

New Evidence of Canthariphily: *Tilloidea transversalis* (Coleoptera: Cleridae) Sequestering Cantharidin From *Lydus trimaculatus* (Coleoptera: Meloidae)

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Abstract

Cantharidin (CTD) is a defensive compound autogenously and exclusively produced by two phylogenetically related beetle families: Meloidae and Oedemeridae. Although this molecule usually acts as a strong deterrent against potential predators and parasites, some arthropod species, collectively named 'canthariphilous species', are attracted to CTD. Some species can sequester CTD from the CTD-producing species, using it as a chemical defense against enemies. The present paper focuses on the first-ever description of canthariphilous interactions between a checkered beetle species (Coleoptera: Cleridae) and a CTD-producing species. Field observations revealed individuals of the phytophagous beetle *Tilloidea transversalis* (Charpentier, 1825) (Coleoptera: Cleridae) biting individuals of the blister beetle *Lydus trimaculatus* (Fabricius, 1775) (Coleoptera: Meloidae). Laboratory behavioral experiments followed to verify if this peculiar behavior of *T. transversalis* also occurs on other co-occurring species. Moreover, chemical analyses were performed to assess whether *T. transversalis* can sequester CTD. Our results show that *T. transversalis* only attacks CTD-producing species. However, while chemical analyses prove that *T. transversalis* can sequester CTD from the hemolymph of *L. trimaculatus*, some clues (based on a CTD-baited traps sampling) suggest that this beetle, contrarily to other canthariphilous species, does not appear to show a high attraction to pure synthetic CTD. Thus, other unknown signals, alone or in combination with CTD, could be implicated in triggering the canthariphilous behaviors of *T. transversalis*.

Graphical Abstract



Key words: cantharidin, canthariphilous species, compounds sequestration, interspecific interactions, secondary metabolites

Many organisms use chemical compounds as a defense against potential predators and parasites (Berenbaum 1995, Torres and Schmidt 2019). Some species do not produce these compounds themselves but can sequester secondary chemicals from other organisms (Petschenka and Agrawal 2016, Zvereva and Kozlov 2016). Even though most of these interspecific interactions have been observed in phytophagous insects sequestering chemicals from their host plants, a greater diversity of interaction pathways exists in nature (Savitzky et al. 2012).

Cantharidin (CTD) is a secondary chemical exclusively produced by two families in Tenebrionioidea: blister beetles (Coleoptera: Meloidae) and false blister beetles (Coleoptera: Oedemeridae) (Carrel and Eisner 1974, Carrel et al. 1986). This toxic terpene acts as a strong feeding deterrent against potential predators and parasites of CTD producers in all phases of their development (Carrel and Eisner 1974, Smedley et al. 1996, Dettner 1997, Carrel 1999, Whitman et al. 2019). It is also used as a nuptial gift in some species, although such function seems negligible, at least in some Oedemeridae (Holz et al. 1994, Nikbakhtzadeh et al. 2007, Hashimoto et al. 2016). Despite its renowned high toxicity (Schmitz 1989, Li and Casida 1992), popular pharmacology of different ancient cultures has traditionally used CTD for many purposes for more than two millennia (Bologna 1991), and because of its antitumoral properties, scientific interest in CTD has been renewed in the last decades (Ren and Kinghorn 2021).

Of note, several arthropod species, collectively named ‘canthariphilous species’, are attracted to CTD, and some of these can sequester this compound from CTD-producing beetles (Hemp and Dettner 2001, Hashimoto and Hayashi 2014). More than 300 canthariphilous species have been recorded in several orders of Hexapoda (Coleoptera, Diptera, Hymenoptera, Hemiptera), and one case has been reported in Opiliones (Hemp and Dettner 2001, Hashimoto and Hayashi 2014). However, most canthariphilous species have been identified just because they are attracted to either CTD-baited traps or individuals of Meloidae and Oedemeridae (e.g., Bologna and Havelka 1985, Hemp and Dettner 2001, Horiuchi et al. 2018) or because CTD was detected in their body (e.g., Islami and Nikbakhtzadeh 2009); thus, the ecological significance of canthariphily for most of these remains unknown (Schütz and Dettner 1992, Frenzel and Dettner 1994, Holz et al. 1994, Dettner 1997, Hemp et al. 1997, Hashimoto and Hayashi 2014).

