

EGG-HATCHING BENEFITS GAINED BY POLYANDROUS FEMALE LOCUSTS ARE NOT DUE TO THE FERTILIZATION ADVANTAGE OF NONSIBLING MALES

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Several studies suggest that polyandrous females bias paternity in favor of unrelated males to avoid inbreeding depression. Here we tested whether the migratory locust biases sperm usage toward unrelated males by analyzing the paternity of offspring from females mated with either two siblings, or two nonsiblings, or a sibling and a nonsibling in either order. We found that the eggs of females mated only with siblings had decreased hatching success. When females mated with both a nonsibling and a sibling, egg hatchability was significantly increased. Subsequent paternity analyses found no evidence that females could avoid fertilization by sibling males. Therefore, improvement of the hatchability of eggs sired by siblings suggests that rather than biased fertilization by females toward genetically compatible or superior males, male-induced maternal effects or direct effects of male ejaculates might influence the survival of offspring sired by related males.

KEY WORDS: Inbreeding avoidance, *Locusta migratoria*, microsatellite, polyandry, sperm competition.

Females of numerous species mate more often than necessary to ensure fertilization of their eggs, although the mating is often associated with the costs of general time and energy, the increased risk of physical injury, parasite/pathogen infection, or predation rates (Arnqvist and Nilsson 2000). A variety of hypotheses have been proposed to explain why natural selection should favor the evolution of polyandry. With the exception of forced copulation, these hypotheses fall into two broad but distinct categories of material and genetic benefits (Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Zeh and Zeh 2001; Ivy and Sakaluk 2005). Polyandrous females may gain such material benefits as an adequate sperm supply (Ridley 1988), nutrient donations from spermatophores or seminal fluid (Zeh and Smith 1985), or addi-

tional paternal care (Davies 1992). The main potential genetic benefits of polyandry include increasing the offspring diversity (Loman et al. 1988), producing competitively superior sons (Keller and Reeve 1995), minimizing the risk and/or cost of fertilization by genetically incompatible sperm (Zeh and Zeh 1996, 1997), compensating for a poor-quality mate in socially monogamous species (Kempnaers et al. 1992; Hasselquist et al. 1996), increasing the probability that eggs are fertilized by high-quality sperm (Birkhead et al. 1993; Radwan 2003), or diminishing the cost of inbreeding (Stockley et al. 1993).

The generality of the claim that polyandry allows for inbreeding avoidance via postcopulatory mechanisms is largely untested (Jennions et al. 2004). To date, several studies have tested the inbreeding hypothesis. Polyandry, as a mechanism for inbreeding avoidance, has been demonstrated in lizards (Olsson et al. 1996),

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ascidians (Bishop et al. 1996), fruit flies (Mack et al. 2002), crickets (Tregenza and Wedell 2002), and birds (Pizzari et al. 2004). In contrast with these studies, no evidence for inbreeding avoidance through postcopulatory mechanisms has been found in shrews (Stockley 1997) and crickets (Simmons 2001; Jennions et al. 2004). However, conclusions of most of these studies mentioned above were deduced from hatching success. To investigate whether polyandry allows for inbreeding avoidance via postcopulatory mechanisms, there have been few studies in which molecular paternity analyses were conducted (Jennions et al. 2004), except for a study on crickets by Bretman et al. (2004).

The migratory locust, *Locusta migratoria*, is a very good model to study the possible benefits of polyandry because both sexes have high mating frequency over their lifetime (Gregory 1965). Eight polymorphic microsatellite loci in the locust have been developed by Zhang et al. (2003), which can be used to determine the paternity of the offspring. In this research, we established a set of unrelated full-sibling families and designed block experiments to try to test whether the female locust can use polyandry as a mechanism for inbreeding avoidance.

Material and Methods

LOCUSTS AND FAMILY ESTABLISHMENT

Fifth instar nymphs were collected from the field in Huanghua County, Hebei Province, China, in 2004. Nymphs were reared in 30 × 30 × 30 cm cages at 30 ± 1°C under a 14L:10D photoperiod in an insectary, and freely provided with wheat and wheat bran ad libitum. Upon their adult eclosion, males and females were separated to maintain their virginity.

