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SUMMARY

Group living and the trade-off between its costs and benefits has been deeply studied in vertebrate as much as in invertebrate groups. Eusocial insects represent the highest level of social organisation in Arthropods and a highly valuable model to study group living dynamics. In this thesis, different species of Hymenopteran social insects are used as a model to study the adaptations to cope with one of the main issues of the group living lifestyle, the potential increase of disease risk. In fact, the high density of related and interacting individuals coexisting in the reduced area occupied by a social insect nest facilitates the spread of parasites among colony members. Despite this, the evolutionary success of social insects indicates that they evolved effective responses to the biotic pressure imposed by parasites. These responses result from the combination of individual and collective immune adaptations, and rely on physiological, behavioural and organizational mechanisms which, through collective dynamics, compose the integrated colonial strategies referred as social immunity. This PhD thesis examines some aspects of social immunity by facing questions experimentally. Social immunity in insects has a fundamental role in colony survival but empirical evidence about its expression and its interactions with the physiological status of individuals is still scattered. The tendency to focus on single aspects of social immunity and on specific colony components complicates the understanding of the phenomenon and a balanced overview of its costs and benefits. An ant species, the acrobat ant *Crematogaster scutellaris*, has been investigated with a particular effort in the aim of describing the existence of immunization in queens and of trans-generational immunization in their worker offspring. A methodological study quantified the long-term consequences of microbiological and manipulative stress on queen survival and on the timing and success colonial development. In a second study the occurrence of trans-generational immunization against the entomopathogenic fungus *Metarhizium anisopliae* has been demonstrated for the first time in ants using this model organism. In a third study we found that both claustral and worker-right *C. scutellaris* queens do not express immunisation against the same pathogen; conversely, a first exposure increases mortality during the challenge. These results indicate that immunization and trans-generational immunization are uncoupled phenomena in this host-parasite system since foundresses exposed to *M. anisopliae* can elicit an increased resistance in the offspring without providing themselves with an increased immune response. A study on the integration between physiological and behavioural components of social immunity have been carried out on in the ant *Temnothorax unifasciatus*, showing the particular habit of nesting in small and promiscuous cavities. In the absence of direct contact, *T. unifasciatus* colonies inhibited fungal growth inside their nests, presumably through volatile compounds and colony size was positively correlated with this fungistatic activity. In a second experiment, we found that small colonies presented with contaminated fibres showed an increased removal of both contaminated and non-contaminated items compared to small colonies presented with control fibres only. Conversely, larger colonies removed items regardless of the presence of the spores inside the nest. This demonstrated that colony size qualitatively affects removal behaviour. The behavioural component, in particular, the hygienic behavior of social grooming, has

been also studied in *Apis mellifera*. Using a highly multidisciplinary approach we characterized this behavior and individuals expressing it, elucidating the relevance of a trade-off between behavioural specialization and phenotypical plasticity. The possibility to study a newly discovered nesting association between the stingless bee *Paraponera clavata* and the bullet ant *Partamona testacea* led to the production of a study about the peculiar interactions occurring between associated colonies of these two species.

Chapter 1

Introduction

1.1 Social insect colonies as resources for predators and pathogens

Living in a social group involves several advantages for colony members respect to individuals conducing a solitary lifestyle, nevertheless the counterparts of the two distinct social conditions are commensurate to the benefits. In social insect societies, cooperation grants a more efficient brood care, supply of resources and an effective protection against adversities and predators (Wilson, 1971). This efficiency in eusocial species results in structures, nests, hosting up to thousands of individuals, with a large quantity of energetic resources in terms of stored food and harmless brood. This opulence never remained unnoticed, and several organisms adapted their biology to exploit it as predators, parasites and pathogens (Alexander, 1974; Hamilton, 1987). The micro-environmental conditions characterizing social insect nests are generally more stable and compensated respect to the external environment, hand in hand with the social complexity of the observed species. Nest structure and behavioural responses of colony members are integrated in order to ensure optimal conditions in terms of temperature, humidity and ventilation for the overall colony health. Furthermore, overall genetic variability among colony members is relatively low, resulting in a homogeneity also in terms of susceptibility. These conditions, together with the high concentration of individuals and the high rate of social interactions among them, essential in colonial life, create a suitable and virtually optimal environment for the establishment and the diffusion of micro and macro-parasites (Hamilton, 1987; Schmid-Hempel, 1998; Shykoff and Schmid-Hempel, 1991). Nevertheless, the evolutionary success of social insects is undeniable (Lach et al., 2010; Wilson, 1971), indicating that they evolved effective responses also to the biotic pressure imposed by parasites (Evans, 1974). These responses result from the combination of individual and collective immune adaptations, relying in physiological, behavioural and organizational mechanisms which, through collective dynamics of synergy, shape the integrated colonial strategies referred as Social Immunity (Cremer et al., 2018, 2007).

1.2 Storyboard and evolutionary biology of social immunity

The focus on the relatively recent topic of social immunity rapidly drove to considerable advances in understanding why and how colonies of social insects effectively protect themselves from infections and infestations (Cremer et al., 2007; Cremer and Sixt, 2009; Schmid-Hempel, 1998). Social immunity definition has been debated and gradually tuned with time. Cremer et collaborators (2007) defined social immunity as collective actions or altruistic behaviours resulting in avoidance, control or elimination of parasitic infections in eusocial insect colonies. In 2009 Wilson-Rich and collaborators (Wilson-Rich et al., 2009) provided a more general definition of social immunity, as the set of collective defences against parasites and pathogens expressed in colonies of eusocial insects. Cotter & Kilner (S. Cotter and Kilner, 2010) opened to other forms of group living organisms, proposing that ‘any type of immune response that has been selected to increase the fitness of the challenged individual and one or more recipients’ is an expression of social immunity, thus including also mechanisms, like parental cares, sanitary behaviours and herd

immunity, expressed by other group living species, even if non eusocial (Boos et al., 2014; John and Samuel, 2000; Nalepa and Bell, 1997).

Two different hypotheses about the role covered by social immunity in the evolution of group living, defined as eusocial and group-living framework (Van Meyel et al., 2018), took form over time. The eusocial framework represents the main approach embraced by socio-biologists in the past years, which identify social immunity as the respective in social insect colonies of the individual immunity in multi-cellular organisms (Cremer et al., 2018; Cremer and Sixt, 2009; Masri and Cremer, 2014), fitting with the concept which regards social insect colonies as super-organisms (Kennedy et al., 2017). This approach gives way for the conception of social immunity as a secondarily derived adaptation in eusocial systems contributing to its stabilization (Cremer et al., 2018; Schmid-Hempel, 2017, 1998). On the other hand, a more recent approach emerged, identified in the group-living framework, which introduces social immunity in a more flexible and ancestral context in evolutionary terms. Basing on evidence that components of social immunity can be found in many group-living organisms which are not eusocial, this framework supports that social immunity is heavily involved in the early emergence and maintenance of group living from a solitary lifestyle (S. Cotter and Kilner, 2010; Meunier, 2015). The group-living framework includes among social immunity phenomena the collective defences, like communal defences against diseases and parental cares, adopted by species living in non-eusocial groups against parasites (Cremer et al., 2018; Schmid-Hempel, 2017). This point of view supposes the existence of a continuum between eusocial and non-eusocial group living species to unravel the evolutionary pathways of social immunity across group living species (S. Cotter and Kilner, 2010; Meunier, 2015). This approach enlarges the conception of social immunity and opens to a more comparative approach through different levels of sociality across a broader range of models. This recent shift which moves the attention of the evolutionary processes from a eusocial to a group-living framework has generated the necessity to re-define the boundaries of social immunity. In this optic, a new definition has been introduced by (Meunier, 2015) and refined by (Van Meyel et al., 2018) which describes social immunity as 'any collective or personal mechanism that has emerged and/or is maintained at least in part partly due to the anti-pathogen defence it provides to other homospecific group members'.

1.3 Immunity mechanisms at group and colonial level

The dynamic of parasite infections in social insect colonies and in group living organisms can be resumed in three main steps (Cremer et al., 2007; Wilson-Rich et al., 2009) during which colony members can interfere to constrain or virtually eliminate the parasite infection. First, the parasite has to be carried inside the nest, which implies its uptake from the external environment and its intake inside the nest environment. Second, the parasite must establish itself inside the nest profiting of the suitable micro-environment. Third, it has to spread among colony/group members, and in an advanced stage among other colonies/groups. Each intervention at individual or collective

level aimed to reduce the parasite success inside the colony/group could be considered as an expression of social immunity.

2) Limiting parasite uptake from the environment: There are two main routes of parasite transmission between colonies or groups: parasites can be encountered by a colony member in the external environment, for example during foraging or patrolling, and carried to its own nest; otherwise the parasites can be directly transmitted from a mother colony to the daughter one. Cremer and collaborators (2007) defined these two routes respectively horizontal and vertical transmission (see fig. 1). In social insects there are many cases reported of collective and individual strategies performed to limit parasite contact or its transport inside the nest during external activities (Tranter et al., 2014). Individual defences reducing the risk of infection are recorded in foragers of different species, for example, in ants and termites foragers tend to avoid areas with high parasite densities (Diehl-Fleig and Lucchese, 1991; Drees et al., 1992; Epsky and Capinera, 1988; Mburu et al., 2009), refrain to cannibalize infected conspecifics (Kramm et al., 1982; Marikovsky, 1962; Zhou et al., 2002) or to consume contaminated food (Baur et al., 1998). Individual avoidance behaviours are reported also in non-eusocial and solitary species (Jaronski, 2013; Kaakeh et al., 1996), whereas other mechanisms are exclusive of eusocial species being dependent on peculiar aspects of eusociality, as those based on the division of labour. For example, leaf-cutting ants of the genus *Atta* present a hitchhiker caste, smaller workers carried back to the nest on the vegetable material transported by cutters, with the role to prevent egg-laying of parasitoid flies on the latter ones (Vieira-Neto et al., 2006). To prevent a parasite intrusion in the nest mediated by foragers, specialised honeybee guards stand at the nest entrance attacking or excluding infected nestmates (Drum and Rothenbuhler, 1985; Waddington and Rothenbuhler, 1976). In species which have the nest (characteristic common in most of eusocial taxa), the nest architecture can represent a barrier to limit parasite income. Especially for parasites which actively penetrate into the colony, as several macro-parasites, parasitoids and social parasites usually do, the limited exposure of the nest core and the number and the shapes of the entrances can heavily reduce the vulnerability of the colony (Pie et al., 2004; Schmid-Hempel, 1998).

2) Containing parasite establishment into the nest: when parasites manage to elude these first barriers and reach the nest environment, the colony infection can still be avoided by preventing their establishment inside the nest. In this situation, any mechanism involved in maintaining nest hygiene is fundamental. This is particularly evident in species nesting in substrates generally rich in parasites, as soil or decaying wood, or in perennial colonies which could easily accumulate parasites over time (Cremer et al., 2007; Hughes, 2005; Schmid-Hempel, 1998). Two main strategies are involved in this process, the use of antimicrobial substances and the performance of sanitary behaviours. Chemical substances can be collected from the external environment and, after different levels of processing, incorporated in the nest as it has been observed in many species of ants and bees (Christe et al., 2003; Farnesi et al., 2009; Gilliam et al., 1988; Simone-Finstrom and Spivak, 2010). Moreover, the production of secretion by the colony members is a fundamental

mechanism in many species, showing exocrine glands adapted to produce antimicrobials (Otti et al., 2014). In ants, the secretion of metapleural glands plays a key role in self-disinfection of individuals but also in nest treatment (Hölldobler and Engel-Siegel, 1984; Ortius-Lechner et al., 2000; Yek and Mueller, 2011), while wasps use for the secretions of the venom gland (Baracchi et al., 2012; Turillazzi et al., 2006) for similar purposes. Notably, the use of antimicrobial and substances into the nest environment has been observed also in non-eusocial species as sub-social beetles and wasps (Cardoza et al., 2006; Kaltenpoth et al., 2005; Otti et al., 2014). Sanitary behaviours are performed to eliminate from the nest material which can represent a suitable substrate for parasite growth (Bot et al., 2001; Diez et al., 2012) or which constitute a blatant source of the infection spread, like corpses of nestmates succumbed to an infection. Inside eusocial colonies, waste is normally moved toward peripheral areas of the nest, outside of it, or accumulated in specific landfills (Ballari et al., 2007; Bot et al., 2001; Trumbo et al., 1997); a similar treatment is reserved to nestmate corpses, which are promptly removed and in some cases accumulated in specific areas, the so called "graveyards" (Howard and Tschinkel, 1976; Wilson et al., 1958). Frass removal has been also observed in sub-social cockroaches (Nalepa and Bell, 1997), crickets (West and Alexander, 1963) and in group-living beetles (Kirkendall et al., 1997). If the removal of the biohazard is not possible or unsustainable, its physical isolation *in situ* might result an effective method to knock down its infectiveness. This can be actuated by encapsulating the risky item with antimicrobial materials. Ants pile fungal spores and seal off them in nest material (Jaccoud et al., 1999; Storey et al., 1991), bees encapsulate with propolis parasite beetles penetrating in the colony (Neumann et al., 2001) and termites treat the risky frass or nestmate corpses with antimicrobial secretions (Chouvenc et al., 2013; Rebeca B. Rosengaus et al., 1998) as also observed in cockroaches and other non-eusocial species (Cotter and Kilner, 2010; Rosengaus et al., 2013; Steiger et al., 2011). The termite *Zootermopsis angusticollis* has been observed to manipulate corpses of nestmates succumbed to nematodes with the aim to exsiccate them to kill the parasites (Wilson-Rich et al., 2007).

3) Limiting parasite spread between group members: parasites can elude the above-mentioned precautionary measures and establish themselves in the nest. In this inauspicious situation, group members can actuate several strategies to contain parasite diffusion among nestmates. Cremer and collaborators (Cremer et al., 2007) categorized these mechanisms depending on which of the three main factors operating in colony infection dynamics they act on: i) the infectiousness of the infected individual; ii) the contact rate and the interaction typology between the infected individual and the other group members; iii) the susceptibility to the infection of non-infected group members.

i) The reduction of the infectiousness of the infected individuals is often operated by physical removal of the parasite and its elimination. Social grooming, the removal of debris and infection particles from the body surface of other group members, is very common in eusocial species (Drees et al., 1992; Kermarrec et al., 1986; Oi and Pereira, 1993; Peng et al., 1987; Rebeca B Rosengaus et al., 1998; Wilson-Rich et al., 2007) and the elimination of risky groomed material can occur by

physical destruction. For example, *Varroa* mites are killed by social groomer Asian honeybees by biting them (Peng et al., 1987), while in ants and wasps groomed parasites are collected in an infrabuccal pocket and neutralized with the secretion of the labial glands (Hughes, 2005; Little et al., 2006).

ii) Contacts between nestmates of social insects do not have a random distribution and determinate dyads of individuals interact more often respect others. This implies social heterogeneity inside the colony, resulting in the formation of partially isolated sub-groups of individuals having a reduced rate of interaction with other colony sub-groups (Fefferman and Ng, 2007; Naug, 2008; Naug and Camazine, 2002). Depending on the biology of the species different levels of compartmentalization between operative sub-groups can emerge as a result of spatial and behavioural constrains which discourage specific social interactions. This division tends to produce a spatial pattern where compartments of individual performing risky tasks are more peripheral while the ones hosting the brood and the reproductive individuals are located in the colony core. The centrifugal polyethism (i.e. the coherence with age dependent tasks and their position in the nest) (Bourke et al., 1995; Schmid-Hempel, 1998), combined with the property of compartments to keep the infection local, creates a multi-shell protection around the most precious colony resources providing a sort of quarantine line and a form of 'organisational immunity' (Naug and Camazine, 2002; Naug and Smith, 2007; Read and Keeling, 2003; Stroeymeyt et al., 2014). The extrapolation of a single infected member from the interaction network of the colony can be actuated by isolation or removal. Entombing is an expression of social isolation where a colony member is physically confined by nestmates, normally buried alive, to impede its interaction with the other colony members inside the nest (Chouvenc et al., 2013; Culliney and Grace, 2000; Rath, 1999). Otherwise the infected individual can be detected inside the nest and expulsed outside by nestmates, as observed in honeybees (Baracchi et al., 2012), or it can actuate self-removal, actively leaving the nest (Bos et al., 2012; Heinze and Walter, 2010; Rueppell et al., 2010).

iii) The susceptibility of the individuals to parasites is at the base of each infection dynamic. Parasite transmission in the group can be limited by reducing the individual susceptibility through different strategies, thus limiting the probability of disease transmission between dyads (the infected individual and the healthy one). Insect societies are expected to show a relatively high rate of genetic similarity which implies also a similar susceptibility to parasites. In species showing multiple-mating or multiple-breeder strategies, overall colony susceptibility to parasites is reduced when genetic diversity is higher among colony members probably because they share differential physiological or behavioural defences (Hughes and Boomsma, 2004, 2006; Ugelvig et al., 2010). The increase of genetic diversity, differentiating susceptibility among colony members, represents a prophylactic defence strategy (Reber et al., 2008; Schmid-Hempel, 1997; Sherman et al., 1988). In the ant *Cardiocondyla obscurior*, workers from more genetically diverse colonies respond more promptly to the presence of entomopathogenic fungal spores respect to workers belonging to more

homogeneous colonies, expressing behavioural responses such as allogrooming and removal of infected items (Ugelvig et al., 2010).

1.4 Individual immune defenses

The personal immune defences are the main factors determining the individual susceptibility to pathogens, defining the tolerance (parasite load that the host can tolerate) and the resistance (host ability in reducing pathogen load) toward parasites of the single colony components (Medzhitov et al., 2012; Schneider and Ayres, 2008). Although, if observed on its own, Individual immunity it does not differ so much between social, group living and solitary insect species, it also represents one of the components of the integrated complex constituting social immunity. Individual immunity is represented by two main components known as innate and acquired immunity. The innate immunity operates through encoded factors for recognition and elimination of parasites and alien corps, while acquired immunity involves enhanced responses toward certain pathogens based on immune memory mechanisms acquired by an individual during its life experience (Fearon, 1997).

Innate immunity in insects comprises mechanisms associated to anatomical and physiological body barriers and the immune system, divided in (i) cell-mediated and (ii) humoral defence responses (Lemaitre and Hoffmann, 2007; Wang et al., 2011). This division depends more on practical reasons than on functional ones, being haemocyte activity often regulated by humoral factors and being many humoral defence molecules produced by haemocytes (Strand, 2008).

i) The cellular immune response involves haemocytes which can phagocytise pathogens or, in case of large organisms as parasitoids, encapsulate them by cell-mediated mechanisms (Gillespie and et al., 1997; Irving et al., 2005; Lackie, 1988; Lavine and Strand, 2002; Strand and Pech, 1995). Cell-mediated responses are carried out by enzymes activated and/or produced by haemocytes. The enzyme phenoloxidase (PO), the activated state of the proPO (prophenoloxidase), is produced by haemocytes and released into the haemolymph (Cerenius et al., 2008). Receptors for pathogen recognition trigger a cascade of serine proteases activating the PO, which catalyses the production of melanin and cytotoxic products (reactive oxygen intermediates) involved in the encapsulation (Cerenius et al., 2008).

ii) Humoral responses involve the release in the haemolymph of soluble molecules with antimicrobial proprieties such as antimicrobial peptides (Strachecka et al., 2018) synthetized in the fat bodies (Imler and Bulet, 2005). Other humoral responses are the activation of enzymes as phenoloxidase and hemolectin respectively regulating melaninization of damaged tissues (Cerenius and Söderhäll, 2004) and clotting. The latter is the aggregation of haemolymph components (like haemocytes) around microorganisms which immobilize and kill them by producing antimicrobial substances (Muta and Iwanaga, 1996; Theopold et al., 2004).

Knowledge about acquired immunity in invertebrates impressively developed in the last decade. Basing on different mechanisms respect to the well-studied immune memory of vertebrates,

invertebrates can also improve their immune responses against the parasites experienced during their life (Chambers and Schneider, 2012; Kurtz, 2005, 2004; Little et al., 2005; Milutinović and Kurtz, 2016). This phenomenon, named immunization, leads to the protection of the host against a disease-causing agent (mostly parasites) upon secondary exposure to the same agent (Janeway et al., 2005). Immunization can rely on a general immune upregulation or on a specific immune memory, called immune priming. The first, provides an unspecific protection toward a broad range of pathogens, included the eliciting one (Moret and Siva-Jothy, 2003), while the immune priming protects the host against the same elicitor parasite, and its specificity can be expressed at genus, species and even strain level (Kurtz and Franz, 2003; Masri and Cremer, 2014; Pham et al., 2007; Roth et al., 2009; Roth and Kurtz, 2009; Sadd and Schmid-Hempel, 2006). Immunization occurs within the lifespan of an individual, even across developmental stages (Masri and Cremer, 2014). In social insects its expression depends on the host-pathogen system observed and on the physiological status of the host at the moment of the exposure (Gálvez and Chapuisat, 2014). Furthermore, immunization can be transmitted from parents to offspring, and takes the name of trans-generational immunization. Also this phenomenon can rely on an unspecific general protection or on specific agents and the transmission can occur among individuals belonging to the same colony (Bordoni et al., 2018; Roth et al., 2009, 2010; Sadd et al., 2005; Sadd and Schmid-Hempel, 2007; Tidbury et al., 2011; Zanchi et al., 2011) or from the mother colony to the daughter one (Moret and Schmid-Hempel, 2001). Immunization also occurs horizontally at the colonial and group level, since it has been observed that the susceptibility of group members is reduced after a first contact of other individuals with a given parasite (Hamilton et al., 2011; Traniello et al., 2002). Immunological mechanisms underlying the immunization in invertebrates, their expression and fitness consequences on individuals are still elusive (Gálvez and Chapuisat, 2014; Masri and Cremer, 2014; Moret, 2000), in this optic the phenomena of individual immunization and its transmission across generations in social insects will be discussed more in detail in the experimental section of this thesis.

1.5 Aim of the thesis

This PhD thesis takes in examination distinct aspects of social immunity in social insects by facing questions experimentally. In fact, although social immunity in insects is considered as a fundamental phenomenon in the survival of the colonies, empirical evidence about its expression and its interactions with the physiological status of individuals is growing but it is still scattered. The tendency to focus on single aspects of social immunity and on specific colony components (e.g. workers or reproductors), makes it difficult to get a comprehensive understanding of the phenomenon and mostly a balanced overview of its costs and benefits. In this thesis different hymenopteran social species (ants and bees) have been used as models to study the expression of different components of social immunity (physiological, behavioural and organizational). One ant species, the acrobat ant *Crematogaster scutellaris*, has been investigated with a particular effort in

the aim of describing the existence of immunization in queens and trans-generational immunization in their worker offspring. A preliminary methodological study has been carried out to quantify the possibility to rear colonies in the lab and the long-term consequences on queens and colonial development associated with microbiological and manipulative stress. A particular attention has been paid in this thesis to avoid a series of theoretical and experimental biases. First, it can be expected that any perturbation (e.g. exposure to parasite, stress) in individuals and colonies can produce its effects both in the long and the short term. For this reason, the effects of different stress including the exposure to the entomopathogenic fungus *Metarhizium anisopliae* in *C. scutellaris* have been monitored through observations during all the first year of colonial development and, in one study, the consequences of the exposure have been also compared between colonies belonging to their first and second year of colonial development. Moreover, studies searching for evidence of immunization in social insects often test immune competence by assessing quantity and quality of the immune cells or parasite density in the organism (typically measured with bacterial clearance assays). Nevertheless, there is empirical evidence that hosts bearing more haemocytes and less parasites are not necessarily the most fit (Graham et al., 2011). In this optic, in the studies about of *C. scutellaris* the effects of stress and the occurrence of immunization have been tested by measuring the colonial development over the long term and the fitness consequences on the host measured in terms of survival, also in different physiological conditions (typically starved and ad libitum feeding). The study on the integration between physiological and behavioural components of social immunity have been carried out on in the ant *Temnothorax unifasciatus*, showing the particular habit of nesting in small and promiscuous cavities. This model appeared particularly profitable to study the nest sanitization in ants with a specific focus on producing volatile antimicrobial substances. In this case, compared with the sole study reporting this phenomenon in ants (Wang et al., 2015), we paid a particular attention in designing and experimental setting maintaining the social integrity in order to avoid potential confounding effects produced by the extrapolation from the colonial context. The behavioural component, the hygienic behavior of social grooming in particular, has been also studied in *Apis mellifera*. Using a highly multidisciplinary approach resulting from the combination of behavioural observations, network analyses, proteomics and immune assessments we tried to characterize this behavior and individuals expressing it, elucidating the relevance of a trade-off between behavioural specialization and phenotypical plasticity. The possibility emerged during the PhD to study a newly discovered nesting association between the stingless bee *Paraponera clavata* and the bullet ant *Partamona testacea* led to the production of a study about the peculiar interactions occurring between associated colonies of these two species. The initial aim of studying the immunological aspects of this association failed because microbiological experiments revealed to be impracticable in the Peruvian field station. Nevertheless, the study of demography, behavior, cuticular hydrocarbon signature of both species and the assessment of bee mitochondrial genetic structure shed light on mechanisms allowing the strict coexistence in these two species.

The description of the specific aims of each study and the related discussions are reported in detail in the experimental sections of this thesis.

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Chapter 2

Long-term assessment reveals the hidden and hiding effects of experimental stress on ant colonies

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Long-term assessment reveals the hidden and hiding effects of experimental stress on ant colonies

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ABSTRACT

Social insects react to stress at both the individual and colonial level by modifying their physiology, behaviour, offspring morphology and colonial productivity. Experimental protocols involve treatments that potentially increase the stress load and may lead to misleading conclusions if not appropriately evaluated. We examined the long-term consequences of an experimental treatment (in vivo cuticular hydrocarbon sampling) and of the exposure to a fungal pathogen (*Metarhizium anisiopliae*) on queen mortality and colonial development in *Crematogaster scutellaris* ants. Cuticular hydrocarbon sampling has been carried out through a widely used non-destructive procedure analogous to the Solid Phase Micro-Extraction (SPME) technique. The exposure to pathogen clearly increased mortality but cuticular hydrocarbon sampling interacted with infection in determining survival. In fact, the manipulation increased the mortality of not-exposed queens but decreased the mortality of queens exposed to the pathogen. Queens subjected to cuticular hydrocarbon sampling also accelerated the emergence of the first worker thus shortening the critical claustral phase. On the other hand, the two treatments did not significantly affect the final number of successful colonies and the number and the morphology of the workers produced in the first season. Our results indicate that each manipulation may be followed by hidden effects becoming evident months after the application of the stress, and that immediate effects may disappear in the long-term. We thus suggest that n pluriannual species it is important to evaluate the effects of the stress covering at least an entire colonial season. Moreover, considering that manipulation can interact with pathogen exposure in determining queen mortality, immunological experiments on social insects should carefully take into account the potentially confounding effect of any experimentally-induced stresses.

SIGNIFICANCE STATEMENT

Organisms adjust their physiology, morphology and behaviour in response to stress and this requires energetic costs. Nevertheless, the effects of experimental stressors on social insects remain largely unknown. We evaluated short and long-term effects on colony development of a manipulative stress during the first year of colony foundation and compared it with the effects produced by an entomopathogenic fungus. The treatments represented two widely used experimental procedures: the exposure to an entomopathogenic fungus and the collection in vivo of cuticular hydrocarbons using a glass capillary. We showed that the two stresses may be followed by hidden effects only evident months after the application. Other immediate effects may disappear in the long-term. Together with unraveling the biological meaning of the observed responses to stress, our results have strong methodological implications in designing experimental protocols involving social insects.

Keywords: allostasis; ants, CHC sampling; colony development; *Metarhizium anisopliae;* stress

1. INTRODUCTION

All organisms modify their physiology, morphology and behaviour in distinct stages of their life cycle. Together with life-cycle events predictable by an organism, unpredictable perturbations which constitute stressors may occur. All the responses to stress aimed at achieving stability through change, defined as allostasis, involve energetic costs which are supplied by endogenous resources or are obtained from the environment (McEven and Wingfield 2003, 2010; Romero et al. 2009). These costs can crucially affect survival during specific phases of the life-cycle requiring high energy costs (McEven and Wingfield 2010).