Canthariphily is well represented among species of antlike flower beetles (Coleoptera: Anthicidae) and fire-coloured beetles (Coleoptera: Pyrochroidae) (Hemp and Dettner 2001, Horiuchi et al. 2018, Kejval and Chandler 2020), which represent the most phylogenetically related groups to the CTD-producing family of Meloidae (McKenna et al. 2019). The ecological significance of canthariphily in these two families has been investigated in more detail than in others, although only on a few species (Schütz and Dettner 1992; Frenzel and Dettner 1994; Holz et al. 1994; Eisner et al. 1996a,b; Dettner 1997; Hemp et al. 1997). Indeed, in these families, it has been demonstrated that CTD plays a crucial role in sexual selection: males sequestering CTD are positively selected by females for mating (Schütz and Dettner 1992; Eisner et al. 1996a,b; Hemp et al. 1997). Males of Anthicidae and Pyrochroidae have peculiar ‘test-organs’, involved in CTD secretion, which they show to females during courtship (Schütz and Dettner 1992; Eisner et al. 1996a,b; Hemp et al. 1997). Moreover, during mating, males can pass CTD to females, which, in turn, can transfer the chemical to the eggs (Holz et al. 1994; Eisner et al. 1996a,b; Dettner 1997; Hemp et al. 1997).

In this paper, we focus on the first-ever description of canthariphilous interactions between a checkered beetle species

(Coleoptera: Cleridae) and a CTD-producing species. Scientific literature concerning canthariphily in Cleridae is scarce, limited to only two individuals of *Cymatodera* sp. from Mexico and individuals of three East African species (*Pallenothriocera rufimembris* Pic, 1949 and other two unidentified species) collected in CTD traps (Chandler 1976; Hemp et al. 1999a,b). Moreover, Hemp et al. (1999a) hypothesized the presence of CTD-secreting glands in males of *P. rufimembris*, but the authors themselves asserted that their hypotheses needed further investigations. Besides, CTD has been detected in *Trichodes apiarius* (Linnaeus, 1758) (Coleoptera: Cleridae) even though this species has never been collected in CTD traps (Fietz et al. 2002). *Trichodes apiarius* is a generalist omnivore species, also feeding on blister beetles and false blister beetles, that probably evolved a tolerance to CTD as observed in other generalist predators of insects (Bologna 1985, Kelling et al. 1990, Carrel 1999, Fietz et al. 2002, Heneberg 2016). Thus, up to date, *T. apiarius* is not considered a canthariphilous species (Fietz et al. 2002, Bocakova et al. 2015).

The checkered beetle *Tilloidea transversalis* (Charpentier, 1825) (Coleoptera: Cleridae), contrary to most checkered beetles, is a phytophagous species, feeding primarily on some genera of Apiaceae and Asteraceae (Crovetti 1961, Luna Murillo and Obregón 2014, Bocakova et al. 2015). An unexpected field observation of interspecific interactions between *T. transversalis* and *Lydus trimaculatus* (Fabricius, 1775) (Coleoptera: Meloidae) triggered our research, aimed at exploring the possible existence of canthariphilous behavior in this species of Cleridae. Our observational, behavioral, and chemical data contribute to shed light on the evolution of canthariphily in Cleridae and, more generally, in canthariphilous species.

Materials and Methods

Field Sampling

All specimens were collected in derived pastures within *Quercus* woodlands at the western base of the Tolfa Mountains (Italy, Lazio, Roma, Tolfa, Rio Fiume valley, 42.053816N, 11.936530E, 28 m a.s.l.).

Specimens of *Tilloidea transversalis* (Coleoptera: Cleridae) were hand-collected while feeding on capitula of *Cynara cardunculus* Linnaeus, 1753 in July 2020 ($N = 133$ individuals for behavioral analysis). In the same area and period, beetles from six locally abundant species of different families were also collected to perform behavioral experiments on the interspecific interactions of *T. transversalis*: *Larimus cynarae* (Fabricius, 1787) (Coleoptera: Curculionidae), *Lydus trimaculatus* (Coleoptera: Meloidae), *Oedemera flavipes* (Fabricius, 1792) (Coleoptera: Oedemeridae), *Oxythyrea funesta* (Poda, 1761) (Coleoptera: Scarabaeidae), *Rhagonycha fulva* (Scopoli, 1763) (Coleoptera: Cantharidae), *Stictoleptura cordigera* (Fussli, 1775) (Coleoptera: Cerambycidae). All collected specimens ($N = 7$ individuals for each species except for *L. trimaculatus*: $N = 14$) were kept alive at room temperature in fauna boxes (28 × 20 × 14 cm), where species were reared separately and fed with the same flowers on which they were collected. After the experiments, all the surviving specimens were returned to the sampling area.

