Intersexual Transfer of a Toxic Terpenoid during Copulation and Its Paternal Allocation to Developmental Stages: Quantification of Cantharidin in Cantharidin-Producing Oedemerids (Coleoptera: Oedemeridae) and Canthariphilous Pyrochroids (Coleoptera: Pyrochroidae)

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In whole body extracts of the canthariphilous pyrochroid *Schizotus pectinicornis* and the cantharidin-producing oedemerid *Oedemera femorata* cantharidin contents were determined by means of quantitative gas chromatography. Adults and all developmental stages of both species contain this terpenoid, but in considerably different amounts. Cantharidin contents differ significantly between the sexes of adult *O. femorata:* 8.4 μg/female, 3.5 μg/male. Values for *Sch. pectinicornis* are more than 30-fold lower than those of *O. femorata* and no intersexual difference in the cantharidin content could be detected. Eggs of *O. femorata* contained cantharidin and larvae of this species are capable of synthesizing this terpenoid. The total amount of cantharidin increases in successive instars. In *Sch. pectinicornis,* the content of cantharidin decreases from egg to first instar.

Feeding and copulation experiments with [$^2H_2$]cantharidin indicate that in *O. femorata* no or only very small amounts of cantharidin are transferred from males to females at copulation. Thus, males do not invest in the protection of their offspring by cantharidin gifts to the mate. For *Sch. pectinicornis* an intersexual transfer of labelled cantharidin during copulation was detected. About 45% of the entire cantharidin content in mated females derived from fed males. Analyses of eggs and first instar larvae show that a paternal allocation of ^{2}[H_2]cantharidin to developmental stages exists for this canthariphilous pyrochroid.

Introduction

The terpenoid cantharidin represents one of the most famous and oldest known toxins from insects. The only natural source are blister beetles (Meloidae) and false blister beetles (Oedemeridae). Some blister beetle species contain up to 11 mg cantharidin per individual (Capinera *et al.,* 1985) and values of 38 μg have been determined for oedemerids (summary in Frenzel and Dettner, 1994). These beetles are capable of synthesizing this unusual vesicant, deterrent and antifeedant (Carrel *et al.,* 1986; Carrel and Eisner, 1974), which also acts as a systemic poison to higher vertebrates (LD_{50} to humans: 0.5 mg/kg) (McCormick and Carrel, 1987). Recent investigations revealed the cytotoxic effect of cantharidin: From very different organisms (*e.g.* yeast, certain arthropods, mammalian tissues) a protein phosphatase 2A (PP2A) has been isolated which dephosphorylates other enzymes (Cohen, 1989; Cohen *et al.,* 1989). Cantharidin inhibits this cytosolic protein (Honkanen, 1993; Li and Casida, 1992) and injures important cell functions like glycogen metabolism. However, several organisms – *e.g.* frogs and Japanese quail – are known to be not harmed after ingestion of cantharidin crystals or cantharidin-containing diet (Eisner *et al.,* 1990; Kelling *et al.,* 1990; Carrel, 1971). These organisms and, as expected, cantharidin-producing meloids and oedemerids must have evolved mechanisms which prohibit the binding between the probable ubiquitous PP2A and cantharidin in order to avoid cytotoxic effects. Possibly, they have at least one cantharidin-binding protein which may be re-

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sponsible for transport and detoxification of this terpenoid.

Moreover, cantharidin is a potent attractant for various insects which belong to different insect orders (Fig. 1) (Mafra-Neto and Jolivet, 1994; Frenzel *et al.*, 1992; Dettner, 1989; Young, 1984; LeSage and Bousquet, 1983; Havelka, 1978; Fey, 1954; Hemp, pers. comm.; Holz, unpubl. data). Until now two taxa were unknown to be cantharidin-philous: chrysomelids of the tribe Galerucini (Mafra-Neto and Jolivet, 1994; Hemp, pers. comm.) and staphylinids (Holz, unpubl. data). In the field cantharidin-philous insects feed on living or dead meloids (Mafra-Neto and Jolivet, 1994; LeSage and Bousquet, 1983; Havelka, 1978), oedemerids (Frenzel and Dettner, 1994) or their cantharidin-containing excrements. In the laboratory cantharidin-philous insects often consume offered cantharidin crystals without any recognizable irritation or toxification (Frenzel and Dettner, 1994; Schütz and Dettner, 1992). They seem to be cantharidin-tolerant and must have evolved detox-