Many papers showed that social insects react to stress and disturbance at both the individual and colonial level (Cremer and Heinze 2003; Eeva et al. 2004; Linksvayer and Janssen 2009 and literature therein; Schneider et al. 2016). Unraveling the effects of different types of stress not only has implications for understanding the biology of a given species, but also has methodological applications since experimental protocols involve treatments that are likely to increase the level of stress in the organisms under study. Nevertheless, the effect of experimentally-induced stress on the development of social insect colonies remains largely unknown and is rarely tested in experimental protocols (Hranitz et al. 2010). Here we argue that an inappropriate evaluation of experimentally-induced stress may produce misleading conclusions. We compared the long-term consequences of a widely used manipulative treatment with the effects produced by a fungal infection and we evaluated their costs and the allostatic responses of the ant queens in coping with them.

As a model organism, we selected the acrobat ant *Crematogaster scutellaris.* This is one of the commonest ants in the Mediterranean basin and plays a pivotal role in community dynamics (Santini et al. 2007). Queens are single mated and colonies are founded by single fertilized queens (haplometrosis) with a claustral strategy (Frizzi et al. 2015). In haplometrotic and claustral species establishing the new colony is the most critical phase in a queen life cycle. This phase encompasses the nuptial flight, the search for a hibernation site and the survival to winter in temperate species, the preparation of the nest and the production of the first workers. Claustral queens raise the first

brood only relying in their body reserves and only eat after the emergence of the first worker (Brown and Bonhoeffer 2003). The success of colony foundation for queens may be very low and, for this reason each colony produces many queens in each reproductive cycle (Hölldobler and Wilson 1990).

It is thus reasonable to presume that any form of stress can strongly affect incipient colonies of *Crematogaster scutellaris*. We evaluated and compared the effects on queen survival and colonial development of a manipulative experimental stress with the consequences of the exposure to the entomopathogenic fungus *Metarhizium anisopliae.* As a manipulative stress, we selected the Solid Phase Micro Extraction (SPME) method representing the most commonly used non-destructive procedure for collecting cuticular hydrocarbons (CHCs) (Monnin et al. 1998; Ferreira-Caliman et al. 2014). SPME extraction is done by rubbing a glass capillary on the insect abdomen cuticle. Holding the ants for two minutes and rubbing them with a capillary has a high potential as a stressor and it can also return adaptive responses. In fact, this procedure can simulate a predatory event and it has been shown that similar non-lethal physical stress can trigger physiological and behavioural responses in insects (Mowlds et al. 2008; Bateson et al. 2011).

M. anisopliae is a generalist entomopathogenic fungus which spores germinate and iphae penetrate in the host through the cuticle. On insect, *M. anisopliae* is an obligate killer since sporulation happens only after the death of the host. The mortality peak usually occurs within the first 10 days after the infection (Jaccoud et al. 1999). *M. anisopliae* is a widely used model in understanding the responses of social insect to fungal infections (e.g. Rosengaus et al., 1999; Ugelvig and Cramer, 2007; Yanagawa, and Shimizu, 2007; Bos et al., 2012; Okuno et al. 2012; Reber and Chapuisat, 2012). However, most papers focused on survival and/or behavioural responses to *M. anisopliae* exposure in the very short-term (usually comparable with the mortality peak period) while studies focusing on long term effects of this fungus on social insect colonies are rare (e.g. Okuno et al. 2012). There is also potential for the existence of interactions between a manipulative stress and the immune responses to *Metarhizuim*. In fact, it has been found in the larvae of the moth *Galleria melonella,* that a manipulative stress increases the resistance to *Candida* fungal pathogens (Mowlds et al. 2008).

We observed the first year of colonial development, from the first hibernation of the queen to the onset of the second one. We evaluated the effects of the two treatments on queen mortality and on parameters of colonial development such as the success in colony foundation, the time required to produce the first larva, pupa and worker, the final number of workers and their quality, measured with morphological size and shape. We found that there are short-term responses revealing much lower magnitude when observed in a longer time-span and hidden costs (Moret and Schmid-Hempel 2000) only detectable several weeks after the treatment.

2. MATERIALS AND METHODS

Collection

In Autumn 2015, at the onset of their hibernation period we collected 120 fertilized queens of *Crematogaster scutellaris* from oak and poplar galls in the area around Florence (Italy). We transferred the individual ants to small plastic boxes (2 cm in diameter x 1.5 cm height) and allowed them to hibernate in controlled conditions (7 °C temperature, 70% humidity) until 22nd February.

Treatments

We divided the queens into 4 groups of 30 individuals. Each group was characterized by a different combination of two treatments as follows: i) stress induced by a widely used procedure for collecting cuticular hydrocarbons (CHCs). This was done by rubbing the queen abdomen with a glass capillary for two minutes, as per Solid Phase Microextraction (SPME). SPME is a non-lethal method of collecting CHCs from live insects, and thus allows for repeated collection of CHCs from the same individual at different times (Moneti et el. 1997; Monnin et al. 1998; Sledge et al. 2001; Ferreira-Caliman et al. 2014). The procedure was repeated four times during our experiment (late hibernation, 9th February; onset of egg laying, 16th February; emergence of first larvae, 16th March; emergence of first workers, 20th June). The second treatment was: ii) exposure to a heavy dose of the entomopathogenic fungus *Metarhizium anisopliae* (Met52® Monsanto). Since commercial product spores have a low and not standardized germination, we exposed *C. scutellaris* individuals to the commercial product thus obtaining an invigorated and more specific fungus

stock. In the late hibernation period (9th February) we applied a 0.5 µl of a suspension of 10^5 live spores/ml of *M. anisopliae* in a sterile solution of water and Triton x-100 (0.01% vol) on the queen thorax. The two treatments were combined in the four groups as follows: SM group, subjected to capillary rubbing and exposure to *M. anisopliae*; SC group, capillary rubbing and Triton x-100 solution; CM group, *M. anisopliae* exposure and no rubbing; and CC group, Triton x-100 solution and no rubbing.

Colony development

The containers were kept in a fridge at 7 °C until 9th February before being transferred to a thermostatic room with the natural photoperiod and a temperature of 18°C; on 18th February the temperature was raised to 20°C. From 22nd February to 14th September we recorded the mortality of queens and the number of eggs, larvae, pupae and workers in each colony every three days. At the emergence of the first worker, representing the end of the claustral phase, we added two new chambers to the colonies, in the form of two plastic Petri dishes of 5 and 9 cm diameter, respectively. The larger Petri dish, representing the foraging area, contained *ad libitum* water, sugar and proteins (finely chopped dry dog food). The colonies with workers and the alive queen at the end of the experiment, representing the onset of the hibernation period, were considered as successful colonies.

Differences in survival trends among queens were tested by Cox proportional hazards regression models, as implemented in the "coxph" function of the "survival" R package. We used a full factorial design using exposure to *M. anisopliae* and CHC sampling as fixed factors together with their interaction. To verify if the survival analysis would have produced different results at different times, we performed separated tests for survival in the first 30, 60, 90, 120, 150, 180 and 210 observation days (the end of the experiment).

For the queens that produced workers and survived until the end of the observation period we compared the differences among the two treatments in terms of the day of emergence of the first larva, pupa and worker by using full factorial Generalized Linear Models (GLM). The most appropriate models have been identified by comparing AIC values. Depending on the distribution

of the response variables we selected a Poisson or a Gaussian family. For response variables showing a Poisson distribution, we checked for overdispersion of data by using the "dispersiontest" function (Cameron and Trivedi 1990) as implemented in the "AER" R package. In case of significant overdispersion values, we used a negative binomial family GLZ as implemented in the "glm.nb" function of the "MASS" R package. Since the number of cases were not balanced among groups, we used type III sum of squares as returned by the "Anova" function of the "car" R package. In the same way, we compared the final number of workers produced by each colony. Depending on the status at the end of the experiment, we divided the queens into four gruops: i) dead claustral, queens died before the emergence of first worker; ii) alive claustral, queens alive but still in claustral condition; iii) unsuccessful colonies, queens emerged from claustral phase but which died; iv) successful colonies, queens emerged from claustral phase and founded the colony. By using Chi Square Test, we compared the proportion of queens belonging to these four groups among the four combinations of treatments (SM, SC, CM, CC). To summarize the outcome between stressed and non-stressed groups, we performed a second series of Chi Square Test by comparing stressed (SC+SM+CM) and non-stressed groups (CC).

Offspring morphometry

At the end of the experiment we randomly collected a maximum of four workers per colony for morphometric analyses. We excluded the colonies where the queen had died before the end of the observation period (14th September) and those which produced less than three workers (mean number of worker per colony 3.94±0.24 standard deviation). There is a rich body of research indicating head width as the best estimator of body size in ants (Grześ et al. 2016; Fjerdingstad 2005). We removed the heads from the bodies and took frontal photographs of the heads using an Optika stereomicroscope together with an Optika View camera. Three authors (LD, AB, MAM) measured the maximum width using ImageJ (https://imagej.nih.gov/ij/) independently and blindly. The measures taken from the three authors turnout out to be highly repeatable (Pearson $r > 0.992$ and $p = 2.2e^{-16}$ for each comparison).

A combination of landmarks and sliding semi-landmarks (Bookstein 1997) was applied to the head profile of the workers. The eight points on the outline that could be identified precisely were taken as landmarks (type II and type III, Bookstein 1997), whereas 18 other points were allowed to slide along the outline trajectory (sliding semi-landmarks). We digitalized landmarks and semi-landmarks by using TPSDIG2.28 (life.bio.sunysb.edu for all the programs of the TPS family) and a definition of sliders was made using TPSUTIL 1.69. With TPSRELW1.65 we applied Generalized Procrustes Analysis (GPA) to the landmark data to remove non-shape variation, to superimpose the objects in a common coordinate system (Bookstein 1997). By using MORPHOJ 1.06d (Klingenberg 2011) we removed the effect of asymmetry by creating medium symmetric dispositions with respect to the sagittal axis of the head. In MORPHOJ we tested for the occurrence of differences in shape among the four groups using Canonical Variate Analysis (CVA). Significant effects in shape differences among groups was investigated using permutation tests for pairwise Procrustes distances among groups with 10,000 iterations. We investigated the effects of the two treatments on worker head width using a mixed General Linear Model. Head width of the queens were also included in the model and colony membership, nested into the four experimental groups (SM, SC, CM, CC), was used as a random factor. The best model was selected by comparing AIC values among models obtained by using the "lmer" function of the "lme4" R package. Then the effects and the p-value for the best model was assessed by using the "Anova" function of the "car" package by using type III sum of squares.

3. RESULTS

Queen survival

At the end of the experiment the mortality of queens belonging to different groups differed SM=44.8%, SC=41.4%, CM=56.7% CC=10.0% (Table 1, Fig. 1). A series of Cox regressions carried out in different periods after the beginning of the experiment showed that the pattern changed during the observation period. This was mainly due to the trend of the SC group (only subjected to CHC sampling). In the first 60 days, the only significant effect was ascribed to *M. anisopliae* treatment with queens exposed to the fungus showing a lower survival (Fig. 1, Table 2).

From day 90, the effect of *M. anisopliae* alone disappeared, but a significant interaction between *M. anisopliae* exposure and CHC sampling emerged and it maintained a significant effect to the end of the experiment (Fig. 1, Table 2).

The experimental period covered the first year of colony development from colony foundation to the onset of hibernation. Some of the queens which survived did not go beyond the claustral condition during this period, since they failed to produce the first worker (Table 1). It is worth noting that all the colonies that did not produce workers also failed to produce pupae, making it unlikely for them to go beyond the claustral phase before the beginning of the hibernation. The proportion of queens that produced at least one worker (successful and unsuccessful colonies in Table 1) did not differ among the four groups (χ^2=0.824, p=0.844, Fig. 2). Only five queens died after producing the first worker (unsuccessful colonies) and they all belonged to the stressed groups (Table 1), however there were no significant differences in their proportion against the successful colonies among the four groups (χ^2=2.298, p=0.513, Table 1, Fig. 2). Finally, there were significant differences among the proportion of queens that died before the emergence of the first worker (dead claustral) and those that survived to the end of the experiment without producing any worker (alive claustral) among the four groups (χ^2=8.583, p=0.035, Table 1, Fig. 2).

Group	**n**	**Dead claustral**	**Alive claustral**	**Unsuccessful colonies**	**Successful colonies**
SM	29	11	4	2	12
SC	29	12	4	2	11
CM	30	16	4	1	9
CC	30	6	10	0	14

Table 1: Final observation on queen mortality and colony success. Group: SM, capillary rubbing and exposure to *M. anisopliae*; SC, capillary rubbing and Triton solution; CM, *M. anisopliae* exposure and no rubbing; CC, Triton solution and no rubbing; n, sample size; dead claustral, queens died before the emergence of first worker; alive claustral, queens alive but still in claustral condition; unsuccessful colonies, queens emerged from claustral phase but which died; successful colonies, queens emerged from claustral phase and founded the colony.

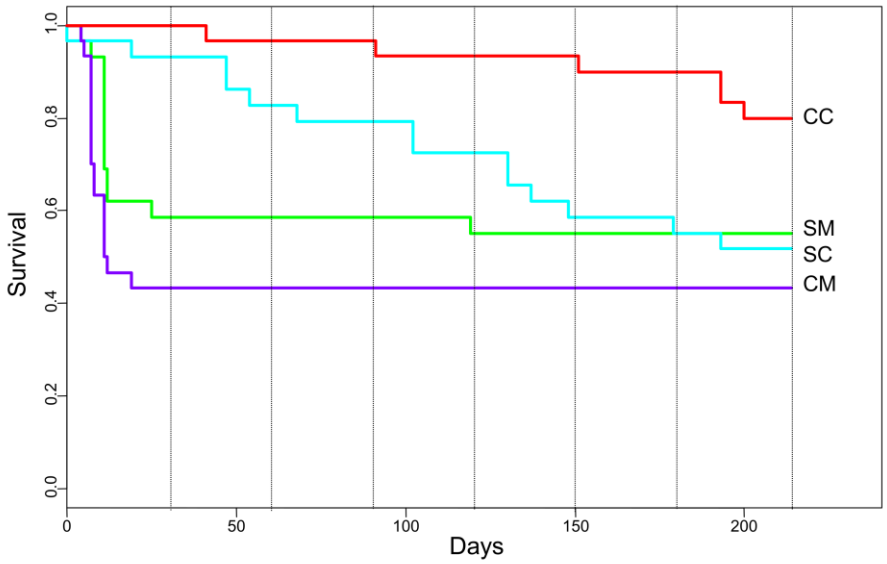

Figure1: Survival plot for the four groups during the observation period. Vertical lines indicate periods when Kaplan Mayer tests were performed

	Day 1-30		Day 1-60		Day 1-90		Day 1-120		Day 1-150		Day 1-180		Day 1-210	
	z	P	z	P	z	P	z	P	z	P	z	P	z	P
Met	**-2.550**	**0.011**	**-1.998**	**0.046**	-1.770	0.077	-1.521	0.128	-0.691	0.490	-0.484	0.628	-0.444	0.657
CHC	1.479	0.139	1.491	0.136	1.493	0.135	1.322	0.186	1.316	0.188	1.314	0.189	1.131	0.258
Int	-0.005	0.996	1.940	0.052	**-2.128**	**0.033**	**-2.260**	**0.024**	**-2.831**	**0.005**	**-2.829**	**0.005**	**-2.357**	**0.018**

Table 2. z and P values for Cox regression for queen survival carried out at different times after the beginning of the observation period. The effects of *Metarhizium* exposure (Met), CHC sampling (CHC) and their interaction (Int) are reported; significant effects are highlighted in bold.

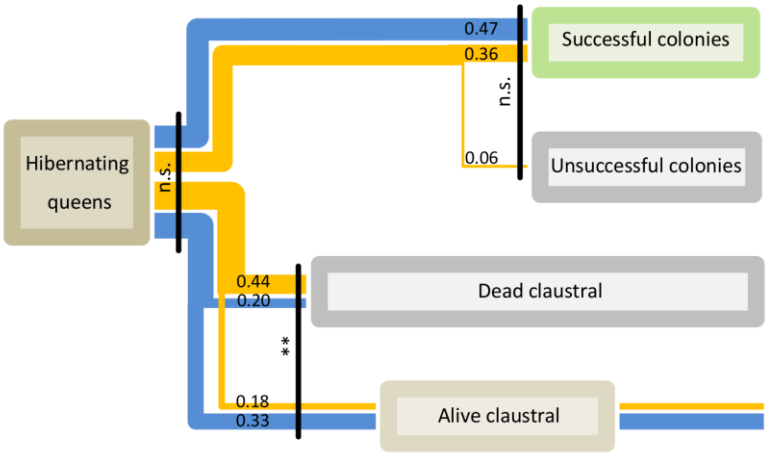

Figure 2. Proportion of non-stressed (CC, blue) and stressed (SM+SC+CM, orange) queens belonging to the four groups identified in Table 1. Chi Squared tests (vertical black lines) comparing the proportions of different combinations of queens are also reported.

Offspring appearance and development

The emergence of the first larva showed a Poisson distribution without evidence for overdispersion (dispersion = 0.940, z = -0.127, p = 0.550). A stepwise AIC based GLM did not show any significant effect in the full factorial model (Figure 3a, Table 3). The emergence day of the first pupa showed a Poisson and significantly overdispersed distribution (dispersion = 8.882, z = 2.797, p = 0.003). AIC selection on a negative binomial GLM entered both types of treatment in the selected model, but only the capillary rubbing revealed a significant effect on the emergence of the first pupa with queens sampled for CHCs producing earlier pupae (Fig. 3b, Table 3). A similar result was obtained for the emergence of the first worker (dispersion = 7.195, z = 3.600, p < 0.001) where only the capillary rubbing entered the model with a significant effect (Fig. 3c, Table 3). At the end of the experiment the two different treatments did not affect the final number of workers per colony (dispersion = 3.308, z = 4.976, p < 0.001) and in the negative binomial GLM none variable was selected by AIC (Fig. 3d, Table 3).

	LR Chisq	Df	p
First larva			
Metarhizium	2.002	1	0.157
First pupa			
Metarhizium	2.105	1	0.146
CHC sampling	9.393	1	0.002
First worker			
CHC sampling	11.188	1	<0.001
Final worker number			
No variables selected	-	-	-

Table 3: The results for GLM (type III sum of square) estimating the effect of *Metarhizium* exposure and CHC sampling on the emergence of the first larva, pupa, worker, on the final number of workers. Likelihood ratio chisquare (LR Chisq), degrees of freedom (Df) and p values (p) are provided.

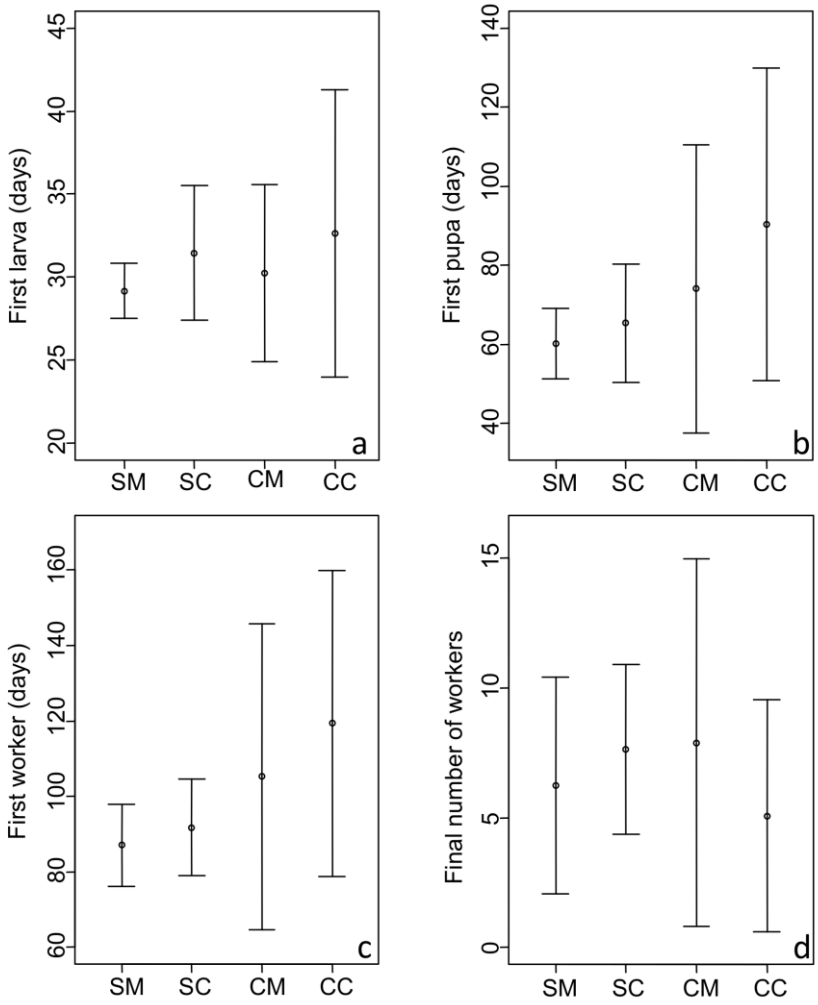

Figure 3. Mean and standard deviation for the emergence day of first larva(a), pupa (b) and worker (c) and the final number of workers (d) for the four groups.

Offspring morphometry

AIC selection on a mixed GLM only entered queen head as an explanatorily variable for worker head width (Fig. 4a), although without a significant effect (Chi Square = 0.9341, df = 1, p = 0.334) (Fig. 4b). The CVA using landmark based shape (Fig. 4a) as a variable and treatment as a discriminant factor revealed no differences in shape among the four groups (permutation tests for Procrustes distances among groups $p>0.050$ for each test, Fig. 4c).

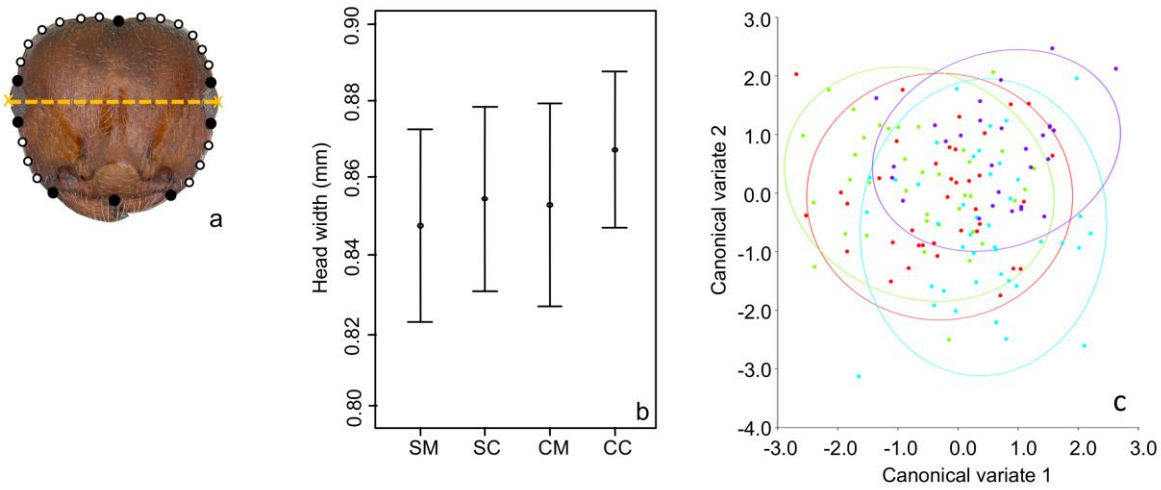

Figure 4. Representation of head width (orange dashed line) and of landmarks (black dots) and semi-landmarks (white dots) used for morphometric analyses (a). Mean and standard deviation for head width of workers in four types of colonies (b). Canonical variate scatterplot showing no difference in head shape among the four groups (c), red, CC; cyan, SC; green, SM; purple, CM.

4. DISCUSSION

The manipulative stress of collecting cuticular hydrocarbons in vivo, considered in literature as a non-destructive procedure, induced strong effects on *Crematogaster scutellaris* foundresses in terms of survival and colonial development. Such consequences have been in some cases even stronger than those produced by the infection of an entomopathogenic fungus. Although SPME extraction and exposure to *M. anisopliae* are two widely used experimental procedures, experiments aimed to detect their effects in the long term are lacking. By observing the first year of colonial development, we found that some effects of the two stresses were only evident at an early stage while others only appeared in the long-term. Consequently, a clear understanding of the effects of stress on the survival of queens was only possible at an advanced stage of the survey. In fact, CHC collection started to interact with pathogen exposure only three months after the beginning of the experiment. Although the treatments differently affected mortality trends during the experimental period, the final success in terms of establishment of successful colonies did not differ among experimental groups. At least under our experimental settings, the higher number of queens which died after the two treatments was compensated in the control group by a higher number of queens which did not successfully found the colony, but which survived until the end of

the experiment. Given that it is unlikely that a queen failing to produce at least one worker in the first season would survive a second hibernation and found a colony in the next season, these queens must be considered "walking dead". The CHC sampling also induced a shorter claustral phase with an earlier emergence of the first worker, but the final number of workers and their quality, at least as it can be measured with morphological traits, did not differ among treatments. In the following sections, we discuss the biological significance of the results and their methodological implications.

Effects of experimental stress on queens and their responses

In our study, most of the queens which had produced the first worker, survived until the end of the experiment. In species showing claustral foundation, the period from the departure from the maternal colony to the production of the first worker(s) is the most challenging stage in the queen life cycle (Hölldobler and Wilson 1990). Since the queen does not eat before the emergence of workers, any increase in energy expenditure during the claustral phase can strongly affect its survival (Camargo et al. 2012). Stress induces a series of allostatic responses to cope with and these responses are costly in terms of energy resources. We found that queens affected by a manipulative stress and those exposed to *M. anisopliae* died in higher numbers before colony foundation. The mortality peak for queens exposed to *M. anisopliae* occurred between 7 to 12 days after the exposure. Since this time corresponds to the typical mortality-time of *M. anisopliae* (Jaccoud et al. 1999), we can assume that these queens died due to the fungal infection. On the other hand, the mortality observed following physical stress may have been produced by mechanical damage caused by experimental handling. But, we did not observe a peak in mortality after the collection of CHCs. The protracted latency makes more probable that the energetic costs related to the allostatic reactions (Mowlds et al. 2008; Bateson et al. 2011) increased mortality in non-infected queens. In line with the allostatic reaction hypothesis, we found that CHC sampling interacted with parasite infection in determining survival, since the manipulation decreased the mortality of the queens exposed to *M. anisopliae*. Larvae of the wax moth *Galleria mellonella* subjected to physical stress increase their haemocyte density and the mRNA levels of genes coding for two antimicrobial peptides and produce specific peptides in the haemolymph (Mowlds et al. 2008). As a result, they show a reduced susceptibility to the fungal pathogen *Candida albicans* (Mowlds et al. 2008).

Similarly, CHC sampling could have triggered a generalized immune response in queen ants, favouring the individuals exposed to *M. anisopliae* but, in the long term, the costs associated to such response could have induced a higher mortality in control queens (see Moret et al. 2000 for bumblebees).

We also found that the emergence time of the first pupa and worker occurred earlier in colonies subjected to physical stress. Since the emergence time of the first larva did not differ among groups, the variation in development time was due to a shortening of the length of the larval period. Although the difference in average emergence time of the first worker between the extreme SM and CC groups was close to 30 days (Fig. 3c), the minimal time necessary for stressed queens to produce a worker was included in the variance seen in the control group (Fig. 3c). In practice, queens subjected to physical stress tended to reduce the time necessary to produce the first worker to the minimum allowed by species-specific biological constraints. We conclude that shortening the length of the immature stage of the first offspring is a functional response of stressed queens aimed to minimize the length of the critical claustral period in a condition of increased energetic demand. The mechanisms by which the larval period can be shortened in this species are unknown but it is known in ants that the larval stage can vary depending on environmental conditions (Abril et al. 2010). During the claustral phase *C. scutellaris* queens may modify nutrition rate, manipulate larvae with pheromone emission or induce maternal effects in offspring and these mechanisms may imply additional energetic costs. In our experiment 5 colonies out of 51 colonies failed due to the death of the queen after the emergence of the first worker (Fig. 2) and they all belong to treatment groups. These events may have been a direct effect not only of the stressors but also of energy costs needed to cope with them.