Furthermore, additional specimens of *T. transversalis* ($N = 32$ males and $N = 18$ females) and *L. trimaculatus* ($N = 32$) were collected on 23rd June 2021 to perform chemical analyses.

Behavioral Experiments

After field observation, laboratory-based behavioral experiments were conducted to verify if, beyond *L. trimaculatus*, *T. transversalis* was attracted to other beetles co-occurring in the sampled area (Table 1).

Table 1. Schematic representation of *Tilloidea transversalis* behavioral experiments

Species (Order: Family)	Replicates						
<i>Larinus cynarae</i> (Coleoptera: Curculionidae)	-	-	-	-	-	-	-
<i>Lydyus trimaculatus</i> * (Coleoptera: Meloidae)	+	+	+	+	+	-	+
<i>Oedemera flavipes</i> * (Coleoptera: Oedemeridae)	+	-	-	+	-	-	+
<i>Oxythyrea funesta</i> (Coleoptera: Scarabaeidae)	-	-	-	-	-	-	-
<i>Rhagonycha fulva</i> (Coleoptera: Cantharidae)	-	-	-	-	-	-	-
<i>Stictoleptura cordigera</i> (Coleoptera: Cerambycidae)	-	-	-	-	-	-	-

Experiments were staged in Petri dishes by placing *T. transversalis* together with other beetles from different families. Each experimental replica lasted 5 min. CTD-producing species are marked with an asterisk (*); replicates in which attacks of *T. transversalis* occurred are marked with a plus (+).

Experiments were staged in Petri dishes (90 × 20 mm) whose inner bottom was covered with a paper disc to provide a suitable substrate for insect locomotion. Three specimens of *T. transversalis* ('recipient') were placed on the dish. After 5 min of acclimatization, experiments started with the placement of an individual from one of the abovementioned beetle species ('source') on the dish. Interactions were observed for 5 min. Seven replicates were performed for each species, using new individuals each time. An additional set of seven replicates was performed to record the duration of the biting time of *T. transversalis* into *L. trimaculatus*. Potential differences in the attack rate of *T. transversalis* against the other species were tested using a *Chi-square* test.

Chemical Analyses

In order to assess if *T. transversalis* can sequester CTD from *L. trimaculatus*, specimens collected on 23rd June 2021 were stored in fauna boxes (39 × 28 × 28 cm) for 24 h, divided into two groups (i.e., treated group and untreated or control group), both comprising 25 individuals ($N = 16$ males; $N = 9$ females). In the treated group, *T. transversalis* individuals were stored together with 32 individuals of *L. trimaculatus*, while, in the control group, individuals of *T. transversalis* were isolated from any source of CTD. Fauna boxes were lined internally with paper sheets and set up with crumpled papers to increase the three-dimensionality of the arena and provide a suitable substrate for insect locomotion. A 10% sucrose solution was provided to both groups in a tube capped with cotton wool and leaned on a Petri dish inside the fauna boxes. After 24 h, treated and control individuals of *T. transversalis* were frozen at -80°C . Because of the tricky sex determination, individuals were sorted by sex only after the freezing phase to avoid overstressing the specimens. To explore possible differences in CTD storage throughout the body, specimens were dissected, isolating head and prothorax (HP) from the rest of the body (BR), then lyophilized for 48 h using Telstar LyoQuest -55/230 V 50 Hz. To allow a better quantification of CTD content, we pooled the specimens in a total of eight samples: TM-HP (treated males—head and prothorax; $N = 16$), CM-HP (control males—head and prothorax; $N = 16$), TM-BR (treated males—rest of the body; $N = 16$), CM-BR (control males—rest of the body; $N = 16$), TF-HP (treated females—head and prothorax; $N = 9$), CF-HP (control females—head and prothorax; $N = 9$), TF-BR (treated females—rest of the body; $N = 9$), CF-BR (control females—rest of the body; $N = 9$). Extraction and detection of CTD were performed following the protocol described in Gisoni et al. (2019). Each sample was ground using a pestle and mixed with 50 ml of ethyl acetate (AcOEt, 99.8%; Sigma Aldrich, Darmstadt, Germany) in a round-bottom flasks at reflux temperature (80°C) for 48 h and subsequently filtered, after cooling down, with polytetrafluoroethylene filters (13 mm × 0.2 μm Acrodisc MS Syringe Filter; Pall Corporation, Port Washington, NY) to separate the organic solution from the insoluble remains (e.g., small cuticular fragments). The solvent was then evaporated under reduced pressure (BUCHI

