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Original article

Three substances ejected by *Apis mellifera* drones from everted endophallus and during natural matings with queen bees*

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Abstract – Until now, all reports state that during eversion of the drone endophallus, two substances are ejected, viz. semen and mucus, and that the mating sign of a queen bee consists of the bulb of the endophallus filled with mucus. I examined substances ejected during eversion of drone endophalli, as well as substances present in the mating sign. In the fully everted endophallus, creamy semen was found near the chitinized plates, amorphous white mucus was located further distally and a transparent-whitish condensed substance appeared at the end of the everted endophallus. In mating signs, white mucus was found near the bursa copulatrix of the queens and a transparent-whitish condensed substance in the distal part of the sign. Microscopic examinations showed that the transparent-whitish substance consisted of fragments of epithelial membranes sloughed from mucus glands. Thus, not two substances, but three, viz. semen, mucus and epithelial membranes, are ejected during endophallus eversion and natural mating with queen bees.

endophallus / eversion / mating sign / drone / queen

1. INTRODUCTION

Instrumental insemination of queens enables to genetically control the offspring of honey bees. This technique is used not only in research laboratories but has also found entry into practical beekeeping. In order to improve animal breeding stocks, the Polish government subsidizes instrumental insemination of queen bees. According to official reports for Poland, prepared by the National Centre of Animal Breeding, 68 000, 70 000, 70 000 and 77 000 queen bees were instrumentally inseminated in the years 2005, 2006, 2007 and 2008, respectively. In addition to these figures, several more queens are instrumentally inseminated in Poland each year, and comparable figures can probably be found in countries with active research programs and beekeeping industries all over the world. Thus, worldwide hundreds of thousands of queens are inseminated each year. Furthermore, since semen from about 8 drones is generally required to inseminate a single queen, an even larger number of drones, certainly over a million, must be provoked to evert their endophalli for semen collection.

Due to the commercial importance of instrumentally inseminated queens it is important to know the exact composition of the substances ejected during endophallus eversion and injected into the queens. All authors describing provoked eversion of drone endophallus, and collection of semen for instrumental insemination of queen bees state that two substances, semen and mucus are ejected (Bishop, 1920; Watson, 1927; Mackensen and Roberts, 1948; Fyg, 1952; Woyke, 1955, 1958b; Laidlaw and Eckert, 1962; Ruttner, 1966; Mackensen and Tucker, 1970; Laidlaw, 1977; Schley, 1983; Holm, 1986; Moritz,

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1989; Laidlaw and Page, 1997). Other substances have not been mentioned.

When Woyke (1958a) found fragments of epithelial membranes inside the end of the bulb of uneverted endophalli, he did not suppose that the membranes are ejected from everted endophallus. However, during instrumental inseminations of more than 1000 queens, I noticed a transparent-whitish substance at the end of fully everted drone endophalli. This induced me to conduct the present investigation to find out, what the substance may be.

During natural mating of *Apis mellifera,* the drone endophallus is everted and the semen is ejaculated into the queen's common oviduct, and both lateral oviducts. The semen is pushed out of the endophallus by mucus from the drone's accessory glands. The mucus creates part of the mating sign, with which the queen returns from the mating flight.

Arnhart (1938, p. 67) found 175 papers concerning mating and the mating sign. The first scientists to describe the mating sign were the blind man Huber (1792), with the help of his assistant. They found that the mating sign of a queen bee was part of a drone endophallus, namely the bulb, filled with a white mass. He also noted that the bulb of the mating sign inside the queen's sting chamber was not everted. Next, the mating sign was examined by Siebold (1854) and by Leuckart (1860). Dzierzon (1853, p. 96) found mating signs, in some of the about 400 observed queens performing the mating f1ights. All authors described the mating sign as consisting of the bulb of the drone endophallus filled with mucus (Bishop, 1920; Zander, 1921, 1922; Fyg, 1952; Woyke, 1955; Ruttner, 1956; Snodgrass, 1956; Woyke and Ruttner, 1958; Laidlaw and Eckert, 1962; Laidlaw, 1977; Koeniger, 1986; Koeniger and Ruttner, 1989; Laidlaw and Page, 1997). Arnhart (1935) also examined celloidin sections of a mating sign inside the abdominal tip of a queen. Nobody ever mentioned, however, that in addition to the mucus, another substance may be present within the mating sign.

