MATING EXPERIENCE AFFECTING MALE DISCRIMINATION BETWEEN SEXES AND FEMALE MORPHS IN *ISCHNURA SENEGALENSIS* (RAMBUR) (ZYGOPTERA: COENAGRIONIDAE)

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Ischnura senegalensis ♀ ♀ exhibit colour dimorphism, appearing as andromorphs and gynomorphs. Binary choice experiments between sexes and morphs were conducted in the laboratory. Virgin ♂ ♂ reared separately from ♀ ♀ showed no preference between sexes or between morphs, suggesting that virgin ♂ ♂ were unable to recognize potential mates and had no innate mating preference for a particular ♀ morph. After enclosure with a single ♀ in a small cage, ♂ ♂ that had experienced copulation significantly preferred the same ♀ morph with which they had copulated, while ♂ ♂ that failed to copulate with the ♀ showed no preference. The ♂ ♂ that had experienced copulation significantly preferred ♀ ♀ over ♂ ♂. Therefore, ability of ♂ ♂ to discriminate between sexes and morphs was confirmed by their copulation experience.

INTRODUCTION

Differences in reproductive interests between sexes can lead to sexual conflict over mating frequency (PARKER, 1979), potentially causing a sexually antagonistic co-evolution (HOLLAND & RICE, 1998). GAVRILETS & WAXMAN (2002) suggested that one of the outcomes in sexually antagonistic coevolution is female divergence within a population, e.g. female polymorphism. In zygopterans, female colour polymorphism involves an andromorph, resembling conspecific males, and gynomorphs, exhibiting different colouration from the males (e.g. ROBERTSON, 1985).

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Because frequency of female morphs in a population varies spatially and temporally (BERGSTEN et al., 2001; CORDERO RIVERA & EGIDO PÉREZ, 1998; CORDERO RIVERA & SÁNCHEZ-GUILLÉN, 2007), forming a search--image of the common female morph may be advantageous for males if it increases their searching efficiency (FINCKE, 1994). Males have to adjust their mating preference to the change in the morph frequency. In female-polymorphic species, frequency-dependence in male mating preference for female morphs has been reported in *Ischnura elegans* (Vander Linden) (VAN GOSSUM et al., 1999) and in *I. senegalensis* (Rambur) (TAKAHASHI & WATANABE, 2009). Such preferential mating attempts with common female morphs might lead to negative frequency-dependent selection, which is a potential driving force for maintaining the multiple female morphs within a population (SVENSSON & ABBOTT, 2005).

Males encounter various insects in their habitat throughout their lifespan, including potential mates as well as unsuitable individuals such as sexually immature females, conspecific males or heterospecific individuals. Since attempts to mate with unsuitable individuals are costly in terms of time wasted (STOKS & DE BRUYN, 1998), males have to distinguish between potential mates and unsuitable individuals as well as between morphs in order to increase their mating success. In *I. elegans*, males preferentially attempted to mate with sexually mature females rather than with sexually immature females (VAN GOSSUM et al., 2001a) or conspecific males (VAN GOSSUM et al., 2005).

In *Enallagma civile* (Hagen), a previous experience by males of encountering a particular female morph increased their mating preference for that morph (MILLER & FINCKE, 1999). VAN GOSSUM et al. (2001b) showed that *I. elegans* males reversibly change their mating preference based on the experience. Although MILLER & FINCKE (2004) suggested that an encounter experience elicited a change in the males' mating preferences, the mating preferences might be affected by the type of interactions with the individuals encountered. In the present study, we examined the innate male mating preference for female morphs and sexes, and clarified the effects of mating experience on mating preferences.

METHODS

STUDY SPECIES − *Ischnura senegalensis* (Rambur) females exhibit colour dimorphism, with a conspicuous blue andromorph and a brown gynomorph, though males are monomorphic with green thoracic colour. Female morphs were controlled by a single autosomal locus having two alleles, with expression limited to females (Y. Takahashi & M. Watanabe, unpubl. data), as in the case of *I. demorsa* (Hagen) (JOHNSON, 1964) and *I. damula* Calvert (JOHNSON, 1966). Both sexes stay at the water's edge all day long. Copulations start in early morning and terminate around noon (TAKAHASHI & WATANABE, 2009). Females oviposit alone during the afternoon without copulation, although males make many attempts to mate with them.

