



Experimental demonstration of possible cryptic female choice on male tsetse fly genitalia

R.D. Briceño, W.G. Eberhard*

Escuela de Biología, Universidad de Costa Rica, Ciudad Universitaria, Costa Rica

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ABSTRACT

A possible explanation for one of the most general trends in animal evolution – rapid divergent evolution of animal genitalia – is that male genitalia are used as courtship devices that influence cryptic female choice. But experimental demonstrations of stimulatory effects of male genitalia on female reproductive processes have generally been lacking. Previous studies of female reproductive physiology in the tsetse fly *Glossina morsitans* suggested that stimulation during copulation triggers ovulation and resistance to remating. In this study we altered the form of two male genital structures that squeeze the female's abdomen rhythmically in *G. morsitans centralis* and induced, as predicted, cryptic female choice against the male: sperm storage decreased, while female remating increased. Further experiments in which we altered the female sensory abilities at the site contacted by these male structures during copulation, and severely altered or eliminated the stimuli the male received from this portion of his genitalia, suggested that the effects of genital alteration on sperm storage were due to changes in tactile stimuli received by the female, rather than altered male behavior. These data support the hypothesis that sexual selection by cryptic female choice has been responsible for the rapid divergent evolution of male genitalia in *Glossina*; limitations of this support are discussed. It appears that a complex combination of stimuli trigger female ovulation, sperm storage, and remating, and different stimuli affect different processes in *G. morsitans*, and that the same processes are controlled differently in *G. pallidipes*. This puzzling diversity in female triggering mechanisms may be due to the action of sexual selection.

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1. Introduction

One of the most sweeping of all evolutionary patterns in animal morphology is for male genitalia to diverge especially rapidly compared with other body parts (Eberhard, 1985, in press; Hosken and Stockley, 2003). One hypothesis to explain this pattern is that male genitalia function as courtship devices, and diverge rapidly because they are under sexual selection by cryptic female choice (Eberhard, 1985). Sexual selection by cryptic female choice (CFC) can occur when the females of a species modulate reproductive processes under their control that occur after copulation has begun, and thus favor the paternity of males that have certain traits (such as a particular genital morphology) over that of others (Thornhill, 1983; Eberhard, 1996). The female could gain from biasing paternity by producing sons whose genitalia are better able to induce such female responses. An alternative hypothesis to explain this divergence is that male–male competition to manipulate female reproductive processes results in male-

imposed damage to the female's reproduction, and that selection on females to avoid this damage results in females and males being engaged in sexually antagonistic coevolution (SAC) (Arnqvist and Rowe, 2005).

Experimental modification of the male's genitalia in the tsetse fly *Glossina pallidipes*, and of the receptors in the portions of the female that they contact during copulation showed that stimuli from two male genital structures trigger three different female reproductive processes that could result in cryptic female choice: ovulation; sperm storage; and female avoidance of remating (Briceño and Eberhard, 2009). The present study describes the results of a complementary set of experimental alterations of male genital form and of corresponding female receptors in a second species of tsetse fly in the same subgenus, *G. morsitans centralis*. These modifications included removal of a derived male genital structure, the median cercal hook, which is present only in *G. morsitans* and its sister species *G. submorsitans*.

Glossina morsitans is widely though somewhat patchily distributed in Africa, where it is an important vector of trypanosomiasis in humans and domestic animals. It shows clinal variation, differences among different geographic populations, and gene flow between such populations; different forms have been variously recognized as species, subspecies, and races of subspecies (Buxton,

* Corresponding author at: Escuela de Biología, Universidad de Costa Rica, Ciudad Universitaria, Costa Rica. Tel.: +506 2228 0001; fax: +506 2228 0001.

E-mail address: william.eberhard@gmail.com (W.G. Eberhard).

1955; Gooding and Krafur, 2005). Copulation in *G. morsitans* lasts about 45–120 min (Saunders and Dodd, 1972; Wall and Langley, 1993). A spermatophore is transferred toward the end of copulation (almost never before 45 min) (Saunders and Dodd, 1972). The mouth of the spermatophore is placed in the mouth of the spermathecal duct in *Glossina*, which is distant from the spermathecae (Buxton, 1955; Pollock, 1974). Transfer of a spermatophore may not always be associated with transfer of sperm to the spermathecae, as some discarded spermatophores of *G. austeni* contained “considerable quantities” of sperm (Pollock, 1970). Only a single egg is ovulated in each reproductive cycle, and ovulation of the female’s first egg is triggered by her first copulation. The egg is fertilized in the female’s uterus, where the larva hatches and feeds and develops, leaving only when it is mature and ready to pupate (Newstead et al., 1924).

Previous experiments concerning induction of ovulation employed interrupted copulations, copulations with and without spermatophore transfer, insertion of glass beads into the uterus, haemolymph transfusions from mated females, copulations with males rendered aspermic by either repeated previous copulations and or severed ejaculatory ducts, males with modified genitalia, implants of male fat body, testes, ejaculatory ducts, and accessory glands, and implantations of full and empty spermathecae from other females (Saunders and Dodd, 1972; Dodd, 1973; Chaudhury and Dhadialla, 1976; Gillott and Langley, 1981). They showed that the stimuli which induce ovulation in *G. morsitans* are not chemical. Ovulation was not triggered by transfer of sperm, deposition of the spermatophore in the female, male fat body, secretions of the male’s testes, accessory glands or ejaculatory ducts, or from humeral factors from spermathecae of inseminated females (Saunders and Dodd, 1972; Gillott and Langley, 1981). Instead, mechanical stimulation received during copulation seemed to induce ovulation, with the effects accumulating gradually during copulation (Saunders and Dodd, 1972). The nature of these mechanical stimuli was not determined. Artificial stimulation of the uterus with a glass bead

increased ovulation, but not as much as natural copulation (Chaudhury and Dhadialla, 1976).

