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Paussus favieri a model species for behavioral, chemical and acoustical investigations of ant nest beetles (Coleoptera, Carabidae, Paussini).

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1. S. Fattorini, **E. Maurizi** and A. Di Giulio. Tackling the taxonomic impediment: a global assessment for the ant-nest beetle diversity (Coleoptera, Carabidae, Paussini). Biological Journal of Linnean Society, *in press* (accepted 16 September 2011).
2. A. Di Giulio, **E. Maurizi**, P. Hlaváč and W. Moore, 2011. The long-awaited first instar larva of *Paussus favieri* (Coleoptera: Carabidae: Paussini). European Journal of Entomolology, 108: 127–138.

Papers published or prepared in the course of the PhD

Paper 1 – **E. Maurizi**, S. Fattorini, W. Moore and A. Di Giulio. Behavior of *Paussus favieri* (Coleoptera, Carabidae, Paussini) a myrmecophilous beetle associated with *Pheidole pallidula* (Hymenoptera, Formicidae). Psyche on the special issue "Ant and their parasites" (accepted with minor revisions 3 November 2011,and re-submitted 24 November 2011).

Paper 2 - Chemical mimicry of the ant nest beetle *Paussus favieri* (Carabidae, Paussinae), associated with *Pheidole pallidula* (Formicidae, Myrmicinae). **E. Maurizi**, P. d'Ettorre, W. Moore and A. Di Giulio (in preparation).

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Paper 4 – A. Di Giulio, **E. Maurizi**, M. V. Rossi Stacconi & R. Romani. Functional structure of antennal sensilla in the myrmecophilous beetle *Paussus favieri* (Coleoptera, Carabidae, Paussini). Micron, *in press* (accepted 20 October 2011, DOI information: 10.1016/j.micron.2011.10.013).

Paper 5 – S. Fattorini, **E. Maurizi** and A. Di Giulio. Tackling the taxonomic impediment: a global assessment for the ant-nest beetle diversity (Coleoptera, Carabidae, Paussini). Biological Journal of Linnean Society, in press (accepted 16 September 2011).

Paper 6 - A. Di Giulio, **E. Maurizi**, P. Hlaváč and W. Moore, 2011. The long-awaited first instar larva of *Paussus favieri* (Coleoptera: Carabidae: Paussini). European Journal of Entomolology, 108: 127–138.

Preface

The thesis is structured in:

1. Briefly introduction on the issue of the Ph.D. project, explaining the communication system in social insect, and how the myrmecophiles can exploit it. In particular, addressing the attention towards the ant nest beetles and the study species, *Paussus favieri*, a myrmecophilous beetle associated with *Pheidole pallidula.*

Chapters are structured as manuscripts with briefly introductions, materials and methods and discussion, including tables and figures.

2. Chapter 1 is focused on the investigation of inter- and intra-specific behaviors performed by *P. favieri* inside the ant nests maintened in captivity conditions.

3. Chapter 2 is centered on the chemical strategy adopted by the beetle to deceive the recognition system of its host, *Pheidole pallidula.*

4. Chapter 3 investigate the role of the acoustical communication in *P. favieri* both in the relationships with its host, and intra-specific interactions.

5. Chapter 4 is focused on the morpho-functional analysis of the antennal sensory system of *P. favieri.*

6. Conclusions section briefly synthesize and link the mainly results of the thesis, and suggest directions for future researches.

Additionally, abstract is separated by the rest of the thesis, in other pdf file. Chapters 1 and 4 totally correspond to Paper 1 and 4 (see p. IV), respectively, already accepted. Thus, I included also the abstracts and the references prepared for journal submission. Chapters 2 and 3 correspond to Paper 2 and 3 (see p. IV), respectively, in preparation. The abstracts and the references are not included. References include all the citations of the introduction, the chapters 2 and 3.

Introduction

Communication in social insects

All organisms from the simplest (unicellular) to the most complex (multicellular) depend on communication. Communication occurs when a sender produces a signal in attempt to influence the behavior of a second actor (receiver) in order to increase its chances of survival or reproduction (Brenowitz 1994). Zuk and McKean (2000) categorized the consequences of the communication considering the relationship between costs and benefits to either senders and receiver, noting that the type of signals involved depend on this relationship (Fig. 1). Hence, senders and receivers can obtain mutual benefits of the interactions by using an 'honest' signal; nevertheless in some communication systems senders manipulate or exploit the receivers (or vice versa) by using 'dishonest' signals (Markl 1985).

		Benefit to receiver	
		Positive	**Zero (or Negative)**
Benefit to Sender	**Positive**	Honesty (Mutual benefits)	Deceit
	Zero (or Negative)	Exploitation	Spite

Table 1. Honest and dishonest signals in animal communication.

In animal groups or societies where many members interact and collaborate at the same time inside a discrete place, the system of communication is expected to be more complex than in solitary animals. The highest degree of specialization is achieved in social insects, especially in ant societies, characterized by communities in which all members are related and are able to maximize their fitness by sophisticated division of labor between reproductive and non-reproductive castes (Hölldobler and Wilson, 1990). Their social organization and behaviors are finely regulated by continuous exchange of signals and cues through different sensory channels (chemical, visual and mechanical). Effective communication in ants is based on the exchange of the honest signals; dishonesty should

disrupt the perfect stability and cohesion of the society (Heinze and d'Ettorre, 2009).

A signal can be composed by distinct components, transmitted simultaneously or in sequence, and can be characterized by the use of more than one sensory modality. Hence, the ant communication system is defined as multicomponent and multimodal or multisensorial (Hölldobler and Wilson, 1990; Hölldobler 1999; d'Ettorre and Moore, 2008).

The mechanical channel includes tactile signals (antennation and grooming), widely recognized as important in ant communication (Hölldobler and Wilson, 1990), and acoustical signals (stridulation and drumming). The acoustical channel is generally considered as 'weakly developed' in ants (Hölldobler and Wilson, 1990; Keller and Gordon, 2009); however, recent findings support a basic role of this channel in the intraspecific communication, often integrating, amplifying and modulating chemical cues (Markl and Hölldobler, 1978; Baroni-Urbani *et al*., 1988; Hölldobler 1999). The visual channel seems to have a secondary importance in the darkness of the nests where they spend most of the time (Kirchner 1997), while the main channel that drives almost all the ant's activities is chemical, mediated by pheromones and cuticular hydrocarbons (Hölldobler and Wilson, 1990). These last, owing to their stability, low volatility and diversity of structure, are widely acknowledged to represent the main source of recognition cues in ants. Their peculiar blend would work like a 'signature' for the members of the same colony, allowing the recognition between nestmates and intruders (Howard 1993). The colony odor (according to the 'Gestalt' model by Croizer and Dix, 1979) is homogenized among nestmates by physical contact and by grooming and throphallaxis (Soroker *et al*., 2003). A combination between both cuticular hydrocarbons (critical signals) and volatile chemicals (transient signals) was also proposed for the ant recognition system (Akino and Yamaoka, 2000).

Myrmecophily

Many animals, and especially insects, are strongly attracted by the

ant nests, that represent stable and protected environments, rich in suitable microhabitats and valuable resources (ants, their broods stored food, waste materials, etc.). Several adaptive strategies (morphological, behavioral, chemical and acoustical) have been evolved by the so called myrmecophiles to infiltrate the nests and exploit the resources (Wilson 1971; Kistner 1982). Most of these strategies are aimed to circumvent the host recognition systems in order to avoid or deter ant attacks (Lenoir *et al*., 2001).

The multitude of myrmecophilous species belonging to very different animal taxa (especially arthropods) show different degrees of interaction with the host ants, that many authors tried to categorize. However, most of the classifications of myrmecophiles are not completely satisfying, considering that one species can fit more than one category in different life stages, or in different phases of the interaction.

The first tentative classification was that of Wasmann (1894), who, basing on the degree of acceptance into a social system of the host ants, recognized five categories: 1) synechthrans, predators treated in hostile manner by the ants; 2) synoeketes, predators or scavengers ignored by their hosts; 3) symphiles, symbionts totally accepted and integrated in ant society; 4) trophobionts, species that supply their hosts with rewarding secretions, obtaining protection from enemies; 5) ectoparasites and endoparasites, conventional parasites that damage the hosts. This classification, though considered outdated (Holldobler and Wilson, 1990; Geiselhardt *et al*., 2007), is still frequently employed as a kind of shorthand in the literature of social symbioses. Kistner (1982) suggested an ecological classification, in two categories: 1) non-integrated species, living in the ant nests, but not included in the social system, and 2) integrated species, included in the social system. Witte *et al.* (2002) recognize two categories: Type 1, which live inside the nest, and Type 2, which live in the surroundings of the nests.. Today it is common to distinguish the myrmecophiles in: facultative, that are occasionally tended by ants; and obligate, that depends on ants (Geiselhardt *et al*., 2007). This last relationship can range from mutualism to parasitism.

Ant parasites

Recent estimates suggest that about 10% out of 100,000 myrmecophilous species, such as butterflies, crickets, beetles and flies, are specialized ant parasites (Thomas *et al.*, 2010) that establish relationships with ants for a considerable part of their life cycle and depend on the host ants for their survival and reproduction.

In the host-parasite association can be recognized different phases of the integration process (Passera and Aron, 2005; Nash and Boomsma, 2008):

1. Ant parasites have to **locate** the host colony, following the cues produced by the ants and regularly released on the trails. This ability has been detected in several species, as in Staphilinidae beetles, *Homoeusa acuminata*, that intercept the trails of *Lasius fuliginosus* (Quinet and Pasteels, 1995); in the paussid beetle *Paussus favieri* (Carabidae, Paussinae) host of *Pheidole pallidula,* that preferentially follows the pheromone trail produced by the poison glands of its host ant, discriminating these from pheromones of non-host ant species (Cammaerts *et al*., 1990; Cammaerts and Cammaerts, 1992); and in lycaenid butterfly *Maculinea teleuis,* the exclosed fourth instars actively search the trails of *Myrmica rubra* until the nest enter, not be carried into the nest by the ants as in other *Maculinea* species (Hölldobler and Wilson, 1990).

2. The parasites have to **enter** the nest facing the aggressivity of the ants and **access** the resources of the colony. During these integration phases ant parasites have to corrupt the honest signals of the host ants (Thomas *et al.*, 2010) disguising their true nature and appearing as member of the colony. They exploit different sensory channels, in particular the chemical channel (Hölldobler and Wilson, 1990; Passera and Aron, 2005; Nash and Boomsma, 2008). The aggression toward intruders can be avoided by parasites imitating the ant cuticular profiles. They are able to imitate the ant cuticular hydrocarbon profiles through either an active synthesis of the hydrocarbons (chemical mimicry) (*sensu* Dettner and Liepert, 1994; Howard *et al.*, 1990; Orivel *et al*., 2004;

Geiselhardt *et al*., 2006; Witte *et al*., 2008), or by passively acquiring the chemical profile (chemical camouflage) (*sensu* Dettner and Liepert, 1994).

The chemical mimicry has been demonstrated in a few parasitic species: in caterpillars of lycenid butterflies *Maculinea rebeli*, guest of *Myrmica schencki*; in hoverfly larvae of *Microdon piperi* associated with *Camponotus modoc*; and in larva of ladybird *Thalassa saginata* guest of *Dolichoderus bidens* (Howard *et al*., 1990; Akino *et al*., 1999; Orivel *et al*., 2004). Camouflage can be achieved by physical contact (Vander Meer and Wojcik, 1982; Akino *et al*., 1996, 2002; Elmes *et al.*, 2002), by the exchange of food (trophallaxis), or by host larval consumption (Dinter *et al.*, 2002; Pierce *et al*., 2002). Among beetles the chemical camouflage seems to be the most used strategy, as suggested in *Myrmecaphodius excavaticollis* (Scarabaeidae) associated to *Solenopsis* spp. (Vander Meer and Wojcik, 1982); *Zyras comes* (Staphylinidae) and *Diaritiger fossulatus* (Pselaphidae) both associated to *Lasius fuliginosus* (Akino *et al.,* 2002).

Another strategy used by the parasites is to refrain from expressing any identification cue (chemical insignificance), in order to become chemically "invisible" to the ants (Lenoir *et al*., 2001; Witte *et al.*, 2008). This result can be accieved by reducing the number or the amount of cuticular hydrocarbons.

Multiple strategies can be combined by the same parasite, like in the case of two staphilinids beetles, *Lomechusa strumosa* and *Atemeles pubicollis,* close associates of two species of *Formica*, to be accepted by the ants: chemical, producing defensive and appeasing secretions; and behavioral, imitating the tactile solicitation to obtain regurgitation by the ants. Moreover, the larvae mimic the larval brood pheromone of the host ants, soliciting in this way the worker's trophallaxis and cares (Hölldobler and Wilson, 1990).

Another strategy employed by parasites associated with ants able to stridulate (Ponerinae, Nothomyrmecinae, Pseudomyrmecinae, Myrmicinae, Ectatomminae), is to mimic the ant's acoustical cues, often in combination with other chemical strategies. For example, a

social manipulation is performed by larvae and pupae of *Maculinea rebeli,* that achieve the initial acceptance in the nests of *Myrmica schenki* by chemical mimicry and camouflage, and than the acoustical mimicry is employed to elevate the caterpillars towards a higher social status, like the queen level (Barbero *et al*., 2009a, b; Thomas *et al*., 2010).

Ant nest beetles (Carabidae, Paussinae, Paussini)

Paussinae, a carabid subfamily mainly distributed in tropical regions, includes about 772 species, are nocturnal predators, characterized by a specialized mode of chemical defence in adults (together with brachinines they are considered as 'bombardier beetles) and highly derived larvae with unusual and unique habitus (Di Giulio 1999, 2007; Di Giulio and Vigna Taglianti, 2000; Di Giulio *et al*., 2003, 2011; Di Giulio and Moore, 2004; Moore and Di Giulio, 2006; Moore *et al*., 2011). Paussinae includes the following five tribes: two basal tribes, Metriini and Mystropomini, Ozaenini, all with free-living and predatory life style, and two derived tribes, Protopaussini, and Paussini, obligate myrmecophiles.

The monogeneric Metriini, probably sister group of the rest (Di Giulio *et al*., 2003; Di Giulio and Moore, 2004), with only 3 species in North America and 1 in China; Mystropomini, with 2 species endemic to Australia; the Ozaenini, with about 180 species some of them associated with ants (Moore 2008; Moore *et al*., 2011), mainly distributed in Neotropical Region; the monogeneric Protopaussini, with 8 described species restricted to the Oriental Region, and the myrmecophilous Paussini (monophylethic), commonly known as ant nest beetles, with the center of species diversity in Afrotropical Region (572 species) (Nagel 1987; Fattorini *et al*. in press).

The tribe **Paussini** is mainly tropical and subtropical with very few species known in the Palearctic Region (see Nagel, 2003 for an updated list). Only two species are distributed in Europe, *Paussus turcicus* Frivaldszky von Frivald, 1835 and *P. favieri* Fairmaire, 1851. They share striking adaptations, such as greatly modified

antennae (flattened, enlarged, lenticular, globular, concave, elongate etc.), slender or compact bodies, elongate or flattened legs, stridulatory organs and peculiar 'myrmecophilous organs' composed of trichomes (tufts of hairs) connected to exocrine glands for the release of chemical secretions.

The host specificity of Paussini is still matter of debate, since most specimens are collected by light traps and not in the ant's nests, and a limited set of data, not always reliable, is available for the host ants (Luna de Carvalho 1989; Di Giulio *et al*., 2011). In general, they seem to be mainly associated with Myrmicinae (especially with the genus *Pheidole*) and Formicinae (Geiselhardt *et al*., 2007).

Paussine beetles are generally considered as ant parasites since they prey on ants and their broods without a clear benefit for the colonies (Escherich 1907; Le Masne 1961a, 1961b; Geiselhardt *et al*., 2007; Di Giulio *et al*., 2011a). On the basis of their morphological adaptations, two functional types are generally recognized in Paussini: the 'protective' type, characterized by a compact body with hard and smooth surfaces and retractable appendages, that defend the beetle against the ant aggressions; and the 'symphilous' type, characterized by slim bodies with long slender appendages and many trichomes covering the body, that show a full integration in the ant societies. The symphilous type is generally present in the most derived taxa.

Paussini, like other ant parasites, are typically rare insects living in concealed environments which makes it difficult to observe their behavior in nature. Therefore, while they have been extensively studied from a taxonomic point of view, information about their interactions with hosts and their life cycle is limited and largely indirect (i.e. inferred from their structural adaptations) with few ethological observations (Di Giulio and Moore, 2004). Although several attempts have been made to rear Paussini with their host ants, this has proven to be particularly difficult, and promising results have been achieved only for a few *Paussus* species (5 out of 572 Paussini species!) (Geiselhardt *et al*., 2007).

Paussus favieri Fairmaire, 1851

Paussus favieri, guest of the facultatively polygynic ant *Pheidole pallidula* (Nylander, 1849), is one of the two species of Paussini distributed in Europe. It is an Atlanto-Mediterranean species (Nagel, 1987, 2003) present in southwestern France, Spain, Portugal, Morocco, Algeria and Tunisia (Casale *et al*., 1982; Nagel, 1987; Di Giulio *et al*., 2011a). There are some records of *P. favieri* from Sicily and Sardinia (Casale *et al*., 1982), and one from Corsica (Zerche, 1990), however, no specimens have been collected on these islands during the past 100 years.

For over 150 years this species has attracted the intense interest of researchers and collectors, due to its rarity and its bizarre structural adaptations to a myrmecophilous lifestyle. Escherich (1898) reported the first descriptive observations in captivity of *P. favieri* mainly emphasizing its feeding strategy on larvae of the host ants, and some interactive behaviors between beetles and ants (e.g. dragging, grooming, aggressive behaviors). Later, Le Masne (1961a, b, c) reared in captivity *Paussus favieri*, adding valuable and detailed information to the knowledge on its biology. Le Masne mainly focused his observations to the predatory strategy of *P. favieri* while feeding on adults and broods of ants and to the mechanisms of adoption of the beetle inside the nest. According to the observations by Escherich (1899) and Le Masne (1961b) this beetle is readily accepted and fully integrated within the colony without hostility.

Paussus favieri was also object of recent researches. Cammaerts *et at*. (1990) and Cammaerts and Cammaerts (1992) showed that this beetle preferentially follows the pheromone trail produced by the poison glands of its host ant, discriminating these from pheromones of non-host ant species. Additionally, Di Giulio *et al*. (2011a) reared and described the first instar larva of *P. favieri* that, like other *Paussus* larvae, shows remarkable adaptations to a myrmecophilous lifestyle (e.g. shortened and degenerated head capsule, reduced mouthparts, partial atrophy of legs, fused terminal disk), with specialized feeding behaviors that suggest the larvae are fed by the ants through trophallaxis.

However, many gaps are still present in our knowledge on the life history of *P. favieri*, and in general of Paussini.

Aim and objectives

Aim of the present Ph.D. thesis is to contribute to the still limited knowledge about the Paussini, and more in general about the myrmecophiles, analysing from different perspectives (morphological, behavioral, chemical, acoustical), a model species representative of the most derived ant nest beetles, *Paussus favieri*, obligate guest of the ant *Pheidole pallidula*.

In particular, the main objectives of this study are:

1) to clarify the mechanisms underlying host-parasite relationships between *P. favieri* and its host ant *Pheidole pallidula*, analysing the **interspecific and intraspecific behaviors** performed by the beetles inside the ant nests;

2) to investigate the **chemical identity of** *P. favieri* and of its host ant, through a comparative analysis of their cuticular hydrocarbons in order to evaluate which chemical strategy (chemical mimicry, camouflage, insignificance) is adopted by this beetle to deceive the host ant recognition systems;

3) to evaluate the role of the **acoustical communication** of *P. favieri*, both in the relationship with the host ant (acoustical mimicry?) and in the intraspecific interactions (partner attraction, mating behavior);

4) to analyze the fine **morphology** and the ultrastructure of the antennal sensilla in *P. favieri* in order to verify the presence, number, distribution, and possible functions of the sensorial structures involved in the perception of the chemical and acoustical signals, serving the inter- and intraspecific communication.

CHAPTER 1

Behavior of *Paussus favieri* (Coleoptera, Carabidae, Paussini), a myrmecophilous beetle associated with *Pheidole pallidula* (Hymenoptera, Formicidae) [1]

Abstract. Several specimens of the myrmecophilous beetle *Paussus favieri* were reared in ant nests of *Pheidole pallidula*. Their interactions were recorded and all behaviors observed are described. Duration and frequency of five behaviors of *P. favieri* were analyzed with ANOVA and post hoc Tukey tests; these comprised rewarding, antennal shaking, antennation, escape, and 'no contact'. Significant differences in duration and frequency among behaviors were detected. The main result is that the rewarding behavior, during which the beetle provides attractive substances to the host, is performed significantly more frequently than all others. This result strongly supports the hypothesis that the chemicals provided by the beetles and licked by the ants are great importance for the acceptance and the full integration of *P. favieri* in the ant society. This result also suggests that, contrary to previous findings and interpretations, the myrmecophilous strategy of *P. favieri* is very similar to the symphilous strategy described for *P. turcicus*. The occasional interactions of some beetle specimens with the *Pheidole pallidula* queen were recorded, illustrated and discussed, indicating the possibility of a more complex strategy of *P. favieri* involving a chemical mimicry with the queen. In addition, the courtship performed by the beetle is here described for the first time, together with a peculiar 'cleaning' behavior, which we hypothesize functions to spread antennal chemicals over the body surfaces.

1. Introduction

Ant nests are very attractive for many organisms, because they represent well protected and stable environments that are rich in various resources (ants, their brood, stored food, waste materials, etc). In particular, a large number of insects establish relationships

[1] This chapter corresponds to Paper 1 in press.

with ants for a considerable part of their life cycle [1, 2, 3] and are classified as true myrmecophiles [4]. Insect-ant interactions range from commensalism to specialized predation, parasitism and mutualism [1]. The most specialized myrmecophiles are able to deceive the complex communication and recognition systems of the ants, infiltrating their societies and exploiting their resources [1, 4, 5]. These ant parasites represent about 10% (~10,000 species) of known myrmecophilous insects and most are members of Coleoptera, Lepidoptera, Orthoptera and Diptera [6]. They show several refined adaptations (e.g. chemical and morphological mimicry, specialized feeding behaviors, structural modifications) to avoid ant attacks, to be accepted by ants, and to develop and reproduce within ant nests [7].

