

# Experimental modifications imply a stimulatory function for male tsetse fly genitalia, supporting cryptic female choice theory

R. D. BRICEÑO\* & W. G. EBERHARD\*†

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

†Smithsonian Tropical Research Institute

## Keywords:

tsetse fly;  
cryptic female choice;  
genital function;  
sexual selection.

## Abstract

One of the most sweeping of all patterns in morphological evolution is that animal genitalia tend to diverge more rapidly than do other structures. Abundant indirect evidence supports the cryptic female choice (CFC) explanation of this pattern, which supposes that male genitalia often function to court females during copulation; but direct experimental demonstrations of a stimulatory function have been lacking. In this study, we altered the form of two male genital structures that squeeze the female's abdomen rhythmically in *Glossina pallidipes* flies. As predicted by theory, this induced CFC against the male: ovulation and sperm storage decreased, while female remating increased. Further experiments showed that these effects were due to changes in tactile stimuli received by the female from the male's altered genitalia, and were not due to other possible changes in the males due to alteration of their genital form. Stimulation from male genital structures also induces females to permit copulation to occur. Together with previous studies of tsetse reproductive physiology, these data constitute the most complete experimental confirmation that sexual selection (probably by CFC) acts on the stimulatory properties of male genitalia.

## Introduction

One of the most sweeping of all evolutionary patterns is for the morphology of male genitalia in species with internal insemination to diverge especially rapidly when compared with other body parts (Eberhard, 1985, in press; Hosken & Stockley, 2003). The male genitalia of many species are much more elaborate than seems necessary for sperm transfer, and in a wide variety of animals (including nematodes, snakes, insects, monkeys, spiders, mites and many others) taxonomists have used genitalia as key characters to distinguish closely related species. One possible explanation for this evolutionary pattern is that males use their genitalia as courtship devices, and that genital morphology diverges rapidly because it is under sexual selection (Eberhard, 1985). Sexual selection can occur if females modulate repro-

ductive processes that occur after copulation has begun, favouring the paternity of some males over that of others, and if this bias is correlated with differences among males with respect to particular traits (such as stimulation provided by genital morphology) (Thornhill, 1983; Eberhard, 1985, 1996). A female could gain from biasing paternity by producing sons with traits (e.g., genitalia) better able to induce such female responses (resulting in cryptic female choice or CFC) (Eberhard, 1985); or, if male stimulation is damaging to the female's reproductive output, she could gain from avoiding male manipulations (potentially resulting in sexually antagonistic coevolution or SAC) (Arnqvist & Rowe, 2003).

Abundant data give indirect support for the CFC hypothesis (Eberhard, 1985, in press), but there have been few direct tests of the effects of a male's genital morphology on his reproductive success, and none of these was focused specifically on stimulation of the female. Most direct tests involved only correlations between the sizes of certain male genital structures and paternity when a female mates with two males, and did not document cause and effect experimentally (Arnqvist

Correspondence: William Eberhard, Escuela de Biología, Universidad de Costa Rica, Frente a Agronomía, Ciudad Universitaria, Costa Rica.  
Tel.: +506 2228 0001; fax: +506 2228 0001;  
e-mail: william.eberhard@gmail.com

& Danielsson, 1999; Danielsson & Askenmo, 1999; House & Simmons, 2003; Wenninger & Averill, 2006). Only one study (Rodríguez *et al.*, 2004) complemented a correlation of this sort with an experimental test that suggested that the male genital structure itself, rather than some other male trait or traits correlated with genital morphology, was the cause of the paternity differences. No previous study has shown that stimulation of the female by the male's genitalia is responsible for inducing female responses that produce paternity biases. The present study of the tsetse fly *Glossina pallidipes* constitutes the most diverse set yet performed of experimental alterations of the forms of male genital structures and of female receptors that could sense these forms; it also reports the greatest variety of effects on female reproductive responses to male genital modifications documented to date.

Copulation in *G. pallidipes* lasts about 30 min, and a spermatophore is transferred during the last approximately 30 s (Jaensen, 1979). A single egg is ovulated and fertilized in each reproductive cycle. It hatches in the female's 'uterus', where the larva feeds and grows. The larva finally leaves the female only when ready to pupate (Newstead *et al.*, 1924). Previous experiments have shown that ovulation in the closely related species *Glossina morsitans* (Potts, 1970; Chen *et al.*, 1999) is triggered by mechanical stimuli associated with copulation, and that these stimuli are not derived from the transfer of sperm, deposition of the spermatophore in the female, secretions of the male's testes, accessory glands or ejaculatory ducts, or humeral factors from the spermathecae of inseminated females (Saunders & Dodd, 1972). Saunders & Dodd (1972) concluded that mechanical stimuli received during copulation must induce ovulation, but did not determine what these stimuli might be. Artificial stimulation of the uterus with a glass bead increased ovulation, but not as much as natural copulation (Chaudhury & Dhadialla, 1976).