Fine-tuning regulation of offspring morphology has been reported as a response to stress and represents a typical cost of reproducing under stressful conditions (McEven and Wingfield 2003, 2010; Badyaev 2005, Romero *et al*. 2009). Nevertheless, we observed that offspring from stressed and control groups were similar in quality, at least based on their size and head shape. This datum, together with the observation that the final number of workers did not differ among treatments, indicates that although queens subjected to CHC sampling had to accelerate the production of their

first worker, there does not have to be trade-off between the developmental time of workers and their number, size and shape.

Similarly, although the mortality showed a complex pattern among treatments, in all four groups more than half of the queens failed to establish a successful colony and their proportion did not differ among treatments. Specific studies are necessary to clarify the factors determining this outcome, but it is reasonable to hypothesize that after hibernation many queens do not have a physiological status allowing them to cope with different stresses and/or to produce the first cohort of workers, at least under our experimental settings. If this holds true, the stresses we induced only selected at an earlier stage the fittest individuals having the physiological features allowing them to rise the first cohort of offspring also through allostatic responses to stress (Fig. 2).

Methodological implications

The proportion of queens founding a successful colony and the workers produced by them did not differ among groups. On the other hand, the temporal pattern of these parameters was clearly affected by the two stressors. Queens affected by physical stress established earlier colonies which were similar at the end of the experiment to the control colonies in terms of quality and number of workers. Had queen survival and colonial parameters been compared before the end of the first colonial season, the conclusions drawn about the effects of different stressors are likely to have been different.

In practice, a researcher measuring the effects of stressors one month after the treatment would have concluded that only *M. anisopliae* had a strong impact on queen survival and that the two stresses had no impact on colony development since all the queens that survived laid eggs and the emergence date of the first larva was not affected by the two stressors. Later, the effect of CHC sampling on queen survival became evident and the more rapid emergence of the first workers in the colonies of stressed queens may have led us to conclude that the physical stress induces a higher proportion of queens to complete the claustral phase, which, at the end of the experiment also appeared to be a transitory phenomenon. If some early effects of experimental stressors may disappear completely at the end of the season, other hidden effects can only be detected in the long-

term. As a first methodological indication, we warn that it would be opportune, for manipulative protocols dealing with annual or pluriannual insect colonies, to extend the observation period to cover at least one entire season.

Another precaution would be to adjust the protocol to guarantee appropriate controls for all manipulated groups. The use of control groups for the effects of CHC sampling (and of handling procedures in general) is virtually absent in the literature (e.g. Monnin et al. 1998; Sledge et al. 2001 but see also Hranitz et al. 2010) but the effects of the stress, producing substantial changes in the individual physiology and colony development, could generate misleading conclusions. The use of control groups is instead a common procedure in experiments involving exposure to pathogens (e.g. Ugelvig and Cremer 2007; Reber and Chapuisat 2012; Schneider et al 2016). However, as already found in solitary and group-living insects (Mowlds et al. 2008; Kohlmeier et al. 2016), we showed that manipulative stress can interact with pathogen exposure in determining survival trends. Depending on the magnitude of the immune response produced by the manipulative stress, it could hide or reduce differences between control (uninfected and manipulated) and experimental (infected and manipulated) groups in the observed responses to pathogens, returning type II errors. In the same order of ideas, our results showed that there are two kinds of queens, probably differentiated based on their innate physiological features: some more resistant to stress and likely to produce a successful colony and others less resistant and likely to fail in the foundation. Since it is impossible to discriminate these two groups before worker emergence, the experiments aimed at investigating immune competence of queens will inevitably mix them. This can produce a high variance in resistance to pathogens under different experimental conditions, not only determined by the treatment and then returning, other potential type II errors. As a possible example, it can be found in literature that the enhanced resistance to pathogens upon a second exposure (immune priming) is much weaker, when not apparently absent, in pre-founding queen ants compared to other social insects (Reber and Chapuisat 2012; Gálvez and Chapuisat 2014; Milutinović and Kurtz 2016). The phenomena observed in our study can represent confounding variables hiding existing mechanisms of resistance to pathogens.

Authors' contributions

S.T., A.B. and L.D. conceived the study; A.B., L.D. and S.T. designed the experiments; all authors collected the data; A.B., M.A.M. and L.D. carried out the analyses and drafted the manuscript; all authors critically revised the paper, approved the final version of the manuscript and agree to be held accountable for the content therein.

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Chapter 3

Trans-generational immunization in the acrobat ant *Crematogaster scutellaris*

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Trans-generational immunisation in the acrobat ant *Crematogaster scutellaris*

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ABSTRACT

Trans-generational immunisation is defined as the transmission of an enhanced resistance to a pathogen from parents to offspring. By using the host-parasite system of the ant *Crematogaster scutellaris* and the entomopathogenic fungus *Metarhizium anisopliae,* we describe this phenomenon for the first time in ants. We exposed four groups of hibernating queens to different treatments i) a non-lethal dose of live conidiospores in Triton, ii) a dose of heat-killed conidiospores in Triton, iii) a control Triton solution, and iv) a naïve control. We exposed their first workers to a high dose of conidiospores and measured mortality rates. Workers produced by queens exposed to live conidiospores survived longer than those belonging to the other groups, while exposure to Triton and dead spores had no effect. Starved workers showed a significantly higher mortality. The treatments did not influence queen mortality, nor the number of offspring they produced at the emergence of the first worker, showing no evidence of immunization costs - at least for these parameters in the first year of colony development. We propose that trans-generational immunisation represents an important component of social immunity that could affect colony success, particularly during the critical phase of claustral foundation.

Key-words: Trans-generational immunisation, *Crematogaster scutellaris*, ants, *Metarhizium anisopliae*

1. INTRODUCTION

Among insects, social species show a vast series of collective behavioural and organisational defenses against parasites, grouped under the term social immunity (Cremer et al., 2007). Social insects also possess several physiological mechanisms to avoid parasites and cope with infections (Milutinović and Kurtz, 2016). The immunisation phenomenon is a physiological process that improves the resistance to a pathogen after a first exposure (Contreras-Garduño et al., 2016; Masri and Cremer, 2014; Milutinović and Kurtz, 2016). When the immunisation is specific to the particular parasite it is referred as immune priming (Masri and Cremer, 2014). Immunisation can be transmitted both vertically and horizontally among colony members (Hernandez Lopez et al., 2014; Sadd et al., 2005; Salmela et al., 2015; Traniello et al., 2002; Ugelvig and Cremer, 2007), and when it occurs from parents to offspring it is referred to as trans-generational immunisation (TGI).

Although the transmission of immune factors is hypothesized to represent an important component of social immunity (Cremer et al., 2007), the occurrence of TGI has only been demonstrated in bees and termites (Hernandez Lopez et al., 2014; Sadd et al., 2005; Salmela et al., 2015; Traniello et al., 2002). In ants, there is evidence for the existence of immunisation in a few species (Gálvez and Chapuisat, 2014; Reber and Chapuisat, 2012), but definitive evidence of its transmission is still lacking. Ugelvig and Cremer (Ugelvig and Cremer, 2007) found that workers of *Lasius neglectus* showed a higher resistance to the entomopathogenic fungus *Metarhizium anisopliae* after five days of coexistence with nestmates exposed to live conidiospores of this pathogen, but this was due to transfer of the pathogen among individuals rather than by physiological mechanisms (Konrad et al., 2012).

We investigated whether the exposure to *Metarhizium anisopliae* elicits TGI in the acrobat ant *Crematogaster scutellaris* during the crucial phase of colony foundation. Colony foundation in this species is claustral by a single mated queen (Bordoni et al., 2017). In species with claustral foundation, future queens rely only on physiologically stored reserves to found the nest and rear the first cohort of workers. Under laboratory conditions, a large fraction of *C. scutellaris* foundresses fail in producing workers and die before winter, but if a single worker is produced, queens usually survive and produce successful colonies (Bordoni et al., 2017; Hölldobler B and Wilson EO, 1990). A trans-generational transmission of immune factors could protect the crucial investment represented by the first workers.

2. MATERIALS AND METHODS

In winter 2016-2017 we collected hibernating foundresses of *C. scutellaris* around Florence, Italy. We allowed them to hibernate in round plastic containers (2cm×1.5cm) in controlled conditions (7°C temperature, 80% humidity). On February 10th, we used 60 of these foundresses to assess the lethal-dosages (LD) of *M. anisopliae* conidiospores (Monsanto Met52®, see supplementary methods). On February 28th, four groups of 35 hibernating foundresses each were prepared. Three treatments (Triton solution, live conidiospores and dead conidiospores) where differently combined in the four groups as follows; we dipped the foundresses of the first (Mt-spores) group in the LD10 suspension (3×10^5CFU/ml) of Triton and live spores for three seconds. We dipped the foundresses of the second group (dead Mt-spores) in the same suspension after killing the conidiospores by autoclaving at 120ºC for 20 minutes and plating the solution to verify the absence of CFU. The third group (Triton) was dipped in the Triton solution (0.01%). The fourth naïve group did not receive any treatment. To facilitate the germination of conidiospores, the foundresses, excluding the naïve group, were kept at 100% humidity. After two days when the conidiospores had firmly attached to the host's cuticle and can no longer be transferred to nestmates (Konrad et al., 2012), we moved the foundresses to sterile plastic containers. The foundresses were then reared in darkness in a thermostatic room (20°C-25°C temperature, 80% humidity). We recorded queen mortality on a weekly basis until August 8th (150 days). In each colony, at the emergence of the first adult worker, we counted the number of immature offspring, and added a plastic Petri dish (5cm diameter) where we supplied *ad libitum* water, sugar and chopped dry dog food. We exposed the foraging chamber to a 12-12hLD cycle and covered the queen chamber with aluminum foil to maintain dark conditions.

The next stage of the experiment involved exposing workers to a high dose of *Metarhizium* conidiospores. We did not remove a worker until a younger worker had emerged to maintain queens in colonial phase. To standardize the age of workers, we used workers once they had reached 5-10 days of age. For each colony, we used a maximum of eight workers. Workers were dipped for 3 seconds in the *Metarhizium* LD80 suspension (3×10^4CFU/ml) and then isolated in a small plastic container with a plaster base to maintain 90% humidity. Because there can be costs of immunization only evident in starvation (Moret, 2000), we randomly assigned workers in each colony to two different diet treatments after the challenge: starved and fed with *ad libitum* sugar. After the trial, we checked mortality daily for two weeks. All the exposed ants showed an outgrowth of the fungus after their death.

We modelled the survival of queens and workers by using Cox regressions. For foundresses, by using the "coxph" function of the "survival" R package, we tested the effect of the three treatments: Triton (administered to Triton, Mt-spores and dead Mt-spores queens vs naïve queens); live spores (administered to Mt-spores queens) and dead

spores (administered to dead Mt-spores queens). For workers, we also included, in a mixed effects Cox regression ("coxme" function of the "coxme" package), the diet treatment, its interactions with the treatments, the order of emergence and two random factors: colony membership and day of challenge. We performed analyses of deviance on Cox regressions using the "anova" function to assess the effects of the variables and their interactions and the "summary" function to evaluate, for each variable, the ratio in death hazard between treatments (hazard ratio). We compared the count variable of immature offspring produced by queens under different treatments with a negative binomial GLM using the "glm.nb" function of the "MASS" package and obtained a type III analysis of variance table by using the "Anova" function of the "car" package. The datasets and the scripts are available as supplementary material.

3. RESULTS

None of the three treatments revealed a significant influence on queen survival during the 150 days of observation (Fig.1a, Table 1). The hazard of workers produced by the queens exposed to live conidiospores was less than half of that of workers of the other treatments, resulting in a significantly lower mortality for the live spores group (Fig.1b, Table 1). Starvation increased mortality focused in the third and fourth days after infection, without any interaction with treatments (Table 1, Fig.S1). Nevertheless, the hazard ratio between starved workers and fed workers was quite low (1.110, Table 1). Treatment of queens with Triton and heat-killed conidiospores did not affect worker survival (Fig.1b, Table 1). The three treatments did not affect the number of immature offspring occurring in the colonies at the emergence of the first worker (Fig.2, Table 2).

	Queens				Workers			
Variables	Hazard R.	ChiSq	D.f.	p	Hazard R.	ChiSq	D.f.	p
Mt-spores	0.523	1.795	1	0.180	**0.496**	**6.947**	**1**	**0.008**
Dead Mt-spores	1.279	1.064	1	0.302	0.861	0.524	1	0.469
Triton	1.502	0.682	1	0.435	1.155	0.590	1	0.442
Diet	-	-	-	-	**1.110**	**6.146**	**1**	**0.013**
Order	-	-	-	-	0.991	0.041	1	0.840
Triton*Diet-S	-	-	-	-	1.054	0.015	1	0.904
Mt-spores*Diet-S	-	-	-	-	1.454	0.164	1	0.686
Dead Mt-spores *Diet-S	-	-	-	-	1.651	2.430	1	0.904

Table 1. Hazard ratios and analysis of deviance for Cox regression of 140 queens exposed to a combination of three treatments (Triton, Mt-spores, dead Mt-spores) (left) and of 223 workers (right) produced by foundresses exposed to the same treatments. The effects of order of emergence, worker diet (starved-fed) and its interactions are also reported. Significant effects are highlighted in bold.

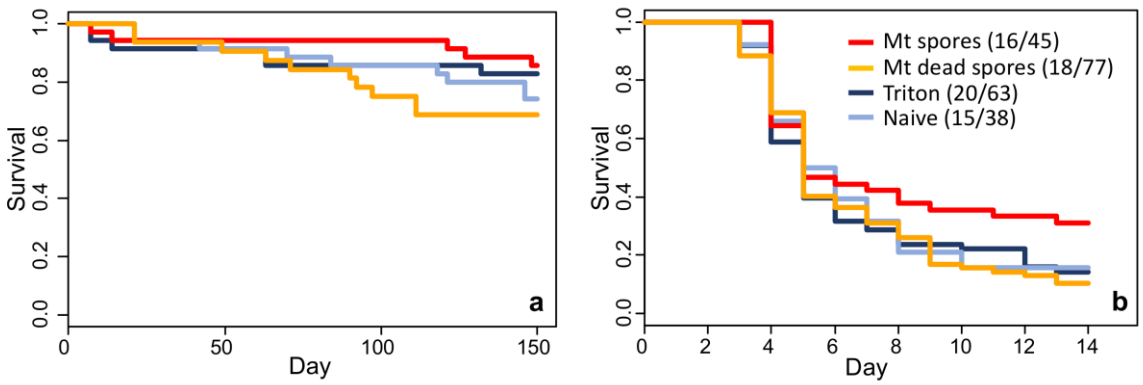

Figure 1. Survival plot for the four groups of queens during the 150 days of observation period (a) and for the four groups of workers during the 14 day challenge with a LD80 dose of *M. anisopliae* (b).

Variables	LR Chisq	D.f.	p
Triton	2.384	1	0.123
Mt-spores	1.319	1	0.251
Dead Mt-spores	0.174	1	0.676

Table 2. Type III analysis of variance table for the negative binomial GLM testing for the effect of the three queen treatments on the number of immature offspring occurring at the emergence of the first worker.

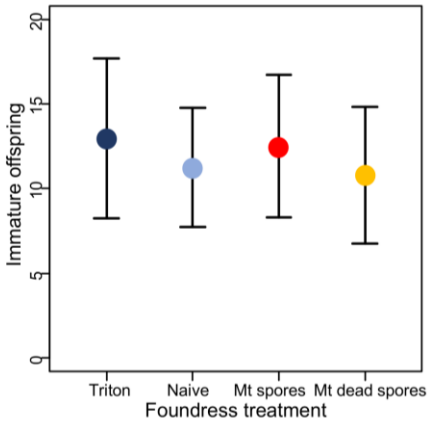

Figure 2. Means and standard deviations of the number of immature offspring occurring in the colonies belonging to the four groups at the emergence of the first worker.

4. DISCUSSION

Crematogaster scutellaris foundresses exposed to a non-lethal dose of *M. anisopliae* did not show reduced survival and produced a similar number of offspring compared to control groups in the first colonial season. Offspring produced by queens exposed to *M. anisopliae* showed a lower mortality than workers belonging to the other treatments. The hazard of Mt spores workers was halved with respect to the other groups, with a final survival of 31.1%

compared to 13.1% in the other treatments. Such a survival increase of 18.0% is similar to values reported in TGI literature (11% in *Tenebrio molitor* beetles (Moret, 2006); 23% in *Penaeus monodon* shrimps (Huang and Song, 1999), 26% in honeybees (Hernandez Lopez et al., 2014)).

In ants, the immunisation response varies among species and depends on the physiological or social status of individuals. Workers, virgin and mated queens of *Formica selysi* did not show higher resistance to the entomopathogenic fungus *Beauveria bassiana* after previous exposure (Reber and Chapuisat, 2012). *Lasius niger* mated queens, as opposed to conspecific virgin queens, showed evidence of immunisation against the same pathogen (Gálvez and Chapuisat, 2014). In our experiment the overall immune response was affected by the status of workers since fed individuals coped better with the parasite in the first days of the infection. Diet treatment, however, did not interact with the immunisation phenomenon, increasing survival in all the treatment groups similarly.

The fact that many studies used infection routes differing from natural ones (e.g. systemic vs oral or epicuticular), and that pathogens used to elicit immunisation have been applied either alive or dead but rarely in both forms, has complicated the understanding of immune response mechanisms (Milutinović and Kurtz, 2016). In other studies, microbes were introduced into the insects via injection, thus producing an immunological recognition of dead pathogens (Milutinović and Kurtz, 2016). In our study heat-killed conidiospores did not induce TGI through the natural epicuticular infection route, thus the induction of TGI in *C. scutellaris* likely involves pathogen infection through an active penetration of the host cuticle.

Although it is theorized that immunization implies some form of cost, they have been rarely assessed in social insects. Queens exposed to the pathogen did not show reduced survival during our observations, including all the claustral phase and the first growing season of the colony. As such, if some costs are incurred in queens and workers, they are not so critical as to increase mortality. Finally, exposed queens did not produce a lower number of offspring, excluding a possible trade-off between surviving with a parasite and investing in reproductive output, at least during the claustral phase.

In conclusion, we found, for the first time in ants, that founding queens exposed to a non-lethal dose of an entomopathogenic fungus produce offspring with an increased resistance to the same pathogen. This phenomenon did not reveal apparent costs to foundresses and workers, at least with respect to their survival and reproductive output. Although TGI can also represent an adaptive phenomenon occurring in mature colonies, we argue that an enhanced resistance during initial colony founding can protect the fundamental resource represented in the first cohort of workers which crucially contribute to queen and colony survival (Bordoni et al., 2017; Sadd et al., 2005).

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Authors' contributions

S.T., B.P. and S.R.B. conceived the study; A.B., B.P., S.R.B., L.D. and S.T. designed the experiments; all authors collected the data; A.B. and L.D. carried out the analyses and drafted the manuscript; all authors critically revised the paper, approved the final version of the manuscript and agree to be held accountable for the content therein.

Data accessibility: data and scripts are available as supplementary material.

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Competing interests: We have no competing interests.

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SUPPLEMENTARY METHODS AND RESULTS

M. anisopliae spore suspension and growth conditions

To verify the virulence of *Metarhizium anisopliae* commercial spores (Met52© Monsanto) against ants, we rinsed a series of ten foundresses of *C. scutellaris* in a suspension of the commercial product. After the death of the foundresses, we collected the fungal spores by washing the ant bodies showing growth of mycelium in Triton solution (0.01% in distilled water). The conidiospore suspension was plated on Maltose Extract Agar (MEA, OXOID) in Petri dishes and incubated at 30°C for some days. Conidia from individual colonies were recognized as belonging to *M. anisopliae* under microscope according to their morphology and then collected in Triton solution. Dilutions of spore suspension were plated on MEA and incubated at 30°C. The number of - Colony-Forming Units (CFU) were counted to determine the spore viable title (CFU/ml).

Determination of the Lethal dosage for foundresses and workers

To assess the lethal dosages of *M. anisopliae* conidia on foundresses, we prepared suspensions with different conidia concentrations (3×10^3, 3×10^4, 3×10^5, 3×10^6, 3×10^7 CFU/ml), and a control Triton solution, and then rinsed ten foundresses for 3 seconds in each suspension.
Ants were reared in the darkness in a thermostatic room (20°C-25°C temperature, 80% humidity). To facilitate the germination of the spores, the foundresses were kept at 100% humidity during the first two days (cit). After 14 days, we evaluated the mortality of the foundresses revealing the following values:
Triton solution = 0%
3×10^3 CFU/ml = 0%
3×10^4 CFU/ml = 20%
3×10^5 CFU/ml = 10%
3×10^6 CFU/ml = 20%
3×10^7 CFU/ml = 50%
Although the concentration of 3×10^4 CFU/ml resulted in a mortality of 20%, the comparison of the other values suggested that 3×10^5 CFU/ml can be considered a reliable LD10, and thus it was used as the low-dosage to expose foundresses.
On January 30th we treated 20 foundresses with Triton solution (0.01%) and allowed them to found colonies in spring conditions (darkness, 25°C-18°C temperature, 80% humidity). In each colony, at the emergence of the first worker, we added a plastic petri dish (5cm diameter) where we supplied *ad libitum* water, sugar and chopped dry dog food. We covered the queen chamber with aluminium foil to maintain darkness, while we exposed the foraging chamber to a 12-12h LD cycle. On May

23rd we collected 30 workers and we assessed the *Metarhizium* lethal dosage by rinsing ten workers in each of the following spore suspensions:

3×10^3 CFU/ml = 20%

3×10^4 CFU/ml = 80%

3×10^5 CFU/ml = 90%

We used the 3×10^4 CFU/ml as LD80 suspension for the challenges.

Treatment	n	Dead claustral	Live claustral	Producing suitable workers	mean workers ± s.d.
Triton	35	6	3	20	3.2±1.7
Naïve	35	6	2	15	2.5±1.1
Mt spores	35	4	2	16	2.8±1.1
Dead Mt spores	32*	8	3	18	4.3±2.4

Table S1. Number of queens used in the study for each treatment (n), queens dead before day 150 without producing the first worker (Dear claustral), live queens at day 150 which did not produce the first worker (Live claustral), queens which produced workers suitable for the challenge (at least two worker produced with a difference in adult age of less than 10 days, see methods), mean and standard deviation of workers used for the challenges for each treatment. In the dead Mt spores groups, three queens died in the day of the treatment and they have been removed from the analysis.

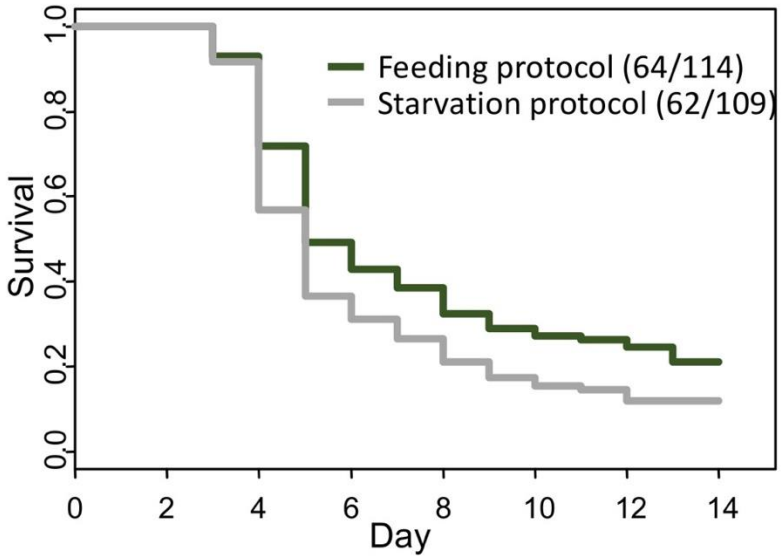

Figure S1 Survival plot for the workers subjected to the two protocols (feeding and starvation) during the 14 day challenge with a DL80 dose of *M. anisopliae*. In parentheses number of colony having produced suitable workers and total number of workers tested for each protocol.

Chapter 3

Annex1

Setting up an experimental protocol to study acquired immunity in the ant *Crematogaster scutellaris*

This preliminary study has been presented as a poster to the XVI National Congress of the AISASP, Rome, 14-15/09/2017, Rome. (Winner of the prize for best student poster contribution)

Setting up an experimental protocol to study acquired immunity in the ant *Crematogaster scutellaris*

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1. INTRODUCTION

In the last decade the existence of acquired immunity in invertebrates has been demonstrated. This phenomenon, called immunisation or immune priming (the second one is against a specific pathogen), is represented by a higher resistance to pathogens upon secondary exposure (Milutinović and Kurtz, 2016). In some species, the transmission of immunisation among individuals has been also demonstrated (Sadd et al., 2005). The transmission of acquired immunity among nestmates in social insect colonies presumably has a relevant role in the health of the colony. While it is not experimentally difficult to verify the occurrence of this phenomenon in a given host-parasite system, understanding the underlaying mechanisms requires the application of elaborated experimental designs. It has been recently shown that *Crematogaster scutellaris* queens transmit acquired immunity to their offspring (trans-generational immunisation) in response to the entomopathogenic fungus *Metarhizium anisopliae* (Bordoni et al., 2018). This study is aimed to understand the physiological mechanisms at the basis of this phenomenon. Furthermore, we tried to discern whether immune factors were transmitted horizontally, for example by trophallaxis, and/or vertically before egg laying.

2. METHODS

The experimental design has been shaped based on the biology of the model species. *Crematogaster scutellaris* is a monogynic species, mature colonies in the wild normally show a peculiar structure represented by a colony core, containing the queen with brood and workers, and some satellite units, hosting workers and brood. These colony sub-units are in constant communication during the warm season and remain isolated during the hibernation (Santini et al., 2011). We simulated these conditions in an artificial environment, arranging the colony components (queen, brood and workers) in different combinations to verify whether the trans-generational immunisation occurs vertically, by maternal effect, or horizontally through contacts with the queen or the nurses.

- 41 colonies 1 year old, after overwintering in controlled conditions, have been divided in two groups: group **M**, which queens have been treated with a non-lethal dose of *M. anisopliae* (n=25); and group **T** (control), which queens have been treated with Triton (n=16) (group 1 in Fig. 1).
- After the treatment, the queen of each colony has been kept isolated for 3 days. Successively, we created a new colonial sub-unit by moving the queen to a new nest together with two workers. The orphan colony was indicated as sub-unit A (group 2 in Fig. 1).
- We allowed the queen lay 8/9 eggs, then we moved the queen and the two workers to a new nest, which represented the colony sub-unit C (group 3 in Fig. 1).
- Eggs separated from the queen have been reared by two workers of sub-unit A, this new combination results in the sub-unit B (group 4 in Fig. 1).