A second response of female *G. morsitans* to copulation is a diminished receptivity to additional mating attempts by males. Undetermined mechanical stimuli during copulation (as well as male accessory gland substances and distension of the uterus) also trigger this female response (Gillott and Langley, 1981). Still another possible female response to copulation is transfer of sperm to the spermathecae, as suggested by evidence from *G. pallidipes*; modification of female ability to sense male genital structures resulted in reduced sperm storage in the spermathecae (Briceño and Eberhard, 2009). Both ovulation and sperm transfer to the spermathecae sometimes fail to occur in otherwise apparently normal copulations of *G. morsitans* (Buxton, 1955; Saunders and Dodd, 1972). There are also intimations that female *G. morsitans* affect sperm transfer to the spermathecae; when Saunders and Dodd (1972) interrupted copulations after 2 h, 19 of 26 females entirely lacked sperm in their spermathecae, while only 1 of 19 pairings that separated spontaneously in the same period (1–2 h after initiation) failed to result in insemination ($\text{Chi}^2 = 20.4$, $p < 0.001$).

Numerous stimuli associated with copulation could induce these female responses. Males of *G. morsitans* perform energetic and sustained courtship behavior during copulation (Wall and Langley, 1993), and males also squeeze the female with vigorous, rhythmic, sustained movements of their genitalia; the temporal pattern of genital squeezing differs from that in *G. pallidipes* (Briceño and Eberhard, unpub.), as would be expected if genital squeezing is under sexual selection. Several portions of the male’s genitalia that contact the female have morphological modifications that appear designed to stimulate the female, including the cerci, the surstyli, the inferior claspers, and the abdominal sternite 5 (Briceño et al., 2007; Briceño and Eberhard, unpub.).

In nature *Glossina* copulate near feeding sites (large mammals) (Wall and Langley, 1993). Field data are not sufficient to

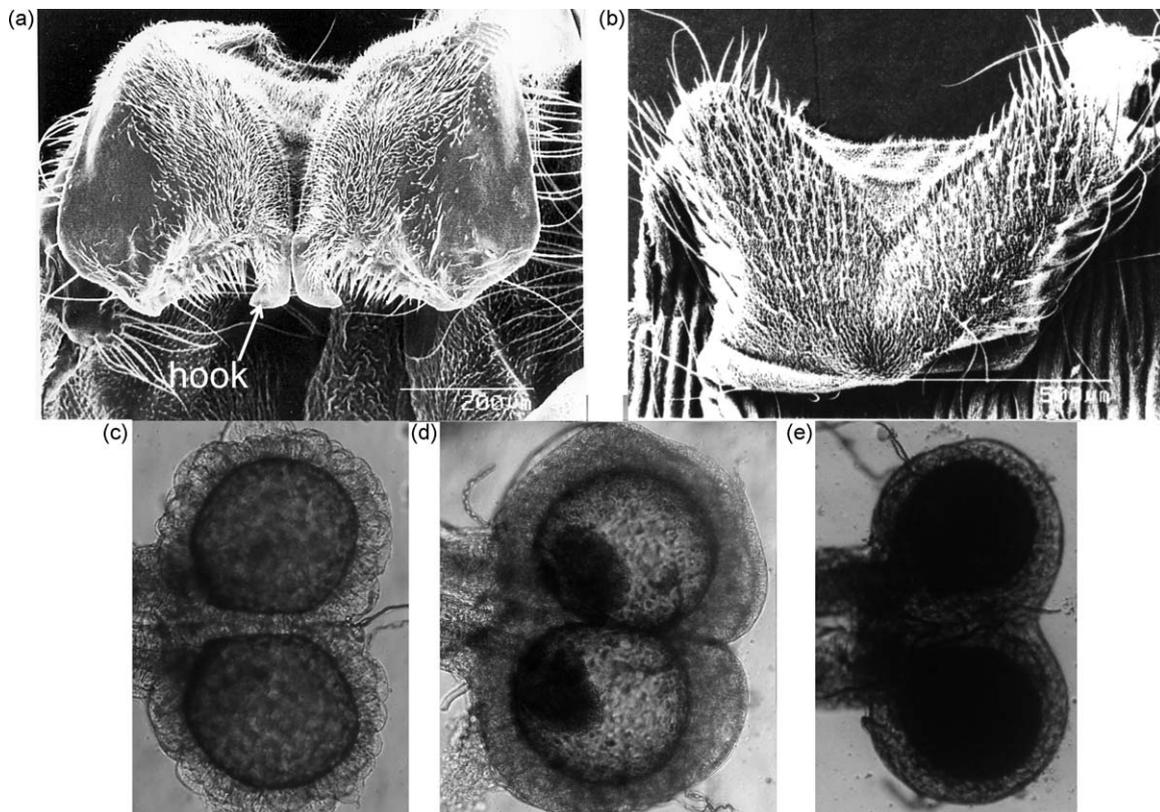


Fig. 1. Male cerci (a) and sternite 5 (b) of *G. morsitans centralis*, and the spermathecae of *G. pallidipes* (c–e) illustrating 0% (c), 15–20% (d) and 100% (e) filling with sperm.