PREPARATION OF TEST SPECIMENS − In order to obtain test specimens, artificial oviposition by females collected from the field was conducted in the laboratory. Sexually mature females

were put individually into petri dishes (φ90 mm) for several days with a wet filter paper as an oviposition substrate. Eggs laid on the filter paper were kept in a petri dish filled with water at room temperature, and most hatched 10-11 days after oviposition. The first instar larvae were moved to large plastic containers (8×12 cm, height 5 cm) with a submerged polypropylene mesh (5×5 cm, weave size 1 mm) as their perching sites. They were fed on live brine shrimps (*Altemia* sp.). When they had grown to approximately 3 mm in body length, they were placed individually in plastic bottles (φ3.5 cm, height 5.8 cm) in order to avoid cannibalism. Medium-sized larvae (>5 mm) were fed for three months on live *Tubifex* spp. as their main food until emergence. A twig about 10 cm in length was provided in each bottle as a support for the emergence of the final instar larva.

An individual identification code was marked on the right hindwing of each adult with a fine felt--tipped pen after the wings had hardened. The sexes were separated in respective flying cages (40×40×50 cm) with wooden frames covered by polypropylene mesh (weave size 1 mm). Less than ten adults were reared in each cage, in order to decrease the risk of cannibalism. The adults were fed on fruit flies, *Drosophila* spp., which had been cultured. To increase the humidity as well as to supply water, we misted inside of the cage with water 2-3 times per day.

PROCEDURE OF BINARY CHOICE EXPERIMENT – Five to nine days old sexually mature adults of both sexes were used as the test specimens in the binary choice experiment. They were already sexually mature, judging from the body colour and the wing transparency. Since the male's mate choice behaviour might be affected by mate refusal behaviour as well as by unknown female mate choice behaviour, it was necessary to use immobile specimens in the binary choice experiment. Sexually mature virgin adults reared in flying cages were killed by being placed in a freezer for two minutes. After that, their abdomens were straightened, and their wings were kept closed. Before the experiment, two specimens were pinned with a micro-needle (φ0.18 mm) on the upper frame in the experimental cage (30×30×30 cm wooden frames covered by polypropylene mesh, weave size 1 mm) in the perching posture. To evaluate the male's response to each colour morph, both types of specimen were pinned next to each other 3 cm apart. The site of each specimen was changed in every trial. Since the body colouration of the freshly killed specimens deteriorated by the evening, new specimens were prepared for each experimental day.

To investigate innate mating preference for morphs and sexes, a binary choice experiment was conducted using a virgin male that was reared separately from females after emergence. In the morning (07:00-09:00), the male was introduced into an experimental cage in which a pair consisting of an andromorph and gynomorph, an andromorph and male, or a gynomorph and male was pinned. Each binary choice experiment was stopped when the male 'attacked' one specimen, i.e., when it dashed to a female and tried to form a tandem with it. The males' 'attacking' behaviour in the direction of a pinned specimen was judged as an indicator of the male's preference. If a male had chosen neither specimen within five minutes, the male was discarded from the experiment.

To determine the effect of the copulation experience of the male on mating preference for morphs or sexes, a single virgin male was introduced into the experimental cage with a single sexually mature female, either an andromorph or a gynomorph, in the morning. The male's mating attempts and copulation behaviour in the cage were observed during the enclosure period for about 4 hours. Just after removal of the coexisting female at noon, the binary choice experiment between andromorph and gynomorph, andromorph and male, or gynomorph and male was conducted for the males.

STATISTICAL TEST – Outcomes of binary choice experiments were analyzed by performing binominal tests. Differences and changes in preference of males were analyzed by the Chi-square tests. The program used was the R version 2.7.0 (R Development Core Team, 2008).

RESULTS

MATING PREFERENCE OF VIRGIN MALES

In each binary choice experiment, most males 'attacked' the killed specimen presented as soon as they were introduced into the experimental cage. The males grasped and tried to form a tandem with the specimen. Some males hovered around the specimens just before they 'attacked' them. Such behaviour suggested that the males recognized the paired specimens simultaneously and that they were able to choose freely one of the specimens in this experiment.

