Functional Morphology of Copulation in Chrysomelidae-Criocerinae and Bruchidae (Insecta: Coleoptera)

Susanne DÜNGELHOEF & Michael SCHMITT
Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany

Abstract. Until now, the genitalia of beetles have been investigated mainly under taxonomic aspects while there exist few studies on the functional morphology of genital structures. Many questions concerning the position of male genitalia inside the female during copulation, the functional role of parameres and endophallus, the mechanical coupling of the mates as well as sperm transfer remain unsolved for most species of Coleoptera. This article presents results gained from investigations of pairs of Acanthoscelides obtectus (Bruchidae), Oulema melanopus, Oulema duftschmidi and Lilioceris lilii (Criocerinae) fixed in copula. During copulation parameres remain outside the female in Acanthoscelides. In Criocerinae, the flagellum is placed near the entrance of the spermathecal duct. Campaniform sensilla were found on the surface of the endophallus in Lilioceris.

Key words. endophallus, flagellum, aedeagus, sperm-transfer, parameres, bursa copulatrix, Acanthoscelides obtectus, Lilioceris lilii, Oulema duftschmidi, Oulema melanopus

1. INTRODUCTION
Most evolutionary variations can be interpreted as a means to increase fitness. Anything connected with reproduction is of central importance for this increase. The functional morphology of copulation, however, is unknown for most species of beetles. There exist studies on the functional morphology of genitalia in insects in general (HEBERDEY 1928, 1931) and in several beetle species. NYHOLM (1969) for example, analysed the structure and function of the copulatory organs of Cyphon species (Coleoptera: Scirtidae). EBERHARD (1993) studied courtship and genital mechanics of three species of Macroproctus (Coleoptera: Scarabaeidae). Spermatozoon morphology and sperm transfer of the staphylinid beetle, Aleochara curtula (Coleoptera: Staphylinidae), are well understood (GACK & PESCHKE 1994; FÖRSTER et al. 1998). HAUBRUGE et al. 1999 found that in Tribolium castaneum (Coleoptera: Tenebrionidae) the male removes the sperm of previous males from the female tract by means of its median lobe. In Cicindela (Coleoptera: Cicindelidae) as well, the males seem to clear the spermatheca and the spermathecal duct with its flagellum before they place the spermatozoon (FREITAG et al. 2001). Since all these groups are not closely related to the Chrysomelidae, the results may not be applicable to our group.

One of the first studies on the functional morphology of genitalia in Chrysomelidae is that of HARNISCH (1915) who described the copulatory apparatus of Chrysomela populi, Clytra quadripunctata and Plateumaris sericea. RODRIGUEZ et al. (2004) found that females of Chelymorpha alternans (Chrysomelidae: Cassidinae) prefer males with a longer flagellum. This result confirms the cryptic female choice hypothesis (THORNHILL 1983; EBERHARD 1985, 1997). CRUDGINGTON & SIVA JOTHY (2000) observed that the males of Callosobruchus maculatus (Coleoptera: Bruchidae) penetrate the wall of the female genital tract to prevent re-mating of the female. (The “Bruchidae” are, in terms of phylogeny, simply a subtaxon of the Chrysomelidae, as discussed by SCHMITT 1996).

The latter works show impressively that studies on functional morphology of genitalia can provide new insights on sexual selection and sexual conflict. Our aim is to investigate the functional role of genital structures in all subfamilies of Chrysomelidae to learn more about these phenomena in the whole family. Hopefully, the results will lead to new ideas on speciation processes in this large beetle family. In this paper we present some first results gained from three species.

2. MATERIALS AND METHODS

2.1. Beetles

Bruchidae

Acanthoscelides obtectus (Say, 1831):
Breeding of these beetles is very uncomplicated, in which a preserving glass was used, filled with beans and with a lid consisting of a membrane permeable to air. We received seed-beetles from the Institute for Plant Diseases, Bonn and used the bush beans Montano or Maja as substrate. Every three months we removed dead beetles and replaced a portion of the old beans with fresh ones.

Chrysomelidae-Criocerinae

Lilioceris lilii (Scopoli, 1763):
These beetles were caught in private gardens around Bonn and Kevelaer (lower Rhine province near the Dutch border) in April and May 2003. We kept them in Drosophila-tubes, where they fed on leaves of Lilium sp. To achieve an improved climate, a layer of 1-2 cm of gypsum was poured into the bottom of the rearing tubes.

