Integrative Biosciences 11: 141-146, 2007

Integrative
Biosciences
http://www.ibiosci.or.kr

Further Evidence for the Role of Cantharidin in the Mating Behaviour of Blister Beetles (Coleoptera: Meloidae)

Mahmood Reza Nikbakhtzadeh[1,*], Claudia Hemp[2] and Babak Ebrahimi[3]

[1]*Department of Medical Parasitology & Entomology, Tarbiat Modares University, Tehran, Iran;* [2]*Department of Animal Ecology II, University of Bayreuth, 95440 Bayreuth, Germany;* [3]*Department of Entomology, The Ohio State University, Columbus, OH 43210, USA*

Abstract: Cantharidin is produced by blister beetles (Coleoptera: Meloidae) and smaller oedemerid beetles (Coleopetra: Oedemeridae) and is found in hemolymph and various tissues. The function of cantharidin in the courtship behaviour of meloids had never been fully established. Our studies show a correlation between density of cuticular pores and cantharidin titre of the scape and pedicel segments of male specimens of the East African species of *Epicauta nyassensis* (Haag-Rutenberg, 1880) (Coleoptera: Meloidae). Light microscopy of semi-thin cross sections of the male scape and pedicel indicates that there are many canal shaped structures that stretch from the antennal hemolymph to the antennomere surface. These structures may be tubules, which transport cantharidin circulating in the hemolymph to the surface, where the compound can be released via cuticular pore openings. Analyses of the head capsule and antennal segments of *E. nyassensis* females which had been copulated with males revealed low titre of cantharidin in the first two antennal segments. The density of the scape and pedicel pores of females was to some extent higher than the density of these pores on flagellum; however it was considerably lower than that of the males. Interestingly, no tubular cell or other transport structures were found in the cross sectioning of the female antennomeres or on the integument surface. During mating, male antennomeres, as well as cantharidin containing pores which are located on the 1st and 2nd antennomeres, come into direct contact with the female antennae and may release cantharidin to their surface. Female *E. nyassensis* may be able to discriminate the opposite sex with abundant reserves of cantharidin prior to mating. This is another evidence that cantharidin function in close range sexual selection.

Key words: Meloidae, blister beetle, *Epicauta nyassensis*, cantharidin, cuticular pore, sexual selection

*To whom correspondence should be addressed.
Tel: 98-91-27973497; Fax: 98-21-88013030
E-mail: nikbakht_m@excite.com

Cantharidin is produced by blister beetles and smaller oedemerid beetles (Col: Oedemeridae) and is found in hemolymph and various tissues (Dixon et al., 1963; Carrel et al., 1986; Carrel et al., 1993; Frenzel and Dettner, 1994; Dettner, 1997). In most blister beetles, females possess cantharidin but cannot produce it (Carrel et al., 1993). During copulation, males transfer large amounts of cantharidin along with sperm to the female (Sierra et al., 1976). The involvement of cantharidin in courtship behaviour has been already confirmed for certain cantharidin insects. For example, it was shown that males of *Neopyrochroa flabellata* (Coleoptera: Pyrochroidae) secrete cantharidin from a cephalic gland that females sample during courtship. Females mate preferentially with males possessing higher titres of cantharidin (Eisner et al., 1996a, b). Cantharidin has been reported as a sexual attractant and stimulates copulation in *Atrichopogon* (Diptera: Ceratopogonidae) (Frenzel et al., 1992; Frenzel and Dettner, 1994). Moreover, females of *Notoxus monocerus* (Coleoptera: Anthicidae) bite several times into a pair of notch-like structure on the apices of male elytra containing considerable amount of cantharidin. This behaviour provides the female with the information whether or not the males will be able to transfer sufficient amounts of cantharidin during copulation (Schütz and Dettner, 1992). Paired tufts of hairs around the glandular pores on the elytra of *Pallenothriocera rufimembris* (Coleoptera: Cleridae) may play a role in courtship behaviour by releasing cantharidin (Hemp et al., 1999). Pinto (1974, 1975) considered male cuticular pores being involved in the courtship behaviour of blister beetles. McCormick and Carrel (1987) suggested that cantharidin might be used by female meloids while selecting a mate at close range. However, the function and intrinsic role of cantharidin in the courtship behaviour of meloids has been never fully established. Working on different species of blister beetles,

we came across to *Epicauta nyassensis* (Haag-Rutenberg, 1880) (Coleoptera: Meloidae) from the Eastern Africa with remarkable morphologic and chemical features. We hereby provide some further evidence that cantharidin may act as an infochemical in courtship behaviour of meloid beetles.