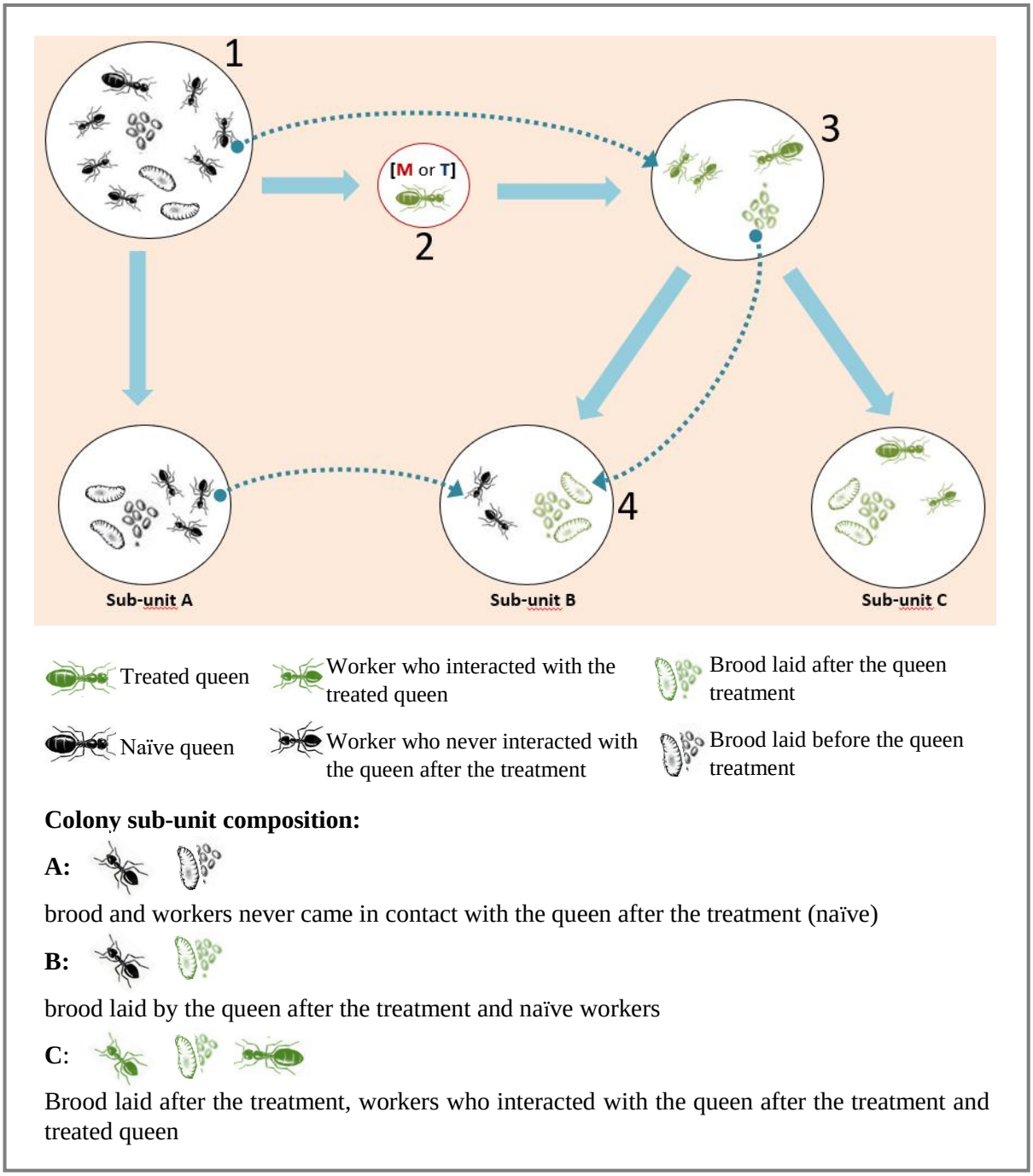

Figure 1. The experimental design.

Melanisation capability assay of brood haemolymph: to highlight the potential differences in terms of immuno-competence between brood belonging to each colony sub-unit (A, B and C) of the two experimental groups (M and T), we compared the level of activate Phenoloxidase (PO) in larval haemolymph. PO is directly involved in the physiological processes of melanisation and encapsulation of *M. anisopliae* hyphae when they penetrate the host body (Roth et al., 2010; Wang et al., 2011).

Melanisation assay protocol:

- Collection of a pool of haemolymph from larvae belonging to the same sub-unit (total vol: 0.5 µl)
- Suspension of the pools in TRIS buffer solution.
- Reaction with the substrate L-Dopa in controlled conditions (37°C)
- Record of the change of Optical Density (OD) using a TECAN plate reader (10 on 30 min,490nm).
- PO Vmax has been calculated for each sample (maximum speed of substrate conversion).

3. RESULTS

The experimental protocol designed to obtain three distinct colony sub-unit in controlled environment partially failed. Because in colony sub-unit B workers did not care the brood. Furthermore, a high colony failure occurred in the colony sub-unit C of both experimental groups.

The following colony sub-units were suitable for the enzymatic assay:

- M (experimental group colonies, queen exposed to *M. anisopliae*): A=4, B=0, C=3
- T (control group colonies, queen treated with Triton): A=4, B=0, C=3

Some of these haemolymph samples have been used to tune the experimental protocol of the enzymatic assay (optimal amount of haemolymph, optimal record interval and total duration of the OD recording)

Samples used for the optimized enzymatic assay:

- M (experimental group colonies, queen exposed to *M. anisopliae*): A=3, B=0, C=3
- T (control group colonies, queen treated with Triton): A=3, B=0, C=3

We compared the PO Vmax values of the haemolymph samples from the C[M], C[T] and A colony sub-units (boxplot). The sample number is too limited to state conclusions. Nevertheless, preliminary data suggest that the haemolymph of the larvae produced by queens exposed to *M. anisopliae* (C[M]) tend to show a higher PO activity (Fig. 2) (Kruskal-Wallis chi-squared test, p = 0.0686).

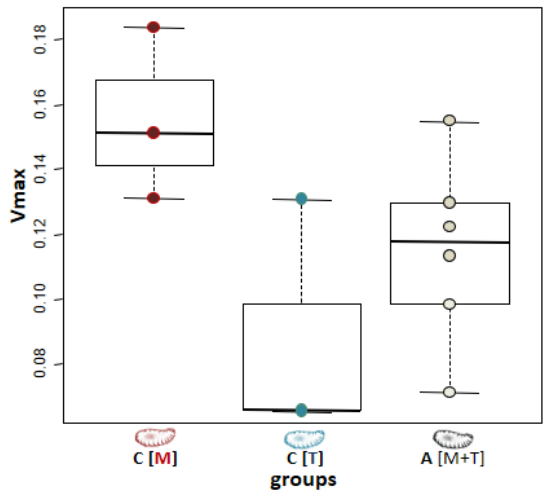

Figure 2. Median and relative quartiles of PO Vmax values obtained from group C in control and triton queen treatment (C[M] and C[T]) and in A groups [M+T].

4. CONCLUSIONS

The experimental design based on the biology of the model species characterized by a nest compartmentalization in colony sub-units did not show the expected success. Colony sub-units of type B failed because naïve workers did not nurse brood laid by the queen after the treatment. We can speculate that the excessive manipulation negatively affected the workers, placed in an alien nest hosting eggs never encountered before, even laid by their mother queen. Consequently, it has been not possible to obtain information about the modality of transmission from adults to offspring. Nevertheless, a protocol to assay the enzymatic activity of phenoloxidase in the brood haemolymph has been successfully set. Despite the reduced sample size, impeding to make a definitive statement, there is a strong tendency suggesting a higher PO activity in haemolymph of larvae produced by queens exposed to *M. anisopliae.* Higher levels of activated phenoloxidase can indicate an increased immune physiological response of melanisation, which is a possible explanation for the evidence of trans-generational immunisation found in the same host- parasite system.

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Chapter 4

Queen immunization and trans-generational immunization are uncoupled phenomena in acrobat ants (*Crematogaster scutellaris*).

This manuscript represents a preliminary report of an ongoing study

Queen immunization and trans-generational immunization are uncoupled phenomena in acrobat ants (*Crematogaster scutellaris*).

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ABSTRACT

Like vertebrates, invertebrates evolved acquired immunity based on memory-like mechanisms, known as immunization. Immunization and its transmission among individuals have been reported in different insect orders, suggesting a phylogenetically ancient and conserved phenomenon. Physiological mechanisms are still largely unknown, and the high variability in responses in different host-parasite systems led to different conclusions. In social insect species, the complex organization of colonies further complicates the interpretation of the immune responses. In ants, evidence of immunization is ambiguous since it has been shown that the expression of immunization depends on species, caste and physiological status of individuals, revealing in some cases the absence of the phenomenon. In this study, we investigate the occurrence of immunization on queen of *Crematogaster scutellaris* ants using as elicitor the fungus *Metarhizium anisopliae*. Foundation in *C. scutellaris* is claustral and monogynic, allowing us to test the existence of the phenomenon in two distinct physiological conditions, starvation and ad libitum feeding, corresponding to the natural claustral and colonial phases of queens. Queens challenged with heavy doses of the pathogen showed higher mortality if previously exposed to light doses, indicating the absence of immunization in queens and foundresses in our experimental settings. On the other hand, evidence of the trans-generational immunization in the same host-parasite system has been recently found, where workers produced by queens exposed to light doses of *M. anisopliae* survived longer than those belonging to the control group. Current results suggest that immunization and trans-generational immunization are uncoupled phenomena in this host-parasite system and that foundresses exposed to *M. anisopliae* can elicit an increased resistance in the offspring without providing themselves with an increased immune response.

1. INTRODUCTION

Immunization leads to the protection of the host against a disease-causing agent, mostly represented by parasites, upon secondary exposure to the same agent (Janeway et al., 2005; Milutinović and Kurtz, 2016). Physiological mechanisms determining this response can operate through i) general upregulation of the immune system, which provides to the host an unspecific protection toward a broad range of pathogens, included the eliciting one (Moret and Siva-Jothy, 2003) or ii) specific immune memory, the so called immune priming, which is effective against the same parasite which elicited it, characterized by different levels of specificity, like as parasite genus, species and even strain (Masri and Cremer, 2014). Anyway, immunological mechanisms underlying the immunization in invertebrates are still elusive (Masri and Cremer, 2014; Milutinović and Kurtz, 2016) but is known that immunization can occur within the lifespan of an individual, even across developmental stages (Masri and Cremer, 2014). In social insects, and in ants in particular, the expression of immunization strongly depends on the host-pathogen system and on the physiological status of the host (Gálvez and Chapuisat, 2014; Reber and Chapuisat, 2012). Indeed, immunization is not without physiological costs (Moret, 2000) and according to a species biology, in some particular phases of a life cycle and under certain conditions of pathogen pressure it could not be convenient to maintain a prompt immune system (Graham et al., 2011). Accordingly, in *Lasius niger* ants it has been found that immune priming does not occur in virgin queens, but it appears after fecundation (Gálvez and Chapuisat, 2014; Reber and Chapuisat, 2012).

In this study we focused on the immunization on queens belonging to different physiological status of the acrobat ant *Crematogaster scutellaris* (Myrmicinae). This species, widespread in the Mediterranean Basin, is characterized by monogynic and claustral foundation (Frizzi et al., 2015). During the claustral phase queens only rely on their limited physiological resources until the emergence of the offspring, determining the beginning of the colonial phase (Bordoni et al., 2017; Hölldobler B and Wilson EO, 1990). The claustral phase of this species lasts around six months. It begins after mating, when the queen starts the hibernation in a shelter and terminates when the first adult worker emerges and starts to forage and feed the queen, determining the beginning of the colonial phase. A large fraction of foundresses dies during the first year of life but at least in lab experiments almost all the queens that reach the colonial phase survive, even if subjected to different stresses (mechanical and biological) (Bordoni et al., 2017). In this optic, the emergence and the survival of the first cohort of workers is critical for queen survival and the overall colony success. In line with this hypothesis, trans-generational immunization has been demonstrated since workers produced by queens exposed to light doses of the entomopathogenic fungus *M. anisopliae* showed a higher survival when challenged with the same pathogen (Bordoni et al., 2018).

Being the pathogen exposure of the mother queen necessary to elicit the trans-generational immunization, it is possible to suppose that queen triggers some physiological reactions to enhance

the offspring immunity. With the aim to understand if the pathogen also activates mechanisms enhancing the individual immunity of the exposed queens, and if the expression could be affected by the queen physiological status, we tested the occurrence of immunization in *C. scutellaris* queens belonging to claustral and colonial conditions using as elicitor *M. anisopliae.*

2. MATERIALS AND METHODS

In winter 2016-2017 we collected hibernating young colonies and foundresses of *Crematogaster scutellaris* around Florence, Italy. We allowed them to conclude the hibernation in plastic containers (2cm x 1.5cm) and controlled conditions (7°C temperature, 80% humidity) (Bordoni et al., 2018). In February 2017, we used 60 foundresses to assess the lethal-dosages (LD, see supplementary methods for lethal-dosage assessment) of Met52® *Metarhizium anisopliae* conidia (see supplementary methods for fungus rearing conditions). In March 7th, 2017, two groups (Triton and Mt-spores) of 30 foundresses queens, and two groups (Triton and Mt-spores) of 30 queens belonging to young (one year) colonies, for a total of 120 queens. As a first treatment (exposure), we rinsed the queens of the Mt-spore groups in the LD10 suspension (10^5 CFU/ml) for three seconds. The Triton groups were rinsed in the Triton solution (0.01%). In the same day, we moved the queens and the colonies to a thermostatic room simulating spring conditions (12-12h L-D period, 25°C-18°C temperature, 80% humidity). To facilitate the germination of the spores, queens have been kept at 90% humidity during the first two days (Bordoni et al., 2018). Queens belonging to colonies have been also kept separated by workers and brood during these two days, to avoid spore removal through allogrooming by workers (Konrad et al., 2012). Thereafter, we lowered the humidity to 80% and we reintroduced the mother queens to the respective colonies. We daily monitored queens until the second treatment. The second treatment, corresponding to the challenge, has been performed after two weeks for half of the individuals of each experimental group and after four weeks in the other half of them. The challenge has been carried out by rinsing the queens for 3 seconds in the LD80 suspension of *M. anisopliae* (10^8 CFU/ml). Queens were rinsed and then isolated in small plastic containers with the same conditions as after the first treatment. After two days, we reintroduced the mother queens in the respective colonies and we daily checked mortality of foundresses and mother queens for the two weeks following the challenge.

We compared survival of queens through the observation period by using a Cox regression as implemented in the "coxph" function of the "survival" R package. We tested the effect on survival of the first treatment (Triton vs non-lethal dose of *M. anisopliae*), of time between exposure and challenge (2 vs 4 weeks) and on the status of the queen (foundress or mother queen). We also tested for the interactions between these factors. The datasets and the R scripts are available as supplementary material.

3. RESULTS

A significant interaction between queen status and treatment was produced by the observation that the only group showing a final survival higher than the expected 20% (the LD80 has been assessed on foundresses) were the unexposed mother queens (Table 1, Figure 1). Interval between first exposure and challenge also entered two interactions, mostly because unexposed mother queens showed a higher survival when challenged with a LD80 two weeks after the first exposure compared to those challenged one month after the exposure (Table 1, Figure 1). Although the presence of significant interactions between status, treatment and time, complicate the interpretation of the main effects factors, it can be recognized that the groups of queens tested in the presence of workers had a lower mortality of the claustral ones, although the exposure to *M. anisopliae* had clearly lowered the survival of worker-right queens Accordingly, the queen showed a significant effect in explaining survival to a LD80 dose of *M. anisopliae* in the Cox Regression, with mother queens showing a higher survival than foundresses (Table 1).

	Coef.	Hazard ratio	S.E.(Coef.)	Z	p
Treatment	-0.378	0.685	0.902	-0.420	0.680
Interval	-0.003	0.997	0.026	-0.100	0.920
Status	-4.961	0.007	2.011	-2.470	**0.014**
Treatment*Interval	0.037	1.038	0.037	1.010	0.310
Treatment*Status	7.032	1132.610	2.213	3.180	**0.002**
Interval*Status	0.166	1.181	0.072	2.310	**0.021**
Treatment*Time*Status	-0.225	0.798	0.081	-2.770	**0.006**

Table 1. Cox regression on survival in a LD80 Challenge of 120 queens exposed to two different first exposure treatments (Triton, Mt-spores), at different intervals between exposure and challenge (2 or 4 weeks) and belonging to claustral or colonial conditions. The effects of interactions are also reported. Significant effects are highlighted in bold.

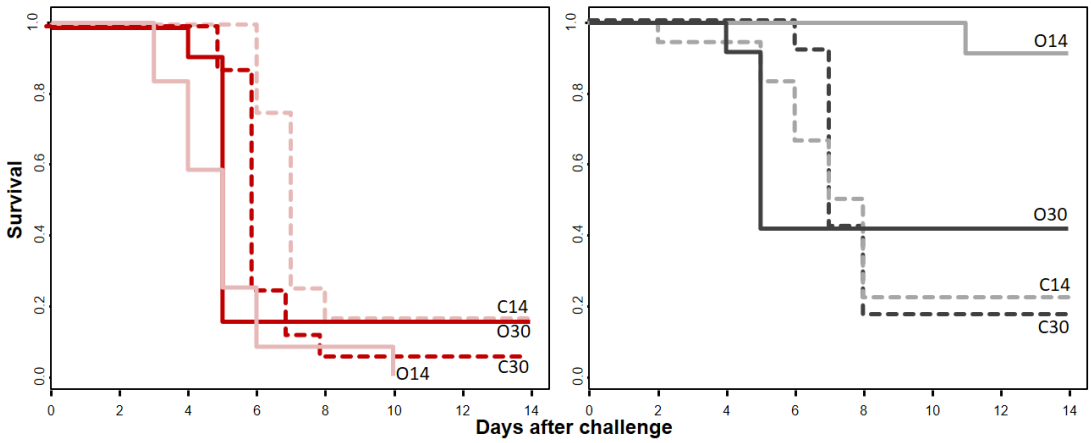

Figure 1. Survival plots for the four groups of queens previously treated with LD20 of *M. anisopliae* during the 14 days after the challenge with LD80 of *M. anisopliae* (a) and for the four groups of queens treated with Triton (b). C14 and C30, claustral queens challenged 14 and 30 days after the first exposure, respectively; O14 and O30 queens attended by workers challenged 14 and 30 days after the first exposure, respectively.

4. DISCUSSION

It is evident that, dealing with experimentally induced infections is energetically costly for acrobat ants. By applying to workers different feeding protocols (ad libitum feeding or starvation), it has been demonstrated, that food intake explains a conspicuous part of the differential survival to *M. anisopliae* infection (Bordoni et al., 2018). Current results on queens exposed to the same parasite are in the same line; in fact, the only group that showed lower mortality than the expected value (20% respect to the 80% expected with the administrated LD80) was represented by queens attended by workers which never experienced the pathogen before. Consequently, we can also affirm that a first non-lethal exposure did not induce immunization in queens, conversely it decreased the survival after the challenge. This effect was likely due to a physiological deficit of an unknown nature which had not been restored at least one-month after the first exposure. It is widely known that, in claustral ant species, foundresses rely on limited energetic resources, slightly sufficient to establish a new colony (Hölldobler B and Wilson EO, 1990). A clear example can be found in the foundresses of the leafcutter ant *Atta sexdens* during the colony foundation; the energetic resources available to these foundresses are sufficient to dig only in the soil one hole deep enough to establish the first colony chamber (Camargo et al., 2011).

It Is necessary to take in account different parameters to understand the evolutionary meaning of host responses when exposed to a pathogen. Graham et al. (2011) argued that, to evaluate the impact of the immune response on the overall host fitness, three factors and their interactions must be considered: i) host fitness, ii) parasite density and iii) relevant immune responses. Taking for granted that the magnitude of the immune system is necessarily positively correlated with the host fitness represents a potentially misleading approach to the problem which could lead to wrong conclusions.

In fact, when physiological resources of the host are heavily impacted by the immune system activation, the individual might have no more means to be invested on fitness (Bonneaud et al., 2012; Graham et al., 2011; Marzal et al., 2007). In bumblebees it has been demonstrated that immunization is costly, but these costs can determine a higher mortality only in starved workers (Moret, 2000). In our experimental system a cost-benefit trade-off is likely at the basis of the observed results, with the peculiar occurrence of trans-generational immunisation in workers (Bordoni et al., 2018) without evidence of immunisation in mother queens (this study). In *C. scutellaris* queens, the immune system could require unaffordable costs to potentiate or activate immunity responses. Claustral foundresses are highly starved queens, and they could not afford the energetic costs of immunization. Workers might likely have a different optimum in this trade-off, indeed, they don't have to invest energies in oogenesis; moreover, they experience a higher risk to get in contact with parasites during foraging activity. Shortening worker life expectancy has much less consequences on the colony success compared to shortening queen lifespan; finally, *M. anisopliae* is an obligate killer pathogen producing infective spores only after the death of the host. Consequently, the immunisation of workers protects the queen from the spread of this pathogen inside the colony (Ebert and Weisser, 1997). This overview could explain the uncoupled pattern of the immunization responses observed in the acrobat ant, expressing transgenerational immunization from queen to workers, and not the immunization of the queen against the same pathogen.

Authors' contributions

S.T. and A.B. conceived the study; A.B. and L.D. designed the experiments; A.B., I.T. and L.D. collected the data; A.B., and L.D. carried out the analyses and drafted the manuscript; all authors critically revised the paper, approved the final version of the manuscript and agree to be held accountable for the content therein.

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Chapter 5

Linking individual to social immunity: enhanced bacterial clearance in a social immunity specific-task in honeybees.

This manuscript represents a preliminary report of an ongoing study

Linking individual to social immunity: enhanced bacterial clearance in a social immunity-specific task in honeybees.

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ABSTRACT

Insect societies are characterized by an elevated risk of disease transmission, which selects for effective immune-defensive strategies. Division of labour, with some individuals specializing in immunity related tasks, strongly concurs to increase immune ability of insect societies. A trade-off however may exist between phenotypic specialization to increase efficiency and maintenance of phenotypic plasticity to cope with a changing environment. Here we investigated this trade-off by using social grooming in the honeybee, *Apis mellifera*, where this hygienic behavior is assumed to be potentially effective in lowering ectoparasites load. We adopted an integrated approach encompassing behavioural observation at individual and social network level, bacterial clearance based immuno-assays and antennal proteomics, to characterize the behavioural and physiological phenotype of social groomers. We found that social groomers have higher immune capacity compared to same age control bees, while they do not differ in their chemosensory proteomic profile. Behaviourally, they show rather slightly differences in the task performed, while they clearly differ in their connectivity within the colonial social network, having a higher centrality than same age control bees. This demonstrates the presence of an immune-specific physiological and social behavioural specialization in individuals involved in social immunity related tasks, thus linking individual to social immunity. It also suggests that division of labour might lead to physiological specialization narrowly tailored upon the task performed while maintaining an overall plasticity.

Keywords: social grooming, allogrooming, phenotypic specialization, plasticity, proteome, odourant-binding proteins, immunocompetence

1. INTRODUCTION

Living in crowded societies has as a major drawback to increase the risk of parasite and pathogen transmission (Naug and Camazine, 2002; Naug and Smith, 2007; Nunn et al., 2006; Schmid-Hempel, 1998), due to the high levels of spatial proximity and intimate interactions among group members (Fries and Camazine, 2001; Otterstatter and Thomson, 2007; Schmid-Hempel, 2017, 1998). Disease pressure represents a major challenge for the survival of insect societies and natural selection has favoured the evolution of a plethora of different physiological and behavioural strategies (Cremer et al., 2018, 2007; Ezenwa et al., 2016; Naug, 2008; Naug and Camazine, 2002; Naug and Smith, 2007), at both the individual (i.e. production and use of antimicrobial molecules; (Baracchi et al., 2011; Stow et al., 2007) and the collective level (i.e. socio-spatial compartmentalization to limit disease spread (Baracchi and Cini, 2014; Cremer et al., 2018). Division of labour is defined as the pattern of specialization by cooperative individuals of a social group performing different tasks or roles depending on their different morphology (polyphenism) or behavior (polyethism) (Alexander, 1974; Freeland, 1976; Hughes, 2005; Smith and Szathmary, 1997; Wilson, 1985). Division of labour is a fundamental organizational component of social immunity in social insect colonies (Stroeymeyt et al., 2014). In some species, specialized workers patrol the entrance of the colony (Breed et al., 2004; Moore et al., 1995) to spot and repel sick or parasitized individuals, thus preventing the entrance of pathogens and parasites inside the colony (Baracchi et al., 2012; Cappa et al., 2016, 2014). Hygienic and undertaking behaviours (i.e. destructive disinfection and/or removal of infected individuals) evolved in some ants and bees (Baracchi et al., 2012; Julian and Cahan, 1999; Pull et al., 2017; Robinson and Page Jr, 1988; Sun and Zhou, 2013). These task specializations often occur with a suite of behavioural and physiological correlates, some of which are specific phenotypic specializations that increase the aptitude, the efficiency and/or decrease the costs of the task performed (Currie et al., 2006, 2003; Little et al., 2006; Powell, 2008; Šobotník et al., 2012). However, the phenotypic specialization associated with division of labour is expected to be under contrasting selective forces: a colony might benefit from having sets of workers with highly specialized phenotypes, more efficient and apt to perform a specific task. At the same time, such phenotypic specializations may limit plasticity therefore reducing performance of other tasks when needed (Mertl and Traniello, 2009; West-Eberhard, 1989). Thus, division of labour should show an adequate degree of flexibility to allow the colony to rapidly reallocate its resources in response to the environmental demands (Bourke et al., 1995; Tofts and Franks, 1992). Understanding the degree of phenotypic specialization in group of workers performing specific tasks has the potential to unravel the trade-off between phenotypic plasticity and specialization. Social grooming, also known as allogrooming (Hölldobler and Wilson, 1990), is a hygienic behaviour in which a worker uses its mouth parts to remove debris from the body of other colony members. This behaviour, observed in some species of eusocial insects, is generally assumed to play a role in defence against parasites and pathogens (Evans and Spivak, 2010; Hughes et al., 2002; Kermarrec et al., 1986; Oi and Pereira, 1993;

Rosengaus et al., 1998; Yanagawa et al., 2008). In honeybees, social grooming represents an important resistance mechanism that seems to limit ectoparasites load, especially mites, within colonies (Aumeier, 2001; Boecking and Spivak, 1999; Guzman-Novoa et al., 2012; Kruitwagen et al., 2017; Pritchard, 2016) and its expression depends on genetic and environmental factors (Pettis and Pankiw, 1998; Zaitoun and Al-Ghzawi, 2009). In *Apis cerana*, social grooming is performed at a high rate and appears to be a particularly effective counter-adaptation against the major worldwide threats for honeybee colonies and apiculture, the parasitic mites *Varroa destructor* (Aumeier, 2001; Boecking and Spivak, 1999; Nazzi and Le Conte, 2016; Pritchard, 2016). The relatively recent introduction of this parasite in Europe heavily impacted on *Apis mellifera* (Cervo et al., 2014; Nazzi and Le Conte, 2016) but effective strategies to deal with this emergency are still lacking. Being social grooming expressed with efficacy against *V. destructor* in *Apis cerana*, its characterization on *Apis mellifera* may clarify its possible effectiveness to control parasite load at colony level. Despite the potential value that social grooming could have in maximizing colony resistance to parasites and disease transmission, very scarce attention has been given to the behavioural and physiological specializations of social grooming at the individual level. Thus, the degree of specialization of individuals performing this task is still unclear and behavioural and physiological correlates of social groomers are largely unknown. Here, we investigated the degree of phenotypic specialization of social groomers in the honeybee *Apis mellifera*. Empirical evidence reported in literature about the temporal expression of social grooming and the degree of behavioural specialization in individuals expressing is contrasting. According to some authors, the expression of social grooming is temporally restricted from the 1st to the 20th day post-emergence (Seeley, 1982) while others observed workers performing social grooming during their entire life (Kuswadi, 1992; Winston and Punnett, 1982). To assess the timing of social grooming expression, we thus performed detailed behavioural observations on a large cohort of workers along their lifespan inside the hive (until the onset of foraging) to assess the timing of social grooming expression. We then investigated the degree of behavioural specialization of social groomers, focusing on their individual behavioural profile (i.e. the array of tasks performed) and on the role they play in the colonial social network. Indeed, it is not clear to what extent specialization in social grooming implies a different behavioural repertoire in social groomers compared to same age non-grooming nestmates. Previous studies showed that social groomers are specialized individuals performing social grooming at a consistently higher frequency compared to other tasks typical of same-age workers (Kolmes, 1989), while others reported that this behaviour is unfrequently expressed also by the small number of individuals performing it, also performing the set of tasks typical of their age (Van der Blom, 1993). We thus compared behavioural repertoires of social groomers and of same age non-grooming bees, predicting that if social grooming is a strong specialization the behavioural repertoire will differ with social groomers showing reduced performance of the typical in-hive tasks (prediction 1). Moreover, we lack information on how social groomers behave within the colonial social network. The nature and structure of animal

social network affect the likelihood and the dynamics of disease transmission as well as the efficiency of prophylactic strategies (Naug, 2008). In *A. mellifera*, individuals have been shown to occupy different positions (more or less central, i.e. more or less connected) within the colonial social network, according to their caste, age and task (Baracchi and Cini, 2014), producing a compartmentalized structure that likely reduces disease transmission (Stroeymeyt et al., 2014). If social groomers are behaviourally specialized, we expect that their social network behaviour differs from that of same age non-grooming bees. In particular, as this hygienic behaviour would be advantageous if social groomers inspect and groom many bees within the hive, we might expect them to be more central in the colonial network than same age, non-grooming bees (prediction 2).