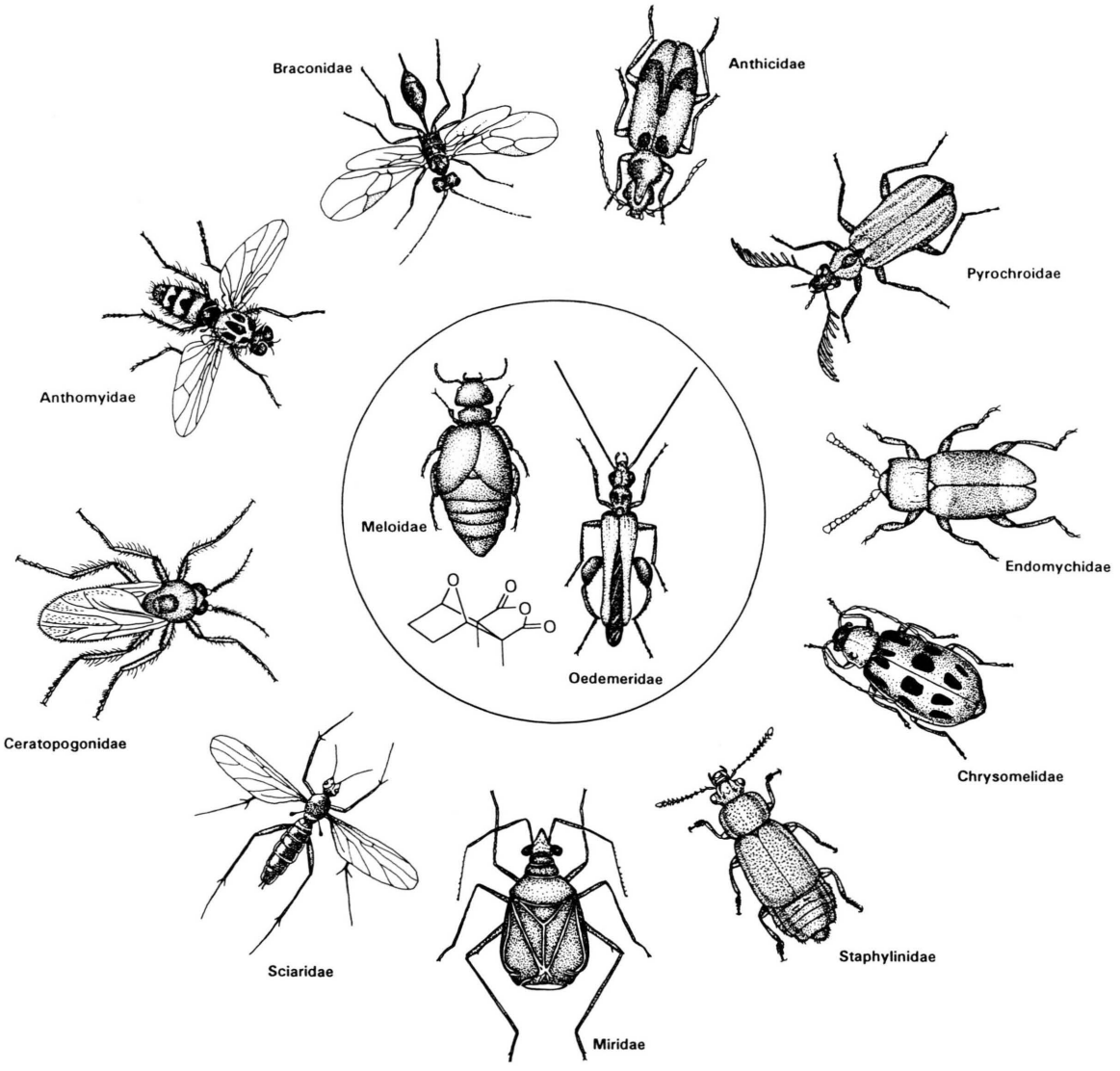

Fig. 1. Cantharidin producers (in the circle) and representatives of cantharidin-philous taxa belonging to different insect orders: Coleoptera, Heteroptera, Diptera, Hymenoptera.

ification mechanisms similar to cantharidin-producing beetles. As cantharidin uptake has no nutritional function, it seems to be a case of pharmacophagy as it was described for pyrrolizidine alkaloid-containing insects (Boppré, 1986).