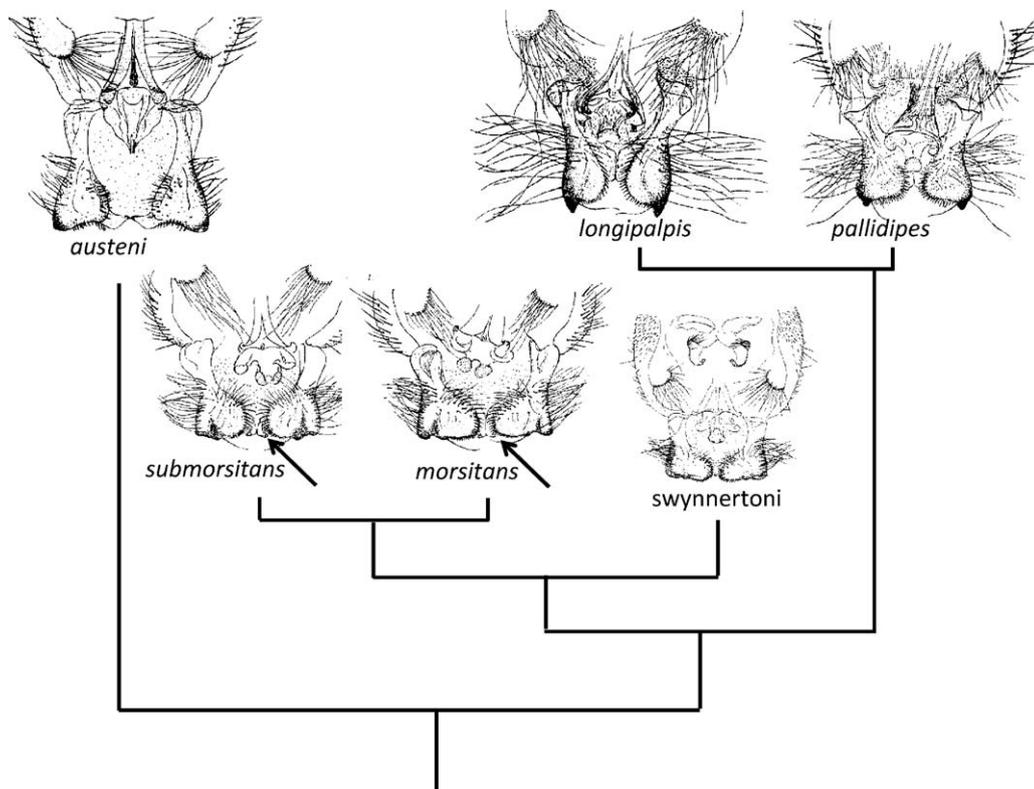


Fig. 2. Male cercus morphology (after Potts, 1970) arrayed on the probable phylogeny of the species in the *morsitans* subgenus of *Glossina* (after Chen et al., 1999). Median cercal hooks (arrows) occur only in the sister species *morsitans* and *submorsitans*.

determine whether female *G. morsitans* mate more than once during a normal lifetime in the field, but they do remate in captivity (Gillott and Langley, 1981; below). Flash-freezing of copulating pairs as well as direct behavioral observations show that the male genitalia of *G. morsitans* perform the same two basic mechanical functions (in addition to possible stimulation) that have been documented in *G. pallidipes*: one set of structures squeezes the external surface of the tip of the female's abdomen in a powerful grip; a second set is introduced deep into the female's vagina (VanderPlank, 1948; Briceño and Eberhard, unpub.). The present study concerns the structures that squeeze the male's cerci (Figs. 1 and 2), whose distal margins press powerfully against the featureless membrane on the ventral surface of the female's abdomen; and his highly modified, sexually dimorphic sternite 5 (Figs. 1b and 3), whose dense covering of stout setae (the "hectors" of older publications—Buxton, 1955) is pressed against the

posterior dorsal surface of her tergite 6 by the squeezing action of his cerci. The male's cerci rhythmically squeeze the female during much of the copulation (Briceño and Eberhard, unpub.). The substantial force exerted by cercal squeezing causes the ventral wall of the female's abdomen to bend inward so sharply and deeply that the entire cercus is generally hidden from view (VanderPlank, 1948; Briceño et al., 2007).

