Chapter 15
Species-Specific Behavioral Differences in Tsetse Fly Genital Morphology and Probable Cryptic Female Choice

R.D. Briceño and W.G. Eberhard

Abstract A long-standing mystery in morphological evolution is why male genitalia tend to diverge more rapidly than other structures. One possible explanation of this trend is that male genitalia function as "internal courtship devices," and are under sexual selection by cryptic female choice (CFC) to induce female responses that improve the male's chances of fathering her offspring. Males of closely related species, which have species-specific genital structures, are thought to provide divergent stimulation. Testing this hypothesis has been difficult; the presumed genital courtship behavior is hidden from view inside the female; appropriate experimental manipulations of male and female genitalia are often technically difficult and seldom performed; and most studies of how the male's genitalia interact with those of the female are limited to a single species in a given group, thus limiting opportunities for comparisons of species-specific structures. In this chapter, we summarize data from morphological, behavioral, and experimental studies of six species in the tsetse fly genus *Glossina*, including new X-ray recordings that allowed visualization of events inside the female during real time. Species-specific male genital structures perform dramatic, stereotyped, rhythmic movements, some on the external surface of the female's abdomen and others within her reproductive tract. Counting conservatively, a female *Glossina* may sense stimuli from the male's genitalia at up to 8 sites on her body during some stages of copulation. As predicted by CFC theory, these movements differ among closely related species;

R.D. Briceño (✉) · W.G. Eberhard
Escuela de Biología, Universidad de Costa Rica, Ciudad Universitaria, Costa Rica
e-mail: rbriceno@biologia.ucr.ac.cr

W.G. Eberhard
e-mail: william.eberhard@gmail.com

W.G. Eberhard
Smithsonian Tropical Research Institute, Panama, Republic of Panama

W.G. Eberhard
Biological Sciences and Museum of Natural History, Louisiana State University, Baton Rouge, USA

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some of the species-specific male genital structures that are apparently designed to stimulate the female move with different rhythms against different portions of the female's body in different species. In no case does female morphology show signs of counter adaptations to avoid or reduce male stimulation or to fit mechanically with male structures, as predicted by some alternative hypotheses to explain rapid divergent evolution of genitalia; for most male structures, the corresponding portion of the female is featureless and uniform in different species. Experimental modifications of one species-specific genital structure (the cercus), and of the possible female sense organs in the portion of the female that this male structure contacts during copulation, elicited female reproductive responses in two species (reductions in sperm transport, ovulation, and resistance to further copulations) that could result in cryptic female choice favoring this male structure.

15.1 Introduction

The male genitalia of animals with internal fertilization tend to diverge rapidly, probably because they are under one or more types of sexual selection (Eberhard 1985, 1996, 2009; Hosken and Stockley 2003; Leonard and Córdoba-Aguilar 2010). This pattern of rapid divergence is amply demonstrated in the taxonomic literature on many groups of animals; the morphological differences between the male genitalia of closely related species are often greater than those between other body parts of the same species. The ways in which sexual selection has acted on male genitalia function are much less studied. One major hypothesis is that they are used as internal courtship devices by males to stimulate females. The male's genitalia are thought to be under selection to induce the female to use the male's sperm to fertilize her eggs, and thus to exercise cryptic female choice in his favor (Eberhard 1985). Testing this hypothesis and the several alternatives (see Eberhard, this volume; also Discussion below) has been difficult, however, because the presumed genital courtship behavior during copulation is generally hidden from view inside the female.

Nearly, all studies of the possible functions of genital structures have relied on static views of the form of the male's genitalia, and their positions within the female (for Diptera, see references to studies of 43 species in Table 1 of Eberhard 2004a, also Briceño et al. 2007).

This chapter summarizes data from morphological studies of the erect male genitalia during copulation, video recordings of genital movements both outside and inside females, and experimental modifications of the species-specific details of male genital structures and of female sense organs in the areas that are contacted by these structures in different species of the tsetse fly genus *Glossina*. Because of the abundant background data that are available due to the medical and economic importance of tsetse flies, these studies provide one of the most extensive comparative views of the functional genital morphology ever published. Tsetse studies are also unique in providing the first direct behavioral observations in an arthropod of how male genital structures move within the female.

15.2 Background: The Natural History of *Glossina*

Glossina (tsetse) flies are important vectors of diseases of humans and domestic animals in sub-Saharan Africa (Gooding and Krafsur 2005; Feldman et al. 2005), and their taxonomy, biogeography, habitat preferences, genome composition, and reproductive physiology have been studied intensively (e.g., Newstead et al. 1924; Buxton 1955; Mulligan 1970; Tobe and Langley 1978; Gillot and Langley 1981; Gooding and Krafsur 2005; International *Glossina* Genome Initiative 2014). The genus is composed of approximately 20 species. Their reproduction is unusual. Females ovulate only a single egg at a time; it is fertilized and then retained in her modified oviduct (the "uterus"); the larva hatches here and feeds on material secreted by the female's milk gland until it is mature. A single larva is raised at a time, and only finally emerges from the female when it is ready to pupate. A female can raise several larvae (up to an estimated 11 cycles in field-collected females of *G. pallidipes* and *morsitans*) (Hargrove 2012) in a lifetime in the field.

Both males and females feed by sucking blood from vertebrates (generally, but not always, large mammals) (Hoppenheit et al. 2013), and males apparently lurk near hosts to grab females in the air when they come to feed. *Glossina* females are probably effectively isolated from heterospecific crossings by different diurnal activity cycles, habitats, and geographic ranges, and by species-specific surface hydrocarbons that allow males to distinguish the sex and species identity of females prior to copulation (Huyton et al. 1980; Wall and Langley 1993). Although females may occasionally be subject to brief chases or strikes by heterospecific males, they are apparently not normally subject to intromission attempts by cross-specific males in nature. Dissections of field-captured females of *G. pallidipes* and *morsitans morsitans* indicate that females do not begin mating until they are several days old and that some females mate more than once (Hargrove 2012). Some female *G. palpalis* also mate more than once in the field (Squire 1951). Multiple matings by females are reproductively significant, because both first and second males sometimes sired offspring in twice-mated females of *G. morsitans* (Kawooya 1977).

Females need to feed several times to rear a single larva, so there are opportunities for a female to copulate repeatedly. Males seize and attempt to copulate with objects coated with species-specific female surface hydrocarbons (Wall and Langley 1993). Copulation is long (normally about 30–120 min, but rarely up to 24 h Saunders 1970), and in at least some species, it is so long that the male's genitalia are designed to allow the female to defecate during copulation, thus avoiding the danger of her intestinal tract becoming plugged (Pollock 1974). Sperm transfer occurs just before separation, at the end of copulation (Jaensen 1979a, b). The male first constructs a spermatophore which is surrounded by a diffuse male accessory gland product and whose tip is inserted into the lower portion of the common spermathecal duct; sperm is then transferred into the spermatophore and (in smaller quantities) up the spermathecal duct (Pollock 1970, 1974).

Females probably participate actively in moving the sperm up the spermathecal ducts and into the spermathecae, as is the general rule in insects (summaries

in Eberhard 1996; Simmons 2001): Spermatophores that are discarded by females commonly contained "considerable" amounts of sperm (Pollock 1974 on *G. austeni*); and there is a negative effect on sperm transfer to the spermathecae of blinding female sense organs to male stimuli (Briceño and Eberhard 2009a, b on *G. pallidipes* and *G. morsitans*). Away from hosts, tsetse flies are rare, and the densities of their populations are quite low (Feldmann et al. 2005). The flies are relatively large (>1 cm long; their bite is painful) and are robust and can survive rough handling, facilitating experimental manipulations.

The phylogenies of both the bacterial gut symbionts (which may have been necessary to permit *Glossina* as well as related hippoboscoid flies to evolve to feed on vertebrate blood—McAlpine 1989), and also of the ribosomal ITS-2 sequence, as well as other, morphological traits and habitat preferences of the flies indicate that there are three subgenera: The *morsitans* and *palpalis* subgenera are more closely related to each other than they are to the *fusca* group (Newstead et al. 1924; Potts 1970; Aksoy et al. 1997; Chen et al. 1999).

15.3 Copulatory Courtship Behavior

Male *G. pallidipes* performed six highly repetitive male behavior patterns during copulation that are likely to stimulate the female (Jaensen 1979a, b; Briceño et al. 2007): "Peep" (a high pitched whine produced by rapid vibration of the male's wings while they were folded over his abdomen; "wing buzz" (both wings were repeatedly brought forward to extend laterally and buzzed for about 0.5 s; usually the male made two wing-forward movements in each burst of buzzing); "raised legs II"(both middle legs were raised dorsally and anteriorly at the initiation of a bout of wing buzzing); "rub with legs I" (the front legs rubbed or tapped repeatedly on the pronotum and/or dorsal surface of the head of the female for about 0.5 s/burst); "rub with legs II" (the middle legs rubbed or tapped repeatedly on the sides of the female's thorax, her head or her abdomen); and "rub with legs III" (the hind legs rubbed or tapped repeatedly on the ventral surface of the female's abdomen). None of these male behaviors had any obvious mechanical consequences for copulation (e.g., they did not help the male hold onto the female); they appear to be designed instead to stimulate the female during copulation. They resembled the copulatory courtship behavior of many other insect species (Eberhard 1994, 1996).

