

## Origin, transfer and distribution of cantharidin-related compounds in the blister beetle *Hycleus scabiosae*

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**Abstract:** Cantharidin provides chemical protection for the coleopteran families Meloidae and Oedemeridae. In the present study, it was observed that cantharidin concentration in *Hycleus scabiosae* was slightly decreased from mated females (mean = 0.011 mg/mg of dry weight) to males (mean = 0.010 mg/mg) and considerably diminished in relation to virgin females (mean = 0.005 mg/mg). Significant concentrations of palasonin (21.69 ng/mg among virgins and 17.49 ng/mg in mated females) and palasoninimide (14.62 ng/mg in virgins and 9.17 ng/mg in mated females) were found in *H. scabiosae*. Palasonin, palasoninimide and cantharidinimide content of eggs were measured as 5.61, 7.69 and 7.80 ng/mg respectively. Surprisingly, males showed no trace of cantharidin-related compounds (CRCs); therefore CRCs in *H. scabiosae* could not be transferred from males to females and based on experiments employing its deuterated form, cantharidin is probably independently synthesized in females from the male nuptial transfer. An inseminated female incorporates about 38.5 ng of cantharidin (0.34% of the maternal content), 196.35 ng of palasonin (91.82% of maternal content) and 269.15 ng of palasoninimide (96.70% maternal content) into each egg mass during oviposition. It seems that eggs of this meloid species exploit a different array of protective chemicals by increasing the ratio of CRCs versus cantharidin. CRCs are less toxic than cantharidin; therefore, such compounds might have been deposited in eggs as a safer substitute for cantharidin to provide effective protection, but does not simultaneously harm the susceptible embryo.

**Key words:** cantharidin, Meloidae, blister beetle, *Hycleus*, chemical defense.

### INTRODUCTION

Cantharidin (C<sub>10</sub>H<sub>12</sub>O<sub>4</sub>) is among the most widely known insect defensive products (1, 2). Autogenous producers of cantharidin occur exclusively within the coleopteran families of Meloidae and Oedemeridae (1, 3-12). The family Meloidae currently has 125 genera and 3000 species, with the greatest diversity in temperate steppe and arid regions, and in subtropical and tropical savannas (13, 14).

Cantharidin is a monoterpene anhydride, and is highly toxic to most mammals, birds and frogs (2, 15). It is well established that in many species of blister beetles, females possess but cannot

produce cantharidin (11, 16). Females acquire it from males through frequent copulation and it passes thence to eggs for chemical protection (15, 17).

A few substances structurally similar to cantharidin, known as cantharidin-related compounds (CRCs), have been found in blister beetles. The function of CRCs in meloid beetles is not yet clear. It is unknown whether this series of compounds are cantharidin metabolites or if they are synthesized via an independent biochemical pathway. There are also many questions concerning the method of CRC synthesis and transfer in male and female blister beetles. The present study has tried to answer some of these

questions and thus resolve a few more pieces of this complex biological puzzle.

## MATERIALS AND METHODS

### Field Collection, Insect Identification and Dissection of Specimens

Specimens of *Hycleus scabiosae* (Olivier, 1811) (Coleoptera: Meloidae) were collected in July 2008 from Mahabad county (36.41N, 45.57E) and along Sarv road (37.38N, 44.59E) in northwestern Iran, while resting on flowers or stems of different weed shrubs in the families Compositeae and Leguminoseae. Several batches of eggs were also collected from the captured females.

Preliminary identification was done in the Department of Medical Entomology (TMU) using a key to the Old World genera of Meloidae (14). Subsequently, specimens were identified at the species level by Dr. Marco Bologna at Roma Tre University in Rome, Italy.

Live beetles were sexed by observing their external genitalia (male aedeagus vs. female valvifers) (Figure 1). The external genitalia of female specimens were additionally examined for fresh spermatophores, which indicate a recent copulation. CO<sub>2</sub>-anesthetized females were also dissected in deionized water and their ovaries and receptacles were examined with the aid of a binocular microscope (Stemi-SR®, Zeiss, Germany) in order to determine their precise copulation background. Frozen materials were transported in separate vials to the laboratory at Tarbiat Modares University (TMU), Tehran, Iran, where the work was performed.

