

**Transfer and Distribution of Cantharidin
within Selected Members of Blister Beetles
(Coleoptera: Meloidae) and Its Probable
Importance in Sexual Behaviour**

**Dissertation
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1. Introduction

Insects with more than one million described species constitute 90% of known arthropods. They are among the oldest creatures on the Earth, which appeared first 350 million years ago in Devonian era (Borror et al., 1989). Order Coleoptera (beetles) with more than 300,000 species is the largest among insects. The first records of beetle presence on the earth return back to about 285 million years (Gullan and Cranston, 1994). Beetles have 500 families and subfamilies which are classified under the four following sub orders:

Archostemata, Myxophaga, Adephaga and Polyphaga (Arnett et al., 2002).

Beetles are among the insects of medical importance, mainly due to the broad spectrum of chemical substances within their hemolymph. The most medically important families within Coleoptera are Meloidae, Staphylinidae, Oedemeridae, Tenebrionidae and Dermestidae, however a very tiny fraction of other families may be considered as medically important insects (Alexander, 1984; Blum, 1981; Eisner and Meinwald, 1966; Harwood and James, 1979; Theodorides, 1950).

1.1 Family Meloidae

This family of beetles which is a major one in superfamily Tenebrionoidea are commonly called “Blister beetles”. Adults of these distinctive terrestrial phytophagous beetles can be recognized by characters such as soft body, bright colouration, rather elongate, head deflexed with narrow neck, pronotum not carinate at sides, heteromerous tarsi, smooth integument (Arnett et al., 2002; Bologna, 1991; Borror et al., 1989).

1.1.1 Family Description

Most members of the family elongate with long legs, mostly between 10-20 mm. Colour variable, from opaque to metallic, short and very sparse. Antennae usually with 11 segments, but 7-10 in tribes Mylabrini and Cerocomini, mostly filiform or moniliform, often modified in males (Arnett et al., 2002; Borror et al., 1989). Pronotum generally narrower than base of elytra, narrowest in apex at most. Tarsal formula 5-5-4. Elytra entire or short ended. Abdomen soft with 6 visible sterna. Male genitalia with aedeagus elongate, either with 1-2 distal dorsal hooks and one ventral endophallic hook or with hooks absent. Parameres fused at base only or entirely fused. Female genitalia short, lacking long membranous tube-like ovipositor.

Apart from subfamily Eliticinae, larvae are parasitoids of grasshoppers' eggs and eggs and larvae of wasps and bees and the development is hypermetamorphic with the various instars differing considerably in morphology and behaviour. The first instar larvae of blister beetles are heavily sclerotized, campodiform, prognathous, and highly mobile. They are often referred to as triungulins. This instar is followed by four grub-like, weakly sclerotized and hypognathous scarabaeiform instars referred to as feeding grubs. The feeding grub is followed by instar VI, the coarctate mostly a heavily sclerotized diapausing larva with aborted mouthparts and legs. Instar VII, or the second grub is morphologically similar to V, but does not feed. The second grub is followed by a typical exarate pupa (Arnett et al., 2002).

1.1.2 Status of Classification

The most recent family classifications of Meloidae were made by Bologna (1991) and Selander (1991). By placing the Tetraonycini in the Nemognathinae, Selander classified all meloids within three subfamilies, but Bologna's classification which has been followed here recognizes four subfamilies: Eleticinae, Meloinae, Tetraonycinae and Nemognathinae (Bologna, 1991). Allocation of tribes within subfamilies listed in table 1; however classification from tribe to lower taxa remains unsettled. Recent cladistic analyses support four subfamilies within family Meloidae (Arnett et al., 2002). All species which have been used throughout this study are allocated to subfamily Meloinae.

Table 1: Tribes within different subfamilies of Meloidae; (Bologna, 1991)

Eleticinae	Meloinae	Tetraonycinae	Nemognathinae
Morphozonitini	Lyttini	Tetraonycini	Horiini
Derideini	Pyrotini		Sitarini
Spasticini	Eupomphini		Nemognathini
Eleticini	Cerocomini		
	Epicautini		
	Mylabrini		
	Meloini		

1.2 Biology and Life Cycle in Sub family Meloinae

Meloid adults are grouped in two categories, those which fed on leaves and petals and the others which are mainly pollen feeders. Immature stages of leaf feeders are parasites of honey bees, wasps and attack the egg, larvae and the stored food within bee hives, whilst larvae of the other group are parasites of grasshoppers' eggs (fig. 2). Different species of *Melanoplus* (Insecta: Orthoptera) have been recorded as the preferred host of meloids, such as *M. differentialis*, *M. femurrubrum*, *M. atlantis*, *M. bivittatus* and *Oedipoda sulphurea* (Selander, 1986). A member of tribe Meloini (genus *Cyaneolytta*) which has been studied in the present survey belongs to the leaf feeding group whereas other studied species (members of tribe Mylabrini) are among the pollen eating group.

Eggs may be present in the field from shortly after the beginning of adult season until shortly after its end. Larvae feed during the first instar of the triungulin phase and the four or five following instars, in which the larva is grublike in appearance (first grub phase). On completing feeding, the first grub larva prepares a cell in the soil apart from the grasshopper egg pod on which it fed and typically becomes, at ecdysis, a heavily sclerotized, and immobile coarctate larva. Unless it is to take part in the production of a second generation during the same season, this larva enters diapause and passes the winter or other unfavourable period of the year in the coarctate phase. When near the beginning of the next season of adult activity, the larva undergoes ecdysis, it again becomes grub like in form (second grub phase). This phase, which entails only one instar is followed shortly by pupation and adult emergence (fig. 1).

New adults generally rest for three or four days following emergence. Feeding begins at the adult age of 4-6 days and sexual behaviour normally appears at a mean age of about 10 days. The minimum age of males within Epicautini, Lyttini and Mylabrini at first courtship is 6 days. Adults indicate a periodical activity in nature, presumably in response largely to varying conditions of heat and humidity. In hot arid areas, it has been found that adults tend to remain on or near the ground, under dense foliage for the greater part of the day (Adams and Selander, 1979).

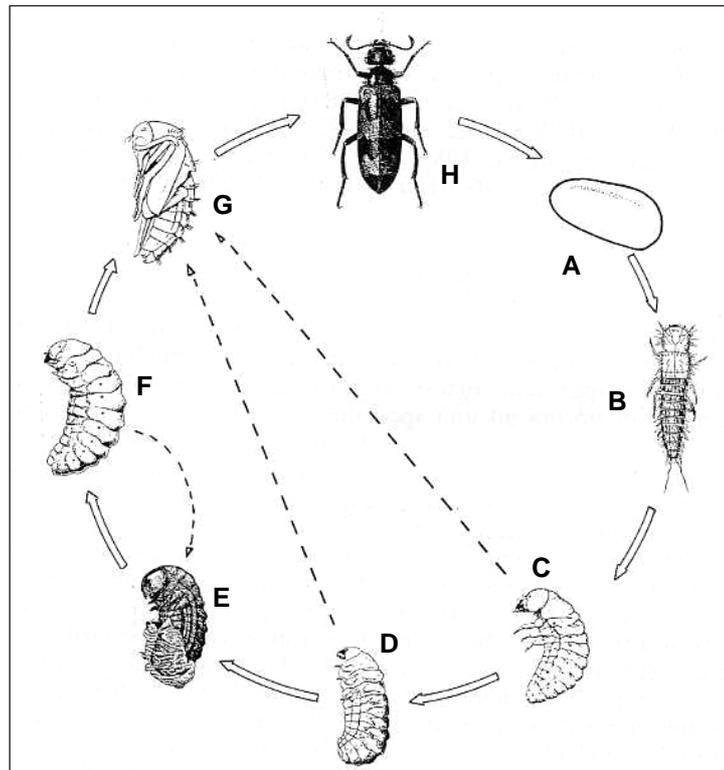


Fig. 1: Life cycle of *Mylabris variabilis* (Pallas), Meloinae (Bologna, 1991)

A: egg, B: triungulin, C: first grub 1 (FG1), D: FG2, E: coarctate larva (CL), F: 2nd grub (SG), G: pupa, H: adult beetle. Arrows indicate the normal life cycle but it can be shorter in some meloids, for example they can jump from FG1 and FG2 to pupa without passing SG and CL instars or can change from SG to CL in a reverse trend. These shifts from the normal life cycle have been shown by the lined arrows.

Sexual behaviour in Subfamily Meloinae is readily divided into *courtship* or the sexually oriented activities of both sexes occurring before the genitalia are coupled and *copulation* or behaviour during the period of genital coupling. Females become sexually receptive only periodically. Male courtship display involves touching or stroking of the female with the antennae, mouthparts and the abdomen, including the sclerotized portion of genitalia as well as the legs. Females tend to become sexually responsive soon after ovipositing. Courtship occurs during both the day and night. Females normally oviposit in burrows which they excavate with

the mandibles and legs and subsequently refill (Church, 1967; Selander, 1964; Pinto, 1972a, 1972b, 1974, 1975). Characteristically, the eggs produced at a given oviposition are deposited in a compact mass. Eggs are yellow and weakly adherent to each other. Burrows depth is about 1.5-2.5 cm inside the soil. Females oviposit repeatedly throughout the life (Church, 1967).

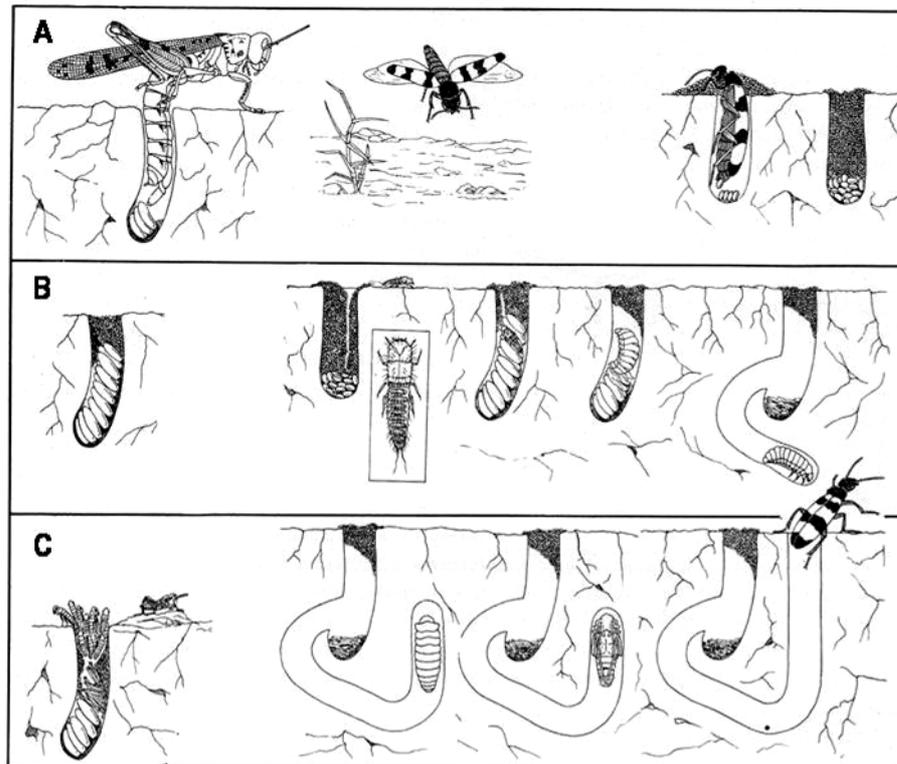


Fig. 2: Association of *Mylabris variabilis* to grasshopper in nature (Touring Club Italiano, 1959), **A:** Grasshoppers lay their egg pods in a cavity of about 5-8 cm depth (according to species) by their long ovipositors and cover it. Adult *Mylabris* lay their own eggs in small holes at the same environment. **B:** Triungulins leave the hole in search for grasshoppers' eggs to feed and will change to 1st grub (FG1) in grasshoppers' cavity or an extension to the main cavity. **C:** Intact eggs of grasshoppers will hatch to 1st nymphal instar and meanwhile FG1 moulted to other larval instars and eventually to pupa within the soil. Adult emerges from the underground cavity.

1.2.1 Habitats and Distribution

Adult meloids are phytophagous and feed on leaves and flowers of several families of plants, particularly Asteraceae, Leguminosae, Compositae, Umbeliferae and Solanaceae (Arnett et al., 2002). Larval hosts are provisions and immature stages of wild bees and in Epicautini and Mylabrini, grasshoppers' eggs. Larvae of most Meloinae directly attain their food source. Larval Nemognathinae, Tetraonycinae and some of the Meloinae, which are all bee parasitoids, reach their host nests by phoresy, attaching to the adult bees visiting flowers. Larvae of several species of *Cyaneolytta* are phoretic on carabid beetles (Di Giulio et al., 2003), although their actual hosts remain unknown (Adams and Selander, 1979).

This family is widespread throughout the world except for New Zealand and Antarctic (Arnett et al., 2002). Diversity is greatest in arid and semiarid regions. The family has currently 120 genera and 3000 species (Dettner, 1997). Distribution of Meloid genera over the different zoogeographical regions indicated in table 2 (Bologna, 1991).

Table 2: Distribution of blister beetles' genera over the main zoogeographical regions

Zoogeographical Regions	Number of Genera (G)	Number of Endemic Genera (EG)	EG/G %
Palaeartic	40	12	30
Mediterranean/Saharan	39	8	20.51
Nearctic	21	1	4.76
Afrotropical	40	15	37.50
Oriental	22	3	13.64
Australasian	5	2	40

1.3 Economic Importance of Blister beetles

Not all species of blister beetles are pests. Almost all economic pests within Family Meloidae are members of Vittata group (Meloinae: Epicautini) which are known as pests of garden and field crops, mostly in new world (Adams and Selander, 1979). Damage to potato, tomato and alfalfa is the most commonly mentioned in the literature, but soybeans, sugar beet, cotton and a variety of truck crops and garden vegetables are also attacked (Towsend, 2000; Sansone, 2002). All species of the Vittata group, found in USA (*Epicauta vittata*, *E. occidentalis* and *E. temexa*) are of economic importance (Adams and Selander, 1979). Blister beetles are seldom found dispersed generally through an area of food plant, but rather tend to aggregate which make them to be an agricultural pest. The mechanism by which the gregarious behaviour of blister beetles is brought about is unknown, although it is not improbable that either one or both of the sexes produces an attractant chemical substance (Selander, 1969).

1.4 An Overview to Insect Chemical Defence

Insects have a variety of ways to defend themselves. Many insects survive through natural products which are compounds that are often limited in their known distribution to a few species. The possessors of these highly adaptive discharges must often tolerate the presence of the toxic compounds in these exudates which often cover a large area of the producer's body. It seems that many insects have admirably exploited their potential as natural product chemists, to deter the ubiquitous and omnipresent predators with which they share their world. However the repellent secretions of arthropods generally originate in exocrine glands, other glandular and non glandular discharges have also frequently been adapted to serve the role of highly effective defensive exudates. Hemolymph itself is often discharged as a defensive vehicle and in some cases it contains highly toxic compounds which have been synthesized either *de novo* by the insect itself (Blum, 1981) or via bacterial symbiosis.

1.4.1 Reflex Bleeding

When agitated, adult meloids release droplets of hemolymph in so-called reflex bleeding. Discharge of blood, constitutes a defensive reaction which is highly effective in deterring aggressive organisms.

Over eighty years ago, it was clearly established that the reflex blood of beetles in several unrelated families as well as some species of grasshoppers is enriched with compounds which rendered it odoriferous and/or distasteful. They are gustatory repellents (Blum, 1981): predators rapidly learn to discriminate against these autohemorrhagic species as food items. Cantharidin which is found throughout the meloid body is considered responsible for the repellent properties of meloid blood against a wide variety of predators (Carrel and Eisner, 1974). Meloids reflex blood from the femorotibial joints and generally only discharge blood from the leg which is tactually stimulated (Alexander, 1984; Blum, 1981) but larvae exude a milky solution containing cantharidin out of their mouth (Alexander, 1984).

1.5 Medical Importance of Cantharidin

Cantharidin, which is mainly found in blister beetles, is among the most widely known insect natural products (Dettner, 1997; McCormick and Carrel, 1987). Its reputation derives principally from descriptions of its physiological activities, most notably as an aphrodisiac for humans and livestock that are traced from oral history. Its mode of action as an aphrodisiac is by inhibition of phosphodiesterase and protein phosphatase activity and stimulation of β -receptors which irritates the genital mucosa, therefore enhancing sensation (Sandroni, 2001). Cantharidin is also the blistering agent (fig. 3) that earned these beetles their common names (McCormick and Carrel, 1987). Eighteen species of meloids are reported as important blistering agents in the field (Alexander, 1984). Different species of *Mylabris*, *Lytta* and *Epicauta* are responsible for many cases of dermal blisters and dermatitis reported every year from India and south west USA (Alexander, 1984; Scott, 1962).

For more than 2000 years, blister beetles in powdered or tincture form have been used medicinally in Europe, China and elsewhere. In western cultures these materials are called Cantharides, derived from the Greek word for beetle (*Kantharos*) (Wang, 1989; McCormick

and Carrel, 1987). They are generally referred to as Spanish fly, the common name of the iridescent green meloid, *Lytta vesicatoria* from Europe (McCormick and Carrel, 1987).

The ancient Greeks and Romans consumed cantharides as a diuretic and abortifacient as well as an aphrodisiac. The use of the cantharides grew in western societies until the nineteenth century, however in the course of the development of modern medicine, investigations produced evidence that cantharides have little therapeutic or aphrodisiac affect except for removal of certain warts (Moed et al., 2001). A recent medical movement in the USA, along with some pieces of new research may change the position in near future to add cantharidin once more in the FDA conventional list of pharmaceuticals (Moed et al., 2001). In China and South Korea, cantharidin has been commercially formulated along with laboratory evaluation and clinical trials to be prescribed as anti tumour and anti cancer agent in humans (Wang, 1989; Pemberton, 1999; Huh et al., 2004).



Fig. 3: The blistering symptom of *Epicauta* sp.

1.6 Cantharidin: Properties and Natural Source

Cantharidin ($C_{10}H_{12}O_4$) with molecular weight of 196.2 is a monoterpene anhydride with the following chemical formula: 2-endo,3-endo- dimethyl-7-oxabicyclo(2.2.1) heptane- 2-exo,3-exo- dicarboxylic anhydride.

It is highly toxic to most animals (LD_{50} for human 10-60 mg/kg and intraperitoneal mouse LD_{50} 1.0 mg/kg) (Dettner, 1997). Cantharidin is an effective feeding deterrent at concentrations as low as 10^{-5} M, that is 100 times lower than physiological cantharidin concentrations in blister beetles (Carrel and Eisner, 1974). Cantharidin has been shown to be toxic for mammals, birds and frogs (Schmidt, 2002) and thus has great advantage not only for nonmobile stages, eggs and pupae but also for larvae which are usually devoid of defensive glands. It also bears potent antifungal activities against *Trichophyton* and *Microsporium* species and may therefore protect developing embryos and all immature stages from entomopathogenic fungi (Carrel and Eisner, 1974; Dettner, 1997). Among vertebrates, numerous records exist of species which take up cantharidin containing prey and are not injured (Eisner et al., 1990). Among mammals, only primitive insectivorous species such as hedgehogs and to some extent bats, armadillos and rabbits may tolerate cantharidin (Kelling et al., 1990; Dettner, 1997).

In animal kingdom, cantharidin is only known to be produced by blister beetles (Col: Meloidae) and the smaller oedemerid beetles (Col: Oedemeridae) where it 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). Cantharidin has not been discovered in plants; however, insecticidal seeds of the Indian tree, *Butea frondosa* (Leguminosea) contain demethyl cantharidin (Palasonin), where the 3-methyl group of cantharidin is missing (fig. 4) (Bochis, 1960; Raj and Kurup, 1966; Dettner, 1997). Up to now, palasonin has been also recorded from four species of meloid beetles, *Hycleus lunatus* (Dettner et al., 2003) and *Hycleus polymorphus*, *Mylabris quadripunctata* and *Cyaneolytta* sp. (Nikbakhtzadeh, unpublished data) however unlike the plant source; the former species produces palasonin of low ee with (R)-(+)-enantiomer prevailing (Fietz et al., 2002).

It is well established that in many species of blister beetles, females possess but can not produce cantharidin. During copulation, males transfer large pockets of cantharidin along with sperm to the female. That is a form of nuptial gift which provide the beetle with chemical protection by passing on cantharidin to the eggs (Schmidt, 2002). The possibility that cantharidin is transferred from male into female during mating was suggested long ago (Beauregard, 1890) and more recently confirmed (Sierra et al., 1976; McCormick and Carrel, 1987; Holz et al., 1994).

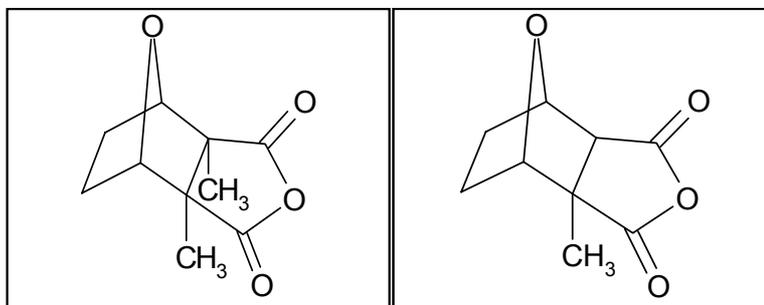


Fig. 4: Chemical structure of cantharidin (left) and palasonin (right)

1.6.1 Cantharidin Mode of Action

The reversible phosphorylation of protein is a major mechanism in the regulation of cellular process. The phosphorylation or dephosphorylation of amino acids is indicative of the balance between the activities of protein kinases and protein phosphatases (PPs). In mammalian tissue, at least 4 types of PPs have been identified: PP₁, PP_{2A}, PP_{2B} and PP_{2C} (Cohen, 1989; Knapp et al., 1998). Cantharidin and also palasonin inhibit the activity of both PP₁ and PP_{2A} (Liu et al., 1995; Knapp et al., 1998; McCluskey et al., 2001; McCluskey et al., 2002).

Cantharidin and its hydrolysis product, cantharidic acid, are structurally similar to highly toxic commercial herbicides, e.g. endothall and endothall thioanhydride (fig. 5) (Knapp et al., 1998).

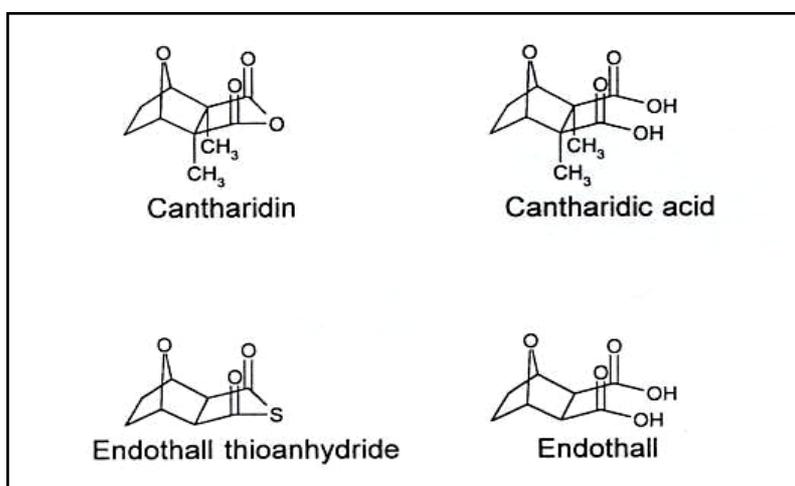


Fig. 5: Chemical structure of cantharidin, cantharidic acid and some commercial herbicides

1.7 Goals of the Project

There are some publications concerning cantharidin transfer and distribution within canthariphilous taxa; however no report has been published on the distribution and pharmacodynamics of cantharidin in the natural producers, e.g. blister beetles. Therefore the current project was defined in order to clarify the following points:

1. Cantharidin distribution in male's and female's sexual organs.
2. Pharmacodynamics of cantharidin in the genital organs of the both sexes based on a time sequence method.
3. If any labelled analogue of cantharidin, particularly labelled palasonin is found in the beetles's tissue following introduction of deuterated cantharidin.
4. The relationship between cantharidin and cuticular pores in *Cyaneolytta* sp.
5. If cantharidin is used by *Cyaneolytta* sp. as a precopulatory pheromone

2. Materials and Methods

2.1 Beetle Collection

Meloid beetles cannot be collected in Bayreuth or in other parts of Upper Franconia (Bavaria, Germany) (Frenzel et al., 1992). There are some meloid species reported from Germany (table 3), but they have often low abundance and been registered under the endangered list which makes the field collection difficult.

Table 3: Meloid species of Germany (Koch, 1989)

No.	Species	Location
1.	<i>Mylabris polymorpha</i> (Pall.)	Bavaria
2.	<i>Mylabris crocata</i> (Pall.)	Hanover
3.	<i>Lytta vesicatoria</i> (L.)	Germany except for Northwest
4.	<i>Cerocoma schaefferi</i> (L.)	Germany except for Northwest
5.	<i>Meloe proscarabaeus</i> (L.)	Germany*
6.	<i>Meloe violaceus</i> Marsh	Rhineland
7.	<i>Meloe cicatricosus</i> Leach	Hessen, Baden
8.	<i>Meloe coriarius</i> Br. Er.	Hessen, Baden
9.	<i>Meloe decorus</i> Br. Er.	Rhineland, Baden
10.	<i>Apalus bimaculatus</i> (L.)	Baden
11.	<i>Sitaris muralis</i> (Forst.)	Thüringen, Baden, Hessen, Rhineland

* This species is found throughout the country

That is why the required beetles had to be collected from other geographical locations which are listed in table 4. Accordingly, European species collected in Southern France (fig. 6). South African species collected by Prof. G. Gaede at the campus of University of Cape Town, Cape Town, South Africa. *Cyaneolytta* was collected by Dr. C. Hemp from Croton-Calodendrum forest, Karen, Nairobi, Kenya while the beetles were sitting on the shrubs of *Solanum aculeatissimum* (Solanaceae). All other species were collected by inspection while sitting on flowers or stem of different shrubs of family Astraceae, Compositeae and Leguminoseae.

Table 4: Collecting locations of blister beetles for the present study (2001-2004)

Species*	Country/ Region	Date of Collection	Collecting Site	Geographical Location	Remarks
MQ	Southern France	July 2002	Camping site, Mas de la Cam	St. Jean du Gard Departement Gard	Approx. 200-250 m
HP	Southern France	July 2002	Can de l'Hospitalet	Departement Lozère	Approx. 1050 m
HP	Southern France	June 2003	Gardon d'Anduze	Departement Gard	N 44° 1' E 4° 3'
HP	Southern France	June 2003	Can de l'Hospitalet	Departement Lozère	Approx. 1050 m
MQ	Southern France	June 2003	Macchie, Southwest of Mt. Bouquet	South East Alès, Departement Gard	Approx. 250 m
HL	South Africa	Feb. 2002	Cape Town	Campus of Cape Town University	1 st package
HL	South Africa	Jan. 2003	Cape Town	slope of Table Mountain	S 33° 56' E 18° 21'
CY	Kenya	May 2002	Croton-Calodendrum forest	Karen, Nairobi	S 1° 19' E 36° 42'

- MQ: *Mylabris quadripunctata* (Linné, 1767)
- HP: *Hycleus polymorphus* (Pallas, 1771)
- HL: *Hycleus lunatus* (Pallas, 1782)
- CY: *Cyaneolytta* Péringuey, 1909. The type species is *Lytta signifrons*



Fig. 6: *Hycleus polymorphus* (left) and *Mylabris quadripunctata* (right) Southern France, 2002

2.2 Transportation of Insect Material

Those beetles which were on the plants or fallen on the ground were picked up and placed in small net ported plastic boxes with the dimensions 18×13×6 cm, net ported and covered bottom by a layer of kitchen paper. Not more than 10 beetles should be kept in such a box; otherwise mortality rate will be increased. Water sprayed over the covering layer and flowers of the habitat along with some vegetation provided for the beetles as natural food resource. Besides, small plastic Petri dishes (30 mm diameter) of artificial diet were supplied as a complementary food source (fig.7).



Fig. 7: Plastic box for transportation of field collected beetles, contained a few flowers on which the adults were feeding and small Petri dishes of artificial diet

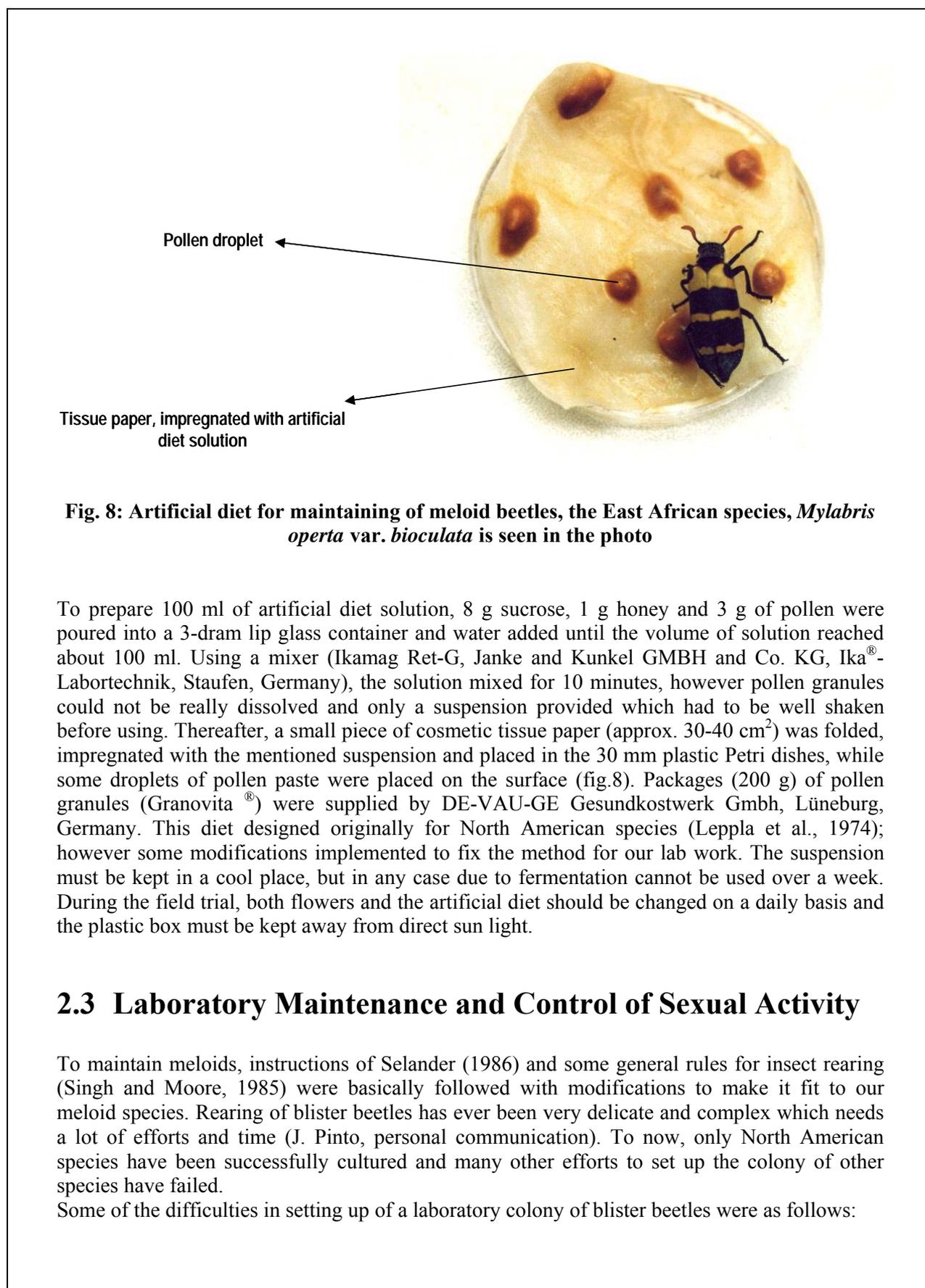


Fig. 8: Artificial diet for maintaining of meloid beetles, the East African species, *Mylabris operta* var. *bioculata* is seen in the photo

To prepare 100 ml of artificial diet solution, 8 g sucrose, 1 g honey and 3 g of pollen were poured into a 3-dram lip glass container and water added until the volume of solution reached about 100 ml. Using a mixer (Ikamag Ret-G, Janke and Kunkel GMBH and Co. KG, Ika[®]-Labortechnik, Staufen, Germany), the solution mixed for 10 minutes, however pollen granules could not be really dissolved and only a suspension provided which had to be well shaken before using. Thereafter, a small piece of cosmetic tissue paper (approx. 30-40 cm²) was folded, impregnated with the mentioned suspension and placed in the 30 mm plastic Petri dishes, while some droplets of pollen paste were placed on the surface (fig.8). Packages (200 g) of pollen granules (Granovita[®]) were supplied by DE-VAU-GE Gesundheitswerk GmbH, Lüneburg, Germany. This diet designed originally for North American species (Leppla et al., 1974); however some modifications implemented to fix the method for our lab work. The suspension must be kept in a cool place, but in any case due to fermentation cannot be used over a week. During the field trial, both flowers and the artificial diet should be changed on a daily basis and the plastic box must be kept away from direct sun light.

2.3 Laboratory Maintenance and Control of Sexual Activity

To maintain meloids, instructions of Selander (1986) and some general rules for insect rearing (Singh and Moore, 1985) were basically followed with modifications to make it fit to our meloid species. Rearing of blister beetles has ever been very delicate and complex which needs a lot of efforts and time (J. Pinto, personal communication). To now, only North American species have been successfully cultured and many other efforts to set up the colony of other species have failed.

Some of the difficulties in setting up of a laboratory colony of blister beetles were as follows:

1. They are univoltine and thus bear not more than a generation per year.
2. Meloids have hypermetamorphosis, so that each larval stage requires specific conditions.
3. Their larval stages are mostly parasites of grasshoppers' eggs or bee and wasps' eggs and their provisions.
4. Eggs are laid beneath soil surface. Similarly prepupae and pupa also live in soil.
5. There are several steps of quiescence and diapause in larval and pupal stage.
6. They are very sensitive to food diet and environmental conditions.
7. Adults have usually host plant specificity which is hard to supply in captivity.
8. Mass rearing of blister beetle is not possible because of larval cannibalism.

2.3.1 Maintenance Conditions of Blister Beetles

The maintenance procedure is complicated and needs a great deal of work and patience. It is mainly suitable for *Mylabris quadripunctata*, *Hycleus polymorphus* and *Hycleus lunatus*, whereas the other species like *Cyaneolytta* sp. need additional requirements or some modifications to the instruction. In order to identify the collected blister beetles of Southern France, Bologna's keys were used (Bologna, 1991). The two other species from South Africa and Kenya had been already identified by Prof. G. Gaede and Dr. C. Hemp respectively.

Adults were transported from the field and kept in a screened cage or in a plastic box provided with a screened port with dimension of 20×30×40 cm. As flooring, kitchen paper was used. Females may oviposit on the floor or egg masses may be laid on plant material. The flooring material changed daily in order to lessen the probability that eggs would be contaminated with the faeces on the floor.

In order to control the courtship and copulating, the sexes were separated immediately and 16-20 adults of the same sex were kept in each cage, since excessive crowding may decrease longevity due to hyperactivity and injuries resulting from fights. On the other hand it may reduce the frequency of oviposition or inhibit it completely. Cages were placed in climate chambers with fully environmental control. A constant temperature of 27-28° C, relative humidity (RH) of 40-45% and a daily photoperiod similar to the natural habitat (14 L: 10 D) were applied. Meloids are capable of obtaining all the water they need from their food (Selander, 1986). Therefore, fresh food was given in abundance, to avoid any separate source of water.

Depending on the species, meloid adults eat pollen, leaves or the floral parts. As the fresh food diet for leaf and flower eaters (*Cyaneolytta*), potato and tomato shrubs were provided on a daily basis, while pollen eaters prefer plants of Compositae or Leguminosae. The artificial diet which was mentioned before proved to be acceptable for adults of different species. The flowers and other plant materials were offered in bouquet, with the stem in sucrose-water solution 2% to keep them fresh for a longer time at high temperature. All adults were counted exactly and the cages dated not to miss any individual.

Adult females lived for 2-3 months and produced egg masses periodically (intervals of 1-2 weeks). The number of eggs per mass varied from 30-40 according to body size. Eggs of various species were also different in size and colour, for example eggs of *Mylabris quadripunctata* were bigger and yellowish (fig. 9A), while those of *Cyaneolytta* were smaller and more whitish. Egg masses were yellow, cream or white in colour, relatively large (1.5-2 mm long) and were easily recognized without magnification. Females laid eggs on or beneath the paper covering the floor or on plant materials.

Eggs were lifted carefully by a very fine painting brush (Pelikan, No.2) and dropped individually into clean glass vials of 8 mm internal diameter (ID)×50 mm. If it was a danger for

eggs to be handled, the substrate was cut out and transferred with the egg mass to a vial. All vials were marked from outside with a label including the name of species, date of oviposition and a serial number. All glassware were well washed and then disinfected by 10 % Extran MA 04 (Merck KGaA, Darmstadt, Germany). Using a vacuum desiccator jar (21 cm ID), eggs were incubated at 28° C and 100% RH in darkness. At such a temperature, eggs usually hatched within 2-3 weeks. The heavy glass lid of the jar fitted, but an opening of about 10 mm in diameter left at the top of the lid. Unfertilized eggs soon became cloudy and the damaged eggs developed fungal growth. These were removed as soon as possible.

Newly emerged triungulin larvae are strongly gregarious and tend to be quiescent (fig. 9B). The same environmental conditions as eggs applied for triungulins. Triungulins were fed with three different diets: fresh eggs of *Schistocerca gregaria*, the pollen paste and finally fresh eggs of blister beetles. Grasshoppers' eggs were freed from the pods and offered in loose form. Meloid eggs were offered only if the larvae were not attracted by the two mentioned alternatives.



Fig. 9: A. Egg Batches of *Mylabris quadripunctata* (2 X), B. Triungulin of *M. quadripunctata* (28 X)

2.3.2 Establishing of Grasshopper Colony

In order to provide triungulins of meloids with the fresh eggs of grasshoppers, a large colony of *Schistocerca gregaria* was cultured, at first 40 specimens (sex ratio 1:1) received from BTBE Insektenzucht GmbH, Schützenstraße 3, 89194 Schnüpfingen, Germany (www.insektzucht.de). A rearing cage measuring 100×50×50 cm made from wood, equipped with two screened port was used. In order to minimize mortality, not more than 50 individuals were kept per cage. The cages were placed in the same climate chamber as blister beetles, so the same environmental factors implemented, except for the light intensity which enhanced by adding an extra 60 W lamp. This source of light installed nearby the cage to provide them with both light and enough heating. On the other hand, some little shady areas obtained where the insects could mate in privacy (fig 10A). Eggs were kept at the same temperature as adults, on a moist sand bed, contains 15-20% water.

To feed grasshoppers, fresh leaves of maize (*Zea mays*) and Flamenettle (*Coleus blumei*) were offered (fig. 10B); however they are polyphagous and can usually feed on many different grasses. A dry food diet such as wheat germ mixed with the vitamin was helpful to provide them with a source of protein. In this way, prepacked wheat germ which could be easily purchased from the market used as dry diet (Weizenkeime, Schapfenmühle GmbH and Co. KG, Ulm, Germany).