In the binary choice experiment between andromorph and gynomorph, every virgin male immediately attacked to mate with the female specimen. Virgin males that were offspring of the andromorphs exhibited no preference for female morphs (binomial test, P=0.215) (Tab. I). Also no preference was found in virgin males that were offspring of gynomorphs (binomial test, P=1.0); nor did the offspring of unknown female morphs (binomial test, P=1.0). The selectivity was not significantly different between males derived from andromorphs and those derived from gynomorphs (χ^2=0.739, P=0.390), indicating that virgin males did not distinguish between morphs irrespective of their maternal morph.

As shown in Table II, virgin males exhibited no preference between male and andromorph (binomial test, P=0.860) and between male and gynomorph (binomial test, P=0.122). Every male attempted to mate with the specimens presented irrespective of sex, and formed a tandem by attaching their anal appendages to the pronotum of the specimen. These results indicated that virgin males could not discriminate potential mates from conspecific males.

DISCRIMINATION BETWEEN FEMALE MORPHS

When virgin males were enclosed with a single virgin or a mated female in the morning, they repeatedly tried to mate with it, and sometimes grasped it. Few mated females accepted the male mating attempt throughout the enclosure period, showing a mate refusal display, i.e. fluttering their wings and bending their abdomens. On the other hand, most virgin females accepted the males after sev-

Table I

Binary choice experiment between an andromorph and gynomorph for virgin males
(P-values refer to binomial tests)

Maternal morph of males tested	No. males that chose andromorph	No. males that chose gynomorph	P
Andromorph	12	20	0.215
Gynomorph	9	9	1.000
Unknown	37	36	1.000

Table II

Binary choice experiment between an andromorph and gynomorph for virgin male
(P-values refer to binomial tests)

	No. males that chose male	No. males that chose female	P
Between male and andromorph	17	15	0.860
Between male and gynomorph	18	9	0.122

eral mating attempts. Each copulation lasted for about three hours and terminated around noon. Figure 1 shows the outcome of the enclosure just before the binary choice experiments between an andromorph and gynomorph. Out of 22 males that were enclosed with an andromorph, ten males succeeded in copulating, and 12 males failed to copulate. Out of 36 males that were enclosed with a gynomorph, 24 males succeeded in copulating, and 12 males failed to copulate.

In the binary choice experiment in the afternoon following the enclosure, males 'attacked' the dead specimen of each morph as soon as they were introduced into the cage, except four tested males, probably due to handling damage or weariness. Males that had succeeded in copulating with an andromorph subsequently tended to choose andromorphs and this was significant (binomial test, P=0.008) (Fig. 1). Similarly males that succeeded in copulating with a gynomorph tended to choose gynomorphs, again significatnly (binomial test, P=0.007). However, males that had failed to copulate with an andromorph or a gynomorph in the enclosure period showed no preference between morphs in the afternoon (binomial test, P=1.0 and P=0.754, respectively).

DISCRIMINATION BETWEEN SEXES

Binary choice experiments between the sexes after the enclosure in the morning were conducted for males that had succeeded in copulating with virgin females. In the binary choice experiment between a female andromorph and male, males that had copulated with andromorphs significantly chose the andromorph (binomial test, P=0.003) (Fig. 2). On the other hand, in the binary choice experiment between a female gynomorph and male, although males that had experienced copulation with a gynomorph were apt to prefer to mate with gynomorphs, no significant difference was found (binomial test, P=0.308). The proportion of males that chose a gynomorph among the experienced males (62.5%) was significantly higher than that among the virgin males (33.3%) (χ^2=4.339, P=0.037), indicating that males were able to improve their precision of mate recognition through their copulation experience.