All members of the ground beetle tribe Paussini (Coleoptera, Carabidae, Paussinae) are myrmecophiles and are considered to be ant parasites [8]. Like many other parasites of ants, they show striking adaptations, such as greatly modified antennae (flattened, enlarged, lenticular, globular, concave, elongate etc.), slender or compact bodies, elongate or flattened legs and peculiar 'myrmecophilous organs' composed of trichomes (tufts of hairs) connected to exocrine glands for the release of chemical secretions.

Paussini (known as 'ant nest beetles') are typically rare insects living in concealed environments which makes it difficult to observe their behavior in nature [7]. Therefore, while they have been extensively from a taxonomic point of view (Fattorini et al. in press), information about their interactions with hosts and their life cycle is limited and largely indirect (i.e. inferred from their structural adaptations) with few ethological observations [9]. Although several attempts have been made to rear Paussini with their host ants, this has proven to be particularly difficult, and promising results have been achieved only for a few species (5 out of the currently recognised 572 Paussini species) [8].

The first observations of paussine behaviors in captivity were reported by Péringuey [10, 11] for *Paussus lineatus* Thunberg, 1781 and *P. linnaei* Westwood, 1833 and, to a lesser extent, for *P. burmeisteri* Westwood, 1838. Other early ethological notes were reported by Escherich [12] for *P. turcicus* Frivaldszky 1835, *P. favieri* Fairmaire, 1851 [13], and *P. arabicus* Raffray 1885 [14]. These authors carefully reported their annotations mainly emphasizing the obligate association of these beetles with the ants

(especially the ant genus *Pheidole*), their feeding strategy on larvae of the host ants, and some interactive behaviors between beetles and ants (e.g. dragging, grooming, aggressive behaviors). According to this first, though limited and speculative set of information gathered in captivity, and to previous anecdotal observations in nature reported by several authors [e.g. 15, 16, 17, 18, 19], Escherich [14] tentatively categorized the strategies of the members of the genus *Paussus* in three main levels of interactions, referring to the Wasmann's [20, 21] myrmecophilous categories: synectrans (e.g. *P. linnaei*), synecoetes (e.g. *P. arabicus* and *P. lineatus*) and symphilous (e.g. *P. turcicus*). Later, Le Masne [22, 23, 24] successfully reared *Paussus favieri*, adding valuable and detailed information to the knowledge on the biology of this species which is a guest of the facultatively polygynic ant *Pheidole pallidula* (Nylander, 1849). Le Masne mainly focused his observations on the predatory strategy of *P. favieri* while feeding on adults and ant larvae [22, 24], and on the mechanisms of adoption of the beetle inside the nest [23]. More recently, the Escherich's [14] classification has been reviewed and updated by Geiselhardt et al. [8], and three different strategies have been identified, exemplified by three *Paussus* species: 1) the strategy of *Paussus arabicus* reported by Escherich [14] is considered the most basal, since the initial contact with the ants triggers their aggression; however, the attacks cease after the contact with the ants [10, 12, 14, 25], and for this reason the authors speculated that a chemical camouflage might occur in this species [8]; 2) the costly strategy of *Paussus turcicus*, which is actively groomed by its host ants, to which the beetle supplies an attractive and possibly rewarding antennal secretion [12, 13]; 3) the strategy of *P. favieri*, which is considered the most derived, since it has no apparent costs. According to the observations by Escherich [13] and Le Masne [23], this beetle is readily accepted and fully integrated within the colony without hostility. It is usually ignored by the ants, only rarely touched, quickly groomed and dragged, and it moves undisturbed within the nest, free to feed on brood and adults. Probably, an advanced chemical mimicry mediates the mechanism of this association [8].

Paussus favieri was also the object of recent researches, being one of the most common species of Paussini in Northern Africa and one of the two species present in Europe. Cammaerts [26, 27, 28] showed that *P. favieri* preferentially follows the pheromone trail produced by

the poison glands of its host ant, discriminating this from pheromones of non-host ant species. Lastly, Di Giulio et al. [7] reared and described the first instar larva of *P. favieri* that, like other *Paussus* larvae, shows remarkable adaptations to a myrmecophilous lifestyle (e.g. shortened and degenerated head capsule, reduced mouthparts, partial atrophy of legs, fused terminal disk), with specialized feeding behaviors that suggest the larvae are fed by the ants through trophallaxis.

To clarify the mechanisms underlying host-parasite relationships between *P. favieri* and its host ant *Pheidole pallidula*, we investigated the interspecific and intraspecific behaviors performed by the beetles inside the ant nests maintained in laboratory. In particular, our aims are: (1) to describe the main behaviors performed by *P. favieri* and its host ant; (2) to analyze the duration and frequency of the behaviors performed by the beetles; and (3) to discuss the possible functional and adaptive significance of the observed behaviors.

2. Materials and Methods

2.1 Material examined and rearing conditions

During an expedition to Morocco (High Atlas Mountains) in May 2010, adults of *P. favieri* were collected under stones, in nests of *Pheidole pallidula* (Fig. 1). Beetles and ants were then transported in the laboratory for behavioral experiments. Each beetle was reared with the ants from the nest in which it was found; when multiple specimens of *P. favieri* were found in the same nest, all specimens were reared together. Ants and beetles were kept under controlled conditions (21 – 24°C; 12h:12h light:dark; 60% humidity) in transparent glass boxes (32 × 22 × 15 cm) lined with a layer of plaster. The boxes were kept open to facilitate observations. The colonies were maintained on a diet of sugar or honey, and fruit flies or moth caterpillars three times per week. Ten colonies were established but we used only five, well-structured colonies with at least 100 nestmates (70% workers, 30% soldiers and queen) for behavioral observations.

Figure 1 - *Paussus favieri* with worker and soldier of *Pheidole pallidula* (Photo by P. Mazzei).

2.2 Descriptions of behaviors

Host-parasite interactions and intraspecific behaviors (cleaning and mating) were observed under natural light and recorded with a NV GS120EG Panasonic video camera for a total of 20 hours. Because beetle manipulation could have unpredictable effects on the host-parasite interactions, beetles and ants were not marked or sexed. For the analysis of the host-parasite interactions we selected 14 beetles for which recording sessions of at least 15 minutes were available.
All behaviors of the beetles and the ants were described and classified into five categories (see Results). The behaviors performed by the *P. pallidula* during the interactions with the beetles were described following the behavioral repertoire suggested by Hölldobler and Wilson [1], Passera and Aron [29] and Sempo and Detrain [30]. Beetle cleaning and sexual behaviors were described after analyzing the videos in slow motion.

2.3 Statistical analyses of behaviors

We statistically analyzed the five behaviors performed by the beetle while interacting with the host ant (see § 3.2). Recording sessions were analyzed using the observation transcription tool EthoLog 2.2 [31] to continuously record the time that the beetle spent performing

different behaviors. We tested whether different behaviors of beetles have significantly different durations, i.e. if there are differences in the amount of time a beetle spends engaged in different behaviors when it interacts with ants. Differences between behavior duration were tested using a main effect ANOVA. A total of 1030 measurements of behavior duration (dependent variable) were analyzed. Because the beetles were not obtained by rearing but were directly collected from ant nests in the field, we have no information about possible inter-individual variation due to genotypic differences, or previous experience with ants, age, days of fasting, etc. Thus, we combined all of these unknown factors into the concept of 'individuality'. To control for this 'individuality', beetles were numbered from 1 to 14 and 'beetle identity' was introduced as a second factor in the ANOVA. Therefore the beetle that which exhibited a behavior, and the type of behavior (classified into five categories, A–E, see results) were used as categorical predictors (factors). Post hoc comparisons were performed using Tukey HSD tests. To determine whether different behaviors were performed more frequently than others, we executed analogous analyses on the recorded frequency of the behaviors. Statistical analyses were performed with Statistica for Windows version 7.2 (StatSoft Inc., Tulsa, OK, USA).

2.4 Scanning Electron Microscopy

Morphological structures of *P. favieri* (Fig. 2) involved in the interactions with host ants and with others conspecifics were studied using a Philips XL30 scanning electron microscope at L.I.M.E. (Interdepartmental Laboratory of Electron Microscopy, University "Roma Tre", Rome). Specimens used for morphological study were kept overnight in a detergent water solution, cleaned by ultrasounds for 15 seconds, rinsed in water, dehydrated through a series of EtOH baths of increasing concentration (70, 80, 90, 95 and 100%), critical point dried (Bal-Tec CPD 030), mounted on a stub (by using self adhesive carbon disks) and sputter-coated with gold (Emitech® K550 sputter coater).

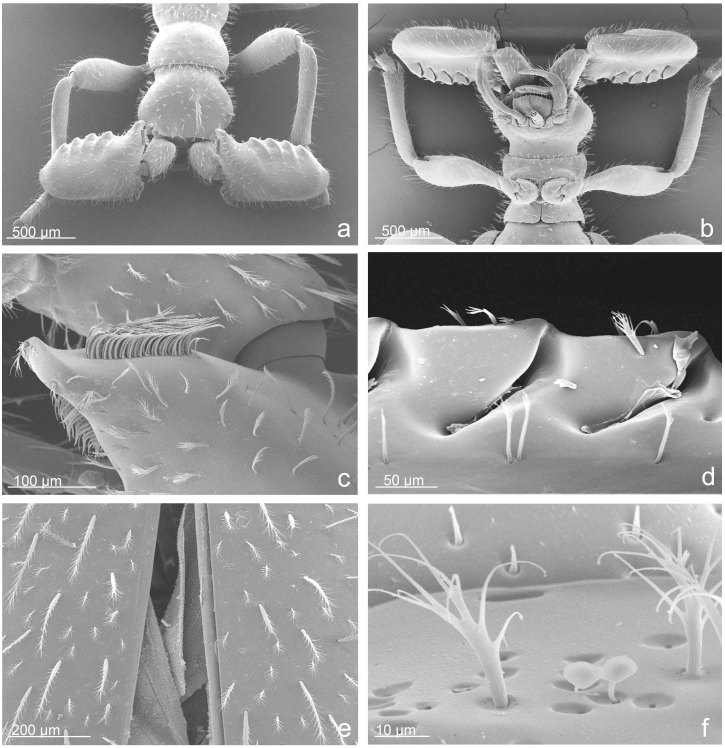

Figure 2 - SEM micrographs of *Paussus favieri*: A) antero-dorsal view of head and thorax; B) ventral view of head and thorax; C) basal spur of the antennal club, dorsal view; D) ventral antennal pockets with visible secretion; E) elytra with modified sensilla chaetica; F) modified sensilla chaetica on head with glandular pores.

3. Results

3.1 General morphology of *Paussus favieri*

The beetle is small (length ~ 4 mm), much bigger than workers of *Pheidole pallidula*, with intermediate dimensions between soldiers and queen (Figs. 1 and 3). The body is slim with slender elongated legs and bulged modified antennae. The colour is light brown, similar to that of soldiers and workers of host ant, with shining, oily appearance. The head is sub-hexagonal with elongate palpi and dark

eyes, bearing dorsally a long medial tuft of trichomes (Fig. 2a-b). The antennae are particularly modified, composed by three joints: 1) a cylindrical and slightly elongated scape; (2) a globular, ring-like pedicel; and (3) a wide 'antennal club' sub-triangular, swollen, and strongly asymmetrical resulting from the fusion of the 9 flagellomeres (Fig. 2a-b). The scape and the antennal club are covered by several modified trichomes, and glandular pores (Fig. 2d), while chemoreceptors are mainly distributed apically. Additionally, the antennal club shows a pointed basal spur with two tufts of trichomes (myrmecophilous organs, see Fig. 2a-c), and ventral pockets (Fig. 2d) where the glandular secretion is stored. The prothorax is elongated, of about the same width of the head, strongly constricted in the middle, without tufts of trichomes. Like the other *Paussus* species, a stridulatory organ is present on the ventral side, composed by finely ridged pars stridens on the hind femora and a plectrum (row of cuticular spines) on the basal part of the abdomen. The elytra are parallel, covered by elongate, branched trichomes. The pygidium is truncate with short fringed trichomes. The ventral side of the body is smooth, without trichomes.

3.2 Description of *Paussus favieri* behaviors interacting with ant host

A. Rewarding. The beetle remains still, while it is antennated and actively licked by ant workers and soldiers (Fig. 2a-b; see video in additional materials). This behavior is generally associated with movements of the beetle's hind legs, either singly or in combination.
B. Antennal shaking. The beetle vibrates the antennae, quickly shaking them forward and backward in the vicinity of the ants. This behavior mostly occurs after a long period of rewarding (see above).
C. Antennation. The beetle moves its antennae in a slow, alternate, vertical way, oriented toward the object of interest. The beetle usually explores an ant's body with the apices of the antennae, which are particularly rich in sensorial structures.
D. Escape. The beetle tries to elude the host ant in a temporary negative reaction. This behavior is not connected with aggression by the host, but rather in most cases it is a consequence of the presence of a high numbers of excited ants antennating and licking the beetle, or after an extended rewarding period.

E. No contact. The beetle does not interact with the ants. This state includes many different activities like exploring, resting, cleaning, interacting with partners, mating etc.

Figure 3 – Interactions between *Paussus favieri* and queen of *Pheidole pallidula* (Photo by P. Mazzei).

Feeding and mating behaviors were observed rarely. The beetle feeds on ant larvae by piercing the integument with its mandibles and carrying around the victim while sucking blood and soft tissues from the abdomen. In these situations, the ants do not react aggressively against the beetle.
Beetles were observed directly interacting with the queen (Fig. 3a-b). In a few cases, the beetles remained in the queen's chamber for some days, antennating and rubbing against the queen's body without any aggressive reaction from the workers.

3.3 Description of *Pheidole pallidula* behaviors interacting with beetles

A. Antennation. The ants touch the beetles with their antennae on all exposed parts of the body, but especially on the beetle's antennae (Fig. 2a-b).
B. Alarm. The ants antennate frenetically and widely open their mandibles, similarly to alarm behaviors performed during dangerous situations [1, 31]. This behavior is rarely observed against beetles but when it is, it is not followed by biting.
C. Licking. The ants lick all exposed parts of the beetle's body that are rich in trichomes (antennae, head, legs, elytra and pygidium) (Fig. 2a, e and f). This licking behavior (Fig. 3a-b) is very similar to the ants' allogrooming behavior [30 and references therein]. The ants spend most time licking the trichomes on the basal spur and the pockets of the antennae (Fig. 2c-d). This activity can be performed simultaneously on one beetle by many ants (workers and soldiers), and it is the reciprocal behavior to 'rewarding' by *P. favieri* (see § 3.2).
D. Dragging. The ants, mostly workers, occasionally bite the antennal club of *P. favieri* and quickly drag the beetle around the nest. The beetles, though much bigger than ants, do not resist being dragged around. In two cases we observed workers dragging a beetle inside the queen's chamber. We also observed workers moving the queen in a similar way to that described by Brown & Traniello [32] for *Pheidole morrisi*.

3.4 Cleaning behavior of *Paussus favieri*

The cleaning behavior is characterized by the following phases (see video in additional materials):
1. **Antennal cleaning**. The fore legs clean the antennae one at time, starting from ventral to dorsal side of antennal surface. In particular, the tarsus and the hairy apical part of tibia rub the apex and the posterior part of the antennal club, the ventral pockets (Fig. 2b and d) and the posterolateral teeth (Fig. 2a), with numerous quick movements. The tarsus also rubs against the whole antennal club, moving laterally from base to apex with slow movements. Additionally, the tibia cleans the dorsal side of antennal surface, with a single movement. During this phase, the antenna is highly movable and it is rotated according to the side to be cleaned.
2. **Head cleaning**. One of the fore legs moves over the head, rubbing the apical tuft of long sensilla (Fig. 2a). This behavior has been rarely observed.
3. **Leg cleaning**. This cleaning is performed mutually by pairs of legs of the same side, the fore against the middle, and the middle against the hind legs. The tarsus and the tibia of one leg slowly rubs the reciprocal leg from the base to the apex. In addition, the tarsi are rubbed together (fore-middle, middle-hind) repeatedly.
4. **Elytral cleaning**. The elytra are cleaned in the antero-posterior direction with slow repeated movements of the middle and hind tibiae and tarsi of the same side. The tarsi of the middle and posterior legs also rub the lateral surface of the abdomen.

3.5 Mating behavior of *Paussus favieri*

The mating behavior of *P. favieri* is characterized by two distinct phases: courtship and copulation (see video in additional materials).
Courtship. Males actively search for females, approaching them by antennal contact (antennal approach) in one of two different ways: (a) with a slow alternate vertical movements of his antennae touch the female's antennae (frontal approach); (b) his antennae touch laterally the side of the female's elytron (lateral approach). After the lateral antennation, the male fore legs are moved up and down, touching the female elytra and pronotum. Female replies by moving her antennae and the hind legs. After this preliminary antennal approach, the male climbs upon the female's body, dorsally positioning himself in the opposite direction of the female, touching his antennae to the apex of the female's abdomen. This dorsal

inverted phase lasts a few seconds; afterwards the male turns 180°, reaching the typical mating dorsal phase. During this phase the partners reciprocally touch their antennae, and the female often moves her hind legs. In the 10 sequences analyzed, the dorsal phase lasts from 5 to 12 minutes and, in a few cases, it was followed by copulation attempts.

Copulation. From the dorsal phase the male of *P. favieri* slides backwards, bends the abdominal apex downward, extrudes the aedeagus and tries to insert it into the female's genitalia. The antennae of the male are frenetically moved up and down. The copulation with complete insertion of genitalia was observed only once. In fact, the female often rejects the male and avoid copulation.

During mating the ants frequently interact with the beetles, antennating them and/or actively licking their antennae and legs.

3.6 Analyses of behaviors of *Paussus favieri* during interactions with its ant host

The following behaviors of *P. favieri* were analyzed statistically: (A) rewarding, (B) antennal shaking, (C) antennation, (D) escape, and (E) no contact. We detected significance differences in the time a beetle spends performing different behaviors (Table 1). Post-hoc Tukey tests showed significant differences between E vs A, B, C and D ($P<0.0001$ in all pairwise comparisons). Individuality was not significant, which indicates that behavioral patterns do not vary significantly among individuals. Differences in the mean duration of different behaviors are shown in Fig. 4.

Table 1 - Results of a main effect ANOVA for values of times spent performing different behaviors by beetles. *d.f.* = degrees of freedom; *SS* = sum of squares; *MS* = mean sum of squares; *F* = Fisher *F*; *P* = probability.

Effect	d.f.	SS	MS	F	P
Individuality	13	6183.000	475.620	0.848	0.609
Behavior	4	27389.200	6847.310	12.201	**0.000**
Error	1012	567925.800	561.190		

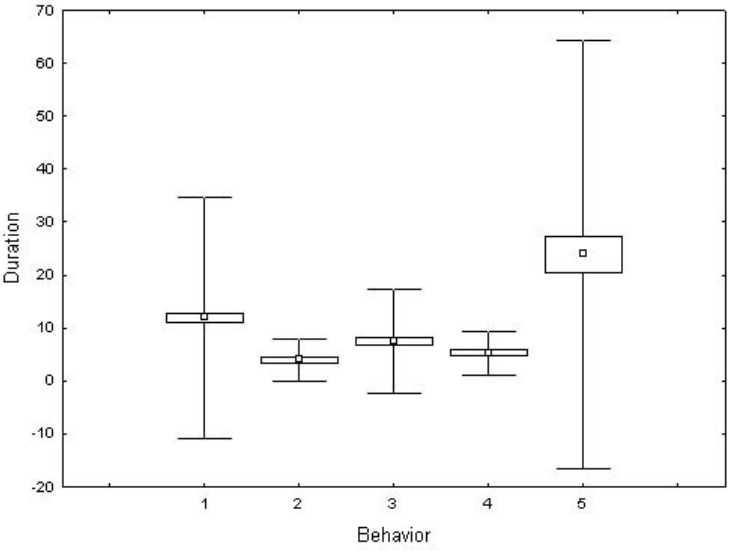

Figure 4 - Differences in the duration of behaviors performed by beetles. Mean values are shown by squares, standard errors as boxes and standard deviations as whiskers. A= rewarding; B= antennal shaking; C= antennation; D= escape; E= no contact.

We found significant differences for the frequencies with which different behaviors are performed (Table 2). Post-hoc Tukey tests showed significant differences for A vs B, C, D and E ($P<0.0001$) and for C vs B and D ($P<0.05$).
Differences in the mean values of frequencies of different behaviors are shown in Fig. 5

Table 2 - Results of a main effect ANOVA for values of frequency of different behaviors by beetles when interacting with ants. *d.f.* = degrees of freedom; *SS* = sum of squares; *MS* = mean sum of squares; *F* = Fisher *F*; *P*= probability.

Effect	**d.f.**	**SS**	**MS**	**F**	**P**
Individuality	13	643.238	49.480	1.027	0.437
Behavior	4	4498.345	1124.586	23.336	**0.000**
Error	66	3180.655	48.192		

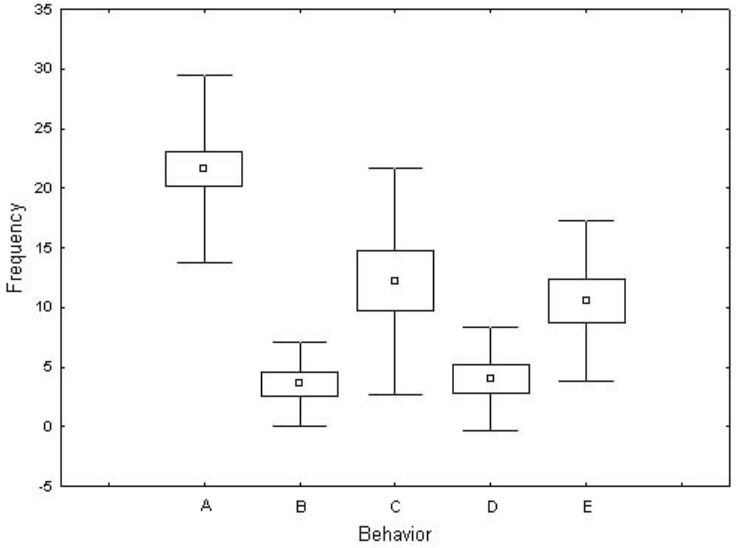

Figure 5 - Differences in the frequencies of behaviors performed by beetles. Mean values are shown by squares, standard errors as boxes and standard deviations as whiskers. A= rewarding; B= antennal shaking; C= antennation; D= escape; E= no contact.

