

Division of labor in anti-parasite defense strategies in ant colonies

Claudia Missoh

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Division of labor in anti-parasite defense strategies in ant colonies.

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PhD thesis in Ecology and Evolutionary Biology

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1. ABSTRACT

Division of labor is a key characteristic of social insects and contributes to their ecological success. Especially in disease defense, the intra-colony partitioning of sanitary work can reduce disease transmission, keep nestmates available for other tasks and reduce costs associated with sanitary task performance (i.e. at the behavioral and physiological level). Factors internal and external to the individual affecting sanitary task allocation are not well known and most studies investigated genetic differences between workers performing behavioral sanitary work. In the first two studies I addressed whether individual experience (through repeated exposure to a sanitary hazard or performance of the task) can generate interindividual differences in the performance of behavioral sanitary tasks. Repeated parasite exposure is a common threat in colonies of social insects, posing selection pressures on colony members to respond with improved disease-defense performance. In the clonal ant Platythyrea punctata, I tested whether experience gained by repeated tending of low-level fungus-exposed (Metarhizium robertsii) larvae alters the performance of sanitary brood care. I found that ants trained both with sham- and fungus-treated larvae groomed the brood longer than naive ants. Increased grooming of fungus-treated larvae resulted in more effective fungal removal, thus making trained ants better caretakers under parasite attack of the colony.

Decomposing cadavers pose a sanitary risk to social insect colonies, necessitating cadaver management. In the second study I investigated whether cadaver management (i.e. cadaver grooming and transports) is divided among workers and task allocation affected by recent individual experience or worker size in the polymorphic and polygynous ant *Cataglyphis velox*. Many individuals performed cadaver management infrequently and few individuals dominated task performance. Our results suggested low division of labor for cadaver grooming and transport and a reduced modulation of these behaviors by recurrent exposure to nestmate cadavers.

Polyandry increases the diversity of group members and thereby favours division of labor within the colony. Colonies with increased genetic diversity can also be more resistant towards disease, but the mechanisms underlying increased disease resistance are not well understood. I analyzed in *C. velox* whether patriline differences among workers could affect their investment into the immune enzyme phenoloxidase. I did neither find heritability for this trait, nor for worker size. Environmental determination of variation in worker size could be advantageous with respect to division of labor, by permitting colonies to adapt worker size ratios to changing conditions if worker size predicts task performance. Environmental determination of the level of phenoloxidase might confer increased disease defense only to individuals performing the riskiest tasks within the colony and thereby limiting costs of immune investment at the colony level. My work suggests that individual experience may influence the performance of sanitary tasks and that tasks may vary in their degree of plasticity. It further demonstrates the importance of future research effort to understand the

underlying mechanisms of interindividual variability and the benefits and costs associated with increased nestmate diversity.

Key words: ecological immunology, division of labor, phenotypic plasticity, experience, brood care, parasite exposure, cadaver management, phenoloxidase, polyandry, heritability, *Platythyrea punctata, Metarhizium robertsii, Cataglyphis velox*

2. RÉSUMÉ

La division du travail est une caractéristique clé chez les insectes sociaux et contribue à leur succès écologique. En ce qui concerne les tâches sanitaires, la division du travail au sein d'une colonie peut permettre de réduire la transmission des maladies, de libérer certaines ouvrières pour d'autres tâches, permettant de diminuer les couts associés à l'exécution des tâches sanitaires (sur le plan comportementale et physiologique). Les facteurs externes et internes aux individus déterminant leur participation aux tâches sanitaires ne sont pas bien connus. La plupart des études portent sur l'importance des différences génétiques entre ouvrières. Dans les deux premières études, j'ai examiné le rôle de l'expérience des individus (par exposition répétée à des déchets sanitaires ou à l'exécution d'une tâche) sur la mise en place de différences interindividuelles dans l'exécution d'une tâche sanitaire comportementale. L'exposition à un parasite est une menace fréquente au sein de colonies d'insectes sociaux. En utilisant la fourmi clonale Platythyrea punctata, j'ai voulu savoir si une exposition répétée des individus à des larves portant une faible quantité de conidiospores du champignon Metarhizium robertsii affectait la performance des soins sanitaires portés au couvain. J'ai trouvé que la durée de nettoyage des larves était plus élevée chez des fourmis entrainées, aux larves exposées ou non exposées au champignon, que chez des fourmis inexpérimentées. Un temps de nettoyage plus élevé améliorait l'élimination des conidiospores. Ainsi les fourmis entrainées pourraient être plus efficaces pour éliminer les conidiospores lors d'une attaque parasitaire de la colonie.

La décomposition des cadavres représente un risque sanitaire dans les colonies d'insectes sociaux, nécessitant une gestion de cadavres. Dans la deuxième étude, j'ai étudié la possibilité d'une division du travail dans la gestion des cadavres (c'est-à-dire le nettoyage et le transport) chez les ouvrières de la fourmi polygyne et polymorphe *Cataglyphis velox*. J'ai plus spécifiquement testé si la propensité d'accomplir ces tâches était en rapport avec une récente expérience individuelle ou avec la taille des ouvrières. Nos observations ont montré que la majorité des individus de la colonie n'effectuait que rarement des tâches de gestion de cadavres même si quelques individus pouvaient être impliqués plus fréquemment, au moins sur un laps de temps cours, dans l'exécution de ces tâches. Les résultats suggèrent une faible division du travail dans le nettoyage et le transport des cadavres et une faible modulation de ces tâches par l'exposition répétée des ouvrières à des cadavres.

La polyandrie accroît la diversité entres les membres d'un groupe et par ce fait pourrait favoriser la division du travail au sein de la colonie. Les colonies ayant une plus grande diversité génétique pourraient être plus résistantes aux maladies, mais les mécanismes sousjacents permettant une meilleure résistance aux maladies restent mal connus. J'ai analysé chez *C. velox* si les différences génétiques entre ouvrières appartenant à des lignées paternelles différentes avaient un effet sur le niveau de l'enzyme immunitaire phénoloxydase dans l'hémolymphe et sur la taille des ouvrières. Aucune héritabilité n'a pas pu être prouvée, ni pour le niveau de l'enzyme phénoloxydase, ni pour la taille de ouvrières. Si des ouvrières de différentes tailles n'ont pas la même probabilité d'effectuer une tâche, l'effet de l'environnement sur la taille des ouvrières pourrait alors être avantageux pour la division du travail, en permettant aux colonies d'adapter la distribution de la taille de leurs ouvrières aux conditions environnementales fluctuantes. Un effet environnemental, en particulier de la taille, sur le niveau de la phénoloxydase pourrait contribuer à accroitre la défense aux maladies seulement chez les individus de la colonie effectuant les tâches les plus risquées; ce qui aurait pour conséquence de limiter l'investissement immunitaire au niveau de la colonie.

Cette thèse suggère que l'expérience individuelle pourrait moduler la propensité à exécuter des tâches sanitaires, et que les tâches peuvent différer dans leur niveau de plasticité. Mon travail démontre l'importance de développer de futures recherches afin de pouvoir comprendre les mécanismes entraînant une variabilité interindividuelle et les bénéfices et coûts associés à une variabilité accrue des ouvrières.

Mots-clés: immunologie écologique, division du travail, plasticité phénotypique, expérience, soin de couvée, exposition aux parasites, gestion des cadavres, phénoloxydase, polyandrie, héritabilité, *Platythyrea punctata, Metarhizium robertsii, Cataglyphis velox*

3. INTRODUCTION

Studies on division of labor in parasite defense combine exciting issues: the variability among nestmates of social insects to invest into physiological and behavioral disease defense mechanisms, both at individual and collective level. Ongoing research investigates how variability in immune defense arises and is maintained within social groups. This thesis aimed to investigate whether individual experience and genetic background, both sources of interindividual variability, affect the investment into immune defenses at the individual ants. These aspects were investigated for behavioral and physiological defenses at the individual and collective level. The following introduction presents a short overview on immune defense and interindividual variability in social insects.

3.1 Disease resistance in social insects

A major cost of social life is the increased threat imposed by macro- (e.g. helminth worms and arthropods) and microparasites (e.g. fungi, bacteria, viruses and some protozoa; Schmid-Hempel 1998; Tella 2002; Godfrey et al. 2006). Parasites can have various effects on their hosts, such as the suppression of the host's immune system (Edlund et al. 1976; Dupas and Boscaro 1999; Richards and Parkinson 2000; Wang and St. Leger 2006) or the manipulation of host behavior to improve parasitic spread (reviewed in e.g. van Houte et al. 2013), thereby often leading to disease and death. There are numerous ways in which social insect workers (i.e. ants, termites, social bees and wasps) can get in contact with infective microorganisms, for instance in their nest material, through contact with nestmates (Rosengaus and Traniello 1997; Hughes et al. 2002; Cremer et al. 2007; Konrad et al. 2012) or by sharing the same foraging grounds with infectious individuals of the same or other species (Durrer and Schmid-Hempel 1994). A particularity of social insect colonies is that the transmission of infective agents is facilitated among a high density of locally confined, closely related and frequently interacting nestmates. Furthermore, colonies achieve homeostatic nest conditions (in temperature and humidity) and provide resources in form of brood or honey, which facilitate the establishment of parasites within them (Shykoff and Schmid-Hempel 1991; Rosengaus and Traniello 1997; Schmid-Hempel 1998; Cremer et al. 2007; Fefferman et al. 2007). In consequence of the increased selection pressure imposed by parasites, natural selection favored the evolution of sophisticated and collective defense strategies, which complement individual behavioral and physiological defense mechanisms (Schmid-Hempel 1998; Cremer et al. 2007; Evans and Spivak 2010). In social insects, selection acts both at the individual and at the collective level and affects traits which significantly influence colony phenotype and thereby colony fitness (Jeanson and Weidenmüller 2013).

Understanding the kind of parasites which endanger social insect colonies and the evolution of natural defense mechanisms is of great importance for conservational, agricultural and economic reasons. Honeybees and bumblebees are important pollinators and their services used in agricultural settings but wild populations have declined during the last years, which is in part linked to emerging infectious diseases (Fürst et al. 2014 and the references therein). Research on the heredity of immune defense components will allow improved selection programs. Ants and termites are also important members of terrestrial ecosystems and comprise more than 50 % of the biomass in some tropical habitats (Hölldobler and Wilson 1990). Some species are considered as major pest insects and damage control requires integrated pest management strategies (e.g. with the help of entomopathogenic fungi: Shah and Pell 2003).

3.1.1 Individual physiological immune defenses

The physiological immune system serves as an ultimate defense mechanism against parasites when higher levels (e.g. individual sanitary behaviors such as self-grooming or collective defense mechanisms (see 3.1.2)) do not prevent infection. Workers of most ant species are able to spread antimicrobial secretions of their metapleural glands onto the cuticle (reviewed in Yek and Muller 2010), thereby increasing boundary cuticle defense to hinder parasite entrance into the body. If the parasite nevertheless breaches the cuticle, host receptors recognise conserved microbial patterns such as peptidoglycans and lipopolysaccharids from bacteria and β-1,3-glucans from fungi (Gillespie et al. 1997; Söderhäll and Cerenius 1998; Tzou et al. 2002; Siva-Jothy et al. 2005). Upon recognition, an individual can employ different defense mechanisms, which can be constitutively expressed (always present, unspecific and immediate) or induced at parasite presence (specific but more delayed; Schmid-Hempel 2005a; Hamilton et al. 2008). Such defense mechanisms comprise pathways leading to the secretion of antimicrobial factors like lysozyme, antimicrobial peptides and cytotoxic molecules, as well as responses including melanization, phagocytosis of small and encapsulation of larger microbes through hemocytes (reviewed in Gillespie et al. 1997; Tzou et al. 2002; Schmid-Hempel 2005a; Siva-Jothy et al. 2005; Lemaitre and Hoffmann 2007). Viruses are detected on the basis of their double-stranded RNA and can be defended with the help of the RNA interference pathway, producing small interfering RNAs which inhibit the replication of viral RNA (reviewed in Kingsolver et al. 2013). The phenoloxidase cascade is presumably the main frontline defense against parasites and anti-microbial peptides are applied at later stages if parasites remain within the host (Haine et al. 2008). The phenoloxidase cascade leads to the production of melanin, which is used for cuticle hardening, wound healing and to protect against microbes (Gillespie et al. 1997; Söderhäll and Cerenius 1998; Cerenius et al. 2008). As melanin and intermediates of the melanin pathway are cytotoxic and can lead to autoimmune costs in form of self-harm (e.g. Sadd and Siva-Jothy 2006), phenoloxidase is stored in its inactive form and only activated on demand (Söderhäll and Cerenius 1998; Cerenius et al. 2008).

Autoimmune reactions and the requirement of resources are examples for maintenance and usage costs of physiological immune defense mechanisms (e.g. Moret and Schmid-Hempel

2000; Sadd and Siva-Jothy 2006). Evolutionary costs comprise negative genetic covariance relationships, where the expression of an immune parameter negatively affects other fitness-relevant traits (e.g. reproduction, growth) and leads to trade-offs among them (reviewed in Schmid-Hempel 2003). Evolutionary ecology assumes that an organism's resources are limited and must be distributed and traded off among immunocompetence and life-history traits (Sheldon and Verhulst 1996; Rolff and Siva-Jothy 2003; Schmid-Hempel 2003, 2005a), as well as among different immune mechanisms (e.g. phenoloxidase and antimicrobial peptides: Ruiz-González et al. 2009). Given the trade-offs and fitness costs of immune defenses, individuals are expected to optimise physiological immune defenses and only invest if benefits outweigh the costs, which leads to a considerate plasticity in immune responses over individual states and parasitic pressures (Sheldon and Verhulst 1996; Schmid-Hempel 2003).

3.1.2 Collective immune defenses

When comparing the highly social honeybees with non-social *Drosophila melanogaster* and *Anopheles gambiae*, individual honeybees possess fewer immune genes, which might reflect their strength of collective defense mechanisms (Evans et al. 2006), but could also be driven by the fact that honeybees possess other immune genes than these dipteran species which were not detected in this study. The colony-level protection achieved by organisational, behavioral and physiological mechanisms performed by cooperating social insect nestmates is termed 'social immunity' (e.g. Cremer et al. 2007; Wilson-Rich et al. 2009; Evans and Spivak 2010; Stroeymeyt et al. 2014) and these mechanisms can both be prophylactic or activated on demand (reviewed in Cremer et al. 2007). Modelling demonstrated that disease threat within the colony is increased through frequent and homogenous contact among nestmates and reduced by colony structuring. Structuring arises through spatial nest complexity and heterogeneity among workers as well as in their social interactions (Pie et al. 2004; Naug and Camazine 2002; 'organizational immunity': Naug and Smith 2007; Stroeymeyt et al. 2014).

