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An examination of competitive gametic isolation mechanisms between the damselflies *Ischnura graellsii* and *I. elegans*

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Recent findings suggest that postmating prezygotic isolation (i.e. gametic barriers) could be an important factor preventing hybrid formation. Competitive gametic barriers emerge when a female is inseminated by a conspecific and a heterospecific male. We examined whether sperm proportions after double matings and copulation duration impede hybrid formation. For this, we used females of *Ischnura graellsii* that mated with one conspecific and one heterospecific (*I. elegans*) male and vice versa, and calculated paternity of the second male by using RFLPs. Values of paternity (although preliminary because of a small sample size) suggest no bias in paternity towards conspecific males. However, proportion of sperm stored in the bursa and spermatheca of the female was biased towards the conspecific male when the heterospecific male was the first male, while copulation duration did not differ between conspecific and heterospecific males. Our results suggest that the relative sperm volumes may play a role as a gametic barrier in this species. However, cryptic female choice mediated by the preferential use of the conspecific sperm, although not detected, could not be discarded owing to small sample sizes in some cases.

Keywords: prezygotic barriers; competitive gametic mechanisms; paternity; RFLPs; copulation duration; sperm volumes; ischnurines

Introduction

Classically, the study of prezygotic isolation has been focused on premating barriers (Coyne & Orr, 1998). However, several findings (Eady, 2001; Fricke & Arnqvist, 2004; Howard, 1999) suggest that postmating prezygotic barriers (also known as gametic barriers) could be an important factor. Gametic barriers are divided into non-competitive, when the female has been inseminated only by a heterospecific male, and competitive, when the female has doubly mated, with a conspecific and a heterospecific male (in any order).

Competitive gametic barriers are important isolation processes for preventing hybrid formation between closely related species (Howard, 1999), and have been reported in several insect taxa, including Coleoptera (Hewitt, Mason, & Nichols, 1989; Rugman-Jones & Eady, 2007), Orthoptera (Bella, Butlin, Ferris, & Hewitt, 1992; Gregory & Howard, 1994) and Diptera (Price, 1997). Competitive gametic barriers can be due to three different types of fertilization barriers: (1) competition between ejaculates for fertilization (namely sperm competition), which favours conspecific sperm due to higher competitiveness (Coyne & Orr, 2004); (2) male-driven adaptations

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by the mating male to increase fertilization chances over those of rivals; and (3) cryptic female choice due to preferential storage or usage of conspecific males' sperm (Coyne & Orr, 2004). We are largely unaware of the mechanistic basis that underlies each of these barriers. In particular, two mechanisms have been scarcely explored: the relative contribution of sperm volumes by each male (male-drive mechanisms) and copulation duration as a form of copulatory courtship that females may use to bias paternity (cryptic female choice; Coyne & Orr, 2004).

The blue tailed damselflies *Ischnura elegans* and *I. graellsii* show remarkable morphological (Monetti, Sánchez-Guillén, & Cordero Rivera, 2002) and genetical (Nei's genetic distance is only 0.2%; Sánchez-Guillén, Van Gossum, & Cordero-Rivera, 2005) similarities. Interestingly, unidirectional hybridization, mainly due to differences in secondary genitalia, between *I. graellsii* females and *I. elegans* males has been shown (Monetti et al., 2002; Sánchez-Guillén et al., 2005; Sánchez-Guillén, Wellenreuther, Cordero-Rivera, & Hansson, 2011). A previous study using *I. elegans* and *I. graellsii* assessed the absolute and relative importance of 19 premating prezygotic, postmating prezygotic (non-competitive gametic barriers) and postmating postzygotic barriers (Sánchez-Guillén, Wellenreuther, & Cordero-Rivera, 2012). Notice that relative sperm volume contribution and copulation duration (competitive gametic barriers) were not assessed in this previous study, although these mechanisms may be relevant in damselflies and arthropods in general. Thus more studies in the context of both conspecific and heterospecific males mating with the same female (competitive gametic barriers) are needed. In all these animals, sperm volumes transferred during mating are controlled by the mating male and are explained by sperm competition: males transfer large sperm volumes with a previously mated female to increase the chances of fertilization with respect to those of previous mating males (reviewed by Simmons, 2001). On the other hand, copulation duration is known to be used by insects to bias paternity, favoring males that prolong copulation (for damselflies see Andrés & Cordero Rivera, 2000; Cordero, 1990; Uhía & Cordero Rivera, 2005; for other insects see review by Simmons, 2001). Thus, in the context of ischnurine damselflies, we predict that hybrid formation can be impeded by reduced heterospecific sperm volume and/or shorter copulation duration of the heterospecific male.