4. Discussion

According to Wasmann [33, 34], two defensive structural types are generally recognized in myrmecophile morphology: the 'protective' type, characterized by a compact body with hard and smooth surfaces, and retractable appendages; and the 'symphilous' type, characterized by slim bodies with long slender appendages and many trichomes covering the body and/or crowded in myrmecophilous organs [8, 36]. These body forms suggest different strategies for entering the nests and for avoiding ant attacks. Both body types are present in the Paussini, sometimes with intermediate forms, with the symphilous type generally present in the most derived taxa that are considered to be the well-integrated into the ant colony [1, 36]. *Paussus favieri* is clearly assignable to the latter type, showing all the distinctive characters noted above. Our observations confirm that *P. favieri* is fully integrated in the host ant society since almost no

aggressive behaviors against the beetles were observed. On the contrary, ants were strongly attracted by the beetle's secretions.

The results of our statistical analyses show that beetles and ants spend a significantly longer amount of time not interacting (no contact, E) than the time they spend interacting with one another in a specific behavior. The state of no contact (E) can be the effect of a temporary withdrawal of the beetle, or the absence of caring by the ants. This is an expected result, since it is reasonable that the beetle spends most time in a number of activities that do not involve host interactions (i.e. exploring, mating, cleaning, resting, etc.).

Concerning the behaviors performed by the beetle during the interactions with its host ants, the analysis of duration showed that rewarding (A), antennal shaking (B), antennation (C) and escape (D) are performed for similar amounts of time. However, it is notable that frequency of rewarding behavior (A) is significantly greater than that of all other behaviors. During the rewarding behavior, *P. favieri* is antennated and actively licked by the ants, especially near the antennal symphilous organs (Fig. 2c). This is consistent with the fact that the primary role of the highly modified antennae of *P. favieri* is glandular, producing substances that are highly attractive to the ants [37]. These substances are mostly stored inside the antennal pockets (Fig. 2d). The chemical nature of this secretion is unknown, but it seems to be important for the acceptance and survival of the beetles within the ant nest [37] and for the success of the parasitic interaction. It has been speculated that, similar to other social parasites [1, 38], the chemicals secreted by Paussini beetles may have an appeasing function [8, 34]. Another hypothesis is that these substances provide a protective or rewarding food for the ants and their brood [8]. The rewarding behavior is generally associated with movements of the beetle's hind legs, an action possibly connected to the emission of stridulations. The high frequency of the rewarding behavior recorded in our experiments is quite in contrast with the previous observations by Escherich [13] and Le Masne [23], who reported that the ants only occasionally groom the beetle (see Introduction). According to our observations, the myrmecophilous strategy of *P. favieri* seems very similar to that of *P. turcicus* [12, 13], and the supposition that there is a more derived (less costly) level of integration for *P. favieri* [8] seems unjustified.

The quick shaking of the antennae (antennal shaking (B)), not noted by Le Masne and never recorded in other Coleoptera, has has been

occasionally observed in another species of *Paussus* [15]. Our observations suggest that antennal shaking might be correlated with the glandular activity of the antennae, facilitating the spread of the viscous exudates from the antennal surface, or, most probably, with the spray of volatile allomones whose presence needs to be confirmed.

The antennation behavior (C) was described by Le Masne [22, 24], who interpreted it as a precursor to predation. Le Masne [24] observed that through antennation the beetle finds the ant's abdomen. Once found, the beetle pierces the abdomen with its sharp mandibles and feeds on the ants' hemolymph. However, in the videos analyzed for the present work we never observed predation following the antennation behavior.

The occasional observations of some beetles interacting with the queen (Fig. 3a-b), also for a prolonged time, seems to be of particular interest. We hypothesize that the physical interaction could supply a chemical queen camouflage to the beetle and/or the beetle could spread some of its attractive substances on the queen's body. In both cases a chemical combination of beetle and queen odors could be reached, resulting in a deception of the nestmates, allowing the beetle to achieves a higher social status inside the nest. The dragging of *P. favieri* inside the nest by *Pheidole pallidula* workers and soldiers, is a behavior usually deserved to the queen, and could be related to this possible mimicry. However, further research are required to confirm that this is a regular interaction, and that an exchange of cuticular hydrocarbons or other substances is involved.

The cleaning and mating behaviors performed by *P. favieri* inside the nest of *P. pallidula* have been observed and described in this work for the first time. Péringuey [10] mentioned a similar 'brushing' behavior by fore and hind legs performed by males of *P. lineatus* after copulation. The complex cleaning behavior of *P. favieri* is quite different from the simple cleaning of Carabidae [39] which mainly involves rubbing the comb organ of the fore legs (a row of spines positioned in an emargination of the inner edge of the fore tibiae) against antennae and mouthparts. In fact, the typical comb organ of ground beetles is vestigial or absent in Paussini [39, 40]. In *P. favieri* the antennae show a primary glandular function [37] secreting a great amount of attracting substance. We interpret the rubbing of the fore legs against the antennae and then against middle and hind legs, head, elytra and abdomen, as a means of

spreading antennal substances throughout the body. This is also supported by the fact that the ants actively lick not only the antennae but also head, legs and elytra, suggesting that the attractants are present also on these body parts.

Little is known about the sexual behavior of Carabidae [41, 42], while no information is available for the Paussini except for a brief note of Péringuey [10] on *P. lineatus*. In this species the male fixes his mandibles in the prothoracic excavation of the female, and, with the hind legs, pulls the abdominal apex of the female towards him; in order to strengthen his position on the female's back, the male passes its antennae under the female, keeping this position for several hours. Serrano et al. [43] observed in Portugal two specimens of *P. favieri* in copulation in an ant nest of *Pheidole pallidula,* confirming that this beetle mates inside the colony, as is reported for other myrmecophilous beetles [44]. In captivity we observed the specimens of *P. favieri* mating in the ant nests several times and for a long duration. The pre-copulatory behavior is carried out through the exchange of tactile signals by antennae and legs, though it cannot be excluded that chemical signals are also involved. Unlike observations of *P. lineatus* [10], in both pre-copulatory and copulatory behaviors the mandibles are not used by *P. favieri*, while the dorsal positon is maintained only by the male's legs. Of particular interest is the presence of an 'inverted' dorsal phase (not noted in *P. lineatus*) that may be unique among Carabidae.

Our experiments also suggest that acoustic signals are probably exchanged during the pre-copulatory behavior, since the female has been observed repeatedly moving the hind legs, a behavior possibly connected to the emission of stridulations (see § 3.2). However, the actual role of the acoustical communication in intra- and inter-specific behaviors is still unknown.

In conclusion, the importance of the rewarding behavior confirms the primary role of the antennal secretions, possibly spred by a complex 'cleaning' behavior, for the successful acceptance and integration of *P. favieri* inside the host colony. The identification of the secretions would be very important to verify their appeasing/rewarding properties, providing a more complete understanding of the myrmecophilous strategy of *P. favieri* and of other members of this tribe.

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SUPPLEMENTARY MATERIAL

Additional Materials may be found in the electronic Appendix A.

CHAPTER 2

Chemical mimicry of the ant nest beetle *Paussus favieri* (Carabidae, Paussinae), associated with *Pheidole pallidula* (Formicinae, Myrmicinae).[2]

1. Introduction

The ability to recognize nest-mates from intruders (non nest-mates) is essential for maintaining the cohesion and survival of the social insect colonies (Hölldobler and Wilson, 1990). The recognition cues are mainly chemical (Hölldobler 1995), mostly represented by a peculiar blend of cuticular hydrocarbons that works like a 'chemical signature' for the members of the same colony (Howard and Blomquist, 2005), as suggested by recent studies on bees (Breed 1998, Chaline *et al*., 2005), wasps (Dani *et al*., 2001), ants (Hefetz 2007; d'Ettorre and Lenoir, 2010; Bos *et al*., 2010; Guerrieri *et al*., 2009) and termites (Dronnet *et al.*, 2006).
Many animals, and especially insects, are strongly attracted by the valuable resources (eggs, larvae, pupae, adults and stored food) present in the ant colonies, and several adaptive strategies (structural, behavioral, chemical, acoustical etc.) have been evolved by the so called myrmecophiles (ant's symbionts) to avoid or deter ant attacks in order to circumvent the host recognition systems (Lenoir *et al.*, 2001; Geiselhardt *et al*., 2007). Recent estimates suggest that about 10,000 myrmecophilous species, such as butterflies, crickets, beetles and flies, are specialized ant parasites (Thomas *et al.*, 2010). They are able to imitate the ant cuticular hydrocarbon profiles corrupting the honest signals of their host (Hölldobler and Wilson, 1990; Dettner and Liepert, 1994; Lenoir *et al*., 2001). This result can be achieved through either an active synthesis of the hydrocarbons (chemical mimicry) (*sensu* Dettner and Liepert, 1994; Howard *et al.*, 1990; Orivel *et al*., 2004; Geiselhardt *et al*., 2006; Witte *et al*. 2008), or by passively acquiring the chemical profile (chemical camouflage)

[2] This chapter corresponds to Paper 2, in preparation.

(*sensu* Dettner and Liepert, 1994). Camouflage can be achieved by physical contact (Vander Meer and Wojcik, 1982; Akino *et al*., 1996; 2002; Elmes *et al.*, 2002) or by host larval consumption (Dinter *et al.*, 2002; Pierce *et al*., 2002). Another strategy used by the parasites is to refrain from expressing any identification cue (chemical insignificance), in order to become chemically "invisible" to the ants (Lenoir *et al*., 2001; Witte *et al.*, 2008). In holometabolous insects, more than one strategy can be adopted by the same species in different semaforonts (larva, pupa, adult) or during the different phases of the integration within the ant society (Akino *et al*., 1999; Schönrogge *et al*., 2004).

The knowledge about the chemical strategies of the myrmecophilous beetles is still scarce, most works being focused on their morphological and behavioral adaptations (Hölldobler and Wilson, 1990; Cammaerts *et al*., 1989, 1990; Cammaerts and Cammaerts, 1992; Quinet and Pasteels, 1995). The cuticular hydrocarbon profile has been analysed in comparison with that of the host ant only in a few beetle species, only in *Thalasssa saginata* (Coccinellidae) larvae and pupae being demonstrated a chemical mimicry with *Dolichoderus bidens* broods (Orivel *et al*., 2004). Three other beetle species, instead, adopt a chemical camouflage to integrate in host ant colony: *Myrmecaphodius excavaticollis* (Scarabaeidae) associated to *Solenopsis* spp. (Vander Meer and Wojcik, 1982); *Zyras comes* (Staphylinidae) and *Diaritiger fossulatus* (Pselaphidae) both associated to *Lasius fuliginosus* (Akino *et al*., 2002). No indication of chemical mimicry or camouflage has been found in three rove beetles species (Staphilinidae) of the genus *Pella* associated with *Lasius fuliginosus* (Stoeffler *et al*., 2011), while *Trachidonia leptogenophyla* (Staphilinidae) (Kistner et al., 2003) was reported to match (without any discussion about the chemical strategy) the host chemical signature of the army ant *Leptogenys distinguenda* (Witte *et al*., 2008) Recently a chemical camouflage has been reported for two myrmecophagous ground beetles: in larvae of *Termophilous sexmaculatum* (Dinter *et al*., 2002) and in adults of *Siagona europaea* (Talarico *et al*., 2009), both predators of several ant species.

Among Carabidae, all members of the tribe Paussini (subfamily Paussinae) are highly specialized myrmecophiles accepted and integrated in the ant societies, mainly associated with Myrmicinae (especially with the genus *Pheidole*) and Formicinae (Geiselhardt *et*

al., 2007). The host specificity of Paussini is still matter of debate, since most specimens are collected by light traps and a few data, not always reliable, are available for the host ants (Luna de Carvalho 1989). Paussine beetles are generally considered as ant parasites since they prey on ants and their broods without a clear benefit for the colonies (Escherich 1907; Le Masne 1961a, 1961b; Geiselhardt *et al*., 2007; Di Giulio *et al*., 2011a). The present knowledge about myrmecophilous adaptations, relationships with host ants, behavior, and life cycle of these beetles is still very lacking, and has been recently summarized by Nagel and collaborators (Geiselhardt *et al*., 2007). As far as known, different levels of integration in an ant colony can be exhibited by adults Paussini (Reichensperger 1948) assignable to two main types of integration exemplified by: 1) the strategy of *Paussus arabicus*, considered the most basal since the initial contact with the ants trigger their aggression; however, the attacks cease after the contact (Escherich 1898, 1907; Péringuey 1833; Raffray 1886); it has been speculated that a chemical camouflage can occur in this species (Geiselhardt *et al*., 2007); 2) the strategy of the only two European species, *P. turcicus* (Escherich 1898, 1899) and *P. favieri* (Escherich 1899; Le Masne 1961a, b, c; Maurizi *et al*., in press), readily accepted and fully integrated in ant societies, that involves the costly supplying of attractive and possibly rewarding antennal secretion to the ants. It was assumed that an advanced chemical mimicry could mediate the mechanism of this association (Geiselhardt *et al*., 2007). However, currently, no chemical investigation exploring the possible congruency between the hydrocarbon profiles of Paussini and host ants is available.
Aim of the present study is to identify and compare the cuticular hydrocarbon profiles of *Paussus favieri* Fairmaire 1851, and its host ant *Pheidole pallidula* (Nylander, 1849) from a Moroccan population, in order to evaluate which chemical strategy is adopted by this myrmecophilous beetle to deceive the host ant recognition system.

2. Materials and methods

2.1 Collection data

During spring 2010 we collected several specimens of *Paussus favieri* and its host ant *Pheidole pallidula* from natural colonies,

under stones, located in open and sunny fields on the High Atlas Mountains in Morocco. A total of 25 *P. favieri*, 50 soldiers, 50 workers, 20 larvae and pupae, and 1 queen of *P. pallidula* were collected and analysed from five colonies. We found between 4 and 6 beetles per ant colony.
Additionally, in two colonies of *Pheidole pallidula* parasitized by *Paussus favieri*, a few specimens of *Paussus olcesii* (Fairmaire, 1856) were collected, and only 1 was analysed for the present work. It is worth noting that the young adults of *P. olcesii* were collected by digging deeply under the nests, and not together with the ants like the *P. favieri* specimens. For these reasons it is possible that they pupated in isolated chambers under the nest, and they have not been in contact with the host ants as adults.

2.2 Chemical analysis

The cuticular compounds of whole ants and beetles, killed by freezing, were extracted by immersing: for the beetles and ant queen, each individual in 200 µl of pentane for 10 min; and for the ants, 10 individuals (workers, soldiers and larvae) in 200 µl of pentane for 10 min. The extract was then transferred into a 200 µl glass insert and the solvent allowed to evaporate before storing the extracts at -20°C. Before chemical analysis, the extracts were re-dissolved in 50 µl of pentane, and 2 µl were injected into an Agilent Technologies 6890N gas-chromatograph, equipped with a HP-5MS capillary column (30 m x 250 µm x 0.25 µm), a split-splitless injector; carrier gas: helium at a flow rate of 1 ml/min^{-1}. The GC was coupled with a 5975 Agilent Technologies Mass Spectrometer with 70eV electron impact ionization. After an initial hold of one minute at 70ºC, the temperature of the column rose to 200ºC at 30ºC/min and then finally to 320ºC at 5ºC/min, held for 10 minutes. Compounds were identified on the basis of their retention time and mass spectra.

2.3 Statistical analysis

The areas under GC peaks that were in common between *P. favieri* and the ants (workers and soldiers) were integrated and transformed according to Aitchison (1986). The formula applied was:

$$Z_{i,j} = \mathrm{Ln}\,[Y_{i,j}/\, g(Y_j)]$$

where $Y_{i,j}$ is the area of peak *i* for the individual *j*, $g(Y_j)$ is the geometric mean of the areas of all peaks for individual *j*, and $Z_{i,j}$ is the transformed area of peak *i* for individual *j*. These transformed peak areas were used as variables in a principal component analysis (PCA) to investigate the possible similarity between ants and beetles. To analyse colony specificity in the cuticular profile of the beetles, we used another PCA, only on the beetles' data, followed by a discriminant analysis (DA). Unfortunately, for technical problems the same analysis was not performed with the ants. For all statistical analyses we used Statistica v.7.0 for Windows (StatSoft Inc., Tulsa, OK, U.S.A.).

3. Results

3.1 Chemical analysis of *Paussus favieri* and its host *Pheidole pallidula*

The cuticular chemical profile of the beetles is characterized by 24 regularly occurring hydrocarbons, principally methyl branched and linear alkanes, with chain length between 17 and 31 carbon atoms, although one long chain unsaturated hydrocarbon was also identified ($C_{31:1}$) (Table 1). The ants show a more complex cuticular profile, characterized by 55 hydrocarbons, methyl branched and linear alkanes ranging between 17 and 34 carbon atoms, including several unsaturated hydrocarbons (Table 1). Figure 1 shows the qualitative difference between the typical cuticular profile of beetles and ants.
We performed a PCA on the peaks that were in common between the ants and the beetles, these were 22 in total (including $C_{31:1}$) (Table 1). The PCA analysis produced four principal components with eigenvalues higher than 1, explaining the 80,50% of the original variance. A plot of the first two factors of the PCA (explaining together the 66,6% of the variance, (Fig. 1) shows that the beetles and the ants are clearly distinct, even when considering only the hydrocarbons that the two species have in common. The difference is based on relative proportions of some hydrocarbons, especially 11-MeC_{23}, C_{25}, 11+13-MeC_{27} and 5,13diMeC_{27}, which load very highly (> 0.9) on factor 1. Looking at the relative proportions of the hydrocarbons common to the ants and the beetles, it appears clearly that some compounds are more abundant on the beetles (Fig. 2), in particular, 11-MeC_{23} (beetles 29,44 ± 4,78; ant workers 1,64 ± 0.86,

mean ± SD), C_{25} (beetles 7,21 ± 2,57; ant workers 2,97 ± 1,25), C_{27} (beetles 14,38 ± 9,91; ant workers 5,39 ± 3,49), and 11+13-MeC_{27} (beetles 17,81 ± 4,29; ant workers 5,22 ± 2,10). While in the ants two compounds are clearly more abundant 3-MeC_{27} (ant workers 6,31 ± 2,27; beetles 1,06 ±0,32) and $C_{31:1}$ (ant workers 22,45 ± 8,10; beetles 0,85 ± 0,33).

Table 1 – List of cuticular hydrocarbons present in the chemical profile of *Paussus favier*i and its host *Pheidole pallidula,* both workers and soldiers, + = presence; - = absence.

Peak	RT	Identification	*P. favieri*	Worker	Soldier
1	**7.125**	**x,y-diMeC17**	+	+	+
2	**7.406**	**5,7-diMeC17 + 7,9-diMeC17**	+	+	+
3	**8.763**	**5,7-diMeC19 + 7,9-diMeC19**	+	+	+
4	8.868	unknown	-	+	+
5	9.148	?triMeC19	+	+	+
6	10.953	unknown	+	-	-
7	**11.463**	**5,9-diMeC21 + xMeC21**	+	+	+
8	**11.613**	**C22**	+	+	+
9	**12.856**	**C23**	+	+	+
10	**13.360**	**11-MeC23**	+	+	+
11	**13.580**	**unknown**	+	+	+
12	**14.101**	**unknown**	+	+	+
13	**15.489**	**C25**	+	+	+
14	**15.952**	**11+13MeC25**	+	+	+
15	**16.455**	**3MeC25**	+	+	+
16	**16.817**	**C26**	+	+	+
17	**16.922**	**unknown**	+	+	+
18	17.263	11+13-MeC26	+	-	-
19	17.832	C27:1	-	+	+
20	**18.193**	**C27**	+	+	+
21	**18.624**	**11+13-MeC27**	+	+	+
22	18.816	5-MeC27	-	+	+
23	**19.126**	**3-MeC27**	+	+	+
24	**19.214**	**5,13-diMeC27**	+	+	+
25	19.917	?triMeC28	-	+	+
26	20.273	4-MeC28	-	+	+
27	20.494	C29:1	-	+	+
28	**20.764**	**C29**	+	+	+

29	20.973	11,15-diMeC29	-	-	+
30	**21.169**	**11+13+15-MeC29**	+	+	+
31	21.542	11,17-diMeC29	-	+	+
32	21.717	3-MeC29	-	+	+
33	21.792	5,15-diMeC29	-	+	+
34	22.035	C30	-	+	+
35	22.118	10,12,14-triMeC30	-	+	+
36	22.451	10,16-diMeC30	-	+	+
37	22.784	4,6,12-triMeC30	-	+	+
38	**23.022**	**C31:1**	+	+	+
39	23.127	C31:1	-	+	+
40	23.279	C31	-	+	+
41	23.478	9,13-diMeC31	-	+	+
42	23.770	9,13,17-triMeC31	-	+	+
43	23.952	13,17-diMeC31	-	+	+
44	24.192	3-MeC31	-	+	+
45	24.251	5,13-diMeC31	-	+	+
46	24.554	5,9,13-triMeC32	-	+	+
47	24.946	8,12-diMeC32	-	+	+
48	25.197	8,12,14-triMeC32	-	+	+
49	25.468	C33:1	-	+	+
50	25.862	4,8-diMeC33+ x,14-diMeC32	-	+	+
51	26.114	9,13,15-triMeC33	-	+	+
52	26.877	10,14-diMeC34	-	+	+
53	27.224	8,12-diMeC34	-	+	+
54	27.486	8,12,16-triMeC34	-	+	+
55	27.725	x,y-diMeC34	-	+	+

*Hydrocarbons in common between beetles and ants are in bold.