Although an intersexual transfer of cantharidin and its parental allocation to developmental stages have already been discussed for canthariphilous pyrochroids (Eisner, 1988), the aim of this investigation was to substantiate this consideration by experimental data. With cantharidin-producing oedemerids, no studies have been made so far. First of all we detected cantharidin contents in field-caught specimens of *Oedemera femorata* (Scop.) (Oedemeridae) and *Schizotus pectinicornis* L. (Pyrochroidae), respectively, and of their offspring to determine the "natural" cantharidin concentrations. Until now it was unknown, whether canthariphilous pyrochroids of Middle Europe contain the terpenoid. We were interested whether larvae of both species are capable of synthesizing cantharidin or whether the content of the terpenoid decreases in successive instars. As individuals of both investigated species contained the terpenoid, males were fed with $[^2H_2]$cantharidin. By that, we were able to investigate intersexual cantharidin transfer at copulation and paternal endowment of cantharidin upon developmental stages. Similar studies have already been made with pharmacophagous arctiid moths *(Utetheisa ornatrix)* and danaine butterflies *(Danaus gilippus)* in connection with pyrrolizidine alkaloids (Dussourd *et al.*, 1989; Dussourd *et al.*, 1988).

Materials and Methods

Insects and breeding conditions

Oedemeridae: *Oedemera femorata* were caught in Upper Franconia (Bavaria) during June 1993 from flowers of composites (*e.g.* marguerites, blackberries, camomiles). 25 males and 20 females were immediately frozen (−25 °C), while the other specimens were sexed and kept in plastic boxes where the beetles were given the opportunity to feed on pollen granulate. Boxes with females were provided with stems of *Heraclium sphondylium* for depositing eggs. Every day water was sprayed on the filter-paper in the boxes. 26 eggs were frozen immediately after depositing and five larvae of each instar stage which were reared at room temperature were frozen one day after moulting.

Pyrochroidae: *Schizotus pectinicornis* were caught in Upper Franconia (Bavaria) with cantharidin traps in the field in the middle of May 1993. A folded filter-paper disk (diam.: 5.5 cm) soaked with a diluted cantharidin-acetone solution was placed at the bottom of a rectangular plastic box (10×10×6 cm) under gauze. Thus, an intake of cantharidin by the attracted beetles was impossible. In the laboratory 9 males and 5 females were immediately frozen. Further specimens of *Sch. pectinicornis* were kept alive under the same conditions mentioned above; the beetles had free access to honey water. Preliminary investigations showed that plant or tree stems were not necessary as substrate for depositing eggs; females of *Sch. pectinicornis* laid their eggs under filter-paper or dishes with honey water. As the larval development of *Sch. pectinicornis* takes at least two years, only first instar larvae were analyzed.

Synthesis of deuterated cantharidin

$[^2H_2]$Cantharidin was synthesized by a DIELS-ALDER reaction of furane with 2,5-dihydrothiophene-3,4-dicarboxylic acid anhydride (Dauben *et al.*, 1980) in an ethereal solution of 5 M $LiClO_4$ (Grieco *et al.*, 1990). The mixture of *exo-* (15%) and *endo*-isomers (85%) was separated by chromatography on SiO_2 using ether/pentane (4:1, v:v) as eluent. The product was hydrogenated in THF using Pd-C (10%) as catalyst. Desulfurization was achieved by refluxing the thioether with freshly prepared RANEY-Ni in CD_3OD. Commercial RANEY-Ni failed to give cantharidin. Overall yield was 16%. Spectroscopic data of $[^2H_2]$cantharidin: ^1H NMR (250 MHz, $CDCl_3$): d 1.2–1.20 (s, br., 4H); 1.85 (m, 4H); 4.73 (s, br., 2H). – IR (KBr): 2963, 2180 br., 1841, 1780, 1454, 1430, 1262, 1018 cm^{-1}. – MS (EI 70 eV): see Fig. 2b. – MS (CI, CH_4): 201 (18), 200 (32), 199 (100), 198 (52), 197 (47), 196 (22), 195 (11), 171 (13), 153 (21), 152 (19), 127 (10), 125 (14), 109 (51), 108 (24), 97 (41), 96 (15), 59 (25).

Feeding and copulation experiments with deuterated cantharidin

Male *O. femorata* ($n = 11$) were given the opportunity to feed continuously on deuterated can-

tharidin crystals (20 μg/mg pollen) for seven days. Male *Sch. pectinicornis* (n = 19) had free access to a filter-paper disk impregnated with 17 μg deuterated cantharidin solved in chloroform for a two days period. (Offered [$^2H_2$]cantharidin contained less than 12% authentic cantharidin.) Ten males of *Sch. pectinicornis* were frozen after feeding. Fed males of both species were put together with the respective unfed females. Plastic boxes with *O. femorata* contained stems of *Heraclium sphondylium* for egg deposition. After copulation and oviposition, the beetles were removed and immediately frozen. One half of the egg clutches was frozen, whereas the other eggs developed to first instar before freezing.