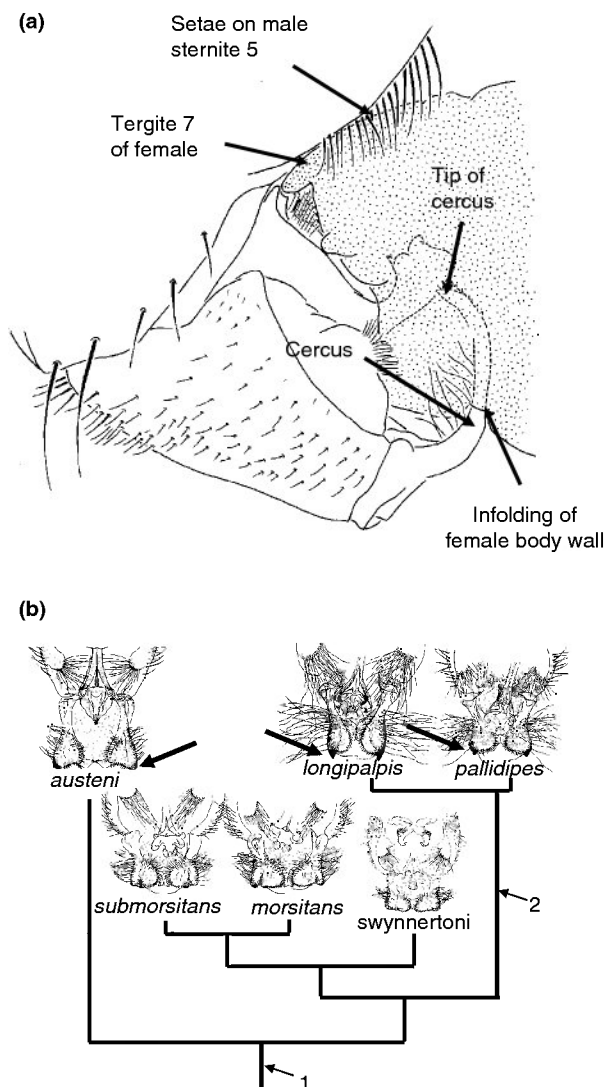
A second response of female *G. morsitans* to copulation is reduced receptivity to further mating. Undetermined mechanical stimuli during copulation also trigger this female response (as do male accessory gland substances and distension of the uterus) (Gillot & Langley, 1981). There are less direct indications that female responses affecting intromission and sperm transfer may also be influenced by stimuli from the male (Briceño *et al.*, 2007). Both ovulation and sperm transfer to the spermathecae sometimes fail to follow apparently normal copulations in *Glossina* and females sometimes mate repeatedly (Buxton, 1955), so male ability to increase these female responses may be selectively important.

There are many stimuli associated with copulation in *G. pallidipes* that could induce female responses. Males perform six different kinds of energetic and sustained courtship behaviour during copulation; these include the production of sounds and potential visual stimuli with their wings, and stylized rubbing movements on different

parts of the female with all three pairs of legs (Jaensen, 1979; Briceño *et al.*, 2007). Males also squeeze the female with vigorous, rhythmic, sustained movements of their genitalia (Briceño *et al.*, 2007). Several male genital structures contact and move against the external surface of the female during these squeezes, and six of these have morphological modifications appropriately designed to stimulate her (Briceño *et al.*, 2007). One non-genital behaviour ('male jerking' – Jaensen, 1979) probably also causes one of these structures, the male sternite 5, to rub vigorously against the female during one stage of copulation.

In nature, female *Glossina* copulate near feeding sites (large mammals) (Wall & Langley, 1993). Scanty field data suggest that female *G. pallidipes* mate several times during a normal lifetime (Jaensen, 1979), and females in captivity also sometimes mate several times. The male genitalia of *G. pallidipes* perform two general mechanical functions (in addition to possible stimulation): one set of structures grasps and squeezes the external surface of the tip of the female's abdomen; a second set thrusts deep into the female's vagina, and deposits the sperm-filled spermatophore at the entrance to her spermathecal duct (VanderPlank, 1948; Jaensen, 1979; Briceño *et al.*, 2007), from where the sperm move or are moved to the spermathecae. This study concerns two of the squeezing structures (Fig. 1a): the male's cerci, whose tips press powerfully against the membranous ventral surface of the female's abdomen; and his sternite 5 which, along with his inferior claspers, is pressed against her posterior dorsal surface by the squeezing action of his cerci. Ventrally, the male cerci clamp the tip of the female's abdomen, and deliver rhythmic squeezes for much of the approximately 30 min copulation. The force exerted by the cerci causes the ventral wall of the female's abdomen to bend inward deeply, and the entire male cercus is generally hidden from view throughout copulation (VanderPlank, 1948; Briceño *et al.*, 2007) (Fig. 1a). Dorsally, the stout setae or 'hectors' (Buxton, 1955) that cover the male's highly modified sternite 5 press against the female's abdominal tergite 6, and the groove in the tip of each of his inferior claspers cradles the rear edge of this female tergite. The male cerci of *G. pallidipes* are plate-like structures joined medially by a membrane (Fig. 2a). Cercal morphology varies among species of *Glossina* (Fig. 1b). In *G. pallidipes*, each cercus has a row of stout spines along its distal margin, and a strong, dark 'lateral tooth' at its distal lateral corner (Figs 1b, 2c). Elongate, strengthened lateral cercal teeth of this sort are apparently derived structures within the genus *Glossina*, and occur only in *G. pallidipes* and its sister species *G. longipalpis* (Fig. 1b).

The sites of attachment of muscles associated with the cerci, plus direct observations of the bases of the cerci during copulation indicate that the cerci move in at least two different ways during copulation (Briceño *et al.*, 2007). The cerci repeatedly flex ventrally, producing the



**Fig. 1** (a) Schematic lateral view of male genitalia (unstippled) and tip female abdomen (stippled) during copulation. The male's cerci squeeze the tip of the female's abdomen, causing its membranous ventral surface to invaginate sharply, and press the setae on male sternite 5 against the dorsal surface of her tergite 6 (after Briceño *et al.*, 2007). (b) Relationships in the *morsitans* subgenus of *Glossina*, showing male cercus morphology for each species (arrows indicate lateral cercal teeth). 1. Lateral cercal teeth present but not elongate or strengthened (black); 2. Lateral cercal teeth elongate, strengthened (changes in morphology based on outgroup comparisons with *Glossina* species in the other two subgenera). (phylogeny from Chen *et al.*, 1999; drawings of genitalia from Newstead *et al.*, 1924).

strong, rhythmic squeezing movements just mentioned. In addition, the cerci rock against each other at a distal median articulation (Fig. 2c); medial movements of their bases result in lateral movements of the lateral teeth.