MATERIAL AND METHODS

Field collection of beetles and transport to laboratory

The East African blister beetles, *E. nyassensis*, were collected in May 2006 in Nairobi, Kenya from flowers and stems of *Solanum aculeatissimum* (Solanaceae). *Mylabris quadripunctata* and *Hycleus polymorphus* were located in southern France (Table 1). They were collected manually while sitting on different shrubs of the families Astraceae, Compositeae and Leguminoseae. The insects were transported to the Medical Entomology Department at Tarbiat Modares University, where they were maintained in the laboratory.

Laboratory maintenance and control of sexual activity

Thirty females and males were respectively determined by the presence of valviferes or an aedeagus on the external genitalia. Virgin females were recognized by inspecting the genital opening for the absence of a spermatophore (Gerber et al., 1971). Males and virgin females were kept in separate screened cages to prevent mating. Colonies were maintained based on procedures from Selander (1986) and Singh and Moore (1985). Cages were kept in environmental chambers at constant temperature and humidity (27-28°C, RH of 40-45%) using a photoperiod of 13 L: 11 D.

Observation of the pre- and postcopulatory behaviour

After two weeks of laboratory maintenance, five males and five virgin females out of 30 were selected and placed in a screened cage (40 × 40 × 40 cm) until the sexual activity was observed. Subsequently, they were sacrificed and dissected two days following copulation. They were used to measure the cantharidin component of the antennal segments. Another group of 5 individuals of each sex was used for electron microscopy and calculating the cuticular pore density. In the last group, the whole antennae were completely removed and analyzed, and their cantharidin titre was compared to that of the sexual organs and external appendages.

Sample preparation and extraction

To determine the total cantharidin component, hydrolysis and extraction of the dissected tissues were carried out according to Holz et al. (1994).

Quantitative GC-MS

Cantharidin was quantified by GC-MS using a Varian Saturn 2000.40 equipped with a ZB-5 capillary column coated with 5% phenyl polysiloxane (Phenomenex: FT 0.25 μm, ID 0.25 mm, Length: 60 m). A 1079 injector was used and samples (1 μl) were injected (split/splitlessly) using a Varian autosampler 8200 CX. Trap and transfer line were kept at 175°C and 260°C, respectively. Mass spectra were taken at 70 eV (EI mode) at 1 scan sec^{-1} from *m/z* 30 to 350. Data were processed by a Saturn® GC/MS Workstation package, Saturn view™ version 5.2.1, 1989-1998, Varian Associates, Inc. Helium at constant pressure served as the carrier gas (1.8 ml min^{-1}). The elution of compounds was programmed from 40°C (2 min) to 130°C at 100°C min^{-1}, then to 195°C at 3°C min^{-1}, followed by rapid heating to 250°C at 100°C min^{-1} kept for 2 min prior to cooling. Authentic cantharidin (purity 98%, SIGMA-ALDRICH Chemical Co., UK) served as a standard for identification and calibration. Analyses were made in the normal EI-MS mode. EI Ionization provides mass spectra with characteristic fragments of cantharidin at *m/z* 96 and *m/z* 128 (M^+: 197).

Scanning electron microscopy (SEM)

The antennae of five males and females of *E. nyassensis* were dissected under a stereomicroscope and individually fixed on the probe plates. The samples were gold-coated using an Edwards Sputter Coater S 150 B under the following conditions: Argon pressure: 3×10^{-1} atm, Voltage: 1-1.5 KV and AC: 40 mA. Ten random areas of the gold coated samples were examined under a Cambridge electron microscope (Cambridge Instruments®) and photograhps were taken with a Nikon Coolpix 995 digital camera. To determine pore density within any frame of a sample, cuticular pores were counted over an area of 24.5 μm^2. To understand the internal structure of such pores, a semi-thin cross section was prepared from the antennal segments and the stained tissues were studied by light microscopy at 400 fold magnification.