### Artificial Diet

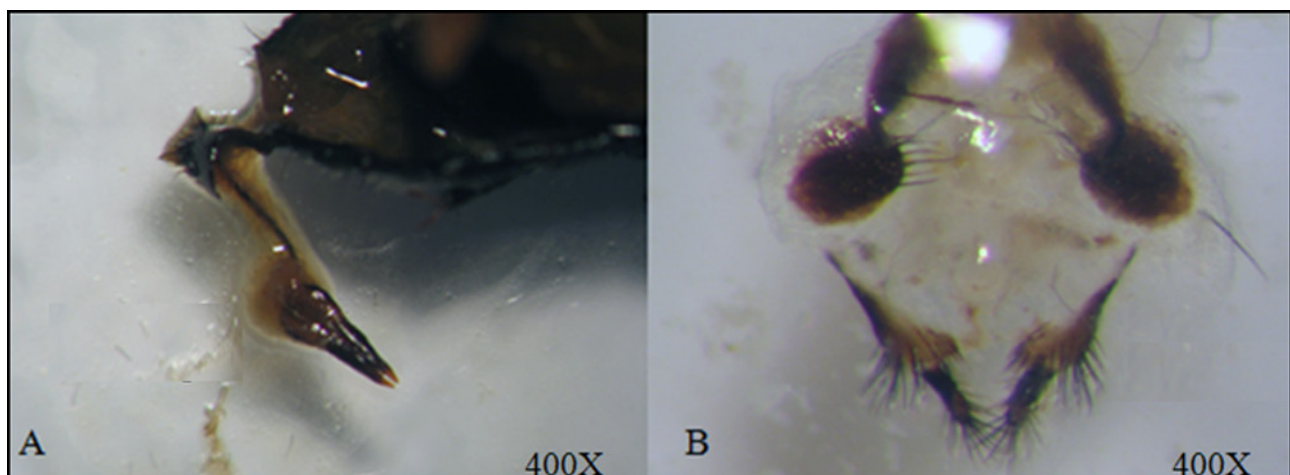
To prepare the artificial diet, 3 g of pollen granules (Swanson bee pollen, USA) was mixed with water to form a pollen paste. Then 2 g of honey and 1 g of sucrose were added to the paste and mixed thoroughly (18). In a lab experiment, we separated twenty mated females of *H. scabiosae* and fed them the aforementioned artificial diet. Two egg batches were laid in a three-week interval. After the first oviposition, two beetles were chemically analyzed weekly, and their total palasonin titers were measured.

### Feeding of Male Beetles on Deuterated Cantharidin (D<sub>2</sub>C)

One thousand nanograms (ng) of synthesized deuterated cantharidin (D<sub>2</sub>C, see acknowledgement), in which a single hydrogen atom in each of the two angular methyl group had been replaced by a deuterium atom, was introduced into male individuals by feeding them the artificial diet. D<sub>2</sub>C produces characteristic MS fragments (100% rel. intensity) and hence can be easily differentiated from a natural background of cantharidin (19). Males on a five-day diet of D<sub>2</sub>C were paired with virgin females for five days. The D<sub>2</sub>C intake by male individuals was verified by GC-MS analysis of their feces.

### Extract Preparation and Chemical Analysis

To make an extract, the whole tissue of each beetle was transferred into a silanized (by dimethyldichlorosilane in heptane 5%, Merck, Germany) test tube and the dry weight (DW) determined after 24 hours of freeze drying