To test the above, we first investigated any paternity bias toward the conspecific male in females doubly mated with a conspecific and a heterospecific male. Second, since non-competitive barriers (e.g. an inability of a heterospecific male to fertilize eggs) may explain paternity biases rather than relative sperm volume contribution and copulation duration, the fertility of the doubly mated females (with two conspecific males or one conspecific and one heterospecific male and vice versa) was compared. Notice that when Sánchez Guillén et al. (2012) estimated fertility to investigate non-competitive gametic barriers, a clearly reduced rate of fertility was detected in heterospecific crosses. Third, we inferred sperm proportions (conspecific versus heterospecific) from both female sperm storage organs (bursa and spermatheca). Notice again that Sanchez-Guillén et al. (2012) estimated sperm volumes but this was done to assess the ability to inseminate and the ability of rival's sperm removal by the mating male. In this study we use this information to infer the contribution by each male towards exceeding the sperm volumetric contribution of a rival male. Fourth, we estimated, using conspecific and heterospecific matings of *I. graellsii* and *I. elegans*, copulation duration.

Material and methods

Laboratory rearing

Final instar larvae of both species were collected from a sympatric region in north-west Spain in June 2000: for *Ischnura elegans*, larvae collection was made at Louro (42°69′08.8″N, 8°66′03.5″E) and for *I. graellsii* was made at (42°34′35.29″N, 9°4′30.52″E), Lanzada

(42°25′44.46″N, 8°52′20.20″E) and Alba 156 (42°26′29.39″N, 8°38′40.99″E) localities. Larvae were transported to the laboratory and reared to adulthood (for methodological details see Sánchez-Guillén et al., 2005; Van Gossum, Sánchez-Guillén, & Cordero Rivera, 2003). Males and females of both species were maintained in separate insectaries until they reached maturity (6 days for males and 8–10 days for females) (Sánchez-Guillén et al., 2005, 2012) when they were used in the different experiments.

Paternity in doubly mated females

We estimated paternity using nine females of *I. graellsii* mated to a conspecific and a heterospecific male with an inter-mating interval of one day, as obtaining second matings in the same day is extremely difficult. In three cases the conspecific male was the first mate, while in the remaining six the heterospecific male was the first mate. Again, this inequality was due to the difficulty in getting second matings under laboratory conditions. One day after the second mating, the female was placed in a small jar, with humid filter paper, to oviposit. All females oviposited during three consecutive days or until they reached their third clutch. Eggs were incubated in Petri dishes at room temperature (23–25°C) until hatching (which normally took from 12 to 15 days). We randomly selected 15 larvae of each of the three clutches of each female for paternity analyses (details in Table 1). Additionally and in order to compare paternity in heterospecific and conspecific matings, we used data from Cordero, Santolamazza Carbone, and Utzeri (1998) which consisted of six doubly mated females of *I. graellsii* with conspecific males ($P_2 = 0.82 \pm 0.10$; mean $\pm$ SE).

For paternity between species we used a diagnostic sequence, which includes a partial fragment of the small nuclear ribosomal DNA unit (18 nrDNA), the spacer ITS1, 5.8S gene, the spacer ITS2 and part of the large nuclear ribosomal DNA unit (28 nrDNA) (GenBank accession numbers AF461239 and AJ488545). This sequence is identical in both species except for one position of the ITS1, which shows a transition (T/C) that allows the identification of both species. First we tested the reliability of the selected marker to identify the parental species and the hybrids, and to this end we amplified 14 samples of *I. elegans*, 15 of *I. graellsii*, and 67 laboratory hybrids. After that, we amplified the DNA of 337 larvae from the nine females, to estimate the proportion of *I. graellsii* larvae (sired by the conspecific male) and the number of hybrid larvae (sired by the heterospecific male).