The behavioral defense mechanisms employed can vary greatly within and between species (Cremer et al. 2007), ranging from parasite avoidance (Epsky and Capinera 1988, Diehl-Fleig and Lucchese 1991) and social exclusion of infected individuals and brood (Arathi et al. 2000; Renucci et al. 2010; Ugelvig et al. 2010; Baracchi et al. 2012) to intensified sanitary care (Waddington and Rothenbuhler 1976; Rosengaus et al. 1998b; Walker and Hughes 2009). Nest sanitation is a prophylactic mechanism to avoid microbial growth within the nest and comprises the application of gland- or bacteria-derived antimicrobials (e.g. metapleural gland secretions: reviewed in Yek and Mueller 2010; Fernández-Marín et al. 2006; secretions from a mutualistic bacterium: Currie et al. 1999; salivary gland secretions: Lamberty et al. 2001). The strength of immune defense mechanisms can be adjusted to parasitic pressure, as the level of hitchhiking in leaf-cutting ants is adjusted to parasite abundance (minor workers hitchhike on leaves to protect major workers against parasitic flies; Feener and Moss 1990)

and the quantity of antimicrobial secretions is positively correlated with parasite virulence (metapleural gland: Yek et al. 2012). Nest sanitation is further achieved by incorporating faecal pellets (termites: Rosengaus et al. 1998a) or collected plant resin (ants: Christe et al. 2003; honeybees: Simone et al. 2009) into nest material, as well as by cadaver management (Howard and Tschinkel 1976, Robinson and Page 1988, Hart and Ratnieks 2001). Behavioral processes directed towards the dead differ between social insect species, depending on nest ecology and feeding habits (Neoh et al. 2012) and comprise behaviors such as cadaver avoidance (e.g. Kramm et al. 1982; Franks et al. 2005), grooming (Wilson et al. 1958; Neoh et al. 2012), burial (Renucci et al. 2010; Sun et al. 2013), cannibalism (especially common in termites: Neoh et al. 2012) and necrophoresis (also called 'undertaking', i.e. the removal of cadavers from the nest). Another way to keep parasites at bay is the performance of pro-active self-grooming (Morelos-Juárez et al. 2010). If the infective agent has already entered the nest, individuals can warn nestmates of its presence through alarm behavior (vibratory displays in termites: Rosengaus et al. 1999a; Myles 2002). Grooming is an effective behavior to remove infectious particles from the cuticle of brood (Drees et al. 1992; Tragust et al. 2013a), one-self (Hughes et al. 2002), nestmates (Rosengaus et al. 1998b; Hughes et al. 2002; Yanagawa et al. 2008) or a mutualistic fungus (Currie and Stuart 2001) and thereby increases resistance of the individual (Rosengaus et al. 1998b, Hughes et al. 2002, Yanagawa et al. 2008). It also serves to spread antimicrobial substances from exocrine glands over the insect cuticle (e.g. Fernández-Marín et al. 2006; Tragust et al. 2013a).

Some of the behavioral defense mechanisms seem to be triggered by chemical cues (e.g. hygienic behavior: Martin et al. 2002; Swanson et al. 2009). Necrophoresis is elicited by accumulating decomposition products (e.g. oleic acid: Wilson et al. 1958; Haskins and Haskins 1974; Gordon 1983; Lopez-Riquelme et al. 2006; Diez et al. 2013b) in some species or through the disappearance of cues showing the vitality of the individual (dolichodial and iridomyrmecin: Choe et al. 2009). Honeybee cuticular hydrocarbon profiles (essential in recognition processes and chemical communication of social insects: reviewed in Howard and Blomquist 2005) are modified by immune system activation (using bacterial lipopolysaccharide injections: Richard et al. 2008) and might be a cue for differential treatment of infected individuals.

Social and individual immune defense are tightly linked. For instance, exocrine gland secretions serve both individual and collective sanitary defense (e.g. Do Nascimento et al. 1996; Bot et al. 2002; Fernández-Marín et al. 2006; Tragust et al. 2013a) and the presence of nest resin requires lower individual immune investment (Castella et al. 2008; Simone et al. 2009).

3.2 Division of labor in social insect colonies

How the behavior and interactions of up to several millions of individuals in social insect colonies (the so-called "superorganism") are integrated and form the colony phenotype is one of the central questions in insect sociobiology (Wilson 1971; Robinson 1992). Without central colony control ('self-organization': Bonabeau et al. 1997), workers perform tasks in response to an array of different task-related stimuli in their local environment whose occurrence depends on the workers' occupied space, intranidal task needs and the behavior of nestmates (reviewed in Beshers and Fewell 2001). Performing a task, workers change the associated stimulus level and thus shape the environment of their nestmates and thereby collective behavior (e.g. Beshers and Fewell 2001). Division of labor, where individuals perform different tasks from the group repertoire, is a complex social phenotype and found across a diversity of social taxa (Oster and Wilson 1978; Bednarz 1988; Wilson 1971; Stander 1992; Patterson et al. 2004). It is especially pronounced in social insect colonies (e.g. Oster and Wilson 1978; Gerber et al. 1988; Hölldobler and Wilson 1990) and assumed to be a key factor responsible for their remarkable ecological success (Oster and Wilson 1978; Wilson 1971). The primary division of labor is reproductive division of labor, where only one or a few individuals (usually the queen caste) monopolize reproduction and all other individuals (the worker caste) help to raise the offspring and perform daily colony tasks (reviewed in Wilson 1971; Oster and Wilson 1978; Hölldobler and Wilson 1990). Labor can again be divided within the worker caste, with individuals showing different probabilities to perform certain tasks or sub-tasks, if a task is partitioned (Wilson 1971; Oster and Wilson 1978; Robinson 1992). Some ant species and most termites possess distinct worker castes that can differ morphologically (e.g. in size; i.e. polymorphism) and show distinct behavioral profiles (Wilson 1971; Oster and Wilson 1978; Hölldobler and Wilson 1990). Soldiers of leaf-cutting ants possess bigger and stronger mandibles and specialize in colony defense (Hölldobler and Wilson 1990). Most species show temporal polyethism, where a worker sequentially performs different tasks within its life, starting with intra-colony duties when young and changing to outdoor activities with a higher mortality rate when older (ants: Hölldobler and Wilson 1990; Tripet and Nonacs 2004; Mersch et al. 2013; honeybees: Seeley and Kolmes 1991; termites: Gerber et al. 1988; bumblebees: Cameron 1989). Age-related transitions between tasks are coupled with physiological and neurological changes (Robinson 1987, 1992; Withers et al. 1993; Schulz and Robinson 1999; Fahrbach et al. 2003; Manfredini et al. 2014). Age polyethism can co-occur with worker polymorphism, within physical castes (Hölldobler and Wilson 1990). In addition to age and morphology, further factors can increase inter-individual differences between workers and 'fine-tune' division of labor within the colony. Physiological differences among workers contribute to age-related or morphological variance, but can by itself generate subgroups among workers of the same size and age (Robinson 2009).

Response-threshold models (for review see Beshers and Fewell 2001) are the current paradigm to explain the emergence of division of labor in social insects. For any given colony

task, workers are assumed to engage in task performance once their stimulus level (response threshold) for that task is met (e.g. Bonabeau et al. 1996; Beshers and Fewell 2001). Response thresholds for a particular task differ between nestmates and thereby determine task allocation within the colony (Robinson and Page 1989; Bonabeau et al. 1996; Fewell and Page 1993, 2000; O'Donnell and Foster 2001; Weidenmüller 2004). Workers with the lowest response thresholds will be the first to engage in task performance and thereby lower the corresponding stimulus level within the colony (e.g. Bonabeau et al. 1996), thus decreasing the need for other workers to perform the same task. As their thresholds will be repeatedly met when the stimulus level increases, these workers will become task specialists (e.g. Theraulaz et al. 1998). Task specialists (i.e. workers which perform tasks more frequently or longer than their nestmates) are more abundant in larger societies (Bourke 1999; Thomas and Elgar 2003; Jeanson et al. 2007; Holbrook et al. 2011) and also in societies with higher genetic diversity (Oldroyd and Fewell 2007). They are expected to perform tasks more efficiently than their non-specialized nestmates (e.g. Oster and Wilson 1978; Jeanne 1986; Dukas and Visscher 1994; Trumbo and Robinson 1997; Julian and Cahan 1999), but literature is conflicting (e.g. see Dornhaus 2008). The diversity of response thresholds within the workforce can be achieved through interindividual differences in caste (e.g. Detrain and Pasteels 1991; Pankiw and Page 1999), sex (Pankiw and Page 1999) and genotype (e.g. Pankiw and Page 1999; Kryger et al. 2000; Masterman et al. 2001; Jones et al 2004; Scheiner and Arnold 2010). They can further underlie modulation with age (Pankiw and Page 1999), nutrition (Pankiw et al. 2001), season (Scheiner et al. 2003), hormone levels (Scheiner et al. 2002; Barron et al. 2002), rearing conditions (Weidenmüller et al. 2009), recent experience of the individual (Plowright and Plowright 1988; Theraulaz et al. 1998; Weidenmüller et al. 2004; Westhus et al. 2013) and depend on the rate of stimulus increase (ants: Yousif 2005; bumblebees: Westhus et al. 2013).

Plasticity is a key characteristic of the division of labor found in social insect colonies and has been linked to colony success (Schmid-Hempel 1991, cited in Trumbo et al. 1997). Colonies are able to adjust the workforce engaged in different tasks to changing internal and external conditions (e.g. changing age demography: Huang and Robinson 1992, 1996; disturbances: Gordon 1989) because of the behavioral flexibility of individual workers (Theraulaz et al 1991; Robinson 1992; Gordon 1996). Workers are able to pass through age-typical tasks at different rates, performing tasks precociously (Huang and Robinson 1996) or reversing back to tasks they had performed when younger (Robinson et al. 1992).

3.3 Division of labor in disease resistance and causes of interindividual variability in the workforce

Division of labor for sanitary tasks can be advantageous, as it increases behavioral and spatial compartmentalisation, as well as heterogeneity in the interaction network, thereby decreasing parasite transmission among workers (Schmid-Hempel and Schmid-Hempel 1993; Schmid-

Hempel 1998; Naug and Camazine 2002; Pie et al. 2004; Naug and Smith 2007; Stroeymeyt et al. 2014). Spatial and social isolation of sanitary task performers protects individuals of higher lifetime ergonomic value (the queen, brood and young workers; reviewed in Stroeymeyt et al. 2014) and can even be reinforced through aggressive interactions (Hart and Ratnieks 2001; Ballari et al. 2007). Without variability in sanitary task performance, workers might focus all efforts on some tasks (e.g. sanitary brood care) while not performing others (e.g. cleaning of the nest and removing cadavers) so that sanitary risks might increase without being attended to. Nestmates could further interfere in task performance (as has been demonstrated in necrophoric honeybees: Trumbo and Robinson 1997), reducing task efficiency (Jeanson and Weidenmüller 2013). Even though immune defense and division of labor in social insects have both been studied comprehensively, knowledge on the allocation of sanitary work loads within the worker caste is limited.

The probability of a worker to engage in a sanitary task may be affected by a variety of factors, which have been shown to affect allocation to other colony tasks. These factors can be of internal or external nature and include an individual's biotic and abiotic environment, its spatial location, age, morphology, genotype, physiology and individual experience (reviewed for non-sanitary tasks in Beshers and Fewell 2001; Robinson 2009). Individual age, the genetic background and worker size are factors influencing sanitary task allocation in highly evolved honeybees and leaf-cutting ants (Frumhoff and Baker 1988; Robinson and Page 1988, 1995; Arathi et al. 2000; Breed et al. 2002; Ballari et al. 2007; Camargo et al. 2007; Pérez-Sato et al. 2009; Waddington and Hughes 2010; Waddington et al. 2010; Eyer et al. 2013a). Less research effort has been performed on sanitary-task allocation in simpler societies (sensu Bourke 2001), with reduced morphological differences among nestmates or among workers and reproductives, simpler nests and communication systems and smaller colony size (but see Turnbull et al. 2012; Diez et al. 2013a). It is also not well understood how experience affects the performance of sanitary behaviors in social insect colonies (see point 3.3.2).

When responding towards a sanitary hazard, workers can show variability in task performance itself (e.g. the behavioral speed, efficiency, duration, precision or location of the behavior) and in their responsiveness towards the sanitary stimulus (Jeanson and Weidenmüller 2013). An individual's responsiveness is characterised by several possibly independent parameters (Weidenmüller 2004): the time an individual takes to respond, a worker's response threshold (the level of the sanitary stimulus at which the response is initiated) and its response probability (the probability to respond once the individual's response threshold is exceeded by the stimulus; Weidenmüller 2004; Jeanson and Weidenmüller 2013). Task performers might further differ in their degree of behavioral plasticity (Dingemanse and Wolf 2013). Inter-individual variability in the before mentioned behavioral response parameters is of great importance for colonies as they underlie division of labor, increase colony productivity and allow colonies to flexibly and robustly respond to

changing external and internal conditions (Jeanson and Weidenmüller 2013). In the context of social immune defenses, research has mainly focussed on behavioral frequency, duration or efficiency (e.g. Trumbo and Robinson 1997; Julian and Cahan 1999; Ugelvig et al. 2010; Tragust et al. 2013b). Less information is available on individual responsiveness, but research demonstrated increased olfactory sensitivity for diseased brood in honeybee workers performing hygienic behavior (Masterman et al. 2001; Gramacho and Spivak 2003). Interindividual variability might be increased through intraindividual variability, where one worker changes its response or responsiveness over time, for instance due to acquired experience (see point 3.3.2).

Individuals with greater physiological immune investment and thus reduced susceptibility could constitute a subgroup among workers (division of labor arising through physiological differences among workers: Robinson 2009) and serve as barriers against the spread of diseases, which require direct contact between nestmates (see e.g. Cremer et al. 2007; Stroeymeyt et al. 2014). One could thus consider physiological immune investment a worker task. In the ant *Camponotus pennsylvanicus*, individuals which mounted an immune response even transferred antimicrobial compounds in their trophallactic droplet to nestmates (Hamilton et al. 2011). The selection acting on the expression of immune parameters may differ among species depending on the relative importance of life history traits under certain conditions. In bumblebees, foraging activity decreased immune function (measured as the encapsulation response; König and Schmid-Hempel 1995; Doums and Schmid-Hempel 2000). Higher immune investment of more exposed task groups is however suggested by the findings that *Cataglyphis velox* foragers possess higher levels of active phenoloxidase (Bocher et al. 2007) and waste workers of Atta sexdens rubropilosa bigger metapleural glands than workers engaged in less risky tasks (Lacerda et al. 2010). But whether task performance results in higher immune levels (as suggested by Bocher et al. 2007) or whether workers possessing higher levels are more likely to engage in task performance remains to be investigated. To understand the conditional expression of traits and thus phenotypic plasticity, it is important to investigate the relative importance of environmental and genetic factors, as well as their interplay.

3.3.1 Genetic variability

Even though multiple mating is rare in social insects, polyandry (a female mates with several males) occurs in some highly eusocial species such as honeybees and the herein studied *Cataglyphis velox* (Boomsma and Ratnieks 1996; Strassmann 2001; Eyer et al. 2013b). Polyandry, as well as polygyny (several functional queens present within one colony) and genetic recombination (intra-chromosomal recombination; e.g. Sirviö et al. 2006), increase the genetic diversity among the workforce. As multiple mating events and increased genetic diversity of the offspring can be disadvantageous for both the female reproductive and the collective unit, hypotheses concerning the evolution of polyandry are diverse (for review see

Palmer and Oldroyd 2000; Crozier and Fjerdingstad 2001). Genetic diversity is believed to confer several advantages to social insect colonies, such as the reduction of parasite loads and improved disease resistance (e.g. Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999; Schmid-Hempel and Crozier 1999; Baer and Schmid-Hempel 2001; Hughes and Boomsma 2004; Tarpy and Seeley 2006; Seeley and Tarpy 2007; Ugelvig et al. 2010; but see e.g. Wilson-Rich et al. 2012). Mechanisms underlying these relationships remain to be investigated. Polyandry modulates division of behavioral sanitary defense in bees and ants, as patriline (the offspring of a single male) origin influences the probability of an individual to engage in guarding the nest entrance (Robinson and Page 1988; Oldroyd et al. 1994), waste management (Waddington et al. 2010; Eyer et al. 2013a), necrophoresis (Robinson and Page 1988, 1995), allogrooming (Frumhoff and Baker 1988) and hygienic behavior (Pérez-Sato et al. 2009). These task preferences of some patrilines potentially arise through lower response thresholds for sanitary stimuli. Higher sensitivity for diseased brood was determined for workers performing hygienic behavior (Masterman et al. 2001; Gramacho and Spivak 2003). Genetically based differences in task performance among nestmates benefits colonies by increasing colony homeostasis (e.g. Jones et al. 2004) and generating a stable and resilient system of division of labor when faced with environmental perturbations (e.g. Oldroyd and Fewell 2007).

