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Male-produced sex pheromone in *Tribolium confusum*: Behaviour and investigation of pheromone production locations

P.-O. Christian Olsson[a,*], Camilla Ryne[a], Rita Wallén[b], Olle Anderbrant[a], Christer Löfstedt[a]

[a]*Department of Ecology, Lund University, Ecology Building, Sölvegatan 37, SE-223 62 Lund, Sweden*
[b]*Department of Cell and Organism Biology, Lund University, Helgonavägen 3, SE-223 62 Lund, Sweden*

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Abstract

It is generally accepted that mating in flour beetles of the genus *Tribolium* is mediated by a male-produced aggregation pheromone. The pheromone production site in *T. castaneum* is believed to be glands on the ventral side of the femur. Behavioural experiments with the confused flour beetle *T. confusum* were conducted using extracts of beetles and different body parts as well as live beetles to investigate the responses to the complete odour bouquet released from the beetles. In our experiments, females but not males were attracted to male-produced volatiles, corresponding to the definition of a sex pheromone rather than an aggregation pheromone. SEM pictures confirm the occurrence of glands on all three pairs of legs of male *T. confusum*, but behavioural experiments show that females are attracted not only to extracts of male legs but also to whole body extracts and extracts of bodies without legs. These data suggest that in this species attractive compounds are produced not only in the glands on the femurs but also at multiple locations. Thus the pheromone systems involved in mating of flour beetles may be more complex than previously reported and differ among closely related species.
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Keywords: Tenebrionidae; Sex pheromones; Body extracts; Leg extracts; Walking bioassay; SEM

*Corresponding author. Tel.: +46 46 222 0126; fax: +46 46 222 4716.
E-mail address: christian.olsson@ekol.lu.se (P.-O. Christian Olsson).

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1. Introduction

Flour beetles (*Tribolium* spp.) are some of the most abundant pests in the world. The beetles feed both as larvae and adults, and cause damage resulting in high economic losses. Knowledge of the chemical communication of the beetles is valuable for the development of alternative control strategies, which are required now that many conventionally used pesticides are being withdrawn. *Tribolium castaneum* (Herbst) has gained most attention because of its worldwide distribution. However, *T. confusum* (J. du Val) is just as abundant in some countries and the odour-mediated communication system of *T. confusum* has not received the same attention. According to the literature, males of both *T. castaneum* and *T. confusum*, produce an aggregation pheromone, 4,8-dimethyldecanal (DMD), and it attracts conspecific males and females (Suzuki and Sugawara, 1979; Suzuki, 1980; Suzuki et al., 1984). The production of an aggregation pheromone occurs in the exocrine glands beneath the setiferous sex patches on the ventral side of the first pair of femurs (Faustini et al., 1981). Faustini and co-workers tested secretions from the structure and found that both females and males were attracted to beetles with secretions and also to secretions alone. The sex patches were first described by Hinton (1942), and are only present in adult male *Tribolium* beetles. Even though potential production sites are visible on the legs of *T. castaneum*, Bloch Qazi et al. (1998) reported that attractive substances are produced in glands elsewhere on the body of *T. castaneum* and not only on the legs. Thus, the reports on pheromone production sites in *T. castaneum* are contradictory. *Tribolium confusum* males have the same setiferous patches located on their femurs; however, they are not only on the first pair of legs, but on all three pairs of legs (Hinton, 1942; Sokoloff, 1972; Faustini and Halstead, 1982). The production and emission of the pheromone seem to be coupled with feeding activity in *Tribolium*. Feeding increases both the production and emission of the pheromone (Obeng-Ofori and Coaker, 1990). Since the identification of the aggregation pheromone, pheromone analysis and different trapping experiments have mostly focused on 4,8-DMD, in spite of numerous other substances being produced and emitted from the beetles (e.g. Suzuki et al., 1975; Keville and Kannowski, 1975). Therefore, extracts of entire beetles and parts of beetles were used in this study in order to include the whole chemical bouquet that could influence the beetles' behaviour.