DNA was extracted from larval by proteinase K digestion followed by a standard phenol/chloroform-isoamylalcohol extraction (Sambrook, Fritsch, & Maniatis, 1989). The small sub-unit of rDNA (18 nrDNA), ITS1, 5.8S, ITS2 and part of the large sub-unit of rDNA (28 nrDNA) were amplified using the primers described by Samraoui, Weekers, & Dumont (2002): LITS and H28S. DNA amplification was done in a total reaction volume of 20 μl. The amplification conditions were as follows: 50 ng of DNA (1 μl), 1 unit (0.2 μl) of Taq DNA polymerase (Ecogen, Barcelona, Spain), 2 μl 10× of reaction buffer (Ecogen), 0.5 μl of $MgCl_2$ (50 mM) (Ecogen), 0.5 μl of dNTPs Mix Sigma (200 μM), and 1 μl of each primer (10 pmol). All PCR reactions were completed in a "GeneAmp PCR system 2700" thermocycler (Applied Biosystems, Valencia, Spain). The PCR program had an initial cycle of 95°C for 3 min, the annealing temperature for 1 min, and an elongation period at 72°C for 45 s, followed by 34 cycles at 95°C for 30 s, with annealing for 45 s, and an elongation phase at 72°C for 45 s, and a final extension phase at 72°C for 10 min. The PCR products (10 μl) of 18 samples (six of *I. graellsii*, six of *I. elegans* and six F_1 hybrids) were sent to an external sequencing service (University of Valencia) where bidirectional sequencing reactions were conducted using Bigdye™ terminator cycle sequencing kit (Applied Biosystems, Valencia, Spain) using an automatic sequencer ABI3100 (Applied Biosystems, Valencia, Spain). Forward and reverse sequences were edited in Codon Code Aligned (CodonCode, Dedham, MA, USA). Variable positions were revised by eye, and only high quality sequences

were considered. PCR products of the remaining samples were digested for 1 h at 37°C with 2 μl of buffer (10×) and 0.2 μl of the enzyme Mbo II (Amershan Bioscience, Barcelona, Spain) which showed: (1) in *I. elegans*, three cutting points, resulting in four fragments of 290, 221, 73 and 46 bp; (2) in *I. graellsii*, four cutting points with five fragments of 290, 116, 105, 73 and 46 bp; and (3) in hybrids, five cutting points with six fragments of 290, 221, 116, 105, 73 and 46 bp. All specimens (*I. elegans*, *I. graellsii* and F_1 hybrids) were unequivocally identified, demonstrating that this methodology can be used to estimate paternity in hybrid crossings. To detect differences in the paternity of the two female groups, and given that the experimental unit is the crossing and not the male, we computed a GLM with binomial errors corrected for overdispersion, assuming that each crossing is independent (the same pair of males is not repeated).

Fertility data (from Sánchez-Guillén et al., 2012) of doubly mated females with two conspecific males, or with one conspecific and one heterospecific male and vice versa were analysed using a GLM with a binomial distribution and a logit-link function, with the number of hatched eggs as the response variable, clutch size as binomial totals and treatment as the predictor variable.