Rotavapor R II, Flawil, Switzerland, or Edwards RV3, Burgess Hill, United Kingdom) to obtain a yellowish sticky oil covering the bottom of flasks. After the extraction, the samples were injected in a gas chromatography system equipped with capillary column SLB-5 ms (30 m × 0.25 mm × 0.25 μm) coupled to a mass spectrometer (GC-MS; QP2010S, Shimadzu, Kyoto, Japan) carrying out five replicates for each sample. The analysis program follows a temperature ramp from 50 to 260°C with a $10^{\circ}\text{C}/\text{min}$ rate. The quantification was performed by mathematical integration of the area underneath the target peak provided by GC-MS Solution software (version 2.7; Shimadzu). Since samples were unitary, results for each sample represent the mean concentration of CTD of several individuals, but we have no data about the variability within samples, and thus our chemical results are only qualitative. Box plots were drawn on R Statistical Software (v4.1.2; R Core Team 2021) using five instrumental replicates of each sample.

Results

Field Observation

During a sample survey aimed at collecting canthariphilous species through CTD-baited traps, two individuals of the phytophagous species *T. transversalis* (Crovetti 1961; personal observation) were observed being stuck with their mandibles on the abdominal tergites of one individual of *L. trimaculatus* feeding on a sweet scabious [*Sixalix atropurpurea* (L.) Greuter and Burdet, 1985] (3 July 2020; Italy, Lazio, Roma, Tolfa, Rio Fiume, 42.058720N, 11.941320E, 44 m a.s.l.) (Fig. 1; Supp. Video 1 [online only]). Both species are common in the area but localized on their host plants, locally mainly represented by both sweet scabious and cardoon (*Cynara cardunculus* L.) (Mayer, 2015) for *L. trimaculatus* and *T. transversalis*, respectively. The two individuals of *T. transversalis* remained several minutes on *L. trimaculatus*, which, in the meantime, kept feeding on its host plant inflorescence. Afterwards, the two checkered beetles flew away independently.

Behavioral Experiments

In laboratory experiments, attacks of *T. transversalis* were recorded only against *L. trimaculatus* (in six out of seven replicates) and *O. flavipes* (in three out of seven replicates) (the presence of CTD has previously been documented in both species: Frenzel and Dettner 1994, Gisoni et al. 2019) (Table 1). Although the rate of attack is not significantly dissimilar (*Chi-square* statistic = 2.8; $\text{df} = 1$; $P = 0.09$), we observed a different behavior of *T. transversalis* on the two species. When interacting with *L. trimaculatus*, *T. transversalis* showed a peculiar behavior: it firmly attached itself to the body of the host (legs, abdomen, or elytra) through its mandibles, folded legs, and antennae under its own body and rhythmically moved the maxillae to ingest the hemolymph bleeding from the prey (Fig. 2;



Fig. 1. Field observation of two *Tilloidea transversalis* individuals extracting hemolymph from *Lydus trimaculatus* on *Sixalix atropurpurea* (L.) Greuter and Burdet. The two images show different shots of the same individuals.



Fig. 2. Lateral (left) and ventral (right) view of *Tilloidea transversalis* attacking *Lydus trimaculatus*. The checked beetle firmly sticks its mandibles to the blister beetle and folds legs and antennae under his own body.

[Supp. Video 2](#) [online only]). This behavior could last up to 48 min (27.81 ± 13.11 min [mean \pm SD]; $N = 7$). At the end of the bite, the beetles left the host which, apparently, continued its activities undamaged. In three additional laboratory experiments, *T. transversalis* displayed this same behavior toward *Mylabris variabilis* (Pallas, 1781) (Coleoptera: Meloidae). Contrastingly, when *T. transversalis* attacked *O. flavipes* in captivity, the behavior mentioned above changed rapidly to ordinary predation, in which the prey was dismembered and, at least partially, ingested. Both males and females of *T. transversalis* exhibited predatory behaviors on *O. flavipes* ($N = 2$ males; $N = 1$ female) and *L. trimaculatus* ($N = 7$ males; $N = 6$ females).