Preparation of interior segments of the antennae

To study the internal architecture of antennal segments, whole antennae of both sexes were removed and divided into segments; thereafter, fixation, dehydration, and post-fixation steps were carried out according to the method of Adam and Czihak (1964). For microtome cuttings, samples were well coated with a thick layer of paraffin (non-caking, Merck). Using a diamond knife on an ultramicrotome (Leica RM 2035, Leica Instruments GmbH), semi-thin cross sections of about 10 μm were taken from antennal samples embedded in a paraffin block. The sections were rinsed with distilled water at 35-38°C for a few seconds and then placed on pre-treated microscopy slides (Super Frost® Plus, Menzel Gläser®, Germany). The paraffin was removed by placing the samples first for 20 min in xylol, followed by a passage through a graded isopropanol series and finally

Table 1. Chemically examined species of blister beetles (Coleoptera: Meloidae) and cantharidin contents of antennal segments of the both sexes

Species	Country/Region	Collecting site	n	Cantharidin[d] (♂)	Cantharidin[d] (♀)
MQ[a]	Southern France	St. Jéan du Gard, Departement Gard	5	2473	1618
HP[b]	Southern France	Can de l'Hospitalet, Departement Lozère	5	3091	2301
EN[c]	Kenya	Nairobi	4	58402	10578

[a]: *Mylabris quadripunctata* (Linné, 1767)
[b]: *Hycleus polymorphus* (Pallas, 1771)
[c]: *Epicauta nyassensis* (Haag-Rutenberg, 1880)
[d]: Median value (ng/mg DW)

distilled water (10 min). Staining was performed using kernechtrot-aluminiumsulfate and pikroindigocarmin (Adam and Czihak, 1964). Final dehydration of the samples was achieved by passage through a graded isopropanol series (isopropanol 70-95% for several seconds and isopropanol 100% for 10 min) and then pure xylol for 20 min.

Statistical analyses

The data of the internal distribution of cantharidin were analysed by Kruskal-Wallis ANOVA test. If the test showed any significant difference within an experiment, the statistically significant group(s) was determined by a Tukey Kramer test. Repeatability of chemical experiments was tested over 5 samples (95% confidence level), and for determination of the cuticular pore density, 10 random locations of each of 5 samples were inspected at 99.9% confidence level. Cuticular pore density in experiments with more than two groups was analysed by a one-way ANOVA as a parametric test, and significant means were separated with Tukey *HSD* posthoc test at the $P < 0.01$ and $P < 0.001$ levels. Whenever cuticular pore density was compared within two groups, student *t*-test was used to indicate the significance at $P = 0.001$ level, unless variable P was more than 1 and therefore analysed by Mann-Whitney *U*-test. Apart from the Tukey-Kramer test, the statistical analyses were carried out using Statistica Package (Kernel version 5.5 A, Statsoft Inc., 1999, USA).

RESULTS

Cantharidin is present in the somatic tissues and hemolymph of blister beetles, but occurs at higher concentrations in male and female sexual organs. Chemical analyses of the antennal segments of many species of meloid beetles have indicated extremely low amounts of cantharidin (Table 1). In contrast, *E. nyassensis* showed high concentrations of cantharidin in male antennae. By analysing the head capsule and antenna, it was found that most of cantharidin accumulated in the antenna itself (Nikbakhtzadeh, 2004). Dissection of the male antenna showed that cantharidin was not uniformly distributed over the segments, but accumulated