The male cerci of *G. morsitans* are plate-like structures joined medially by a membrane, with strong setae along their distal margins (Fig. 1a). Each cercus has a sharp hook-like, laterally directed projection near its distal median corner (Figs. 1 and 2). This structure (the "median cercal hook" hereafter) has small setae on its base, but lacks setae distally. This hook is an apparently derived structure within the genus *Glossina*, and is present only in the sister species *G. morsitans* and *G. submorsitans* (Fig. 2). The cerci of *G. morsitans* apparently articulate against each other near their

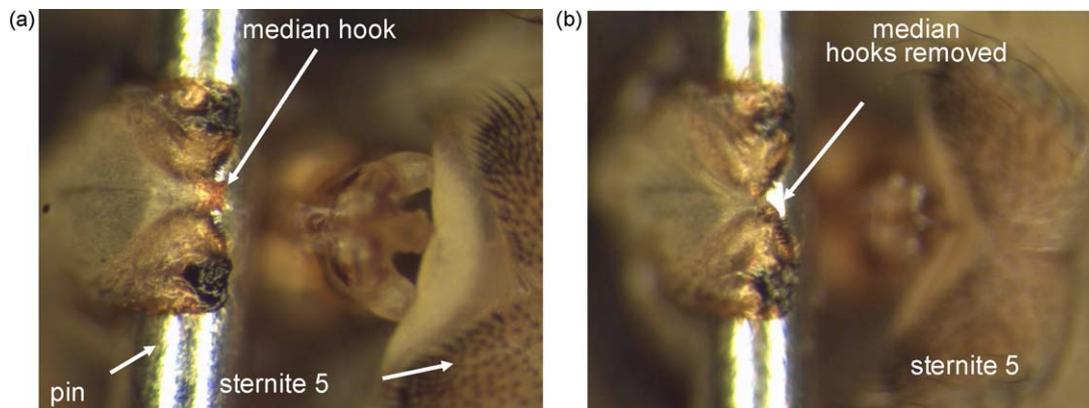


Fig. 3. Male cerci that are held away from the body with a pin illustrate the median cercal hooks in a control male (a) and their absence in an experimental male (b).

tips, and are moved by muscles connecting their bases (Eberhard and Briceño, unpub.).

2. Materials and methods

2.1. Flies

All flies were 10–12-day-old virgins of a mass reared stock at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria, which was founded at least 10 years previously with specimens collected in Tanzania. All experimental flies were kept at $23.5 \pm 24^\circ\text{C}$ and $75 \pm 78\%$ relative humidity, with lights on at 08:00 and off at 16:00, and were offered a blood meal of frozen and thawed bovine blood through a silicone membrane three times per week throughout the experiments. Copulations occurred when recently fed flies in a room at $24.5\text{--}25^\circ\text{C}$ and $53\text{--}55\%$ humidity were introduced into a glass tube 7.5 cm long and 2.5 cm in diameter. The male was removed immediately following copulation, and the female was kept individually in a glass cylinder covered at the ends with open-meshed cloth that allowed her to be fed as above. The relatively few pairs in which copulation lasted less than 5 min were classified as not mating.

2.2. Experimental manipulations

Male cerci were modified by restraining the unanesthetized male ventral side up under a dissecting microscope by using an open-weave cloth to hold him against the paraffin-coated floor of a Petri dish. The cloth was positioned so that the male's cerci were under a hole in the weave. The tips of the cerci were raised by sliding an insect pin under their ventral surfaces, and the median hooks were clipped off using a scissors (Fig. 3b). The hooks are nearly solid cuticle, and their removal never resulted in appreciable bleeding. In a second experiment, the possible effect of movement at the central articulation between the cerci was tested by cutting the articulation with a scissors as just described. Control males in both of these experiments were immobilized, and their cerci were touched with the scissors. Males were allowed at least 1 day to recover before being mated.

The male's sternite 5 was modified by restraining the fly as above, and applying clear nail polish to the array of strong setae on the surface of the sternite with a fine calligraphy brush. This made the surface relatively smooth. Control males were restrained in the same way, and nail polish was applied to sternite 4, which does not squeeze the female.

Two female "sensory blocking" experiments to modify the possible stimuli that the female could receive during copulation were performed as follows. In one, the area on the ventral surface of her abdomen where the tips of the male's cerci pressed during copulation was modified by applying nail polish while the female was restrained as above. Control females received a similar amount of nail polish on the ventral surface just anterior to this area. In the other experiment, the posterior portion of the female tergite 6, where the male sternite 5 presses during copulation, was painted with nail polish; control females received nail polish on tergite 5. Each experimental and control female was mated to a normal male after being allowed at least 1 day to recuperate.

2.3. Measurements

Ovulation and sperm storage in the spermathecae following copulation were assayed by dissecting females 9–10 days after they copulated. The spermathecae were removed and placed on a glass slide, and the degree to which they were filled with sperm was estimated visually under a compound microscope (Fig. 1c–e), and then averaged for the two spermathecae, as in other studies of

sperm transfer in *Glossina* (Abila et al., 2003; Briceño and Eberhard, in press). Two measures of sperm storage are reported below: the frequency with which both spermathecae were empty; and the degree of filling of the spermathecae when at least some sperm were present (zero values excluded; "degree of filling" hereafter). Females with sperm in their spermathecae but without a larva in the uterus were judged not to have ovulated despite having been inseminated; those with a larva in the uterus obviously had been inseminated and had ovulated. Females without sperm were not included in our calculations of "ovulation rates", which may thus be underestimates of total ovulation rates.

Female receptivity to remating was tested in a separate set of females following copulations with control and experimental males. Each female was placed with a 7-day-old virgin male in a 7.5 cm \times 2.5 cm glass vial for 3 min on each of the 11 days following her first mating. Means are followed by \pm one standard deviation.

3. Results

Most results are summarized in Table 1. They will be discussed separately for each experiment.