We then analysed the chemical profile of beetles alone, to see whether they would cluster according to the colony of origin. A PCA performed on the beetle's hydrocarbons (24 peaks) produced five principal components with eigenvalues higher than 1, explaining 86.2% of the original variance. We then performed a discriminant analysis based on factors extracted by the PCA (we included 7 factors, explaining together the 93.3% of the variance). The DA clearly differentiated the chemical profiles of the beetles according to their colony of origin (5 different ant colonies, Wilk's λ = 0.00141, $F_{28,51}$ = 9.5825, $p < 0.00001$; Fig. 3). All individual beetle's profiles were 100% correctly classified to their group (colony of origin).

The cuticular profile of the larvae of *P. pallidula* was only partially identified and qualitative comparisons were not always possible, because of low concentrations of several hydrocarbons and some impurities present in most of larval samples. For these reasons the statistical treatment was not performed. However, a descriptive analysis was performed to serve as base for future investigations. We were able to identify with certainty 41 hydrocarbons of the larval profile, characterized by a blend of linear alkanes (from C_{22} to C_{29}), methyl branched alkanes, and also four long chains of unsaturated hydrocarbons ($C_{25:1}$, $C_{27:1}$, $C_{29:1}$ and $C_{31:1}$). The hydrocarbons in common with *P. favieri* are 15, mostly linear alkanes (C_{27} : 6%, C_{29} : 27%) and few methyl branched alkanes (Fig. 4a-b see in additional material).

The chemical profile of queen of *P. pallidula* is partially identified for some impurities present in the sample. For this reason the statistical treatment was not performed. The descriptive analysis show that the profile is characterized by 32 hydrocarbons, linear alkanes and methyl branched alkanes, chain length between C_{17} and C_{34} carbon atoms, and also two long chains of unsaturated hydrocarbons ($C_{27:1}$ and $C_{29:1}$). The hydrocarbons in common with *P. favieri* are 22, 7 linear alkanes and 15 methyl branched alkanes. The most aboundant are C_{25} (7%), C_{27} (21%), 3MeC_{27} (7%) and C_{29} (7%) (Fig. 5a-b see in additional material).

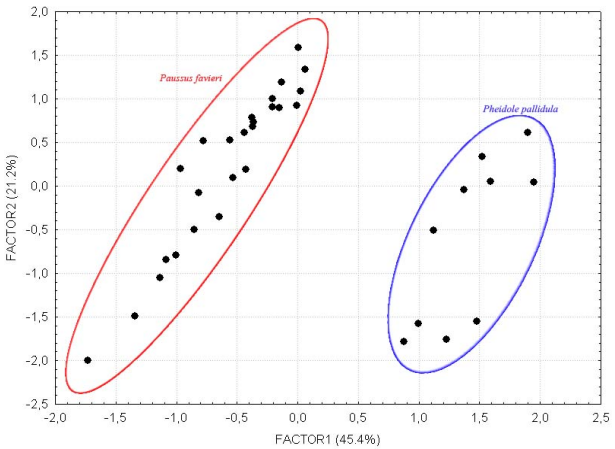

Figure 1 – Plot of the first two factors of the PCA, based on 22 peaks in common between the ants and the beetles.

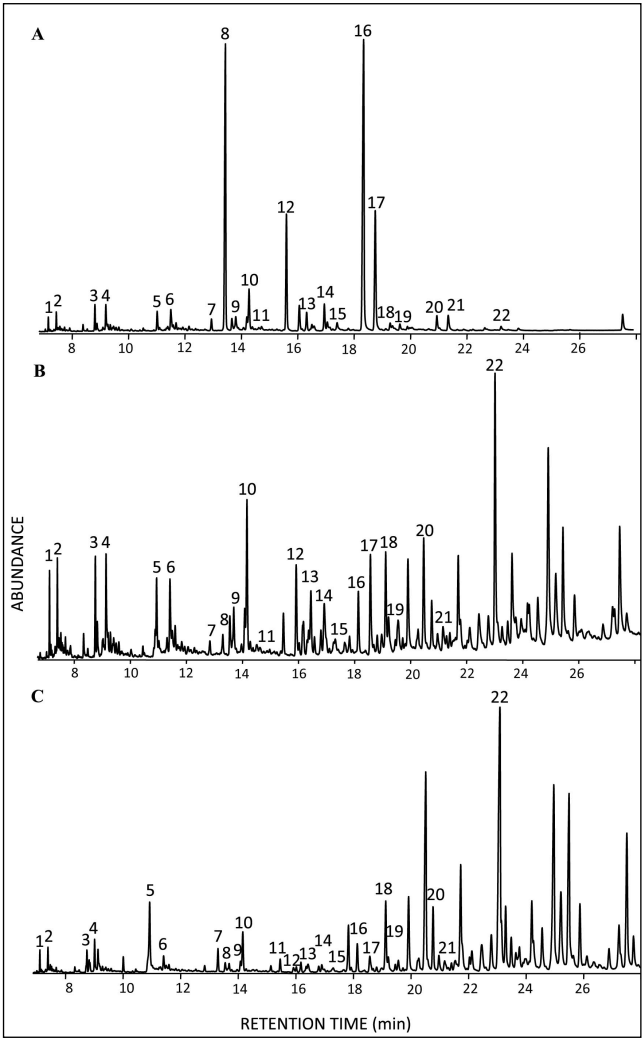

Figure 2 – Gas chromatogram of the cuticular hydrocarbon profile of: A. *Paussus favieri*; B. workers of *Pheidole pallidula* and C. soldiers of *Pheidole pallidula*. The peaks in common among beetles and castes of ant are marked with numbers.

3.2 Chemical analysis of *Paussus olcesii*

The interesting co-occurrence of two myrmecophilous species of *Paussus* in the same nest of *Pheidole pallidula* brought us to investigate the chemical profile of *Paussus olcesii*, though only one specimen was available for such analysis.
The prelimary descriptive result shows that the hydrocarbon profile of *P. olcesii* is characterized by 46 compounds, composed principally by linear alkanes, methyl branched alkanes, with chain length between 17 and 31 carbon atoms, and including three unsaturated hydrocarbons ($C_{23:1}$, $C_{25:1}$ and $C_{31:1}$) (Fig. 6a-b see in additional material). In Fig. 6b is clearly evident that some hydrocarbons are more abundant than other, in particular C_{23} (21% of the whole spectrum area), 11-MeC_{23} (6%), C_{24} (10%), 11+13MeC_{24} (5,50%), $C_{25:1}$ (8%), C_{25} (6,50%), 11+13MeC_{25} (8%), 9,13-diMeC_{25} (5%), and 3-MeC_{25} (4,50%). The pattern of the cuticular hydrocarbons of *P. olcesii* is quite similar to that of *P. favieri*, with 21 hydrocarbons in common, out of 19 hydrocarbons in common with workers and soldiers of *P. Pallidula.*

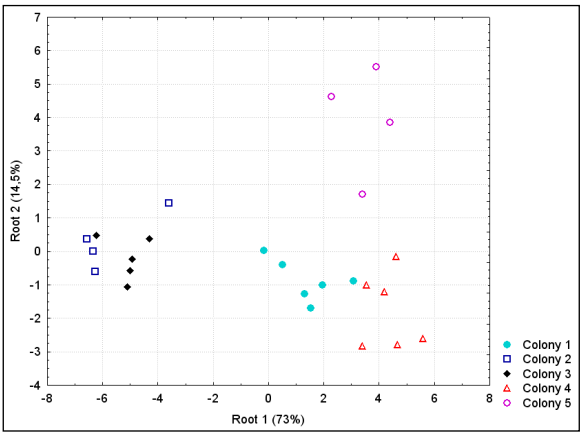

Figure 3 – Plot of DA bases on the factors extracted by the PCA performed on the beetle's hydrocarbons (24 peaks).

4. Discussion

In insects the primary function of the cuticular hydrocarbons (CHCs) is to act as a protective barrier against dehydration and microorganisms (Lockey 1988), while secondarily this layer have assumed a central role for the recognition system (Akino *et al*., 2002), the complex mixture of compounds (linear alkanes, methyl-branched alkanes, and alkenes) being highly species-specific (Howard 1993; Howard and Blomquist, 2005). In social insects, especially in ants, the pattern of CHCs can vary both qualitatively among species and quantitatively among different colonies of the same species, and for this reason it is used to discriminate between nestmates and intruders (Guerrieri *et al*., 2009; Lenoir *et al*., 1999, 2001). However, the role of different classes of CHC in social recognition system remains a controversial issue (Dani *et al*., 2001; Schönrogge *et al*., 2004; Chaline *et al*., 2005; d'Ettorre and Moore, 2008), not all the compounds of the blend representing true chemical signals (Lenoir *et al*., 2001; d'Ettorre and Moore, 2008). For example, branched or unsaturated hydrocarbons seem to be more informative than linears in some ant species (Akino *et al*., 2004; Martin *et al*., 2008), while in others linear alkanes would play a key role in the recognition (e.g. Greene and Gordon, 2007).

Our analyses show that the cuticular profile of *Pheidole pallidula* (workers and soldiers) is complex, composed by 55 hydrocarbons, most of them methyl-branched alkanes, with one long chain unsaturated hydrocarbon ($C_{31:1}$) extremely abundant. The profiles of larvae and queen of *P. pallidula*, though not statistically analyzed, show a chemical pattern very similar to that of the other castes, workers and soldiers (Figs. 5 and 6). On the contrary, *Paussus favieri* shows a simplified cuticular profile, characterized by 24 hydrocarbons, mostly linear and methyl-branched alkanes, with four main peaks (11-MeC_{23}, C_{25}, C_{27}, and 11+13-MeC_{27}). The comparison of the chemical profiles indicates that 22 (15 in ant larvae) out of 24 hydrocarbons (92%) of the beetles are shared with those of the ants. However, the relative proportions of the compounds in common are very different, and for this reason the PCA analysis shows that the chemical identities of beetles and ants (workers and soldiers) are clearly distinct. The congruence of these results with the different

chemical strategies usually proposed for myrmecophiles (camouflage, insignificance, chemical mimicry) is analized in the following.

1. The chemical camouflage has been indicated as better explain the observed chemical pattern in most studies on myrmecophilous beetles (see Introduction). However, even though *P. favieri* feeds on ant larvae and adults of *P. pallidula* (Le Masne 1961a, c), a chemical camouflage can be excluded to be the main myrmecophilous strategy, considering that only a subset of the ant cuticular hydrocarbons are shared with the beetle, and their different relative abundance does not allow a matching point-by-point of the observed peaks, like for example in *Zyras comes* (Staphylinidae) and *Diaritiger fossulatus* (Pselaphidae) showing a perfect overlap of their cuticular profiles with that of *Lasius fuliginosus,* achieved exchanging the cues by physical contact with the workers (Akino *et al*., 2002).

2. The chemical insignificance was never recorded in myrmecophilous beetles, but it was only found in social parasitic ants (*e.g.* Lenoir *et al*. 2001). Usually very few CHCs are present in these species, or their amount is so low that the receivers are unable to detect them. Interpreting the strategy of *P. favieri* as insignificance, we should hypothesize that the 22 hydrocarbons shared with the host ants (43% of the ant's CHCs) are not employed as recognition cues, and this seems rather unlikely. Additionally, the relative abundances of these compounds is significant, with at least four of them (see § 3.2) highly represented. Finally, behavioral observations in captivity (Maurizi *et al*. in press) show that the beetle is not ignored by the ants; actually, the ants are strongly attracted by the beetles and frequently contact them. For all these reasons a chemical insignificance does not seem the myrmecophilous strategy of *P. favieri* .

3. The chemical mimicry involves the active biosynthesis of the host cuticular hydrocarbons, a process that is apparently very unlikely to take place in phylogenetically distant taxa. However, according to Dettner and Liepert (1994), a prolonged coevolution can explain the ability to synthesize the same compounds even in unrelated taxa. In fact, this strategy has been recorded in a few ant parasites: in hoverfly larvae of *Microdon piperi* (Howard *et al*., 1990), in larvae and pupae of ladybird *Thalassa saginata* (Orivel *et al*., 2004), and in lycenid caterpillars of *Maculinea rebeli* (Akino *et*

al., 1999). In this last example, it was observed a partial qualitative congruence between the profiles of parasites (12 CHCs) and host larvae (26 CHCs), that share 10 CHCs (83% of the caterpillar's CHCs and 38% of the ant's CHCs). The comparison between the cuticular profiles of *P. favieri* and *P. pallidula* shows a similar pattern, sharing 92% of the beetle's CHCs, though differences in relative abundances of these compounds are present between ant and beetle. This quantitative difference, also detected in the *Maculinea rebeli-Myrmica schencki* system, could be explained by the phylogenetic distance between mimic and model, since it is unlikely that insects of different orders can follow similar physiological pathways that bring exactly to the same chemical result. For this reasons we are of the opinion that a chemical mimicry with active secretion of cuticular chemicals better explain the symbiotic interaction between *P. favieri* and *P. pallidula*.

Observations carried out in captivity show that *P. favieri* is continuously touched and actively licked by workers and soldiers of *P. pallidula*, especially on the antennae, which show a primary glandular function (Di Giulio *et al*., 2009; Maurizi *et al*. in press). This fact suggests that the chemical mimicry of *P. favieri* could be reinforced by the spreading of attractive antennal secretions, as reported for the larvae and pupae of *Thalassa saginata* (Orivel *et al*., 2004). The chemical nature of the antennal secretion is still unknown, but it seems to be important for the acceptance and for the success of the parasitic interaction (Di Giulio *et al*., 2009). Several authors have speculated that the chemicals secreted by Paussini beetles may have an appeasing or a rewarding function for the ants (Geiselhardt *et al*., 2007). Further investigations are necessary to identify the antennal secretions of Paussini and their role in the association with the host ants.

Though the specimens of *P. favieri* here analyzed were collected in the same locality and thus they belong to the same population, the DA clearly differentiated the chemical profiles of the beetles according to their colonies of origin (5 different ant colonies),. The perfect clustering of the profiles in different groups, each matching the colony of origin suggests the possibility of a secondary passive acquisition of the ant 'colony odour' by the beetles, an acquisition maintained by the regular exchange of chemicals between beetles and ants. In other words, they could actively synthesize the mimetic compounds necessary to avoid the ant aggression, and then by

feeding on larvae and adults of the host ants, and by physically interacting with ants and ant nest, could obtain other compounds distinctive of the individual colony, refining their integration inside the individual nest. In larvae of *Maculinea rebeli*, Akino *et al*. (1999) suggested a combination of two chemical strategies, mimicry and camouflage (*sensu* Dettner and Liepert, 1994), to enter and access the resources of the host colony; the larvae post-adoption (after few days from entering) showed a cuticular profiles more complete that the larvae pre-adoption. A similar strategy can be suggested also for *P. favieri* but additional investigations on beetles in pre-adoption, isolation and nest crossing are required.

The interesting finding in co-occurrence of two species of *Paussus* belonging to two very different subgenera in the same nest of *Pheidole pallidula*, *P. (Flagellopaussus) favieri* and *P. (Klugipaussus) olcesii*, stimulated us to analyse comparatively their cuticular profiles, though in a merely descriptive way. This preliminary chemical investigation (based only on a single newly exclosed specimen of *P. olcesii*) shows that the cuticular profile of *P. olcesii* is characterized by 46 CHCs, 21 (45%) shared with *P. favieri*, and a total of 19 (41%) in common with the ants (workers and soldiers). However, the young adult has been found together with some larvae and pupae deeply digging the nest, and it is likely that it was never in contact with the host ants, even though its cuticular profile shows high similarity with that of workers and soldiers.

In conclusion, the results of the present analysis clearly suggest that chemical mimicry is the strategy evolved in *P. favieri* to deceive the host recognition system, possibly reinforced by the attractive antennal secretion that contribute to the successful acceptance and integration of the beetles inside the host colony. Moreover, the passive exchange of chemical cues between host and parasite might allow a constant maintaining of the 'colony odour' to the beetles, refining the main chemical mimicry through a partial camouflage. However, additional chemical investigations would be necessary to provide a more complete understanding of the myrmecophilous strategy of *P. favieri* and of other members of this tribe.

SUPPLEMENTARY MATERIAL

Additional Materials may be found in the electronic Appendix B.

CHAPTER 3

Role of the acoustical communication in the myrmecophilous beetle *Paussus favieri* associated with *Pheidole pallidula.*[3]

1. Introduction

Ants can exploit various communication channels (chemical, visual and mechanical), to exchange cues, signals and information at the base of their social organization and behaviors. The main channel that drives almost all the ants' activities and behaviors is chemical, mediated by pheromones and cuticular hydrocarbons (Hölldobler and Wilson, 1990). The mechanical channel includes tactile cues, widely recognized as important in ant communication (Hölldobler and Wilson, 1990), as well as acoustic cues (stridulation and drumming). The visual channel seems to have secondary importance in the darkness of the nests where ants spend most of their time (Kirchner 1997). The acoustic channel is the least studied way of communication in ants (Barbero *et al.*, 2009a,b) and is generally considered 'weakly developed' (Hölldobler and Wilson 1990; Keller & Gordon 2009). Some recent findings, however, support a basic role for this channel in intraspecific communication, often integrating, amplifying and/or modulating chemical cues (Markl and Hölldobler 1978; Baroni-Urbani *et al*., 1988; Hölldobler 1999). As far as we know, only the adults of five ant subfamilies (Ponerinae, Nothomyrmecinae, Pseudomyrmecinae, Myrmicinae, Ectatomminae) are able to produce low frequency sounds, such as stridulations (Markl 1965; Taylor 1978; Ferreira *et al*., 2010). The general shape of the stridulatory organs is similar in these subfamilies, being composed by two parts: a minutely ridged stridulating *pars stridens* placed meso-dorsally on the posterior edge of the fourth abdominal segment, and a *plectrum*, consisting in a prominent spine projecting medially under the anterior edge of the third abdominal segment. Sounds are emitted by rubbing the *plectrum* against the *pars stridens* during the movements of the gaster (Giovannotti 1996; Grandi 1966;

[3] This chapter corresponds to Paper 3 in preparation.

Pavan *et al*., 1997; Grasso *et al*. 1998; Ruitz *et al*., 2006) and consist in a series of 'chirps'. These vibratory signals are transmitted by the substrate, while it is matter of debate if, and at which level, ants are able to perceive the air-born components of the stridulations (Roces *et al*., 1993; Roces and Tauz, 2001; Hickling and Brown, 2000, 2001; Hölldobler & Wilson, 2009). The frequency of the stridulations can vary from a few kilohertz, as in *Myrmica* spp. (Barbero *et al.*, 2009a, b), up to 84 kHz, as in *Pachycondyla apicalis* (Pavan *et al.*, 1997). Acoustical signals are involved in several behavioral aspects of the ant society, such as social organization (Barbero *et al*., 2009a, b), food recruitment (Hölldobler *et al*., 1978; Baroni-Urbani *et al*., 1988; Roces *et al*., 1993), trophallaxis (Stuart and Bell, 1980), mating (Mark *et al.,* 1977), inter- and intra-specific conflicts (Grasso *et al*., 1980; Markl 1965), as well as in nest emigration (Markl 1965).

It has been demonstrated that the stridulations are also employed by myrmecophilous riodinid and lycaenid butterflies to circumvent the acoustical communication systems of their hosts (Barbero, 2009a, 2009b; De Vries 1990, 1991a, 1991b, Thomas *et al*., 2010). In particular, social parasites are generally more specialized than others symbionts, showing several adaptations (behavioral, morphological, chemical, and acoustic) for corrupting the honest signals of the ants (Thomas *et al*., 2010).

All members of the ground beetle tribe Paussini (Carabidae, Paussinae) are highly specialized myrmecophiles, integrated in the ant societies (mainly Myrmicinae and Formicinae: Geiselhardt et al 2007). They are generally considered ant's parasites since they prey on ants and their broods without any obvious benefit for the ant colonies (Escherich 1899; Le Masne 1961a, 1961b; Geiselhardt *et al*., 2007; Di Giulio *et al.,* 2011a). Our understanding of the myrmecophilous adaptations, behavior, relationships and life cycle of these beetles is scanty, (Geiselhardt et al. 2007). The presence of stridulatory organs in the Paussini has long been known, but somewhat hidden in the specialistic literature (Westwood 1874; Luna de Carvalho 1951, 1989; Darlington 1950), not being mentioned in any review of stridulatory organs of Coleoptera (Arrow 1942; Wessel 2006). Three main types of organs have been recognized, involving specialized cuticular structures (*plectrum* and *pars stridens*) respectively placed on: 1) abdomen and metafemora (type I, subtribe Paussina); 2) metasterna and mesofemora (type II, genus

Euplatyrhopalus); 3) mesofemora and metasterna (type III, genus *Platyrhopalopsis*). However, due to the rarity of these beetles and the challenges in rearing them in captivity, sounds emitted from their stridulatory organs have never been investigated, and their biological significance remains matter of speculation (Geiselhardt *et al.,* 2007). In particular, both intra- (sexual), and inter-specific (host attractive, host appeasing, defensive, etc.) roles have been hypothesized for stridulations (Geiselhardt *et al*., 2007).
In this work we used *Paussus favieri* Fairmaire, 1851, a representative of the stridulatory organ type I, and of its host ant *Pheidole pallidula* (Nylander, 1849), to investigate the role of acoustic communication in sexual behavior and in relationships with the host ants. More in particular, the objectives of the present study are: 1) to record and comparatively analyse the stridulations produced by *P. favieri* and its host ant; 2) to describe and illustrate the fine structure of the three types of stridulatory organs present in the Paussini and those of *Pheidole pallidula* (workers, soldiers and queen).