Oulema melanopus (Linnaeus, 1758)/ Oulema duftchmidt (Redtenbacher, 1874):
Specimens of Oulema came from a wheat field near Bonn in May 2003. They were kept in Drosophila-glasses like L. lilii and fed wheat plants from the native field. As the two species are only distinguishable by their genitalia and lack external differences, the first author caught and worked with both forms. The results for each species were pooled and therefore are presented as a whole and not separately.

2.2. Behaviour

To initiate and observe copulations we put pairs or small groups of beetles together in a tube. The behaviour was visible with the naked eye. For some details we used a dissecting microscope. Ten copulations of different individuals of A. obtectus were watched closely. In Criocerinae we observed fewer copulations accurately from beginning to end (n≤3). Duration of copulation could be estimated by keeping an eye on the tubes while occupied with work elsewhere. We define copulation time as the time during which the aedeagus is inserted in the female.

2.3. Fixation in copula

To fix the copulating beetles we used chloroethyl spray (Chloroethyl “Dr. Hennig”, Dr. Georg Friedrich Hennig, Chemische Fabrik Walldorf GmbH, D-69190 Walldorf, Germany). Shock freezing by this spray works well, but sometimes the mating pairs separated partly or completely by the pressure of spraying. In these cases, the mates had not been coupled sufficiently. We fixed 8 – 12 pairs of each species. Frozen beetles were dissected or stored in 70% ethanol at –12 °C for three weeks.

2.4. Histology

After three weeks the entire beetles or their abdomina were transferred from 70% to 100% ethanol (30 min each in 80%, 90%, 95%, 100% ethanol). Each was immersed in a second soaking in 100% ethanol overnight. Resin was prepared with the “Low Viscosity” set from Electron Microscopy Sciences (Hatfield, Pennsylvania). Objects were infiltrated by a further series (at intervals of 30 min aceton : resin 1:1, aceton : resin 1:3 and 60 min pure resin) and degased in an exsiccator. The Resin was put in silicon moulds (6x12x5 mm or 6x14x4 mm), the objects added and adjusted. The resin polymerised over night at 69 °C.

The embedded objects (1-3 in copula fixed pairs of each species) were cut with a microtome in slices of 1.0 μm or 1.5 μm. Every fifth section was put on a slide, heat-dried and dyed with Richardson-dilution (BÖCK 1989). To study the slices we used a Nikon Eclipse E600.

2.5. Scanning electron microscopy

We used the SEM to study in detail the shape and surface of the endophallus. Three exemplars, one of each species, were prepared from in copula-fixed pairs. Therefore, the endophallus was everted in an authentic way in all preparations. Objects were transferred from 70% to 100% ethanol and dried using HMDS (Hexamethyldisilazane) (BOCK 1987). After fixing them on a stub they were coated with a 35 nm gold-layer using a HUMMER VII sputter (ANATECH LTD). We used an HITACHI S-2460 N SEM.

3. RESULTS

3.1. Acanthoscelides obtectus

Pairs of A. obtectus mate for about ten minutes. After this time, the male often leans backwards until it is almost lying on its back, after which it separates from the female. Females do not seem to be willing to remate directly after copulation but require a refractory period.

It is striking that fixed pairs separate very easily during dissection. The linking between the dead male and female is much less strong than in Criocerinae.

As shown in Figure 1, the parameres in A. obtectus remain outside the female and lay on the last female sternite during copulation while the median lobe is inserted. The photo is taken from a pair fixed in copula. One of us (SD) could observe this also while watching live in-
individuals of *A. obtectus* through a dissecting microscope: The male moves the parameres up and down on the last female sternite during copulation and pushes only the tip of the median lobe into the female genital opening. Before inserting the median lobe, the male strikes the female genital opening with the setae of his parameres by moving them up and down.

In Figure 2, the considerable size of the everted endophallus is visible. It is formed like a balloon and fills the whole bursa copulatrix during copulation. The close-up view (Fig. 3) shows that the parameres are nearly flush with the median lobe. That means that even during copulation, the median lobe and tegmen are hardly moved against each other.

The surface of the endophallus bears teeth-like structures on its central region that are obviously sclerotised and appear quite sharp (Fig. 4). These teeth are absent on the apical region; in the basal region they are longer, less sharp structures.

### 3.2. *Oulema melanopus/duftschmidtii*

In this species, pairing lasts longer than in *A. obtectus*. The males insert their median lobes for about twenty minutes or even longer. After copulation, males continue to sit on the females’ elytra, guarding their mates. Females try to get rid of them by kicking. Both sexes may remate directly after copulation.