One virgin female was mated to a single male to create a sibling family. Twenty-five unrelated full-sibling families were established.

MATING TRIALS

Sexually mature virgin adults (25 days old) were allowed to mate according to the experimental design. The mating experiments were conducted in 20 × 20 × 20 cm cages, and the floor of each cage has a hole of 11.5 cm diameter and 9.8 cm deep through which the female deposited eggs into a sterile sand-filled container.

The experiments were arranged in blocks of four females and four males following the protocol of Tregenza and Wedell (2002); six of these were siblings (four females and two males from one family), and two were male siblings from another family. In each block, two males (S1, S2) were full siblings (brothers) of the four females and two males (N1, N2) were not related to the females, but were brothers to one another. Females were assigned to one of four mating regimes that mated them with: (1) S1 then S2; (2) S2 then N1; (3) N2 then S1; or (4) N1 then N2. Therefore, all females and males mated twice during the experiment. Males were

used in one block only. In the first round of mating, each female mated with a virgin male. After a successful mating, the pair was separated immediately. To prevent a mating plug effect from the first male, the second round of mating was conducted after the first egg pod was laid (Zhu and Tanaka 2002). According to our unpublished data, male locusts could recover their ejaculates within two days. In our experiment, the time interval between the first and the second mating is at least three days. We conducted 34 blocks in this experiment. During the first round of mating, five females failed to mate in three blocks. In addition, two females failed to lay fertilized eggs in another two blocks. Three females and one male died during the experiment in another four blocks. These nine blocks were abandoned. Finally, 25 blocks were successfully conducted.

Cages were inspected every 30 min for copulations. Immediately after mating, males were taken out within 30 min of termination of copulation. During the dark period, a small red flashlight was used for observation (Zhu and Tanaka 2002).

HATCHING SUCCESS

Sand containers were checked daily for egg pods. Egg pods were excavated and transferred to a sterile moist sand-filled plastic cup and maintained under the same conditions as adults. Eggs were checked daily for hatching until three days after the last emergence, by which time eggs that had not hatched would have begun to breakdown. All hoppers were counted, as were eggs that had failed to develop to the hatching stage. Each female in 10 blocks was allowed to deposit up to seven egg pods to determine how the hatching success varied over time.

PATERNITY ANALYSIS

Adults, hatchlings, and unhatched eggs were kept at −20°C until DNA extraction. Genomic DNA was extracted following the protocol described by Vavre et al. (1999) with slight modifications. Four polymorphic microsatellite loci (LmIOZc19, LmIOZc29, LmIOZc35, and LmIOZc67) in the locust, *L. migratoria*, developed by Zhang et al. (2003) were used to determine the paternity. Polymerase chain reaction (PCR) amplifications were carried out in a 10 μL reaction mixture containing 0.1–0.2 μM of each primer, 200 μM of each dNTP, 1.5 mM MgCl₂, 10 ng genomic DNA, 0.6 U of Taq DNA polymerase (TaKaRa), and 1 μL enzyme buffer supplied by the manufacturer. PCR products were analyzed on an ABI-377 automated sequencer. The number of eggs in each pod was from 35 to 111 (78.47 ± 0.74; *N* = 400). Parents and 50% randomly sampled hatchlings per female (sample size range: 19–48; mean ± SE: 35.23 ± 1.01, *N* = 48) from 12 randomly sampled blocks were genotyped; all unhatched eggs per female of these blocks (range: 1–33; mean ± SE: 7.59 ± 1.24, *N* = 44) were also genotyped.