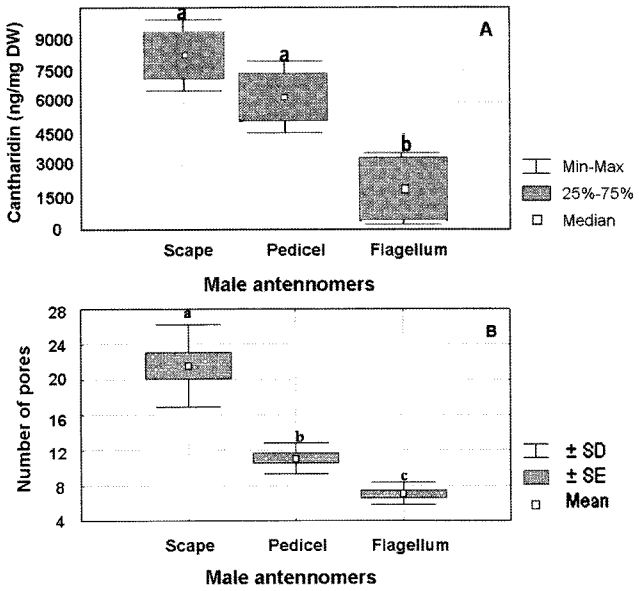

Fig. 1. A: Median (± minimum and maximum data, n = 5) titre of cantharidin (ng/mg dry weight) in the male antennal segments of *Epicauta nyassensis*. Kruskal-Wallis ANOVA test, $P < 0.05$. B: Mean number (±SD, n = 5) of cuticular pores on the male antennal segments of *Epicauta nyassensis* per 24.5 μm^2 of SEM visible field. Rectangles with different letter on top indicate statistically significant differences from each other using ANOVA, Tukey *HSD*-test at $P < 0.001$ level of confidence.

in the scape and the pedicel (Fig. 1A). Examination of male antenna by SEM revealed a comparable pattern of cuticular pores (Fig. 4) over the surface of the scape, pedicel and flagellum (Fig. 1B). As shown in Fig. 5A, the cuticular canals extend from the surface of the integument to the hemolymph in the scape and pedicel segments. The antennal segments of *E. nyassensis* females, which had copulated with males, were studied as accumulation sites for cantharidin. We found that cantharidin was not preferentially accumulated in the first two antennal segments (Fig. 2A) and just a relatively higher concentration of pores was observed on the scape and pedicel (Fig. 2B). No conducting structure was observed in female antennae (Fig. 5B). Comparing the two sexes, the first two antennomeres in males display a higher pore density (Fig. 3A, B).

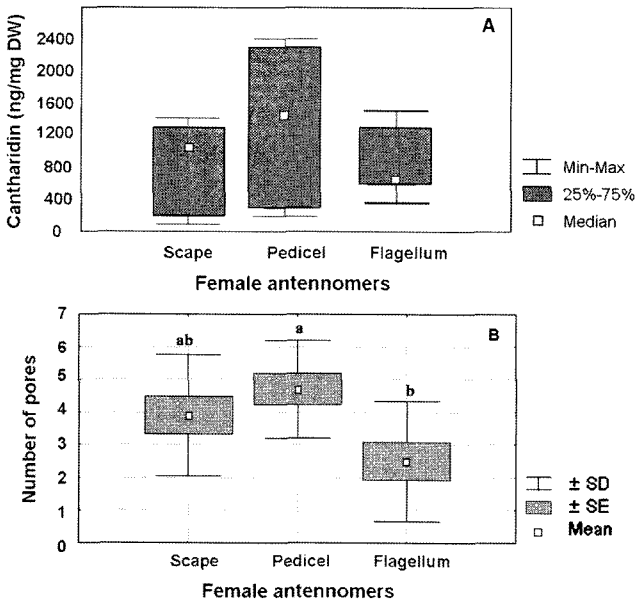

Fig. 2. A: Median (± Minimum and maximum data, n = 5) titre of cantharidin (ng/mg dry weight) in the female antennal segments of *Epicauta nyassensis* following copulation with a male. Kruskal-Wallis ANOVA test, $P > 0.05$. B: Mean number (±SD, n = 5) of cuticular pores on the female antennal segments of *Epicauta nyassensis* per 24.5 μm^2 of SEM visible field. Rectangles with the same letter on top are not statistically different from each other using ANOVA, Tukey *HSD*-test at $P < 0.05$ level of confidence.

