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## Movements and morphology under sexual selection: tsetse fly genitalia

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SHÖN (2009, *Ethology Ecology & Evolution* 21: 161–172) pointed out that in order to understand the functional morphology of sexually selected structures that are used as signaling devices in birds, it is crucial to understand how these structures move during sexual interactions. This insight applies not only to bird feathers, but also to many other types of possible signaling devices, including male genitalia. This note highlights the need for studies of the behavior of genitalia, and describes two promising techniques, using a tsetse fly as an example. Observations of this species revealed otherwise cryptic, highly rhythmic and forceful thrusting, pinching, pressing, and scraping movements by the male's genitalia within the female's body that have no obvious relation to sperm transfer. Thus even though on the outside the male's body is nearly motionless during long lapses during copulation, the female is subject to a barrage of possible stimulation from his genitalia during copulation. Similar studies are needed in other groups to understand the functional significance of genital morphology.

KEY WORDS: sexual selection, signals, genitalia, display traits.

Recently SHÖN (2009) argued in these pages that the functional significance of signal colors in birds cannot be understood without understanding the bird's behavior. The ways in which colored feathers are moved will have strong effects on the stimuli that they provide to other individuals. This argument surely applies to other signaling systems, including structures, such as male genitalia, that stimulate the female by making direct contact with her. Because genitalia are frequently useful in distinguishing

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species, their morphology has been carefully studied by taxonomists in a very wide diversity of animals. These studies virtually never include, however, information on how genitalia move. This lack of understanding of how genitalia move represents a gaping hole in the understanding necessary to understand how they function, and why they so consistently tend to diverge especially rapidly and assume elaborate shapes compared with other body parts.

Although male genitalia are often more or less hidden inside the female during copulation, there are several ways to obtain information about their movements. The most effective technique is direct observation of male genital structures that are not introduced into the female, or that are periodically withdrawn (DEWSBURY 1972, 1988; UHL et al. 1995; EBERHARD & KARIKO 1996; PERETTI et al. 2006; BRICEÑO et al. 2007; AISENBERG & EBERHARD 2009; EBERHARD & GELHAUS 2009). Many male genital structures remain inside the female during copulation, however, and the movements of these structures have scarcely been explored. In a few cases it is possible to observe internal movements through the semi-transparent body wall of the female (EBERHARD 1993). A second possibility is to isolate the male and observe the behavior of his intromittent genitalia by stimulating them artificially (summary in EBERHARD 1996; also BRENNAN et al. 2007; BRICEÑO et al. in prep.). A third technique is to employ X-ray video recordings (WESTNEAT et al. 2003; SOCHA et al. 2007). The purpose of this note is to demonstrate techniques that can provide behavioral insights into the functional morphology of the intromittent genitalia of a small animal, using tsetse flies as an example.

The complex male genitalia of the tsetse fly *Glossina pallidipes* include several species-specific structures (POTTS 1970). Use of direct observations of copulating pairs and flash freezing of copulating pairs has shown that some male genital structures press against the female's outer surface and squeeze or brush against her rhythmically during copulation; several of these male structures appear designed to stimulate her (BRICEÑO et al. 2007). Experimental modifications of two of these male structures and of the female receptors that they contact showed that stimuli from these structures during copulation serve to induce the female to store the male's sperm, to ovulate, and to reject subsequent attempts to mate with her (BRICEÑO & EBERHARD 2009a, 2009b).

A second set of male structures is introduced into the female's reproductive tract, where they deposit a spermatophore near the entrance to her common spermathecal duct during the last minute or so of the approximately 60 min copulation (SAUNDERS & DODD 1972; DODD 1973; JAENSON 1979; BRICEÑO et al. 2007). These intromittent genital structures (Fig. 1) include a cylindrical phallosome that bears a pair of inflatable, spine-covered sacs at its tip (pneumopophyses) and a triangular aedeagal sclerite near its tip; the sclerite bears an opening and contains a small folded sac that is everted into the mouth of the female's spermathecal duct (BRICEÑO et al. 2007). The functional significance of these structures is the focus of the present study.