I investigated herein which substances are ejected by drones during provoked eversion of the endophallus and compose the mating sign.

2. MATERIAL AND METHODS

Two issues were addressed:

1. What substances are ejected by drones during artificially provoked eversion of the endophallus for collection of semen, used for instrumental insemination of queen bees?

2. What substances are present in the mating sign of naturally mated queens?

To investigate item 1, sexually mature drones were caught at hive entrances. In the laboratory, the drones were then stimulated by holding one of their hind legs whi1e they attempted to fly away. Next, the thorax was squeezed, which resulted in the eversion of the endophallus. I examined in detail endophalli of about 100 drones. However, a whitish-transparent substance at the end of the endophalli, I noticed in more than 1000 drones during instrumental insemination of queen bees.

To investigate item 2, queens returning from successive mating flights were collected. To do this, special mating nuclei were prepared. Plastic transparent tubes (2.5 cm × 10 cm) were fixed in front of the entrances. A slit was cut near the distal end of the tube, into which a small piece of queen excluder was inserted. The observations of queen flights were undertaken during three consecutive years (2007, 2008 and 2009). Mating f1ights of queens were watched daily from 12 h till 17 h. When a queen appeared in the tube, the queen excluder was removed. After the queen flew out, the excluder was slipped back into the slit. Queens returning with a mating sign were caught and anaesthetized with ethyl acetate. Altogether, mating signs of 40 queens were examined. Substances ejected by drones or present in mating signs were collected with the help of preparatory needle or with watchmaker forceps. They were collected from different places of partly or fully everted endophalli, as well as from different portions of the mating signs.

All samples were immediately examined microscopically. To do this, they were immersed in water or physiological solution in a small porcelain evaporator dish with blue bottom, or in a watch-glass block. After stirring, the samples were examined by means of a Nikon stereo microscope, at magnification 5–80 times. Samples containing epithelial membranes were characteristic by their whitish transparent color, in contrast to the white mucus.

Epithelial samples were further examined microscopically. After stirring in physiological solution, the aqueous solution was removed and replaced by Gilson fixative. Subsequently, the membranes

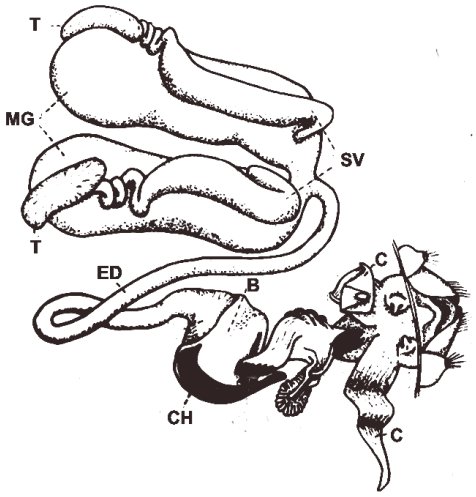

Figure 1. Reproductive organs of a drone. B bulb, C cornua, ED ejaculatory duct, CH chitinized plates, MG mucus glands, SV seminal vesicles, T testis.

were rinsed in water and then stained with Delafield hematoxylin. After another rinse in water, a drop containing fractions of epithelial membranes was put on a microscope slide and cover slipped. The slides were examined under a light microscope equipped with phase contrast at 420 × magnification.

3. RESULTS

The bulb of the endophallus in the abdomen of young or old drones was empty (Fig. 1). Its lateral walls were close to each other and the sack was invaginated inside the lumen of the bulb. The chitinized plates of the bulb appeared at the left side of the drone's abdomen.

