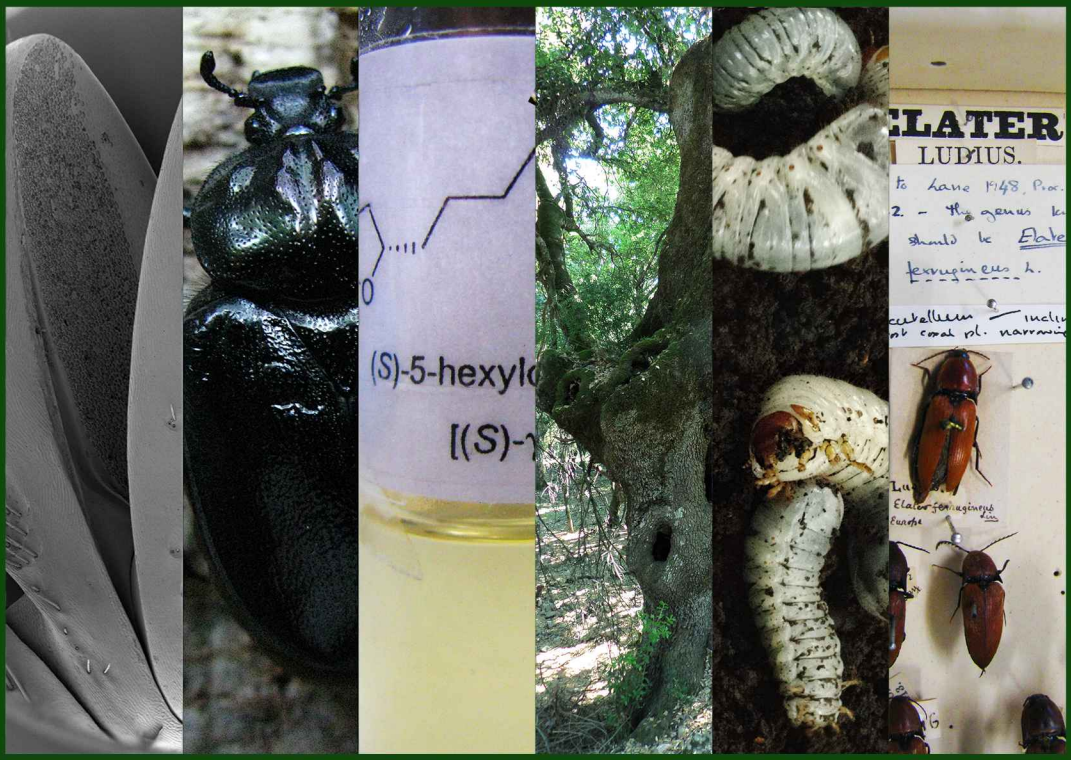
Chemical communication and conservation ecology of two rare saproxylic beetles: *Osmoderma eremita* (sensu lato) and *Elater ferrugineus*



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Section: Biodiversity and Ecosystem Analysis
XXVII Cycle, A.A. 2014/2015

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Comunicazione chimica ed ecologia della conservazione di due rari coleotteri saproxilici: *Osmoderma eremita* (sensu lato) ed *Elater ferrugineus*

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A.A. 2014-2015

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Front cover: *Osmoderma eremita* antenna, *Osmoderma eremita* male, vial containing pheromone, hollow tree, *Osmoderma eremita* larvae, *Elater ferrugineus* in an entomological box at Natural History Museum London.

Thesis defense 27th February 2015 in front of the following jury of European committee members:

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ABSTRACT

Osmoderma eremita (Coleoptera, Scarabaeidae) and *Elater ferrugineus* (Coleoptera, Elateridae) are threatened saproxylic beetles associated with old hollow broad-leaved trees in mature forest ecosystems of Europe. The larvae of *O. eremita* feed on decaying wood, while those of *E. ferrugineus* are predators of the immature stages of large Scarabaeidae, including *O. eremita.* The females of *E. ferrugineus* are attracted to the *O. eremita* male-emitted sex pheromone, (*R*)-(+)-γ-decalactone, and exploit this compound as a kairomone in order to locate suitable tree cavities in which to lay eggs. In addition, the males of *E. ferrugineus* are strongly attracted to the sex pheromone emitted by conspecific females (7-methyloctyl (*Z*)-4-decenoate). The overall goal of the present project is to increase the knowledge about *O. eremita* and *E. ferrugineus* ecological relationships and their chemical communication system.

The antennal sensilla of both species were studied using scanning electron microscopy. *O. eremita* antennae did not show any sexual dimorphism concerning the distribution of sensilla placodea on the antennal club, the ones responsible for pheromone reception. In fact, both the sexes are attracted by the same pheromone. In contrast, *E. ferrugineus* showed a strong sexual dimorphism, with one type of thricoid sensillum occurring only on the male antenna. Probably these sensilla are responsible for the reception of female-emitted sex pheromone, to which only males are attracted.

A mark-recapture study was performed using traps baited with the two pheromones. On the whole, 13 *O. eremita* and 1,247 *E. ferrugineus* were trapped. For *E. ferrugineus,* 7-methyloctyl (*Z*)-4-decenoate was a much more efficient lure than the kairomone, and 1% of the individuals dispersed farther than 1,600 m from their natal site. In contrast to some studies on these beetles conducted in northern Europe, the activity pattern was not influenced by variation in temperature during the season.

Scent collection and analysis on *O. cristinae*, endemic to Sicily, showed that this species uses exactly the same sex pheromone compound as used by *O. eremita,* demonstrating a strong conservation of this sexual trait within the genus. Data on mtDNA cytochrome C oxidase I gene (*COI*) and morphology of male genitalia supported the divergence of the two species and suggested a species status for *O. cristinae*.

“Dum loquimur fugerit invida aetas:

carpe diem, quam minimum credula postero”

[Quintus Horatius Flaccus, Odi 1, 11, 8]

CONTENTS

Preface and thesis overview

Chapter 1. Introduction
The first chapter gives a presentation of the two target species with notes on their systematics, distribution and biology, followed by a brief discussion on their chemical ecology and conservation biology. At the end of the chapter, the aims of the thesis are explicitly stated.

The three subsequent chapters (2-4) consist of the following four published or submitted papers identified with roman numbers:

Chapter 2. Sensing the mates, a matter of structures

I. Zauli A, Maurizi E, Chiari S, Svensson GP, Carpaneto GM, Di Giulio A. Fine morphological analysis of the antenna in the threatened saproxylic beetle *Osmoderma eremita* (Coleoptera, Scarabaeidae). Submitted to *Journal of Morphology*.

II. Zauli A, Maurizi E, Carpaneto GM, Chiari S, Svensson GP, Merivee E, Di Giulio A. Scanning electron microscopy analysis of the antennal sensilla in the rare saproxylic beetle *Elater ferrugineus* (Coleoptera, Elateridae). In preparation for *Zoomorphology*.

Chapter 3. Using semiochemicals to sample rare beetles

III. Zauli A, Chiari S, Hedenström E, Svensson GP, Carpaneto GP (2014). Using odour traps for population monitoring and dispersal analysis of the threatened saproxylic beetles *Osmoderma eremita* and *Elater ferrugineus* in central Italy. *Journal of Insect Conservation*, 18: 801-813. (Paper reproduced with the permission of Springer International Publishing).

Chapter 4. *Osmoderma cristinae*, scent on the island

IV. Zauli A, Carpaneto GM, Chiari S, Manicni E, Nyabuga FN, Redolfi De Zan L, Romiti F, Sabbani S, Audisio P, Hedenström E, Bologna MA, Svensson GP. Assessment of the species status of *Osmoderma cristinae* (Coleoptera: Scarabaeidae), endemic to Sicily, using pheromonal, genetic and morphological analyses. In preparation for *Systematic Entomology*.

Chapter 5. Conclusions
The chapter includes a discussion and a brief synthesis of the major outcomes of this thesis, in the light of the aims stated.

1. Introduction

1.1. The genus *Osmoderma*: systematics, distribution and biology

The genus *Osmoderma* Lepeletier & Serville, 1828 (Coleoptera, Scarabaeidae, Cetoniinae) is widespread throughout the Palaearctic and Nearctic Regions with eleven species described (Audisio et al., 2007). The European species avoid high latitudes being associated to a wide range of broad-leaved trees (Ranius et al., 2005).On the basis of the most recent systematic revision, where sequence data for the mitochondrial cytochrome oxidase subunit I gene have been used (Audisio et al., 2009; Svensson et al., 2009; Landvik et al., 2013), four species of *Osmoderma* occur in Europe (Fig.1-2): *O. eremita* (Scopoli, 1763); *O. barnabita* Motschoulsky, 1845; *O. lassallei* Baraud and Tauzin, 1991; *O. cristinae* Sparacio, 1994 (a fifth taxon, *O. italicum* Sparacio, 2000, is probably a subspecies of *O. eremita*).

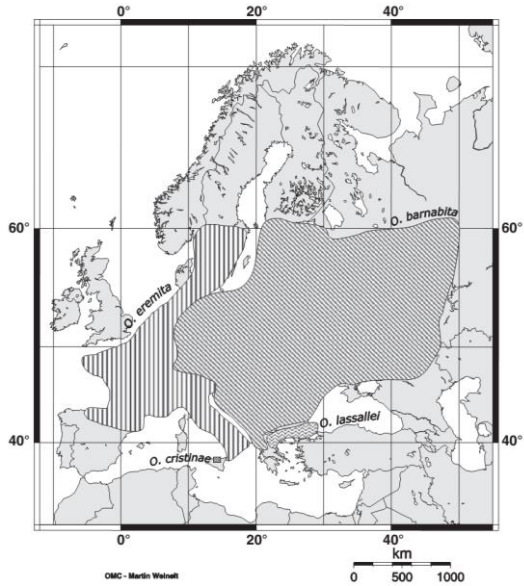

Fig.1. Geographical distribution of species within the *Osmoderma eremita* species complex in Europe. *O. eremita*, widespread in western Europe; *O. barnabita*, widespread in eastern Europe; *O. cristinae*, confined to Sicily; *O. lassallei*, distributed in NE Greece and European Turkey (Audisio et al., 2009).

Fig.2. Geographical distribution of species within the *Osmoderma eremita* species complex in Italy. *O. eremita*, northern and central Italy; *O. italicum*, southern Italy; *O. cristinae* endemic to Sicily.

Many ecological data are available for *O. eremita* (e.g. Ranius et al., 2005) and we can hypothesize that most of the general biological information reported hereafter for *O. eremita* is also relevant for the other European species.

O. eremita (Fig.3A) is a large-sized beetle (20-39 mm), black to dark brown with a metallic sheen. The males are easily recognized for the deep longitudinal furrow on the middle of the pronotum, which is lacking in the females (Fig.3C-D). Other morphological characters distinct to males are: the dorsal excavation of the head; the strongly raised borders of the clypeus, which form two small horns near to the antennal insertion; the more rounded pygidium (Tauzin, 1994a; 1994b; Schaffrath 2003a; 2003b).

The larvae are 'white grubs', similar to those of other Cetoniinae (Fig.3B). They live inside tree hollows where develop in wood mould (i.e. loose rotten wood often mixed with fungi and frass). Normally, after two years they build a cocoon made of rotten wood and frass, and become pupae. The adults emerge the following year and fly in summer, from June to August. Thus, the biological cycle usually takes three years, and the adults have a life expectancy of about one month (Ranius et al., 2005).

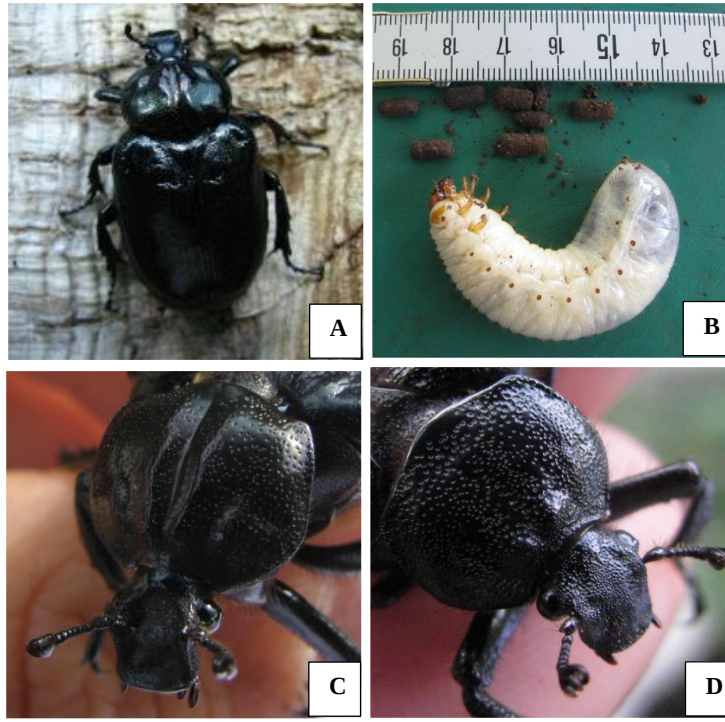

Fig.3. *Osmoderma eremita*. A: male habitus; B: third instar larva; C: male head and pronotum; D: female head and pronotum (Photo: A. Zauli).

Ecological studies on *Osmoderma* beetles have mostly been conducted in Sweden (e.g. Ranius 2001; 2002; Hedin and Ranius, 2002; Jönsson et al., 2004; Hedin et al., 2008; Larsson and Svensson, 2009; Svensson et al., 2011) but in the last few years studies have also been performed in Italy (Audisio et al., 2009; Carpaneto et al., 2010; Chiari et al., 2012; 2013a; 2013b), France (Dubois and Vignon, 2008; Dubois et al., 2009; Dubois et al., 2010; Le Gouar et al., 2014), Poland (Oleksa et al., 2007; Svensson et al., 2009; Oleksa et al 2012; Hilszczański et al., 2014), and Finland (Landvik et al., 2013). These recent studies examined different aspects, such as population ecology, dispersal, chemical ecology, habitat requirements, conservation strategies, and the current distribution of different species.

In Sweden *O. eremita* inhabits large trees and population studies there have shown a metapopulation structure (sensu Levins, 1969) on a small scale where each hollow tree is a habitat patch, potentially sustaining a local population (Ranius, 2000; 2001). Individual dispersal did not usually exceed a few hundred meters, with only a small fraction of individuals moving from the natal tree (Ranius and Hedin, 2001; Hedin and Ranius, 2002; Hedin et al., 2008; Svensson et al., 2011). In contrast, in Italy, where the species lives in smaller trees, studies have shown a different situation with greater distances covered by individuals (up to 1,500 m) (Chiari et al., 2013a; 2013b). This difference could be attributed to different environmental conditions, in particular the resource distribution in the landscape. In fact, a single smaller hollow tree is often not able to host a sufficient number of individuals to represent a population in itself. This is due to the modest volume of the cavities with a small amount of wood mould, which is one of the most important parameters for habitat suitability for *O. eremita* (Ranius and Nilsson, 1997; Ranius and Jansson, 2000; Ranius et al., 2009; Chiari et al., 2012). Therefore, in Italy a single population is more spread out in the landscape, and as a consequence, more beetles move from the natal tree and the dispersal distances covered are greater than for Swedish populations (Chiari et al., 2013b).

1.2. *Elater ferrugineus*: systematics, distribution and biology

The genus *Elater* shows a very wide distribution range and occurs in the Palaearctic Region (nine species), in the Indo-Malayan Region (four species), in the Australian Region (one species), in the Nearctic Region (four species) and in the Neotropical Region (two species) (Schimmel and Tarnawski, 2010; Platia et al., 2011). *E. ferrugineus* Linnaeus, 1758, (Fig.4B) occurs in Europe from Spain to the Caucasus, and from Sweden to Italy.

Pronotum and elytra of this species are variable in colour. Four different colour varieties can be recognized in different areas but there is no clear distinction because more than one variety can occur in the same population. The nominal variety (var. *ferrugineus*), with red pronotum and elytra, seems to be more abundant in southern Europe; the var. *occitanus* du Buysson, with black pronotum and red elytra, is more abundant in north-central Europe, for example in Sweden and France; the var. *morio* Schilsky, entirely black, occurs in

some populations of northern Italy, France and Poland; finally, var. *fumatus* Piton, with red pronotum and black elytra, is very rare (Hansen, 1966; Leseigneur, 1972).

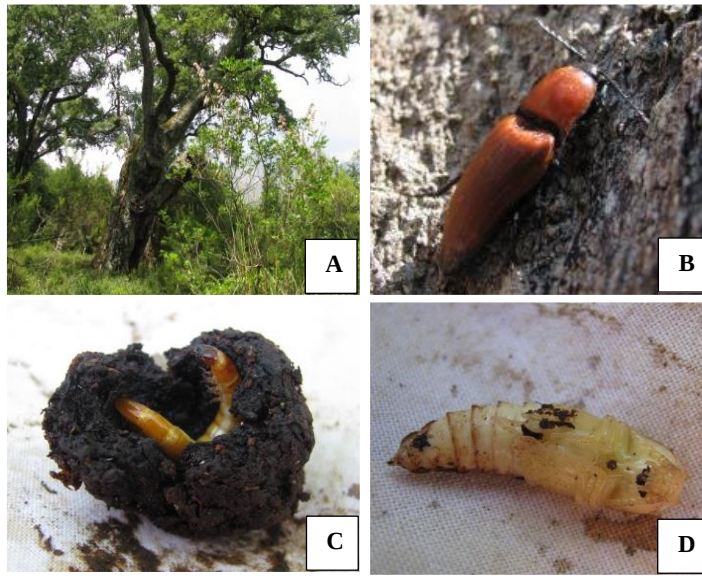

Fig.4. *Elater ferrugineus* habitat and life cycle. A: hollow tree potential harboring *E. ferrugineus*; B: male habitus; C: last instar larva in the cocoon; D: pupa (Photo: A. Zauli).

Much of *E. ferrugineus* biology is still unknown or debated. It is reported to be associated with many broad-leaved trees as *Salix*, *Castanea*, *Fagus*, *Fraxinus*, *Quercus*, *Ulmus*, *Aesculus* (Iablokoff, 1943; Hansen, 1966; Allen, 1966). The female has shorter antennae, a larger body and a more rounded lateral side to the pronotum and elytra.

The larvae of *E. ferrugineus* (Fig.4C) are carnivorous, preying on the larvae and pupae of cetoniid beetles like *Osmoderma*, *Cetonia* and *Protaetia* that live in hollow trees (Fig.4A), but they can also eat larvae of their own species (Iablokoff, 1943, Hansen, 1966; Tolasch, 2007).

The life cycle of *E. ferrugineus* can last up to six years, depending on the abundance of prey in the breeding substrate (Tolasch, 2007). At the end of their development, the larvae build a cocoon similar to

but smaller than that of *O. eremita* (Fig.4C). The larvae pupate in hollow trees in late May or early June. When pupation begins they group together in a corner of the cavity far from the larvae of *O. eremita* (Fig.4D) (Iablokoff, 1943; Zauli pers. obs.).

The recent identification of the female-produced sex pheromone of *E. ferrugineus* (Tolasch et al., 2007) (See paragraph 1.3) has greatly facilitated the collection of information on the distribution of the species in Europe. New populations have been recorded for example in Latvia (Barševskis and Nitcis, 2011), Lithuania (Meržijevskis and Tamutis, 2010), Spain (Fernández de Gamboa, 2010) and central Italy (A. Zauli, pers. obs.). In addition, pheromone trapping has aided in gathering detailed ecological data on *E. ferrugineus* (Larsson and Svensson, 2011; Musa et al., 2013; Andersson et al., 2014). For example, in Sweden a high spatial and temporal correlation in flight activity of the predator *E. ferrugineus* and its prey *O. eremita* was observed (Larsson and Svensson, 2011).

At landscape scale, the occurrence of *E. ferrugineus* was better explained by the density of large hollow and non-hollow trees, particularly pedunculate oak (*Quercus robur*), than by the presence in the area of small hollow trees (Musa et al., 2014). This means that tree-size is a more important parameter than the presence of hollows for the habitat suitability of this species (Musa et al., 2014). The potential function of *E. ferrugineus* as umbrella species has also been studied (Andersson et al., 2014), showing that it is a good indicator of rare saproxylic beetles, co-occurring with many of them. Therefore, the knowledge of its habitat requirements is particular important as it can be used also for detection and monitoring of other species (Andersson et al., 2014; Musa et al., 2014).

1.3. Chemical ecology of *Osmoderma eremita* and *Elater ferrugineus*

The close interaction between *O. eremita* and *E. ferrugineus* has been noticed from entomologists since long time ago, before the studies on their chemical ecology (Iablokoff, 1943; Hansen, 1966; Platia, 1994; Schaffrath, 2003b).

The chemical ecology of this predator-prey system was revealed with the identification of the male emitted sex pheromone in *O. eremita* as (*R*)-(+)-γ-decalactone (Larsson et al., 2003). Since then, further studies have been conducted using this compound in traps

(Svensson and Larsson, 2008; Larsson and Svensson, 2011; Svensson et al., 2012; Chiari et al., 2013a). Field experiments showed that both females and males are attracted to this compound but usually the captures of males are less abundant (Chiari et al. 2013a; Larsson et al., 2003). In field trials it was noticed that the traps baited with (*R*)-(+)-γ-decalactone also attracted females of *E. ferrugineus*. Therefore, it was hypothesized that *E. ferrugineus* uses the sex pheromone of *O. eremita* as a kairomone in order to locate suitable hollow trees where to lay eggs (Svensson et al., 2004).

Pheromone gland extracts from *E. ferrugineus* females were examined and found to contain a blend of four esters: 7-methyloctyl 5-methylhexanoate, 7-methyloctyl octanoate, 7-methyloctyl 7-methyloctanoate, and 7-methyloctyl (*Z*)-4-decenoate in a ratio of approximately 1:1:3:3, and the four-component blend was found to be highly attractive to conspecific males (Tolasch et al., 2007). However, a subsequent study revealed that only one of the esters (7-methyloctyl (*Z*)-4-decenoate) is active as attractant, and this compound is a much more efficient lure than the prey kairomone when monitoring *E. ferrugineus* (Svensson et al., 2012). The identification of the sex pheromone was based on analyses of german and swedish populations but subsequent studies have shown that 7-methyloctyl (*Z*)-4-decenoate is a potent attractant throughout the distribution range of the species.

Further studies have shown that the (*S*)-enantiomer of γ-decalactone is not a behavioral antagonist for *O. eremita* or *E. ferrugineus* (Svensson and Larsson, 2008). From an applied perspective, this fact is very important for the development of a cost-efficient semiochemical based monitoring system, because pure (*R*)-enantiomer of γ-decalactone is much more expensive than a racemic mixture (Svensson and Larsson, 2008). In fact, many studies and surveys of *Osmoderma* beetles have used the racemic mixture instead of the pure (*R*)-enantiomer.

A recent study (Svensson et al., 2009) showed that also males of *O. barnabita* produce only the (*R*)-enantiomer of γ-decalactone and conspecific females are equally attracted to the (*R*)-enantiomer and to the racemic mixture indicating that also *O. barnabita* is anosmic to the (*S*)-enantiomer.

1.4. Threats and conservation

Saproxylic insects are dependent, at least during some parts of their life cycle, upon wounded or decaying woody material from living, weakened or dead trees (Speight, 1989; Alexander, 2008; Stockland et al., 2012). Within this ecological guild, *O. eremita* (s.l.) and *E. ferrugineus* are considered to be particularly threatened.

Modern forest practices are focused on timber trade, determining structural simplification of the habitat through the removal of hollow trees or dead trunks (Davies et al., 2008). Therefore, as these species are restricted to veteran trees, any activities that destroy these habitats are strongly detrimental (Nieto and Alexander, 2010).

From a long-term perspective, structural changes in the environment such as the decline of heterogeneity in the age classes of tree populations and their decline in density can affect diversity and abundance of saproxylic species. In fact, these two factors lead to a deficiency of habitat connectivity, to which these species are particularly susceptible due to their more or less limited dispersal capability (Ranius and Hedin, 2001; Ranius, 2006; Larsson and Svensson, 2011; Chiari et al., 2013b).

In central Europe, *O. eremita* (s.l.) and *E. ferrugineus* are regarded as "Urwald relict species" i.e. associated to primeval forest structures and features (Müller et al., 2005). Nevertheless, they are often found also in old trees along rural avenues and canals within an intensive agricultural landscape, or in urban parks where wood harvest is not allowed (Oleksa et al., 2007; Dubois et al., 2009; Carpaneto et al., 2010; Sebek et al., 2012; 2013).

O. eremita (s.l.) and *E. ferrugineus* can be used as indicators for old and species-rich deciduous forests (Schmidl and Bussler, 2004). In addition, according to Ranius (2002), *O. eremita* seems to be both an indicator of saproxylic species richness and a keystone species, while *E. ferrugineus* was discovered to co-occur with many red-listed saproxylic beetles (Andersson et al., 2014). Thus, the preservation of *O. eremita* (s.l.) and *E. ferrugineus* could be important for the survival of the whole invertebrate community associated with hollow trees in Europe (Ranius, 2002; Svensson et al., 2003, Ranius et al., 2005; Andersson et al., 2014).

O. eremita (s.l.) is protected under the Habitat Directive and Bern Convention and is listed together with *E. ferrugineus* in the IUCN Red List of threatened species (Table 1). Generally, the most

important conservation measure to be recommended for these species is the protection of large and old broad-leaved trees, in the management of the habitat to ensure that there is a constant or increasing supply of such veteran trees (Nieto and Alexander, 2010).

Tab.1. *Osmoderma eremita* (s. l.) and *Elater ferrugineus* categories of protection under different IUCN Lists, the Habitat Directive and Bern Convention (Nieto and Alexander, 2010).

	IUCN INT	**IUCN EU**	**IUCN IT**	**HD**	**BC**
O. eremita	NT	NT	VU	II/IV	II
O. barnabita	NT	NT	-	II/IV*	II*
O. cristinae	EN	EN	EN	II/IV*	II*
O. lassallei	EN	EN	-	II/IV*	II*
O. italicum	EN	EN	EN	II/IV*	II*
E. ferrugineus	-	NT	VU	-	-

IUCN INT: Interational IUCN Red LIST (The IUCN Red List of Threatened Species); IUCN EU: European Red List (Nieto and Alexander, 2010); IUCN IT: Italian Red List (Audisio et al., 2014). HD: Habitat Directive; BC: Bern Convention; *as part of *O. eremita.*

Other conservation tools are currently under evaluation, for instance, the use of boxes full of woody debris and mimicking tree hollows (Jansson et al., 2009; Hilszczański et al., 2014): these boxes might be particularly useful in case of the entire loss of hollow trees or could have the role of 'stepping stones' in case of a lack of habitat continuity for the beetles. In addition, pollarding trees showed to trigger a rapid formation of tree hollows. Such an active management can be a very important tool in improving the habitat quality for the conservation of fauna associated to hollow trees (Sebek et al., 2013).

1.5. Aims of the study

Research on the chemical ecology of insects took off in the 1950s with the identification of the silk moth sex pheromone (Butenandt et al., 1959). Since then, pheromones have been used in traps for monitoring populations of pest insects in forestry, plantation and crop management, and also in direct control via mating disruption (Witzgall et al., 2010). The advantages in the use of pheromones in pest control are many, e.g. they are selective, specific and very efficient at low population density of the target species (Witzgall et

al., 2010). These features are also useful when studying threatened or rare species, but the use of semiochemicals in conservation biology started only recently and was addressed to the study of *O. eremita* and *E. ferrugineus* (Svensson and Larsson, 2008; Musa et al., 2013; Andersson et al., 2014). These studies were performed on Swedish populations, while the present project is a contribution to their ecology in Mediterranean woodlands.

The overall goal of the present project is a contribution to the knowledge of *O. eremita* (s.l.) and *E. ferrugineus* ecological relationships and to their chemical communication, specifically pursuing the following aims:

1) Describing the antennal microstructures deputed to the pheromone reception in *O. eremita* (paper I) and *E. ferrugineus* (paper II);
2) Gathering information on their ecology: population size, dispersal rates and distances, phenological data and adult activity in relation to seasonal weather conditions (paper III);
3) Assessing sampling efficacy of pheromone traps for *E. ferrugineus* (paper III);
4) Identifying the sex pheromone of *O. cristinae*, a species endemic to Sicily (paper IV).

The information collected in this work can serve different purposes:

1) The scanning electron microscopy study on *O. eremita* antenna describes in detail the morphology and distribution of the structures suggested to be responsible for pheromone reception. A parallel analysis of *E. ferrugineus* is a preliminary work for electrophysiological studies aimed at investigating the sensitivity of the different type of sensilla to semiochemicals.
2) Information on the ecology of these species, especially *E. ferrugineus*, which was until now studied only in Sweden, can help to take science-based decisions for their conservation.
3) The evaluation of trap efficiency can contribute to assess the occurrence of *E. ferrugineus* and the threat status of its populations in monitoring programs.
4) The study on *O. cristinae*, which is endemic to Sicily, is meant to constitute the first step of research aimed at gathering ecological

information on its populations, and to confirm the taxonomic rank of this species.

