The Possible Role of Antennal Cuticular Pores in the Sexual Behaviour of *Cyaneolytta* sp. (Coleoptera: Meloidae)

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Abstract: Die mögliche Rolle der antennalen Cuticularporen beim Sexualverhalten von *Cyaneolytta* sp. (Coleoptera: Meloidae)

Ölkäfer (Col: Meloidae) gehören zu den Insekten mit medizinischer Bedeutung. Cantharidin, das im gesamten Körper (v.a. in den Sexualorganen beider Geschlechter) der Käfer zu finden ist, verursacht Blasen auf der Haut. Die mögliche Rolle des Cantharidins im Sexualverhalten der Ölkäfer ist von besonderem wissenschaftlichem Interesse. *Cyaneolytta* sp. wurde in Karen, Kenia, gesammelt. Nach der Sektion wurden die Insektenkompartimente hydrolysiert und mittels quantitativer GC-MS chemisch analysiert. Um Poren der Kutikula und andere äußere Merkmale zu untersuchen, wurde ein Rasterelektronenmikroskop verwendet. Für Dünnschnitte der inneren Gewebe wurden Mikrotomschnitte angefertigt. Statistische Analysen wurden mit dem Softwarepaket Statistica durchgeführt. In den Antennen von *Cyaneolytta* sp. Péringuey, 1909 finden sich extrem hohe Cantharidinmengen. Die Daten zeigen eine deutliche Korrelation zwischen der Dichte der Kutikularporen und dem Cantharidingehalt der Antennenglieder Scapus und Pedicellus der *Cyaneolytta*-Männchen. Es existieren zahlreiche kanalartige Strukturen, die sich von der Hämolymphe der Antennen zur Oberfläche erstrecken, wo sich die Kutikularporen befinden. Betrachtet man den Cantharidingehalt der Antennensegmente, so enthalten die Glieder der Männchen viel größere Mengen als die der Weibchen. Auch besitzen die Kutikularporen der Weibchen eine deutlich geringere Dichte auf den Antennensegmenten. Die kanalartigen Strukturen auf den ersten beiden Antennensegmenten der Weibchen könnten uni- oder multizelluläre Tubuli sein, die das in der Hämolymphe zirkulierende Cantharidin an die Oberfläche bringen, aber die Poren auf den Antennen der Weibchen müssen eine andere Funktion haben. Während des Balzverhaltens berühren sich beide Geschlechter mit den Antennen, wobei die cantharidinhaltigen Poren in direkten Kontakt mit den weiblichen Antennen gelangen. Es wird vermutet, dass Cantharidin an die Oberfläche der Antennen der Männchen abgegeben wird. Demnach könnte der porenhaltige Bereich der männlichen Antennen eine Struktur sein, die Cantharidin abgibt, während der porenhaltige Bereich der weiblichen Antennen ein Rezeptorfeld darstellen könnte, dessen Chemorezeptoren einen bevorzugten Geschlechtspartner erkennen. Damit wurden weitere Hinweise für die Hypothese gesammelt, dass Cantharidin eine Rolle bei der sexuellen Selektion im Nahbereich spielt.

Key Words: *Cyanelolytta* sp., Meloidae, blister beetle, cantharidin, sexual selection

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Cantharidin, which is mainly found in blister beetles (Coleoptera: Meloidae), is one of the most intensively studied natural products of insect (Dettner, 1997; McCormick & Carrel, 1987). The involvement of cantharidin in courtship behaviour has been already confirmed for certain canthariphilous insects (Eisner & al. 1996a,b; Frenzel & Dettner 1994; Frenzel & al. 1992; Schütz & Dettner, 1992; Hemp & al. 1999).

The function and intrinsic role of cantharidin in the courtship behaviour of Meloids has been never fully established. McCormick & Carrel (1987) only suggested that cantharidin might be used by female meloids when selecting a mate at close range. Pinto (1974, 1975) was, in fact, the first to consider male cuticular pores as being involved in the courtship behaviour of species from the genus *Linsleya* and *Tegrodera* (Meloidae). Based on morphology and chemical analyses of *Cyaneolytta* sp. (Coleoptera: Meloidae), we have hereby provided some further evidences that cantharidin may act as an infochemical in courtship behaviour of meloid beetles.