Chemical Analyses

Gas chromatography–mass spectrometry analyses showed the presence of CTD in the body of *T. transversalis*. Results are summarized in [Fig. 3](#). Concentrations were qualitatively higher in all treated groups than in control groups and the highest

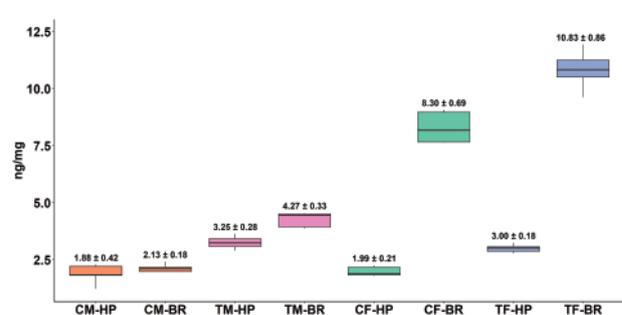


Fig. 3. Means content of CTD (ng/mg) in tissues of *Tilloidea transversalis*. Boxes of the same color indicate different tissues (head and prothorax; rest of the body) from the same specimens. Mean and standard deviation were calculated on five instrumental measurements. CM, control males; TM, treated males; CF, control females; TF, treated females; HP, head and prothorax; BR, rest of the body. $N = 16$ (CM, TM); $N = 9$ (CF, TF). Total dry weight: CM-HP = 44.3 mg; CM-BR = 148.3 mg; TM-HP = 44.8 mg; TM-BR = 166.1 mg; CF-HP = 32.7 mg; CF-BR = 123.3 mg; TF-HP = 32.1 mg; TF-BR = 147.7 mg.

concentrations were observed in the rest of the body of treated females (Fig. 3).

Discussion

Our field observation showed a peculiar feeding behavior of the phytophagous species *T. transversalis* on the blister beetle *L. trimaculatus* (Fig. 1; Supp. Video 1 [online only]). Our laboratory experiments, performed on a representative subset of the beetle community co-occurring in the area, clearly showed that this behavior occurs only on CTD-producing species (Table 1). Moreover, chemical analyses confirmed that males and females of *T. transversalis* can sequester CTD from *L. trimaculatus* (Fig. 3). This evidence demonstrates that *T. transversalis* likely represents a newly ascertained canthariphilous species, revealing for the first time canthariphilous interactions between checkered beetles and CTD-producing species.

Although not statistically significant in terms of occurrence, a higher number of attacks of *T. transversalis* was observed on *L. trimaculatus* than on *O. flavipes*, and, surprisingly, this behavior led to different outcomes depending on the attacked species. Notably, although starting similarly (i.e., the checkered beetle tried to extract hemolymph from the host), when attacking *O. flavipes*, the behavior of *T. transversalis* changed into destructive predation involving the ingestion of other tissues and leading to the death of the prey. Further investigations are needed to clarify whether *T. transversalis* commonly attacks species of Oedemeridae in the wild or if captivity conditions bias our observations. On the contrary, the highly specialized behavior of ingesting the hemolymph of *L. trimaculatus* (Fig. 2; Supp. Video 2 [online only]), together with our field observation (Fig. 1; Supp. Video 1 [online only]), demonstrated that *T. transversalis* actively extracts hemolymph from this species of blister beetle. Those results, together with our additional observation on *M. variabilis*, and a photographic record of *T. transversalis* biting *Zonitis immaculata* (Olivier, 1789) (Coleoptera: Meloidae) on their shared host plant *C. cardunculus* (Romano 2006), suggest that this checkered beetle commonly attacks blister beetles in the wild. According to this evidence, and because of the absence of attacks of this phytophagous species on non-CTD-producing species, it could be inferred that *T. transversalis* evolved a detection mechanism allowing to locate CTD-producing species from which (at least in *L. trimaculatus*) sequestering CTD.