In order to establish the relationship between sender and receiver in the odour communication system of *T. confusum*, we observed the immediate response of separated males and females in behavioural tests. This is in contrast to previous studies where beetles have been tested in groups over a longer period of time, in which it is only possible to observe the end-product of numerous responses, including intraspecific interactions (Suzuki and Sugawara, 1979; Faustini et al., 1981; Suzuki et al., 1984; Trematerra et al., 2000). We tested extracts of beetles to elucidate if a natural material could elicit higher responses than synthetic stimuli used earlier (Levinson and Mori, 1983; Suzuki et al., 1984). Based on previous studies, we hypothesised that the male would produce a pheromone that attracts both males and females (aggregation pheromone). We also hypothesised that extracts of male legs would be more attractive than other body part extracts to both sexes, due to the earlier reports of putative pheromone-producing structures found on the femurs. Scanning electron microscopic (SEM) pictures were taken to define the location of the possible femoral production sites in the beetles, before making extractions from the legs.

2. Materials and methods

2.1. Insects

A culture of *T. confusum* was established using adult beetles purchased from the Central Science Laboratory, Slough, England. The beetles were reared on a mixture of wheat flour (100 g) and brewer's yeast (5 g). Males and females were separated at the pupal stage (Ho, 1969). Approximately 200 pupae were placed in separate jars and kept in a climate chamber with light–dark cycle L12:D12, temperature 30 °C and relative humidity of 50%.

2.2. Scanning electron microscopy (SEM)

Female and male beetles and excised legs of both sexes were fixed and dehydrated in 70–100% ethanol for 2 days followed by critical point drying. Beetles were mounted ventrally on aluminium stubs and sputter coated with gold/palladium (80:20) in a Polaron E 5400 high-resolution sputter coater to enhance conductivity. Specimens were examined in a JEOL-T330 SEM operated at 15 kV (described in Ochieng et al., 1998).

2.3. Bioassay

Behavioural studies were conducted in an open arena walking bioassay olfactometer (Lanne et al., 1987 and references therein; Table 1). The arena consisted of a glass plate covered with paper

Table 1
Summary of the behavioural experiments carried out with *Tribolium confusum*

Experiment	Test beetles	Stimulus
1	Females (unfed)	Control (air)
		5 Live males
	Females	Control (air)
		5 Live males
2	Males (unfed)	Control (air)
		5 Live females
	Males	Control (air)
		5 Live females
3	Males	Control (air)
		5 Live males
		5 Live males + 5 live females
4	Females	Control (hexane)
		Male body extracts, dose–response 1–15 ME
5	Females	Control (hexane)
		Male leg extracts, dose–response 3–20 ME
6	Females	Control (hexane)
		Male body without legs extracts, dose–response 1–15 ME

In the experiment with leg extracts, 1 male equivalent (ME) corresponds to 6 legs.

that was replaced after each trial. On the paper a large circle of diameter 50 cm was drawn with a pencil. Directly in front of the source and upwind, a smaller circle (diameter 2 cm) was drawn. Virgin adult beetles, 1–2 months old, were used in bioassay tests, to avoid the risk that the beetles were not behaviourally active due to young age (Obeng-Ofori and Coaker, 1990). Ten beetles were placed under an upside-down plastic cup in the middle of the larger circle and were allowed to acclimatise in the arena for 1 min before the test started. The beetles were then released downwind in a laminar airflow of approximately 1 m/s, as measured by displacement of $TiCl_4$-smoke. This was replicated 5 times for each stimulus tested. A 3.33 µl capillary (Drummond Scientific Co.) filled with odour substance generated the odour plume. Beetles reaching the smaller circle at the odour source within 3 min were counted as responding animals and were immediately removed from the arena, to minimise the risk of insects successfully locating the source by being attracted to other insects. Beetles leaving the larger circle were given a second try, since there was a risk that they had not encountered the odour plume. If beetles did not respond to the odour source in the second trial, they were considered to be non-responding. When live beetles were used as the odour source, 5 beetles without any food medium were put in a 10 ml plastic syringe (Plastipak, Becton Dickinson). The openings of the syringe were covered with a small piece of cloth to avoid the escape of the beetles. The air passing through the syringe was cleaned with active charcoal, and then led via a Teflon tube into the olfactometer at the same position as the capillary was positioned in other trials. To test the effect of starvation the responding beetles were deprived of food for 3 days prior to the behavioural tests. The proportion of responding animals was arcsin $\sqrt{x}$-transformed for normalisation before applying ANOVA, followed by Tukey's post hoc test.