Females of genus *Schistocerca* are larger in size and can easily be distinguished from the male by checking the sternal tip of abdomen. Their females have a relatively large flat area which is considerably smaller in males. Females laid egg batches inside a sand surface in a depth of about 7-10 cm. Eggs were stored in a place with a constant temperature of about 27-30 °C with enough relative humidity because they were sensitive to dryness. Eggs have the size of rice grains and are yellowish in colour (fig. 10C). A frothy secretion over the eggs hardens to form an egg pod. Every female lays 5-7 egg pods with an interval of about a week between each pod, every pod comprises of 40-80 eggs. In such a condition, *Schistocerca gregaria* needs a developmental duration of approximately 2 months from egg to the adult stage.

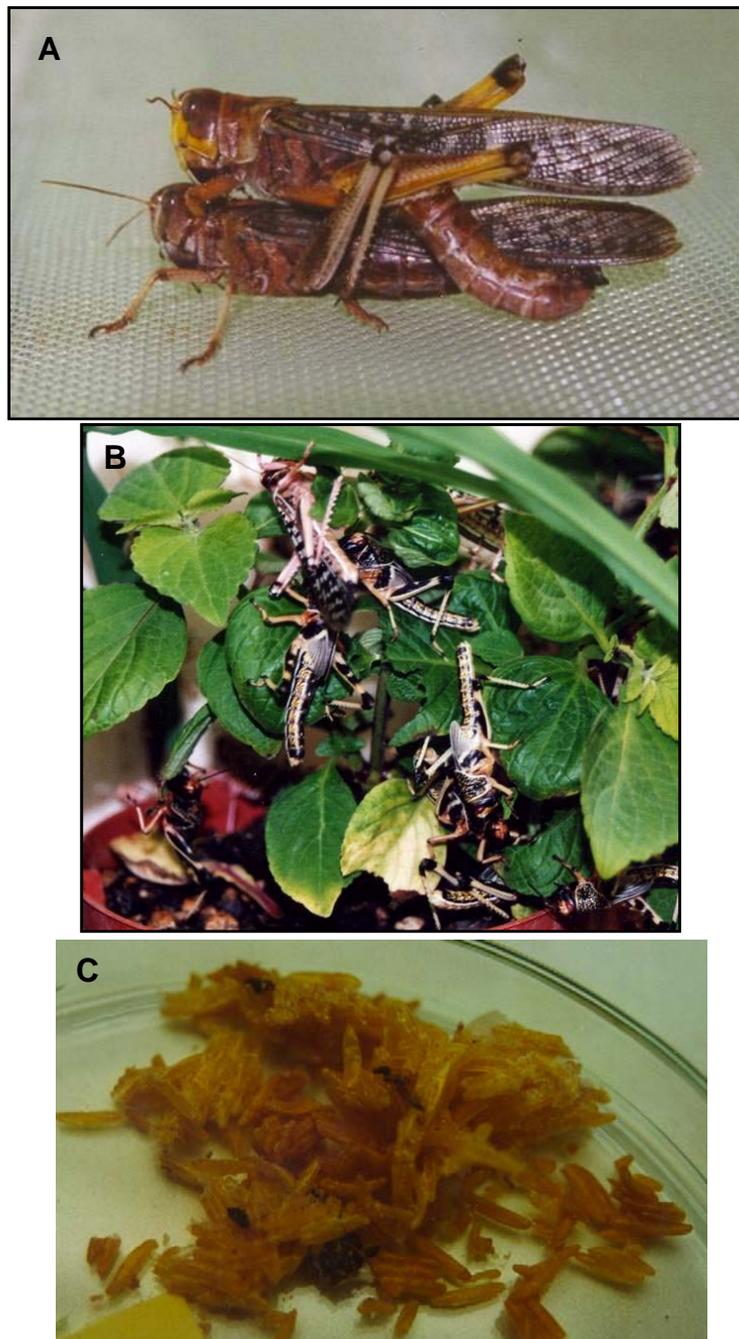


Fig. 10: A. Adults of *Schistocerca gregaria* in copulation, B. Fifth nymphal stage of *Schistocerca gregaria*, C. Eggs of *Schistocerca gregaria*, released from the pod

2.4 Sex Differentiation in Meloid Beetles

There are three different methods of sex differentiation in blister beetle. Among them the third one because of its accuracy is preferred and was used in this study:

- By relative size of females which are larger than males
- By the Y shape incision on the last visible abdominal sternite of males which does not exist in females (M. Bologna, personal communication).
- By exact observing of external genitalia, females bear a pair of valvifers (fig. 11A) while males indicate pairs of aedeagus/parameres (fig.11B).

Prepared samples were placed on a microscope slide (Super Frost[®], 76×26 mm, Menzel Gläser, Braunschweig, Germany), treated with droplets of polyvinyl lactophenol (Schmid GmbH and Co., Köngen am Neckar, Germany) and covered by a glass cover (18×18 mm, Menzel Gläser, Braunschweig, Germany).

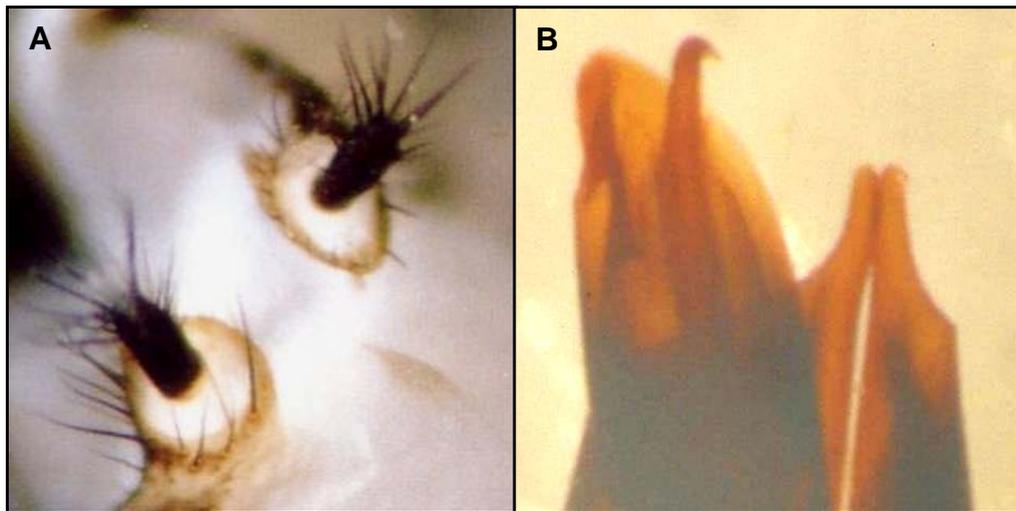


Fig. 11: A. A pair of valvifers of *Mylabris operta* var. *bioculata* which are part of female external genitalia, B. Pairs of aedeagus and parameres in male external genitalia of *Hycleus lunatus*

2.5 Introducing of Marker, Deuterated Cantharidin (D₂C), into Beetle's Body

Since it was not possible to rear meloids in the laboratory, we could not go further in our research just based on the cantharidin measurement. Cantharidin titre in meloids depends on several factors such as species, age, feeding condition, sex, mating records and so on. So, all these matters must be taken into account to prevent any mistake in data interpretation. In order to avoid such complexities and make the measurement more reliable, deuterated cantharidin (fig. 13D) in which two hydrogen atoms had been replaced by two deuteriums (D₂C) was introduced into individuals by different methods. D₂C had been synthesized in the lab of Professor W. Boland (Max Planck Institute for Chemical Ecology, Jena, Germany) and kindly supplied to us by him. Due to an absolute lack of this synthetic compound in nature, such a marker is highly identical (W. Boland, personal communication). On the other hand, it produces characteristic base peaks in mass spectrometry and hence can be easily traced and differentiated from the natural cantharidin of beetles or any other chemical. Another advantage of D₂C is the fact that it has the greatest similarity to cantharidin, so it is expected to behave *in vivo* as the beetle made compound.

In general, the following methods used for D₂C introduction:

- Injection of D₂C dissolved in dimethylsulfoxide (DMSO) into beetles' abdomen from intersternite membranous tissue by a microsyringe (fig. 12). The solvent for D₂C was first suggested by Holz (1992) and since then has been ever indicated as the solvent of choice (Huh et al., 2004).
- Feeding of target beetles by D₂C-Chloroform based solution which was mixed with modified artificial diet (fig. 13).

The physical condition of such a diet is to some extent different from the one used for maintenance of blister beetles in the lab. Since D₂C quantification is of great importance in this study, we had to develop another method for mixing of artificial diet with the marker. First, 10 pollen granules were mixed with water droplets to form a pollen paste. The paste was added to 2 g of honey, 1 g of sucrose and 6 µl of D₂C chloroform based solution (conc. 2000 ng/µl) in a Petri dish and mixed well. The mixture divided into 5 equal parts, each formed a droplet of diet containing 2000 ng D₂C. The diet was exposed to the beetles after the solvent had evaporated and the beetles were dissected three days after the exposure. In this way, we were able to measure both the up-taken marker by beetles and the remainder to have a better analysis.

- The Ringer's solution experiment (fig. 14)

At first, sexual organs of males and females were carefully dissected under the stereomicroscope to be placed into a glass vial bearing a mixture of Ringer solution, D₂C-DMSO and inert sand which was shaken at 191 r/min. Every two hours samples of different organs were taken until 6 hours. Liquid samples from the solution were also taken by a microsyringe simultaneously. In order to control the interaction of different parts of sexual organ on each other and also marker incorporation to these parts in a more realistic approach, the whole abdomen of both sexes was also placed into the solution and liquid sampling taken every two hours. To prepare D₂C solution, 1.2 mg of D₂C dissolved in 750 µl of DMSO which makes a solution of 1600 ng D₂C per 1 µl of DMSO.

These time dependent series of experiments were based on the fact that different organs of a beetle are physiologically active, even after dissection, if they are provided with a medium which is chemically similar to hemolymph to let the cells indicate their normal function in short

time interval; that is why Ringer solution was chosen (table 5). Important chemicals and instruments used for marker introduction have been shown in table 6 and 7 respectively.

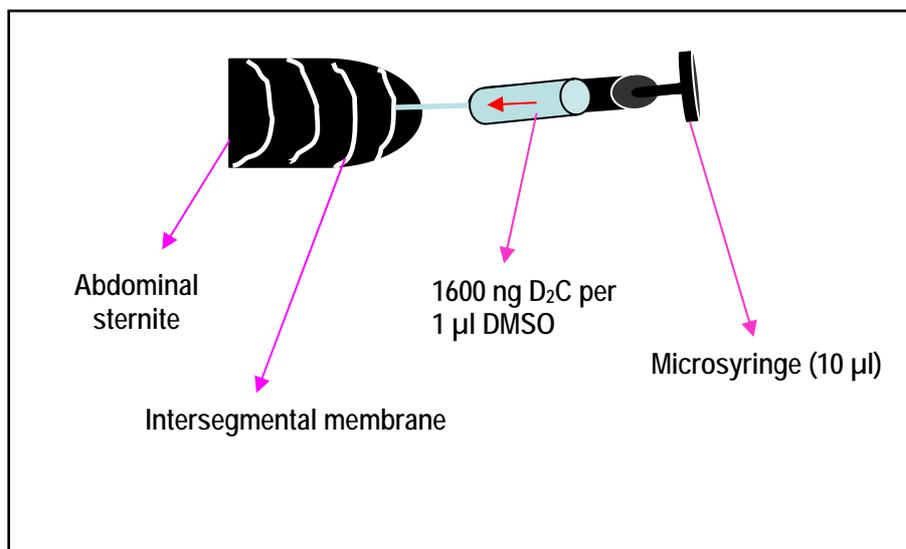


Fig. 12: Schematic view of the experiment in which deuterated cantharidin dissolved in DMSO was injected into the beetle's hemocoel via a microsyringe

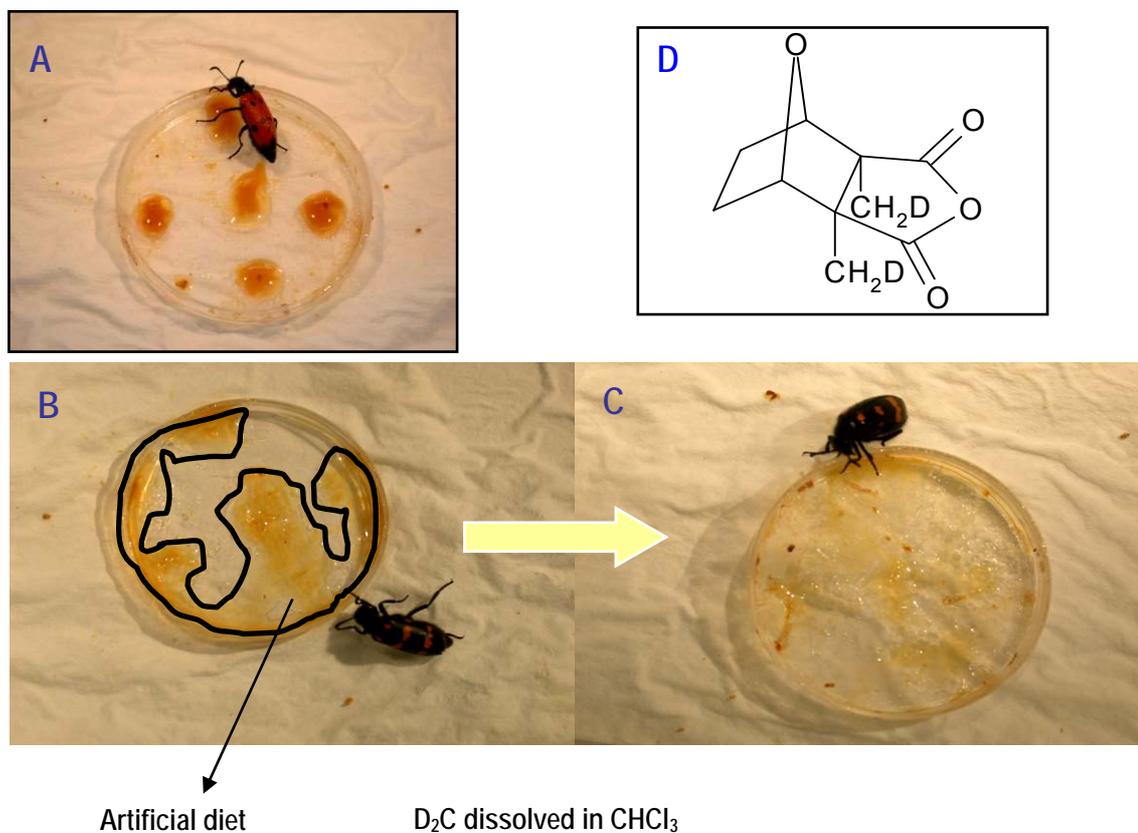


Fig. 13: Feeding experiment with D_2C , A: Droplets of artificial diet mixed with D_2C before thawing, B: *H. polymorphus* during feeding, C: Petri dish of artificial diet after experiment duration, D: Structural formula of D_2C ($C_{10}H_{10}D_2O_4$)

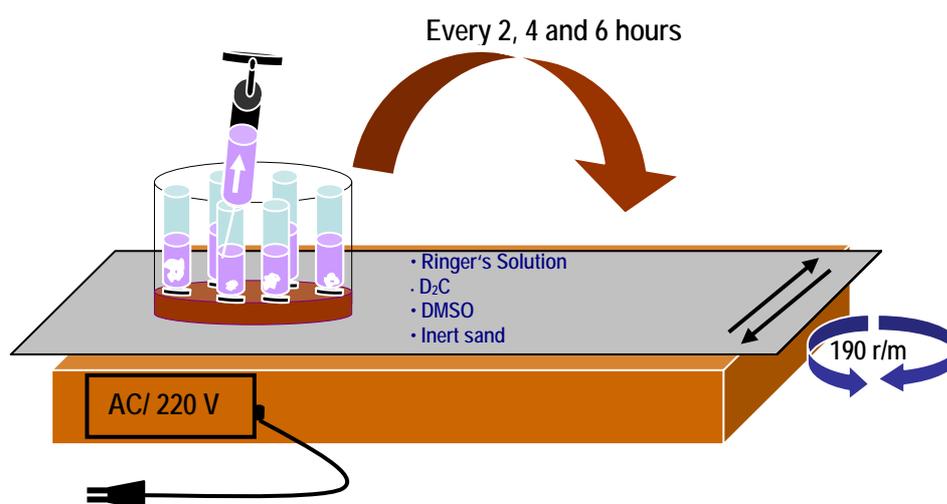


Fig. 14: Schematic view of Ringer experiment in which beetles' organs (white masses) were placed into a solution of Ringer, DMSO and D_2C (solution is shown in violet)

Table 5: Required components to prepare 200 ml of Ringer solution

No.	Substance	Volume (ml)	Concentration(g/l)
1.	NaCl	19.8	79.8
2.	KCl	2	37.5
3.	MgCl ₂	2	20
4.	Na ₂ HPO ₄	2	18
5.	NaHCO ₃	5	50
6.	Distilled Water	167	-
7.	D-glucose	0.4 g	-
8.	CaCl ₂	2	30

Table 6: Important chemicals used for introducing of marker into beetles' body

Chemical	Remarks
DMSO	Dimethylsulphoxide, for synthesis, Purity: 99%, Merck, Hohenbrunn, Germany
CHCl ₃	Suprasolv, for organic trace analysis, Merck, Darmstadt, Germany
NaCl	p.a., Purity: 99.5%, Merck, Darmstadt, Germany
KCl	p.a., Purity: 99.5%, Merck, Darmstadt, Germany
MgCl ₂	p.a., MgCl ₂ and 6 H ₂ O, Purity of complex matrix: 99- 102%, Merck, Darmstadt, Germany
Na ₂ HPO ₄	Diabasic Anhydrous, Min purity: 99%, Sigma Chemical Co., St. Louis, MO, USA
NaHCO ₃	p.a., Purity: 99.5%, Merck, Darmstadt, Germany
D-glucose	D(+)- Glucose monohydrate, C ₆ H ₁₂ O ₆ and H ₂ O, Merck, Darmstadt, Germany
CaCl ₂	Calcium chloride Dehydrated, Granulated, Purity (KT): 97%, Fluka Chemie AG, Neu-Ulm, Germany
Inert sand	0.1- 0.5 mm particle size, BayWa AG, Bindlach, Germany

Table 7: Important instruments used for introducing of marker into beetles' body

Instrument	Remarks
Shaker	Infors TR- 125, INFORS AG, CH- 4130, Bottmingen
Microsyringe	1 µl, SGE, Australia
Microsyringe	10 µl, Hamilton, Reno, Nevada, P.O.Box 10030, USA

2.6 Chemical Analyses

The important task after sampling has been chemical analysis which means searching for cantharidin, D₂C and cantharidin metabolites in different organs of meloid species. Besides, we had to measure the exact amount of these compounds. Since quantitation has had an important role in this study and because of the method which has been partly developed during the current project, methodology has been described in details.

2.6.1 Sample Preparation and Extraction

Tissue samples were put into test tubes and their dry weight (DW) determined after 36 hours of freeze drying (-50 °C, 9×10^{-2} mbar) by a LYOVAC GT2-E freeze-dryer (AMSCO/ FINN-AQUA Co. Ltd.). Cantharidin which is present in insects found in two different forms, free compound and the bound one. Free compound can be directly measured by dissolving into solvent, while the bound part needs to be first freed from the conjunctive tissue and thereafter is detectable. So, all body fragments as well as faeces were hydrolyzed in small fused test tubes using 100- 300 µl 6 N Hydrochloric Acid (Technical HCl, 31-33%, AUG. Headinger, Stuttgart, Germany) at 120 °C for 4 hours in order to remove biomatrix and to set the bound cantharidin free. Following a short period of cooling down, an equivalent amount of chloroform (100- 300 µl) was added and each sample was vigorously shaken on a Vortex mixer for 60 seconds. Afterwards, samples were centrifuged (Medifuge centrifuge, Heraeus Sepatech GmbH, Osterode, Germany) at 3000 r/m for 5 minutes. Using Pasteur pipette, the organic phase (chloroform-based compounds) of each tube which stands at the bottom, filtered and transferred into a conical 3-dram lip glass vial. To reduce evaporation of chloroform, a few droplets of deionised water were added into each vial (Holz at al., 1994), samples labelled and kept in the freezer until the injection time.

Similar to tissue samples, liquid ones of Ringer experiments were first dried by freeze drying to get rid of water and later on treated with 150 µl of chloroform. The solution was shaken for 60 sec. on a Vortex mixer, labelled and transferred to the freezer for further studies.

According to Seidl, 1999; Palasonin (3-demethylcantharidin) sticks to the inner surface of glass vials, so all glassware were first silanized by Dimethyldichlorosilane solution I in Heptane 5% (C₂H₆Cl₂Si, Fluka Chemie AG, Neu Ulm, Germany).

2.6.2 Quantitative Gas Chromatography

Relatively high volatility and good thermal stability are those characters of cantharidin which makes GC analysis the method of choice. Packed column GC analyses have been performed effectively on not only commercial cantharidin and D₂C but on that derived from biological samples too. Fortunately, capillary GC sensitivities are very good and the typical high resolution achieved with capillary GC permits analyses of substances from biomatrices with minimal sample preparation. Characterization of cantharidin by Electron Impact Ionization (EI) provides valuable information (McCormick and Carrel, 1987).

In order to detect and quantify total cantharidin, D₂C, palasonin or any probable labelled analogue of cantharidin, as indicated below, GC-MS in three different models was used and for the first two machines, 0.1- 0.5 µl of each sample injected (split/splitlessly) by a 1 µl SGE microsyringe into the injector.

1. GC 6000, Vega Series 2, Carlo Erba gas chromatograph equipped with a HT8 (8% phenyl polysiloxane-carborane, non-polar) bounded phase fused silica capillary column (SGE: FT 0.25 μm , ID 0.32 mm, OD 0.43 mm, Length: 25 m) was used which in turn connected to a Finnigan MAT Ion Trap Detector (ITD). Constant Helium pressure was 53 mbar and injector temperature defined as 230 °C.

Electron Impact Ionization (EI 70 eV) provides mass spectra with a characteristic fragmentation of cantharidin: the base peak with m/z 96 and two other fragments of m/z 128 and 67 (M^+ : 197). D_2C provides mass spectrum with m/z 98, 130, 69 (M^+ : 199). Base peaks of palasonin are m/z 114 and m/z 82 (M^+ : 183). EI mass spectra of these three compounds have been indicated in fig. 16, 17 and 18.

Oven temperature programme has been indicated in table 8. Under such a programme and the detector delay time of 10.00 minutes, retention time (rt) for cantharidin, D_2C and palasonin will be 15:53, 15:52 and 15:01 minutes in Ion Trap (IT) data system and 15:62, 15:57 and 14:72 based on Xcalibur software respectively. Total mass spectra analysed by either IT Data System, (version 4.00) or Xcalibur software (Xcalibur TM, Home Page version 1.2, Finnigan Corp. 1998-2000) and base peaks were compared by NBS registry of mass spectral data bank.

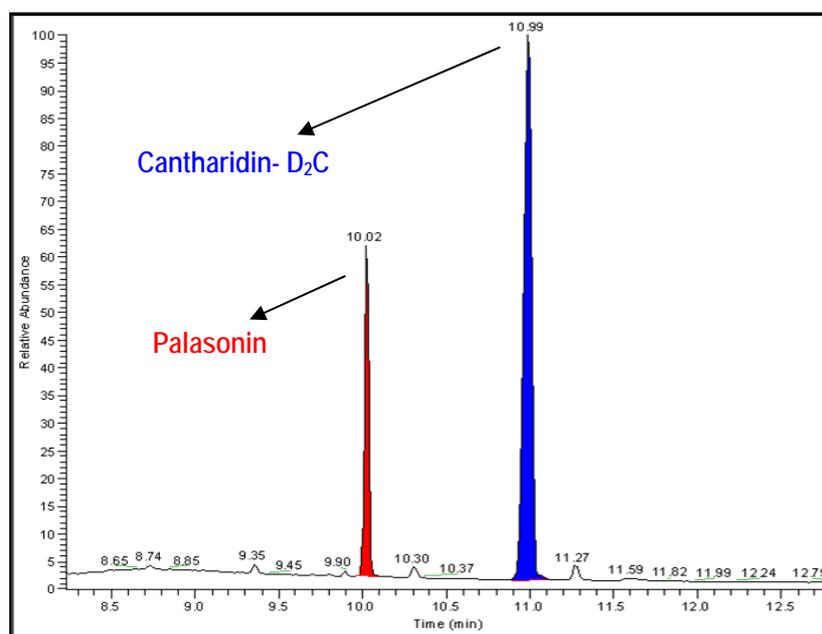
Table 8: Oven temperature programme in GC 6000, Vega Series 2 in order to separate cantharidin from D_2C

Temperature (°C)	Rate (°C/min)	Holding time of temperature (min)	Time (min)
60	10	0	21.6
275	0	10	10
60	100	0	2.16
-	-	-	Total: 33.76

2. GC-Q Finnigan MAT gas chromatograph equipped with a HT5 (5% phenyl polysiloxane-carborane, non-polar) bounded phase fused silica capillary column (SGE: FT 0.1 μm , ID 0.32 mm, OD 0.45 mm, Length: 25 m) connected to an Ion Trap System (GCQ). According to the following oven programme (table 9) and the detector delay time of 8.00 minutes, cantharidin, D_2C and palasonin will be eluted at RT 11:00, 10:59 and 10:02 minutes respectively (fig. 15). Mass spectra were taken at 70 eV (in EI mode) with scanning speed of 1 scan/ sec from m/z 50 to 250. Constant Helium velocity was 40 cm/sec (Helium pressure: 53 mbar) and injector temperature set at 230 °C. Ion source and transfer line temperature were 150 °C and 275 °C respectively. The data were processed using GCQ analysis software package, 1997, Version 2.2 (Build 173), Finnigan Corporation.

Table 9: Oven temperature programme in GC-Q in order to separate cantharidin from D₂C

Temperature (°C)	Rate (°C/min)	Holding time of temperature (min)	Time (min)
60	10	0	10
160	0	3	3
300	10	5	19
60	100	0	2.4
-	-	-	Total: 34.4

**Fig. 15: Total ion chromatograms of palasonin (RT 10:02) and cantharidin/D₂C (RT 11:00) separated by a GCQ gas chromatograph**

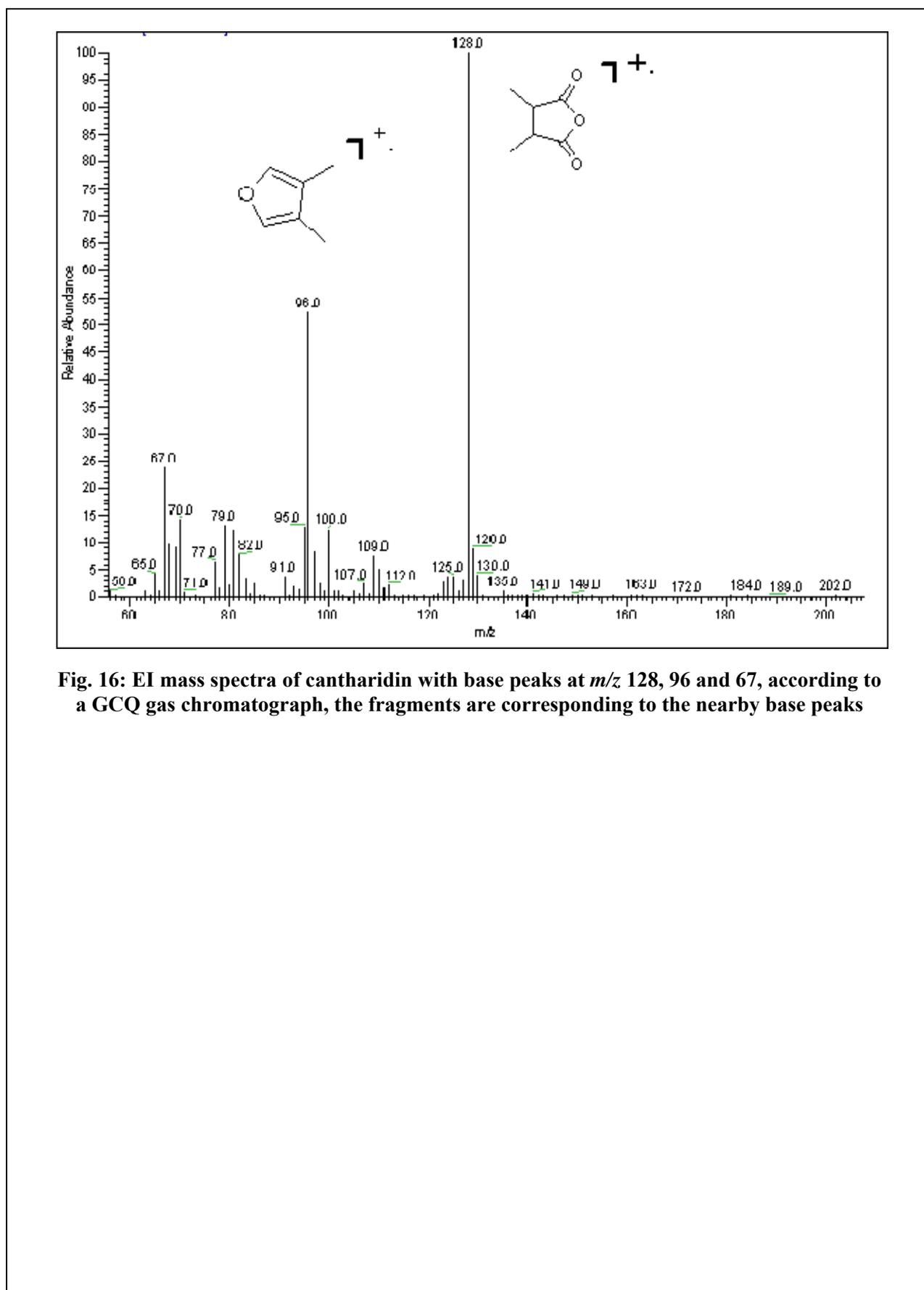


Fig. 16: EI mass spectra of cantharidin with base peaks at m/z 128, 96 and 67, according to a GCQ gas chromatograph, the fragments are corresponding to the nearby base peaks

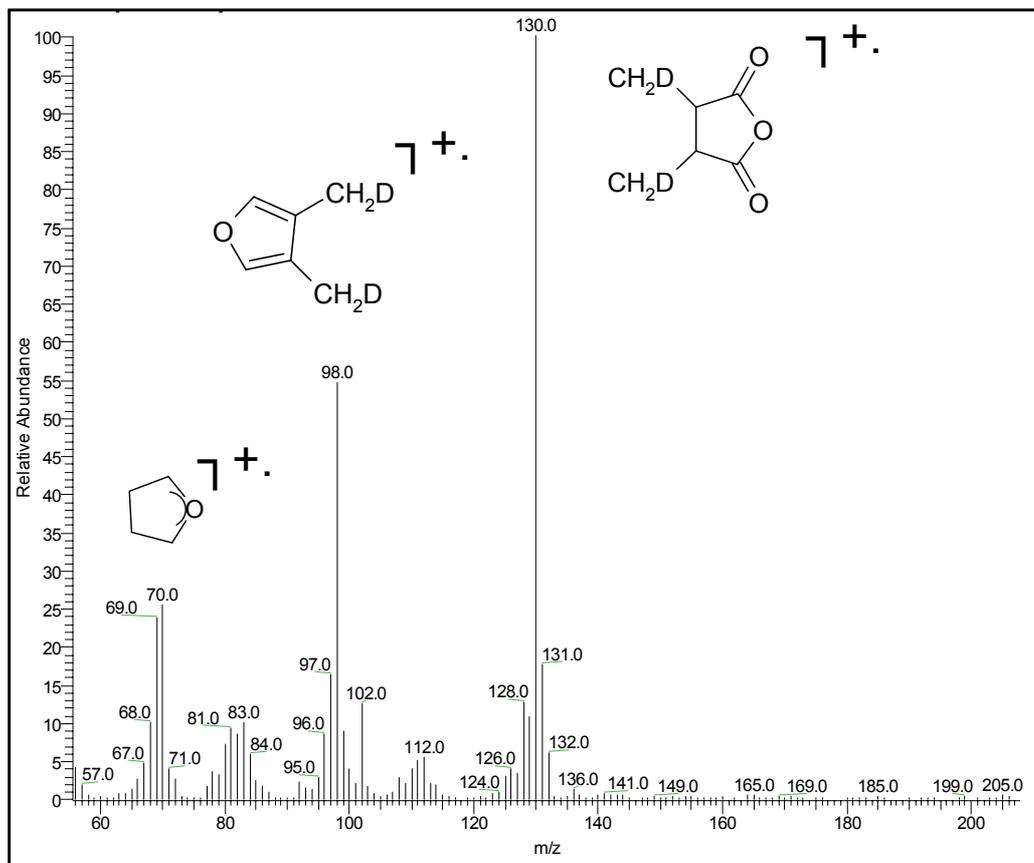


Fig. 17: EI mass spectra of D₂C with base peaks at m/z 130, 98 and 70 according to a GCQ gas chromatograph, the fragments are corresponding to the nearby base peaks

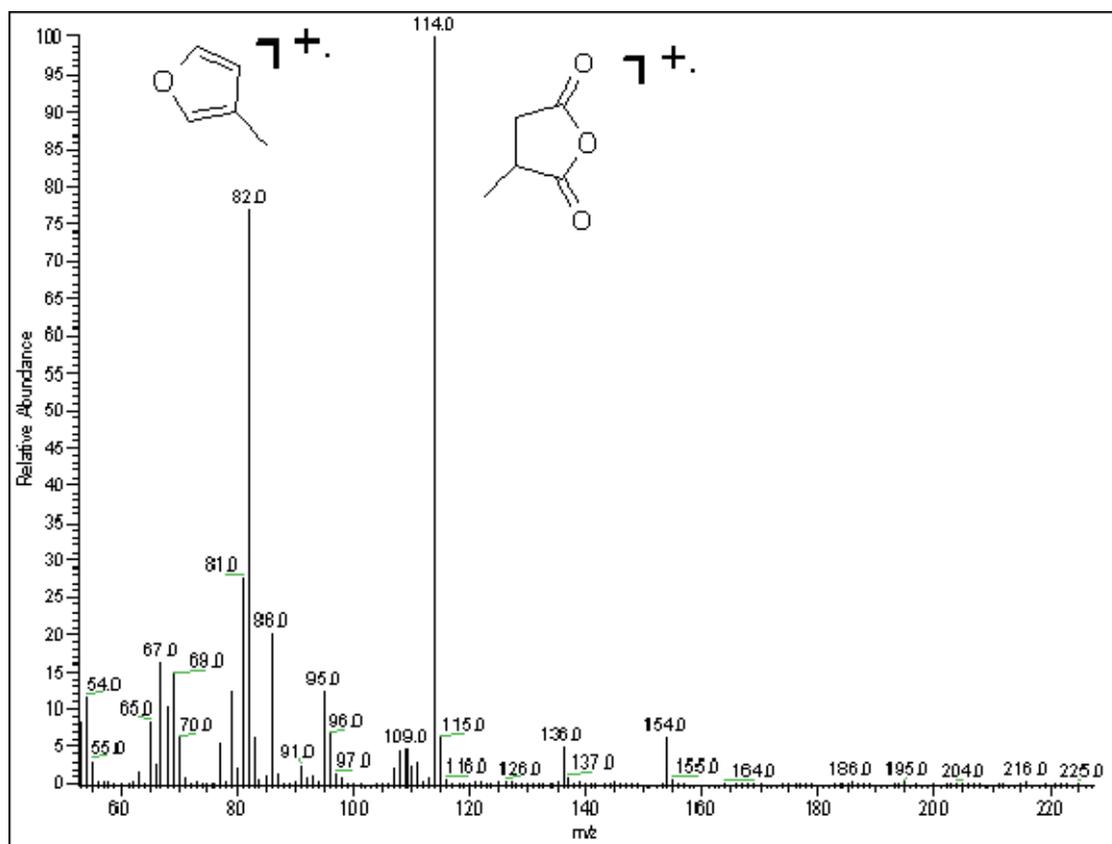


Fig. 18: EI mass spectra of palasonin with base peaks at m/z 114 and 82 according to a GCQ gas chromatograph, the fragments are corresponding to the nearby base peaks

3. GC-MS Varian Saturn 2000.40 which was equipped with a ZB-5 (5% phenyl polysiloxane, non-polar) column (Phenomenex: FT 0.25 μ m, ID 0.25 mm, Length: 60 m) and a 1079 injector. Trap and transfer line temperature were 175 $^{\circ}$ C and 260 $^{\circ}$ C respectively. Mass spectra were taken at 70 eV (in EI mode) with scanning speed of 1 scan/sec from m/z 30 to 350. GC-MS data were processed by a Saturn[®] GC/MS Workstation package, Saturn view[™] version 5.2.1, 1989-1998, Varian Associates, Inc. Other system specifications were as follows:

Constant Helium flow: 1.8 ml/ min. Oven programme has been shown in table 10. One μ l of all samples was injected using a Varian autosampler 8200 CX.

Table 10: Oven temperature programme in GC-MS Varian in order to separate cantharidin from D₂C

Temperature ($^{\circ}$ C)	Rate ($^{\circ}$ C/min)	Holding time of temperature (min)	Time (min)
40	0	2	2
130	100	0	0.9
195	3	0	21.66
250	100	2	24.21
40	100	0	2.1
-	-	-	Total: 26.31

As reference compound and also for calibration equations, technical form of cantharidin (Purity 98%) supplied by SIGMA-ALDRICH Chemical Co., Gillingham, Dorset, UK was dissolved in chloroform and injected into all above mentioned GC-MS systems in different concentrations. Palasonin and D₂C were kindly supplied by Professor W. Boland (Max Planck Institute for Chemical Ecology, Jena, Germany).

2.6.2.1 Cantharidin/ D₂C Separation

Because of the small mass difference between cantharidin and D₂C, the two compounds cannot be well separated by gas chromatography to produce two separate ion chromatograms. Different oven programmes or column types were tried to see if they had any effect on the approximate coeluting cantharidin/D₂C. Since quantitation is based on the peak area of a single compound, the exact measurement of these two chemicals was faced with a technical limitation. In order to remove the burden, a kind of MS/MS technique called MRM (Multiple Reaction Monitoring) was used to separate cantharidin from D₂C for the first time. However many different GC-MS systems are able to do MS/MS, not all can cope with MRM running. That was why, a Varian GC-MS system was used to achieve the mentioned goal. MRM collects MS/MS data on more than one compound in the same time window of the chromatogram. It is especially useful when coeluting compounds have different parent ions, e.g. a target compound and its coeluting isotopically-labeled standard.

Another advantage of MRM was the fact that after MS/MS, several compounds could be directed towards different channels based on the defined parent ion which made quantitation not only easier but more reliable. In every channel of MRM, data can be viewed and integrated independently. So, cantharidin and D₂C viewed independently by designating the appropriate channels in the file selection dialog (fig. 19, 20). It is even possible to send up to 10 different parent ions in each chromatographic segment.