DISCUSSION

JOHNSON (1975) suggested that males of *I. demorsa* and *I. damula* having an andromorphic genotype and males having a gynomorphic genotype innately had a mating preference for andromorphs and for gynomorphs, respectively. If mating preference of males depends on their own genotype, the preference was partially affected by maternal genotypes. In the present study, however, males showed no mating preference irrespective of maternal morphs, suggesting that no virgin males have innate preference for a particular female morph, as in the case of *Enallagma* spp. (FINCKE et al., 2007). In addition, no virgin males dis-
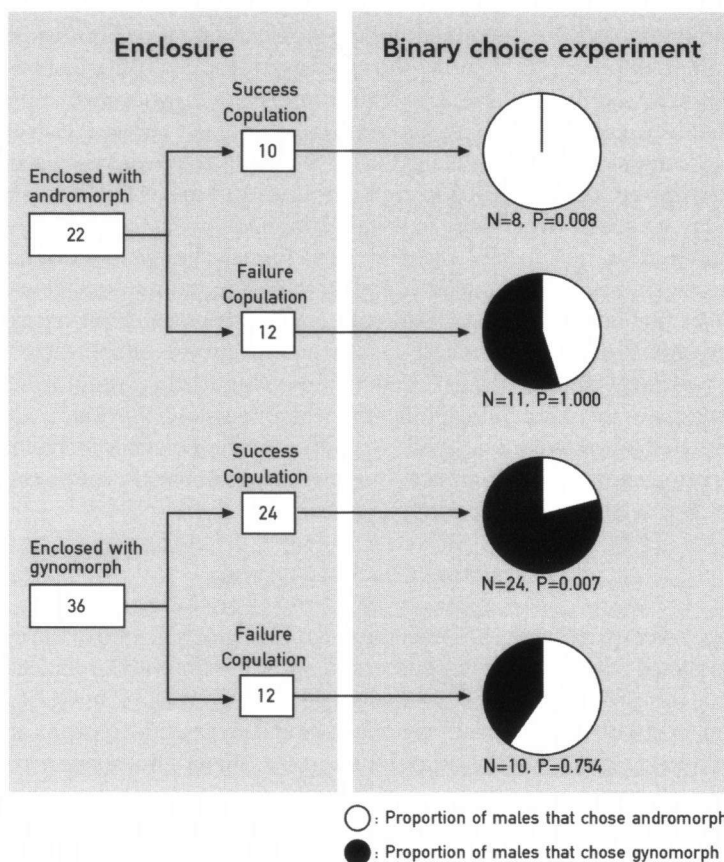

Fig. 1. Male preference for female morphs in the binary choice experiments following enclosure with a female. Numerals in the boxes indicate the number of males that were enclosed with a female and the results of their mating attempt. N represents the number of males that chose the female morph in the binary choice experiments. P-values were calculated using binomial tests.

tinguished conspecific males from females, indicating that virgin males cannot recognize sexes and morphs.

FINCKE (1994) showed that males learned to recognize potential mates through their prior experience. When *I. elegans* males had been enclosed with a particular female morph, the males changed their mating preference in accordance with this previous enclosure experience (VAN GOSSUM et al., 2001b). MILLER & FINCKE (1999) suggested that males switched their mating preference with the frequency of female morphs encountered. However, in *I. senegalensis*, exposure to a particular female morph without copulation did not affect subsequent mating behaviour, while males that experienced copulation preferred to mate with the morph with which they had previously mated. Therefore, our results revealed that copulation experience elicited a change in the mating preference of *I. senegalensis* males, but encounters did not. In the present study, some males failed
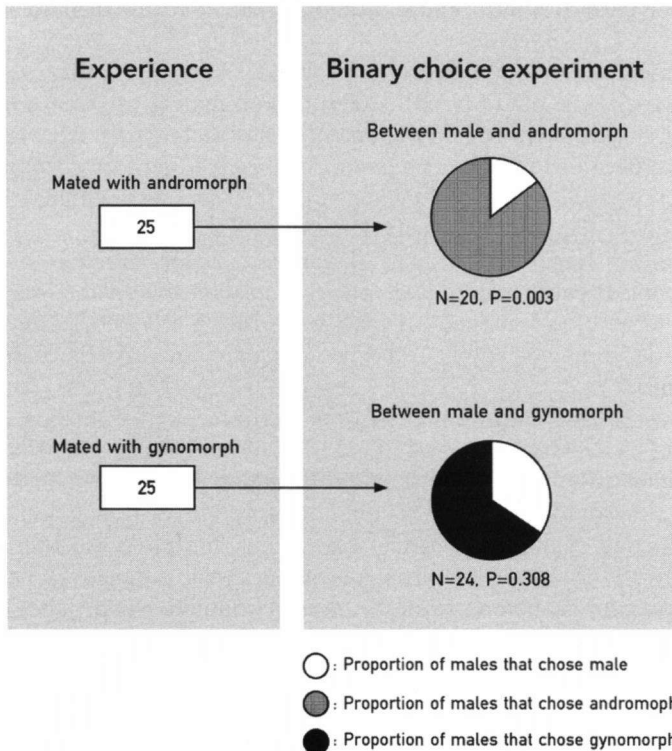