3.1. Modify median cercal hooks and the female counterpart

3.1.1. Remove the median cercal hooks

Experimental removal of the median hooks did not impede the male's ability to copulate (90% of 101 experimental pairs mated, as compared to 88% of 87 controls; $\text{Chi}^2 = 0.02$, $p = 0.89$). All males attempted to mate in this and other experiments. Removal of the cercal hooks did not significantly affect the frequency with which females ovulated, but reduced the frequency with which sperm were found in the spermathecae, and decreased the relative filling of the spermathecae in those females in which sperm was present. The female's tendency to remate increased.

3.1.2. Cover the ventral surface female abdomen contacted by male cerci

When the female was mated to an intact male after the area of her abdomen with which the tips of the male cerci came into contact during copulation was covered, the results resembled those when the male's median cercal hooks were removed. The frequency of ovulation was unchanged, and the frequency with which sperm was present in the spermathecae decreased. There was no significant effect, however, on the degree of filling of the spermathecae which received sperm. Female rejection of male copulation attempts was not affected (30% of 123 experimental females rejected the male compared with 28% of 117 control females; $\text{Chi}^2 = 0.15$, $p = 0.70$).

3.1.3. Cover median cercal hook

Covering the median cercal hook with nail polish did not result in significant changes in the frequency of ovulation, sperm present in the spermathecae, or in the degree of filling of the spermathecae with sperm.

3.1.4. Damage the distal articulation between the cerci

There was no effect of damaging the articulation between the cerci on ovulation, the likelihood that sperm would be present in the spermathecae, or the degree of filling of the spermathecae.

3.2. Modify male sternite 5 and the female counterpart

3.2.1. Cover setae of sternite 5

When the setae on male sternite 5 were covered with nail polish, there was no effect on female ovulation, the frequency with

Table 1

Results of experiments with *G. morsitans centralis*. Numbers in parentheses are the percentages of the total number of pairs in which genital coupling occurred but last <5 min and were thus not considered.

	Female ovulated		Sperm in spermathecae		% fill sphcae	Female remated in <11 days	
	Yes	No	Yes	No		Yes	No
Expt.: Remove median hooks of cerci							
EXPERIMENTAL (2.2%)	58	19	77	24	30 ± 15 (N = 78)	32	10
CONTROL (2.5%)	77	13	90	11	39 ± 15 (N = 90)	35	48
Statistical test	$\chi^2 = 2.80, p = 0.094$		$\chi^2 = 5.84, p = 0.0157$		$Z = -2.5, p < 0.05$	$\chi^2 = 13.0, p = 0.0157$	
Expt.: Cover area of female abdomen contacted by cercal hooks with nail polish							
EXPERIMENTAL (4.4%)	55	10	65	26	44 ± 19 (N = 65)		
CONTROL (5.1%)	64	11	75	9	45 ± 21 (N = 75)		
Statistical test	$\chi^2 = 0.01, p = 0.90$		$\chi^2 = 8.71, p = 0.0032$		$Z = -0.37, p > 0.71$		
Expt.: Cover median hook with nail polish							
EXPERIMENTAL (3.9%)	92	6	98	12	43 ± 22 (N = 98)		
CONTROL (5.0%)	65	11	76	4	47 ± 22 (N = 76)		
Statistical test	$\chi^2 = 3.39, p = 0.065$		$\chi^2 = 2.10, p = 0.1476$		$Z = -1.09, p > 0.27$		
Expt.: Damage articulation between cerci							
EXPERIMENTAL (3.2%)	47	14	61	6	71 ± 34 (N = 61)		
CONTROL (1.8%)	40	15	55	5	74 ± 31 (N = 55)		
Statistical test	$\chi^2 = 0.29, p = 0.591$		$\chi^2 = 0.02, p = 0.901$		$Z = -1.31, p = 0.18$		
Expt.: Cover male sternite 5 with nail polish							
EXPERIMENTAL (1.3%)	45	29	74	13	25 ± 12 (N = 74)		
CONTROL (2.4%)	52	29	81	10	28 ± 14 (N = 81)		
Statistical test	$\chi^2 = 0.21, p = 0.649$		$\chi^2 = 0.62, p = 0.43$		$Z = -1.08, p > 0.27$		
Expt.: Cover area of female tergite 6 contacted by male sternal setae							
EXPERIMENTAL (0%)	30	6	36	29	42 ± 22 (N = 36)		
CONTROL (0%)	51	13	64	10	47 ± 21 (N = 64)		
Statistical test	$\chi^2 = 0.20, p = 0.65$		$\chi^2 = 16.58, p = 0.000$		$Z = -1.14, p > 0.25$		
Expt.: Remove median cercal hooks and cover male sternal setae with nail polish							
EXPERIMENTAL (1.4%)	62	9	71	32	29 ± 11 (N = 71)		
CONTROL (1.6%)	58	3	61	10	32 ± 15 (N = 61)		
Statistical test	$\chi^2 = 2.39, p = 0.122$		$\chi^2 = 6.62, p = 0.01$		$Z = -0.52, p = 0.59$		

which sperm was present in the spermathecae, or the degree of filling of the spermathecae.

3.2.2. Cover female tergite 6 (contacted by male sternal setae)

When the area of the female abdomen with which the setae of the male's sternite 5 come into contact during copulation was covered and the female was mated with an intact male, ovulation was not affected, but the frequency with which sperm were present in the female's spermathecae decreased significantly. There was no significant effect on the degree of filling of the spermathecae.