The present study shows that stimuli from the cercal squeezing movements serve to induce the female to

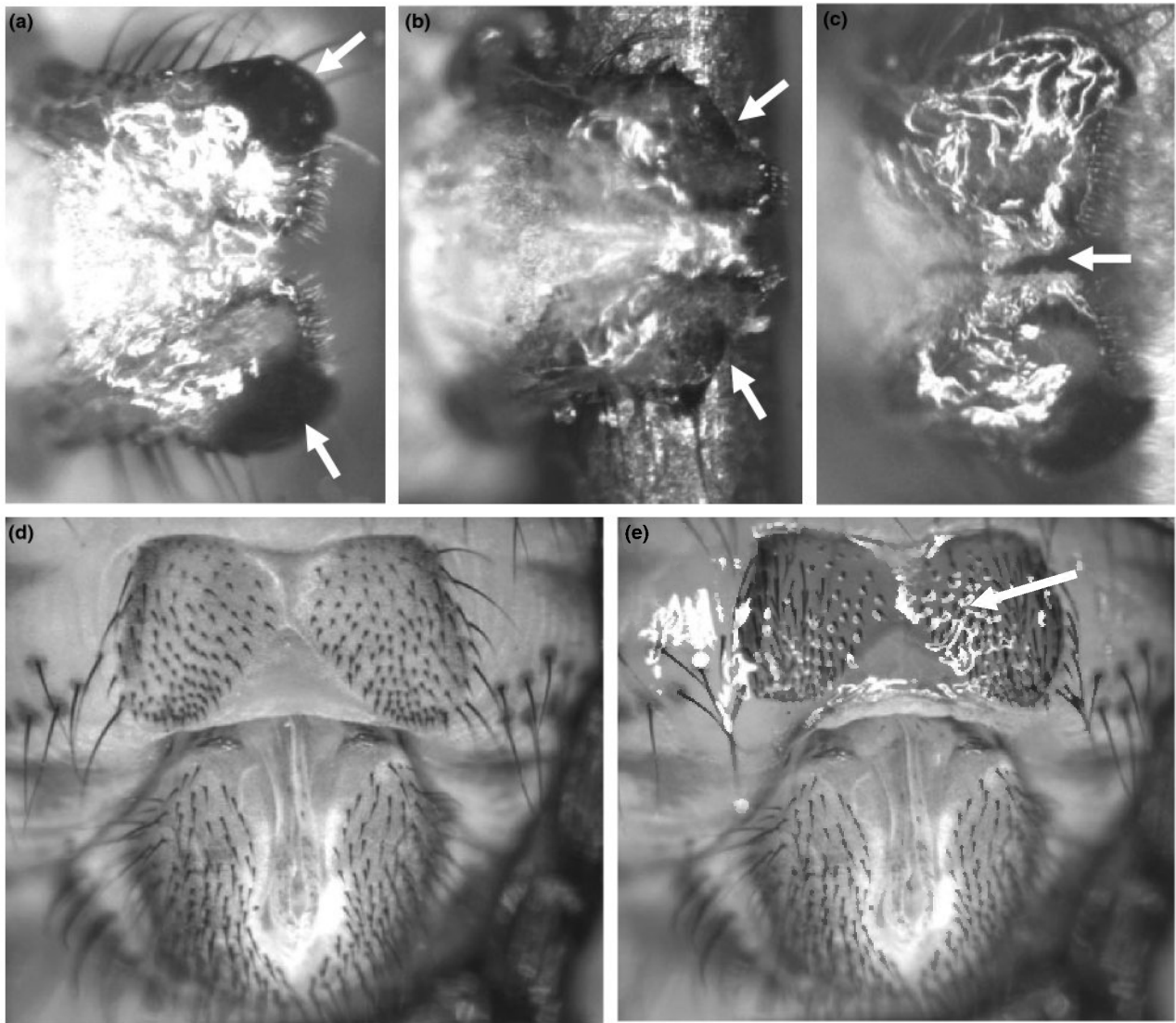
ovulate, to move sperm into her spermathecae, and to refrain from remating. Potential stimuli were altered by experimentally changing the form of male structures; controls for possible effects of these alterations on the male's behavior included sham operations on males, and sensory 'blinding' of the female in the areas contacted by these structures.

## Methods

All flies were mated when they were 10 to 12-day old virgins of a mass reared stock at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria, which was founded with specimens collected in Tororo, Uganda, kept in Amsterdam for 2–3 years, and then maintained in Seibersdorf since 1980. 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 involved recently fed flies in a room at  $24.5\text{--}25^\circ\text{C}$  and 53–55% humidity. As in previous studies on the effects of copulation on females used in matings (Saunders & Dodd, 1972; Gillott & Langley 1981), we staged copulations in glass vials (7.5 cm long and 2.5 cm in diameter). However, the duration of copulation may vary with the setting in which it occurs (Jaensen, 1979; Briceño *et al.*, 2007), so we also used a second setting for some experiments (male added to  $15 \times 19.5 \times 11$  cm plexiglass cage 5 min after the female). Unless otherwise specified, mating occurred in glass vials. The male was removed immediately following copulation, and the female was placed individually in an approximately 5 cm dia and 15 cm long cylinder covered at the ends with open-meshed cloth which allowed her to feed as described above. A few pairs broke apart after < 5 min of copulation, and were omitted from the analyses (sperm transfer is unlikely in these cases – Buxton, 1955).