2.4. Extracts

Male beetles used to produce leg and body extracts were at least 6 months old. The beetles were kept on ice until dissection or put into hexane. However, to minimise the risk of the release of alarm substances, e.g. benzoquinones, which the beetles release in response to cold treatment (Markarian et al., 1978), the beetles were first sedated with CO_2. Before legs and bodies without legs were extracted, the beetles were mounted dorsally onto a sticky surface, and legs were removed from the sedated beetles with micro tweezers and extracted in 30 µl redistilled hexane for 30 min. All three pairs of legs were used in all bioassays, i.e. six legs equals one male equivalent (ME) and legs taken from 3, 5, 10, 15 and 20 male beetles, respectively, were tested. One, 3, 5, 10 and 15 bodies, respectively, with and without legs were extracted in 60 µl redistilled hexane for 30 min before the bodies were removed from the extraction vials. Extracts were kept in a freezer until experiments, but never longer than 48 h. Redistilled hexane was used as a control.

3. Results

SEM pictures show that a patch with more hairs is visible on the ventral side of the femur of the first pair of legs (prothoracic femurs, Fig. 1a). These structures are found on all three pairs of legs of the males (Figs. 1a–c), whereas no corresponding patches were found on females (Fig. 1d).

Females of *T. confusum* were more attracted to 5 live male *T. confusum* than the control (clean air). The attraction was even greater when the females had been starved for 3 days (Experiment 1,