Background on stridulaton in Coleoptera

The presence of stridulatory organs is relatively common in adults and larvae of Coleoptera, being known so far at least 20 different types of organs, independently evolved in 30 beetle families (see Wessel 2006, for a detailed list of taxa, types of organs and references). Stridulation in beetles is performed by an amazing diversity of cuticular structures, variously placed on body and appendices, and more or less specialized for this use (Gahan 1900; Arrow 1904; Wessel 2006). Despite many works treated the morphology of the beetles' stridulatory organs and emphasized their taxonomic and systematic value, little attention was paid to the nature and the diversity of beetle stridulations, bioacoustic studies being available only for a limited number of species. If compared to other orders like Orthoptera and Auchenorrhyncha, very few is known about the physiological mechanisms influencing sound production (Tembrock 1960) and sound reception (references therein Wessel 2006) in Coleoptera. The biological meaning proposed for beetle stridulations widely varies among species and life stages, and can involve both intra- and inter-specific signaling. In the past it was stressed a functional role of stridulations during courtship, acting as

an isolating mechanism preventing interspecific matings (Wessel 2006). Recent works, however, indicate that stridulations play important roles also in other behaviors like deterring, attracting, distressing or defensive behaviors (Dumortier 1963; Bauer 1976; Masters 1979, 1980; Lewis and Cane, 1990; Schmitt and Traue, 1990; Riede and Stueben, 2000). In several beetle families it has been demonstrated that different types of sounds can be played even by the same structure, serving multiple functions including courtship, aggression, defense and aggregation (Wessel 2006). Additionally, a few cases of acoustic mimicry have been reported for some predators, the beetle stridulations simulating the sounds of protected insects (*e.g.* Silphidae miming *Bombus* bees, see Rothscild 1965) or acting as an acoustic warning to certain vertebrate predators (*e.g.* Carabidae avoiding shrews predation, see Claridge 1974).
Despite the extraordinary number of myrmecophilous Coleoptera, stridulatory organs have been observed only in members of the ground beetle tribe Paussini belonging to Paussina (*Paussus s.l.*, *Granulopaussus*, *Hylotorus*) and Platyrhopalina (*Platyrhopalus*, *Euplatyrhopalus*) subtribes, usually considered as sister taxa and the most derived within the tribe (Geiselhardt *et al*., 2007).

2. Materials and Methods

2.1 Beetles, ants and their rearing

Adults of *P. favieri* were collected under stones, inside the nests of *Pheidole pallidula* in sunny open fields, on the High Atlas Mountains, close to Marrakesh, Morocco (2000 m a.s.l.) in May 2010. Each beetle was reared together with ants from the same original nest; when more specimens of *P. favieri* were found in the same nest, all specimens were reared together. Ants and beetles were kept under controlled conditions (21 – 24°C; 12h: 12h light: dark; 60% humidity) in plaster layered transparent glass boxes (32cm x 22cm x 15cm) maintained on a diet with sugar and fruit flies or moth caterpillars offered three times per week. The nest boxes were kept open to facilitate observations. Five well-structured colonies with about 100 nestmates (workers and soldiers) and queen were used.

2.2 Morphological analysis of the stridulatory organ

A total of 12 beetles (6 females and 6 males) and 15 ants (2 queens, 5 workers and 4 soldiers) from 5 different Moroccan colonies were dissected and the segments containing the pars stridens were kept overnight in a detergent water solution, cleaned by ultrasounds for 15 seconds, rinsed in water, dehydrated through a series of EtOH baths of increasing concentration (70, 80, 90, 95 and 100%), critical point dried (Bal-Tec CPD 030), mounted on a stub (by using self adhesive carbon disks) and sputter-coated with gold (Emitech® K550 sputter coater) and observed at FIB/SEM (Dualbeam Helios – FEI, L.I.M.E. lab, University "Roma Tre", Rome, Italy). Measurements of the stridulatory organ were obtained from the digitalized SEM images. Five variables of *pars stridens* were measured by using the specific software Cell^D SIS (Soft Imaging System GmbH, Münster, Germany): maximum length, maximum width, numbers of ridges, ridges width and inter-ridges distance, taken in the medial part of the pars stridens (Table. 1). Five measures were taken from each morphological variable, and the mean value was computed. Also, as an estimate the ant's size (Elmes, 1976), the head width was measured, while for beetles' size the maximum width and length of the pronotum were considered. The pictures were obtained by using Stereo Microscope LEICA Z16 APO.
Additionally, the three types of stridulatory organs (pars stridens and plectrum) present in Paussini of *Paussus favieri* (1 female and 1 male), *Platyrhopalopsis picteti* (Westwood, 1874) (1 female: "Laos, Ban Kheun, 70 km N.W. Vientiane, 20.10.1968", Museum of Natural History, Paris; 1 male: "Laos, Ban Na Hin, 15-21.5.2007") and *Euplatyrhopalus vexillifer* (Westwood, 1874) (1 male "British Bootang, Maria Basti, 1889") were also investigated at FIB/SEM.

Table 1 - Average values of the morphological parameters of the stridulatory organs of the beetles and ants.

Pars stridens		**Maximal length (µm)**	**Maximal width (µm)**	**Ridge width (µm)**	**Inter-ridge distance (µm)**
Paussus favieri	F	186,05±18,95	120,55±8,66	1,28±0,12	1,78±0,34
	M	156,72±20,83	119,2±7,08	1,57±0,14	1,62±0,43
Pheidole pallidula	Queens	495,09±14,42	208,94±33,38	0,48±0,05	0,63±0,07
	Soldiers	228,27±11,06	96,15±4,07	0,43±0,04	0,59±0,08
	Workers	141,06±3,82	59,47±2,76	0,41±0,04	0,49±0,06

2.3 Sound recording and Analysis

Stridulations produced by *P. favieri* (N= 5; 2 males and 3 females) as well as by ants *Pheidole pallidula* (from two different colonies), workers (N= 5), soldiers (N= 3) and queens (N= 3), have been recorded by means of the recording device described by Barbero et al. (2009).
This recording equipment consisted of a 12.5cm x 8cm x 2cm recording chamber with miniature microphone localized in the centre and connected to noise reduction system. Every recording sessions lasted 15 minutes. Before each session, both the ants and the beetles were allowed to acclimate for 10 minutes inside the chamber.
The sound tracks were recorded directly on a laptop computer using Audacity v. 1.3 Beta (Unicode), and to further reduce ambient noise and interference, the equipment was powered by a 12V gel cell battery, while the recording chamber and microphone were placed inside an anechoic chamber. All residual background noise was removed using iZotope Rx.
Dominant frequency (DF; Hz), pulse repetition frequency (the reciprocal of the duration of one pulse; PRF; s–1), pulse length (PL; s) and sound intensity (dB) were measured on stridulations of both beetles and ants using Audacity 1. 3 Beta (Unicode) (Table 2).

Table 2 - Average values of the four sound parameters for the beetles and the castes of the ants.

Acoustical signal		Pulse lenght(s)	PRF (s^{-1})	Frequency (Hz)	Intensity (dB)
Paussus favieri	Pa	0.034 ± 0.005	14.491 ± 7.424	2100.500 ± 378.251	-33.817 ± 4.951
	Pb	0.015 ± 0.004	40.896 ± 21.450	957.463 ± 375.417	-38.083 ± 9.628
	Pc	0.030 ± 0.008	2.332 ± 1.261	2019.007 ± 253.538	-41.857 ± 6.923
Pheidole pallidula	Queens	0.066 ± 0.019	9.546 ± 10.118	1390.736 ± 177.419	-15.250 ± 7.629
	Soldiers	0.065 ± 0.020	15.280 ± 6.064	1455.473 ± 468.327	-19.775 ± 3.913
	Workers	0.057 ± 0.012	16.734 ± 3.390	1204.832 ± 399.465	-33.144 ± 4.477

To test whether sound differed among ant casts and between Paussini males and females we used the analysis of variance (ANOVA and Mann-Whitney, respectively).

To further test whether the sounds produced by beetles were similar to those emitted by the queens, soldiers or workers of the host ants, we plotted the first and second factor of a principal components analysis and we estimated differences among groups by the analysis of variance, testing for pairwise comparisons by Post hoc least significant differences (LSD). Moreover we performed SIMilaritiesPERcentage analysis on the matrix of pairwise Bray Curtis similarities and we used the ANalysis Of SIMilarity implemented in Primer v. 6.1.12 (Primer-E Ltd.) to estimate the significance of the differences calculated between groups. We included in our statistical analysis also the sound parameters estimated on the stridulation emitted by *Myrmica scabrinodis* queen and worker, as out-groups. Samples of *Myrmica* ants, collected in the site of Caselette (NW Italy, TO), have been recorded using the aforementioned recording device with the same experimental setting. Additionally, to test whether the stridulatory organs differed among ant casts and between *P. favieri* males and females we used Mann-Whitney U test.

3. Results

3.1 Morphological description of stridulatory organs in Paussini

The following three different types of organs have been recognized in this tribe, and analyzed by using a three model species:
Type I. "Abdomen-femur" type (Luna de Carvalho 1951; 1989; Darlington 1950) was analysed in *Paussus favieri*. This type is composed by an active *pars stridens* with a stridulatory file (slightly raised, finely ridged area) positioned basally on the inner face of the hind femora, rubbing against a fixed *plectrum* composed by a curved row of small spines positioned at both sides of the proximal abdominal sternite (stridulatory file *sensu* Darlington 1950, considered as *pars stridens* by Geiselhardt *et al*., 2007). This highly derived type is the most common type found in Paussini present in all species of the derived subtribe Paussina except *Leleupaussus* (Geiselhardt *et al.*, 2007) (Fig. 1).

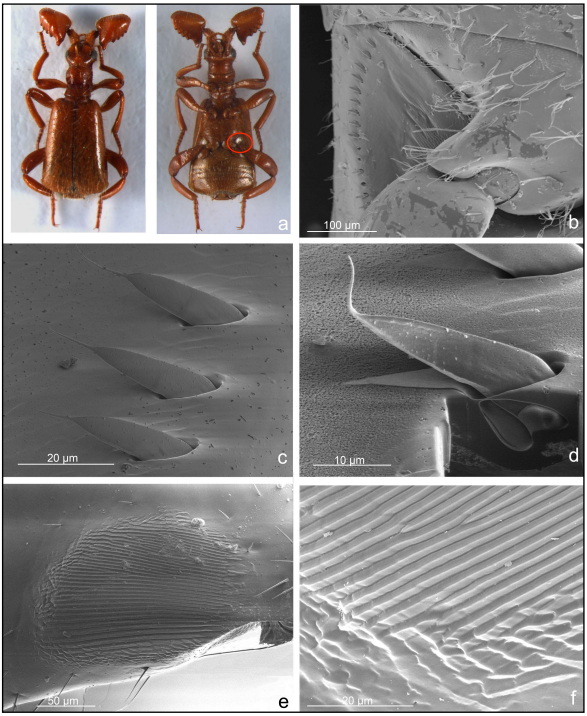

Figure 1- SEM micrographs of *Paussus favieri* (female). a. dorsal and ventral view of body, red circle shows the position of the stridulatory organ Type I; b. *plectrum* positioned on the left side of the proximal abdominal sternites; c. row of small spines of the *plectrum*; d. cross section of the cuticular spines analyzed by using the FIB; e. *pars stridens* positioned basally on the inner face of hind femora; f. particular of the ridges.

Type II. "Thorax-femur" type present only in the genus *Euplatyrhopalus* (6 species), was analysed in detail in *Euplatyrhopalus vexillifer*. This type was first described by Westwood (1874) without any functional interpretation. Later it was recognized as a stridulatory organ and considered as a sexual character by Fowler (1912). It is composed by a fan of 9-12 short costae (*pars stridens*), strongly raised and widely separated one another, sub-basally located on the internal side of each mesofemur, rubbing against a *plectrum* basally present on each side of

metasternum, composed by two curved parallel rows of 5-6 pointed setipherous protuberances of the cuticle. (Fig. 2a).

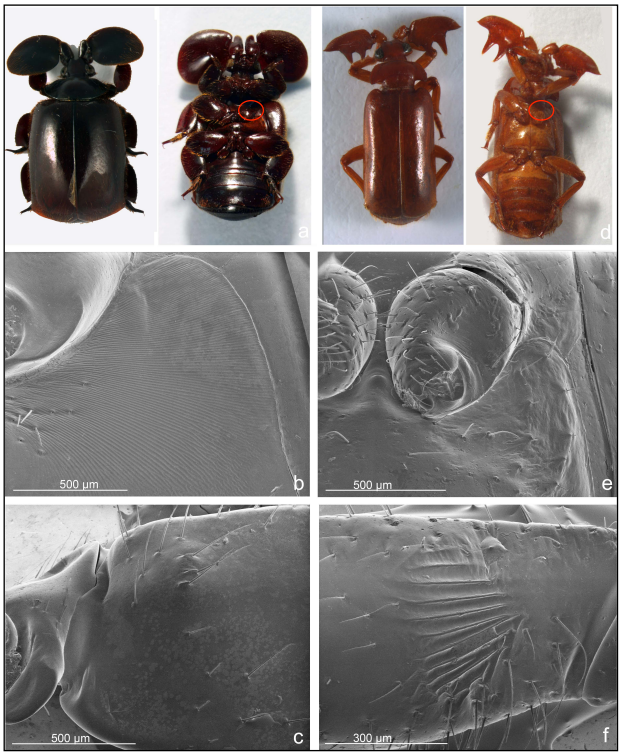

Figure 2 - SEM micrographs of a. ventral and dorsal view of *Platyrhopalopsis picteti,* red circle shows the position of the stridulatory organ Type III; b. *pars stridens* on metasternum; c. *plectrum*; sub-basally positioned on the internal side of mesofemur; d. ventral and dorsal view of *Euplatyrhopalus vexillifer*, red circle shows the position of the stridulatory organ Type II; e. *plectrum* basally present on metasternum; f. *pars stridens* sub-basally located on internal side of mesofemur.

Type III. "Femur-thorax" type present in the small genus *Platyrhopalopsis* (3 species) (Luna de Carvalho 1951), and analysed in *Platyrhopalopsis picteti.* This type is composed with the two parts, *plectrum* and *pars stridens*, inverted compared to type II. It is composed by a fan of several (about 100) fine costae (*pars stridens*), present at each side of metasternum, rubbing against a *plectrum* sub-

basally positioned on the internal side of each mesofemur, and composed by a row of 4 small setipherous keels of the cuticle (Fig. 2b). Only the first type was considered as "complete" by Luna de Carvalho (1951; 1989) since in types II and III the authors were not able to find a distinct *plectrum*.

3.3 Sound analysis

Members of all three castes of *P. pallidula* are able to produce sounds (Fig. 3B-D). We recorded Σ72 sound sequence measurements averaged within individuals separately for queens (Σ107), workers (Σ112) and soldiers. The stridulations consist of a series of pulses that are repeated from few (1.524 s) to about ten seconds without interruption. Both the queens' and the soldiers' trains of impulses contain about twenty pulses, while the workers' trains are on average longer, of about thirty pulses.

We found that sounds produced by *Pheidole pallidula* queens and workers are distinct ($F_{Pulse\ Length}$=10.011, F_{PRF}=70.089, $F_{Frequency}$=13.131, $F_{Intensity}$=231.031, $p<0.001$). Queens' stridulations are much more similar to those emitted by soldiers in terms of Pulse length and Dominant Frequency (Table 5 see in additional material). The stridulation emitted by workers are substantially dissimilar from the other two castes, apart from their PRF, which is not distinguishable from that produced by soldiers. Taking into account sounds produced by *P. pallidula* it is possible to single out three separate groups corresponding to the three castes (Fig. 4; Table 3 and Table 5 see in additional material).

We recorded Σ657 pulses for *Paussus favieri* (males and females). *P. favieri* demonstrated to be able emit at least 3 statistically different kinds of pulses (Fig. 3A) ($F_{Pulse\ Length}$=196.377, F_{PRF}=691.560, $F_{Frequency}$=234.921, $F_{Intensity}$=16.248, $p<0.001$ post hoc pairwise comparison show no differences only between the Dominant Frequency of Pa and Pc, Fig. 4, Table 3 and Table 5 see in additional material). Type a and b pulses are found alternating in trains, while pulses of type c are emitted subsequently to form other trains. Sequences of pulses lasted approximately 3 seconds, on average.

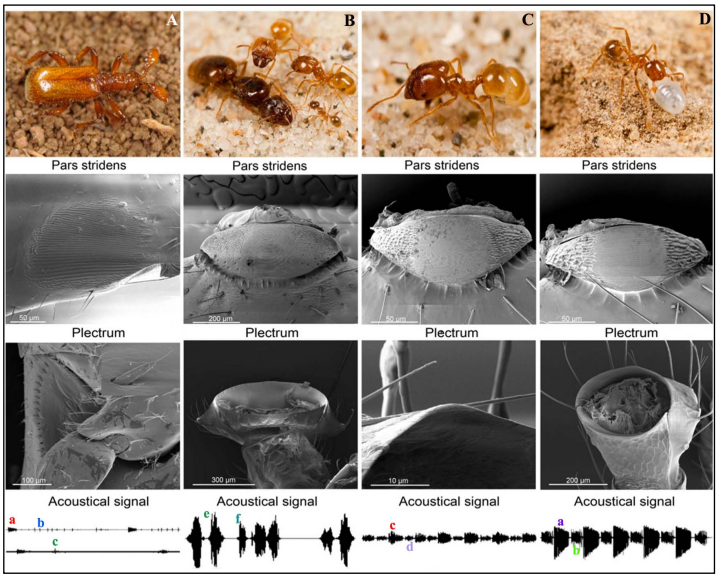

Figure 3 – A. Picture, scanning electron mycrographs of plectrum and pars stridens (stridulatory organ), and acoustical signals recorded (pulses a, b and c) of *Paussus favieri*. B-D. Picture, scanning electron mycrographs of plectrum and pars stridens (stridulatory organ), and acoustical signals recorded (different pulses for caste) of queens (B), soldiers (C) and workers (D) of *Pheidole pallidula.* (Photo of *P. favieri* by P. Mazzei; Photo of *Pheidole pallidula* by H. Darras).

All the three kind of pulses produced by *P. favieri* (a, b, c) showed higher pairwise similarities with the sounds produced by host ants than within themselves (Table SIMPER), and they were completely distinct from the *Myrmica* acoustical emissions. Also the overall acoustics produced by the three castes of *P. pallidula* showed high degrees of dissimilarity with respect to those of *Myrmica*. In particular, *P. favieri* 'type a' is closer to *P. pallidula* queen and soldiers, 'type b' to the workers and 'type c' to the queens. It is worth noticing that the level of resemblance between pulse 'type c' and queens is higher than the percentage of similarity observed between soldier and worker ants (Table 4 see in additional material). Sounds emitted by *P. pallidula* queens and pulses 'type a' produced by *P. favieri* are similar in Dominant Frequency and PRF and they are, therefore, the only group which are not separated by factor 1 of PCA (Fig. 4 and Table 6 see in additional material).

Interestingly we found a difference in the stridulations emitted by *P. favieri* males and females (Mann-Whitney $U_{Pulse\ Length}$=110.5, Mann-Whitney U_{PRF}=450.0, Mann-Whitney $U_{Frequency}$=86.5, N=59, p<0.05). Although this has to be tested on a larger sample size, differences in the sound emitted by males and females are consistent with those observed in the morphology of their respective stridulatory organs (Mann-Whitney $U_{RidgeWidth}$= 1596.50, N=133, P=0.008; Mann-Whitney $U_{Inter\text{-}ridge\ distance}$= 1576.000, N=127, P=0.039).

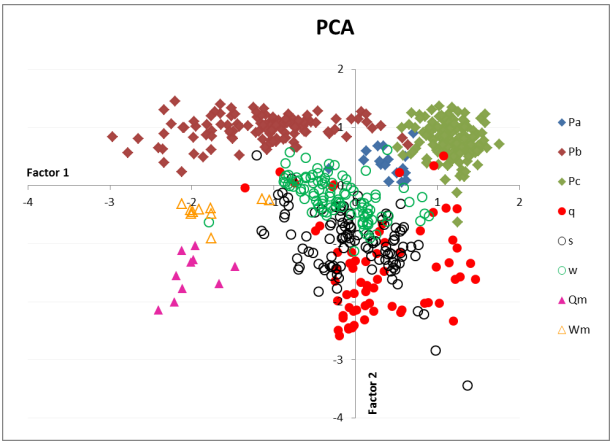

Figure 4 -:Comparisons of the overall acoustics of *Pheidole pallidula* queens, soldiers and workers and *Paussus favieri* pulses. The combined effect of the four sound parameters is shown as the first and second component plot of a principal components analysis over all individual measurements. Beetles: *Paussus favieri* Pa=pulse type a, Pb= pulse type b, Pc= pulse type c; Ants: *P. pallidula* q=queens, s= soldiers, w=workers; *Myrmica scabrinodis* Qm=queens, Wm=workers.

Table 3 - Component Loadings of Principal Component Analysis (PCA).

	Components Loadings	
	Factor 1	**Factor 2**
Pulse lenght	0.288	-0.851
PRF	-0.894	-0.037
Freq	0.884	0.011
Intensity	0.233	0.869
Percent of Total Variance explained	42.927	37.022
ANOVA (N=585)	F= 260.031 P<0.001	F=362.267 P<0.001

4. Discussion

In this paper we analyzed the myrmecophilous species *Paussus favieri* and its host ant *Pheidole pallidula* to infer about the role of the stridulatory organs in Paussini. We recorded the stridulations produced by this species (the first ever recorded for the Paussini) and the stridulations produced by different castes (worker, soldiers and queen) of the host ants. We also analyzed at SEM all recognized types of organs (I-III), giving a synthetic comparative description and illustration of their fine morphology. Formerly, only the type I was considered "complete" by Luna de Carvalho (1951; 1989), since in types II and III the authors were unable to find a distinct *plectrum*. Actually, the present analysis clearly shows the presence of minute plectra in Platyrhopalina, represented by setipherous cuticular protuberances on the metasternum (type II, *Euplatyrhopalus*) and on mesofemora (type III, *Platyrhopalopsis*). Moreover, this study demonstrates that apparently identical stridualtory organs are present both in males and females; they are also surprisingly similar to those of the ants (especially type I), both composed by a finely ridged suboval pars stridens and some distinct cuticular prominence working as plectrum.