Fig. 2. Male preference for female morphs in the binary choice experiments following enclosure with a female that involved successful mating. Numerals in the boxes indicate the number of males that had mated with a female of each morph. N represents the number of males that chose the specimen presented in the binary choice experiments. P-values were calculated using binomial tests.

to copulate after tandem formation due to female rejection. Effect of the tandem formation without copulation on mating preference needs to be investigated.

CORDERO RIVERA & SÁNCHEZ-GUILLÉN (2007) indicated that the mating frequency of andromorphs was lower than that of gynomorphs in *I. elegans*. ROBERTSON (1985) suggested that *I. ramburi* (Selys) males potentially preferred gynomorphs because andromorphs resemble conspecific males, and thus the males have difficulty recognizing an andromorph as a potential mate due to male-mimic colouration. However, in *Coenagrion puella* (L.), both the colour and pattern of andromorphs differed from those of conspecific males (JOOP et al., 2007), indicating that the males might be able to distinguish andromorphs from conspecific males. FINCKE et al. (2007) also reported that the reflectance spectrum of the thorax in andromorphs differed from that in males for *E. ebrium* and *E. hageni* (Walsh). In the present study, experienced males distinguished females from males irrespective of the morphs of the females, indicating that the colour and pattern alone of the andromorphs did not cause them to be mistaken for males in *I. senegalensis*.

In the field, *E. civile* (MILLER & FINCKE, 2004) and *I. senegalensis* males (TAKAHASHI & WATANABE, 2009) had no mating preference in the early morning, suggesting that temporal bias of males' mating preference disappeared overnight. Because the mating preference of the males conformed with their copulation experience, wild males might change their mating preference in response to their day-to-day copulation experience. This mating preference could help the males easily distinguish individuals who are suitable for mating from individuals who are not. Because mating attempts with unsuitable individuals are probably costly in terms of time wasted (STOKS & DE BRUYN, 1998), learned mate recognition depending on copulation experience might increase the mating success of the males.

In *I. senegalensis*, most females in the field accepted copulation during the morning (TAKAHASHI & WATANABE, 2009). If males mated randomly in the morning with females of either morph with no preference, then the majority of the males would mate with the common morph in the population. Therefore, the proportion of males that prefer each female morph in the afternoon must coincide with the proportion of morphs in the female population. This suggests that changes in the preference on the basis of copulation experience might lead to the frequency-dependent prevalence of male mating preference for each morph (TAKAHASHI & WATANABE, 2009).

In *I. senegalensis*, females forage and oviposit alone after daily copulation activity, i.e., in the afternoon. The males tried to mate with foraging and ovipositing females, but few females accepted the copulation (SAWADA, 1999). The attempt by males to mate in the afternoon might interfere with the behaviour of females of searching for oviposition sites (CÓRDOBA-AGUILAR, 1993) or with ovipositing itself (FINCKE, 1987). Because males that harassed the com-

mon morph would outnumber males that harassed the rare morph, the common morphs were more frequently harassed by males encountered (TAKAHASHI & WATANABE, 2010). This suggests that the common morph might have their reproductive success decreased more than the rare morphs. In *I. elegans*, females of each morph exhibited a negative frequency-dependence in their reproductive success (SVENSSON et al., 2005). Therefore, in *I. senegalensis*, experience-based behavioural change in individual males might drive maintenance of female multiple morphs within a population under negative frequency-dependent selection.

ACKNOWLEDGEMENT

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