15.4 Clasping Male Genitalia

15.4.1 Morphology of Male Clasping Genitalia

Several male genital structures have long been known to have species-specific forms (Fig. 15.1) and are well illustrated in the taxonomic literature on *Glossina*

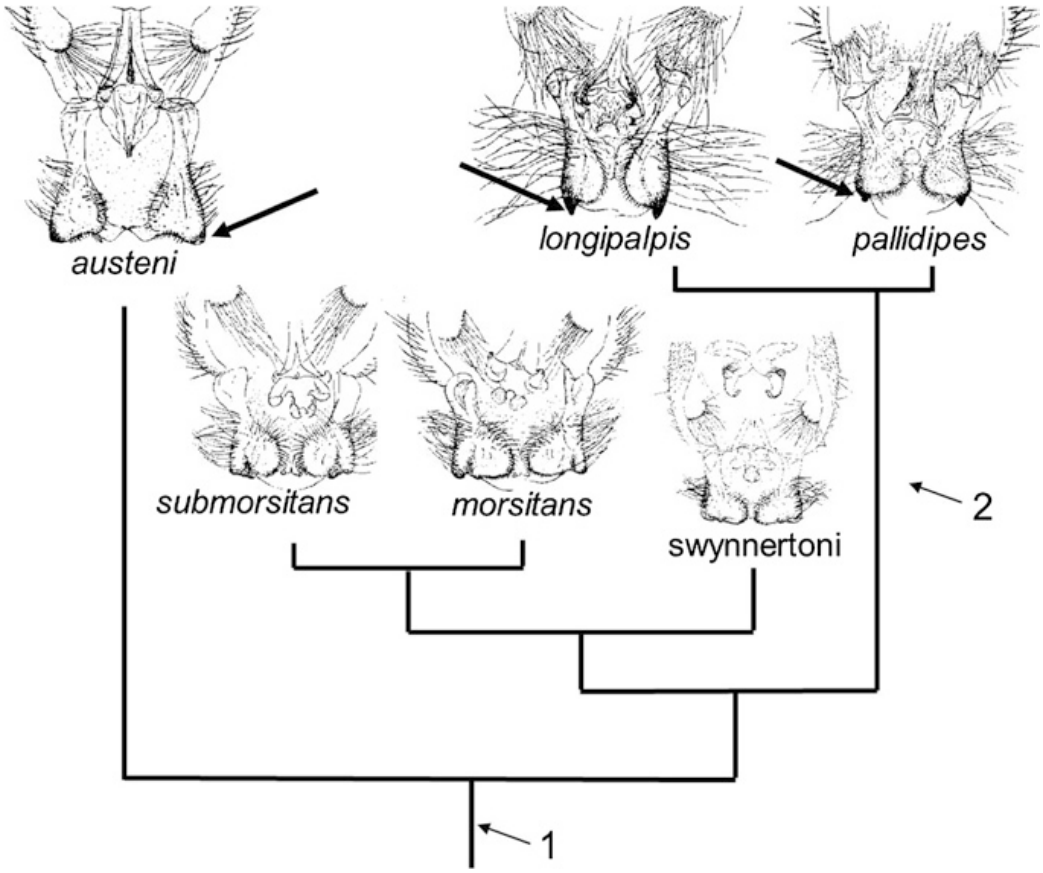

Fig. 15.1 Drawings of the male genitalia (cerci) of *Glossina* species in the *morsitans* subgenus (from Newstead et al. 1924) superimposed on the phylogenetic relations between these species (after Chen et al. 1999). At *1*, there were lateral cercal teeth but they were not elongate or strengthened (*heavy arrow* to *austeni*). At *2* these teeth, whose function was studied experimentally in *G. pallidipes* by Briceño and Eberhard (2009a), were elongate and strengthened (*heavy arrows* in *longipalpis* and *pallidipes*) (changes deduced from outgroup comparisons with the other two subgenera of *Glossina*) (from Briceño and Eberhard 2009a)

(summary in Potts 1970). The male genitalia are highly derived with respect to those of other flies, and the homologies of some structures are not clear (D.M. Wood, pers. comm.). Most taxonomic illustrations of male genitalia include only their positions at rest, rather than to the everted positions that they assume during copulation. Thus, several of the male sclerites and processes long known to differ between species were not understood with respect to their mechanical relationships to each other and to the female during copulation until recently.

We have observed copulating pairs of six species *G. pallidipes*, G. *morsitans*, and *G. swynnertoni* of the *morsitans* subgenus; *palpalis* and *fuscipes* of the *palpalis* subgenus; and *G. brevipalpis* of the *fusca* subgenus under a dissecting microscope; in addition, we have flash-frozen pairs and then dissected them (Briceño et al. 2007, 2015; Briceño and Eberhard 2009a, b), Pollock (1974) sectioned more than 70 pairs of *G. austeni* (in the *morsitans* subgenus) that were flash frozen during

copulation. The following description of clasping in the best-studied species, *G. pallidipes*, applies in at least in its major details to all seven species (and probably for species throughout the genus).

The male genitalic structures of *Glossina* can be classified in two categories: those which function to grip or press against the external surface of the female's abdomen—the cercus, the fifth abdominal sternite, and the inferior clasper of the male (we include male's modified sternite 5 as part of his genitalia, though strictly speaking this is not correct); and those inserted into her genital tract—the phallobase or phallosome (e.g., Patton 1936; Pollock 1974) (with its associated structures) (Fig. 15.2). There are species-specific traits on both sets of male structures, though the morphology of the gripping structures is better studied (they are larger and are more easily visible when the male genitalia are not everted). The mechanical interactions of the male's grasping structures with the female are also better understood (Figs. 15.2 and 15.3) than those of his intromittent genitalia.

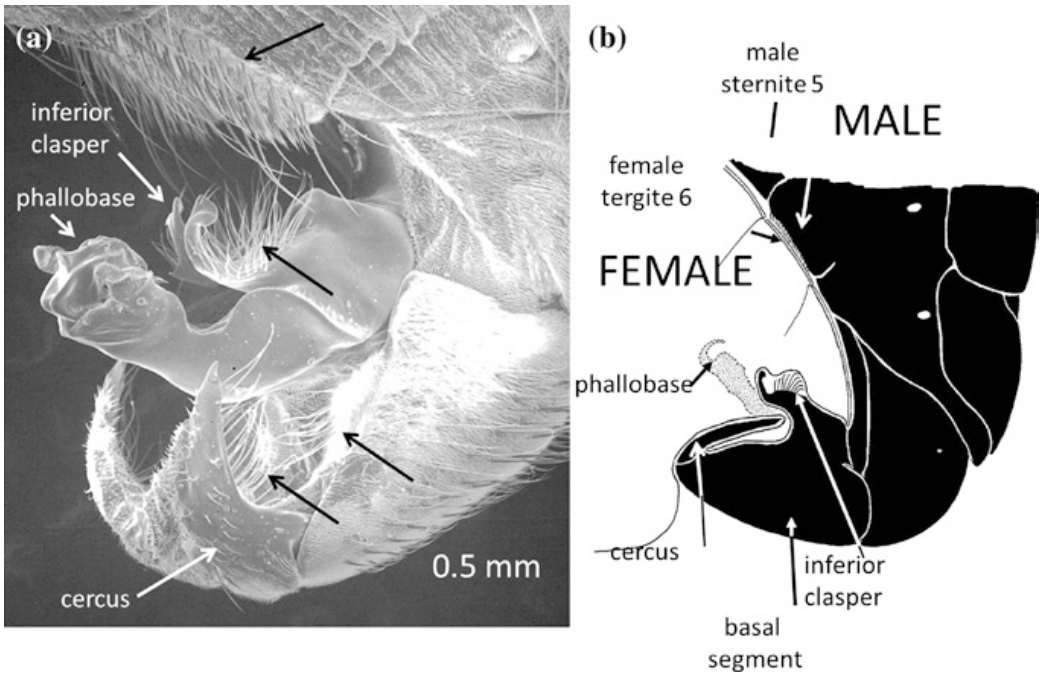

Fig. 15.2 **a** SEM portrait of the everted male genitalia of *G. fuscipes*. The *heavy arrows* indicate groups of setae that rub against the female when the male squeezes her with his cerci. **b** Schematic representation of the male (*black*) and female genitalia of *G. fuscipes* during copulation. The male's phallobase is deep inside the female's vagina, his tightly folded cerci pinch the ventral wall of the female's abdomen tightly, the dense, stout setae on his sternite 5 press on her tergite 6, and the curved processes of the his inferior claspers and their brush of setae press on the external surface of her abdomen just ventral to the posterior tip of her tergite 7 (the tips of the inferior clasper setae were not observed directly; probably they are curved as pictured, given their lengths, the tight quarters, and the substantial pressures that males apply when squeezing females, as evidenced by deformation of the ventral surface of the female's abdomen) (from Briceño et al. 2015)