STATISTICS

Statistics were performed using SPSS 11.0 software. All percentages were arcsine transformed before statistical analysis. Datasets were tested for deviation from normal distribution by using the Kolmogorov–Smirnov test. Paired *t*-tests were performed to test for differences between the duration of the first copulation and that of the second copulation. Randomized block analyses of variance were used to test for the effects of: (1) mating treatment on mating behavior, (2) mating treatment on egg number, (3) mating treatment on hatchability, (4) mating treatment on paternity, (5) block on egg number, (6) block on hatchability, and (7) block on paternity. Post-hoc analysis (Tukey's test) was conducted to examine the effect of mating treatment on hatchability. Spearman rank correlations were applied to test for a relationship between the proportion of offspring sired by the second male and its relative copulation duration.

Results

EGGS AND HATCHABILITY

In the first egg pods when females only have one mate, there was no significant effect of mating with either a nonsibling or a sibling on egg number ($F_{1,24} = 0.824$, $P = 0.373$), and the hatchability was significantly higher for nonsibling matings than sibling matings ($F_{1,24} = 38.119$, $P < 0.001$). The mean hatchability of eggs sired by the sibling males was $79.94 \pm 3.03\%$ ($N = 50$), and the mean hatchability of eggs sired by the nonsibling males was $95.71 \pm 1.31\%$ ($N = 50$). However, a significant effect of block on egg number ($F_{24,24} = 2.721$, $P < 0.01$) was observed.

In the second egg pods when females have two mates, no significant effect of mating treatment on egg number was found ($F_{3,72} = 0.642$, $P = 0.59$), but there was a significant effect of block on egg number ($F_{24,72} = 4.067$, $P < 0.001$). There was a significant effect of mating treatment on hatchability ($F_{3,72} = 15.667$, $P < 0.001$) (see Fig. 1), but hatchling success did not differ significantly between experimental blocks ($F_{24,72} = 1.339$, $P = 0.170$). Post-hoc analysis of egg hatching indicated that the significant effect of treatment was associated with the lower hatching success of females mated to two siblings relative to females mated to at least one nonsibling (Tukey's test: NN versus SN or NS, all $P > 0.105$; SS mating versus other treatments, all $P < 0.001$) (see Fig. 1).

From the second to the seventh egg pods, the total hatchability was $88.89 \pm 0.24\%$, $92.73 \pm 0.48\%$, $97.08 \pm 0.12\%$, and $98.49 \pm 0.20\%$ ($N = 10$ each) in the SS, NS, SN, and NN mating treatment, respectively. There was a significant effect of mating treatment on total hatchability ($F_{3,27} = 5.314$, $P < 0.01$), and laying order had no significant effect on the hatchability during the observing period ($F_{5,45} = 1.408$, $P = 0.240$) (see Fig. 2). Total hatchling success did not differ significantly between experimental blocks ($F_{9,26,265} = 1.000$, $P = 0.464$).

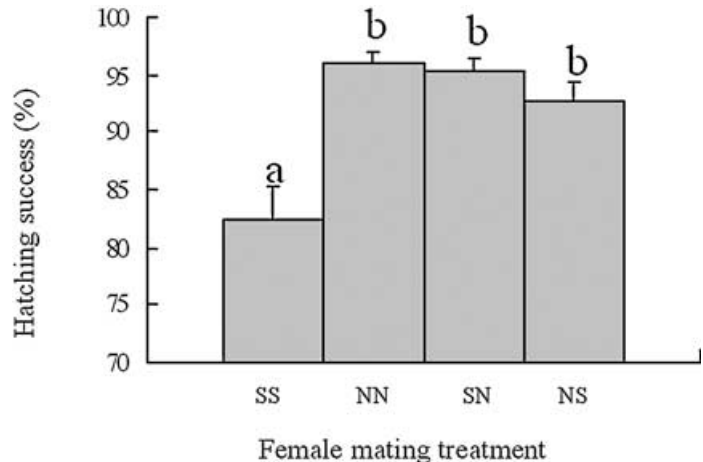


Figure 1. Relative hatching success of eggs in the second egg pod. Eggs were from females mated either to two siblings (SS), two nonsiblings (NN), a sibling followed by a nonsibling (SN) or a nonsibling followed by a sibling (NS) (means and standard errors).