**Figure 1.** External genitalia of *Hycleus scabiosae* (A) aedeagus in male, and (B) paired valvifers in female.

( $-50^{\circ}\text{C}$ ,  $9 \times 10^{-2}$  mbar) using a Alpha 1-4 freeze dryer (Christ, Germany). Cantharidin and CRCs in insects are found in free and bound forms. The free form can be directly measured by dissolving the insect tissue in an appropriate solvent, while the bound part first must be liberated from the tissue (12). All body parts were hydrolyzed in small fused test tubes using 400  $\mu\text{L}$  of 6 N hydrochloric acid (Technical HCl, 37%, Merck, Germany) at  $120^{\circ}\text{C}$  for four hours. After cooling, 400  $\mu\text{L}$  of chloroform ( $\text{CHCl}_3$  for HPLC, 99.8%, Merck, Germany) was added to each sample which was then vigorously shaken by a Vortex mixer for 60 s. Samples were centrifuged (Kubota KN70, Germany) at 3000 rpm for five minutes, and the organic phase was removed from the bottom of the test tube (11). Feces were treated in the same manner but with 100  $\mu\text{L}$  each of hydrochloric acid and chloroform.

### Cantharidin Standards

Authentic cantharidin (purity 98%, Sigma, Germany) was used as an external standard. Because of the higher concentration of naturally occurring cantharidin relative to any of the CRCs, two independent serial dilutions of cantharidin were prepared. Six ascending concentrations of cantharidin (50-1000 ng/ $\mu\text{L}$ ) were used to create a correlation curve ( $Y = 2 \times 10^{-6} + 138.2$ ,  $R^2: 0.950$ ) for cantharidin quantification. To quantify the rational mass of each CRC, authentic cantharidin was applied to another set of six ascending concentrations (0.1-100 ng/ $\mu\text{L}$ ) to provide the second correlation curve ( $Y = 2 \times 10^{-6} - 1.29$ ,  $R^2: 0.998$ ).

### Quantitative Gas Chromatography-Mass Spectrometry

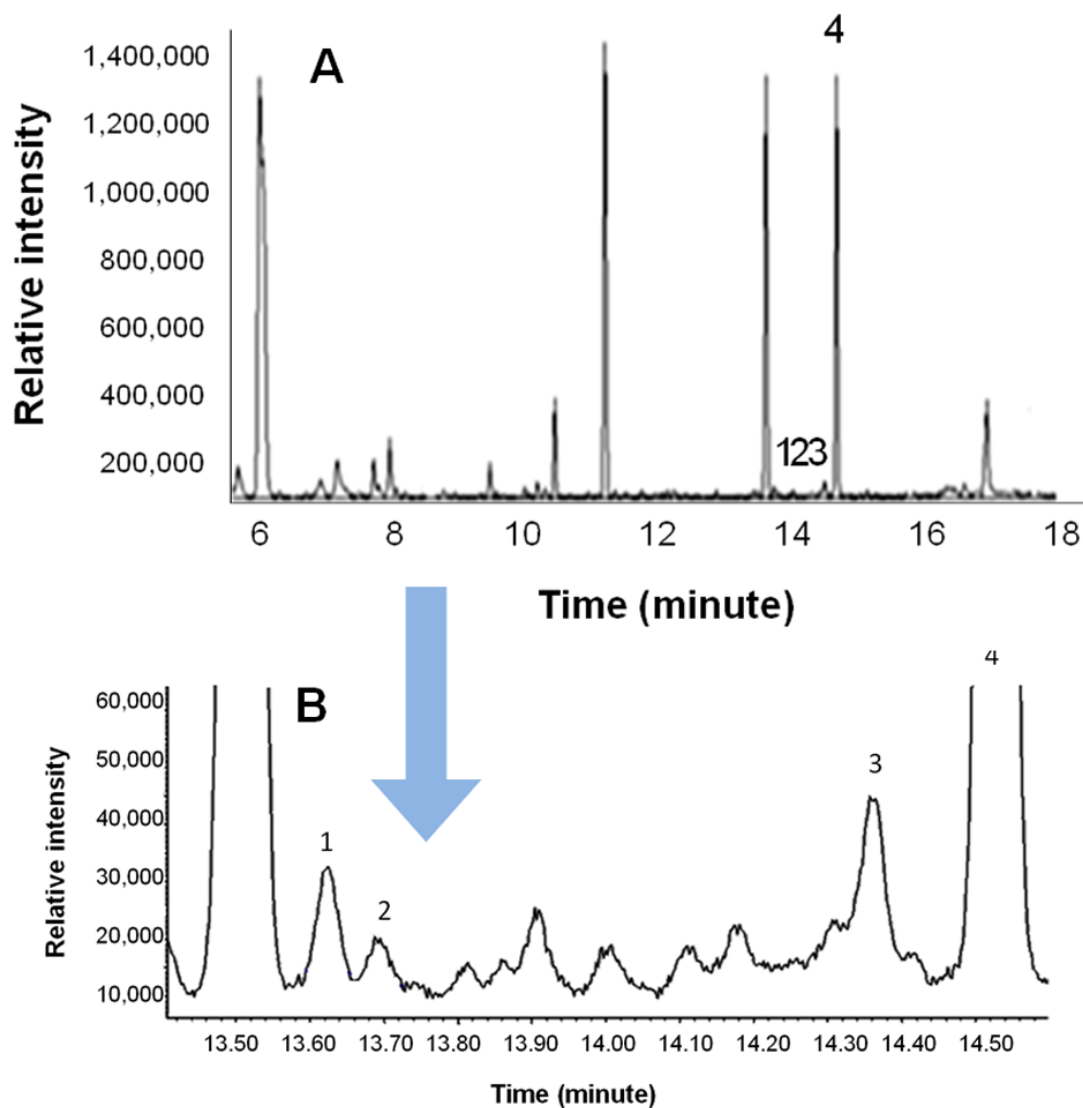
Cantharidin and CRCs of each sample were measured ten times by quantitative GC-MS ( $n = 10$ ). One microliter of each extract was injected in non-split mode into an Agilent 6890 N GC instrument, connected to a 5973 N mass detector (Agilent technologies, USA). Regular negative controls (chloroform blanks) were injected between runs to ensure that none of the detected records was due to a prior contamination. Vials of insect extracts and external standards were vortexed for 30 seconds before any injection. The gas chromatograph was equipped with a HP5 (5% phenyl and 95% methylsiloxane, non-polar) bonded-phase fused-silica capillary column (30