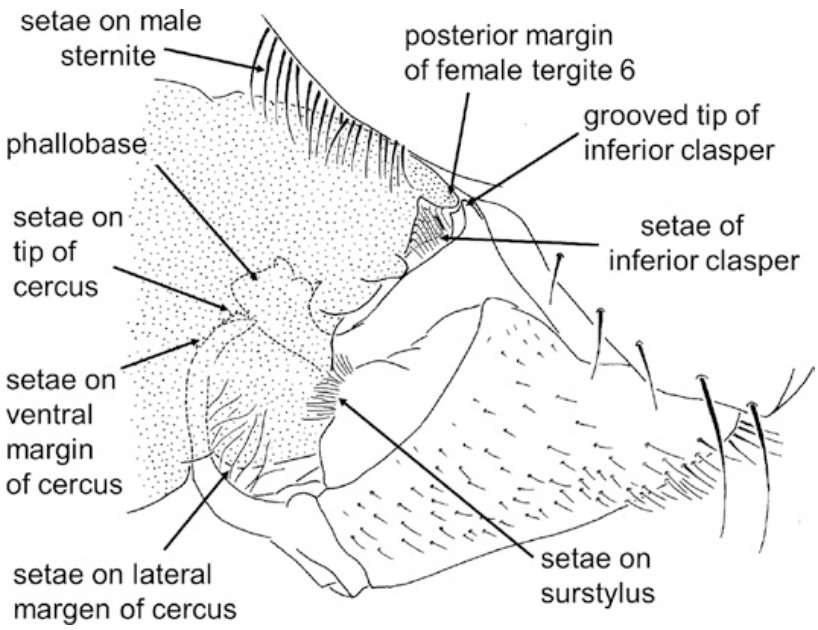

Fig. 15.3 Diagrammatic view of the genitalia of a copulating pair of *G. pallidipes* showing multiple points where apparently stimulatory male structures contact and move against the female. Six groups of male setae that press or rub against the female during copulation, as well as the inferior clasper contact with the posterior margin of her tergite and his intromittent phallobase, are all indicated (from Briceño et al. 2015)

The male's cerci and his sternum 5 clasp the external surface of the female in a pliers-like grip. The plate-like cerci articulate basally with the thick "basal segment," which houses powerful muscles (Fig. 15.4). The tips of the cerci press against the membranous ventral surface near the tip of the female's abdomen, causing it to invaginate sharply (Fig. 15.2). The other arm of the "pliers" is the highly setose ventral surface of the male's sternite 5 (which is sexually dimorphic); it presses on dorsal surface of tergite 6 or 7 of the female abdomen. Studies of *G. pallidipes* (Briceño et al. 2007) showed that the genitalic clasp and the subsequent movements of the male genitalia cause five different groups of setae on the male genitalia and a further group on his sternite 5 to rub or brush against the external surface of the female's abdomen (Fig. 15.3).

There are minor differences between the subgenera in the positions of the male's genitalia on the female during copulation. Males in the *morsitans* subgenus were positioned slightly more posteriorly on the female. In *G. brevipalpis*, G. *palpalis*, and *G. fuscipes*, the cerci folded tightly against the ventral surface of the basal segment, making an angle of substantially less than 90° with the basal segment (Fig. 15.2), while in the *morsitans* subgenus this angle was closer to 90° (Fig. 15.3).

Details of the contact between the inferior claspers of the male genitalia and the surface of the female's abdomen during copulation also varied, and the members of the *morsitans* subgenus again differed from the other species. The inferior claspers of the *morsitans* subgenus pressed on the tip of tergite 6 itself, and the tergite tip fit into a groove in the inferior clasper (Fig. 15.3), while the inferior

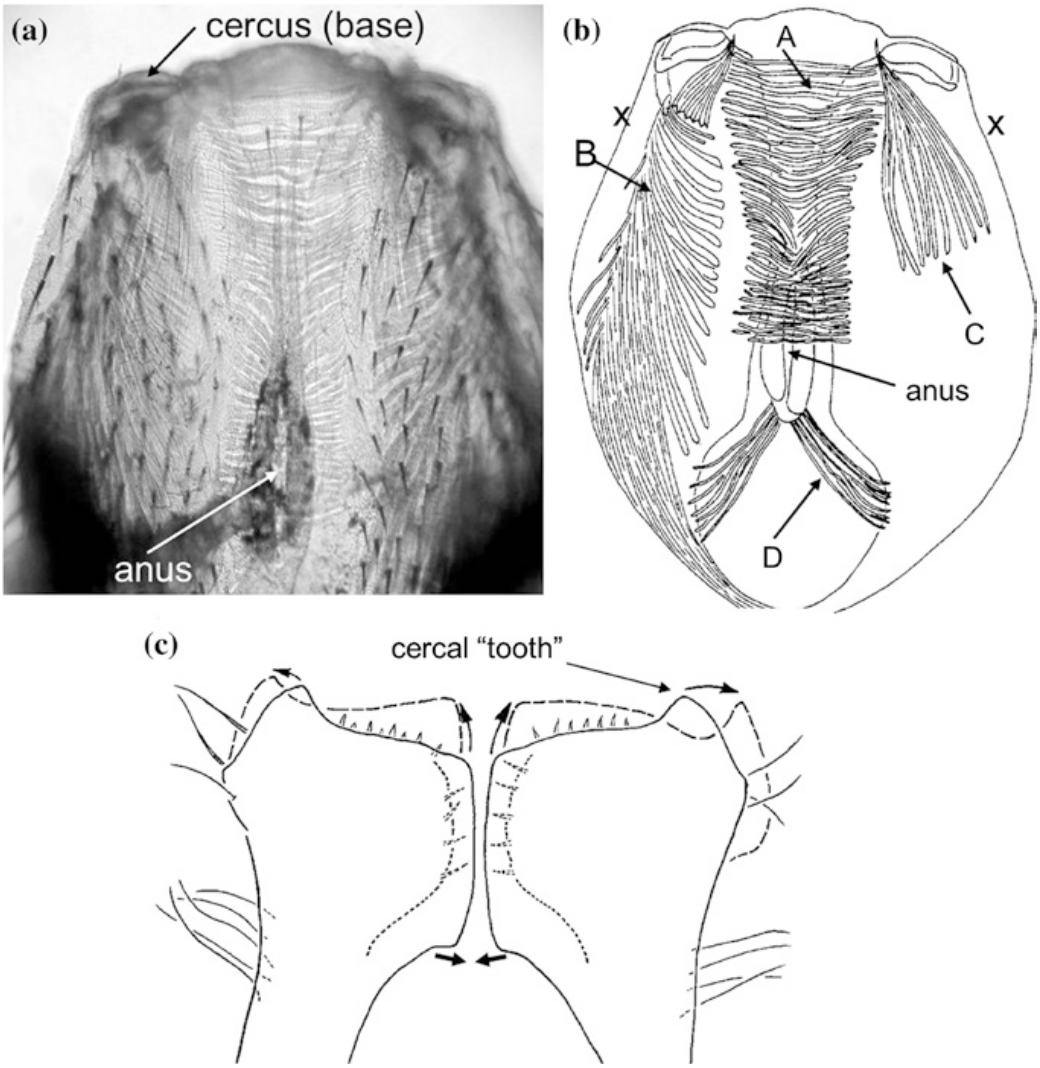

Fig. 15.4 The powerful muscles of the male genitalia of *G. pallidipes* provide indirect evidence for further movements that could not be directly observed during copulation. When the dorsal wall of the male's basal segment is made transparent (**a**), several groups of muscle fibers are visible (*A–D* in **b**). The most massive group of fibers (*B*) apparently serves to move the two cerci, as shown diagrammatically in (**c**) (artificially squeezing the cerci at points "x" produced the rocking motion shown in **c**). Rocking movements would cause each cercal tooth to scrape against or stretch the abdominal cuticle of the female during copulation (the tips of the cerci are at the bottom in **a** and **b**, at the top in **c**) (after Briceño et al. 2007)

claspers and their dense setae in the males of the *palpalis* and *fusca* subgenera pressed on the membranous body wall of the female, directly ventral to the tip of her abdomen (tergite 7) (Fig. 15.2) (Briceño et al. 2015). In *brevipalpis*, in contrast, each inferior clasper bears a flat plate-like extension and lacks strong setae; the medial surfaces of these planar processes pressed on the female's body wall just ventral to the tip of her tergite 7 (Briceño et al. 2015).

There are no external differences in the females that corresponded to any of these differences in the morphology and positions of the male genitalia. The

ventral surface of her abdomen that was grasped by the cerci, the tip of her tergites 6 and 7, and the membranous area just ventral to the tergite that were contacted by the male inferior clasper are all featureless and at least superficially similar in the different species, except for a transverse membranous cleft dorsal to the vulva of *G. austeni* into which the inferior claspers fit (Pollock 1974).

15.4.2 Behavior of Male Grasping Genitalia. Observations of the Behavior of Cerci and Associated Structures

Direct observations under the dissecting microscope showed that the cerci squeeze the distal tip of the female's abdomen rhythmically in long bursts of activity (lasting many minutes). These squeezes exert substantial force on the female. They cause the membranous ventral surface of her abdomen to invaginate sharply (Fig. 15.2) and bend the lower portion of her internal reproductive tract sharply (Vanderplank 1947; Pollock 1974; Briceño et al. 2007); in *G. palpalis*, the male cerci produce small areas of abrasion on the membranous female abdomen (Squire 1951). Another indication of strong forces is that the distal margins of the cerci in field-collected males sometimes have a "distinctly chipped or worn appearance" in *G. morsitans* (Newstead et al. 1924, p. 38).

