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## Beetles “in red”: are the endangered flat bark beetles *Cucujus cinnaberinus* and *C. haematodes* chemically protected? (Coleoptera: Cucujidae)

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### Abstract

Two native species of the genus *Cucujus* show a wide geographic distribution in Europe, *Cucujus cinnaberinus* (Scopoli, 1763) and *C. haematodes* Erichson, 1845. Although data on the distribution and ecology of these rare and endangered species are increasing, there are few reports on their biology and behaviour, and some aspects of their feeding ecology remain problematic. Our aim was to study, for the first time, the cuticular chemical profiles of these two beetles to (i) investigate the presence of chemicals potentially involved in defence by pathogens and (ii) lay the foundation for understanding the role of their bright red colour. The analysis of the cuticular profile was performed in-vivo by solid phase microextraction coupled with gas chromatography-mass spectrometry. In the cuticular profiles of the two species we identified 24 compounds belonging to different classes of molecules, i.e. hydrocarbons, aldehydes, esters, n-alkyl morpholines, and a high number of organic acids. Qualitative differences in terms of both signal intensity and detected compounds were found between the two species. As reported in other insects, the remarkable array of avoidance substances suggests a strict relationship with the bright red colour of the adults, which probably acts as an aposematic or warning signal. European *Cucujus* species are probably well protected against enemies because some identified chemicals, particularly fatty acids, are related to an anti-predatory strategy to fight off predators that use their sense of smell to locate their prey. Other substances found on the cuticular layer of these beetles are probably involved in an antimicrobial and antifungal function, as demonstrated in other insects living in habitats that host many pathogens.

**Keywords:** *Cuticular profiles, solid phase microextraction, aposematism, insect anti-predator strategies*

### Introduction

The flat bark beetles of the genus *Cucujus* are an example of extreme adaptation to the under-bark microhabitat. Their extremely flat bodies allow them to crawl through the thin space that opens between the xylem and phloem of decaying dead trees. Adults and larvae share a broad, flat head with enlarged genae that provide an enhanced insertion for the muscles of a pair of robust mandibles. The 14 species described so far are defined as “saproxyllic” by Speight (1989) and are distributed in the Holarctic Region, but only one lives in North America. Nearly all are bright red but some, like *C.*

*mniszehi* Grouvelle and *C. nigripennis* Lee and Satô, are dark blue or dark brown (Lee & Satô 2007). The European species *C. cinnaberinus* (Scopoli, 1763) is strictly protected and enclosed in Annexes II and IV of the EU Habitats Directive 92/43, with the aim of maintaining existing populations. It has been classified as “near threatened” in Europe by the International Union for Conservation of Nature (IUCN) organisation and “vulnerable” in Italy (Carpaneto et al. 2015). Recently, there have been reports on the role of *Cucujus cinnaberinus* and *Clinidium canaliculatum* as possible biodiversity indicators. In Southern Italy, they provide a simple and

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useful tool for periodic diversity monitoring in forest nature reserve networks (Mazzei et al. 2018).

Another related species, *C. haematodes* Erichson, 1845, is even more at risk (“endangered” in Carpaneto et al. 2015), and is currently very rare in Europe, apart from in Calabria.

Although there is plenty of data on the distribution and ecology of these cucujid beetles, there are very few reports on their biology and behaviour, and those that exist are contradictory. Adults of all European species share a bright red colour (Figure 1). They live for several months under bark and are active outside for a relatively short period from April to July (Horák & Chobot 2009).

