

Cryptic female choice by female control of oviposition timing in a soldier fly

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There is substantial evidence that cryptic female choice (CFC) is present in numerous taxa. Several mechanisms have been proposed for CFC; however, we only have experimental evidence for a few of them. Female control of oviposition timing is a potentially widespread mechanism of CFC, but it has never been experimentally demonstrated. The aims of this study are to test 2 critical predictions of the hypothesis that CFC through control of oviposition timing occurs in the soldier fly *Merosargus cingulatus*: 1) to determine if *M. cingulatus* females are less likely to oviposit immediately after mating when the male does not perform copulatory courtship than when he does and 2) to determine if failure to immediately oviposit by the female results in lower reproductive success for the male she just mated with. To answer the first question, I compared the oviposition behavior of females that mated with control males versus females that mated with manipulated males that could not perform copulatory courtship. I showed that *M. cingulatus* females fail to oviposit immediately after copulation when males do not perform copulatory courtship. To answer the second question, I showed that there is last male sperm precedence in *M. cingulatus*. Because the last male to mate fertilizes most of the female's eggs, a male will benefit when females oviposit immediately after mating with him and before remating with another male. **Key words:** copulatory courtship, cryptic female choice, oviposition timing, postcopulatory sexual selection, soldier fly, Stratiomyidae. [*Behav Ecol*]

Postcopulatory sexual selection is potentially important in polygamous species, where the number of copulations by males does not necessarily predict their reproductive success. There are 2 primary mechanisms: sperm competition and cryptic female choice (CFC). CFC is a female-controlled, postcopulatory process that biases paternity toward males that have a preferred trait over males that lack that trait, when the female has mated with both (Eberhard 1996). Whereas there is abundant evidence for sperm competition (Birkhead and Møller 1998; Simmons 2001), there are far fewer studies that provide direct evidence for CFC (see Table 1). However, there is substantial indirect evidence for CFC in numerous taxa (Eberhard 1996).

Several potential mechanisms have been proposed for CFC (Eberhard 1996). Although indirect evidence suggests that there may be more than 20 mechanisms, there is experimental evidence for only a few: female adjustment of the number of eggs laid, female control of copulation duration and sperm transfer, and internal sperm manipulation by the female (see Table 1). Other potential mechanisms have not been demonstrated but are likely to be widespread. One such mechanism is female control of oviposition timing. A female can bias paternity toward a male by ovipositing soon after mating with him and before she mates with another male, if last male sperm precedence occurs (i.e., the last male to mate with a female fertilizes most of her eggs). This means that if a female mates with a second male before ovipositing, she will greatly decrease the first male's reproductive success. Female control of oviposition timing is a potentially important mechanism of CFC because last male sperm precedence is widespread among insects (Thornhill and Alcock 1983). However, there are no known examples of CFC through oviposition timing. The goal of this study is to experimentally test

the hypothesis that CFC by female control of oviposition timing occurs in the soldier fly *Merosargus cingulatus* (Diptera: Stratiomyidae).

In *M. cingulatus*, copulations occur at oviposition sites such as piles of rotting fruit or recently cut grass, in which the larvae develop, and where males patrol and defend a territory. Oviposition sites attract large numbers of males and females. Male territories are relatively small, with neighboring males typically about 10–15 cm apart (Barbosa F, personal observation). The male attempts to grab and copulate with any female that flies near his territory. Females do not appear to have any opportunity to choose a mate and do not seem to resist mating once they are grasped by a male. It is unclear if females come to the territories primarily for mating or oviposition. Males perform copulatory courtship throughout the copulation. Copulatory courtship involves 2 distinct behaviors: Males tap the female's abdomen with their hind legs and wave their legs in the air.

After mating, females usually lay eggs deep in the rotting vegetable matter in or close to the male's territory. When females leave a male's territory without ovipositing, they will likely mate again before they have the chance to oviposit elsewhere. Male density at the oviposition sites is high, and if a female is detected by a male, he will likely grab her and mate (Barbosa F, personal observation). Observations of marked animals show that both males and females mate multiply in the field (Barbosa F, personal observation).