3.1. Provoked eversion of the endophallus and ejected substances

After the drones were provoked by squeezing the thorax, one of three main phenomena occurred:

1. Drones that were stimulated and provoked by only mildly squeezing the thorax did not evert endophallus out of the abdomen. However, the bulb did fill with semen from seminal vesicles and with the content of the accessory mucus glands (Fig. 2). The semen was

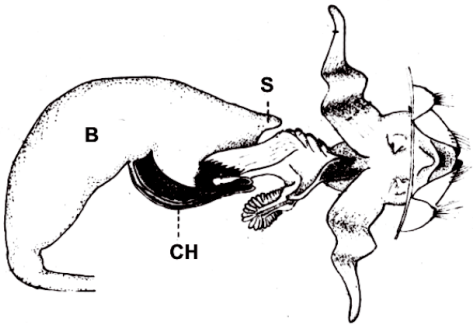

Figure 2. Bulb of uneverted endophallus filled with semen and contents of mucus glands. B bulb, CH chitinized plates, S sac.

located in the sack (Fig. 2, marked as S) and in the posterior part oft he bulb. The content of the mucus glands was located in the anterior (cranial) part of the bulb.

2. In drones which had been more strongly stimulated and provoked, partial eversion of the endophallus occurred. At first the vestibulum everted, the empty bulb was pushed inside it and then filled with semen and the content of the mucus glands. The chitinized plates of the bulb appeared at the left side of the everted vestibulum. Further pressure caused the bulb to turn along its oblong axis in such a way that the chitinized plates moved to the dorsal side. Additional gentle squeezing of the drone's abdomen resulted in further gradual eversion of the endophallus. As a result, a drop of creamy semen appeared at the end of the endophallus. This stage of endophallus eversion is the best for collecting semen into the tip of a syringe for instrumental insemination of queen bees (Woyke, 2008, Fig. 23).

3. In drones that were very stimulated and provoked, squeezing of the thorax resulted in full eversion of the endophallus (Fig. 7). The creamy white semen was located near the chitinized plates. Further towards the end of the everted endophallus, the amorphous white mucus was present.

3.2. Two ejected substances analyzed microscopically

The semen presents a creamy substance. By means of phase contrast microscopy,

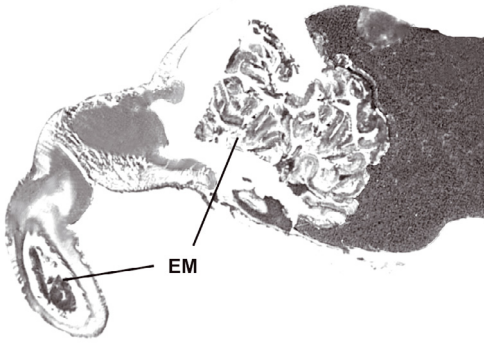

Figure 3. Sagittal section through the distal part of the bulb of an uneverted endophallus (see Fig. 2) filled with epithelial membranes from mucus glands. EM epithelial membranes.

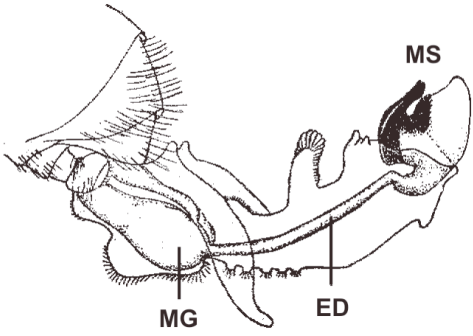

Figure 4. Everted endophallus with mating sign slipping out of it. ED ejaculatory duct, MG mating gland, MS mating sign.

spermatozoa were visible in semen collected from the sack of filled uneverted endophalli, from the sack of partly everted endophalli, and also from fully everted endophalli.

The white mucus consists mainly of a water-soluble proteinaceous secretion (Colonello and Hartfelder, 2003). When dissolved in water and stirred, mucus collected from uneverted or everted endophalli appeared as white clumps and under the stereo microscope, granules of different size were visible (Fig. 8). Under the light microscope, depending upon the fixative and staining, specimens stained with hematoxylin, appeared as black masses (Fig. 3) or black granules (Woyke, 1958a, b). No cell nuclei or parts thereof were found in the mucus.