The squeezing behavior of *G. pallidipes* is highly structured, with long rhythmic series that vary consistently at different stages of copulation (Briceño et al. 2007). Species apparently differ in the relative strengths and durations of squeezes (Table 15.1). Although different strengths were only classified in loose, probably somewhat imprecise categories, and although the squeezes at different stages of copulation were combined, some differences were so clear that they surely reflect real behavioral differences between species. For instance, both the strong and very strong squeezes by *G. pallidipes* were much longer than those of *G. fuscipes*; small squeezes were common in *G. pallidipes*, rare in *G. brevipalpis* and absent in *G. fuscipes* and *palpalis*.

Artificial manipulation of the cerci of *G. pallidipes* by squeezing their bases together gently with a forceps showed that the two cerci could rock against each other (Fig. 15.4c); this movement would cause the cercal teeth to scrape against or stretch the female's abdominal cuticle during copulation. These movements were not verified by direct observations (as can be seen in Fig. 15.3, the cercal tips are out of view in the deep fold in the female's abdomen), but the heavy musculature in the basal segment (muscles A, B, C in Fig. 15.4b) and the sustained, rhythmic narrowing movements of the basal segment during copulation in *G. pallidipes* imply that such movements do occur (Briceño et al. 2007).

X-ray videos (Briceño et al. 2010, Fig. 15.8) of all species of the *morsitans* subgenus consistently revealed a pair of retractable spines arising near the bases of the cerci that pinched the surface of the female's abdomen against the cerci, just posterior to the distal tips of the male's cerci, causing a small, rounded portion of the female's abdomen to be pinched off between the tip of the spine and the tip of the male's cercus (Briceño et al. 2015). This pinch was constant, and the spine did not move, except when the male extended his cerci and relaxed his squeeze on the

Table 15.1 Durations (in seconds) of different types of squeezing movements by the male's cerci in five species of *Glossina*

	G. pallidipes	*G. morsitans morsitans*	*G. morsitans centralis*	*G. brevipalpis*	*G. fuscipes*	*G. palpalis*
Relative strength of the squeeze						
Very strong	$62.6 \pm 44.1a_1a_2a_3a_4$ (256)	$26.6 \pm 27.4a_1a_5a_6a_7b_1$ (376)	$13.5 \pm 16.7a_2a_5a_8a_9a_{10}$ (195)	$6.3 \pm 5.97a_3a_6a_8a_{11}a_{12}$ (280)	$3.92 \pm 21.4a_4a_7a_9a_{11}a_{13}$ (51)	$131.1 \pm 13.0b_1b_2a_{10}a_{12}a_{13}$ (13)
Strong	$50.4 \pm 53.5a_1c_1a_2a_3$ (119)	$53.5 \pm 33.0a_1a_4a_5a_6$ (50)	$16.5 \pm 15.1c_1a_4a_7a_8$ (18)	–	$0.64 \pm 0.47a_2a_5a_7a_9$ (409)	$4.47 \pm 6.67a_3a_6\ a_8a_9$ (279)
Medium	$22.0 \pm 9.0a_1a_2$ (112)	$6.7 \pm 6.2a_1$ (20)	–	$5.6 \pm 3.7a_2$ (218)	–	–
Mean number of sequences	$14.8 \pm 8.9b_1a_1$ (55)	$9.4 \pm 9b_1$ (20)	–	$7.8 \pm 5.1a_1$ (211)	–	–
Small	0.17 ± 0.03a1a2 (150)	0.12 ± 0.16a1a3a4 (150)	0.14 ± 0.13a3 (50)	$0.23 \pm 0.04a_2a_4$ (45)	–	–
Mean number of sequences	33.6 ± 20.3a1a_2 (160)	27.6 ± 26.1a_1a_3 (288)	30.6 ± 14.9a_4 (160)	$3.1 \pm 1.1a_2a_3a_4$ (30)	–	–
N (pairs)	22	19	9	5	4	5

The relative strengths of squeezes were determined by qualitative judgements of how deeply the male cerci folded the ventral surface of the female's abdomen inward. Numbers in parentheses refer to the number of squeezes measured. The values in the same row with the same letter and number (e.g., a_1, a_2) were significantly different with Mann–Whitney U tests (a = $p < 0.001$; b = $p < 0.01$; c = $p < 0.05$)

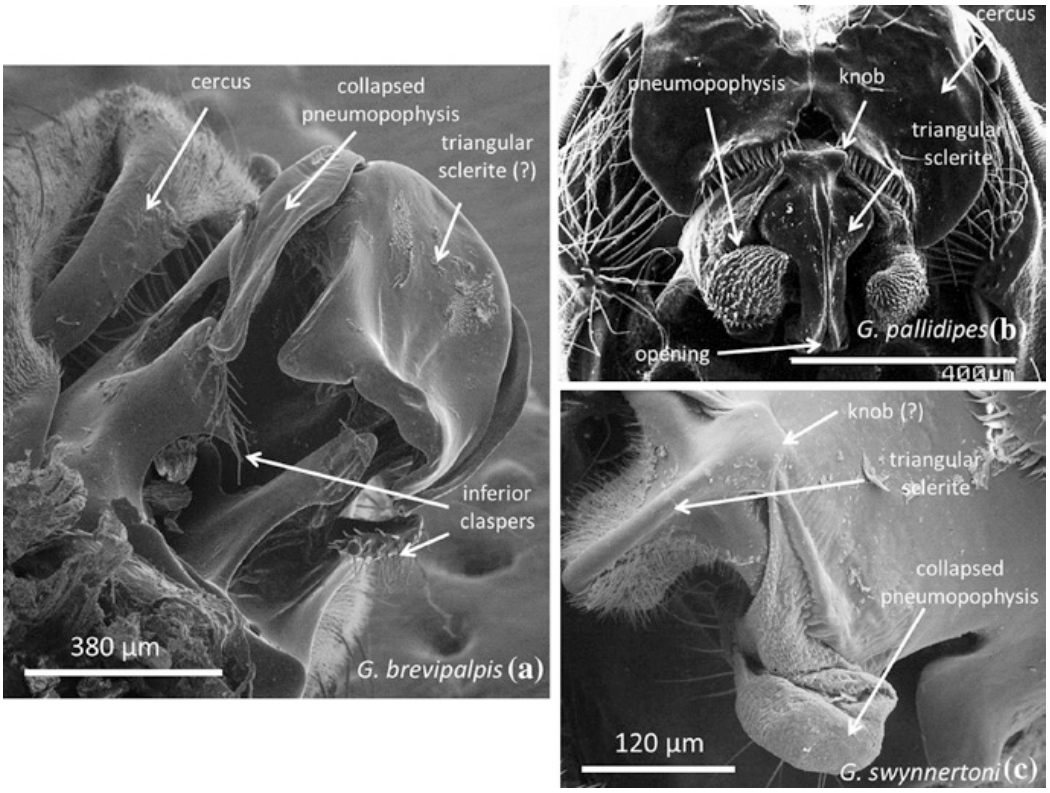

Fig. 15.5 SEM portraits of selected aspects of the distal portion of the phallobase in *G. brevipalpis* (**a**), *G. pallidipes* (**b**), and *G. swynnertoni* (**c**) that illustrate types of inter-specific differences. *G. pallidipes* and *G. swynnertoni* are in the same subgenus, and some homologous though differently shaped structures are recognizable (e.g., the triangular sclerite and the knob); both species have pneumopophyses, but those of *G. pallidipes* are heavily spined while those of *G. synnertoni* are nearly smooth, at least in the distal portion. In *G. brevipalpis*, which is a member of a different subgenus, some traits, such as the triangular plate and knob are absent or so different in form that they are not easily recognizable; other homologous traits, such as the pneumopophyses, are recognizable but have different forms and lack spines. In **b**, the cerci of *G. pallidipes* press forcefully against the curved surface of the basal portion of the phallobase (the female tissue which was between these male structures in the copulating pair—abdominal cuticle plus one wall of the oviduct—has been removed) (after Briceño et al. 2015)

female's abdomen, when the spines were withdrawn toward the bases of the cerci. Despite the consistency with which these pinches were seen in the X-ray videos, they never occurred in flash-frozen pairs, and indeed, no such pinches were seen in the extensive study of *G. pallidipes* (Briceño et al. 2007). Presumably the spines were withdrawn when the copulating pair was frozen.

In X-ray videos of two species, *G. pallidipes* and *G. brevipalpis*, the tips of the cerci sometimes repeatedly flexed posteriorly (with respect to the female) with small amplitude movements. Because the tips of the cerci were near the basal, cylindrical portion of the phallobase during copulation (Fig. 15.5b), the effect of these flexions was to rub or knead the female tissue between the male's cerci and his phallobase.

One type of movement seen in X-ray videos of one pair of *G. swynnertoni* was not observed in any other species. A pointed sclerite between the male's cercus

and his phallobase rhythmically stabbed the female, probably on the membrane or a sclerite just ventral to her vulva (Briceño et al. 2015). The phallobase of this male was not inserted in the female's vagina; it was not clear whether it had been withdrawn after a previous insertion or had not yet been inserted into the female.

15.5 Deducing the Functions of Clasping Genitalia from Morphology and Behavior

The morphological designs of genital structures and their positions with respect to the female during copulation suggested that several non-intromittent structures function to stimulate the female (Briceño et al. 2007). Six different groups of modified setae on and near the male genitalia of *G. pallidipes* rub directly against particular sites on the female's body during the squeezing behavior that accompanies clasping (Fig. 15.3) (Briceño et al. 2007). Differences in the designs of these setae correlate with the probable force with which they press on the female and the probable sensitivity of the female surfaces that they contact (e.g., the setae are robust where the male presses strongly on a female abdominal tergite, thin where they brush against female membranes) (Briceño et al. 2007).