m, 320  $\mu\text{m}$  ID, 1  $\mu\text{m}$  FT). Helium was used as the carrier gas at 2 mm/min velocity. The injector, ion source and transfer line temperatures were set at 230, 150 and  $275^{\circ}\text{C}$ , respectively. A post run, during which the temperature was increased to and maintained at  $280^{\circ}\text{C}$  for five minutes, was used to clean the injector, inlet and column from any non-degraded particle. The detector was set for 5.00 min; mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan/sec from  $m/z$  50–250. Autointegration was achieved by Agilent Enhanced Chemstation® (version D.00.38, 2001).

### Chemical Characterization

The identification was based on the spectra from the NIST library (NIST Mass Spectrometry, 2007), and the previous studies (20, 21). Cantharidin (98% purity, CAS 56-25-7) were supplied by Sigma, but palasonin ( $\text{C}_9\text{H}_9\text{O}_4$ ), palasoninimide ( $\text{C}_9\text{H}_{10}\text{O}_3\text{N}$ ), cantharidinimide ( $\text{C}_{10}\text{H}_{13}\text{O}_3\text{N}$ ) and  $\text{D}_2\text{C}$  ( $\text{C}_{10}\text{H}_{10}\text{D}_2\text{O}_4$ ) were synthesized in the lab (see acknowledgements). One microliter of these compounds was injected into the same GC-MS instrument to confirm our identification by relative retention time and the produced mass fragments. Cantharidin was eluted after 14.53 minutes in insect-derived and standard samples (Figure 2), and mass spectra indicate its characteristic fragments at  $m/z$  128, 96, 70 and 67 ( $\text{M}^+$ : 197). The  $\text{D}_2\text{C}$  peak was apparent at 14.52 minutes with mass fragments at  $m/z$  98, 130, 69 ( $\text{M}^+$ : 199). Due to the absolute lack of synthetic  $\text{D}_2\text{C}$  in nature, such a labeled compound is highly identical. Another advantage of  $\text{D}_2\text{C}$  is the fact that it has greater similarity to cantharidin, and is expected to behave *in vivo* as the natural compound (19).