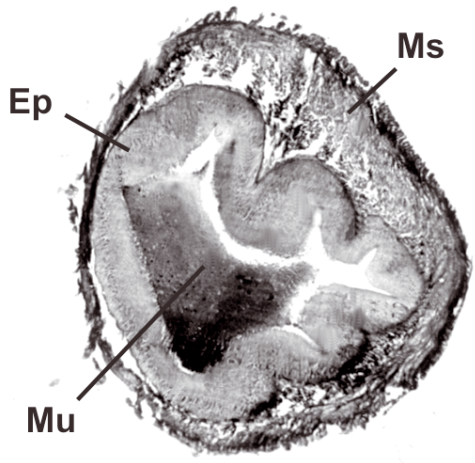

Figure 5. Cross section of a mucus gland. Ms muscles, Ep epithelium, Mu mucus.

Figure 6. Cross section of a mucus gland after eversion of the endophallus. The gland is deprived of epithelium. Ms muscles. Above is seminal vesicle SV with epithelium.

3.3. The third substance

At the tip of the fully everted endophallus, a whitish-transparent substance was noticed (Fig. 7). Presence of such a substance at the end of everted endophallus had not been

mentioned in any previous scientific literature. Therefore I investigated it in more detail. Under stereomicroscopic magnification (15–25 ×), this substance can be distinguished from the mucus because of its whitish transparency, whereas the mucus is white. The substance forms a spiral filament, while the mucus is amorphous. The volume of the substance accounts for about 1/4 to1/3 of the total volume (mucus and semen) of the ejected mass. After the substance was put into water and stirred with a preparatory needle or pipette, fractions of whitish-transparent membranes were visible (Fig. 9). Microvilli were noticed at one border of the membranes. Microvilli are hair-like folds in the plasma membrane that extend from the surface of secretory cells. They are most clearly visible with an electron microscope, but their "brush border" appearance can also be seen under a stereo (Fig. 10) or light microscope. By means of phase contrast it was also possible to evidence the shape of the cells, as well as cell structures, such as nuclei and cytoplasm (Fig. 11). Thus, the third substance ejected during eversion of the endophallus clearly consisted of fragments of epithelial membranes that could be sucked into the tip of syringe used for instrumental insemination of queen bees (Fig. 12). Epithelial membrane fragments were also present in the cranial part of the bulb of uneverted, as well as of partially everted endophalli.

3.4. Mating sign

In queens returning from successive mating flights, the semen is in their lateral oviducts and the mating sign sits in the sting chamber (Fig. 13). The mating sign consists of a part of the bulb of the last drone's endophallus. Chitinized plates of the bulb are situated at the ventral side of the sting chamber. I did not find any epithelium of the endophallus in the mating sign. The mating sign slides out of the everted endophallus (Fig. 4) and the epithelium remained inside the everted endophallus after the mating sign had slid out of it.

Both sides of the mating sign were covered with a yellow substance. Investigations under way showed that it consists of epicuticle covered by a yellow sticky mass that originated from the yellow cornua of the endophallus. However, the endocuticle as well as the epithelium remain at the cornua after the endophallus everted and the mating sign had slid out of it. Thus, neither endophallic nor cornua epithelium was present in the mating sign.

Inside the mating sign, white mucus is present. In all the queens, the mucus was present in the proximal half of the sting chamber only, near the bursa copulatrix. Neither glandular cells, nor any cell fragments, such as nuclei, were present in the mucus.

The distal part of the mating sign protruding from the sting chamber, as well as the thin thread at its end behind the chitin plates consisted of a whitish-transparent substance (Fig. 13). The volume of this substance was about 1/3 of the total volume of the mass inside the mating sign.

After the mating sign was immersed in water, physiological saline or alcohol, the mucus in the proximal part remained white. However, the distal part of the sign became more transparent, than when observed in air (Fig. 14). After a while, the mucus started to dissolve in the water. The transparent distal part of the sign, however, coagulated when in alcohol and became white like the mucus.