In order to maximize grooming beneficial effects, social groomers would benefit from detecting which nestmates need to be groomed. Since many stressors included pathogens and parasites alter the odour of workers in *A. mellifera* (Baracchi et al., 2013; Cappa et al., 2016; Richard et al., 2012), the perception of such chemical cues could be a mechanism involved in a differential expression of social grooming. Thus, it would be advantageous for social groomers to be equipped with particular chemosensory abilities. Recent proteomic investigation showed that bee task influences the antennal profile of proteins involved in olfaction, with proteins such as Odorant binding proteins (OBPs), Chemosensory proteins (CSPs) and Niemann-Pick type C2 (NPC-2), being differently expressed (Iovinella et al., 2018). These soluble olfactory proteins play a crucial role in the first steps of odour recognition (Pelosi et al., 2014, 2006) by transporting hydrophobic odorants through the sensillar lymph and interacting in a combinatorial way with odorant-receptors (ORs) of the olfactory neurons (Leal, 2013). Moreover, two OBPs have been reported among biomarkers linked to social immunity by Guarna and coworkers (2015). In particular bees of colonies selected for hygienic behaviour were found to overexpress OBP16 and OBP18 in their antennae; both these two proteins have shown good affinity towards ligands released by decaying insect corpses. We thus investigated the expression of these proteins in the antennae, predicting that if social groomers have any degree of chemosensory specialization, this would be reflected in a different antennal proteomic profile, with a different expression level of olfactory proteins (prediction 3).

Performing social grooming is likely to increase the risk for social groomers to come in contact with pathogens and parasites. Social groomers would benefit from having an increased immunocompetence compared to same age non-grooming bees, in order to cope with a higher risk of infection. We tested this prediction by comparing immunocompetence ability between social groomers and age-matched non-grooming bees, predicting a higher level of immunocompetence in the formers (prediction 4).

Overall, our work characterizes for the first time the social groomers phenotype through an integrated approach encompassing behavioural observations, proteomics and immuno-assays. We believe our study paves the way toward a better understanding of the drivers of this fascinating defensive strategy. Moreover, it suggests that phenotypic specialization may occur in those

phenotypic traits that are strictly related to the task performed while an overall phenotypic plasticity is retained, to allow flexibility in task performance.

2. MATERIAL AND METHODS

Insect collection, rearing and general procedures

Experiments were conducted between June and July, when expression of social grooming is higher (Zaitoun and Al-Ghzawi, 2009), in 2014 (behavioural observations for individual behaviour characterization), 2015 (antennal proteomics and bacterial challenge) and 2016 (behavioural observation for social network analysis). All studies were performed using standard one-frame observation hives maintained in laboratory (Department of Biology, University of Florence) where bees were free to forage outside. Observation combs were taken from colonies belonging to a local beekeeper (Azienda Cristofori). We screened 15 colonies each year in order to identify colonies with relevant social grooming rates (at least 30 social grooming acts per comb during 30 minutes of observation. From each selected colony we took a comb containing stored honey and pollen, open and sealed brood, queen and around 2000 workers and transferred it to the observation frame. Experiments were performed on four observation hives for the behavioural experiments (two for prediction 1 and two for prediction 2), three hives for antennal proteomics (prediction 3) and four observation hives for the bacterial challenge (prediction 4). Overall, observation combss were issued from a total of 8 colonies.

Individual bee marking procedure

To obtain house bees of known age needed for the experiments, we collected newly emerged bees directly from the comb by gently removing them with forceps. Bees were a) individually marked with plastic coloured numbered tags on their thorax (predictions 1 and 2, for which we needed to follow individual behaviour over several days) or b) marked with a spot on the thorax with UniPosca® paint markers using different colours according to day of collection and hive of origin (predictions 3 and 4, where individual age and colony of origin were sufficient as social groomers were immediately removed from the comb and used for analyses as soon as they performed the grooming act). Marked bees were gathered in plastic cylindrical containers (Ø 10 cm x h 10 cm), they were then lightly dusted in icing sugar before being gently reintroduced in their natal colonies to favour acceptance by older nestmates (time between collection, marking and reintroduction was between 12 and 20 hours). More than 200 newly emerged workers per observation comb were marked for testing predictions 1 and 2 and more than 100 per comb for testing predictions 3 and 4.

Temporal dynamics of social grooming expression

We evaluated the temporal dynamics of social grooming expression along individual worker lifespan using an all occurrences sampling method (Altmann, 1974). Starting the day after re-introduction of newly emerged marked bee and until day 25 post emergence (average life expectancy during summer, Woyciechowski and Kozłowski, 1998) we counted the occurrences of all social grooming events performed by a marked bee by observing combs every odd day for 30

minutes for each side. Observations took place during central hours (between 11:00 and 14:00) according to Pettis and Pankiw (1998). A total of 12 h of observation was performed on each hive. Departure from randomness of the temporal expression of social grooming was tested with a Runs test.

Prediction 1. Social groomers show a specific behavioural repertoire.

We determined the behavioural repertoire of social groomers and same age non-grooming bees using focal animal sampling (Altmann, 1974). Every marked bee observed performing an act of social grooming for at least 30 seconds (hereafter called social groomer) was followed continuously for 10 minutes during the same day (between 2 and 5 pm). Ten minute observation periods were divided into 20 intervals of 30 seconds each, and for each interval the main behaviour performed by the focal bee was recorded, thus obtaining 20 observation scans for each focal groomer. The same procedure was applied to same age non-grooming bees, which were marked bees of the same age of focal groomers and that have never been observed, during the current or previous days, performing an act of social grooming. If bees chosen as same age non-grooming bees were later observed performing social grooming they were removed from the dataset. The number of same age non-grooming bees chosen was the same as the number of focal groomers. This procedure allowed the comparison of behavioural specialization between social groomers and same age non-grooming bees at each age. Overall, we obtained 53 focal groomers (first hive, n=26; second hive, n=27) and 62 focal same age non-grooming bees (first hive: 26; second hive=36) of age range 4-15 days post-emergence. For each bee we calculated: a) the behavioural repertoire size as the number of different tasks a bee performed at least once; this measure has been used as a proxy of behavioural plasticity, i.e. opposite to behavioural specialization (Jandt et al., 2009; Johnson, 2010); b) the performance rate of each behavioural item, as the number of observations scans in which the focal bee performed that behaviour. Moreover, we controlled for possible differences in behavioural repertoire due to different activity rates, by comparing activity rate (number of scans during which the bee was performing any behaviour other than inactivity) between groomers and same age non-grooming bees. Behavioural repertoire size and performance rates for each behavioural item were compared between social groomers and same age non-grooming bees using the non-parametric two-samples Mann Whitney test, as data departed from normality. Statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL) and Past v3.20 (Hammer et al., 2001).

Prediction 2. Social groomers are more central in the colonial social network

An association network was built on the basis of the spatial proximity of bees, following the protocol in Baracchi and Cini (2014). We considered two bees interacting when they were at a distance minor of the length of a honey body (~ less than 3 cells). Interaction among marked bees where recorded from each observation colony taking 25 photos on both sides, over a period of four days. Photos were taken every hour during the central hours of the day (approximately 10 am-4 pm). In order to define the association network, each individual bee was considered as a node and

an interaction between two individuals was taken as an edge existing between these two nodes, thus resulting in a weighted and not directed network. The following measures of centrality were calculated for each node: degree and betweenness (Everett and Borgatti, 2005). Degree is defined as the sum of the strength of all ties connected to a node; betweenness is centrality measure that is defined as the total number of shortest paths between pairs of nodes that pass through the considered node. It is thus an index of liaising otherwise separate parts of the network. We focused on these two measures as they catch different aspects of network centrality (respectively the size of the social neighbourhood of a bee, i.e. the individual potential to groom many bees (degree) and to influence the passage of pathogens through the network (betweenness). Both these measures have shown to differ according to age and task in *A. mellifera* (Baracchi and Cini, 2014). Centrality measures were computed using Ucinet 6 and differences in centrality measures between social groomers and same age non-grooming bees were assessed using a generalized linear model (GLZ) with negative binomial distribution and log-link function. The full factorial model included hive of origin as a random factor and category (social groomers vs same age non-grooming bees) as a fixed factor as well as their interaction. Statistical analyses were performed with Ucinet 6 (Borgatti et al., 2014).

Prediction 3. Social groomers have a differential expression of antennal olfactory proteins compared to same age non-grooming bees

Social groomers and control bees (defined as above) were collected from 3 observation hives. As this same study shows social grooming is mainly performed by bees within a well-defined age-interval (Fig. 1) we sampled marked bees within this range. Once identified as social groomers, bees were gently removed from the comb with forceps and immediately stored at -20°C. Flagellum from antennae were dissected and pooled immediately before protein extractions from pools of 5 individuals randomly sampled from the three colonies. Five biological replicates for each sample were prepared. Reagents and procedure used for protein extraction, digestion, purification and shotgun analysis, as well as protein identification and quantification are described in Iovinella et al. (2018). Data were searched against databases downloaded from Uniprot containing all *Apis mellifera* proteins as well as those from common honeybee pathogens and parasites (*Nosema*, *Paenibacillus larvae*, *Varroa*, *Melissococcus*, *Ascosphaera*). A Venn diagram was drawn between groomers and same age non-grooming workers, considering "Unique+Razor" peptides (i.e. those exclusively shared by the proteins of the same group) identified in at least 3 of the 5 biological replicates of one group. Differential protein expression was evaluated after filtering data for proteins quantified in at least 3 replicates (out of the 10). Hierarchical clustering analyses were performed using average Euclidean distance and the default parameters of Perseus (300 clusters, maximum 10 iterations). Analysis of differential expression of single proteins was performed using Mann–Whitney test with a Benjamini-Hochberg correction (p=0.005). Statistical analyses were performed with Perseus software (version 1.5.1.6) and "stats" R package.

Prediction 4. Social groomers have higher immunocompetence compared to same age non-grooming workers

Social groomers and same age non-grooming bees were collected from 4 observation hives following the same criteria used to sample bees for antennal proteome analysis (see Prediction3). We compared the ability to remove bacterial cells from their haemolymph (i.e. bacterial clearance) between social groomers and same age non-grooming bees by injecting bees with the Gram-negative bacteria *Escherichia coli*, an immune elicitor commonly used to test immunocompetence in insects (Cappa et al., 2015; Cini et al., 2018; Manfredini et al., 2010; Yang and Cox-Foster, 2005). We measured bacterial clearance as a good proxy of workers immunity since different parameters linked to antimicrobial immune response are positively correlated in insects' immunity (Gillespie and et al., 1997; Lambrechts et al., 2004; Schmid-Hempel, 2005). Moreover, injection of live bacteria provides an integrative view of the activation of the organism immune system (Charles and Killian, 2015). *E. coli* is not naturally found in *A. mellifera*, thus, we could exclude its presence in our workers prior to artificial infection. Bacterial culture and injection were carried out following the same procedure used by Cini et al. (2018) (see also supplementary material). After injection, bees were separated for category into plastic cylindrical containers (Ø 10 cm x h 10 cm) provided with *ad libitum* honey as food and maintained under controlled conditions, (~ 30°C; 55% RH). Twenty-four hours later each worker was inserted in a sterile plastic bag with 10 mL of PBS after removing the sting and the venom sac and processed with a Stomacher® 400 Circulator (230 rpm x 10 min) to homogenize the bee body in the PBS. Afterwards, 0.1 mL of undiluted and serially diluted PBS suspensions (dilutions 10^{-1}, 10^{-2}) of each sample were plated on LB solid medium added with tetracycline (10 µg/mL) and incubated overnight at 37°C. The following day, the colonies grown on the plate surface were counted and the viable bacterial count was expressed as Colony Forming Units (CFUs) per bee. At least 8 same age non-grooming bees per colony for each category were injected with 1 µL of PBS, homogenized and plated following the same procedure of *E. coli*-infected workers, to ensure absence of other bacterial strains capable of growing on our LB agar plates added with tetracycline. A total of 235 bees were infected with *E. coli* and plated: (i) groomers, N = 108, (ii) same age non-grooming bees, N = 127. Bacterial challenge data (number of CFU) were analyzed with a generalized linear model (GLZ) with negative binomial distribution and log-link function. The full factorial model included hive of origin as a random factor and category (social groomers vs non-grooming bees) as a fixed factor. Statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL) and Past v3.20. (Hammer et al., 2001).

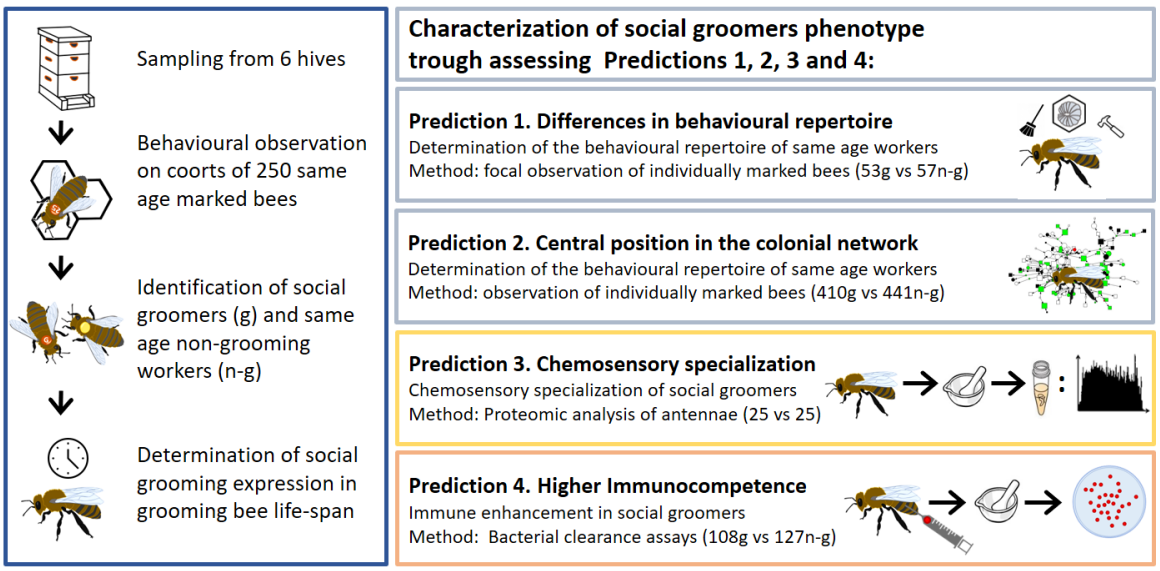

Figure 1. The experimental design of the study.

3. RESULTS

Temporal dynamics of social grooming expression

Occurrence of social grooming clearly varies with bee age (Fig.1), occurring within an age-range of 3 to 15 days, and being especially common in the range 6 to 11 days (76% of social grooming events was observed within this range). The age dependency of this temporal trend is supported by Runs test, which shows significant departure from randomness (Runs test, number of runs=3, p=0.013). Furthermore, the fraction of social groomers that were seen grooming at least once over the total amount of marked bees varies with bees age, showing the same range of to 15 days for grooming expression and with largest part of groomers having age within 6 and 11 days (78% of groomers). Percentage of marked bees that performed at least once social grooming was 1.50 over the entire observation period and rose up to 3.60 in the peak range (6 to 11 days). The large majority of social groomers (57.1 %) was observed performing only once social grooming, and the number of grooming acts per individual rapidly decreased.

Prediction 1. Social groomers show a specific behavioural repertoire.

Repertoire size (the number of different tasks a bee performed at least once) did not differ between social groomers and same age non-grooming bees (Mann Whitney test, U=1559,5 Z=-0.485, p=0.628, N=53 vs 62; median and interquartile range: 3.0, 2.0 for social groomers and 3.0, 2.0 for same age non-grooming bees). Social groomers and same age non-grooming bees did not differ in the performance rate (number of observations scans in which the focal bee performed that behaviour) for any of the behavioral task considered (see table 1), except from social grooming. Moreover, overall activity rates (number of scans during which the bee was performing any behaviour other than inactivity) did not differ between social groomers and same age non-grooming bees (Mann Whitney test, U=1619.5, p=0.894, N=53 vs 57).

Behaviour code	Behaviour category	U	Z	p.
BC	brood care	1539.0	-0.589	0.556
SI	social interaction	1531.5	-0.646	0.518
AG	self-grooming	1392.5	-1.514	0.130
SG	social-grooming	1395.0	-3.155	0.002
EX	external activity	1638.0	-0.124	0.901
WL	walking	1551.0	-0.530	0.596
IN	inactivity	1619.0	-0.134	0.894
OT	other activities	1590.0	-1.313	0.189

Table 1. Behaviours considered in order to describe the behavioural repertoire of same age grooming and non-grooming bees.: U, Z, and p. values of Mann Whitney test for all comparisons.

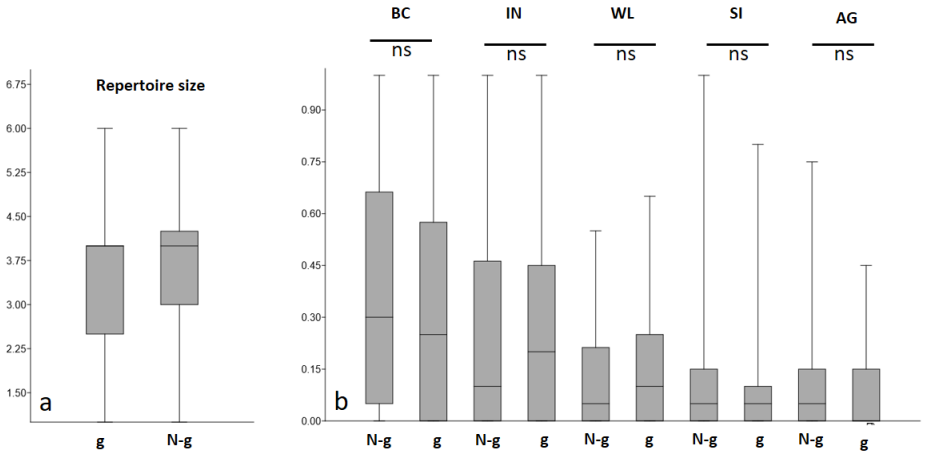

Figure 2. Comparisons between groomers (g) and non-groomers (N-g) in the repertoire size (a) and in five behaviours (b), for abbreviation and statistic results see in Table 1.

Prediction 2. Social groomers are more central in the colonial social network

Social groomers showed higher degree centrality than same age non-grooming bees, both for degree (Chi-square= 12.452, df=1, p<0.001) while they did not show differences in their betweenness (Chi-square= 2.719, df=1, p=0.099) (Fig 3). While colony of origin had a significant effect on both degree (Chi-square=22.132, df=1, p<0.001), and betweenness (Chi-square=316.401, df=1, p<0.001), the interaction between colony of origin and bee category did not have a significant effect on neither degree (Int Chi-square=0.557, df=1, p=0.456) nor betweenness centrality (square=0.032, df=1, p=0.858).

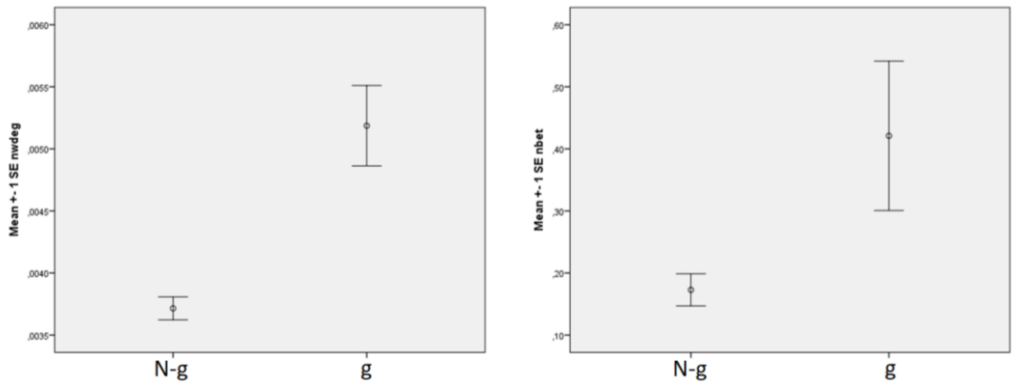

Figure 3. Mean and standard error values of centrality (left) and betweenness (right) between groomers (g) and non-groomers (N-g).

Prediction 3. Social groomers have a differential expression of antennal olfactory proteins compared to same age non-grooming bees

Overall, the "shotgun" approach applied on crude extracts of antennae of same age non-grooming bees workers and groomers bees identified 428 and 426 proteins respectively. The global distribution of the identified proteins and their expression level are very similar between the two groups (Fig S1). This high degree of overlap is also reflected considering the numbers of proteins belonging to each gene ontology (GO categories), both for molecular function and for biological process, Pfam and Interpro. Only two proteins were exclusive in same age non-grooming workers: a dehydrogenase (id A0A087ZVG0) and the Polyprotein Kakugo virus, this last suggesting that control bees could be more infected than groomers; while four proteins were exclusively identified in groomers (Fig S1): a protein belonging to the purine-rich element binding (Pur) family (id A0A087ZNG2), a ras-related protein (id A0A087ZV73), an uncharacterized protein (id A0A088A9I2) and the neprilysin-2 isoform X1 (id A0A088AKD7), but none of them has ever been reported to be involved in olfaction nor in immune-response. Besides the global expression pattern of antennal proteins, our primary aim was to understand if the groomers could have a chemosensory specialization reflected in a different profile of olfactory proteins with respect to control bees. The expression profiles of the soluble olfactory proteins are reported in Figure 4. None of them is significantly more expressed in groomers or control bees and therefore groomers do not seem to be specialized in the very first steps of odour perception. Quantitative differences in protein expression between control bees and groomers were evaluated through Mann–Whitney test with a Benjamini-Hochberg correction (p=0.005). Table 2 reports the proteins exclusively expressed in groomers and control bees and the 8 proteins differentially expressed between groups: a lysosomal aspartic protease (id A0A087ZY53), two uncharacterised proteins (id A0A088AH61, A0A088ACF4), a myoneurin-like isoform X1 (id A0A088ARU1), an arginine kinase (id A0A088ARZ8), a small ubiquitin-related modifier (id H9KID5), a putative acyl-CoA-binding protein (id A0A087ZSM9) and a malate dehydrogenase (id A0A087ZYQ1).

None of these proteins has been reported to be involved in odour perception nor in immune response, disease-resistance and other immune-related tasks.

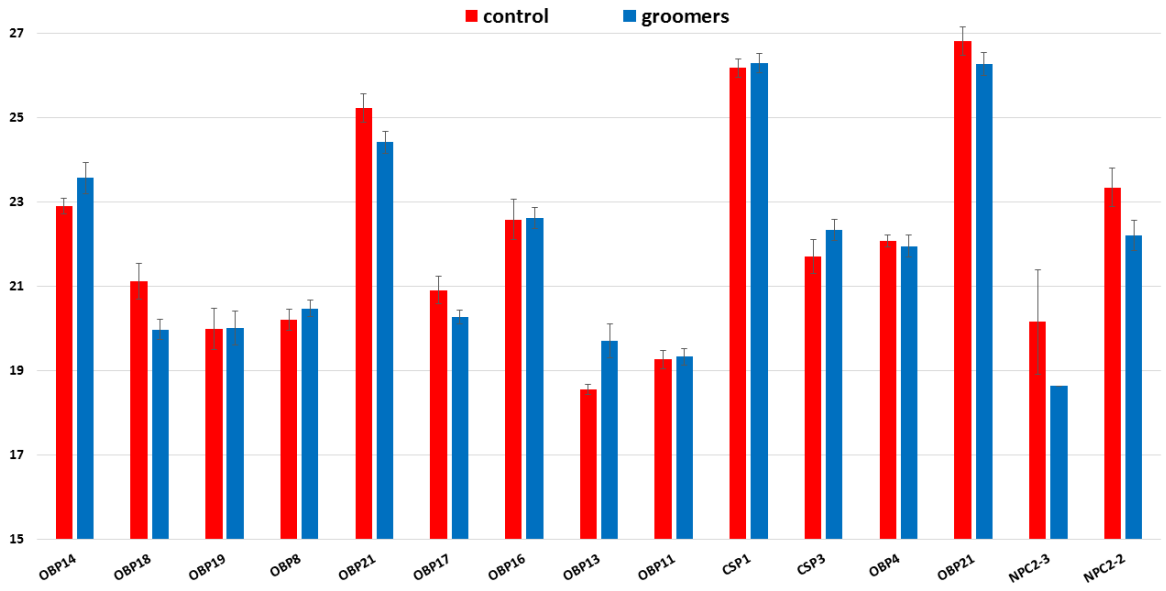

Figure 4. Bar charts showing log2 LFQ (Label-free quantification) intensities of the identified olfactory proteins, averaged among the biological replicates of groomers (blue bar) and control bees (red bar) respectively. For each sample standard error is also reported.

Proteins exclusively expressed in groomers				
Protein ID	**Description**	**Pfam**	**Peptides**	
A0A087ZNG2	Uncharacterized protein	PurA	1	
A0A087ZV73	ras-related protein Rab-7a	P-loop_NTPase	1	
A0A088A9I2	Uncharacterized protein		1	
A0A088AKD7	neprilysin-2 isoform X1	MetalloPept_cat_dom	1	
Proteins exclusively expressed in control bees				
Protein ID	**Description**	**Pfam**	**Peptides**	
A0A087ZVG0	very long-chain specific acyl-CoA dehydrogenase, mitochondrial	Acyl-CoA_dh_1	1	
Q0EDH4	Polyprotein Kakugo virus		4	
Proteins more expressed in groomers than control bees				
Protein ID	**Description**	**Pfam**	**Peptides**	**p**
A0A087ZY53	lysosomal aspartic protease	Asp	5	0
A0A088AH61	Uncharacterized protein	/	1	0
A0A088ARU1	myoneurin-like isoform X1	DUF4775	2	0

A0A088ARZ8	Arginine kinase	ATP-gua_Ptrans	15	0
H9KID5	Small ubiquitin-related modifier	Rad60-SLD	1	0
A0A087ZSM9	putative acyl-CoA-binding protein	Acyl-CoA-binding protein	2	0.002
A0A087ZYQ1	Malate dehydrogenase	lactate/malate dehydrogenase, NAD binding domain	7	0.004
A0A088ACF4	Uncharacterized protein	Hsp70 protein	18	0.004

Table 2. List of proteins exclusively expressed in groomers or control bees and proteins significantly more expressed in groomers with respect to control bees according to Mann–Whitney test.

Prediction 4. Social groomers have higher immunocompetence compared to same age non-grooming workers

Category (groomers vs same-age non-grooming workers s) had a significant effect (Wald chi-square=7.752, df=3, p=0.005), with groomer bees being on average 1.54 times more immunocompetent than same age non-grooming bees (means +- SE; groomers: 103.19+-10.47; non-groomers: 158.56+-18.43) (Fig. 5). Hive of origin did not have a significant effect (Wald chi-square=2.649, df=3, p=0.449). There was no significant interaction between hive of origin and category (Wald chi-square=0.941, df=3, p=0.816).