Identical peaks of palasoninimide, palasonin and cantharidinimide appeared in the total ion chromatogram of a beetle extract and synthetic standard at 13.62, 13.69 and 14.34 minutes, respectively (Figure 2). Synthetic CRCs produced the same diagnostic mass fragments (Figure 3) as the insect-derived ones (palasonin: 82, 114, palasoninimide: 113, 67, 81, 83 and cantharidinimide: 127, 70, 96, 79) when injected into the Agilent GC-MS instrument (Table 1). Deuterated palasonin (DP:  $\text{C}_9\text{H}_8\text{DO}_4$ ) and deuterated palasoninimide (DPI:  $\text{C}_9\text{H}_9\text{DO}_3\text{N}$ ) were coeluted with palasonin and palasoninimide



**Figure 2.** (A) Total ion chromatogram of *H. scabiosae* eggs, analyzed by an Agilent 6890 N GC instrument. Peaks of choice are numbered based on their elution time. (B) The same chromatogram, zoomed at RT 13:40-14:60. 1: palasoninimide (RT 13:62), 2: palasonin (RT 13:69), 3: cantharidinimide (RT 14:34), 4: cantharidin (RT 14:53). RT: retention time (minute).

and identified based on their identical base peaks ( $m/z$  83, 115 for DP and  $m/z$  68, 114 for DPI).