Male cerci were modified by restraining the unanesthetized fly under a dissecting microscope, ventral side up against the paraffin-coated floor of a Petri dish using an open-weave cloth. The cloth was positioned so that the male's cerci were under a hole in the weave. The tips of his cerci were exposed by sliding an insect pin under their ventral surfaces. The lateral tips of the cerci were clipped off using a scissors (Fig. 2b); these 'teeth' are nearly solid cuticle, and their removal never resulted in appreciable bleeding. In a second experiment, the central articulation between the cerci was cut with a scissors (Fig. 2c). Control males in these experiments were treated in a similar way: the fly was immobilized, and his 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 its surface with a fine calligraphy brush. This produced a relatively



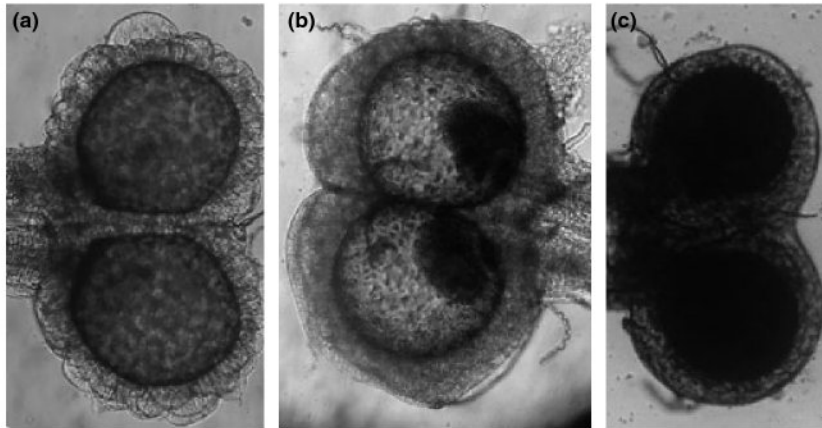
**Fig. 2** Male genitalia and associated structures of *Glossina pallidipes*. (a) extended intact cerci (solid arrows indicate the two dark lateral teeth, dotted arrow indicates articulation between the cerci); (b) extended cerci with lateral teeth removed (arrows); (c) extended cerci with cut damaging articulation (arrow); (d) ventral view of distal portion of male abdomen at rest, showing stout setae on the modified sternite 5 (the cerci are hidden from view in this position); (e) similar view of sternite 5 covered with clear nail polish (male cerci are supported on a pin in a–c)

smooth surface (Fig. 2d). Control males were restrained in the same way, and nail polish was applied to sternite 4, which is not in contact with the female during copulation.

The possible stimuli that the female could receive in the area on the ventral surface of her abdomen where the tips of the male's cerci press during copulation was modified by applying clear nail polish while the female was restrained as above. Control females received a similar amount of nail polish on the ventral abdominal surface just anterior to this area. In other females, we attempted to completely inactivate the female sense organs in the region contacted by the male cerci by

briefly pressing a red hot needle to this area (< 1 s). This treatment did not break the external surface of the female's abdominal wall. Control females were touched in a similar way with a hot needle just anterior to the site contacted by the male cerci. Females were given 2–3 days to recover before being mated to a normal male.

Ovulation and storage of sperm following copulation were assayed by dissecting females 9–10 days after they copulated. The paired spermathecae were removed and placed on a glass slide under a compound microscope, where their semitransparent walls made it possible to estimate degree to which they were filled with sperm (Fig. 3). Data were averaged for the two spermathecae.



**Fig. 3** Different degrees of filling of spermathecae with sperm: empty (a); estimated 15–20% full (b); and full (c)

The degree of filling of the spermathecae is reported below only for those females whose spermathecae were not empty. A female with sperm in her spermathecae but without a larva in her uterus was judged not to have ovulated despite being inseminated; those females with a larva in the uterus had obviously ovulated (females without sperm were not included in calculations of ovulation rates).

Female receptivity to remating was tested following copulations in some experiments as follows. The female was placed in a  $7.5 \times 2.5$  cm glass vial for 3 min with a 7 day-old virgin male on each of the next 11 days post-mating, and then on days 25, 50 and 75 post-mating. All these males attempted to mate. Means are followed by  $\pm$  SD.

## Results

### Removal of lateral cercal teeth

Removal of the lateral teeth of the cerci did not appear to affect the male's ability to grasp the female, as the frequency with which experimental males failed to achieve genital union was not different from that with which control males failed (Table 1a) (all males made behavioral attempts to copulate in all experiments). Removal of the lateral teeth also did not appear to impede the male's ability to hold on to the female once copulation had begun, and the durations of copulations with experimental males were not shorter. The durations of copulations with experimental males in plexiglass cages were significantly longer than the durations of copulations of control males in these same cages in both treatments ( $Z = -5.22$ ,  $P < 0.0001$  for experimental pairs;  $Z = 3.56$ ,  $P < 0.001$  for controls).