After I collected the whitish-transparent substance and put it into water or physiological saline, stirred it and examined it under the stereo microscope, I found epithelial membranes (Figs. 9–10) similar to those ejected by the drones. By light microscopy, the cellular structure of the epithelial membranes was confirmed (Fig. 11). The posterior part of the mating sign as well as the final thread consisted exclusively of fragments of epithelial membranes. The thread at the left side of the mating sign shown in Figure 14 is curved towards the broad end of the chitinized plates.

4. DISCUSSION

The question is, where does the third substance originate from? The third substance, that is the epithelial membrane fragments, was present at the tip of the fully everted endophallus, as well as in the distal end of the mating

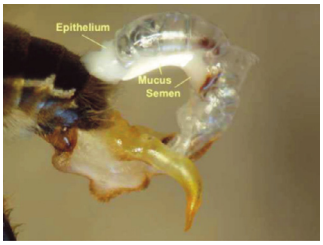

Figure 7. Fully everted endophallus.

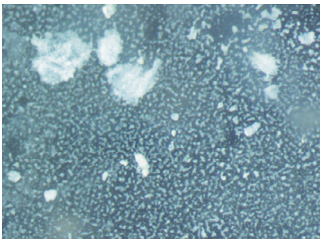

Figure 8. Mucus dissolved in water.

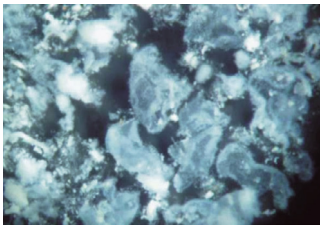

Figure 9. White mucus and transparent fractions of epithelial membranes.

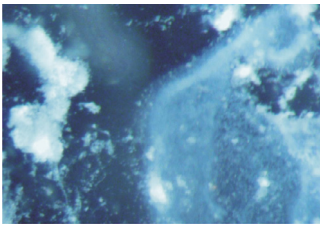

Figure 10. Small microvili appearing as a "brush border" on one side of epithelial membrane.

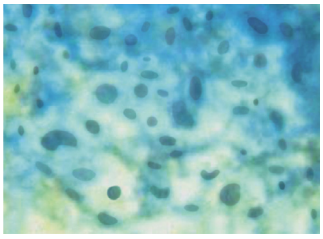

Figure 11. Cellular structure of the epithelial membranes.

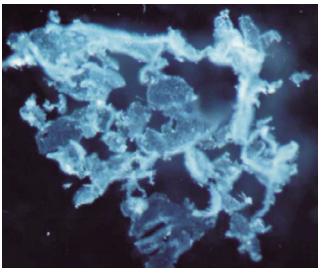

Figure 12. Epithelial membranes sucked into the tip of the syringe for instrumental insemination of queen bees.

Figure 13. Mating sign inside sting chamber of a queen.

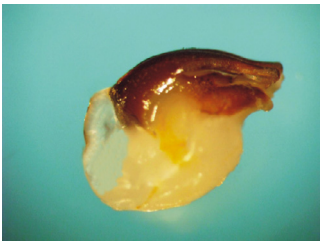

Figure 14. Mating sign.

sign. Woyke (1958a) showed that till the eversion of the endophallus the gland epithelium and the mucus are present beneath the muscle layer of the mucus glands (Fig. 5). Woyke (1955, 1958a) furthermore reported, that after the drone contracts its abdomen, the mucus is pushed out of the mucus glands and the epithelium is torn off the gland. As a result, the gland is now deprived of mucus and epithelial layer (Fig. 6). The epithelium is pushed into the ejaculatory duct and the distal end of the bulb of the endophallus, as shown in Figure 3, which was taken from a drone that was only slightly stimulated and the first phenomenon occurred, that is, the bulb was filled but not everted outside the abdomen (Fig. 2).