Patriline origin further affected disease resistance (Hughes and Boomsma 2004) and investment into constitutive antibacterial activity and metapleural gland size in leaf-cutting ant workers (Hughes et al. 2010; Armitage et al. 2011). In honeybees however, subfamilies (offspring of different patrilines) did not differ in phenoloxidase investment or in their capacity to encapsulate a foreign body (Wilson-Rich et al. 2012). Determining the heritability of immune traits advances the understanding of how traits will adapt to natural selection and changing environmental conditions. In honeybees, heritability of hygienic behavior was determined to be high (Padilha et al. 2013) and candidate genes that affect an individual's propensity to perform this task are currently investigated (Lapidge et al. 2002; Oxley et al. 2010). Heritability estimates of honeybee immune traits can be used by breeders to select colonies with higher resistance towards disease-causing agents.

The sequencing of the honeybee genome (The Honeybee Genome Sequencing Consortium 2006) and subsequent sequencing of several ant (Bonasio et al. 2010; Nygaard et al. 2011; Smith et al 2011a; Smith et al. 2011b; Suen et al. 2011, Wurm et al. 2011) and one termite species (Terrapon et al. 2014) was a major milestone in genetic and genomic analyses of social insects in recent years and will increase the understanding of division of labor and its underlying evolutionary and mechanistic molecular processes (Smith et al. 2008b). Social insects are excellent model organisms for sociogenomic studies and sequenced genomes together with advanced molecular tools (e.g. expressed sequence tags, reverse transcription PCR, DNA microarrays, RNA interference) have demonstrated differential gene expression in conserved pathways (associated with nutrition, foraging behavior, maternal care and

reproduction) corresponding to division of labor between workers (mainly foraging versus intranidal; reviewed in Smith et al. 2008b). In honeybees for instance, the age-related transition from intranidal tasks to foraging correlated with an increase in the expression of the foraging gene (Ben-Shahar et al. 2002). Other epigenetic factors such as DNA methylation, histone modification and RNA editing, seem to be important for behavioral differences between eusocial castes and developmental stages, suggesting considerate genomic plasticity (e.g. Smith et al. 2008b; Bonasio et al. 2012; Simola et al. 2013; Li et al. 2014). Allelic variation and epigenetic factors could both modulate phenotypic plasticity and increase interindividual variation within the workforce.

3.3.2 Phenotypic variability

Eusocial insects are able to generate worker phenotypes with distinct morphological, physiological and behavioral phenotypes from the same genome through a variety of nonmutually exclusive mechanisms. In social insects, immune defense varies at the colony and at the individual level and worker immune investment has important fitness consequences as it may affect colony disease rate and productivity (e.g. Evans and Pettis 2005; Baer and Schmid-Hempel 2006). Several immune components underlie plasticity, depending on environmental conditions and factors such as task performance, age, diet or parasite exposure (Rosengaus et al. 1998b; Jaccoud et al. 1999; Doums and Schmid-Hempel 2000; Amdam et al. 2005; Bocher et al. 2007; Castella et al. 2008, 2010; Simone et al. 2009; Walker and Hughes 2009; Kay et al. 2014). An individual's degree of plasticity in response to these factors is determined by the level of heritability of a trait (including a potential effect of plasticity-regulating loci which exert environmentally-dependent control over structural gene expression: Schlichting and Pigliucci 1993). Factors leading to phenotypic variability among the workforce are dynamic and enable colonies to more rapidly adjust to changing internal and external conditions, compared to solely genetic trait determination (Passera et al. 1996; McGlynn and Owen 2002; Hughes et al. 2003; Bargum et al. 2004; Jeanson and Weidenmüller 2013).

Worker specialization on waste management, necrophoresis and hygienic behavior depends not only on genetic factors (Robinson and Page 1988, 1995; Pérez-Sato et al. 2009; Waddington et al. 2010; Eyer et al. 2013a), but is further influenced by individual age as task performers are of middle age (older than nurses but younger than foragers; Arathi et al. 2000; Breed et al. 2002; Mersch et al. 2013; Camargo et al. 2007; Waddington and Hughes 2010). Necrophoric individuals are presumably developmentally advanced, as they start foraging at an earlier age (Trumbo et al. 1997) and possess higher levels of juvenile hormone than bees of the same age performing other age-typical tasks (Huang et al. 1994). It has been demonstrated that physiological immune defenses of social insect workers change with age (immunosenescence; Doums et al. 2002; Amdam et al. 2004, 2005; Schmid et al. 2008; Moret and Schmid-Hempel 2009) and can be linked to the age-dependent performance of indoor or outdoor tasks (Amdam et al. 2005; Bocher et al. 2007).

The social environment

The social environment creates opportunities for feedback loops between nestmates (direct interactions) and between individuals and their shared environment (indirect interactions; Jeanson and Weidenmüller 2013), which can affect the investment into individual and social immune defenses of workers and amplify interindividual differences. The different spatial regions workers occupy depending on age and tasks performed lead to interindividual differences in connectivity to nestmates and to a differential exposure to environmental stimuli (e.g. Jeanson 2012; Mersch et al. 2013; Stroeymeyt et al. 2014). Individuals with fewer social contacts are less likely to both transmit (e.g. individuals performing sanitary tasks) and receive (e.g. valuable colony members) infectious agents to and from nestmates (reviewed in Stroeymeyt et al. 2014). An individual's position in the interaction network will thus reinforce asymmetry between nestmates (Jeanson and Weidenmüller 2014; Stroeymeyt et al. 2014). Agonistic interactions among nestmates could also reinforce interindividual differences and contribute to sanitary task allocation. Biting interactions affected an individual's propensity to forage in the wasp Polybia occidentalis (O'Donnell 2003, 2006) and were directed towards waste-contaminated workers of the ant Atta cephalotes, thereby reinforcing sanitary division of labor (Hart and Ratnieks 2001). Stress within social groups arising through reproduction-based dominance interactions led to immunosuppression in ants (Bocher et al. 2008). Sanitary task performance by some workers will change the task-related stimulus level for nestmates, and might affect their probability to engage in the same task. In honeybees, an individual's performance of hygienic behavior and task partitioning depended on the proportion of hygienic bees (individuals with low response thresholds for diseased brood and high propensity of task performance) present in the colony (Arathi and Spivak 2001; Gempe et al. 2012).

Social interactions within colonies directly affect the physiological susceptibility of individual group members and can increase their resistance towards a parasite ('social immunisation': Traniello et al. 2002; Ugelvig and Cremer 2007; Konrad et al. 2012; Hamilton et al. 2011). Immunity can be socially transferred (reviewed in Masri and Cremer 2014), by passing antimicrobial molecules between workers (horizontal transfer, e.g. Hamilton et al. 2011) or to the offspring (vertical transfer, e.g. Sadd and Schmid-Hempel 2007). In leaf-cutter ants, mutualistic *Pseudonocardiaceae* bacteria (produce antibiotics controlling a fungal parasite of their symbiotic fungus) are not present on major *Acromyrmex octospinosus* workers directly after eclosion, but transferred from older to freshly eclosed workers (Poulsen et al. 2003). Trophallaxis, which can serve to transfer antimicrobial substances between nestmates (Hamilton et al. 2011), could also affect sanitary task allocation by permitting the transfer of behavior-activating or –inhibiting substances (as has been described in the context of

foraging: Huang and Robinson 1996; Leoncini et al. 2004). Nestmates are able to increase survival rates of workers exposed to parasites by removing and killing the parasite through grooming and the application of antimicrobial secretions (e.g. Rosengaus et al. 1998b; Hughes et al. 2002; Traniello et al. 2002; Yanagawa et al. 2008; Tragust 2013a). During social contact, small quantities of a parasite can be transmitted, causing low-level infections in the recipient resulting in increased immune gene expression and a better protection at secondary exposure (Konrad et al. 2012).

Theoretical (foraging context: Linksvayer et al. 2009) and empirical research (hygienic behavior: Arathi and Spivak 2001; Gempe et al. 2012) demonstrates that trait expression of individuals is modulated by the combination of surrounding phenotypes. The mere presence of nestmates affects the expression of immune genes (e.g. genes coding antimicrobial peptides) in bumblebees (Richter et al. 2012). However, a short-term absence of nestmates did not affect immunocompetence (the level of phenoloxidase) in leaf-cutting ants (Armitage and Boomsma 2010). Social evolution is linked with increasing group size and potentially increasing individual density, which have been shown to affect individual immune status (e.g. Doums and Schmid-Hempel 2000; Ruiz-González et al. 2009; Turnbull et al. 2011) and both increased group size (e.g. Rosengaus et al. 1998b; Hughes et al. 2002) as well as demographic differences among nestmates (Rosengaus and Traniello 2001) can be beneficial for individual survival.

Individual experience

Social insects gain experience in various life activities when responding to external stimuli, such as aggression, nursing, temperature control, predator avoidance, social interactions, sexual behavior, navigation, colony emigration and food acquisition (e.g. Dukas and Visscher 1994; Langridge et al. 2004, 2008; Leadbeater and Chittka 2007; Weidenmüller 2004; Ravary et al. 2007; Dukas 2008; Weidenmüller et al. 2009; Muscedere et al. 2013; Westhus et al. 2013). For certain tasks, experienced individuals have been shown to work more efficiently and thus benefit colony performance (but see e.g. Dornhaus 2008). With increasing experience, honeybee, wasp and bumblebee foragers showed higher foraging success (Dukas and Visscher 1994, O'Donnell and Jeanne 1992, Raine and Chittka 2008). Experienced ant workers were more efficient in colony emigration (Temnothorax albipennis: Langridge et al. 2004; Langridge et al. 2008) and thermal brood relocation (Camponotus rufipes: Weidenmüller et al. 2009) and thereby lead to a better output of the whole group. Recent experience is presumably one of the most important parameters modulating individual behavior and thereby increasing interindividual differences between nestmates. Workers can acquire experience either passively by being exposed to a sanitary stimulus or actively by performing the corresponding task (Jeanson and Weidenmüller 2013). Especially in longlived social insect colonies, individuals are likely repeatedly confronted with the same infectious microorganisms (Schmid-Hempel 1998; Schmid-Hempel 2005b), by contracting them from nestmates (Rosengaus and Traniello 1997; Hughes et al. 2002; Cremer et al. 2007; Konrad et al. 2012; Tragust et al. 2013b) or picking them up from their surroundings and bringing them back to the colony (e.g. Durrer and Schmid-Hempel 1994). How repeated exposure to infectious microorganisms affects the physiological and behavioral immune defense of individual workers is a relatively new research field which remains largely unexplored and empirical evidence that experience contributes to mitigate disease risk is still scarce. Recent advances demonstrate that the physiological immune system of social insects is capable of immune memory ('immune priming'), providing survival benefits upon secondary exposure to the same parasite (termites: Rosengaus et al. 1999b; bumblebees: Sadd and Schmid-Hempel 2006; ants: Konrad et al. 2012) through micro-infections and upregulation of physiological immune defense (Konrad et al. 2012). The produced antimicrobial substances can be transferred to nestmates via trophallaxis ("passive social immunization"; Hamilton et al. 2011) and individuals can thus benefit from parasitic exposure of nestmates. Also the behavioral anti-parasite defenses of social insects were found to be upregulated after first parasite exposure, but studies were performed at the collective level. Ant colonies that had previous contact to the fungal parasite *Metarhizium* show increased expression of allogrooming towards sham-treated (Reber et al. 2011) and infectious nestmates, resulting in their increased survival (Walker and Hughes 2009). In the special case of social parasites, previous exposure to a slavemaker scout (Protomognathus americanus ant) lead to increased aggression in host colonies of Temnothorax longispinosus ants (Pamminger et al. 2011) and these colonies were able to rescue a higher proportion of brood during a later colony raid (Kleeberg et al. 2014).

Experience could improve the behavioral performance of sanitary tasks (e.g. Walker and Hughes 2009; Reber et al. 2011) by (i) reducing individual response thresholds and enabling individuals to react sooner towards a sanitary threat, (ii) increasing their response probability and making it more likely for them to perform the sanitary task once their response threshold is reached, (iii) leading to a faster transition from perception of the sanitary stimulus to the final response, (iv) increasing response duration or (v) improving motoric capacities (e.g. more efficient grooming of fungal spores or preening of nematodes). Individuals specialized on necrophoric behavior removed cadavers faster and more successfully than less experienced individuals (*Apis mellifera*: Trumbo and Robinson 1997; *Acromyrmex versicolor*: Julian and Cahan 1999), but an effect of experience was not demonstrated (Trumbo and Robinson 1997).

Theoretical models propose that response thresholds can be modulated via positive feedback ('self-reinforcement'), where successful task performance results in decreased response-thresholds, leading to an increased propensity to perform the task and thereby promoting task specialization. On the other hand, response thresholds are assumed to increase when the task is not performed, decreasing the propensity of task performance (Plowright and Plowright 1988; Theraulaz et al 1998; reviewed in Beshers and Fewell 2001). Empirically, it has been demonstrated that foraging success increased the probability of individual ants to repeat this

task (Ravary et al. 2007; Robinson et al. 2012) and thereby impacted colony organization (Ravary et al. 2007). Evidence concerning a modulation of response thresholds through recent experience is scarce and conflicting (Jeanson and Weidenmüller 2013). Whereas fanning response thresholds decreased with repeated task performance in thermoregulating workers of the bumblebee *Bombus terrestris* (Weidenmüller 2004; Westhus et al. 2013), a modulation which depends on the time interval between task performances (Westhus et al. 2013), fanning response thresholds were not affected by experience in the bumblebee *Bombus impatiens* (Duong and Dornhaus 2012). Both these results and the finding that spatial memory lasted only 7 minutes for necrophoric *Myrmica rubra* workers (Diez et al. 2011), suggest short-term modulation for infrequent tasks. If the stimulus level increases and thereby the sanitary risk (e.g. through parasitic attack), either "all hands on deck" might be needed (e.g. Dornhaus 2008; Chittka and Muller 2009) to deal with the sanitary hazard or this situation might provide ample opportunity for experience effects to arise.

3.4 Aims of the thesis

Despite a lot of knowledge on both division of labor and immune defense in social insect colonies, both research fields have only been combined in recent years. Previous studies have mainly focused on differences between colonies or between castes in disease susceptibility and defense mechanisms (e.g. Spivak and Reuter 2001; Poulsen et al. 2002, 2006; Gramacho and Spivak 2003; but see e.g. Bocher et al. 2007). But even within morphological castes or age cohorts, interindividual differences between workers can modulate individual immune investment and sanitary-task allocation at the colony level. The allocation of workers to sanitary tasks is not well understood and previous research effort has mainly investigated direct and indirect genetic effects on the performance of hygienic and necrophoric behavior (e.g. Robinson and Page 1988; Arathi and Spivak 2001). As social insect colonies may repeatedly face the same parasites (Schmid-Hempel 1998; Schmid-Hempel 2005b), individual experience (acquired through the exposure to a sanitary hazard or through the performance of sanitary work) could be similarly important for task performance and contribute to sanitary task allocation. In the first chapter I investigate whether experience shapes the expression of sanitary brood care, i.e. larval grooming and effectiveness of conidiospore (Metarhizium robertsii) removal. The ponerine and clonal ant P. punctata allowed studying an effect of individual experience in the absence of genetic differences between individuals (Schilder et al. 1999a). In the second chapter, I analyze whether experience affects sanitary task allocation and thus division of labor by determining if recent experience increases the propensity of an individual to perform cadaver management behaviors. I determine if the workload associated with cadaver management is equally distributed among workers and whether worker size affects task performance in the polymorphic ant *Cataglyphis velox*. The genetic diversity of group members is important for disease dynamics, providing increased resistance of the colony (e.g. Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999, 2001). The underlying mechanisms of this relationship remain to be investigated and could comprise differential immune investment of patrilines (as suggested by Hughes and Boomsma 2004; Armitage and Boomsma 2010). Using individuals of known age and genotype, I analyze in the polyandrous and polymorphic ant *C. velox* whether there is a direct link between genotype and immunocompetence (the level of phenoloxidase) in the **third chapter**. Patriline-level comparisons allow separating genetic and environmental influences on traits as workers from different patrilines share maternal genes and environmental conditions.