## METHODS

Movements of the genitalia of isolated males were video-taped by removing the male's head, then restraining him on his back under a dissecting microscope with an open-weave cloth. The cloth was positioned so his genitalia were exposed. A pin was used to lift the cerci and expose the intromittent genitalia. Movements of male genitalia inside the female during copulation of 13 pairs were video-taped using synchrotron phase contrast X-ray images of the type used in previous studies to allow real-time visualization of the movements of structures inside small living animals (WESTNEAT et al. 2003; SOCHA et al. 2007). The female was glued to the tip of a vertically oriented

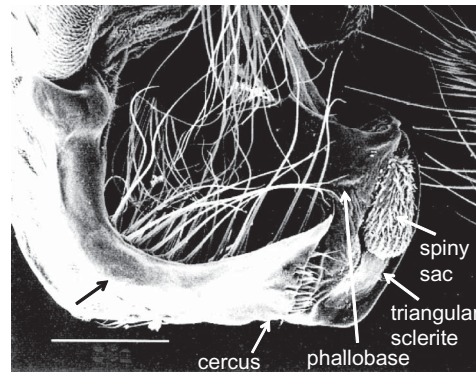


Fig. 1. — The inflatable spiny sac (pneumopophysis) and triangular sclerite on the phallosome of *G. pallidipes*, structures which are inserted into the female reproductive tract during copulation (scale line 200  $\mu\text{m}$ ).

plastic rod, and the male mounted her after being introduced from an adjacent chamber. The X-ray setup was similar to that used by SOCHA et al. (2007) except in the following respects: the measurements were carried out at the TOMO-TOPO beamline, ANKA Synchrotron Facility, Karlsruhe, Germany. A white synchrotron beam produced by a bending magnet source passed through a 3 mm thick silicon filter. The effective photon energy was about 25 keV and the flux density was  $3 \cdot 10^{11}$  ph/sec/mm<sup>2</sup> at 100 mA. The phase-contrast enhanced images (up to 250 images/sec for up to 5 min) were acquired by a single crystal thin layer scintillator plate (LuAG:Ce) positioned 1 m behind the sample. The images were magnified by an optical system coupled to a fast thermoelectrically cooled and stabilized CMOS camera (Photron Fastcam SA-1) capable of taking image sequences with a maximum speed of 5400 fps at  $1024 \times 1024$  pixels. The effective pixel size was equal to 5.5  $\mu\text{m}$ . False colors were added manually to one sequence of images by subtracting consecutive frames from the original sequence, to increase contrast between male and female structures.

## RESULTS

Eversion of the phallobase was elicited by gently touching setae on the inferior claspers with a fine forceps. Similar stimulation of intact males failed to evoke genital behavior, perhaps because the brain inhibits the spontaneous activity in the terminal ganglion of the ventral nerve cord that controls genital movements, as in other insects (ROEDER 1967). Two types of rapid, repeated movements were elicited more or less reliably in headless males: inflations of the spiny sacs (pneumopophyses), and distal flexion of the terminal sclerite of the phallobase (Fig. 2). Inflations always preceded distal flexions, and some inflations occurred without a distal flexion.

X-ray videos of copulating pairs revealed that even though there was little external movement in copulating pairs other than periodic squeezes with the male's cerci (BRICEÑO et al. 2007), the male's intromittent phallobase made several types of stereotyped movement within the female's body. The phallosome performed rhythmic, piston-like thrusting movements (Fig. 3) that lasted about 0.3 sec and occurred about once every 2.3 sec. The basal portion of the elbow-like basal structure, which drove the phallobase into the female, pressed against the female's anal plate with each inward movement. This movement must cause the arc of long setae on the male's inferior genital claspers to press against the membranous groove around the female's anal plates and