The endophallus of *O. duftschmidtii* consists of a membranous and a sclerotised part, the so-called flagellum (Fig. 5). The flagellum is tubular, at least apically (Fig. 6). During copulation, the median lobe is inserted completely in *O. melanopus*. The endophallus fills the whole bursa copulatrix (Fig. 7). A close-up view (Fig. 8) shows how well the walls of bursa and endophallus match. Every bulge of the bursa is filled by the endophallus. Figure 8 shows clearly that not only the distal part of the flagellum is tubular but also the whole structure is tubular. An analysis of the neighbouring sections revealed that the flagellum ends at the entrance of the spermathecal duct.

The surface of the endophallus of *O. duftschmidtii* bears comb-like structures (Fig. 9) or something similar. They are not sclerotised, unlike the “teeth” of *A. obtectus*. The structures are directed towards the median lobe.

### 3.3. *Lilioceris lili*

As in *O. melanopus*, copulation in *L. lili* takes more than twenty minutes. The male clings to the female’s back for several hours while the male organ is intromitted.

The everted endophallus of *L. lili* is about three times the diameter of the median lobe (Fig. 10). This fact obviously facilitates a perfect mechanical coupling of *Lilioceris*-pairs. The fixed pairs could hardly be separated. As in *Oulema*, the endophallus is divided into a membranous and sclerotised part. The sclerotised part includes the flagellum as one can see in the SEM photo (Fig. 11). The tip of the flagellum is also tubular (Fig. 12).

The sagittal section of *Lilioceris* (Fig. 13) represents the mates during copulation. Even if one cannot see a connection between the male body and the median lobe in this figure, one can imagine that the median lobe is inserted. The endophallus fills the bursa. The sclerotised part of the endophallus is visible lying close to the basal part of the spermatheca. We can track this on the preceding and following sections. Thus, in *L. lili* as well as in *O. melanopus* the tip of the flagellum is positioned very close to the entrance of the spermatheca.

The surface of the endophallus of *L. lili* bears the same comb-like structures as *O. duftschmidtii*. But there are additional structures in *L. lili* (Fig. 14), closely resembling sensilla campaniformia, receptors responding to pressure. These receptors occur in rows on parts of the surface of the endophallus. After we had found these structures we searched for dendrites in the sections. And indeed, we found structures crossing the wall of the endophallus, which could be interpreted as dendrites.

### 4. DISCUSSION

#### 4.1. General aspects

We focused on three species that were easily available. Furthermore, we decided to work with one species possessing parameres and two species without in order to obtain an idea on the functional role of these structures. We chose closely related species to enable a meaningful comparison.

In future studies, the methods will be maintained but have to be expanded. Paraffin sections for example will be necessary to demonstrate sperm by giemsa-staining. In our sections we found structures inside the flagellum of *Oulema* that seem to be spermatzoa, however we are presently uncertain. Besides that, many more pairs of each species will have to be fixed for a comparison of different states of copulation. This will permit us to reconstruct the process of copulation.

#### 4.2. Sperm-transfer

We assume that in *L. lili* and *O. melanopus/duftschmidtii* the flagellum is positioned near the spermatheca to guarantee a directed sperm transfer towards the female sperm-storage. The results show that the flagellum in *O. melanopus*/
melanopus/duftschmidi as well as in L. lili is tubular with a distal opening. Therefore, one could imagine that it is involved in sperm transfer. Berti & Rapilly (1976) studied the endophallus of L. lili under a taxonomic aspect and figured the ejaculatory duct ending in the flagellum tube. That means that in L. lili sperm transfer actually takes place through the tubular flagellum. It is self-evident that the same may be the case in O. melanopus/duftschmidi, though we have not found the ejaculatory duct in the sections. The idea of the flagellum as a sperm transferring structure is corroborated by the positioning of its tip: the males presumably adduct their flagellum close to the basal piece of the spermatheca to increase the probability that their sperm arrives at the entrance. Rodríguez et al. (2004) consider possible that the flagellum of Chelymorpha alternans (Chrysomelidae: Cassidinae) is pushed up into the spermathecal duct to release sperm there. Tactics of males to transport their sperm close to or even into the spermatheca have been investigated in other cases as well, for example in the staphylinid Aleochara curtula (Gack & Peschke 1994). It is conceivable that the long and thin flagellum of O. duftschmidi may reach even into the spermathecal duct during copulation. The spermathecal duct of this form is longer than in O. melanopus (Berti 1989). One could suppose that females have in the course of evolution lengthened the distance from the spermatheca to test the males’ quality. Males responded by extending their flagellum. In Chelymorpha species (Rodríguez et al. 2004) the length of the flagellum is actually adapted to the length of the spermathecal duct. Females of this species prefer males with a longer flagellum that reaches the spermatheca.