3.5 The study systems

3.5.1 Platythyrea punctata

The genus *Platythyrea* belongs to the subfamily Ponerinae and consists of 37 species worldwide (Bolton 1995). *Platythyrea punctata* Smith, 1958 (Fig. 1) occupies a wide geographical range from southern Texas to Costa Rica and from Florida to most islands in the West Indies and the Bahamas (Seal et al. 2011). Preferred habitats are relatively undisturbed, wooded areas and colonies nest in preformed cavities in the soil, in dead branches in trees or in rotten wood on the ground (Heinze and Hölldobler 1995; Kellner 2009). Nest cavities contain organic material such as dead plant particles, prey remnants and empty cocoon cases (Schilder 1999). Occupying these nest environments might expose colonies to naturally occurring parasites, for instance to fungi and bacteria developing on decomposing organic material or to their resting forms in the soil.



Fig. 1 *P. punctata* worker photographed by Simon Tragust.

Solitary foragers hunt for small live and dead arthropods and feed larvae of their colony on these prey items (Schilder 1999; Torres 1984). Colonies are relatively small in size, comprising few to some hundred workers (mean size = 35 individuals/colony (range 2 - 475) in 189 field colonies determined by Kellner et al. (as cited in Kellner and Heinze 2011a)). They presumably reproduce by splitting (fission or budding) and disperse by walking over land (Seal et al. 2011). Various alternative reproductive tactics and female phenotypes co-occur in *P. punctata*: sexually reproducing queens (alate and dealate) and gamergates (mated, egg-laying workers), parthenogenetically reproducing workers as well as intercastes

(phenotypes between workers and queens; Schilder et al. 1999b). In most populations (including the populations used for this thesis), the queen caste is absent and reproduction is monopolized by usually one unmated worker, even though all workers are potentially able to reproduce (Heinze and Hölldobler 1995; Hartmann and Heinze 2003; Schilder et al. 1999b). Reproductive monopolization probably evolved to increase colony efficiency and is achieved by worker policing (Heinze and Hölldobler 1995; Hartmann et al. 2003). Unmated reproductive workers reproduce by thelytokous parthenogenesis (i.e. the production of clonal females from unfertilized eggs) and recombination events are extremely rare in this species, leading to genetically homogeneous colonies (Heinze and Hölldobler 1995; Schilder et al. 1999a; Hartmann et al. 2005; Kellner et al. 2010; Kellner and Heinze 2011b). A low level of division of labor among workers is based on age, with older non-reproductives switching from intranidal tasks to foraging (Hartmann and Heinze 2003).

3.5.2 Cataglyphis velox

The genus *Cataglyphis* (subfamily Formicinae) comprises more than 100 species occurring in arid habitats in Asia, Europe and Africa. Especially the Mediterranean *C. bicolor, C. cursor* and *C. velox* are valued model species to investigate the elaborate navigational abilities and thermal adaptations typical for this genus. One of the most polymorphic species is *Cataglyphis velox*, Santschi, 1929, with a continuous worker length range of 4.5 -12 mm (Cerdá and Retana 1997). This species is endemic to the Iberian Peninsula and found in an elevation range between 0 and 2400 m (Tinaut 1990) (Fig. 2). Colonies have small to medium size, containing from hundreds to a few thousands of workers and one to several queens (worker number = 1220 individuals/colony (range 230-3650); queen number = 5 queens/colony (range 1-14) in 25 field colonies). The colonies occupy an underground nest containing several chambers close to the soil surface, containing empty cocoon cases and cadavers. Waste dumps with cadavers were also found exterior to the colony, about 15 to 20 cm away from the nest exit (observation by C. Haussy).



Fig. 2 Typical habitat of *Cataglyphis velox* at 1300 m altitude in the Sierra Nevada, close to Grenada, Spain (on the left). Individuals enter and exit the nest through a single tunnel and small exit hole (on the right; diameter ca. 1.5 cm).

With high maximal activity temperature values (44 to 50°C), *C. velox* workers (Fig. 3) are able to forage at temperatures deadly for other insects, scavenging on fresh cadavers of small arthropods and rarely incorporating plant material into their diet (Cerdá and Retana 1997). Thermal tolerance of *C. velox* is size related: the longer legs of large workers allow them to withstand higher temperatures, achieve higher running speeds and shorter foraging durations than smaller workers. In the field, large foragers are most abundant during the hottest hours of the day, whereas smaller and medium foragers scavenge for food in the early and late hours of the day. This division of labor based on worker size enables a longer daily activity period (Cerdá and Retana 1997; Cerdá 2001). Workers of *Cataglyphis* species have been described to follow the classical age-dependent polyethism (reviewed in Lenoir et al. 2009). In *C. cursor* however, only one third of the workers follow the classical age-dependent polyethism, with the remainder showing either fixed behavior or irregular deviations from the expected scheme (Retana and Cerdá 1991).



Fig. 3 *C. velox* forager returning with collected bait (cookie crumb) to the colony. Thermophile foragers scavenge individually for food.

Within a single colony, *C. velox* queens reproduce sexually to produce workers and use parthenogenesis to produce queens (thelytokous parthenogenesis) and males (arrhenotokous parthenogenesis). *C. velox* thus benefits from the advantages of both sexual and asexual reproduction (Pearcy et al. 2004a). Most queens within the colony are essentially clones, they mate with multiple males (generally with 2-5 males) from different genetic lineages (Eyer et al. 2013b), thus increasing genetic diversity in the workforce.

3.5.3 Metarhizium robertsii

Insect parasitic fungi play an important role in agriculture and public health. Especially the entomoparasitic fungi *Metarhizium spp*. (Fig. 4) and *Beauveria bassiana* have been applied as biological pesticides to control insects classified as agricultural pests (e.g. locusts in Africa) or malaria-causing parasites in mosquitoes (reviewed in Thomas and Read 2007). The general insect pathogenic fungus *Metarhizium robertsii* (Bischoff et al. 2009) is a natural pathogen of ants (Rath et al. 1992; Keller et al. 2003; St. Leger et al. 2011) and occurs in soils worldwide (St. Leger et al. 2011). Entomopathogenic fungi invade hosts by direct penetration of the cuticle. When conidiospores of *Metarhizium* adhere to the cuticle, they germinate under high-humidity conditions and penetration occurs by a combination of physical force and cuticlula-degrading enzymes after approximately 24 hours (Clarkson and Charnley 1996; Hajek and St. Leger 1994; Ugelvig et al. 2010).



Fig. 4 A *Lasius neglectus* cadaver without fungal growth and with *Metarhizium* spp.. Hyphae (in white) and cylindrical green spore packages (picture on the right). Picture provided by Martina Klatt.

Inside the hemocoel, *Metarhizium* produces yeast-like blastospores to spread the infection within the insect body and evades insect immune responses by a collagenous protective coat (Wang and St. Leger 2006). The production of fungal toxins (e.g. destruxins) and nutrient depletion weaken the host and usually cause death within several days; the speed of killing depends on parasite dose, the host-parasite combination, environmental conditions (Clarkson and Charnley 1996; Hänel 1982; Hajek and St. Leger 1994; Thomas and Blanford 2003), secondary infections and host defense reactions. Upon the death of the host, the fungus penetrates host tissues and mycelia emerge from the intersegmental regions of the cadaver cuticle under humid conditions (Arthurs and Thomas 2001: optimal sporulation of *M. anisopliae* at relative humidity of >96% and at temperatures between 20 to 30° C). Conidiospore packages are asexually produced on the cadaver (Fig. 4) and passively dispersed in the environment. Under unfavourable conditions (e.g. harsh temperatures), *Metarhizium* can produce thick-walled resting spores (Chlamydospores) which persist in soil for long periods of time (reviewed in Shah and Pell 2003).

CHAPTER 1

Increased grooming after repeated brood care provides sanitary benefits in a clonal ant.

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Abstract Repeated pathogen exposure is a common threat in colonies of social insects, posing selection pressures on colony members to respond with improved disease-defense performance. We here tested whether experience gained by repeated tending of low-level fungus-exposed (Metarhizium robertsii) larvae may alter the performance of sanitary brood care in the clonal ant, Platythyrea punctata. We trained ants individually over nine consecutive trials to either sham-treated or fungus-exposed larvae. We then compared the larval grooming behavior of naive and trained ants and measured how effectively they removed infectious fungal conidiospores from the fungus-exposed larvae. We found that the ants changed the duration of larval grooming in response to both, larval treatment and their level of experience: (1) sham-treated larvae received longer grooming than the fungus-exposed larvae and (2) trained ants performed less self-grooming but longer larval grooming than naive ants, which was true for both, ants trained to fungus-exposed and also to sham-treated larvae. Ants that groomed the fungus-exposed larvae for longer periods removed a higher number of fungal conidiospores

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from the surface of the fungus-exposed larvae. As experienced ants performed longer larval grooming, they were more effective in fungal removal, thus making them better caretakers under pathogen attack of the colony. By studying this clonal ant, we can thus conclude that even in the absence of genetic variation between colony members, differences in experience levels of brood care may affect performance of sanitary brood care in social insects.

Keywords Host-parasite interactions · Sanitary brood care · Experience · Grooming · *Platythyrea punctata · Metarhizium* fungus

Introduction

Organisms benefit greatly from the ability to respond to repeated extrinsic stimuli by improved task performance. Insects are known to gather experience and to be able to learn in different contexts (Dukas 2008), such as foraging (Dukas and Visscher 1994; Ravary et al. 2007) and thermal brood relocation (Weidenmüller et al. 2009). Experience could for instance result in (i) a faster reaction time to the stimulus (e.g., Weidenmüller et al. 2009), (ii) a higher probability to perform the task (e.g., Ravary et al. 2007), and (iii) a more efficient task performance (e.g., O'Donnell and Jeanne 1992; Dukas and Visscher 1994; Raine and Chittka 2007), or a combination of these.

Colonies of social insects (wasps, bees, ants, and termites) are a good model system to study the effects of experience in social groups, as they are characterized by a strong division of labor with different group members specializing on particular tasks (Oster and Wilson 1978). Task specialization, the long-term behavioral bias toward the performance of a task (Oster and Wilson 1978), can be affected by factors such as age (age polyethism; reviewed, e.g., in Hölldobler and Wilson 1990;

Robinson 1992: Mersch et al. 2013) and genetic background (e.g., patriline: Robinson and Page 1988, 1989; Waddington et al. 2010; Schlüns et al. 2011; genetic colony composition: Arathi and Spivak 2001) and seems further modulated by experience (Theraulaz et al. 1998; Ravary et al. 2007). Response-threshold models propose that task specialization within groups arises from inter-individual variation in response thresholds for task-associated stimuli. Individuals with the lowest thresholds most likely become specialists, as they will be the first to respond and thereby decrease the stimulus level present in the nest, so that the higher thresholds of their nestmates will not be met (reviewed in Beshers and Fewell 2001). Furthermore, task performance of the acting individuals can further lower their future reaction threshold, increasing the propensity of future task performance and further increasing specialization (self-reinforcement: e.g., Theraulaz et al. 1998; Weidenmüller 2004; Westhus et al. 2013).

Whereas previous work studied the effect of experience on activities such as foraging, nursing, temperature control, and colony emigration in social insects (e.g., Dukas and Visscher 1994; Langridge et al. 2004, 2008; Weidenmüller 2004; Ravary et al. 2007; Dukas 2008; Weidenmüller et al. 2009; Muscedere et al. 2013; Westhus et al. 2013), it is not well understood how experience in anti-pathogen defense affects the performance and efficiency of sanitary behaviors in the colonies of social insects. Yet, this is an important factor particularly in the long-lived colonies of ants and termites, where individuals likely are repeatedly confronted with the same pathogens within their lifespan of several months to vears (Schmid-Hempel 1998; Schmid-Hempel 2005). Repeated pathogen exposures can occur because individuals pick up the same infectious particles recurrently from their surroundings or by contraction from their sick nestmates inside the colony (Rosengaus and Traniello 1997; Hughes et al. 2002; Cremer et al. 2007; Konrad et al. 2012).

Repeated pathogen exposure may affect both the physiological immune system and the behavioral defense line, which in social insects can occur both at the individual and group level (e.g., Siva-Jothy et al. 2005; Cremer et al. 2007; Wilson-Rich et al. 2009). The physiological immune system of social insects (bumblebees: Sadd and Schmid-Hempel 2006; ants: Rosengaus et al. 2013) shows lower susceptibility to a previously encountered pathogen upon secondary contact (referred to as immune priming in invertebrates; Kurtz and Armitage 2006). At low exposure doses, an infection can even lead to a protective immune upregulation (termites: Rosengaus et al. 1999; ants: Konrad et al. 2012), and such low-level exposures may be contracted during social contact with a diseased nestmate ("social immunization"; Konrad et al. 2012).

Also, the behavioral anti-pathogen defenses of social insects were found to be upregulated after first pathogen exposure. Ant colonies that had previous contact to the fungal pathogen *Metarhizium* show increased expression of collective sanitary behaviors, such as allogrooming (Walker and Hughes 2009; Reber et al. 2011). During allogrooming, social insects efficiently remove infectious particles from the body surface of their nestmates (Rosengaus et al. 1998; Hughes et al. 2002; Yanagawa et al. 2008; Tragust et al. 2013a). To our knowledge, it has not been studied, however, if repeated pathogen encounter of particular individuals changes their propensity to perform sanitary brood care of diseased brood. Brood care is an integral part of sociality in insect societies and is of particular importance in the context of disease defense, as the brood is, on one hand, particularly susceptible to disease (Patterson and Briano 1993) and, on the other hand, very valuable, as it represents the next generation of workers and sexuals in the colony.

Social insects react to pathogen-exposed brood either by intensive brood grooming (ants: Ugelvig et al. 2010, Tragust et al. 2013b), by cannibalism (termites: Rosengaus and Traniello 2001), or by brood removal from the colony, the latter being termed "hygienic behavior" (bees: Rothenbuhler and Thompson 1956; Wilson-Rich et al. 2009; ants: Ugelvig et al. 2010; Tragust et al. 2013b). Whereas little is known for other social insects, such hygienic behavior is performed by middle-aged individuals in honeybees (Arathi et al. 2000). Moreover, the propensity to perform the task depends on the genotype, the genetic colony composition, and is higher in individuals with increased olfactory sensitivity (Arathi and Spivak 2001; Masterman et al. 2001; Gramacho and Spivak 2003; Arathi et al. 2006). In general, hygienic tasks do not seem to be equally performed by all individuals of a colony, but rather by a subset of specialized workers (Trumbo et al. 1997; Arathi et al. 2000). This task specialization promotes behavioral and spatial nest compartmentalization, which is predicted to decrease the risk of pathogen transmission (Schmid-Hempel and Schmid-Hempel 1993; Naug and Camazine 2002; Cremer et al. 2007), yet the factors underlying hygienic task specialization and division of labor are not well understood.

