First-male sperm precedence and precopulatory and postcopulatory rituals in the parasitoid wasp *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae)

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Abstract—Sperm competition generates selection for male traits to prevent it. These traits remain unclear in species where males compete for a virgin who is briefly receptive. Males of the parasitoid wasp *Ooencyrtus kuvanae* Howard (Hymenoptera: Encyrtidae) compete over females following emergence from host egg masses. Males engage virgins in a precopulatory ritual, mate, and then immediately perform a postcopulatory ritual after which the female becomes unreceptive. Often, sneaker (M_2) males copulate with a female while she is engaged in the postcopulatory ritual, and they also perform the postcopulatory ritual. We investigated (i) paternity of M_1 and M_2 males using DNA microsatellite analysis, (ii) copulation and postcopulatory behaviour of both males, and (iii) morphological adaptations of the aedeagus for sperm removal. Eighty-eight percent of M_1 males sired all daughters when they were first to perform the precopulatory and postcopulatory ritual, suggesting a linked effect of both rituals on paternity. The number and length of copulations by both males did not affect paternity, and the shape of the aedeagus does not seem to facilitate sperm removal. Our results are consistent with the hypothesis that postcopulatory rituals represent forms of mate guarding that function to increase paternity in the context of sperm competition.

Résumé—La compétition spermatique entraîne une sélection des traits mâles qui permettent de l'éviter. Ces traits restent obscurs chez les mâles qui font compétition pour une femelle vierge qui n'est réceptive que sur une courte période. Les mâles de la guêpe parasitoïde *Ooencyrtus kuvanae* Howard (Hymenoptera: Encyrtidae) se font compétition pour les femelles lors de leur émergence des masses d'œufs de l'hôte. Les mâles entreprennent un rituel précopulatoire avec les femelles, s'accouplent et complètent immédiatement un rituel post-copulatoire à la suite duquel les femelles ne sont plus réceptives. Souvent, des mâles intrus (M_2) s'accouplent avec la femelle durant son rituel post-copulatoire et pratiquent aussi eux-mêmes le rituel postcopulatoire. Nous avons étudié *i*) la paternité chez les mâles M_1 et M_2 par analyse des microsatellites d'ADN, *ii*) la copulation et le comportement postcopulatoire chez les deux types de mâles et *iii*) les adaptations morphologiques de l'édéage pour le retrait du sperme. Quatre-vingt-huit pour cent des mâles M_1 engendrent toutes les guêpes filles lorsqu'ils sont les premiers à compléter les rituels copulatoire et postcopulatoire, ce qui laisse croire à un lien effectif entre ces deux rituels et la paternité. Le nombre et la durée des accouplements chez les deux types de mâles n'affectent pas la paternité et la forme de l'édéage ne semble pas faciliter le retrait du sperme. Nos résultats s'accordent avec l'hypothèse selon laquelle les rituels postcopulatoires représentent une forme de surveillance du partenaire dont la fonction est d'augmenter la paternité dans une situation de compétition spermatique.

Introduction

Sperm competition, the process by which spermatozoa of two or more males compete to fertilise the egg(s) of a lone female, occurs when females mate with multiple males in a single breeding bout (Parker 1970; Simmons 2001). Evolutionary responses that help males avoid or

Received 29 June 2013. Accepted 3 October 2013. First published online 28 March 2014.

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Subject editor:Heather Proctor
doi:10.4039/tce.2014.7

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https://doi.org/10.4039/tce.2014.7 Published online by Cambridge University Press

cope with sperm competition include morphological, physiological, and behavioural adaptations (Knowlton and Greenwell 1984; Simmons 2001).

In species with internal fertilisation, a female may store sperm from competing males in her reproductive tract or sperm storage organ (*e.g.*, spermatheca) for some time before fertilisation, thus setting the stage for intense and potentially prolonged sperm competition. In response, males may achieve first-male sperm precedence if they reduce competition from subsequent rival sperm by (i) depositing copulatory plugs to prevent female re-mating (Simmons 2001), (ii) reducing a female's attractiveness to other males through substances in the seminal fluid (Simmons 2001), (iii) prolonging the duration, or increasing the frequency, of copulations (Thornhill 1984), and (iv) by engaging in postcopulatory interactions such as grasping or guarding the mated female (Gwynne 1984; Alcock 1994). Precopulatory mate guarding also favours first-male sperm precedence; *e.g.*, in species where females trigger male competition as a mechanism of mate choice and females choose to mate with the strongest or fastest male (Brown *et al.* 1997), but postcopulatory rituals may represent an adaptation to sperm competition if mated females would otherwise remain receptive (Alcock 1994).

Prolonged interactions, whereby a male will stay near, or remain in contact with, a receptive female following insemination, may evolve in response to direct competition from rival males attempting to mate with that female (Alcock 1994). Under this scenario, postcopulatory interactions are expected to take longer when receptive females are limited and difficult to secure (Parker 1974; Alcock 1994; Simmons 2001), and to intensify when the second or last male to mate (henceforth M_2 male) is likely to deposit sperm that will be used to fertilise a greater proportion of eggs than sperm from the first male (henceforth M_1 male) due to last-male sperm advantage (Boorman and Parker 1976; Simmons 2001).