Materials and Methods

Field Collection of Beetles and Transport to Laboratory. The East African blister beetles, *Cyaneolytta* sp. (Coleoptera: Meloidae), were collected in May 2002 in Nairobi, Kenya from the flowers or stems of the shrub *Solanum aculeatissimum* (Solanaceae). The insects were transported soon afterwards to Germany, where they were maintained in the laboratory.

Laboratory Maintenance and Control of Sexual Activity. The sexes were distinguished by their external genitalia, separated and kept in different screened cages to control the courtship and copulation behaviour. Cages were kept in environmental chambers at constant temperature and humidity (27-28°C, RH of 40-45%) using a photoperiod similar to that of the natural habitat (14 L: 10 D).

Administration of deuterium labelled Cantharidin. To obtain reliable data, independent of the natural variation of cantharidin, deuterium labelled cantharidin (CAN-d_2) was injected into hemocoel of the beetles (Holz & al. 1994). 10 µl of a concentrated solution of CAN-d_2 in dimethylsulfoxide (1600 ng/µl) was injected through the intersternite membranous tissue using a microsyringe. Beetles were then kept in isolation for two days. Pre-treated beetles which died earlier than two days were excluded to keep the experimental conditions uniform. Surviving males and females were dissected after two days into parts including internal and external organs.

Sample Preparation and Extraction. Tissue samples were placed into test tubes and their dry weight (DW) was determined after 36 hours of freeze drying (-50 °C, at 9×10^{-2} mbar). All body fragments were first hydrolyzed for 4 h at 120 °C in small fused test tubes containing 100-300 µl 6 N hydrochloric acid (Technical HCl, 31-33%). After cooling, the sample was treated with the same amount of chloroform (100-300 µl) and vigorously shaken for 60 sec on a Vortex mixer. Layers were separated by centrifugation at 3000 r/m for 5 min. The organic phase was removed from the bottom of the tube by a Pasteur pipette, filtered and transferred into a conical 3-dram lip glass vial.

Quantitative Gas Chromatography. CAN-d_2, 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. Synthetic CAN-d_2 (Holz & al. 1994) served as standards for identification and calibration.

Quantification of Cantharidin and CAN-d_2. Owing to the small mass difference between cantharidin and CAN-d_2, the two compounds cannot be fully separated and quantified by gas chromatography. Reliable quantification of the overlapping peaks was achieved by "Multiple Reaction Monitoring" (MRM). MRM collects MS/MS data from different compounds in the same time window and is especially useful when coeluting compounds have different parent ions, e.g. a target compound and its coeluting isotopically-labeled standard. MS/MS provides a signal at *m/z* 82 for cantharidin and at *m/z* 84 for CAN-d_2 which could be linked via MRM with their parent ions. Both channels of MRM can be viewed and integrated independently. Analyses were performed until RT 21:20 in the normal EI-MS mode and then in the MRM mode (cantharidin and CAN-d_2 coeluted at RT 22:20). EI Ionization (70 eV) provides mass spectra with characteristic fragments for cantharidin at *m/z* 96 and *m/z* 128 (M^+: 197) and for CAN-d_2 at *m/z* 98 and *m/z* 130 (M^+: 199).

Scanning Electron Microscopy (SEM). The antennae of males and females of *Cyaneolytta* sp. 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. Random areas of the gold coated samples were examined with a Cambridge electron microscope. To determine pore density, cuticular pores were counted over an area of 24.5 μm^2. To understand the function of such pores, a semi-thin cross section was prepared from the male antennal segments and the stained tissues were studied using light microscopy at 400 fold magnification.