PATERNITY ANALYSIS

In the 12 blocks we genotyped, for all hatchlings and most unhatched eggs (72.22% in the SS treatment, 83.33% in the SN treatment, 79.41% in the NN treatment, 90.12% in the NS treatment) we successfully extracted their DNA. Less than 50% DNA-extracted offspring in one family in the SS treatment were genotyped with four microsatellite loci. This family was excluded from the analysis. Two females produced only the first male's offspring; one was in the NN treatment, and the other was in the SN treatment. In such cases, the second males paired might have failed to transfer their sperm, as this often occurs even in single mating pairs (Reinhardt and Meister 2000; this study). These two families were also excluded from the analysis. The value of P_2 (proportion of offspring sired by the second male) in successfully genotyped offspring (both nymphs and unhatched eggs) was $91.54 \pm 3.39\%$ in the SS treatment ($N = 11$), $82.59 \pm 7.72\%$ in the NN treatment ($N = 11$), $81.48 \pm 6.67\%$ in the SN treatment ($N = 11$), and $91.33 \pm 3.31\%$ in the NS treatment ($N = 12$) (see Fig. 3). Randomized block analysis of variance showed that there was no significant effect of mating treatment on P_2 ($F_{3,30} = 1.100$, $P = 0.365$), and no significant effect of block on P_2 ($F_{11,30} = 1.417$, $P = 0.216$). Post-hoc analysis of P_2 did not reveal any difference in fertilization success between sibling and nonsibling males (Tukey's test: NS versus other treatments, all $P > 0.695$; SN versus other treatments, all $P > 0.502$).

MATING BEHAVIOR

For the male, no significant difference was observed in the length of time from introducing it into the mating cage to the onset of its mounting on the back of the female between sibling and nonsibling matings both in the first ($F_{1,24} = 0.589$, $P = 0.450$) and the second mating ($F_{3,72} = 0.476$, $P = 0.700$). For the female, the

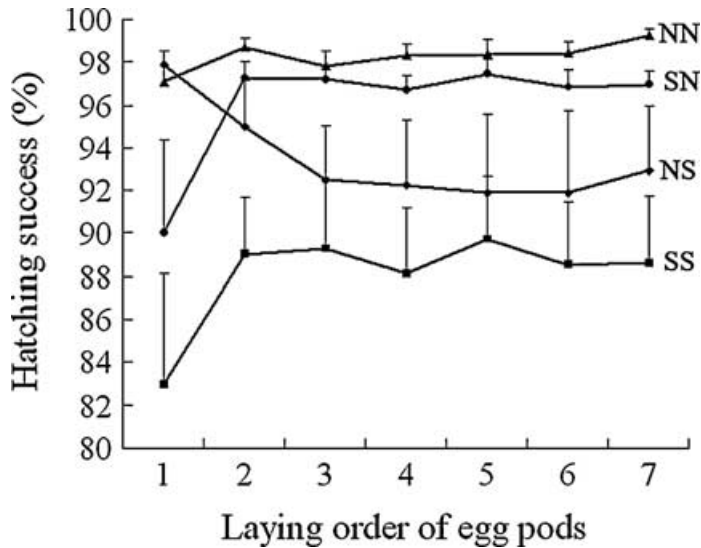


Figure 2. Changes of hatchability in the observed reproductive period.

length of time from the onset of male's mounting to the beginning of copulation did not differ significantly between sibling and non-sibling matings both in the first ($F_{1,24} = 1.295$, $P = 0.266$) and the second matings ($F_{3,72} = 0.713$, $P = 0.548$). The copula duration was very long (7.41 to 15.29 h) in the locust (see Table 1), and the length of copulation did not differ significantly with respect to the relatedness of mates both in the first ($F_{1,24} = 0.236$, $P = 0.632$) and the second mating ($F_{3,72} = 0.975$, $P = 0.409$). However, for both males and females, the duration of the second copulation was significantly longer than that of the first copulation whether data were paired by male or female identity (males: $t_{99} = -6.453$, $P < 0.001$; females: $t_{99} = -6.531$, $P < 0.001$).