Removal of the lateral cercal teeth resulted in a reduction in the frequency with which the female ovulated following matings in glass vials, and in a similar, nearly significant trend following matings in plexiglass cages. The time a female spent nurturing the

larva, once ovulation had occurred, was not affected by removal of the lateral cercal teeth, as females mated to experimental and control males did not differ in the amount of time between copulation and production of the first pupa. Removal of the lateral cercal teeth reduced the fraction of females that had sperm in their spermathecae 9–10 days after mating in both glass vials and cages. The relative degree to which the spermathecae of these females were filled with sperm was significantly lower in experimental pairs that mated in glass vials, but not those that mated in cages. Finally, removal of the lateral cercal teeth increased the likelihood that the female would remate. Of those females which did remate within 10 days after the first mating, experimental females took longer.

### Cover female abdomen at the site contacted by male cerci with nail polish

This experiment represents a 'control' for the possibility that the changes seen in the first experiment were due to other changes in the male's behavior that resulted from cutting his cercal teeth (Table 1b). Coating the area of the female's abdomen which the male grasped with his cerci did not reduce his ability to grasp, and he folded this portion of her body wall inward as in normal copulations. But coating this area resulted in the female being more likely to reject male copulation attempts. In those experimental females which did copulate, the frequency of ovulation, the likelihood of having sperm in the spermathecae, and the degree to which the spermathecae were filled with sperm were all reduced.

### Damage female abdomen at site contacted by cerci with hot needle

This experiment represents a second, probably more radical control of the type just described (Table 1c). The effects of pressing a hot needle to the area of the female abdomen is normally contacted by the cerci were similar

**Table 1** Results of the experiments.

	Yes/No/Mean	Stat. test
<b>(a) Remove lateral cercal teeth</b>		
Female prevented genital coupling (vial)		
Expt.	24 130	$\chi^2 = 0.87$
Cont.	31 123	$P = 0.35$
Duration (min) cop. (vial)		
Expt.	19.6 ± 6.1 (50)	$Z = 0.93$
Cont.	18.7 ± 4.1 (50)	$P = 0.10$
Duration (min) cop. (cage)		
Expt.	26.9 ± 6.9 (50)	$Z = 3.86$
Cont.	22.1 ± 5.1 (50)	$P = 0.001$
Female ovulate (vial)		
Expt.	39 22	$\chi^2 = 4.38$
Cont.	60 15	$P = 0.037$
Female ovulate (cage)		
Expt.	24 16	$\chi^2 = 3.62$
Cont.	37 10	$P = 0.057$
Sperm spermathecae (vial)		
Expt.	61 25	$\chi^2 = 6.89$
Cont.	75 11	$P = 0.0087$
Sperm spermathecae (cage)		
Expt.	40 10	$\chi^2 = 4.33$
Cont.	47 3	$P = 0.037$
% Fill spermathecae (vial)		
Expt.	64 ± 31 (61)	$Z = -3.07$
Cont.	80 ± 24 (75)	$P < 0.05$
% Fill spermathecae (cage)		
Expt.	61 ± 22 (40)	$Z = -0.64$
Cont.	66 ± 22 (47)	$P > 0.1$
Female remate w.i. 75 days (vials)		
Expt.	42 47	$\chi^2 = 5.61$
Cont.	28 65	$P = 0.018$
Time (days) pupa laid after cop. (vials)		
Expt.	17.8 ± 7.6 (59)	$Z = -0.07$
Cont.	18.3 ± 8.1 (67)	$P = 0.93$
Time (days) remate if female remated within 10 days (vials)		
Expt.	5.4 ± 3.7 (91)	$Z = 3.12$
Cont.	2.9 ± 2.9 (90)	$P = 0.0018$
<b>(b) Coat female abdomen at site contacted by cerci</b>		
Female prevented genital coupling		
Expt.	40 79	$\chi^2 = 7.31$
Cont.	11 63	$P < 0.007$
Female ovulate		
Expt.	43 20	$\chi^2 = 5.43$
Cont.	50 8	$P = 0.019$
Sperm in spermathecae		
Expt.	63 16	$\chi^2 = 4.22$
Cont.	58 5	$P = 0.04$
% Fill spermathecae		
Expt.	68 ± 28 (63)	$Z = -2.68$
Cont.	84 ± 22 (58)	$P < 0.05$
Female remate		
Expt.	32 34	$\chi^2 = 15.6$
Cont.	8 47	$P = 0.0001$
<b>(c) Damage site female abd. contacted by cerci with hot needle</b>		
Female prevented genital coupling		
Expt.	71 4	$\chi^2 = 54.9$
Cont.	19 46	$P < 0.0001$
Female ovulate		
Expt.	8 8	$\chi^2 = 7.04$
Cont.	31 7	$P = 0.005$