The aim of the current study was to test whether individual experience in sanitary brood care-gained by repeated contact of workers to pathogen-exposed broodmay affect the performance or effectiveness of their hygienic actions. As a model system, we used the parthenogenetically reproducing (thelytokous) ant Platythyrea punctata (Schilder et al. 1999) and the general insect pathogenic fungus Metarhizium robertsii (Bischoff et al. 2009), a natural pathogen of ants (Rath et al. 1992; Keller et al. 2003; St. Leger et al. 2011). We chose this ant species that forms small colonies by clonal reproduction to study the effect of experience on task performance in groups with a low level of division of labor (Hartmann and Heinze 2003) and in the absence of genetic variation among workers. In our experimental approach, we repeatedly confronted ant workers to Metarhizium-exposed or sham-treated larvae. We then compared ants before and after training, i.e., at first contact with the larvae (trial 1) and after nine consecutive contacts of the same type of larvae (all fungus-exposed or all controls; trial 9) to test whether trained as compared to naive ants may show (i) a faster response to the stimulus, (ii) increased grooming performance, and (iii) higher removal of fungal infectious particles, or a combination of these. This experimental design allowed us not only to determine whether experience may affect the performance or efficiency of sanitary brood care in the clonal ant *P. punctata*, but also to disentangle a general effect of repeated brood care (trial 1 vs. 9) from a specific effect of repeated pathogen exposure (interaction between trial and treatment).

Materials and methods

Ant colonies

Colonies of the thelytokous ant *P. punctata* were collected from two populations in Puerto Rico (2005, authorized by the Departamento de Recoursos Naturales y Ambientales DRNA), one from the Dominican Republic (2006, authorized by La Dirección General de Vida Sylvester y Biodiversidad) and one from Barbados (2007, authorized by the Ministry of Agriculture and Rural Development), as detailed in Kellner et al. 2013. Ants were reared in the laboratory under standard conditions (24 ± 2 °C, 60–65 % humidity, 12:12 h light/dark cycle; see Hartmann and Heinze 2003). All ants used in the experiments had eclosed in the laboratory and were therefore naive to the fungal pathogen.

Eight colonies with confirmed clonal colony structure (microsatellite analysis; Kellner and Heinze 2011) were used as source colonies to set up two subcolonies from each. All subcolonies consisted of 15 individually colormarked (enamel paint, PinRestore) adult ants with brood (ten larvae and pupae). The size of these subcolonies lies in the natural range of colony size of this ponerine ant species (Kellner and Heinze 2011). To ensure that only non-reproductive workers were used in the following experiment, the single reproductive individual that established in each subcolony within 2 weeks after set up was determined by observation of egg-laying. It remained within its respective subcolony over the whole course of the experiment. Twenty additional colonies were used as "larval donors" to provide brood for the experiments, as colonies of P. punctata readily accept alien brood (Kellner et al. 2010). We excluded the smallest and largest larval stages, i.e., L1 and L5, respectively, and preferentially used L3 larvae (mean weight 5.01± 1.11 mg standard deviation as revealed from weighing of 116 larvae in a preliminary experiment).

Fungal pathogen exposure

As a pathogen, we used the entomopathogenic fungus M. robertsii (strain ARSEF 2575 (Fang et al. 2006), previously named Metarhizium anisopliae, but now recognized as a sister species (Bischoff et al. 2009)). The non-sexually produced fungal transmission stage (conidiospores/conidia) was freshly harvested from malt agar plates 3 to 4 days before the start of each experiment (Konrad et al. 2012), suspended in the sterile surfactant Triton X-100 (0.05 %; Sigma) at a concentration of 10⁶ conidiospores/ml and the fungal germination rate determined (>97 % in all cases). In the fungus exposure treatment, ant larvae were exposed to 500 conidiospores of the pathogenic fungus by applying 0.5 µl of the conidiospore suspension, while the sham control was treated with the same amount of Triton X solution. Treated larvae were air-dried on sterile plastic for 1 h before start of each experiment. This exposure dose was chosen to (i) provide a stimulus that likely approximates a natural exposure dose from surrounding soil (Keller et al. 2003: 10⁴ colony forming units of *M. anisopliae* per gram soil) and to (ii) prevent tending ants from contracting the disease so we could study behavioral changes based on repeated hygienic brood care experience rather than sickness of the tending workers.

Experimental procedure

We randomly assigned one subcolony per source colony to the sham control and the other subcolony to fungus exposure. From each subcolony, eight intra-nest workers were taken from the brood chamber, isolated from the colony for 1 h, during which they were placed individually in an arena (petri dish, diameter 5.5 cm), where they encountered a larva (a Triton X-treated larva for ants from the control-subcolony and a fungus-exposed larva for ants from the fungus-subcolony). Despite their isolation from the colony, ant workers readily engaged in normal brood care behavior. We repeated this procedure with exactly the same eight ants per colony over five consecutive days, with two trials per day (morning and afternoon training session) on the first 4 days and one on the fifth day (morning session only), always using a new larva and arena, so that each ant was trained for nine consecutive trials (Fig. 1). Trials 1 and 9 could thus be compared without any potential confounding effect of time of day, which is a known factor influencing behavior (Blackmer and Byrne 2008). After each trial, the ants were placed back into their subcolony and left undisturbed until the next trial. We performed the experiments for the two subcolonies (sham control and fungus exposure) of each source colony at the same time and the eight experiments/ colonies in consecutive order within a period of 8 weeks.



Fig. 1 Experimental design. *Platythyrea punctata* ants were individually confronted with either one sham-treated control larva (*blue* group) or fungus-exposed larva (*green* group) in nine consecutive trials (each of 1 h). Behavioral observations were performed for naive ants at their first encounter with the respectively treated larva (trial 1; *light colors*) and for trained ants after repeated encounters (trial 9; *dark colors*). Conidiospore number of fungus-exposed larvae was counted after trial 1 and trial 9 to test for changes in conidiospore removal capacity of the ants due to experience

Behavioral observations

"Naive ants" (trial 1) and "trained ants" (trial 9) were video-recorded immediately after the larvae were placed into the arena containing the ants (Logitech QuickCam Sphere AF). Videos were analyzed blindly with respect to larval treatment, using the software BioLogic (http:// sourceforge.net/projects/biologic/). We determined the time to first contact (mostly antennation behavior) of the ant to the larva, as well as the time to first performance of sanitary brood care (larval grooming; Ugelvig et al. 2010) after the start of the experiment. We also determined the frequency (number of events/hour) and duration of larval grooming and worker self-grooming during the complete hour of the trial. We did not detect any trophallaxis behavior between the adult ants and the larvae. In very rare cases, ants also showed mandible-opening behavior, which is generally classified as slightly aggressive behavior. Due to its rareness, it was not included in the analysis.

Conidiospore removal

To determine how many of the originally applied 500 conidiospores were removed by the ants during grooming, we washed off conidiospores from the surface of fungusexposed larvae (as in Hughes et al. 2002) after the 1-h contact to either naive (trial 1) or trained (trial 9) ants and counted them. To account for potential differences in fungal attachment to the larval cuticle (Vestergaard et al. 1999), we determined the baseline number of conidiospores that could be washed off from additional fungus-exposed larvae for each source colony after 1 h in the absence of any ants, both at day 1 (trial 1) and day 5 (trial 9). Washes were performed by vortexing each larva for 2 min in 50- μ l Triton X solution. After larval removal, the conidiospore suspension was concentrated to 10 μ l and the number of conidiospores determined by counting five droplets of 1 μ l each per sample under a stereomicroscope (at × 400 magnification).

Disease contraction by adult ants

At the end of the experiment, we determined whether adult ants had contracted the disease during contact with the larvae. To this end, all ants were killed by freezing for 6 min at -20 °C, which does not affect conidiospore germination (M. Klatt and CW unpublished data). To check for internal fungal infection, each ant was surface-sterilized (Lacey and Brooks 1997) and monitored for outgrowth of *M. robertsii* hyphae and conidiospores over 3 weeks under humid conditions at 24 °C.

Data analysis

We used the program R (v. 2.12.1) for all statistical analyses.

Behavioral observations We analyzed a mean of five (2-8) ants per colony and treatment, at both their first and ninth trial, to compare the same ant in its naive and trained state (total of 80 ants, 40 of which were trained to sham-treated larvae and 40 to fungus-exposed larvae; total 160 h of observations). Ninety-three percent (74/80) of the ants (37/40 in both groups) had contact to the larva in both trials 1 and 9 of the experiment, and only these ants were subjected to further statistical analvsis. We compared the occurrence of larval grooming performed by naive and trained ants toward sham-treated and fungus-exposed larvae with Fisher exact tests and corrected the significance level α to 0.025 due to multiple testing (Bonferroni correction). We analyzed the time to first larval contact and the time delay between first contact and first grooming using survival analysis (Cox proportional regression, generating Wald statistics), as data were censored (R package "survival" (Therneau 2012)). Larval treatment (sham control vs. fungus exposure) and experience of the ants (trial 1 vs. 9) were included as fixed effects, with individual ants nested within their source colony as random effect using the frailty function in coxph. Non-significant interaction terms were removed from the final models. For behavioral frequencies and durations, we performed linear mixed-effects models (LMM) to analyze the effects of treatment (sham control vs. fungus exposure) and experience (trial 1 vs. 9) as fixed effects and their interaction, with individual ants nested within their source colony as random effect, thereby controlling for repeated measures of the same individual (R package "nlme" (Pinheiro et al. 2012)). Data either showed normal error distribution and homogeneity of variance or reached it by log(x+1)-transformation (applied for larval grooming frequency and frequency and duration of worker self-grooming). From the full model, the significance of the interaction and each fixed factor were tested by removing the factor of interest

(likelihood-ratio test) to obtain a minimal adequate model only containing significant terms.

Conidiospore removal We tested for a potential difference in the basal fungal attachment rates in the absence of ants in trials 1 and 9 by performing a LMM with trial as fixed factor (trial 1: n=31, trial 9: n=37) and source colony as a random effect. Following, we determined whether the number of conidiospores washed off from fungus-exposed larvae depended on experience (trial 1 vs. 9) and grooming duration performed by the ants during the respective trial (n=24 ants,each in trial 1 and 9), by performing a LMM including trial as a fixed factor and grooming duration as covariate and individual ants and source colony as random effect. Conidiospore number was log-transformed to obtain a normally distributed error structure and homogeneity of variance. For display, we show the number of conidiospores removed by grooming (i.e., the applied 500 minus the count value of conidiospores still to be washed off after the experiment).

The data sets supporting the results of this article are available in the DRYAD repository (doi:10.5061/dryad. NNNNN).

Results

Performance of sanitary behavior

Occurrence of sanitary brood care Ninety-two percent of the ants that had larval contact performed larval grooming (trial 1: sham control: 36/37, fungus exposure: 32/37; trial 9: sham control: 36/37, fungus exposure: 33/37), with no difference between larval treatments (Fisher exact test with α -level of 0.025; sham control vs. fungus exposure: P=0.056) or experience level of the ants (trial 1 vs. trial 9: P=1.000).

Time to response Naive and trained ants (trials 1 and 9) of both treatment groups (sham control and fungus exposure) first approached larvae approximately 6 min after start of the experiment (Fig. 2a). Hence, there was no significant effect of treatment or experience in the time to first contact (Cox regression, treatment: Wald $\chi^2=0.71$, df=1, P=0.400; trial: Wald $\chi^2=0.33$, df=1, P=0.560). Ants of all groups then started grooming approximately another 6 min after the first contact (Fig. 2b). There was thus also no effect of larval treatment or experience of the ant on the delay period between the first contact to the larva and the first grooming (treatment: Wald $\chi^2=0.30$, df=1, P=0.440).

Frequency and duration of larval grooming The frequency of larval grooming did differ neither across treatments (sham control vs. fungus exposure) nor between naive and trained ants (trial 1 vs. 9; LMM: treatment: $LR_{1,4}=2.807$, P=0.094; trial: $LR_{1,5}=0.118$, P=0.731; interaction treatment*trial: $LR_{1,6}=0.0003$, P=0.987; Fig. 3a). Grooming duration, however, was significantly affected by larval treatment (LMM: $LR_{1,5}=6.941$, P=0.008) with sham-treated larvae being groomed longer than fungus-exposed larvae. Moreover, trained ants groomed the larvae longer than naive ants, such that ants in the sham control increased larval grooming duration from trial 1 to 9 by 14 % and ants in the fungus treatment even by 32 % (Fig. 3b; $LR_{1,5}=4.473$, P=0.034). There was no significant interaction between treatment and trial ($LR_{1,6}=0.253$, P=0.615).

Frequency and duration of worker self-grooming The frequency of worker self-grooming was significantly affected by trial (LMM: trial: LR_{1.4}=19.942, P<0.0001) with naive workers self-grooming more frequently than trained ants, irrespective of larval treatment (LMM: $LR_{1,5}=0.041$, P=0.840), with no significant interaction between treatment and trial (LR_{1,6}=0.715, P=0.398; mean±SE in events/ hour; sham control: trial 1 56.6 \pm 3.5, trial 9 35.6 \pm 3.6; fungus exposure: trial 1 57.9±6.8, trial 9 34.2±2.9). Similarly, self-grooming duration was significantly affected by trial (LMM: LR_{1.4}=4.683, P=0.031) with naive workers self-grooming longer than trained ants but remained unaffected by larval treatment (LMM: LR_{1.5}=0.112, P= 0.738). Again, no significant interaction was found between treatment and trial (LR_{1.6}=0.718, P=0.397; mean± SE in min; sham control: trial 1 5.9 ± 0.7 , trial 9 4.0 ± 0.5 ; fungus exposure: trial 1 5.6 \pm 0.8, trial 9 4.6 \pm 0.7).

Conidiospore removal

We found that the basal attachment rate of the fungal conidiospores to the larvae, as measured in the absence of any workers, showed a non-significant trend of being higher in trial 9 vs. trial 1 (LMM: LR_{1,4}=3.712, P=0.054). Despite this tendency toward stronger fungal attachment, trained ants removed a significantly higher number of conidiospores from the fungus-exposed larvae than naive ants (mean±SE of proportion conidiospores removed in trial 1 63.1±0.07 % and trial 9 76.2±0.05 %; LMM: LR_{1.5}=4.243, P=0.039). Moreover, ants expressing longer larval grooming duration removed a higher number of conidiospores than ants that groomed the larvae shorter (Fig. 4; LMM: LR_{1,5}= 20.937, P < 0.0001). There was no significant interaction between the experience of the ants (trial 1 vs. 9) and their grooming duration (LR_{1,6}=0.098, P=0.754), indicating that trained ants did not remove more conidiospores per unit time than naive ants.



Fig. 2 Time to response of brood care. Neither **a** the time to first contact of the ants to the larvae after start of the experiment nor **b** the delay between the first contact and performance of sanitary brood care (larval grooming) depended on larval treatment (sham control: *blue*; fungus

Disease contraction by adult ants

None of the ants tending fungus-exposed larvae (n=40) showed any fungal outgrowth of *M. robertsii* after surface sterilization, and the same was true for the ants tending sham-treated larvae (n=40). We could therefore not detect any signs of internal infections of ants handling fungus-exposed brood.



Fig. 3 Frequency and duration of larval grooming. **a** Larval grooming frequency did differ neither between naive and trained ants (trial 1: *light colors*, trial 9: *dark colors*) nor across larval treatments (sham control: *blue*; fungus exposure: *green*). **b** Larval grooming duration, however, was significantly higher toward sham-treated (*blue*) than fungus-exposed (*green*) larvae and was performed significantly longer by trained (trial 9, *dark colors*) than naive (trial 1, *light colors*) ants. *Bars* depict mean \pm SEM



exposure: green) or experience of the ants (naive ants in trial 1: light colors, trained ants in trial 9: dark colors). The numbers of cases across all replicates are shown as a heatmap with respective median values indicated as square in the color of the group

Discussion

We tested whether repeated contact to either sham-treated or fungus-exposed larvae would affect the performance of sanitary brood care in the clonal ant P. punctata. The experience of individual ants did not affect their probability or time to respond. Independent of their experience and larval treatment, ants thus (i) were equally likely to contact and groom the larvae, (ii) showed no difference in time to detection (first contact; Fig. 2a) and reaction (first contact to first grooming; Fig. 2b), and (iii) performed larval grooming at equal frequencies (Fig. 3a). However, we found a plastic response for the duration of grooming behavior. Ants groomed sham-treated larvae longer than fungus-exposed ones and trained ants in their ninth trial performed longer grooming than naive ants in both treatments (Fig. 3b), thereby removing a larger number of infectious conidiospores from the brood (Fig. 4). Repeated brood care therefore led to a more effective anti-pathogen defense in P. punctata. Yet, this increased sanitary brood care came along with reduced individual worker hygiene, as trained ants performed less self-grooming.