Chemical analysis

Extraction

Prior to extraction dry weight (dw) was determined for all samples. Whole specimens, reproductive organs, eggs, and larvae were hydrolyzed in small fused test tubes with 50–300 μl 6 N hydrochloric acid (HCl) at 120 °C for 4 h in order to resolve all body structures and to set bound cantharidin free. Afterwards an equivalent amount of chloroform (50–300 μl) was added and each sample was vigorously shaken on a Vortex mixer for 30 s. Then, the samples were centrifuged at 2000×g for 3 min. The organic phase was filtered and transferred to a vial. To reduce evaporation of the chloroform during storage, water was added.

Quantitative gas chromatography

A Carlo Erba Mega HRGC 5160 gas chromatograph with on-column injector, equipped with a CP-Sil 19 CB fused silica capillary column (Chrompack, 12 m×0.32 mm I.D.; 0.25 μm phase thickness) was used for quantitative analysis. The analytical column was connected to a deactivated precolumn (1.5–0.6 m). Chromatographic conditions were as follows: initial temperature 55 °C for 1 min, temperature increase of 15 °C/min up to 200 °C, then with 20 °C/min to 270 °C for 5 min. Temperature of detector (FID) was 300 °C. Helium was used as carrier gas (70 kPa). The pressure of hydrogen was 60 kPa and 150 kPa for air.

GC-MS (gas chromatography-mass spectrometry)

The proportion of authentic and deuterated cantharidin in the samples of the feeding and copulation experiments was determined by using GC-MS analysis.

1 μl of each sample was injected by using split/splitless injection in a Carlo Erba Vega Series 2, GC 6000 gas chromatograph equipped with a CP-Sil 19 CB fused silica capillary column (Chrompack, 12.5 m×0.32 mm I.D.; 0.25 μm phase thickness) and connected to a Finnigan MAT Ion Trap Detector (ITD). Electron impact ionization (EI, 70 eV) provides mass spectra with a characteristic fragmentation of cantharidin: the base peak with m/z 96 and a fragment with m/z 128 (Fig. 2a). Double deuterated cantharidin provides a mass spectrum with m/z 98 and m/z 130 (Fig. 2b).

The labelled cantharidin cannot be distinguished from the natural one by gas chromatography, but by mass spectrometry (GC-MS) (Fig. 2c). The intensities of the fragments m/z 96 and 98 were used to determine the proportion of the authentic and deuterated cantharidin in a sample by means of calibration curves.

Statistical analysis

Non-parametric methods were performed with the programme CSS: Statistica of StatSoft, Inc. and median and average deviation were used. The latter is another measure of variability. It is calculated as the sum of absolute deviations (mean for respective variable minus raw score) divided by N (number of valid cases).

For statistical comparison, the two-tailed Mann-Whitney U-test was used.

Results and Discussion

Total content of cantharidin in adults of O. femorata and Sch. pectinicornis

Oedemerid beetles are the only known natural source of cantharidin in Upper Franconia, the area where our field studies were carried out. They were found on flowers of Compositae, whereas pyrochroids were caught with cantharidin traps in the field. Until now it was unknown, whether cantharophilous pyrochroids in Middle Europe contain cantharidin.