3.2.3. Remove median cercal hooks and also cover male sternite 5

When both male structures were modified, the effect was similar to that of removing the median cercal hooks. The rate of ovulation was unaffected, while the frequency of sperm present in the spermathecae was reduced. There was no effect, however, on the degree of filling of the spermathecae.

4. Discussion

4.1. Effects of median cercal hooks

Removal of the median cercal hooks in male *G. morsitans centralis* resulted in an increase in female receptivity to subsequent mating, and a reduction in two variables associated with female sperm storage: a decrease in the frequency with which sperm were present in the spermathecae decreased; and a decrease in the degree of filling of the spermathecae in those females that had sperm. The frequency with which sperm were stored was also reduced in a sensory blinding experiment in which the area contacted by the distal tips of the male's cerci was covered; in contrast, sensory blinding of the distal portions of the male cerci did not affect sperm

storage. These results suggest that stimulation from the male's median cercal hooks elicits female responses that affect sperm storage. The female sensory blinding experiment probably altered the sensations received by the female from the cerci during copulation, but left the male's morphology and (presumably) behavior unaltered; in contrast, the sensory blinding experiment on the male altered the stimuli he sensed through his cerci (as may have occurred in the female sensory blinding experiment).

4.2. Effects of male sternite 5

Smoothing the rough surface of the male's sternite 5 by coating its strong setae with nail polish did not alter the likelihood that the female would ovulate or have sperm in her spermathecae. However, sensory blinding of the female to this male structure by covering the surface of her tergite 6 with nail polish sharply reduced the likelihood of sperm storage. The more pronounced responses to modification of the female's sensory abilities than to changes in the form of the corresponding male structure ($\chi^2 = 16.4, p < 0.001$) may be because the "sensory blinding" treatment resulted in a more radical alteration of the stimuli she received. It seems likely that sperm storage is affected by stimulation of the female tergite by the male during copulation, but further tests are needed to confirm this.

4.3. Sperm transfer to the spermathecae

There appear to be two processes associated with the arrival of sperm in the spermathecae that are at least partially independent of each other: a qualitative, all-or-none process that sometimes excludes all sperm (this could result, for example, from prevention of the deposition of a spermatophore—see below); and a

quantitative effect on the numbers of sperm that are taken up when at least some sperm do arrive in the spermathecae. For instance, sensory blinding of the female tergite 6 reduced the frequency with which females had sperm in their spermathecae, but had no effect on the degree of filling of spermathecae in those females which had sperm in their spermathecae. These facts, combined with the substantial distance sperm must travel from the spermatophore to the spermathecae suggest that active female transport of sperm may be necessary for sperm to arrive in the spermathecae. Males often lack direct access to female spermathecae in other species of Diptera (Graham-Smith, 1939; Lewis and Pollock, 1975; Solinas and Nuzzaci, 1984; Kotrba, 1993; Lachmann, 1996; Eberhard and Pereira, 1995; Eberhard and Huber, 1999; Hosken et al., 1999; Fritz and Turner, 2002), and several types of evidence imply that female flies actively move sperm into their spermathecae (Linley and Simmons, 1981; Camacho, 1989; Hosken and Ward, 2000; Fritz and Turner, 2002).

Several possible mechanisms could have been responsible for reductions in sperm storage. It might be that the male refrained from producing spermatophores or from filling them with sperm, due to a lack of internal female responses allowing him to position his genitalia appropriately at the opening of the spermathecal duct (Briceño et al., 2007). Alternatively, males may have successfully deposited spermatophores filled with sperm, but females may have failed to transport the sperm to their spermathecae, or discarded spermatophores before their sperm entered her spermathecal ducts. Fragmentary results of previous studies suggest a possible active female role: in 7% of 28 *G. morsitans* females which had received a spermatophore from a normal male (a spermatophore was discarded after copulation), there were no sperm in their spermathecae (Saunders and Dodd, 1972); spermatophores discarded by females of the related *G. austeni*

often contained “considerable quantities” of sperm (Pollock, 1970); 1 of 11 female *G. austeni* with a spermatophore in her uterus 24 h after copulation did not appear to have sperm in her spermathecae (Pollock, 1970). The reduction in the frequency with which sperm were present in the spermathecae of females whose sensory capacities were modified in the present study indicates that a female response some sort is involved.

4.4. Stimuli that elicit female responses

Covering the median cercal hook with nail polish did not elicit any changes in female responses, while removing the hook did. Covering the hook probably had several simultaneous effects. The coating altered the profile of the hooks, smoothing over their sharp lateral tips and extending the entire distal edge of the cercus; it probably also united the cerci into a single mechanical unit incapable of independent movements. Interpretation of the lack of effects of covering the hooks on the female is thus not entirely clear. The apparent lack of importance of movements of the cerci relative to each other is in accord with the similar lack of effect on the female when the articulation between them was destroyed.

Ovulation was not affected by modifications of either the male cerci or his sternite 5, or of the corresponding areas of the female where these structures make contact during copulation. Thus the male effect on ovulation documented by Saunders and Dodd (1972) that resulted from stimulation during copulation is apparently due to other stimuli in *G. morsitans centralis*. There are many other mechanical stimuli during copulation, including male copulatory courtship behavior such as wing vibrations and rubbing with his legs (Wall and Langley, 1993; Briceño and Eberhard, unpub.), as well as complex thrusting movements of the male's phallosome within the female's reproductive tract (Briceño et al., in prep.).