Postcopulatory interactions may affect fertilisation in species with cryptic mate choice, whereby females manipulate sperm storage and select sperm from particular partners for egg fertilisation (Gromko *et al.* 1984; Eberhard 1996). Such interactions may also help advertise a male's quality before egg fertilisation (Simmons 1990), or help ensure that mated females are less receptive to other males (Eberhard 2009).

A specific form of postcopulatory interaction and postcopulatory rituals occur in some species of parasitoid wasps (Mackauer 1969). Such rituals generally resemble courtship interactions, involving more or less stereotypical behaviour directed towards females by males, but they occur only after mating, and their adaptive significance has remained largely enigmatic (Viggiani and Battaglia 1983; van den Assem 1986; King and Fischer 2005).

Several hypotheses have been proposed to help explain the evolution of postcopulatory rituals. In some parasitoid wasps, postcopulatory rituals may have evolved in response to selection pressure from rival males (van den Assem *et al.* 1980) who attempt to mate with a female while she is still in a receptive state from her interaction with the first-mating male. These rituals then function as a form of mate guarding by reducing the efficacy of mating attempts by rival males and/or by leading to reduced female receptivity (Allen *et al.* 1994; King and Fischer 2005). For example, following copulation, males of the parasitoid *Pteromalus puparum* Linnaeus (Hymenoptera: Pteromalidae) continuously move the female's abdomen, apparently to better detect a rival's attempt at copulating with her (Thornhill and Alcock 1983), and males of the parasitoid *Cotesia rubecula* Marshall (Hymenoptera: Braconidae) implement female mimicry to distract their rivals (Field and Keller 1993).

In general, rival males who are not detected or distracted could possibly mate with a female. In such circumstances, some males may attempt to increase the chances that their sperm is selected by strategically repositioning or removing rival sperm from entering the female's storage organ (Gromko *et al.* 1984; Thornhill 1984; Simmons 2001), via prolonged duration *in copula*, multiple copulatory bouts, and/or specialised morphological structures (Thornhill and Alcock 1983). Generally, successful sperm removers and sperm repositioners require between seven seconds and 20 minutes of copulation time (Waage 1984); most parasitoids do not devote that much time to the copulatory stage within a mating sequence (Gordh and DeBach 1978; Allen *et al.* 1994; King and Fischer 2005; Ablard *et al.* 2011).

Multiple matings are rare among parasitoids (Gordh and DeBach 1978; Ridley 1993; Benelli *et al.* 2013). Males that reposition and/or remove

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sperm tend to achieve last-male sperm precedence. Last-male sperm precedence is rare and the mechanism is ambiguous. For example, males of the parasitoid wasp *Diachasmimorpha longicaudata* Ashmead (Hymenoptera: Braconidae) achieve sperm precedence when they re-mate a female after 24 hours; the underlying mechanism could be the loss of M_1 sperm from the female's storage organ between copulations (Simmons 2001), with M_1 male sperm not being used or retained by the female (Martinez-Martinez *et al.* 1993). It is also unclear whether *D. longicaudata* males who achieve first-male sperm precedence also perform a precopulatory ritual, and/or a postcopulatory ritual that functions as a form of mate guarding, as evidenced in males of the parasitoid wasps *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae), *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), and *Lariophagus distinguendus* Förster (Hymenoptera: Pteromalidae) (Holmes 1974; van den Assem *et al.* 1989; Allen *et al.* 1994; Kuhbandner *et al.* 2012; Benelli *et al.* 2013).

Both precopulatory and postcopulatory rituals exist in the quasi-gregarious (with one offspring per aggregated host), 2-mm egg parasitoid wasp *Ooencyrtus kuvanae* Howard (Hymenoptera: Encyrtidae). Courtship and mating take place on egg masses of host gypsy moth, *Lymantria dispar dispar* Linnaeus (Lepidoptera: Erebidae). An egg mass measures 2–3 cm across and contains several hundred eggs covered in setae (Brown 1984). Eggs in the uppermost layer are parasitised by female wasps that insert a single egg into each accessible host egg. By fertilising an egg, arrhenotokous females produce a daughter, and by not fertilising it, they produce a son. Haploid sons derive their entire genome from their mother, whereas daughters are diploid and receive genes from both parents. Between three to four weeks, wasps complete development inside host eggs and emerge *en masse* as sexually mature adults that can live four to six weeks. Females emerge up to one day later than males, are immediately receptive to mating, and are about twice as numerous as males. However, a local, adult male-biased sex ratio occurs frequently among nonsiblings (Somjee *et al.* 2011) because males typically remain on the host egg mass as long as there are mating opportunities, whereas mated females disperse within 24 hours, seeking new gypsy moth egg masses (Brown 1984).

Courtship is mediated by a close-range sex pheromone that attracts males to females (Ablard *et al.* 2012). Males then implement one of two alternative mating tactics. They either pheromone-tag a female and at a later time engage her in the mating sequence, or they immediately engage her in the mating sequence (Ablard *et al.* 2013). The mating sequence consists of a brief (~4 seconds) precopulatory ritual, mating (4–9 seconds), and a relatively longer (15–67 seconds) postcopulatory ritual (Ablard *et al.* 2011).