In a recent work on the behavior of *P. favieri* (Maurizi *et al*., in press.) we doubtfully related the movements of the hind legs up and down, singly or in combination, to the emission of stridulations. We observed that this movement of the legs is performed by both male and females of the beetle mainly in two cases, when the beetle interacts with ants during the 'rewarding' behavior (beetle antennated and licked by the ants) and when the female interacts with male during mating behavior. We finally demonstrated here that during this behavior *P. favieri* actually produces stridulations, and that stridulations of male and female are slightly different (se2 results).

Before the present analysis on the bioacoustics of *P. favieri* and *P. pallidula*, three competitive hypotheses were possible to interpret the evolution of stridulatory organs in Paussini: a) an acoustic mimicry with the host ant for the beetle advantage; b) an involvement of the stridulatory organs in the beetle mating behavior; c) both acoustic mimicry and mating behavior performed by the same structures. Several arguments, brought us to consider as very likely the presence in the Paussini of an acoustic mimicry, like it was recently stated in

larvae and pupae of myrmecophilous butterflies (see Introduction). In the case of *P. favieri*, the stridulations produced by these beetles could be used for the exploitation of the acoustic channel of the host ants. Pro acoustic mimicry hypothesis are the following points:
1) Though the knowledge about host ants of Paussini is still scanty, most paussine species with stridulatory organs have been found in association with Myrmicinae, especially *Pheidole*, and Ponerinae, both subfamilies able to stridulate; while most non-stridulating paussines (belonging to basal subtribes) are associated with non-stridulating ants (Formicinae and Dolichoderinae).
2) It is evident from their different morphology and position on the body that in Paussini the stridulatory organs evolved independently three times, one in Paussina (stridulatory organs type I) and twice in Platyrhopalina (types II and III). Though not automatically this implies an acoustic mimicry, an independent evolution of stridulatory organs in parasites of stridulating ants would be compatible with the presence of similar selective pressures in unrelated taxa. It is worth to note that within the subfamily Paussinae no other genera or species show stridulatory organs except for these myrmecophilous taxa.
3) In Paussini, very similar stridulating organs are present both in males and females of the same species. Darwin (1877) and later authors, considered this as sufficient argument to demonstrate that the function of a stridulating organ is not devoted to mating activities since generally only males were supposed to attract the females by using acoustic signals. Though this assumption has been recently challenged by several exceptions in different beetle families (see Wessel 2006), and the presence of similar organs is not automatically considered as related to a similar use, this would represent another indirect clue of the involvement of the stridulations in the interspecific interactions.
4) In *P. favieri* reared in captivity, stridulations have been observed very frequently during the interactions with the host ants (Maurizi et al. in press).
5) The fact that only the most derived taxa (Paussina and Platyrhopalina) use this channel would also be compatible with this hypothesis: an exploitation of the acoustic channel of the host is likely to be part of a complex and refined strategy of extremely specialized parasites, coevolved with host ants for a long time.

Similarly, a few arguments can be supplied for supporting the mating hypothesis:
1) Unlike the case of acoustic mimicry of Licaenidae and Riodinidae (De Vries *et al*., 1993; Barbero *et al*., 2009a, b), in Paussini is the adult stage that stridulates. Since many activities of the adults are shaped by the sexual selection and are aimed to the reproduction, the involvement of the acoustic communication in the courthship seems likely. We can speculate that for the meeting of the partners inside the crowded ant nests acoustical signals could be more effective than visual or chemical signals.
2) The morphometric analysis of the stridulatory organs of *P. favieri* showed the presence of significant differences between organs of males and females.
3) In *P. favieri* reared in captivity, stridulations are performed also during the pre-mating behavior, especially by females (Maurizi *et al*., in press).
In order to verify the former hypotheses and evaluate if both could be compatible in Paussini, we analyzed comparatively the stridulations of *P. favieri* (representative of the organ type I) and those of its host ant *Pheidole pallidula*. Additionally, we included as outgroup in the analysis also the sounds emitted by *Myrmica scabrinodis* queen and workers.
Interestingly, the present acoustical analysis shows that:
1. The sound produced by *P. pallidula* queens are much more similar to those emitted by soldiers than those of the workers.
2. *P. favieri* is able to emit three different types of pulses (a, b, c); in particular, type a resulted to be closer to queens + soldiers, type b to the workers, and type c to the queens. It is worth to note that the level of resemblance between pulse type c and queens is higher than the percentage of similarity observed between soldier and workers ants. Moreover the three types show a higher pairwise similarities with the sounds produced by the host ants than within themselves.
3. Stridulations emitted by *P. favieri* males and females are significantly different, a result consistent with the differences observed in the morphology of their respective pars stridens.
4. The three types of stridulations of *P. favieri* and the acoustical emissions of the three castes of *P. pallidula* are completely distinct from those of *M. scabrinodis*.

These results clearly demonstrate that an acoustic mimicry is present between *P. favieri* and the ant *Pheidole pallidula*. This represents the second example of acoustic mimicry ever recorded in myrmecophiles and the first for beetles. The other case recently reported by Barbero *et al*., (2009a, 2009b) involved the acoustical mimicry between larvae of *Maculinea* spp. and *Myrmica schencki*. Though these models involve taxa very distant phylogenetically, and two different life stages (larvae and pupae in lycenids and adults in *P. favieri*) are involved, several similarities are surprisingly evident. In both cases the stridulations of the queen are different from those of the other castes, in particular from those of the workers. This distinction among castes allows the possibility for the parasites to selectively imitate one target specific caste. Even more refined is the strategy of *P. favieri* that by using the same structure (possibly moving the hind legs singly or in combination or in any other way) is able to "talk" different languages, of queen or workers, by modulating the emissions of different types of pulses. In particular by pulses type a and c the parasitic beetle could elevate its social status to that of the queen, exploiting a more effective attraction and cares, and possibly acquiring a free access to any chamber of the nest.

Interestingly, the recent observations on several specimens of *P. favieri* reared in captivity (Maurizi *et al*., in press) revealed occasional interactions between beetles and the queen, also for a prolonged time. In that case, the physical interaction was interpreted as a possible behavior connected to a queen chemical camouflage by the beetle. The present findings seem to represent another piece of the puzzling strategy of *P. favieri*: we can imagine that a combination between chemical and acoustical mimicry could be very advantageous for the beetle's integration in the host society.

Additionally, our results suggest an involvement of stridulations in the mating behavior, since the differences observed between the sounds emitted by males and females are consistent with the results of the morphological analysis of their stridulatory organs. The behavioral observations in captivity reported above (Maurizi et al. in press) are in agreement with these results, since the stridulations are frequently emitted during the pre-mating behavior. In the past it was doubted the presence in the small antennal pedicel of Paussini of a Johnston's organ, widely acknowledged to be the seat of the acoustical reception in insects (Bailey 1991; Fedorova & Zhantiev

2009; McIver 1985). However, in a recent study a well developed Johnston's organ was found in *P. favieri*, confirming the ability of this beetle (both male and female) to perceive acoustic stimuli (Di Giulio *et al*., in press). This represents another indirect proof that the acoustical communication can be used in the intraspecific communication.
In conclusion, the involvement of the stridulations in both inter- and intraspecific behaviors (hypothesis c) is supported by the present morphological and bioacoustic analysis and by a former behavioral study. Additional researches on other stridulating paussines, especially those representative of types II and III, would be very welcome to demonstrate that this of *P. favieri* exemplifies a generalized use of the acoustical emission in these highly derived ant symbionts. This research also outline once again the importance of the acoustical communication in ants, possibly modulating behavioral responses caste-specific. Further behavioral assays and playback experiments could be useful to confirm some details of this remarkable parasitic strategy.

SUPPLEMENTARY MATERIAL

Additional materials may be found in the electronic Appendix C.

CHAPTER 4

Functional structure of antennal sensilla in the myrmecophilous beetle *Paussus favieri* (Coleoptera, Carabidae, Paussini)[4]

Abstract
The evolution of a myrmecophilous lifestyle in beetles is often associated with morphological alterations. In particular, the antennae of all members of the myrmecophilous ground beetle tribe Paussini are greatly modified, with flagellomeres flattened or crassate, frequently reduced in number from 9 to 5 or even 1 single "antennal club". The enhanced glandular function of the antennal club has been recently described by scanning (SEM) and transmission (TEM) electron microscopy in *Paussus favieri* Fairmaire, 1851, where the antenna has become a complex glandular organ, supplying rewarding substances to the ants. In the present work, the antennal sensilla of *P. favieri* are investigated by SEM, TEM and Focused Ion Beam (FIB/SEM) technology. Most sensilla of scape and antennal club are highly modified mechanoreceptors (*i.e.* multipointed, fringed, branched, brush-like, sickle-shaped), singly or grouped in tufts ("antennal symphilous organs"). These "trichomes", here assigned to 8 different morphotypes of sensilla chaetica (Ch.1– Ch.8), show a variable number of basal pores (present also at the base of the taste sensilla Ch.9), which spread dense substances of unknown chemical composition on the seta. Although hygro-, thermo- and chemoreceptors are reduced in number as compared with non-myrmecophilous relatives, and mainly relegated to the apex of the antennal club, their diversity is comparable to that of other carabid beetles: two types of sensilla trichodea (Tr.1– Tr.2); three types of basiconica (Ba.1– Ba.3); one type of campaniformia (Ca); one type of coeloconica (Co) and one type of Böhm sensilla (Bo). Contrary to the hypothesis that *Paussus* species lack a Johston's organ, a non-connective chordotonal organ composed of 9 groups of scolopidia has been found inside the pedicel. A comparison between sensilla of *P. favieri* and those of other non-myrmecophilous and myrmecophilous ground beetle species is provided.

[4] This chapter corresponds to Paper 4 in press.

1. Introduction

The main function of the insect antennae is sensorial, though glandular activity has been recently described in several species of different orders, with particular functions like intra-specific communication (sexual communication, social integration etc.), host recognition (in parasitic species), protection and lubrication for antennal sensilla or antennomere joints, bacteria cultivation organs etc. (see Di Giulio *et al*., 2009). In these species, peculiar structures for storing or spreading the secretions are often visible on the antenna, connected to epidermal glandular systems which are more or less complex and integrated; nevertheless, no extensive structural changes of the antenna or alterations from its primary sensorial function have been noticed.

A strong modification of the antennae, with flagellomeres flattened or crassate and often reduced in number, is a feature shared by all members of the myrmecophilous (ant symbiont) ground beetle tribe Paussini (Coleoptera, Carabidae, Paussinae). In several genera of this tribe the 9 joints of the flagellum are completely fused into a unique piece ("antennal club"), which can be structurally highly modified even in species of the same subgenus (antennal club big, small, flattened, lenticular, globular, shell-like, sickle shaped, elongate etc.). Such an astonishing diversity of shapes attracted the attention of several scientists who comparatively studied the antennae of this group from morphological and taxonomical points of view (Darlington, 1950; Di Giulio *et al.,* 2009; Luna de Carvalho, 1989; Nagel, 1979; Young, 1938). The adaptive significance of this morphological diversity seems to be connected to the exceptional glandular activity of the antennae and to their functional role in the interactions with the host ants (Di Giulio *et al.*, 2009; Geiselhardt *et al*., 2007; Wasmann, 1903; Young, 1938).

As in other myrmecophilous insects, glandular organs (called "antennal symphilous organs" or "myrmecophilous organs") are present on the antennal surface; these organs, composed of brushes of setae, are actively licked by the ants, and their function is clearly related to the acceptance and survival inside the ant colonies. Nevertheless, both the chemical composition of the epidermal gland secretions (fatty compounds, proteins, sugars, etc.) and their specific effects (appeasing allomones, food reward, protective substances

etc.), still remain matter of speculations (Geiselhardt *et al*., 2007; Wasmann, 1890).
The antennal sensilla of 8 species of the genus *Paussus* were comparatively examined with the SEM by Nagel (1979), while an extensive SEM analysis of antennal microstructures (sensilla, microsculpture, pores etc.) of more than 50 representative species of Paussinae (both myrmecophilous and non-myrmecophilous) is still in preparation (Di Giulio *et al.* in prep.). The unique TEM investigation of Paussini (Di Giulio *et al.*, 2009) was focused on the antennal glandular system of *Paussus favieri* Fairmaire, 1851,, obligate parasite of the ant *Pheidole pallidula* (Nylander, 1849); nevertheless, the sensorial structures of this species remained undescribed.
Aims of the present paper are: (1) to analyze and describe the structural diversity of the antennal sensilla in *P. favieri* through SEM, TEM and FIB/SEM (Focused Ion Beam technology); (2) to assess the degree to which the evolution of myrmecophily, and in particular the related intense glandular activity of the antennae, influences the sensorial system in terms of types, number and distribution of sensilla on the antennal club; (3) to analyze the eventual presence of sexual dimorphism on the microstructures of the antennal club; 4) to compare the microstructures found in *P. favieri* with homologous structures observed on the antennae of both myrmecophilous and non-myrmecophilous carabid species.

2. Materials and Methods

2.1 Material examined

This study is based on the analysis of 10 specimens (five males and five females) of *P. favieri* (Fig. 1), collected in Morocco (High Atlas, Tizi-n-Test, 2063 m a.s.l., 30,87288° N – 8,36204° W, 5.V.2009) in nests of *P. pallidula*. The material is preserved in the A. Di Giulio collection (Rome, Italy).

Figure 1 - *P. favieri* male, habitus, anterolateral view (Picture by P. Mazzei).

2.2 Scanning Electron Microscopy (SEM)

For SEM analysis, the antennae from 3 females and 3 males were removed, kept overnight in a detergent water solution, cleaned by ultrasound for 15 seconds, rinsed in water, dehydrated in a graded ethanol series, critical point-dried in a CPD 030 (Balzers Union, Fürstentum, Liechtenstein) unit, gold coated in a K550 (Emitech Technologies Ltd., Kent, England) unit, and examined with a XL30 (FEI Company, Eindhoven, The Netherlands) SEM microscope at the L.I.M.E. (Interdepartmental Laboratory of Electron Microscopy, 'Roma Tre' University, Rome, Italy). The micrographs obtained by this instrument were used for characterizing number, distribution and measurements (mean ± SD) of sensilla by using the specific software Cell^D SIS (Soft Imaging System GmbH, Münster, Germany).

2.3 Focused Ion Beam/Scanning Electron Microscope (FIB/SEM)

The antennae prepared for the SEM were also analyzed with the Dualbeam (FIB/SEM) Helios Nanolab (FEI Company, Eindhoven, The Netherlands) at the L.I.M.E. ('Roma Tre' University, Rome, Italy). This instrument was used for both obtaining high resolution images of sensilla and to investigate their internal cuticular structure. 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. This apparatus is capable of selectively ablating (milling process) a previously marked region of the sample by using a focused ion current from a gallium source. The milling process can be interrupted every few nanometers to take high resolution pictures to the cross sections by the SEM column.
For the study of the antennal cuticular microstructures of *P. favieri* the FIB was operated at 30 kV and 0.92 nA; SEM pictures were taken to the cross sections approximately every 100 nm of milling (horizontal feed), with an operating voltage of 5 kV and an applied current of 0.17 nA. Short movies representing the milling process, are supplied on the electronic version of this paper. Selected SEM pictures representing the milling process have been cropped and edited in sequence.

2.4 Transmission electron microscopy (TEM)

For TEM analysis, four specimens (two males and two females) were CO_2 anesthetized and immediately immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer + 5% sucrose, pH 7.2– 7.3 at 4 °C. The antennae were removed from the head, the antennal club was cut transversally, placed in the fixative to improve penetration, and held for 2h at 4 °C. They were then rinsed overnight in cacodylate buffer, post-fixed in 1% osmium tetroxide for 1 hour at 4 °C and rinsed in a cacodylate buffer. Samples were dehydrated in a graded ethanol series followed by embedding in Epon-Araldite with propylene oxide as a bridging solvent. Thin sections were cut with a diamond knife (Drukker, Cuijk, The Netherlands) on an LKB-Nova (LKB, Bromma, Sweden) ultramicrotome, and mounted on Formvar-coated 50 mesh grids. Sections were examined with a EM 208 (FEI Company, Eindhoven, The Netherlands) TEM microscope after staining with uranyl acetate (20 min, room temperature) and lead citrate (5 min, 21– 23 °C) at the C.U.M.E. (Universitary Center of Electron Microscopy, Perugia University, Italy). Digital pictures (1280 x 1024 pixels, 16-bit, uncompressed greyscale Tiff files) were obtained using a high resolution digital camera ColorView III (Soft Imaging System GmbH, Münster, Germany).
The terminology of Schneider (1964) and Zacharuk (1985) was used to classify sensilla. And we also referred to classifications reported in recent papers on antennal sensilla of Coleoptera, Carabidae (e.g.

Merivee *et al.*, 2000, 2001, 2002; Ploomi *et al.*, 2003). In Fig. 2 a schematic view of both dorsal and ventral surfaces of the antenna is provided with acronyms identifying functional (glandular/sensorial) areas that will be used throughout this paper and in Table 1 (see in additional material).

3. Results

3.1 Gross morphology of the antennae

The antennae of *P. favieri* (length 1.2 ± 0.02 mm; Fig. 1) are composed of three joints: (1) a cylindrical and slightly elongated scape (A1, Fig. 2; SC, Fig. 3A) (length 0.40 ± 0.05 mm, width 0.23 ± 0.01 mm), articulated basally with the antennal fossa by a posterior condyle and apically with (2) a small, globular, ring-like pedicel (A2, Fig. 2; PD, Fig. 3A) (width 50 ± 8.9 μm); (3) a third joint, called "antennal club" (AC, Fig. 3A), resulting from the fusion/reduction of the 9 flagellomeres (A3–A11, Fig. 2) into a wide, sub-triangular, swollen, and strongly asymmetrical piece (length 0.85 ± 0.04 mm, width at base 0.61 ± 0.02 mm). The anterior margin of the antennal club is keeled and slightly concave (Fig. 2); the posterior margin appears medially four-toothed (Figs. 2 and 3A,E), and with a conspicuous, pointed basal spur (Fig. 3A and C). The dorsal surface is strongly convex (Fig. 3A), while the ventral surface appears concave posteriorly and convex anteriorly furrowed in the posterior half by four transverse, deep pockets (length about 0.13 ± 0.01 mm) (Fig. 4A), ending at the tip of each tooth. The apex is broadly rounded (Fig. 5A). No significant differences were found in the antennal shape or dimensions between males and females.

The surface of the antenna appears completely smooth (Fig. 3B,C and E), except for the presence of appendages of cuticular plates on the condyle of the scape, on the basal part of the pedicel and on the condyle of the antennal club. Most of the surface appears cribrate and riddled with pores (Fig. 3F), crowded in special hairless cuticular areas, surrounding the base of the setae or positioned inside deep pockets that store the secretions, with whitish fine fibers of dense material arising from them.

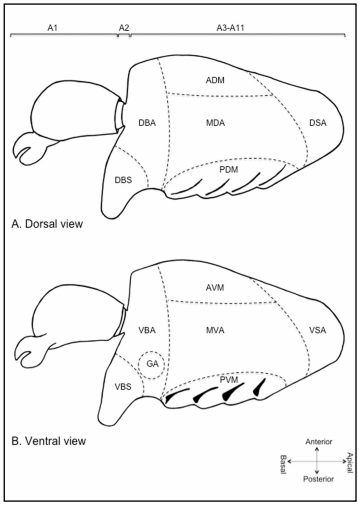

Figure 2 - Line drawings of the antennal topography of *P. favieri* with acronyms of functional areas. A1= scape, A2= pedicel, A3- A11= antennal club. A) Right antenna, dorsal view: DBA= dorsal basal area; DBS= dorsal basal spur; ADM= anterior dorsal margin; MDA= medium dorsal area; PDM= posterior dorsal margin; DSA= dorsal sensorial area. B) Left antenna, ventral view: VBA= ventral basal area; VBS= ventral basal spur; GA= glandular area; AVM= anterior ventral margin; MVA= medium ventral area; PVM= posterior ventral margin; VSA= ventral sensorial area.

3.2 Antennal sensilla

According to their morphological features, we identified 9 different types of sensilla chaetica, 3 types of sensilla basiconica, 2 types of sensilla trichodea, Böhm sensilla, campaniform and coeloconic sensilla. The morphological features, the distribution and the mean number of antennal sensilla are summarized in the Table 1 (see in additional material) (c.f. Merivee *et al.*, 2002).

3.2.1 Sensilla chaetica.

Modified bristles inserted in a distinct socket with variable number of cuticular pores surrounding their base. The basal part of the sensilla is slightly grooved longitudinally and oval in cross section. The apical part is frequently smooth, flattened and variously fringed or branched. Cross sections of sensilla chaetica show thick cuticular walls and a narrow internal space (Fig. 6A). The sensilla are

articulated to the antenna by means of a joint membrane, a tubular body is present just below the socket level (Fig. 6B).