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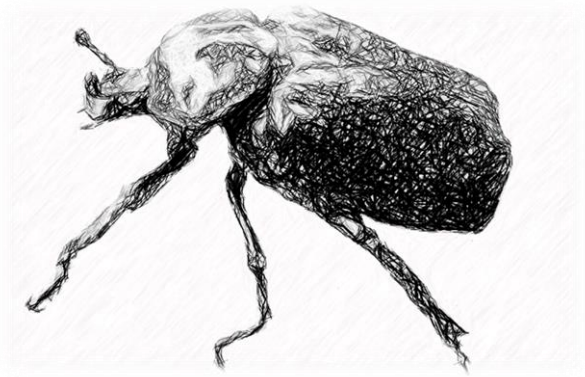
PAPER I



Fine morphological analysis of the antenna in the threatened saproxylic beetle *Osmoderma eremita* (Coleoptera, Scarabaeidae)

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Abstract

The aim of this study was to characterize the antennal morphology of *Osmoderma eremita,* a threatened scarab beetle inhabiting tree hollows. *O. eremita* males produce a sex pheromone, (*R*)-(+)-γ-decalactone, responsible mainly for the attraction of females, but also other males. Gross morphology and fine morphology of microstructures including sensilla, microsculpture and pores were analyzed using Scanning Electron (SEM) and Focused Ion Beam (FIB) Microscopy technologies. The antenna of *O. eremita* showed the typical lamellicorn shape of scarab beetles, with a basal scape, a pedicel, a funicle composed of five antennomeres and a club composed of three lamellae. Six different types of sensilla chaetica (Ch.1−6), one type of Böhm sensilla (Bo), one type of sensilla basiconica (Ba.1), two types of sensilla coeloconica (Co.1−2), two types of sensilla placodea (Pl.1−2), pores and peculiar folds were described in this species for the first time. The two sexes did not show any apparent differences in the occurrence, number and density of the sensilla placodea (Pl.1), known to be responsible for the pheromone reception. Instead, some sexual differences were found on the occurrence and topology of three different microstructures: 1) one type of sensillum chaeticum (Ch.2) occurring on the pedicel only in males; 2) a characteristic pore occurring on the fourth antennomer of the funicle, only in males; 3) a peculiar fold occurring on different antennomeres of the funicle in the two sexes, on the fourth in males and on the fifth in females. The big pore occurring on males antenna is connected underneath to a deep septate channel, and it is likely to have a glandular function. A comparison between sensilla of *O. eremita* and those of other Scarabaeoidea is provided.

Keywords Antennal sensilla; placoid sensilla; lamellate antenna; scarab beetles; SEM; FIB

Introduction

Scarab beetles have a lamellate antenna with a distinctive club composed of the last three to seven antennomeres. These segments are modified into flattened plates, called lamellae, and can be expanded like a fan or folded together, to protect their inner surfaces where many sensilla occur.

The inner surfaces of lamellae are the best known part of the scarab beetle antenna, considered the most interesting part of this structure, due to their olfactory function, in all the tribes of scarab beetles studied so far (Cetoniini, Dynastini, Hopliini, Melolonthini, Rutelini, Coprini and Geotrupini) (Table 1).

In scarab beetles, most of the studies on olfaction and semiochemicals have concerned Melolonthini and Rutelini and have revealed the use of female-released pheromones, whereas studies on Scarabaeini (*Kheper*) (Burger et al., 1983; 2002; 2008; Burger and Petersen, 2002) and Dynastini (*Oryctes, Scapanes, Strataegus*) have provided evidence of male-released pheromones (Gries et al., 1994; Hallett et al., 1995; Rochat et al., 2002; 2004; Vuts et al., 2014). In Cetoniini, chemical ecology studies proved the occurrence of female-released pheromones in genera *Pachnoda* and *Tropinota* (Bengtsson et al., 2011; Imrei et al., 2012), and male-released pheromones in genus *Osmoderma* (Larsson et al., 2003).

Osmoderma eremita (Fig. 1) is a scarab beetle belonging to the functional group of saproxylic organisms i.e. species that depend, during at least some parts of their life cycle, upon wounded or decaying woody material from living, weakened or dead trees (Speight, 1989; Alexander, 2008; Stokland et al., 2012). It is considered one of the most endangered inhabitants of old hollow trees in European deciduous forests (Ranius et al., 2005), being listed in the IUCN Red List of Threatened Species (Nieto and Alexander, 2010) and protected under the European Union's Habitats Directive as a priority species of community interest (Anonymous, 1992). Moreover, *O. eremita* is considered both an indicator of saproxylic species richness and a keystone species (Ranius, 2002a; Jönsson et al., 2004). The main threat affecting local populations of *O. eremita* is habitat loss, i.e. the loss of veteran trees, that are particularly vulnerable due to intentional removal for logging, land clearing, agricultural intensification, fire management and human safety (Lindenmayer et al., 2012).

Table 1. Occurrence of sensillum typology on the lamellae in different tribes of superfamily Scarabaeoidea according to literature data, compared to the one described for *Osmoderma eremita* in the present study. When the sensillum type in a species is homologue to one of that described for *O. eremita* its name is reported in one of the relative columns, otherwise is set in the column other type. Sensilla basiconica and auricillica were not found in *O. eremita* but are present on the lamellae in other species. Notes on the role of sensilla in sexual dimorphism are given.
† Sensilla responsible for the pheromone reception (always placodea but with different morphological characteristics).
Dash in a cell means that a sensillum type is not reported by the authors for the species analyzed.

Tribe	Species	Coeloconica			Placodea						
		Co.1	***Co.2***	**Other type**	***Pl.1***	***Pl.2***	**Other type**	**Basi-conica**	**Auri-cillica**	**Sex. dim.**	**Ref.**
											
Cetoniini	*Cotinis nitida*	Coelo-conica	-	-	-	placodea	-	basiconica	-	not rep.	Baker and Monroe, 2005
	Pachnoda interrupta	grooved -peg	smooth-peg	-	smooth placodea	grooved placodea†	-	-	-	not rep.	Bengtsson et al., 2011
	Pachnoda marginata	grooved -peg	smooth-peg	-	smooth placodea	grooved placodea†	-	-	-	not rep.	Bengtsson et al., 2011
	Pachnoda marginata	peg-shaped	peg-shaped	-	smooth placodea	placodea in heteroge-nous area	-	-	-	no	Stensmyr et al., 2001

Table 1 (continued)

Tribe	Species	Coeloconica			Placodea						
		Co.1	***Co.2***	**Other type**	***Pl.1***	***Pl.2***	**Other type**	**Basi-conica**	**Auri-cillica**	**Sex. dim.**	**Ref.**
Dynastini	*Adoryphorus couloni*	peg-like	-	-	-	-	placodea with wrinkled/granu-late surface	-	-	no	McQuillan and Semmens, 1990
	Oryctes elegans	-	-	-	-	placodea without pores	placodea with pores	-	-	no	Al-Dorsay, 2009
	Oryctes rhinoceros	cs1	cs2	-	SP3	-	SP1 rugged plate, SP2 ridged furrow	-	-	no	Renou et al., 1998
Hopliini	different genera	-	-	-	-	-	PLAS I, II, III, IV, V VI, VII	BAS I, II, III, IV, V, VI, VII, VIII	AUS I, II, III, IV, V, VI, VII	not rep.	Romero-López et al., 2013
Melo-lonthini	*Antitrogus parvulus* male	-	-	-	-	-	with a central cup-like pore plate	peg in a socket	-	yes	Allsopp, 1990
	Antitrogus parvulus female	-	-	-	-	-	-	pegs with surface smooth, wrinkled or granulate	-	yes	Allsopp, 1990
	Dasylepida ishigakiensis	type IV (peg-shaped)	-	type V (dome-shaped)	-	-	type I (shallow dish), II (table-shaped), III (shallow cup)	-	-	yes	Tanaka et al., 2006

Table 1 (continued)

Tribe	Species	Coeloconica			Placodea						
		Co.1	***Co.2***	**Other type**	***Pl.1***	***Pl.2***	**Other type**	**Basi-conica**	**Auri-cillica**	**Sex. dim.**	**Ref.**
Rutelini	*Lepidiota negatoria*	-	-	-	-	-	placoid cup-shaped	central smooth-peg or pointed peg	-	no	Allsopp, 1990
	Phyllophaga anxia	type I	-	type II	-	-	placodeum	-	Auricil-lica	not rep.	Ochieng et al., 2002
Rutelini	*Phyllopertha diversa*	coeloc onica	-	-	-	-	smooth†, longitudinal row	-	-	not rep.	Hansson et al., 1999
	Phyllopertha horticola	pit-organs	campanif orm	-	placodea	-	-	-	-	yes	Ågren, 1985
	Popilia japonica	C/B	-	-	-	D with periferal ditch	P tortoise shell-shaped†	-	-	yes	Kim and Leal, 2000
	Hylamorpha elegans	sco I, II, III	-	-	-	spc in smooth surface	spc in heterogenous surface	-	-	yes	Mutis et al., 2014
Coprini	*Copris pecuarius*	-	-	-	-	-	-	basiconica	-	no	Inouchi et al., 1987
Geo-trupini	*Geotrupes auratus*	-	-	-	-	-	-	basiconica	-	no	Inouchi et al., 1987

In the last few decades, several studies have been carried out to investigate different aspects of the biology of *O. eremita*, such as the population ecology (Larsson and Svensson, 2009; Ranius, 2001, 2002b; Chiari et al., 2013a), the habitat preferences (Ranius and Nilsson, 1997; Ranius, 2002c; Chiari et al., 2012), the dispersal patterns (Ranius and Hedin, 2001; Svensson et al., 2011; Chiari et al., 2013b), the value as bioindicator (Ranius, 2002a), as well as the chemical ecology (Svensson and Larsson, 2008). In fact, *O. eremita* males release a sex pheromone, (*R*)-(+)-*γ*-decalactone, with a characteristic plum-like or peach-like scent, which attracts mainly females (Larsson et al., 2003). In *O. eremita* the antennae of males and females responded identically to (*R*)-(+)-*γ*-decalactone in electroantennographic (EAG) recordings, and did not show any apparent sexual dimorphism in the presence and distribution of olfactory sensilla (Larsson et al., 2003). In addition, recordings from sensilla on the inner lamellae of the antennal club (Svensson and Larsson, 2008), revealed that (*R*)-(+)-*γ*-decalactone sensitive olfactory receptor neurons (ORNs) are sparsely distributed over the whole surface of the antennal inner lamella in both sexes, but are mainly found in a smooth area close to the ventral edge.

Most antennal sensilla of *O. eremita* are specialized to receive chemical signals that play a fundamental role for inter- and intra-specific communication. Therefore, a detailed morpho-functional study of the antennae, preferably integrated with electrophysiological analyses, is a prerequisite to understand other behavioral and biological aspects of the species. Although some electrophysiological studies on *O. eremita* were recently carried out (Larsson et al., 2003; Svensson and Larsson, 2008), its antennal sensilla were not described in detail.

The goal of this work is to improve the knowledge of the chemical communication system in *O. eremita* through a fine morphological analysis of the antennal microstructures through standard SEM and FIB/SEM technologies. In particular, the aims of this work are the following:

1. To analyze, describe and illustrate morphology and topology of the antennal sensilla, pores and microsculpture, in particular the ones present on the club;
2. To analyze the sexual dimorphism at gross and fine scale of the antenna;
3. To compare the antennal microstructures found in *O. eremita* with homologous structures observed in other scarab beetles.

Materials and methods

Material examined
This study is based on the analysis of 4 specimens (2 males and 2 females) of *O. eremita* (Fig. 1), collected by using pitfall traps placed inside tree hollows or using interception traps suspended from tree branches baited with a neat racemic mixture of *γ*-decalactone, in Central Italy (for further details on beetle collection see Chiari et al., 2013a). We used only two specimens per sex, collected at the end of their activity (end of July/beginning of August), in order to avoid affecting the small Italian populations of this species. The material is preserved in Carpaneto G. M. and Di Giulio A. collections (Rome, Italy).

Scanning Electron (SEM) and Focused Ion Beam (FIB) Microscopy
For SEM analysis, the antennae were removed from the specimens, kept overnight in a detergent water solution, cleaned by ultrasounds three times for ten seconds, rinsed in water, dehydrated in a graded ethanol series, and critical point-dried in a CPD 030 unit (Balzers Union, Fürstentum, Liechtenstein), gold coated in a K550 unit (Emitech Technologies Ltd., Kent, England), and examined with both Philips XL 30 and Dualbeam (FIB/SEM) Helios Nanolab (FEI Company, Eindhoven, The Netherlands) at the L.I.M.E. (Interdepartmental Laboratory of Electron Microscopy, University Roma Tre, Rome, Italy).

The FIB/SEM is equipped with two columns including one electron beam (SEM column) and one ion beam (FIB column), oriented at 52°, and focused on the same point of the sample. The FIB column was used to selectively ablate (milling process) a previously marked region of the sample using a focused ion current from a gallium source. The milling process was paused every few nanometers while high-resolution images were taken of the cross sections with the SEM column. The antennae of *O. eremita* prepared for the SEM were also analyzed and partially dissected in correspondence to sensilla and pores by the ion beam (FIB operated at 30 KV and 0.92 nA) to investigate the internal cuticular structure.

The SEM micrographs obtained by using the standard XL 30 (especially for the antennal habitus) and the Dualbeam FIB/SEM (for close-up of the microstructures) were used to characterize size, number, distribution and morphological features of antennal sensilla by using the specific software Cell D̂ SIS (Soft Imaging System GmbH, Münster, Germany).

We followed the terminology of Schneider (1964) and Zacharuk (1985) to classify the sensillum types, and the terminology of Harris (1979) to classify the types of microsculptures. We also referred to classifications reported in other papers on antennal sensilla of Coleoptera Scarabaeidae (Ågren, 1985; Leal and Mochizuki, 1993; Renou et al., 1998; Kim and Leal, 2000).

Fig. 1. Male of *Osmoderma eremita*, habitus in dorso-lateral view (picture by Romiti F.).

Results

Gross morphology of the Osmoderma eremita *antenna*

The antenna of *O. eremita* shows a lamellicorn shape (Fig. 2A–B) typical of the Scarabaeiformia (Meinecke, 1975) and it is composed of ten antennomeres (Fig. 2A, B), modified and arranged in four distinctive functional parts from base to apex: scape (= scapus), pedicel (= pedicellum), funicle (= funiculum) and a lamellate club (= clava). The latter is formed by the last three antennomeres, modified into three plates (= lamellae) that form a fan-like sensorial structure.

Scape (SC) (length 1.40 ± 0.01 mm, both sexes; Fig. 2A, B): a twisted, pipe-like antennomer, expanded at base and apex, and sub-basally constricted, articulated basally with the antennal fossa by a globular condyle.

Pedicel (P) (length 400 ± 1 μm, in male; 440 ± 10 μm in female; Figs. 2A, B and 5C, D): a mushroom-shaped antennomer, characterized by a bulged, cylindrical distal part, and a thin elongate base, loosely inserted inside the scape, giving a high mobility to the articulation between scape and pedicel.

Funicle (F1–F5) (length 1.30 ± 0.03 mm in male, 1.30 ± 0.07 mm in female; Fig. 2A, B): composed of five cup-shaped antennomeres which give support and mobility to the terminal club; F1–F3 are small, cylindrical, subequal, tightly stuck to one another; F4–F5 are larger, widely transverse, asymmetrical, the anterior part being wider than posterior.

Lamellate club (L1–L3) (maximum length 1.70 ± 0.10 mm in male, 1.30 ± 0.05 mm in female; Figs. 2A, B; 4B and 5A, B): formed by three elongate and plate-like antennomeres; outer surface of L1 and L3 strongly convex; inner surface of L1 and L3, and both surfaces of L2 slightly concave or flattened; L2 very thin on ventral side, slightly but distinctly thicker on dorsal side. In resting position, the lamellae are closely pressed to one another, well protecting their inner sensory surfaces. No relevant sexual dimorphism has been recognized in gross morphology of the antennae while some differences in fine morphology regarding the distribution of sensilla and cuticular pores on the surface of P, F4 and F5, were found.

The cuticular microsculpture varies significantly between different portions of the antennae. Most of the antennal surface is covered by a more or less deeply reticulate microsculpture, composed of polygonal, partly imbricate meshes. The condyle of the scape shows a spinulate sculpture (Fig. 3A), while its sub-basal portion is glabrous and smooth. On the edges of the lamellae, the reticulate

microsculpture presents transverse and elongated wrinkles (Fig. 4A), while both inner and outer surfaces of the lamellae are glabrous among the placoid sensilla (Fig. 4A,C−F).

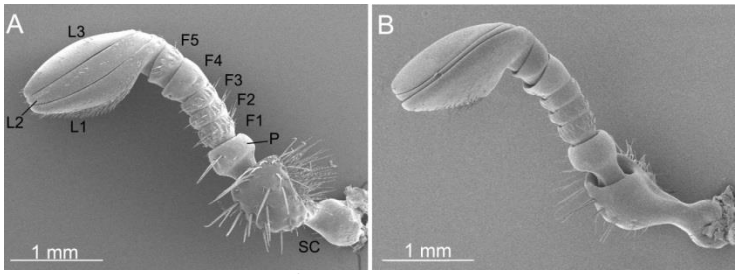

Fig. 2. SEM micrographs of male *Osmoderma eremita* general antennal shape: (A) right antenna, dorsal view; (B) left antenna, ventral view. SC: scape; P: pedicel; F1–F5: antennomeres of the funicle; L1–L3: lamellae of the antennal club.

Fine structure and distribution of sensilla

We identified twelve types of sensilla, according to their fine structure, as defined by Schneider (1964): six different types of sensilla chaetica (Ch.1−6), Böhm sensilla (Bo), one type of sensilla basiconica (Ba.1), two types of sensilla coeloconica (Co.1−2) and two types of sensilla placodea (Pl.1−2).

Sensory bristles (sensilla chaetica), and sensory pegs (sensilla basiconica) are widely distributed on the external surface of antennal segments, but almost absent on the ventral side of antennae. Böhm sensilla (Bo) are located at the base of the scape. All other sensillum types are found on the inner surfaces of the lamellae.

The morphological features and measures of antennal sensilla are summarized in Table 2. The density and percentage of area occupied on the lamellae by sensilla placodea reported in the results are the mean values calculated between the lamellae of the two individuals per sex.

Sensilla chaetica type 1 (Ch.1)

Short and stout, brush-like sensilla (Fig. 3A; Table 2), with the basal part of the stem almost straight or slightly curved, entire and laterally compressed; medial and apical part irregularly frayed by thin projections with sharp tips. The whole surface of the sensillum is smooth. These bristles appear prostrate toward the antennal surface

and are inserted in a deep wide socket, generally with one cuticular pore close to the base, and they are distributed on the condyle of the scape, dorsally and ventrally, among the Böhm sensilla (Bo) (Fig. 3A).

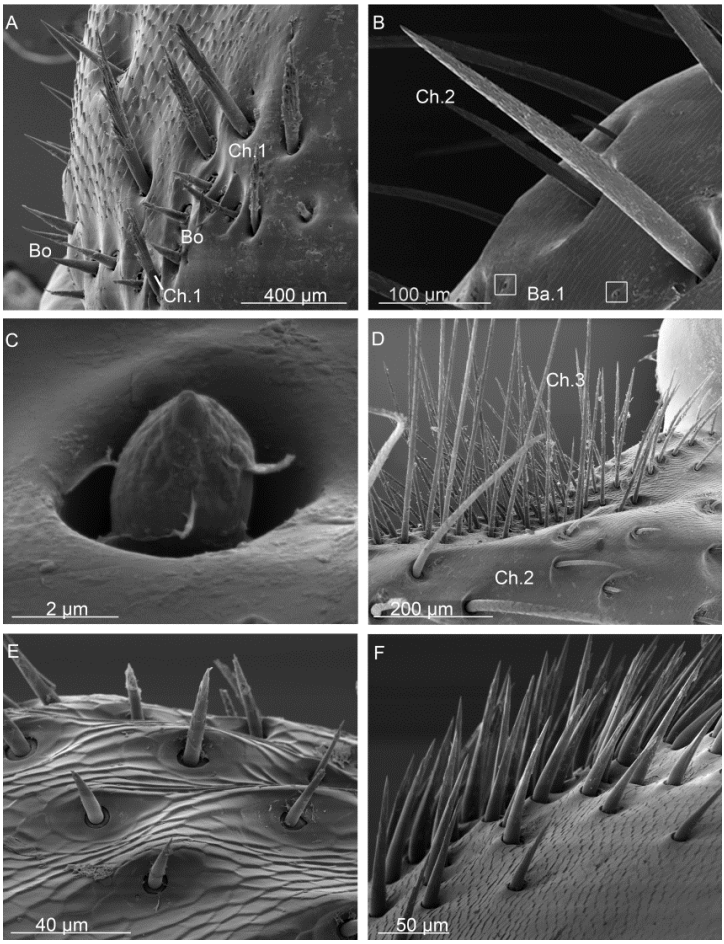

Fig. 3. SEM micrographs of *Osmoderma eremita* antennal sensilla: (A) condile of the scape showing spinulate microsculpture, sensilla chaetica Ch.1, and Böhm sensilla Bo; (B) dorsal view of the distal part of the scape showing the reticulate microsculpture, sensilla chaetica Ch.2 of different lengths and sensilla basiconica Ba.1; (C) sensillum basiconicum Ba.1 on apical part of the scape; (D) dorso-lateral view of the apical portion of the scape showing sensilla chaetica Ch.2 and Ch.3; (E) lateral margin of

antennomere F1 showing sensilla Ch.4; (F) outer surface of first lamella (L1) of the antennal club showing sensilla chaetica Ch.6 densely arranged in a brush-like structure.

Table 2. Overview of morphological characteristics of the antennal sensilla in *Osmoderma eremita*.

Type	Sex	Length (µm)†	Diameter (µm)†	N‡	Tip	Wall	Shape	So*	Fig.
Ch.1	♂	99.01 ± 27.90	5.68 ± 0.85	13	Sharp	S	Brush-like	W	3A
	♀	84.79 ± 37.42	6.91± 1.80	19	Sharp	S	Brush-like	W	
Ch.2	♂	50.74 ± 19.85	6.99 ± 1.28	8	Blunt	S/Ro	Hair-like	W	3B, D
short	♀	41.32 ± 15.02	7.08 ± 1.90	16	Blunt	S/Ro	Hair-like	W	
Ch.2	♂	160.41 ± 80.00	10.80 ± 5.78	7	Blunt	S/Ro	Hair-like	W	3B, D
med.	♀	156.36 ± 43.81	14.30 ± 3.16	7	Blunt	S/Ro	Hair-like	W	
Ch.2	♂	410.06 ± 74.19	20.44 ± 3.55	11	Blunt	S/Ro	Hair-like	W	3B, D
long	♀	349.43 ± 54.74	18.27 ± 2.40	15	Blunt	S/Ro	Hair-like	W	
Ch.3	♂	202.97 ± 32.58	7.28 ± 0.44	8	Sharp	S/Wo	Spine-like	N	3D
short	♀	235.78 ± 23.56	7.27 ± 0.75	8	Sharp	S/Wo	Spine-like	N	
Ch.3	♂	434.56 ± 41.30	10.81 ± 1.50	9	Sharp	S/Wo	Spine-like	N	3D
long	♀	361.26 ± 41.77	9.85 ± 1.73	4	Sharp	S/Wo	Spine-like	N	
Ch.4	♂	21.91 ± 5.14	4.29 ± 0.58	11	Splitted	S	Cone-like	N	3E
	♀	29.66 ± 14.19	4.48 ± 0.88	8	Splitted	S	Cone-like	N	
Ch.5	♂	69.67 ± 26.73	5.84 ± 1.19	22	Sharp	S	Pin-like	N	4A; 5A–B
	♀	41.79 ± 14.53	5.74 ± 1.26	21	Sharp	S	Pin-like	N	
Ch.6	♂	84.54 ± 12.69	10.52 ± 1.48	11	Sharp	S	Cone-like	N	3F
	♀	76.66 ± 11.88	11.17 ± 0.88	8	Sharp	S	Cone-like	N	

†Length and Diameter are expressed as mean ± standard deviation.

‡Number of sensilla measured.

* So = Socket, W = Wide, N = Narrow, Near. Circ. = Nearly circular, S = Smooth, Ro = Rough, Wo = Worn, Gr = Grooved, Por = Porous

Table 2. (continued)

Type	Sex	Length (µm)†	Diameter (µm)†	N‡	Tip	Wall	Shape	So*	Fig.
Bo	♂	23.06 ± 27.90	4.48 ± 0.855	8	Sharp	S	Thorn-like	W	3A
	♀	24.84 ± 7.11	4.18 ± 0.59	9	Sharp	S	Thorn-like	W	
Ba.1	♂	3.28 ± 0.23	4.21 ± 0.14	3	Blunt	S	Peg-like	W	3B–C, 5E
	♀	3.43 ± 0.47	3.88 ± 0.73	5	Blunt	S	Peg-like	W	
Co.1	♂	–	7.42 ± 0.83	41	–	Gr	Onion-like	–	3B, D
	♀	–	8.03 ± 1.03	38	–	Gr	Onion-like	–	
Co.2	♂	–	6.67 ± 0.83	14	–	S	Dome-like	–	4A, C, E–F
	♀	–	6.70 ± 1.22	22	–	S	Dome-like	–	
Pl.1	♂	–	12.05 ± 1.13	56	–	Por	Oval	–	4A, C–D
	♀	–	10.77 ± 1.00	65	–	Por	Oval	–	
Pl.2	♂	–	16.02 ± 1.30	60	–	Por	Near. Circ.	–	4C, E
smaller	♀	–	13.17 ± 1.03	53	–	Por	Near. Circ.	–	
Pl.2	♂	–	8.13 ± 0.57	31	–	Por	Near. Circ.	–	4C, E
larger	♀	–	10.81 ± 0.57	31	–	Por	Near. Circ.	–	

†Length and Diameter are expressed as mean ± standard deviation.

‡Number of sensilla measured.

* So = Socket, W = Wide, N = Narrow, Near. Circ. = Nearly circular, S = Smooth, Ro = Rough, Wo = Worn, Gr = Grooved, Por = Porous

Sensilla chaetica type 2 (Ch.2)

Hair-like sensilla (Figs. 2A, 3B, D and 5D; Table 2), elongated, flattened, and tapered towards the apex, which is blunt and curved, frayed at tip. The basal stem is smooth and stout, inserted in a wide socket with one cuticular pore associated, and emerging at 90° from the antennal surface. The medial and distal portion of the hair is characterized by an irregularly worn, rough microsculpture. These hairs are inserted in pits of the antennal cuticle. Though the general shape is quite constant, the length of such hairs varies significantly between different positions, enabling recognition of three size classes (short, medium-sized, long) (Table 2). There is a tendency for

increasing hair length towards the apex of the scape. These hairs are distributed on the dorsal sub-apical portion of the scape (SC), among the basiconic sensilla (Ba.1) (Fig. 3B), and on the pedicel (P). The pedicel of the females has no chaetic sensilla, while that of males shows medium-sized Ch.2, single or in couple, located in the dorso-lateral surface (Figs. 2A and 5C−D).

Sensilla chaetica type 3 (Ch.3)
Spine-like sensilla (Fig. 3D; Table 2), straightened and tapered towards the apical part, which is thin and sharp, with the base inserted in a shallow socket. The stem is smooth basally and worn-looking on the rest of the surface. These sensilla project straight from the antennal surface at 45°-90°. They are arranged in a crowded group of about 50 sensilla, and located on the lateral margin of the scape. These bristles are also spread dorsally and laterally on the funicle F1−F5 (Figs. 2A and 5C−F).

Sensilla chaetica type 4 (Ch.4)
Cone-like sensilla, set in a narrow socket and associated with one cuticular pore (Fig. 3E; Table 2). Their stem is basally smooth and apically provided with small and short pointed projections, tapered toward the apex. They are inserted in pits of the antennal cuticle, emerging at 90° from the antennal surface, distributed through the dorsal and lateral margin of the funicle (F1−F5), mostly on F1 and F2, and scattered on F3−F5.

Sensilla chaetica type 5 (Ch.5)
Pin-like sensilla, straight and tapered toward the apex, sharp-tipped, smooth-walled emerging at 90° from the antennal surface (Figs. 4A and 5A, B; Table 2). Their base is tightly set in a deep socket, sometimes with one pore associated. These sensilla often occur in pairs and are distributed dorsally on the external edges of the lamellae L1−L3.