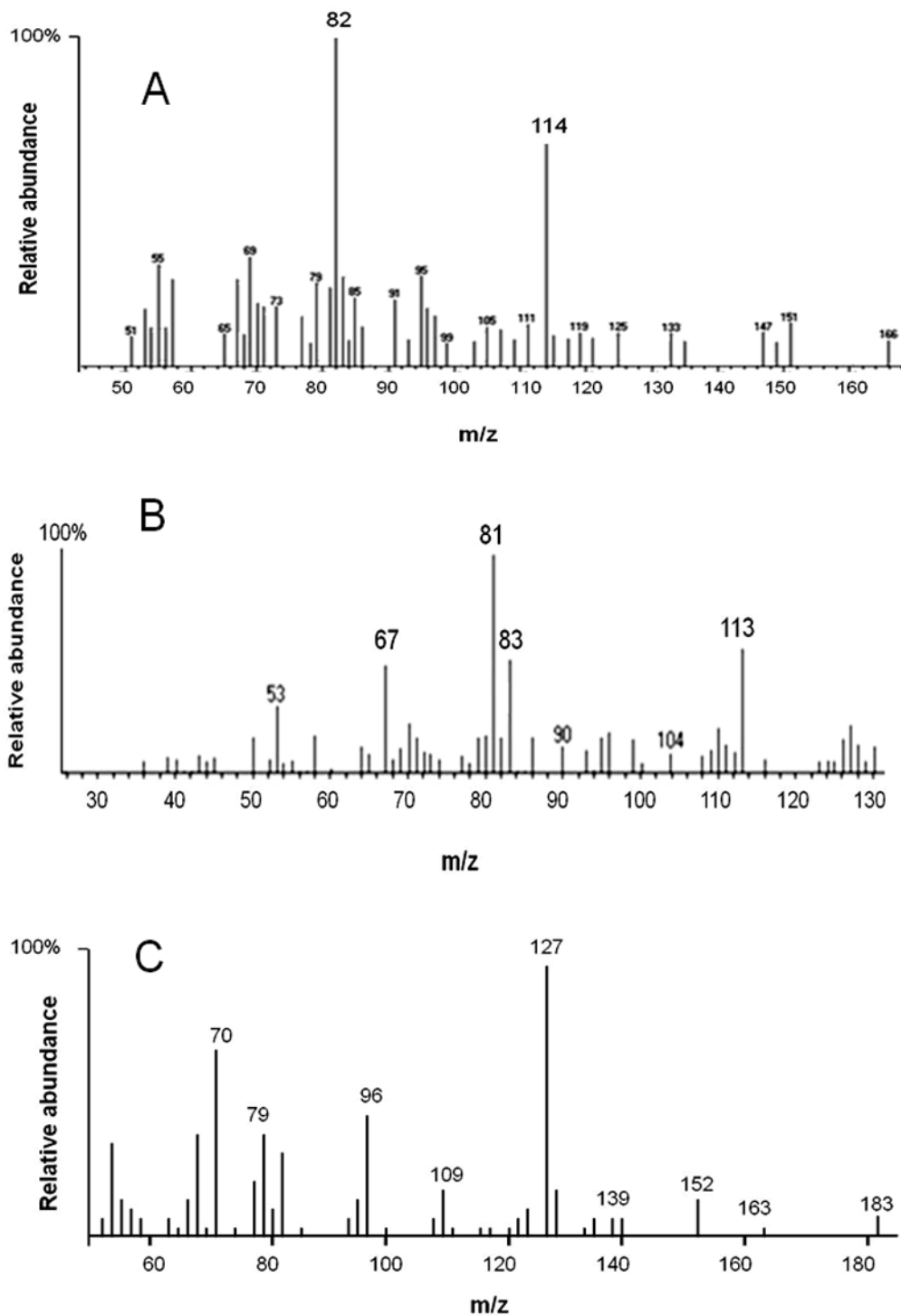
### Statistical Analyses

Variance homogeneity of data was verified by Levene's test ( $p < 0.05$ ) and the Kolmogorov-Smirnov test was used in order to verify the normal distribution of data. Since the data were not normal, the non-parametric Mann-Whitney  $U$ -test was applied to test for statistical differences ( $n = 10$ , 95% confidence level). All aforementioned analyses were accomplished with SPSS ver. 16.00 (USA).

## RESULTS

### Quantification and Distribution of Cantharidin and CRCs

Our data show that the average concentration of cantharidin was highest in mated females (mean = 0.011 mg/mg DW,  $n = 10$ ) and then slightly lower in males (mean = 0.010 mg/mg DW,  $n = 10$ ). Cantharidin concentration was lowest in virgin females (mean = 0.005 mg/mg DW,  $n = 10$ ) (Table 1). These concentrations in males and mated females were significantly higher than in virgin females (Mann-Whitney  $U$  test,  $n = 10$ ,  $p <$



**Figure 3.** Electron-impact (EI) mass spectra of cantharidin-related compounds, detected in *Hycleus scabiosae* (Meloidae). (A) Palasonin with base peaks at  $m/z$  82 and 114; (B) palasoninimide with base peaks at  $m/z$  81, 113, 67, 83 and (C) cantharidinimide with base peaks at  $m/z$  127, 70, 96 according to an Agilent 5973 N mass detector.

**Table 1.** Physicochemical properties, toxicities and measured concentrations of cantharidin and CRCs in *Hycleus scabiosae* (Coleoptera: Meloidae), based on quantitative gas chromatography/electron impact mass spectrometry

Cantharidin & detected CRC <sup>a</sup>	RT (min)	Chemical formula	Molecular weight	LD <sub>50</sub> <sup>b</sup>	Diagnostic mass fragments	Concentration <sup>c</sup> in life stages	
Palasoninimide	13.62	C <sub>9</sub> H <sub>10</sub> O <sub>3</sub> N	180.18	> 400	113, 67, 81, 83	Egg	7.69
						♀ <sub>V</sub>	9.17
						♀ <sub>M</sub>	14.62
						♂	–
Palasonin	13.69	C <sub>9</sub> H <sub>9</sub> O <sub>4</sub>	181.17	3.1	82, 114	Egg	5.61
						♀ <sub>V</sub>	21.69
						♀ <sub>M</sub>	17.49
						♂	–
Cantharidinimide	14.34	C <sub>10</sub> H <sub>13</sub> O <sub>3</sub> N	195.21	> 400	127, 70, 96, 79	Egg	7.80
						♀ <sub>V</sub>	–
						♀ <sub>M</sub>	–
						♂	–
Cantharidin	14.53	C <sub>10</sub> H <sub>12</sub> O <sub>4</sub>	196.20	1	128, 96, 70, 67	Egg	1.1
						♀ <sub>V</sub>	5.060 × 10 <sup>3</sup>
						♀ <sub>M</sub>	11.523 × 10 <sup>3</sup>
						♂	10.466 × 10 <sup>3</sup>