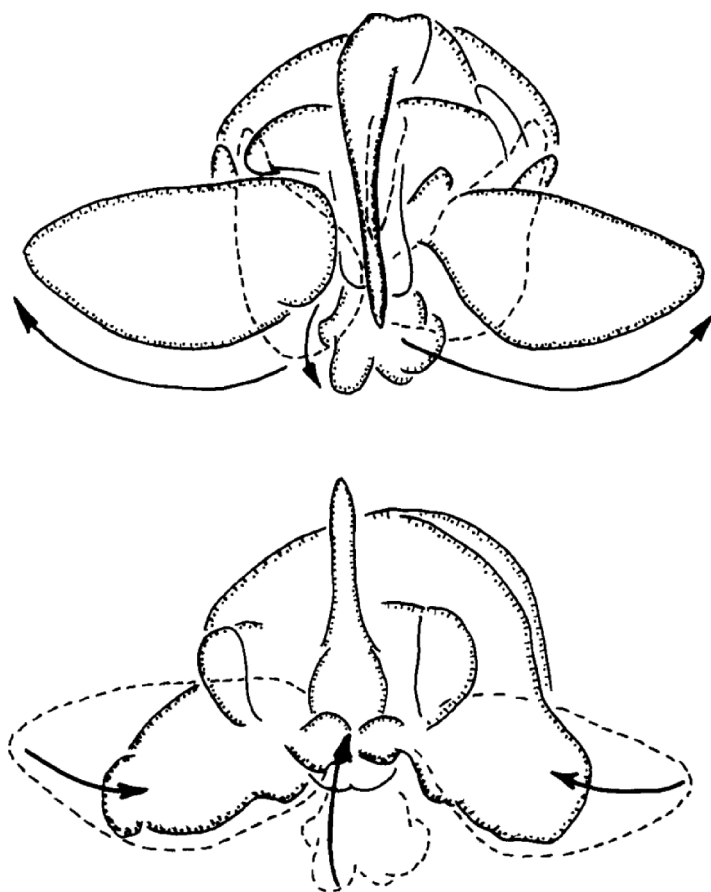


Fig. 2. — Movements of the spiny sacs (pneumopophyses) and the distal, triangular sclerite of *G. pallidipes*, traced from video recordings of movements induced by stimulation of setae on the inferior claspers.

her sternal plate (BRICEÑO et al. 2007). Membranous structures inside the female were largely invisible, and we could not check for movements of the female reproductive tract or of the male's inflatable spiny sacs.

The phallobase was only partially withdrawn following most thrusts, but during especially strong squeezes with the male's cerci it was withdrawn completely, and then thrust in again after the squeeze ended. Distal flexions of the moveable distal triangular sclerite (Fig. 1) appeared to occur at the apex of many thrusts; they resembled, in truncated form, the distal flexion movement performed by isolated males (Fig. 2).

A pair of long, retractable spines apparently arising near the base of the cerci was everted, and their tips pinched the surface of the female's abdomen tightly against the distal tip of the male's cerci (Fig. 3). This pinch was mostly constant, but the spines were withdrawn temporarily when the male relaxed the squeeze by extending his cerci distally; when the cerci resumed their squeezing action, the spines were everted and pinched the female again. The tips of the cerci sometimes flexed posteriorly (with respect to the female) making rapid, small amplitude movements (approximately 13.8/sec) during an inward thrust of the phallobase, presumably scraping the female tissue that

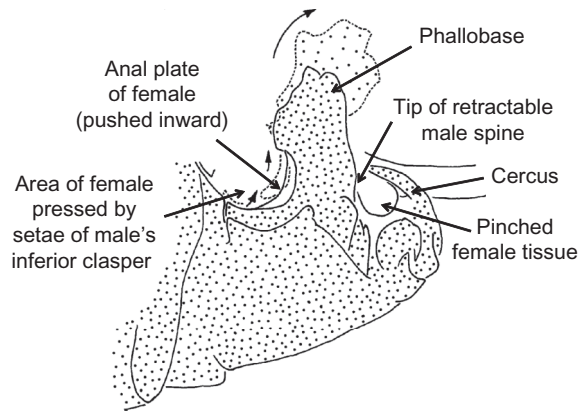


Fig. 3. — A single inward thrust by the phallobase of *G. pallidipes*, drawn from false-color images in an X-ray video recording of a copulating pair (male is stippled). The dotted lines followed the continuous lines by 0.16 sec. The thick arrow indicates the out-pocketing of the female's abdominal surface that was pinched by the male near the tip of his cercus.

was being tightly squeezed between the basal portion of the phallobase and the tips of the cerci. Still another type of movement of the phallobase occurred at the inward apex of some thrusts. Possible outlines of briefly inflated male pneumopophyses were visible during inward thrusting movements in false-color images, and may correspond to the inflations of the spiny sacs observed in isolated males.