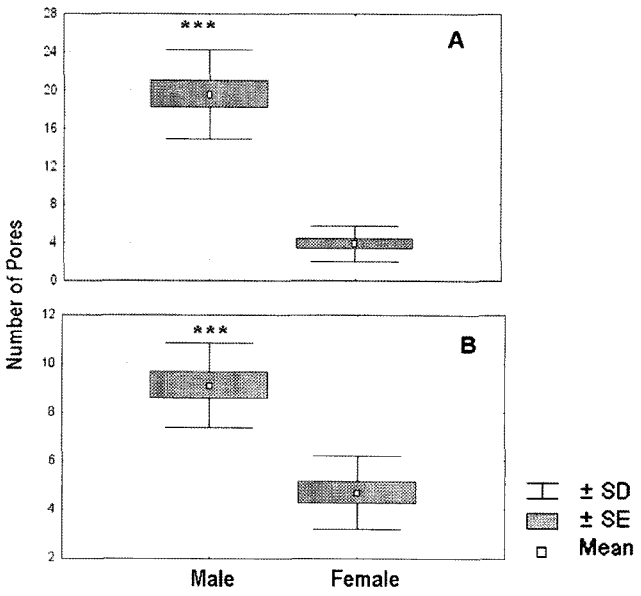

Fig. 3. Mean number (±SD, n = 5) of cuticular pores on the scape (A) and pedicel (B) segments of male and female antennae per 24.5 μm^2 of SEM visible field, *Epicauta nyassensis* ***: statistically significant difference using Mann-Whitney *U*-test, at $P < 0.001$ level of confidence.

DISCUSSION

Insect antennae are not only involved in the reception of signals, but also produce and emit chemicals (Bin et al., 1999). Based on external and internal morphology of antenna and chemical analysis, this study provides further evidence of the role cantharidin may have as an infochemical during courtship in the blister beetle, *E. nyassensis*. A positive correlation was observed between the density of cuticular pores and the titre of cantharidin in the scape and pedicel segments of male *E. nyassensis*. Although high

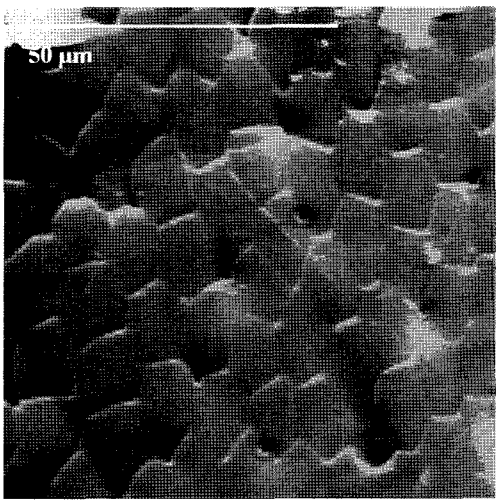

Fig. 4. SEM of cuticular pores on the pedicel of male antenna, *Epicauta nyassensis*, 895 ×.

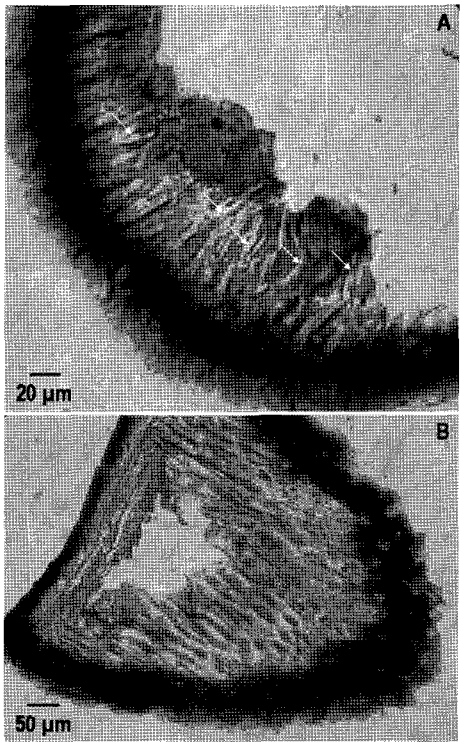

Fig. 5. Semi-thin cross section of (A) a scape segment of male and (B) a pedicel segment of female *Epicauta nyassensis*. Arrows in (A) indicate cuticular canalas of the male antenna for which no comparable pendant exist in the female organ. Light microscopy at 400 × magnification.