Our results contrast to previous findings in *Camponotus* ants, where experience led to a faster response in brood



Fig. 4 Conidiospore removal depending on larval grooming duration. Both naive (trial 1, *light green*) and trained ants (trial 9, *dark green*) removed significantly increasing numbers of conidiospores from the fungus-exposed larva with increasing larval grooming duration. Trained ants removed significantly more fungal conidiospores from the larvae than naive ants. *Trend lines* are shown for both groups (trial 1 and trial 9) in the respective *color*

thermoregulation (Weidenmüller et al. 2009). Yet, they are in line with previous studies on *P. punctata* and other ants that whilst not testing for experience but reaction to first pathogen contact—also found that ants did not differentiate between healthy and contaminated larvae during transport of larvae into their brood chamber (Ugelvig et al. 2010; Tragust et al. 2013b), even if the larval exposure dose used in the current experiment was thousandfold lower. Whereas it is known that social insects can contract *Metarhizium* infections from highdose-exposed nestmates (Rosengaus and Traniello 1997; Hughes et al. 2002; Konrad et al. 2012), none of the ants in our low-dose experiment showed any signs of infection with *M. robertsii* or died from the disease.

Many studies report an upregulation of grooming in response to pathogen exposure (adults: Rosengaus et al. 1998; Jaccoud et al. 1999; Walker and Hughes 2009; Reber et al. 2011; brood: Ugelvig et al. 2010; Tragust et al. 2013b; fungus garden: Currie and Stuart 2001). The lower grooming of fungus-exposed brood was therefore unexpected, particularly since P. punctata ants do show increased grooming of Metarhizium-exposed brood when the applied exposure dose is thousandfold higher and the ants are in a colony context rather than in isolation (Tragust et al. 2013b). This suggests that the reduction in grooming toward fungus-exposed compared to sham-treated larvae in our experiment likely is a result of either the low exposure dose or the fact that ants have been removed from their colony context to be trained individually. Our experimental setting may thus have rather reflected the situation of an ant encountering the larvae outside of the nest, where avoidance of infectious items is common in social insects (Epsky and Capinera 1988; Diehl-Fleig and Lucchese 1991; Mehdiabadi and Gilbert 2002; Fouks and Lattorff 2011).

Despite these different basal grooming levels in the two treatments, brood grooming duration significantly increased by repeated brood care for both the sham-treated larvae (by 14 %) and the fungus-exposed larvae (by 32 %; Fig. 3b), whilst worker self-grooming was reduced. The absence of a significant interaction between trial and treatment for grooming duration indicates that both treatment groups reacted similarly to experience and that the presence of a pathogen neither caused nor interfered with prolonged brood grooming. It thus seems that experienced ants prophylactically upregulate their expression of sanitary brood care at the expense of individual hygiene. It was shown that ant colonies that have previously encountered Metarhizium fungi increase allogrooming toward adult nestmates that are either shamtreated (Reber et al. 2011) or fungus-exposed (Walker and Hughes 2009) and fungus-growing ants whose fungus garden has experienced pathogen threat increase self-grooming (Morelos-Juárez et al. 2010). The novelty of our study is that P. punctata ants intensify brood grooming after repeated brood care even in the absence of any previous pathogen encounter, thereby deviating from *Pheidole* ants, where nursing-experienced individuals did not perform more brood care than their naive nestmates (Muscedere et al. 2013).

While we can exclude that the presence of the fungal pathogen M. robertsii at the used low exposure level triggered the prolonged larval grooming by experienced ants, we cannot disentangle whether the intensified grooming may have been caused by repeated confrontation with the brood per se or with the detergent Triton X. The detergent is required to bring the hydrophobic fungal conidiospores into suspension and was thus the closest control to our fungus-exposure treatment. Some studies have reported that treatment with a detergent elicits the same grooming intensity as fungus exposure (Graystock and Hughes 2011; Reber et al. 2011; Tragust et al. 2013b; but see, e.g., Rosengaus et al. 1998; Ugelvig and Cremer 2007; Walker and Hughes 2009; Ugelvig et al. 2010; Tragust et al. 2013b), suggesting a potential effect of the detergent on the performance of sanitary behaviors. Intensified grooming in our experiment could thus be the result of either repeated nursing itself or repeated contact to a potentially irritating compound that may even act as a "danger signal" (Matzinger 1994), causing potential damage to the host cells through its detergent properties. Our study moreover revealed that fungal attachment might be affected by the storage time of the conidiospore suspension, highlighting that this should be strictly controlled for in experimental work. The cause may be an increase in RNA and protein synthesis during soaking, leading to faster swelling and germination when later coming in contact with the insect cuticle (Dillon and Charnley 1985; Hassan et al. 1989; Dillon and Charnley 1990).

As expected from previous work (Rosengaus et al. 1998; Hughes et al. 2002; Yanagawa et al. 2008; Tragust et al. 2013a), ants removed higher conidiospore numbers when grooming the larvae longer (Fig. 4). Experienced ants, which increased larval grooming duration (Fig. 3b), thus removed the fungal pathogen more effectively than naive ants. Such improved performance through experience is not self-evident, given that, e.g., honeybee undertakers did not improve in cadaver removal with experience (Trumbo and Robinson 1997) and brood-care-experienced Pheidole dentata ants were not more efficient nurses (Muscedere et al. 2013). Higher conidiospore removal by brood-care-experienced individuals is likely key to reduce the infection probability of exposed larvae (as is known for adults; Walker and Hughes 2009), as well as the transmission of the pathogen within the colony. Furthermore, social immunity, i.e., sanitary care by nestmates, can be beneficial for the helper, as it can bestow a survival benefit upon future exposure to the same pathogen (Traniello et al. 2002; Ugelvig and Cremer 2007; Hamilton et al. 2011; Konrad et al. 2012).

We can conclude that repeated brood care in *P. punctata* ants modulates their brood care activities, increasing their potential to fight fungal disease. As experience-dependent
behavioral modulation is reversible and for instance depends on the time delay between task performances (Plowright and Plowright 1988; Theraulaz et al. 1998; Westhus et al. 2013), absence of stimuli could again lead to decreased intensity of brood care. Such experience-modulated expression of sanitary brood care likely allows colonies to respond flexibly to incoming pathogens. Response threshold models postulate that inter-individual variation in response thresholds for taskassociated stimuli can lead to division of labor in the colony and individuals with lower response thresholds are more likely to become specialists for the task (reviewed in Beshers and Fewell 2001). Task specialization (i.e., the tendency of some workers to perform tasks more frequently or longer than their nestmates) may be more pronounced in larger ant societies (Bourke 1999; Thomas and Elgar 2003; Jeanson et al. 2007) and also in societies with higher genetic diversity (reviewed by Oldroyd and Fewell 2007). Yet, already in the absence of large colonies and genetic variation, as in the studied clonal ant P. punctata, repeatedly performed brood care makes ants better caretakers and may promote divergence between group members.

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Ethical standards The performed experiments comply with European laws.

Conflict of interest The authors declare that they have no conflict of interest.

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CHAPTER 2

Necrophoresis is not everything: cadaver groomings and intranidal transports in the ant *Cataglyphis velox*.

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In preparation

TITLE

Necrophoresis is not everything: cadaver groomings and intranidal transports in the ant *Cataglyphis velox*.

ABSTRACT

A major cost of social life is an increased exposure to parasites, leading to the evolution of sophisticated social immune defenses, which complement the physiological immune system of social insects. Decomposing cadavers pose a special danger to eusocial societies, as they exist in densely populated, enclosed and perennial nests with a high genetic proximity between individuals. To prevent parasite establishment within them, social insects evolved cadaver management behaviors, including cadaver avoidance, grooming, carrying, burial and necrophoresis (the removal of cadavers from the nest). Workers managing cadavers might be at higher risk of contracting disease due to potential parasite contact and leaving the confines of the nest. In this study I investigated whether there is division of labor for cadaver management and whether task allocation is affected by recent individual experience and worker size in the thermophilic ant Cataglyphis velox. Recurrent introduction of four nestmate cadavers of different size into nests of cadaver groups in ten trials demonstrated that nestmates were significantly skewed in the performance of cadaver management with few individuals dominating task performance. Many task performers performed the behaviors only once over all trials. Individuals performing one type of cadaver management were also more likely to perform the other and cadaver transporters showed a high propensity to also engage in foraging. Cadaver size did not modulate cadaver management behaviors. The repeated occurrence of cadavers did not affect the number and duration of groomings and transports a cadaver received, but lead to an increasing number of grooming individuals per cadaver. When reunited with less experienced individuals, the probability of experienced individuals to groom and transport a cadaver was increased in only one out of four colonies. Together, these results suggest low division of cadaver management between nestmates and a reduced modulation of these behaviors by recurrent cadaver exposure.

INTRODUCTION

Division of labor is a key feature of social insects and largely contributes to their ecological success (Oster and Wilson 1978; Hölldobler and Wilson 1990). It occurs when individuals specialize on different colony tasks, such as foraging and brood care. A central question about division of labor is how individuals decide what task to execute (Gordon 1996). The concept of self-organization assumes that division of labor is an emergent property of interacting individuals which obey simple behavioral rules (Bonabeau et al. 1997; Page and Mitchell

1998; reviewed e.g. in Theraulaz et al. 2003; Duarte et al. 2011). Social insect workers are assumed to engage in a task when the task-associated stimulus exceeds their internal response threshold (response threshold models reviewed in Beshers and Fewell 2001) and thresholds differ among nestmates (e.g. Bonabeau et al. 1996; Weidenmüller 2004; Duong and Dornhaus 2012). Inter-individual variability in response thresholds can arise through several factors, such as genotype (Pankiw and Page 1999; Kryger et al. 2000; Masterman et al. 2001; Jones et al 2004; Scheiner and Arnold 2010), age (Pankiw and Page 1999), rearing conditions (Weidenmüller et al. 2009) and experience (e.g. Plowright and Plowright 1988; Theraulaz et al. 1998; Weidenmüller 2004; Westhus et al. 2013) and enables a graded and flexible colony response adjusted to current task needs. Individuals with the lowest response thresholds for a task are assumed to engage in task performance more frequently and thereby become task specialists (e.g. Theraulaz et al. 1998), reducing stimulus levels so that response thresholds of their nestmates will not be met. With increasing stimulus levels (due to more frequent or bigger task loads), task specialists become insufficient to reduce stimulus levels and individuals with higher response thresholds will also engage in task performance (Beshers and Fewell 2001). Task specialization presumably increases colony-level efficiency by reducing costs associated with task switching (Wilson 1976; Jeanne 1986; Goldsby et al. 2012) and improving task performance through learning (O'Donnell and Jeanne 1992; Langridge et al. 2008; Chittka and Muller 2009; but see: Trumbo and Robinson 1997; Dornhaus 2008).

Experience is an additional factor which can lead to short- or long-term modulation of individual behavior and thereby affect division of labor within the colony (e.g. Plowright and Plowright 1988; Theraulaz et al. 1998; Weidenmüller 2004; Ravary et al. 2007; Jeanson and Weidenmüller 2013). Workers can acquire experience either passively through repeated exposure to a stimulus, or actively through performance of the corresponding task. The acquired experience can then either modulate task performance itself (i.e. behavioral speed, efficiency, precision or location) and/or affect individual responsiveness towards a taskassociated stimulus (Jeanson and Weidenmüller 2013). Parameters of individual responsiveness comprise an individual's response threshold, its probability to respond once the threshold is reached, the time an individual takes to respond, the duration of response and the intensity (or efficiency) of the response, which can be independent processes (Weidenmüller 2004; Weidenmüller et al. 2009; Jeanson and Weidenmüller 2013). Successful task performance can provide a positive feedback factor, leading to selfreinforcement and increasing the probability of an individual to repeat the task, thereby promoting specialization (e.g. Plowright and Plowright 1988; Theraulaz et al. 1998). In the ants Temnothorax albipennis and Cerapachys biroi, foraging success increased the probability of individuals to repeat this task (Ravary et al. 2007; Robinson et al. 2012) and thereby impacted colony organization (Ravary et al. 2007). Theoretical models predict a modulation of response thresholds through recent experience ('threshold reinforcement'; Plowright and Plowright 1988; Theraulaz et al. 1998), but empirical evidence is scarce and conflicting (Jeanson and Weidenmüller 2013). Whereas fanning response thresholds decreased with repeated task performance in thermoregulating workers of the bumblebee *Bombus terrestris* (Weidenmüller 2004; Westhus et al. 2013), a modulation which depends on the time interval between task performances (Westhus et al. 2013), fanning response thresholds were not affected by experience in the bumblebee *Bombus impatiens* (Duong and Dornhaus 2012).

A major cost of social life is an increased exposure to parasites due to geographic and genetic proximity between frequently interacting individuals in enclosed and perennial nests under homeostatic conditions (e.g. Shykoff and Schmid-Hempel 1991; Rosengaus and Traniello 1997; Schmid-Hempel 1998; Cremer et al. 2007; Fefferman et al. 2007). To counter the selection pressure imposed by parasites, social insects evolved sophisticated group-level defenses which comprise behavioral, spatial and physiological mechanisms, complementing the individual physiological immune system (i.e. 'social immunity': Cremer et al. 2007; Wilson-Rich et al. 2009; Evans and Spivak 2010; Stroeymeyt et al. 2014). Some defense mechanisms are prophylactic to avoid the establishment of parasites within the nest, such as the incorporation of antimicrobial plant resin into nests of ants and honeybees (Christe et al. 2003; Chapuisat et al. 2007; Simone et al. 2009) or cadaver management. Individuals which have died of age or from infection within the nest pose a sanitary risk to nestmates, as they might grow bacteria and fungi under the often warm and humid nest conditions. Waste material is harmful to leaf-cutting ants and their mutualistic fungus (Fisher et al. 1996; Bot et al. 2001; Hughes et al. 2004; Brown et al. 2006) and Myrmica rubra ant workers suffer increased mortality if they cannot remove their dead (Diez et al. 2014). Social insects thus evolved cadaver management behaviors (reviewed in Sun and Zhou 2013), such as cadaver avoidance (e.g. Kramm et al. 1982; Franks et al. 2005), grooming (Wilson et al. 1958; Visscher 1983; Neoh et al. 2012), intranidal transport (Wilson et al. 1958; Visscher 1983; Sun et al. 2013), burial (Renucci et al. 2010; Sun et al. 2013), cannibalism (especially common in termites: Neoh et al. 2012) and necrophoresis (also called 'undertaking', i.e. the removal of cadavers from the nest). Necrophoresis, which is most prevalent in ants and honeybees, has been studied since over 50 years (e.g. in ants: Wilson et al. 1958; Gordon 1983; Ataya and Lenoir 1984; Julian and Cahan 1999; in honeybees: Visscher 1983; Trumbo et al. 1997; Breed et al. 2002). Research has focused on the description of the behavior, on death recognition cues and task allocation, mainly in highly evolved social insect species with large colony sizes, such as honeybees and leaf-cutting ants. Less information is available on cadaver-management behaviors which occur before cadavers are either rejected from or buried within the nest.