During the precopulatory ritual, the females are placed in a "trance-like" (unmoving, unresponsive) state (henceforth "trance") that persists for some time after copulation (Ablard *et al.* 2011). The behavioural mechanisms underlying the postcopulatory ritual resemble those of the precopulatory ritual; the male interlocks the female's antennae with his and then proceeds to strike her antennae with his legs. In contrast to the precopulatory ritual, he uses his forelegs in a random rather than repetitive or synchronous pattern of strikes. The female then strikes back at the male with her forelegs. If she is interrupted and becomes motionless, the male resumes his strikes. Thus, the postcopulatory ritual may function as a form of mate guarding to accelerate the "awakening" of the entranced mated female, who then rejects all mating attempts by other males over the course of her lifetime, ensuring paternity of the M_1 male (Ablard *et al.* 2011).

Following our reports (Ablard *et al.* 2011, 2012, 2013) that *O. kuvanae* females mate only once and then become unattractive to males, we most recently noticed "sneaker" (M_2) males in the *O. kuvanae* mating system (K.M.A., personal observation). In highly competitive settings, these M_2 males do not directly compete for mating opportunities. Instead, M_2 males copulate with an in-trance female, when she is either *in copula* with a M_1 male, or postmating when she is coming out of the trance and engaged in the postcopulatory ritual with the M_1 male, thus possibly siring some or all of the female's daughters.

Interpretation of the adaptive significance of male and female mating behaviour in *O. kuvanae*, and other species with postcopulatory rituals, depends critically on patterns of sperm precedence and use. In this study, we investigated the presence of sperm competition in *O. kuvanae* by testing paternity of M_1 and M_2 males using

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DNA microsatellite analysis. We predicted that (1) there is first-male sperm precedence, as reported in parasitoids where, like in *O. kuvanae*, males perform a precopulatory and a postcopulatory ritual, and mated females remain receptive only briefly; (2) males will not have multiple or lengthy copulatory bouts that are often associated with long periods of receptivity in females and last-male sperm precedence; (3) the postcopulatory ritual represents a male tactic to reduce sperm competition; and (4) that males do not possess morphological adaptations for removal of rival male sperm because such adaptations are associated with lengthy, rather than brief, copulatory bouts.

Materials and methods

Experimental insects

A new colony of *O. kuvanae* was started with specimens field collected from *Quercus* Linnaeus (Fagaceae) hardwood forests in the town of North East, Maryland, United States of America (39°36′ N, 75°55′W). All insects were reared under a 16:8 hour light:dark photoperiod at 22–25 °C and 50–70% relative humidity (Hofstetter and Raffa 1997) in the Global Forest Quarantine Facility of Simon Fraser University, Burnaby, British Columbia, Canada. They were contained in Plexiglass cages (40 × 40 × 30 cm) and provided with cotton wicks (1 × 10 cm; Richmond Dental, Charlotte, North Carolina, United States of America) soaked in a 30% honey water solution (w:v) every two days. Ten gypsy moth egg masses, supplied by the United States Forest Service (Hamden, Connecticut, United States of America), were introduced to be parasitised by female wasps. Fourteen days later, parasitised egg masses were removed and 1000 eggs were placed singly into translucent plastic cups (103.5 mL) and secured with a lid. Emergent insects were separated by sex and size under a microscope and used in the experiment within one day of emergence to avoid adverse effects associated with ageing (van den Assem 1996).

Attaining twice-mated females

To produce twice-mated females, four males without prior contact with a female were confined with one virgin female (n = 10) in a Petri dish (30 mm diameter). This competitive setting increased the likelihood that the female would be mated by a sneaker (M_2) male while she was still in the trance and receptive state following the precopulatory ritual and copulation, and before the completion of the postcopulatory ritual with the M_1 male. Following the completion of the postcopulatory ritual by the M_1 and/or M_2 male, the two males and the twice-mated female were removed from the arena; the two males that did not mate were discarded. Two observers using a digital voice recorder equipped with a time tracker continuously tracked and documented the mating order of the two males that mated, their number of copulatory bouts, time spent *in copula*, and the number of postcopulatory ritual bouts and time spent engaged in the postcopulatory ritual. Due to the small size of the insects, morphological markers were not used. Mated females were placed singly in glass jars provisioned with food and 40 gypsy moth eggs, which according to pre-experiments provide sufficient oviposition opportunities to a twice-mated female (K.M.A., personal observation). After 21 days, the females were removed and the 40 eggs were placed singly into plastic cups to prevent mating between offspring. Emergent daughters and sons were counted. Parents and daughters were stored singly in 2.0 mL Qiagen® sterile microcentrifuge tubes (Company, Toronto, Ontario, Canada) at − 80°C until DNA extraction.