Our findings contribute to knowledge on the complex and still deeply undisclosed phenomenon of canthariphily, the evolution of which remains a puzzling question for evolutionary biologists. Dettner (1997) proposed the hypothesis that the presence of canthariphilous species within phylogenetically related taxa to CTD-producing species could indicate a plesiomorphic condition of CTD production, retained in CTD-producing species (Meloidae and Oedemeridae) and lost secondarily in canthariphilous species (Anthicidae and Pyrochroidae). However, this hypothesis does not justify the presence of canthariphily in other phylogenetically distant taxa such as Cleridae. Moreover, the presence of canthariphilous species in other orders and classes of Arthropoda suggests that canthariphily evolved independently several times (Dettner 1997, Hemp and Dettner 2001, Hashimoto and Hayashi 2014). Previous studies suggest that some phytophagous species, frequently trapped in CTD-baited traps, may have evolved a canthariphilous behavior due to the similarity of CTD to other plants and fungal chemicals used by species in food search (Frenzel et al. 1992, Dettner 1997, Tallamy et al. 1999, Hashimoto and Hayashi 2014, Horiuchi et al. 2018). It is noteworthy that our field observation occurred in the frame of a longitudinal sampling

of canthariphilous species by CTD-baited traps and, surprisingly, despite the high occurrence of *T. transversalis* in the area, this species seems not to be attracted to traps since only one individual was collected during all the sampling season. Thus, in this species, canthariphilous behavior and attraction to CTD-producing species may have evolved differently from other canthariphilous phytophagous species. Although further investigations are needed to ascertain the lack of attraction to pure CTD in *T. transversalis*, our observation seems to corroborate the hypothesis proposed in Dettner (1997) that other unknown signals, alone or in combination with CTD, are involved in the identification of CTD-producing species by some canthariphilous species. Comparing our results with those of previous studies suggests that canthariphily (and CTD tolerance) could have also evolved independently within the Cleridae family. In fact, *T. transversalis* is neither clearly attracted to pure CTD, as in the case of *P. rufimembris* (Hemp et al. 1999a), nor it is a generalist omnivore species feeding on CTD-producing species, as in the case of *T. apiarius* (Fietz et al. 2002).

The results of chemical analyses showed the presence of CTD in *T. transversalis*. The presence of CTD even in the control groups probably indicates that some of the individuals within the samples had already sequestered CTD in the wild before being collected. Although preliminary, results of the treated groups seem to suggest that CTD is stored more in some BR than HP tissues (Fig. 3). Several mating events have been observed during the experiments and the higher concentration of CTD in the females BR (both in control and treated groups), together with its low concentration in CM-BR (which have no access to CTD), could indicate that males passed BR-stored CTD to females during the copula (Fig. 3). If confirmed, this scenario would be congruent with what has already been observed in other canthariphilous and CTD-producing species, eventually suggesting a role of CTD in courtship behavior and offspring defense in *T. transversalis* (Frenzel and Dettner 1994; Holz et al. 1994; Eisner et al. 1996a,b; Dettner 1997; Hemp et al. 1997; Nikbakhtzadeh et al. 2007). However, our protocol does not allow us to discern from alternative hypotheses; therefore, further chemical and behavioral investigations are needed to deeply investigate the role of CTD in the courtship behavior and offspring defense of *T. transversalis*. The present manuscript provides selective interspecific interaction of a phytophagous species toward CTD-producing species, proving for the first time ever the evolution of canthariphily in Cleridae through behavioral experiments in a species not strongly attracted to CTD-baited traps.

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Author Contributions

M.M.: conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing—original draft; writing—review and editing. L.S.: investigation; methodology.

S.G.: methodology; writing—review and editing. T.G.: methodology; resources; supervision. A.D.G.: resources; validation; writing—review and editing. E.M.: methodology; project administration; supervision; validation; writing—review and editing. M.A.B.: funding acquisition; methodology; project administration; resources; supervision; validation; writing—review and editing.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Video 1. Field observation of two *Tilloidea transversalis* individuals stick their mandibles to *Lydus trimaculatus* on its host plant *Sixalix atropurpurea* (3 July 2020; Italy, Lazio, Roma, Tolfa, Rio Fiume, 42.058720N, 11.941320E, 44 m a.s.l.).

Video 2. Details of the canthariphilous behavior of *Tilloidea transversalis* attacking *Lydus trimaculatus*. The checkered beetle firmly sticks its mandibles to the blister beetle and folds legs and antennae under his own body while rhythmically moves his maxillae to ingest the hemolymph bleeding from the prey.

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