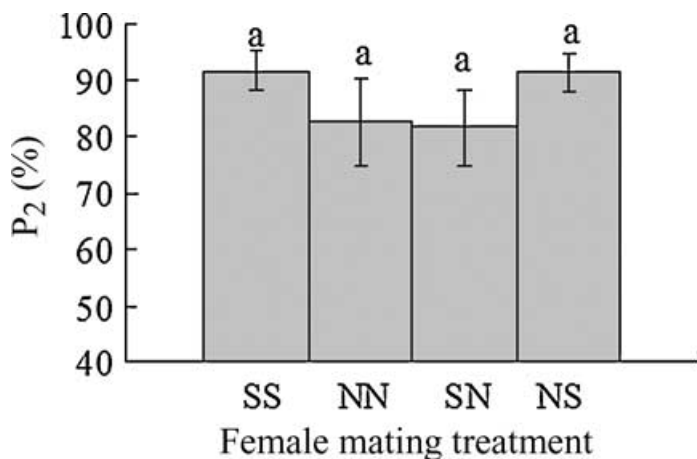


Figure 3. Proportion of successfully genotyped offspring (both nymphs and unhatched eggs) sired by the second mated male (P_2) in four mating treatments (means and standard errors).

PATERNITY AND COPULA DURATION

In the study carried out by Zhu and Tanaka (2002), the length of the second copulation was found to show a significant correlation with P_2 ($N = 16$). However, based on our present data that have triple the samples, the correlation of P_2 with the length of the second copulation was not significant ($r_s = 0.271$, $N = 45$, $P = 0.072$). In addition, there was no relationship between P_2 and the relative length of copulation (the second copula duration/the first copula duration) ($r_s = 0.216$, $N = 45$, $P = 0.154$).

Discussion

INBREEDING DEPRESSION AND BENEFITS OF POLYANDRY

Previous studies have found evidence that polyandry can bring females fitness benefits through increasing egg or offspring viability in adders (Madsen et al. 1992), sand lizards (Olsson et al. 1996), cuis (Keil and Sachser 1998), pseudoscorpions (Newcomer et al. 1999), crickets (Simmons 2001; Tregenza and Wedell 2002), and fruit flies (Mack et al. 2002). Our study provides the first evidence for inbreeding depression in hatching success in the locust. Inbreeding between full siblings caused a significant decrease in hatching success. However, when females mated with both a sibling and a nonsibling without reference to the mating order, egg hatchability was significantly improved. Therefore, polyandrous female locusts could reduce inbreeding cost.

SPERM COMPETITION

Our data showed that the sperm competition pattern in the locust was the last male precedence with one mating per oviposition cycle. The high P_2 in the present studies was consistent with data obtained by Parker and Smith (1975), which showed that the sperm competition pattern was the last male precedence with one mating per oviposition cycle. The range of copula duration was from 4 h to 34 h, and we did not find a correlative relationship between P_2 and the length of copulation. In the study carried out by Zhu and Tanaka (2002), however, the average P_2 was 52.5% and the length of the second copulation was found to show a significant correlation with P_2 . This discrepancy may be related to the fact that 2 h genital contact was regarded as successful copulation in the latter study (Zhu and Tanaka 2002). In the locust, sperm are delivered through the spermatophore and reach the spermatheca 90 to 105 min after the onset of copulation (Gregory 1965). Sperm transfer lasts until the end of the copulation that takes 5–18 h (Gregory 1965). If copulation was disrupted during the early period by the startling of the observer, the last mated male would fail to transfer the whole spermatophore, and subsequently lose precedence in sperm competition under such conditions.