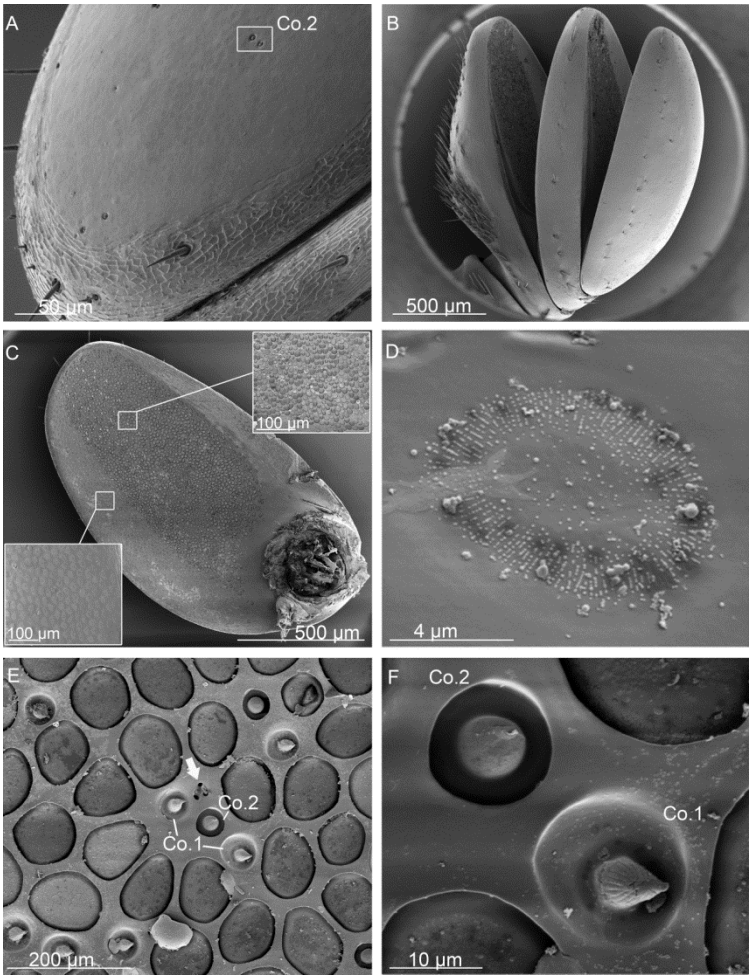

Fig. 4. SEM micrographs of *Osmoderma eremita* antennal club: (A) outer surface of the third lamella (L3) in ventral view showing on the edge the reticulate microsculpture with transverse and elongated wrinkles, sensilla chaetica Ch.5 and pores. On the central portion of the lamella are present sensilla placoidea Pl.1 and some coeloconica Co.2, in this portion the surface is smooth; (B) general view of the female right antennal club composed by three lamellae (L1–L3), on dorsal view; (C) inner surface of the lamella L1, where it is possible to identify two areas: a homogenous one, more ventral, with sensilla placodaea Pl.1 and a heterogeneous one, more dorsal, comprising mainly sensilla placodea Pl.2 and some scattered Pl.1, coeloconica Co.1, Co.2 and pores; (D) a sensillum placodeum Pl.1 from the

inner surface of lamella L1; (E) heterogeneous sensorial area on the inner surface of L2 with sensilla placodea Pl.2, coeloconica Co.1, Co.2 and pores (white arrow); (F) sensilla coeloconica Co.1 and Co.2, from the inner surface of lamella L2.

Sensilla chaetica type 6 (Ch.6)
Cone-like, relatively short, strong, smooth-walled, sharp-tipped sensilla, densely arranged in one group (about 60-70 sensilla) on the outer surface of the first lamella (L1), forming altogether a brush-like structure (Figs. 3F and 5A, B; Table 2). The shape of each sensillum is conical or spiniform, emerging 45-60° from the cuticular surface, distinctly bent distally towards the surface, and abruptly tapered at the sharp apex. The base is stout, inserted in a tight, adherent socket. One cuticular pore is associated with some of these sensilla.

Böhm sensilla (Bo)
Thorn-like bristles (Fig. 3A; Table 2), sharp-tipped, straight or slightly curved, emerging almost at 90° from the antennal surface, set in wide socket. They are present around the base of the scape.

Sensilla basiconica type 1 (Ba.1)
Thick and short conical pegs, smooth-walled, deeply inserted in a wide round socket (Figs. 3B, C; Table 2) and showing an apical pore. They are present in both sexes on the dorsal sub-apical portion of the scape (SC), scattered among the sensilla chaetica Ch.2. In females they are also present dorsally on the fourth antennomere of the funicle (F4) interspersed with pores (Fig. 6C).

Sensilla coeloconica type 1 (Co.1)
Short grooved pegs, sunken in a large circular socket (Fig. 4E, F; Table 2). The cuticular walls of the sensillum present longitudinal grooves. They occur exclusively on the inner surface of lamellae L1−L3, in both sexes and are frequently associated with Co.2, both scattered among the Pl.2 (Fig. 4E), and contribute to create a heterogeneous region in the chemo-sensorial areas on the inner surface of lamellae (Fig. 4C).

Sensilla coeloconica type 2 (Co.2)
Dome-like pegs, sunken in a circular socket (Fig. 4A, E, F; Table 2). They are distributed exclusively on the inner surface of lamellae L1−L3 where they are associated with Co.1. Both are scattered in the area of Pl.2 and contribute to create a heterogeneous region in the

chemo-sensorial areas on the inner surface of lamellae L1−L3. They are also present in pairs on the borders of the outer surface of lamella L3, scattered between Pl.1.

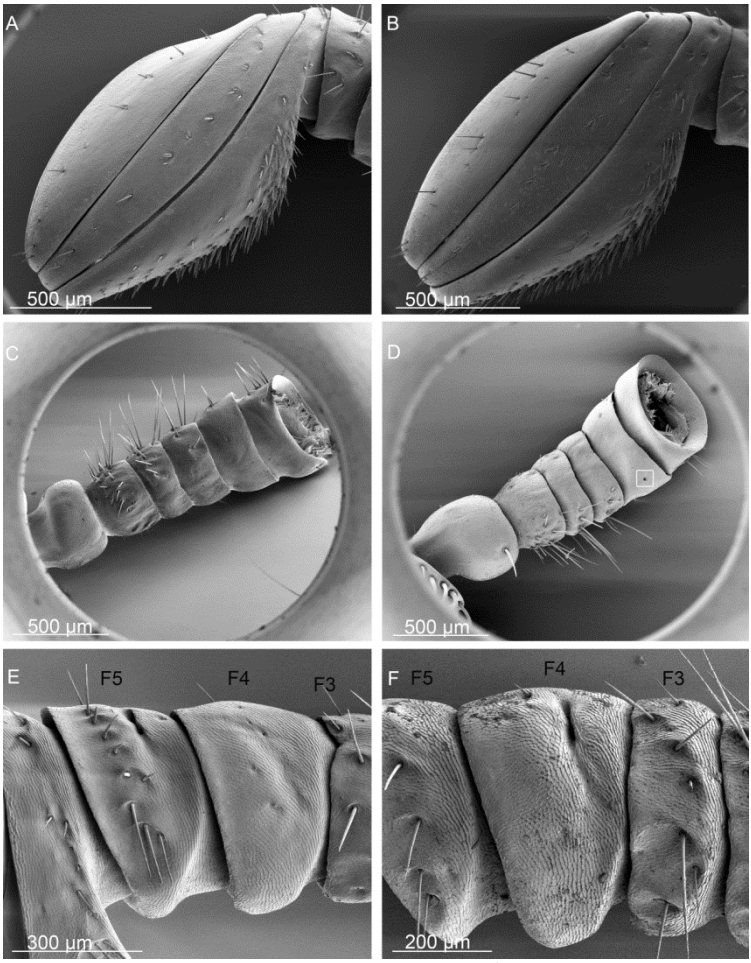

Fig. 5. SEM micrographs showing a comparison of characters between sexes of *Osmoderma eremita* (A, C, E female; B, D, F male): (A) female antennal club in dorsal view; (B) male antennal club in dorsal view; (C) female pedicel without any elongated sensilla chaetica and antennomeres of the funiculum (F1-F5) in ventro-lateral view, showing the reduced number of sensilla in ventral portion; (D) male pedicel showing a sensillum chaeticum Ch.2, and antennomeres of the funiculum (F1–F5) in ventro-lateral view,

showing the reduced number of sensilla in ventral portion, on F4 is it possible to notice a big pore; (E) detail of female antennomeres (F3–F5) of the funiculum in dorso-lateral view, F4 showing a group of four pores and three sensilla basiconica Ba.1, F5 showing a peculiar fold dorsally; (F) detail of male antennomeres (F3–F5) of the funiculum in dorso-lateral view, F4 showing a peculiar fold dorsally.

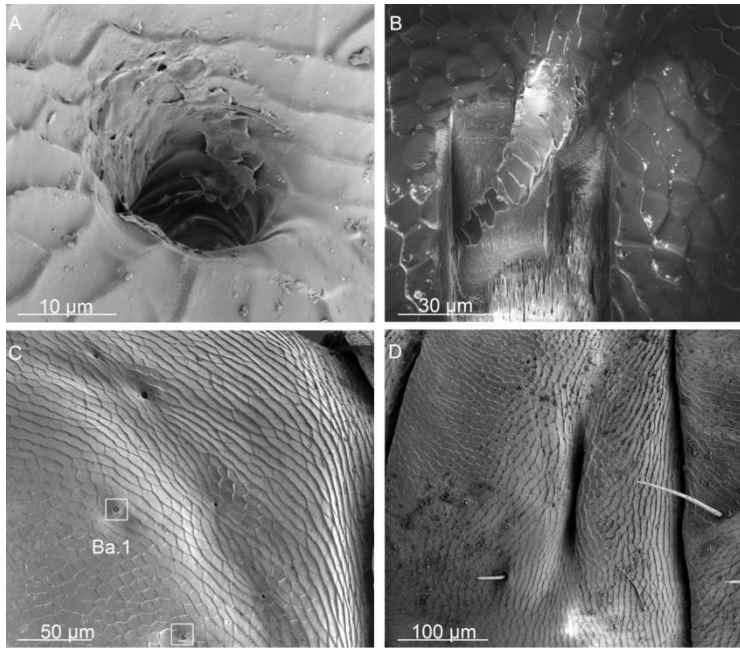

Fig. 6. SEM micrographs of cuticular pores (A,C), folds (D) and cross section of the pore (B) of *Osmoderma eremita*, obtained with Dualbeam FIB/SEM: (A) a big pore (16 µm diameter c.a.) occurring only in male on the ventral portion of F4; (B) a cross section in correspondence of the pore occurring in male on F4, showing a deep channel with inner walls consisting of rings; (C) a group of four pores occurring only in female on the dorsal surface of F4, with interspersed Ba.1; (D) a peculiar fold occurring on F4 in male.

Sensilla placodea type 1 (Pl.1)
Oval plate sensilla, with sensory cuticle nearly smooth and flat, at the same level of the antennal surface, without a deep surrounding furrow (Fig. 4A,C, D; Table 2). Their cuticular surface presents a small central pore and a series of radially aligned pores. They are densely distributed, with a mean ± S.D. of $(46 \pm 9)\cdot 10^2$ sensilla/mm^2 in male and $(42 \pm 2)\cdot 10^2$/mm^2 in female, arranged in a homogenous chemo-sensorial area on the inner surface of lamellae L1 and L3, and on both surfaces of lamellae L2. In L3 they also occur on the outer surface. They occupy a different surface (± S.D.) in the three lamellae, in male 35% (± 1) of L1, 34% (± 1) of L2, 28% (± 3) of L3, in female 36% (± 1) of L1, 40% (± 1) of L2, 30% (± 1) of L3.
In the inner surfaces of all the lamellae, from the ventral to the medial part, they are quite sharply substituted by Pl.2, and then both disappear near the dorsal margin. Some of them are scattered in the heterogeneous area among the Pl.2. In both sexes, they occur isolated or in very sparse groups of 2-3 on the outer surface of L1.

Sensilla placodea type 2 (Pl.2)
Nearly circular flat pegs, surrounded by a furrow (Fig. 4C, E; Table 2). They can be divided in two main size classes (Table 2). Usually the smaller are more rounded and show a broader furrow. The flat surface shows a series of fine pores from where a secretion comes out. They occur in both sexes and are quite densely distributed, with a mean ± S.D. of $(44 \pm 5)\cdot 10^{-4}$ sensilla/μm^2 in male and $(43 \pm 10)\cdot 10^{-4}$ sensilla/μm^2 in female, in the heterogeneous chemo-sensorial area on the inner surface of lamellae L1 and L3, and of both surfaces of L2. They occupy a different surface (± S.D.) in the three lamellae, in male 65% (± 2) of L1, 66% (± 7) of L2, 72% (± 1) of L3, in female 64% (± 1) of L1, 60% (± 1) of L2, 70% (± 1) of L3.

Cuticular pores and folds
Cuticular pores occur at the base of various types of sensilla mainly chaetica but also basiconica (Ch.1−2, Ch.4−6, Ba.1) and on the lamellae both on the inner surfaces and on their outer edge. Here their diameters vary from 0.70 μm to 2.50 μm. These structures are more abundant on the inner surfaces of the lamellae in the heterogeneous area, scattered among Co.1, Co.2 and Pl.2 (Fig. 4E). In males, a big pore (16 μm diameter c.a.) (Figs. 5D and 6A) connected to a deep channel occurs on the ventral portion of F4. The channel connected to the pore is at least 57 μm deep and has the inner

walls consisting of rings each one 4.5−6.0 μm deep (Fig. 6B). Instead in females, on F4 a group of four pores of different diameters (2.0−4.5 μm) occur on the dorsal surface (Fig. 6C). In both sexes, peculiar folds formed by invaginated cuticle are present on the lateral surface of the antennomeres, F4 in male and F5 in female (Figs. 5E, F and 6D). These folds form openings on the surface of the cuticle that are connected to an underlaying (about 60 μm deep) pocket.

Discussion

Gross morphology

The antenna of *O. eremita* is very similar in male and female. The inner surfaces of L1 and L3 are not flat but a little concave forming two narrow chambers between them and L2, as was observed also by Ågren (1958), in the sagittal section of the male antennal club of *Phyllopertha horticola*.

In both sexes, the second lamella shows a dorso-ventral asymmetry being very thin in ventral view. A similar asymmetry on the club was observed by using a stereo microscope (Olympus SZ11, maximum magnification 110x), in other European Cetoniinae of the genera *Valgus*, *Gnorimus*, *Trichius*, *Tropinota*, *Oxythyrea*, *Cetonia*, *Protaetia* and *Aethiessa* (G.M. Carpaneto, Pesonal communication). Another trait shared by *O. eremita* and the previously cited genera is the chaetotaxy strongly reduced over the ventral surface of the antenna, which is almost glabrous. Therefore, the reception of external stimuli should mainly occur in the dorsal side of the antenna. A third trait shared by these beetles is the high density of bristles forming an irregular fringe on the outer lateral surface of the scape.

Fine morphology

Sensory bristles (Ch. 1–6) are the most widespread sensilla, more or less densely occurring in all the antennomeres of *O. eremita*. They do not present deep external modification from the typical bristles described by Schneider (1964). The most modified bristles are Ch.1, present at the base of the scape, interspersed among the Böhm sensilla (Bo), with thin projections extruding from their surface. We can infer from their position that Ch.1 co-operate with the Böhm sensilla to mechanical proprioception. Up to now, these sensilla were never described in scarab beetles. Concerning the Ch.2, positioned at the apex of the scape, we found a wide variability in length (we divided them in three main different classes of size). The absence of

TEM data prevents us from making hypothesis on their function, although the involvement in the reception of mechanical stimuli is very likely. In other scarab beetles, such as *Antitrogus parvulus* and *Lepidiota negatoria* (both Melolonthini) the pedicel is glabrous (Allsopp, 1990). On the contrary, the micrographs published by some authors (Ågren, 1985; Kim and Leal, 2000; Al-Dorsay, 2009) show at least some bristles on the pedicel of *Oryctes elegans* (Dynastini), *Popilia japonica* and *Phyllopertha horticola* (both Rutelini). The third type of sensory bristle, Ch.3, was observed either on the lateral side of the scape or on the funicle of *O. eremita*. The occurrence of a dense group of straight and long bristles, as a fringe on the lateral side of the scape, was deduced by us from the micrographs of the antennae of *Oryctes rhinoceros* (Dynastini), *Antitrogus parvulus*, *Lepidiota negatoria* (both Melolonthini), *Popilia japonica* and *Phyllopertha horticola* (both Rutelini) (Ågren, 1985; Allsopp, 1990; Renou et al., 1998; Kim and Leal, 2000). These bristles are likely proprioceptors providing the beetles with information about the position of the antenna with respect to the head as when the antennae are brought toward the side these bristles can came in contact with an area near the eye. The fourth type of sensory bristle, Ch.4, the smallest type detected, is widespread on the funicle of *O. eremita* and it is different from all other types described in scarab beetles.

The sensory bristles, Ch.5, are spread on the external edges of lamellae in *O. eremita*. Observing the micrographs published in the literature, even if not explicitly reported in the texts, similar sensory bristles occur on the edges of lamellae in many species: *Pachnoda interrupta* and *P. marginata* (Bengtsson et al., 2011; Stensmyr et al., 2001;), *Adoryphorus couloni* (McQuillan and Semmens, 1990), *Oryctes elegans* (Al-Dorsay, 2009), *Oryctes rhinoceros* (Renou et al., 1998), *Amorphochelus retusus* (Romero-López et al., 2013), *Antitrogus parvulus* (Allsopp, 1990), *Dasylepida ishigakiensis* (Tanaka et al., 2006), *Lepidiota negatoria* (Allsopp, 1990), *Anomala cuprea* (Leal and Mochizuki, 1993), *Phyllopertha diversa* (Hansson et al., 1999), *Phyllopertha horticola* (Ågren, 1985), *Popilia japonica* (Kim and Leal, 2000). The occurrence of these sensory bristles in several groups of Scarabaeidae throughout the continents led us to suggest that this bristle type has a very important function. Their function may involve mechano-reception, but somehow linked to the mechanism deputed to open lamellae exposing their inner surface to olfactory cues. The bristles Ch.6 form a brush-like structure on the outer surface of L1, densely clumped as Ch.2 on the scape. Similar

bristles occur also in *Oryctes elegans* (Al-Dorsay, 2009), *Oryctes rhinoceros* (Renou et al., 1998) and *Phyllopertha horticola* (Ågren, 1985) in the same antennal area.

Böhm sensilla, described for the first time in Lepidoptera (Böhm, 1911) are typical bristles found in areas opposite the intersegmental membrane between head and scape, as well as between scape and pedicel, and are probably homologue in all insects (Schneider, 1964). In *O. eremita* we observed them only at the base of the scape and not between scape and pedicel.

Only one type of sensilla basiconica, Ba.1 occurs on the antenna of *O. eremita*, commonly in association with Ch.2 on the apex of the scape in both sexes, and also with pores arranged in a transverse row across F5 only in females. Homologue structures were found in ground beetles (Carabidae), in *Paussus favieri* (Di Giulio et al., 2012) and in *Platynus dorsalis* (Merivee et al., 2001). Unfortunately, the lack of ultrastructural data in the mentioned studies makes it difficult to infer about their function. In contrast with other studies regarding scarab beetles, in which sensilla basiconica have been found on the inner surface of the lamellae (Table 1), in *O. eremita* these sensilla are completely absent from the club.

Concerning sensilla coeloconica and placodea, they were both found only on the lamellate club of *O. eremita*. In other studies regarding Cetoniini, Hopliini, Melolonthini, Coprini and Geotrupini on the lamellae have also been found sensilla basiconica and similarly in Hopliini and Melolonthini sensilla auricillica have been described (Table 1). Overall, the lamellate club seems to be a successful structure in the evolutionary history of scarab beetles, showing a steady structure with minor changes in some apomorphic versions.

Coeloconic sensilla in *O. eremita* (Co.1 and Co.2) occur in low number on the inner surfaces of lamellae, in close association with the placoid sensilla. These peg-like sensilla are very similar to those described in other scarab as in Cetoniini, Dynastini, Melolonthini and Rutelini (Table 1). In other scarab beetles, Ochieng et al. (2002) suggested these pegs on the lamellae are likely olfactory sensilla, even if Kim and Leal (2000) excluded the possibility of being pheromone receptors. The lack of an unequivocal nomenclature for sensilla coeloconica and basiconica makes it challenging to compare our results with other studies. As pointed out by Mutis et al. (2014) sensilla coeloconica have been described as basiconica (Allsopp, 1990; Romero-López, 2004; 2010) or sometimes the authors were

uncertain if assigning a particular sensillum to basiconicum or coeloconicum type (Kim and Leal, 2000). Some have been confused with campaniform types: for example, Ågren (1985) named a coeloconicum type as "probable campaniform". In some studies these pegs are simply described as pit-organs (Ågren, 1985) or peg-like sensilla (McQuillan and Semmens, 1990; Tanaka et al., 2006), without further characterization (Table 1).

Sensilla placodea in *O. eremita* only occur on lamellae, mostly on their inner surfaces. Two different placoid types have been found: the first type (Pl.1) occurs on the inner surfaces of all the lamellae and on the outer surface of the last one; the second type (Pl.2) is only present on the inner surfaces. Pl.1 and Pl.2 are probably homologues to those described in other scarab beetles of different subfamilies and tribes. As revealed by the study of Svensson and Larsson (2008), in *O. eremita* as well as in other scarab beetles (Table 1) the pheromone receptors are the placoid sensilla of the lamellate club. On the contrary, most studies on Lepidoptera, Diptera and other Coleoptera (e.g. Elateridae and Carabidae) showed that pheromone reception is performed by the trichoid sensilla (Keil and Steinbrecht, 1984; Zacharuk, 1985; Hansson et al., 1986; Kaissling et al., 1989; Hallberg et al., 1994; Merivee, 1998; 1999; Hallem et al., 2006). An electrophysiological study on the second lamella (L2) (Svensson and Larsson, 2008) showed that γ-decalactone-sensitive ORNs occur mainly in the smooth area close to the ventral edge of the lamella, corresponding to the area where there is the maximum concentration of Pl.1. Also in other species sensilla homologues to Pl.1 are mainly concentrated in the ventral portion of each lamella, as in Cetoniini, i.e. *Pachnoda interrupta* and *P. marginata* (Stensmyr et al., 2001; Begtsson et al., 2011), and Rutelini, i.e. *Anomala cuprea, Phyllopertha diversa, Phyllopertha horticola* and *Popilia japonica* (Ågren, 1985; Leal and Mochizuki, 1993; Hansson et al., 1999; Kim and Leal, 2000; Larsson et al., 2001).

The sensorial areas located in the inner lamella surfaces are protected because these beetles are able to fold the lamellae forming a compact club when are either resting or crawling through vegetation or on the ground. However the ability to perceive olfactory stimuli is not entirely lost when the club is closed, because the outer surface of L3 with Pl.1 is still exposed (Ågren, 1985).

In most scarab beetles, as in the generality of insects, sex pheromones are released by the females to attract males, and therefore the number of pheromone-sensitive sensilla (placodea, in

case of scarab beetles) is much greater in males (Leal, 1998; Kim and Leal, 2000). A similar sexual dimorphism seems to be a rule in Rutelini (Ågren, 1985; Kim and Leal, 2000; Mutis et al., 2014) and Melolonthini (Allsopp, 1990; Romero-López et al., 2004; 2010) (Table 1), but is not observed in *O. eremita*. Different density and distribution of olfactory sensilla in various species may reflect different ways in the use of sexual pheromones for intraspecific communication. In general, as reported by some authors (Allsopp, 1990; McQuillan and Semmens, 1990), the long-range attraction of pheromones is correlated with a strong sexual dimorphism in both density and diversity of sensilla involved in pheromone reception. On the contrary, the lack of sexual dimorphism is associated with short-range attraction and to close-range sexual encounters, for example at feeding sites where individuals congregate (Allsopp, 1990; McQuillan and Semmens, 1990). Larsson et al. (2003) hypothesized that the γ-decalactone of *O. eremita* could function as an intraspecific long-distance odour signal, attracting both males and females, but the range of attraction and mating behaviour are still to be clarified.

Traits of sexual dimorphism in *O. eremita* were observed mainly in pedicel and funicle being not directly involved in the pheromone reception. In the males examined the pedicel showed one or two sensilla chaetica Ch.2 while F4 has a wide pore in ventro-lateral position. These traits are absent in females where instead we found a series of four smaller pores in dorso-lateral position of F4. A big pore (16 μm) as that found in male F4 has never been described in scarab beetle antennae. The FIB analysis showed a long duct under the pore that may be associated to a secretive gland. Even if the main function of the insect antennae is sensorial, glandular activity has been described for some species and in some cases identified as the site of the pheromone secretion (Bin et al., 1986; 1999; Bartlet et al., 1994; Skilbeck and Anderson, 1994; Isidoro et al., 1999; Romani et al., 2005; Di Giulio et al., 2009). At present, abdominal pheromone glands in scarab beetles have only been described from three taxonomic groups (Rutelinae, Melolonthinae, Dynastinae) (Hoyt et al., 1971; Tada and Leal, 1997; Leal, 1998; Kim and Leal, 2000; Rochat et al., 2000; Romero-López et al., 2009; 2011). The possible connection of these large antennal pores with antennal glands and the eventual significance of the secretions should be object of further TEM investigations.

In *O. eremita*, small glandular openings are also found between the sensilla placodea, from which a secretory substance seems to be

produced (Fig. 4E). These structures are particularly abundant on the inner surfaces of the lamellae where the secreted substances could be possibly used to facilitate the folding and the adhesion of the lamellae one another. Similar glandular openings occur also in *Phyllopertha horticola*, and TEM analyses revealed that pore canals are linked to these openings and run through the cuticle, often branching downward (Ågren, 1985).

Another type of pores was observed in *O. eremita* at the base of sensilla chaetica Ch.1, near the proximal end of the scape; some authors inferred that their secretion may serve to reduce friction between sclerotized parts (Martin, 1977; Skilbeck and Anderson, 1994).

To conclude, *O. eremita* did not show any sexual dimorphism for the placoid sensilla, that previous studies demonstrated to be responsible for the pheromone reception, and this match the fact that both sexes are attracted by the pheromonal compound. Even if the low number of specimens examined (two for each sex) cannot give a reliable description of individual variation and sexual dimorphism, it was however possible to describe new structures for scarab beetles i.e. two types of sensilla chaetica (Ch.1 and Ch.4), a big pore on the antenna of males, and peculiar folds on the funicle.

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PAPER II

Scanning electron microscopy analysis of the antennal sensilla in the rare saproxylic beetle *Elater ferrugineus* (Coleoptera, Elateridae)

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Abstract

In this work we supplied the first morphological analysis (both at gross and fine level) of the antennal structures in the genus *Elater* (Coleoptera, Elateridae). In particular, the typology, number and distribution patterns of the antennal sensilla in the rare saproxylic *Elater ferrugineus* (both male and female) were studied by using scanning electron microscopy (SEM). The serrate antennae of *E. ferrugineus* consisted of a scape, a pedicel, and nine flattened flagellomeres. Overall, ten types of sensilla were identified according to their morphological features: one type of sensilla chaetica (Ch), one type of Böhm sensilla (Bo), three types of sensilla trichodea (Tr.1-3), two types of sensilla basiconica (Ba.1-2), one type of sensilla styloconica (St), one type of grooved peg sensilla (Gp) and one type of sensilla campaniformia (Ca). A marked sexual dimorphism was found both at gross and fine scale. The male antenna was bigger (8.6 mm) than the female antenna (7.0 mm) and showed one type of sensilla trichodea (Tr.2) absent in female antennae, possibly responsible for reception of the female-emitted sex pheromone. The female antenna showed a higher number of sensilla (~ 9,800) than the male antenna (~7,000), with more abundant sensilla chaetica (Ch) and basiconica (Ba.1).

Keywords Antennae, sexual dimorphism, fine morphology, cuticular microstructures, SEM

Introduction

The rusty red click beetle *Elater ferrugineus* Linnaeus, 1758 (Coleoptera, Elateridae) inhabits hollow trees of Europe. It is a saproxylic organism i.e. depending, during part of its life cycle, upon decaying woody material from living, weakened or dead trees (Speight 1989; Alexander 2008; Stokland et al. 2012). Forest practices oriented to the removal of dead or old trees cause a serious threat to *E. ferrugineus* as well as other saproxylic organisms (Grove 2002; Davies et al. 2008; Thomas et al. 2009). Consequently, *E. ferrugineus* is listed as Near Threatened (NT) in the IUCN European Red List of Saproxylic Beetles (Nieto and Alexander 2010) and as Vulnerable (VU) in the Italian Red List of Saproxylic Beetles (Audisio et al. 2014).