<sup>a</sup> Cantharidin-related compound; <sup>b</sup> mg/kg, intraperitoneal injection into mouse (see ref. 21, 22); <sup>c</sup> mean value (n = 10), ng/mg dry weight, M: mated, V: virgin.

0.05). An egg, on the other hand, had an average concentration of 1.18 ng/mg DW, which was significantly lower than any of the above three groups (Mann-Whitney U test, n = 10, p < 0.05).

Males of *H. scabiosae* had no traces of CRCs, but chemical analysis revealed the presence of considerable concentrations of palasonin and palasoninimide in virgin and mated females, and cantharidinimide in eggs. The average mass (n = 10) of the three chemically characterized CRCs (Figure 3), their retention time, and the diagnostic mass fragments (*m/z*) of males, females and eggs of *H. scabiosae* are summarized in Table 1. Palasonin and palasoninimide are present in both virgin and mated females, but a statistically significant difference was only observed in the palasoninimide content of mated females, who had accumulated higher titers of this chemical

relative to virgins (Mann-Whitney U test, n = 10, p < 0.05). Compared to mated and virgin females, each egg had a considerably lower reserve of palasonin (Table 1).

### Precursors of CRCs

In an interesting experiment, we paired D<sub>2</sub>C-fed males with virgin females of *H. scabiosae* for five days. Cantharidin and D<sub>2</sub>C, but not CRC, were detected in the analyzed male specimens and their feces. When mated females were similarly analyzed, not only were cantharidin, D<sub>2</sub>C, palasonin, and palasoninimide identified, but also DP and DPI. Therefore, such compounds could not be transferred from male to female specimens, and must be synthesized in a female organ from the males' nuptial deposit.

## DISCUSSION

### Cantharidin

The total volume of cantharidin varies depending on the species, sex and age of individuals. In our study we found that the cantharidin concentration of *Hycleus scabiosae* ranged from 0.16 to 1.39 mg per individual (mean = 0.53 mg, n = 12). Mebs *et al.* (23) found 0.2 and 1 mg of cantharidin in *H. tinctus* and *H. aculatus*, respectively.

Males transfer high amounts of cantharidin to females during copulation. This considerably increases the total cantharidin reserve in mated females, which is why no significant difference is observed between the total titer of cantharidin in these two groups when they are collected randomly from the field. On the other hand, virgin females have significantly lower cantharidin reserves than conspecific males and mated females because they do not synthesize any cantharidin after the larval stage (24). It is interesting to note that each egg of *H. scabiosae* has an average cantharidin reserve of  $1.1 \times 10^{-6}$  mg/mg DW, but all adults, even the virgin females, present a much higher amount. The sharp increase ( $10^4$  folds) of total cantharidin in males and mated females can be explained by the active biosynthesis of cantharidin in males and its direct transfer to females during copulation. Virgin females also increased their total amount of cantharidin up to 5000 fold, which is not surprising because cantharidin is synthesized by both sexes of meloids in the larval stage (8). Therefore, it is likely that the significant difference in cantharidin concentration between virgin females and eggs is due to cantharidin biosynthesis during the larval phase.