MATING BEHAVIOR

It is not known how common matings between full siblings are in natural populations of the locust. In our research, no evidence of

Table 1. Mean (\pm SE) values of the length of time from introducing the male into the mating cage to the onset of its mounting on the back of the female (LTM), the length of time from the onset of male's mounting to the beginning of copulation (LTF), and the length of copulation (LC).

| | | LTM (h) | LTF (h) | LC (h) |
|-----------------------------------|--------------------------|-----------------|-----------------|------------------|
| Copulation with sibling at the | first time ($N = 25$) | 2.72 \pm 0.55 | 0.20 \pm 0.08 | 7.41 \pm 0.73 |
| | second time ($N = 25$) | 3.35 \pm 0.56 | 0.32 \pm 0.26 | 15.29 \pm 1.24 |
| | combined ($N = 50$) | 3.04 \pm 0.39 | 0.26 \pm 0.14 | 11.35 \pm 0.82 |
| Copulation with nonsibling at the | first time ($N = 25$) | 2.14 \pm 0.31 | 0.46 \pm 0.22 | 7.98 \pm 0.87 |
| | second time ($N = 25$) | 3.17 \pm 0.60 | 0.10 \pm 0.07 | 12.98 \pm 0.98 |
| | combined ($N = 50$) | 2.66 \pm 0.34 | 0.28 \pm 0.12 | 10.48 \pm 0.70 |

behavioral difference by both sexes in response to the relatedness of mates was found, and the locusts copulated readily with siblings. Hence mating with relatives may occur commonly in nature. Although there is a body of evidence pointing to the existence of recognition cues (e.g., pheromone; Simmons 1990), which allow animals to recognize relatives, several other studies have failed to find pre-copulatory differences in behavior in relation to mate relatedness (Tregenza and Wedell 2002; Pizzari et al. 2004). Species and population differences in mating preferences may arise due to variation in the strength of selection to avoid copulations with relatives (Stockley 1999).

The duration of the second copulation was significantly longer than that of the first copulation in the locust. This was not due to the immaturity of the locusts, because 15 days are enough for the locust to be matured (Reinhardt and Arlt 2003). In our study, all individuals were 25 days old when the mating experiment began, and most males started to mate within 3 h. One possible explanation was that males could detect the female mating status, and the long copula duration was probably related to elicit rival sperm ejection in the locust. Similar phenomena have also been reported in wartbiters (Wedell 1992), bugs (Wang and Millar 1997), and damselflies (Uhía and Rivera 2005).

HATCHABILITY OF POLYANDROUS FEMALES

Hatchability enhancement in polyandrous females, which mate with both sibling males and nonsibling males, is thought to have arisen only when there is differential fertilization success of sperm in favor of the ejaculate of unrelated males (Tregenza and Wedell 2002). The earlier studies provide evidence for postcopulatory inbreeding avoidance in crickets with females apparently avoiding using sperm from their brothers (Bretman et al. 2004). Our data, however, did not show any difference in fertilization success between sibling and nonsibling males. It is possible that the relatively low impact of inbreeding on hatching success (10–15%) combined with low statistical power leads to the lack of difference in hatching success between the NS treatment and the SN treatment. Although our sample size ($N = 11$ – 12 blocks) was small for testing whether there was biased

sperm usage, the direction of the trend was towards greater P_2 for NS matings, thus a reasonable increase in sample size is unlikely to yield a result in which P_2 for SN is greater than for NS. High P_2 suggests that the scope for differential sperm use may be more limited in the locust than in species with P_2 closer to 50% if it does occur.