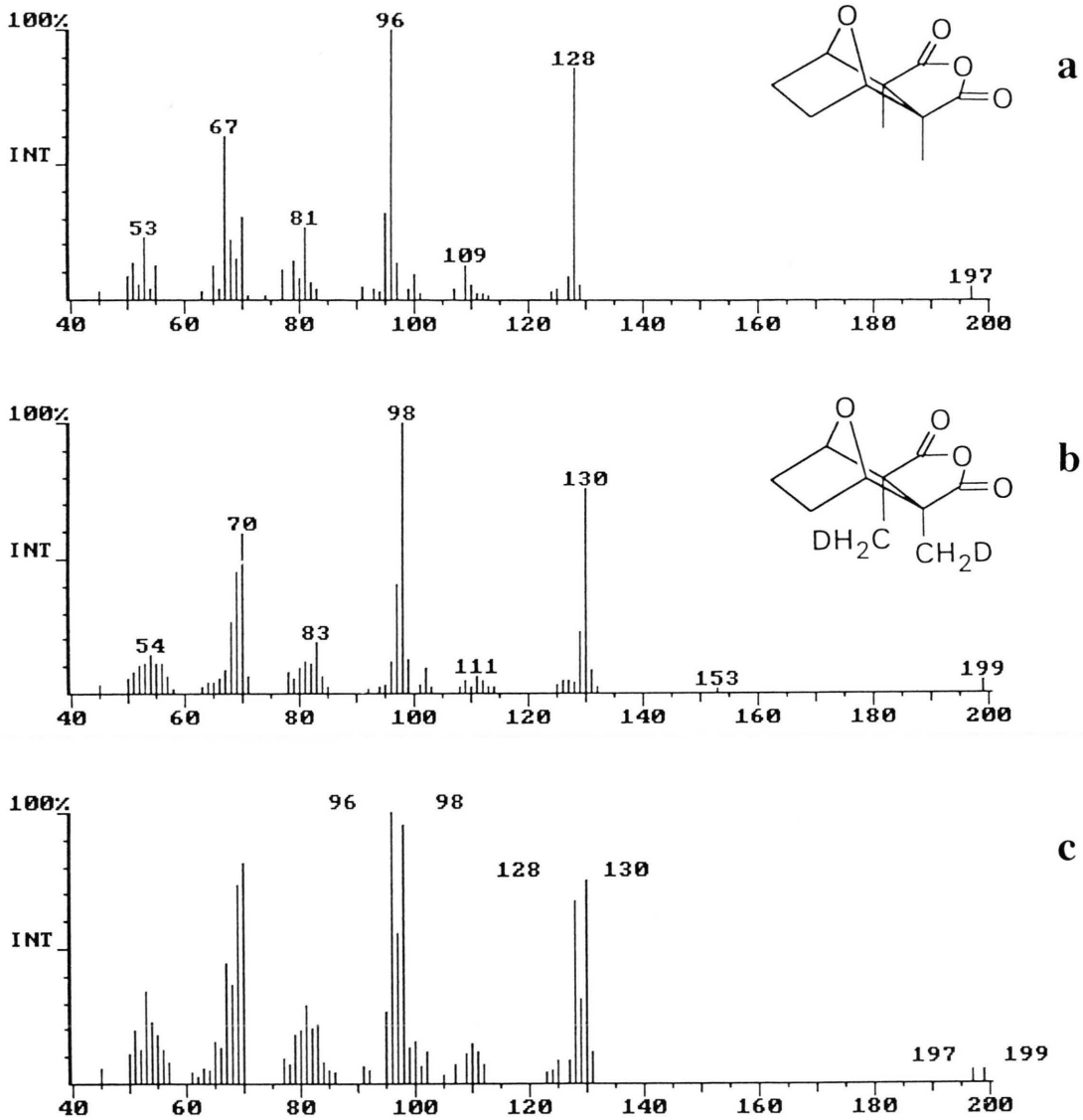

Fig. 2. EI-mass spectra of authentic cantharidin (a), $^{2}[H_2]$cantharidin (b) and a mixed solution of a and b (3:1) (c).

Fig. 3 shows average contents of cantharidin in field-collected males and females of *O. femorata* and *Sch. pectinicornis*. Individuals of both species contained cantharidin, but in considerably different amounts: Males of *O. femorata* contained about 32-fold more cantharidin than male *Sch. pectinicornis* (30-fold in terms of concentration), females even about 63-fold (30-fold, respectively). These differences can be explained with the capability of *O. femorata* to synthesize cantharidin. Even one day old adults emerged in the laboratory contained considerable amounts of the terpenoid, although they had no access to cantharidin-containing diet (Streil, unpubl. data). External precursors with a cantharidin-like skeleton are probably not necessary for the cantharidin synthesis in adults of *O. femorata*. Furthermore, oedemerids have never been found at or in cantharidin baits in the field. Accordingly, cantharidin sources do not attract oedemerids.

On the contrary, both sexes of *Sch. pectinicornis* are attracted to cantharidin traps in the field.