**Table 1** (Continued).

	Yes/No/Mean	Stat. test
Sperm in spermathecae		
Expt.	16 8	$\chi^2 = 4.52$
Cont.	38 1	$P = 0.03$
% Fill spermathecae		
Expt.	67 ± 28 (16)	$Z = -3.19$
Cont.	86 ± 20 (38)	$P < 0.01$
<b>(d) Cover distal tips lateral cercal teeth with nail polish</b>		
Female ovulate		
Expt.	42 5	$\chi^2 = 0.05$
Cont.	49 5	$P = 0.82$
Sperm in spermathecae		
Expt.	47 10	$\chi^2 = 2.12$
Cont.	54 5	$P = 0.15$
% Fill spermathecae		
Expt.	85 ± 17 (47)	$Z = 1.24$
Cont.	79 ± 19 (54)	$P > 0.1$
<b>(e) Cover distal border of cerci with nail polish</b>		
Female ovulate		
Expt.	71 1	$\chi^2 = 0.84$
Cont.	48 2	$P = 0.36$
Sperm in spermathecae		
Expt.	72 6	$\chi^2 = 5.84$
Cont.	50 14	$P = 0.01$
% Fill spermathecae		
Expt.	86 ± 24 (72)	$Z = 1.5$
Cont.	76 ± 22 (50)	$P > 0.1$
<b>(f) Damage distal articulation between cerci</b>		
Female ovulate		
Expt.	63 7	$\chi^2 = 0.54$
Cont.	68 11	$P = 0.46$
Sperm in spermathecae		
Expt.	70 8	$\chi^2 = 0.53$
Cont.	79 6	$P = 0.47$
% Fill spermathecae		
Expt.	71 ± 34 (70)	$\chi^2 = -0.30$
Cont.	74 ± 31 (79)	$P > 0.1$
<b>(g) Cover male sternite 5 with nail polish</b>		
Female ovulate		
Expt.	32 3	$\chi^2 = 0.21$
Cont.	47 3	$P = 0.65$
Sperm in spermathecae		
Expt.	35 18	$\chi^2 = 5.34$
Cont.	50 9	$P = 0.02$
% Fill spermathecae		
Expt.	45 ± 33 (35)	$Z = 0.51$
Cont.	59 ± 34 (50)	$P > 0.1$
<b>(h) Cover female tergite 6 with nail polish</b>		
Female ovulate		
Expt.	19 9	$\chi^2 = 8.92$
Cont.	52 4	$P = 0.003$
Sperm in spermathecae		
Expt.	28 18	$\chi^2 = 12.93$
Cont.	56 5	$P = 0.0003$
% Fill spermathecae		
Expt.	59 ± 25 (28)	$Z = -1.91$
Cont.	69 ± 25 (56)	$P > 0.1$
<b>(i) Remove lat. cercal teeth + cover sternite 5 with nail polish</b>		
Female ovulate		
Expt.	22 3	$\chi^2 = 0.0$
Cont.	53 7	$P = 0.97$

**Table 1** (Continued).

	Yes/No/Mean		Stat. test
Sperm in spermathecae			
Expt.	25	50	$\chi^2 = 36.97$
Cont.	60	12	$P < 0.0001$
% Fill spermathecae			
Expt.	57 ± 27 (25)		$Z = -1.62$
Cont.	67 ± 31 (60)		$P > 0.1$

Some sample sizes (given in parentheses) differ within a treatment for several reasons: data for ovulation and percent filling of the spermathecae included only those females which had sperm in their spermathecae; remating experiments involved two separate samples of females (for  $\leq 75$  days and  $\leq 10$  days); copulation duration was only recorded for subsets of 50 experimental and control females; and the females in the  $\leq 75$  days experiment that were used to determine time until pupa production in some cases died or failed to produce pupae. The females counted to determine prevention of genital coupling were in the  $\leq 75$  days and  $\leq 10$  days remating experiments, but not all were counted.

to those of coating this area with nail polish. Experimental females were more likely to reject mating. This rate of failure was greater than the failure rate when this area was covered with nail polish ( $X^2 = 38.0$ ,  $P < 0.0001$ ). Other effects of sensory 'blinding' of the female in this way were to decrease the ovulation rate, decrease the probability that sperm would be present in the female's spermathecae, and decrease the degree of filling of the spermathecae of females which had sperm.

#### Cover lateral cercal teeth, or cover distal margins of cerci with nail polish

Changes in female responses when the tips of the male cerci were removed might be due to changes in the male's behavior (for instance, lack of ejaculation or reduction in the volume of sperm transferred) that stemmed from lack of proprioceptive input normally received by the male from his cerci during copulation. This possibility was tested in two further 'control' experiments, using nail polish to cover either the lateral teeth of his cerci (Table 1d), or the distal borders of the cerci (Table 1e). These treatments were designed to eliminate or severely reduce proprioceptive stimuli from the distal portions of the cerci when the male grasped the female's abdomen. Neither treatment resulted in changes in ovulation, sperm present in the female's spermathecae, or the degree of filling of spermathecae similar to the reductions seen when the cercal teeth were removed.

#### Damage distal articulation between cerci

When the median articulation between the cerci was destroyed, there was no effect on whether the female ovulated, whether there were sperm in her spermathe-

cae, or the relative degree of filling of her spermathecae (Table 1f).

#### Cover male sternite 5 with nail polish

When the strong setae on male sternite 5 were covered, the likelihood that the female would ovulate was not affected, but the likelihood that she would have sperm in her spermathecae decreased (Table 1g). The relative filling of the spermathecae of females with sperm was also reduced, but not significantly.

#### Cover dorsal surface female tergite 6 with nail polish

The site on the female contacted by male sternite 5 was the rear dorsum of her tergite 6. When this surface was covered by nail polish and the female copulated with an intact male, ovulation decreased, and the fraction of females with sperm in the spermathecae decreased. The degree of filling of the spermathecae in those females with some sperm in their spermathecae was slightly but not significantly lower (Table 1h).

#### Remove lateral cercal teeth and also cover sternite 5 with nail polish

Modifying both male genital structures produced mixed effects (Table 1i). Female ovulation was not affected, but the fraction of females with sperm in their spermathecae decreased sharply. The degree of filling of the spermathecae of females with sperm was slightly but not significantly lower.