15.6 Experimental Tests of the Functions of Male Genital Structures

Experimental modifications utilizing two techniques demonstrated that in *G. pallidipes* and *G. morsitans* two species-specific structures on the male cerci stimulate virgin females to ovulate, to transport sperm, and to refrain from remating following copulation (Briceño and Eberhard 2009a, b). In some pairs, the male cerci were modified experimentally by cutting off the large teeth on the cerci (see arrows in Fig. 15.1); control males were restrained as if to produce the experimental modifications but were left untreated. In other pairs, the male was left intact, but any sense organs in the area of the female's abdomen that is contacted by the male during copulation were blocked (by coating them with nail polish) or inactivated (by pressing a red hot needle briefly (<1 s) against her abdomen) (in both experiments adjacent segments were modified in similar ways in control females).

Sperm transfer to the spermathecae and ovulation were checked by dissecting females 9–10 days following copulation. Sperm transfer was estimated by the degree of filling of the spermathecae (Fig. 15.6). Sperm are thought to be deposited by the male in a spermatophore at the mouth of the common spermathecal duct (as in other *Glossina*); spermathecal filling (or lack of filling) may be influenced by female processes (sperm transport, sperm dumping), or by sperm motility). Ovulation was verified by checking whether there was a developing larva in the uterus 9–10 days later. Female receptivity to further mating was tested by placing the female in a glass vial

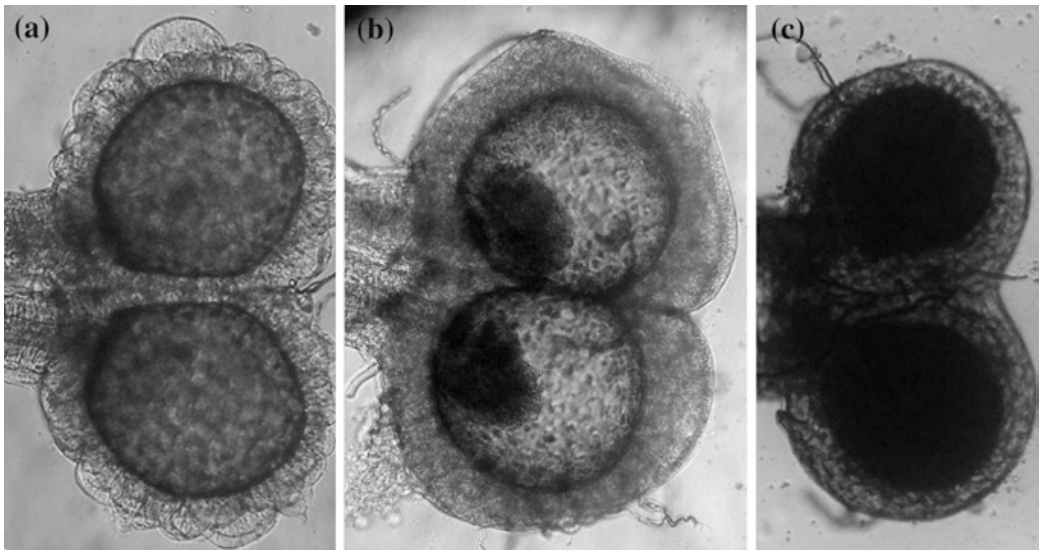

Fig. 15.6 Different degrees of filling of the spermathecae of *G. pallidipes*: **a** empty; **b** 15–20 % full; **c** full (after Briceño and Eberhard 2009a)

for 3 min with a 7-day-old virgin male. Remating tests were conducted (until the female accepted a mating) on each of the first 11 days after copulation, and then on days 25, 50, and 75 post-mating. All males attempted to mate in these trials.

As predicted by CFC theory, these manipulations of both the males and the females induced female reproductive responses that would likely produce biases against the male's chances of paternity: Ovulation and sperm storage decreased, while female remating increased. The fact that experimental blocking of female sense organs elicited the same responses as modifying the males showed that responses to altered male genital morphology were due to changes in tactile stimuli received by the female from the male's genitalia, rather than to other possible changes in the males that resulted from alterations of their genitalia. Experimental modifications of females also revealed a previously unappreciated female response. When unable to sense male contact, females prevented clasping, by keeping their wings folded posteriorly (an occasional response noted previously in intact females by Squire 1951). In combination with previous studies of tsetse reproductive physiology, these data constitute the most complete experimental confirmation that sexual selection (probably by CFC—see below) acts on the stimulatory properties of male genitalia (Briceño and Eberhard 2009a, b).

In similar experiments, in which the strong setae on the male sternite 5 of *G. pallidipes* were covered with nail polish, the likelihood that the female would ovulate was not affected, but the likelihood that she would have sperm in her spermathecae decreased (Briceño and Eberhard 2009a). The reciprocal experiment, in which the male was left unaltered, but nail polish was applied to the dorsal surface of the female tergite which is contacted by male sternite 5 during copulation, gave similar results: The fraction of females with sperm in the spermathecae decreased. In addition, ovulation decreased significantly (Briceño and Eberhard 2009a).

In one additional, unpublished experiment, nail polish was applied to the pneumopophyses of *G. pallidipes* (inflatable sacs on the male phallobase—see below), smoothing their surfaces but allowing at least some expansion. This treatment resulted in reduced sperm transfer to the female's spermathecae (7 of 12 females mated to experimental males lacked sperm in their spermatheca 9–10 days after copulation; 2 of 19 females mated to control males lacked sperm; $chi^2 = 4.27$, $p = 0.04$.). The roles of the two sexes in these experiments remain undetermined, however. It is possible, for instance, that expansions of the pneumopophyses help drag the male's phallobase deeper into the female or position it correctly to transfer sperm, but they might also serve to stimulate the female and induce a response favorable to the male's reproduction (or both). The significance of these results regarding possible cryptic female choice is thus uncertain.

15.7 Intromittent Male Genitalia

15.7.1 Morphology of Intromittent Male Genitalia

Additional species-specific male genital structures of *Glossina* are inserted into the female's vagina. The intromittent male genital structure is approximately cylindrical phallosome, which bears several structures that have been less completely characterized (see, however, Patton 1936). All five species examined had a pair of small membranous sacs (pneumopophyses); their sizes and shapes differed; and some but not others were covered with short spines (Fig. 15.5) (Briceño et al. 2015). The positions of the two pneumopophyses varied even on the two sides of the phallobase of the same male in copulating specimens (e.g., Fig. 15.5b), so these sacs are mobile during copulation (see also direct observations below). All species also had a sclerite at the tip of the phallobase; in some, it was more or less triangular, with the distal portion produced into a point bearing an opening through which sperm are probably transferred (Fig. 15.5b). This opening contained a small folded sac that, in some specimens of *G. pallidipes* that were flash frozen during copulation, was everted into the mouth of the female's spermathecal duct (Briceño et al. 2007). In *G. austeni*, the male phallobase is inserted into the lower portion of the common spermathecal duct (Pollock 1974). Taxonomic studies that have focused on the phallobase have documented substantial inter-specific variation (Newstead et al. 1924; Patton 1936).

15.7.2 Behavior of Intromittent Male Genitalia

The male's genitalia are hidden inside the female during copulation, so their behavior is difficult to study and is poorly understood. There are nevertheless two sources of data. One technique involved removing the male's head, positioning

him immobile with his cerci lifted to expose his phallobase under a dissecting microscope, and then eliciting movements of his genitalia by gentling deflecting the setae on his interior claspers (stimulation of these setae in intact males failed to evoke genital behavior). Movements of genital sclerites and the pneumopophyses were videotaped through the microscope. A second more direct technique was to make X-ray recordings of movements that occur inside the female during copulation (Briceño et al. 2010, in press). For obtaining X-ray videos, virgin male and female flies were placed together in small chambers; recording began approximately 30 s or less after the male had mounted the female and seized her abdomen with his genitalia. Intromission was not always immediate (as also determined in some pairs of *G. austeni* and *pallidipes* by interrupting copulations—Pollock 1974; Briceño et al. 2007). The process of intromission, which, judging by some other fly species (Eberhard 2002), may involve female movements that expose her genital opening, has not been studied.

Each technique has limitations (behavior outside the female is not necessarily the same as that inside; and the resolution of the X-ray recordings was limited by the size of the genitalia and differences in X-ray opacity). It was clear, however, that the male genitalia move actively inside the female during copulation. Some movements are clearly rhythmic and are not mechanically necessary to bring the phallobase to the probable site of sperm deposition; instead, they seem likely to stimulate the female (Briceño et al. 2010, in press).

15.7.3 Pneumopophyses

When headless males of all five species were stimulated, some everted their intromittent genitalia and repeatedly inflated and collapsed their pneumopophyses rapidly. These inflations were directed laterally and basally. The shapes and positions of the fully inflated pneumopophyses appeared to be consistent intra-specifically and to vary between species (Fig. 15.7; Briceño et al. 2015). The pneumopophyses could not be seen in the X-ray videos of copulation; they were visible when the male genitalia were outside the female, but not when they were inside her. The mechanical result of pneumopophysis inflation inside the female is presumably either to brace the male's phallobase within the female's vagina, or to push it deeper inward; both types of movement seem likely to stimulate the female via stretch receptors (if they are present) in the walls of the oviduct.