Table 2
Summary of effects of male on female reproductive processes in *Glossina morsitans*.

Male structure/trait	Female process			Reference
	Ovulation	Sperm storage ^a	Resist remate	
Male pneumopophysis	Yes			Dodd (1973)
Empty spermatheca	No			Saunders and Dodd (1972)
Spermathecae full of sperm	No		Yes	Saunders and Dodd (1972); Gillott and Langley (1981)
Male fat body	No		No	Gillott and Langley (1981)
Testes	No		No	Saunders and Dodd (1972); Gillott and Langley (1981)
Male accessory gland	No			Saunders and Dodd (1972)
Entire male reproductive tract	No		Yes (some)	Gillott and Langley (1981)
			Yes (some)	Saunders and Dodd (1972)
Repeated short copulations				
<3 min			Yes	Gillott and Langley (1981)
45 min	Yes			Saunders and Dodd (1972)
Glass bead in uterus	Yes (some) ^b			Chaudhury and Dhadialla (1976)
			Yes	Gillott and Langley (1981)
Longer copulation		Yes ^c		Saunders and Dodd (1972)
Spontaneous end (<2 h)		Yes ^c		Saunders and Dodd (1972)
Copulate without inseminate (aspermic male)	Yes			Saunders and Dodd (1972)
Spermatophore	No			Saunders and Dodd (1972)
Copulate with male of another species	Yes (some)	Yes (some) ^c		Saunders and Dodd (1972)
Sham operation (cut open)	Yes (some)			Saunders and Dodd (1972)
Male mount but not clasp female			No	Gillott and Langley (1981)
Cover tip female abdomen with wax	No		No	Gillott and Langley (1981)
Hemolymph from a mated female	No		No	Gillott and Langley (1981)
Inject saline	Yes (some)			Gillott and Langley (1981)
Stimulation from median cercal hook	No	Yes	Yes (?) ^d	Present study
Stimulation from male sternite 5	No	Yes (?)	(?)	Present study

^a Includes both lack of sperm in spermathecae and percent filling of spermathecae.

^b Dodd (1973) found no effect of bead.

^c Could be due to lack of sperm transfer by male, or to lack of cooperation by female.

^d Effects of stimulation per se by blocking female receptors were not tested.

4.5. Multiple female cues and their consequences in *Glossina* spp.

There is a high intra-specific diversity of stimuli that trigger female reproductive processes in *Glossina morsitans* (Table 2). For instance, the female decision to resist remating is affected by products emitted by spermathecae filled with sperm (Gillott and Langley, 1981), male accessory gland products (Gillott and Langley, 1981), repeated sessions of stimulation during the first 3 min of a male's copulation (Gillott and Langley, 1981), glass beads in the uterus (Gillott and Langley, 1981), and male cercal hooks (this study). Similarly, multiple factors trigger ovulation, and different sets of stimuli affect remating and ovulation.

Why should so many different cues be used in different ways by the female to register the apparently simple message that copulation has occurred? One possible explanation is that there has been sexual selection on males to evolve new ways to influence these female reproductive processes, and subsequent female evolution to favor particular male cues (which may have originally arisen due only to weak, incidental effects on a given female process). A second, non-exclusive explanation that focuses at the level of mechanisms rather than ultimate causes is that multiple factors are important because they affect a common mechanism (Gillott and Langley, 1981).

Whatever the reason for the female's susceptibility to diverse stimuli in triggering her reproductive responses, the existence of this diversity opens the door to the evolution of multiple male mechanisms of manipulation of the female when the males becomes subject to sexual selection to trigger such female responses (Eberhard, 1996). In turn, the existence of multiple possible avenues of manipulation makes inter-specific diversification in such male mechanisms more likely to arise over evolutionary time, because it reduces the likelihood that the same suite of male techniques for influencing female processes will evolve in different evolutionary lines. It is not clear whether or not this diversity in female sensitivity and responses in *Glossina* is unusual; multiple female cues are known in several other insects (Eberhard, 1996).

Comparison of the results of this study with those of similar experiments on the closely related *G. pallidipes* shows that the triggering mechanisms that induce female responses to copulation in the genus *Glossina* are complex. In *G. pallidipes*, stimuli from both the male's cerci and his sternite 5 induced female ovulation, while neither type of stimulus had an effect on ovulation in *G. morsitans centralis*. In *G. pallidipes* both a change in the morphology of the distal edge of the male cercus and blocking female sensitivity in the area contacted by this cercal margin during copulation strongly increased the frequency with which virgin females rejected male attempts to mate, while neither modification had such an effect in *G. morsitans centralis*. And in *G. pallidipes*, smoothing the surface of the male sternite 5 with nail polish lowered the degree to which sperm filled the spermathecae while it had no similar effect in *G. morsitans centralis*. This surprising diversity in control mechanisms in closely related species could be the result of sexual selection acting on female abilities to bias male paternity. Whatever its origin, it is likely to result in diversity in the male traits used to trigger female controls. On the other hand, the results of experimental manipulations in *G. morsitans centralis* resembled those in *G. pallidipes* in several respects, presumably as a result of their common ancestry: sperm storage and female tendency to remate were reduced when a derived, species-specific aspect of cercus form was altered, and sperm storage was reduced by "sensory blinding" of the female in both the area contacted by the cerci and the area contacted by the male's sternite 5.