DNA library construction, screening, and enrichment

Methods for DNA library construction, enrichment, and screening are published elsewhere (Jones *et al.* 2002) and were applied by Genetic Identification Services (GIS, Chatsworth, California, United States of America). Genomic DNA was partially restricted with a cocktail of seven blunt-end cutting enzymes. Fragments that ranged between 300 and 700 base pairs in length were adapted and subjected to magnetic bead capture (CPG, Lincoln Park, New Jersey, United States of America), using biotinylated capture molecules. Libraries were prepared in parallel, using Biotin-CA(15), -AAG(12), -AAT(12), and -ATG(12) as capture molecules in a protocol provided by CPG (Lincoln Park, New Jersey, United States of America). Captured molecules were amplified and restricted with HindIII to

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remove the adapters. The resulting fragments were ligated into the HindIII site of pUC19. Recombinant molecules were electroporated into *Escherichia coli* (Enterobacteriales: Enterobacteriaceae) DH5α. Recombinant clones were selected at random for sequencing, and enrichment levels were expressed as the fraction of sequences that contained a microsatellite. Sequences were obtained on an ABI 377 or an ABI 3730, using ABI Prism Taq dye terminator cycle sequencing methodology. Microsatellite-containing sequences were identified by inspection, polymerase chain reaction (PCR) primers were designed using DesignerPCR version 1.03 (Research Genetics Inc., Huntsville, Alabama, United States of America), and they were purchased from Integrated DNA Technologies (Coralville, Iowa, United States of America). The optimal amplification reaction mix for all primer pairs consisted of 1× Biolase© Buffer from a 10× stock solution supplied by Bioline (Taunton, Massachusetts, United States of America), 2 mM $MgCl_2$, 0.2 mM of each dNTPs, 6 µM of each primer, 0.025 U/µL Biolase DNA Polymerase (Bioline), and 0.2 ng/µL template DNA in a 50-µL final reaction volume. Samples were amplified in a Perkin-Elmer-Cetus thermal cycler by an initial denaturation at 94 °C (180 seconds), followed by 35 cycles of 94 °C (40 seconds), 55 °C (40 seconds), and 72 °C (30 seconds), with a final extension of 72 °C (240 seconds). DNA was extracted using the DNeasy Blood and Tissue® kit (Qiagen, Germantown, Maryland, United States of America) according to the manufacturer's protocol. Microsatellite loci were amplified in 10-µL reactions in the following reaction mix: $MgCl_2$, 2 mM; dNTPs (premixed), 0.2 mM each; primers, 0.3 µM each; Biolase DNA Polymerase® (Bioline), 0.025 U/µL; template DNA, 0.2 ng/µL. PCR was conducted in a RoboCycler Gradient 96® thermocycler (Stratagene, La Jolla, California, United States of America), using the same protocol as above. PCR products were separated on 3.5% agarose gels, and stained with ethidium bromide to identify polymorphic loci; six loci were polymorphic (A1, A3, A106a, A107, B105, and D106) and limited to this study.

DNA extraction

Frozen-stored specimens were transferred to a bed of ice before being crushed with a sterile plastic micropipette tip. Immediately postcrushing, DNA was extracted using the microLysis®-Plus kit (Gel, San Francisco, California, United States of America) following the manufacturer's protocol except that 40 instead of 20 µL of microLysis®-Plus were used.

PCR

DNA paternity analyses were based on four microsatellite loci. Genomic DNA was amplified with PCR blends that contained 5.15 µL ddH_2O, 1.0 µL of 10× enzyme buffer, 1.0 µL of 25 mM of $MgCl_2$, 0.8 µL of 2.5 mM of dNTPs, 0.3 µL mix of 10 mM forward-labelled primer [700 series] (Integrated DNA Technologies), and unlabelled forward primer, 0.3 µL of 10 mM reverse primer, 0.05 µL of *Taq* DNA polymerase (GenScript, Piscataway, New Jersey, United States of America), and 1 µL of 2 ng/µL template DNA. The sequences of the designed primers were as follows: A1-F: 5′-CCC GTA TTA TAG ACG TTC GTA C-3′; A1-R: 5′-GCA AAA TTG CAC ATA TAC ACA G-3′; A106a-F: 5′-AGA GCA TAA GCC GTC GTC-3′; A106a-R: 5′-GCG AAG CAC ACA CAA CTG-3′; A107-F: 5′-TTG GTC TCT CTT TCT CTC CTG-3′; A107-R: 5′-GCA GTG CTG TTG CTG TTA C-3′; B105-F: 5′-TCG CTC TCT CGC TTG TTC-3′; B105-R: 5′-AGT TGG TCA GGA GGG TGA G-3′. PCR reactions were denatured at 94 °C (180 seconds), followed by 30 cycles of 94 °C (40 seconds), 58 °C (40 seconds), 72 °C (30 seconds), and a final extension step of 72 °C (320 seconds). We added 2 µL of formamide and bromophenol blue loading dye to PCR mixtures that were electrophoresed through a 10% polyacrylamide gel with a 1× TBE buffer at 1500 V and 45 °C for 1.5–2.0 hours on a LI-COR 4300 genetic analyser (Lincoln, Nebraska, United States of America). Products were visualised for paternity analysis on LI-COR gel images. Parents and offspring were run with a positive control generated from the initial testing of the primers.