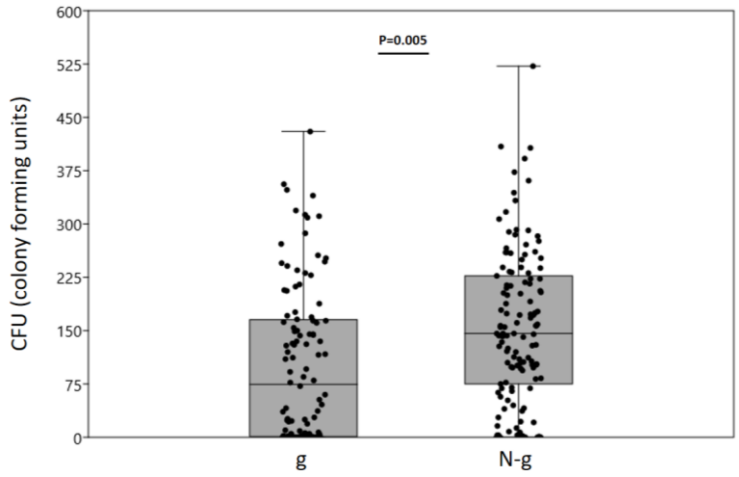

Figure 5. Counts of colony forming units (median and quartiles) in social groomer (g) and non-groomer bees (N-g). Dots represent the individual values.

4. DISCUSSION

Through an integrated approach involving behavioural observations and physiological assays we here demonstrate that social grooming in the honeybee *Apis mellifera* is a transient behavioural specialization, mirrored by an increased immunocompetence but without a chemosensory specialization in terms of antennal overexpression of chemosensory proteins. Our observations clearly show that social grooming is age-dependent, with a peak of expression within 6 and 11 days

of worker age. Moreover, our results suggest that social grooming is a weak specialization. The behavioral repertoire of social groomers and age-matched non-groomers is overall very similar: apart from the defining activity of social grooming, which is by the way poorly expressed in terms of frequency, social groomers perform the same set of tasks, and with similar frequency when compared to same age non-grooming bees. This suggests that, apart from the instances of social grooming, social groomers indeed perform all the tasks expected from their age polyethism. Our results contrasts with some of the previous findings, such as those of Kolmes (1989) and Moore et al. (1995) which observed individual bees performing social grooming at significant rates for their entire life, without undertaking the typical polyethism path (Huang and Robinson, 1996; Robinson, 1987). A possible explanation is that variation in social grooming expression also exists within the category of social groomers itself, with a small percentage of individuals showing a hyper-specialization in this task, as suggested by Kuswad (1992). Our results suggested however that, if this was the case, these bees nonetheless represent a tiny percentage of social groomers in the colony. Overall, our behavioural investigation, which specifically compared a large sample of social groomers and age matched non-grooming bees, allows to characterize social grooming as a transient and weak behavioral specialization. The finding that behavioral plasticity is maintained by social groomers despite their specific task is indeed not puzzling in the view of colony task organization (Robinson, 1992). In fact, previous work has shown that the worker temporal polyethism is highly flexible, responding to both genetic influences and colony social requirements (Amdam et al., 2005; Amdam and Omholt, 2003; Breed et al., 2004; Huang and Robinson, 1996; Omholt and Amdam, 2004; Robinson, 1992; Robinson et al., 1992). Despite the individual behaviour of groomers in terms of task performance is rather similar to that of same age non-grooming workers, these two categories show significant differences in their position within the colonial social network suggesting a spatial specialization in social groomers. Groomers are more connected, i.e. have higher network centrality, compared to same age non-grooming workers, which translates into contacting a higher number of colony mates. From a proximate perspective, this might be due to a different use of space by groomers. Individual network position has been shown to depend on spatial behaviour in bees (Baracchi and Cini, 2014). While our results show that they are no more active than non-groomers, they might have less fidelity to specific parts of the comb and/or move faster across the comb. From an ultimate perspective we might speculate that a higher centrality could be beneficial as it allows groomers to screen a higher number of colony members for parasites. Interestingly, the possible increased costs of such higher network centrality (increased exposure to pathogens, (Stroeymeyt et al., 2014) might be reduced in groomers thank to their increased levels of immune ability (our results). The peculiar task of social groomers might be supported by differential chemosensory abilities that could help in identifying unhealthy or disease or pathogen carrying individuals to better direct social grooming towards the most suitable targets inside the colony (prediction 3). Our results, however, do not support this hypothesis since at least for soluble olfactory proteins no differences were found. There are two possible explanations for

this finding. First, social groomers might not need a chemosensory specialization. Bees in need of being groomed can be recognized through other channels, such as the tactile one or by their behaviour. Indeed, grooming is sometimes solicited through the so called "grooming invitation dance", whereby bees shake the whole body from side-to-side producing specific vibrations which increase the probability of being groomed by a nestmate (Land and Seeley, 2004). Moreover, social grooming might also be performed on specific age-class individuals rather than on parasitized individuals, and thus recognition tools might be the ones already developed in normal workers. Alternatively, we should also recognize that chemosensory abilities do not only depends on the expression of olfactory proteins in the antennae, as the olfactive perception process is a long way from the binding of odorant molecules at the level of chemosensillar lymph to the integration in the central nervous system. The absence of specialization at the antennal proteome level is thus not, per se, a definitive proof for the absence of chemosensory specialization. Future studies are needed to assess the perceptive abilities of social groomers at different levels and using different techniques (proboscis extension reflex, electroantennography). Moreover, the overexpressed antennal proteins found in groomers (4 exclusive and 8 more expressed) have very diverse functions which are not directly involved in immunity and suggest a link with other unpredicted physiological conditions. The picture is clearer in bees selected for hygienic behaviour (Guarna et al., 2015) and *Varroa* sensitive hygiene (Hu et al., 2016) for which 7 and 78 proteins, respectively, were more expressed in antennae and were considered by authors as protein biomarkers for these specific selective breeding. While antennal proteome is not peculiar in social groomers, a clear and striking difference in immunocompetence has been found between social groomers and same age non-social groomers, with the former being more able to clear bacterial cells from their hemolymph. Interestingly, the magnitude of the difference in immunocompetence between social groomers and same age non-grooming bees is large respect to what previously found when comparing nurse bees to foragers (Cappa et al submitted). Among honeybee workers, social interactions appear to increase towards nestmates showing signs of potential infections (Richard et al. 2008; 2012). Grooming parasitized or sick nestmates to free them from parasites, debris, spores or other infective agents might increase the risk to be infected, therefore, the strong physiological specialization in individual immunity showed by social groomers in our immune assays, might contribute in carrying out their hygienic task inside the hive. Our study did not clarify whether the higher immune ability of groomers is part of their specialization toolkit (as it is the case for other physiological specialization in bees, such as increased JH level in guard bees, Huang et al., 1994; Pearce et al., 2001) or rather a consequence of the increased exposure to pathogens due to their increased network centrality. Future studies should address this issue, possibly by directly manipulating individual exposure to pathogens and through non-lethal sampling of hemolymph to follow ontogenetic development of immune ability in groomers and non-groomers. The implications of our study are twofold. First, we provide evidence to solve the controversy about the degree of specialization of social grooming in *Apis mellifera* which appears as a weak and transient

behavioural specialization, characterized by a marked physiological specialization, i.e. increased immunocompetence. Since the expression rate of the behavior appears to be limited also at the colony level in terms of individuals performing the behaviour respect the total number of individuals, we can summarize that social grooming is expressed in *Apis mellifera* but not as much it is necessary to effectively contrast the parasite pressure of *Varroa destructor*. Despite this evidence might be discouraging, strategies to increase the overall colony rate of social grooming in order to cope with this very impacting pest might still be considered as reasonable. The second insight from our results is that the physiological specialization of social groomers is specifically related to immune phenotype. Our results indicate an enhanced immune response in social groomers respect to the same age non-groomers nestmates. Is necessary to take in account that from our study is not possible to discern whether the higher immunocompetence found in social groomers is characteristic of this weak and transient specialization or if it is a consequence of the higher rate of encounters with pathogens and parasites due to the task (immune general enhancement due to a previous contact with immune stimulating factors). Overall, our results demonstrate the presence of an immune-specific physiological and social behaviour specialization in individuals involved in social immunity related tasks, thus linking individual to social immunity. It also suggests that division of labour might lead to physiological specialization narrowly tailored upon the task performed while maintaining an overall plasticity.

Authors' contributions

R.T., S.T. and A.C conceived the study; A.B., R.C. and A.C. designed behavioural experiments, A.B. and A.C. collected behavioural data; F.C. designed and performed the immunological assay; F.R.D. and I.I. designed and performed the proteomic experiment. A.C., A.B., F.C., I.P., F.R.D. and I.I. carried out the data analyses and drafted the manuscript; all authors critically revised the paper and agree to be held accountable for the content therein.

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SUPPLEMENTARY METODS AND RESULTS

Detailed protocol for antennal proteome characterization

Reagents

Ammonium bicarbonate, DTT, iodoacetamide, sodium chloride, formic acid, acetonitrile, trifluoroacetic acid, acetic acid, thiourea were from Sigma-Aldrich (Milano, Italy), while Tris and urea from Euroclone. Trypsin was purchased from Promega (Sequencing Grade Modified Trypsin) and Lys-C from Thermo Scientific (MS grade). The hand-made desalting/purification STAGE column were prepared using three C18 Empore Extraction Disks (3M).

Protein Sample Preparation and Digestion

Antennae were dissected and pooled immediately before protein extractions from pool of 5 individuals. Five biological replicates for each sample were prepared. The extracts were prepared crushing the tissue in a mortar under liquid nitrogen and the proteins extracted with 6M Urea/2M Thiourea in Tris-Cl 50mM pH 7.4. The protein extracts were centrifuged at 14.000 rpm for 40 minutes at 4°C and the supernatants were collected for the analysis. The total amount of protein in each sample was assessed by the Bradford colorimetric assay, with the "Bio-Rad Protein Assay" kit using serial dilutions of bovine serum albumin to generate a standard curve. Protein sample concentration was measured by Infinite PRO 200 reader (TECAN).

Protein digestion was carried out on 15 μg protein extracts. Reduction of disulfide bridges was performed by treating samples with DTT (1 μg of DTT/50 μg of proteins for 30 min at RT), followed by alkylation (5 μg of iodoacetamide/ 50 μg of proteins for 20 min at RT in the dark), as described by Foster and co-workers (Foster et al., 2003). Protein samples were diluted 3 times with 500 mM ammonium bicarbonate, to increase pH and reduce the concentration of urea/thiourea. A first enzimatic digestion was performed by incubating the samples with LysC in a ratio 1:50 (w/w) for 3 hours at 37°C. The digestion products were then incubated with trypsin in a ratio 1:50 (w/w) overnight at 37°C. The digested samples were then acidified by adding trifluoracetic acid and desalted on STop And Go Extraction (STAGE) tips (Rappsilber et al., 2007). The eluates were concentrated and reconstituted to 20 μL in 0.5% acetic acid, prior to HPLC-MS analyses.

Mass Spectrometric Analysis

The peptide mixture of each sample was submitted to a nanoLC-nanoESI-MS/MS analysis on an Ultimate 3000 HPLC (Dionex, San Donato Milanese, Milano, Italy) coupled to a LTQ-Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany). Fractions containing 2.25 μg protein were injected directly on a homemade nano column packed with Aeris Peptide XB-C18 phase (75 μm i.d. × 15 cm, 3.6 μm, 100Å, Phenomenex, Torrance, CA, USA) and eluted with a flow rate of 300 nl/min. The elution mobile phases composition was: H2O 0.1% formic acid/CH3CN 97/3 (phase A) and CH3CN 0.1% formic acid/ H2O 80/20 (phase B). The elution program was: 0 minute, 2% B; 40 minutes, 2% B; 68 minutes, 15% B; 168 minutes, 25% B; 228 minutes, 35% B; 273 minutes, 50% B; 274 minutes, 90%B; 288 minutes, 90% B; 289 minutes, 2% B; 309 minutes, 2 % B. Mass spectra were acquired in positive ion mode, setting the spray voltage at 1.8 kV, the capillary voltage and temperature respectively at 45 V and 200°C, and the tube lens at 130 V. Data were acquired in data dependent mode with dynamic exclusion enabled (repeat count 2, repeat duration 15 seconds, exclusion duration 30 seconds); survey MS scans were recorded in the Orbitrap analyzer in the mass range 300-2000 m/z at a 15,000 nominal resolution at m/z = 400; then up to five most intense ions in each full MS scan were fragmented (isolation width 3 m/z, normalized collision energy 30) and analyzed in the IT analyzer. Monocharged ions did not trigger MS/MS experiments.

Data processing

The identification of proteins was performed using MaxQuant software (version 1.5.2.6) (Cox and Mann, 2008). The derived peak list was searched with Andromeda search engine (Cox et al., 2011). We used as database all the proteins of Apis mellifera from Uniprot merged with a set of commonly observed contaminants, such as human keratins, bovine serum proteins, and proteases. Data were also searched against databases from common honeybee pathogens and parasites (*Nosema*, *Paenibacillus larvae*, *Varroa*, *Melissococcus*, *Ascosphaera*) downloaded from Uniprot. In parameter section, we set as enzyme Trypsin and Lys-C, allowing up to two missed cleavages. The minimum required peptide length was seven amino acids. Carbamido-methylation of cysteine and oxidation of methionine were set as variable modifications. As no labeling was performed, multiplicity was set to 1. During the main search, parent masses were allowed an initial mass deviation of 4.5 ppm and fragment ions were allowed a mass deviation of 0.5 Da. PSM (Peptide Spectrum Match) and protein identifications were filtered using a target-decoy approach at a false discovery rate (FDR) of 1%.

Relative, label-free quantification (LFQ) of proteins was done using the MaxLFQ algorithm integrated into MaxQuant for samples from tarsi and basal part of forelegs. The match between runs option was enabled with a match time window of 4 min and an alignment time window of 20 min. For protein quantification we used 1 as Minimum ratio count, "Unique+Razor" peptides (i.e. those exclusively shared by the proteins of the same group), peptides with variable modifications, and selected "discard unmodified counterpart peptide".

Annotations regarding gene onthology (GO) categories, Protein family (Pfam) and Interpro were downloaded from the link (http://141.61.102.106:8080/share.cgi?ssid=0q4b6sT) available in Perseus software (version 1.5.1.6) and each protein identifier was associated with these categories if available. The data were filtered to eliminate hits to the reverse database, contaminants and proteins only identified with modified peptides.

Detailed protocol for bacterial clearance assays

Bacterial culture and artificial infection

Bacterial cultures of *E. coli* tetracycline-resistant strain XL1 Blue (Stratagene, La Jolla, CA, USA) were grown aerobically in Luria-Bertani (LB) complex medium (Miller, 1972) containing tetracycline at a concentration of 10 µg/mL overnight at 37 °C in a shaking incubator. After centrifugation, bacteria were washed twice in phosphate-buffered saline (PBS), resuspended and diluted to the desired concentration with PBS (~1.5 x 10^8 cells/ml). The approximate amount of bacterial cells in the solution was determined using a haemocytometer (Neubar) and confirmed by plating the bacterial solution on LB agar (dilutions 10^{-6}, 10^{-7}) and counting the colony forming units (CFUs) that grew overnight at 37 °C. Each bee was infected by injecting 1 µL of inoculum, containing ~1.5 x 10^5 cells, with a Hamilton™ micro syringe between the 2nd and the 3rd tergite. Before injection, workers were cooled down in a refrigerator (T 8°C) to facilitate their manipulation. After infection, bees were introduced in groups of about 10-20, separated for category, into plastic cylindrical containers (Ø 10 cm x h 10 cm) provided with *ad libitum* honey inside pearced Petri dishes (Ø 2,5 cm) as food. Bees were then maintained under controlled conditions (~ 30°C; 55% RH) for 24 hours until subsequent homogenization and plating.

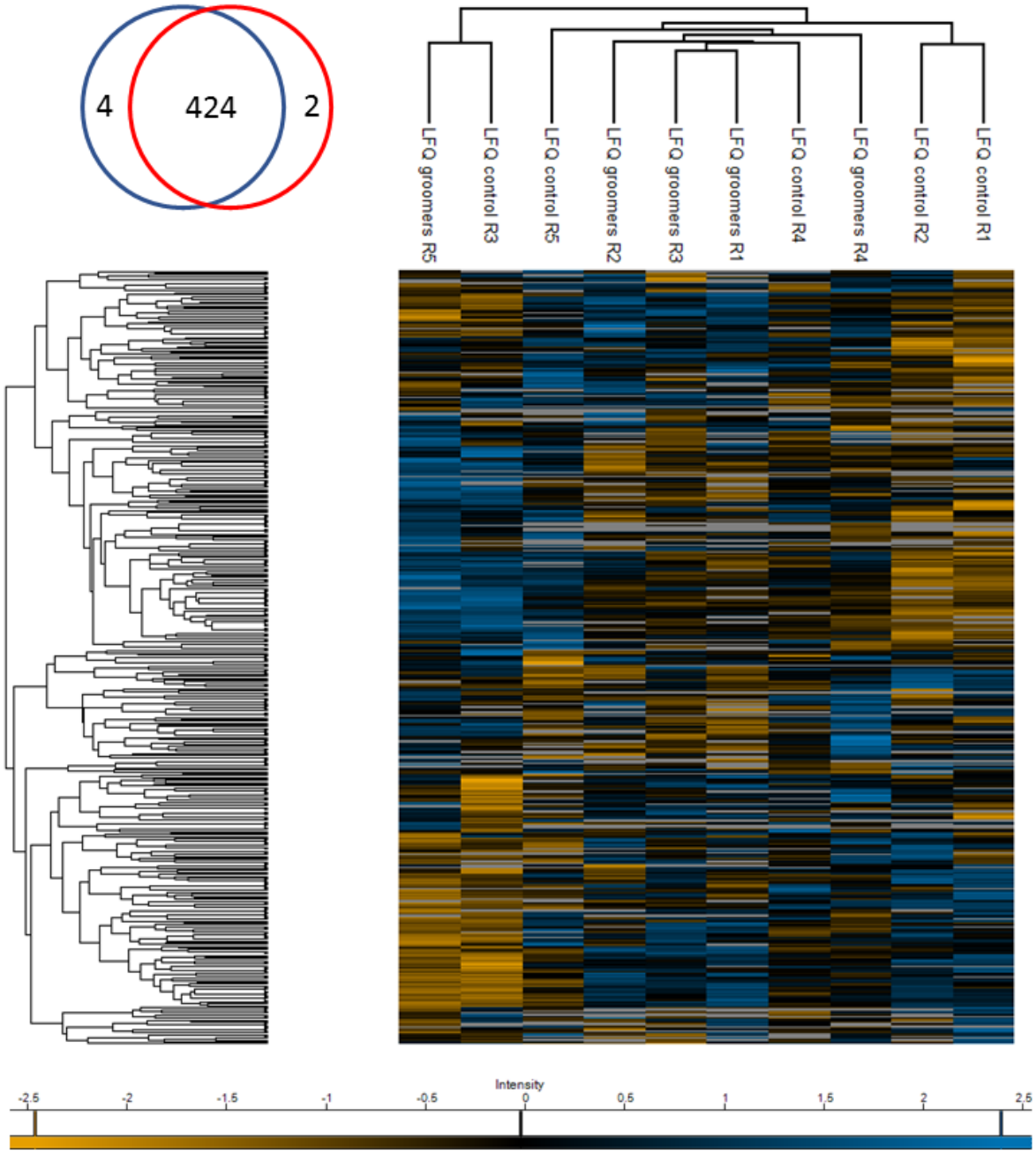

Figure S1. Heatmap representation of the expression of single protein levels between groomers and control bees. The map has been built making an unsupervised hierarchical clustering (300 clusters, maximum 10 iterations) based on LFQ (Label-free quantification) values of proteins with at least 3 observations (out of 10). Colour scale reports Z-score $\log_2$ transformed LFQ intensity values. Missing data are reported in grey. No differences have been highlighted between the two groups as displayed in the cluster grouping biological replicates. In the top left a Venn diagram is reported, drawn considering "Unique+Razor" peptides (i.e. those exclusively shared by the proteins of the same group) identified in at least 3 of the 5 replicates of one group. Groomers are represented with a blue circle, while control bees with a red circle.

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Chapter 6

Home economics in an oak gall. Behavioural and chemical strategies against a fungal pathogen in *Temnothorax* ant nests.

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Home economics in an oak gall. Behavioural and chemical immune strategies against a fungal pathogen in *Temnothorax* ant nests.

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ABSTRACT

Nest architecture contributes to immune strategies of social insects by shaping and determining their efficacy. The arboreal ant *Temnothorax unifasciatus*, nests in small cavities, such as oak galls, where the entire colony lives in a unique small chamber. Compartmentalization should play a marginal role in colonial immunity and physiological and behavioural strategies likely prevail and are presumably linked with colony size. We designed two experiments to study chemical and behavioural immune strategies against the entomopathogenic fungus *Metarhizium anisopliae* in colonies of varying sizes. In the first, we compared spore germination and length of germinal tubes inside artificial nests that hosted colonies of different size and inside empty nests. In the absence of direct contact between ants and the fungus, *T. unifasciatus* colonies inhibited fungal growth inside their nests, presumably through volatile compounds. Colony size was positively correlated with this fungistatic activity indicating a similar per capita effort in producing such substance by workers of small and large colonies. In the second experiment, we focused on the removal behaviour of contaminated and un-contaminated items introduced inside the nests of colonies of different size. Small colonies presented with contaminated fibres showed an increased removal of all the items (both contaminated and non-contaminated) compared to small colonies presented with control fibres only. Conversely, larger colonies removed items regardless of the presence of the spores inside the nest. We conclude that colony size qualitatively affects removal of waste items, resulting in responses in small colonies that optimize the reduced workforce, while removal behaviour in larger colonies reveals to be expressed constitutively.

Key words. *Temnothorax unifasciatus*, waste removal, *Metarhizium anisopliae*, alternative strategies, colony size, antimycotic

1. INTRODUCTION

Social insect colonies are faced with several consequences of sociality. Crowded nests, high rates of social interactions, and high relatedness among individuals increase the risk that pathogens spread among colony members (Schmid-Hempel, 1998). To cope with these risks, social insects show a large array of behavioural, physiological and organizational strategies, collectively indicated as social immunity (Cremer *et al.*, 2007). Nest architecture together with the structure of the interaction network and division of labour, constitute the organizational component of the social immunity (Baracchi and Cini, 2014; Stroeymeyt et al., 2014; Tranter and Hughes, 2016). A wide range of social and spatial compartmentalization strategies have evolved, strongly affecting infection dynamics inside the colony (Quevillon et al., 2015). Many ants build their colonies with complex structures. Nests of the leafcutter ants, *Atta* sp., contain several inner areas of spatial and social compartmentalization, serving to protect the core of the colony represented by the chambers containing the fungus they rear (Little et al., 2006). Many ant species show a much simpler nest architecture. In extreme cases, this is represented by a single chamber of a few cubic centimetres hosting all colony members. In these conditions, compartmentalization is expected to play an inconsequential role in colonial immunity, and physiological and behavioural strategies have evolved to compensate for it.

Colony size has a positive relationship with general colony efficiency (Bourke et al., 1995; Colin et al., 2017; Hölldobler B and Wilson EO, 1990; Luque et al., 2013). Social insects often employ self-organization algorithms in collective behaviours requiring communication, task allocation and consensus building. Models predict that an efficient actuation of these algorithms can be strongly favoured in large groups (Anderson and Ratnieks, 1999; Gautrais et al., 2002). Moreover, colonies of different size often use alternative strategies to solve the same problems (Dornhaus and Franks, 2006) also in response to infection risk (Leclerc and Detrain, 2018). Individuals can qualitatively change their behaviour depending on colony size (Jeanne and Bouwma, 2002; Leclerc and Detrain, 2018). Small colonies are expected to be more risk-averse and more likely to adopt less probabilistic behaviours to improve efficiency of individuals compared to large colonies (Herbers, 1981). Compared with solitary species, social insects heavily invest in antimicrobial compounds to reduce microbial growth (Hoggard et al., 2011; Stow et al., 2007; Wang et al., 2015), and many ant species have evolved glands specialized for this task (Yek & Mueller 2011). Large colonies are expected to rely in antimicrobial production, due to a low cost per individual, while smaller colonies could invest more resources on behavioural responses, due to the high per-capita costs of producing sufficient quantities of antimicrobials (Karlik et al., 2016).

We used *Temnothorax unifasciatus* as a model to investigate the integration between physiological and behavioural strategies adopted in coping with parasites in an ant species with extremely reduced compartmentalization. These ants nest among rocks and under stones and they also frequently occur

under bark and in hollow plant stems (Czechowski et al., 2002) and in other wooden tree structures like cynipid oak galls (Espadaler and Nieves-Aldrey, 1983; Mackay, 2000). In these structures, the colonies are usually established in a single cavity where all the individuals live in close contact. In these conditions, strategies of compartmentalization and worker relocation to peripheral tasks as quarantine methods are presumably unaffordable (Heinze and Walter, 2010). Instead, *Temnothorax unifasciatus* exibits several behavioural strategies to cope with the spread of infections among nestmates of the obligate-killer pathogen *Metarhizium anisopliae*. These include social withdrawal of dying workers (Heinze and Walter, 2010), the removal of infected corpses and items (Colin et al., 2017) and brood reallocation in nests exposed to pathogens (Karlik et al., 2016). Some of these strategies have also been shown to be dependent on colony size (Colin et al., 2017).

In this study we verified whether *Temnothorax unifasciatus* displays behavioural and biochemical strategies against the entomopathogenic fungus *Metarhizium anisopliae*. Furthermore, we evaluated the effect of colony size in the expression of distinct strategies and in determining the different the effort invested by the work force.

2. MATERIAL AND METHODS

Study species and laboratory conditions

In spring 2017 and 2018 we collected colonies of *Themonthorax unifasciatus* in oak woods. All colonies were found in galls generated by oak cynipids of the genus *Andricus spp.* (mainly *A. quercustozae*). Once in the lab, galls were opened, and colonies relocated to artificial nests composed of two rounded plastic containers organized in an upper colony chamber (2cm Ø) and a lower peripheral one (5cm Ø), containing *ad libitum* water, sugar, and proteins (finely chopped dry dog food). The two chambers were connected by a hole (2-3mm Ø). All colonies contained the queen, the brood, and a number of workers ranging from about ten to a maximum of approximately two hundreds. Colonies were kept in a thermostatic room (20–25°C temperature, 80% humidity).

Waste management experiment

Colonies used for this experiment spent at least three days in laboratory conditions before the beginning of the experiment. To study the management of pathogen-contaminated material we introduced differently treated nylon fibers (2mm length, 0.3mm Ø) in the upper colony chamber. As a pathogen we used commercial spores (Met52© Monsanto) of the entomopathogenic fungus *Metarhizium anisopliae.* The spores had been re-freshed on ants, then collected, suspended in a sterile Triton solution (0.01% in distilled water) and plated on Mat Extract Agar (Oxoid) to count the number of Colony-Forming Units (CFUs) and determine the spore viable titre (CFU/ml). A final spore suspension in a concentration of

10^8 CFU/ml was then used. To distinguish treatments during the observations we used fibres with different colours (blue, red, black, brown and grey). Once sterilized, experimental nylon fibres were subjected to two treatments in groups of 200, i) immersion in 1ml of the *Metarhizium anisopliae* spore suspension (contaminated fibres), ii) immersion in 1ml of the water and Triton solution (0.01%) (control fibres). Contaminated and control fibres were dried by evaporation for 48 hours. To discern if ants managed differently exposed, control fibres and control fibres co-occurring in the nest with exposed ones, we introduced 20 fibres to upper colony chambers in two different combinations i) 10 contaminated fibres of one colour and 10 control fibres of another colour or ii) 10 control fibres of one colour and 10 control fibres of another colour. This resulted in three kinds of fibres involved in different experiments: i) contaminated fibres (m fibres), ii) control fibres co-occurring with contaminated fibres (cm fibres), iii) control fibres only (cc fibres).