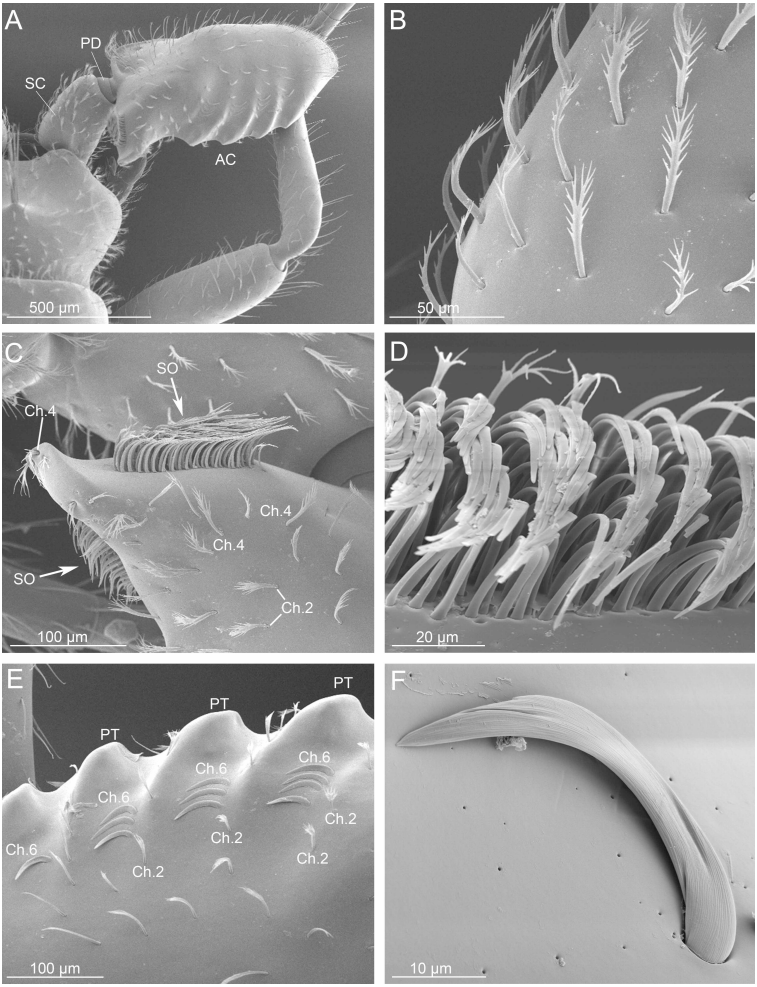

Figure 3 - SEM micrographs of *P. favieri*: A) right antenna, postero-dorsal view; B) sensilla Ch.1 on anterior side of scape; C) basal spur of antennal club, dorsal view; D) sensilla Ch.7; E) posterior margin of antennal club, dorsal view; F) sensillum Ch.6. SC = scape; PD = pedicel; AC = antennal club; SO = symphilous organ; PT = posterior teeth.

Sensilla chaetica type 1 (Ch.1): The general shape is fishbone-like (53.07 ± 18.71 μm long; N= 90), with a simple, almost straight, cylindrical basal part (diameter: 3.29 ± 0.84 μm; N= 90) and a flattened or concave, expanded and multi-lobed distal part abruptly bent towards surface (projection: 61.94 ± 19.07°) (Fig. 3A, B; Table 1 see in additional material). The medial lobes are spine-like and emerge almost symmetrically from both sides of the setae like bones of a fish. The apex is irregularly multilobed. These sensilla are regularly distributed on the whole surface of the scape, increasing in length from the base to the apex. In particular, the sensilla at the apex of the scape show longer and straighter basal parts and less regular distal branching. These setae are not present on the antennal club.

Sensilla chaetica type 2 (Ch.2): The general shape (Figs. 3C,E and 4D; Table 1 see in additional material) is brush-like (37.02 ± 7.65 μm long; N= 46), with a simple and straight basal part (diameter: 2.50 ± 0.34 μm; projection: 100.73 ± 19.77°; N= 46), bearing an apical tuft of long and thin cuticular projections, often secondarily branched. The setae can be sub-apically bent. They are present on both dorsal and ventral basal part of antennal club, mostly on the posterior margin.

Sensilla chaetica type 3 (Ch.3): The general shape is arborescent (Fig. 4C and E; Table 1 see in additional material), with flexible and thin stem (thickness gradually decreasing toward apical part) (91.31 ± 18.77 μm long; N= 100), laterally and apically branched by thin and elongate projections, often secondarily ramified. The bases of the Ch.3 (diameter: 3.20 ± 0.49 μm; N= 100) project from the antenna at 87.31 ± 36.14°. They are mostly present on the basal part of the antennal club, especially crowded on the anterior corner.

Sensilla chaetica type 4 (Ch.4): These sensilla are completely prostrate or bent with various degrees from surface (projection: 155.52 ± 11.90°; N= 20). This type includes sensilla strongly modified (Figs. 3C and 4A,B; Table 1 see in additional material), with a flattened stem (37.36 ± 12.76 μm long; diameter: 2.15 ± 0.40 μm; N= 20), corrugated through whole the surface, often irregularly twisted or curved and abruptly bent apically. The apical part is expanded and more or less deeply lobate. They are scarcely represented on the antennal club, mostly on the basal part (dorsally and ventrally), ventrally around the pockets and on the basal spur.

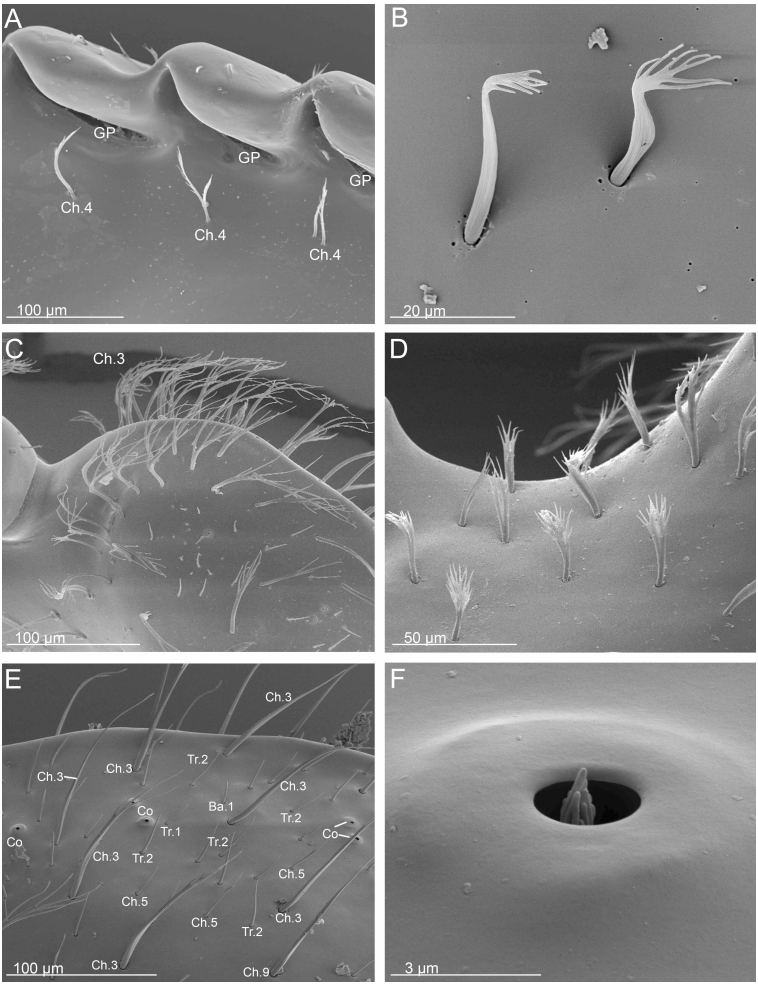

Figure 4 - SEM micrographs of *P. favieri* antennal club, ventral view: A) posterior margin glandular pockets; B) sensilla Ch.4 on sub-basal part; C) sensilla Ch.3 on basal anterior corner; D) sensilla Ch.2 on concavity between basal spur and first tooth of posterior margin; E) antero-ventral margin; F) sensillum Co on antero-ventral margin. GP = glandular pockets.

Sensilla chaetica type 5 (Ch.5): The general shape is furcate, bi- or tri-dentate apically, slightly curved, round in cross section (Figs. 4E and 5E; Table 1 see in additional material). The bristle bases (diameter: 1.40 ± 0.22 μm; N= 80) project about 139.65 ± 27.92° from the antenna surface. The stem (19.26 ± 6.38 μm long; N= 80) is smooth or slightly grooved, with one cuticular pore close to the base. They are distributed mainly on the anterior part of the antenna, both dorsal and ventral surface from the basal to the apical area.

Sensilla chaetica type 6 (Ch.6): These peculiar sensilla (53.10 ± 5.04 μm long; N= 16) are prostrate, laying on the antennal surface (Fig. 3E,F; Table 1 see in additional material). They are sickle-shaped, curved and strongly flattened, longitudinally grooved and tapered to the sharp apex. Scattered pores are visible around the basal insertion (diameter: 5.48 ± 1.28 μm). They are present dorsally on the posterior part of antennal club in four rows (in correspondence to four teeth) composed of 1– 4 setae laying in parallel, with apices directed towards the antennal apex.

Sensilla chaetica type 7 (Ch.7): These bristles are tightly arranged in groups (antennal symphilous organs, see Nagel 1979) located subapically, dorsally and ventrally, to the basal spur of the antennal club (Fig. 3C,D; Table 1 see in additional material). Each sensillum (55.97 ± 8.32 μm long; N= 55) is flexible, elongate, and tightly inserted in a socket surrounded by many wide pores. Pores-like openings of glandular ducts are present inside the sockets of Ch.7 as well as outside. The basal part of these sensilla (diameter: 3.04 ± 0.47 μm) is smooth or only slightly longitudinally grooved and irregular in cross section; while the apical part is smooth, flattened and tapered toward the tip. Several bristles are laterally and apically branched with irregular thin projections. The tips of the setae are stuck together like bristles of a brush.

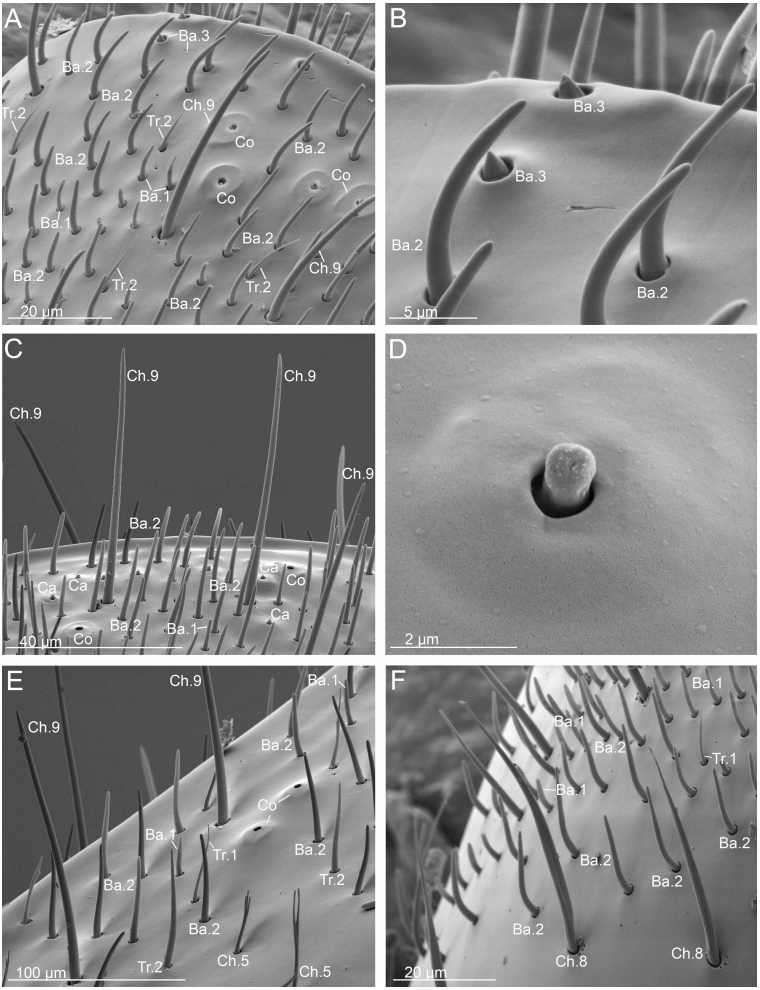

Figure 5 - SEM micrographs of *P. favieri* antennal club, apical sensorial area: A) apical margin, postero-ventral view; B) close-up of Fig. 5A; C) apical margin, dorsal view; D) sensillum Ca; E) antero-dorsal margin close to apical sensorial area; F) basal part of the apical sensorial area, dorsal view.

Sensilla chaetica type 8 (Ch.8): These sensilla are whip-like, set in socket wider than in other sensilla chaetica, usually bi- or tri-sinuate, simple (not branched) and longitudinally grooved (Fig. 5F; Table 1 see in additional material). Their stem (44.97 ± 10.73 μm long; N= 32) is stout basally, curved or irregularly twisted, then straightened, flattened and tapered towards the apical part, which is elongate, thin, flattened and flexible. The base (diameter: 2.43 ± 0.17 μm) is round in cross section, surrounded by a crown of pores. Ch.8 projects 127.05 ± 14.50° from the antennal surface. They are present on the anterior part of the antennal club from the base to the apical sensorial area.

Sensilla chaetica type 9 (Ch.9): These sensilla are thorn-like (56.91 ± 14.02 μm long; diameter: 2.91 ± 0.64 μm; N= 38), straight or slightly curved, slightly longitudinally grooved, with sharp tip (Figs. 4E and 5A,C,E,F; Table 1 see in additional material). It is remarkable that glandular pores surround also the base of these unmodified sensilla. Cross-sections at the cuticular shaft level show a single-walled sensillum with a variable thickness of the cuticle that changes from the tip to the base, going from 0.5 μm to 1.1 μm. Five dendritic processes extend in the hair lumen up to the tip of the sensillum (Fig. 6C). The sensillum is inserted in the antennal wall through a large socket, where it is suspended by means of a joint membrane (Fig. 6C). At this level, a sixth sensory neuron becomes evident, giving rise distally to a typical tubular body (Fig. 6D). The six sensory neurons innervating each sensillum are wrapped with a dendritic sheath, which divides the mechanoreceptor neuron from the five chemoreceptor neurons. It is noteworthy that the mechanosensory neuron appears much bigger (diameter ≈ 0.3 μm) than the remaining four neurons (diameter ≈ 0.1 μm) (Fig. 6D). The Ch.9 sensilla are regularly distributed through in the apical area of antennal club, on both dorsal and ventral surfaces.

3.2.2 Sensilla trichodea

Sensilla trichodea type 1 (Tr.1): These sensilla are small and thin (13.51 ± 3.83 μm long; N= 45), flexuous, usually prostrate, with blunt tip, with smooth walls (Figs. 4E and 5E,F; Table 1 see in additional material). The base (diameter: 1.08 ± 0.20 μm) is round in cross section, without pores. These hairs project outwards at 162.36 ± 25.29°. They are present on the anterior part of the antennal club from the base to the apical sensorial area (included).

Sensilla trichodea type 2 (Tr.2): Their stem (20.77 ± 4.36 μm long; N= 57) is slightly sinuate, distinctly tapered towards the blunt tip, with smooth walls (Figs. 4E and 5A,E; Table 1 see in additional material). The bristle base (diameter: 1.86 ± 0.26 μm) is sub-oval in cross section, without pores and set in a tight socket. Tr.2 protrudes at 138.98 ± 19.89° from the antenna surface. They are distributed on the anterior part of the antennal club, mostly ventrally, and in the apical sensorial area.

3.2.3 Sensilla basiconica

Sensilla basiconica type 1 (Ba.1): These sensilla (Figs. 4E, 5A,C,E,F and 6E,F; Table 1 v) are represented by classical basiconic pegs (7.24 ± 1.45 μm long; N= 238), blunt-tipped and smooth-walled, slightly to strongly curved towards to the antennal shaft. The base of the pegs (diameter: 1.51 ± 0.22 μm; N= 238) is tightly set in the socket, projects at 23.44 ± 14.65°. Cross sections show a constant thickness of the cuticular wall from the base to the tip (≈180 nm). The sensillar lumen is filled with a variable number of dendritic processes, ranging from 9 to 12, that show a certain degree of branching within the shaft (Fig. 6E). The dendritic branches vary greatly in dimensions. At the socket level, the sensillum is rigidly inserted on the antennal wall without any joint membrane. Below the socket level, two outer dendritic segments are bounded by a thick dendrite sheath (Fig. 6F). Ba.1 are densely distributed in the apical sensorial area (both dorsal and ventral) where they represent the most common type of sensilla and ventrally with few elements on the antero-basal sensorial field.

Sensilla basiconica type 2 (Ba.2): They are similar to Ba.1 by their shape and structure (Figs. 4E and 5A,C,E,F; Table 1 see in additional material), but they are relatively larger (14.37 ± 4.00 μm long; diameter: 1.76 ± 0.24 μm; N= 280) and less curved (projection: 35.75 ± 16.41°). They are almost exclusively in the apical sensorial area (both dorsal and ventral).

Sensilla basiconica type 3 (Ba.3): They are thick conical pegs (2.30 ± 0.80 μm long, diameter: 1.51 ± 0.38 μm; N= 9), smooth walled, deeply inserted in a wide socket (Fig. 5A,B; Table 1 see in additional material). They are present only on the antennal club, with about 10 sensilla disposed along the distal margin of the apical sensorial area and just one in the basal area.

3.2.4 Böhm sensilla (Bo)

They are thorn-like bristles, sharp or blunt tipped, straight or slightly curved, set in wide socket (Table 1 see in additional material). They are present on the condyle of the scape and at the base of pedicel.

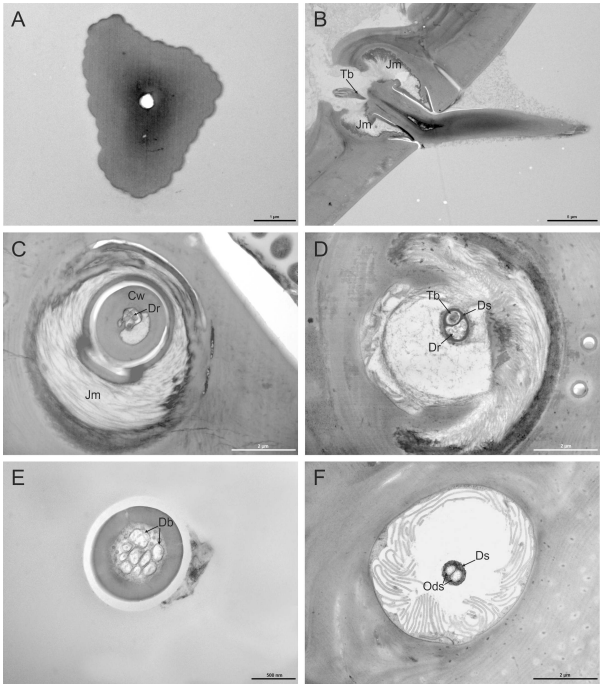

Figure 6 - TEM micrographs of *P. favieri* antennal sensilla: A) Cross section through the peg of Ch.3, showing the empty internal space. B) Longitudinal section of a Ch.3 through the socket. The tubular body (Tb) ends just below the base of the peg, with is suspended on the antennal wall by means of a joint membrane (Jm). C) Cross section of Ch.9 close to the base. The cuticular wall (Cw) of the peg is smooth and relatively thick. Five dendrites (Dr) can be counted within the lumen. A joint membrane (Jm) encloses the base of the sensillum. D) Cross section of Ch.9 taken at a deeper level. A sixth dendrite ending in a tubular body (Tb) in now present close to the five dendrites (Dr). All the sensory neurons are enclosed by a dendrite sheath (Ds) that keeps separated the tubular body from the rest of the dendrites. E) Cross section of Ba.1 showing the sensillum lumen with several dendritic branches (Db). F) Cross section of Ba.1 below the socket level, two outer dendritic segments (Ods) are enclosed by a thick dendrite sheath (Ds).

3.2.5 Sensilla coeloconica (Co)

They are typical pit organs composed of a round or sub-oval smooth cuticular plate, raised above the antennal surface, with a pit orifice (diameter: 1.40 ± 0.34 μm; N= 34) in the middle (Figs. 4E,F, 5A,C,E and 7B; Table 1 see in additional material). The discoidal plate (diameter: 7.78 ± 0.61 μm; N= 34) is flat or slightly concave and appears slightly raised around the pit orifice. From the orifice, which diameter is greatly variable, it is possible to see the tip of coniform peg which sits deeply in the pit and is distally produced into a bunch of cuticular ridges. In some cases the tip emerges outside of the orifice. The FIB analysis shows the presence of about 10 ribs radially surrounding the medial peg, only partially visible from outside. Cross sections at the peg base level show a double-walled organization (Fig. 7B). The two cuticular walls show a different thickness, ranging from 0.05 μm (inner wall) to 0.2 μm (outer wall). Two distinct cavities are defined by the two walls, an innermost and an outermost. The innermost has a diameter of about 0.6 μm and presents the outer dendritic segments of the sensory neurons closely packed together and completely filling the lumen (Fig. 7B). The outermost cavity completely surrounds the innermost cavity and it is filled with electrolucid vesicles (Fig. 7B).

Sections of the apical part of the sensillum show a different organization, with the inner and outer walls merged together and the presence of about ten ridges with pores (Fig. 7A). Dendritic branches end before reaching the sensillum tip where a star-shaped cuticle without internal space is visible (Fig. 7A). At its base, the peg is inserted on the antennal wall without a socket, and the outer dendritic segments are enclosed with the dendrite sheath (Fig. 7C). This sensillum is innervated by four sensory neurons (Fig. 7D). Co are distributed exclusively on the antennal club, dorsally only on the sensorial apical area frequently lined along the margin, and ventrally on the sensorial apical area and scattered along the whole anterior antennal margin often grouped into two or three sensilla.