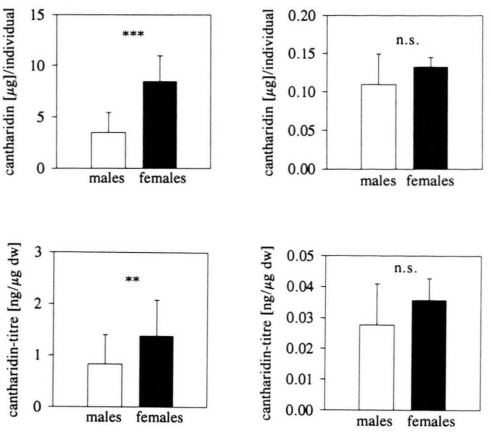

Fig. 3. Average content and concentration of cantharidin (both median) in males and females of *O. femorata* (left) and *Sch. pectinicornis* (right). Bars: average deviation. Sample sizes: *O. femorata:* males $n = 25$, females $n = 20$; *Sch. pectinicornis:* males $n = 9$, females $n = 5$. Abbreviations: dw, dry body weight; n.s., not significant; **, $p \leq 0.01$; ***, $p \leq 0.001$. Differences between amounts and concentrations of cantharidin were tested by using two-tailed Mann-Whitney U-test.

Probably, the cantharidin content of this cantharidin-philous species and of other cantharidin consumers depends on the sympatric occurrence of oedemerids which represent the only known natural source of this terpenoid at the study site. Investigations of Schütz and Dettner (1992) show that the geographically varying cantharidin titre of the cantharidin-philous beetle *Notoxus monoceros* (Anthicidae) is correlated with the presence of meloid and oedemerid beetles in the respective area.

Besides these differences between oedemerids and pyrochroids, intraspecific distinctions can be made for the investigated species (Fig. 3). Female *O. femorata* contained significantly more cantharidin in absolute amounts than males (8.4 µg/females, 3.5 µg/males; $p \leq 0.001$, Mann-Whitney U-test). Referring to the dry body weight females had nearly twice as much cantharidin than males ($p \leq 0.01$). The intersexual difference in the cantharidin content of *O. femorata* corresponds to the results of Carrel *et al.* (1986). They showed that females of the oedemerid species *Heliocis repanda* and *Oxycopis thoracica* contained two to three times more cantharidin than males of the same species. The total amounts of cantharidin we detected in *O. femorata* are lower than those found in other oedemerid and meloid species (Frenzel and Dettner, 1994; Carrel *et al.*, 1986). Probably, the intraspecific difference in the cantharidin content of adult *O. femorata* can be explained by the parental endowment of cantharidin upon developmental stages. This will be discussed below in connection with our results from the copulation experiments.

In contrast, the cantharidin content of adult *Sch. pectinicornis* do not differ significantly between the sexes, neither in terms of total amounts nor in cantharidin titres (Fig. 3).

Content of cantharidin in developmental stages of O. femorata and Sch. pectinicornis

In the laboratory field-caught individuals of both species were given the opportunity to copulate and deposit eggs. These eggs and hatched larvae were analyzed for their cantharidin content.

In eggs and larvae of both species cantharidin was detected. The total amount of cantharidin in developmental stages of *O. femorata* significantly increases in successive instars ($p \leq 0.05$), with the exception of the first to second instar (Fig. 4). This result indicates undoubtedly that larvae of *O. femorata* are capable of synthesizing cantharidin, especially as in stems of *Heraclium* (the only diet of larvae) no cantharidin was determined at all. Except for the first instar larva the cantharidin concentration in elder larvae (L2–L5) of *O. femorata* shows a constant value of about 4 ng/µg dry body weight. This indicates that the increase of the cantharidin content is positively correlated with the increase in biomass in successive instars. The significant increase of cantharidin content from egg to first instar can be explained by field and laboratory observations: In the laboratory, females of *O. femorata* deposited on average 35 eggs in one clutch, but per stem of *Heraclium* only one or two larvae were observed in the field. If the observed clutch size is not an artefact due to laboratory conditions, how can the decrease from 35 eggs to only 1–2 larvae be explained? It seems probable that first instar larvae do not hatch simultaneously and in that way the firstly hatched larva gets the chance to incorporate cantharidin from cantharidin-containing eggs. That an intraspecific egg cannibalism actually exists for *O. femorata* was observed in experiments with L1 larvae and eggs