Woyke (1955, 1958a) suggested, that the epithelial membranes may push the semen and the mucus during eversion of the endophallus. However, I did not realize then that the membranes are co-ejected during full eversion of the endophallus. The present investigation shows that after the endophallus is fully everted, the epithelium inside the bulb is the last (third) substance to be ejected by the drone, and therefore it should appear at the tip of the fully everted endophallus (Fig. 7).

A similar sequence occurs during natural mating. When the drone everts the endophallus, the semen is the first component to be ejaculated into the queen's oviducts. Next, the mating sign filled with mucus slits out of the endophallus and the mucus is ejected into the bursa copulatrix and the sting chamber. The present investigation shows that the last step in the sequence is the ejection of the third substance, *viz.* the epithelial membranes, and therefore these appear at the distal end of the mating sign protruding from the queen's abdomen. According to Zander (1921, 1922) a piece of torn off ejaculatory duct hangs down from the end of the mating sign. Other authors claim that the thread consists of coagulated mucus. However, I could now show that the thread at the end of the mating sign consists exclusively of fragments of epithelial membranes that are torn off the mucus glands.

I herein presented evidence for microvilli appearing at one border of the torn off epithelial membranes collected from everted endophallus or the mating sign (Fig. 10). According to Moors et al. (2005), the apical cell membrane of the secretory cells of mucus glands is differentiated into a microvillar border. This corroborates the present conclusion, according to which the fragments of the epithelial membrane originate from the mucus glands.

The phenomenon of the epithelium being torn off the walls of the mucus glands, resembles an enforced holocrine secretion process, in which the whole cells are released into the lumen of the glands. This is a novelty. The contracting muscles of the gland push fragmented epithelial membranes into the ejaculatory duct and into the bulb. The ejaculatory duct and the endophallus of a drone are deprived of muscles. Thus, the gland muscles may take over the role of the muscles in reproductive organs of other animals.

The role played by the epithelial membranes inside the bulb for natural mating of the queens is still unclear. Their presence in the bulb, however, increases the volume of the mass inside it. This means that during natural mating, the epithelial membranes inside the ejaculatory duct and in the bulb may push all the mucus out of the endophallus resulting in ejaculating the semen with great force into the vagina and oviducts of the queen. Ejection of the semen with great force is important during multiple mating, when the semen of successive drones is ejaculated into the queen's vagina and lateral oviducts, which already contain semen from previous drones.

There is circumstantial evidence that a similar process may occur in *Apis cerana* and in *A. koschevikovi,* namely that the third substance (parts of the epithelial membranes) is ejected by the drones and is also present in the mating signs. Cross sections trough the everted endophallus of *A. cerana* showed the ejaculatory duct filled with epithelial fragments (Ruttner et al., l973). In mating signs of *A. cerana* (Woyke, 1975) and *A. koschevikovi* (Koeniger and Koeniger, 2000), spiral filaments were visible. The spirals are similar to those observed at the end of the fully everted endophallus of *A. mellifera* drones. It is, thus, possible that in all these species, the filaments consist of parts of epithelial membranes originating from the mucus glands.

Already 50 years ago I described the presence of epithelial membranes inside the filled bulb of uneverted endophalli (Woyke, 1955, 1958a). Even though semen for instrumental insemination of queen bees is collected from thousands of drones each year, it is surprising that the presence of the epithelial membranes at the end of fully everted endophalli has gone unnoticed, despite the fact that its consistency and whitish-transparent color are easily distinguished from the amorphous white mucus.

When genotyping mating signs for microsatellite loci, Franck et al. (2002) found PCR products in five out of six samples, indicating the presence of nuclear material in the secretion. No explanation for the lack of PCR products in the sixth mating sign was presented. The remnants of the drone endophallus creating the mating sign - the chitinized plates with the amorphous substance reinforcing them, the bow and the cornual epicuticle with the yellow substance - are deprived of epithelium. Furthermore, the results of the present study show that neither cell nuclei nor parts of nuclei are present in the mucus contained in the mating sign. Thus, no PCR products should be found when amplifying these components of the mating sign. However, as the distal part of the mating sign consists of epithelial membranes with cell nuclei, these may generate PCR products. This indicates, that the Franck et al. (2002), although not knowing this, de facto genotyped nuclei of the epithelial membranes in the five mating signs. In the sixth mating sign, they probably did not examine the protruding part of the mating sign, which contains cell nuclei of the epithelial membranes, and therefore no PCR products were found. The presence of PCR products in the five mating signs, corroborates the results of the present study, according to which, not only mucus, but also epithelial membranes are components of the mating sign.