15.7.4 Distal Triangular Sclerite of Phallobase

In headless males, the distal sclerite periodically swung about 180° so that its distal end projected distally. In all species, extension of this sclerite occurred only while the pneumopophyses were extended. Similar movements, though only

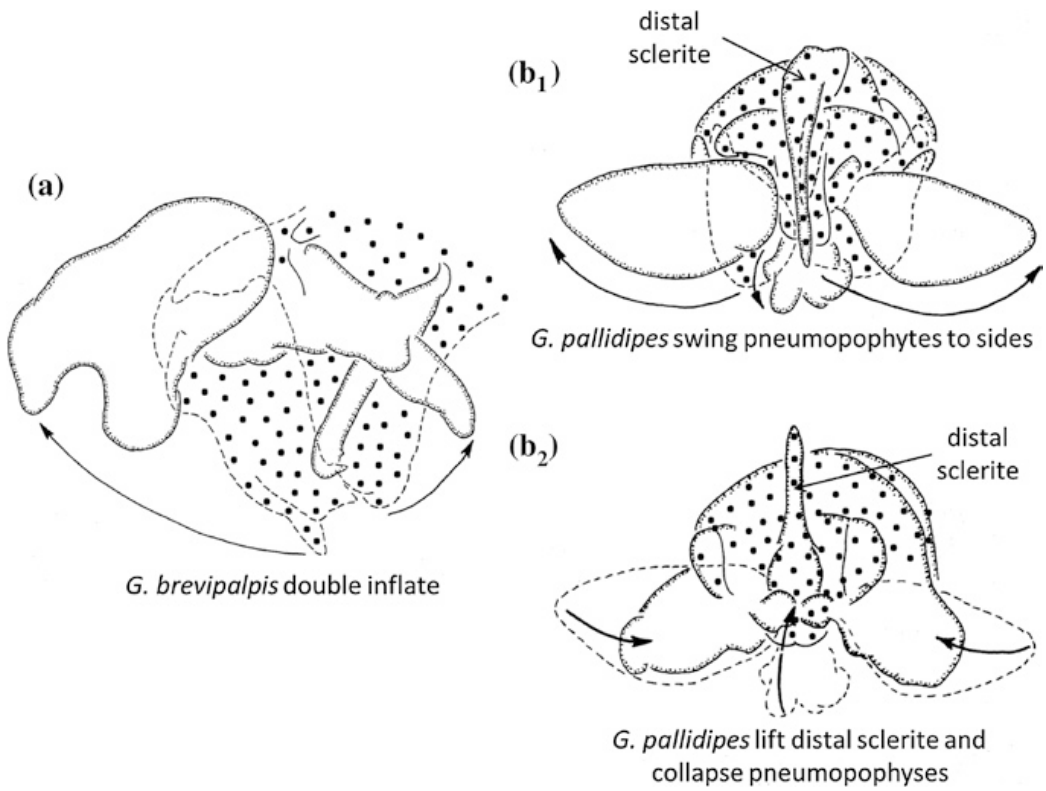

Fig. 15.7 Schematic drawings of different movements and shapes of the inflated pneumopophyses of *G. brevipalpis* (**a**) and *G. pallidipes* (**b**). The stippled portions show the basiphallus; the *curved arrows* indicate the movements of the pneumopophyses immediately preceding the positions shown in the drawing (**a** in lateral view, $\mathbf{b_1}$ and $\mathbf{b_2}$ in caudal view). The distal sclerite of *G. pallidipes* also moved just prior to $\mathbf{b_2}$ (*G. brevipalpis* from Briceño et al. 2015; *G. pallidipes* from Briceño et al. 2010)

incomplete in scope and less clearly resolved, may have occurred sometimes when the phallobase was at the apex of a thrust in the X-ray videos (Briceño et al. 2015). It is possible that these movements exert strong forces. In *G. austeni*, the "hook-like aedeagus" (perhaps homologous with the distal sclerite) is provided with powerful muscles and appears to push strongly against the female's genital papilla on the wall of the uterus (Pollock 1974).

15.7.5 Phallobase as a Unit

The phallobase did not move as a unit in headless males, but it made long series of thrusting movements as a unit inside the female in the X-ray video recordings of five species (Fig. 15.8) (Briceño et al. 2015). Some patterns of phallobase movements in X-ray recordings, including the thrusting movements of the entire phallobase, as well as an unidentified sclerite, pulsing movements of the phallobase,

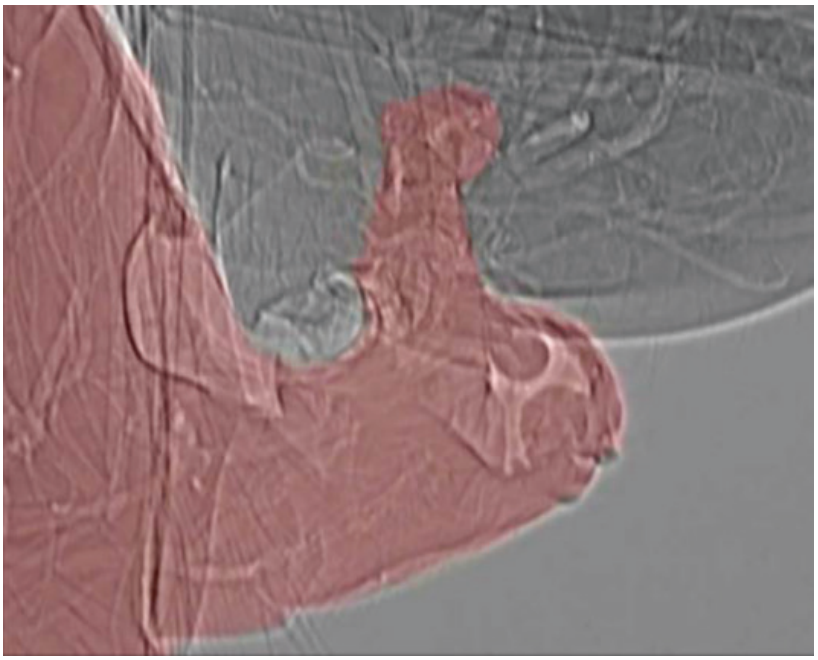

Fig. 15.8 An X-ray image of a copulating pair of *Glossina pallidipes* (male shaded pink) showing the male's intromittent phallobase inside the female. The outlines of the phallobase were determined by analysis of movement patterns during thrusting behavior (see Briceño et al. 2015)

and withdrawal of the phallobase during strong cercal squeezes, and rubbing movements of the tips of the cerci against the central cylinder of the phallobase, were seen in some species but not others (Briceño et al. 2015). There were also some probable species-specific differences in the durations of individual thrusts of the phallobase; for example, the mean thrust duration was 0.28 ± 0.16 s in *G. pallidipes*, but only 0.04 ± 0.01 s in *G. brevipalpis*) (Briceño et al. 2015). The small sizes of some samples and their incomplete coverage of the entire copulation period preclude confident conclusions regarding the absence of particular types of movements in any given species; nevertheless, it appears that there are differences between species in relative frequencies.

Thrusting movements caused the dorsal surface of the basal portion of the phallobase to press and move structures on the female's external surface near her vulva, such as the sternal plate just ventral to her anus. In *G. pallidipes,* these movements presumably caused the arc of long setae on the male's inferior claspers to press deeper into or against the membranous groove around the anal plates and the sternal plate (this contact occurred in flash-frozen pairs) (Briceño et al. 2007). Thrusting movements of the basal portion of the phallobase also applied pressure to the curved projections of the inferior claspers of *G. palpalis*, which in turn pressed against the female. The rhythmic thrusting movements (about 1 every 3 s) of a long thin, hinged structure in *G. palpalis* caused the tips of the inferior claspers to rub across a surface (probably the intersegmental membrane of the female) (Briceno et al. in press). The phallobase was also temporarily withdrawn entirely from inside the female for periods of up to 49 s during especially strong cercal squeezes in *G. pallidipes* and *morsitans* (Briceño et al. 2015).

15.8 Discussion

15.8.1 Stimulation of the Female: Implications from Morphological Designs and Behavior

Several kinds of data confirmed two cryptic female choice predictions for *Glossina*: Males stimulate females during copulation; and the stimuli received by the female diverge rapidly over evolutionary time. Even though in external view the abdomen, legs, wings, and head of a male *Glossina* tsetse fly move only sporadically during copulation, the female receives a continuous barrage of stimuli from his genitalia, which move actively and in a sustained manner, both inside and on the external surface of her body. Externally, the male's cerci pinch and squeeze the ventral surface of the tip of her abdomen rhythmically. The squeezing movements are not mechanically necessary to restrain the female. They cause one patch of setae on the male's sternum and five other patches on his genitalia to rub against the female (Fig. 15.3). Internally, the male's phallobase also executes sustained, rhythmic thrusting, pulsing and extension movements in the female's vagina and her oviduct; there are also probable movements of his pneumopophyses, the distal triangular sclerite of his phallobase and a long, thin sclerite, and rhythmic movements of the basal portion his phallobase also push against other parts of the female. In addition to these genital movements, the male executes bursts of other rhythmic movements of his legs and wings, brushing, tapping, and vibrating other parts of her body during copulation (Jaensen 1979a, b; Briceño et al. 2007; Briceño and Eberhard 2009a, b). A female *Glossina* may sense stimuli from the male at up to ten sites on her body at once during some moments of copulation!