The question if L. lili and O. melanopus/duftschiemdi form spermatophores as well, is unsolved so far as for many other Chrysomelidae. Males of C. alternans transmit a spermatophore in addition to the pretended sperm transfer via the flagellum (Rodríguez et al 2004). In the Criocerinae we have so far not found any trace of a spermatophore in the sections (which does not necessarily mean that spermathophores do not exist). Males of Acanthoscelides obtectus are able to produce a spermatophore (Huignard 1978, 1983), which we also could identify in the sections.

It is an open question if Criocerinae males remove previous males’ sperm by means of their genitalia, as males in many other groups do (Haubrúge et al. 1999; Freitag et al. 2001). It can’t be excluded that the flagellum has this additional function. In Acanthoscelides, it is improbable that the males dispose other males’ sperm from the female genital tract because the median lobe is hardly inserted and no structures of the endophallus seem to be able to perform such a function.

4.3. Parameres

Harnisch (1915) has already observed that in Plateumaris sericea (Chrysomelidae: Donaciinae) the parameres remain outside the female genitalia. He suggests that they act as a grasping organ to connect the male more intensively to the female during copulation. Our results show that the parameres of A. obtectus can’t fulfill this function because the lever formed by them is too short to stabilize. Another possible function of the parameres is to position the apical orifice of the median lobe opposite the opening of the female genital tract as Kingsolver (1970) suggests. SD observed in live A. obtectus individuals that the male touched the female genital area with the setae of the parameres before inserting the median lobe. It is hard to decide if this touching serves the male to orientate the position of the median lobe or to stimulate the female. Such kind of paramere-movement has been observed in other groups as well: males of the carabid beetle Pasimachus punctatius tap their parameres rhythmically upon the apical edge of the female last abdominal sternite. After a while, the genital orifice of the female opens and the male inserts his median lobe (Alexander 1959). If these movements actually serve as stimulation, this would support the cryptic female choice hypothesis (Eberhard 1985).

4.4. Surface of the Endophallus

The comb-like structures on the surface of the endophallus in L. lili and O. duftschmidt are directed towards the median lobe. We assume that the functional role of these structures is to support the mechanical coupling of the mates. They may make it more difficult to disconnect the median lobe forcibly out of the bursa or without the agreement of the male, respectively. If the females want to stop copulation and get rid of the aedeagus, they use their legs to kick the male away as is common for many other species of Chrysomelidae as well (Dickinson 1997; Jolivet 1999; Crudginton & Siva-Jothy 2000). The everted matching endophallus with its microstructures may prevent separation. Another possible function of the ultrastructures of the endophallus may be stimulatory. To verify this idea there should be receptors on the interior wall of the bursa copulatrix.

The other structures on the endophallus of L. lili are without much doubt receptors. The existence of such receptors means probably that the males are able to control the eversion and positioning of the endophallus very well. The sensilla campaniformia may sense that correct position inside the bursa to provide the best opportunity for the flagellum to perform direct sperm transfer.

Acanthoscelides obtectus males have teeth-like structures on the surface of their endophallus. Kingsolver (1970) suggests that these structures or “endophallus
"armature" serve as holding devices during copulation. This seems to be a good idea, especially because the shape of the endophallus of *A. obtectus* is not suitable to guarantee its foothold inside the female. But in contrast to the little combs in the Criocerinae, the structures in *A. obtectus* are sclerotized and may have another function as well. CRUDGINGTON & SIVA JOTHY (2000) found that the armed endophallus of *Callosobruchus maculatus* penetrates the bursa wall during copulation. This damage of the genital tract is costly for the female because it has to repair the wall. The study even points out that copulation frequency has a life-history cost for females: doubly mated females died significantly younger than singly mated females. Hurting the female is thus a strategy of males to ensure that females will not remate. Maybe the same is the case in *A. obtectus*. As our observations of live individuals showed, females of *A. obtectus* do not remate after copulation for a fairly long time (unlike females of *O. melanopus*). To reach clarity, we shall have to analyse the bursa wall after copulation.