Under bark, *C. cinnaberinus* is associated with Silvanidae, Carabidae, red and protected Pyrochroidae (*Pyrochroa coccinea*, Nardi & Bologna 2000) and non-coleopteran taxa such as ants from the genus *Lasius*, mites, flies, centipedes and spring-tails (Horák et al. 2011a). *Cucujus haematodes* is also associated with Acarina and Formicidae, Carabidae, Histeridae and Chrysomelidae (Horák et al. 2011b). The majority of flying activities in *C. cinnaberinus* occur from mid-spring to mid-summer (e.g. Bussler 2002; Horák & Chobot 2009; Horák et al. 2010; Straka 2017). Schlaghamerský et al. (2008) recorded flying activity using window traps in April and May only; Marczak (2016) reported that the adults are much more active during the night than in the diurnal hours but, more recently, adults of *C. cinnaberinus* have been observed in copula in the morning on tree surfaces (*Populus* sp.) in the Danube floodplain forest (Lower Austria) (Straka 2017).

There is little data on the food preferences of either adults or larvae of these beetles, and that which is available is still unclear. Smith and Sears



Figure 1. *Cucujus haematodes* (left) and *C. cinnaberinus* (right) adults. Scale bar: 1 mm.

(1982), examining the gut contents of the North American species *Cucujus clavipes*, reported predatory feeding habits of larvae. Ślipiński (1982) believes that the *Cucujus* spp. adults are facultative predators. Lawrence (1991) considers the larvae of *Cucujus* to be facultative predators after finding insect parts, but also plant and fungal material, in the dissected gut. Mamaev et al. (1977) indicated *Cucujus* species are scavengers, only occasionally preying on larvae and pupae of other beetles. Zdeněk et al. (2012) reported on the opportunistic omnivore behaviour of *C. cinnaberinus*. Other preliminary studies showed the cannibalistic and predator habits of *C. cinnaberinus* larvae under artificial conditions (Palm 1941; Mazzei et al. 2011; Bonacci et al. 2012). *Cucujus haematodes* is known to be a scavenger, feeding on pupae and larvae of other insects (Mamaev et al. 1977) and on other subcortical beetles (Burakowski et al. 1986). Necrophagous and predacious feeding behaviour of *C. haematodes* was reported by Nikitskiy et al. (2008) and Horák and Nakládal (2009).

In a few cases, adults of *C. cinnaberinus* and *C. haematodes* were observed to be motionless under bark (Palm 1941) and showed thanatosis when disturbed (Horák & Chobot 2009, T. Bonacci personal observations), a behaviour displayed by many insects when potential threat or danger occurs. No studies have reported other defence strategies used by these species against enemies. Larvae and adults of both species are closely associated with moribund or dead trees (Horák et al. 2010) with wood-inhabiting fungi and microbes.

This study addresses two fundamental questions: (i) Why do *C. cinnaberinus* and *C. haematodes* display aposematic colour patterns? (ii) Could these beetles have developed cuticular defences against pathogens inhabiting pine bark?

Although there is little doubt that bright colour is often an anti-predatory strategy (Joron 2003), why has conspicuous colour (aposematism) evolved in these species if they spend most of their life under bark? How can these species live in habitats full of micro-organisms, such as the bark of the endemic pine, *Pinus laricio* var. *calabrica*?

As regards aposematism in insects, Joron (2003) raises some questions, e.g. whether aposematic colours are signals that help predators learn to better differentiate inedible from edible prey, or whether bright colours are more easily memorised and associated to bad taste by predators. According to Rowe (2001), aposematism is simply the correlation between conspicuous signals, such as bright colour, and prey unprofitability. Many aposematic insects use chemicals as repellents for protection from enemies, generally synthesised in particular glands or

acquired from food resources (Bowers 1992). The kind of defensive chemicals and the amount present in an unpalatable insect may determine its degree of unpalatability and its potential for protection from enemies (Howard et al. 1982).

The bright colour of European *Cucujus* spp. (Figure 1) was the primary factor that led us to make cuticular chemical investigations.

