboratory due to their long generation time and aquatic larval stage.

Overall I am able to show that frequency-dependent harassment in that the tendency of males to attempt to form tandem (and eventually succeed) with each morph depends (directly or indirectly) on their frequency. Such behaviours may well manifest itself as frequency dependent selection in the sedge sprite damselfly *Nehalennia irene*, thereby serving to maintain the polymorphism. For example, both my stick and my cage work suggest that gynomorphs are harassed by males more than andromorphs when gynomorphs are common, yet they are harassed at far lower rates when they are rare. As such, gynomorphs may increase in frequency when they are rare because of their low rate of harassment, but when they become more common they may be harassed more than andromorphs, generating a form of balancing selection. Whether (as implied by my stick experiments) or not (as implied by my cage experiments) andromorphs are harassed at greater rates when they are common compared to when they are rare is largely immaterial – given strong frequency-dependent selection on the gynomorph, then a balanced polymorphism is almost inevitable.

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