The larvae of *E. ferrugineus* are predaceous, feeding on immature stages of large beetles, mostly those of Scarabaeidae Cetoniinae (Iablokoff 1943; Platia 1994; Schaffrath 2003b; Tolasch et al. 2007). Males of *E. ferrugineus* are strongly attracted to the sex pheromone (7-methyloctyl (*Z*)-4-decenoate) emitted by conspecific females (Tolasch et al. 2007; Svensson et al. 2012). Females are attracted to the sex pheromone (*R*)-(+)-γ-decalactone emitted by males of a major prey species, the scarab beetle *Osmoderma eremita*. This compound thus functions as a kairomone (prey or host signal) allowing *E. ferrugineus* to locate suitable hollow trees in which to lay eggs so as to ensure their larvae the presence of prey (Svensson et al. 2004). The recent identification of the *E. ferrugineus* sex pheromone (Tolasch et al. 2007) has greatly facilitated the collection of information on its population ecology and distribution in Europe (Svensson et al. 2012; Larsson and Svensson 2011; Musa et al. 2013; Andersson et al. 2014; Zauli et al. 2014). New populations have recently been recorded in Latvia (Barševskis and Nitcis 2011), Lithuania (Meržijevskis and Tamutis 2011), Spain (Fernández de Gamboa 2010), Poland (Kadej et al. 2014) and Italy (Zauli et al. 2014).

Although some papers have shed light on antennal structures and reception of environmental stimuli in Elateridae, the understanding of the olfactory communication system is still poor in this family. Limited information on morphology and physiology are available for a restricted number of species. The identification and description of antennal sensilla through scanning electron microscopy (SEM) has been performed only for a few species, e.g. *Agriotes obscurus* (Merivee et al. 1997), *Limonius aeruginosus* (Merivee et al. 1998),

Melanotus villosus (Merivee et al. 1999) and *M. cribricollis* (Guandi et al. 2012). These studies are the prerequisite for any electrophysiological research and can suggest possible functions of sensilla by considering their morphology, number, distribution and presence or absence on the antennae of the two sexes. For *Tetrigus lewisi* both antennal fine morphology (by SEM) and sensilla ultrastructure (by TEM) have been described (Ren et al. 2014). In this case, the fine structure of sensilla, and the neuron dendrites associated made it possible to deduce their different functions as chemoreceptors, mechanoreceptors and thermo and hygroreceptors. For only one species, *A. obscurus* (Tooming et al. 2012), an electrophysiological study was performed on sense of taste, showing the occurrence of salt- and sugar-sensitive neurons, and of a mechanoreceptor neuron in its hair-like gustatory sensilla. No studies of fine morphology, ultrastructure and physiology have so far been performed on the genus *Elater*.

In this work we carried out a fine morphological analysis of the antennal microstructures of *E. ferrugineus* by scanning electron microscopy, which may serve as a base for future ultrastructural and electrophysiological studies. The main aims of this work are:

1. To outline the antennal gross morphology of *E. ferrugineus*;
2. To describe the fine morphology of the antennal sensilla (shape, size, number, distribution) and other microstructures (such as pores and microsculpture);
3. To analyze the sexual dimorphism at gross and fine scale of the antenna;
4. To compare the antennal microstructures of *E. ferrugineus* with homologous structures observed in other genera of click beetles (*Agriotes*, *Melanotus*, *Limonius*, *Tetrigus*) already studied by other authors.

Materials and methods

Material examined

The antennae of four specimens of *E. ferrugineus*, two males and two females, were analysed. Each couple of click beetles was collected respectively in two beech forests of Central Italy, in Latium region: Allumiere (42°09’N 11°54’E, 520–630 m a.s.l.) and Monte Venere (42°21’N12°11’E, 580–840 m a.s.l.) during the summer 2012. The sampling was performed by using odour-baited interception traps

suspended from tree branches (for further details on beetles collection see Zauli et al. 2014).

Scanning Electron Microscopy (SEM)
For SEM analysis, the antennae were removed from the specimens, kept overnight in a detergent water solution, cleaned by ultrasounds three times for ten seconds, rinsed in water, dehydrated in a graded ethanol series, and critical point-dried in a CPD 030 unit (Balzers Union, Fürstentum, Liechtenstein), gold coated in a K550 unit (Emitech Technologies Ltd., Kent, England), and examined with both Philips XL 30 and Dualbeam (FIB/SEM) Helios Nanolab (FEI Company, Eindhoven, The Netherlands) at the L.I.M.E. (Interdepartmental Laboratory of Electron Microscopy, Roma Tre University, Rome, Italy).

The SEM micrographs were analysed by using the specific software Cell D̂ SIS (Soft Imaging System GmbH, Münster, Germany) in order to characterize the size, number, distribution and morphological features of antennal sensilla. All values in the text are reported as mean ± standard deviation (S.D.).

We followed the terminology and classification reported in other papers on antennal sensilla of Coleoptera Elateridae (Merivee et al. 1997; 1998;1999; Ren et al. 2014). For the orientation of the antenna we referred to the work of Merivee (Merivee et al. 1997).

Results

Gross morphology of the Elater ferrugineus *antenna*
E. ferrugineus (male and female) shows serrate antennae composed of eleven segments (Fig. 1-2) arranged in three different functional parts: (1) an elongated scape (SC), articulated basally with the antennal fossa by a globular condyle, sub-basally constricted, and apically elongated (length 0.94 ± 0.10 mm in males and 1.09 ± 0.01 mm in females, Fig. 2); (2) a globular cup-shaped pedicel (P) (length 0.29 ± 0.06 mm in males and 0.30 ± 0.01 mm in females, Fig. 2); (3) the flagellum (F) made up of nine flattened flagellomeres (F1-F9, length 7.40 ± 0.21 mm in males and 5.63 ± 0.13 mm in females, Figs. 2-3).

In both sexes, flagellomeres F2-F8 show a triangular shape with a ventral expansion forming an acute angle on the margin (40-45° in males and 55-60° in females). In both sexes, F1 is more similar to the pedicel than to other flagellomeres regarding its shape and sensillar types. The last flagellomere (F9) in both sexes shows an apically

tapered profile, narrower in males, and a broadly rounded expansion directed downwards (Figs. 2-3).

The females are overall bigger than males (mean pronotum width: males = 5.80 mm, females = 7.00 mm; mean elytra length: males = 13.60 mm, females = 14.20 mm).

Fig. 1 Male of *Elater ferrugineus*, habitus in dorsal view (picture by AZ).

Fine structure and distribution of sensilla

In both sexes, the microsculpture of the scape is spinulate on the condyle and reticulate on the remaining part. The same reticulated pattern is also observed on the pedicel, on the F1 and on the flat discal surfaces of F2-F9, both in anterior and posterior views.

In females, the ventral angled expansions, as well as the latero-dorsal edges of F2-F8, present a microsculpture formed by bulged cuticular areoles; microsculpture of F5-F8 is almost flat on the latero-dorsal edge and completely flat on the discal surface. F9 shows a bulged areolate microsculpture all around the margins. In males, the distribution of the microsculpture is the same as in females, but less rough.

Each antennal segment (excluding P) shows at least a hundred sensilla, with an increase in number and types of sensilla from the scape to F9. We identified ten types of sensilla according to their fine structural features, homologous to those already recognized and

described in other click beetle genera (Merivee et al. 1997; 1998; 1999): one type of sensilla chaetica (Ch), one type of Böhm sensilla (Bo), three types of sensilla trichodea (Tr.1-3), two types of sensilla basiconica (Ba.1-2), one type of sensilla styloconica (St), one type of grooved peg sensilla (Gp) and one type of sensilla campaniformia (Ca).
Morphological features and measures of antennal sensilla are summarized in Table 1, and the number of sensilla for each antennomere are reported in Fig. 6A-H.

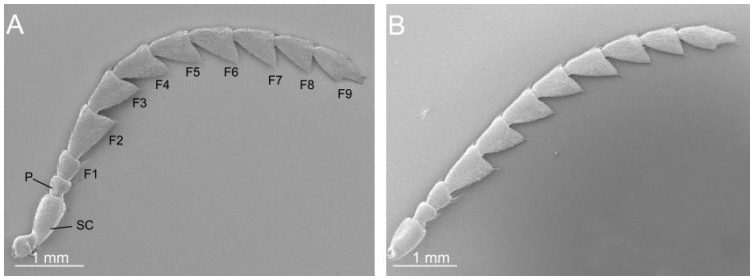

Fig. 2 *Elater ferrugineus* female antenna general shape. (A) anterior view, left antenna; (B) posterior view, right antenna. SC: scape; P: pedicel; F1-F9: first to ninth flagellomeres.

Sensilla chaetica (Ch)
These are long sickle-shaped bristles, sharp-tipped, with longitudinal grooves from the base to the apex, inserted in a wide socket and movable at their base (Figs. 4A-D; 5E; 6A; Table 1). Above the insertion, the sensilla bend toward the antennal apex so that they are directed almost parallel to the antennal longitudinal axis. Each Ch is associated with a small pore (diameter about 1 µm) close to the articular membrane and to a large pore (diameter about 6 µm), about 8 µm from the base of the sensillum (Fig. 4A-D; 5E). These sensilla are more abundant in females than in males but in each sex they are evenly distributed on all the surface of the antennomeres (Fig. 6A). These sensilla are quite variable in length, but two different sizes can be recognized: a longer type (length about 130 µm) on SC, P and F1, and a shorter type (length about 85 µm) on F2-F9 (Table 1).

Böhm sensilla (Bo)
These are small thorn-like bristles (Fig. 5A), sharp tipped, straight or slightly curved, emerging almost at 90° from the antennal surface, set

in a wide socket, occurring on the condyle of the scape and in the proximal area of the pedicel.

Sensilla trichodea type 1 (Tr.1)
These are blunt-tipped hairs with strong longitudinal grooves and are curved toward the antennal shaft. They protrude between 50-80° from the antennal surface, and their base is tightly inserted into a small cuticular socket (Figs. 4B, E, 6B; Table 1). Tr.1 sensilla occur singularly, or in small flocks, formed by up to six hairs, in both sexes, on the distal portion of each antennomere.

Sensilla trichodea type 2 (Tr.2)
These are blunt-tipped, nearly straight hairs, with longitudinal grooves in their basal part, tightly located in a small socket (Fig. 4F, 6C; Table 1). They taper toward the apex and project outwards at 80-90°. These sensilla (about two thousands on each antenna) occur only in males, evenly distributed on the anterior and posterior side of F2-F9, grouped on the lateral expansion of the ventral margin, and on the distal margin of each flagellomere.

Sensilla trichodea type 3 (Tr.3)
These are blunt-tipped straight hairs with smooth surface (Figs. 4A, D, F, 6D; Table 1), inserted in a very tight socket. They taper toward the apex and project outwards at 70-90°. They are shorter than Tr.2 (see Table 1), and occur in both sexes on F2-F9, almost in the same number on the anterior and the posterior side. In females, they are grouped along the margin of the ventral expansion of the flagellomeres and on their distal margin. In males, they are mostly grouped on at the distal edge of the ventral expansion.

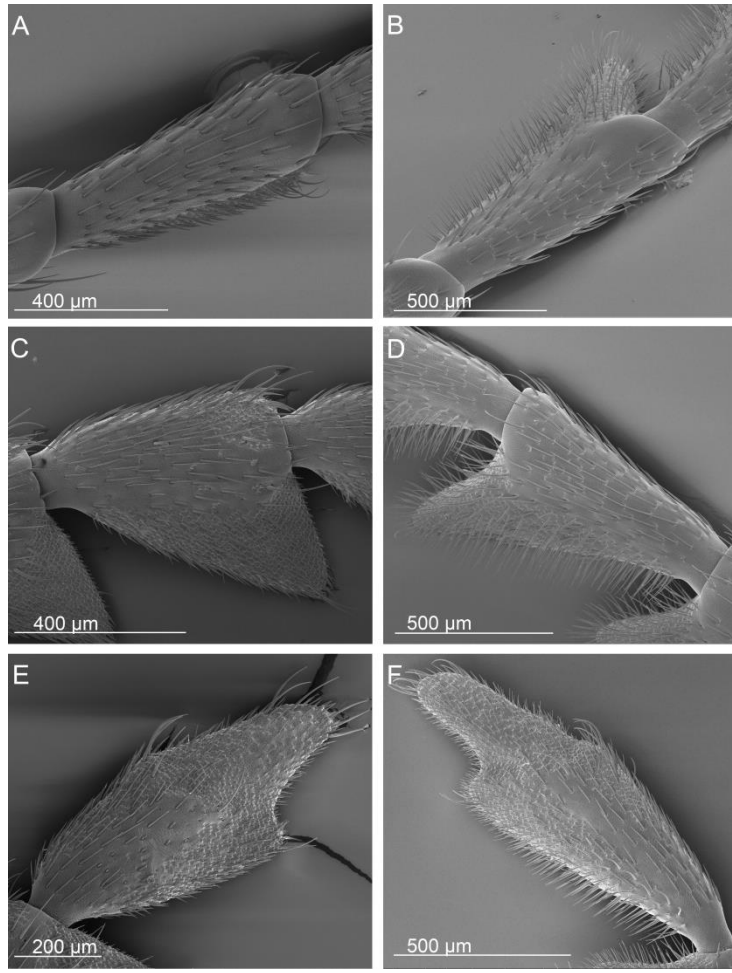

Fig. 3 *Elater ferrugineus* antennal flagellomeres in female (A, C, E) and male (B, D, F). (A-B) second flagellomere (F2) in dorsal view; (C) sixth flagellomere (F6) in posterior view; (D) third flagellomere (F3) in posterior view; (E-F) last flagellomere (F9) in anterior view.

Table 1 Overview of morphological characteristics of the antennal sensilla in *Elater ferrugineus*. Measures reported as mean ± S.D.; N = number of sensilla measured.

Sensillum type			Morphological characteristics of sensilla					
			Length (µm)	Diameter (µm)	N	Socket	Wall	Tip
Sensilla chaetica	*Ch short*	♂	85.55±13.50	6.42±0.77	90	Wide	Deep grooved	Sharp
		♀	86.10±17.82	6.64±1.07	90	Wide	Deep grooved	Sharp
	Ch long	♂	115.275±32.81	5.67±0.98	35	Wide	Deep grooved	Sharp
		♀	148.78±32.29	7.19±1.08	35	Wide	Deep grooved	Sharp
Böhm's bristles	*Bo*	♂	9.96±1.55	1.85±0.33	6	Wide	Smooth	Sharp
		♀	14.03±2.07	2.40±0.35	6	Wide	Smooth	Sharp
Sensilla trichodea	*Tr.1*	♂	112.87±18.81	6.75±7.04	17	Tight	Deep grooved	Blunt
		♀	136.09±16.01	8.11±0.53	17	Tight	Deep grooved	Blunt
	Tr.2	♂	114.29±15.88	6.56±0.62	52	Tight	Basally grooved	Blunt
		♀	-	-	-	-	-	-
	Tr.3	♂	35.43±4.68	3.91±0.39	21	Tight	Smooth	Blunt
		♀	32.73±5.40	3.56±0.23	21	Tight	Smooth	Blunt
Sensilla basi-conica	*Ba.1*	♂	7.77±1.38	2.41±2.72	90	Tight	Smooth	Blunt
		♀	9.93±1.42	2.44±0.33	90	Tight	Smooth	Blunt
	Ba.2	♂	9.30±0.97	1.96±0.13	15	Tight, Raised	Smooth	Blunt
		♀	10.61±1.05	1.98±0.14	15	Tight, Raised	Smooth	Blunt
Sensilla stylo-conica	*St*	♂	8.15±1.30	3.35±0.36	10	Wide, Raised	Smooth, porous	Tip nipple
		♀	7.37±0.50	3.41±0.32	10	Wide, Raised	Smooth, porous	Tip nipple
Grooved pegs	*Gp*	♂	5.21±1.01	2.71±0.43	7	Open	Apically grooved	Sharp
		♀	6.55±0.68	2.23±0.09	7	Open	Apically grooved	Sharp
Sensilla campani-formia	*Ca*	♂	†1.58±0.18	‡8.57±1.44	7	Wide, Raised	Smooth	Cap-shaped
		♀	†2.13±0.31	‡9.25±1.17	7	Wide, Raised	Smooth	Cap-shaped

† internal cap diameter
‡ collar diameter

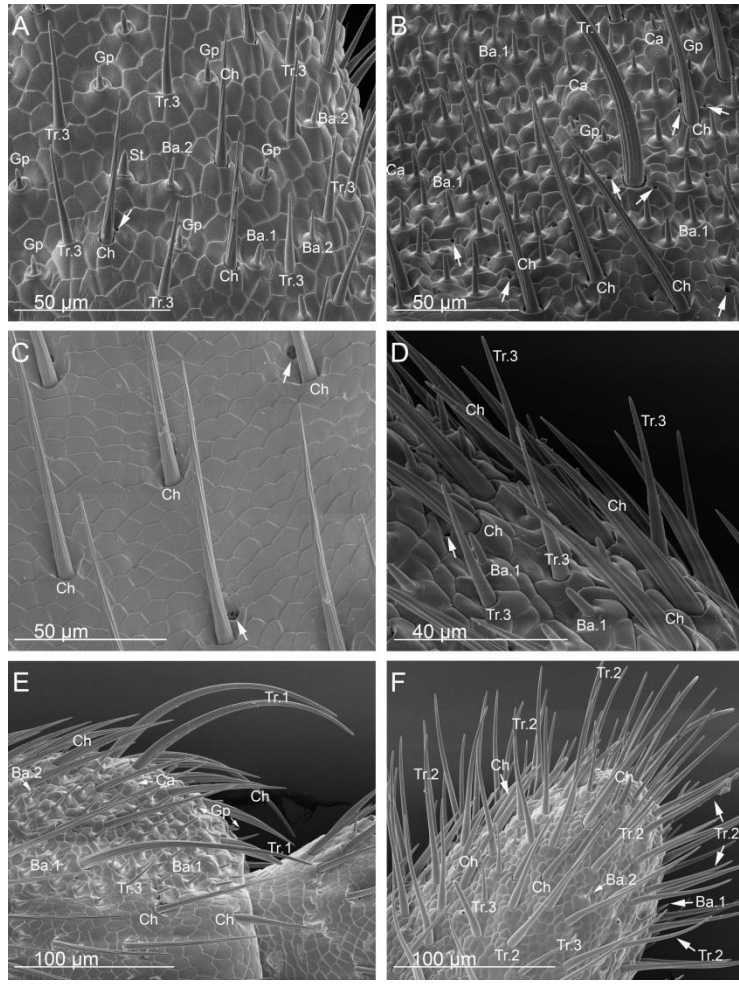

Fig. 4 *Elater ferrugineus* antennal sensillum types: (A) female last flagellomere (F9), it is possible to notice grooved sensilla chaetica Ch, smooth sensilla trichodea Tr.3, sensilla styloconica with an apical nipple St, sensilla basiconica Ba.1 and Ba.2 the latter overall slender, grooved peg sensilla Gp, grooved at the apex and inserted in a wide socket; (B) female last flagellomere (F9) it is possible to notice grooved sensilla chaetica Ch, with multiparous structures at their base (white arrows),the curved base of the long sensilla trichodea Tr.1, the stout sensilla basiconica Ba.1, grooved peg sensilla Gp and the dome-shaped sensilla campaniform, Ca; (C) discal surface of the female seventh flagellomere (F7) in posterior view, it is possible to notice grooved sensilla chaetica Ch, with multiporous structures

at their base (white arrows) and the reticulated microsculpture; (D) antero-dorsal margin of the female ninth flagellomere (F9) it is possible to notice grooved sensilla chaetica Ch with multiparous structures at their base (white arrows), sensilla basiconica Ba.1, smooth sensilla trichodea Tr.3. (E) antero-dorsal view of the sixth flagellomere (F6) it is possible to notice grooved sensilla chaetica Ch, the blunt-tipped sensilla trichoid Tr.1, with strong longitudinal grooves occurring near the distal margin of the flagellomere, both types of sensilla basiconica Ba.1 and Ba.2, groved peg sensilla Gp and campaniform sensilla Ca; (F) angle of the ventral expansion on the ninth flagellomere (F9) in male, it is possible to notice grooved sensilla chaetica Ch, sensilla tichodea Tr.2 grooved at their base and occurring only on the male antenna, smooth sensilla tichodea Tr.3 and both types of sensilla basiconica Ba.1 and Ba.2.

Sensilla basiconica type 1 (Ba.1)
These are blunt-tipped smooth-walled and relatively stout pegs, inserted in a tight socket. Straight or slightly curved towards the antennal shaft (Figs. 4A-B, D-F, 5D, 6E; Table 1). They occur in dense groups on the ventral expansion of F2-F9 in both sexes, but also on the dorsal portion of F4-F9. In females their number is higher than in males.

Sensilla basiconica type 2 (Ba.2)
These are blunt-tipped, smooth-walled slender pegs, almost straight or slightly curved. They have a relatively wide base but get thinner at the apex. They are slightly thinner and in most cases longer than Ba.2 (Fig. 4A, E, F, 6F; Table 1), and occur in both anterior and posterior sides on the ventral expansion of F2-F9, interspersed among Ba.1. In females, they are almost twice as abundant as in males.

Sensilla styloconica (St)
These are short, smooth and porous walled conical pegs, having an apical nipple, each inserted in a wide socket, and emerge 90° from the antennal surface (Figs. 4A, 5C; Table 1). They only occur on the anterior and posterior side of F9, in similar number in both sexes (12 ±1 in males and 10 ±1 in females). They are mostly crowded in the apical portion of the last antennomere.

Grooved pegs (Gp)
These sharp-tipped pegs are thinner at their apices, and differ from classical basiconic sensilla because deep longitudinal grooves are present only on their apical part. They are typically inserted in a wide cuticular socket (Figs. 4A, B, E, 5B, 6G; Table 1) and occur in both

sexes; on the posterior side of F2-F9, interspersed among St and Ba.2, while on the anterior side they occur only on F9.

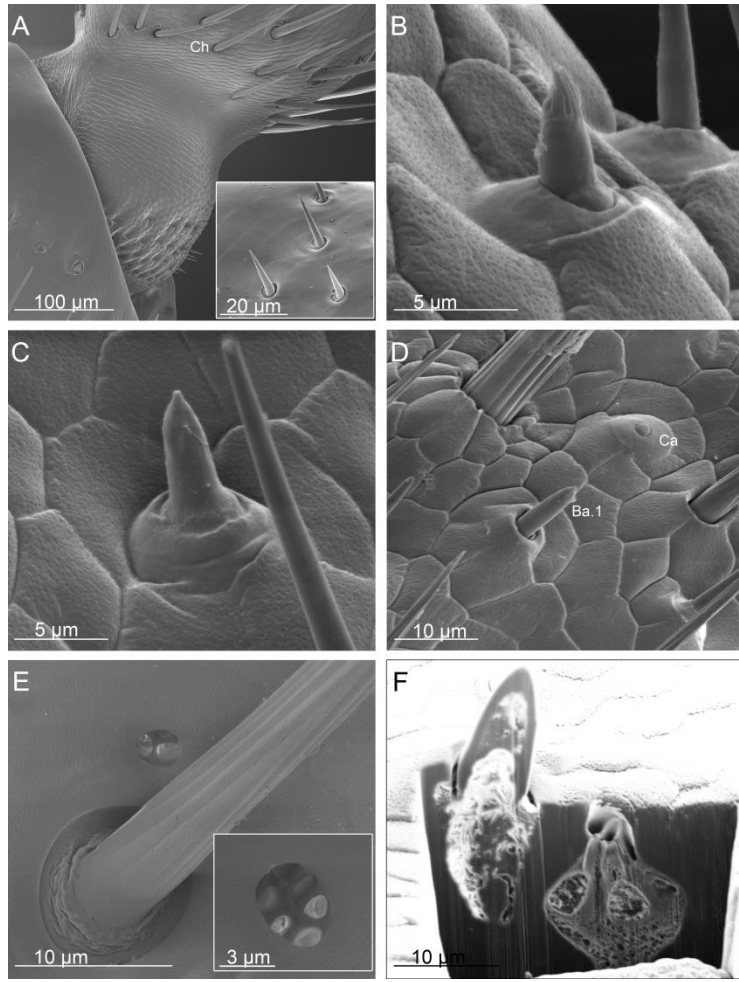

Fig. 5 *Elater ferrugineus* antennal sensilla: (A) Böhm sensilla at the base of the pedicel; (B) grooved peg sensillum; (C) sensillum styloconicum with an apical nipple; (D) sensillum basiconicum (Ba.1) and campaniform (Ca); (E) base of the sensillum chaeticum Ch, with its insertion in the socket and a multiporous structure at its base; (F) cross section, obtained with the

Dualbeam FIB/SEM, of the sensillum chaeticum and the multiporous structure at its base.

Sensilla campaniformia (Ca)
These are dome-shaped sensilla. The cuticular collar surrounding the central small cap forms a slightly raised dome (Figs. 4B, E, 5D, 6H; Table 1). They show the same distribution as the grooved pegs, occurring in both sexes on the posterior side of F2-F9; and on the anterior side only on F9.

Cuticular pores
These cuticular pores occur at the base of various types of sensilla, mainly chaetica (Ch) and trichodea (Tr.1). Two types of pores were observed; small pores (diameter about 1 µm) associated to Ch and Tr.1, and large pores (diameter about 6 µm) with multiple openings usually underlying Ch and located about 8 µm from their base (Figs. 4A-C, 5E-F). These large pores are also interspersed among Ba.1.

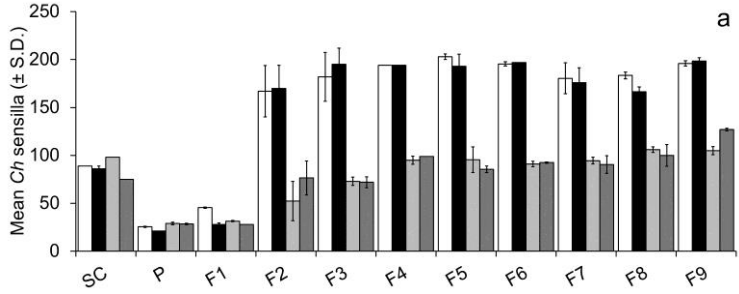

Fig. 6A Number of sensilla chaetica (Ch) for each antennomere of *Elater ferrugineus* (Elateridae). White and black bars correspond to the anterior and posterior sides of females, instead light and dark grey correspond to the anterior and posterior sides of males.

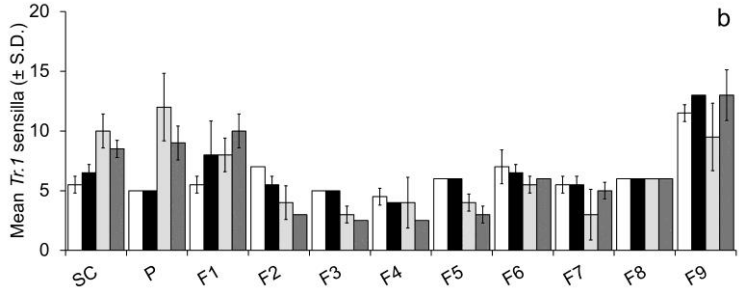

Fig. 6B Number of sensilla trichodea (Tr.1) for each antennomere of *Elater ferrugineus* (Elateridae). White and black bars correspond to the anterior and posterior sides of females, instead light and dark grey correspond to the anterior and posterior sides of males.

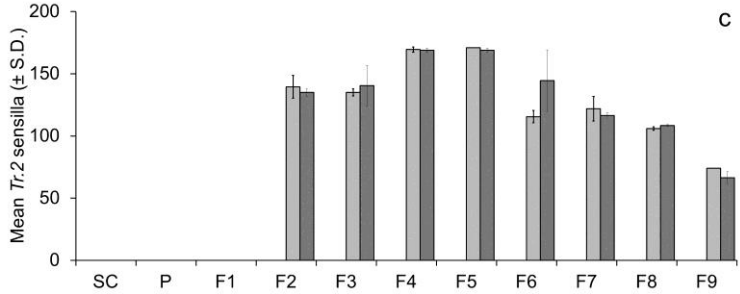

Fig. 6C Number of sensilla trichodea (Tr.2) for each antennomere of *Elater ferrugineus* (Elateridae). Light and dark grey correspond to the anterior and posterior sides of males.

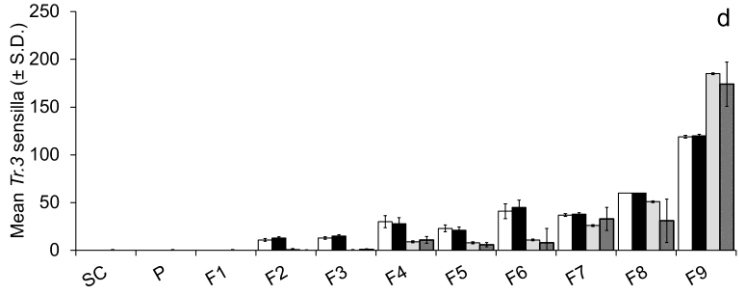

Fig. 6D Number of sensilla trichodea (Tr.3) for each antennomere of *Elater ferrugineus* (Elateridae). White and black bars correspond to the anterior and posterior sides of females, instead light and dark grey correspond to the anterior and posterior sides of males.