Behavioral processes directed towards the dead differ between social insect species, depending on nest ecology and feeding habits (Neoh et al. 2012). But plasticity in cadaverdirected behaviors also exists within a single colony, depending on the quantity of the cadavers present (Breed et al. 2002), the cadavers' origin (nestmate or foreign; Ataya and Lenoir 1984; Renucci et al. 2010; Diez et al. 2013b), and the associated sanitary risk (e.g. decomposition time, the presence of harmful microorganisms; Neoh et al. 2010; Renucci et al. 2010; Fan et al. 2012; Sun and Zhou 2013). Recognition of dead individuals appears to be achieved through chemical cues (necromones), either accumulating decomposition products (e.g. oleic acid: Wilson et al. 1958; Haskins and Haskins 1974; Gordon 1983; Lopez-Riquelme et al. 2006; Diez et al. 2013b) or disappearing cues showing the vitality of the individual (dolichodial and iridomyrmecin: Choe et al. 2009).

Division of labor for sanitary tasks can be advantageous, as it increases behavioral and spatial compartmentalisation and can, together with heterogeneity in the interaction network, decrease parasite transmission among workers (Schmid-Hempel and Schmid-Hempel 1993; Schmid-Hempel 1998; Hart and Ratnieks 2001; Naug and Camazine 2002; Pie et al. 2004; Cremer et al. 2007). But how colonies allocate risky sanitary tasks such as cadaver management is not well understood. Sanitary tasks vary in the pathogenic exposure risk of the task-performing individual, in the frequency with which they occur (i.e. the time interval between task performances; Diez et al. 2013a), in their workload (e.g. the quantity of cadavers which occur at the same time; Diez et al. 2013a) and their motoric complexity (i.e. whether an individual is able to transfer motor skills from a non-sanitary task) or in the level of physiological adaptations required for task performance. We expect stronger division of labor between nestmates for tasks which occur frequently, involve a big workload (Diez et al. 2013a) and a high exposure risk or require physiological specializations of task performing individuals. Division of labor is further expected to increase with genetic diversity of the workforce (reviewed in Oldroyd and Fewell 2007), colony size (Bourke 1999; Thomas and Elgar 2003; Jeanson et al. 2007) and spatial complexity (Tofts and Franks 1992; Bourke and Franks 1995) which determines at what rate and in which intensity nestmates are exposed to stimuli. Some of these conditions are found in colonies of the highly evolved leaf-cutting ants and honeybees, for which worker specialization on necrophoresis and waste management has been reported and seems to be influenced by age, size and genotype of individuals (e.g. Visscher 1983; Robinson and Page 1988, 1995; Trumbo et al. 1997; Julian and Cahan 1999; Julian and Fewell 2004; Ballari et al. 2007). Waste workers of leaf-cutting ants are not only spatially segregated, but also socially isolated, which is reinforced through aggressive interactions (Hart and Ratnieks 2001; Ballari et al. 2007). To my knowledge, no reports on cadaver management specialization exist to date in termites (Sun and Zhou 2013) and how cadaver management is allocated among workers in "more basic" ant species remains to be investigated (but see Diez et al. 2013a). It is also not well understood how experience in sanitary tasks affects the performance of sanitary behaviors in social insect colonies.

Experience could improve sanitary task performance (e.g. Walker and Hughes 2009; Reber et al. 2011; Westhus et al. 2014) by (i) reducing individual response thresholds (enabling individuals to react sooner towards a sanitary threat), (ii) increasing their response probability (making it more likely for them to perform the sanitary task once their response threshold is reached), (iii) leading to a faster transition from perception of the necrophoric stimulus to the

final response, (iv) increasing response duration (Westhus et al. 2014) or through (v) improved motoric capacities (e.g. more efficient grooming of fungal spores or preening of nematodes). In the leaf-cutting ant Acromyrmex echinatior, a previous encounter with a pathogenic fungus resulted in individuals being more likely to allogroom re-introduced fungus-exposed nestmates, thereby increasing their likelihood of survival, compared to controls in naïve nests (Walker and Hughes 2009). Repeated brood care resulted in longer grooming duration and thereby higher removal of a pathogenic fungus in the ant *Platythyrea* punctata (Westhus et al. 2014). The previously mentioned behavioral modifications by experience could increase the performance of cadaver management by experienced individuals, leading to individual specialization and division of labor for the management of cadavers. These individuals might also become more efficient in cadaver management through repeated task performance. Individuals specialized on necrophoric behavior removed cadavers faster and more successfully than less experienced individuals (Apis mellifera: Trumbo and Robinson 1997; Acromyrmex versicolor: Julian and Cahan 1999), but an effect of experience was not demonstrated (Trumbo and Robinson 1997). However, if the occurrence of intranidal cadavers is rare and infrequent, which is expected in healthy colonies (Diez et al. 2013a), only a short-term modulation of cadaver management is expected. Indeed, in the ant Myrmica rubra, short-term spatial memory of necrophoric individuals was demonstrated within a time frame of 7 minutes (Diez et al. 2011). As the modulation of response thresholds depends on the time interval between task performances (Theraulaz et al. 1998; Westhus et al. 2013), increased time intervals will presumably not lead to self-reinforcement when the task is successfully performed. Even though cadaver management presumably occurs with low frequency within a healthy colony, the sanitary risk imposed by cadavers (e.g. Diez et al. 2014) might have a strong impact on colony fitness, especially under parasitic attack.

I studied cadaver management in the ant *Cataglyphis velox*, Santschi, 1929. Intranidal cadaver management could be of importance for these ants inhabiting extreme thermal environments (maximal activity temperature 46 °C: reviewed in Cerdá 2001; reviewed in Lenoir et al. 2009) with increased extranidal mortality risk. Additionally, their diet consisting of fresh cadavers of small arthropods (Cerdá and Retana 1997) might lead to increased cadaver management behaviors. This species further offers polygynous colonies, allowing the establishment of queenright subcolonies for empirical studies. Workers are highly polymorphic, enabling division of labor in foraging with the biggest workers foraging during the hottest hours of the day (Cerdá and Retana 1997; Cerdá 2001). *Cataglyphis* species generally show division of labor based on age, with workers passing from intranidal tasks when young to foraging when older (reviewed in Lenoir et al. 2009).

I studied necrophoresis (i.e. the removal of cadavers from the nest) but also cadaver grooming and intranidal transports of cadavers, which have rarely been addressed in current literature. Nestmate cadavers present a natural stimulus and stimulus quantity can easily be manipulated. My objectives were: (i) to determine whether division of labor exists for cadaver management within the colony by providing eight cadavers per day over a period of 5 days, (ii) to evaluate the relative influence of both worker and cadaver size on cadaver management and (iii) to test whether worker experience affects cadaver management by comparing workers from subcolonies that have been differentially exposed to cadavers. I predicted that if cadaver management were a result of stimulus exposure (i.e. opportunity) or recent task performance, individuals from subcolonies which were recently exposed to cadavers would be more likely to perform cadaver management than individuals from subcolonies without this exposure. Moreover, bigger cadavers might provide a stronger stimulus (e.g. because of an increased surface area), potentially eliciting stronger cadaver management behaviors and faster removal from the nest.

MATERIALS AND METHODS

Laboratory colonies

Four polygynous colonies of *Cataglyphis velox* were collected from a Sierra Nevada population near Grenada, Spain in May 2011 (37°08' N, 3°29' W, 1300 meters altitude). Colonies occupied an underground nest containing from 700 to 1900 workers and six to 11 queens. They were reared in a climate chamber under LD 12 : 12 h photoperiod, at 35-50% relative humidity and at a mean temperature of 28°C (24.4-29.9°C) over a period of 2 months and fed 3 times/week with a mixed diet of frozen crickets, live meal worms, fruit (apple, orange, banana), and *ad libitum* sugar and water. Except cadaver obtention, experimental steps were performed in the same climate chamber.

Establishment of subcolonies

Eight weeks after collection, two subcolonies were established from each of four stock colonies (four treatment replicates). Subcolonies contained one queen, 100 workers and few pupae. In *C. velox*, workers exhibit strong and continuous size variation from 4.5 to 12 mm, but can be grouped into four size classes for experimental convenience (Tinaut 1990; Cerdá and Retana 1997). I kept the proportion of each size class constant in the eight subcolonies based on their mean proportion estimated from three different stock colonies (very small workers: 23%; small workers: 25%; medium workers: 32%; large workers: 20%). These proportions were similar to the ones detected by Cerdá and Retana (1997). At the time of subcolony establishment, workers of the two subcolonies were individually marked (Uni Paint Marker) with a unique color combination on the thorax and gaster. Very small workers (4.5 - 6 mm body length) could not be color-marked due to practical limitations such as difficulties of color identification on computer screens and during scans as well as successful color removal by ants when color spots were very small. Even though the smallest workers were too small for individual observations but still comprised 23% of the colony, they were

deliberately kept in the colony to not disturb worker size distribution and division of labor within the group.

The eight subcolonies were maintained in plastic boxes $(37 \times 27 \times 11 \text{ cm})$ lined with plaster and coated with fluon, containing a plastered petri-dish nest (diameter 9 cm) allowing direct observations of intranidal activities. Four holes were drilled through the top of the petri dish nest to introduce cadavers in the nests with minimal nest disturbance during the experiment. Subcolonies of the same stock colony were randomly assigned to either control (without cadavers) or cadaver group.

Experimental procedure

The experiment was divided into two steps (Fig. 1). The **first step** of five days consisted of two trials per day, one in the morning and one in the afternoon (with at least 1.5 hours between the morning and afternoon trial). At the beginning of each trial, I placed four cadavers (one of each size class, originating from the same stock colony) into the nest of the *cadaver groups*. The cadavers were inserted in the nest centre through holes in the petri-dish cover using forceps. To control for potential stress effects due to forceps insertion, the same forceps were similarly inserted into the nest of the *control groups* but without the introduction of cadavers. Nest areas of cadaver groups were filmed from cadaver insertion until all cadavers had been taken out, or until a maximum filming duration of three hours (Sony HDR-XR520VE camera). After the last daily trial, cadavers were removed from the foraging arena of cadaver groups to avoid unobserved cadaver transport within the foraging arena. The subcolonies were observed after food was given for 10 minutes after the last trial of each experimental day and during one week after subcolony establishment (in total 9-12 times per group) to identify foragers.



Figure 1: Experimental design. *Cataglyphis velox* colonies were split into cadaver and control group of 100 individuals each. The first experimental step started 2.5 weeks after splitting and consisted of 5 days with two trials per day. In each trial, four cadavers were inserted into the nest of the cadaver group. At the same time, forceps were inserted into the nest of the control group to control for colony disturbance. During the second step, the subcolonies were reunited on day 6 of the experiment and ants present within the foraging arena recorded the next day. On day 8 of the experiment, eight cadavers were inserted into the nest of the reunited colonies in two trials (four in each trial).

The **second step** was to reunite the control and cadaver groups of the same stock colony. Before reunification, I placed them into a refrigerator at 4°C for 1.5 hours to decrease aggression towards introduced ants. For two colonies (B and C), I transferred the control group into the box of the cadaver group. The reverse was done for the two other colonies (A and D). I preferred not to use a new box as colony installation into a previously unused nest takes time and would not have allowed us to perform the observation just after reunification. The reunited colonies were left undisturbed the following day, during which the ants present outside the nest were noted three times (morning, noon and afternoon) and foragers were observed during one 10 min interval to determine whether both splits groups were equally well established within the nest. On the following day, I introduced eight cadavers into the reunited nests in two trials in the same way as in the first step of the experiment. The nests were video-recorded and the videos later analyzed according to the first experimental step.

Up to five ants died in the subcolonies (cadaver groups A: 3, B: 3, C: 1, D: 2, control groups A: 0, B: 5, C: 3, D: 3) after marking and before the first experimental step (during 2.5 weeks) and a maximum of two dying ants (control groups A: 1, B: 0; C: 2, D: 2; cadaver groups A: 0, B: 0; C: 1, D: 2) were recorded during the first step of the experiment (5 days). These comprised marked ants and emerging callows and were soon after occurrence removed. The insertion of 40 nestmate cadavers during the first step of the experiment thus increased the

daily occurrence of cadavers (0.147 cadavers naturally occurred in all subcolonies daily) by 5574 % in cadaver groups.

Cadaver obtention

To obtain cadavers, 100 workers (25 per size class) of each field colony were killed by freezing (10 min at -20 °C) at the time of colony splitting. They were deposited in an open plastic box in a climate chamber (22 - 25 °C, 50 - 80 % humidity) for 13 days, until the start of the experiment. At this point, cadavers were transferred to a refrigerator (4 °C) to avoid stimulus change over the course of the experiment.

Size measurements

The body size of an individual was estimated using the tibia length of the right posterior leg as described in Bocher et al. (2007). Tibias were photographed with a Sony XCD-SX910CR camera connected to a binocular microscope and tibia length was measured with the ImageJ software (version 1.40, Wayne Rasband, National Institutes of Health, USA).

Video analysis

During video analysis, the identity of worker ants and the duration of their behavior directed towards each cadaver were noted. As every cadaver belonged to a different worker size class (1-4), they could be followed individually. I noted cadaver grooming (licking a cadaver with mouthparts) and intranidal cadaver transport (lifting or dragging a cadaver with mandibles and displacing it) during a maximum of 30 min after cadaver insertion. Necrophoric events were noted during the whole observation time (180 min). Once the cadaver was transported out of the nest, it was no longer considered as the video recorded only the nest area.

Data organization and statistical analysis

The worker force was divided into four task groups: foragers, cadaver groomers, cadaver transporters and other workers. Foragers were defined as ants that had been observed feeding in the arena. Cadaver groomers or transporters were observed grooming or transporting (within and outside of the nest) a cadaver at least once during the 10 trials and are termed cadaver-managing individuals. Cadaver transporters contained both necrophoric workers (individuals which transported a cadaver outside the nest) and intranidal cadaver transporters because (i) intranidal chambers comprising cadavers were observed in the field, (ii) several cadavers were deposited onto an intranidal waste pile in colony D, (iii) 59% of necrophoric workers (cadaver group A: 42.9 %, B: 55.6 %, C: 87.5 %, D: 50 %) also performed intranidal transports and (iv) only few necrophoric workers were observed during the first experimental step (cadaver group A: 7, B: 9, C: 8, D: 4 workers), preventing separate statistical analyses.

Other ants which performed neither foraging nor cadaver management are termed "other workers". Each subcolony contained 77 marked workers and the percentages given are based on these workers.

Unmarked individuals performed 8.9 % necrophoric events during the first experimental step (cadaver group A: 32.4 %, B: 0 %, C: 3.4 %, D: 0 %). Whereas in colonies B and C nearly all cadaver transports (97.2 %) and groomings (98.7 %) in the first experimental step were performed by marked individuals, unmarked individuals performed 34.9 % cadaver transports (intranidal and necrophoric transports) and 11.9 % groomings in cadaver groups A and D.

Slightly aggressive behavior was directed towards the cadavers in that some individuals were observed standing in the vicinity of the cadavers showing mandible opening behavior during the whole observation time. Infrequently, cadaver biting occurred, especially during and right after cadaver insertion into the nests. Aggressive acts directed towards cadavers during and after insertion were probably elicited by the introduction of a foreign object and were not necessarily linked to the fact that this object was a cadaver. Two cadavers (1.4 %, one in each cadaver group A and B) were dismembered during the experiment.

Pattern of cadaver removal.

I analyzed the time to cadaver removal using survival analysis (parametric survival regression model), as data were censored (R package "survival" (Therneau 2012)). The size of the cadaver (factorial), cadaver group (factorial) and trial were included as fixed effects. Non-significant interaction terms were removed from the final models.

Performance of cadaver management.

I analyzed the performance of cadaver management from two different perspectives, from the perspective of a worker performing the tasks and from the perspective of a cadaver receiving the behavioral acts.