### CRCs

Cantharidin has never been detected in plants, but the first CRC, palasonin (demethylcantharidin), which lacks one of the angular methyl groups of cantharidin, was characterized in 1960 (25). It was initially isolated from the seeds of the Indian tree, *Butea frondosa* (Leguminosae) (26). Fietz was the first to detect palasonin in bodily extracts from a blister beetle (*Hycleus lunatus*) and a clerid beetle (*Trichodes apiaries*, Cleridae) (27). Dettner *et al.* (21) reported the second CRC, palasoninimide, from the South African species *H. lunatus*. Cantharimides, whose anhydride

oxygen atoms are replaced by basic amino acids, and cantharimide dimers, which consist of two cantharimide units combined with a tri-, tetra-, or penta-methylene group, have been reported in the Chinese meloid *Mylabris phalerata* Pall. (28, 29). A low amount of cantharidinimide was found in bodily extracts from *Mylabris impressa stillata* (20). Variable titers of palasonin were recently detected in two other southern African species, *Hycleus oculatus* and *Hycleus tinctus* (23). Although there are a few studies on palasonin, the CRCs' function, concentration, transfer or production site have never been discussed. The impact of sex or mating status on the diversity and frequency of such compounds was also unclear.

Our observation that palasonin and palasoninimide were found in virgin and mated females but not in males indicates that CRCs do not have the same transfer pattern as cantharidin and thus, unlike cantharidin, could not have been transferred from males to females. Two alternative routes can be envisaged for the origin of palasonin and palasoninimide in females. Palasonin might be originated directly by oxidative demethylation of cantharidin or derived from cyclization of a previously demethylated 12-nor-farnesol, the sesquiterpene precursor of cantharidin (9, 30). Since chemical analysis of male extracts did not detect any CRC, the labeled palasonin and palasoninimide must be directly synthesized in the female body from demethylation of D<sub>2</sub>C. We also found that the female palasonin reserve is considerably reduced following each oviposition, but gradually restored to about the same level before the next batch of eggs is laid. This provides further evidence that females are able to actively synthesize palasonin. It has been indicated that unlike the plant source, insect-derived palasonin has a low enantiomeric excess (ee), with the (R)-(+)-enantiomer prevailing (30); therefore, palasonin uptake from hitherto unknown plant sources seems highly unlikely (21).

Apart from cantharidin; palasonin, palasoninimide and cantharidinimide were also found in the eggs of *H. scabiosae*. The mean value of cantharidinimide was slightly higher than palasonin and palasoninimide content of eggs (Table 1). Despite the high structural similarity of cantharidinimide to cantharidin, this CRC was not found in adults and we still do not know how it might have appeared in eggs.

Palasonin and palasoninimide have significantly higher titers per egg than cantharidin (Table 1). Our data indicate that inseminated females transfer 91.82% and 96.70% of their palasonin and palasoninimide reserves to each egg batch, respectively, while only 0.34% of their maternal cantharidin is transferred to an egg mass. The eggs are characteristically deposited as a compact mass and the number of eggs per mass varies with body size (approx. 35 eggs/mass). Mated females survive for several months and produce egg masses periodically (18). A female incorporates about 38.5 ng of cantharidin, 196.35 ng of palasonin and 269.15 ng (dry weight values in ng/mg) of palasoninimide into an egg mass during each oviposition. As it was shown earlier, a gravid female must have had a higher titer of palasonin and palasoninimide than an already-oviposited female. Inseminated females periodically oviposit; therefore, their lifelong accumulated titers of CRCs should be much higher than those of a virgin female.

CRCs have been so far found in less than 0.5% of the described meloid species. Meanwhile, the diversity of such compounds varies greatly among species. It seems that eggs of this meloid species take advantage of a different array of protective chemicals by increasing the ratio of CRCs versus cantharidin. While only a few meloid species have been examined for CRCs, it is not known how widespread these compounds are within the large number of cantharidin-producing species. CRCs, which have a lower toxicity than cantharidin (Table 1), are already known to have the same effect on protein phosphatases as cantharidin (31-33). Thus, it might be hypothesized that such compounds are deposited in eggs as a safer substitute for cantharidin to provide effective protection, but do not simultaneously hurt the susceptible embryo.

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#### CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

#### FINANCIAL SOURCE

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