This peculiarity and other unknown aspects of their biology suggest that the species lend particular interest to defensive behaviour towards potential predators. Furthermore, it is known that cuticular defences include antimicrobial peptides produced in many cells and glands of insect bodies (Meister et al. 2000; Schmid-Hempel 2005). Polar compounds found on the cuticle of many species of arthropods are the main defence against fungi and bacteria (Gillespie & Kanost 1997; Turillazzi et al. 2006). Microbiological investigations have shown that cuticular polar extracts of *Rhynchophorus ferrugineus* (Coleoptera Dryophthoridae) inhibit the growth of some microorganisms (e.g. gram-positive bacteria *Bacillus subtilis* and *B. thuringiensis*) (Mazza et al. 2011). Polar chemicals identified on the cuticle layer of this invasive coleopteran species end the germination of *Beauveria bassiana*'s spores. Mycelial growth of fungal species has been prevented by caprylic and lauric acid, both identified in *Liposcelis bostrychophila* (Psocoptera: Liposcelidae) cuticular extracts (Lord & Howard 2004).

In the grain beetles of the genus *Cryptolestes*, formerly included in Cucujidae but now part of the related family Laemophloeidae, the only chemicals detected are aggregation pheromones named “cucujolides”, which are a mixture of macrocyclic lactones (Oehlschlager et al. 1987; Vanderwel & Oehlschlager 1987) that may include fatty acids as a precursor in their biosynthesis (Francke & Dettner 2005). Fatty acids are substances of fundamental importance in the defensive strategies of many insect species. They play a role as precursors in the biosynthesis of pheromones, waxes and eicosanoids, and as components of defensive secretions (Stanley-Samuelson et al. 1988).

Gas chromatography (GC) is a powerful separation technique, particularly when combined with the extreme selectivity and sensitivity of mass spectrometric detection (Guido et al. 2012). Indeed, mass spectrometry (MS) has long been used in the development of methods for investigation of biomolecules (Lo Feudo et al. 2011) and biomarkers, especially when it is applied as tandem mass spectrometry (Monteleone et al. 2012; Naccarato et al. 2013).

In this work, we describe, for the first time, the cuticle chemical compositions of *C. cinnaberinus* and

*C. haematodes*, with the aim of providing more insight into the possible role that some chemicals could play in the biology of these red beetles. This purpose has been achieved using solid phase micro-extraction (SPME) as the analytical sampling approach. The use of SPME allows the simultaneous extraction and preconcentration of the analytes in a single step and without the use of organic solvents (Naccarato et al. 2014; Naccarato & Pawliszyn 2016). Furthermore, this microextraction technique allows the performance of in-vivo analysis of the cuticular profile of the beetle, thus providing a better assessment of the molecules produced by the investigated beetles, and is compatible with the release of protected beetles into their original habitat.

## Material and methods

### *Taxonomy and biogeographic background*

The genus *Cucujus* F. is distributed throughout the Holarctic region and highly concentrated in Asia, with many endemics especially in India, Nepal, Myanmar, China, Taiwan and Japan (Lee & Pütz 2008; Horák & Chobot 2009). Only two native species were known from Europe, *C. cinnaberinus* (Scopoli, 1763), and *C. haematodes* Erichson, 1845, but lately a third species, *C. tulliae* Bonacci et al. 2012, from Calabria has been described. *C. cinnaberinus* is a saproxylic beetle endemic to Europe, living in several countries including Spain, Eastern France, the Netherlands, Ukraine, Russia and Sweden. Its populations are more densely distributed in Eastern Europe, from Austria and Bavaria eastwards (Horák & Chobot 2009; Teunissen & Vendrig 2012; Fuchs et al. 2014). Moreover, in the last 10 years, a re-expansion of its habitat along riverine forests has often been reported (Hörren & Tolkiehn 2016). Although the beetle was thought to be extinct in Italy after 1960, in the last decade it has been found in Piedmont and on the Alburni mountains in the Campania Region, by Biscaccianti et al. (2009), and, after 49 years of absence, in the Sila National Park in Calabria (Mazzei et al. 2011). *Cucujus haematodes* is a palearctic species spanning areas from Bavaria and Southern Italy to Japan; it comprises at least one well-differentiated subspecies, *C. h. opacus* Lewis, 1988, found in Japan and Taiwan, whereas the nominal form is known from Southern Italy (Calabria), Greece and Eastern Europe to the Primorskiy Region of Far Eastern Russia and China (Horák & Chobot 2009). Another form, *C. h. caucasicus* Motschulsky, 1845, is known from Armenia, Georgia, and Russia, although the status of this subspecies was considered more dubious (Horák & Chobot 2009), because Mamaev et al. (1977), in a