The reproductive consequences for the male of a reduction in the chances that his sperm will be stored in the female may be less in *G. morsitans centralis* than in *G. pallidipes*. In control copulations

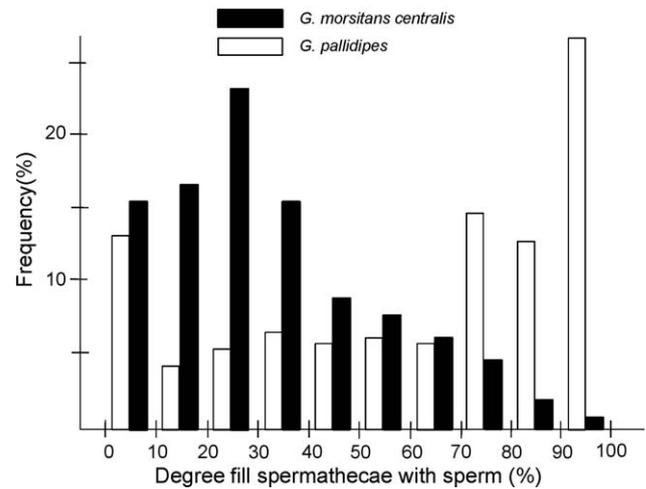


Fig. 4. The degree of filling of the spermathecae in matings of *G. morsitans centralis* in which at least some sperm was stored (control data from all experiments combined), compared with similar data from a previous study of *G. pallidipes* (Briceño and Eberhard, in press). The mean degree of filling in *G. morsitans centralis* ($39 \pm 21\%$) was significantly lower than that for *G. pallidipes* ($71 \pm 28\%$) ($Z = 17.2$, $p < 0.001$ with Mann-Whitney U -test).

(intact males and females) in which at least some sperm was stored in the spermathecae, the degree of filling of the spermathecae was much less complete in *G. morsitans centralis* (Fig. 4). This suggests that the reproductive payoff to a male from achieving sperm storage may be larger in *G. pallidipes* (no data on sperm precedence patterns are available in either species, however).

4.6. Limitations of present study

Our experiments have several limitations. We do not know exactly how a coat of nail polish modifies the sensations that a female perceives from stretch receptors on the membranous area on the ventral surface of her abdomen when it is bent inward by the male's cerci. Stimuli from the male's cerci were probably only partially eliminated by the nail polish. Nail polish on more rigid surfaces, such as male sternite 5, the cercal hooks and the tips of the cerci, and the female's tergite 6, probably immobilized all the setae on these structures, thus eliminating most if not all sensations resulting from their movements. The coating probably bent many setae toward the cuticle, however, and may have produced other sensations. We cannot be certain that coating the median hooks with nail polish was an appropriate control for the possible effects on the male's behavior of the changes in stimulation that he received when mating with a female with nail polish on the ventral surface of her abdomen where his hooks contacted her. We do not know the significance (if any) of the trend (not statistically significant) of males with this treatment to elicit ovulation (Table 1).

A second important limitation stems from the very crude nature of our experimental manipulations. This study shows that females respond to the absence of median cercal hooks by altering a post-copulatory process in ways that reduce the male's chances of paternity, as predicted by CFC theory. This does not mean, however, that females respond selectively to the much smaller differences between the forms of cercal teeth of present-day males of *G. morsitans*. Thus a prediction of the theory was confirmed, but CFC was not demonstrated directly among the forms of modern males. We have presumed that larger amounts of sperm in the female's spermathecae translate into greater probable paternity, but not sperm precedence studies are available in *Glossina*.

Our data offer only a partial evaluation of the SAC hypothesis. Use by the female of diverse, multiple cues is predicted by SAC as

well as by CFC (e.g., Holland and Rice, 1998). The fact that the cerci of some species of *Glossina* (*palpalis*, *fuscipes*, *brevipalpis*) leave mating scars on the female where they scrape or pierce the ventral surface of her abdomen (Squire, 1951; Briceño and Eberhard, unpub.) also suggests possible SAC in this genus. Nevertheless, careful examination of the female cuticle of *G. morsitans* revealed that the median cercal hooks do not produce any perceptible damage to the female. In addition, the area on the female abdomen that is contacted by the male cerci is featureless throughout *Glossina*; this area has not been used by taxonomists to distinguish species (Newstead et al., 1924; Potts, 1970). Thus this portion of the female has not coevolved defensively with the male cerci, as would be expected under the physically coercive version of SAC (Alexander et al., 1997; Arnqvist and Rowe, 2002a,b). Discrimination between CFC and an alternative, sensory trap version of SAC (Arnqvist, 2006) depends on evaluation of the balance for females between the costs of mating, and the benefits from selective fertilization (Orteiza et al., 2005; Eberhard, in press) that result from female changes in responsiveness to male stimuli; such data are not available.

Finally, it should be noted that the lack of morphological female coevolution with the male cerci clearly fails to fit the classic lock-and-key hypothesis for genital evolution in *Glossina* (Shapiro and Porter, 1989).

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