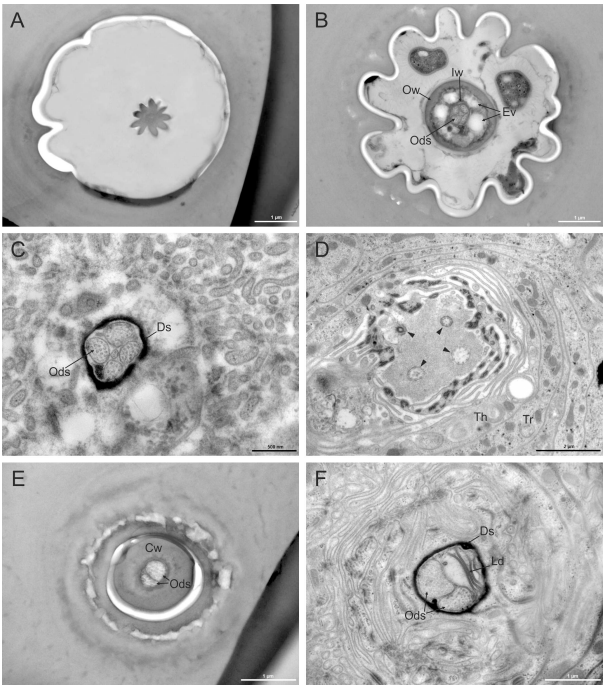

Figure 7 - TEM micrographs of *P. favieri* antennal sensilla: A) cross section of Co close to the tip, the stellate appearance due to the presence of the external grooves appears evident. B) Cross section of Co close to the base of the peg, showing the outer wall (Ow) and the inner wall (Iw). The inner space is filled by outer dendritic segments (Ods) while the outer space presents electronlucid vesicles (Ev). C) Cross section of Co below the cuticle showing four outer dendritic segments (Ods) enclosed by a thick dendrite sheath (Ds). A small dendritic branch can be seen just in the middle. D) Section of the same sensillum taken at the ciliary constrictions level, the presence of four sensory neurons (arrowheads) appears now clear. Th, thecogen cell; Tr, trichogen cell. E) Cross section of Ca at the peg level. The thick, poreless cuticle (Cw) defines a cavity filled by two outer dendritic segments Ods). F) Cross section of Ca below the socket, the dendrite sheath (Ds) encloses three outer dendritic segments (Ods), one of which presents several lamellae. Ld, laellated dendrite.

3.2.6 Sensilla campaniformia (Ca)

They are represented by cuticular caps (1.15 ± 0.23 long; diameter: 0.88 ± 0.19 μm; N= 22), peg-like, emerging from large sockets in the middle of raised circular dome (diameter: 4.38 ± 0.46 μm; N= 22). The cap is cylindrical and sub-truncate at apex, with a small molting pore in the middle (Fig. 5C,D and 7E,F; Table 1 see in additional

material). Cross sections show a peg in pit sensilla occupying a round cavity. The cuticular walls of the sensillum are thick (480 nm) and poreless, the internal space is completely filled with two dendrites (Fig. 7E). Below the insertion level three sensory neurons are evident, one of them presents several lamellated branches (Fig. 7F). Ca are distributed on the antennal club on the sensorial apical area both dorsally and ventrally, frequently associated with Co. Additionally, from 1 to 3 Ca are present ventrally on the basal sensorial area associated with Co.

3.2.7 Johnston's organ

A non-connective Johnston's organ is situated inside the pedicel. It is made up of 9 groups of chordotonal organs (Cho) placed along the entire circumference of the antennal segment (Fig. 8A). Each group is composed of a variable number of scolopidia, from 2 to 5 (Fig. 8B,C). All the scolopidia are mononematic heterodynal type, being inserted in an electron dense cap and innervated by three sensory cells (Fig. 8D).

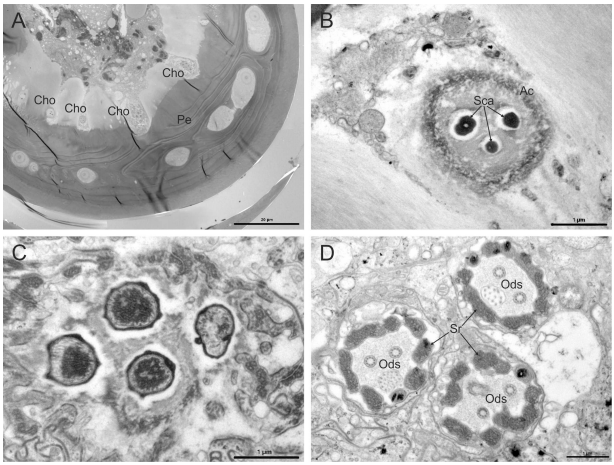

Figure 8 - TEM micrographs of *P. favieri* antennal sensilla: A) Cross section of the pedicel (Pe) showing four chordotonal organs (Cho) close to the internal side of the antennal wall. B) Detail of one Cho with three scolopale caps (Sca) surrounded by the attachment cell. C) Close up view of one Cho with four scolopale caps belonging to four different scolopidia. D) Cross section of one Cho taken more proximally, showing three scolopidia, each one innervated by three outer dendritic segments. At this level, scolopale rods (Sr) formed by the scolopale cell, are clearly visible

3.3 FIB/SEM analysis

Through the FIB/SEM microscope, several antennal sensilla of *P. favieri* have been dissected and analyzed: Ch.3 (Fig. 9 see in additional material), Ch.6 (Fig 10 see in additional material), Ch.7, Böhm sensilla (Fig. 11 see in additional material), Co, Ca. The FIB/SEM serial sectioning of these sensilla gives three-dimensional information useful to describe basal insertions of the setae (joint membrane, suspension fibers, socket, etc) and structures which are hidden or difficult to characterize with only TEM, because the hard cuticle often hampers serial reconstruction. In particular, the FIB analysis shows that the flattened outer part of sensilla Ch.6 is nothing more than the distal part of the seta, tangentially emerging from the cuticle surface, while the basal part is round and its true socket is deeply embedded inside the cuticle (Fig. 10 see in additional material). Under each Ch.6, two large pores are hidden by the prostrate seta, representing the end of adjacent glandular ducts that run straight from two deep exocrine glands (Fig. 10 see in additional material). Additionally, it is possible characterize by FIB analysis the glandular ducts that lead to the secretions inside the sockets of Ch.7, and the structure of the medial conical peg and its ribs of Co, which are usually concealed under the cuticular plate.

4. Discussion

The morpho-functional adaptations to an obligatory myrmecophilous lifestyle in the genus *Paussus* has led to an almost complete reorganization of the antenna from an organ primarily sensorial with a little or no glandular role, to an organ mainly glandular, with a primary role in the interaction with the host ants (Di Giulio *et al*., 2009). In fact, if compared to the moniliform antennae of a non-myrmecophilous ground beetle, the antenna of *P. favieri* (and of all species of the same genus) is drastically modified both at gross and fine levels. Many myrmecophilous beetles show structural modifications of the antennae, frequently combined with the presence of the so called “trichomes”, modified setae fringed, flattened, spatulated, branched, twisted, spinulated, brush-like etc., often crowded in tufts. These trichomes can be present also in other

areas of the body (especially the head, pronotum and abdomen), and their primary function is to enlarge the surface to be wetted by capillary action with secretions which spread out from basal pores, which represent the end of epidermal glandular cells; they also protect the beetles from from attack by the ants, since the aggressiveness and attention of the ants is addressed on these organs (Geiselhardt *et al.*, 2007). A sensorial function to these cuticular structures has traditionally been denied, neglected or only hypothesized (Geiselhardt *et al.*, 2007; Nagel, 1979). In a recent paper, Di Giulio *et al.* (2009) clearly demonstrated that the trichomes of *P. favieri*, though highly modified externally, are instead typical mechanoreceptors, innervated by a single neuron with apical tubular body ending at the base of the seta. The mainteinance of the mechanoreceptive function of these modified setae has been interpreted as a feedback control system for the release of the secretions (Di Giulio *et al.*, 2009). It is also possible that the external apparatus of the mechanoreceptive structures is preferentially recruited for assisting the glandular function since it represents the most plastic structure to be modified, having less morpho-functional constraints than setae and pegs of chemoreceptors.

In *P. favieri*, the different types of trichomes have been assigned to the category of sensilla chaetica (types Ch.1– Ch.8). They all share some features such as: variously modified seta (fishbone-like, brush-like, arborescent, sickle-shaped, twisted, branched etc.); variable number of pores surrounding the base of the seta and/or opening inside its socket; surface more or less corrugated at base and smoothed apically; presence of a tubular body. Groups of trichomes with the same modification and similar length are usually present on the same functional area of the antenna. A particular shape and position of the antennal mechanoreceptors matches a selective function to respond to different mechanical stimuli (Keil, 1997); it is likely that different modifications of the seta are suitable to spread a different mix of chemical secretions for the ants. This would agree with the morphological complexity of the glandular system, represented by at least three different types of glands (GhA– GhC), with different positions inside the antennal club (Di Giulio *et al.*, 2009).

While almost the entire antennal surface of *P. favieri* is covered by modified mechanoreceptors and glandular pores, most of the other sensilla types are “relegated” (with some exceptions) to the thin

apical sensorial area of the antennal club. Similar distribution pattern of sensilla seems to be widespread in all species of the genus *Paussus*, and in most species of Paussini (Di Giulio personal observation; Nagel, 1979). Looking at the different sensilla in *P. favieri*, sensilla chaetica type 9 (Ch.9) are characterised by the presence of a long cuticular shaft sticking out from the rest of the sensilla. The presence of five sensory neurons running within the shaft along with the basal joint membrane housing a tubular body are consistent with a possible, bi-modal, mechano- and chemosensory function (this latter of the gustatory type). Such sensilla have been described for other ground beetle species [ch.2 in *Platynus dorsalis* Pontoppidan (Merivee et al., 2001) and *Bembidion properans* Steph. (Merivee et al., 2002)], for which the role in the perception of salts, sugars and pH variations has been demonstrated (Merivee et al., 2004; 2005; 2007; 2008; Milius et al., 2006).

Sensilla trichodea type 1 and 2 are distributed on the anterior part of the antenna, with some differences. They are characterised by a thin and slender, blunt tipped cuticular shaft and they do not show evident pores. The absence of TEM data makes it difficult to hypothesize about the function of these sensilla, although the involvement in the olfaction seems to be possible. In fact, the presence of cuticular pores it is very difficult to highlight using only the SEM approach. However, the involvement of Tr.1 and Tr.2 in mechanoreception cannot be ruled out. Similar sensilla have been reported in *Bembidion properans* as sensilla trichoid type 1 (s.t.1) (Merivee *et al*., 2002), for which a mechanosensory function was hypotesized.

In *P. favieri*, the deep structural and functional modification of the antenna is reflected also on the distribution of some classes of sensilla. It is the case of sensilla basiconica type 1 (Ba.1) and type 2 (Ba.2), that are mostly concentrated on the dorsal and ventral apical sensorial area. As regards their structure, they are typical basiconic sensilla with porous cuticle, they present dendritic branches within the cuticular peg and not flexible socket. Sensilla of the same type were commonly found in non myrmecophilous groud beetles (Merivee *et al*., 2000; 2001; 2002), for which the involvement in the perception of volatiles (olfactory chemoreceptors) was hypothesized. In *P. favieri*, Ba.1 and Ba.2 could play a key role either in intraspecific interactions and/or relationships with the ants.

Besides Ba.1 and Ba.2, in *P. favieri* a third class of basiconic sensilla was found, termed as Ba.3. They are present in very low number along the distal margin of the apical sensorial area and are structurally very similar to the sensillum basiconicum type 3 of *Platynus dorsalis* (Merivee *et al*., 2001). Unfortunately, the absence of TEM data makes it difficult to reveal their function, that at present remains unknown.

The apical sensorial area revealed also the presence of sensilla coeloconica (Co). These sensilla were described in several order of insects (Tominaga and Yokohari, 1982; Iwasaki *et al*., 1995; Ruchty *et al*., 2009), showing a typical conserved structure consisting of a subcuticular chamber, housing a grooved peg and communicating with the outside through a single aperture. The Co we found in *P. favieri* antennae show a structure similar to the sensilla coeloconica described in other ground beetle species (Merivee et al., 2000; 2001; 2002). As regards their function, sensilla coeloconica have been proved to act as thermo- or thermo-hygroreceptors (Altner and Prillinger, 1980; Altner *et al*., 1981; Yokohari, 1983; Altner and Loftus, 1985; Ruchty *et al*., 2009), as well as combined thermo- and chemoreceptors (Altner *et al*., 1977, 1981; Davis, 1977; Hansson et al., 1996) and exclusively chemoreceptors (Boeckh, 1967; Pophof, 1997; Pophof *et al*., 2005).

The antennal apical sensorial area of *P. favieri* is characterized by the presence of sensilla campaniformia (Ca), occurring in low number and in close association with the Co. Such sensilla revealed to be of the aporous type with inflexible socket, innervated by three sensory neurons. In ground beetles, these sensilla were described for several species (Merivee et al., 2000; 2001; 2002). Although their function as mechanoreceptors was misinterpreted because of the absence of TEM and electrophysiological data, it has been proven later through electrophysiology that these sensilla respond to variation in humidity and temperature (the three neurons are of the dry, moist and cold type) (Merivee *et al*., 2003; 2010; Must *et al*., 2006a; 2006b; 2010).

The apical sensilla in *P. favieri* (Ch.9, Tr.1– Tr.2, Ba.1– Ba.3, Co, Ca) are representative of all main types present in other ground beetles, and they must play a key role in detecting environmental cues, as well as host-produced substances and semiochemicals acting at intraspecific level.

The antennal sensilla of Carabidae have been studied by SEM, TEM and, more recently, electrophysiology in only about 20 (out of 40,000) non-myrmecopilous species, not representative of their great diversity: *Loricera pilicornis* (Altner & Hintzpeter, 1984), *Nebria brevicollis* (Dalay & Ryan, 1979), *Carabus fiduciarius* (Kim & Yamasaki, 1996), *Damaster spp.* (Kim & Yamasaki, 1998), *Bembidion* spp. (Merivee *et al.*, 2000; 2002; Ploomi *et al.*, 2003), *Platynus* spp. (Merivee *et al.*, 2001; Must *et al.*, 2006a; Ploomi *et al.*, 2003; Weis *et al.*, 1999), *Siagona* spp. (Giglio *et al.*, 2005; 2008), *Pterostichus* spp. (Merivee *et al.*, 2003, 2004, 2005, 2010; Milius *et al.*, 2006; Must *et al.*, 2006b; Symondson & Williams, 1997), *Poecilus cupreus* (Must *et al.*, 2006b). If compared to the species of Carabidae listed above, *P. favieri* shows: 1) general reduction in number of the antennal sensilla of all types; 2) greater asymmetry and irregular distribution of sensilla in functional areas that parallel the antennal structural asymmetry (dorsal *versus* ventral, anterior *versus* posterior, proximal *versus* distal); 3) great number of morphotypes of sensilla chaetica (Ch.1– Ch.9); 4) sensilla chaetica with a socket relatively narrower; 5) sensilla campaniformia Ca atypical, more similar to the coelocapitular sensilla of the honey bee (Yokohari, 1983) than to true campaniformia of other Carabidae; nevertheless, the homology with campaniformia is here tentatively based on the comparison with homologous structures of more basal, non-myrmecophilous Paussinae (Di Giulio personal observation).

Antennal structures of 8 *Paussus* species (*P. humboldti pilosus*, *P. procerus*, *P. armatus*, *P. spinicoxis*, *P. cilipes*, *P. sphaerocerus*, *P. centurio* and *P. arabicus*) have been comparatively studied at SEM by Nagel (1979). In his pioneer work, Nagel (1979), according to the available classifications and descriptions of sensilla (Callahan, 1975; Dethier, 1963; Harbach & Larsen, 1977; Schneider & Steinbecht, 1968; Snodgrass, 1935, 1956) identified: one type of sensillum trichodeum (composing the "antennal symphilous organs"); three main types of sensilla chaetica: Ch.1 (including two sub-types, Ch.1a and Ch.1b), Ch.2 and Ch.3; two main types of sensilla basiconica: Ba1 (including two sub-types, Ba1a and Ba1b), and Ba2; one type of sensillum coeloconicum. The SEM analysis of a larger taxon sampling (more than 50 representative species in the whole subfamily Paussinae), including non-myrmecophilous tribes (Di Giulio *et al.* in prep.), as well as the present thorough SEM/TEM/FIB analysis of the species *P. favieri* reported in this

work, lead us to reconsider some of the preliminary Nagel's (1979) interpretations. In particular, the following main similarities/differences were recognised: (1) the sensilla of the antennal symphilous organs of various species have been considered as trichodea by Nagel (1979); nevertheless, we found a clear homology of these sensilla with other modified sensilla chaetica; for this reason, we prefer to assign these structures to a particular morphotype of sensilla chaetica (Ch.7); (2) the Nagel's Ch.1a are similar to the fishbone-like (Ch.1) here described for *P. favieri* and this type seems to be widespread in the genus *Paussus*; (3) the sickle-shaped (Ch.6) of *P. favieri* seems to be homolog to the Ch3 (Nagel, 1979) of *P. spinicoxis*, and to the Ch1b (Nagel, 1979) of *P. procerus*; (4) the marginal Ch2 described by Nagel (1979) are not present in *P. favieri*; (5) the Ba1a (Nagel, 1979) includes actually sensilla very variable in size and could be homologous to both Ba.1 and Ba.2 of *P. favieri*; (8) sensilla tentatively identified by Nagel (1979) as coeloconica (or even as large secretory pores), actually include two types of very different sensilla, campaniformia and coeloconica, also present in *P. favieri* and here identified respectively as Ca and Co.

A sexual dimorphism concerning the gross antennal structure has long been known in many species of the genus *Paussus*. Nagel (1979) clearly outlined in *P. centurio* and *P. arabicus* that a sexual dimorphism can also be present at the level of the antennal sensilla, at least in several species of this genus. In fact, numerous Ba1a (corresponding to Ba.1 of *P. favieri*), with a possible role in the intraspecific communication, are present on the antennal surface of the males (giving an opaque looking at the stereoscope), while they are almost absent on the same part of the antennae of the females (antennae shining). In *P. favieri*, neither a macro-structural dimorphism of the antennae, nor a clear difference in number, types and distribution of any type of sensilla have been observed.

Another evident modification of all paussine species is the strong reduction of the pedicel to a small globular ring. This last, tightly and rigidly encircling the basal process of the antennal club, forms with the scape a highly effective ball-and-socket joint that increases the antennal mobility (Geiselhardt *et al.*, 2007; Luna de Carvalho, 1989; Nagel, 1979). Earlier it was assumed that the reduction of the pedicel caused the disappearance of the Johnston's organ, which controls the antenna's tactile function and the flight speed, bringing the authors

to the speculative conclusion that the antennae of Paussini are not receptors of mechanic stimuli (Darlington, 1950; Luna de Carvalho, 1989; Nagel, 1979; Geiselhardt *et al.*, 2007). The present analysis shows that in *P. favieri* a Johnston's organ is present inside the pedicel. The structure is composed of 35– 40 scolopidia attached to the distal half of the pedicel, close to the joint zone with the flagellum.

Paussini are able to fly, and, as already described in other insect orders (Gewecke, 1967, 1970; Gewecke & Niehaus, 1981; Johnson, 1956; Heran, 1959; Yagodin, 1980), the presence of a chordotonal organ in the pedicel aids in flight. The flagellum receives air current stimuli, generated during flight, and conducts the aerodynamic forces to the pedicel. The Johnston's organ perceives the movements of the flagellum with respect to the pedicel (Gewecke, 1974). Another widely acknowledged function of the Johnston's organ in insects is acoustical, detecting the sound waves that generate vibrations of the antenna (Bailey, 1991; Fedorova & Zhantiev, 2009; McIver, 1985). *P. favieri* (male and female) and most species of Paussini, are able to produce sounds by means of stridulatory organs, also present in their host ants (Di Giulio *et al.*, 2011; Di Giulio *et al.,* in prep.). The presence of a Johnston's organ inside the pedicel would confirm the possibility of receiving acoustic stimuli at the level of the antennae.

Finally, the FIB/SEM has revealed to be a useful tool for the morphological characterization of cuticular microstructures like antennal sensilla, or structures connected to the glandular system, offering complementary information to those obtained by TEM. This is possible thanks to its exceptional imaging and the precise and fast localization of the subject through the SEM column, combined to the powerful FIB column that works like a nano-scalpel for the serial cross sectioning of the cuticle, and allows a 3D reconstruction of the structure. The short movies representing the milling process (see electronic version of this paper), and Figs. 9 and 10 give an idea of the potential applications of this new tool in arthropod investigations, even for cuticular structures extremely rare on cuticle, or partially or totally concealed.

7. References

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Conclusions

The present contribution represents a pioneer study on a myrmecophilous model particularly difficult to investigate, Paussini are extremely rare and difficult to rear in captivity. For this reason behavioral studies have been tried by very few authors in the past, with scarce success. No chemical studies have been performed before, and no information was available about the biological meaning of the stridulatory organ. Through performed on a single species, *Paussus favieri,* representative of the most derived type present in Paussini, this work supplies several new and more general information on the biology and functional morphology of this interesting group of beetles.

The results confirm that several sensory channels are involved in the different phases of the process of integration of *P. favieri* in the host ant society. The behavioral, morphological, chemical and acoustical adaptations discovered in *P. favieri* represent different aspects of an extraordinarily complex strategy that can be tackled only by an integrated multidisciplinary approach, combining different techniques, methodologies and analyses. For example, without the ultrastructural analysis of the antennae, the presence of the Johnston's organ would have not been detected. This finding has allowed, instead, to recognize the ability of *P. favieri* to perceive acoustic stimuli, confirming that the acoustical communication can actually be involved in the mating behavior.

The integration of *P. favieri* into host ant *Pheidole pallidula* colonies is achieved by chemical mimicry, probably reinforced by the attractive antennal secretions that contribute to the successful acceptance and integration of the beetle inside the host colony. The beetles are continuously touched and actively licked by the workers and soldiers, especially on the antennae, which show a primary glandular function. Moreover, the beetles seem to obtain other cuticular compounds distinctive of the individual colony (colony odour) by feeding on larvae and adults of the host ants, and by physically interacting with different castes, refining the main chemical mimicry through a partial camouflage.

Surprisingly, an advanced acoustic mimicry is involved in the relationship between *P. favieri* and *P. pallidula*. By using the same stridulatory organ, the beetle is able to "talk" different languages, of

queen, soldiers or workers, by modulating the emissions of different types of pulses.
The acoustical mimicry with the queen also suggest that the beetle can raise the highest hyerarchical status, and join of the advantages. Additionally, the frequent interactions/contacts with the queen, highlighted in the behavioral analyses, suggests that a combination between chemical and acoustical mimicry could be likely.
Another aspect of this research is that investigating in detail how do the parasite exploit the communication channels of the host, we gather relevant information on the communication system of the ants outlining its complexity and weakness.
However, further behavioral, chemical and acoustical investigations are needed to show whether a similar pattern of communication is involved also in other paussine species, or different degrees of interactions can be recognized in this myrmecophilous tribe.

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