A total of 157 wasps were genotyped. This large sample size is extraordinary given the requisites (i) to develop a novel and effective experimental protocol for testing paternity in an egg parasitoid wasp using DNA microsatellite markers, (ii) to visually track and attain twice-mated females (see above) and their morphologically identical mates within highly competitive settings (one female: four males),

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(iii) to establish and maintain large controlled broods for daily experimental replicates, and (iv) to extract DNA from extremely small and delicate specimens. Replicates (n = 10) consisted of 30 parents (10 females and 20 males) and 127 daughters, totaling 157 wasps, which were genotyped. This large dataset proved appropriate for the application of robust statistical analyses.

Microstructure: the male aedeagus

The microstructure of the males' aedeagus was examined by means of photomicrographic imaging and environmental scanning electron microscopy (ESEM). Aedeagi of one-day-old virgins (n = 4) protruded without force when males were placed on dry ice. Photomicrographic images were obtained with a Nikon Microphot-FX EPI microscope (Japan) and SPOT software v. 4.6 (SPOT Imaging Solutions, Sterling Heights, Michigan, United States of America). ESEM images were obtained by mounting insects onto a peg (12.7 mm diameter) covered with a conductive carbon adhesive tab, and by imaging with the ESEM FEI Quanta FEG 4000 (FP Innovations, Hillsboro, Oregon, United States of America) at magnifications of 1500×, 2500×, and ⩾5000× within a chamber kept at ambient temperature, using 1.5 T pressure, an accelerating voltage of 15 kV, and a Gaseous Secondary Electron Detector with a 1-mm aperture.

Statistical analyses

A paired *t*-test was used to compare the mean number of copulations, the mean duration *in copula,* and the mean duration of the postcopulatory ritual recorded from M_1 and M_2 males. The data were tested for normal distribution using a Kolmogorov–Smirnov test. A Pearson's correlation was used to test for a linear relationship between (i) the duration of copulation and the number of daughters sired by M_1 and M_2 males, and (ii) the duration of the postcopulatory ritual and the number of daughters sired by M_1 and M_2 males.

Fragment sizes (base pair) were scored from LI-COR gel images and assigned paternity probabilities with the computer program COLONY v 2.0 (Jones and Wang 2010), which assigns paternity based on maximum-likelihood. To accurately assign paternity, COLONY requires additional information regarding the mating and genetic system of the species. For these analyses, females were considered polygamous because they could mate with more than one male, while males were considered monogamous because they were constrained to mate with only one female in our experiment. This programme also allowed us to specify the genetic background of the species, which is haplodiploid. Other parameters were constrained to reflect the facts that the female in the experiment was the only possible mother and that each male had a 50% chance of being the sire of the offspring. In addition, we specified a low genotyping error rate (0.00001) and indicated that inbreeding may occur in this species.

A male was assigned paternity for each daughter within a brood if the COLONY-issued probability was 1.000, except for replicates 3, 8, and 10 where paternity was assigned to a total of 14 males whose overall probability of paternity did not equal 1.000, but averaged 0.70. Replicate 3 resulted in an average probability of 0.50 for four daughters; replicate 8 resulted in an average probability of 0.80 for eight daughters; and replicate 10 resulted in an average probability of 0.70 for two daughters. All probabilities were individually tested for each replicate using a binomial distribution. For each replicate, exclusion probabilities were calculated in Microsoft Excel (Microsoft Canada, Mississauga, Ontario, Canada) on alleles of an individual locus; a mean was then calculated for all loci. Descriptive statistics were used to calculate P_2 values, and a paired *t*-test was used to compare the mean number of offspring sired by M_1 and M_2 males. Nonpaternity assignment analyses were run with PASW v. 18.0 software. The confidence interval for all tests was set at 95%.

Results

Postcopulatory ritual behaviour of M_1 and M_2 males

Seventy percent of M_2 males engaged the female in a postcopulatory ritual, either by performing the ritual concurrently (n = 3) with M_1 males, or after they physically prevented the ritual (n = 4) of M_1 males. The time M_1 and M_2 spent engaged in the postcopulatory ritual differed (M_1:$\overline{X}$ = 18.30 ± 2.825 seconds, M_2:$\overline{X}$ = 5.90 ± 1.574 seconds; t_9 = 3.730, P = 0.005) (Table 1).

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Table 1. Precopulatory ritual (precop) and postcopulatory ritual (postcop) duration (seconds) of the first male (M_1) and the second male (M_2) to mate.