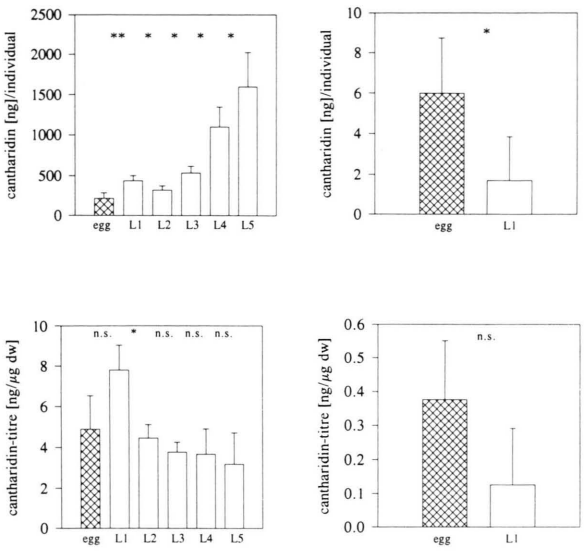

Fig. 4. Average content and concentration of cantharidin (both median) in developmental stages of *O. femorata* (left) and *Sch. pectinicornis* (right). Bars: average deviation. Sample sizes: *O. femorata:* eggs $n = 26$ (pooled in 5 samples), each instar $n = 5$; *Sch. pectinicornis:* eggs $n = 172$ (pooled in 10 samples), L1 larvae $n = 261$ (pooled in 10 samples). Abbreviations: dw, dry body weight; n.s., not significant; *, $p \leq 0.05$; **, $p \leq 0.01$. Differences between amounts and concentrations of cantharidin were tested by using two-tailed Mann-Whitney U-test.

of this species (Streil, unpubl. data). Similar conditions have been found in the pharmacophagous caterpillar *Utetheisa ornatrix* (Bogner and Eisner, 1991). Larvae of this moth are evidently prone to cannibalize eggs in the laboratory and the presence of pyrrolizidine alkaloids in the egg can increase its vulnerability to cannibalism. Further investigations with *O. femorata* must show whether larvae selectively prey on conspecific eggs/larvae that contain cantharidin.

In the case of *Sch. pectinicornis* the total amount of cantharidin decreases significantly from egg to first instar (Fig. 4). Referring to the concentration no significant difference was observed between egg and first instar. However, there is a tendency for a decrease in the cantharidin titre ($p \leq 0.059$). Possibly, L1 larvae excrete or metabolize the terpenoid in considerable amounts. Moreover, it seems possible that cantharidin is mainly incorporated in the egg shell and first instar larvae does not feed on it.

The comparison of cantharidin content in developmental stages between *O. femorata* and *Sch. pectinicornis* gives the same result as our investigations with adults do (Fig. 3). Obviously, eggs and L1 larvae of *O. femorata* contained considerably more total cantharidin than those of *Sch. pectinicornis* (Fig. 4): eggs 36-fold more cantharidin (13-fold in terms of cantharidin titre). L1 larvae even about 217-fold (46-fold, respectively).

Additionally, in both species cantharidin titres of developmental stages are much higher than in corresponding adult beetles (Figs. 3 and 4). This indicates that in cantharidin-producing oedemerids and cantharidin-consuming pyrochroids the most vulnerable stages of their offspring are protected by high cantharidin concentrations. Further investigations must show whether a high cantharidin content serves the developmental stages of both species effectively in defense against their predators. Carrel and Eisner (1974) have found that cantharidin is a highly effective feeding deterrent to certain predaceous insects.

Transfer of deuterated cantharidin during copulation

To investigate a possible intersexual transfer of cantharidin, males were given the opportunity to feed on [$^2H_2$]cantharidin for some days. After an intake of deuterated cantharidin and copulation with unfed females it was possible to investigate whether labelled cantharidin is transferred from males to females at copulation and whether females incorporate the terpenoid in the eggs.

Males of *O. femorata* incorporate offered deuterated cantharidin crystals which were added to the pollen diet. After copulation with unfed females males contained between 14.2 and 83.6% deuterated cantharidin of the entire cantharidin content in whole body extracts (Fig. 5). The analysis of mated females showed no clear result: In whole body extractions of 6 females no deuterated cantharidin could be detected. However, when the *receptaculum seminis* of further mated females was analyzed, in two of four samples labelled cantharidin was found (30 ng and 175 ng [$^2H_2$]cantharidin). This result indicates that in the species *O. femorata* no or only very small amounts of cantharidin are transferred from males to females at copulation. As field-caught females contained significantly