larval key, considered the pre-imaginal characters to be those of a separate species. In this respect, Bonacci et al. (2012) have re-evaluated the status of this taxon and have finally considered *C. h. caucasicus* to be a distinct subspecies.

#### *Cucujus* sampling and rearing

Samples of *C. cinnaberinus* (N = 4) and *C. haematodes* (N = 5) larvae were collected by hand in Natura 2000 SAC ("Special Areas of Conservation") forest sites of the Sila National Park (Calabria, Southern Italy) from May to July 2016, with careful inspections under the bark of fallen or standing dead Calabrian pine trees. The beetles were kept alive in the lab and stored in controlled conditions in a thermostatic chamber with natural substrate (dead wood and bark), at constant temperature (15°C), 12:12 L:D light rhythm, and constant adequate humidity values of the decaying wood and bark layer. The larvae were fed with small pieces of chopped veal and *Tenebrio molitor* until pupation, in accordance with Bonacci et al. (2012). After chemical analysis in the laboratory, some of the emerged beetles were released into the sample sites.

#### Instrumentation

GC-MS analysis was performed using a TSQ Quantum GC (Thermo Fischer Scientific) system constituted by a triple quadrupole mass spectrometer (QqQ-MS) Quantum and a TRACE GC Ultra equipped with programmable temperature vaporiser (PTV) injector. Chromatographic separation of the analytes was performed using a Restek Rxi-5MS capillary column 30 m × 0.25 mm inner diameter, 0.25 µm film thickness (95% polydimethylsiloxane, 5% polydiphenylsiloxane). The GC oven temperature was initially held at 100°C for 3 min, then ramped at 16°C/min to 280°C and held at this temperature for 15 min. The carrier gas was helium (99.999%, purity) at 1 mL/min. The transfer line and ionisation source temperatures were set at 280 and 250°C, respectively. For SPME analysis, a Thermo PTV straight Liner 0.75 mm × 2.75 mm × 105 mm was used as GC inlet liner. The analyses were performed in splitless mode with the injector temperature set at 280°C. The MS was operated in electron ionisation (EI) and full scan modes (40–500 m/z as mass range). The filament emission current was set at 25 µA. Instrument control and data processing were performed using Xcalibur software, version 2.0.0 (Thermo Fisher Scientific). Experimental data were evaluated with Microsoft Excel software, USA.

#### Analytical procedure

The cuticular organic profile of adult beetles (*C. cinnaberinus*: N = 4 males; *C. haematodes*: N = 5 males) was investigated. All individuals were analysed 5 days after adult emergence, without feeding. The survey of cuticular compounds was performed in vivo by solid phase microextraction coupled with gas chromatography-mass spectrometry (SPME-GC-MS). The analysis was carried out using a polydimethylsiloxane 100 µm (PDMS) fibre according to the following procedure: the SPME fibre was exposed and gently rubbed against the body of the live insect for about 1 min. Afterwards, the fibre was withdrawn into the needle and then exposed into the injector port of the gas chromatograph. The detected compounds were identified by matching the EI+ spectra against the NIST 02 database (NIST/EPA/NIH Mass Spectral Library, version 2.0 and by comparison of measured retention indices (RIs) with data collections of Kovats RIs.

## Results

#### Cuticular profiles

The compounds detected on the cuticle of the investigated *Cucujus* species are listed in Table I.