### Discussion

Both removing the lateral cercal teeth of the male genitalia, and smoothing the bristly surface of his sternite 5 with a coat of nail polish resulted in lower frequencies of ovulation and sperm transfer to the spermathecae; modification of the cerci also reduced female avoidance of remating. At least two and probably all three of these female responses are independent of each other. The presence or absence of sperm in the spermathecae had no effect on ovulation when spermathecae were implanted in virgin females (Saunders & Dodd, 1972), and in no case in the present study did the degree of filling of the spermathecae differ between females that ovulated compared with those in the same test that did not ovulate (p-values ranged from 0.31 to 0.93 with Mann-Whitney *U*-Tests). In addition, ovulation was not affected while sperm storage was strongly affected in one treatment (when both the cercal teeth removed and the sternum was coated – Table 1i). With respect to female receptivity, transfer of hemolymph from mated females to virgins did not affect the virgins' receptivity, and repeated matings without sperm transfer were capable of inducing female resistance to mating (Gillott & Langley

1981), arguing against a strong link between remating and sperm in the spermathecae.

There are two non-exclusive interpretations of the effects of our experimental modifications of male genitalia: (1) Male behavioural change. Modifying the male's morphology may have caused him to alter his behavior. For instance, he might have changed his elaborate copulatory courtship (Briceño *et al.*, 2007), failed to ejaculate or transferred smaller amounts of sperm, altered the elaborate movements of his intromittent genitalia within the female (R.D. Briceño, E. Chinea-Cano, D. Węgrzynek & W.G. Eberhard, unpublished), or he may have been debilitated by immune reactions that were induced by cutting his cerci. Some of these types of changes might have resulted, for example, from a lack of normal proprioceptive feedback from his cerci or his sternite. (2) Female stimulation. Changes in female responses to modified males may have been due to changes in the stimuli that the female received from the modified male structures during copulation. For instance, absence of stimuli from the lateral cercal teeth during copulation may have induced the female to more often fail to transfer sperm (or allow transfer) to her spermathecae.

The results of the experiments that were designed to discriminate between these hypotheses favoured the female stimulation hypothesis over the male behavioural change hypothesis. When possible female receptors of stimuli from the male's cerci were masked by coating them with nail polish, these 'sensorially blinded' females responded in the same ways (reduced ovulation, reduced sperm in the spermathecae, increased remating) as if the male lacked the cercal teeth. Similar results were obtained using a second method of 'blinding' (contact with a hot needle). An additional 'control' experiment of the same type which masked possible female sense organs that could be stimulated by the male's sternal setae also resulted in a reduction in sperm presence in the spermathecae that was similar to that produced by modification of the male sternite 5. These results thus controlled for the possibility that the changes in female responses to experimentally altered male structures were due to possible changes in the male or his behavior that resulted from our manipulations of these structures (removing his lateral cercal teeth, covering his sternal setae). Controls of this sort are missing even in some classic sexual selection experiments (Andersson, 1982; Møller, 1988). Similar sensory blinding of intact males, in which we covered either the male's cercal teeth or the distal edges of his cerci, did not result in similar changes in female responses. This also indicates that possible male responses to reductions in proprioceptive stimuli that resulted from our experimental alterations of his genitalia were not responsible for the changes in female responses to copulation.

These results support the female stimulation hypothesis, that changes in female reproductive responses to the

loss of male cercal teeth and to smoothing of male sternite 5 were triggered by stimuli received from these structures during copulation. As expected, if CFC favours these male structures, the female responses to the experimental alterations all reduced rather than increased the male's chances of paternity (SAC does not predict this trend, but is not contradicted by it).

Stimuli from the male's genitalia are also apparently important at an earlier stage of male–female interactions. 'Blinding' female sense organs stimulated by the cerci on the ventral surface of her abdomen resulted in sharp increases in female rejections of male copulation attempts. The female seems to require the sensation of being grasped by the male's cerci to allow genital coupling, although she apparently does not discriminate at this stage whether his cerci have lateral teeth, or whether his sternal surface is relatively smooth. The presumably more minor changes in female stimulation that resulted from removal of the lateral cercal teeth and coating of sternite 5 had no effect on the likelihood that the female would allow copulation to occur.

Discrimination of such details may not be selectively important for the female at early stages of interactions with males, to avoid cross-specific copulations, because *Glossina* species are probably effectively isolated by several other differences, including diurnal activity cycles, habitat, and geographic range, and species-specific surface hydrocarbons that allow males to distinguish the sex and species identity of females prior to copulation (Huyton *et al.*, 1980; Wall & Langley, 1993). Although they may occasionally suffer brief chases or strikes by heterospecific males, females may not normally be subject to intromission attempts by cross-specific males; field data are lacking, however. Female sensitivities at later stages of copulation to removal of the lateral cercal teeth and smoothing of the sternite are thus unlikely to represent adaptations to avoid cross-specific pairing, and more likely represent mechanisms of female bias among conspecific males.

Stimulation from the male's cercal teeth is mechanically linked with stimuli from his sternite 5, since both structures are pressed against the female by the male's rhythmic, highly persistent genital squeezes during copulation (Briceño *et al.*, 2007). Interactions between these stimuli are apparently complex. When we experimentally modified one male structure, stimuli from the other were probably largely unaffected. For instance, removal of the lateral cercal teeth did not prevent the male from squeezing the female's ventral surface with the central portions of his cerci and thus delivering very similar if not identical dorsal stimuli to the female with his sternite 5. Similarly, covering sternite 5 did not prevent the male's cerci from grasping and squeezing the female's abdomen. While both treatments resulted in less frequent presence of sperm in the spermathecae, ovulation was affected by removing the cercal teeth but not by covering sternite 5. Modifying both male structures at the same time



produced still different effects: ovulation showed no change (the effect of removing the lateral cercal teeth was lost), while the reduction in sperm presence in the spermathecae was accentuated ( $\text{Chi}^2$  values in comparisons with modifications of only the cercal teeth, or only the sternite 5 were 24.3 and 13.3 respectively, both  $P < 0.001$ ). The effects of the two male structures on these female reproductive processes are thus to some extent independent; such independence could result in complex selection on male signals in these flies.