Most of these stimulatory male structures and their movements are not utilitarian, in the sense that they are not needed to hold or to penetrate the female. Even the thrusting movements with the phallobase did not result in progressively deeper penetration in the species observed with the X-ray technique (Briceño et al. 2015). In *G. austeni*, the phallobase has also already arrived at the mouth of the spermathecal duct only half way through copulation (Pollock 1974). It seems inevitable, however, that the friction of the male's intromittent genitalia with the walls of the vagina and the oviduct must stimulate the female. In at least three of the four species in the *morsitans* subgenus (including *G. pallidipes*), the male's intromittent genitalia are periodically withdrawn completely or nearly completely from the female's vagina when the male's cerci begin to squeeze strongly on the ventral surface of her abdomen and are then reinserted after the cercal squeeze ends.

The possibility that the movements of the pneumopophyses function to stimulate the female is supported by the reduction in sperm transfer when the pneumopophyses of *G. pallidipes* were covered with nail polish (above). In addition, the likelihood that copulation would induce ovulation was nearly extinguished when the pneumopophyses of *G. morsitans* were cauterized (Dodd 1973). The possibility that sperm transfer by the male (rather than some female response to the male) was altered by these pneumopophysis treatments was not checked, however.

Only a few of the male structures that contact the external surface of the female's abdomen are also mechanically necessary to restrain the female and hold her abdomen in a position that permits him to insert his phallobase. It is highly likely that the male's powerful grasp of the tip of the female's abdomen between his cerci and his fifth sternite functions to restrain the female. And it could be argued that the dense setae on his fifth sternite function as anti-slip devices that make his grip more effective. But the complex squeezing and flexing behavior of his cerci, the species-specific teeth on the tips of his cerci, and the multiple patches of setae on his genitalia at points where they rub on the female during the squeezing movements, all seem useless in terms of mechanical restraint. The groove in the inferior clasper that receives the edge of female tergite 6 in species of the *morsitans* group also seem superfluous with respect to a hold-fast function, as the cerci hold the female in a powerful, apparently unbreakable grip. The inferior claspers of *G. palpalis* (Squire 1951) and *G. austeni* (Pollock 1974) have been hypothesized to lift the female sternum and expose her vulva. But even if this occurs, the differences in their widely divergent forms seem mechanically irrelevant, given the relative uniformity of female morphology.

In sum, the functions of nearly all of the different types of genital movements and many of the setose genital structures described here are probably not directly related to restraining the female or to transferring sperm; in contrast, their designs seem appropriate to stimulate the female.

It is important to emphasize the fragmentary nature of our observations of phallobase behavior, even in the one species for which there is an appreciable sample size (*G. pallidipes*). Copulation in *Glossina* lasts up to two hours, while the observations with X-rays were limited to a few minutes because the X-rays damaged the animals (Socha et al. 2007). Judging by the clear differences in the copulatory courtship behavior of the legs and wings of male *G. pallidipes* that occur during the course of a normal copulation (Jaensen 1979a, b; Briceño et al. 2007), as well as in the patterns of squeezing behavior of the male's cerci (Briceño et al. 2007), the behavior of the phallobase may also vary during the course of a copulation. Such changes seem particularly likely if, as appears to be the case, the male sometimes succeeds in grasping the female's abdomen with his cerci, but is not immediately able to introduce his phallobase into the female's vagina. In *G. austeni* pairs that were flash frozen about half way through copulation and then sectioned, the male seemed to exert outward rather than inward force on some portions of the female's reproductive tract, and the distal portion of his basiphallus seemed to "grasp" a projection of the uterus (the genital papilla) (Pollock 1974). We suspect that the otherwise unique rhythmic stabbing movements observed in *G. swynnertoni* (Briceño et al. 2015) were attempts by the male to achieve intromission and that we may have failed to see similar movements in the other species because we did not happen to record pairs that were in that stage of copulation. It has long been known that some "copulations" in *Glossina* do not result in sperm transfer (Buxton 1955; Pinhão and Grácio 1973; Pollock 1974). Another stage that may show different behavior patterns is ejaculation (there was very distinctive copulatory courtship behavior in *G. pallidipes* during ejaculation—Jaensen 1979a, b;

Briceño et al. 2007); this stage was probably not included in any of the X-ray recordings. In summary, our list of positions and movements of the phallobase is likely to be incomplete.

15.8.2 Female Responses to Male Stimuli

If cryptic female choice is the reason why male genital morphology and behavior are elaborate and species-specific in *Glossina*, then it should be true that females respond to the male stimulation by altering their reproductive processes in ways that improve the male's chances of paternity. This prediction was tested experimentally in two species for two of the several structures mentioned above, but the results clearly confirmed the prediction. Both removing the male cercal tooth and smoothing his setae on sternite 5 and incapacitating female sense organs in the areas in which they are likely to be stimulated by these structures elicited female responses that were likely to decrease the male's chances of paternity: reduced sperm transfer to her spermathecae; reduced ovulation; and greater receptivity to subsequent male mating attempts (Briceño and Eberhard 2009a, b).

Some female responses were greater when the possible female sense organs in the area contacted by the male were blocked than when the male structure was altered. This is not surprising, because the female alterations were likely to eradicate rather than reduce stimulation from the male structure and may also have reduced or eliminated stimuli from male squeezing behavior.

It is revealing to place these observations of the triggering of ovulation in the context of previous detailed research on female reproductive physiology in *Glossina*. Saunders and Dodd (1972) concluded, on the basis of extensive experiments, that ovulation was not triggered by transfer of sperm, deposition of the spermatophore in the female, secretions of the male's testes, his accessory glands or his ejaculatory duct, or by humeral factors from the spermathecae of inseminated females. They speculated, by elimination, that stimuli received during copulation must induce ovulation. These physiological studies thus reinforce the likely importance of the stimuli from the male's genitalia in inducing ovulation.

15.8.3 Species Specificity and Evolutionary Transitions

One prediction of cryptic female choice is that different aspects of the behavior of genitalia that are presumed to be used as internal courtship devices, such as the squeezing movements of the cerci, the thrusting movements, the inflations of pneumopophyses, and the flexion of the triangular sclerite, are likely to differ among even these closely related species (as, of course, is typical of courtship behavior in general). As far as the available data go, it appears that this prediction is fulfilled and that many of the stimuli delivered by male *Glossina* differ

in different species. As shown above and by Briceño et al. (in press), there are many differences in both the morphology and the behavior of the male genitalia of different species of *Glossina*. The morphological differences were partially documented in earlier taxonomic studies, while the differences in their behavior and physical interactions with the female's genitalia have been documented only recently, and much less completely. Differences are especially clear in the stimuli from the cerci and the inferior claspers.

It is possible to trace some of the probable evolutionary transitions of morphological and behavioral genital traits we have described, using the phylogenetic relations of species in *Glossina*. Several genital traits that are shared between species in the *palpalis* and *fusca* subgenera, but not with those in the *morsitans* subgenus are probably synapomorphies in the *morsitans* group. These putatively derived traits of the *morsitans* subgenus include the central joining of the cerci (as argued by Potts 1970), pressing the female tergite 6 rather than tergite 7 with the male's modified sternum 5 during copulation, folding the cerci less sharply to make a relatively large angle (approximately 90°) with the basal segment during copulation, stabbing the ventral surface of the female's abdomen so as to fold off a small round mass of female tissue near the tip of the cercus during copulation, and pressing the inferior clasper against the posterior tip of the female's tergite 6 rather than against the membranous surface that is directly ventral to this tip.

Male stimulation of the female in the *G. morsitans* spp. lineage during copulation thus appears to have become more concentrated on female tergite 6. Experimental covering of the female tergite of *G. pallidipes* confirmed that stimulation of this tergite during copulation induces the female to ovulate, to store sperm, and to resist further copulation (Briceño and Eberhard 2009a) and that it induces sperm storage in *G. morsitans* females (Briceño and Eberhard 2009b). The positions and forms of large cercal teeth have also changed in this lineage (Fig. 15.1), and experiments in two species have shown that stimulation from these teeth increase ovulation, sperm storage and sexual receptivity responses in ways that could improve the male's chances of paternity.

15.8.4 Alternative Explanations for the Evolution of Genital Morphology and Behavior in Glossina

15.8.4.1 Lock and Key

Three other commonly cited hypotheses to explain rapid divergent evolution of animal genitalia can be examined in light of the data presented here (see Eberhard, this volume). The species isolation hypothesis proposes that divergence in male genital morphology is driven by selection on females to avoid receiving sperm from heterospecific males. The avoidance mechanism could be via a mechanical lock-and-key mechanisms, in which heterospecific male genitalia are mechanically excluded from those of the female ("mechanical lock and key"), or via

female discrimination against heterospecific males on the basis of the stimuli that she receives from their genitalia ("sensory lock and key") (see Eberhard 1985, Simmons 2014). The mechanical version is clearly contradicted as an explanation for the species-specific forms of the male cercus and inferior clasper in *Glossina*. Both the male cerci and the inferior claspers contact featureless, smooth membranous portions of the female abdomen that are mechanically incapable of excluding the genitalia of heterospecific males; the female structures show no perceptible differences between species. Thus, female morphology clearly does not mechanically exclude the species-specific cerci and inferior claspters of heterospecific males, and the mechanical lock-and-key hypothesis cannot explain their rapid divergence.