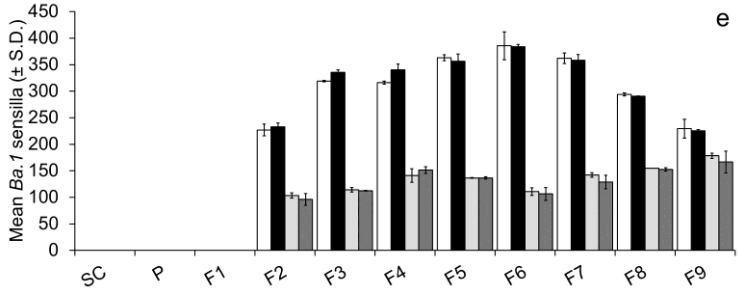

Fig. 6E Number of sensilla basiconica (Ba.1) for each antennomere of *Elater ferrugineus* (Elateridae). White and black bars correspond to the anterior and posterior sides of females, instead light and dark grey correspond to the anterior and posterior sides of males.

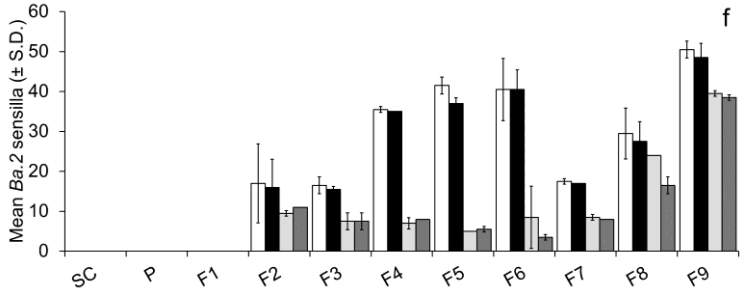

Fig. 6F Number of sensilla basiconica (Ba.2) for each antennomere of *Elater ferrugineus* (Elateridae). White and black bars correspond to the anterior and posterior sides of females, instead light and dark grey correspond to the anterior and posterior sides of males.

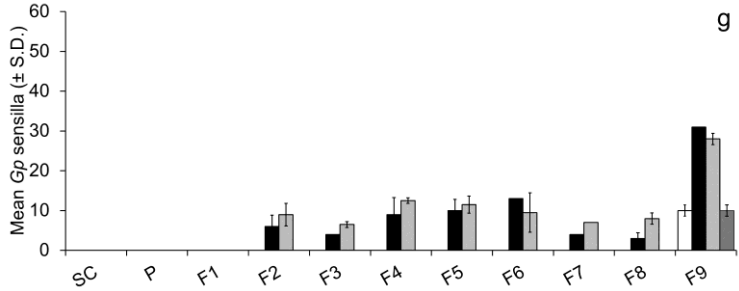

Fig. 6G Number of grooved peg sensilla (Gp) for each antennomere of *Elater ferrugineus* (Elateridae). White and black bars correspond to the anterior and posterior sides of females, instead light and dark grey correspond to the anterior and posterior sides of males

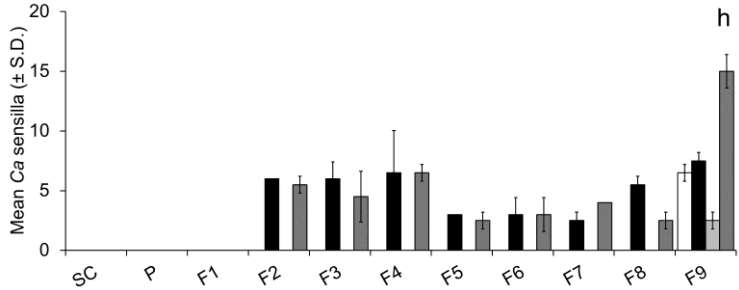

Fig. 6H Number of sensilla campaniformia (Ca) for each antennomere of *Elater ferrugineus* (Elateridae). White and black bars correspond to the anterior and posterior sides of females, instead light and dark grey correspond to the anterior and posterior sides of males.

Discussion

The distribution and types of antennal sensilla of *E. ferrugineus* are similar to what has been described in other genera and species of Elateridae: *A. obscurus* (Merivee et al. 1997), *L. aeruginosus* (Merivee et al. 1998), *M. villosus* (Merivee et al. 1999), *M. cribricollis* (Guandi et al. 2012) and *T. lewisi* (Ren et al. 2014). The different types of sensilla hereby described show two major patterns of distribution: 1) different number on the anterior and posterior surfaces of the antenna, and this difference is common to both sexes (mainly for Ca and Gp); 2) similar number on the anterior and posterior surfaces, but the number differs between sexes (Ch, Tr.2, Ba.1 and Ba.2). Most probably all the types of sensilla that differ between sexes are connected to the need to perform different sensory-guided behaviors in males and females.

The sensilla increase in type, complexity and heterogeneity from the first to the last antennomere. In fact, some types of sensilla occur on all antennomeres (i.e. Ch and Tr.1), others occur from F2 to F9 (i.e. Tr.2, Tr.3, Ba.1, Ba.2, Ca, Gp), while one type (St) occurs only on F9. Except for Ch, the sensilla are mostly grouped on the lateral expansion of the antennomeres.

Type Ch, here subdivided in two different size classes, has been described also for *A. obscurus*, *L. aeruginosus* and *M. villosus* (Merivee et al. 1997; 1998; 1999). In electrophysiological experiments Ch responded to mechanical stimuli in *A. obscurus* and *L. aeruginosus* (Merivee unpublished in Merivee et al. 1998). Similar sensilla chaetica occur also in other beetle families: in Buprestidae, for example, they may regulate movement or rotation of the entire antenna (Crook et al. 2008); in Cerambycidae, they were described as having a double function being both mechanoreceptors and protecting the olfactory sensilla under their shaft (Chen et al. 2014; Zhang et al. 2011; Liu et al. 2012; Crook et al. 2003); in Chrysomelidae, sensilla with a similar shape were found to have a combined gustatory and mechanosensory function that is responsive to hostplant chemicals (Isidoro et al. 1998). Usually gustatory sensilla have a blunt tip with a large terminal pore, and the apical part of the gustatory hair is thick enough to house several unbranched dendritic endings of sensory neurons which reach up to the terminal pore. By contrast, for Ch in *E. ferrugineus* it was not possible to detect the apical pore and Ch seem to taper apically to a point. Therefore, these sensilla in *E. ferrugineus*, occurring in large number, evenly distributed on the antennal surface and having a wide

articulary socket, most probably function as touch receptors. In *E. ferrugineus* these sensilla are twice as abundant in females than in males, which may reflect some kind of different sensory-driven behaviors in the two sexes.

Sensilla similar to trichodea Tr.1 of *E. ferrugineus* were most intensively studied in Carabidae and were demonstrated to have a mechanosensory and gustatory function. These sensilla are usually innervated by five neurons: four chemoreceptor neurons and one mechanoreceptor neuron. In previous studies it has been demonstrated that the first chemoreceptor neurons responds to various salts (Merivee et al. 2004); that the second neuron is sensitive to pH (Merivee et al. 2005; Milius et al. 2006); that the third neuron responds to various plant and aphid honeydew sugars and amino acids (Merivee et al. 2007; Merivee et al. 2008; Merivee et al. 2012); that the fourth neuron responds to plant alkaloids and glucosides (Milius et al. 2011); that the fifth neuron responds to bending of the hair (Merivee et al. 2008). In other Elateridae, sensilla Tr.1 of *E. ferrugineus* shares similar morphological characteristics to sensilla trichodea Tr.1 of *A. obscurus* (Merivee et al. 1997; Tooming et al. 2012), T1 of *L. aeruginosus* (Merivee et al. 1998), T1 of *M. villosus* (Merivee et al. 1999) and Tr.1 of *T. lewisi* (Ren et al. 2014). The ultrastructure of these sensilla was described for *T. lewisi* as having thick cuticular walls. In *E. ferrugineus*, as in all the species investigated so far, Tr.1 always occurs in the same position on each antennomere, and their number (about 70 sensilla) is constant. It has been demonstrated that, in *A. obscurus*, a salt and sugar-sensitive neuron and a mechanoreceptor neuron innervate this type of sensilla, in order to respond to host-plant chemicals (Merivee et al. 1997; Tooming et al. 2012). Therefore, we suspect that also in *E. ferrugineus* this type of sensilla may have a mechanosensory and gustatory function.

Sensilla Tr.2 occur only on male antennae. Homologous sensilla were described as T2 on male antenna of *A. obscurus* (Merivee et al. 1997) and were shown by electrophysiological methods (Merivee unpublished in Merivee et al. 1997) to be pheromone receptors. Similar sensilla were described as s.t.2, and found in high numbers on the male antenna of *M. villosus*, indicating that they are probably responsible for the detection of pheromones at long distance (Merivee et al. 1999). Comparable sensilla were analyzed by TEM and described as tr. 2 on the antenna of *T. lewisi*, with a thick wall and a low density of wall pores (Ren et al. 2014). In this species tr. 2

were assumed to be contact pheromone receptors, being more abundant in females than males. Thus, the Tr.2 observed by us in *E. ferrugineus* are probably responsible for the reception of the sex pheromone compound 7-methyloctyl (*Z*)-4-decenoate, emitted by females and to which males are strongly attracted. These results are also in accordance with previous studies on Lepidoptera, Diptera and other Coleoptera, indicating that pheromone reception is performed by similar trichoid sensilla (Zacharuk 1985; Hansson et al. 1986; Kaissling et al. 1989; Hallberg et al. 1994; Hallem et al. 2006).

Sensilla Tr.3 of *E. ferrugineus* are similar to those described for *M. villosus* (Merivee et al. 1999), where their number was higher in males than in females, indicating that these sensilla in *M. villosus* may be related to pheromone communication. However, in *E. ferrugineus* these sensilla occur in the same number in the two sexes, and further investigations are needed to elucidate their function.

Sensilla basiconica Ba.1 of *E. ferrugineus* match the description of sensilla basiconica type 2 of *M. villosus* (Merivee et al. 1999), B1B2 of *A. obscurus* (Merivee et al. 1997), s.b. 1 of *L. aeruginosus* (Merivee et al. 1998) as well as Ba.1 of *T. lewisi* (Ren et al. 2014). These are the most abundant sensilla to be observed on the antennae of *E. ferrugineus*, though their number in females is almost twice as many as in males. In the work on *T. lewisi*, sensilla basiconica were analyzed by using TEM, showing that these sensilla have a thin cuticle wall, a high density of dendritic branches inside the lymph lumen and a series of pores and pore kettles on the cuticular wall. This kind of structure probably serves an olfactory function as indicated by previous works (Lopes et al. 2002; Steinbrecht, 1997; Chen and Fadamiro 2008; Been et al. 1988; Zacharuk 1980; 1985).

Sensilla basiconica Ba.2 of *E. ferrugineus* occur in similar numbers in both sexes and share similar morphological characteristics with sensilla basiconica Ba.2 of *T. lewisi* (Ren et al. 2014), s.b.1 of *M. villosus* (Merivee et al. 1999), and s.b.2 of *L. aeruginosus* (Merivee et al. 1998). Further investigations are needed to elucidate their function.

Sensilla homologous to St of *E. ferrugineus* have been described in *L. aeruginosus* as basiconicum type 5 (Merivee et al. 1998), as styloconicum in *M. villosus* (Merivee et al. 1999) and in *T. lewisi* (Ren et al. 2014). In the latter species this sensillum was analysed by TEM revealing its thin cuticular wall and its joint membrane, which anchors it inside a raised socket. In the three species considered in literature as well as in *E. ferrugineus* these sensilla occur only on the

last flagellomere, on both anterior and posterior sides, representing a very small fraction of the entire sensory equipment of the antenna. A thermo and hygroreceptive function for this sensillum type has been proposed for *T. lewisi*, and it is possible that also St in *E. ferrugineus* may serve the same function.

Grooved pegs described by us for *E. ferrugineus* are homologous to s.b.4 of *L. aeruginosus* (Merivee et al. 1998) occurring in the same location and to g.p. of *M. villosus* (Merivee et al. 1999). These sensilla were interpreted as coeloconica in *T. lewisi* (Ren et al. 2014). In *E. ferrugineus* and in *L. aeruginosus* grooved pegs have a peculiar distribution being present only on posterior side of F2-F9 and anterior side of F9. A cross section of this sensillum in *T. lewisi* showed that it is double-walled, and probably has an olfactory function. The same function can be hypothesized for the grooved pegs observed by us in *E. ferrugineus*.

Sensilla campaniformia Ca observed in *E. ferrugineus* were found in the same position in *M. villosus* (Merivee et al. 1999), *L. aeruginosus* (Merivee et al. 1998) and *T. lewisi* (Ren et al. 2014). Campaniform sensilla are usually considered proprio-receptors that perceive mechanical stress in the cuticle, and are predominantly located near the joints (Keil 1997; Zacharuk 1985; Zill and Moran 1981; Gnatzy et al. 1987). Nevertheless, in honeybees these sensilla allow the perception of temperature, CO_2 and humidity (Dietz and Humphreys 1971). In many Carabidae it has been demonstrated several times that the cold neuron, innervating antennal campaniform sensilla, is highly sensitive to temperature fluctuations (Merivee et al. 2003; Must et al. 2006a; b; 2010). In addition to the thermoreceptor neuron, in carabid beetles these sensilla house two hygroreceptor neurons, the moist and dry air neuron, antagonistically responding to changes in humidity (Merivee et al. 2010). Thus, due to the peculiar position of Ca in *E. ferrugineus* and other click beetles, antennal campaniform sensilla are most probably thermo- or hygroreceptors instead of serving a proprioception function.

The porous structures found mainly at the base of Ch in *E. ferrugineus* appear to be clusters of small pores, and may be openings of cuticular ducts, part of glands associated with Ch, probably responsible for secretion of mucous-like substances for lubrication (Giglio et al. 2005). These porous structures are similar to those described in carrion beetles that have the antennae covered with organic secretions (Crook 2008).

In conclusion, we have found marked sexual differences on the antennae of *E. ferrugineus* both in gross and fine morphology. Both males and females show serrate antennae, which differ in length and shape. The antennae of the females are shorter than those in males in proportion to body size. In fact, the ratios between antennal length and pronotum width, and between antennal length and elytron length are greater in males than in females (antennal length / pronotum width is 1.38 in males and 1.00 in females; antennal length / elytron length is 0.59 in males and 0.49 in females). The expansion on the ventral margin of F2-F9 is more prominent in females and forms a less acute angle. In comparison, the antenna of males has a slender aspect. The numbers of sensilla in the two sexes are very different: ~7,000 counted on the antenna of males versus ~9,800 on the antenna of females. However, the most prominent differences between the two sexes at are fine scale: 1) the presence of Tr.2 only in males; 2) the higher abundance of sensilla Ch and Ba.1 in females. Our study showed that the morphology and pattern of distribution of the antennal structures of *Elater* is similar to those described in other click beetle genera. Future functional electrophysiological studies are needed in order to: 1) confirm the olfactory function proposed for Tr.2 as pheromone receptors in males; 2) to clarify if sensilla chaetica and basiconica are involved in the kairomone reception in females.

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PAPER III

Using odour traps for population monitoring and dispersal analysis of the threatened saproxylic beetles *Osmoderma eremita* and *Elater ferrugineus* in central Italy

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Abstract

Pheromone-based monitoring could be a very efficient method to assess the conservation status of rare and elusive insect species, but there are still few studies for which pheromone traps have been used to obtain information on presence, abundance, phenology and movements of such insects. We performed a mark-recapture study of two threatened saproxylic beetles, *Osmoderma eremita* (Scarabaeidae) and its predator *Elater ferrugineus* (Elateridae), in two beech forests of central Italy using pheromone baited window traps and unbaited pitfall traps. Two lures were used: (i) the male-produced sex pheromone of *O. eremita* (racemic γ-decalactone) to attract females of both species, and (ii) the female-produced sex pheromone of *E. ferrugineus* (7-methyloctyl (*Z*)-4-decenoate), to attract conspecific males. In total, 13 *O. eremita* and 1247 *E. ferrugineus* individuals were trapped. For *E. ferrugineus*, males were detected earlier than females, and 7-methyloctyl (*Z*)-4-decenoate was much more efficient lure compared to racemic γ-decalactone in detecting its presence. The population size at the two sites were estimated to 520 and 1369 individuals, respectively. Our model suggests a sampling effort of ten traps checked for three days being sufficient to detect the presence of *E. ferrugineus* at a given site. The distribution of dispersal distances for the predator was best described by the negative exponential function with 1% of the individuals dispersing farther than 1600 m from their natal site. In contrast to studies on these beetles in Northern Europe, the activity pattern of the two beetle species was not influenced by variation in temperature during the season.

Keywords Conservation, Kairomone, Mark-recapture, Pheromone, Predator-Prey, Temperature

Introduction

Semiochemicals are odorous molecules mediating both intraspecific and interspecific interactions (Gullan and Cranston 2010). The chemical ecology of insects, by the study of semiochemicals, started in 1950s with the identification of the silk moth sex pheromone (Butenandt et al. 1959). Since then, pheromones have largely been used with success for monitoring and control of pest insect in forestry, plantation and crop management (Witzgall et al. 2010). The advantages in the use of pheromones in monitoring of pest species are many, e.g. they are selective and very efficient at low population density (Witzgall et al. 2010). However there are still very few studies for which odour-baited traps have been used to study population parameters in rare and threatened insects. Ongoing research to implement semiochemical-based methods for conservation purposes includes some of the most charismatic and threatened European insects such as the moth *Graellsia isabellae* (Millar et al. 2010), and some beetles such as *Lucanus cervus* (Chapman et al. 2002; Harvey et al. 2011), *Osmoderma eremita* (Larsson et al. 2003; Svensson et al. 2003; Svensson and Larsson 2008), *Osmoderma barnabita* (Svensson et al. 2009), *Elater ferrugineus* (Svensson et al. 2004; Tolasch et al. 2007; Svensson and Larsson 2008; Musa et al. 2013; Andersson et al. 2014), *Morimus asper* (Chiari et al. 2013c) and *Prionus* sp. (Barbour et al. 2011). The use of attractive odour traps has been one of the most accredited approaches for monitoring presence and abundance of threatened beetles, especially those protected under the Habitat Directive.

All the above cited beetles belong to the functional group of the saproxylic, i.e. all the organisms that depend, during some parts of their life cycle, upon wounded or decaying woody material from living, weakened or dead trees (Speight 1989; Alexander 2008; Stokland et al. 2012). Saproxylic beetles are considered one of the most threatened insect groups in Europe mainly due to the modern forestry practices addressed to timber commercial use and dead wood harvesting, that deplete the amount of this resource and negatively affect the habitat quality for these organisms (Grove 2002; Davies et al. 2008; Thomas et al. 2009). Moreover, most of the saproxylic beetles are difficult to detect because they may occur at small population sizes and/or live hidden in tree microhabitats like wood crevices or tree holes. In this context, an efficient odour lure can be

very useful for detecting a species, assessing its population size, studying its dispersal capacity and flight phenology, as well as mapping its geographical distribution through large-scale surveys. Furthermore, as the sampling effort with odour traps could be standardized, it is possible to describe variations in the population parameters of a species in space and time.

The systematic review of Brouwers and Newton (2009) revealed that only a limited number of studies provided measures of movement rate for woodland invertebrates. No studies have been conducted on dispersal ability of forest click beetles but a few studies have estimated dispersal distances in pest click beetles (Yamamura et al. 2003; Blackshaw and Vernon 2006; Arakari et al. 2008; Schallhart et al. 2009). Detailed information on the dispersal ability (i.e. dispersal rate and range) is fundamental for assessing the extinction risk of local populations of threatened species, especially in saproxylic beetles, of which many species are today threatened due to habitat loss and fragmentation (Ranius 2006).

Only a few studies on IUCN listed saproxylic beetles, by means of mark-recapture, have been carried out in the Mediterranean region (López-Pantoja et al. 2008; 2011; Torres-Vila et al. 2012; Chiari et al. 2013a), and none of them was focused on the predator-prey system represented by *E. ferrugineus* (Coleoptera, Elateridae) and *O. eremita* (Coleoptera, Scarabaeidae), which has been extensively studied in northern Europe (Svensson et al. 2004; Larsson and Svensson 2009; Larsson and Svensson 2011).

The larvae of *E. ferrugineus*, live in hollow trees and feed on the immature stages of other saproxylic beetles, mostly those of flower chafers, including *O. eremita* (Iablokoff 1943; Platia 1994; Schaffrath 2003b; Tolasch et al. 2007). Svensson et al. (2004) showed that adult females of *E. ferrugineus* are attracted by the male-produced sex pheromone (*R*)-(+)-γ-decalactone, of *O. eremita*, using this compound as a kairomone in order to locate suitable tree cavities in which to lay eggs. On the contrary, males of *E. ferrugineus* are strongly attracted to the sex pheromone 7-methyloctyl (*Z*)-4-decenoate emitted by conspecific females (Tolasch et al. 2007; Svensson et al. 2012). Both *E. ferrugineus* and *O. eremita* are listed as Near Threatened (NT) in the IUCN European Red List of Saproxylic Beetles (Nieto and Alexander 2010), and *O. eremita* is also listed in the EU Habitat Directive as a priority species of community interest (Anonymous 1992).

The goal of this work was to use different trapping methods to analyse some aspects of the population ecology of *E. ferrugineus* and *O. eremita* at two locations in the Mediterranean region with the following aims: a) testing the efficacy of the traps baited with the racemic γ-decalactone and 7-methyloctyl (*Z*)-4-decenoate in capturing adults of these two species; b) estimating population size of the two species; c) obtaining estimates of dispersal rates and distances; d) obtaining phenological data and investigating the adult activity in relation to the variation in seasonal climatic conditions.

Materials and methods

Study areas

The study was carried out in central Italy (Latium region), in two Sites of Community Importance (SCI): "Boschi mesofili di Allumiere" (hereafter "Allumiere") (IT6030003) (42°09'N 11°54'E, 520-630 m a.s.l.) and "Monte Fogliano e Monte Venere" (hereafter "Monte Venere") (IT6010023) (42°21'N 12°11'E, 580-840 m a.s.l.).

These highly natural forests of beech (*Fagus sylvatica*) represent two secondary old-growth stands of the primeval forests that covered part of the Latium region during the cool and humid phases of Pleistocene (Magri 1998; 2008; Piovesan et al. 2011; Ziaco et al. 2012). These relatively small stands (45 ha Allumiere and 170 ha Monte Venere) occur at lower elevation in respect to the large mountain beech forests still widely spread along the Apenninic range between 900 and 2000 m a.s.l.

Sampling design

A mark–recapture study was conducted between 10th June and 25th July 2012, within an area of two hectares in each forest. Two trap types were used: Pitfall Traps (PT) and Black Cross Window Traps (BCWT). PT were empty jars, without an odour bait, placed inside tree hollows, with the opening (diameter of 7 cm) at the level of the wood mould surface (Ranius 2001). In total 23 PT (17 in Allumiere and six in Monte Venere) were set in all the accessible tree cavities occurring within the selected areas. BCWT, the same as used by Svensson and Larsson (2008) and Chiari et al. (2013a), were suspended from tree branches at 2–4 m height. For each replicate two traps were baited respectively with: a) 1200 µl of neat racemic mixture of γ-decalactone (Sigma-Aldrich, USA) as pheromone to

attract *O. eremita* (both sexes) and as kairomone lure to attract *E. ferrugineus* (mainly females); b) 2 µl of neat 7-methyloctyl (*Z*)-4-decenoate as pheromone to attract *E. ferrugineus* males (Svensson et al. 2012). The racemic γ-decalactone was loaded in a 2 ml plastic Eppendorf tube with cut strings of cotton as wicks. The vials were replaced at every fourth trap checking. The 7-methyloctyl (*Z*)-4-decenoate was loaded in a PCR tube pierced with an insect pin size 3 to release the pheromone (Tolasch et al. 2007; Svensson et al. 2012). In this case, the same vials were used throughout the whole trapping experiment. BCWT within the same replicate were placed at least 10 m apart. Each replicate was separated by at least 100 m to increase spatial independence. The number of BCWT replicates was 27 for each study area. In five cases (three in Allumiere and two in Monte Venere) the PT was set in the same tree as the BCWT. Traps were checked every second day and, to avoid position effects, the relative positions of BCWT within each replicate were changed four times during the study period. To avoid influencing the survival probability of captured adults at the end of the season, traps were removed when the number of capture events showed a clear decline. In addition to trap captures, all beetles encountered outside traps during trap checking (Visual Encounter Surveys, VES) were captured as well (cfr. Chiari et al. 2013a).

At first capture, every beetle was sexed and marked with a unique code: *O. eremita* with fine pits produced by a small drill (Dremel Lithium Cordless 8000JE) on both elytra (Ranius 2001; Chiari et al. 2013a), and *E. ferrugineus* with a fine point permanent marker pen (Uni Paint Marker PX-21) on the ventral sclerites. Beetles were normally released on the trunk of the trap tree, even if most the individuals of *E. ferrugineus* flew away from the operator's hand after being marked.

Data analysis

Population estimates

Population size estimates for each species were generated with male and female data pooled together and repeated captures in the same sampling occasion were counted only once.

The daily population size was estimated by the Jolly-Seber method for open populations, as implemented in Simply Tagging (vers. 2.0.1.27; Pisces Conservation Ltd, 2009). In order to allow

comparison with other studies, the overall population size was estimated using Craig's model for closed populations (Craig 1953). The coefficient of variation (C.V.) was calculated as the ratio between the standard deviation and mean population size. We refer to a closed population as one in which the total number of individuals is not changing through births, deaths, immigration or emigration, and an open population as one that is changing during the course of a study because of any combination the parameters previously cited (Amstrup et al. 2005).

Capture probability and survey effort allocation

Occupancy models for multiple detection methods were used to obtain method specific detection probabilities ($\hat{p}_T$) and to estimate occupancy at large (ψ) and small (θ) spatial scales (Nichols et al. 2008). The ψ parameter estimates the probability that a randomly selected site of the study area is occupied, whereas the θ parameter estimates the probability that the species occurs at the local sample station. Detection probability was modelled as constant over time and detection methods (p), as time-independent but different among methods (p^s), as time-dependent but constant among methods (p_t), or with method as an additive effect with time (p_{s+t}). Small-scale occupancy was modelled as either time-independent (θ) or time-dependent (θ_t). Analyses were carried out using the program PRESENCE (Hines and MacKenzie 2004) with single-season multi-method models that were ranked according to their values of AICc (Akaike Information Criterion corrected for small sample size) or QAIC (Quasi Akaike Information Criterion), with models having more support (low AICc or QAIC value) being highly ranked (Burnham and Anderson 2002).

To evaluate the survey effort necessary to achieve a standard error of 0.05 for the occupancy estimator $\hat{\psi}$, given the calculated ψ and p, the value of s (number of sites to investigate, in our case the number of traps) and K (number of surveys) were evaluated according to the equation of MacKenzie and Royle (2005):

$$\mathrm{var}(\hat{\psi}) = \frac{\psi}{s}\left[(1-\psi) + \frac{(1-p^*)}{p^* - Kp(1-p^*)^{K-1}}\right]$$

Where $p^*=1-(1-p)^K$ is the probability of detecting the species at least once during K surveys of an occupied site.

The purpose of this analysis is to determine what values of s and K are to be used to most efficiently achieve the desired level of precision for the value of $\hat{\psi}$ using the different trap types. The values of ψ and p in the equation were the ones resulted from the best model previously selected.

Dispersal distances and patterns

Straight distances between the traps that captured individual beetles were summed to obtain a conservative measure of the overall movements for each beetle captured more than once. Analyses were carried out by pooling distance values obtained from the two forests. The Mann-Whitney U-test was used to test differences between distances covered by males and females for each species. The distributions of dispersal distances were fitted to a negative exponential function, $P = e^{(-D/k)}$, and a power function,
$P = aD^{-n}$, where P is the probability that an individual covers the distance D or farther, and a, n, and k are constants (Ranius 2006; Drag et al. 2011; Svensson et al. 2011; Chiari et al. 2013b). Analyses were performed with STATISTICA 7.0 (StatSoft Inc., 2004), using a significance level of 0.05 to reject the null hypothesis.

The influence of temperature on beetle activity

To assess the influence of temperature on *E. ferrugineus* individual activity, the daily maximum temperature was correlated to the number of catches registered for each sampling occasion. For each species, activity data of males and females were analyzed separately, but pooled for the two study areas. For Allumiere, data on daily temperatures were obtained by the meteorological station in Allumiere municipality operated by the public regional hydrographic office (Ufficio Idrografico, 2013). Whereas, for Monte Venere, data were obtained from the meteorological station (ILAZIOCA3) in Caprarola municipality, about 3 km from the study area (Wunderground, 2013).