The lack of female responses to damage to the articulation between the male cerci may be due to male ability to move his lateral cercal teeth despite experimental modification of the articulation. Alternatively, it may be that pressing the cercal teeth against the female's abdomen is sufficient to trigger sperm storage, ovulation and remating responses, and that lateral movements of the teeth (see Briceño *et al.*, 2007) do not increase these responses. This experiment was not accompanied by a 'control' in which females suffered 'sensory blinding', but the lack of an effect on the female rendered such a control unnecessary. Although we found, as have others, that mating duration was shorter in glass vials than in cages, the effects of modifying the male's cerci on female ovulation and the presence of sperm in the spermathecae were similar in the two settings.

Our experiments have several limitations. We do not know exactly how a coat of nail polish modifies the sensations a female receives from stretch receptors in membranous areas on her abdomen's ventral surface. Stimuli from the male's cerci may have been only partially eliminated by the nail polish (as implied by the greater effect on ovulation from the probably more complete elimination of sensitivity resulting from contact with a hot needle). Nail polish applied to more rigid surfaces, such as male sternite 5, the tips of cercal teeth, the distal edges of the cerci, and the female's tergite 6, probably immobilized all setae and thus eliminated most if not all sensations resulting from their movements. The coating also bent setae to the cuticle, however (Fig. 1e), and may have produced other sensations.

A second general limitation stems from the crude nature of our experimental modifications. This study shows that females respond by altering post-copulatory processes in ways that reduce the male's chances of paternity, as predicted by CFC theory, to the absence of lateral cercal teeth and to smoothing the bristly surface of sternite 5. This does not mean, however, that females respond in the same way to the much smaller differences between the cercal teeth and sternal bristles of present-day males of *G. pallidipes*. It thus confirmed a prediction of the theory; but CFC on the differences among the forms of modern males was not demonstrated. A third possible limitation is that our observations involved lab-reared rather than wild flies; if females remate more often in captivity than in the wild, CFC may be more intense in captivity. There is no reason, however, to

expect that the female responses to genital stimulation themselves arose in captivity; the genital form of current lab males closely matches that depicted in old taxonomic works (Newstead *et al.*, 1924).

One of our measures of sperm transfer to storage involved only estimates of the fraction of the spermathecal volume occupied by sperm, rather than precise counts of sperm cells. However, our other measure of sperm transfer was more precise (presence/absence of sperm in the spermathecae), and when there were differences, the more precise measure generally showed the clearer trends (Table 1); our conclusions regarding effects on sperm transfer to the spermathecae are thus not weakened by the possible imprecision in estimating volumes.

Finally, our results do not discriminate definitively between CFC and SAC. They do contradict coercive versions of SAC, because the male genital structures do not produce perceptible damage to the female. In addition, female morphology shows no rapid divergence in the areas contacted by either of the male genital structures (both tergite 6 and the ventral abdominal membrane of the female are uniform and nearly featureless in different species of *Glossina*); they thus fail to show the predicted female coevolution with the male. The results do not, however, discriminate directly between CFC and sensory trap versions of SAC (Arnqvist, 2006) explanations for female sensitivity to male stimulation. This type of SAC explanation depends on the supposition that the male effects on females that we have documented are disadvantageous to female reproduction, a supposition for which there is no empirical support. A female ability to evolve altered response thresholds would imply that a male effect on a females reproductive processes is not automatically contrary to the female's best interests.

Cercus morphology has diverged relatively rapidly in *Glossina*, and the male cerci in the *morsitans* species group in which *pallidipes* is included are more species-specific in form than other structures (Buxton, 1955; Potts, 1970). Relatively rapid divergent evolution is a hall-mark of traits under sexual selection (West-Eberhard, 1984; Eberhard, 1985), and the results of this study document that one derived aspect of genital morphology, large lateral cercal teeth, appears to provide stimuli that are under sexual selection. The strong setae on male sternite 5 also induce female reproductive responses, but this sternite shows a contrasting pattern of evolution. The sternite is strongly sexually dimorphic throughout *Glossina*, but its form and its dense array of robust setae differ little or not at all among different species; sternite 5 has not been used to distinguish species or species groups of *Glossina* (Buxton, 1955; Potts, 1970). There are, however, species-specific differences in the patterns of squeezing movements of the male genitalia which cause the sternal setae to press or scrape against the female's abdominal tergite 6 (Briceño *et al.*, 2007).

The male cerci and sternite 5 may represent two extremes in the evolution of stimulatory structures. Judging by their sexually dimorphic form throughout the genus, the setae on sternite 5 have probably continued to stimulate females in ways that are important in inducing favourable female responses for the male, with divergence occurring in behaviour (such as temporal patterns of squeezes) rather than morphology. Males have also elaborated on the stimuli produced by the cerci, in this case (judging by the diversity of forms throughout the genus) (Fig. 1b) by altering their forms as well as their behaviour. This illustrates how male sexual signals under selection by female choice can evolve via the addition of new male innovations such as the large cercal teeth without eliminating the benefits of previously evolved signals (Andersson, 1994).

## Acknowledgments

We thank the International Atomic Energy Agency for the use of flies and facilities, John Christy, David Shuker, and Mary Jane West-Eberhard for comments on the ms, and Andrew Parker, Rudolf Boigner and Mark Vreysen for other help; the IAEA, STRI, and the Univ. de Costa Rica provided financial support.

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Received 17 February 2009; revised 29 March 2009; accepted 4 April 2009