Observations of the morphology of the female's common oviduct, where the male's phallobase is inserted, are less complete. The lining of the female's reproductive tract is relatively soft rather than rigid, so mechanical exclusion seems unlikely a priori. There is, however, is a sclerite in the *fusca* group (the "signum") on the inner surface of a thick symmetrical, transparent, gelatinous structure, the "genital fossae," and the signum exhibits considerable specific diversity of form (Newstead et al. 1924; Patton 1936); the functions of the signum and the genital fossae are apparently unknown.

The mechanical lock-and-key hypothesis also has problems explaining the inter-specific differences in phallobase morphology, because the phallobase slides back and forth within the female; at least during early stages of copulation, and thus does not have a fine physical mesh with the female, at least along a large portion of the lower portion of her reproductive tract. Physical exclusion of heterospecific male phallobases seems unlikely, at least in early stages of copulation. Rejection of mechanical lock and key on the basis of the apparent lack of a female "lock" is less certain for the phallobase, however, because it is possible that the available observations may have missed positions assumed by the male at critical moments (e.g., intromission, ejaculation, or spermatophore deposition). There is wide inter-specific variation in the female signum and the male phallobase (e.g., Patton 1936), and their coupling (if it occurs) remains to be described. The X-ray recordings did not permit detailed analyses of how male genitalia were positioned with respect to details the internal morphology of the female vagina and uterus. A further reason to doubt mechanical lock-and-key arguments for the male's intromittent genitalia is that they fail to explain the elaborate, highly repeated, and divergent stimulatory movements of the clasping and of the intromittent portions of the male's genitalia.

A final reason to doubt both mechanical and sensory lock-and-key hypothesis is that, as explained above, *Glossina* species are probably effectively isolated by differences in diurnal activity cycles, habitat, 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 and Langley 1993) (occasional crosses do occur between subspecies—Curtis et al. 1980). This likely reproductive isolation also implies that the experimentally demonstrated female responses at later stages of copulation to the male's lateral cercal teeth and to his sternite 5 are unlikely to represent adaptations to avoid cross-specific pairing.

In sum, the mechanical lock-and-key hypothesis can be confidently discarded for some species-specific aspects of male genitalia, and could be incorrect for all of them. There is also evidence, though less extensive, against the sensory lock-and-key hypothesis.

15.8.4.2 Sperm Competition

The males of some animals directly influence the fate of sperm from other males, for instance by removing them, diluting them, killing them, flushing them from the female, etc. (e.g., Simmons 2001; Leonard and Córdoba-Aguilar 2010). Such sperm competition may possibly occur in *Glossina*, but none of the copulatory courtship behavior, male genital morphology, or male genital behavior described here has any obvious relation with the sperm of previous or subsequent males. Thus, sperm competition, as usually understood (e.g., Simmons 2001), seems unlikely to explain their evolution.

15.8.4.3 Sexually Antagonistic Coevolution

A third alternative hypothesis to explain the rapid divergent evolution of the behavioral and morphological traits of *Glossina* described here is sexually antagonistic coevolution (SAC). Male–female conflict of interests could result in coevolutionary races between male traits that increase the male's chances of paternity but at the same time reduce the female's ability to reproduce, and female defenses against these traits that increase her ability to resist or overcome the male-imposed damage (Chapman et al. 2003; Arnqvist and Rowe 2005). One version of SAC is that the species-specific male traits function to physically or mechanically overcome species-specific female defensive morphological traits (e.g., Alexander et al. 1997). This version can be confidently rejected for the male cerci and inferior claspers of *Glossina* for the same reasons discussed above for lock and key. In essence, there are simply no defensive female structures in the areas contacted by these species-specific male structures of *Glossina*. The only complementary female morphological trait is the transverse cleft dorsal to the vulva, into which the male's inferior claspers fit in *G. austeni* (Pollock 1974). And this cleft would appear to aid rather than impede the possible lifting action of the inferior claspers on the female's sternal plate that may uncover her vulva. The genus *Glossina* can thus be added to the list of groups in which female morphology does not show the species-specific defensive traits that are predicted by SAC to be common in combination with rapid divergent evolution of male genital structures (Eberhard 2004a, b).

These arguments regarding the mechanical effects of male genitalia can also be applied, though with less confidence, to the inflatable sacs or pneumopophyses of the male phallobase. These sacs were present in all species but showed differences between species in both morphology and behavior. One possible SAC-related function of the movements of the pneumopophyses, suggested by observations

of other insects, is that they (at least those with teeth) rub holes in the lining of the female's vagina (Merritt 1989; Crudgington and Siva-Jothy 2000; Flowers and Eberhard 2006). This could allow male seminal products to escape into her body cavity (Gillot and Langley 1981) where they could induce female responses such as sperm transport and resistance to further copulations (Riemann and Thorson 1969; Chen 1984). Previous studies of the reproductive physiology of *G. morsitans* argue strongly against this alternative, however; introduction of male seminal products into the female's body cavity did not affect ovulation, insemination or remating (Saunders and Dodd 1972; Gillot and Langley 1981).

A second version of the SAC hypothesis emphasizes conflict over stimulation rather than mechanical coupling. Females might defend against damaging male effects by means of species-specific differences in the nervous system, rather than differences in external morphology. Changes in a female's nervous system could make her less likely to respond to male manipulation and might be less costly than changes in her morphology. This version of the SAC hypothesis could explain the rapid divergence in both the morphology and the behavior of male *Glossina* genitalia and is compatible with the lack of species-specific morphological differences in the portions of the female that are contacted by the male. It supposes that male stimulation constitutes a sort of a sensory trap (Holland and Rice 1998; Córdoba-Aguilar 2005; Arnqvist 2006), in which females "cannot help themselves" from responding to the male's stimulation.

This argument supposes that females have not been able to solve the problem of being overly responsive to the male and thus damaging their own reproductive prospects, due to natural selection that favors the female responses in other contexts. But in contrast to other sensory traps, in which female responsiveness to male stimuli is maintained by natural selection (Christy 1995; Córdoba-Aguilar 2005), this SAC argument seems unlikely for *Glossina*. There is no obvious reason why the thresholds for female response to male stimuli in *Glossina* would be constrained by natural selection. That is, a female *Glossina* would seem to be free to adjust her degree of sensitivity to male post- or syn-copulatory stimulation so that her responses are in accord with her own reproductive interests.

This stimulatory version of SAC also depends on the supposition that the effects of the species-specific aspects of the male morphology, which at least in *G. pallidipes* and *G. morsitans* include increased sperm transport, increased probability of ovulation, and increased resistance to further copulations, damage female reproductive output. Specifically, the female must lose future offspring due to the increase in sperm transport that is induced by the male, the increased probability of ovulation that is induced by the male, and her decreased chance of copulations with other males that result from her increased resistance to further copulations. And all three of these losses must be larger than the possible gains she could obtain through the increased abilities of her sons to induce these responses in females of the following generation under the SAC hypothesis. There is no evidence, however, to support any of these SAC suppositions in *Glossina* (though it must be noted that we know of no studies that could have tested any of these possibilities).

All of the female responses to males in *Glossina* that have been documented are likely to be favored by natural selection on females—to store and utilize the gametes received from the current male and to avoid the likely costs of additional copulations with subsequent males. This does not mean that it is not possible that the disadvantages posited by SAC exist, but that there is no empirical support for invoking them.

In sum, the SAC hypothesis cannot be definitively ruled out. But it depends on several less than certain suppositions, for which there is little or no evidence. As is typical (Popper 1970), advances in science often involve gradually discarding hypotheses that require more and more post hoc adjustments to comply with the accumulating data. Use of Occam's razor suggests that SAC is less appealing than CFC as a general explanation for genital evolution in *Glossina*.

15.8.5 Limits of the Techniques Used

This chapter shows that a combination of different techniques was needed to gain understanding of the genital behavior in tsetse flies. These included direct detailed observation of external events (Briceño et al. 2007), dissection of flash-frozen pairs, direct observation of the genitalia of isolated, headless males, and indirect observation of mating with X-ray images. Each technique has advantages and disadvantages, and none gives a complete view. External events can be observed without altering the flies' behavior during the entire 30–120 min copulations, but they fail to reveal internal events. Flash-frozen pairs give detailed snap shots of the positions of different sclerites at particular stages during copulation, but cannot document their movements and may sometimes be incomplete; for instance, the basal spines that were seen with X-rays (Briceño et al. 2015) were missed in an earlier study of *G. pallidipes* (Briceño et al. 2007). The genitalia may be induced to move (as in the headless males of thus study), and these movements can be observed in detail, but the males performed only a subset of the behavior patterns that they are capable of executing (e.g., the rhythmic thrusting movements were entirely absent, eversion of the basal spines near the cerci did not occur). The X-ray video images revealed movements that were otherwise hidden from view, but resolution of details was not possible, some structures such as the pneumonophyses were not visible, and only short segments of copulation behavior could be recorded before the X-rays damaged the flies (Socha et al. 2007). And of course, cyclotron facilities where such X-ray recordings are possible are not widely available. We believe that it is very likely that further details of genital behavior remain to be discovered in *Glossina*.

The ability to visualize internal movements using X-ray images opens a new field of study, the behavior of genitalia during copulation. Results may help illuminate the functional morphology of puzzlingly elaborate genital structures and may provide both additional taxonomic characters for distinguishing closely related species, and tests of sexual selection theory.

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