The aims of this study are to test 2 critical predictions of the hypothesis that CFC through control of oviposition timing occurs in *M. cingulatus*: 1) to determine if females are less likely to oviposit immediately after mating when the male does not perform copulatory courtship than when he does and 2) to determine if failure to immediately oviposit by the female results in lower reproductive success for the male she just mated with. To answer the first question, I conducted a field experiment where I compared the oviposition behavior of females that mated with control males versus females that mated with manipulated males that could not perform copulatory courtship. I showed that *M. cingulatus* females fail to oviposit immediately after copulation when males do not perform copulatory

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Table 1
Studies of CFC where the postcopulatory mechanism could be attributed to the female and was shown to affect male reproductive success

Organism	Female mechanism	Male trait favored by female	References
Cucumber beetle, <i>Diabrotica undecimpunctata howardi</i>	Controlling male access to bursa copulatrix and spermatophore transfer	Intensity of copulatory courtship	Tallamy et al. (2002)
Water strider, <i>Gerris lateralis</i>	Clutch size adjustment	Larger body size	Arnqvist and Danielsson (1999)
Scorpion fly, <i>Harpobittacus nigriceps</i>	Clutch size adjustment	Large body size and large nuptial gift prey item (which are correlated)	Thornhill (1983)
Guppy, <i>Poecilia reticulata</i>	Controlling amount of retained sperm	Relatively colorful males	Pilastro et al. (2004)
Water frog, <i>Rana lessonae-esculenta</i>	Clutch size adjustment	Not hybrid males	Reyer et al. (1999)
Yellow dung fly, <i>Scathophaga stercoraria</i>	Unknown	PGM genotype (preference varies with environment)	Ward (1998, 2000, 2007)
Spider, <i>Argiope keyserlingi</i>	Controlling copulation duration by mate cannibalism	Small body size	Elgar et al. (2000)
Flour beetle, <i>Tribolium castaneum</i>	Controlling sperm transfer and storage	Intensity of copulatory courtship	Edvardsson and Arnqvist (2000) and Bloch-Qazi (2003)
Fly, <i>Dryomyza anilis</i>	Transferring sperm to singlet spermatheca, which is primarily used when ovipositing	Number of postcopulatory genital taps	Otronen and Siva-Jothy (1991)
Field cricket, <i>Gryllus bimaculatus</i>	Clutch size adjustment	Higher dominance status	Bretman et al. (2006)
Decorated cricket, <i>Gryllodes sigillatus</i>	Controlling duration of spermatophore attachment and therefore of sperm transfer	Large spermatophylax size	Sakaluk and Eggert (1996) and Sakaluk (1997)

courtship. To answer the second question, I tested for last male sperm precedence using amplified fragment length polymorphism (AFLP) markers. I showed that the last male to mate with a female fertilizes most of her eggs. This means that a male will benefit when females oviposit immediately after mating with him and before remating with another male.

METHODS

Copulatory courtship

All field observations and experiments were conducted at the Smithsonian Tropical Research Institute field station in Gamboa, Panama, in August 2006 and in July 2007. I set up piles of decomposing fruit peels in open areas by small patches of forest in a residential area. Both male and female *M. cingulatus* were attracted to the fruit. I captured a total of 62 males. Half of them were treated so that they could not perform copulatory courtship by applying black acrylic paint to their hind legs. This treatment made the legs stiff and prevented normal movement during copulatory courtship; I did not observe any other change in behavior caused by this treatment, and they obtained mates as readily as untreated males. The other half of the males received the control treatment: They were caught and handled like the manipulated males, but their hind legs were not painted, allowing for normal movement during copulatory courtship. All males were uniquely marked on the thorax with acrylic paint to allow individual identification and were released immediately after being processed. When males returned to their territories, I observed them until they mated. Not all the marked males returned to their territories and mated; I only collected data from the ones that did so. I filmed the copulation and the female behavior until she left the oviposition site and then collected the male. I allowed each male to mate only once.