4.5. Perspectives for evolutionary biology

The results show that the males of *A. obtectus* on one side and the Criocerinae on the other embark on different strategies. In Criocerinae, the males try to prevent further copulations of the females by mate guarding. They obviously have no additional strategy because females seem to be willing to remate just after copulation if only the males desist from them. In *A. obtectus*, the males do not stay longer on the females than copulation or even the pure transfer of the spermatophore lasts. They do not spend as much time in pairing than the Criocerinae but have a different way to prohibit a remate of the females. As HUIGNARD (1983) described, the deposition of the spermatophore in *A. obtectus* is followed “by stimulation of oogenesis and egg-laying, as well as by a temporary inhibition of female receptivity”. He ascertained that male secretions are transferred from the spermatophore into the female haemolymph through the wall of the bursa. It is unclear if these secretions are the main cause of the refractory period of the females or if damage of the bursa wall like in *C. maculatus* (CRUDGINGTON & SIVA-JOTHY 2000) is an additional male strategy to inhibit a female remating. If there will be no damages in the bursa detectable after copulation, we have to ask for another function of the teeth on the endophallus surface. We then will agree with KINGSOLVER 1970 that they mainly serve as holding devices.

Males in Criocerinae obviously achieve the mechanical coupling with their voluminous inflated endophallus and its additional microstructures. So, if the mechanical coupling in Donaciinae is achieved by the parameres as grasping organs (HARNISCH 1915), it would be interesting to know if their endophallus is less voluminous and possesses no microstructures. An idea could be that all species without anchoring parameres offer a voluminous endophallus or at least an endophallus with holding microstructures.

One could speculate that there exist three types of Chrysomelidae – one without parameres, another with parameres that serve mainly as grasping organs, and a third that uses the parameres for orientation or stimulation.

Acknowledgements. We thank Dr. Bernhard Huber (Bonn) for help in all histological matters, Lasse Hubweber (Bonn) for stimulating discussions, Karin Ulmen (Bonn) for technical assistance at the SEM, Bradley J. Sinclair (Bonn) for linguistic assistance, Sandra Steiger (Freiburg im Breisgau) for her patience in searching for dendrite-like-structures in the sections and two anonymous referees for valuable input.
Fig. 1. Ventral view of a copulating pair of *Acanthoscelides obtectus*, fixed by chloroethyl spray. P: parameres, M: median lobe, ST: last female sternite. Stereomicrograph. – Fig. 2. *A. obtectus*. Aedeagus with everted endophallus (E). M: median lobe, P: parameres. Scanning electron micrograph. – Fig. 3. *A. obtectus*. Close-up view of the aedeagus. E: endophallus, M: median lobe, P: parameres. Scanning electron micrograph. – Fig. 4. *A. obtectus*. Surface of the endophallus. Scanning electron micrograph. – Fig. 5. Aedeagus of *Oulema duftschmidtii*. E: endophallus, F: flagellum, M: median lobe. Scanning electron micrograph. – Fig. 6. *O. duftschmidtii*. Tip of the flagellum. Scanning electron micrograph. – Fig. 7. Sagittal section of *O. melanopus* in copula. B: bursa copulatrix, E: endophallus, M: median lobe. Micrograph. – Fig. 8. Sagittal section of *O. melanopus* in copula. B: bursa copulatrix, F: flagellum, M: median lobe. Micrograph. – Fig. 9. *O. duftschmidtii*. Surface of the endophallus. Scanning electron micrograph. – Fig. 10. Dissected aedeagus of *Lilioceris lilii*. E: endophallus, M: median lobe. Stereomicrograph. – Fig. 11. *L. lilii*. The sclerotised part of the endophallus (E) bears the flagellum (F). M: median lobe. Scanning electron micrograph. – Fig. 12. *L. lilii*. Tip of the flagellum. Scanning electron micrograph. – Fig. 13. Sagittal section of *L. lilii* in copula. B: bursa copulatrix, E: endophallus, M: median lobe, S: spermatheca. Micrograph. – Fig. 14. Campaniform sensillum on the surface of the endophallus of *L. lilii*. Scanning electron micrograph.
REFERENCES


Authors’ address: Susanne DÜNGELHOEF (corresponding author) and Michael SCHMITT, Zoologisches Forschungsmuseum Alexander Koenig, Adenauerallee 160, D-53113 Bonn, Germany; E-mail: s.duengelhoef.zfmmk@uni-bonn.de, m.schmitt@uni-bonn.de