Replicate	Precop (s) M_1	Precop (s) M_2	Postcop (s) M_1	Postcop (s) M_2
1	3	0	19	0
2	5	0	17	0
3	9	1	2	11
4	2	7	9	8
5	4	0	28	14
6	3	0	28	9
7	3	0	21	8
8	3	0	29	0
9	2	0	19	6
10	3	3	11	3
$\bar{x} \pm$ SE	3.70 ± 0.6501	1.10 ± 0.7218	18.30 ± 2.825	5.90 ± 1.574

Paternity assignment

Nearly all (98%) daughters were assigned to sires. The exclusion probability (probability that potential sires were excluded on genetic incompatibility alone) averaged 84% over all replicates (Table 2). M_1 males sired more daughters than M_2 males (M_1: $\bar{x} = 11.60 \pm 1.899$, M_2: $\bar{x} = 1.10 \pm 0.823$; $t_9 = 4.426$, $P = 0.002$). Mixed paternity was inferred for only two of 10 broods, resulting in an overall low P_2 value (Table 3).

Copulation behaviour of M_1 and M_2 males

Eight out of 10 M_2 males copulated with the female while the M_1 male was engaging her in the postcopulatory ritual. Two M_2 males copulated with the female shortly after the M_1 male had begun copulating. The number of copulations M_1 ($n = 14$) and M_2 ($n = 13$) males attained did not differ (M_1: $\bar{x} = 1.40 \pm 0.221$, M_2: $\bar{x} = 1.30 \pm 0.153$; $t_9 = 1.000$, $P = 0.343$), and copulation durations of M_1 and M_2 males did not differ (M_1: $\bar{x} = 9.40 \pm 1.869$ seconds, M_2: $\bar{x} = 10.30 \pm 1.309$ seconds; $t_9 = -0.462$, $P = 0.655$). There was no correlation between (i) the mean copulation duration and the mean number of offspring sired by M_1 males ($r = -0.199$, $P = 0.582$), and (ii) the mean duration of copulation and the mean number of offspring sired by M_2 males ($r = 0.018$, $P = 0.962$).

Microstructure of the males' aedeagus

The aedeagus (~7 μm in length; SE = 0.04) of males has no morphological characteristics indicative of a function in sperm removal or displacement. The pointed, rather than arched, tip lacks hooks and spines (Fig. 1A). The grappling hooks (Fig. 1B) are likely clasping organs that help grasp the female during copulation.

Discussion

First-male sperm precedence

In the *O. kuvanae* mating system, the high P_1 value (0.91) for the first-mating (M_1) male and the corresponding low P_2 value (0.09) of sneaker (M_2) males are suggestive of strong first-male sperm precedence, assuming that M_2 males did transfer sperm (Martel *et al.* 2008). In other insect species, low P_2 values usually stem from female preference for a M_1 male, low numbers of copulations, short durations *in copula*, adaptations to first-male sperm usage, unsuccessful copulations due to poor performance, or effective postcopulatory guarding by M_1 males (Simmons 2001; Shuster and Wade 2003). In our study, the high fertilisation success of M_1 males was not associated with the number of copulations or time spent *in copula*, however M_1 males of *O. kuvanae* engage the mated female in a postcopulatory ritual as a form of postcopulatory mate guarding which is associated with a state of nonreceptivity in the female (Ablard *et al.* 2011).

Although M_1 males sired all of the offspring in many matings, our observations and microsatellite data show that M_2 males can mate successfully, but sire very few offspring

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Table 2. Loci, alleles of parents, daughter genotypes and their proportions.

Replicate	Loci	Mother		M_1	M_2	Daughter genotypes (genotype %)	P-value, EP (%)
1 (n = 12)	A106a	238	238	250	238	250, 238 (100)	< 0.0001, 0.87
	A107	192	192	192	200	192, 192 (100)	
	B105	249	249	259	249	259, 249 (100)	
2 (n = 7)	A106a	254	254	254	238	254, 254 (100)	= 0.016, 0.83
	A107	200	200	204	194	204, 200 (100)	
	B105	240	240	240	244	240, 240 (100)	
3 (n = 4)	A107	200	200	200	184	200, 200 (25); 184, 200 (75)	= 0.625, 0.91
4 (n = 8)	A106a	246	254	246	254	246, 254 (100)	= 0.008, 0.86
	A107	180	180	198	180	198, 180 (100)	
5 (n = 13)	A106a	252	240	240	252	240, 252 (100)	< 0.0001, 0.72
	A107	206	198	192	198	192, 206 (100)	
	B105	247	244	247	262	247, 244 (100)	
6 (n = 16)	A1	242	248	242	254	242, 248 (100)	< 0.0001, 0.85
	A106a	254	254	254	244	254, 254 (100)	
	B105	251	251	251	235	251, 251 (100)	
7 (n = 21)	A106a	246	246	246	238	246, 246 (100)	< 0.0001, 0.86
	A107	200	200	200	184	200, 200 (100)	
8 (n = 19)	A1	254	246	246	240	246, 246 (37); 254, 246 (63)	< 0.0001, 0.80
	A106a	244	252	252	244	252, 252 (58); 252, 244 (42)	
9 (n = 11)	A106a	242	242	242	252	242, 242 (100)	= 0.001, 0.88
	A107	188	188	200	188	200, 188 (100)	
10 (n = 16)	A106a	242	242	242	250	242, 242 (50); 250, 242 (50)	
	A107	188	204	206	198	198, 204 (25); 188, 188 (6); 198, 188 (13); 206, 188 (38); 204, 204 (6); 206, 204 (6); 198, 200 (6)	= 1.000, 0.84

Note: P-value representing probability that the first male to mate (M_1 male) sired each daughter (n), and mean exclusion probability (EP) proportion for each replicate.