Results

Capture data

No beetles were captured in PT, and all captures thus refer to odour-baited BCWT. In addition, a total of nine males *E. ferrugineus* were found by visual encounter survey (VES) (three in Allumiere and six in Monte Venere). In total, 13 individuals of *O. eremita* were captured (four in Allumiere, nine in Monte Venere), with a total of 22 capture events (four in Allumiere, eighteen in Monte Venere) (Table 1). In contrast, 1247 individuals of *E. ferrugineus* were captured (374 in Allumiere, 873 in Monte Venere), with a total of 2151 capture events (687 in Allumiere, 1464 in Monte Venere) (Table 1). The recapture rate of *E. ferrugineus* males was 55% in Allumiere and 50% in Monte Venere, whereas the recapture rate of females was 10% in both study areas. The number of individuals captured was significantly higher for males than for females in both study areas (Allumiere, $\chi^2 = 443.63$, df = 1, $p < 0.001$; Monte Venere, $\chi^2 = 419.75$, df = 1, $p < 0.001$) and the same pattern was shown for the number of recapture events (Allumiere, $\chi^2 = 7.80$, df = 1, $p < 0.005$; Monte Venere, $\chi^2 = 39.35$, df = 1, $p < 0.001$).

The first capture of *O. eremita* was observed on 3rd July in Allumiere and on 24th June in Monte Venere; whereas, the last capture was observed on 20th July in Allumiere and on 23rd July in Monte Venere. For *E. ferrugineus*, the first male was captured on 15th June in Allumiere and on 19th June in Monte Venere, while the first female was found on 30th June in Allumiere and on 29th June in Monte Venere. The last capture for both sexes was performed on 24th July in Allumiere, while in Monte Venere the last capture of a male was performed on 25th July and of a female on 23rd July.

Of all *O. eremita* captured more than twice, only one female in Monte Venere was observed to come back to a trap of a previous capture. Of all *E. ferrugineus* captured more than twice, 5% in Allumiere and 8% in Monte Venere were observed to come back to a trap of a previous capture. No *O. eremita* and 7% of *E. ferrugineus* were captured more than once during the same sampling occasion.

No *O. eremita* was injured by the trapping procedure, while 20% of *E. ferrugineus* individuals were found dead in the traps. Of these, 36% were found dead at first capture, and 64% at a subsequent capture. The majority of these captures took place at the end of the field season.

Capture probability and survey effort allocation

All *O. eremita* individuals were captured in BCWT baited with racemic γ-decalactone, but due to the low number of captures it was not possible to build occupancy models for this species. All *E. ferrugineus* were captured by BCWT or VES (Table 1). The median number of captures per trap was 20 (range: 5-84) in Allumiere and 53 (range: 18-92) in Monte Venere.

In both study areas, the estimated large-scale probability of occupancy for *E. ferrugineus* was very high (Allumiere $\psi = 1$, S.E. = 0; Monte Venere $\psi = 1$, S.E. = 0). Model selection statistics provided strong evidence that occupancy probabilities, in both study areas, were influenced by sampling method (*s*) and time (*t*) (Table 2). The BCWT baited with 7-methyloctyl (*Z*)-4-decenoate performed much better than those baited with racemic γ-decalactone in detecting the presence of *E. ferrugineus*, showing a model average of $\hat{p} = 0.68$ (S.E. = 0.10) in Allumiere and $\hat{p} = 0.89$ (S.E. = 0.05) in Monte Venere. In contrast, the probability of detecting the presence of *E. ferrugineus* by BCWT baited with racemic γ-decalactone was $\hat{p} = 0.01$ (S.E. = 0.01) in Allumiere and $\hat{p} = 0.11$ (S.E. = 0.05) in Monte Venere (Table 3).

To achieve a S.E. of 0.05 for the estimate of the parameter ψ, using the values obtained for $\hat{p}$, with the BCWT baited with 7-methyloctyl (*Z*)-4-decenoate, it is sufficient to use ten traps which are checked three times during the experimental period in both study sites (Allumiere S.E. $\psi = 0.034$, Monte Venere S.E. $\psi = 0.020$). In contrast, to achieve the same S.E. for ψ using traps baited with racemic γ-decalactone, the field effort should be at least 250 traps for 130 surveys in Allumiere (S.E. $\psi = 0.054$) and 20 traps for 30 surveys in Monte Venere (S.E. $\psi = 0.042$). As the best fitting model for both study areas is the one with *p* changing with time, the p_t values for each sampling occasion were considered in the analysis of the sampling effort and the variation of the S.E. for ψ is reported in Fig. 1a and 1b. In this case, the S.E. variation during the sampling period was obtained by hypothesizing to set ten BCWT baited with 7-methyloctyl (*Z*)-4-decenoate for four days for both study areas. Instead, to obtain the S.E: variation using BCWT baited with racemic γ-decalactone was hypothesize to set 40 traps for 20 days in Allumiere (Fig. 1a), where the population is smaller, and 20 BCWT baited with racemic γ-decalactone for ten days in Monte Venere (Fig. 1b), where the population is larger.

Table 1 Summary of the mark-recapture data obtained during the study of *Osmoderma eremita* and *Elater ferrugineus* in the two beech forests, Allumiere and Monte Venere, of Central Italy with different capture methods. (BCWT Elater = black cross window trap baited with 7-methyloctyl (*Z*)-4-decenoate; BCWT Osmoderma = black cross window trap baited with racemic γ-decalactone; VES = visual encounter survey; PT = unbaited pitfall trap; *Oe* = *Osmoderma eremita*; *Ef* = *Elater ferrugineus*).

Method	Species	Allumiere		Monte Venere	
		Individuals (♂/♀)	Capture events (♂/♀)	Individuals (♂/♀)	Capture events (♂/♀)
BCWT Elater	*Oe*	0/0	0/0	0/0	0/0
	Ef	352/2[a]	657/2	734/2[b]	1295/2
BCWT Osmoderma	*Oe*	1/3	1/3	0/9	0/18
	Ef	5[c,d]/19[e]	5[c]/20	13[f,g]/134[h]	13[f]/148
VES	*Oe*	0/0	0/0	0/0	0/0
	Ef	3[i]/0	3/0	6[i]/0	6/0
PT	*Oe*	0/0	0/0	0/0	0/0
	Ef	0/0	0/0	0/0	0/0

[a] Individuals of which 1 recaptured with BCWT Osmoderma
[b] All individuals previously captured or then recaptured with BCWT Osmoderma
[c] Individuals/captures of which 2 found/performed in traps with at least 1 *E. ferrugineus* female
[d] Individuals of which 3 previously captured or then recaptured with BCWT Elater
[e] Individuals of which 1 recaptured with BCWT Elater
[f] Individuals/captures of which 11 found/performed in traps with at least 1 *E. ferrugineus* female
[g] Individuals of which 8 previously captured or then recaptured with BCWT Elater
[h] Individuals of which 2 recaptured with BCWT Elater
[i] All individuals previously captured or then recaptured with BCWT Elater

Table 2 Summary of the model selection statistics for the models from the data of *Elater ferrugineus* in the two beech forests of Allumiere (*a*) and Monte Venere (*b*) in Central Italy.

Model	*K*	-2Log (*L*)	ΔAIC*c*	*w*
(a) Allumiere				
ψ, θ, p_{s+t}	20	595.84	0.00	0.99
ψ, θ_t, p^s	20	604.40	8.56	0.01
ψ, θ, p^s	4	763.18	135.34	0.00
ψ, θ, p	3	1092.34	462.50	0.00
Model	*K*	-2Log (*L*)	ΔQAIC	*w*
(b) Monte Venere				
ψ, θ, p_{s+t}	20	608.05	0.00	1.00
ψ, θ_t, p^s	20	679.11	25.37	0.00
ψ, θ_t, p_{s+t}	36	592.44	26.43	0.00
ψ, θ, p^s	4	935.89	85.03	0.00
ψ, θ, p_t	19	939.82	116.43	0.00
ψ, θ_t, p	19	1000.13	137.96	0.00
ψ, θ, p	3	1177.12	169.13	0.00

K represents the number of parameters in the model and -2Log(*L*) is twice the negative log-likelihood value. Small sample Akaike Information Criterion (AIC*c*) and Quasi Akaike Information Criterion (QAIC) were calculated for the models relative to Allumiere and Monte Venere, respectively. Relative AIC*c* and QAIC values and Akaike weight, *w*, are reported. ΔAIC*c* and ΔQAIC represents the difference in AIC*c* and QAIC values relative to the top model (Burnham & Anderson 2002). Detection probabilities may vary among methods (*s*) or sampling occasions (*t*).

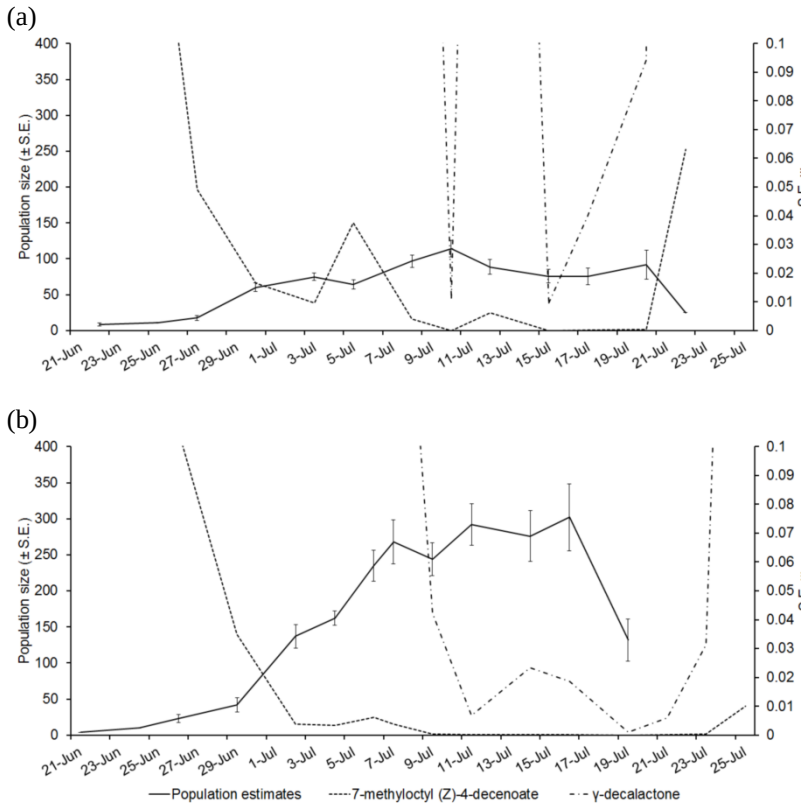

Fig. 1 *Elater ferrugineus* daily estimates of population size and variation of the standard error of the occupancy estimator $\hat{\psi}$ for black cross window traps baited with 7-methyloctyl (Z)-4-decenoate and γ-decalactone during the study period in Allumiere (a) and Monte Venere (b). The population estimates are obtained from the mark-recapture data with males and females data pooled using the Jolly-Seber method in Simply Tagging. The variation of S.E. of $\hat{\psi}$ is evaluated for the black cross window traps baited with 7-methyloctyl (Z)-4-decenoate hypothesizing to use ten traps checked four times for both the study areas. The variation of S.E. of $\hat{\psi}$ is evaluated for the black cross window traps baited with γ-decalactone hypothesizing to use 40 traps for checked 20 times in Allumiere and 20 traps checked ten times in Monte Venere.

Table 3 *Elater ferrugineus* detection probability estimates ($\hat{p}$) and associated standard error (in parenthesis) are given for multi-method models using the black cross window traps baited with 7-methyloctyl (*Z*)-4-decenoate (*Ef*) and racemic γ-decalactone (*Oe*) in Allumiere (*a*) and Monte Venere (*b*) (w = Akaike's weight for each model).

Model	*w*	$\hat{p}_{Ef}$(SE)	$\hat{p}_{Oe}$(SE)
(a) Allumiere			
ψ, θ, p_{s+t}	0.99	0.68* (0.10)	0.01* (0.01)
ψ, θ_t, p^s	0.01	1.00 (00)	0.07 (0.02)
ψ, θ, p^s	0.00	1.00 (00)	0.07 (0.02)
ψ, θ, p	0.00	0.28 (0.01)	0.28 (0.01)
(b) Monte Venere			
ψ, θ, p_{s+t}	1.00	0.89* (0.05)	0.21* (0.06)

* Parameter estimate reported is the median value among the 17 detection probability estimates for each detection method.

Dispersal distances and patterns

Spatial displacements of *O. eremita* were recorded only in Monte Venere, where four females out of nine moved from the site of the first capture to another trap, covering a median distance of 353 m (range: 201-523 m).

Spatial displacements of *E. ferrugineus* in Allumiere were recorded for 149 out of 194 recaptured males (77% of the recaptured males) and two females (100% of the recaptured females). Males moved from the site of first capture covering a median distance of 214 m (range: 10-1561 m), whereas the two females moved 90 m and 299 m, respectively. In Monte Venere, 336 out of 366 males (92% of the recaptured males) and twelve out of 14 females (86% of the recaptured females) moved from the site of first capture covering a median distance of 276 m (range: 10-1507 m) and 317 (range: 123-895 m), respectively. The median dispersal distance did not differ significantly between sexes (Mann-Whitney U-test, U = 3030, Z = 0.69, p = 0.49). Field data on dispersal distances show that, for both sexes, the negative exponential function (Pearson Correlation Coefficient: males $r_p = 0.973$, females $r_p = 0.895$) fits better than the power function (males $r_p = 0.893$, females $r_p = 0.817$) in describing the distribution of dispersal distances (Fig. 2). For both sexes, the first model predicted that about 1% of the individuals will disperse

farther than 1600 m from their natal site; about 51% will disperse farther than 250 m, and about 77% will move farther than 100 m.

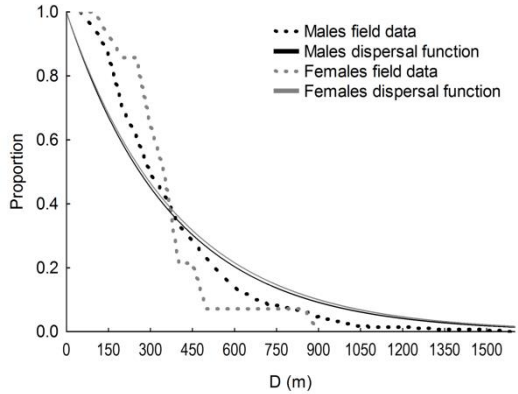

Fig. 2. Cumulative proportion of dispersing *Elater ferrugineus* males and females. Field data from the mark-recapture study combined for the two study areas (Allumiere and Monte Venere) (N_{males} = 485, $N_{females}$ = 14) are compared with the exponential functions $P = e^{(-D/7.54)}$ for males and $P = e^{(-D/7.83)}$ for females, where P is the probability that a dispersing individual exceeds the distance D (m).

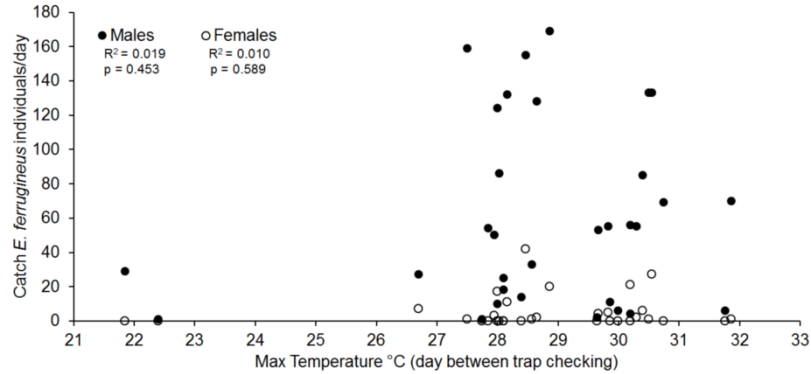

Fig. 3. The relationship between catch rates of *E. ferrugineus* and temperature during the sampling season. Catch rate is an indicator of activity and it is not correlated with the daily maximum temperatures registered during the study period.

The influence of temperature on beetle activity
For *E. ferrugineus*, the number of catches showed no correlation with the daily maximum temperature for any of the sexes (Fig. 3). During the study period, the temperature fluctuated from about 11° to 33° C.

Discussion

We used pheromone-baited traps to obtain estimates of population size, flight phenology, dispersal rates and distances, as well as the effect of temperature on the flight activity in two saproxylic beetles, *O. eremita* and *E. ferrugineus*, at two sites in Italy. Similar analyses have been performed on Swedish populations of these species. The current study and previous Italian studies on *O. eremita* by Chiari et al. (2012; 2013a; 2013b), thus enables comparisons of population parameters in the southern and northern parts of the distribution of these beetles. The differences found between northern (Sweden) and southern (Italy) populations of these beetles mainly concern three aspects: 1) the dispersal rates and distances, which are higher in the south than in the north, probably due to the different distribution of suitable habitat in the two regions (Hedin et al. 2008; Svensson et al. 2011; Chiari et al. 2013b); 2) the hollow trees selected for the oviposition that have a sunny exposure in the north while are not influenced by exposure at south (Ranius and Nilsson 1997 vs Chiari et al. 2012); 3) the flight activity, related to temperature variations during the season at north, while not depending on the same parameter at south, at least for *E. ferrugineus* (Larsson and Svensson 2011 vs present study).

Capture data and population estimates
A much higher number of captures of the predator *E. ferrugineus* was observed compared to the prey *O. eremita*. Even considering only *E. ferrugineus* captures in traps baited with racemic γ-decalactone, the predator/prey ratio was very high (Allumiere 26/4, Monte Venere 161/18). This is in contrast to the results of the studies on Swedish populations of these beetles, where this ratio was less than one (Larsson and Svensson 2011). Even higher would be the predator/prey ratio if we consider the number of individuals captured with the specific pheromone (Allumiere 659/4, Monte Venere 1307/18). This can be explained with the lower consistency of the *O. eremita* populations in the study areas than in Sweden (cf. Ranius

2001) and suggests that in the south *E. ferrugineus* is more generalist than expected in prey selection. Due to the rareness of *O. eremita* at the study sites, we may suppose that *E. ferrugineus* in these forests can also feed on larvae of other scarab beetles as *Cetonia aurata*, *Potosia cuprea*, *Cetonischema aeruginosa, Netocia morio, Gnorimus variabilis*, *Oryctes nasicornis* and/or also on other larvae, e.g. those of darkling beetles. In captivity, the larvae of *E. ferrugineus* were observed to feed on earthworms (AZ personal observation) and therefore we cannot exclude that also other invertebrates are included in their diet.

In this study, the captures of *O. eremita* are much less numerous than those performed in the studies conducted in northern Europe using the same method (Ranius 2001; Svensson et al. 2004; Larsson and Svensson 2011) and in other areas of central Italy (Chiari et al. 2013a), suggesting that these are small populations exposed to local extinction risk (Boyce 1992). In the study of Larsson and Svensson (2011) with a similar number of traps (N = 24-28 during different years), baited with γ-decalactone, between 6 and 30 *E. ferrugineus* captures were observed, probably all females. In the present study, the greatest number of captures (161) was observed in Monte Venere, indicating a larger consistency of this Italian population respect to the Swedish ones. However, other factors such as temperature may influence the catchability of *E. ferrugineus* (Larsson and Svensson 2011) making it difficult to compare the absolute size of populations in different parts of its distribution range.

The use of traps baited with 7-methyloctyl (*Z*)-4-decenoate allowed the capture of a large number of *E. ferrugineus*. The identification of the sex pheromone of this species was based on analyses of German and Swedish populations, and the high catches at the Italian sites show that this compound can be used to survey populations throughout the distribution range of the species. In the study of a Swedish population by Svensson et al. (2012), the recapture rate of males using traps baited with 7-methyloctyl (*Z*)-4-decenoate was 54-63%, which is similar to the 50-55% recaptures found in the present study. The population estimates revealed that the Monte Venere population was three times large as the Allumiere population and this fact is probably due to the greater structural continuity in the former forest than in the latter one. In fact, the beech forest of Monte Venere is wider and connected with other deciduous woodlands. This

favours a larger population size and facilitates connectivity with other populations.

During the season, *E. ferrugineus* males were detected earlier than females in both study areas, suggesting that they emerged in advance, or simply became active as early as two weeks before than females. Protandry is a common feature of species with a restricted breeding season and a multitude of adaptive hypotheses for this mechanism have been proposed during the last 50 years (see Morbey and Ydenberg 2001 for a review). However sex pheromone or kairomone-guided behaviour of insects can be modulated, among other factors, by the mating status of the individuals (Anton et al. 2007). Therefore an alternative explanation for the different timing of catches between sexes could be that females may not respond behaviourally to the kairomonal cue, or will not disperse from their natal tree, until they are mated, which will result in female captures in traps baited with racemic γ-decalactone occurring later in the season compared to male captures in traps baited with 7-methyloctyl (*Z*)-4-decenoate. In a Swedish study on *O. eremita*, a high proportion (79%) of the females captured in pheromone-baited traps were mated (Svensson et al. 2011), which partly supports the hypothesis that they mainly disperse after mating.

Capture probability and survey effort allocation

Pitfall traps have been successfully used to monitor populations of *O. eremita*, both in Sweden and Italy (Ranius 2001; Chiari et al. 2013a). In another Swedish study (Larsson and Svensson 2009) also *E. ferrugineus* was caught in PT, although in low numbers. On the contrary, in the present study none of the two target species was captured with this trap type. The main reason for this difference in PT catches between studies in Sweden and Italy could be that the Swedish sites included larger trees with a higher amount of wood mould in their hollows, suitable to sustain larger population of the beetles. The small volume of wood mould in the trees of the Italian sites could have made less efficient the PT method.

Observations of *E. ferrugineus* by VES were very rare. In total, only nine males were detected by VES versus the 1247 captured by BCWT. Moreover, these nine individuals were found in flight during the trap checking, when BCWT had been lowered at eye level and most probably these males were attracted by 7-methyloctyl (*Z*)-4-decenoate. In this way the beetles attracted by the pheromone could

be caught by hand. There is no information about the height at which *E. ferrugineus* flies, but our pheromone traps placed at a few meters height were efficient in capturing beetles. All the individuals observed to fly soon after the release reached immediately the height of about 10 meters and then took refuge into the tree canopy. Probably, this propensity to fly at high altitude may explain the rareness of direct field observation of this species.

Most (72%) *E. ferrugineus* males captured by BCWT baited with racemic γ-decalactone were found together with at least one female. In one trap it was also possible to observe a pair of mating individuals. This result can be explained in two ways: 1) racemic γ-decalactone is used by *E. ferrugineus* males as an indirect cue to find conspecific females (Svensson et al. 2004); 2) the *E. ferrugineus* females, once captured in the racemic γ-decalactone baited traps, can continue to emit pheromone and attract males to that trap. According to Svensson et al. (2004; 2009; 2011) the (*R*)-(+)-γ-decalactone could be a habitat cue or a territorial signal, emitted by males and exploited by the females of *O. eremita* to track the optimal trees for egg-laying, rather than a classical sexual pheromone. On the contrary, 7-methyloctyl (*Z*)-4-decenoate is a true sexual pheromone that allows the males of *E. ferrugineus* to trace conspecific females.

Similar to the results obtained for Swedish population of *E. ferrugineus* (Svensson et al. 2012), the BCWT baited with 7-methyloctyl (*Z*)-4-decenoate performed much better than those baited with racemic γ-decalactone, and the probability of detecting the species ranged from 64% to 75% in Allumiere and Monte Venere, respectively. This indicating that the own pheromone is the optimal lure for detecting presence of *E. ferrugineus*. In contrast, the probability of detecting *E. ferrugineus* with traps baited with racemic γ-decalactone was 5% in Allumiere and 21% in Monte Venere, showing that this lure is suboptimal for the detection of this species. The analysis on the survey effort allocation showed that it is sufficient to use ten BCWT baited with 7-methyloctyl (*Z*)-4-decenoate for four days to reliably assess the absence of the species (with S.E. ≤ 0.05). In order to assess the presence of *E. ferrugineus*, as the BCWT with racemic γ-decalactone catch mainly the females of this species, that are usually active in the second half of the sampling period (July), we suggest to use these traps for a sampling restricted to the month of July, to obtain the best result with the lowest effort.

As discussed by Svensson et al. (2012), when using an attractive lure in order to sample populations of threatened species, a special care must be taken. Despite the use of all the necessary precautions, such as frequent checking of traps, the use of leaves around the bottles to shade the containers and the positioning of moistened cotton inside the traps to maintain humidity, a significant proportion of captured *E. ferrugineus* (20%) died in the traps. This phenomenon could be due to the high maximum temperatures in our study areas during the sampling period, that usually were about 27-30° C but sometimes reached more than 32°. In studies on Swedish populations, which experience lower temperatures, this negative effect has not been observed (GS personal observation). The death of a few individuals during the end of a single sampling season may not represent a threat for the long-term persistence of a population, because *E. ferrugineus* has a multiyear lasting larval stage (up to six years) (Tolasch et al. 2007). However, as a precautionary principle, we discourage a massive use of these traps for more than one year at the same site, especially in Mediterranean countries, to avoid negative impacts on *E. ferrugineus* populations.

Dispersal distances and patterns

Contrary to what was suggested for the less vagile *O. eremita* (Ranius and Hedin 2001; Ranius 2006; Chiari et al. 2013b), that could be threatened by the loss of genetic variation due to low dispersal capacity (Ranius 2000), most of *E. ferrugineus* males (77% in Allumiere and 91% in Monte Venere) dispersed from the sites of first capture. As a higher dispersal capacity can promote gene flow among populations (Hartl and Clark 2007), and thus reduce inbreeding effects, our data on *E. ferrugineus* suggest that this species is probably less threatened by habitat isolation than *O. eremita*. The longest cumulative distance covered by male and female *E. ferrugineus* during this study was 1507 m and 895 m, respectively. For both sexes the negative exponential function predicted almost the same dispersal pattern with about 51% of individuals moving distances > 250 m. Chiari et al. (2013b) found that a female *O. eremita* was able to travel a distance of 1504 m, and 33% of beetles moved distances > 250 m. These results indicate that both sexes of *E. ferrugineus* are more prone to displacement than *O. eremita*. The greater attitude to displacement for *E. ferrugineus* could facilitate tracking of available prey during the emergency season, and

at a larger temporal scale it could make the species capable to track the successional changes of tree suitability in the landscape from one generation to another.

The maximum dispersal distance (1.5 km) registered in this study for *E. ferrugineus* (a male) corresponds to that registered for a female of *O. eremita* (Chiari et al, 2013b) in a radiotelemetric study. These distances are similar to those registered for other large saproxylic beetles: *Lucanus cervus*, 2.0 km (Rink and Sinsch 2007) and *Rosalia alpina*, 1.6 km (Drag et al. 2012). Data on both the dispersal distances and home range, acquired with the mark-recapture method instead of radio telemetry can be influenced by the trapping grid-size and by the distance among traps. Moreover the sequence of capture events and an attractive lure can interrupt the continuity in the animal movements (Lira and Fernandez 2009). Therefore, it is possible that the distances actually covered by *E. ferrugineus* are longer than those recorded in this study.

The influence of temperature on beetle activity

The flight activity of *E. ferrugineus* was not correlated with the temperature during the study period. In contrast, a study in Sweden, with lower average temperatures, found a positive correlation between the temperature and the flight activity of both *E. ferrugineus* and *O. eremita* (Larsson and Svensson 2011). In our study areas, where the climate is drier and warmer more or less constantly during the summer, these beetles are continuously active. It seems that either in Italy (our study) or in Sweden (Larsson and Svensson 2011) *E. ferrugineus* is active when the mean daily maximum temperature is above 21°C. Probably, this mean temperature value represents a threshold over which this beetle is more or less constantly active. We can suppose that in northern Europe the flight activity of both the target species is higher in warmer days than in colder days, predicting a positive correlation between activity and temperature, and that maximum temperature is never a limiting factor in northern countries. On the contrary, for Mediterranean countries, further investigations could find a second threshold above which the activity of these species is suspended.

Conclusions

The present study contributes important information on the dispersal ecology and adult phenology of *O. eremita* and *E. ferrugineus* populations in Italy and allows comparisons with studies on populations of these species performed in Sweden. Ecological traits expressed by populations over a species range are not constant but may differ due to variation in e.g. availability and size of suitable habitat patches. For conservation purposes, comparing traits such as dispersal capacity and habitat requirements among populations of a species is important to understand potential variation in e.g. vulnerability to habitat fragmentation. Due to this variation not only the management of populations should be adapted to the local conditions but also the sampling strategy used to monitor such populations. This study also highlights the potential problems when using a highly efficient odor lure to study rare and threatened insects. Therefore, to detect the presence of *E. ferrugineus*, thanks to the high efficacy of the lure, it is suggested to reduce the trapping effort (ten traps for three days) during the peak of activity (e.g., the two central weeks of July, in our study areas). Such optimal period could vary according to the climatic conditions for different European countries, i.e. later at higher latitudes and altitude.