Saturn[®] GC/MS Workstation software made it even possible to use two techniques at the same time on a single run which could lead to considerable saving of time. Regarding palasonin with a definitely different *rt* and mass spectra, there was no need to run a MRM method, but a normal MS could be used easily. Therefore the Varian GC-MS was programmed in the way to do first a normal MS and let palasonin to be eluted and then immediately shift into the MRM for the rest of the same run. This feature is shown in fig. 21.

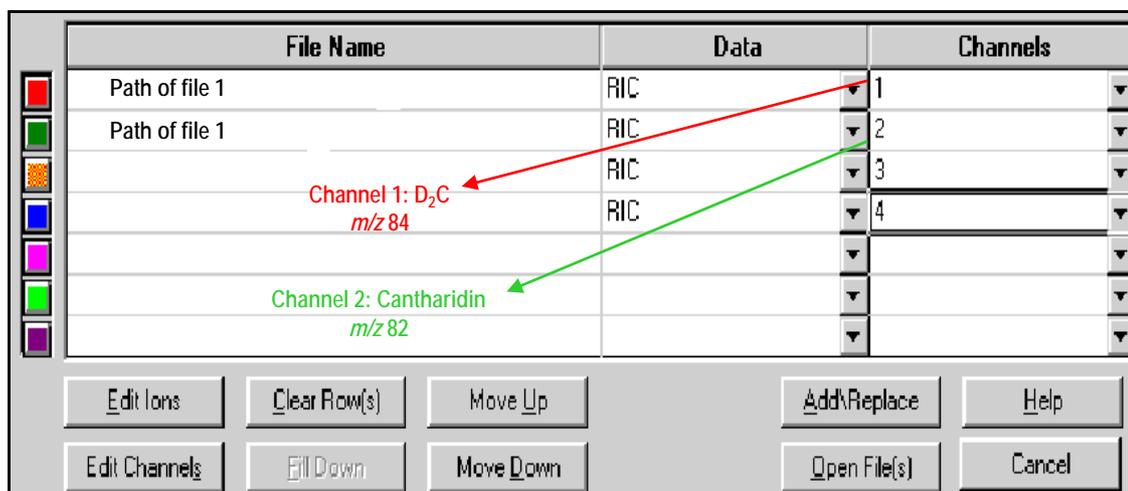


Fig. 19: MRM setting, shows different channel options based on the defined parent ion of cantharidin (m/z 82) and D_2C (m/z 84)

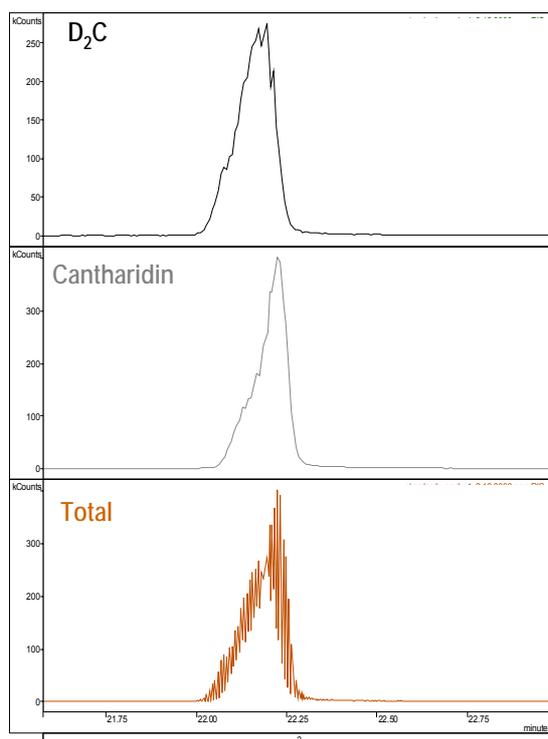


Fig. 20: Three windows of the same segment indicate channel 1 at 1st window (D_2C characteristic peak), channel 2 at 2nd window (cantharidin peak) and finally both peaks have been shown together in the last window

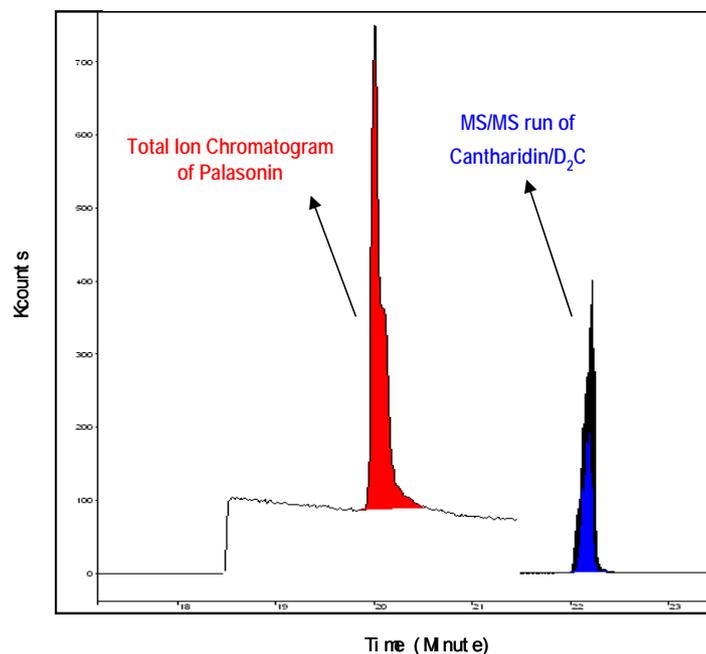


Fig. 21: A GC-MS Varian run: MS run for palasonin (RT 20:00) along with the simultaneous MS/MS run of cantharidin/D₂C (RT 22:20)

2.7 Scanning Electron Microscopy (SEM)

In order to study cuticular pores and other external microscope features in adults of genus *Cyaneolytta* (Col: Meloidae), scanning electron microscope (SEM) used. First, legs and antennae of three males and females were dissected under the stereomicroscope. Then samples were fixed on probe plates individually using narrow aluminium foil bands (8×2 mm) or a small droplet of conductive carbon cement (Leit-C, Neubauer Chemikalien, Münster, Germany) and gold coated in 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

Gold coated probes were thereafter examined with a Cambridge electron microscope (Cambridge Instruments[®]) and photos taken by Nikon Coolpix 995 digital camera. To determine the pore density within any frame of an examined sample, cuticular pores were counted in a fixed arena of 24.5 μm width since the above mentioned SEM was not able to define any area.

2.8 Internal Observations

In order to observe internal tissues, cells or any other structure; samples should be fixed, dehydrated and then coated with paraffin to be cut by microtomy technique. Sections had to be washed off to get rid of paraffin remainders, stained with proper agents and finally fixed on microscope slides. All these steps have been precisely described here and those chemicals consumed which have not been referred in the text, are summarized in table 11.

2.8.1 Paraffin Embedding

To observe antennal segments internally, the whole antennae of both sexes were detached and placed in ethanol 90% for one hour. They afterwards immersed for two days in a solution of 2 parts A (150 ml ethanol 80% and 1 g picric acid) and 1 part B (60 ml formol plus 15 ml acetic acid) as a fixation process. The probes were dehydrated in a graded ethanol series, followed by a post fixative process in certain period of time:

Dehydration → Ethanol 70%: 4- 8 hours

Ethanol 70 %: 4- 8 h

Ethanol 90%: overnight

Ethanol 96%: 24 h

Postfixation → Isopropanol 100%: 2-4 h (depends on the sample size)

Isopropanol 100%: 2-4 h (depends on the sample size)

Methylbenzoate: overnight

Methylbenzoate: 24 h

Xylol 100%: 24 h

Xylol 100%: 24 h

In order to cut the antenna by microtome, it must be well coated by a thick layer of paraffin. To prepare the liquid paraffin, enough paraffin palates (non-cacking, solidification point: 51- 53 °C, Merck KGaA, Darmstadt, Germany) were poured into a measuring beaker and placed inside an oven (Memmert GmbH and Co. KG, Schwabach, Germany) at 60 °C. The whole procedure was done inside the oven in order to prevent liquid paraffin becoming solid again. Thereafter, probes were embedded in different paraffin solution according to these steps and shaken gently several times per day to facilitate paraffin penetration into antennal tissues.

1. Three parts xylol and one part paraffin for 1 day

2. One part xylol and one part paraffin for 1 day

3. One part xylol and three parts paraffin for 1 day

Within silicon blocks (20×20 mm), liquid paraffin added to samples in a way they stand 1-2 mm below the block surface. The filled blocks were left in room temperature for 3-4 hours and then transferred to fridge for the similar period of time to be well hardened.

2.8.2 Tissue Sectioning

Using a diamond knife on an ultramicrotome (Leica RM 2035, Leica Instruments GmbH), thin cross sections of about 10 µm were taken from antennal samples embedded in a paraffin block. The sections were rinsed in water bath (Bemdelin Electronic, Berlin, Germany), filled with distilled water, at 35- 40 °C for a while and then placed on a pre-treated microscope slide (Super Frost® Plus, 25×74×1 mm, Menzel Gläser®, Braunschweig, Germany). Slides were first put vertically on a heating plate (Gerhardt, WP Electronic, Bonn, Germany) at 50 °C for two hours to be dried and then placed on the same surface horizontally over the night.

2.8.3 Staining of Antennal Sections

Since staining procedure contains usage of xylol for a relatively long time, all following steps were done under a fume hood with enough ventilation. This procedure can be divided into three parts which are washing off of paraffin, staining and dehydration (Adam and Czihak, 1964) and thus slides must be immersed in different solvents or staining solutions for specified time.

* Washing off process:

1. Xylol 100% → 10 min
2. Xylol 100% → 10 min
3. Isopropanol 100% → 10 min
4. Isopropanol 100% → 10 min
5. Isopropanol 96% → 5 min
6. Isopropanol 90% → 5 min
7. Isopropanol 70% → 5 min
8. Distilled water → 10 min

* Staining process

1. Kernechtrot- Aluminiumsulfate for 10- 30 min
2. Rising with deionised water for very short time
3. Pikroindigocarmin for 20- 30 sec

To prepare Kernechtrot-Aluminiumsulfate, 0.1 g Kernechtrot was added to 100 ml of Aluminiumsulfate solution 5% and then heated to boiling point. Pikroindigocarmin prepared by adding 0.25 g Indigocarmin to 100 ml saturated water-base picric acid.

* Dehydration process:

1. Isopropanol 70%
2. Isopropanol 90%
3. Isopropanol 95%
4. Isopropanol 100%: twice and each time for 5 min
5. Xylol 100%: twice and each time for 10 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[®], contains isomer mixture of xylene (Merck, Darmstadt, Germany).

Table 11: Chemicals used for paraffin embedding and staining of meloid tissues

Chemical	Remarks
Acetic Acid	$C_2H_4O_2$ (Glacial), purity: 99.8%, p.a., Merck, Darmstadt, Germany
Aluminiumsulfate	$Al_2(SO_4)_3$ and 18 H_2O , crystal, extra pure, Merck, Darmstadt, Germany
Ethanol	C_2H_5OH , purity: 99.8%, p.a., Merck, Darmstadt, Germany
Formol	Formaldehyde solution 37%, stabilized with about 10% methanol, Merck, Darmstadt, Germany
Isopropanol	$CH_3CH(OH)CH_3$ (2- propanol), purity: 99.7%, p.a., Merck, Darmstadt, Germany
Kernechtrot	$C_{14}H_8NNaO_7$, concentration $\geq 90\%$, Merck, Darmstadt, Germany
Methylbenzoate	C_8H_8O , purity: 98%, Fluka Chemie AG, Neu-Ulm, Germany
Picric Acid	$C_6H_3N_3O_7$, p.a., with about 50% water, Merck, Darmstadt, Germany
Indigocarmin	Indigocarmin for Microscopy (Certistain [®]), $C_{16}H_8N_2Na_2O_8S_2$, concentration $\geq 80\%$, Merck, Darmstadt, Germany
Xylol	C_8H_{10} , P-xylol (for synthesis), purity: 99%, Merck- Schuchardt, Hohenbrunn, Germany

2.9 Statistical Analyses

Since cantharidin, D_2C and palasonin do not show a normal distribution in different organs of blister beetles, experiments concerning quantitative internal distribution of these three compounds, 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 experiments for one species was tested over different number of samples (usually 4- 10) according to insect material availability at the 95% or 99% confidence level.

To analyse the data of cuticular pore density model in those experiments with more than two groups, one way ANOVA as a parametric test applied and significant means were separated with Tukey *HSD* posthoc test at the $P= 0.01$ and 0.001 levels. Whenever cuticular pore density 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 rest of statistical analyses have been done using Statistica Package, Kernel version 5.5 A (Statsoft Inc., 1999, Tulsa, OK, USA).

3. Results

In this chapter, the result is presented in three groups of injection experiments, feeding and *Ringer experiments* according to the applied method.

3.1 Injection Experiments

The series of injection experiments were designed in order to get a better understanding of cantharidin distribution in the main internal organs. In this way 10 μl D₂C which had been dissolved in DMSO (concentration: 1600 ng/ μl DMSO), was injected into beetles' hemocoel and they were kept in isolation for two days. Thereafter males (fig. 22) and females (fig. 23) were differentiated, especially internal genitalia. Those injected insects which died earlier than two days were excluded from the experiment to keep the experiment conditions uniform and thus produce a comparable data base.

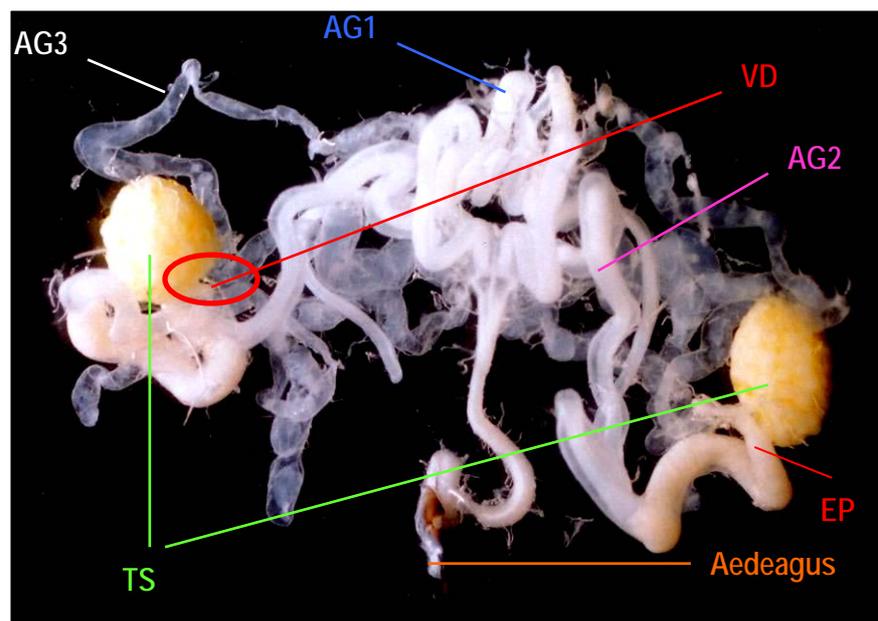


Fig. 22: Male internal genitalia of *Mylabris operta* var. *bioculata*; TS: testis, VD: vas deferens, EP: Epididimis, AG₁: 1st Accessory gland, AG₂: 2nd Accessory gland, AG₃: 3rd Accessory gland

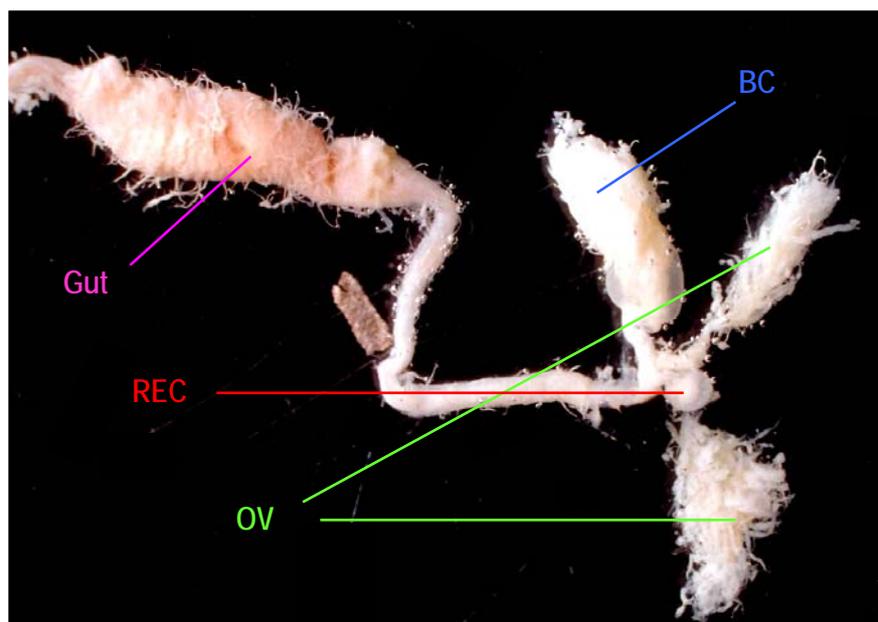
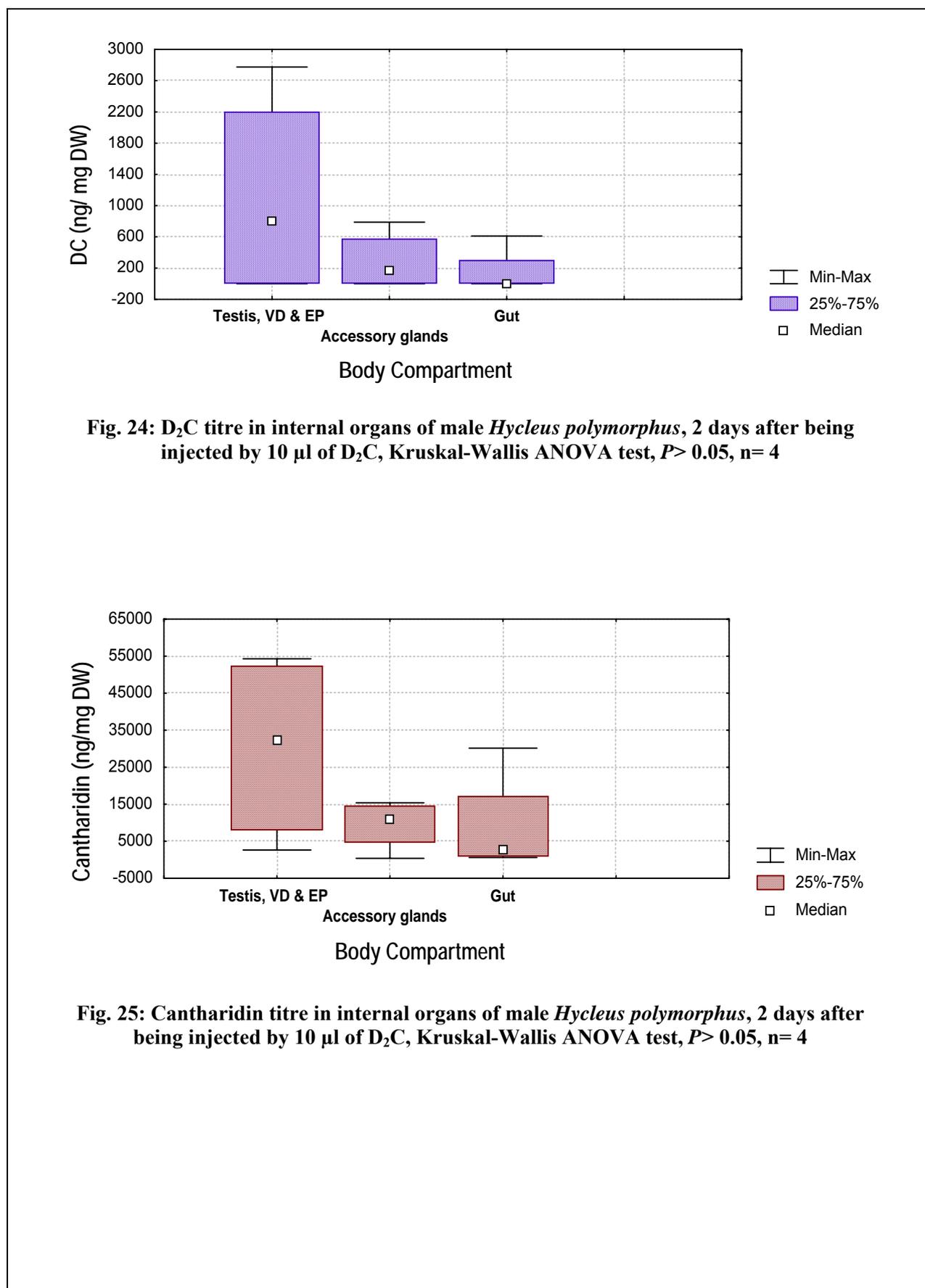
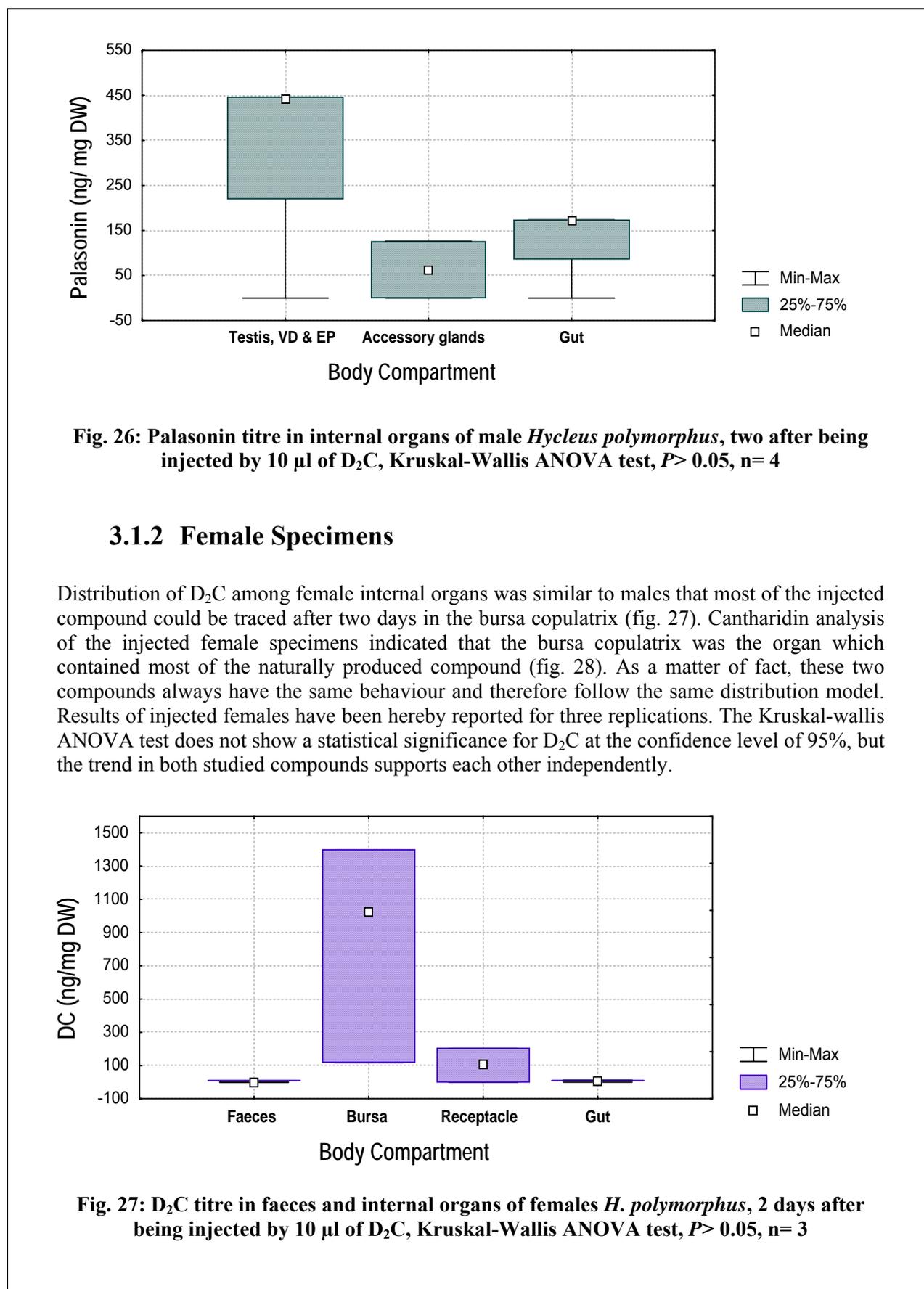


Fig. 23: Female reproductive organ of *Mylabris operta* var. *bioculata*; OV: Ovariols, BC: bursa copulatrix, REC: receptacle

3.1.1 Male Specimens

As indicated in the fig. 24, two days after D₂C introduction into male hemolymph of *Hycleus lunatus*, most of the marker has been clearly incorporated into the testis, vas deferens and epididimidis. The accessory glands and gut were the next organs in D₂C up-taking. This experiment was fulfilled by using 4 replications. Analysis of cantharidin component of the same beetles also indicated a very similar trend in which the testis, vas deferens and epididimidis contained much of the naturally produced compound (fig. 25). Although the non parametric test of Kruskal-Wallis ANOVA did not show any significant difference among the three mentioned groups, a clear tendency towards D₂C and cantharidin incorporation into the testis, vas deferens and epididimidis can be easily observed. Interestingly, Palasonin also shows such a model, however its behaviour is to some extent different from the two other compounds (fig. 26). The vertical bars above and below the histograms indicate maximum and minimum values and the histogram itself is the area which covers 25 to 75% of data on that the median value is located.





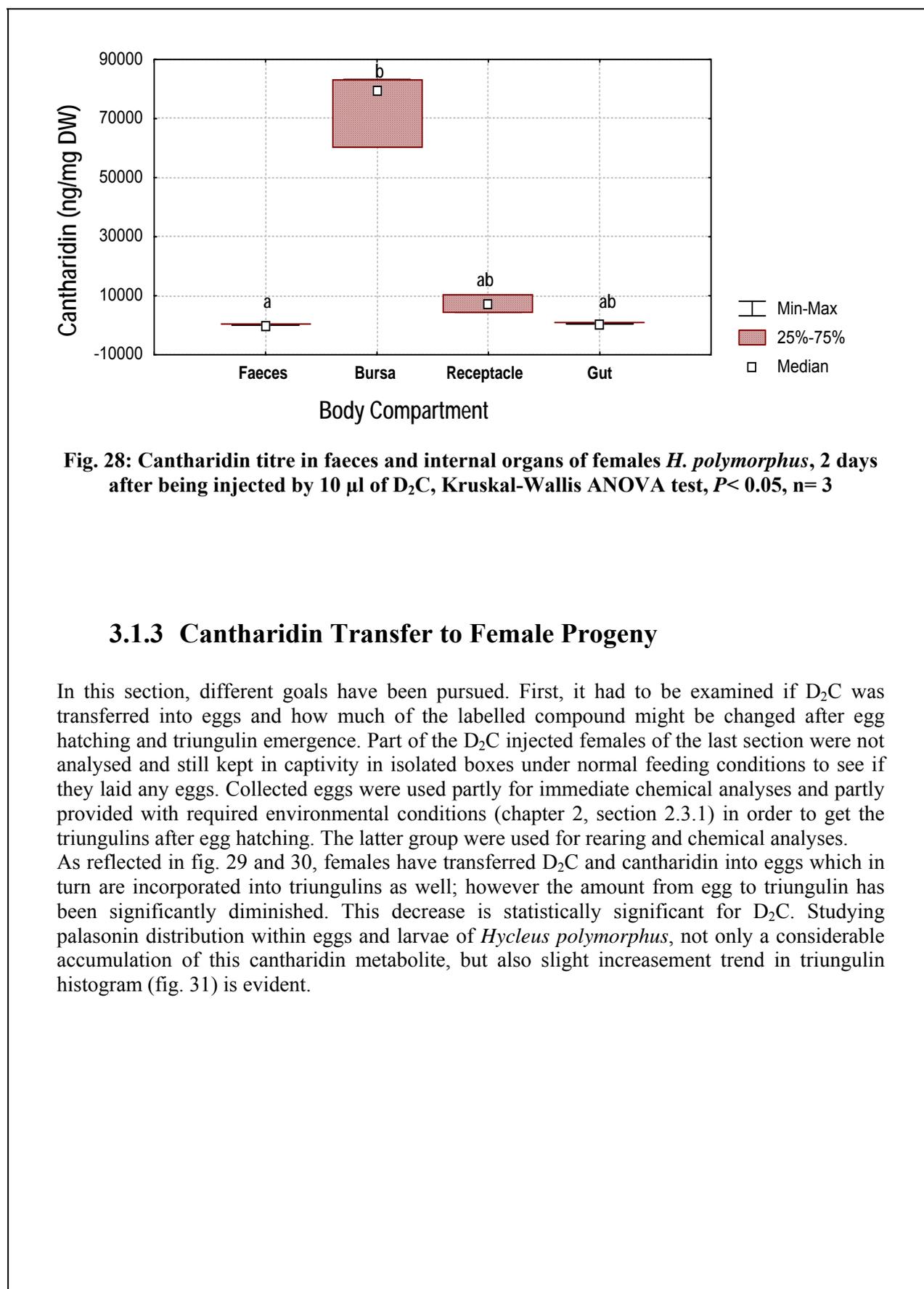


Fig. 28: Cantharidin titre in faeces and internal organs of females *H. polymorphus*, 2 days after being injected by 10 μ l of D₂C, Kruskal-Wallis ANOVA test, $P < 0.05$, $n = 3$

3.1.3 Cantharidin Transfer to Female Progeny

In this section, different goals have been pursued. First, it had to be examined if D₂C was transferred into eggs and how much of the labelled compound might be changed after egg hatching and triungulin emergence. Part of the D₂C injected females of the last section were not analysed and still kept in captivity in isolated boxes under normal feeding conditions to see if they laid any eggs. Collected eggs were used partly for immediate chemical analyses and partly provided with required environmental conditions (chapter 2, section 2.3.1) in order to get the triungulins after egg hatching. The latter group were used for rearing and chemical analyses. As reflected in fig. 29 and 30, females have transferred D₂C and cantharidin into eggs which in turn are incorporated into triungulins as well; however the amount from egg to triungulin has been significantly diminished. This decrease is statistically significant for D₂C. Studying palasonin distribution within eggs and larvae of *Hycleus polymorphus*, not only a considerable accumulation of this cantharidin metabolite, but also slight increasement trend in triungulin histogram (fig. 31) is evident.

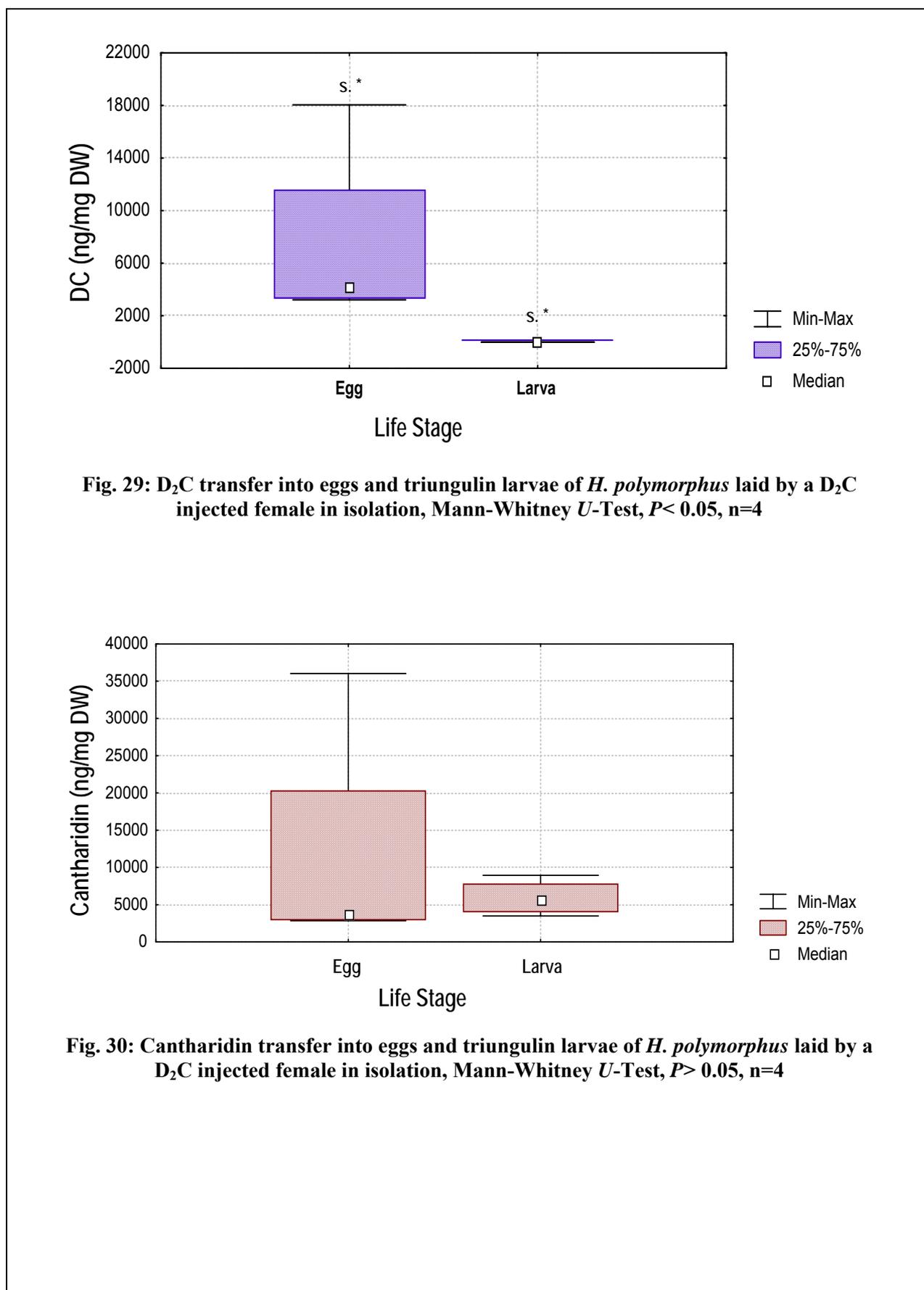


Fig. 29: D₂C transfer into eggs and triungulin larvae of *H. polymorphus* laid by a D₂C injected female in isolation, Mann-Whitney *U*-Test, $P < 0.05$, $n=4$

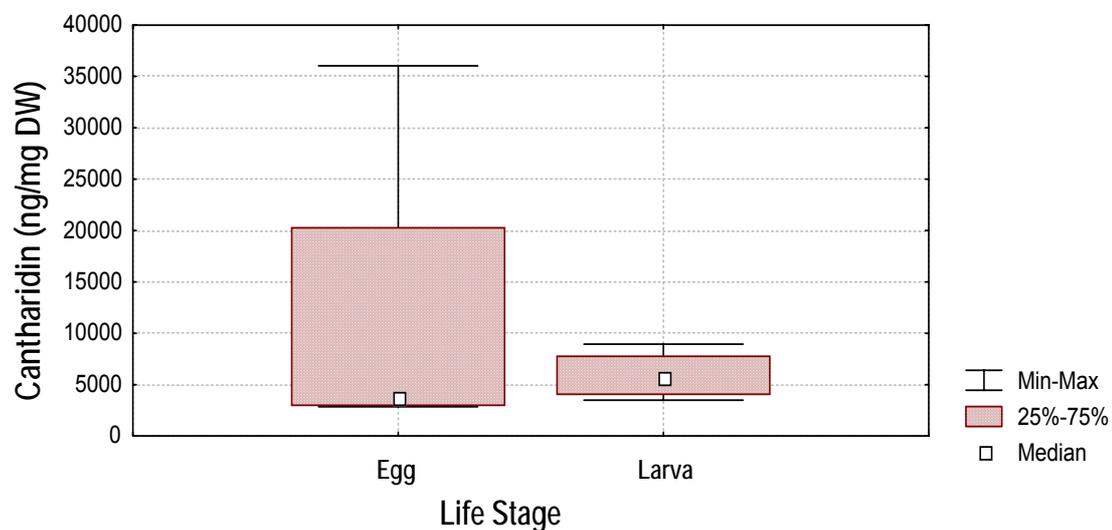
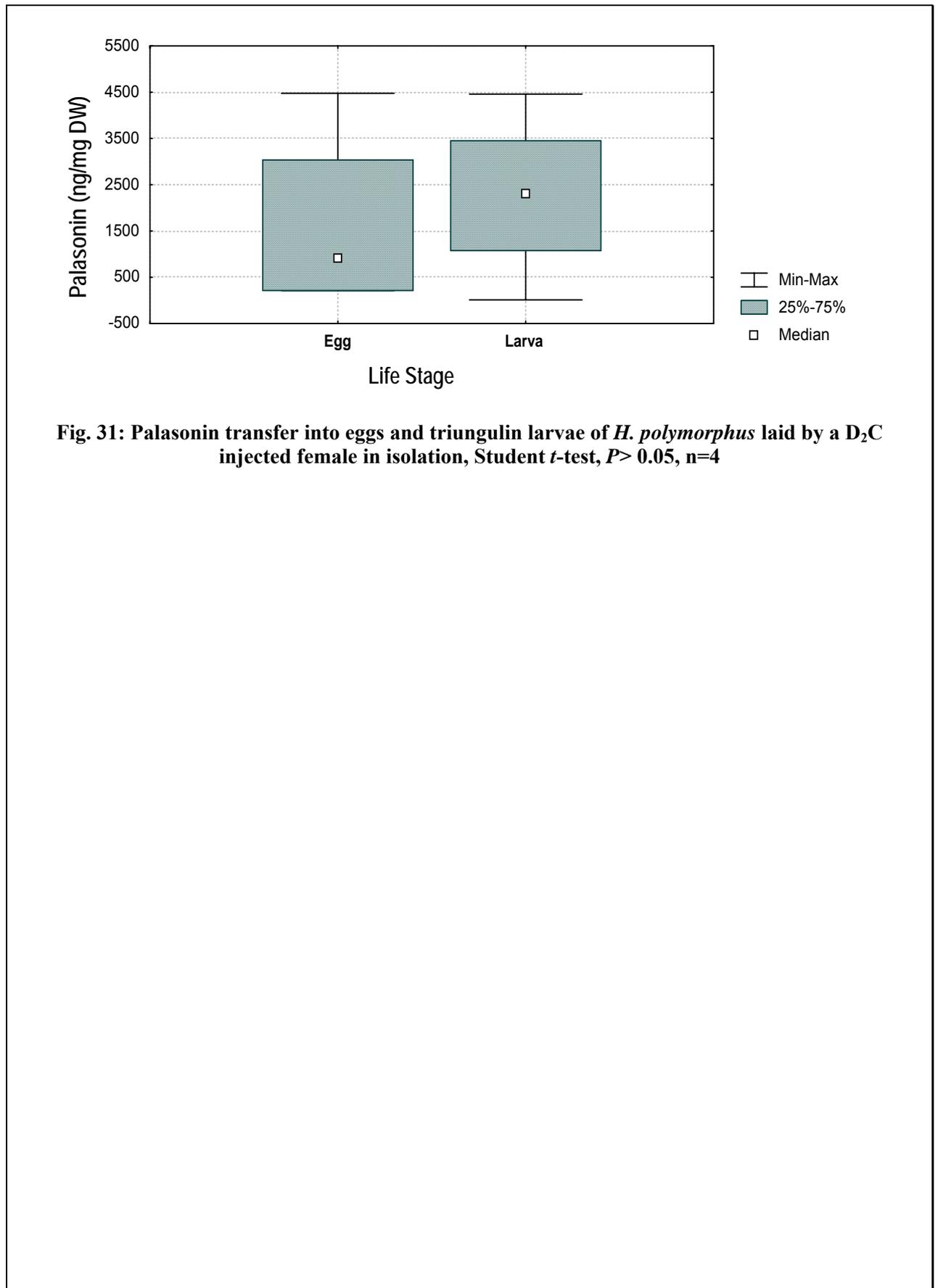


Fig. 30: Cantharidin transfer into eggs and triungulin larvae of *H. polymorphus* laid by a D₂C injected female in isolation, Mann-Whitney *U*-Test, $P > 0.05$, $n=4$



3.2 Feeding Experiments

In spite of several advantages of D₂C injection method, it has a limitation due to considerable shortening of the beetle's life span. It often leads to the exclusion of many experimental animals which have died before the designed period of experiment. In order to avoid such a big loss of insect valuable material, the alternative way of feeding was used in which the beetles took up the pre measured D₂C mixed with the artificial diet (chapter 2, 2.5). In this way, mortality rate was significantly reduced; hence the beetles survived enough to be analysed over a longer period of time. Feeding experiments were performed on *Mylabris quadripunctata* which had been exposed to the mixture of diet and D₂C for three days.