Sperm proportions in doubly mated females

By using mean data on sperm volume transferred after one conspecific mating, one heterospecific mating, and two matings (one conspecific and one heterospecific and vice versa), and sperm volume removed from previous matings in conspecific and heterospecific matings (data from Sánchez-Guillén et al., 2012), we calculated the proportion of heterospecific and conspecific sperm in doubly mated females when the conspecific male was the first male, and when the heterospecific male was the first male. We then inferred the volume of sperm transferred by the first and second male, present in the bursa and the spermatheca of doubly mated females. Volume of sperm of the first male depends on the volume of sperm transferred by the first male, and the volume of sperm removed by the second male. However, the volume of sperm of the second male depends on his ability to remove sperm from a previous mating, his ability to inseminate, and also the maximum volume of sperm that the female can store. Thus the proportion of sperm of the first versus the second male in doubly mated females was inferred by using the following formulae. The inferred sperm volume for the first male is simply the volume of sperm transferred by him minus the volume removed by the second male. The second male can, at most, only transfer a volume of sperm equal to the difference between the maximum volume of the female's sperm storage ogans and the volume still occupied by the first male's remaining sperm. We take the maximum volume to be the mean volume for doubly inseminated females that mated with males in the specified order (Table 2), from Sanchez-Guillen et al. (2012), even though this volume is numerically less than the sperm volume of females singly inseminated by *I. graellsi* males. Thus the volume contributed by the second male is equal to: (volume of a doubly inseminated female) – (volume of the remaining sperm of the first male) = (volume of doubly inseminated female) – (volume transferred by first male – volume removed by the second male). In theory, the second male might not have enough sperm available to fill the female storage organs, but the data of Sanchez-Guillen et al. (2012) show that the amount a male can tranfer is normally much more than he removes from the female's existing store. Sperm volumes are provided in mm^3.

Copulation duration

We measured copulation duration in conspecific matings (of both species) and heterospecific matings of *I. graellsii* females and *I. elegans* males (see sample sizes in Table 3). All mating pairs were isolated in a glass jar to prevent other males from disturbing the copulating pair, which could affect copulation duration (see Cordero, Santolamazza Carbone, & Utzeri, 1995). Time of day was

considered in statistical analyses given that it affects copulation duration (see Córdoba-Aguilar & Cordero-Rivera, 2008). Copulation duration was log10 transformed to fulfill normality criteria. A GLM was used to test whether duration was dependent on the mating cross type, with time of day included as a covariate. Post hoc, between-groups comparisons were carried out using Tukey tests. Data are expressed as mean ± STD.

Results

Paternity in doubly mated females

When the heterospecific male was the last to mate, paternity of the second male (P_2) was 0.26 ± 0.26 (three females; 119 larvae). Conversely, P_2 was 0.80 ± 0.10 (six females, 218 larvae) when the last male was conspecific (Table 1). There was a significant effect of type of mating on paternity (deviance ratio = 3.97, $p = 0.026$), an effect mainly due to the zero paternity of two of the three *I. elegans* males when they were the second mate. However, 58% of *I. graellsii* females that mated only with one *I. elegans* male had no sperm in their sperm storage organs (see Sánchez-Guillén et al., 2012). This observation could explain why in three out of the nine crosses no larvae were sired by the heterospecific male. Since we were not interested in defective insemination, we removed such cases. This resulted in a P_2 of 0.79 (one female; 29 larvae) for the sole heterospecific male that was the last male, similar to the mean value for conspecific males when they were the last in a heterospecific mating ($P_2 = 0.77 \pm 0.11$, five females, 173 larvae). Both values are also similar to those of two conspecific males mated to the same female ($P_2 = 0.82 \pm 0.10$, 6 females, 250 larvae) (Table 1).

Paternity of the second male in doubly mated females with a conspecific and a heterospecific male was similar to paternity of the second male in doubly mated females with two conspecific males. There were no differences in fertility for *I. graellsii* females that mated twice with two conspecifics (0.967 ± 0.009, $n = 7$) against females that mated with one conspecific and one heterospecific male (0.979 ± 0.020, $n = 3$; Tukey test, $p = 0.99$) and females that mated with one heterospecific and then with one conspecific male (0.981 ± 0.011, $n = 6$; Tukey test, $p = 0.99$) (Table 1).

Sperm proportions in doubly mated females

As shown in Table 2, in the first set of females (doubly mated females with two *I. graellsii* males), the inferred proportion of sperm from the first (conspecific) male (67.57%) was

Table 1. Paternity of the second male (P_2) and fertility in the first three clutches of conspecific matings between *I. graellsii* females and *I. graellsii* males, and heterospecific matings between *I. graellsii* females with a conspecific and a heterospecific male and vice versa. G (*I. graellsii*), E (*I. elegans*).