Preparation of Interior Segments of the Antennae. Fixation, dehydration and post fixation steps were carried on according to Adam & Czihak, 1964. Using a diamond knife on an ultramicrotome (Leica RM 2035), semi-thin cross sections of about 10 µm were taken from antennal samples embedded in a paraffin block. The cuttings were rinsed with distilled water at 35-38 °C for a few seconds and then placed on a pre-treated microscope slide (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 distilled water (10 min). Using kernechtrot-aluminiumsulfate and pikroindigocarmin, staining was performed according to Adam & Czihak, 1964. Final dehydration of the samples was achieved by passage through a graded isopropanol series (isopropanol 70 – 95% for a few seconds and isopropanol 100% for 10 min.) and then pure xylol for 20 min. Slides were kept at room temperature for a day and then covered by cover glasses (24×60 mm) using droplets of a rapid mounting media for microscopy, Entelan®.

Statistical Analyses. The data of the internal distribution of CAN-d_2 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 3 samples (95% confidence level) and for determining of the cuticular pore density 10 random locations of each sample 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, then student *t*-test was used to indicate the significance at $P = 0.001$ level, unless variable *P* was more than 1 and therefore analysed with 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).

Results

It is well known that cantharidin is present in different tissues and body fluids of blister beetles, but accumulates in the sexual organs of both sexes. In contrast, *Cyaneolytta* sp. showed an extremely high amount of cantharidin in the antennae of males which was comparable to titre of sexual organs. Analysis of the different body parts of *Cyaneolytta* sp. confirms the presence of extraordinary titres of CAN-d_2 in the male antenna after injection of CAN-d_2 into the male's body. By analysing the head capsule, it was found that most of the CAN-d_2 accumulated in the scape and the pedicel antennomeres. Examination of male antenna by SEM revealed a comparable pattern of cuticular pores over the surface of the scape and pedicel. There are a number of tubular structures extending from the surface of the integument to the hemolymph current of the scape and pedicel segments. Females however do not indicate any extremely high concentration of -the received- CAN-d_2 in the antennae. Similar to the males, the scape and pedicel had a higher density of cuticular pores than the flagellum; however, no conducting structures as found in the males were seen in the females.

Discussion

Based on the external and internal morphology of the antenna and their chemical analysis, the current work provides more evidence that cantharidin acts as an infochemical during courtship within the family Meloidae. We observed a correlation between the density of cuticular pores and the titre of CAN-d_2 of the scape and pedicel segments of male *Cyaneolytta* sp.. Although a high density of pores on the first two antennomeres is not very common, there are a number of confirmed cases among insects (WCISLO, 1998; ISIDORO & al. 2000). The abundant canal shape structures, which extend from the antennal hemolymph to the antennomere surface, are assumed to be involved in the transport of cantharidin from the hemolymph to the surface which will be ultimately released via the openings of the cuticular pores.

Studying the CAN-d_2 pretreated females of *Cyaneolytta* sp., the compound was not preferentially found in the first two antennal segments. Calculating cuticular pore density (the only visible feature on the female antennae), a non-significant higher concentration of pores was readily observed on scape and pedicel (ANOVA, Tukey *HSD* posthoc test, $P < 0.05$). Since tubular cells or other conducting structures such as tyloids were absent in cross sections of female antennomeres or on the integument surface, we assume that the function of the female pores, which are also located on the 1st and 2nd antennomeres, must be different from that of the males. In the tribe Meloini (Meloinae) males and females show antennation during the precopulatory phase of the courtship behaviour (BOLOGNA, 1991). If a similar behaviour is recorded here, it can be concluded that the cuticular pores located on the male's 1st and 2nd antennomeres come into direct contact with the female antennae and release cantharidin onto their surface. In such a case, the porous area of male antennae can be considered as a cantharidin releasing structure, while the female organ represents a multiporous chemical receptor, which recognizes the cantharidin titre of the sexual partner. It is probable that a natural system has developed in the females of *Cyaneolytta* sp., enables them to select the mate with high titres of cantharidin. Hence, comprehensive behavioural studies are required in order to closely monitor the courtship in *Cyaneolytta* sp. prior to any further hypothesizing.

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