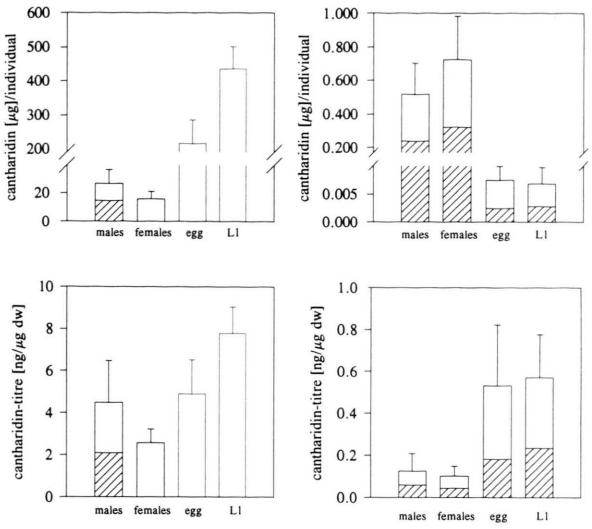

Fig. 5. Average amount and concentration of cantharidin (both median) in mated males and females, eggs and L1 larvae of *O. femorata* (left) and *Sch. pectinicornis* (right) after feeding males with deuterated cantharidin. Amounts of $[^2H_2]$cantharidin are hatched. Bars: average deviation. Sample sizes: *O. femorata:* males $n = 7$, females $n = 6$; *Sch. pectinicornis:* males $n = 10$, females $n = 9$, eggs $n = 198$ (pooled in 10 samples), L1 larvae $n = 303$ (pooled in 10 samples). Abbreviations: dw, dry body weight; n.s., not significant; *, $p \leq 0.05$; **, $p \leq 0.01$. Differences between amounts and concentrations of cantharidin were tested by using two-tailed Mann-Whitney U-test. In eggs and L1 larvae of *O. femorata* no deuterated cantharidin was detected and total amounts were not analyzed by GC. Inserted values of *O. femorata* (eggs and L1 larvae) are taken from Fig. 4.

more cantharidin than unfed males (Fig. 3), there seems to be no need for an intersexual transfer of cantharidin at copulation. Moreover, the amounts of eventually transferred, deuterated cantharidin are much lower than the cantharidin content found in one egg of *O. femorata* (Fig. 4). Even the entire content of cantharidin (authentic and deuterated terpenoid) in the male reproductive organs corresponds only to the amount of cantharidin found in 1–2 eggs. Obviously, males of *O. femorata* do not invest in the protection of their offspring by cantharidin endowment. In the cantharidin-producing meloids the conditions are different: In *Lytta vesicatoria,* for example, only males synthesize cantharidin. They store the terpenoid in the accessory glands of their sexual organs and transfer cantharidin to females during copulation (Sierra *et al.,* 1975).

The qualitative and quantitative results of cantharidin in the cantharidin-bearing *Sch. pectinicornis* show that males of this species transfer high amounts of deuterated cantharidin to females at copulation (Fig. 5). Males ingested high amounts of $[^2H_2]$cantharidin prior to copulation: The entire cantharidin content was 0.84 µg/male ($n = 10$) and 85% was labelled cantharidin. After copulation the entire cantharidin content in fed males decreased to 0.52 µg/individual ($n = 9$). In mated females on average 0.32 µg $[^2H_2]$cantharidin per individual ($n = 9$) could be detected (45% of the entire cantharidin content). Because females had no opportunity to feed on deuterated cantharidin it must be transferred during copulation with fed males. Mated females incorporated this nuptial gift in the eggs (Fig. 5). They contained on average 2.48 ng $[^2H_2]$cantharidin which represents 33% of their entire cantharidin content in one egg. In samples of L1 larvae deuterated cantharidin was found in nearly the same amount. These results demonstrate that a cantharidin transfer from males to females and from females to eggs exists in the cantharidin pyrochroid *Sch. pectinicornis.* Males of this species invest in the protection of their offspring by cantharidin gifts which represent about 50% of the entire cantharidin content in fed males. To these data of transferred $[^2H_2]$cantharidin an unknown amount of authentic cantharidin must be added, because authentic cantharidin derived from males was transferred and incorporated as well, but could not be distinguished from authentic cantharidin of females. Our experimental results of *Sch. pectinicornis* confirm undoubtedly the report of Eisner (1988) who described a cantharidin transfer during copulation for pyrochroids. Further investigations with *Sch. pectinicornis* must show whether females select those males for copulation which have a high cantharidin content.

Investigations of Frenzel and Dettner (1994) showed that a high cantharidin content can increase the individual fitness. Cantharidin-containing individuals of *Atrichopogon oedemerarum* and *A. trifasciatus* (Ceratopogonidae) were rejected by the predaceous empidid fly *Platypalpus* spec. as compared with unfed ceratopogonids. Whether cantharidin serves as a deterrent and antifeedant against specific predators of *O. femorata* and *Sch. pectinicornis* must be investigated in further experiments. Probably, developmental stages of

these species are chemically protected against predators through the capability of synthesizing cantharidin *(O. femorata)* or the intersexual transfer of the terpenoid during copulation *(Sch. pectinicornis)*.

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