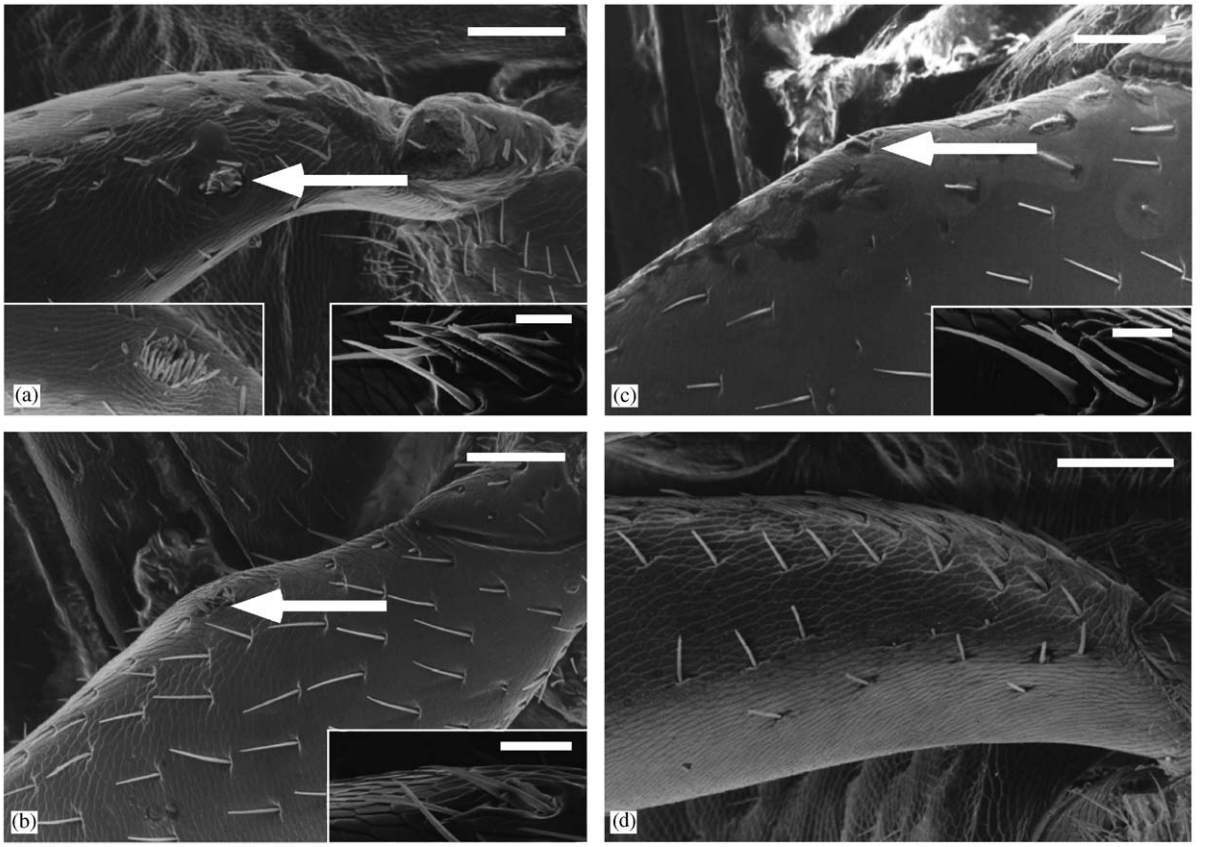

Fig. 1. Electron micrographs showing the setiferous patch on the prothoracic (a), mesothoracic (b) and metathoracic (c) femurs on male *T. confusum*. The structure is indicated by an arrow, and is suggested to be the pheromone production site. Scale bar is 50 μm. The small inserted figures to the right in all large figures show the structure in higher magnification; scale bar is 10 μm. The small figure inserted to the left in (a) shows the corresponding structure found on the prothoracic legs on *T. castaneum* males. Females of both species lack the corresponding structure, as indicated by the micrograph of the prothoracic femur of female *T. confusum* (d).

Fig. 2a). Males also showed a higher tendency to choose female odours compared to clean air when they had been starved for 3 days prior to the test. However, this response was not significantly different from response to the control (Experiment 2, Fig. 2b). Males did not attract other males, neither was attraction to males presented together with females significantly different from the control (Experiment 3, Fig. 2c). Since females were attracted to live males, a dose–response test was conducted, using extracted whole bodies of males. Females were attracted to extracts derived from 1–15 males (Experiment 4, Fig. 3a). Extracts from legs, derived from different numbers of males (3–20), were not attractive to the females (Experiment 5, Fig. 3b). Neither did any single dose of extracts of male bodies without legs reveal any significant treatment effect when females were tested for attraction to them (Experiment 6, Fig. 3c). We did not find any significant dose-dependent effect in our experiments (ANOVA, body extracts: $F = 0.37$, df $= 4$, $P > 0.05$; leg extracts $F = 0.13$, df $= 4$, $P > 0.05$; extracts of bodies without legs: $F = 0.35$, df $= 4$,