3.2.1 Male Specimens

The two natural compounds (cantharidin and palasonin) were first measured in different male sexual organs to get a portrait of their natural distribution within the male specimens. As indicated in fig. 32, the 2nd and 3rd pairs of the accessory glands, the testis and gut apparently contain higher amounts of cantharidin in comparison to the other organs; however only 2nd pair of the accessory glands show a significant difference to the vas deferens, epididimis or the 1st pair of the accessory glands. The data of the testis scattered more than the other organs due to the higher standard deviation. Considering the median, 2nd and the 3rd the accessory glands contain higher amounts with a significant difference to vas deference, epididimis and 1st pair of the accessory glands using Median test at the 95% confidence level.



Fig. 32: Cantharidin titre in male *Mylabris quadripunctata* which had received artificial diet without any D₂C (control), Kruskal-Wallis ANOVA Test, $P < 0.05$, $n = 6$

Following D₂C introduction along with the food source, cantharidin distribution changed which has been shown upon 10 replications. As seen in fig. 33, 2nd pair of the accessory glands has still one of the highest amounts with statistical difference to the 1st pair, vas deference, epididimis and gut. The interesting point is the clear shift of medians in the 2nd and 3rd pairs of the accessory glands before and after of D₂C introduction. Using Median test, a significant difference was recorded at $P= 0.01$ level between these two pairs of accessory glands and the remaining of experimental groups. Interestingly, the vas deference and epididimis contain much lower amounts than the 2nd and 3rd pairs of the accessory glands after D₂C introduction, which is different from control specimens.

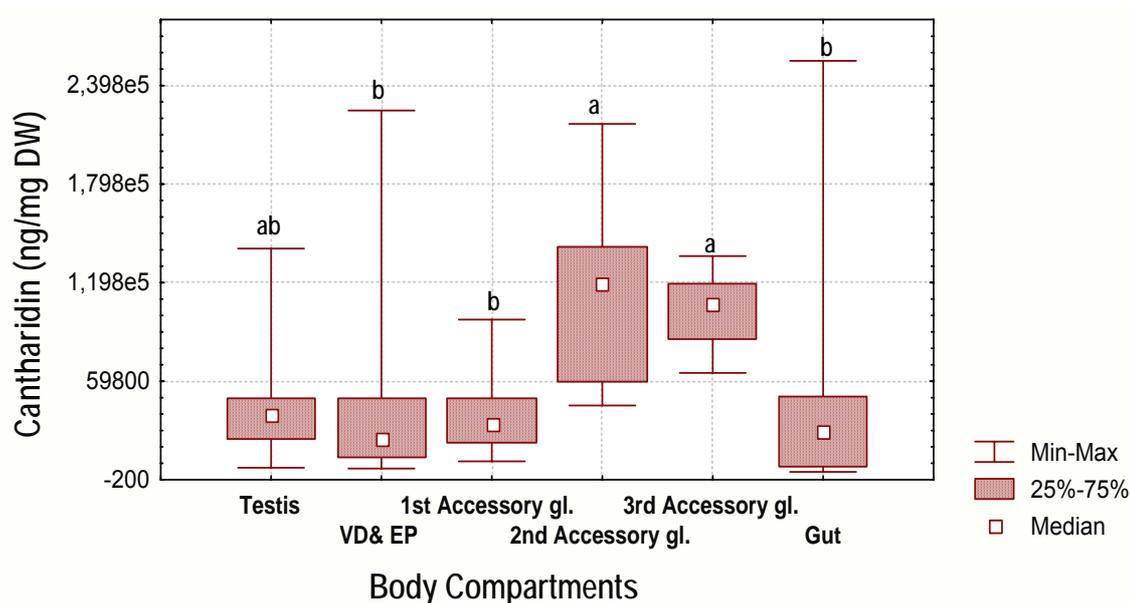


Fig. 33: Cantharidin titre in males *M. quadripunctata* which were fed by artificial diet mixed with D₂C (54 ml/ dish) for 3 days, Kruskal-Wallis ANOVA test, $P < 0.001$, $n = 10$

Considering D₂C incorporation into male internal organs, all internal organs shown in fig. 34 show about the same level of D₂C accumulation with 10 replications, but the 1st pair of the accessory glands and gut with apparently lower titre than others and significantly lower than the 2nd pair of the accessory glands at 99% confidence level (Kruskal-Wallis ANOVA Test) upon 10 replications.

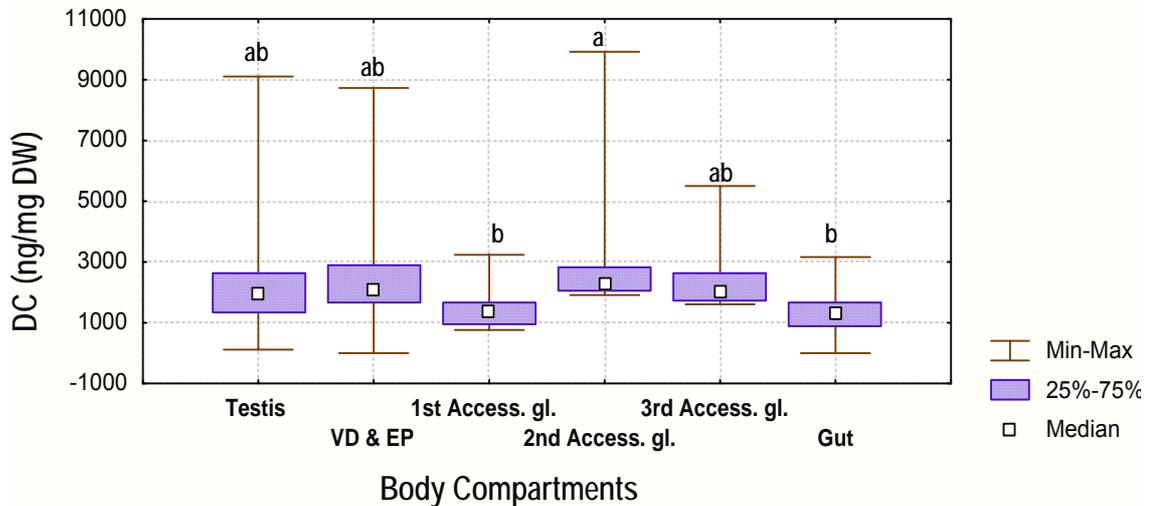


Fig. 34: D₂C titre in males *M. quadripunctata* which were fed by artificial diet mixed with D₂C (54 ml/ dish) for 3 days, Kruskal-Wallis ANOVA test, $P < 0.01$, $n = 10$

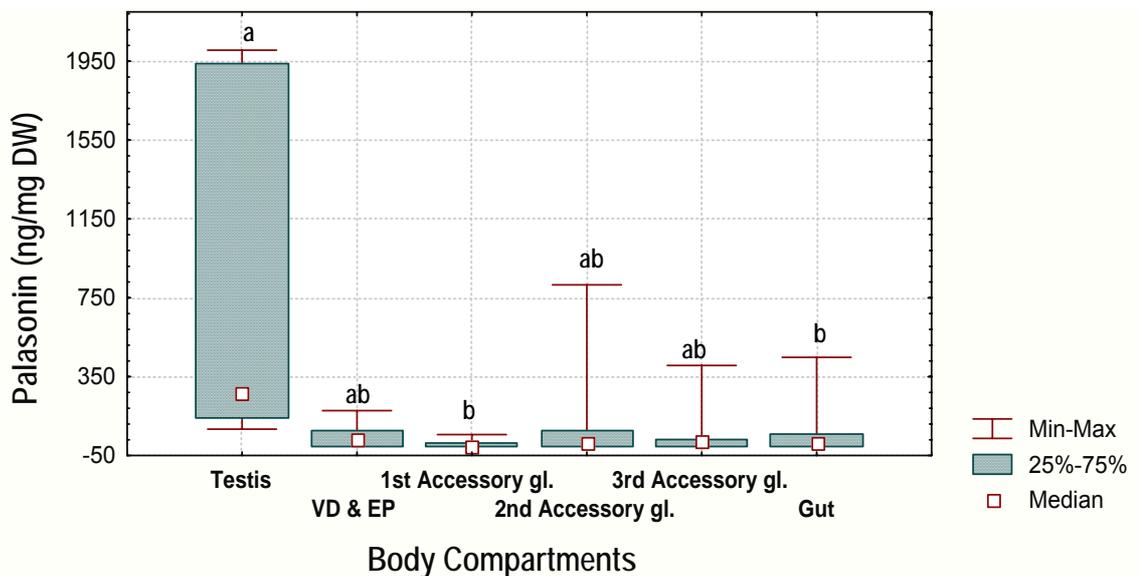


Fig. 35: Palasonin titre in male *M. quadripunctata* which had received artificial diet without any D₂C (control), Kruskal-Wallis ANOVA test, $P < 0.05$, $n = 6$

Palasonin measurement in control samples (without D₂C intervention), indicates a higher rate of accumulation in the testis, while the statistical analysis indicated no difference between the testis on one hand and the vas deference and epididimis and 2nd and 3rd pairs of the accessory glands on the other hand (fig. 35). The only statistical difference is observed between the testis and the 1st pair of the accessory glands and gut. D₂C treated samples experienced a sharp shift in the rate of palasonin accumulation within the testis which decreased from the median level of

about 250 ng/mg dry weight to median of zero. At the same time, much more palasonin was incorporated into the vas deference and epididimidis after D₂C introduction (fig. 36). Another point is the palasonin level in the 3rd pair of the accessory glands and gut which is almost zero (median point value).

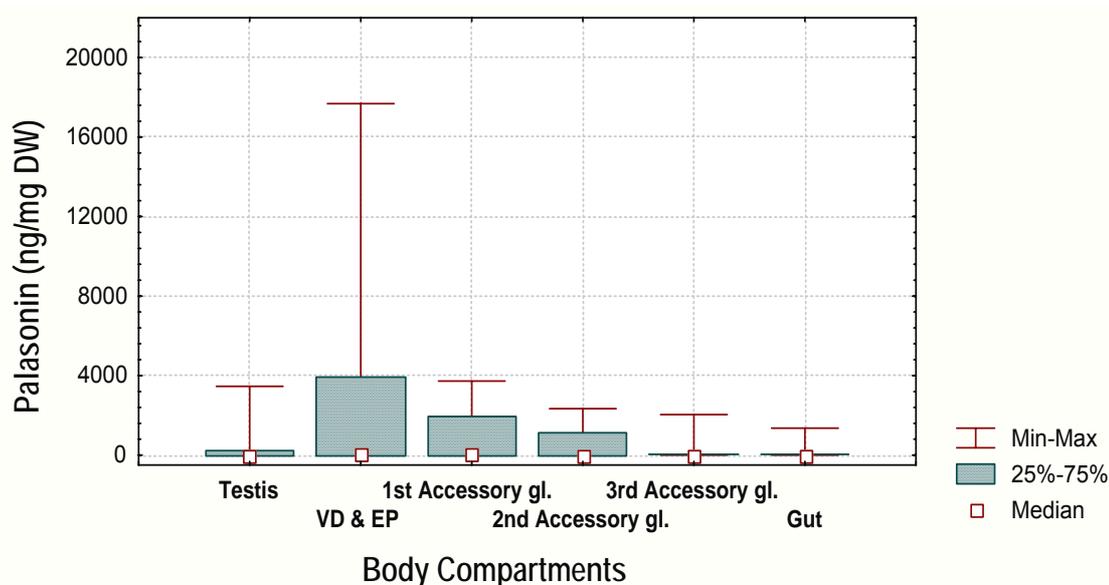


Fig. 36: Palasonin titre in male *M. quadripunctata* which were fed by artificial diet mixed with D₂C (54 ml/ dish) for 3 days, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 10$

Faeces of male *Mylabris quadripunctata* were also studied during the 9 days after D₂C treatment in which faeces of every two days were analysed for D₂C separately, with 6 replications. The result of this survey which has been summarized in fig. 37 indicates the presence of D₂C in the excretory materials of fed males which is a sound proof of taking-up of the marker by the beetles. According to fig. 37, titre of marker was at the highest rate just a day after introduction, but almost the same (zero ng/mg dry weight) for the following days until the 9th day of examination.

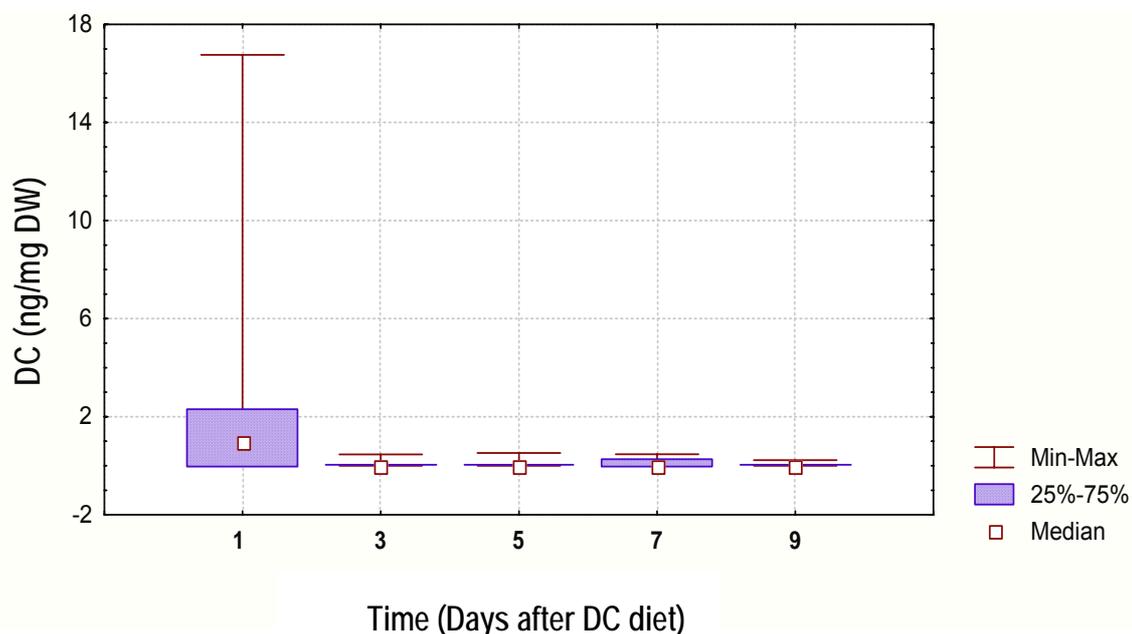


Fig. 37: D₂C titre in faeces of male *M. quadripunctata* which were fed by artificial diet mixed with D₂C (54 ml/ dish) for 3 days, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 6$

3.2.2 Female Specimens

In injection experiments, it was not possible to pair a non-injected female with an injected male to study the marker transfer after mating because the longevity of injected individuals was relatively short and therefore females injected separately. In feeding experiments, those females had fed on normal diet were paired to D₂C fed males for three days to see if there was any transfer of the labelled compound and then to study the distribution of marker within female sexual organs. Apart from D₂C, the distribution of cantharidin and palasonin were also studied. As seen in fig. 38, cantharidin is not distributed into different internal organs uniformly, but much of the compound accumulates in the bursa copulatrix; however there is no statistical difference to eggs and ovaries. D₂C incorporation (fig. 39) is even more interesting, since it not only has the same distribution pattern as cantharidin, but a clear statistical difference between the three categories. Using D₂C, it is confirmed that most of the compound accumulated in the bursa copulatrix. The experiment was carried out using 6 specimens and the data analysed by Kruskal-Wallis ANOVA test at the confidential level of 95%. Considering palasonin, a tendency of accumulation in bursa is observable (fig. 40); it is close to being statistically significant.

Faeces of the females were also examined to see how D₂C excretion changed by time (fig. 41). Faeces collected 1, 4, 6 and 8 days after pairing of females with D₂C fed males and analysed for D₂C. As indicated in fig. 41, most D₂C excretion occurred on the first day after pairing whilst titre of the excreted marker decreases on the following days significantly and remains almost at the same level on the 2nd, 4th and 8th days.

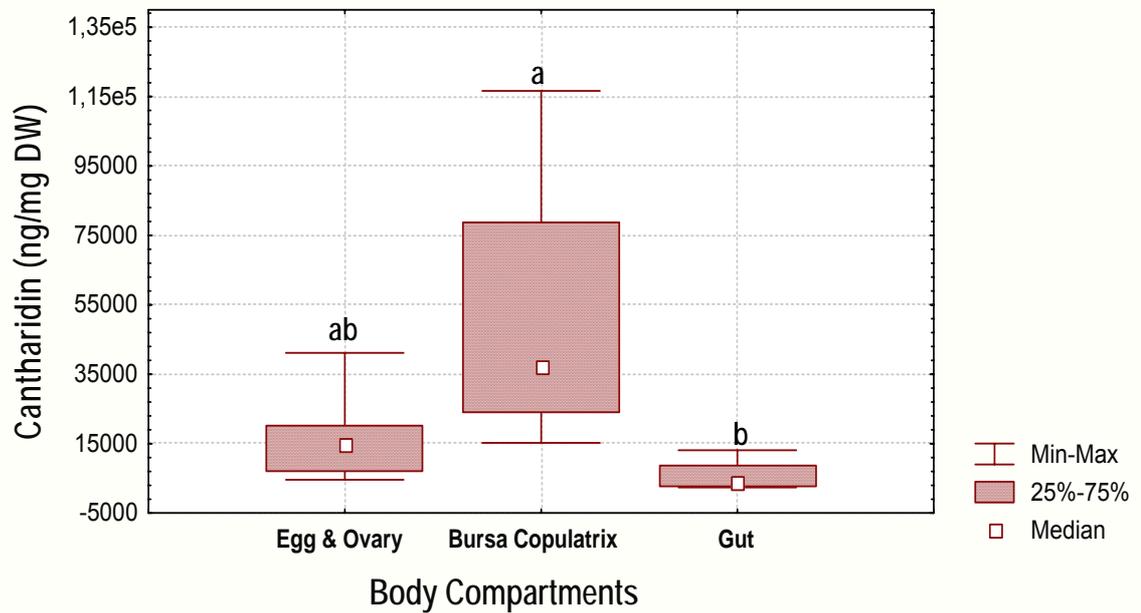


Fig. 38: Cantharidin titre in those females of *M. quadripunctata* paired with D₂C fed males for 3 days, Kruskal-Wallis ANOVA test, $P < 0.05$, n= 6

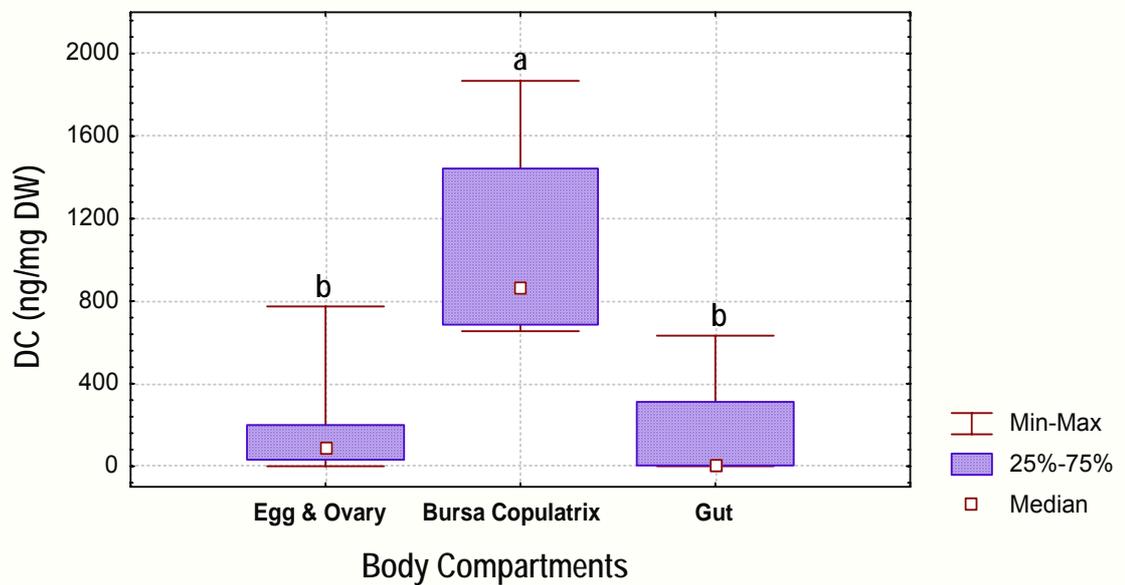


Fig. 39: D₂C titre in those females of *M. quadripunctata* paired with D₂C fed males for 3 days, Kruskal-Wallis ANOVA test, $P < 0.05$, n= 6

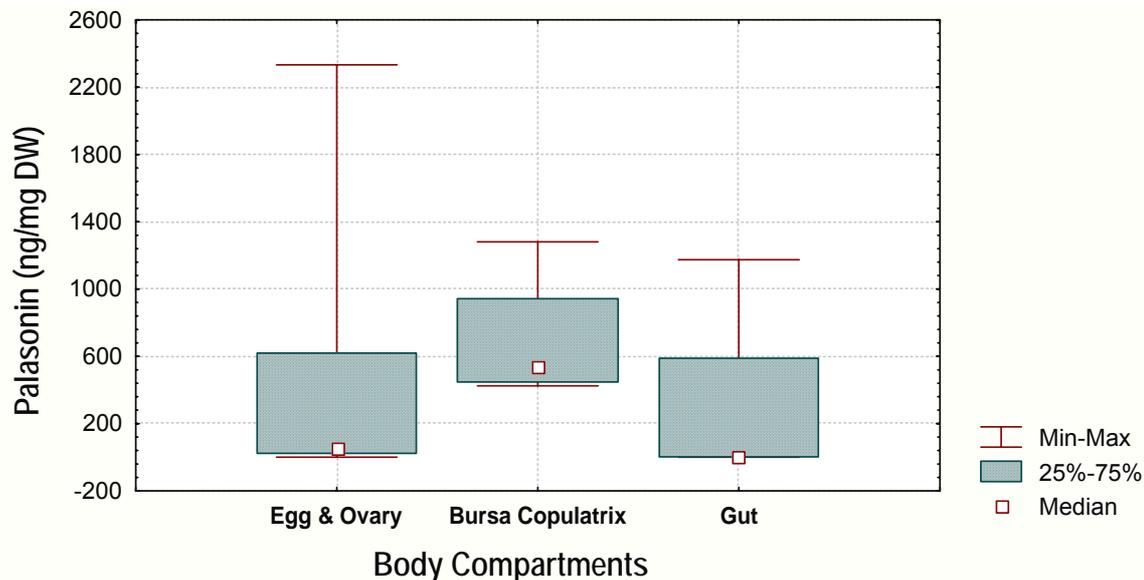


Fig. 40: Palasonin titre in those females of *M. quadripunctata* paired with D₂C fed males for 3 days, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 6$

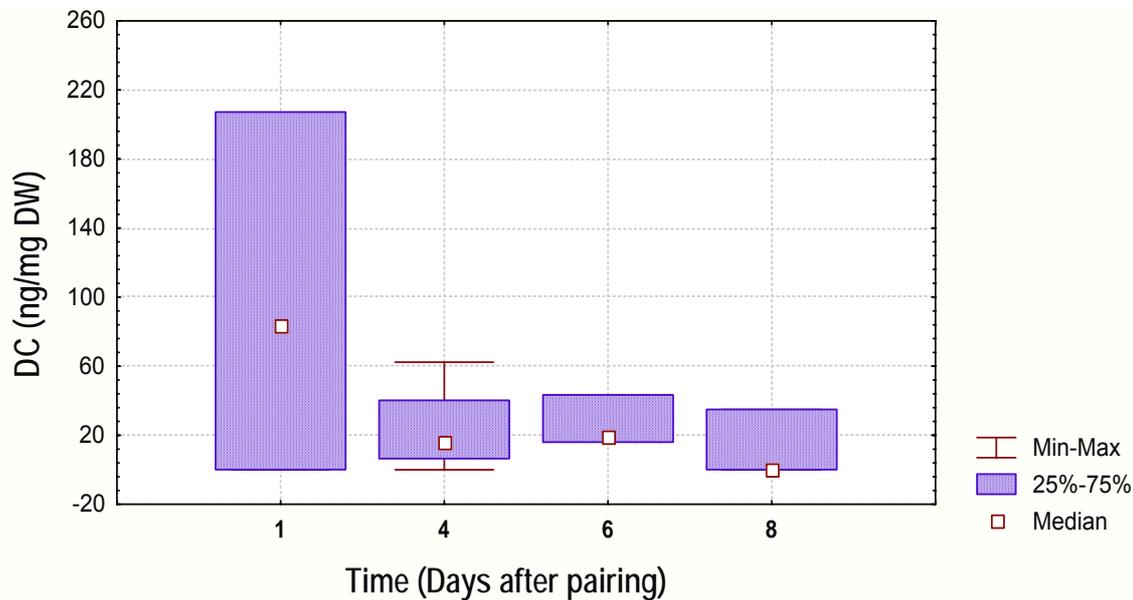


Fig. 41: D₂C titre in faeces of those females of *M. quadripunctata* paired with D₂C fed males for 3 days, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$ (histograms 1 and 6 days), $n = 4$ (histograms 4, 8 days).

3.3 Ringer Experiments

This series of experiments which are briefly referred to as *Ringer experiment* have been specially designed to complete the observation made on the two former groups of experiments (sections 3.1 and 3.2) or confirm part of their derived data. Nevertheless injection and feeding experiments display the distribution of cantharidin and marker (D₂C) within internal organs of both sexes; they are unable to reveal a sequential time profile of cantharidin and/or D₂C movement through sexual organs due to the methodology. In the two former methods, beetles were frozen and analysed later on which gives only a static portrait of distribution, whilst in *Ringer experiments* dissected organs active in Ringer solution for several hours let us study any chemical changes in composition of the solution and furthermore quantify the target compounds at each time sequence (chapter 2, section 2.5).

Ringer experiments were done on *Hycleus lunatus*, which was received from South Africa.

3.3.1 Male Specimens

D₂C was recorded every two hours up to the sixth hour in all treatments indicating a slight increasement in D₂C titre of the testis (fig. 42). In this way, the median value increased from 3500 ng/mg dry weight after two hours to about 4600 ng/mg DW after 6 hours; however neither Kruskal-Wallis ANOVA nor Median test indicates any significant difference for 5 replications at 95% confidence level. Studying the behaviour of the vas deferens and epididimis over the same period of time indicates an obvious reduction in D₂C in these two parts of male sexual organ (fig. 43). The accumulated D₂C fell sharply from the median value of about 3750 ng/mg to about 1250 ng/mg DW in 4 hours and remained at nearly the same level after 6 hours upon 5 replications. The same trend is observable in the accessory glands in which the level of D₂C (median value) fell from over 4000 ng/mg DW to about 1500 and 500 ng/mg DW within 4 and 6 hours respectively (fig. 44). That shows a net decrease of about 3500 ng/mg DW in median value within 4 hours.

In an interesting part of the *Ringer experiment*, the whole male abdomen consisting of sexual organs, complete digestive system, fat bodies and external genitalia was dissected and placed into the Ringer solution to examine the quantity fluctuations of D₂C in the whole system when different organs were in their natural positions. Therefore we had to take sample from the Ringer solution every two hours up to the sixth hour to record the gradual changes in D₂C quantity by time. In this way, the measured titre of D₂C changed from 25 ng/μl of solution to about 35 ng/μl within 4 hours (fig. 45). Although this is only a trend and did not show any significant difference via Kruskal-Wallis ANOVA test over 5 replications at $P= 0.05$ level, considering the standard deviation of time histograms a tendency to D₂C increase after 6 hours is rather clear.

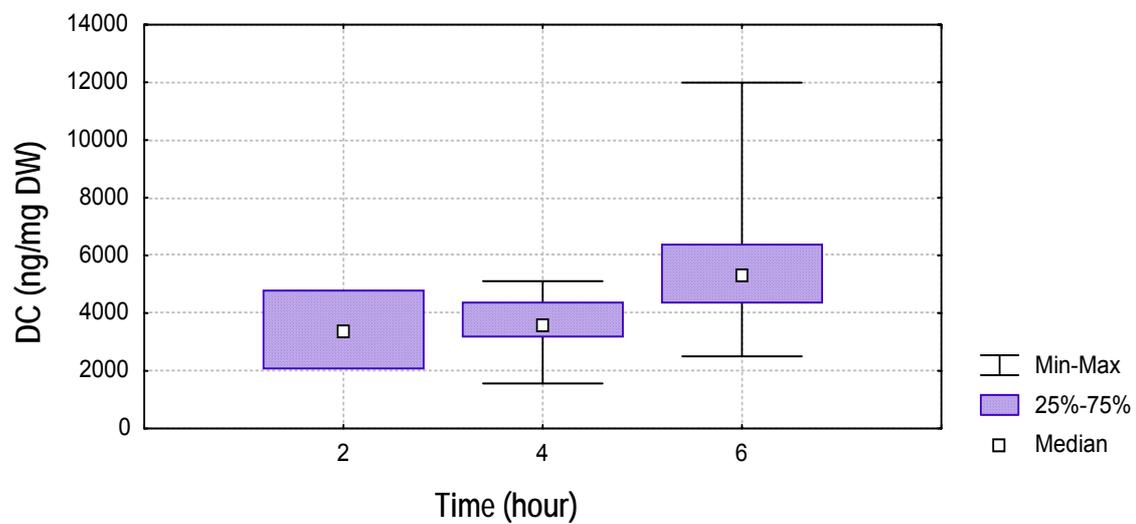


Fig. 42: D₂C titre in the testis of male *Hycleus lunatus* by time, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 5$

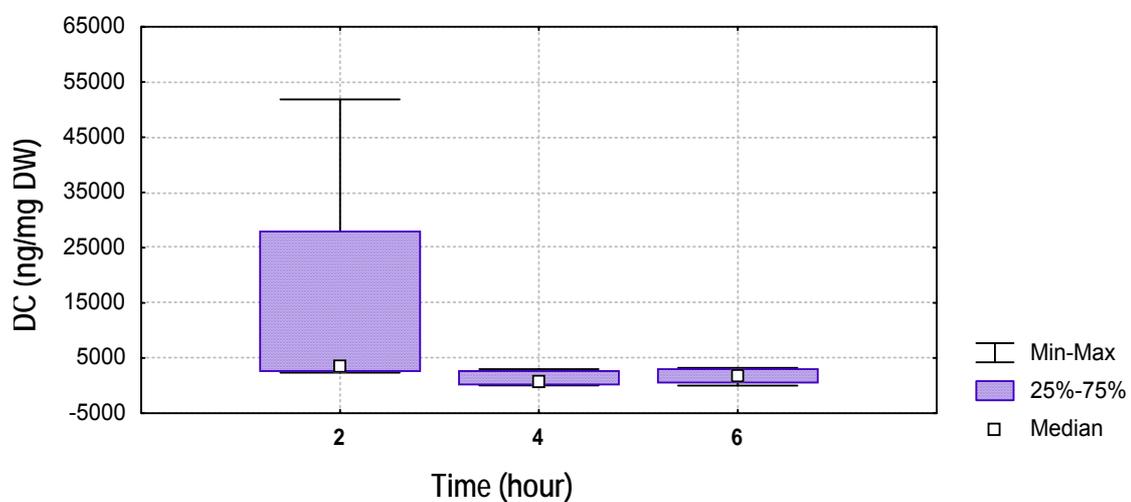


Fig. 43: D₂C titre in the vas deferens and epididimis of male *Hycleus lunatus* by time, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 5$

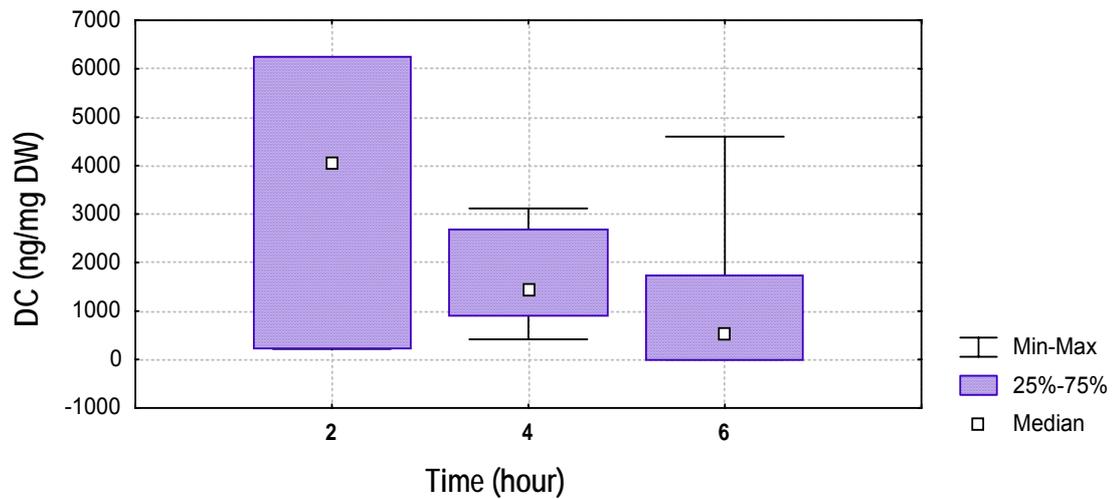


Fig. 44: D₂C titre in the accessory glands of male *Hycleus lunatus* by time, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 5$

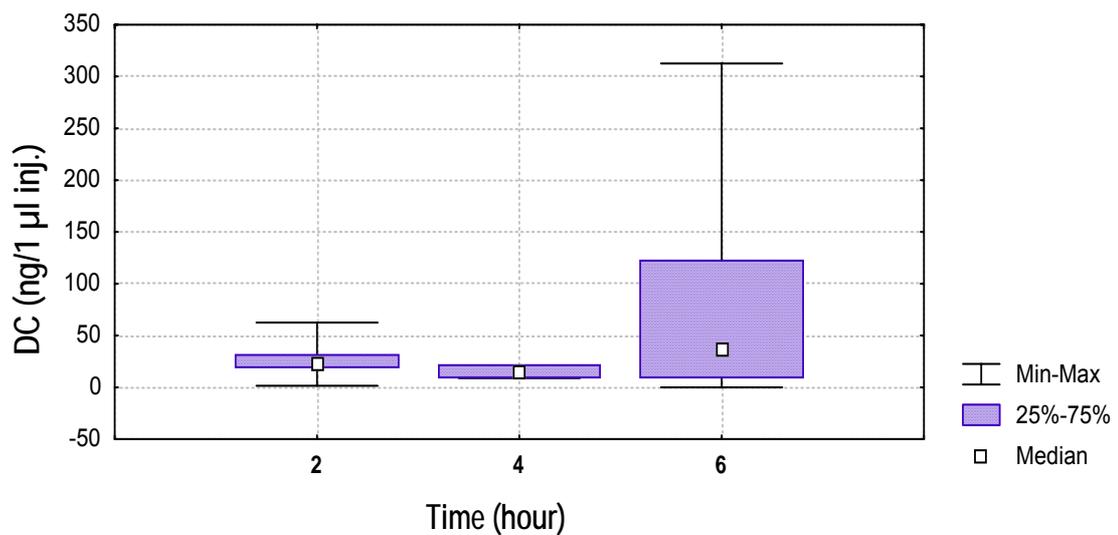


Fig. 45: D₂C titre of Ringer solution in which the whole abdomen of male *Hycleus lunatus* had been placed, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 5$

3.3.2 Female Specimens

As seen for the males of *Hycleus lunatus*, in this series of experiments different parts of female sexual organs were dissected and then placed into Ringer solution along with D₂C to assess the absorption capacity of all these parts independently by time. Samples were taken every two hours up to the sixth hour after the experiment starting time. D₂C was added into the Ringer solution, so no marker was at first present in beetles' tissue. This fact made it possible to study absorption activity of marker for all these tissues. This part of experiments has been shown by the unit of ng/mg of the tissue dry weight. Besides, liquid samples were taken simultaneously to control if any changes in the original quantity of added marker occurred. This not only explains the chemical modification of the solution, but confirms what had been already reported from the tissue itself. These experiments have been shown by the unit of ng/1 µl of injection.

In addition, in another group of experiments the whole female abdomen was placed in Ringer solution plus the marker in order to understand the performance of the whole system together. Time windows of two hours helped us to get the sequential portrait of D₂C fluctuations within the beetle abdomen.

Studying the bursa copulatrix as part of the female sexual organ gives an active up-take of the marker which increased from the median value of 1600 ng/mg DW to about 2700 ng/mg DW within 4 hours (fig. 46). Although the Median test does not show any significant difference among the time sequence of this experiment, D₂C incorporation into the bursa indicates a clear increasing trend. On the other hand solution sampling of bursa reflects continuous reduce in the marker reservoir of Ringer solution (fig. 47) which has been confirmed by Kruskal-Wallis ANOVA test upon 5 replications at the 95% confidence level.

Observing D₂C exchange between eggs and ovaries expresses a steady reduction in the level of marker in the tissue (fig. 48) and a continuous increase in the titre of marker within the solution over the time (fig. 49). While the ovaries and eggs had at first an accumulated level of about 2200 ng/mg DW (median value), it decreased to less than 1000 ng/mg DW within just 4 hours. Meanwhile solution experienced an increase of median value within the same time interval which was recorded from 8 ng/µl injection to around 25 ng/µl. The same model has been repeated for the receptacle which shows continuous decrease in D₂C titre of the tissue itself (fig. 50) and evident increase of the marker within the solution (fig. 51). Although these changes are illustrated clearly in the mentioned figures, none shows a statistically significant difference.

Whole abdomen experiments upon 8 replications provided data which indicate an obvious increase in D₂C level of the solution (fig. 52) which exceeded over 150 ng/µl injection within four hours (median value).