A total number of 88 *Temnothorax unifasciatus* colonies were tested in three different replicates (30, 24 and 32 colonies, respectively). Fibres of each colour were allocated to distinct fibre treatments in different replicates. In replicates 2 and 3 (54 colonies in total) we also recorded the dimension of the colonies ($n<50$ workers, small; $50<n<100$ medium; $n>100$ large). Using colours allowed us to check the number of fibres belonging to distinct treatments moved from the upper colony chamber to peripheral ones after 1, 2, 4, 6 and 24 hours.

Fungal growth inhibition experiment

To test whether *Temnothorax unifasciatus* affects microbiological growth in the nest environment, we designed specific artificial nests. These nests allowed the growth of *Metarhizium anisopliae* in the nest environment without any physical contact with ants. Artificial nests were composed of a single plastic container (2cm Ø), divided in an upper and a lower chamber (0.5cm and 1.8cm height, respectively) by a nylon net (300µm mesh). All next components were sterilized with alcohol and UV light (30min exposure).

Colonies used for this experiment spent one day of adaptation in these experimental structures before the beginning of the treatment. The lower chamber hosted either the whole ant colony (experimental group), or nothing (control group), while in the upper chamber we placed fungal spores. A thin layer of 10 µl of Maltose Extract Agar was applied on a glass cover slide where the spores (1µl of suspension of 4x10^8 CFU/ml of *M. anisopliae* spores in 0.01% Triton solution) were plated. The slide was flipped and fixed with Parafilm® on the roof of the upper chamber where a hole of 1 cm of diameter was previously made (see Fig. 3a for nest structure). Spores were let to grow in experimental and control nests for 24 hours in controlled conditions (20–25°C temperature, 90% humidity). After 24 hours, we collected the slides and took two photographs of the central area of the structure by a camera connected to a microscope (600x magnification). We replicated the experiment twice, using 20 colonies of ants and 20 controls in each occasion.

Two measurements were taken by an experimenter unaware of the experimental group of the images. First, germinated spores and non-germinated spores were counted in each picture. Spores which could not be attributedto either group were excluded from the count (e.g. in areas out of focus, in the presence of optical artefacts or in the occurrence of clustered spores). The germination ratio between number of germinated and total spores was then calculated. Second, to evaluate the length of the germinal tubes, a maximum of 10 randomly chosen germinated spores were examined from each picture. By using ImageJ the length in pixels was measured by using a segmented line (usually 1-6 segments). For each photo, the average length between measured spores was calculated. The dimension of each colony was scored as per the the waste management experiment above (small=1, medium=2, large=3), while we attributed size equal to zero (empty control nests). The experiment was then replicated on further 40 artificial nests and pictures were taken after 48h.

Statistical analyses

The count variable of moved fibres showed a negative binomial distribution. For this reason, we applied the "glmmadmb" function in the "glmmADMB" package to fit a Generalized Linear Mixed Model (GLMM). As predictor variables we used i) treatment of the fibres (m, cm, cc); ii) time of observation (1-2-4-6-24h); iii) colour of the fibres; iv) size of the colony as an ordered variable among small, medium and large, and iv) the interaction between time, treatment and colony size. Colony membership was used as a random factor nested within replicates. We used type III sum of squares as returned by the "Anova" function of the "car" R package. Post hoc tests among main effects, estimated marginal means and their standard errors were calculated with the "emmeans" function of the "emmeans" package. Since the size variable was only scored on a subsample of the colonies, we first performed a GLMM with all the colonies to test the effect of colour, fibres treatment and time. Then, we ran a second GLMM on the subset of colonies for which we knew the size to test the effect of colony size and its interactions with the other predictors. The germination ratio and the average length of gemination tubes were normally distributed and we included them as a response variables in two GLMM ("GlmmPQL" function of the MASS library) using size as an ordered predictor (which also included the zero value for controls) and replicate as a random factor. Scripts and original data are available in Appendix S1.

3. RESULTS

Waste management experiment

The mixed linear model comparing the removal of different kinds of fibres in the total sample of 88 colonies, revealed that treatment, time and colour had significant effects, while there was no interaction between treatment and time (Table 1). Post hoc tests revealed a significant difference between m and cc fibres (estimate, -1.757; S.E., 0.337; z ratio, -5.211; $P < 0.001$) and between cm and cc fibres (estimate, -1.631; S.E., 0.336; z ratio, -4.852; $P < 0.001$) but not between m and cm fibres (estimate, 0.127; S.E.,

0.334; z ratio, 0.380; P =0.924) (Fig. 1a). Among colours, the overall significant effect was due to a difference between blue and brown fibres (estimate, 1.426; S.E., 0.153; z ratio, 0.9.286; P <0.001) (Fig. 1b) while the other comparisons did not show significant effects. In the subset of 54 colonies for which size was assessed, GLMM also returned a significant effect for colony size and for the interaction between size and treatment. Estimated marginal means of moved fibres belonging to different treatments and to colonies of different sizes (Fig. 2), revealed that the significant interaction between size and treatment was produced by small colonies differentially moving cm and m fibres more than cc fibres, while medium and large colonies expressed a similar effort in moving the three kinds of fibres (Fig. 2).The significant interaction between treatment and size showing that small colonies differentially remove fibers when *M. anisopliae* spores occur in the nest compared to medium and large colonies complicates the interpretation of the significant effects for size and treatment. However, in figure 2 it can be detected that larger colonies actually remove a higher fraction of fibers as suggested by the significant effect of the size variable. A post hoc test among the three ordered classes of size revealed that large colonies significantly moved more fibres than small colonies (estimate, -0.969; S.E., 0.302; z ratio, -3.212; P =0.004), while no significant differences were found between small and medium (estimate, -0.357; S.E., 0.351; z ratio, -1.014; P =0.568) and between medium and large colonies (estimate, -0.612; S.E., 0.348; z ratio, -1.760; P =0.183).

	Variable	Df	Chisq	P
Subset 54 colonies	Colour	3	336.507	<0.001
	Time	1	3.825	0.050
	Treatment	2	8.127	0.017
	Size	2	11.947	0.003
	Time*Treatment	2	5.406	0.067
	Time*Size	2	1.677	0.432
	Treatment*Size	4	13.141	0.011
	Treatment*Size*Time	4	3.716	0.446
Total dataset 88 colonies	Colour	4	4010.511	<0.001
	Time	1	5.210	0.022
	Treatment	2	34.931	<0.001
	Time*Treatment	2	0.011	0.995

Table 1. ANOVA table (tipe III sum of squares) for a generalized linear mixed model comparing the number of moved fibers against fiber colour (Colour), time of check (time), treatment (cc, cm, m) for the total dataset of 88 colonies (below) and for the subset of 54 colonies for which size has been assessed (above).

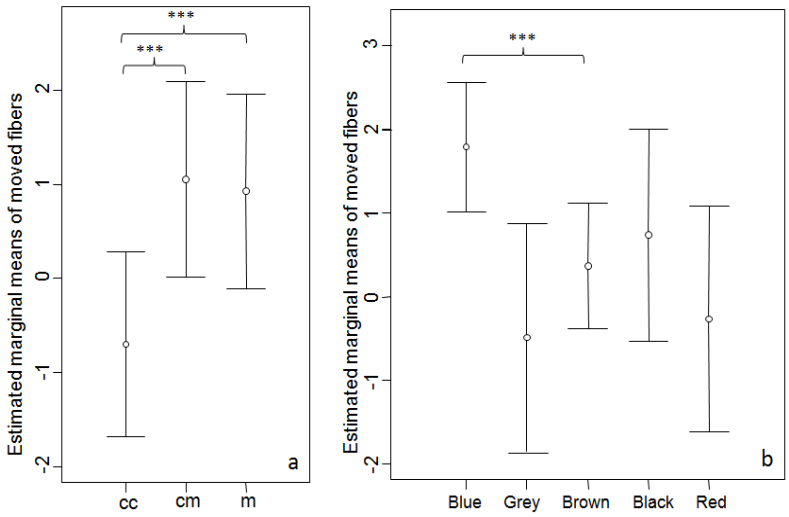

Figure 1. Estimated marginal means after a GLMM of moved fibers when belonging to different treatments (a) and to different colours (b).

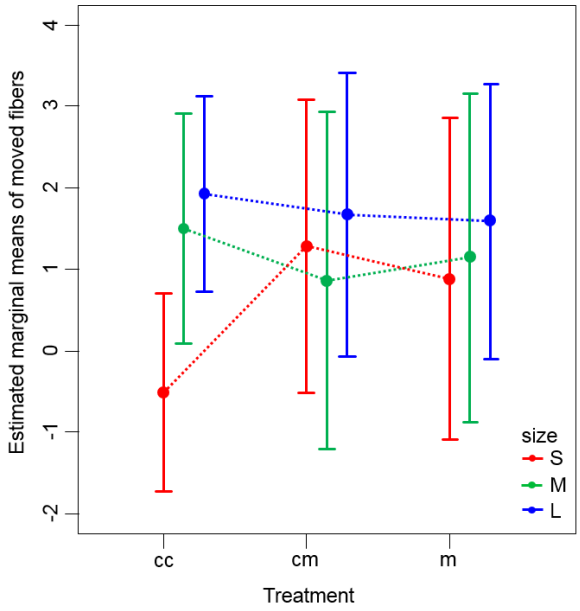

Figure 2. Estimated marginal means after a GLMM with standard error for moved fibers belonging to different treatments and moved by colonies of different size (S, small, red line; M, medium, green line; L, large, blue line).

Fungal growth inhibition experiment

The general mixed models comparing the germination ratio and average length of germination tubes revealed that colony size significantly affected both germination rate (Chisq, 16.477; Df 3; P<0.001) and average length of germination tubes (Chisq, 56.949; Df 3; P<0.001). Post hoc tests revealed that tubes were longer in empty nests than in all ant nests (control vs small, Estimate, 2.035; S.E., 0.740; df, 72; t-ratio, 3.117, P=0.014; control vs medium, Estimate, 3.158; S.E., 0.640; df, 72; t-ratio, 4.935,

P<0.001; control vs large, Estimate, 3.893; S.E., 0.608; df, 72; t-ratio, 6.402, P<0.001), while no significant differences emerged among ant nests (Fig. 3c). Post hoc tests for germinating ratios revealed differing patterns. Small colonies did not differ when compared to empty nests (Estimate, 0.129; S.E., 0.065; df, 72; t-ratio, 1.996, P=0.199), while medium colonies showed a trend (Estimate, 0.142; S.E., 0.056; df, 72; t-ratio, 2.540, P=0.062) and large colonies showed a significant difference (Estimate, 0.181; S.E., 0.053; df, 72; t-ratio, 3.416, P=0.005)(Fig 3b). Fungal growth advanced very rapidly after 24h, and examination of germination and tube length was impossible after 48h in both control and experimental nests.

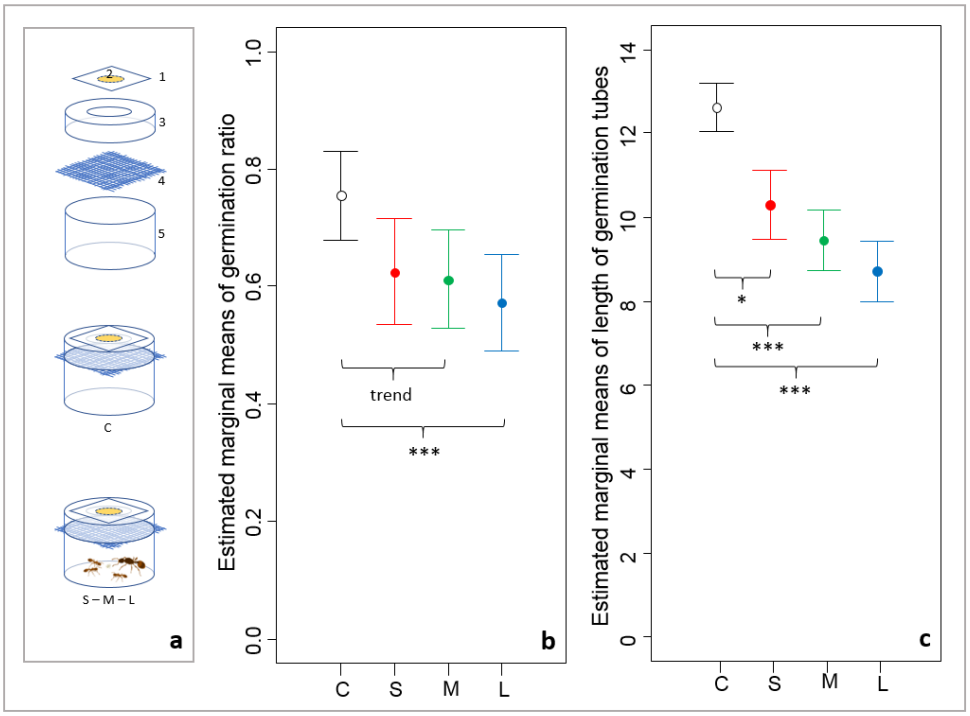

Figure 3. The experimental nests used for estimation of reduction of fungal growth (a); 1, glass cover slide; 2 agar solution with *M. anisopliae* spores; 3 upper chamber; 4 nylon net; 5, lower colony chamber; C, control colony; S-M-L, small, medium and large experimental colonies. Estimated marginal means after a GLMM of germination ratio of spores (a) and of length of germination tubes (b) in empty nests (C) and in small (S), medium (M) and large (L) colonies.

4. DISCUSSION

In our experiments, *Temnothorax unifasciatus* exhibited different immune strategies in dealing with the occurrence of entomopathogenic *M. anisopliae* spores inside nests. The growth of this fungus was inhibited in ant colonies in terms of both germination ratios and length of germinal tubes, and as predicted, such inhibition was stronger in large colonies when compared to small ones. In parallel, only small colonies tested with contaminated fibres showed an increased removal of all fibre types (both contaminated and non-contaminated) compared to colonies presented with control fibres. Interestingly,

a differential removal of fibres of different colours was also observed, possibly, due to differential detectability of blue fibres by the ants. This could occur as a result of varying UV reflection of the blue fibres compared to natural objects. Leaves and soil reflect light dominated by green and yellow light, and typically lack an intense UV light component (Menzel, 1979). This did not significantly impact the results of the experiments as blue fibres were attributed to both control and exposed groups in different replicates.

Inhibition of microorganism growth has been described in several social insects, consisting mainly of application of antimicrobial substances on the surfaces of nests. In many cases, these substances form part of the nest structure. This can take the form of resins collected in the environment (exhibited by many ant (Brühl, 2003; Chapuisat et al., 2007; Christe et al., 2003) and bee species (Ghisalberti, 1979; Simone et al., 2009; Velikova et al., 2000)), chemical compounds such asnaphthalene (Chen et al., 1998) and faeces, as used by some termites (Rosengaus et al., 1998) . Antimicrobial substances are also produced by exocrine glands, and, in ants the metapleural gland plays a fundamental role in their production (Schluns and Crozier, 2009). Alternatively, in the fire ant *Solenopsis invicta*, venom alkaloids have been shown to produce antimycotic activity against the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* (Storey et al., 1991). Sting secretion of the common acrobat ant *Crematogaster scutellaris* also has antimicrobial activity against Gram-positive and Gram-negative bacteria and the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Perito et al., 2018). The volatility of the secretion is also of importance. Whereas non-volatile compounds work passively and require direct contact with the pathogen to be effective, the fumigation of the entire nest with more volatile compounds can largely increase the effectiveness of target substances (Chen et al., 1998). For example, Storey et al (1991) hypothesized that fire ants can diffuse venom alkaloids as an aerosol, which was later demonstrates by Wang and collaborators (2015) where they showed that fire ants inhibited the germination and the growth of the fungus *B. bassiana* by fumigating their nest. This study represented the first evidence for the use of antimicrobial volatiles in ants; although the experiment involved attests of constant number of ants, thus precluding measurements of the potential effects of colony size. In addition, ants were removed from their nests during the experiment and this could have altered their responses. We carried out experiments on whole colonies whit a period of habituation in artificial nests, reducing the effects of stress on the experiment. Interestingly, the fungistatic activity of venom alkaloids of fire ant is temporary in *B. bassiana* and *M. anisopliae*, and the inhibition of germination rate and tube growth is only significant in the first 24 hours (Storey et al., 1991). We also observed that the efficacy of nest fumigation by *Temnothorax unifasciatus* against *Metarhizium anisopliae* is limited to the first 24 hours. Since our experimental nests weren't closed, we can conclude that reduced fungal growth not was due to high concentrations of carbon dioxide and low concentration of oxygen, because the effect was no longer detectable after 48 hours (supplementary materials). The peculiar simplified natural nest of *Temnothorax*

ants, with their unique chamber that hosts the whole colony, is therefore likely to offer a suitable environment to maximise the effectiveness of fumigation with antimicrobial volatiles.

In our experiments colony size showed a positive correlation with fungal growth inhibition, and we found no evidence suggesting increased per-capita production of antimycotic substances in small colonies. This observation is in line proposals by Karlik and collaborators (2016) that smaller colonies should be unable to sustain the increased per-capita cost of producing a sufficient quantity of antimicrobials and that they should rely more on behavioural responses in immune defence.

Future studies are necessary to understand which substances are involved in the fungistatic effect. It is also unknown whether the immune defence is expressed continuously or elicited by the occurrence of the pathogen inside the nest.

Removal and disposal of waste material is a virtually ubiquitous behaviour in ants, in particular when potentially contaminated items occur in the nest (Diez et al., 2012; Hölldobler B and Wilson EO, 1990; Leclerc and Detrain, 2018). Removal of contaminated items is so essential that ants have evolved different behavioural strategies to optimize this task. Many of these have been found to depend on colony size. Large colonies of *Myrmica rubra* react faster to items contaminated with *Metarhizium brunneum* and are more efficient in active removal from the nest in comparison to smaller colonies (Leclerc and Detrain, 2018). On the other hand, a consistent fraction of small colonies display the alternative strategy of evacuating the entire nest, sanitizing it and reintegrating the colony (Leclerc and Detrain, 2018). Removal of wasted material is expected to be a particularly urgent task in small *Temnothorax* nests. Accordingly, we found that medium and large colonies removed infected and uninfected items with the same efficiency while workers of small colonies removed fewer fibres from the nest when contaminated fibres did not occur. This alternative strategy suggests that smaller colonies need to optimize their reduced workforce with respect to medium and larger colonies, resulting in non-contaminated items being disregardes in the absence of infection risk. Optimization of workforce has been found to occur in *Temnothorax* ants. Dornhaus and collaborators (2008) observed that, during colony emigration, small colonies of *Temnothorax albipennis* dedicated a small fraction of workers to item transportation. This restricted workforce transported a higher number of items pro-capita and showed a higher overall efficiency in transport (more items carried per-capita/time). The manner in which individualsin colonies of differing sizes can exhibit varying behavioural strategies is still unclear and largely depends on the context. Dornhaus and Franks (2006) provided three possible mechanisms; i) self-organisation may result in different collective patterns if fewer nestmates participate in them, like in the establishment and maintenance of pheromone trails; ii) individuals could assess the size of their colony and adjust their behaviour accordingly, as it occurs in the decrease of per-capita defensive effort with the increase of colony size in *Polybia occidentalis* (London and Jeanne, 2003); iii) correlation between a trait and the size of the colony may not be due to direct causation, being possible, but rather that colony size is correlated with other factors. Further studies will help to elucidate the mechanisms involved.

Due to their peculiar ecology, nesting habit and social structure, *Temnothorax* ants exhibit several behavioural strategies to deal with pathogens (Heinze and Walter, 2010; Karlik et al., 2016). Here we have highlighted that behavioural and chemical immune strategies are combined to contrast pathogen establishment and transmission inside the reduced dimensions of the nests. Our colonies showed a strong plasticity in optimizing behavioural strategies to remove infected items and this was dependent on the number of workers present. This plasticity is likely to have a fundamental role during colony growth and development, especially in the first phase of colony growth following foundation by queens. At this stage the colony is small, and the reduced workforce must be optimized to maintain colony health in an environment showing a high risk of infection.

Authors' contributions

S.T. and A.B. conceived the study; A.B., Z.M., L.D. and S.T. designed the experiments; all authors collected the data; A.B., Z.K, L.C. and L.D. carried out the analyses; A.B. and L.D. drafted the manuscript; all authors critically revised the paper, approved the final version of the manuscript and agree to be held accountable for the content therein.

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Supplement 1

The interspecific nesting association between the stingless bee *Partamona testacea* and the bullet ant *Paraponera clavate.*

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Dealing with the worst neighbour. The interspecific nesting association between the stingless bee *Partamona testacea* and the bullet ant *Paraponera clavata*.

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ABSTRACT

Nesting in associations with social insect species can provide mutualistic or parasitic protection against predators. Stingless bees are often found nesting association with termites, while association with ants is less common due to the costs related to the high aggressiveness of ants which normally overcome the benefits. Few combative genuses of stingless bees can associate with a larger number of hosts, including ants. Here, we describe a not yet recorded association between a stingless bee (*Partamona testacea*) and the highly aggressive predator ant, the bullet ant *Paraponera clavata*. In the study area colonies of *P. testacea* are strictly associated with the *P. clavata* and *Atta* spp ants. Sequences of COI mitochondrial gene of bees associated with bullet ant and leafcutter ants were compared and no differences emerged, thus discarding the hypothesis that the two nesting strategies represent a case of cryptic speciation and specialization on specific hosts. Bees are not armless respect to the bullet ants; when ants attempted to penetrate in the colony entrance, they have been invariably dragged inside the nest and covered by the bees with a resins-like compound. Behavioural experiments carried on in arenas (focused on ants) and on nest entrance (focused on guard bees) proved that the ants are less aggressive and stressed when an associated bee is presented, and that guard bees are less alarmed when an associated ant is presented. The differential behaviour responses expressed toward associated colony members by both species is likely based on the proximate cues provided by colonial cuticular hydrocarbon signatures. We verified the existence of colony-signatures by Gas Chromatography and Mass Spectrometry analysis.

1. INTRODUCTION

Nests of social insects and their surroundings can represent shelters for other species capable to establish peaceful relationships with them or able to elude their nest defenses. When the nesting association is composed by two species of social insects, two complex and potentially harmful societies are involved a relationship which could range from a mutualistic to a parasitic symbiosis (Wilson, 1971). Stingless bees (Meliponini, Apidae) are eusocial tropical insects establishing perennial colonies composed by up to several thousand workers and a single queen (Michener, 2000) (Sakagami, 1982). Meliponini usually nest in cavities but, since their digging abilities are generally very scarce, they usually exploit pre-existing hollows including abandoned or inhabited nests of other social insects (Roubik, 2006; Siqueira et al., 2012). Several species nest in active colonies of different social insects and these relationships can be obligate (Roubik, 1983; Wille and Michener, 1973) or facultative depending on the stingless bee species, and the relationship is in many cases species-specific (Michener, 2000). Other than an easy nesting opportunity, the association with other eusocial insects provides stingless bees with the protection benefits of nesting near aggressive colonies (Roubik, 1983). However, establish and share the nesting area with a colony belonging to a potentially aggressive species is not always an easy task. Adaptations to mitigate or elude the host nest defense are fundamental to maintain interspecific nesting relationships and these adaptations variate with the nature of the bee-host interaction (Roubik, 2006; Sakagami et al., 1989). Nesting behavior of stingless bees is extremely various, and little is known respect to the conspicuous diversity of this tribe. Depending on the stingless bee species, the relationship can be parasitic or mutualistic, but in most of the cases bees are parasites in terms of lodging and defence, since they do not show aggressive defences and the host do not obtain any evident advantage from the nesting association (Roubik, 2006; Sakagami et al., 1989). On the other hand, a few genuses like *Partamona*, *Plebeia*, *Paratrigona* and *Trigona* are aggressive, participating in the defence of the association and have been found in association with a wider variety of hosts (Roubik, 2006). Willie and Michener (1973) reported that among the studied species a 12% can nest with termites, with an 8% of obligate termite nesters, while species nesting with ants are fewer, with only a 2% involved in an obligated relationship (Sakagami et al., 1989). The preference of host taxa does not appear coherent with stingless bee phylogeny, and many sister species

(e. g. *Trigona cilipes* and *T. mazucatoi;* among the genus *Partamona*) show distinct preference toward nesting with termites or ants (Roubik, 2006). The ecological pressure of ants on the life of other organisms is normally considerable, and its magnitude appears to be even greater in tropical regions (Wilson, 1971). The lower frequency of myrmecophily respect to termitophily can be determined by the generalized higher aggressiveness of ants compared to termites (Sakagami et al., 1989). Colonies involved in regular associations with ants are separated by the outer wall of the bee nest, normally represented by a layer of batumen (Sakagami et al., 1989). Nevertheless, when the shell of bee nests is damaged ants have been reported to invariably attack the bees (all examples for genus *Trigona,* Kerr et al., 1967; Mercado, 1962; Sakagami et al., 1989). These associations seem to rely on an armed peace,and the coexistence seems to be maintained by a high tolerance of the ants toward the bees (Sakagami et al., 1989). This scenario echoes the phenomenon of parabiotic associations, where colonies of different ant species share the same nest. In these cases, ants recognize individuals of the associated colony and tolerate them. The mechanism of recognition is likely based on the chemical signature of their cuticular hydrocarbons. The recognition system of social insects is usually based on the comparison of the cuticular signature shared among nestmates with the signature occurring on encountered individuals. Since in parabiotic associations the two species maintain their species-specific chemical profile, a mechanism different from self vs non-self recognition is required. For this reason, parabiotic associations became models to understand the mechanisms of recognition in insects. Although experiments of recognition and analyses of cuticular profiles have never been carried out, at least to our knowledge, it is possible that similar phenomena can occur also between stingless bees and their associated ant colonies.

Here, we describe for the first time the nesting association between *Partamona testacea* and the bullet ant, *Paraponera clavata* (Fig. 1). *Partamona testacea* is a group of sister/cryptic stingless bee species widespread in South American rainforests (Camargo, 1980). Species of this group have been reported to establish facultative associations with termites of the genus *Syntermes* and with *Atta* leafcutter ants (Camargo and Pedro, 2003). Establish an association with the bullet ant seems to be a great challenge also for the bravest stingless bee. *Paraponera clavata* is notorious for being a generalist predator of arthropods, characterized by a highly

performant predation (Young and Hermann, 1980) besides for its pugnacious nest defense and its extremely potent sting.

We aimed to describe and unravel the mechanisms allowing the relationship between these two social insects by answering to four main questions: Q1) how strict is the association, at least in the study area? Q2) Is host choice between different ant species the result of a cryptic divergence or does it represent alternative nesting strategies of the same species? Q3) Do individuals belonging to associated colonies show a reduced aggressivity respect to individuals belonging to non-associated colonies? Q4) Are cuticular signature a reliable cue for recognition between individuals of associated colonies?

We answered these questions by integrating i) field data about occurrence of associated and non-associated colonies of ants and bee in the study area (Q1); ii) comparison of the mitochondrial COI gene ("barcoding gene") (Hebert et al., 2003) of *P. testacea* colonies nesting in *Atta* and *P. clavata* nests (Q2); iii) behavioural experiments, assessing reciprocal aggressiveness between associated and non-associated colonies; (Q3); and iv) chemical analyses of cuticular hydrocarbons to verify the existence of typical colony signatures in ants and bees (Q4)(Fig.1).