Unfortunately, pheromones or kairomones are unknown for most of the saproxylic species of the Habitat Directive. In such cases other non-destructive methods should be used. Other attractive methods, such as freshly cut log piles, or passive methods, such as emergence traps, were efficiently used for the monitoring of *Morimus asper* and *Limoniscus violaceus*, respectively (Chiari et al, 2013c; Gouix and Brustel, 2012). The advantage of these methods, as in the case of pheromone traps, is the low sampling effort required: few sampling days in an area were sufficient to obtain meaningful information on presence and abundance of the species. When possible, trap methods (active or passive) should be preferred over the ones that cause an alteration of the habitat.

In this study, traps baited with the pheromones of the two species, *O. eremita* and *E. ferrugineus*, allowed us to acquire information on presence, abundance, dispersal patterns and daily activity of *E. ferrugineus*, a rather unknown species of conservation interest. All these parameters resulted in higher values than expected. The simultaneous analysis of the *O. eremita* populations in the same

study areas, with the use of traps baited with racemic γ-decalactone, led us to enlighten the disproportion in abundance of the two species which have always been considered a predator-prey system. In fact, in northern and western Europe, both species are abundant and coexist in many forest stands, being reported to inhabit the same tree hollows. On the contrary, in Mediterranean areas *E. ferrugineus* is much more abundant than *O. eremita* and may occur also when the latter is scarce or absent. This suggests that *E. ferrugineus* may have a greater number of potential preys throughout all its distributional range, and feeding on large size larvae of beetles that live inside tree hollows such as many species of saproxylic scarab and darkling beetles.

In the study areas the populations of *O. eremita* appeared to be reduced to a minimum number of individuals, surely insufficient for sustaining the predator in both forests, and suggests two hypothesis, not mutually exclusive: 1) *E. ferrugineus* exploits a wide spectrum of preys but still shows a strong attraction for the pheromone of *O. eremita* even though the latter cannot be anymore its major prey; 2) the high predation pressure on larvae of *O. eremita* in small areas, is one of the factor affecting negatively its population abundance.

At a local scale the traps baited with γ-decalactone allowed to assess for the first time the presence of *O. eremita* in both the study areas. This scarab species was considered an indicator of rich beetle fauna in tree hollows (Ranius 2002) and *E. ferrugineus* has been associated to its presence by several authors (Larsson and Svensson 2011; Tolasch et al. 2007; Schaffrath 2003b). *O. eremita* has populations more abundant in Sweden, and the probability to detect the species using baited-traps is considerably higher there in comparison with the present study (Andersson et al., 2014). Thus, it should still be considered as a good indicator of both community richness and hollow microhabitat suitability and availability in Northern Europe, in conjunction with *E. ferrugineus*. Nevertheless, in southern Europe, *E. ferrugineus* is much more easily detected than *O. eremita* by specific pheromone traps. For this reason, the former is more likely to be a better indicator of saproxylic beetle richness than the latter when pheromone traps are deployed. Furthermore, because *E. ferrugineus* is a predator of other saproxylic species its abundance is likely to be a good indicator of overall richness of saproxylic assemblages.

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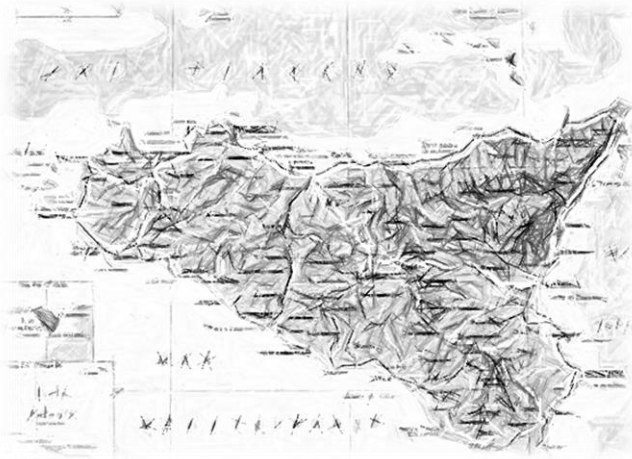
PAPER IV



Assessment of the species status of *Osmoderma cristinae* (Coleoptera: Scarabaeidae), endemic to Sicily, using pheromonal, genetic and morphological analyses

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Abstract

Resolving complexes of closely related and cryptic insect species can be challenging, especially when dealing with rare taxa that are difficult to collect for genetic analyses. Until recently, populations of the genus *Osmoderma* (Scarabaeidae) widespread in Europe were treated as a single species *Osmoderma eremita* (Scopoli, 1763) in spite of observed geographic variation in morphology. A survey using sequence data from the mtDNA cytochrome C oxidase I gene (*COI*) revealed the occurrence of at least two distinct lineages within this species complex: *O. eremita* in the west and *Osmoderma barnabita* Motschulsky, 1845, in the east. Interestingly, beetles confined to Sicily have been described as a distinct species, *Osmoderma cristinae*, Sparacio, 1994, based on morphological traits, but few specimens from this island were included in the genetic analysis, and the results led to a still questionable taxonomic rank for these populations. To explore the robustness of the previous taxonomic arrangement for *O. cristinae*, a combination of pheromonal, genetic and morphological analyses was used. The male-produced sex pheromone was, via scent analysis and field trapping, identified as (*R*)-(+)-γ-decalactone, primarily attracting female beetles, and the (*S*)-enantiomer of the compound had no antagonistic effect on trap catch. In previous studies the same results were obtained for *O. eremita* and *O. barnabita*, demonstrating strong conservation of this sexual trait within the genus. A 617 bp fragment of the *COI* gene, aligned with *O. cristinae* and *O. eremita* sequences already available in GenBank, showed a clear genetic divergence between the two species (interspecific mean distance = 6.6%). In addition, the morphological diagnosis based on the shape of the male genitalia highlighted their clear differentiation in the two species, with the most evident trait to distinguish *O. cristinae* from *O. eremita* being a double crest in the apical part of parameres forming two prominent elevations in *O. eremita*, the same crest being barely outlined in *O. cristinae*. In summary, our genetic and morphological data support the divergence of the two species and suggest species status for *O. cristinae*.

Keywords: Saproxylic, *COI*, genitalia morphology, conservation biology, (*S*)-γ-decalactone synthesis.

Introduction

Semiochemicals play a fundamental role in the communication systems of insects. Among semiochemicals, pheromones are molecules evolved as signals between conspecific organisms and can be used in mate recognition (Nordlund and Lewis 1976; Wyatt 2014). The majority of insect species investigated so far uses multicomponent pheromones, and much fewer use single components (Symonds and Elgar 2008; Riffell et al. 2009; Wyatt 2014). Sex pheromone specificity can be gained by using mixtures including many odorants in unique ratios and/or by using different stereoisomers of the same compound. When similar pheromone blends are shared by more or less closely related taxa other mechanisms intervene to lend specificity to the system, as temporal or spatial isolation (Wyatt 2014). For instance, in the scarab beetles of the genus *Anomala* Schoenherr, 1817, specificity is gained with both a chemical differentiation and temporal or spatial isolation. Females of *A. albopilosa albopilosa* Hope, 1839 attract males using a blend of four components of which the major one is buibuilactone. This compound, together with japonilure, constitutes the sex pheromone of the congeneric *A. cuprea* (Hope, 1839) (Leal et al. 1991; Leal 1996). In this case, the specificity of the chemical signal is enhanced by temporal difference in mating activity and pheromone release by the two species (Leal et al. 1996). In addition, *A. albopilosa sakishimana* Nomura, 1964 uses the same two compounds of *A. cuprea* but cross-attraction is prevented because these two species are geographically isolated (Leal et al. 1994). Similar pheromone parsimony in components of the blend is well documented among many other scarab species (Morin et al. 1996; Ward et al. 2002; Nojima et al. 2003; Alm et al. 2004; Allou et al. 2006; Robbins et al. 2009).

Sibling species that use nonvisual mating signals (e.g. sound, vibration, chemicals, or electrical signals) can be difficult to distinguish solely on the basis of morphological characters, and identifying their pheromones might be a key to resolving complexes of cryptic species (Bickford et al. 2007). In these cases, sampling of specimens by pheromone trapping and subsequent DNA-based analyses can be used when assigning populations to a certain species, and when analysing phylogenetic relationships among taxa within a species complex. The importance of a sound background in species

identification and the consequent correct assignment of populations to different species have a pivotal role in biology, both in general and for protected species in particular. By identifying cryptic taxa within a formerly known rare species, more accurate information regarding the threat status of each species can be gathered resulting in practical implications, e.g. management of protected areas, as Sites of Community Interest under the EU Habitat Directive.

The genus *Osmoderma* (Scarabeidae, Cetoniinae) includes saproxylic beetles inhabiting mature hollow broad-leaved trees (Ranius et al. 2005). Until recently, all populations in Europe were treated as a single species, *Osmoderma eremita* (Scopoli, 1763), but the observation of clear regional differences in morphology has lead to the description of up to five species or subspecies (reviewed in Ranius et al. 2005; Audisio et al. 2007; 2009). A recent molecular survey based on sequence data from the mitochondrial cytochrome oxidase I (*COI*) gene revealed two distinct lineages within the so-called *O. eremita* species complex; the western *O. eremita* and the eastern *Osmoderma barnabita* Motschulsky, 1845 (Audisio et al. 2009). Further subdivision was also observed within each lineage, suggesting additional species or subspecies. Beetles confined to Sicily have been described as a distinct species, *Osmoderma cristinae* Sparacio, 1994, based on morphological traits for both males and females. Moreover, in the molecular analysis of Audisio et al. (2009) Sicilian beetles formed a separate cluster within the *O. eremita* lineage, supporting that *O. cristinae* should be regarded as a separate species. *O. cristinae* is listed as Endangered (EN) in the IUCN Red List of Threatened Species, that includes also other *Osmoderma* species of Europe, due to its very small distribution range and its restricted association to a specific habitat type (Nardi and Micó 2010).

A well-known trait in *Osmoderma* beetles is the strong fruity scent produced exclusively by males. Previous studies on *O. eremita* and *O. barnabita* (Larsson et al. 2003; Svensson et al. 2009) identified the flavour compound as the (*R*)-enantiomer of γ-decalactone, and demonstrated its function as a sex pheromone in both species. In addition, Svensson and Larsson (2008) showed that the opposite (*S*)-enantiomer has no antagonistic effect on the attraction of conspecific females, and a racemic mixture of γ-decalactone, that is much cheaper than the pure (*R*)-enantiomer, has been successfully employed to study *Osmoderma* populations in different areas of its

distribution range: Sweden (Larsson and Svensson 2011; Svensson et al. 2011) and Central Italy (Chiari et al. 2013a; Zauli et al. 2014) for *O. eremita*; Poland (Oleksa et al. 2012) for *O. barnabita*. For conservation purposes, the possibility of monitoring one or several species using a cheap, commercially available and efficient odour lure is of a great importance.

Although available morphological and genetic data suggest that *Osmoderma* beetles confined to Sicily should be regarded as a distinct species, only few specimens from this Mediterranean island were included in previous studies. Therefore, a robust assessment of the species status of *O. cristinae* is needed. In addition, identifying the sex pheromone of the Sicilian *Osmoderma* would greatly facilitate non-destructive sampling of individuals for ecological and genetic studies.

The aim of this study was to assess the species status of *O. cristinae* using a combination of pheromonal, morphological and genetic analyses. The specific goals were: to identify the male-produced sex pheromone of *O. cristinae* via scent analysis and field trapping; to implement genetic data by mitochondrial (*COI*) marker; to revise the morphological criteria used for separating the two species. In addition, the synthesis of the (*S*)-γ-decalactone is reported.

Materials and methods

Study area

The fieldwork was carried out in the Madonie Natural Park (Palermo Province, Sicily) in two Sites of Community Interest (SCI): Gibilmanna (ITA020002, 37°58'N 14°01'E, 800 m a.s.l.) and Piano Zucchi (ITA020016, 37°54'N 13°59'E, 1,100 m a.s.l.). These moderate-grazed woodlands are dominated by oaks in Gibilmanna (*Quercus ilex, Q. pubescens, Q. suber*) and oaks mixed with maples in Piano Zucchi (*Q. ilex, Q. petrea, Acer campestre* and *A. monspessulanum*).

Dispensers and chemicals

Pheromone dispensers for traps were 1.5 ml plastic eppendorf vials with cut strings of cotton as wicks. The (*R*)-enantiomer or racemic mixture of γ-decalactone were purchased from Sigma-Aldrich (USA). (*S*)-γ-decalactone was synthesised via modification of a

published procedure (see Fig. 1) (Shimotori and Miyakoshi, 2006; Shimotori et al. 2007). *rac*-γ-decalactone was reacted with benzyl amine at 20 °C for 5 days and after standard work up and crystallisation from cyclohexane pure *rac*-*N*-benzyl-4-hydroxydecanamide was obtained. The *rac*-*N*-benzyl-4-hydroxydecanamide was resolved in a lipase (CALB) catalysed acylation reaction using vinyl acetate in organic solvent (Et_2O:$CHCl_3$, 5.5:1) at 20 °C. Enantiomeric purity of the (*R*)-substrate, (*S*)-product and the conversion of the reaction was monitored with a GC equipped with a chiral β-dex 120 column. The reaction was stopped after 51 h and the enantiomer enriched product the acetate of (*S*)-*N*-benzyl-4-hydroxydecanamide was separated by MPLC (gradient EtOAc / cyclohexane) from the remaining substrate of (*R*)-*N*-benzyl-4-hydroxydecanamide. The acetate of (*S*)-*N*-benzyl-4-hydroxydecanamide was hydrolysed by refluxing for 2 h in a MeOH solution containing 20% NaOH. The concentrated crude reaction mixture was then acidified with 2 M HCl_{aq} to pH 3 and after standard work up the enantiomer enriched (*S*)-γ-decalactone (90% ee; GC equipped with a chiral β-dex 225 column) was obtained. The (*S*)-γ-decalactone was converted, as described above, back to (*S*)-*N*-benzyl-4-hydroxydecanamide which ones more was subjected to a lipase catalysed reaction to give a pure enantiomer of the acetate of (*S*)-*N*-benzyl-4-hydroxydecanamide. The acetate of (*S*)-*N*-benzyl-4-hydroxydecanamide was treated as above to give a crude (*S*)-γ-decalactone which was further purified by MPLC (gradient EtOAc / cyclohexane) to give 8.99 g of pure (*S*)-γ-decalactone (100% ee; GC equipped with a chiral β-dex 225 column) in a total yield of 35% from *rac*-γ-decalactone. The analytical data for all compounds was similar to the previously reported data (Shimotori and Miyakoshi, 2006; Shimotori et al. 2007).

Odour collection and analysis

We performed headspace collections from 17 individuals of *O. cristinae* (Tab. 1 and 2). One or two beetles of the same sex were placed in plastic oven bags (Toppits, Sweden) or plastic jars (0.5–1.0 l). Controls consisted of air collections in empty oven bags. A Teflon tube (3 mm i.d.) filled with SuperQ (Alltech, Deerfield, IL, USA), and connected to a battery-driven pump (GroTech, Gothenburg, Sweden) via an aquarium hose, was used for odour collection.

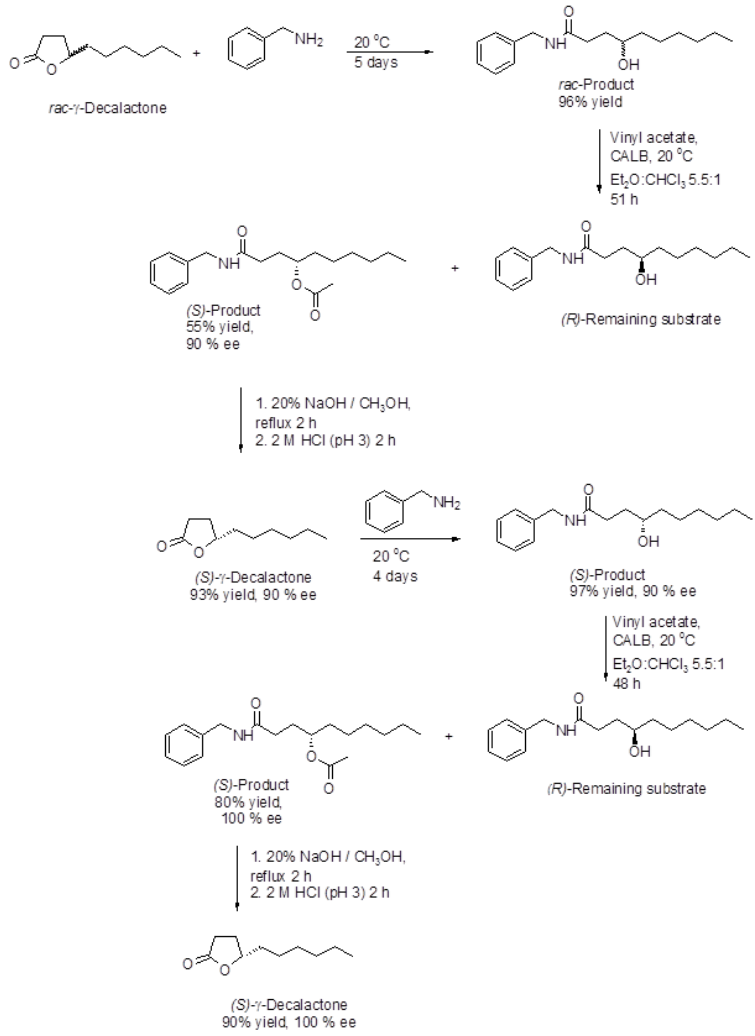

Fig. 1 Synthetic path for the 100% enantiomercally pure (*S*)-γ-decalactone.

The flow-rate of pumps was set to 400 ml/min. Odour collection from the beetles was performed for 4.5 – 5 h under ambient light conditions. After collection, filters were eluted with 100 µl of hexane, and the eluate stored at – 20 °C.

Identification of γ-decalactone in headspace samples was performed using coupled gas chromatography and mass spectrometry (GC-MS). Samples were injected into an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with an HP-5 column (30 m·x 0.25 mm i.d., 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA), and linked to an Agilent 5975C mass spectrometer. Helium was used as carrier gas (flow rate: 0.8 ml/min), injector temperature was 230 °C and transfer line temperature was 280 °C. Oven temperature was programmed at 50 °C for 2 min after injection and then increased at 10 °C/min to 250 °C, and hold for 5 min. The compound was identified by comparison of the retention time and mass spectrum of synthetic γ-decalactone.

To resolve lactone enantiomers, 2 µl of each sample were injected into an HP-5890 Series II Plus gas chromatograph (Hewlett Packard, Wilmington, DE, USA), equipped with a Cyclosil-B chiral column (30 m x·0.25 mm i.d., and 0.25 µm film thickness; J & W Scientific). Hydrogen was used as carrier gas. Oven temperature was maintained at 60 °C for 2 min after injection, then increased by 10 °C/min to 170 °C, followed by 2 °C/min increase to 180 °C, and then 10 °C/min increase to 230 °C. Using this temperature programme, the (*R*)-enantiomer eluted 7.2 s earlier than the (*S*)-enantiomer.

Table 1 Specimens of *Osmoderma cristinae* (OC) and *Osmoderma eremita* (OE) used in the present study for different analyses: chemical (Scent collection), genetic (*COI*) and morphological (Morphology). GC = Giuseppe Carpaneto Collection, MZURT = Museo di Zoologia dell'Università Roma Tre (= Zoological Museum of Roma Tre University).

ID	Region	Province	Site	Collection date	Museum Collection	Scent collection	*COI*	Morpho-logy
OC F01Gi008	Sicily	Palermo	Gibilmanna	2014.06.30	individual released	x	-	-
OC F02Gi003	Sicily	Palermo	Gibilmanna	2014.06.21	individual released	x	-	-
OC F03Gi011	Sicily	Palermo	Gibilmanna	2014.07.04	individual released	x	-	-
OC F04Gi011	Sicily	Palermo	Gibilmanna	2014.07.04	individual released	x	-	-
OC F05Gi006	Sicily	Palermo	Gibilmanna	2014.07.04	individual released	x	-	-
OC F06Gi006	Sicily	Palermo	Gibilmanna	2014.07.04	individual released	x	-	-
OC F07Gi011	Sicily	Palermo	Gibilmanna	2014.07.08	individual released	x	x	-
OC M08Gi021	Sicily	Palermo	Gibilmanna	2014.07.20	individual released	x	x	-
OC M09Gi036	Sicily	Palermo	Gibilmanna	2014.07.11	GC-MZURT	x	x	x
OC M11Gi001	Sicily	Palermo	Gibilmanna	2014.07.16	GC-MZURT	x	x	x
OC M13Gi001	Sicily	Palermo	Gibilmanna	2014.07.18	GC-MZURT	x	x	x
OC M18Gi011	Sicily	Palermo	Gibilmanna	2014.07.20	GC-MZURT	x	x	x
OC F01Pz038	Sicily	Palermo	Piano Zucchi	2014.06.29	individual released	x	-	-
OC F02Pz022	Sicily	Palermo	Piano Zucchi	2014.07.04	individual released	x	-	-
OC F03Pz034	Sicily	Palermo	Piano Zucchi	2014.07.04	individual released	-	x	-
OC M06Pz005	Sicily	Palermo	Piano Zucchi	2014.07.06	GC-MZURT	x	x	x
OC F07nPz007	Sicily	Palermo	Piano Zucchi	2014.07.06	individual released	x	-	-
OC M10Pz022	Sicily	Palermo	Piano Zucchi	2014.07.22	GC-MZURT	x	x	x
OE F01Vf	Abruzzo	Aquila	Val Fondillo	2014.07.26	GC-MZURT	-	x	-
OE M01Di	Abruzzo	Aquila	Bosco della Difesa	2014.07.26	GC-MZURT	-	x	-
OE M02Di	Abruzzo	Aquila	Bosco della Difesa	2014.09.13	GC-MZURT	-	x	-
OE M00POP	Abruzzo	Pescara	Orti di Popoli	2014.08.05	GC-MZURT	-	x	-
OE F02Al53	Latium	Rome	Faggeta di Allumiere	2012.07.03	GC-MZURT	-	x	-
OE F00Fb66	Latium	Frosinone	Forcella Buana	2009.07.25	GC-MZURT	-	x	-
OE F05Fb05	Latium	Frosinone	Forcella Buana	2009.07.03	GC-MZURT	-	x	-
OE F06Fb05	Latium	Frosinone	Forcella Buana	2009.07.03	GC-MZURT	-	x	-
OE M38Fb45	Latium	Frosinone	Forcella Buana	2009.07.25	GC-MZURT	-	x	-
OE M42Fb	Latium	Frosinone	Forcella Buana	2010.07.27	GC-MZURT	-	-	x
OE M00Sv44	Latium	Latina	Sughereta di San Vito	July 2010	GC-MZURT	-	x	-
OE M18SvHM18	Latium	Latina	Sughereta di San Vito	July 2010	GC-MZURT	-	-	x
OE M31Sv	Latium	Latina	Sughereta di San Vito	2010.07.03	GC-MZURT	-	-	x
OE M35Sv	Latium	Latina	Sughereta di San Vito	2010.07.08	GC-MZURT	-	-	x
OE F39Sv13	Latium	Latina	Sughereta di San Vito	2010.07.06	GC-MZURT	-	x	-
OE M40Sv	Latium	Latina	Sughereta di San Vito	2010.07.08	GC-MZURT	-	-	x
OE F41Sv44	Latium	Latina	Sughereta di San Vito	2010.07.28	GC-MZURT	-	x	-

Table 2 Number of *Osmoderma cristinae* individuals captured with different sampling methods in the two study areas in Sicily (Gibilmanna and Piano Zucchi), and number of included in the scent collection. Racemic γ-decalactone = traps baited with the racemic γ-decalactone; (*R*)-enantiomer = traps baited with the (*R*)-enantiomer of the γ-decalactone; (*S*)-enantiomer = traps baited with the (*S*)-enantiomer of the γ-decalactone; control traps = unbaited traps used as control; VES = Visual Encounter Survey, active search of individuals in hollow trees.

Site	Method	Individuals captured		Scent collection	
		Males	Females	Males	Females
Gibilmanna	racemic γ-decalactone	-	5	-	5[d]
	(*R*)-enantiomer	1	6[b]	1	1
	(*S*)-enantiomer	2	-	2	-
	control trap	-	-	-	-
	VES	2	1[c]	2	1
Piano Zucchi	racemic γ-decalactone	1	5[a]	1	2
	(*R*)-enantiomer	1	2	1	-
	(*S*)-enantiomer	-	-	-	-
	control trap	-	-	-	-
	VES	-	1	-	1
Total		7	20	7	10

[a] two of which recaptured;
[b] collected as pupa in the cocoon and reared;
[c] recaptured;
[d] for four individuals (two males and two females) scent collection performed two by two contemporary according to their sex.

Field trials

To get information about the beginning of the flight period of adult beetles, we set ten interception traps with black panels (Black Cross Window Traps - BCWT) at each location from 12th of June 2014. Such non-destructive traps have been used previously by Svensson & Larsson (2008), Chiari et al. (2013), and Zauli et al. (2014) for capturing *Omoderma* beetles. In this initial survey, traps were baited with 1,200 μl of neat racemic γ-decalactone. When the first beetles were captured (29th – 30th June), traps were removed and replaced with traps baited with single enantiomers. From 6th to 23rd of July

2014 ten trap replicates were set in each study area. Each replicate consisted of three BCWT: a) baited with 500 µl of (*R*)-γ-decalactone; b) baited with 500 µl of (*S*)-γ-decalactone; c) unbaited and used as control. The traps were suspended from tree branches at 2-4 meters height. Traps within the same replicate were placed at least 10 m apart, and different replicates were separated by at least 100 m in order to maintain spatial independence. During the whole study period, beetles were also searched for, looking or digging in the wood mould inside the cavities, and captured by hand (Visual Encounter Survey, VES). Traps were checked every second day and captured beetles were sexed. Most of the captured individuals were released soon after the capture, but some of them were kept for few days for the scent collection (see below) and then released. Before the release, each individual was marked on the elytra with fine pits produced by a small drill to enable identification upon recapture (Dremel Lithium Cordless 8000JE) (Ranius 2001; Chiari et al. 2013). In addition, six males used for the morphological analysis (see below) were collected during the last part of the flying season to limit any negative effects of removing individuals from the populations.

Genetic analysis

Genomic DNA was isolated from beetle tarsal samples following a modified cetyltrimethylammonium bromide (CTAB) protocol as described by Reineke et al (1998). For each sample, the tarsus was ground in 500 µl of lysis buffer consisting of 0.1 M Tris, 10 mM EDTA, 2% SDS and 0.2 mg/ml proteinase K. After 1 hr incubation at 58 ^{o}C, 140 µl 5M NacL and 65 µl CTAB were added and the mixture incubated for a further 10 min at 65 ^{o}C. The DNA was extracted once with 700 µl chloroform : Isoamyl alcohol (24 : 1) and 225 µl 5M NH_4Ac added prior to further incubation on ice for 30 min and centrifugation at 14,000 rpm. The DNA was precipitated with Isopropanol, cleaned with 70% ethanol, and re-suspended in 50 µl TE buffer, pH 8.0, and stored at –20 ^{o}C.

The polymerase chain reaction (PCR) was used to amplify a portion of the cytochrome oxidase I (*COI*) coding region of the mitochondrial DNA (mtDNA) using primers OsmoF and OsmoR (Table 3) (Svensson et al. 2009). The PCR reactions were carried out using a Verti 96-w Thermal Cycler (Applied Biosystems). Amplifications were performed in 20 µl reactions, each reaction containing 2 µl 10X Dream Taq PCR buffer (Thermo Scientific), 2 µl

DNTP mix (Thermo Scientific), 0.5 Units of Dream Taq polymerase (Thermo Scientific), 1 µl of each primer and nuclease free water to final volume. Reactions consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Negative controls were performed for all reactions. To assess the success of the amplification, 4 µL samples of the PCR products were analysed on a 1.5% agarose gel (TAE buffer), stained with ethidium bromide, and visualized under UV light using an AlphaImager® EP (Alpha Innotech, Randburg, South Africa).

PCR products were treated with a mixture containing Exo I (Exonuclease I; Thermo Scientific) and FastAP (Alkaline Phosphatase; Thermo Scientific) and incubated at 37 °C for 45 min, then at 80 °C for 15 min. Sequencing was undertaken in both directions using the fluorescent BigDye Terminator Sequencing kit v1.1 (Applied Biosystems). The primers used were OsmoF and OsmoR. Amplification started with a denaturation step at 96 °C for 1 min, followed by 35 cycles of 96 °C for 10 s, 55 °C for 5 s, and 60 °C for 4 min. Purification of extension products was made using the EDTA/ethanol method. Briefly, to each 10 µl of sequencing PCR product 2.5 µl of 0.125 M of EDTA and 40 µl of 95% ethanol was added, and the mixture centrifuged at 3,300 rpm and 4 °C for 45 min. The precipitate was cleaned with 70% ethanol, and products analysed on an in-house ABI PRISM 3130xl genetic analyser (Applied Biosystems).