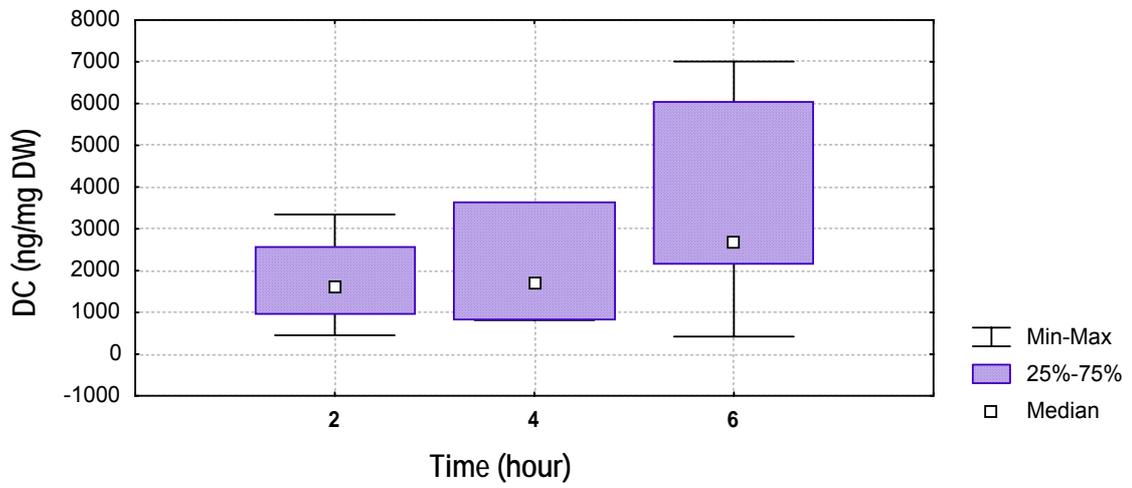


Fig. 46: D₂C titre in the bursa copulatrix of female *Hycleus lunatus* by time, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 5$

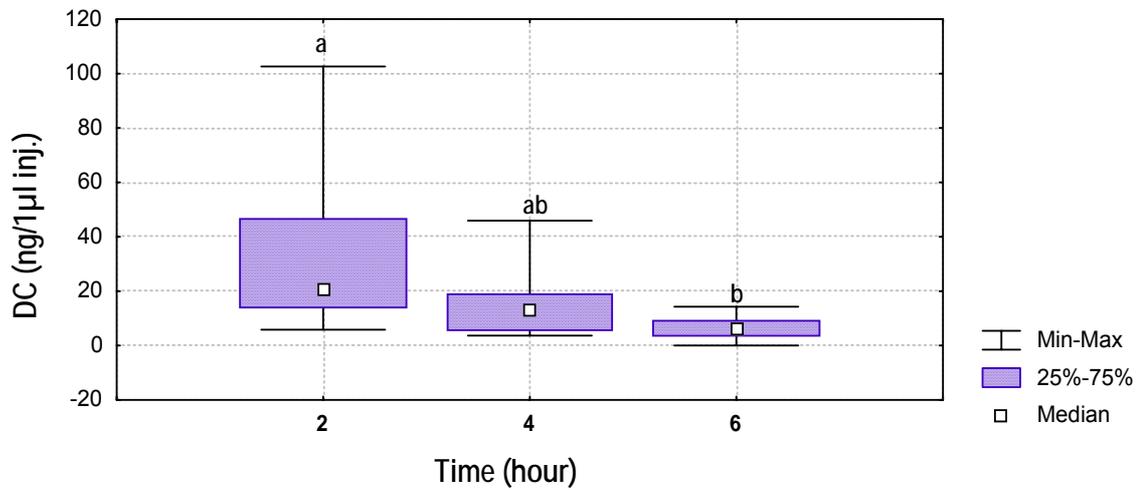


Fig. 47: D₂C titre in Ringer solution in which the bursa copulatrix had been placed, female *Hycleus lunatus*, time dependent experiment, Kruskal-Wallis ANOVA test, $P < 0.05$, $n = 7$

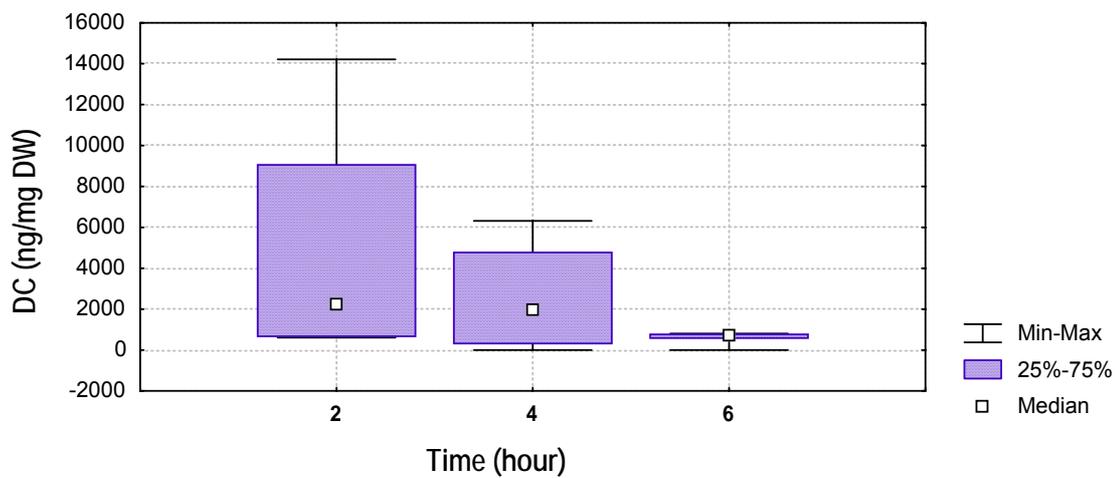


Fig. 48: D₂C titre in eggs and ovaries of female *Hycleus lunatus* by time, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 5$

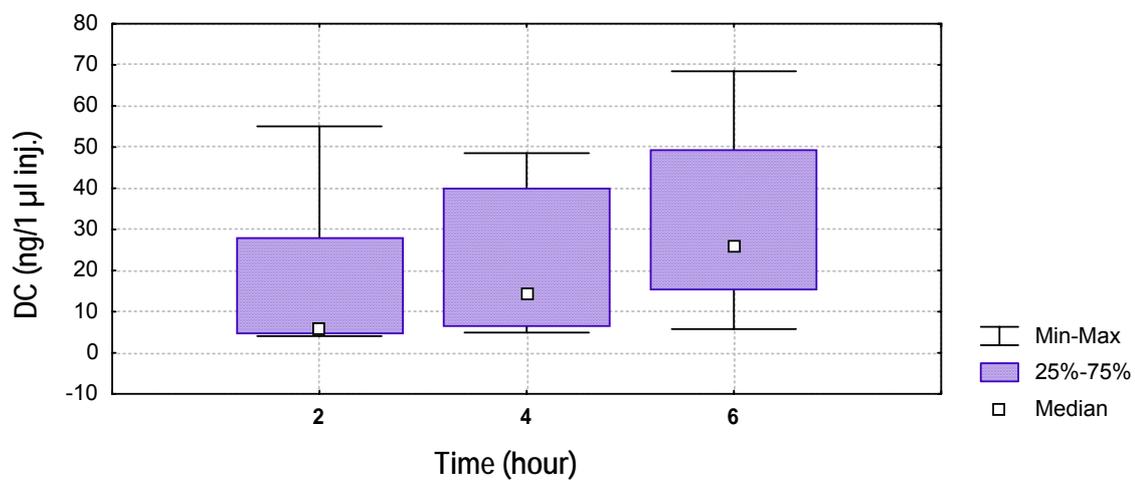


Fig. 49: D₂C titre in Ringer solution in which eggs and ovaries had been placed, female *Hycleus lunatus*, time dependent experiment, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 8$

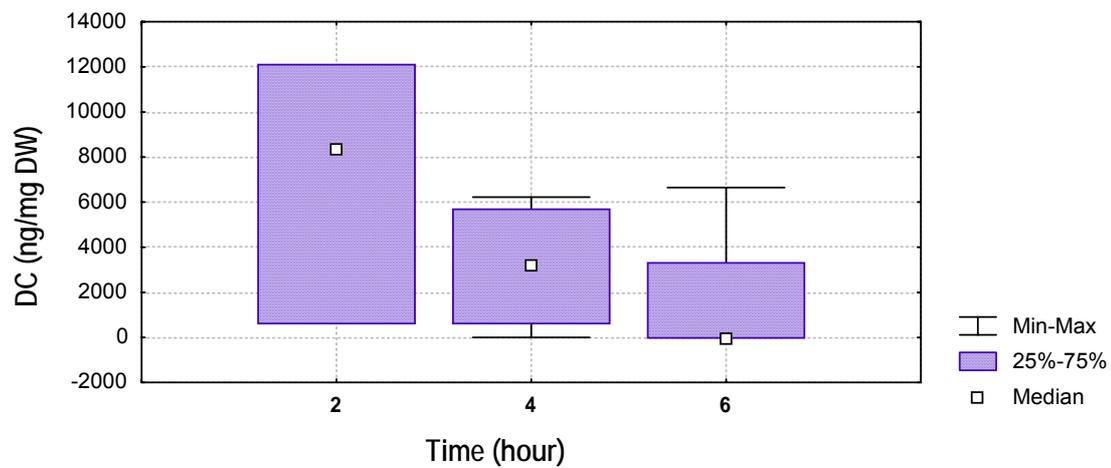


Fig. 50: D₂C titre in the receptacle of female *Hycleus lunatus* by time, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 4$

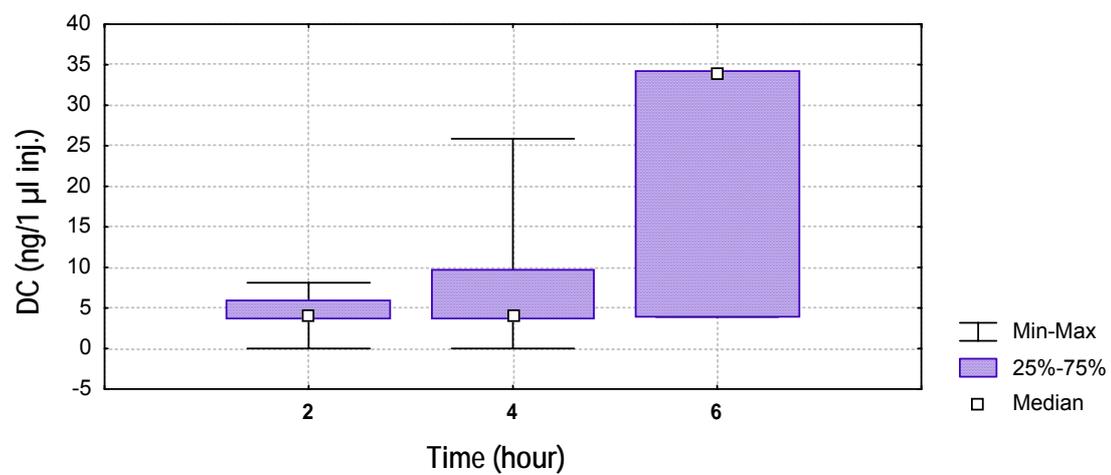


Fig. 51: D₂C titre in Ringer solution in which the receptacle had been placed, female *Hycleus lunatus*, time dependent experiment, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 6$

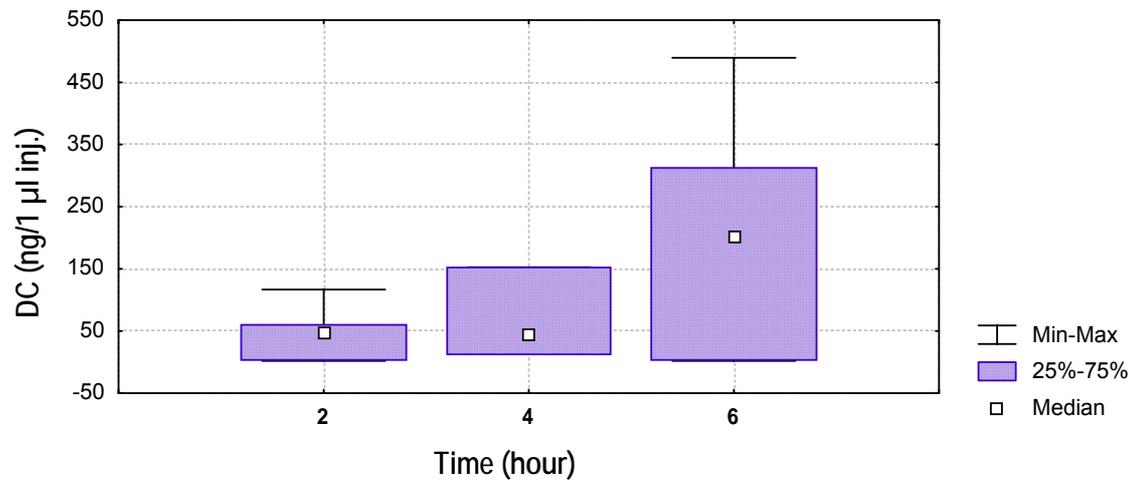


Fig. 52: D₂C titre of Ringer solution in which the whole abdomen of female *Hycleus lunatus* had been placed, Kruskal-Wallis ANOVA test, $P > 0.05$, $n = 8$

3.4 *Cyaneolytta* sp.: A Chemical Approach to the Leg and Antennal Morphology of an East African Species

It has been known since long ago that cantharidin is found throughout different tissues and body fluid of blister beetles particularly in the sexual organs of both sexes. In all external appendages of blister beetles collected from South Africa, southern France or Iran, extremely low amounts of cantharidin was found which was not comparable to the sexual organs of the same species, however the East African species of *Cyaneolytta* sp. (fig. 60), showed a totally different result. There is extremely high amount of cantharidin occurring in the antennae and legs of the both sexes, which is not only comparable to sexual organs but sometimes even higher (fig. 53). This phenomenon which is reported for the first time is indicated in table 11.

Table 11: Studied species of blister beetles (Col: Meloidae) and cantharidin distribution

Species	Geographical location	No. of Replications ¹	Cantharidin ² (♂)	Cantharidin ³ (♀)
<i>Hycleus lunatus</i>	South Africa	4	EL ⁴	EL
<i>H. polymorphus</i>	Southern France	4	EL	EL
<i>Mylabris quadripunctata</i>	Southern France	4	EL	EL
<i>M. impressa</i>	Western Iran	4	EL	EL
<i>M. Schreibersi</i>	Western Iran	2	EL	EL
<i>M. variabilis</i>	Western Iran	4	EL	EL
<i>M. guerini</i>	Western Iran	4	EL	EL
<i>Lydoceras bilineatus</i>	Western Iran	2	EL	EL
<i>Croscherichia</i> sp.	Western Iran	2	EL	EL
<i>Alosimus smyrnensis</i>	Western Iran	4	EL	EL
<i>Muzimes iranicus</i>	Western Iran	4	EL	EL
<i>Calydos alloushei</i>	Western Iran	2	EL	EL
<i>Cyaneolytta</i> sp. ⁵	East Africa	3	EH ⁶	VH ⁷

1. Number of replication per sex

2. Cantharidin titre in antennal or leg segments of male specimens

3. Cantharidin titre in antennal or leg segments of female specimens

4. Extremely Low, 200- 1000 folds less than sexual organs

5. Coleoptera: Meloidae (Meloinae, Meloini)

6. Extremely High, about the same titre as sexual organs

7. Very High, 3- 4 folds less than male of the same species

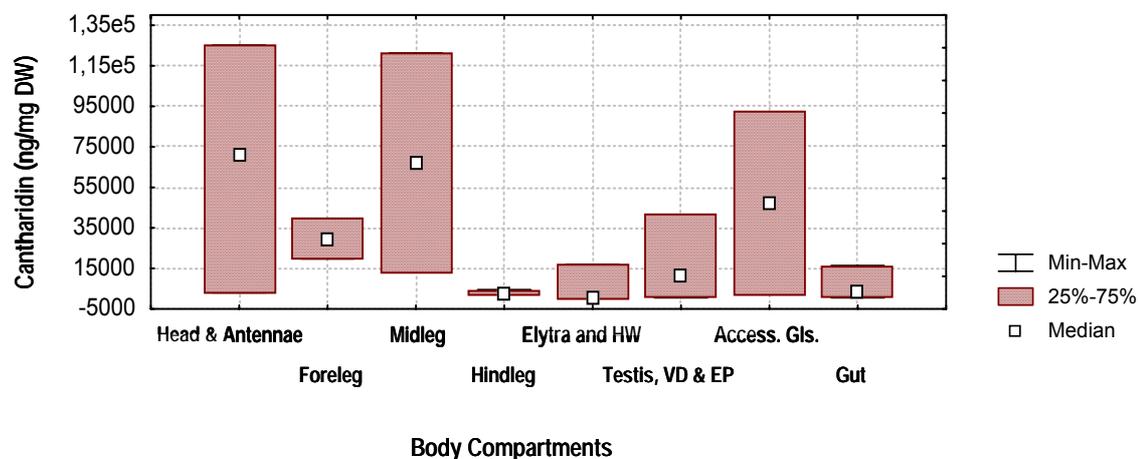


Fig. 53: Cantharidin titre in external and internal organs of male *Cyaneolytta* sp., Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

3.4.1 Male Antennae

As shown in figure 53, there is huge amount of cantharidin which has been stored in the legs, head capsule and antennae of male specimens of *Cyaneolytta*.

To get a better understanding on how cantharidin is distributed in the external appendages of *Cyaneolytta*, D₂C was injected into beetles of both sexes as in section 2.5. D₂C results which are in agreement with cantharidin have been summarized for the males in fig. 54 and confirms the presence of extraordinary titre of D₂C in antenna and the fore, meso and meta legs of the beetle. Analysing of head capsule and antennae separately, it was found that the most compound accumulates in the antennae while head capsule bore much less (20% of antenna). Incorporated D₂C has its highest titre first in antennae and thereafter in the legs, while the titre decreases from the first to the third pair of legs regularly (fig. 55).

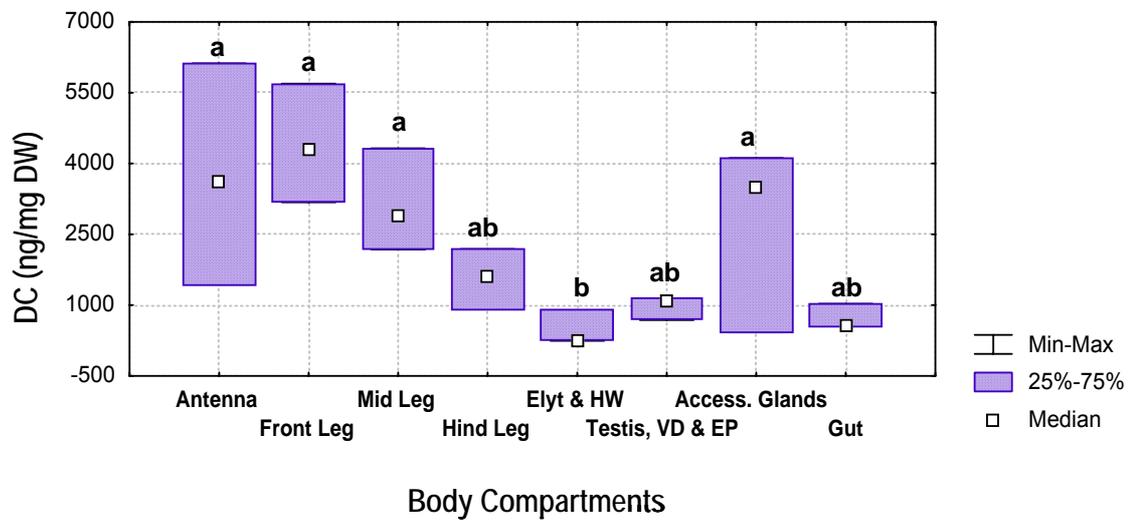


Fig. 54: D₂C titre in external and internal organs of male *Cyaneolytta* sp. after being injected by 10 µl D₂C solution, Kruskal-Wallis ANOVA Test, $P < 0.05$, $n = 3$

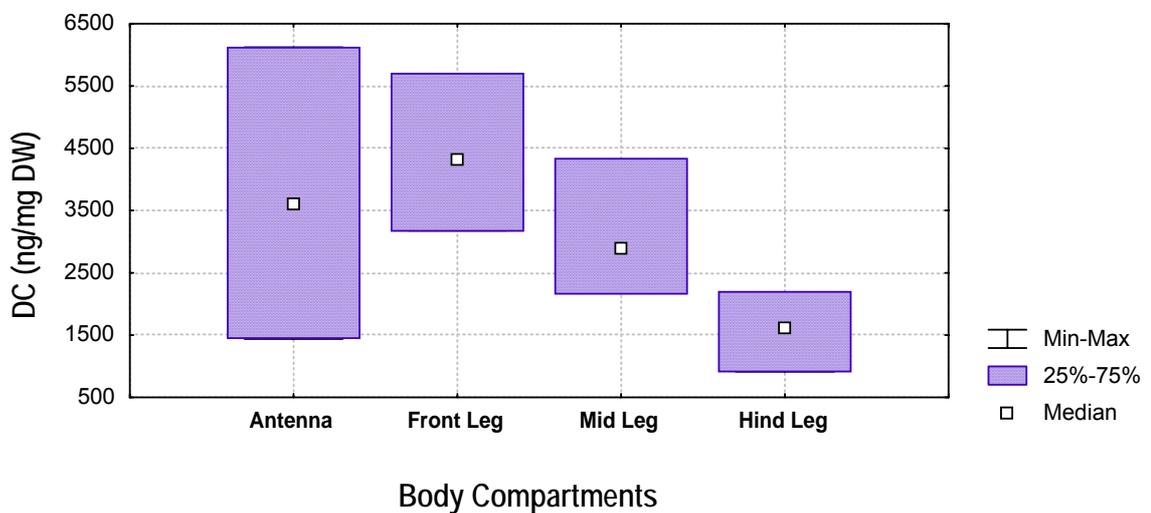


Fig. 55: D₂C titre in external appendages of male *Cyaneolytta* sp. after being injected by 10 µl D₂C solution, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

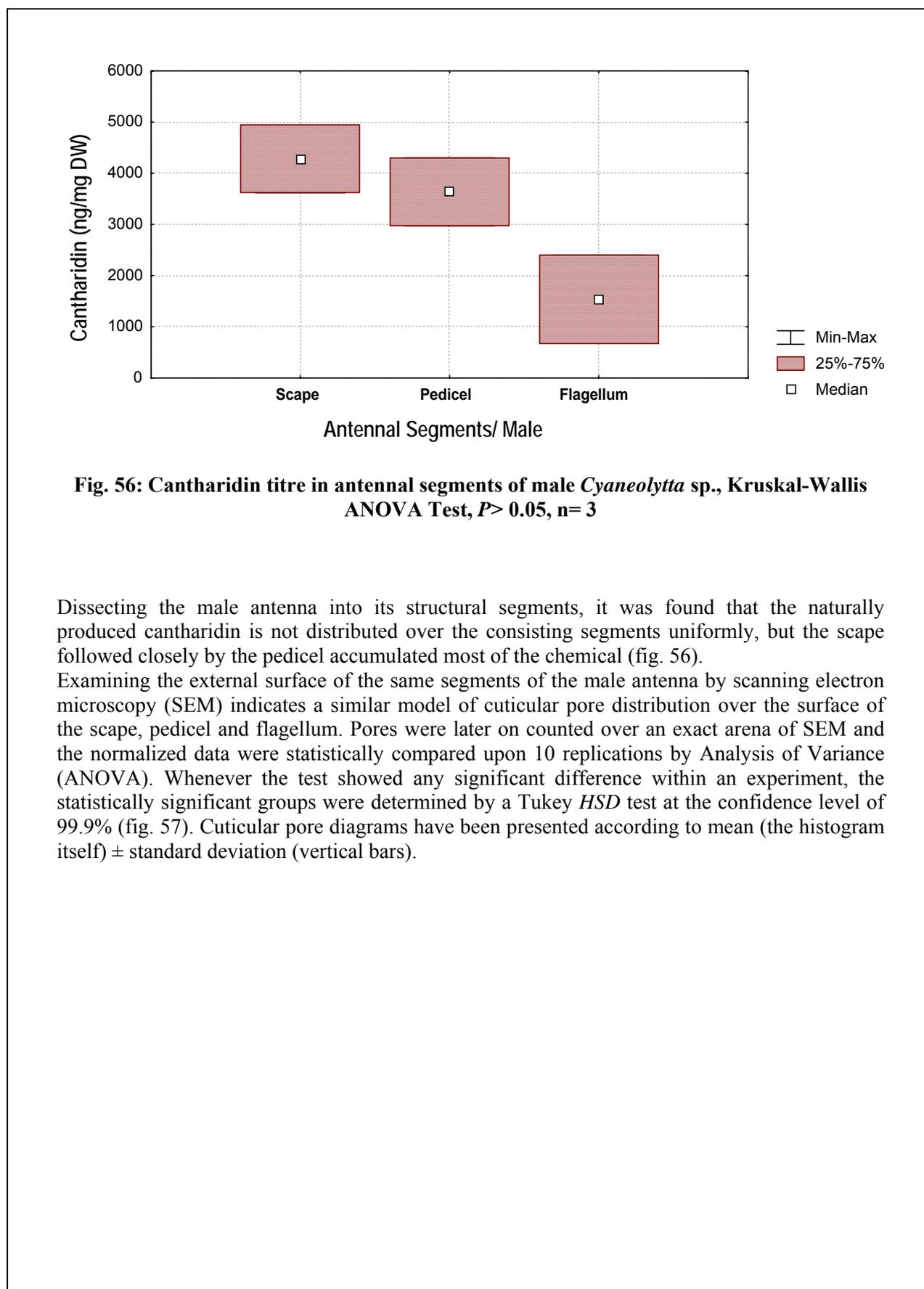


Fig. 56: Cantharidin titre in antennal segments of male *Cyaneolytta* sp., Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

Dissecting the male antenna into its structural segments, it was found that the naturally produced cantharidin is not distributed over the consisting segments uniformly, but the scape followed closely by the pedicel accumulated most of the chemical (fig. 56).

Examining the external surface of the same segments of the male antenna by scanning electron microscopy (SEM) indicates a similar model of cuticular pore distribution over the surface of the scape, pedicel and flagellum. Pores were later on counted over an exact arena of SEM and the normalized data were statistically compared upon 10 replications by Analysis of Variance (ANOVA). Whenever the test showed any significant difference within an experiment, the statistically significant groups were determined by a Tukey *HSD* test at the confidence level of 99.9% (fig. 57). Cuticular pore diagrams have been presented according to mean (the histogram itself) \pm standard deviation (vertical bars).

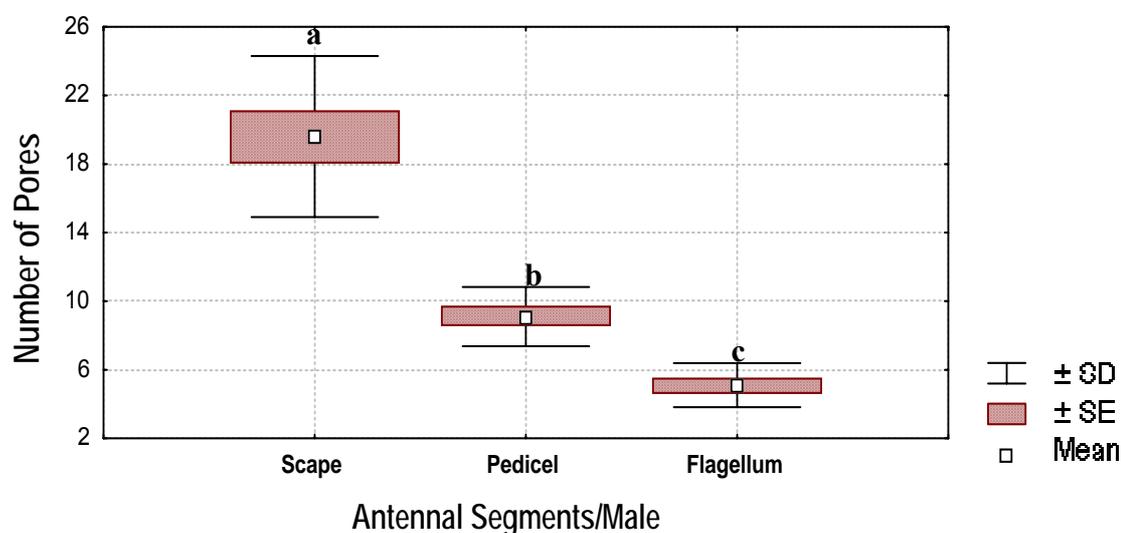


Fig. 57: Number of cuticular pores per 24.5 μm of SEM visible field, male *Cyaneolytta* sp. ANOVA, Tukey *HSD*-Test, $P < 0.001$, $n = 10$

The first two segments of antennae in *Cyaneolytta* are reddish in colour (fig. 58) which is a good landmark for the naked eye observations. The scattered cuticular pores on the male scape have been illustrated in fig. 59 based on the SEM photography. Similarly, SEM photograph of the cuticular pore distribution over the surface of the pedicel are displayed in fig. 60 at the same magnification level.

To understand the function of such pores, a semi thin cross section was prepared from the male antennal segments and the stained tissues were observed using light microscopy at 400 fold magnification. Cross section of male antennal scape has been for instance indicated in fig. 61. According to this sectioning and also the similar one of the pedicel, there are lots of tubular structures which have been stretched from the surface of integument to the hemolymph current of the scape and pedicel segments.

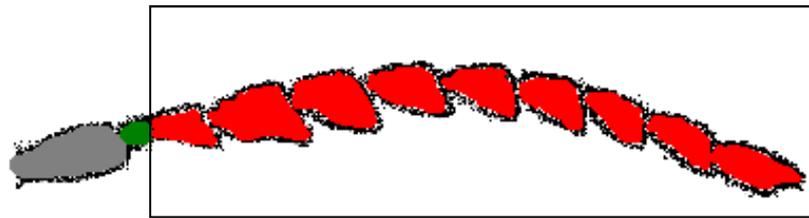


Fig. 58: Schematic view of antennal segments of *Cyaneolytta* sp., the 1st segment, the scape, indicated in grey, the 2nd one, the pedicel, in green and all other antennomeres which form the flagellum are indicated in red colour

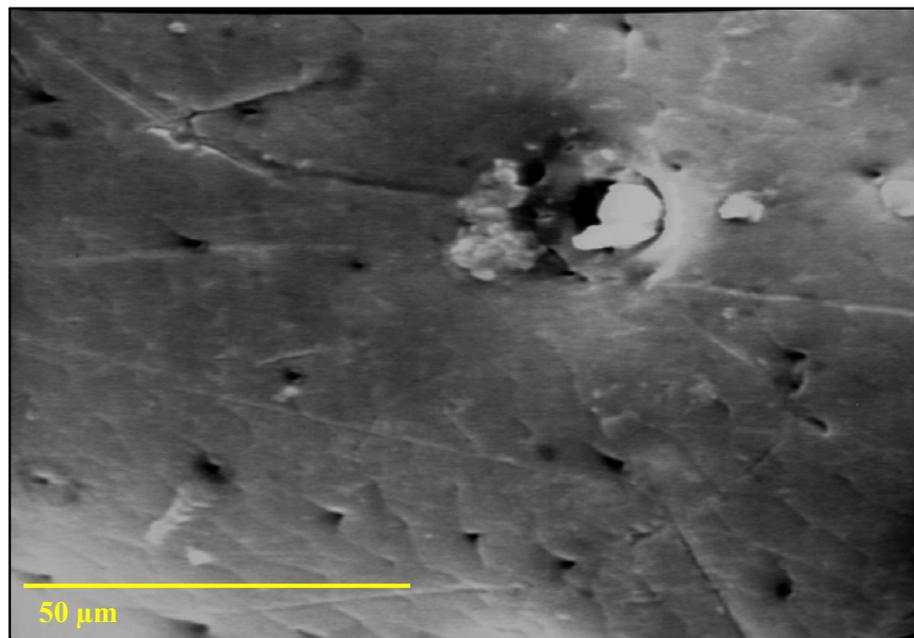


Fig. 59: SEM of cuticular pores on the scape of male *Cyaneolytta* sp., 895 X

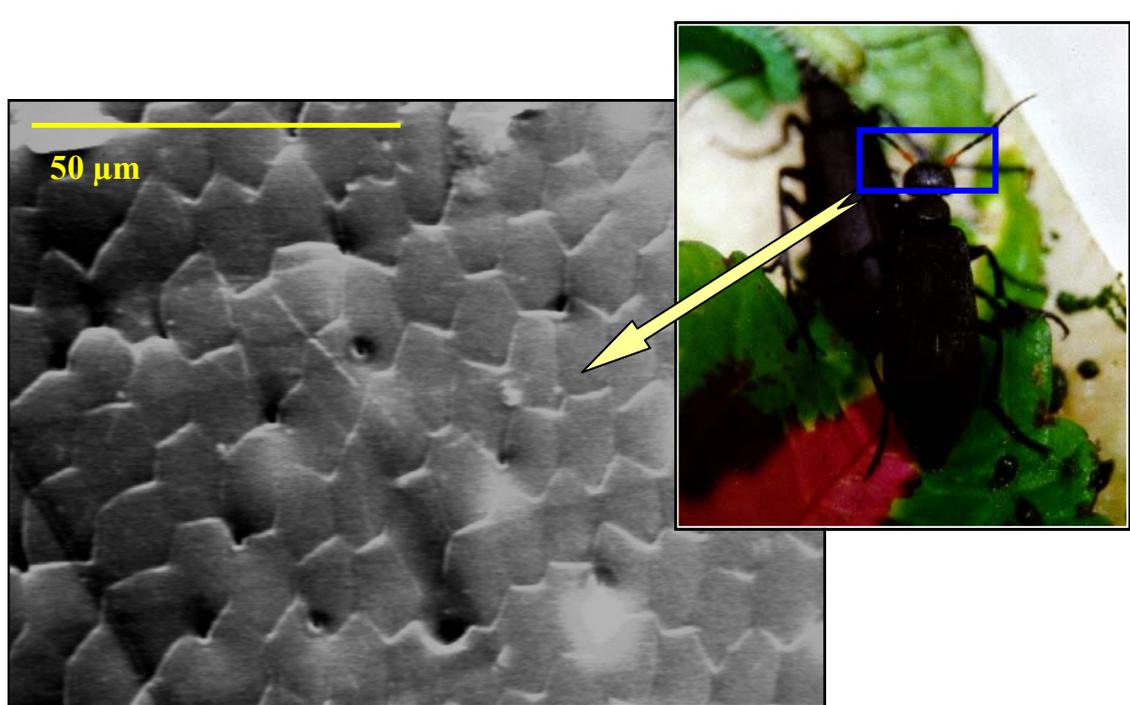


Fig. 60: SEM of cuticular pores on the pedicel of male *Cyaneolytta* sp., 895 X

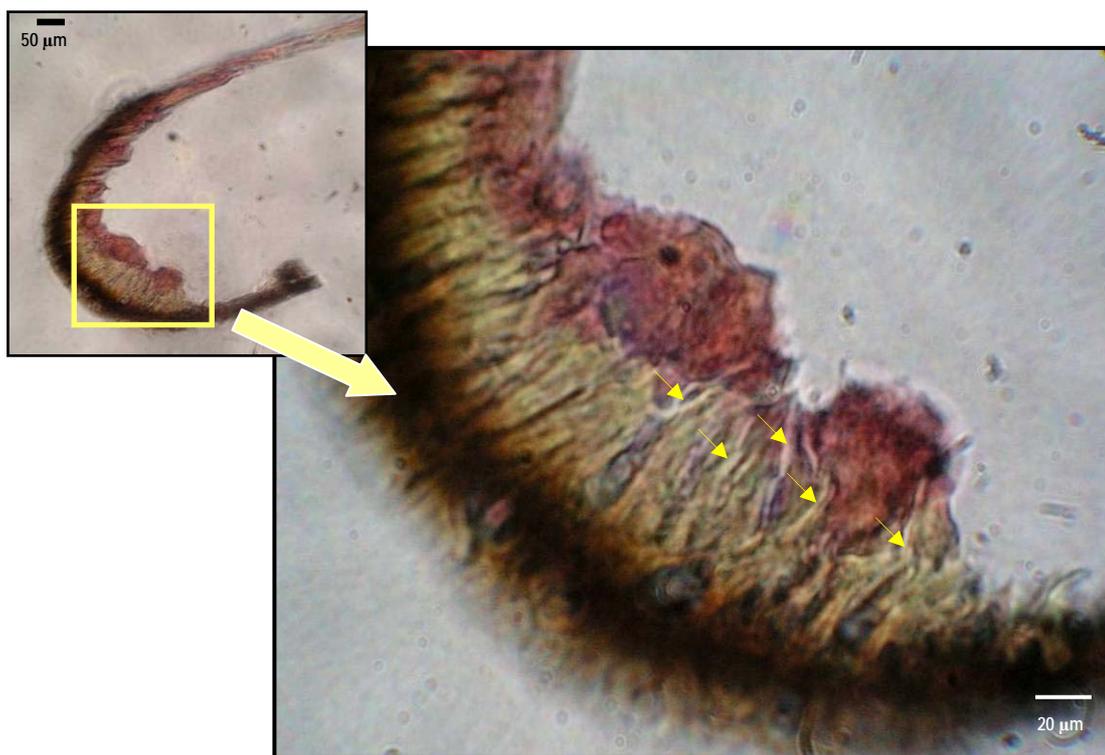


Fig. 61: Cross section of the scape segment (male *Cyaneolytta* sp.), canal shape structures (tubular cells) have been indicated by arrows

3.4.2 Male Legs

In this section, cantharidin distribution within different segments of the three pair of legs has been studied to see if there is any relationship between the density of cuticular pores per surface unit and the incorporated titre of cantharidin within the referred segments. Unfortunately, it was not possible to do the same measurement for D₂C on male legs and antennal segments because of limited insect material (*Cyaneolytta* sp.). All materials used in these series of experiments had been kindly sent to us in limited number by Dr. Claudia Hemp who collected them from Karen, Nairobi, Kenya. Injecting of D₂C requires living beetles, while no more specimen was available to do so.

Cantharidin distribution over the main segments of male foreleg indicates a similar model to cuticular pore density of the same segments. Cantharidin was mostly found in the femur (7300 ng/mg DW for median value) and the tibia (3300 ng/mg DW for median value) of the foreleg which its titre is higher than that of the three other segments of coxa, trochanter and tarsus (fig. 62). Of course, statistical analysis does not show any significant difference among the experimental classes which may be partly to limited number of replications. Interestingly, cuticular pore density of the above mentioned segments revealed very similar pattern in which the femur and tibia bear more pores per measurement unit (24.5 μm); hence using ANOVA and Tukey *HSD*-Test as posthoc, they are placed significantly higher than three other segments at the confidence level of 99.9% (fig. 63).

Figs. 64 and 65 which are based on SEM view, indicating cuticular pores of the femur and tibia of the male foreleg respectively.