pore density on the first two antennomeres is not very common for insects, this morphological feature has been observed in other insects. Cuticular pores are located in a ring in the proximal portion of the 1st and 2nd antennomere in fire ants (*Solenopsis invicta*) in both workers and the queen (Isidoro et al., 2000). The expanded scape of some sphecids (Hymenoptera: Sphecidae) may house glandular openings used during courtship (Wcislo, 1998). The abundant canal-shaped structures may be involved in the transport of cantharidin from the hemolymph to the surface where it is later released via the openings of the cuticular pores. Since tubular cells or other conducting structures such as tyloids were absent in cross sections of female antennomeres or on the integument surface, it can be assumed that the function of the female pores must be different from that of the males. In our laboratory observations males and females engaged in antennation while they were in front of each other, i.e. antennal stroking during the precopulatory phase of the courtship behaviour in which flagellum of the female specimens were repeatedly touched by scape and pedicel of the males. Therefore, male cuticular pores come into direct contact with the female antennae and may release cantharidin onto their surface. In that case, the porous area of male antennae may function as a cantharidin releasing structure (CRS), while the female organ may represent a multiporous chemical receptor (MCR) that recognizes the cantharidin titre of the sexual partner. The three main segments of the female antenna do not show any significant difference in cantharidin titre (Fig. 2A). Furthermore, the female antennomers do not show the same statistical difference in the pore density as seen in the males (Fig. 2B). We believe that part of the female antenna should be involved in cantharidin detection of the males, but have insufficient data to point to a specific region/segment. Combined with the behavioural observations, female flagellum can be regarded as the probable location of the chemical receptors.

As Snead (1985) and Carrel et al. (1993) suggested in their studies, perhaps females of *E. nyassensis* have evolved to select a mate with high titres of cantharidin. We hereby provide further evidence linking the probable role of cantharidin in close range sexual selection in family Meloidae. Further studies are needed to confirm the role of cantharidin as a precopulatory discriminator.

ACKNOWLEDGMENTS

The authors thank Elisabeth Helldörfer and Andrea Beran in the Department of Animal Ecology II and Marion Preiß, Department of Animal Ecology I, University of Bayreuth for their technical support. We also thank Professor M. A. Bologna, University of Rome, Italy for identification of our meloid specimens and Dr. Mansoureh Shaeghi, Tehran University of Medical Sciences, for supporting us by access to the Varian Saturn GC-MS machine. Precise consideration of Adrea Lovejoy (Ohio State University) in reviewing a draft of our manuscript and improving the English text must be acknowledged. This project has been supported by a research grant of Tarbiat Modares University (TMU), Tehran, Iran and partly by Deutsche Forschungsgemeinschaft (DFG) within GRAKO 678, University of Bayreuth, Germany.

REFERENCES

Adam H and Czihak G (1964) Arbeitsmethoden, der makroskopischen und mikroskopischen Anatomie. Gustav Fischer Verlag, Stuttgart, 583 pp.

Bin F, Isidoro N and Romani R (1999) Antennal structures of Hymenoptera: Sensilla or glands? *Atti dell'Accademia Nazionale Italiana di Entomologia Rendiconti* XLVII: 251-263.

Carrel JE, Doom JP and McCormick JP (1986) Identification of cantharidin in false blister beetles (Coleoptera. Meloidae) From Florida. *J Chem Ecol* 12: 741-747.

Carrel JE, McCairel MH, Slagle AJ, Doom JP, Brill J and McCormick JP (1993) Cantharidin production in a blister beetle. *Experientia* 49: 171-174.

Dettner K. (1997) Inter and intraspecific transfer of toxic insect compound cantharidin. In: Dettner K, Bauer G, and Volkl W (eds), Vertical Food Web Interactions, Springer Verlag, Berlin, pp 115-145.

Dixon AFG, Martin-Smith M and Smith SJ (1963) Isolation of cantharidin from *Meloë proscarabeus*. *Can Pharmacol J* 29: 501-503.