Table 3: *COI* primer names and sequences.

COI primers	
OsmoF	5'-GGA GCA GTT AAT TTT ATT ACA ACA-3'
OsmoR	5'-AAA CAT AAT GGA AAT GAG CTA CT-3'

Morphological analysis

The male genital organ (aedeagus) was extracted and described using the following procedure. Dry specimens from collections (Tab. 1) were put into a plastic box containing a layer of cotton soaked in a mixture of ethyl acetate and distilled water and left there for two days to soften their articulations and membranes. Dissection was performed under a stereomicroscope (Leica, MZ12) at a

magnification of 20x using insect pins. The aedeagus was removed from the abdominal cavity through the opening between the pygidium and the last visible sternite. Then, each aedeagus, carefully cleaned, was glued onto a piece of cardboard, and positioned in such a way that the parameres were clearly visible. Pictures were taken in frontal, dorsal and lateral view using a Pentax k5 camera (Pentax 35 mm lens and f 22). A focus stacking technique was used to obtain imagines with a great depth of field. From each view 30 to 200 photos were taken depending on the depth of the field for the specific samples. The multiple images were combined using the software Helicon Focus 6.

Data analysis

Capture data

Differences in the number of captures performed with the three trap types inside the replicates were tested by the χ^2 test applying the Yates' correction.

Molecular data

Sequences were edited with Staden Package ver. 2003.1.6 (Bonfield et al. 2005) and aligned with *O. eremita* and *O. cristinae COI* data from Audisio et al. 2009. Variability was assessed with Mega ver. 6 (Tamura et al. 2013). Best fit of substitution model for the final dataset was performed using Modeltest ver. 3.7 (Posada and Crandall 1998) and the HKY model + G (shape= 0.94) + I (=0.70) was used to compute genetic distances. Bayesian inference was carried out with the selected substitution model with Mr Bayes ver. 3.2.3 (Huelsenbeck and Ronquist 2001): $2x10^6$ generations were run and Markov chains sampled every 1,000 generations, the first 10,000 trees were discarded as "burnin" and the remaining trees used to compute posterior probabilities at nodes.

Results

Odour collection and analysis

GC-MS analyses showed that all male samples except one contained γ-decalactone, whereas the compound was absent in all female samples except one. The subsequent gas chromatographic analysis using a chiral column showed that males produce only (*R*)-(+)-γ-decalactone (Fig. 2A, B). Analysis of the single female sample containing γ-decalactone revealed the presence of both enantiomers. That individual had been captured in a trap baited with racemic lactone the day before the scent collection, and presence of both lactone forms was most probably due to contamination.

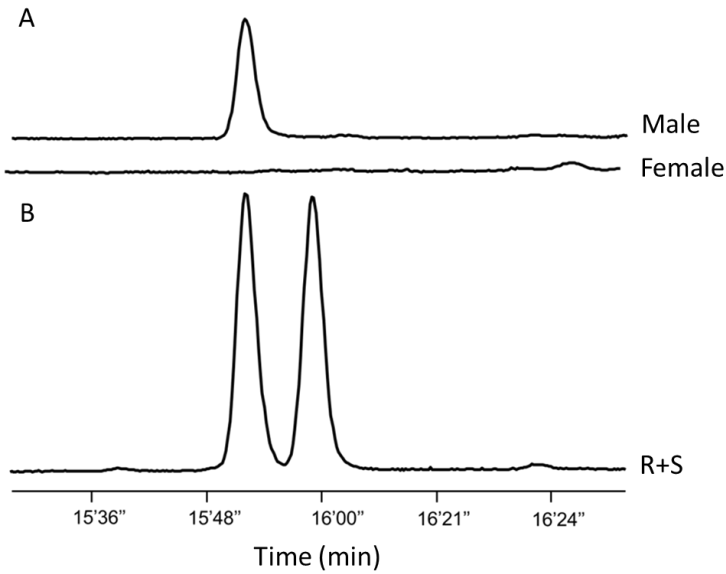

Fig. 2 Enantiomeric resolution of γ-decalactone on a chiral column. (A) Headspace collections from male and female *Osmoderma cristinae*, and (B) racemic γ-decalactone, with the (*R*)-enantiomer eluting earlier than the(*S*)-enantiomer. The peak of the male is in correspondence of the (*R*)-enantiomer.

Field trials

The first capture of *O. cristinae* with the racemic γ-decalactone was performed on the 29th of June. On the whole, from 29th of June to the 5th of July, when traps baited with the racemic γ-decalactone were removed, 11 individuals were captured (Tab. 2). From the 8th to the 23rd of July, when traps baited with individual enantiomers were used, it was possible to collect 12 individuals (Tab. 2), ten captured with the (*R*)-enantiomer and two with the (*S*)-enantiomer. No beetles were captured in control traps. Significantly more captures were performed with traps baited with (*R*)-enantiomer than with the other trap types ((*R*)-enantiomer vs (*S*)-enantiomer: $\chi^2 = 5.79$; $p < 0.02$; d.f. = 1; (*R*)-enantiomer vs control: $\chi^2 = 10.08$; $p < 0.001$; d.f. = 1).

Molecular data

In the alignment, 71 segregating sites were observed. The *COI* sequences of specimens from Sicily and Central Italy were clearly assigned to *O. cristinae* and *O. eremita* (posterior probability = 1.0) respectively (Fig. 3), on the basis of species-specific mutations at 12 nucleotide positions (Fig. 4). Intraspecific distance was 1.2% for *O. cristinae* and 1.3% for *O. eremita*, whereas interspecific genetic divergence was 6.6%.

Morphological diagnosis

The specimens from Central Italy differ from the Sicilian ones regarding the shape of the aedeagus. The parameres of *Osmoderma* are not straight but form an angle that divide them into an apical and a basal portion, which can be observed respectively by a frontal and a dorsal view of the aedeagus. In frontal view, the parameres of *O. eremita* show a high longitudinal double crest along the basal trait of their inner margin, accompanied by scattered and unordered setae (Fig. 5B, frontal view); on the contrary, a low and barely outlined double crest occurs in *O. cristinae* (Fig. 5A, frontal view), accompanied by two short series of roughly aligned setae. The latter ones, starting just below the angle of the parameres (in frontal view), follow a diagonal reaching the outer margin halfway through the parameres. In dorsal view, in *O. eremita*, the posterior trait of the double crest is clearly visible, forming two prominent elevations, which overhang the apex of parameres below (Fig. 5B, dorsal view); on the contrary, in *O. cristinae*, the same elevations are barely

outlined and let observe the two series of setae (Fig. 5A, dorsal view).

In lateral view, the angle between the apical and basal portions of parameres of *O. eremita* is almost right (about 90°) and the double longitudinal crest is high (Fig. 5B lateral view); on the contrary, the angle of *O. cristinae* is obtuse (about 135°) and the crest is just outlined (Fig. 5A, lateral view). Moreover, the series of setae is neatly visible in the latter, also in lateral view, while in *O. eremita* there are only scattered and unordered setae in the same position. At last, both in dorsal and later view, two distinct protuberances are visible in *O. eremita* at the sides of the aedeagus, under the peak of the crest.

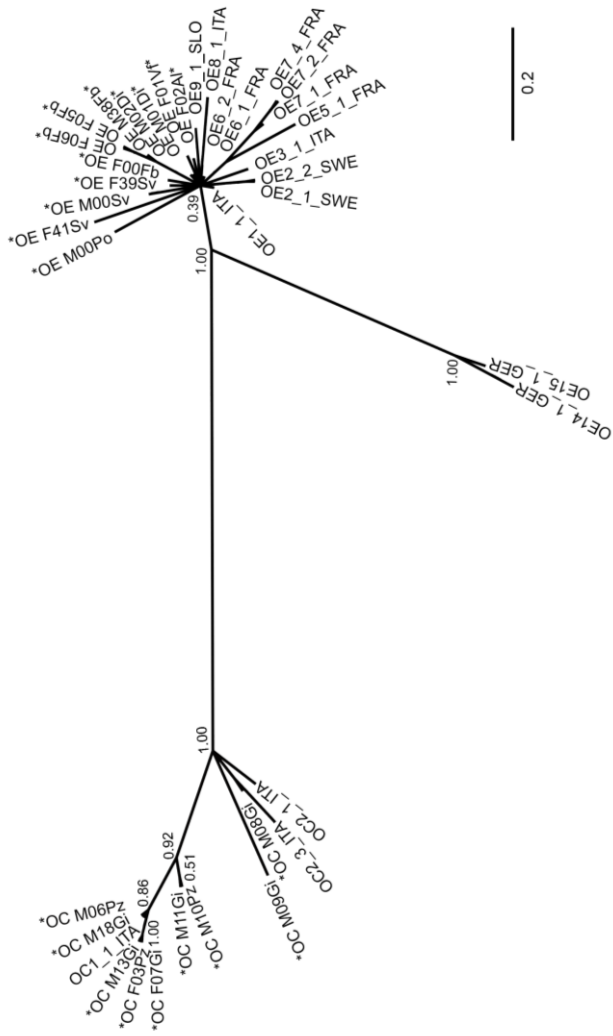

Fig. 3 Unrooted bayesian tree computed on the mtDNA cytochrome C oxidase I gene (*COI*) in *Osmoderma cristinae* and *Osmoderma eremita*. Specimens collected in Sicily cluster with *O. cristinae* instead specimens collected in central Italy cluster with *O. eremita*, previous data are available in GenBank (Audisio et al., 2009). * Samples analyzed in the present work.

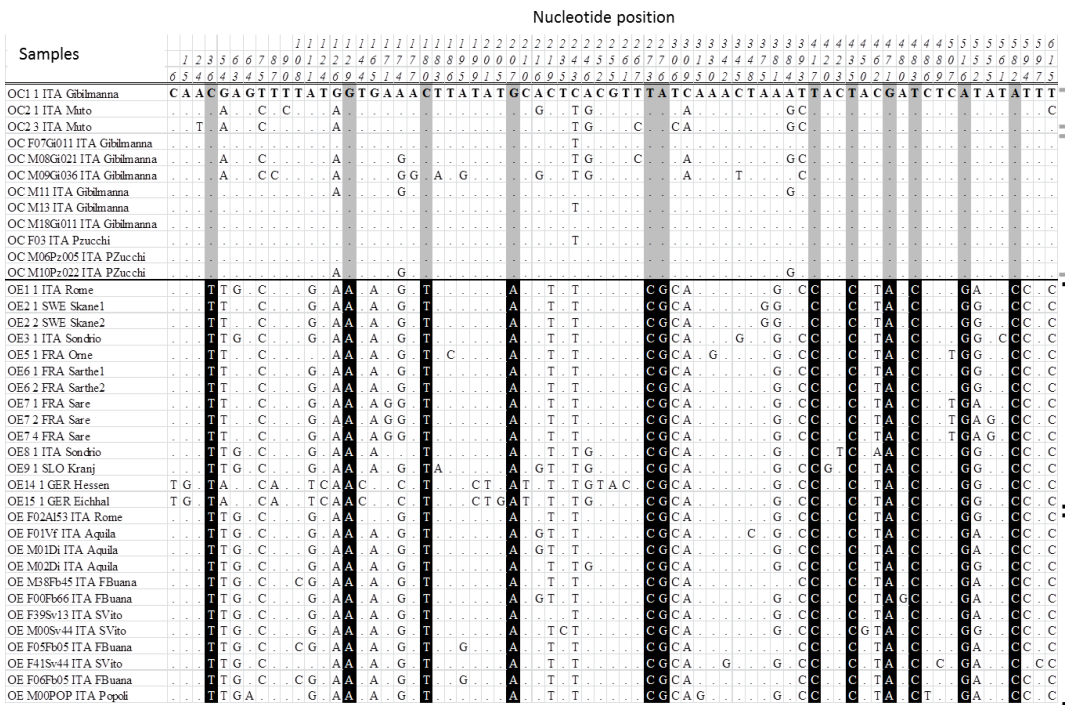

Fig. 4 The 71 segregating sites of the mtDNA cytochrome C oxidase I gene (*COI*) in *Osmoderma cristinae* and *Osmoderma eremita*. Our sequences of the *COI* are aligned with those present in GenBank. In the figure the twelve nucleotide positions presenting species-specific mutation are highlighted (grey for *O. cristinae* and black for *O. eremita*).

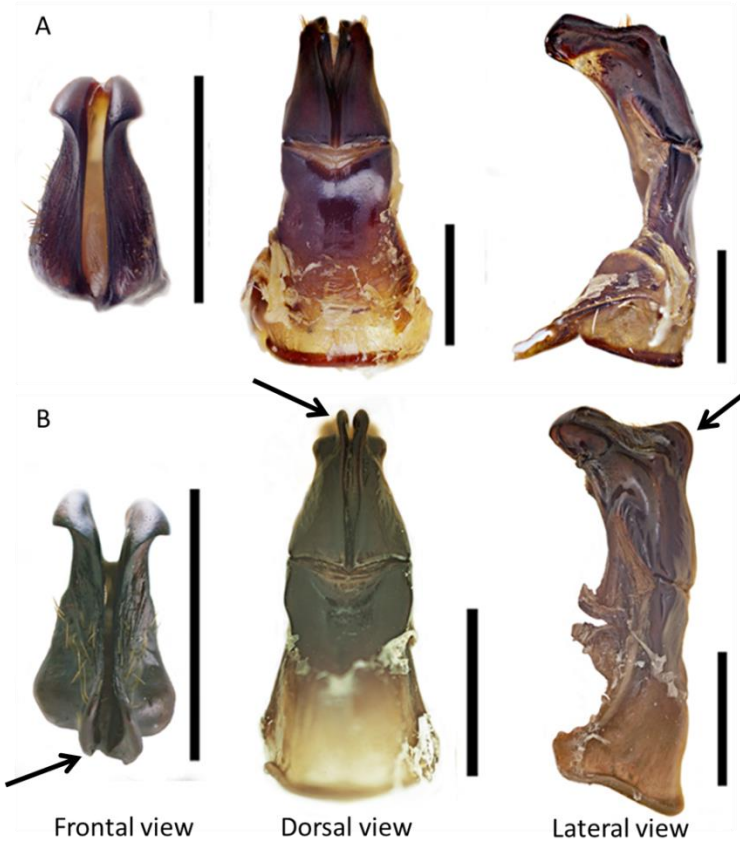

Fig. 5 Male genitalia morphology of *Osmoderma cristinae* and *Osmoderma eremita*. (A) *O. cristinae* parameres in frontal, dorsal and lateral view. (B) *O. eremita* parameres in frontal, dorsal and lateral view. The arrows indicate the most important character for species identification in the three views, that is the double crest occurring in *O. eremita*, that is instead barely outlined in *O. cristinae* (Scale bar = 5 mm)

Discussion

This study supported the divergence of *O. cristinae* and *O. eremita* based on both genetic data (*COI*) and morphological data (male genitalia) and thus suggests species status for *O. cristinae*. In the previous molecular study (Audisio et al. 2009), few Italian specimens were considered in the analysis, two from northern Italy, one from central Italy, one from southern Italy and three from Sicily. New data collected for the present work corroborate the differentiation between beetles collected from central Italy versus those collected on Sicily, and show that specimens collected at our study sites in Sicily cluster together with available *COI* data for *O. cristinae* in GenBank. The intraspecific distance for *COI* within *O. cristinae* was similar to that for *O. eremita* (about 1%). Therefore, *O. cristinae* maintained a high degree of intraspecific genetic variability, which is important for its biological conservation, while for the Swedish population of *O. eremita* was reported as a threat the loss of genetic variation that can increase the extinction risk (Ranius 2000).

Our data confirmed the *COI* gene as a reliable marker for species-level identification in the genus *Osmoderma* (Audisio et al. 2009; Svensson et al. 2009; Landvik et al. 2013).

The distinct genetic divergence between *O. cristinae* and *O. eremita* was correlated with morphological data: the shape of the aedeagus is clearly different between the two species (Fig. 4A, B). This differentiation was reported also by Sparacio (1994) when he described *O. cristinae*. In fact, genitalia often provide taxonomically useful characters for distinguishing organisms at species level, where no other morphological traits show differentiation (Medina et al. 2013), and pinpoint the relatively rapid divergence of this character over evolutionary time (Eberhard 2004; Hosken and Stockley 2004). This is also the case for *Osmoderma* for which a deep study of the morphological characters for both sexes is urgently needed for all species, in order to build a practical identification key.

In contrast to the genetic and morphology data, we demonstrated the absence of a differentiation in the sex pheromone of these species. Interestingly, *O. cristinae* produces and is attracted to the same compound, i.e. the (*R*)-enantiomer of γ-decalactone, used by many other *Osmoderma* species, revealing strong conservation of the sex pheromone within the genus. Previous data have shown that males of *O. eremita* (Larsson et al. 2003), and *O. barnabita*

(Svensson et al. 2009), the North American *Osmoderma eremicola* (Knoch, 1801), and the Japanese *O. opicum* (Lewis, 1887) (G.P. Svensson and M.L. Larsson, unpubl.) all produce the same compound. This seems to be a rare case because generally we find differentiation among sex pheromones used by different species within the same genus (Cardé et al. 1978; Löfstedt 1990 Emelianov et al. 2001; Thomas et al. 2003; Ramírez et al. 2010). As suggested by Svensson et al. (2009), the male-produced pheromone in *Osmoderma* beetles may function as a territorial signal instead of a classical sex pheromone used for species discrimination, and the selection pressure to change the signal may be weak or absent.

Our study showed that traps baited with the neat racemic mixture of γ-decalactone at the beginning of the study period attracted *O. cristinae* indicating that there is no antagonistic effect of the (*S*)-enantiomer. Previous studies revealed anosmia to the non-pheromonal enantiomer also in *O. eremita* and *O. barnabita* (Svensson and Larsson 2008; Svensson et al. 2009). From a methodological point of view, our results demonstrated that monitoring of *O. cristinae* can be achieved by using traps baited with the racemic mixture of γ-decalactone that is less expensive than the pure (*R*)-enantiomer. A higher number of females in comparison to males were attracted to traps baited with either the racemic mixture or the pure (*R*)-enantiomer of γ-decalactone. This was expected, based on previous field experiments on *O. eremita* showing that females are attracted to traps in much higher numbers than males (Larsson et al. 2003; Chiari et al. 2013a). The total number of individuals (n=11) captured by racemic γ-decalactone baited traps, operating for one week after the first captured individual, is similar to the number of individuals (n=10) captured with the (*R*)-enantiomer, in the second period of experimental trials, lasted 16 days. Similar to the studies on other *Osmoderma* species (Larsson et al. 2003; Svensson et al. 2009; Chiari et al. 2013a) the catches in pheromone traps observed in this study are low compared to those observed for other insect species. This is probably due to a combination of a lower innate response to the pheromone in *Osmoderma* species versus other insects, a low dispersal rate in these beetles, and low population densities at the study sites.

However, a rough comparison between the number of individuals captured in the present and previous studies performed in Central Italy on *O. eremita* (Chiari et al. 2013, Zauli et al. 2014), showed that

O. cristinae was easier to detect. The studies conducted in Central Italy were performed in broader areas and based on a higher number of traps. As *O. eremita* is a poor flyer and prefers trees with a high amount of wood mould (Ranius and Nilsson 1997; Ranius and Hedin 2001; Hedin et al. 2008; Ranius et al. 2009; Svensson et al. 2011; Chiari et al. 2013), we expect a similar habit in *O. cristinae*. In the Sicilian study area (the Madonie Natural Park), the trees had a big stem diameter and a high degree of woodland connectivity. Most probably, the ease in captures reflected a higher population abundance attributable to the higher habitat quality in the study area in Sicily respect to Central Italy. Another fact that should be mentioned in this context is that the predator *Elater ferrugineus* (Coleoptera, Elateridae), renowned for its close association to *O. eremita*, and attracted to the pheromone of the latter, was never recorded for Sicily nor collected in our traps. The two captures performed with the (*S*)-enantiomer of the γ-decalactone can be casual or due to a contamination of the traps, and not to an effective attraction of this compound. In fact, the (*S*)-enantiomer was never revealed to be present in the odor samples collected.

Conclusions

The integrative approach used in the present work allowed us to gather information on the chemical communication system of *O. cristinae* and to support its taxonomic specific rank.

Thanks to previous studies on the chemical ecology of the genus *Osmoderma* (Larsson et al. 2003; Svensson et al. 2009; Chiari et al. 2013a), it was possible to by-pass the usual protocol of isolation, identification, and synthesis of compounds, and bioassay confirmation of the activity of such, but to perform all the work in one field season. We demonstrated the production of the sex pheromone (*R*)-γ-decalactone by males, the attraction toward it by individuals (mostly females), and the non-antagonistic effect of the (*S*)-enantiomer. This study suggests odour-based trapping as a promising, effective and cheap method for future population monitoring of this rare species, listed as Endangered by the IUCN (Nardi and Micó 2010). Finally, the use of (*R*)-γ-decalactone as pheromone by the allied species *O. eremita* and *O. barnabita* facilitates large-scale monitoring of *Osmoderma* species in Europe with the same target compound.

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5. Conclusions

In the four contributions of the present research project, various methods and techniques were employed to study some biological aspects of *Osmoderma eremita* (sensu lato) and *Elater ferrugineus*. These methods included scanning electron microscopy to explore antennal microstructures, the capture-mark-recapture technique to study population ecology parameters, odour collection and analysis (including headspace collection, gas chromatography and mass spectrometry), as well as field trials to investigate the use of pheromones. In addition, genetic and morphological analyses were used to assess species status. In the following paragraphs the major results of the project are discussed.

5.1. Antennal morphology mirrors sensory-guided behavior

Both *O. eremita* and *E. ferrugineus* make use of pheromones for intraspecific communication but they rely in different ways on these chemical stimuli. The studies on the antennal morphology (I-II), confirmed the different behavior of the two sexes, of both species, in response to their pheromone compounds.

In *O. eremita*, where the pheromone is produced by males, and where both sexes respond to the pheromone stimulus, we were not able to find any differences between the morphological structures the two sexes use for pheromone reception.

In *E. ferrugineus*, where the pheromone is produced by females and only the males are attracted to this compound, a strong sexual dimorphism was found in the morphological structures of the antenna. In particular, we found that one type of trichoid sensillum occurred only on the antenna of males and we suggested that this type may be responsible for pheromone reception. Like other observations on the possible functions of the antennal microstructures of both species, this needs to be supported by more detailed studies, including the description of sensilla ultrastructure or electrophysiological recordings.

In particular, it would be interesting to carry out a detailed study on the functional role of the antennal microstructures of *O. eremita*, with emphasis on the scape, pedicel and funiculum, as the antennal club has already been investigated in this species as well as in many other scarabs. For *E. ferrugineus*, all the different antennal structures still

need to be studied from a functional perspective. Specifically, it would be interesting to investigate further the olfactory functions of the sensilla and, in this case, those *E. ferrugineus* uses to detect its own pheromone and the kairomone (*R*)-(+)-γ-decalactone.

5.2. Population ecology

The population study on *O. eremita* and *E. ferrugineus* (III) covered a gap of knowledge in some aspects of their biology, particularly about *E. ferrugineus*. Comparing our data on the dispersal distances and patterns of *E. ferrugineus* to those for *O. eremita* in the literature (e.g. Ranius and Hedin, 2001; Ranius, 2006; Chiari et al., 2013) it emerges that *E. ferrugineus* is more vagile. The greater propensity of the predator for dispersal could facilitate the detection of available oviposition sites, signaled by the pheromone of the prey.

Another point emerging from a comparison of the data is that in northern Europe, at higher latitudes than in Italy, the flight activity of both *E. ferrugineus* and *O. eremita* showed a positive correlation with peak temperatures (Larsson and Svensson, 2011). By contrast, our results did not show any correlation between these two variables, probably because the climate in Italy is drier and warmer, inducing a more continuous activity of the beetles over the summer.

Finally, the great number of individuals recorded for *E. ferrugineus* was unexpected, in the light of the notoriously small populations of *O. eremita* in the same study areas. The enormous difference in numbers of captured individuals of the two species can be attributed to two factors: 1) the different degree of attraction that the pheromones exert on conspecifics i.e. the 7-methyloctyl (*Z*)-4-decenoate for *E. ferrugineus* is a more potent lure than (*R*)-(+)-γ-decalactone is for *O. eremita*; 2) a greater tendency to fly for *E. ferrugineus* compared to *O. eremita*. However, the large difference in the number of individuals recorded for the two species reflects a real difference in their population sizes, and cannot be exclusively attributable to these two factors. Moreover, the high abundance of *E. ferrugineus* versus *O. eremita* corroborates the observations of other authors (Iablokoff, 1943, Hansen, 1966) who have suggested that the larvae of *E. ferrugineus* have a wider range of prey, including several species of large saproxylic beetles.

5.3. Difficulties and advantages in studying pheromones of rare or protected beetles

The challenge in studying pheromones of rare or protected species with a restricted breeding season is that these animals are often difficult to find and it is hard to obtain specimens to study. Luckily, *O. eremita* and *E. ferrugineus* were not yet so rare as to prevent scientists from collecting individuals, investigating pheromones and using these compounds in traps to study their populations in different areas of their distribution range. The use of extremely effective traps guarantees more frequent encounters with these species compared to visual surveys. This, and the fact that *O. eremita* and *E. ferrugineus* are associated with a variety of other rare or endangered saproxylic species, makes them good indicators of the richness of saproxylic species and umbrella species (see Ranius, 2002; Andersson et al., 2014). Therefore, actions aimed at the conservation of *O. eremita* are important for the survival of the enormous diversity represented by the invertebrate community associated with veteran forests and in particular with hollow trees in Europe.

Pheromone traps were first designed and employed in pest species management, and aimed for example at controlling populations through mating disruption or mass trapping without attention to the survival of the individuals. By contrast, in the case of rare or protected species, baited traps must be used with extreme care to avoid harming the populations. This may be done in several ways: a) by checking traps frequently followed by the prompt release of the captured individuals; b) using a reduced number of traps; c) concentrating the trapping during the peak of activity; d) avoiding the use of baited traps for consecutive years in the same area (see also Svensson et al., 2012). On the other hand, due to their efficacy, pheromone baited traps can be an expeditious tool to assess the occurrence of a species in a certain area.

Thus, even if some technical precautions should be taken when sampling rare or threatened insects, for some species pheromone traps are probably the only method to obtain detailed information on occurrence, population size, dispersal ability and flight phenology.

5.4. Pheromones, species status assessment and conservation

Sex pheromones can function as species discriminants. However, if sympatric species use the same pheromone compound, other kinds of isolation mechanisms may be present, e.g. a different time of activity. Different species may share pheromones or pheromone blends when they live in different places and there is no selection pressure for them to evolve species-specific signals. This is the case for *O. eremita* and *O. cristinae*, two allopatric species. In fact, our study (IV) revealed that *O. cristinae* uses the same pheromone as *O. eremita* and *O. barnabita*, which occurs in eastern Europe. This reveals a strong conservation of the sex pheromone within the genus. Besides, as suggested by Svensson et al. (2009), the male-produced pheromone in *Osmoderma* beetles may function as a territorial signal instead of a classical sex pheromone used for species discrimination, and the selection pressure to change the signal may be weak or absent.

From a conservation perspective, the use of (*R*)-(+)-γ-decalactone by allied species facilitates large-scale monitoring of the genus *Osmoderma* in Europe since it can be performed with the same target compound.

The integrative approach used in our work (IV) allowed us to gather information on the chemical communication system of *O. cristinae* and to support its taxonomic rank at species level using genetic and morphological analyses. To complement our results on the mtDNA cytochrome C oxidase I gene (*COI*), further analyses based on AFLP (Amplified Fragment Length Polymorphism) are in progress. Preliminary results agree with those based on *COI*. Additionally, the morphological diagnosis based on the shape of the male genitalia highlighted the clear differentiation between *O. cristinae* and *O. eremita*.

In addition, genetic analyses not only enable us to resolve complexes of cryptic species, but also to identify populations within species with a unique genetic setup, e.g. island populations. This is of fundamental importance for the effective targeting of conservation efforts and resources to save as much genetic diversity as possible.

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LIST OF PUBLICATIONS

ISI Indexed Journals

Submitted or in preparation

Zauli A, Maurizi E, Chiari S, Svensson GP, Carpaneto GM, Di Giulio A (submitted). Fine morphological analysis of the antenna in the threatened saproxylic beetle *Osmoderma eremita* (Coleoptera, Scarabaeidae). *Journal of Morphology*.

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