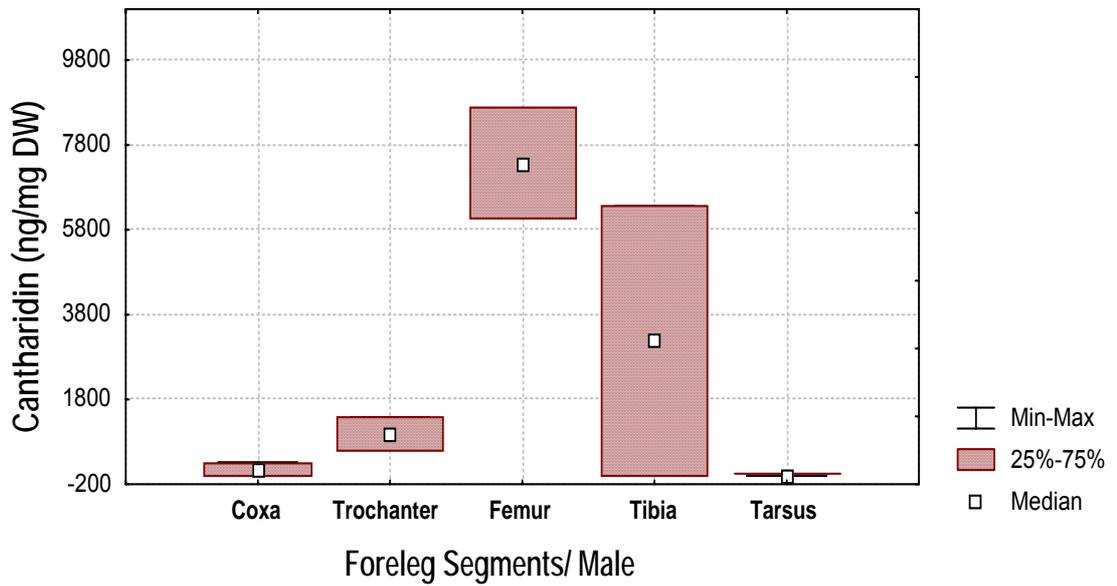


Fig. 62: Cantharidin titre in the foreleg segments of male *Cyaneolytta* sp., Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

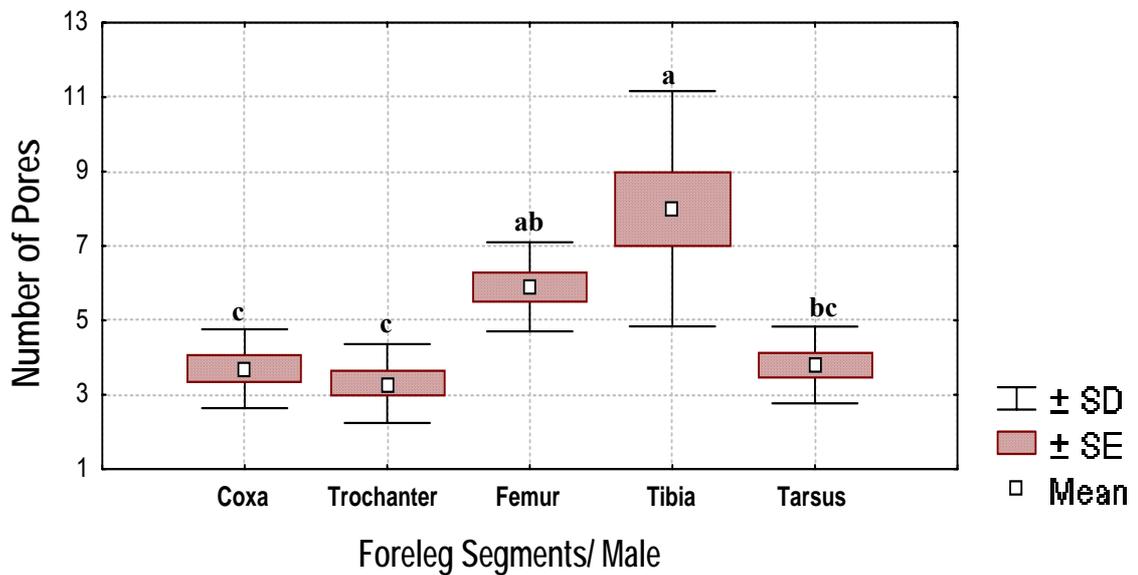


Fig. 63: Number of cuticular pores per 24.5 µm of SEM visible field, male *Cyaneolytta* sp. ANOVA, Tukey *HSD*-Test, $P < 0.001$, $n = 10$

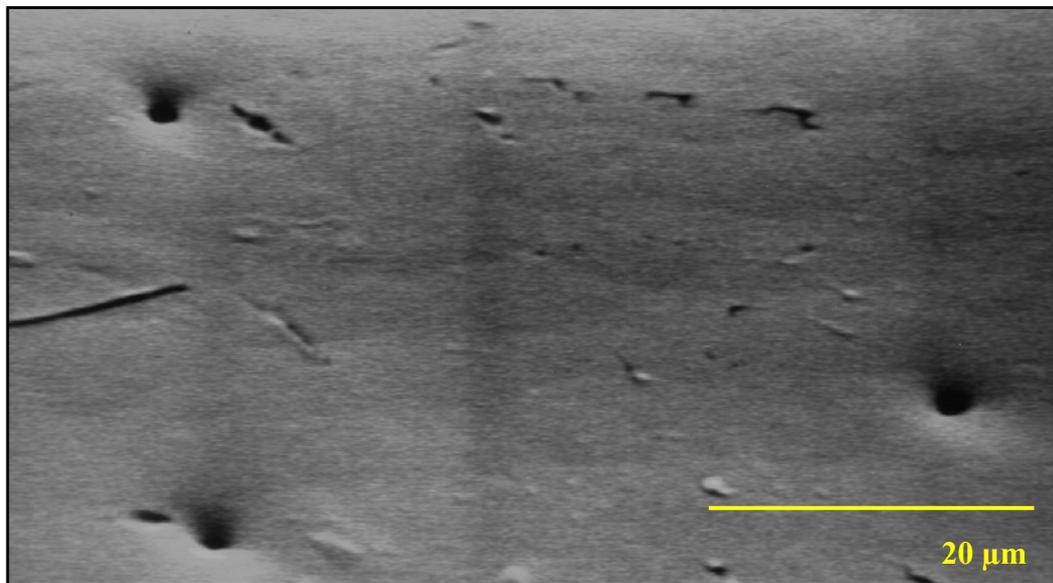


Fig. 64: SEM of cuticular pores on the foreleg femur of male, *Cyaneolytta* sp., 1570 X

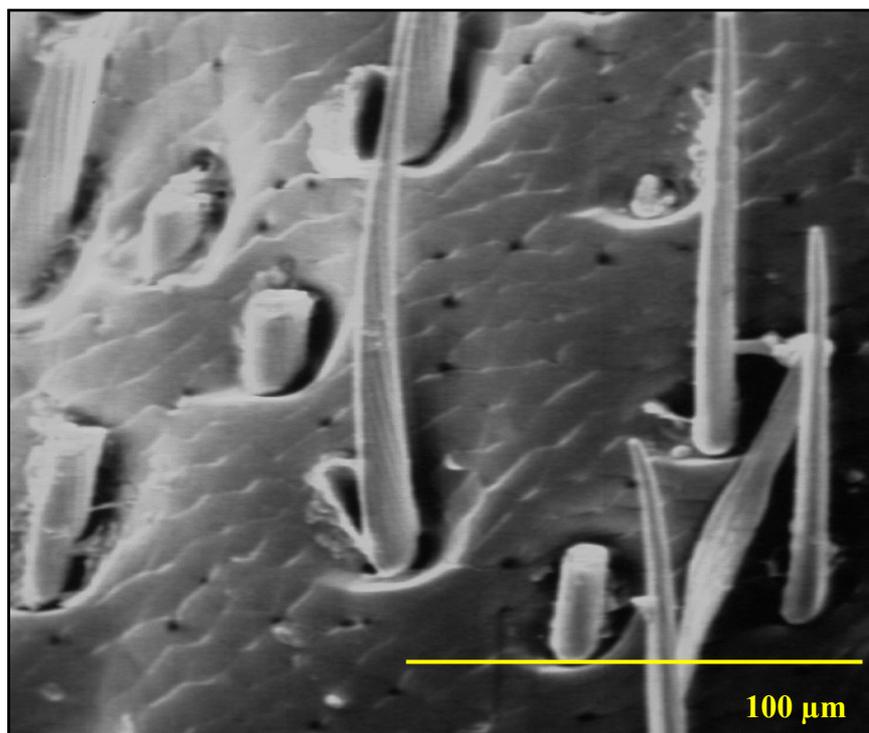


Fig. 65: SEM of cuticular pores on the tibia of male foreleg, *Cyaneolytta* sp., 409 X

There is no relationship between cantharidin incorporation and the cuticular pore density of the midleg segments. As fig. 66 indicates, cantharidin has been mostly accumulated in the coxa, femur and tibia while there is no significant difference of cuticular pore density among the midleg segments (fig. 67).

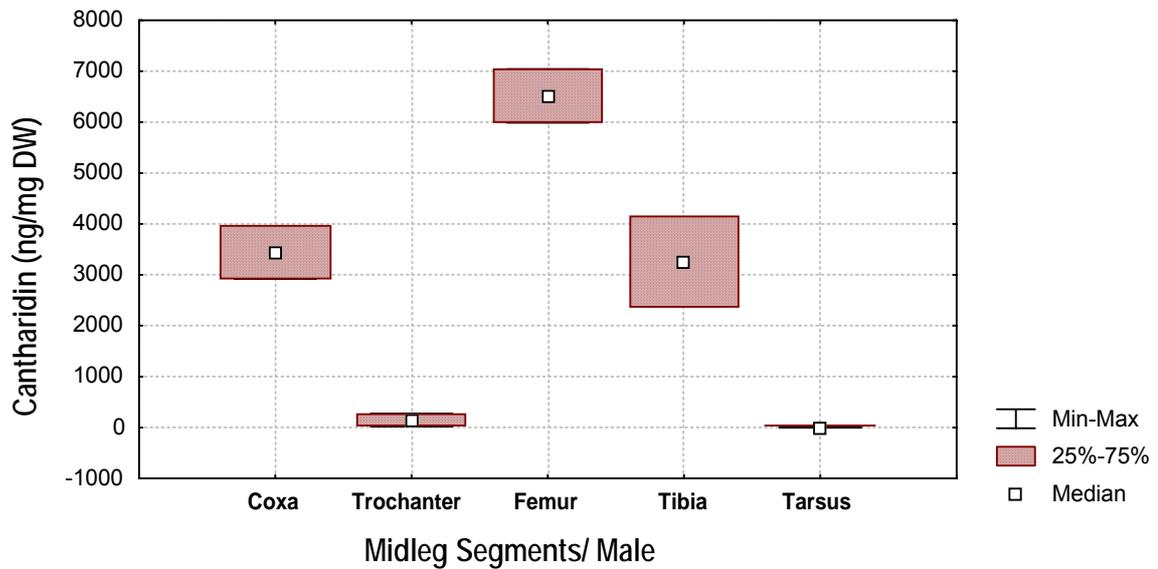


Fig. 66: Cantharidin titre in the midleg segments of male *Cyaneolytta* sp., Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

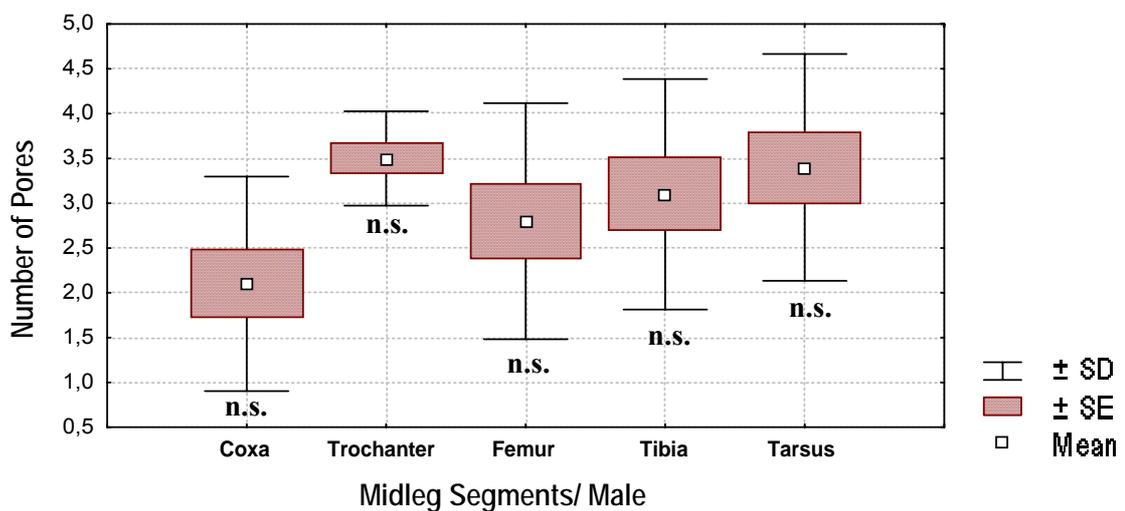


Fig. 67: Number of cuticular pores per 24.5 µm of SEM visible field, male *Cyaneolytta* sp. ANOVA, $P > 0.05$, $n = 10$

Similar to the midleg, the hindleg segments indicate no similarity between cantharidin incorporation and cuticular pore density. While much of the chemical accumulated in the femur comparison to the other hindleg segments (fig. 68), it does not bear more pores per measurement unit (fig. 69). In fact there is no significant difference among all segments of the midleg for cuticular pore density and they are more or less similar to each other.

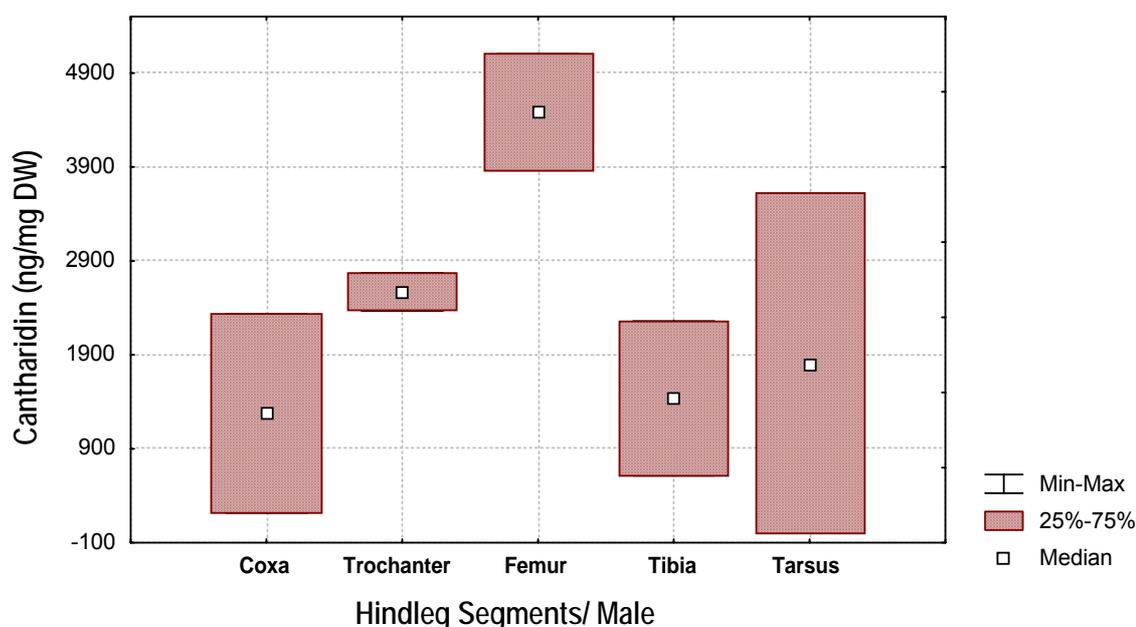


Fig. 68: Cantharidin titre in the hindleg segments of male *Cyaneolytta* sp., Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

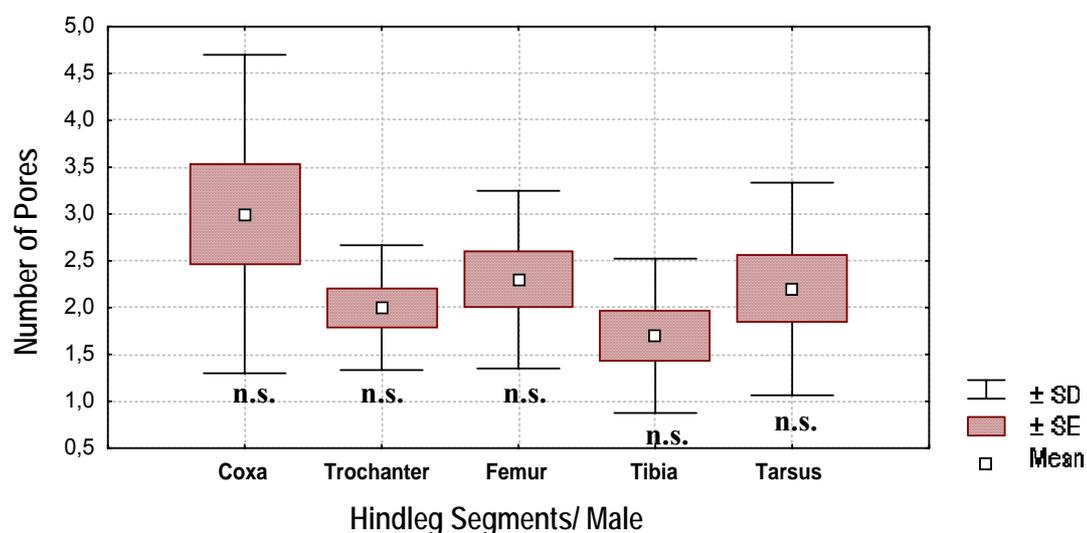


Fig. 69: Number of cuticular pores per 24.5 µm of SEM visible field, male *Cyaneolytta* sp. ANOVA, $P > 0.05$, $n = 10$

3.4.3 Female Antennae

In order to examine cantharidin distribution within female antennal segments, D₂C was injected into the beetles' hemocoel (the same way as section 3.1). Fig. 70 indicates how the marker incorporated into head and antennal segments within a day. It is clearly seen that similar to male specimens, much of the marker compound accumulated in the scape and pedicel, however the difference was not too big to be proved by statistical analysis at the 95% confidence level (Kruskal-Wallis ANOVA). On the other side, cuticular pore density of antenna indicates a significant difference among the main three segments. In this way, the scape and pedicel show higher number of pores per measurement unit of 24.5 μm although there is no significant difference between these two segments themselves (fig. 71).

Fig. 72 shows SEM of antennal pedicel with 1250 folds magnification in which cuticular pores and their openings are clearly observed. Similar to the male antenna, a cross section was also prepared from the female antenna and the stained segments were being observed in search for any probable tubular structures using a light microscope at 400 folds magnification. None of the segments showed such a structure which can be for example seen in fig. 73 for the pedicel.

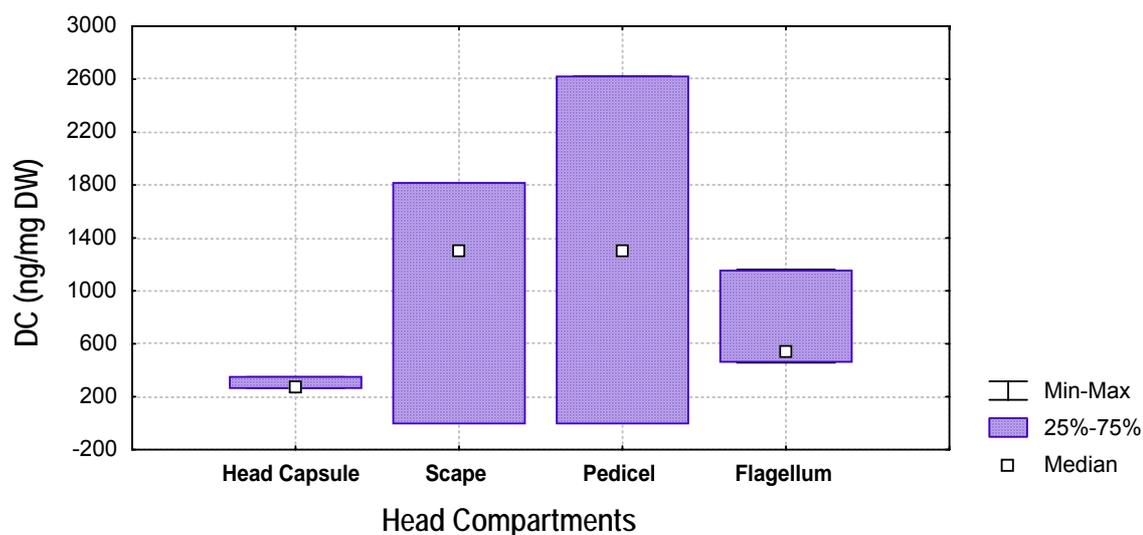


Fig. 70: D₂C titre in head capsule and antennal segments of female *Cyaneolytta* sp., Injected by 10 μl D₂C solution, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

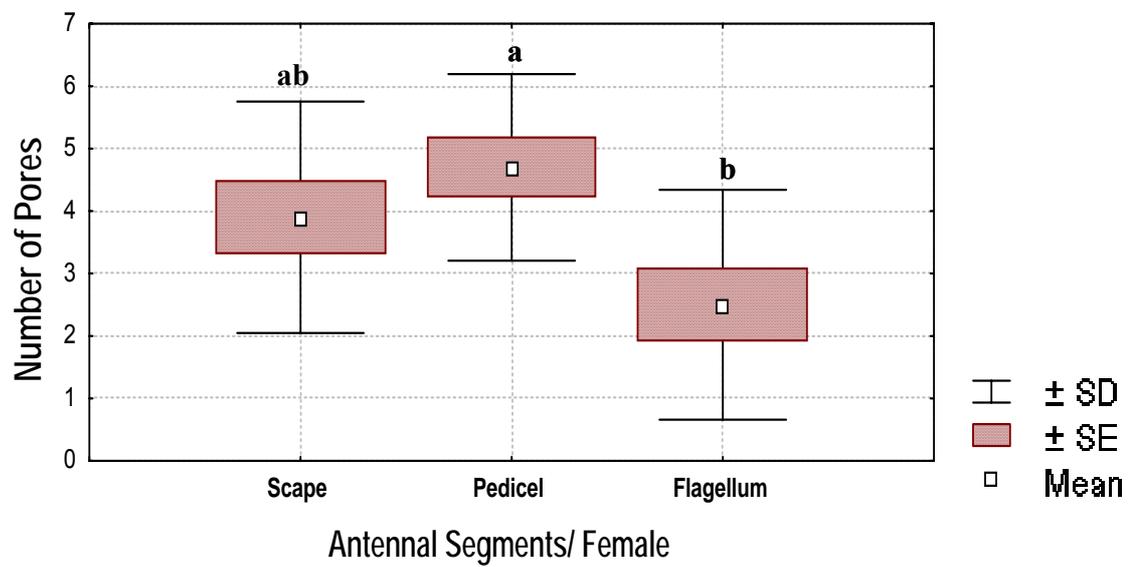


Fig. 71: Number of cuticular pores per 24.5 μm of SEM visible field, female *Cyaneolytta* sp. ANOVA, Tukey *HSD*-Test, $P < 0.05$, $n = 10$

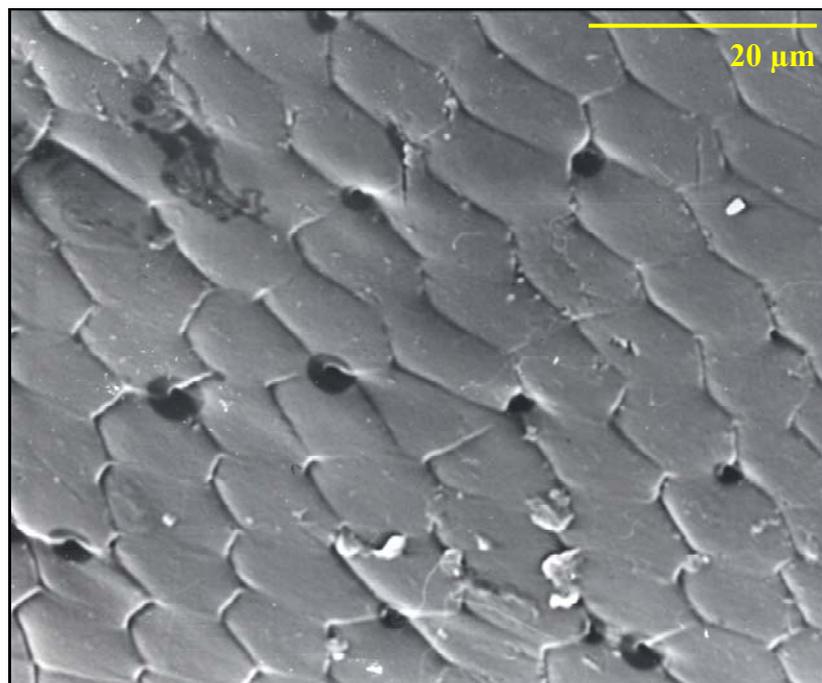


Fig. 72: SEM of cuticular pores on the pedicel of female antennae, *Cyaneolytta* sp., 1250 X



Fig. 73: The pedicel cross section, female antenna, *Cyaneolytta* sp.: cells have not formed any canal or tubular structure

3.4.4 Female Legs

The first point considering all three pair of legs in female *Cyaneolytta* is the correlated behaviour of the two compounds of cantharidin and D₂C which has been often observed in the present study and reflected in the figures 74, 75, 80 and 81. Therefore D₂C distribution within leg segments is also valid for the naturally produced chemical, cantharidin, as well and this is not offered separately. The second point refers to statistical tests (Kruskal-Wallis ANOVA) which never show any significant difference for cantharidin or D₂C distribution within leg segments; however there is an obvious trend which can be easily seen in the figures.

The more important point is searching for any harmony between D₂C distribution and cuticular pore density which has been shown in neither the foreleg nor the midleg. While D₂C has been mostly accumulated in the trochanter of the foreleg (fig. 75), it has the same density of pores per 24.5 μm of measurement as the femur, tibia and tarsus and even significantly less than the coxa (fig. 76). This comparison is even more interesting in the midleg where cuticular pore density over the midleg segments (fig. 79) follows neither cantharidin (fig. 77) nor D₂C distribution model (fig. 78). As the matter of fact, there is no significant difference among the pore density of the midleg segments using an ANOVA test at the confidence level of 95% (fig. 79). The female hindleg is the only leg in which a kind of harmony between D₂C distribution and cuticular pore density is observable. Considering the median value, D₂C is mainly found in the trochanter and tibia (fig. 81) on which cuticular pores have the higher density; however it is not significantly higher than the femur and tarsus using an ANOVA test at the confidence level of

99% upon 10 replications (fig. 82). This point has been also reflected in the cantharidin distribution diagram in which the femur has a closer median value to the trochanter and tibia than others (fig. 80). SEM photograph of the hindleg trochanter of a female *Cyaneolytta* has been shown in fig. 83 at 1050 fold magnification in which cuticular pores and their openings are clearly observable.

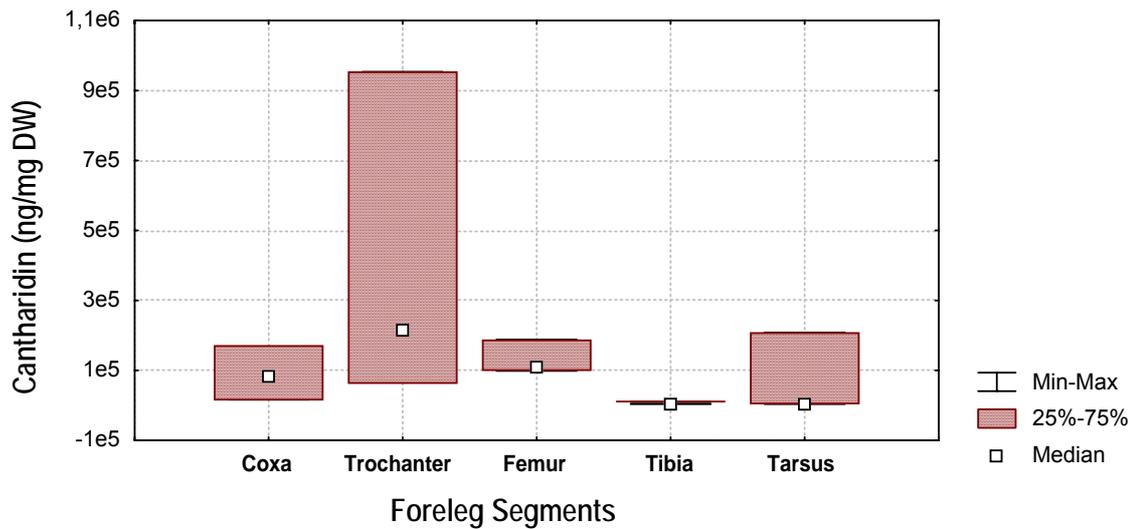


Fig. 74: Cantharidin titre in the foreleg segments of female *Cyaneolytta* sp., Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

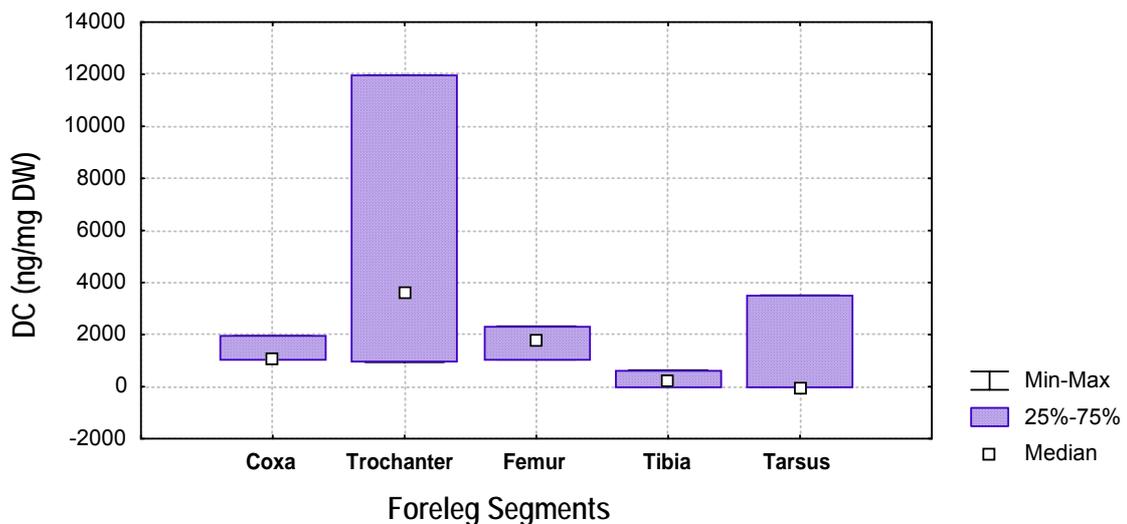


Fig. 75: D₂C titre in the foreleg segments of female *Cyaneolytta* sp., Injected by 10 µl D₂C solution, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

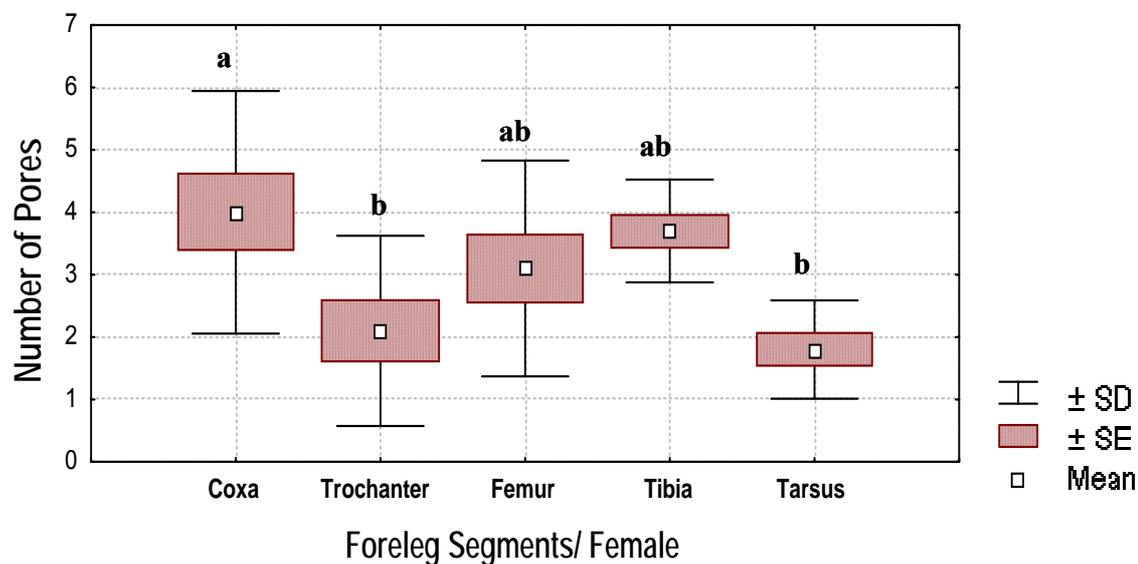


Fig. 76: Number of cuticular pores per 24.5 μm of SEM visible field, female *Cyaneolytta* sp. ANOVA, Tukey *HSD*-Test, $P < 0.01$, $n = 10$

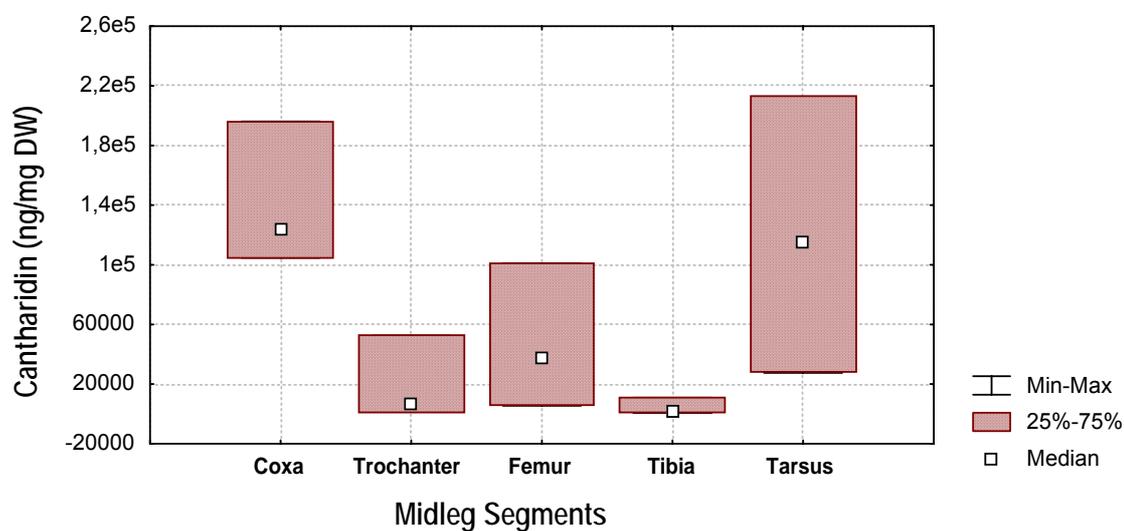


Fig. 77: Cantharidin titre in the midleg segments of female *Cyaneolytta* sp., Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

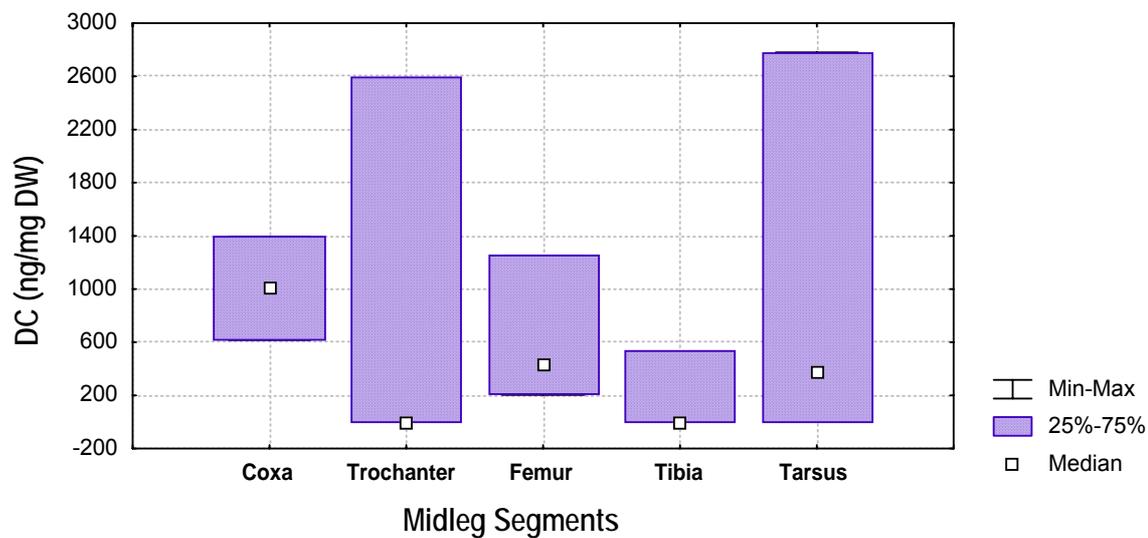


Fig. 78: D₂C titre in the midleg segments of female *Cyaneolytta* sp., Injected by 10 µl D₂C solution, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

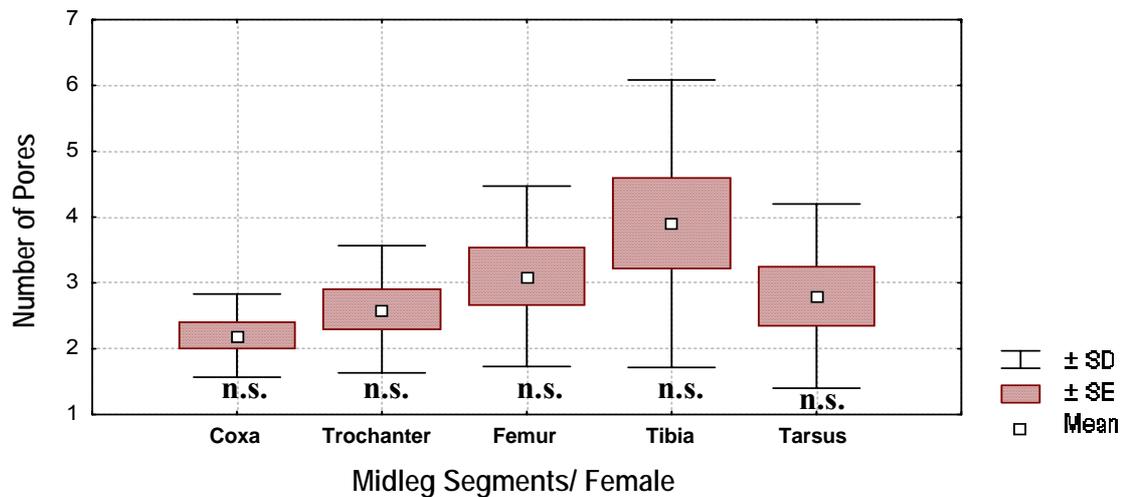


Fig. 79: Number of cuticular pores per 24.5 µm of SEM visible field, female *Cyaneolytta* sp. ANOVA, $P > 0.05$, $n = 10$