Sperm precedence

I collected mated pairs in the field and raised their offspring for paternity analysis in order to assess last male sperm pre-

cedence. Because all individuals were field caught, their previous mating history is unknown. I observed copulations and afterward watched the female as she oviposited. I then collected the male and female and the piece of fruit on which she had oviposited. Because the fruit peels came from fresh fruit and I allowed them to decompose in sealed enclosures, the only eggs present were those laid by the female that I observed mating. I allowed the eggs to develop to 3-week-old larvae and preserved the adults and these larvae in 100% ethanol. I collected a total of 9 "families" (the mated pair and the larvae resulting from that clutch), which had an average of 22.77 offspring per family. Twenty offspring per family were randomly picked for the paternity analysis for the families that had more than 20 offspring; all offspring were analyzed in 4 families with fewer than 20 offspring (number of larvae = 7, 8, 19, and 19).

DNA extraction

I extracted DNA from the tissue from flight muscles and legs of adults. For the larvae, I removed the head and used the rest of the body. I froze the tissues with liquid nitrogen and macerated them with a lysis buffer (250 mM Tris—pH 7.5, 2 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 2.5% sodium dodecyl sulfate and 2% proteinase K), incubating for 2 h at 58 °C. The DNA was purified with a 10% cetrimonium bromide solution, a 24:1 chloroform:isoamyl alcohol solution, and 5 M ammonium acetate. Samples were then treated with a 100 µg/ml solution of RNase A for 30 min at 37 °C. DNA was precipitated with ethanol and resuspended in low TE (0.5 M EDTA, 1 M Tris-HCl). DNA samples were quantified with a NanoDrop N-1000 spectrophotometer and diluted to a final concentration of 50 ng/µl.

Amplified fragment length polymorphism

Generating AFLP markers involved 4 steps. First, I digested the DNA with 2 restriction endonucleases, *EcoRI* and *MseI*. The fragments generated were then ligated to adaptors, which are

small DNA fragments with sticky ends complementary to the enzyme's cutting sites. These 2 steps were combined in a single reaction. The fragments were then submitted to 2 polymerase chain reactions (PCRs): the preselective and the selective amplifications, where the adaptor sequence is used to build the primers. The purpose of the PCRs is to amplify only a subset of the fragments by adding 1–3 arbitrary bases to the primer sequence. The primers used in the preselective PCR have 1 bp added, and the selective PCR primer can have up to 3 bases added to its sequence.