The cuticle of the beetles is mainly composed of hydrocarbons, organic acids and esters. Hydrocarbons are the major compounds; a total of 14 hydrocarbons were detected. There is a remarkable presence of organic acids on *Cucujus* spp. cuticle: n-hexadecanoic acid, oleic acid, octadecanoic acid and dehydroabiatic acid. Seven compounds, namely tetradecanal; one unidentified ester; benzene, (1-methyldodecyl)-; octadecanal; two unidentified hydrocarbons; and cholesterol (Table I), were only detected on the cuticular layer of *C. cinnaberinus*. In comparison, the *C. haematodes* cuticular profile is characterised by the greater presence of hydrocarbons with high molecular weight as well as the presence of atraric acid and heptadecanal (Figure 2).

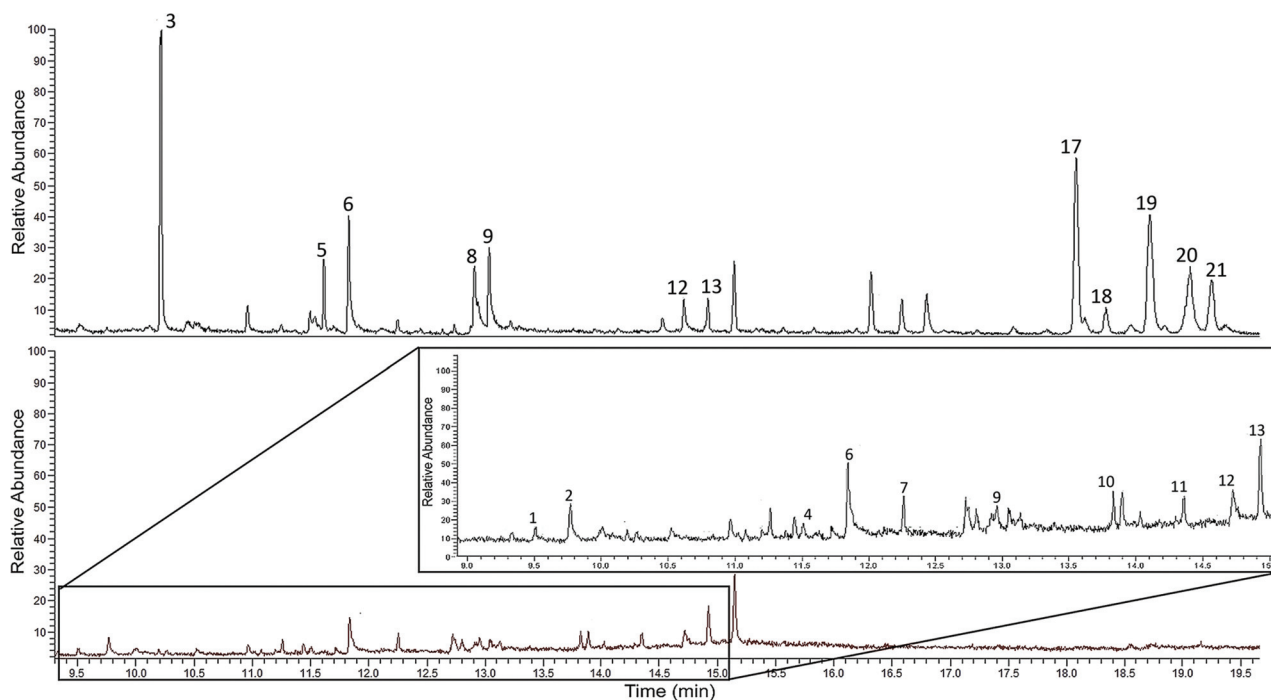
The compounds in common among the *Cucujus* species are three organic acids, namely n-hexadecanoic, oleic and octadecanoic acid, while the only sterol detected, cholesterol, occurs exclusively in *C. cinnaberinus*.

## Discussion and conclusions

During recent decades, there has been a great increase in knowledge of the chemicals occurring on insect

Table I. Chemical compounds identified in adults of *Cucujus haematodes* and *C. cinnaberinus* (tR = retention time).