(low P_2 value). This outcome may be associated with the effective postcopulatory guarding by M_1 males that decreases the receptivity of the female. Alternatively, morphological attributes of the female reproductive tract and/or cryptic sperm choice by females may have influenced the fertilisation success of M_1 and M_2 males.

Copulation behaviour between M_1 and M_2 males

In our study, M_1 and M_2 males of *O. kuvanae* copulated equally often, suggesting that the number of copulations does not function as a male-driven mechanism to increase their fertilisation success. For example, when both the M_1 and the M_2 male copulated twice with the same female in each of replicates 3 and 9, and for circa the same duration each time, the M_2 male in replicate 3 shared paternity and sired more daughters than the M_1 male, whereas the M_2 male in replicate 9 sired no daughters (Table 3). By contrast, competing males of the fly *Dryomyza anilis* Fallén (Diptera: Dryomyzidae), and of the scorpionfly *Panorpa germanica*

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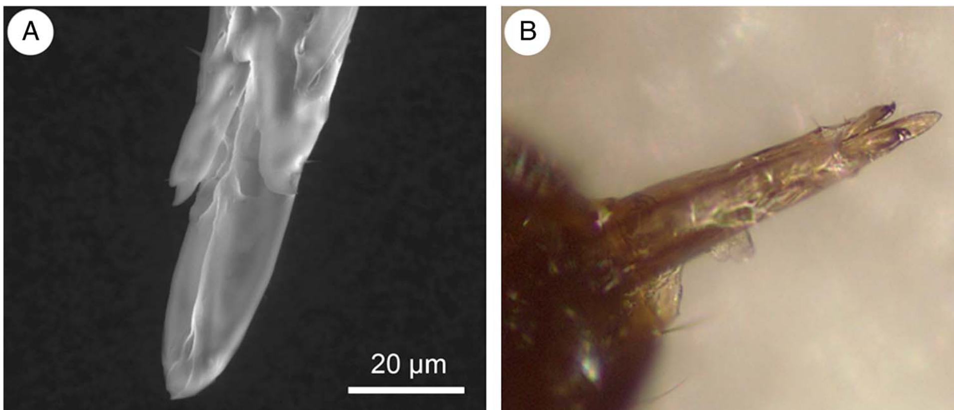
Table 3. Number of daughters sired by the first male (M_1) and the second male (M_2) to mate.

Replicate	Daughters sired M_1	Daughters sired M_2	P_2 values (95% CI limits)	List of key behaviour[1]
1	12	0	0 (0, 0)	M_1 performed pcr first
2	7	0	0 (0, 0)	M_1 performed pcr first
3	1	3	0.75 (0.2194, 0.9868)	M_1 and M_2 copulated with the same female 2 times M_1 was *in copula* 2 seconds longer than M_2. M_2 performed pcr first
4	8	0	0 (0, 0)	M_1 performed pcr first
5	13	0	0 (0, 0)	M_1 performed pcr first
6	16	0	0 (0, 0)	M_1 performed pcr first
7	21	0	0 (0, 0)	M_1 performed pcr first
8	19	0	0 (0, 0)	M_1 performed pcr first
9	11	0	0 (0, 0)	M_1 and M_2 copulated with the same female 2 times and for circa the same duration. M_2 performed pcr first, but M_1 engaged female in pcr 3 additional times
10	8	8	0.5 (0.2551, 0.7449)	M_2 was *in copula* 6 seconds longer than M_1, but M_1 performed pcr first
Total	116	11	0.086 (0.0462, 0.1532)	

Notes: P_2 values (proportions of offspring sired by M_2 male), 95% confidence interval (CI) limits, and list of key courtship and mating behaviour (pcr = postcopulatory ritual).

[1]88% of M_1 males were first to perform the precopulatory ritual; M_2 males performed the precopulatory and postcopulatory rituals in replicates 3, 4, and 10, and had shared paternity in replicates 3 and 10.

Fig. 1. Environmental scanning electron micrograph (A) and photomicrographic image (B) of the proximal tip of a male *Ooencyrtus kuvanae* aedeagus. Note the absence of spines, which could serve in sperm removal.



Linnaeus (Mecoptera: Panorpidae), increase their fertilisation success with the number of copulatory bouts (Otronen 1994; Kock and Sauer 2007).

The duration of a male's copulatory bout can increase the number of offspring he sires (Simmons 2001) but this does not apply to male *O. kuvanae*; M_1 males sired 10 times more

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offspring than M_2 males, yet M_2 males remained *in copula* on average for 50% longer than M_1 males. These results firmly corroborate our prediction that the number of copulations and the time spent *in copula* are not male-driven adaptations to sperm competition.