Trois substances éjectées par les mâles d'*Apis mellifera* lors de l'éversion de l'endophallus et lors des accouplements naturels avec des reines d'abeilles.

endophallus / mâle / substances éjectées / accouplement / reine

Zusammenfassung – Drei Komponenten werden bei der Ausstülpung des Endophallus von *Apis mellifera* Drohnen im Verlauf natürlicher Paarungen mit Königinnen übertragen. Weltweit werden jährlich Tausende von Königinnen künstlich besamt, und dafür muss von einer noch grösseren Anzahl an Drohnen Sperma gewonnen werden. Bisherigen Berichten zufolge werden bei der Ausstülpung des Endophallus zwei Komponenten freigesetzt, Sperma und Mucus. Königinnen, die von einem erfolgreichen Paarungsflug zurückkehren, tragen an der Spitze ihres Abdomens ein Paarungszeichen, das, laut bisherigen Annahmen, aus dem mit Mucus gefüllten Bulbus des Endophallus besteht. Weitere Komponenten, die an der Zusammensetzung des Paarungszeichens beteiligt sein könnten, wurden bisher nicht beschrieben. Ich untersuchte hier, welche Substanzen bei der Ausstülpung des Endophallus freigesetzt und bei der Paarung übertragen werden. Dazu wurden sexuell reife Drohnen durch Pressen des Thorax zur Ausstülpung des Endophallus angeregt und die freigesetzten Substanzen von unterschiedlichen Regionen des Endophallus abgesammelt. Mittels einer besonderen Einrichtung wurden rückkehrende Königinnen am Eingang der Paarungskästchen abgesammelt. Solche mit einem Paarungszeichen wurden anästhesiert, damit die Zusammensetzung der unterschiedlichen Regionen des Paarungszeichens untersucht werden konnte. Sowohl die vom Endophallus abgesammelten Komponenten, als auch die des Paarungszeichens wurden mikroskopisch untersucht. In der Nähe der Chitinplatten des evertierten Endophallus befand sich das Sperma (Abb. 7) und weiter distal der weiße Mucus. Am Ende des Endophallus befand sich jedoch außerdem eine transparente, weißliche Komponente (Abb. 7), die sich als aus Fragmenten von Epithelmembranen bestehend erwies (Abb. 9 und 10). Im Paarungszeichen befand sich der Mucus in der Stachelkammer, in der Nähe der Bursa copulatrix, während der distale Teil, der aus dem Abdomen der Königin herausragte, ebenfalls eine transparente, weißliche Komponente enthielt (Abb. 13 und 14), die aus Membranfragmenten bestand (Abb. 9 und 10). Demzufolge werden bei der Ausstülpung des Endophallus während natürlicher Paarungen mit Königinnen nicht zwei (Sperma und Mucus), sondern drei Komponenten freigesetzt (Sperma, Mucus und Fragmente von Epithelmembranen). Letztere stammen aus der Mucusdrüse (Abb. 5). Nachdem es im Abdomen des Drohns zur Kontraktion kommt, wird das Drüsenepithel von der Wand der Mucusdrüse abgelöst (Abb. 6) und zunächst in den Samenleiter und dann in den distalen Teil des Bulbus gepresst (Abb. 3). Während der Ausstülpung des Endophallus gelangen diese Epithelfragmente demzufolge als letzte Komponente ins Freie und kommen deshalb zunächst am Ende des komplett ausgestülpten Endophallus und dann am Ende des Paarungszeichens zu liegen.

Endophallus / Ausstülpung / Paarungszeichen / Drohn / Königin

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