The protocol used was adapted from Vos et al. 1995. First, 50 ng of DNA was used in a restriction–ligation reaction (1× T4 DNA ligase buffer with ATP, 0.05 M NaCl, 5 µg/ml bovine serum albumin, 5 U *EcoRI*, 1 U *MseI*, 1 U T4 ligase, 50 µM *MseI* adaptor, 5 µM *EcoRI* adaptor) and incubated at 37 °C for 2 h. The adaptors were synthesized by Integrated DNA Technologies (Coralville, IA). The sequence of the adaptor was for *EcoRI* forward: 5'-CTC GTA GAC TGC GTA CC-3' and reverse: 5'-AAT TGG TAC GCA GTC TAC-3' and for *MseI* forward: 5'-GAC GAT GAG TCC TGA-3' and reverse: 5'-TAC TCA GGA CTC AT-3'. The second step was a preselective amplification. The product from the reaction above was diluted 1:10 with low TE; 2.5 µl of it was combined with 1.5 U of *Taq* DNA polymerase, 20 µl of 10× *Taq* DNA polymerase buffer, 1 µl of the preselective amplification primer mix, and 0.5 µl of 10 mM dNTP solution; and this solution was brought to 20 µl with water. The PCR began with a cycle of 72 °C for 2 min; followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min; and a final cycle of 60 °C for 10 min. The structure of the preselective primers used were 5'-GAC TGC GTA CCA ATT CT-3' for the *EcoRI* primer and 5'-GAT GAG TCC TGA GTA AC-3' for the *MseI* primer, and they were both synthesized by Integrated DNA Technologies. The amplification product was diluted 1:5 with low TE and used in a selective PCR. The selective PCR used 1 µl of 10× *Taq* DNA polymerase buffer, 0.75 U of *Taq* DNA polymerase, 2 pmol of the *MseI* selective primer, 0.4 pmol of the *EcoRI*-selective primer, 1.5 µl of the diluted reaction, and 0.25 µl of 10 mM dNTP solution. This mixture was brought to a volume of 10 µl with water. One primer pair combination was identified from a set of 8 primer pairs as the most polymorphic and therefore most informative. The *MseI* primer had 3 selective nucleotides added. It was synthesized by Integrated DNA Technologies, and its structure is 5'-GAT GAG TCC TGA GTA ACA T-3'. The *EcoRI* primer had 2 selective nucleotides added and was fluorescently labeled with 6-fluorescein amidite dye. Its sequence is 5'-GAC TGC GTA CCA ATT CTG-3', and it was synthesized by Applied Biosystems (Foster City, CA). Fragments were separated by gel electrophoresis and detected by an ABI 3730 DNA Analyzer, which uses an automated fluorescence-based detection system. Fragment size was accurately detected by the addition of an internal lane size standard that was also fluorescent marked (Genescan 600 LIZ).

AFLP analysis

Profiles were visualized and scored for presence/absence of fragments ranging from 60 to 400 bp using Softgenetics GeneMarker software. For the paternity analysis, I used Famoz software (Gerber et al. 2003) to calculate the average exclusion probabilities and to determine paternity of each offspring through categorical allocation. The logarithm of odds score (logarithm of the likelihood ratio) of each father–offspring pair was calculated and used to determine if the males analyzed were the fathers of the offspring of the females they mated with. The exclusion probability equations for dominant markers can be found at Gerber et al. 2000.

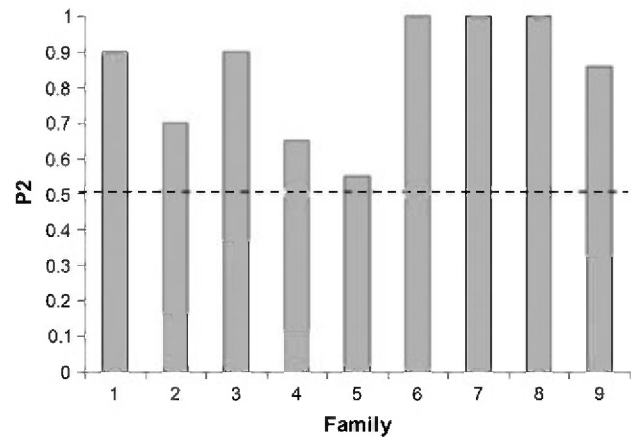


Figure 1

P_2 , or percentage of offspring assigned to the last male to mate with each female. The dotted line shows the P_2 value of 0.5.

RESULTS

Copulatory courtship

None of the 10 manipulated males performed copulatory courtship. Of the 10 females that mated with manipulated males, none oviposited after mating; all flew away soon after copulation ended. All the 13 control males performed copulatory courtship, and all 13 females that mated with controls oviposited (difference in oviposition behavior of females with control vs. manipulated males: Fisher's Exact test, $P < .005$). There was no significant difference in the duration of copulation between manipulated and control males (control group: 95.93 ± 43.97 s [mean \pm standard deviation], manipulated group: 121.10 ± 59.49 ; two-tailed t -test, $P = 0.245$). Samples were checked for normal distribution (Shapiro–Wilk, $P_{\text{control}} = 0.558$, $P_{\text{manipulated}} = 0.745$).