Eisner T, Smedley SR, Young DK, Eisner M, Roach B and Meinwald J (1996a) Chemical basis of courtship in a beetle (*Neopyrochroa flabellate*): Cantharidin as precopulatory enticing agent. *Proc Natl Acad Sci* 93: 6494-6498.

Eisner T, Smedley SR, Young DK, Eisner M, Roach B and Meinwald J (1996b) Chemical basis of courtship in a beetle (*Neopyrochroa flabellate*): Cantharidin as nuptial gift. *Proc Natl Acad Sci* 93: 6499-6503.

Frenzel M, Dettner K, Wirth D, Waibel J and Boland W (1992) Cantharidin analogues and their attractancy for ceratopogonid flies (Diptera: Ceratopogonidae). *Experientia* 48: 106-111.

Frenzel M and Dettner K (1994) Quantification of cantharidin in cantharipHilous Ceratopogonidae (Diptera), Anthomyiidae (Diptera) and cantharidin producing Oedemeridae (Coleoptera). *J Chem Ecol* 20: 1795-1812.

Gerber GH, Curch NS and Rempel JG (1971) The structure, formation, histochemistry, fate, and formations of the spermatophore of *Lytta nuttalli* Say (Coleoptera: Meloidae). *Can J Zool* 49: 1595-1610.

Hemp C, Hemp A and Dettner K (1999) Attraction of the colour beetle species *Pallenothriocera rufimembris* by cantharidin (Cleridae: Coleoptera). *Entomol Gener* 24: 115-123.

Holz C, Streil G, Dettner K, Dütemeyer J and Boland W (1994) Intersexual transfer of a toxic terpenoid during copulation and its parental allocation to developmental stages: Quantification of cantharidin in cantharidin-producing oedemerids (Coleoptera: Oedemeridae) and cantharipHilous pyrochroids (Coleoptera: Pyrochroidae). *Z Naturforsch* 49c: 856-864.

Isidoro N, Romani R, Velasquez D, Renthal R, Bin F and Vinson SB (2000) Antennal glands in queen and worker of the fire ant, *Solenopsis invicta* Buren: first report in female social Aculeata (Hymenoptera: Formicidae). *Insectes Soc* 47: 236-240.

McCormick JP and Carrel J.E. (1987) Cantharidin biosynthesis

and function in meloid beetles. In: Prestwitch GD and Blomquist GJ (eds), Pheromone Biochemistry, Academic Press, Orlando, pp 307-350.

Nikbakhtzadeh MR (2004) Transfer and distribution of cantharidin within selected members of blister beetles (Coleoptera: Meloidae) and its probable importance in sexual behaviour. Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften an der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth. Bayreuth. 105 pp.

Pinto JD (1974) Courtship behaviour in *Linsleya compressicornis* and its taxonomic significance. *Pan Pac Entomol* 50: 1-8.

Pinto JD (1975) Intra and interspecific courtship bahaviour in blister beetles of the Genus *Tegrodera* (Meloidae). *Ann Etomol Soc Am* 68: 275-284.

Schütz C and Dettner K. (1992) Cantharridin secretions by elytral notches of male Anthicid species (Coleoptera: Anthicidae). *Z Naturforsch* 47C: 290-299.

Selander RB (1986) Rearing blister beetles. *Insecta Mundi* 1: 209-220.

Sierra Jr, Woggon WD and Schmidt H. (1976) Transfer of cantharidin during copulation from the adult male to the female *Lytta vesicatoria* (Spanish flies). *Experientia* 32: 142-144.

Singh P and Moore RF (1985) Handbook of Insect Rearing. Vol 1. Elsevier Publications, Amsterdam, 488 pp.

Snead JS and Alcock J (1985) Aggregation formation and assortative mating in two meloid beetles. *Evolution* 39: 1123-1131.

Wcislo WT (1998) Sexual dimorphism of wasp antennal structure in relation to parasitic and non-parasitic behaviour (Hymenoptera: Sphecidae). *J Hym Res* 7: 178-181.

[Received October 22, 2007; accepted November 26, 2007]