Female	First male–second male	N	P_2
Female G 1	Male G1–Male E1	45	0.00
Female G 2	Male G2–Male E2	29	79.31
Female G 3	Male G3–Male E3	45	0.00
Female G 4	Male E1–Male G4	45	97.78
Female G 5	Male E1–Male G5	45	100.00
Female G 6	Male E4–Male G3	45	97.78
Female G 7	Male E5–Male G3	17	88.24
Female G 8	Male E6–Male G6	31	41.94
Female G 9	Male E7–Male G7	35	57.14

Table 2. Sperm proportions of calculations of actual sperm volumes (in mm^3) of first versus second male for both female sperm storage organs (bursa + spermatheca) of doubly mated *I. graellsii* females.

	Sperm volume calculations		Sperm proportion calculations		Sperm proportions (%)	
Male order	First male	Second male	First male	Second male	First male	Second male
Male G–Male G	7.623–3.320	6.323–(7.623–3.320)	4.303 × 100/6.365	2.065 × 100/6.365	67.57	32.43
Male G–Male E	7.623–3.342	6.502–(7.623–3.342)	4.281 × 100/6.502	2.221 × 100/6.502	65.84	34.16
Male E–Male G	5.381–3.320	6.840–(5.381–3.320)	2.061 × 100/6.840	4.779 × 100/6.840	30.13	69.87

higher than from the second (conspecific) male (32.43%). Similarly, the inferred proportion of sperm from the first male (65.84%) was higher than from the second male (34.16%) in the second set of females (doubly mated females: first with *I. graellsii* males and second with *I. elegans* males). However, in the third set of females (doubly mated females: first with *I. elegans* males and second with *I. graellsii* males), the inferred proportion of sperm from the first (heterospecific) male was (30.13%) lower than from the second (conspecific) male (69.87%).

Copulation duration of the first mating

Copulation duration was strongly affected by time of day (Figure 1): matings starting early were longer than those starting late in the afternoon ($F_{1,71} = 27.255$, $p < 0.0001$). Copulation duration varied among groups ($F_{2,71} = 4.919$, $p = 0.01$). In conspecific copulations, mating pairs of *I. graellsii* male and female spent longer times than mating pairs of *I. elegans* male and

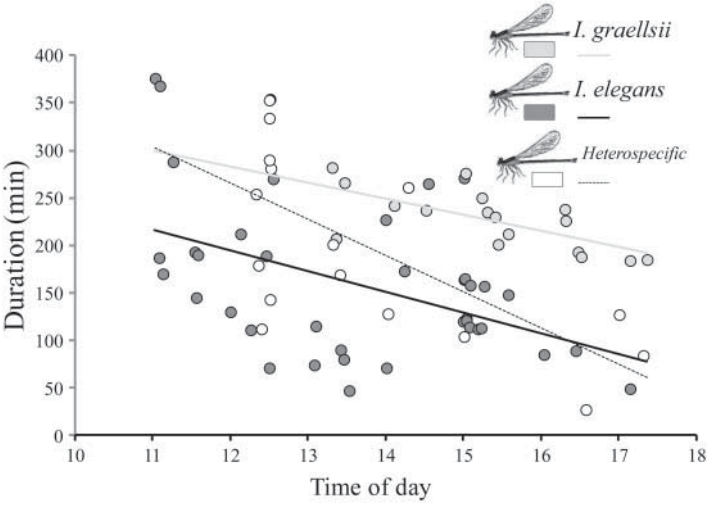

Figure 1. Relationship between time of day and copulation duration (in min) for conspecific matings compared to heterospecific matings between female *I. graellsii* and male *I. elegans*.

Table 3. Copulation duration (mean ± SE; in min) of conspecific matings of *Ischnura graellsii* and *I. elegans* females and in heterospecific mating between *I. graellsii* females and *I. elegans* males in their first mating. G means *I. graellsii* while E means *I. elegans*. Sample size (N) indicates the number of females.