It was not possible to follow the entire time course of a copulation because the flies were apparently damaged by the X-rays, and separated after 5–10 min of exposure.

## DISCUSSION

The major lesson of this study is that some portions of the male genitalia of *G. pallidipes* have previously unsuspected abilities to execute dramatic movements. Any attempt to understand the functional significance of these structures must take these movements into account. A second lesson is that none of the techniques described here was sufficient by itself to fully document the genital movements, and that the techniques need to be combined. The X-ray videos provide limited contrast, and did not reveal soft or membranous structures such as the male's pneumopophyses, the spermatophore, or the lining of the female's reproductive tract. On the other hand, the genital movements observed in isolated males were of apparently much greater amplitude than those performed during copulation; movements of male structures during copulation are probably restrained by the confines of the female reproductive tract.

None of the types of movements we have described is likely to be directly related to sperm transfer. Most if not all types occurred long before ejaculation, and some, such as thrusting and pinching, occurred for many minutes at a time. The thrusting movements did not result in progressively deeper penetration, but rather in friction with the walls of the vagina, much of the time while the phallosome was already apparently deep enough for insemination. The pinching action of the sharp spines against the tips of the cerci was superfluous for holding onto the female, as the strong clamping action of the cerci holds the tip of her abdomen very securely (BRICEÑO et al. 2007).

One possible function of the spreading movements of the pneumopophyses that is suggested by observations of other insects might be to rub holes in the lining of the female's vagina (MERRETT 1989; CRUDGINGTON & SIVA-JOTHY 2000; FLOWERS & EBERHARD 2006). This could function to allow male seminal products to escape into her body cavity where they could induce responses such as ovulation or resistance to further copulation (REIMAN et al. 1967; CHEN 1984). Previous studies of the closely related species *G. morsitans* argue against this alternative, however, because introduction of male seminal products into the female's body cavity did not affect ovulation, insemination or remating (SAUNDERS & DODD 1972; GILLOT & LANGLEY 1981). Another possible function is to stimulate the female (EBERHARD 1996).

All of the movements we described are likely to result in stimulation of the female. Preliminary experiments supported indirectly the possibility that movement of the spiny sacs functions to stimulate the female. When the pneumopophyses of *G. morsitans* were cauterized, the male's ability to induce ovulation by copulating was reduced (DODD 1973). In addition, when nail polish was applied to the spiny sacs of *G. pallidipes* (smoothing their surfaces but allowing at least some expansion), sperm transfer to the female's spermathecae was reduced (one of 21 females mated to control males lacked sperm in their spermatheca 9 days after copulation; six of 21 females mated to experimental males lacked sperm;  $\chi^2 = 4.29$ ,  $P = 0.04$ ). The possibility that this difference was due to changes in sperm transfer by the male rather than in sperm transport by the female was not checked, however.

In summary, direct observation of genital behavior in isolated males and in X-ray videos, combined with dissections of flash-frozen pairs, revealed otherwise cryptic, highly rhythmic and forceful thrusting, pinching, pressing, and scraping movements by the male's genitalia on and within the female's body. Some of these movements were associated with specialized male setae that pressed or rubbed against membranous portions of the external surface of the female. Thus, even though the portions of the male's body that contacted the female's outer surface were nearly motionless, the female was subject to a barrage of possible stimulation from his genitalia during copulation. Observations using the techniques described here promise to illuminate the functional morphology of other puzzlingly elaborate genital structures, and may also provide both additional taxonomic characters for distinguishing closely related species as well as tests of sexual selection theory.

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