Prolonged duration of a copulatory bout may also provide the time needed for sneaker males to remove M_1 male sperm and deliver their own (Simmons 2001). However, similar to *Trichogramma euproctidis* (Girault) (Hymenoptera: Trichogrammatidae) (Damiens and Boivin 2006), this does not apply to the *O. kuvanae* mating system; the aedeagus of males simply lacks any attributes that could facilitate removal or displacement of M_1 male sperm by M_2 males. In contrast, in mating systems with last-male sperm precedence, the males' aedeagus of some species assumes a unique shape or is fitted with spines or hooks capable of displacing a competitor's sperm (Thornhill and Alcock 1983). For example, in the dragonfly *Sympetrum rubicundulum* Say (Odonata: Libellulidae) two long and coiled structures of the males' aedeagus fit into a paired spermatheca and push M_2 sperm deeper into the spermatheca while flushing out M_1 male sperm, resulting in last-male sperm precedence (Thornhill and Alcock 1983).

Mate guarding

As the reproductive success of M_1 and M_2 *O. kuvanae* males was not coupled to the number and duration of copulations with the same female, or aedeagus morphology, the underlying mechanisms of first-male sperm precedence in *O. kuvanae* appear to include both precopulatory and postcopulatory mate guarding as adaptations to reduce sperm competition. The precopulatory ritual is associated with the female entering a "trance" and receptive state (Ablard *et al.* 2011). Effectively, a female exhibits mate choice by engaging in the precopulatory ritual with the first male to contact her. He proceeds to mate with her and then immediately performs the postcopulatory ritual, which results in her exit from the trance and becoming unreceptive (Ablard *et al.* 2011). Males that first encounter a female may be favoured in species such as *O. kuvanae* that engage in intense, time-limited scramble competition among males for matings. Potential sperm choice by females would favour, by default, the first male to perform the postcopulatory ritual, which typically is the same male to have contacted and engaged her in the precopulatory ritual. Whether M_1 males would sire most offspring had they not performed the postcopulatory ritual first has yet to be determined.

In seven out of 10 replicates where the M_1 male sired all of the daughters, he was first to engage the female in the precopulatory ritual and in the postcopulatory ritual (Table 3). Conversely, the M_2 males in replicates 1, 2, 5–7, and 9 who did not sire daughters (Table 3) did not perform the precopulatory ritual; yet all attempted the post-copulatory ritual. These data suggest a linked effect of precopulatory and postcopulatory rituals on paternity. For example, of the three replicates where the M_1 and the M_2 males performed both the precopulatory and postcopulatory rituals, two replicates (3 and 10) (Table 3) resulted in shared paternity. In replicate 3, the M_1 male performed the precopulatory ritual but failed to initiate the postcopulatory ritual before the M_2 male did. In replicate 10, both the M_1 and the M_2 male performed the precopulatory ritual and in the same order engaged the female in the postcopulatory ritual. These results combined clearly indicate that sperm competition can occur in *O. kuvanae.* Observations that (i) males invariably engage a female in the postcopulatory ritual immediately after mating (Ablard *et al.* 2011), (ii) fiercely compete over postcopulatory ritual rights (this study), and (iii) share paternity if the M_2 male performs the precopulatory and postcopulatory ritual, all suggest that first-male sperm precedence is linked to the precopulatory and postcopulatory rituals, and that the completion of the postcopulatory ritual renders the mated female unreceptive and helps prevent sperm competition.

Theoretical models of mating systems predict that males should abandon their mates immediately after mating, if there is strong first-male sperm precedence (Simmons 2001). Such behaviour negates postcopulatory rituals in species where male adaptations to sperm competition could rely solely on precopulatory ritual performance, or chemical substances in seminal fluid of the first male to mate, which immediately inhibit female receptivity (Simmons 2001). In the absence or the presence of competitors, male *O. kuvanae* never abandon their receptive mate immediately after copulation, and complete the postcopulatory ritual even if they then forego

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mating opportunities with other females. This behaviour corroborates the importance of the postcopulatory ritual as a form of mate guarding, which functions to ensure paternity in the context of sperm competition (Simmons 2001).

Conclusion

In summary, our study demonstrates that first-male sperm precedence is most prevalent in *O. kuvanae,* but that "sneaker" males are also capable of achieving paternity. The underlying mechanisms do not entail more frequent or prolonged copulatory bouts, or morphological characteristics of the males' aedeagus. Instead, our data suggest that the precopulatory and post-copulatory rituals may function as an adaptation to sperm competition, with the postcopulatory ritual accelerating the awakening of an in-trance female, thereby effectively and quickly closing her window of receptivity.

Acknowledgements

The authors thank M. Todd, G. Sadowski, F. Fernando, and K. Jones (Genetic Identification Services) for technical assistance with the DNA library construction, screening, and enrichment; U. Somjee for technical assistance with the experiments; J. Drummond for ESEM and photomicrographic imaging; M. Christie for his exclusion probability program; J. Wolfe for formatting the tables; M. Keena (Hamden) for supplying reared gypsy moth eggs; D. Stover for helpful suggestions; and two anonymous reviewers for meticulous reviews and constructive comments. This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) – Discovery Grant and by an NSERC – Industrial Research Chair to G.G., with Contech Enterprises, and Global Forest Science (GF-18–2007–226; GF-18–2007–227) as sponsors.

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