Female	Type of cross	N	mean ± SE
Female G–male G	Conspecific	17	226.6 ± 7.57
Female G–male E	Heterospecific	17	200.1 ± 24.40
Female E–male E	Conspecific	38	158.7 ± 13.06

female (Table 3; Tukey test, $p = 0.011$). Copulation duration in heterospecific matings (200.1 ± 24.40 min) did not differ from either of the two conspecific mating types (p for both after Tukey tests >0.05).

Discussion

Competitive gametic barriers can be important factors preventing hybrid formation. A putative bias in the paternity could be the result of non-competitive mechanisms (i.e. an inability of the heterospecific gametes to fertilize) or two competitive gametic mechanisms (male-driven mechanisms by the mating male and/or cryptic female choice). Our results showed that paternity of the second male in doubly mated females with two conspecific males, and with a conspecific and a heterospecific male, and vice versa did not differ. Paternity was not explained by an inability of heterospecific males to fertilize, because fertility in doubly mated females with two conspecific males and with one conspecific and one heterospecific male and vice versa was similar. However, we cannot rule out a bias in the paternity due to our small sample size.

Interspecific differences in sperm volumes being transferred to females in insects can be explained by the intensity of sexual selection (Simmons, 2001), and in this case may incidentally serve as a competitive barrier of reproductive isolation. In particular, we know little of how sperm numbers of conspecific and heterospecific males, once stored in the female's sperm storage organs, can determine paternity. Actually, *I. graellsii* males transfer more sperm to their own females than *I. elegans* do, but both species are equally able to remove sperm from previous matings (Sánchez-Guillén et al., 2012). Consistent with this observation, we inferred a lower proportion of stored heterospecific sperm in doubly mated females both when the heterospecific male (*I. elegans*) was the first male, and when it was the second male. In our study, we observed that the progeny was mainly sired by the second male, regardless of whether the male was conspecific or heterospecific. This was consistent with previous studies showing that in *I. graellsii* (Cordero & Miller, 1992) and *I. elegans* (Cooper et al., 1996) there is a bias in the paternity toward the second male, and discards in our ischnurines a role of sperm proportions to determinate paternity.

Females may bias paternity in the form of cryptic choice (Coyne & Orr, 2004). One way that this can be done in our ischnurines is via copulation duration, if this is taken as a male copulatory courtship trait. Coenagrionid females use copulation duration as a cue to bias paternity, favoring males that prolong copulation (an effect that is not explained by differential sperm removal; Andrés & Cordero Rivera, 2000). In odonates, copulation is highly variable (Uhía & Cordero-Rivera, 2005); during the first stage the mating male displaces the sperm stored from previous matings from the female's sperm storage organs – the bursa copulatrix and the spermatheca (reviewed by Córdoba-Aguilar, 2003). Interspecific variation in stage I duration has been interpreted as the result of a coevolutionary game between the sexes to retain control of (for the case of females) or have access to (for the case of males) the stored sperm (Cordero-Rivera & Córdoba-Aguilar, 2010). Our results showed that copulation duration of *I. graellsii* tended to be longer than copulation duration of *I. elegans*. This is surprising because in the field, copulations of *I. elegans* are clearly longer (Miller, 1987). This shorter duration in *I. elegans* could be due to the fact that in the laboratory, copulations started relatively late compared to what occurs in the field (see Miller, 1987). Biased paternity by cryptic female choice, however, was not supported by our results, as males of both species spend similar copulation times when mating with *I. graellsii* females. Of course, our results need to be completed by incorporating double matings for each female and/or at least matings of *I. elegans* females with *I. graellsii* males. Although copulation duration may be used as a courtship device, it is by no means the only one. Another candidate for this is sensory stimulation as has been documented in odonates (reviewed by Córdoba-Aguilar & Cordero-Rivera, 2008).

In odonates, no previous studies have elucidated the mechanisms of the competitive gametic barriers. Therefore, according to our evidence it seems that male ability to inseminate the female and remove sperm from previous matings partly explains paternity, and cryptic female choice, mediated by the preferential use of the conspecific sperm, although not detected, cannot be excluded because of our small sample sizes in some cases.

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