Peak no.	tR	Compound	Chemical group	<i>C. haematodes</i> ♂♂	<i>C. cinnaberinus</i> ♂♂
1	9.5	Tetradecanal	Aldehyde		X
2	9.76	Unidentified ester	Ester		X
3	10.2	Atraric acid	Ester	X	
4	11.59	Benzene, (1-methyldodecyl)-	Hydrocarbon		X
5	11.62	Heptadecanal	Aldehyde	X	
6	11.83	n-Hexadecanoic acid	Organic acid	X	X
7	12.26	Octadecanal	Aldehyde		X
8	12.91	Oleic acid	Organic acid	X	X
9	13.04	Octadecanoic acid	Organic acid	X	X
10	13.82	Unidentified hydrocarbon	Hydrocarbon		X
11	14.35	Unidentified hydrocarbon	Hydrocarbon		X
12	14.71	Dehydroabietic acid	Organic acid	X	X
13	14.92	Unidentified hydrocarbon	Hydrocarbon	X	X
14	16.33	Unidentified hydrocarbon	Hydrocarbon	X	
15	16.6	Unidentified hydrocarbon	Hydrocarbon	X	
16	16.8	Unidentified hydrocarbon	Hydrocarbon	X	
17	18.1	Unidentified hydrocarbon	Hydrocarbon	X	
18	18.3	Unidentified hydrocarbon	Hydrocarbon	X	
19	18.7	Unidentified hydrocarbon	Hydrocarbon	X	
20	19.07	Unidentified hydrocarbon	Hydrocarbon	X	
21	19.24	Unidentified hydrocarbon	Hydrocarbon	X	
22	21.03	Unidentified hydrocarbon	Hydrocarbon	X	
23	21.82	Cholesterol	Sterol		X
24	22.02	Unidentified hydrocarbon	Hydrocarbon	X	X

Figure 2. Chemical profiles of *C. haematodes* male (above) vs *C. cinnaberinus* male (below).

cuticle. In this research field, solid phase microextraction provides a significant contribution due to its features, such as the ability to perform the solvent-free extraction of analytes as well as the possibility to carry out in-vivo sampling. In this work, the combined use of

SPME extraction and GC-MS analysis has been applied to investigate the chemical profile of living *Cucujus* individuals through non-invasive sampling. These bright red bark dwellers are characterised by cuticular profiles significantly different from those of

other beetles (Bonacci et al. 2008). Indeed, in addition to the hydrocarbons also found in the cuticle layer of many other insect species (Howard et al. 1995), on the *Cucujus* cuticle we found a substantial number of organic acids. Fatty acids are involved in some important biochemical pathways of insects; indeed, they play a major role in several physiological functions and are used also as pheromones or defensive substances. For example, toxic alkaloids derived from the unsaturated fatty acid, and in particular oleic acid (Attygalle et al. 1994), were identified in coccinellid beetles. Oleic acid (C<sub>18</sub>H<sub>34</sub>O<sub>2</sub>) has been detected only in a few taxa, such as Formicidae, Termites and Chrysomelidae (Wilson et al. 1958). Eggs of the green dock beetle *Gastrophysa cyanea* contain oleic acid in amounts which were demonstrated to deter several species of ants from feeding (Howard et al. 1982). This fatty acid elicits corpse-carrying behaviour in several ants (Wilson et al. 1958; Haskins & Haskins 1974; Lanza et al. 1992) and foraging behaviour in other social contexts. Some coccinellid eggs are fortified with alkaloids derived from fatty acids. In addition, in *Pogonomyrmex* and other ant genera, fatty acids were found to act as a releaser of necrophoric behaviour (Haskins & Haskins 1974). Octadecanoic acid has also been detected in *Graphosoma lineatum* (Heteroptera, Pentatomidae) and is used by this bug as a defensive compound (Durak & Kalender 2009). The same chemical was identified in the defensive secretion of the polydesmoid millipede *Pseudopolydesmus serratus* (Conner et al. 1977; Makarov et al. 2012). Atraric acid has been extracted from a bark sample contaminated by *Parmelia olivetorum* and *P. perlata* (Bourgeois et al. 1999). Other authors have reported on the antimicrobial role of atraric acid in lichen species (Mitrović et al. 2011). The role that some of the hydrocarbons detected could play against microorganisms should not be left out. The surface extract of other insects living in habitats full of pathogens exhibited antimicrobial activity (Butera et al. 2009). Moreover, other authors have shown the action of some hydrocarbons detected in arthropods against entomopathogens (Banerjee & Dangar 1995). Recently, microbiological findings (Mazza et al. 2011) showed that polar substances detected in the red palm weevil are effective against microorganisms.