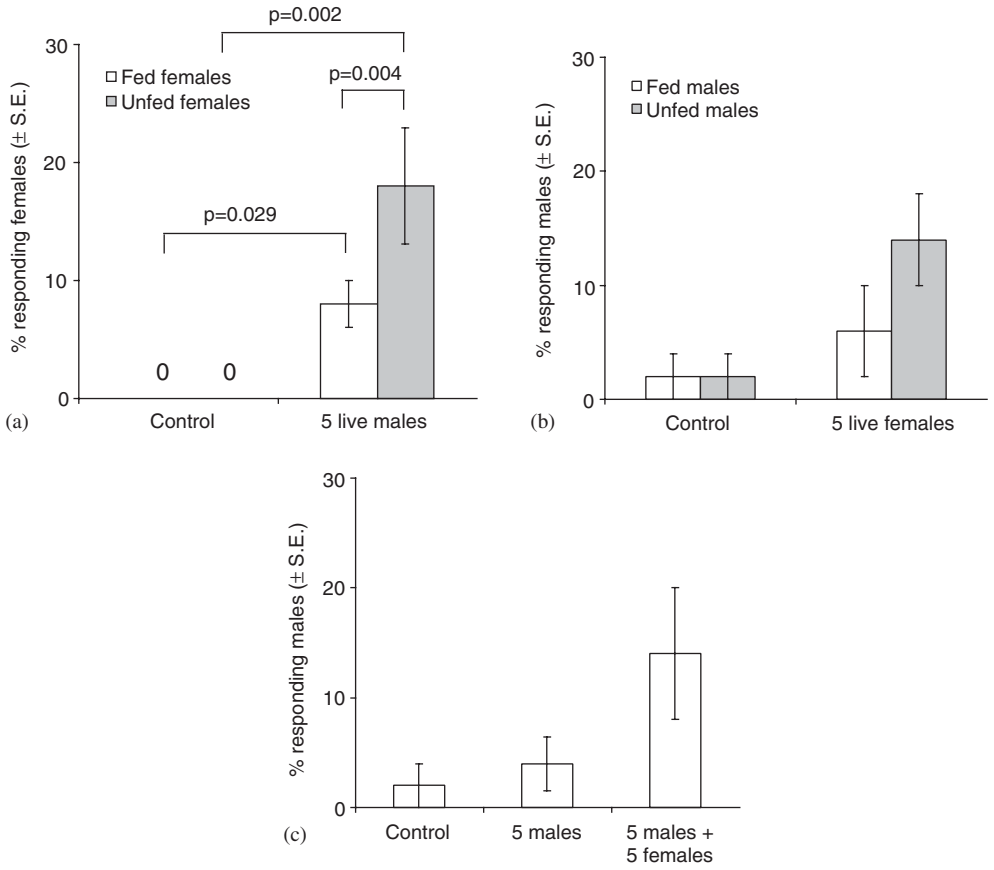

Fig. 2. Proportion of (a) fed and unfed female beetles (±S.E.) responding to live males (experiment 1, ANOVA, $F_{2,17} = 8.14$, $P<0.001$), (b) fed and unfed male beetles (±S.E.) responding to live females (experiment 2, ANOVA, $F_{2,17} = 1.16$, $P>0.05$) (c) fed male beetles (±S.E.) responding to live males and both sexes (experiment 3, ANOVA, $F_{2,12} = 1.74$, $P>0.05$) in the walking bioassay. Five groups with 10 beetles were tested for each stimulus and air was used as the control.

$P>0.05$) and we pooled data from all doses. In the subsequent analysis of the pooled data we found that responses to body extracts and extracts from bodies without legs were significantly different from the control but not the responses to leg extracts (*T*-test, body extracts: $F = 7.15$, df = 28, $P<0.001$; extracts of bodies without legs: $F = 10.87$, df = 28, $P<0.001$; leg extracts $F = 5.41$, df = 28, $P>0.05$).

4. Discussion

Our findings suggest that the volatiles emitted by male *T. confusum* function as a sex pheromone attracting females only. Females were attracted to males, whereas males were not significantly attracted to either males or females. Earlier studies also report that *T. confusum* females are more responsive than males to the reported aggregation pheromone both behaviourally and

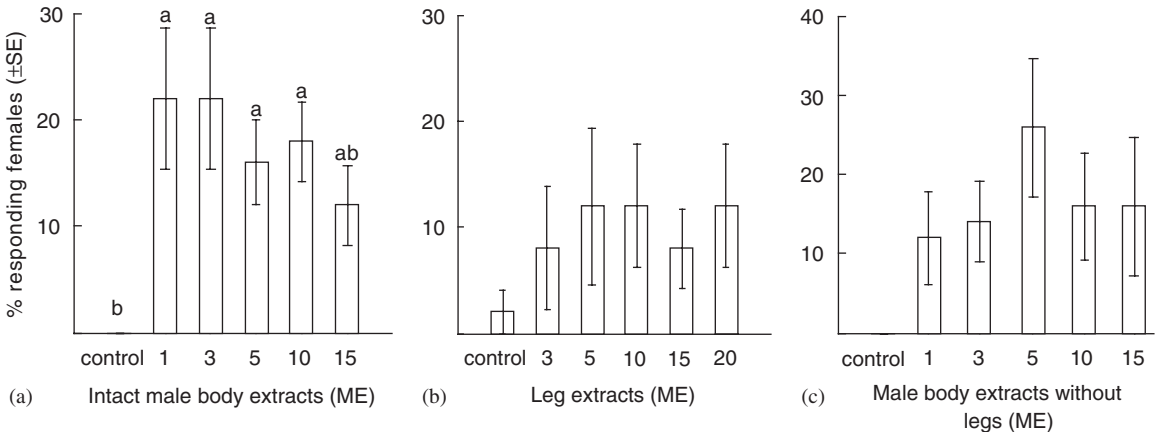

Fig. 3. Proportion of fed female beetles (±S.E.) responding to (a) whole body extracts of males (experiment 4, ANOVA, $F_{5,24} = 4.46$, $P < 0.01$), (b) leg extracts of males (experiment 5, ANOVA, $F_{5,24} = 0.56$, $P > 0.05$), (c) extracts of males with their legs removed (experiment 6, ANOVA, $F_{5,24} = 1.98$, $P > 0.05$) in the walking bioassay. Eighteen legs correspond to 3 male equivalents (ME). Five groups with 10 beetles were tested for each stimulus and hexane was used as the control. Bars with the same letter are not significantly different at the $P < 0.05$ level.