Sperm precedence

The AFLP-selective primer pair used generated 127 polymorphic loci, with an average exclusion probability of 0.996. In the 9 families analyzed, average P_2 , or percentage of offspring sired by the last male, was 0.839 (range = 0.55–1.00) (Figure 1). Because the females used in the analysis were field caught and their mating history is unknown, it is possible that in the 3 cases where P_2 was 1, those females only mated once. The average P_2 when these females are excluded from the analysis is 0.759 (1-sample t -test, null hypothesis: $P_2 = 0.5$, $t = 4.317$, $P = 0.008$). In either case, these results show that there is last male sperm precedence in *M. cingulatus*.

DISCUSSION

CFC is a female-controlled process that biases paternity toward males that have a preferred trait, after having mated with males with and without the trait. In *M. cingulatus*, male copulatory courtship influences female oviposition behavior: Females oviposited immediately after copulating with males that performed copulatory courtship but not with males that performed no copulatory courtship. There is last male sperm precedence in this species, so failure of the female to oviposit before remating will decrease the first male's reproductive success. Because oviposition is under female control, the behavior of female *M. cingulatus* is an expression of CFC. To my knowledge, this study is the first to demonstrate CFC through female control of oviposition timing.

It is important to note that there is an alternative explanation for the observed results that cannot be ruled out with the present data. It is possible that females fail to oviposit in the absence of courtship because sperm transfer (and therefore fertilization) does not happen in that case. Copulatory courtship has been demonstrated to be critical for males to achieve complete penetration (and spermatophore transfer) in a chrysomelid beetle (Tallamy et al. 2002). To distinguish between the 2 scenarios, it would be necessary to determine whether manipulated males that do not perform courtship transfer sperm to the female. However, it should also be noted that females can oviposit even if the last male they mated with does not transfer sperm because they are likely to have sperm stored from previous matings. Therefore, it seems unlikely that females fail to oviposit due to lack of sperm transfer by manipulated males. Another possibility is that manipulated males transfer less sperm than the control males. In that case, the observed female behavior is still an example of CFC by female control of oviposition timing.

Last male sperm precedence is a widespread pattern among insects (Thornhill and Alcock 1983), which suggests that control of oviposition timing is likely to be a common mechanism of CFC. The possibility that females in other species can influence paternity of their offspring by changing oviposition timing has been overlooked and deserves to be explored.

Copulatory courtship is a male trait that is likely under selection by CFC, such that males performing more or superior courtship benefit from increased paternity. Because copulatory courtship occurs only after copulation has begun, it does not play a role in attracting the courted female. In most species, it also does not seem to function in obtaining a second mating with the female being courted, as males leave after a single copulation (Eberhard 1991, 1994). Copulatory courtship is therefore likely to be a trait that influences paternity after mating. The widespread occurrence of copulatory courtship in arthropods (Eberhard 1991, 1994) suggests that CFC is also widespread. A relationship between copulatory courtship and male reproductive success has been found in 2 other species (see Table 1). However, this is the first study to show CFC of copulatory courtship through an external and easily observable female behavior. This discovery increases the possibilities for future studies because such female behaviors can be easily scored.

This is the first study of CFC conducted in the field, rather than in the laboratory. Using field individuals entails some limitations, the main one being that the mating history of those individuals is unknown. However, field experiments provide results that are relevant to natural populations. Female choice can play out very differently in the field and in the laboratory: Factors such as predation risk and the costs of sampling males can make female preferences more costly, which may result in preferences not being expressed as strongly, if at all. In a laboratory setting, these factors are often removed, which in some species is known to lead to a level of female choosiness that would not be expressed in the field (Jennions and Petrie 1997). Thus, field studies are fundamental to assess the relevance of CFC: Only by showing that these female preferences are expressed in natural populations, can we confirm that CFC is an important agent of sexual selection.

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