In summary, this study provides, for the first time, the qualitative chemical cuticular profiles of two endangered species of the genus *Cucujus*. The compounds detected show the profile of living *Cucujus* because the sampling was performed on the beetles in a non-invasive way. In these insects, among the molecules detected we found several fatty acids, which are reported and identified as defensive

substances in other arthropods (Attygalle et al. 1994). In particular, oleic acid is reported to play a defensive or repellent role in many other insects. Our results do not explain the anti-predator or defence function of the chemicals found in these beetles but encourage us to conduct further research on many aspects of their biology and of their defensive mechanisms.

Indeed, the remarkable range of substances found in *Cucujus* suggests a close relationship with the bright red colour of the adults that concurrently acts as an aposematic signal for visual and olfactory predators. Considering tangible data published on the role of colour patterns in animals, we cannot disregard the suggestion that principles applicable to one group of animals, in all conspicuous colouration cases, may be valid also in the case of others (Longley 1917), like the red body pattern of *Cucujus*. Moreover, a literature overview of the gut contents of several insectivore taxa (reptiles, small mammals, birds, bats) shows that bright red beetles like cucujids, pyrochroids and lycids have never been recorded in their predation spectrum (Gaetano Aloise, Zoology Museum, University of Calabria, personal communication). The colour/odour combination probably plays an important role during the spring flying phase and in June and July, when the adults stay motionless on the outer bark side, waiting perhaps for sexual partners or copulating (Straka 2017). Our results support the theory that conspicuous colour and defence chemicals in these two beetles can produce a sufficient aposematic signal to limit attack by ambush and active predators (Matthews 1977). Animals protected by chemical defence are often conspicuously coloured (Alcock 1979), since unpalatability is frequently coupled with warning signals (aposematic colours and odours, Cott 1940; Tullberg et al. 2000). In this case, the fatty acids produced by the species investigated could act as predator/pathogen repellents. Preliminary food choice tests carried out in the laboratory by the authors showed that *Cucujus* larvae prefer *Lasius* (Hymenoptera, Formicidae) pupae and larvae versus other associated potential preys (unpublished data). It is known that many insect species acquire the chemicals by ex novo synthesis (autogenously) or exogenously by dietary sequestration of secondary compounds from food. Thus, the question arises: are flat bark beetles able to acquire their chemicals by preying on other associated insects? This issue should be addressed in future studies aimed at better understanding the complex mechanism of synthesis of the compounds found on the adult cuticle and the significance of habitat association with similar and protected species.

The presence of polar compounds with antimicrobial activity has already been detected on the epicuticular layer of arthropods (Kuhn-Nentwig 2003), including social insects (Hölldobler & Wilson 1990; Turillazzi et al. 2006; Mazza et al. 2011). *Cucujus* spp. live in association with many pathogens, entomo-pathogenic fungi and microbes related to under-bark habitat. Fatty acids and cuticular hydrocarbons with a possible antimicrobial function appear to be fundamental components of the defence biology of the red *Cucujus* beetles, and encourage further investigation.

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No potential conflict of interest was reported by the authors.

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