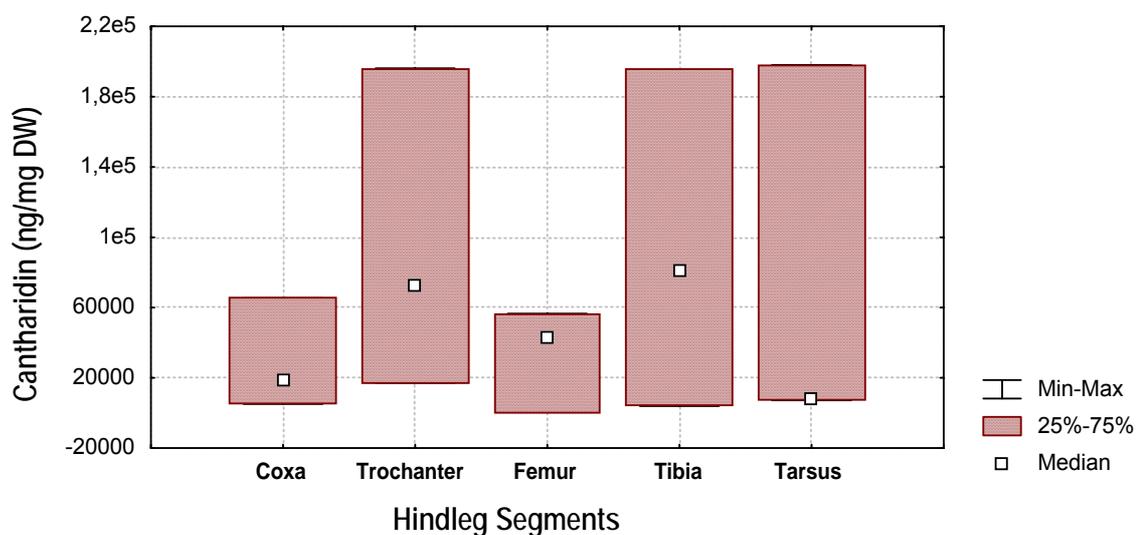


Fig. 80: Cantharidin titre in the hindleg segments of female *Cyaneolytta* sp., Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

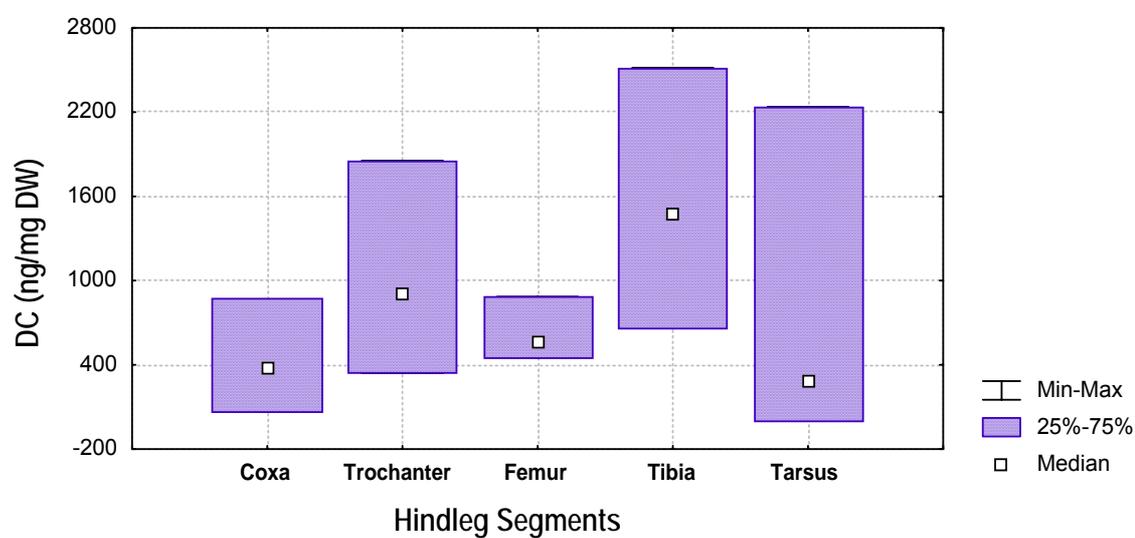


Fig. 81: D₂C titre in the hindleg segments of female *Cyaneolytta* sp., Injected by 10 μ l D₂C solution, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 3$

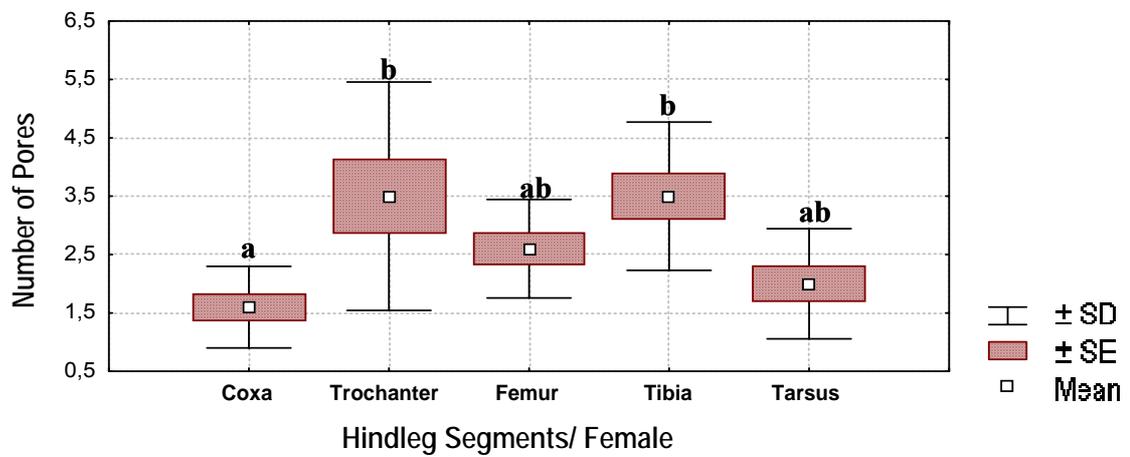


Fig. 82: Number of cuticular pores per 24.5 µm of SEM visible field, female *Cyaneolytta* sp. ANOVA, Tukey *HSD*-Test, $P < 0.01$, $n = 10$

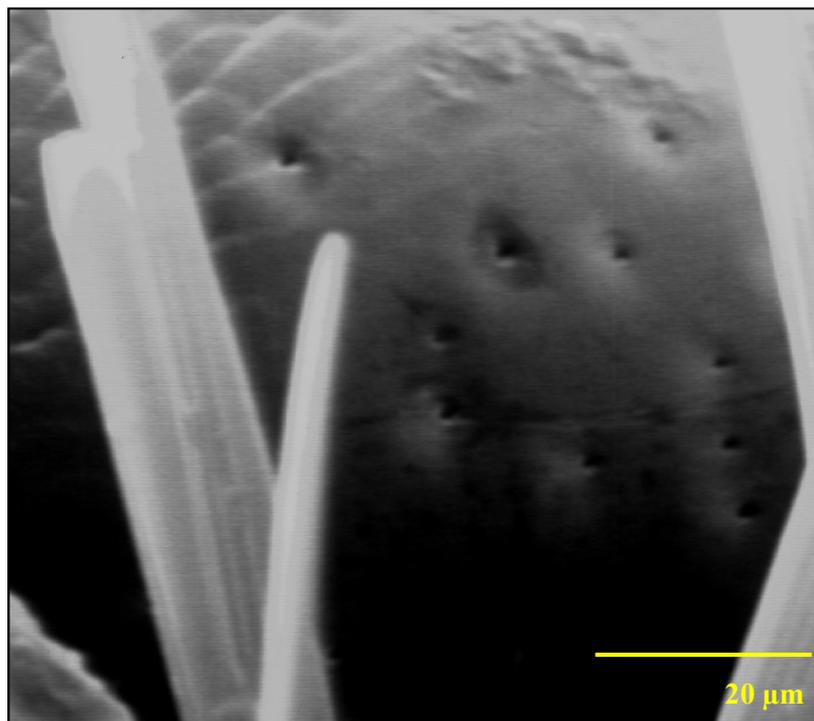


Fig. 83: SEM of cuticular pores on the hindleg trochanter of female, *Cyaneolytta* sp. 1050 X

4. Discussion

4.1 D₂C Separation via MRM Method

There are some interesting points concerning D₂C elution and usage of the MRM method to separate D₂C from cantharidin which can be discussed here. Although D₂C has two mass units more than cantharidin and is in principal eluted later than the naturally produced compound, it always eluted earlier regardless of the applied machine or program setting. The reason returns back to the two Hydrogen atoms which have been replaced by two Deuteriums in the molecule and the effect of these two Deuteriums on spatial angles of the molecule. However the molecular mass is increased by two units; interaction between the internal coating layer of capillary column and the molecule increases and deters it of being released with the same velocity as non-labelled compound (W. Boland, personal communication).

As indicated in the fig. 84, intensities of D₂C parent ions (m/z 84) at any time scan of a MRM run is clearly opposite to cantharidin parent ion (m/z 82); *i.e.* when D₂C has the highest peak, cantharidin has the lowest one. This phenomenon for example has been shown in fig. 84 for a time scan between 22.1 and 22.2 seconds where the difference between the two compounds is well marked by the orange coloured spike. Immediately after reaching the peak point of D₂C, a spectacular turning point happens in which D₂C counted ions are reduced and the cantharidin ions counted by detector increased significantly. In this way, cantharidin gets its peak point at a time scan between 22.2 and 22.3 seconds where D₂C stands far lower than cantharidin peak. Fig. 84 clearly explains the interaction between the naturally produced and the labelled cantharidin by time. Since D₂C has no separate peak area and it is highly integrated within cantharidin, it cannot be simply measured by a normal MS and its affiliated software, either manually or by the autointegration method.

The experiments performed in this study indicated that beetles cease feeding on the artificial diet if it is treated with a titre of the labelled compound which was more than what explained in section 2.5. On the other hand, the mortality rate of beetles is considerably increased when such high titres applied in injection experiments. Therefore, a complexity may arise concerning D₂C measurement in a MS system which is due to the very low amount of introduced marker into the beetle organs. As indicated in fig. 85, the very small peak of D₂C which is less than 50 Kcounts based on the Saturn[®] software has been completely covered by cantharidin huge peak (over 600 Kcounts). This is exactly what happens in a MS system with the difference that the other softwares rather than Saturn[®] could not reveal these two coincided peaks and let the small peak of D₂C being observable. In this way, the other softwares measured mostly cantharidin while the measured amount of D₂C often recorded as zero which is wrong (fig. 85). MRM has been first applied in this study as the preferred method to separate cantharidin from the isotopically labelled cantharidin (D₂C) with high sensitivity.

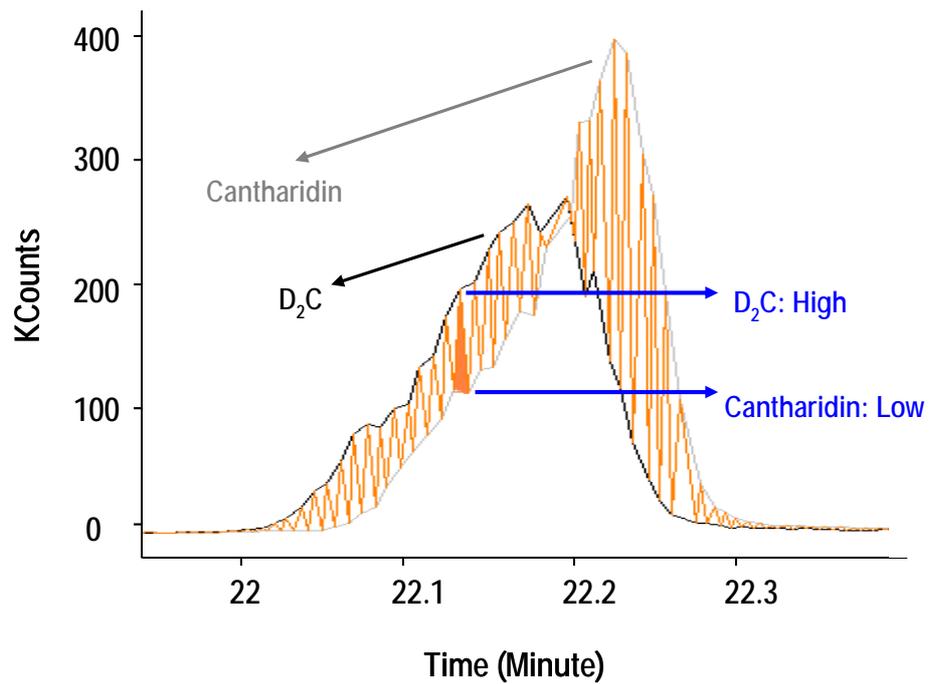


Fig. 84: MS/MS (MRM technique) of a D₂C introduced sample of *H. lunatus*, D₂C chromatogram indicated by black and cantharidin by a grey line

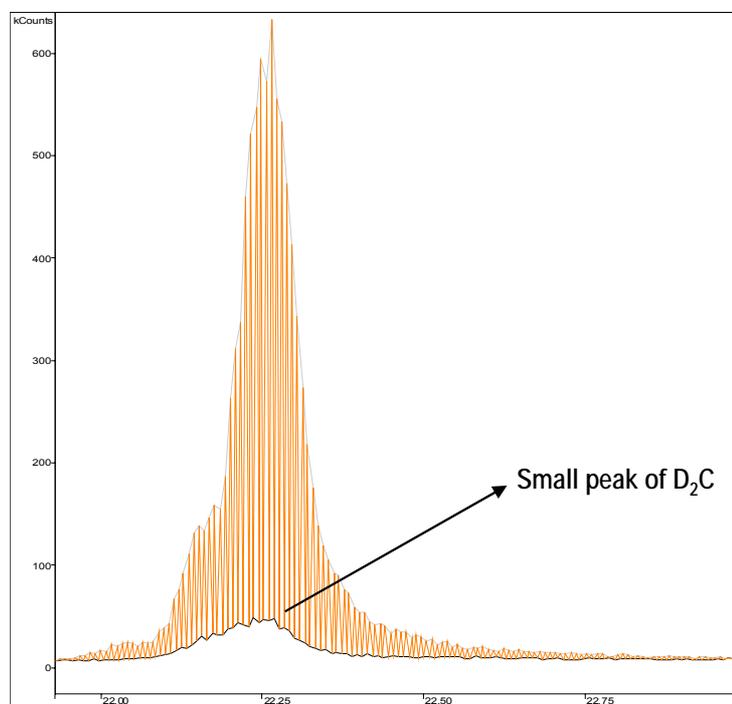


Fig. 85: MRM technique of a D₂C introduced sample of *H. lunatus*, cantharidin chromatogram and the peak area are indicated in grey line with a peak area in orange colour

4.2 Cantharidin Distribution within Male Genital Organs

Taking a look at the natural distribution of cantharidin over the male internal organs (fig. 32), it is found that the compound is not distributed uniformly. Distribution patterns in fig. 32 show the accumulation sites of cantharidin within male specimens of *Mylabris quadripunctata* (n= 6) without any intervention. It is clear that the 2nd and the 3rd pairs of the accessory glands accumulate a higher titre of compound. Although just the 2nd pair are statistically different to the vas deferens, epididimidis and 1st pair of the accessory glands (Kruskal-Wallis ANOVA), the Median test indicates a significant difference between the 2nd and 3rd pairs of the accessory glands on one hand and the vas deferens, epididimidis and the 1st pair of the accessory glands on the other hand (95% confidence level). Considering the whole titre of cantharidin, it can be therefore concluded that the 2nd and 3rd pairs of the accessory glands along with the testis and the whole gut accumulate over 80% of the naturally produced compound in *Mylabris quadripunctata*. Similarly, it has been also reported that 80-92% of cantharidin in male specimens of *Epicauta pestifera* (recently known as *E. funebris*) is only localized in the 3rd pair of the accessory glands (McCormick and Carrel, 1987; Carrel et al., 1993).

If this species fed with a mixture of artificial diet and D₂C, the distribution of cantharidin after marker introduction will be different (fig. 33). Based on the two mentioned figures, it is concluded that following introduction of D₂C, the titre of cantharidin increased not only in the accessory glands (mostly 2nd and the 3rd pairs), but also in the testis, vas deferens, epididimidis and gut. In this way, 2nd and 3rd pairs of the accessory glands accumulate cantharidin significantly higher than all internal organs except the testis at the confidence level of 99.9% (Kruskal-Wallis ANOVA). Interestingly, the 2nd and 3rd pairs of the accessory glands are even significantly different to all other experimental groups as well as the testis using the Median test at 99% confidence level.

D₂C once it introduced into male body via feeding seems to form a more or less uniform distribution (fig. 34). Apart from the 1st pair of the accessory glands and gut which are statistically lower than 2nd pair of the accessory glands, all other studied organs show no difference in their D₂C accumulation capacity at 99% confidence level (Kruskal-Wallis ANOVA). Time dependent sampling of D₂C which was done by the series of *Ringer experiments*, reveals some more details of D₂C distribution behaviour. Since it was shown that the two compounds have very similar behaviour, D₂C movement and incorporation model could be used as a high resolution simulation for the naturally produced cantharidin. According to the *Ringer experiments*, the incorporated titre of D₂C in the testis increased slightly from 3500 ng/mg DW (median value) after two hours to about 4600 ng/mg within 6 hours (fig. 42). At the same time, the three other organs of male internal genitalia followed a different pattern. D₂C slightly decreased within the vas deferens and epididimidis by time. Whilst they had first collected about 3750 ng/mg DW within 2 hours, it finally reduced to about 1250 ng/mg after 6 hours which was one third of the primary reserved titre (fig. 43). The same trend occurred for the accessory glands as well in which the absorbed titre of D₂C fell down sharply from 4000 ng/mg DW within 2 hours to 1500 and 500 ng/mg after 4 and 6 hours respectively (fig. 44). Since the labelled compound does not exist naturally, the measured titre of D₂C within any of these organs could be explained merely by active absorption. By placing the whole male abdomen into the Ringer solution, it was found that titre of D₂C reduced dramatically from 1600 ng/μl injection to not more than 70 ng/μl within 2 hours (fig. 45). It clearly means that the rest of the compound must be actively absorbed by the insect tissues. Following the experiment up, it was observed that this index increased slightly, but never get any value higher than 330 ng/μl which is yet far lower than initial titre of 1600 ng/μl. Cells of insect various tissue or at least majority of them should be active even after 6 hours, otherwise they could not keep the labelled compound any longer and due to diffusion it would be released into the solution rapidly to

produce a much higher titre than what was measured. Slight releasing trend of D₂C indicates that the compound must be discharged from insect tissues into Ringer solution, since no any other source of D₂C was available. Based on what has described before, the vas deferens, epididimidis and the accessory glands were the organs which lost the marker by time, therefore these three organs must have been the source of released D₂C. A question which has ever been asked by many researchers was the route by which cantharidin moves through different parts of internal genitalia. In the other word, it was asked if cantharidin released by any part of the sexual organ into hemolymph and then absorbed by other parts or the compound is able to move internally from one part to the other part without using hemolymph as a transit way (McCormick and Carrel, 1987).

Regarding all *Ringer experiments* hereby done, it is now clear that if not all at least most part of cantharidin transfers to other sexual organs internally and hemolymph is not as the major transit route. Fig. 45 shows a very slight reduction in D₂C titre between the 2nd and 4th hour of whole abdomen experiment. In this way, the median value decreased from 25 ng/μl to 21 ng/μl within two hours which means release of a total load of about 100 ng while the vas deferens, epididimidis and the accessory glands released 5000 ng/mg DW at the same time which is equal to a total load of about 7750 ng (fig. 43, 44). If D₂C of the three mentioned organs had to be first released into the solution and thereafter taken up by the testis, we should have seen a considerable increase in D₂C titre of Ringer solution, but on the contrary only a slight reduction was observed. Therefore D₂C could not be released into Ringer by the vas deferens, epididimidis and the accessory glands. Focusing on the testis, only a slight increase of median point is observed (about 155 ng). This load could not be supplied by the solution, because its D₂C titre was much lower at that time (100 ng) to be considered as D₂C source for the testis. Thus, it is concluded that required D₂C for the testis must have been supplied by the three other organs. It is also evident that the huge titre of D₂C released by the vas deferens, epididimidis and the accessory glands should be also transferred to some other organs rather than the testis. If the natural distribution of cantharidin (fig. 32) is considered again, it may be hypothesized that a considerable part of cantharidin will be sent to the insect alimentary canal. D₂C titre (median value) increased between 4th and the 6th hour of *Ringer experiment* (fig. 45) which indicates a D₂C diffusion of about 300 ng into the solution. At the same time the accessory glands released a total load of about 1600 ng (fig. 44) and the testis actively absorbed 1550 ng (fig. 42) within two hours. It is obvious that the absorbed load of D₂C by the testis could not be supplied by the solution and most probable transferred into the testis by the accessory glands.

This has been summarized in fig. 86 which schematically explains cantharidin movement inside the male sexual organ. Our studies do not provide any information about the site of cantharidin production. Blum (1981) proposed that by analogy with other insect systems it is reasonable to speculate that the site of synthesis may be in tissue associated with the site of storage. McCormick and Carrel (1987) reported the 3rd pair of male the accessory glands as the storage site; however they demonstrated that this part of accessory gland could not be involved in biosynthesis. I indicated here that cantharidin movement in males of the studied species is highly dependent on the time and could be quite different by time proceeding. As the accessory glands absorb a high amount of D₂C in short term, they will eventually accumulate less than the testis. Furthermore, McCormick and Carrel (1987) found very high level of maximally labelled cantharidin in male hemolymph after exposure to labelled water (H₂¹⁸O), but in contrast little labelled one in the accessory glands. This indicates that newly synthesized cantharidin proceeds first to the blood and later on absorbed and accumulated in the accessory glands. It has been implied that post-farnesyl steps in the cantharidin pathway may occur entirely in the male's body outside reproductive system (McCormick and Carrel, 1987; Carrel et al., 1993) but then the ultimate product is transported into male internal genitalia via membrane of the accessory glands which should be permeable to the chemical. It transfers afterwards to the epididimidis and

vas deferens in higher volumes compared to other organs of the male genitalia and is finally absorbed by the testis. However, the testis slowly started to absorb cantharidin; it accumulates a considerably higher titre in comparison to the vas deferens, epididimis or the accessory glands by time. Fig. 24 and 35 which described the distribution of D₂C and cantharidin within male internal organs show the higher accumulation of both chemicals in the testis, vas deferens and epididimis as well.

An extra proof concerning the above interpretation can be provided by studying palasonin distribution in male internal organs. As indicated in figure 35, palasonin tends to be gathered mostly in the testis if no intervention implemented; however it does not show a significant difference to the vas deferens, epididimis and 2nd and the 3rd pairs of the accessory glands (Kruskal-Wallis ANOVA) at 95% confidence level. When *M. quadripunctata* was fed by mixture of D₂C and artificial diet, Kruskal-Wallis ANOVA test did not show any significant difference among experimental groups upon 10 replications (fig. 36).

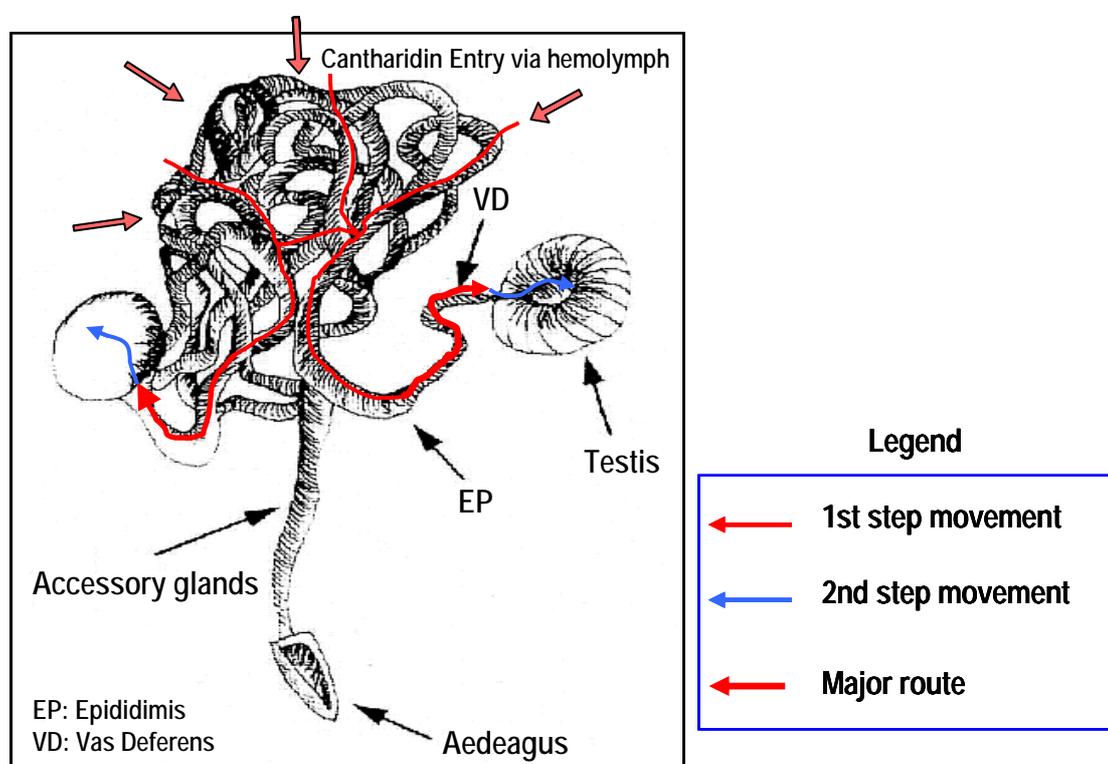


Fig. 86: Schematic view to entry and distribution of cantharidin in male sexual organ

There is an equilibrium between cantharidin and its metabolites within insect various tissues. D₂C Introduction makes the condition imbalanced. It is thought that because of lower toxicity of palasonin, it may be regarded as an intermediate compound. So, palasonin is released from the testis into other organs shortly after D₂C incorporation to keep the balance. It must be noticed that although cells in blister beetles are tolerant to cantharidin and its metabolites, there is a threshold. That is why palasonin is set free and directed into other organs to maintain the maximum toxicity below the threshold line.

Palasonin fluctuation in the testis indicates an increasing trend by time (fig. 87), but no part of the released palasonin into Ringer solution found to be deuterated; therefore it could not be produced by D₂C and had to be synthesized from naturally produced cantharidin as part of a detoxification process.

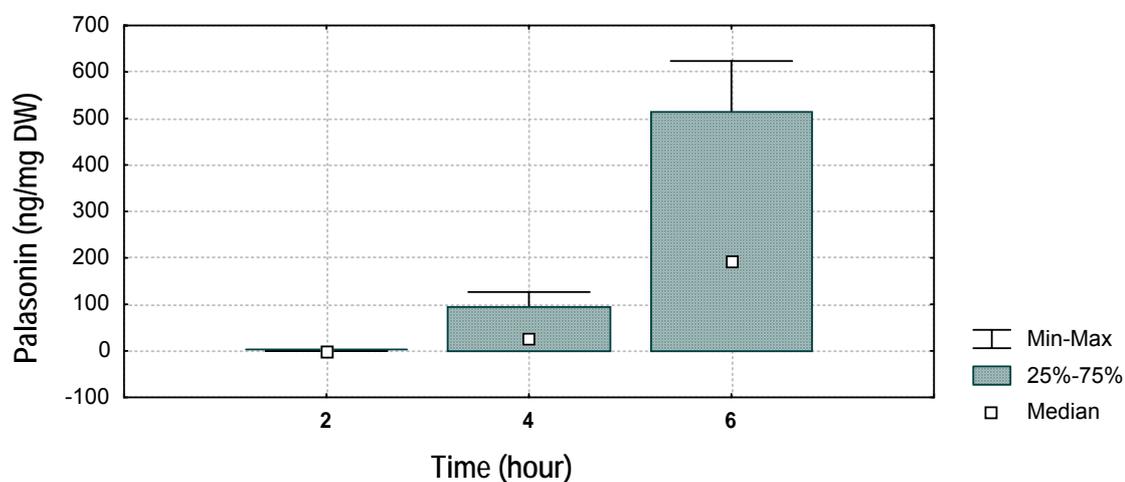


Fig. 87: Palasonin titre in the testis of *Hycleus lunatus* by time, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 5$

McCormick and Carrel (1987) suggested that cantharidin biosynthesis seems to be correlated with mating duration in meloids. For example *Epicauta* males produce much cantharidin before mating and then rapidly transfer this stored material into females towards the end of their copulation period which lasts several hours. In contrast, *Lytta* males appear to make cantharidin slowly before mating, but during 20-24 hours they spend in copulation they synthesize prodigious amounts of cantharidin to be transferred to female. Based on laboratory observations, mating in *H. lunatus* take about 3 hours. As high amounts of cantharidin were always found in this species, it is logical to assume that they follow the same pattern as genus *Epicauta* and store huge amount of cantharidin which has to be transferred to female within a limited period of time.

4.3 Cantharidin Distribution within Female Genital Organs

The distribution pattern of D₂C and cantharidin was first studied in those beetles which had been injected by 10 µl of D₂C-DMSO solution. Three specimens of *Hycleus polymorphus* were being injected (both sexes) to see how the marker distributed within internal organs. Considering the data of fig. 27, D₂C incorporated mostly in the bursa copulatrix and to lesser extent in the receptacle. However a Kruskal-Wallis ANOVA test does not indicate any difference between the experimental groups, they are very close to a statistically significant difference between the bursa copulatrix and the other groups ($P= 0.08$). Any way, the trend is clear enough to indicate a higher accumulation of D₂C within the bursa (median value of 1050 ng/mg DW) in comparison to the 2nd highest group, the receptacle (median value of about 100 ng/mg DW). Distribution of naturally produced cantharidin over female internal organs shows the same pattern as D₂C, in which the bursa accumulated the higher amount of the chemical (fig. 28). The median value for bursa is about 80,000 ng/mg DW which placed it in a much higher rank than the 2nd part of sexual organ, the receptacle, with less than 10,000 ng/mg DW. This difference was also confirmed by a Median test at 99% confidence level; however a Kruskal-Wallis ANOVA did not show a significant difference at 95% confidence level. Data derived independently from the feeding series of experiments emphasize the above mentioned trend. As expressed in the last chapter, females paired for three days with the D₂C fed male to see if any transfer of the labelled compound occurred. Fig. 39 indicates not only D₂C transfer into female genitalia, but a very characteristic distribution over female internal organs. The bursa copulatrix accumulated significantly higher titre of the marker than ovaries, eggs and the gut. This experiment was confirmed by Kruskal-Wallis ANOVA test upon 6 replications at 95% confidence level. Cantharidin distribution has been shown to follow the same trend (fig. 38) in which bursa reserved higher titre than the other experimental groups, although the test only indicates a statistically significance between bursa and gut. The fact that males of *M. quadripunctata* have been able to mate in captivity and thereafter transfer the sperm along with pockets of cantharidin and D₂C into female genitalia is well reflected in fig. 41 which shows the traces of the marker in faeces of female within a period of 8 days after being paired. The detected volume of D₂C in the 1st day has been considerably higher than the other following days which returns back to the fact that D₂C has just once offered to the female and consequently the titre has to be diminishes by time.

Palasonin distribution over female internal organs does almost show the same model as D₂C and cantharidin except for the lower difference between bursa absorbed load in one hand and the ones of ovaries, eggs and gut on the other hand (fig. 40).

Ringer experiments indicate not only a very similar trend, but provide us with plenty of other information concerning cantharidin movement within female sexual organ. D₂C titre which has been incorporated into the bursa copulatrix, increased from 1600 ng/mg DW (median value) to 2700 ng/mg which shows an increase of about 1100 ng/mg during a time scan of 4 hours (fig. 46). Interestingly, samples taken every two hours from the Ringer solution reflects continuous decreasing trend of D₂C titre from 20 ng/µl to about 7 ng/µl which has been confirmed by Kruskal-Wallis ANOVA upon 7 replications at 95% confidence level (fig. 47). Taking the whole volume of solution into account, the total decreased titre of D₂C within 4 hours calculated as 1600 ng. At the same time total volume of absorbed D₂C by the bursa reaches 1940 ng based on the average weight of those bursa applied in the experiment ($n= 5$). Since D₂C has been only present in the Ringer solution and no any other organ rather than bursa has had chemical exchange with it, it can be said that the marker has been actively absorbed by the bursa and the initial volume of marker in Ringer solution has been consequently decreased. An opposite trend is observed for eggs and ovaries where the median value of D₂C titre fell from 2200 ng/mg DW to about 915 ng/mg within 4 hours (fig. 48). On the other hand, the D₂C titre of corresponding

Ringer solution increased from around 8 ng/ μ l to about 25 ng/ μ l (median value) within the same time scan (fig. 49). Similarly, D₂C titre in the receptacle decreases over the time window of 4 hours from about 8500 ng/mg DW (median value) to about 0 ng/mg (fig. 50) and on the contrary, its solution shows an obvious increase of about 4 ng/ μ l to about 34 ng/ μ l (median value) within the same time window (fig. 51). Furthermore data of fig. 52 can create an overview of what has really happened in the whole female genitalia. Considering D₂C density in Ringer solution, a clear increase is observable which made the titre of final level 200 ng/ μ l (median value) from the initial titre of 50 ng/ μ l within 4 hours. It means a total increase of 15,000 ng in the whole solution. Remembering the two organs of eggs and ovaries and the receptacle which set their titre free in the solution, a total released volume of 11,000 ng of D₂C was calculated within four hours. If 2000 ng which was taken-up by the bursa added to 11,000 ng, we reach the approximate volume of 13,000 ng which stands much better against 15,000 ng of the solution. Taking device and human failures into consideration, we may get an equation between up-taken and released quantities of D₂C. All these observations, which have been fulfilled by independent experiments, support the idea that the bursa copulatrix actively absorbs cantharidin, while the two other organs of ovaries (plus eggs) and the receptacle lose the compound over the time. Besides, reviewing the initial titre of D₂C in these three organs of female internal genitalia (fig. 46, 48, 50), it is known that ovaries and the receptacle have started marker collecting earlier than the bursa (got a higher initial level comparing to bursa) while by time (6 hours) bursa absorbs much more and finally stands as the first reservoir of the marker (2700 ng/mg DW for median value). The whole process of cantharidin absorption and transfer within female sexual organ can be simplified as in fig. 88. According to this figure, cantharidin enters into female genitalia by male as a nuptial gift. It is first absorbed by the receptacle in high volume while at the same time goes through ovary and distributed upon eggs; however the up-taken volume by ovaries remains considerably lower than the receptacle. The fact that the cantharidin which has been deposited by a male meloid in the female's spermatophoral the receptacle moves and does not all simply remain there, has been demonstrated for two American species of *Epicauta funebris* and *Lytta polita* (McCormick and Carrel, 1987). This part of movement has been referred in the schematic view as the 1st step movement. Over time these two organs stopped accumulating of cantharidin, whilst the bursa copulatrix starts accumulating the gift actively. This acquired amount is mainly supplied by the receptacle and ovaries directly. In the other word, the required volume of cantharidin which has been incorporated into bursa cannot be obtained by hemolymph, so the internal transfer remains as the main transport. Calculating the data of fig. 46 and 52, it is clearly observed that the median value of the solution is 50 ng/ μ l which remained almost unchanged between the 2nd and 4th hours; however bursa collected 200 ng/mg DW which is equal to 400 ng. If D₂C had been absorbed from solution, it must have effect on the titre of marker and therefore cannot be transferred into bursa by Ringer solution. Based on such a simulation and regarding very similar behaviour of D₂C and cantharidin, the above conclusion can be applied for the naturally produced compound as well.

Transfer of cantharidin from the receptacle and ovaries to bursa has been referred to as the 2nd step of movement in fig. 88 and that is exactly why the cantharidin titre of bursa increases later than the ovaries and receptacle. Following collection of high volume of D₂C in bursa, the reserved titre of palasonin diminished (fig. 89) which seems to be released later into Ringer solution (fig. 90). Since the palasonin titre of ovaries and the receptacle did not show such a decrease, it can be concluded that the released palasonin must be merely originated from bursa. Hereby, palasonin can be once more referred to as an intermediate compound which keeps the balance of total toxicity within a specific organ. It seems that a female meloid not only acquires cantharidin from her mate, but transforms it biochemically into one or more substances useful to

her, to her mate or to their offsprings (McCormick and Carrel, 1987). Palasonin may be regarded as one of these substances.