electrophysiologically (Levinson and Mori, 1983). In our study we recorded the response of each individual beetle, since the responding beetles were removed immediately when entering the response circle, which is more informative than testing groups of beetles over a longer period of time in an olfactometer or in a pitfall bioassay (Suzuki and Sugawara, 1979; Faustini et al., 1981; Suzuki et al., 1984; Trematerra et al., 2000). In all of the studies mentioned, the primary responding beetles might attract other beetles, and thereby synergise the effect of the stimulus tested. In comparison with aggregating bark beetles, where the major function of the aggregation pheromone is to overcome the chemical defence of the attacked tree (Borden, 1974, 1985; Schlyter and Birgersson, 1999), flour beetles do not benefit from aggregation behaviour in an obvious way. The suggested adaptive functions of aggregation pheromone, i.e. optimal feeding, location of oviposition sites and finding mates (Borden, 1985; Levinson and Levinson, 1995) can still be achieved if the pheromone is a sex pheromone. It has been assumed that aggregation pheromones are evolutionary precursors of sex pheromones and that the sexual function has appeared secondarily, when association with feeding was lost (Shorey, 1973; Levinson and Levinson, 1995). An alternative hypothesis is that the communication system evolved from a male-emitted sex pheromone, attracting females only, and that some males could "eavesdrop" on conspecific males to find food and females (Phillips, 1997). The strategy of "sneaking" males can be advantageous, since the sneakers can rely on detection of pheromone-calling male conspecifics and thereby avoid the cost of pheromone production.

The effect of 3-day starvation was most obvious when analysing female response to males. The responsiveness to host odours may increase when beetles are starved, and perhaps also to pheromones. An association of feeding and pheromone perception can be advantageous for the beetles when searching both for food and mates, and should be even more pronounced when they are deprived of food. Starvation, however, had no significant effect on male attraction of females. Starved beetles, however, are not likely to occur in the stored-product environment, where the

beetles commonly occur, since they rarely move outside food sources and disperse to other habitats through human transport of stored products. In a bioassay with relatively small numbers of insects responding, the effect of starvation may produce the extra response accounting for the difference between significant and non-significant effects.

A female sex pheromone has been suggested in *T. confusum*, but with a much lower response rate than the male aggregation pheromone (O'Ceallachain and Ryan, 1977). We could not see any evidence of a female-emitted attractant in our experiments. We did not, however, test the beetles in close range contacts where female-emitted substances could play a role in sex discrimination. However, homosexual copulations have been reported in both *T. castaneum* and *T. confusum* (Wade et al., 1995; Serrano et al., 2000), indicating that if the females emit a sex discriminatory signal it has a minimal effect on the males. It could also indicate that the cost of copulating with another male instead of a female is less than the cost of missing an insemination and a potential fitness increase.

Extracts from legs alone were not enough to significantly attract females, whereas extracts from intact male bodies and bodies without legs were attractive. Our behavioural results confirm that the males are the pheromone-producing and emitting sex. Our electron microscopic study confirmed that *T. confusum* males have setiferous patches on all legs, as described by Hinton (1942), whereas females do not. The corresponding structure in *T. castaneum* which has been reported as a pheromone production site, is only present on the prothoracic pair of legs (Faustini et al., 1981). The structure in male *T. confusum* is most evident and well developed on the prothoracic femurs and not so prominent on the meso- and metathoracic femurs. Transmission electron microscopy (TEM) reveals glandular tubes that exit in the patches found on the femurs, supporting a role in pheromone production (C. Olsson and R. Wallén, unpublished). However, based on our behavioural experiments the femoral structures are not the main production sites of attractive substance. Our study indicates that the male pheromone is produced, or at least emitted, from multiple locations, on the body and possibly on the legs. This conclusion is also applicable to *T. castaneum*, for which it has been shown that neither the amount of pheromone produced nor female attraction are significantly decreased when the prothoracic legs are removed from the males (Bloch Qazi et al., 1998). Morphological studies of putative pheromone-producing glands are of little value if no behavioural tests confirm activity of substances produced and secreted by the glands.

Earlier studies have suggested that the 4,8-DMD is an aggregation pheromone but the repeated responses to even high doses of synthetic DMD were rather low (Levinson and Mori, 1983; Suzuki et al., 1984). We also observed low responses in our behavioural experiments, but we nevertheless believe that working with extracts is more relevant from a biological point of view. Perhaps, the secretion from the femoral glands only works at short distances, not functioning as an aggregation pheromone. During mating, male *T. castaneum* transfer secreted substances by rubbing the legs on the female elytra (Edvardsson and Arnqvist, 2000). The substances might then function as a contact marking pheromone, revealing to subsequent males that the female is already mated. It is also possible that active substances were missed by the extraction method and solvents used.

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