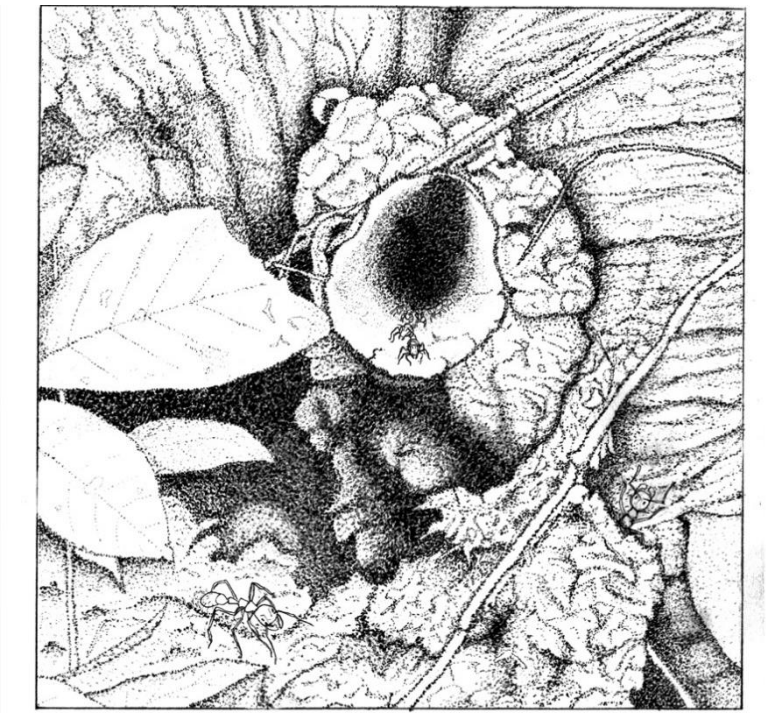

Fig 1. A representation of the association between *Partamona testacea* and *Paraponera clavata* (drawing by GM).

2. MATERIALS AND METHODS

The study area and the experimental colonies:

This study has been carried out in the lands of the Urku Estudios Amazónicos centre, located in Tarapoto (San Martin region, Perù). The study area embraced 13 hectares of tropical rainforest in the buffer zone of the Área de conservación regional Cordillera Escalera. The colonies of *Partamona testacea* and *Paraponera clavata* have been spotted during a two-week survey by three operators (AB, MM and GM). Each encountered colony has been geo-referenced and, in case of nesting association between *P. testacea* and *P. clavata*, the minimal distance between the nest entrances has been measured. The nests have been photo-documented and categorized depending on nesting association as: colonies currently involved in the association, colonies not involved in the association and colonies involved in the association in the past. In fact, the peculiar structures of *P. testacea* and *P. clavata* nests were still detectable if the nests were abandoned in the recent past.

COI characterization

Tissue samples (legs) were obtained and preserved in ethanol at 4 °C. The genomic DNA was isolated using the protocol of "Smarter Nucleic Acid Preparation" (Stratec). The DNA barcoding region of COI was amplified using the LC01490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') / HC02198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') primer pair (Folmer et al., 1994). PCR products were, then, purified using ExoSAP-IT PCR Product Cleanup Reagent (Thermofisher) and sequenced by Macrogen Inc. Chromatograms were checked with SeqTrace 0.9.0 (Stucky, 2012).

Behavioural experiments

Behavioural experiments aimed to ascertain whether associated colonies show peculiar relationships compared to non-associated (alien) colonies have been carried out. In this aim, we performed two distinct experiments focusing on the reaction of ants towards stingless bees and vice-versa.

To compare the reaction of bee colonies toward ants belonging to associated and alien colonies we carried out experiments in the field. For each trial, we collected a single ant from its colony entrance from 5 minutes to 1 hour before the experiment and kept it in a glass drug

container (4 x 2.5 cm). The ant was softly sedated by cooling it for three minutes in a field refrigerator at 4 °C. Then, the ant was placed on the inner base of the selected hive entrance using forceps and we video-recorded the reaction of bees and the interactions between the individuals until the ant left the nest entrance. The focus of the video-recording was on the guard bees. The general reaction of the guard bees was consistent and represented by a retreating, then guard bees hided in the nest entrance while the ant occurred on the structure. Once the ant left the entrance the guard bees repositioned in the nest entrance to the initial position. Coherently with this generalized response, we collected the following parameters: i) number of guard bees visible at the nest entrance before the beginning of the trial, ii) number of guard bees visible in the presence of the ant, iii) number of guard bees repositioned once the ant left the hive entrance, iv) repositioning time, corresponding to the time interval spent in repositioning by the bee guards. We performed all the possible combinations between bee and ant colonies involved in an association, following a time table organized to minimize repetitive stress on the bee colonies.

To test the reaction of ants toward bees belonging to associated or alien colonies, we designed a different experiment. Indeed, the peculiar shape of the entrance of the bullet ant colony appears as a 1-2 cm gap running around the basis of the tree trunk where this species usually nests. This made impossible to identify a localised nest entrance, and generally guard ants are not visible from outside. For this reason, experiments focusing on the ant reaction have been performed in artificial arenas represented by plastic Petri dishes (9 cm diameter). Bees and ants have been collected directly from their colony entrances and individually kept in glass containers. In each trial after a soft sedation (see above), one ant and one stingless bee were placed in the arena. We video-recorded their interactions for three minutes after the first active contact. In this experiment, we focused on ant reactions toward bees and we scored the following events in each trial: i) physical contacts between individuals, ii) mandible openings following contacts, iii) bites and stings (normally coupled) iv) stalking behaviour and v) suction of abdominal secretions. We performed all available combinations between bee and ant colonies involved in an association.

Bee reaction toward intrusion in to the nest

While performing the presentation on the bee nests we occasionally observed that some anesthetised ants which were no able to run away and moved too deep in the nest entrance were attacked by bees. After the conclusion of the behavioural experiments above mentioned we performed further experiments in order to describe this behaviour. We performed one trial per each bee colony with an ant collected from the same nesting association. We highly sedated the ant by refrigerating it and we tight in the junction between the head and the thorax the extremity of a 50 centimetres long nylon cord (0.5 millimeters wide). After an eventual further sedation, we introduced by using forceps the ant in the deepest visible portion of the bee entrance and we video recorded bee reactions. We then rescued the ant by using the nylon cord.

Cuticular hydrocarbon characterization

We extracted cuticular hydrocarbons (CHCs) from *Partamona testacea* individuals belonging to the 6 colonies involved in the experiment (5 of them associated with *Paraponera clavata* and one associated in the past.) Moreover, we collected bees from two colonies associated with colonies of *Atta* spp. leafcutter ants, found nearby the study area. Four individuals per each *P. clavata* and *P. testacea* colony, were sacrificed by freezing and then washed in pentane (500µl for ants, 250µl for bees). The extracts were dried in the field station and eluted in the Florence lab with heptane (100µl for ants, 40µl for bees). One µl of the solution was injected in a Agilent technology instrument 7820 gas chromatograph (GC) coupled to a 5977B mass selective detector (using 70 eV electronic ionization source). An Agilent silica capillary column (30 m x 0.25 mm x 0.25 µm) was installed in the GC. The injector port and transfer line temperatures were set at 280 °C and the carrier gas was helium (at 15 psi head pressure). The temperature protocol was: from 70 °C to 150 °C at a rate of 15 °C/ min held for 3 min and from 150 °C to 310 °C at 5 °C/min held for 16.7min. Injections were performed in split mode. Data acquisition and analysis were done using the Agilent MassHunter Qualitative Analysis Version B07.00 ©Agilent technologies.

Statistical analyses

COI sequences have been aligned using MAFFT v. 7 with auto parameter setting (Katoh and Standley, 2013). The number of mutations and their segregation between *P. testacea* associated with *P. calvata* and *Atta* sp. colonies have been inspected.

All the video records have been analysed with BORIS, the open source software for video/audio coding and live observations created by the University of Turin, Italy. Since the resulting count and time measurements of the behavioural responses showed a Poisson distribution, we used the "glmmadmb" function in the "glmmADMB" package (http://glmmadmb.r-forge.r-project.org/) to fit a Generalized Linear Mixed Model. We included the different count measures as response variables, the relationship of association between colonies as a factorial binomial (alien-associated) predictor and colony membership of bees and ants as two random factors (see Appendix S1 for R scripts). For the petri dish experiments (reaction of ants) the physical contacts between individuals has been entered as an additional predictor in the GLMMs together with alien-associated variable in order to account for a possible increase in responses after an increased number of encounters. In these cases, estimated marginal means to be plotted on graphs have been computed by using the "emmeans" function of the "emmeans" R package. Since the number of cases were not balanced among groups, we used type III sum of squares as returned by the "Anova" function of the "car" R package. CHCs were identified basing on their mass spectra, and equivalent chain length. For the preparation of the dataset used in statistical analysis we only included compounds quantified in at least 25% of specimens belonging to one of the species. To reduce the bias due to the use of compositional data in multivariate analyses, we transformed the area by the following formula (Aitchison, 1982):

$Z_{ij}=\ln (Y_{ij}/g(Y_j))$

where Y_{ij} is the area of peak i for individual j, $g(Y_j)$ is the geometric mean of the areas of all peaks for individual j, and Z_{ij} is the transformed area of peak i for individual j. To visualize the general pattern of similarity among individuals and colonies, we first performed Partial Least Square Discriminant analysis using colony membership as a priori grouping variable for ants and bees separately. Among conspecifics, we also verified if nestmates were chemically more similar to each other than non-nestmates. In this aim, we scaled the

frequencies of each compound (mean = 0, standard deviation = 1) to avoid an unbalanced contribution to the dissimilarity matrix and compared the obtained dissimilarity matrix among individuals with the corresponding membership matrix indicating whether each pair belong to the same colony. Due to the non-independence of data, we used a Mann Withney U test and assessed the P-value by comparing the observed U value with the values obtained after sampling 10000 time the membership matrix. A similar analysis has been done to verify whether bees and ants belonging to the same association show a higher inter-specific similarity. For this analysis we selected the compounds shared among *P. testacea* and *P. clavata* and calculated a dissimilarity matrix as described above. Then, we extracted the rectangular section of the dissimilarity matrix containing the chemical distances among ants and bees. We verified if the pairs of associated pairs ants and bees show a lower chemical distance compared to non-associated ones by using a generalized linear mixed model (Gaussian family, glmmPQL function of the MASS R package) using bee and ants individuals as random factors.

3. RESULTS

Frequency of colony association

In the study area all the colony of *P. testacea* were at least founded in association with ants. In the lands of the Urku reserve we spotted 19 colonies of *P. clavata* and 6 colonies of *P. testacea*. All the colonies of *P. testacea* were associated with active bullet ant colonies except one which was in association with *P. clavata* until 2016, but then the ants disappeared from the colony (MM personal observation). In the surrounding area of the reserve we identified 6 colonies of *P. testacea* associated with *Atta* leafcutting ants.

COI characterization

The COI sequences we obtained were 678 bp long, and correctly translate for 225 amino acids. We recovered a single haplotype (Genbank acc. no XXX; BOLD project XXX) out of 28 sequences belonging to individuals from 7 colonies. In this respect, any further analyses appeared meaningless.

Behavioural experiments

Bee guard reaction

At the end of the experiment we carried out 47 trials (18 among associated bee and ant colonies and 29 between non-associated bee and ant colonies). When the ant was presented on the nest entrance, the guard bees invariably retreated; a few seconds after the ant left the structure of colony entrance, they simultaneously advanced in a line and repositioned. The ratio between guard bees immediately repositioned and the number of guard bees occurring before the arrival of the ant showed a normal distribution with a mean value of 0.576+-0.238 s.d.. This value reveals that almost half of guard bees did not promptly reposition after the occurrence of a bullet ant on the nest entrance. This ratio did not differ between ant belonging to the colony associated to the focus bee colony and to a non-associated ant colony (Df = 1, ChiSquare = 0.055; p=0.814, Fig. 2a). Repositioning time was rather variable among trials (mean 4.4 s ± 4.1 s.d.) and in a Poisson GLMM, bees revealed to have repositioned faster when an ant belonging to a non-associated colony was presented (Df = 1, ChiSquare = 7.790; p=0.005, Fig 2b).

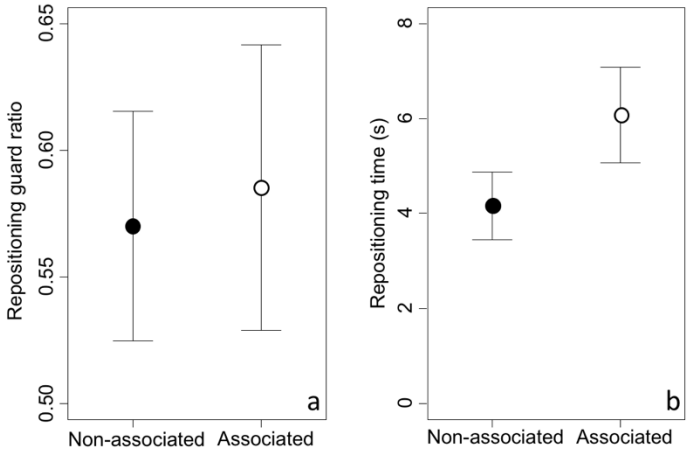

Fig. 2. Comparisons guard bees repositioned after an associated and a non-associated bullet ant left the nest entrance (a) and of the repositioning time showed by guard bees (b).

Bee reaction toward intrusion in to the nest

All the ants introduced in the deepest visible portion of the bee entrance were grabbed by bees hidden behind the nest entrance and dragged inside the vestibular chamber of the bee nest (Camargo and Pedro, 2003). Once rescued, after about 30 seconds following their disappearance inside the nest, ants were covered by a highly gluey substance (presumably composed by resins) hampering their escape by slowing them and sticking them on any contacted surface. This sticky substance was carried by bees on hind legs, using the corbiculae, and manipulated and applied by mouth parts.

Ant reaction

At the end of the experiment we carried out 39 trials (21 among associated bees and ants and 18 between non-associated bees and ants). A Poisson family generalized linear mixed model using ant colony as a random factor showed that associated and non-associated ants and bees did not differ in the number of physical contacts in the arenas (Df = 1, ChiSquare = 0.106; p=0.744, Fig. 3a); number of mandible openings highly depended on number of physical contacts (Df = 1, ChiSquare = 88.977; p<0.001) but was also higher when non-associated bees and ants were examined (Df = 1, ChiSquare = 131.077; p<0.001, Fig. 3b). A similar effect was found in stalking behaviour (physical contacts, Df = 1, ChiSquare = 88.977; p<0.001; associated vs non-associated bees, Df = 1, ChiSquare = 5.086; p=0.024, Fig. 3c). Bites and sting were highly uncommon, and they have been recorded in three cases only. This did not allow to model this behaviour. Suction of abdominal secretion by ants has been performed in six trials and in five cases it occurred when a non-associated bee occurred in the arena. Accordingly, in a binomial GLMM, this behaviour showed a significant relationship with number of physical contacts (Df = 1, ChiSquare = 8.642; p=0.003) and were significantly more frequent when a non-associated bee occurred in the arena (Df = 1, ChiSquare = 6.823; p=0.009).

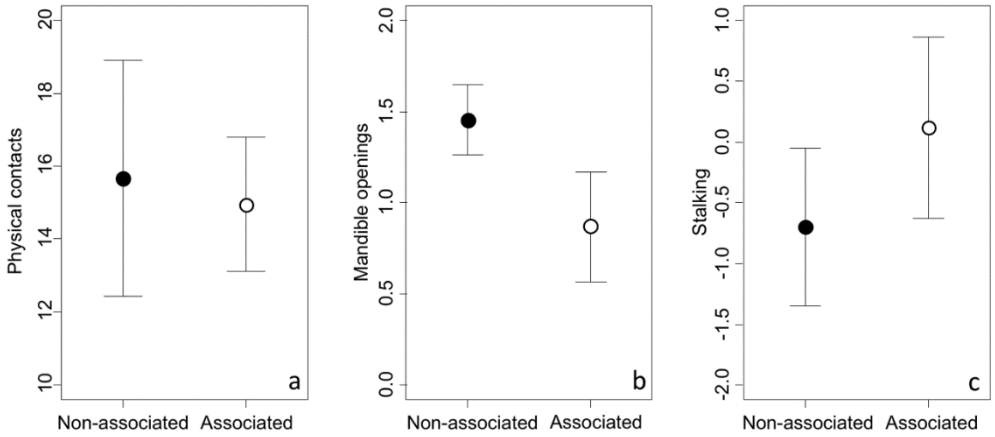

Fig. 3. Comparisons of frequencies of physical contacts (a), mandible openings (b) and stalking behaviour (c) performed by *P. clavata* toward associated and non-associated individuals of *P. testacea*

Cuticular hydrocarbon characterization

We detected 135 peaks in the three species analysed (*P. testacea*, *P. clavata*, *Atta* spp) corresponding to one or more chemical compounds. As expected in a inter-specific comparison, many peaks revealed qualitative differences (Fig. 4a). A partial least square discriminant analysis using species membership as response variable (also separating *P. testacea* living in association with *P. clavata* and *Atta* sp.) confirmed that the three species have different profile and also that *P. testacea* living with bullet ants and leafcutter ants show highly similar profiles (Fig. 4a,b). As it occurs in most social species also *P. testacea* and *P. clavata* showed evidence for the existence of colony-specific signatures as showed by PLSDA (Fig. 4d) and verified with a significantly higher similarity between nestmates compared to aliens (bees, Mann Withney test, W=9382, permutation P<0.001, Fig. 4e; ants Mann Withney test, W=10354, permutation P<0.001, Fig. 4e). A comparison of chemical distances between pairs of ants and bees belonging to associated and non-associated colonies revealed that associated pairs were characterized by a lower chemical distance (GLMM, Estimate = -1.485, Standard Error = 0.438, degrees of freedom = 545, T-value = -3.388, P<0.001).

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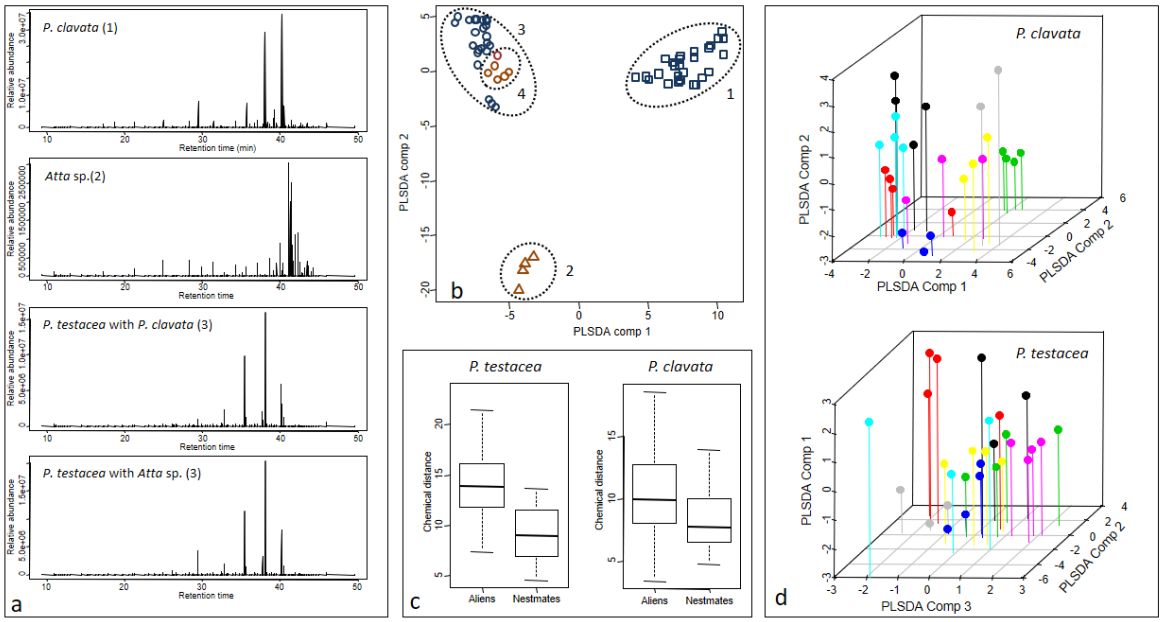

Figure 4. The chemical profiles of *Paraponera clavata*, Atta sp., and of *Partamona testacea* associated to *P. clavata* and *Atta* sp (a). A scatterplot obtained by PLSDA of these four groups of individuals (b) (reference numbers as in a). Comparisons of chemical distances between nestmate and alien individuals in bees and ants (c). PLSDAs of bees and ants grouping individuals of the same colonies based on their chemical profiles (d), different colonies are indicated with different colours.

4. DISCUSSION

In the study area in the Peruvian Amazon, we described for the first time the association between a stingless bee species (*Partamona testacea*) and the bullet ant *Paraponera clavata*. All the bee colonies we spotted have been founded in association with the ants *P. clavata* or *Atta* spp. (Q1). Differences in host choice were neither mirrored by differences in mitochondrial COI sequences (Q2) nor in cuticular hydrocarbon composition of bees (Q4). During behavioral experiments both *P. testacea* and *P. clavata* showed a generalized high tolerance toward the other species, despite in specific circumstances they were both able to effectively fight against individuals of their counterpart. Furthermore, aggressiveness and alertness among individuals belonging to associated colonies were reduced (Q3). As it occurs in most social insect species, cuticular hydrocarbons of both *P. clavata* and *P. testacea* showed differences among colonies in composition. Although species-specific cuticular signatures were preserved, associated colonies revealed a higher similarity in chemical

profiles compared to non-associated ones. The colony-specific profile and the higher similarity among associated colonies are likely the proximate cue allowing the heterospecific recognition system. (Q4).

Partamona testacea is reported as the only species in its genus obligatorily building subterranean nests, usually inside ant and termite nests but also in subterranean cavities not excavated by social insect colonies (Camargo and Pedro, 2003). In our study area, *P. testacea* showed a strict nesting relationship with two ant species (*Paraponera clavata* and *Atta* sp.), presumably depending on the local availability of the different hosts. All the colonies were founded inside cavities produced by active ant colonies and the disappearance of the ant colony from one association did not determine the disappearance of the bee colony. This supports the hypothesis that the association is probably necessary for the foundation but not for the survival of the bee colony.

At least in our study area, host choice is not mirrored by differences in COI sequences. This mitochondrial gene is highly variable, not only among species, but also among conspecific lineages, making it the most used marker for the identification of animal species and to detect incipient phenomena of genetic divergence (Dincă et al., 2015; Hebert et al., 2003; Ratnasingham and Hebert, 2013). In this respect, the absence of a single mutation in 678 COI bps allows us to affirm that nesting habits with different ant species of *P. testacea* is not related to genetic divergence among cryptic species, lineages, or even matrilines and supports literature data indicating that this bee can nest with different hosts (Camargo e Pedro 2003).

According to Roubik (2006) most nesting association between stingless bee species and other social insects should be considered as parasitic relationships. This because stingless bees usually do not participate in defence and ants do not receive any apparent advantage from the association. *P. testacea*, belongs to one of the few genuses for which the association is not considered as a parasitic relationship (Roubik, 2006). Indeed, together with species of the genus *Trigona*, Partamona species are considered aggressive bees contributing in the defence of the nest association. In our behavioural experiments both *Paraponera clavata* and *Partamona testacea* showed to be able to fight against their counterpart but also a generalized tolerance between them emerged.

In the arena experiments, the ants showed a low aggressiveness toward the bees and physical attacks were highly uncommon. In the presentation of ants at the bee colony entrance, the

guard bees invariably retreated and hid when facing the ants and did not attack them except in the few cases when ants moved deep in the entrance. Conversely, when ants were purposely forced to penetrate in the deepest visible portion of the bee nest entrance, even if associated, guard bees reacted by grabbing and drugging them inside the nest and applying on them a sticky substance manipulated with their mouth parts. Nests of species belonging to the genuses *Partamona* and *Plebeia* possess a peculiar chamber between nest entrance and the proper nest (Roubik 2006), the vestibule or false nest, hosting a reticular structure made of soil and resins (Camargo and Pedro, 2003; AB, GM and MM personal observation). The sticky substance applied on the intruder likely trap it inside the vestibule reticular structure. Stingless bees also normally apply on the nest entrance resin and other ant repellent materials (Schwarz, 1948), and this also invariably occurred in *P. testacea* after the intrusion experiments.

In the experiments where the reaction toward associated and non-associated individuals was compared, both ants and bees showed a lower frequency of agonistic behaviour and higher levels of avoidance. Ants showed a lower frequency of mandibular openings (a well-known agonistic behaviour (Guerrieri and d'Ettorre, 2008). Stalking behaviour was higher when an associated bee occurred in the arena; we did not find any interpretation of this bullet ant behaviour in literature but, in our experiments, it has never been followed by aggressive behaviours. In this optic, stalking, in our experiments, appeared as a non-aggressive behaviour which function still needs to be investigated. Experiments on bee conducted in the field on their nest entrance, showed that guard bees show a higher level of alertness when a non-associated ant approaches the nest expressed as a shorter repositioning time after their retreat, when the ant leave the nest entrance structure.

In social insects, the differential behaviour showed toward nestmates and by individuals of associated colonies is generally based on the composition of cuticular hydrocarbons (Dani et al., 2005; Emery and Tsutsui, 2013; Lenoir et al., 2001; van Zweden and d'Ettorre, 2010). Typically, in social insect colonies, common nest odor, (the 'gestalt odor'), is maintained through social interactions homogenizing chemical profiles across individuals (Breed et al., 1985; Lenoir et al., 2001; van Zweden and d'Ettorre, 2010). Nothing was known about the composition and the dynamics of chemical phenotypes in stingless bees associated with other social insect species. Couples of ant species involved in heterospecific nesting association

(parabiosis) do not show a convergence of their species-specific cuticular profiles as it generally occurs in parasitic symbioses, where parasitic species mimic cuticular hydrocarbon signatures of their specific host (Emery and Tsutsui, 2013; Lenoir et al., 2001). *P. testacea* also did not show a chemically insignificant profile, since the hydrocarbon blend was composed by several chemical compounds, most of which are recognized as having a high significance as semiochemicals like the alkenes (Dani et al., 2005). In this perspective, the reduced aggressivity and alertness showed in *P. clavata-P. testacea* associations is likely based on a similar mechanism to that existing in ant parabiotic associations. This rare and particular associations have been used as a model to demonstrated that the recognition process in social insects can be based on learned signatures and not necessarily on a self-referent comparison (Emery and Tsutsui, 2013; Lenoir et al., 2001; Menzel and Schmitt, 2012). Ants species living in parabiotic association show a chemical profile characterized by a high frequency of alkenes with a longer carbon chains compared to congeneric species (Emery and Tsutsui, 2013). The repeated evolution of these unusually long-chain compounds suggests that they are a key trait facilitating heterospecific tolerance (Emery and Tsutsui, 2013; Menzel and Schmitt, 2012). The comparison among the bee chromatograms obtained in this study with those available in literature reveals that *P. testacea* shows a cuticular profile dominated by longer chain alkenes (C29:1, C31:1, C33:1) respect to the stingless bees species not involved in association, C25 and C27 and C27:1 in *Melipona scutellaris* (Kerr et al., 2004), C25 and C27 in *Austroplebeia australis* (Leonhardt et al., 2011) and C27 and C29 in *Frieseomelitta varia* (Nunes et al., 2009). Bees and ants from the same association showed reduced chemical distances compared to non-associated pairs of individuals. Although we did not investigate the mechanisms determining such an increased similarity, it could depend on the acquisition of similar fractions of environmental chemicals existing in the nesting area or by some exchanges of cuticular components. Whatever the mechanism involved, the reduced dissimilarity could represent one of the factors determining a lowered aggressivity.

In conclusion, we showed that the association of *P. testacea* and *P. clavata* is far to be a mere case of facultative nesting in close proximity. In fact, members of associated ant and bee colonies showed to recognize each other's with similar characteristics to the complex parabiotic associations between ants, both in terms of proximate cues (CHCs) and of behavioural responses. The wide generalism showed by *P. testacea* manifests that this

stingless bee species evolved plastic strategies to engage in strict relationships with different ants and termite species. The study of this phenomenon can enlarge our understanding about social insect recognition systems and about mutualistic vs parasitic nesting strategies.

Authors' contributions

M.M., A.B. and S.T. conceived the study; A.B, L.D. and S.T. designed the experiments; A.B., G.M., M.M., L.D., M.B., G.F., A.L. collected the data; A.B. and L.D. carried out the analyses; A.B. and L.D. drafted the manuscript; all authors critically revised the paper, approved the final version of the manuscript and agree to be held accountable for the content therein.

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