Because most eggs were sired by the last mated male, and inbreeding could bring hatchability decline in the locust, then why was the hatchability in the NS treatment significantly higher than that in the SS treatment? Prehatching embryo viability may be affected by the amount of resources laid down in the egg during its development (Simmons 2001), and it seems conceivable that nonsibling males induce maternal effects that increase hatching of eggs fertilized by sibling males in the locust. Maternal effects arise when females vary the amount of resources they provide for their offspring and can have profound influences on offspring performance (Simmons 2005). A good evidence is from Weigensberg et al.'s study (1998) of the cricket *Gryllus rmus*. They used a recessive mutation for pale eye color as a marker to examine paternal genetic effects and maternal effects induced by males on the prehatching embryonic development of offspring produced by females that had mated to both a pale-eyed and a wild-type male. Embryos sired by wild-type males were generally larger than those sired by pale-eyed males at day 10 of development, suggesting that the genetic contribution of the sire is affecting embryonic growth and hatchling body size. Further, a negative correlation was found between the size of the pale-eyed male and the size of all the eggs that the female laid, suggesting an effect of male size on the amount of nutrients the female receives from her mates. Thus, embryonic growth is likely influenced by both genetic sire effects and male-induced maternal effects in this cricket (Weigensberg et al. 1998).

A previous study has shown that male locusts transfer very significant amounts of proteinaceous nutrients through the ejaculate to the females and these are then transferred to the eggs (Pardo et al. 1994). If males of different families vary in the composition or potency of their seminal components, this could lead to variation in the quality of eggs produced by females and thus

the viability of those eggs. In our studies, there was a significant block's effect on the number of eggs laid by females, which might be due to variation between families in their egg-laying ability. Hence, it is reasonable to suppose that the quantity or quality of male gonadotropic substances is different among different families in the locust. In a species in which females can mate many times before ovipositing, a male's investment will influence all the eggs in his mate's clutch regardless of whether he fertilized them (Weigensberg et al. 1998). Polyandry could therefore reduce the variance in quantity and/or quality of seminal products acquired by females and explain the increased embryo viability of polyandrous females without invoking genetic mechanisms (Simmons 2005).

It is also a possibility that some bioactive molecules in the sperm fluid of the nonsibling males enhance the hatchability of eggs sired by the sibling males, although the identity of the causal agent remains unknown. Male accessory reproductive gland secretion generally includes carbohydrates (both free and complexed with protein), some lipid (normally bound to protein), and small amounts of amino acids and amines. Unexpected materials, such as uric acid, prostaglandins, juvenile hormones, and various toxic materials that serve as egg protectants, are also found in the accessory gland in several species (Gillott 2003). If modulators of egg hatchability operate better in unrelated females due to some unknown immunological mechanisms, mating firstly with a nonsibling male will inevitably increase the hatching success of eggs subsequently laid from the females in the NS treatment, despite these eggs being sired by the sibling males.

So far, most evidences that polyandry reduces inbreeding costs come from hatching success (e.g., Jennions et al. 2004), and there are few studies in which molecular paternity analyses were conducted to examine this effect. The first molecular evidence is from the cricket, *Gryllus bimaculatus*. The eggs of females that mate only with siblings have decreased hatching success. However, if females mate with both a sibling and a nonsibling they avoid altogether the low egg viability associated with sibling matings (Tregenza and Wedell 2002). This strongly implies that the nonsibling male preferentially fertilized her eggs. Subsequent paternity tests have confirmed this explanation, although the unrelated male's share of paternity was not positively correlated with the proportion of eggs that hatched, which may reflect the compounding influence of variation in male or female effects on absolute hatching success (Bretman et al. 2004). In the black field cricket, *Teleogryllus commodus*, however, no evidence was provided that female *T. commodus* can bias sperm usage towards unrelated males through postcopulatory mechanisms based on hatching success data (Jennions et al. 2004). Other evidence comes from the viviparous pseudoscorpion, *Cordylochernes scorpioides*. Polyandry diminishes the cost of inbreeding by both reducing the risk of spontaneous brood abortion and increasing

embryonic survival in successful broods. However, polyandry in viviparous pseudoscorpions reduces the inbreeding cost not through paternity-biasing mechanisms favoring outbred offspring, but rather because outbred embryos exert a rescuing effect on inbred half-siblings in mixed-paternity broods (Zeh and Zeh 2006). In this research, we found no evidence that *L. migratoria* females could avoid fertilization by sibling males. Thus, the molecular details of inbreeding avoidance may be more complex than previously thought and could involve certain epigenetic mechanisms.

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