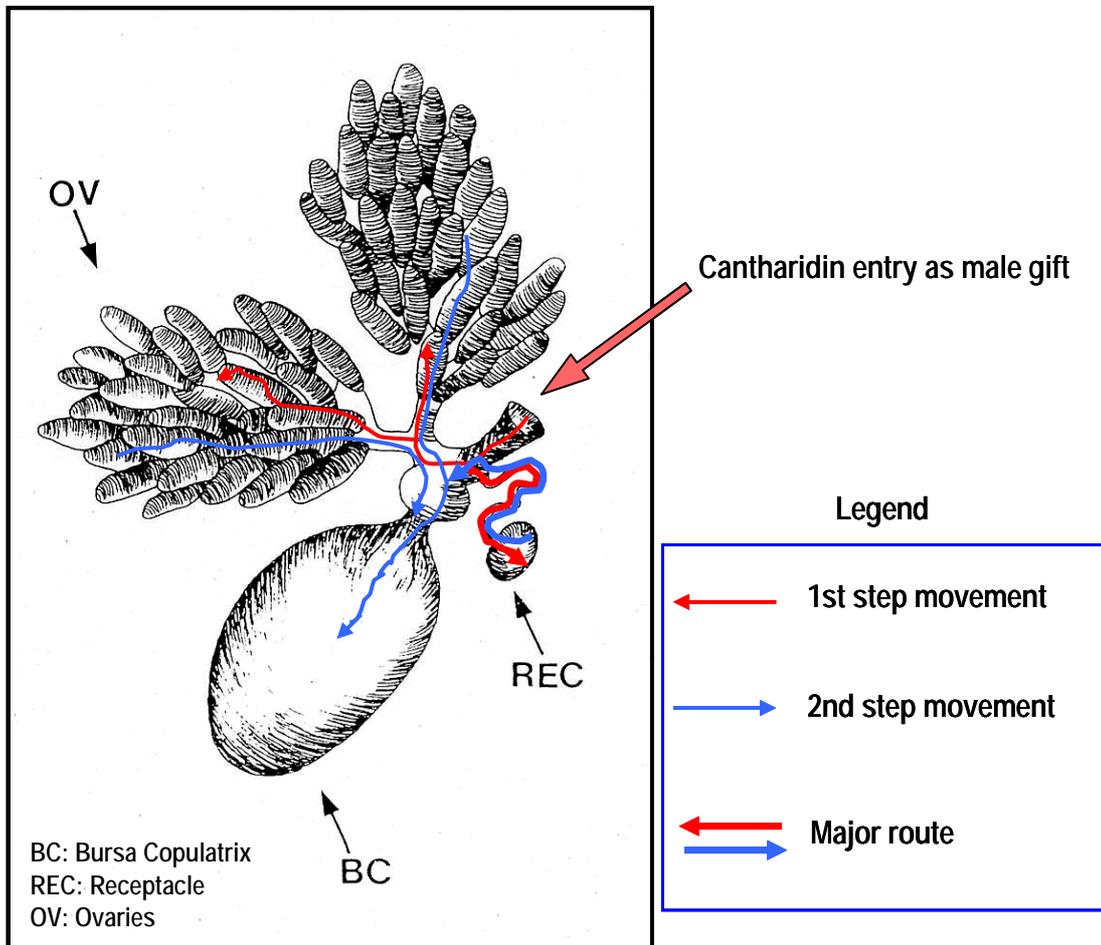


Fig. 88: Schematic view to entry and distribution of cantharidin in female sexual organ

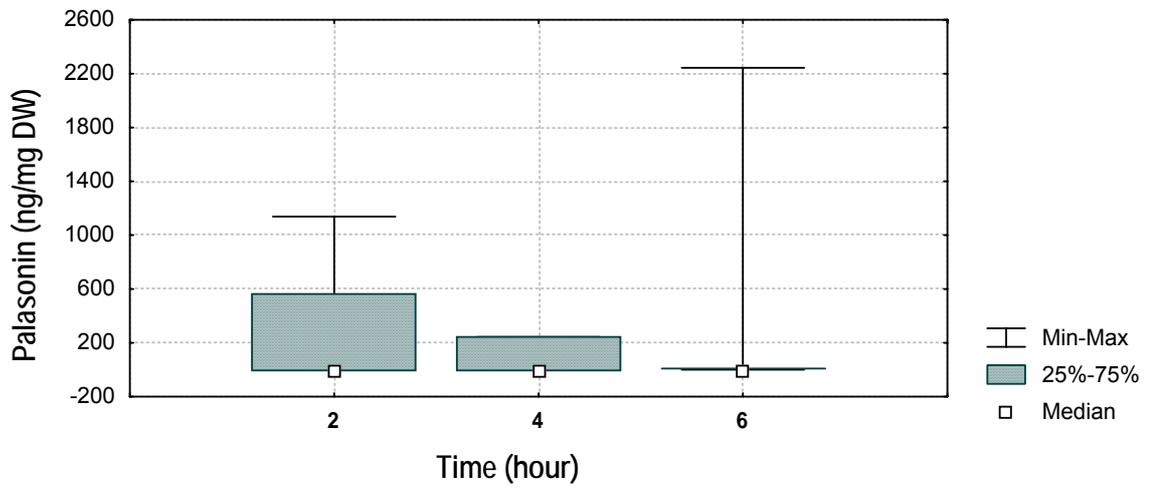


Fig. 89: Palasonin titre in the bursa copulatrix of *Hycleus lunatus* by time, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 5$

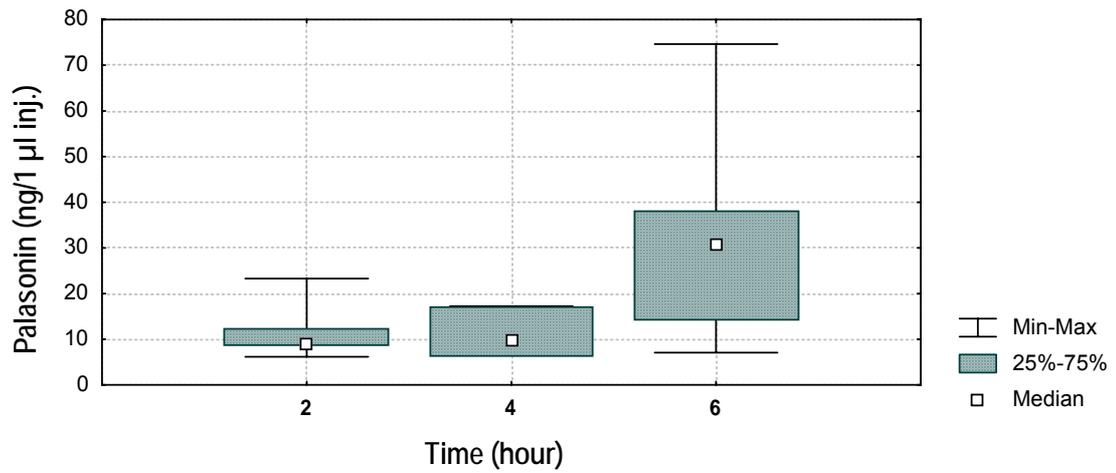


Fig. 90: Palasonin titre of Ringer solution in which the whole female abdomen of *Hycleus lunatus* had been placed, Kruskal-Wallis ANOVA Test, $P > 0.05$, $n = 8$

4.4 Cantharidin Transfer to the Female Progeny

Transfer of cantharidin from females into eggs was studied on a group of females which had been injected by D₂C solution. These females maintained under normal conditions until they laid eggs. Analysis of the eggs laid by D₂C injected females of *Hycleus polymorphus* proves that the marker has been transferred from female to the eggs (fig. 29). In a study it has been determined that some, but evidently not all *Epicauta* species deposit considerable amount of cantharidin with each egg mass (McCormick and Carrel, 1987; Carrel et al., 1993). Incorporated cantharidin will be used as chemical defence against pathogenic fungi, nematodes and predaceous arthropods. Parental chemical defences have been also documented in two lepidopteran families. Alkaloids derived from males are present in eggs of some danaiid butterflies and arctiid moths (Eisner and Meinwald, 1987). After hatching of eggs, part of larvae was also examined for D₂C. The larvae were first examined for any trace of the labelled compound and thereafter their incorporated titre was also measured. As indicated in fig. 29, D₂C has been also incorporated into triangulin larvae; however the titre decreased significantly from egg to larva. Mann-Whitney *U*-Test confirms a significant difference between introduced volume of D₂C in eggs and larvae of *H. polymorphus* upon 4 replications at 95% confidence level. Transfer of naturally produced cantharidin from female to eggs and afterwards to larvae shows exactly the same inoculation trend in which eggs possess much more cantharidin than triangulin larvae; however such a difference is not as high as D₂C and thus is not significant using Mann-Whitney *U*-Test at 95% confidence level.

On the other hand, palasonin shows a bit different trend in which the level of compound from eggs to triangulin larvae has been slightly increased, but Student *t*-test does not demonstrate a statistically significant difference between the two groups. Like cantharidin; palasonin also inhibits PP₁ and PP_{2A}, albeit with much lower efficiency (palasonin LD₅₀: 3.1 mg/kg for mouse). Because of slightly lower toxicity as compared to cantharidin (LD₅₀: 1.0 mg/kg for mouse), it may be speculated that the female incorporates mostly palasonin into egg batches to enhance the chemical defence of this defenceless stage on one hand and reduce any arising risk of cantharidin high toxicity to the eggs' susceptible tissues on the other hand (K. Dettner, unpublished data). Besides, canthariphilous predators may be less attracted to palasonin and thus give more chance to meloid eggs to survive.

4.5 Release Structures of Antennae in Male *Cyaneolytta* sp. With A Hypothesis on Their Function

The possible role of cantharidin in sexual behaviour of blister beetles has been of considerable interest to scientists (Schmidt, 2002). In spite of some evidence on the subject, it has never been indicated that cantharidin has a clear role in courtship within any member of family Meloidae. Demonstrating of such a relationship has failed owing to shortage of enough proof. MacCormick and Carrel (1987) even reported there was no evidence that cantharidin serves as a sex attractant or aggregation pheromone in meloid beetles; however they added that cantharidin might in some very subtle way be used by female meloids when selecting a mate at close range. All findings on cantharidin producers have been merely based on chemical analyses or behavioural studies whereas fewer morphological studies have been made. Cantharidin involvement in courtship behaviour has been confirmed in some of canthariphilous insects. For example it was shown that males of *Neopyrochroa flabellate* (Col: Pyrochroidae) secrete cantharidin from a cephalic gland in which females sample during courtship and mate preferentially with males with higher titre of cantharidin (Eisner et al., 1996 a,b). Frenzel and Dettner (1994) found that cantharidin may either function in bringing sexes of *Atrichopogon* (Diptera: Ceratopogonidae) together or represent meeting places for the two sexes and stimulate copulation (Frenzel et al., 1992). Furthermore it was demonstrated that females of *Notoxus monocerus* (Coleoptera: Anthicidae) bit several times into a pair of notch-like structure on the apices of male elytra which contain considerable amount of cantharidin during sexual display to see if males were able to offer the terpene. Therefore, cantharidin behaves as a discriminative agent in mating selection of family Anthicidae too (Schütz and Dettner, 1992).

Based on our results, there is a spectacular correlation between density of cuticular pores and cantharidin/ D₂C component of the scape and pedicel antennomeres of male *Cyaneolytta* sp. (fig. 54, 55, 56, 57). Although concentrating of pores on the first two antennomeres is not very common, there are a number of confirmed cases among insects. For example, cuticular pores in fire ants are also located as a ring round the proximal portion of the 1st and 2nd antennomere in workers and the queen respectively (Isidoro et al., 2000) and the expanded scape of some sphecids (Hymenoptera: Sphecidae) may house glandular openings which are applied during courtship (Wcislo, 1998). Light microscopy of semi thin cross sections which were prepared from the male scape and pedicel helped me to examine the nature of these pores better. According to fig. 61, there are plenty of canal shape structures which have been stretched from the antennal hemolymph to the antennomere surface where cuticular pores situated. These structures can be uni- or multicellular tubules which bring the circulating cantharidin of hemolymph into the surface where it is later released via the opening of cuticular pores. Insect antennae in fact are not exclusively receivers of signals, but also producers and emitters of chemicals (Bin et al., 1999b). Pinto (1974, 1975) was the first who took the probable role of male cuticular pores in courtship behaviour of genus *Linsleya* and *Tegrodera* (Meloidae) into consideration. After Pinto, we have collected direct evidences of external and internal morphology along with the chemical analyses for the first time to suggest the infochemical role of cantharidin within family Meloidae. As Snead (1985) and Carrel et al. (1993) implied for some meloid species, we also believe that a natural system has been developed in the females of genus *Cyaneolytta* which let them select males with abundant reserves of cantharidin prior to any mating behaviour.

On the other side a similar correlation apparently exists in the tibia of the male foreleg too. If the tibial pores are really involved in any transporting task, it should be proved by any sort of internal or external structures but preliminary observations indicate neither carrying structure

within the tibial cross sections nor any form of integumental tyloids after decolourizing treatment by 10% potassium hydroxide for 30 minutes (pers. observations). Besides, data of male legs are all based on natural component of cantharidin and due to a shortage of insect specimens, it is impossible to confirm these data by D₂C as it was fulfilled for male antennae. Any further conclusion has to be based on the marker administration and/ or behavioural observations which could not be achieved in the present study because of no access to more beetles. In tribes Cerocomini and Epicautini (Meloidae: Meloinae), antennal cleaning is performed by the outside of foretibia that are then passed through the mouthparts, while in the rest of sub family such as in tribe Meloini to which *Cyaneolytta* is belonged, antennal articles are only passed through mouthparts (Turco et al., 2003). Therefore cantharidin could not be found in foretibia because of antennal contamination and this segment of male foreleg appears not to be directly involved in the courtship. However the presence of numerous pores on the tibiae, high content of cantharidin and their frequent use for cleaning of head capsule during the courtship (Turco et al., 2003) suggest a synergy of sexual and cleaning behaviours. In particular, the frequent cleaning activity could increase the diffusion of pheromones (Turco et al., 2003). Matthes (1969) explained the mating behaviour of *Cerocoma schäfferi* (Col: Meloidae) during which fanning forelegs of the male who is sitting on female dorsum were covered by an odour secreted from the 3rd antennal segment and 2nd segment of maxillary palps. A comprehensive behavioural study can help us to know if a similar behaviour exists also in genus *Cyaneolytta*.

As illustrated in fig. 70, the head capsule and antennal segments of those *Cyaneolytta* females which were injected by D₂C inspected for accumulation sites of the marker. Surprisingly, the marker occurred mostly in the two first antennal segments, while the head capsule itself bore much less titre of injected marker. Calculating cuticular pore density (the only visible feature on the female antennae), a higher concentration of pores was readily observed in the scape and pedicel which the latter segment shows even a statistically significant difference to the flagellum (ANOVA, Tukey *HSD* posthoc Test). Therefore it is reasonable to hypothesize a relationship between pores and cantharidin. Considering the total amount of cantharidin in antennal segments of the both sexes of *Cyaneolytta*, male segments bear much higher titre than that of females. Comparing cuticular pore density of male and female, a highly significant difference is considered upon 10 replications at the confidence level of 99.9%. Overall, male segments bear much higher density of pores (fig. 91, 92, 93). Additionally, by the fact that tubular cell or any other carrying structure such as tyloids was found neither in cross sectioning of female antennomeres (fig. 73) nor on integument surface respectively; I believe that the function of the female pores which are also located on the 1st and 2nd antennomeres must be different from the male ones.

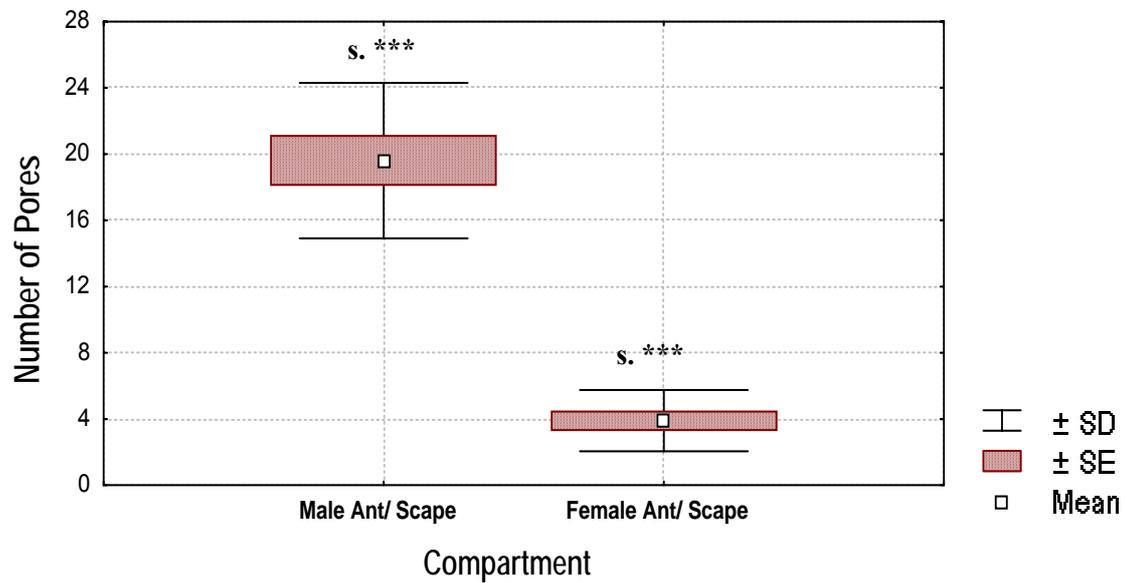


Fig. 91: Number of cuticular pores per 24.5 μm of SEM visible field, *Cyaneolytta* sp. Mann-Whitney *U*-Test, $P < 0.001$, $n = 10$

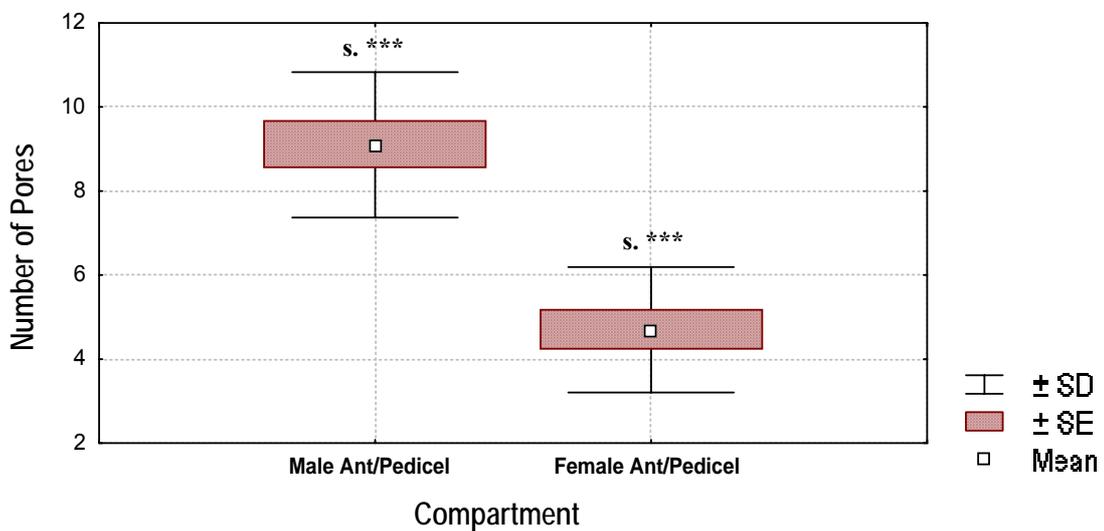


Fig. 92: Number of cuticular pores per 24.5 μm of SEM visible field, *Cyaneolytta* sp. Student *t*-test, $P < 0.001$, $n = 10$

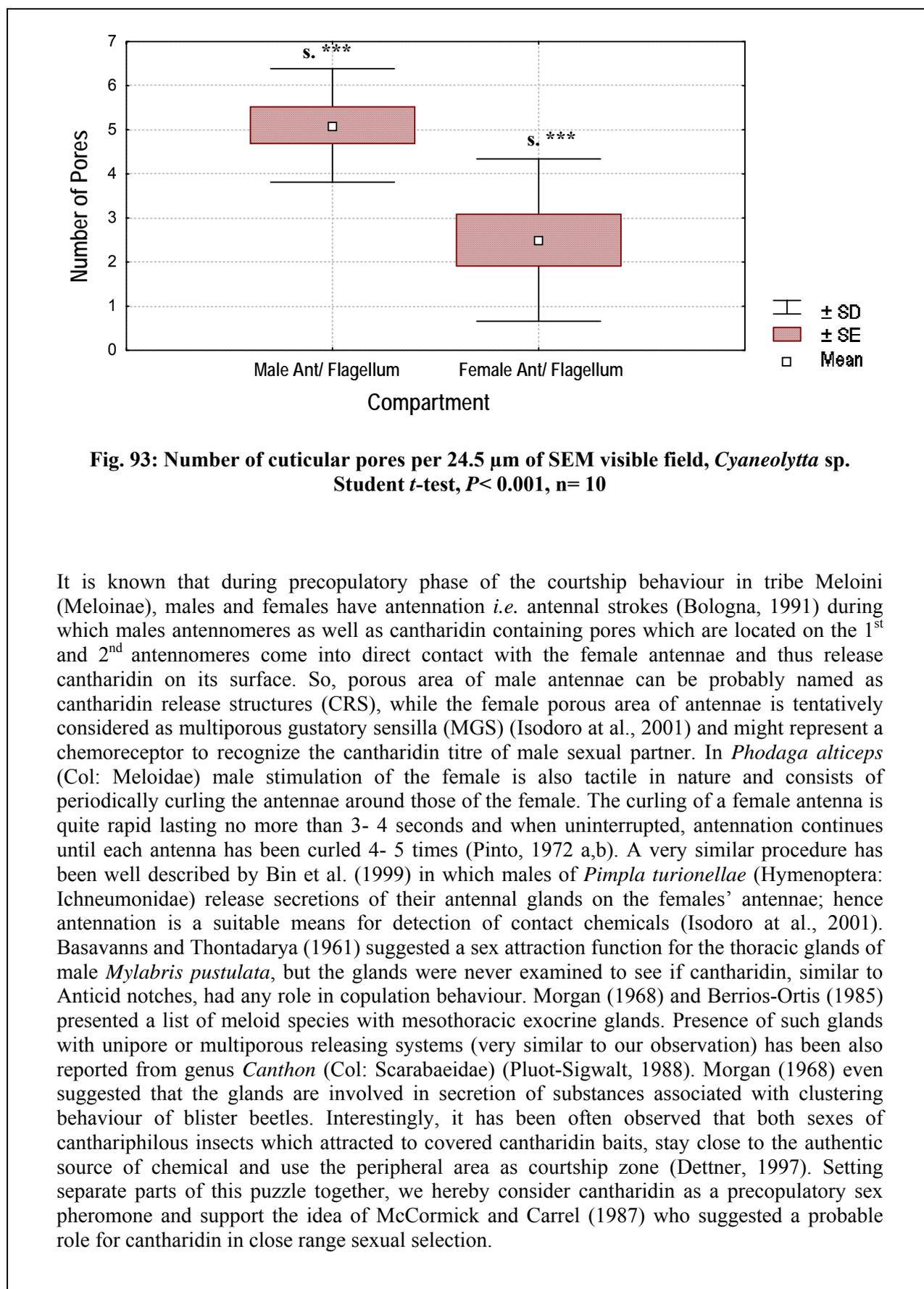


Fig. 93: Number of cuticular pores per 24.5 μ m of SEM visible field, *Cyaneolytta* sp. Student *t*-test, $P < 0.001$, $n = 10$

It is known that during precopulatory phase of the courtship behaviour in tribe Meloini (Meloidae), males and females have antennation *i.e.* antennal strokes (Bologna, 1991) during which males 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 thus release cantharidin on its surface. So, porous area of male antennae can be probably named as cantharidin release structures (CRS), while the female porous area of antennae is tentatively considered as multiporous gustatory sensilla (MGS) (Isodoro et al., 2001) and might represent a chemoreceptor to recognize the cantharidin titre of male sexual partner. In *Phodaga alticeps* (Col: Meloidae) male stimulation of the female is also tactile in nature and consists of periodically curling the antennae around those of the female. The curling of a female antenna is quite rapid lasting no more than 3- 4 seconds and when uninterrupted, antennation continues until each antenna has been curled 4- 5 times (Pinto, 1972 a,b). A very similar procedure has been well described by Bin et al. (1999) in which males of *Pimpla turionellae* (Hymenoptera: Ichneumonidae) release secretions of their antennal glands on the females' antennae; hence antennation is a suitable means for detection of contact chemicals (Isodoro et al., 2001). Basavanna and Thontadarya (1961) suggested a sex attraction function for the thoracic glands of male *Mylabris pustulata*, but the glands were never examined to see if cantharidin, similar to Antacid notches, had any role in copulation behaviour. Morgan (1968) and Berrios-Ortiz (1985) presented a list of meloid species with mesothoracic exocrine glands. Presence of such glands with unipore or multiporous releasing systems (very similar to our observation) has been also reported from genus *Canthon* (Col: Scarabaeidae) (Pluot-Sigwalt, 1988). Morgan (1968) even suggested that the glands are involved in secretion of substances associated with clustering behaviour of blister beetles. Interestingly, it has been often observed that both sexes of canthariphilous insects which attracted to covered cantharidin baits, stay close to the authentic source of chemical and use the peripheral area as courtship zone (Dettner, 1997). Setting separate parts of this puzzle together, we hereby consider cantharidin as a precopulatory sex pheromone and support the idea of McCormick and Carrel (1987) who suggested a probable role for cantharidin in close range sexual selection.

Finally, another interesting correlation is seen between the pore density and the incorporated D_2C in female hindleg (fig. 81, 82). One explanation could be presence of some exocrine glands or ducts in female hindtibia which release cantharidin to the surface via cuticular pores. Since mated females of blister beetles usually receive a large amount of cantharidin via the male insect, bearing high volume of cantharidin in such a gland or reservoir can be a sign of already mated females. Female blister beetles are often unreceptive after mating which takes until egg-laying time. After oviposition, the female inoculates egg batches with plenty of cantharidin, so its total volume of cantharidin and consequently cantharidin titre of any probable gland will be reduced significantly. In this way low titre of such a hypothetical gland implies to a receptive female. Pinto (1972, 1974, and 1975) has indicated that courtship in many species of blister beetles starts when males approach the females from the posterior side and touch the abdominal posterior tip and the hindleg of females by their antennae.

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6. Summary

Meloidae (with 3000 species worldwide) is a major family in superfamily Tenebrionoidea and has commonly called as “Blister beetles”. Larvae of meloids are parasitoids and development is hypermetamorphic with the various instars differing considerably in morphology and behaviour. Meloid adults are grouped in two categories, those which feed on leaves and petals and the others are mainly pollen feeders. Immature stages of leaf feeders are parasites of honey bees, wasps and attack the egg, larvae and the stored food within bee hives, whilst larvae of the other group are parasites of grasshoppers’ eggs. Sexual behaviour is readily divided into courtship or the sexually oriented activities of both sexes and copulation or behaviour during the period of genital coupling. Cantharidin which is found throughout the meloid body is considered being responsible for their repellent properties against a wide variety of predators and thus has great advantage for immature stages. In animal kingdom, cantharidin is only known to be produced by blister beetles and the smaller oedemerid beetles (Col: Oedemeridae) where it is found in hemolymph and various tissues. Males of blister beetles transfer large pockets of cantharidin along with sperm to the female during copulation. Females in turn provide their offspring chemical protection by passing on cantharidin to the eggs.

Cantharidin distribution within male and also its fate when introduced into female’s genitalia was not known. Furthermore, it was not clear whether cantharidin moves from one part of the male or female sexual organs to another one by means of hemolymph or it moves internally within the two organs without using hemolymph as a transit way. The other question which had been occurred in previous pieces of research was finding a high amount of cantharidin in the third pair of the accessory glands; however it was not sure if this part of the male sexual organ would have any role in the terpene production. In the current work, cantharidin distribution was studied in selected meloid species not only upon male sexual organ, but also throughout female one just after its entrance into female genitalia as a nuptial gift. Cantharidin movement through different parts of male and female genitalia was surveyed to get a better time sequence of the pharmacodynamic of this compound. Since cantharidin titre in meloid beetles is highly depended on the age, sex and mating record of individuals, any quantitative study needs working on laboratory reared specimens. In spite of our attempts, a colony could not be established, hence the alternative way of marker introduction was applied to accomplish the goal. Because of its high similarity to the naturally produced cantharidin, deuterated cantharidin (D_2C) was applied as the marker and the experimental specimens of blister beetles were administered by such a labelled compound. Our data indicate a good correlation between physiological behaviour of cantharidin and the marker, so using such a simulation, the presented results for D_2C can be generalized to cantharidin.

All samples were analysed by gas chromatography- mass spectrometry (GC-MS), but because of approximate coelution of cantharidin and D_2C , their exact measurement via normal MS seemed to be very difficult. Therefore, MRM (Multiple Reaction Monitoring) method for cantharidin/ D_2C separation and independent measurement was developed for the first time.

Looking at the natural distribution of cantharidin over the male internal organs, it was found that the compound is not distributed uniformly. Cantharidin pharmacodynamic in male of studied species could be quite different over a period of time. As the accessory glands absorb high amount of D_2C in short term, they will eventually accumulate less amount than the testis. Confirming the previous studies, it has been concluded that post-farnesyl steps in the cantharidin pathway may occur entirely in the male’s body outside reproductive system but the ultimate product is transported into male internal genitalia via membrane of the accessory glands which should be permeable to the chemical. It transfers afterwards to epididimis and the

vas deferens in higher volume comparing to other organs of male genitalia and finally absorbed by the testis. Regarding experiments using intact organs/compartments floating in Ringer solution, it has been shown that if not all at least most part of cantharidin transfers to other sexual organs internally and hemolymph has not been used as the major transit route.

In females, cantharidin enters into genitalia by male as a nuptial gift. It is first absorbed by spermatophoral receptacle in high volume while at the same time goes through ovary and is distributed upon eggs; by the way the up-taken volume by ovaries remains considerably lower than the receptacle. By time these two organs stopped accumulating of cantharidin, whereas the bursa copulatrix starts incorporating the gift actively. This reserved amount should be mainly supplied by the receptacle and ovaries directly, so the internal way of the terpene transfer also remains as the main transport way in females. Analysis of the eggs laid by D₂C injected females of *Hycleus polymorphus* demonstrated that the marker has been transferred from female to the eggs. After hatching of eggs, part of larvae was also examined for D₂C. It was found that D₂C had been also incorporated into triungulin larvae; however the titre decreased significantly from egg to larva. On the other hand, palasonin showed a bit different trend in which the level of compound from eggs to triungulin larvae had been slightly increased. Female perhaps incorporated mostly palasonin into egg batches to enhance the chemical defence of this defenceless stage on one hand and reduce any arising risk of cantharidin high toxicity to the eggs' susceptible tissues on the other hand. Besides, canthariphilous predators might be less attracted to palasonin and thus given more chance to meloid eggs to survive.

The possible role of cantharidin in sexual behaviour of blister beetles has ever been of interest to scientists. Based on our results, there is a good correlation between density of cuticular pores and the cantharidin/ D₂C level of the scape and pedicel antennomeres of the male specimens of *Cyaneolytta* sp. Light microscopy of semi thin cross sections of male scape and pedicel indicates that there are plenty of canal shape structures which have been stretched from the antennal hemolymph to the antennomere surface where cuticular pores situated. These structures can be uni- or multicellular tubules which bring the circulating cantharidin of hemolymph into the surface where later on released via the opening of cuticular pores.

Tracing the head capsule and antennal segments of those *Cyaneolytta* females which were injected by D₂C shows that the marker is found mostly in the two first antennal segments while the head capsule itself bore much less titre of injected marker. A higher concentration of the cuticular pores was also observed in the scape and pedicel. Therefore it is reasonable to hypothesize a relationship between pores and cantharidin. Tubular cells or any other carrying structures such as tyloids were found neither in cross sectioning of female antennomeres nor on integument surface. We suppose a natural system has been evolved in females of genus *Cyaneolytta* which let them select males with abundant reserves of cantharidin prior to any mating behaviour. Males and females have antennation during which males 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 thus release cantharidin on its surface. So, the porous area of male antennae can be considered as cantharidin release structures (CRS). In contrast, female porous area of antennae is apparently regarded as multiporous gustatory sensilla (MGS) which means a chemoreceptor to recognize the cantharidin titre of male sexual partner and is supported by behavioural data from other meloid species.

Considering all data published so far and what has been observed in our study, we hereby refer to cantharidin as a precopulatory sex pheromone and support the idea who suggests cantharidin role in close range sexual selection.

7. Deutsche Zusammenfassung

Die Familie Meloidae wird im Deutschen als Ölkäfer bezeichnet. Die Larven leben parasitisch und ihre Entwicklung ist hypermetamorph mit verschiedenen Stadien, die sich in ihrer Morphologie und Verhalten beträchtlich unterscheiden. Die Imagines der Ölkäfer lassen sich in Blattfresser und Pollenfresser einteilen. Die Larven der Blattfresser sind Parasiten bei Honigbienen oder Wespen, während die Larven der anderen Gruppe Heuschreckeneier parasitieren. Das Sexualverhalten lässt sich einteilen in Balz und Kopulation. Cantharidin, das im gesamten Meloidenkörper vorhanden ist, wird als verantwortlich für die abschreckende Wirkung der Käfer gegenüber zahlreichen Fressfeinden angesehen. Die Verbindung ist giftig für Säuger, Vögel und Frösche, und sie bietet somit den Larvenstadien Vorteile. Im Tierreich wird Cantharidin nach bisherigen Kenntnissen nur von Ölkäfern und den kleineren Scheinbockkäfern (Col.: Oedemeridae) produziert, wo es sich in der Hämolymphe und verschiedenen Geweben befindet. Während der Kopulation übertragen die Männchen der Ölkäfer mit dem Sperma große Cantharidinnengen auf die Weibchen, wodurch diese sowie deren Eier chemisch geschützt werden.

Die Verteilung des Cantharidins im Männchen und auch im Weibchen nach dem Transfer war unbekannt. Weiterhin war nicht klar, ob Cantharidin zwischen den einzelnen Kompartimenten der männlichen bzw. weiblichen Geschlechtsorgane mittels Hämolymphe oder auf anderem Weg verteilt wird. Ein weiteres Problem, das sich aus früheren Arbeiten ergab, war die große Menge an Cantharidin, die in dem dritten Anhangsdrüsenpaar gefunden wurde, zumal nicht klar war, ob dieses Organ eine Rolle bei der Produktion dieses Terpenoids spielt. In vorliegender Arbeit wurde die Cantharidinverteilung nicht nur in den männlichen Geschlechtsorganen, sondern auch innerhalb der Weibchen untersucht, die die Verbindung als „Hochzeitsgeschenk“ erhalten. Andererseits wurde die Verbreitung des Cantharidins in den verschiedenen Kompartimenten der männlichen und weiblichen Geschlechtsorgane beobachtet, um die zeitliche Verteilung der Verbindung aufzuklären. Da der Cantharidintiter bei Ölkäfern stark abhängig ist von Alter, Geschlecht und Kopulationsverhalten der Individuen, sind für quantitative Untersuchungen Labortiere wünschenswert, deren Lebenslauf vollständig bekannt ist. Trotz intensiver Versuche konnte keine Laborkolonie etabliert werden, worauf wir als Alternative deuteriertes Cantharidin (D₂C) als Marker einsetzten, um das Ziel dieser Studie zu erreichen. Aufgrund der großen Ähnlichkeit mit natürlichem Cantharidin wurde D₂C eingesetzt und die Versuchstiere damit behandelt. Unsere Daten zeigen eine starke Korrelation hinsichtlich der physiologischen Eigenschaften zwischen Cantharidin und dem Marker, so dass die Ergebnisse für D₂C auch für Cantharidin gültig sind.

Alle Proben wurden mittels Gaschromatographie/Massenspektrometrie (GC/MS) analysiert, was wegen der ähnlichen Retentionszeiten von Cantharidin und D₂C sehr schwierig schien. Daher wurde erstmals eine MRM (Multiple Reaction Monitoring)- Methode zur Trennung und unabhängigen Messung von Cantharidin und D₂C entwickelt.

Betrachtet man die natürliche Cantharidinverteilung der männlichen inneren Organe, so findet man deutliche Unterschiede. Wir fanden Hinweise, dass die Verteilung bei den Männchen der untersuchten Arten stark zeitabhängig ist. Obwohl die Anhangsdrüsen zunächst große Mengen an D₂C absorbieren, akkumulieren sie letztlich geringere Mengen als die Hoden. Im Einklang mit früheren Arbeiten nehmen wir an, dass die Cantharidin-Biosynthese aus der Vorstufe Farnesyl vollständig außerhalb der Geschlechtsorgane des Männchens abläuft und das endgültige Produkt über cantharidinpermeable Membranen der Anhangsdrüsen in die männlichen Geschlechtsorgane gelangt. Es wandert anschließend hauptsächlich in Nebenhoden und vas deferens und wird schließlich durch die Hoden absorbiert. In unseren Ringer-

Experimenten konnte gezeigt werden, dass zumindest das meiste Cantharidin (wenn nicht alles) intern in den Geschlechtsorganen transportiert wird und die Hämolymphe keinen bedeutenden Transitweg darstellt.

In die Geschlechtsorgane der Weibchen gelangt das Cantharidin als „Hochzeitsgeschenk“. Es absorbiert dort zunächst in großen Mengen im Receptaculum seminis, wandert aber gleichzeitig in das Ovar und verteilt sich in den Eiern, wobei das aufgenommene Volumen in den Ovarien deutlich geringer bleibt als im Receptaculum. Mit der Zeit beenden diese beiden Organe die Cantharidinaufnahme, während die Bursa copulatrix die Substanz aktiv aufzunehmen beginnt. Die aufgenommene Menge dürfte hauptsächlich von Receptaculum und Ovar kommen, so dass auch im weiblichen Geschlechtstrakt der interne Transportweg die entscheidende Rolle spielt. Die Analyse der Eier von *Hycleus polymorphus*-Weibchen, denen D₂C injiziert wurde, zeigte einen Transfer des Markers vom Weibchen auf die Eier. Nach dem Schlupf der Larven wurden auch diese auf D₂C untersucht, wobei ebenfalls ein Transfer auf die Triungulus-Larven (L1) nachgewiesen wurde, wenngleich der Titer vom Ei zur Larve signifikant sinkt.

Palasonin dagegen zeigt einen anderen Trend, indem der Gehalt der Verbindung vom Ei zur Triungulus-Larve leicht steigt. Man kann spekulieren, dass die Weibchen hauptsächlich Palasonin in die Eigelege einbauen, um einerseits die chemische Verteidigung dieser ansonsten schutzlosen Stadien zu verbessern und um andererseits das Risiko einer Selbstvergiftung des empfindlichen Eigewebes durch das stark giftige Cantharidin zu reduzieren. Ferner könnten canthariphile Räuber weniger stark von Palasonin angelockt werden, was die Überlebenschance der Meloideneier weiter erhöht.

Die mögliche Rolle von Cantharidin beim Sexualverhalten der Ölkäfer wurde in der Wissenschaft immer wieder diskutiert. Nach unseren Befunden besteht ein bemerkenswerter Zusammenhang zwischen der Anzahl cuticularer Poren und der gemessenen Cantharidin/ D₂C-Menge in den basalen Antennengliedern Scapus und Pedicellus bei den Männchen von *Cyaneolytta* sp., einer ostafrikanischen Art. Lichtmikroskopische Untersuchungen von Dünnschnitten des männlichen Scapus und Pedicellus zeigen eine Fülle von kanalartigen Strukturen, die sich von der Hämolymphe bis zur Antennenoberfläche erstrecken, wo sich die Poren befinden. Die gefundenen Strukturen könnten ein- oder mehrzellige Tubuli sein, die das in der Hämolymphe zirkulierende Cantharidin zur Antennenoberfläche befördern, wo es später über die Öffnungen der Cuticularporen freigesetzt wird.

Untersuchungen der Kopfkapsel und Antennen von *Cyaneolytta*-Weibchen, denen D₂C injiziert wurde, zeigen, dass der Marker hauptsächlich in den ersten beiden Antennensegmenten gefunden wird, während die Kopfkapsel einen viel geringeren Titer aufweist. Auch bei den Weibchen wurde eine höhere Konzentration von Cuticularporen beobachtet. Daher erscheint ein Zusammenhang zwischen Cantharidin und den Cuticularporen plausibel. Es wurden keine tubulären Zellen oder andere Transportstrukturen wie Tyloide in den Querschnitten der weiblichen Antennenglieder oder auf der Oberfläche des Integuments gefunden. Wir vermuten, dass sich in der Gattung *Cyaneolytta* ein System entwickelt hat, in dem Weibchen vor der Kopulation solche Männchen auswählen, die große Cantharidinnengen aufweisen. Männchen und Weibchen haben Antennenkontakt, währenddessen die cantharidinhaltigen Poren des Scapus und pedicellus in direkten Kontakt mit den weiblichen Antennen kommen und Cantharidin an der Oberfläche freisetzen. Somit können die Poren enthaltenden Strukturen als Cantharidin freisetzende Strukturen (CFS) bezeichnet werden. Dementsprechend kann die gleiche Region auf der weiblichen Antenne als multiporöse gustatorische Sensille (MGS) bezeichnet werden, die einen Chemorezeptor zur Wahrnehmung des Cantharidintiters beim männlichen Geschlechtspartner darstellt.

Betrachtet man alle bislang veröffentlichten Daten und die Beobachtungen unserer Arbeit, so sehen wir Cantharidin als präkopulatorisches Sexualpheromon, das eine Rolle bei der sexuellen Selektion im Nahbereich spielt.

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Erklärung

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

Mahmood Reza Nikbakhtzadeh
Bayreuth, den 10.09.04