Worker level: To address whether nestmates differed in the total number of cadaver-related behaviors (i.e. an individual's absolute number of cadaver-related behaviors within the 30 min observation time, combined for all trials), I analyzed whether (i) all workers of the cadaver group and (ii) task-performers were skewed (i.e. unequal sharing of a task among individuals) in the number of cadaver groomings and transports (intranidal and necrophoric) they by Peter Nonacs performed. I used Skew Calculator the program 2003 (https://www.eeb.ucla.edu/Faculty/Nonacs/PI.html). Estimations were based on 1000 runs and the significance level of alpha was set to be 0.05% in two-tailed tests to determine the confidence interval (CI). I determined the factors associated with the performance of cadaver management using Generalized Mixed Models (LMER) to account for pseudoreplication based on different cadaver groups and binomial distributed errors (family = binomial). I tested whether the binary response variables grooming, transporting or foraging (individuals performed these tasks or not) depended on worker size and on the performance of the other two behaviors grooming, transporting or foraging (as binary covariates).

Cadaver level: To analyze whether cadaver size and trial affected cadaver management by workers, I used Linear Mixed Effects Models (LMM) and analyzed whether the number or duration of cadaver groomings and transports a cadaver received or the number of task performers per cadaver depended on the covariates cadaver size (factorial), trial (numeric), duration the cadaver stayed in the nest (or until 30 min) and number of task performers. I controlled for pseudoreplication by including cadaver group as a random effect. Response variables were log-transformed and insignificant terms were subsequently removed from the full models.

Reunification: treatment effect.

After reunification, workers of the cadaver and of the control group were expected to be present in the colony boxes and forage with the same probability of 50%. To analyze whether the origin of the colony box (whether it previously housed the cadaver or control group) affected this ratio, a chi-squared test was performed per colony on the observed number of individuals, comparing the cadaver and control group. During the experiment, some workers (12%) were not attributable to a subcolony due to partial color loss or marking errors. Partly unmarked individuals, which performed cadaver-directed behaviors during the reunited trials, could not be clearly assigned to a subcolony (even if already observed performing cadaver-directed behaviors in the cadaver group in the first experimental step) and were grouped separately in the analysis. I further analyzed in each colony whether the number of cadaver-grooming and cadaver-transporting (intranidal and necrophoresis) individuals differed between the cadaver and the control group and between the cadaver group and workers which could not be assigned to either subcolony, using an exact binomial test.

All data gathered was analyzed using R (version 2.11.1).

RESULTS

Pattern of cadaver removal

In the first step of the experiment (Fig. 1), over all 10 trials, a total of 40 cadavers were inserted into the nest of a cadaver group. Due to technical difficulties (e.g. electricity shortings in the laboratory), video data could not be obtained for trial 3 in cadaver groups A, C and D (36 cadavers observed) and for trials 3 and 4 in cadaver group B (32 cadavers observed). Soon after cadaver insertions, cadavers were antennated very frequently by at least ten workers, which ceased after several minutes. Nearly all inserted cadavers (85 %) were removed from the nests within the observation period (180 min) except in one cadaver group

(D) in which only 11 % of the cadavers were removed (Table 1). In this colony, cadavers were deposited next to a pile of food waste and pupal exuviae within the nest. On one occasion, a cadaver was brought back into the nest of cadaver group A. In the four cadaver groups, necrophoresis occurred between 32 sec to 30 min 32 sec (Table 1). The time elapsed to cadaver removal did not differ according to cadaver size (survival analysis, chi-two = -0.598, df = 3, p = 0.897) and did not change over the 10 trials (survival analysis, chi-two = -0.005, df = 1, p = 0.944). However, it significantly varied among colonies (survival analysis, chi-two = -93.289, df = 3, p < 0.0001). The significant effect of colony was still observed even after removing the peculiar colony D (survival analysis, chi-two = -10.589, df = 2, p = 0.005).

Table 1: For each cadaver group, the percentage of cadavers removed in the first experimental step is given, together with the minimum and maximum time of removal over all trials (not including unremoved cadavers). I further provide the percentage of cadaver groomers, transporters and the overlap between both, based on the number of marked workers per cadaver group.

Cadaver group	Cadavers removed (%)	Minimal time until necrophoresis	Maximal time until necrophoresis	Groomers (%)	Transporters (%)	Overlap between groomers and transporters (%)
А	94	59 sec	24 min 15 sec	39	30	42
В	81	32 sec	30 min 32 sec	35	25	48
C	81	2 min 22 sec	30 min 21 sec	32	19	38
D	11	3 min 33 sec	19 min 41 sec	19	12	41

Groomings and transports received by cadavers

The number of groomings cadavers received did neither change over trial, nor was it affected by cadaver size or by the duration a cadaver stayed in the nest (LMM, Table 2). Cadavers were groomed more often if they elicited grooming in a higher number of different grooming individuals (Table 2, Fig. 2, LMM, coefficient = 0.331). The number of transports also significantly increased with an increasing number of transporting individuals (Table 2, Fig. 2, LMM, coefficient = 0.134). Trial, cadaver size and the duration a cadaver stayed in the nest did not affect the number of transports received (Table 2).

The number of workers which groomed cadavers increased with trial (Table 2, LMM, coefficient = 0.051) and with the duration a cadaver stayed in the nest (Table 2, LMM, coefficient = 0.001), but was not affected by cadaver size (Table 2, LMM). The number of individuals which engaged in cadaver transports was neither affected by trial, nor by the

duration a cadaver stayed in the nest, but depended on cadaver size (Table 2, LMM). The largest and second smallest cadavers were transported by fewer individuals.

Table 2: The statistical values of Linear Mixed Effects Models (LMM) testing an effect of trial, cadaver size, the number of task performers (groomers or transporters for grooming or transporting respectively) and the duration the cadaver stayed in the nest (rows) on the number and duration of cadaver groomings and transports and on the number of task performers (groomers or transporters) per cadaver (columns).

	Statistical values	Trial	Cadaver size	Number of task performers	Duration the cadaver stayed in the nest
Number of	L	1.573	1.041	209.357	0.688
groomings	Р	0.209	0.791	< 0.0001	0.407
Grooming duration	L	1.113	2.041	128.031	0.513
Grooning duration	Р	0.292	0.564	< 0.001	0.474
Number of	L	5.461	5.367		21.198
groomers	Р	0.019	0.147		< 0.0001
Number of	L	0.002	4.936	32.139	2.967
transports	Р	0.968	0.177	< 0.0001	0.085
Transport duration	L	0.185	2.295	18.897	8.912
	Р	0.667	0.514	< 0.0001	0.003
Number of	L	2.084	12.668		0.668
transporters	Р	0.149	0.005		0.414

The results for the duration of groomings and transports a cadaver received are qualitatively the same compared to the number of these behaviors (Table 2), except that the duration of cadaver transports was negatively affected by the duration a cadaver stayed in the nest (Table 2, LMM, coefficient: -0.001).



Figure 2: Relationship between the number of groomings (A) and transports (B) and the number of grooming (A) and transporting (B) workers for each of 140 cadavers over all trials. Black circles denote colony A, open circles colony B, black triangles colony C and open triangles colony D.

Who is performing cadaver-related tasks?

At the colony level

The percentage of foragers was on average of 43.3 % in all subcolonies and did not differ between cadaver (40.6 %) and control groups (46.1 %; Pearson's chi-squared test with Yates' continuity correction, $\chi^2 = 1.692$, df = 1, p = 0.193). Foragers were smaller than the other marked workers (LMER, z = -2.237, p = 0.025, coefficient = -0.811). In the four cadaver groups, I observed 31 % of workers grooming cadavers and 21% transporting them within and outside of the nest (Table 1). Of these cadaver-managing individuals, 42 % engaged in both cadaver grooming and transports (Table 1). Individuals differed in their propensity to perform cadaver-related behaviors (Fig. 4). The marked workers of the cadaver groups differed significantly in the number of groomings (cadaver group A: B = 0.102, p = 0, ci = 0.191, cadaver group B: B = 0.076, p = 0, ci = 0.183, cadaver group C: B = 0.045, p = 0, ci = 0.056, cadaver group D: B = 0.092, p = 0, ci = 0.114) and transports directed towards cadavers (cadaver group A: B = 0.118, p = 0, ci = 0.359, cadaver group B: B = 0.073, p = 0, ci = 0.173, cadaver group C: B = 0.082, p = 0, ci = 0.103, cadaver group D: B = 0.047, p = 0.006, ci = 0.107). Among individuals transporting cadavers, I also found a significant skew for cadaver groups A (B = 0.088, p = 0, ci = 0.330), B (B = 0.035, p = 0.001, ci = 0.136) and C (B = 0.029, p = 0.001, ci = 0.057), but not among the transporters of cadaver group D (B = -0.043, p = 0.962, ci = 0.035). Among cadaver groomers, I found a significant skew in each cadaver group (A: B = 0.082, p = 0, ci = 0.170; B: B = 0.052, p = 0, ci = 0.159; C: B = 0.019, p = 0, ci = 0.029; D: B = 0.040, p = 0, ci = 0.057).



Figure 4: The number of cadaver-management behaviors for every task-performing individual within each cadaver group (cadaver groups A to D displayed from top to bottom panel). Cadaver groomings of an individual are displayed in black and transports in white within each individual bar. The circles above bars display necrophoric individuals.

At the individual level

Of the cadaver groomers, 38.9% groomed cadavers only once (cadaver group A: 15, B: 8, C: 9, D: 6 individuals) and 52.6% of transporters performed only one transport (cadaver group A: 16, B: 9, C: 4, D: 6 individuals) over all trials. Only seven groomers (mean value; cadaver group A: 5, B: 10, C: 11, D: 2 individuals) and three transporters (mean value, cadaver group A: 1, B: 2, C: 7, D: 1 individuals) performed these tasks in at least three of the 10 trials, not permitting analyzing an effect of experience on individual responsiveness and task performance.

The propensity of a worker to groom cadavers was neither affected by worker size (LMER, z = -0.352, p = 0.725) nor by the performance of foraging (LMER, z = -1.218, p = 0.223). I observed 34.9 % of cadaver groomers (cadaver group A: 33.3 %, B: 37 %, C: 16 %, D: 53.3 %) feeding in the colony box. Cadaver groomers were however more likely to engage in cadaver transports (LMER, z = 6.909, p < 0.0001, coefficient = 2.699). Transporters were more likely to groom cadavers (LMER, z = 6.911, p < 0.0001, coefficient = 2.700) and to forage (LMER, z = 2.087, p = 0.037, coefficient = 0.814). Of the cadaver transporters, 47 % (cadaver group A: 39.1 %, B: 42.1 %, C: 40 %, D: 66.7 %) were observed feeding. Transporters did not differ in size from the other marked workers (the three biggest worker groups; LMER, z = -0.245, p = 0.807).

Effect of experience after reunification

The day following reunification (Fig. 1), both queens of each colony were alive and resided within the nest and no workers were observed in the corners of the ant boxes. Some ants died directly after reunification of the subcolonies, likely due to increased aggression when introducing new ants, and they were immediately removed by the experimenter (col A: 3, col B: 1, col C: 5, col D: 0). In the four colonies, workers of both previous subcolonies were in equal proportion present outside the nest (Pearson's chi-squared test; col A: $X^2 = 0.803$, df = 1, p = 0.370; col B: $X^2 = 3.462$, df = 1, p = 0.063; col C: $X^2 = 2.500$, df = 1, p = 0.114; col D: $X^2 = 0.397$, df = 1, p = 0.529) and engaged in equal proportion in foraging (Pearson's chi-squared test; col A: $X^2 = 0.758$, df = 1, p = 0.384; col B: $X^2 = 3.270$, df = 1, p = 0.071; col C: $X^2 = 0.862$, df = 1, p = 0.353; col D: $X^2 = 2.314$, df = 1, p = 0.128).

During the reunification trials (Fig. 1), a few (2.6 %) marked workers (2-7 workers/colony) which could not be clearly assigned to either control or cadaver group, were observed performing cadaver-related behaviors (Fig. 4). Among the assignable cadaver-managing workers, 62 % groomers (col A: 53 %, col B: 82 %, col C: 67 %, col D: 46 %) and 58 % transporters (col A: 50 %, col B: 83 %, col C: 67 %, col D: 33 %) originated from the cadaver groups (Fig. 4). In three out of four colonies (A, C and D), these workers did not engage in cadaver grooming (exact binomial test; col A p = 1, col C p= 0.152, col D p = 1) or cadaver

transports (exact binomial test; col A p = 0.453, col C p= 0.180, col D p = 0.754) with a higher probability than workers from the control group. In colony B, individuals from the cadaver group had a higher likelihood to groom (exact binomial test, p = 0.013) and transport cadavers (exact binomial test, p = 0.021) than individuals from the control group. However, this significant difference did not hold true if I considered that all non-assignable workers originated from the control group (exact binomial test, grooming p = 0.189, transports p = 0.804), a situation which is however not very likely.

DISCUSSION

In this study I analyzed whether division of labor exists for cadaver management (groomings and transports) in the ant *Cataglyphis velox*. I investigated whether the performance of cadaver management would be affected by repeated cadaver occurrence and the size of the cadaver and whether task performance depended upon the size of the worker or the performance of foraging behavior. I found that nestmates were significantly skewed in the performance of cadaver management acts and few individuals dominated task performance. Many task performers performed the behaviors only once over all trials and could thus not acquire experience over several trials. Individuals performing one type of cadaver management were also more likely to perform the other and cadaver transporters showed a high propensity to also engage in foraging. Cadaver size did not modulate cadaver management behaviors. The repeated occurrence of cadavers did not affect the number and duration of groomings and transports a cadaver received, but lead to an increasing number of groomers per cadaver. When reunited with less experienced individuals, the probability of experienced individuals to groom and transport a cadaver was increased in one out of four colonies.

Cadaver management was previously investigated especially in termites, where they often serve nutritional purposes or are buried (Neoh et al. 2012; reviewed in Sun and Zhou 2013). In ants and honeybees, research has focused on the performance of necrophoresis (e.g. Wilson et al. 1958; Gordon 1983; Visscher 1983; Trumbo et al. 1997; Julian and Cahan 1999; but see e.g. Ataya and Lenoir 1984; Renucci et al. 2010) and groomings and intranidal transports have, if observed, often only been described (e.g. Wilson et al. 1958; Visscher 1983, but see Renucci et al. 2010 and Neoh et al 2012 which studied cadaver-management modulation due to corpses of different origin). A study on *Myrmica rubra* demonstrated that only a few fresh cadavers are removed immediately and 85% remain in the nest up to 6 days postmortem (Diez et al. 2013b). What happens until the occurrence of necrophoresis has rarely been empirically analyzed. In the present study I demonstrated that even old cadavers are groomed and transported within the nest before necrophoresis occurred. To my knowledge, this is also the first study investigating whether nestmate cadavers of various size are treated differently.

Grooming and transport of cadavers

Cadaver grooming was performed by 31 % of nestmates, a behavior which has been described in the ants Pogonomyrmex badius (Wilson et al. 1958) and Lasius niger (Ataya and Lenoir 1984), in some termite species (Neoh et al. 2012; Myles 2002; Renucci et al. 2010) and in honeybees (Visscher et al. 1983). Grooming is a sanitary behavior during which foreign particles such as fungal spores are removed from the insect cuticle (Rosengaus et al. 1998b; Hughes et al. 2002; Yanagawa et al. 2008; Tragust et al. 2013a) and can then be deactivated in the groomer's alimentary tract (Yanagawa and Shimizu 2007). Grooming of nestmate cadavers before removal is not intuitive as it could potentially increase the transmission of infective agents, but it might (i) increase nest hygiene if cadavers are not removed but deposited in a remote location inside the nest, (ii) bestow a future survival benefit upon the groomer if pathogen contact results in micro-infections and priming of the immune system (Konrad et al. 2012), (iii) be less costly than necrophoresis as individuals do not have to leave the safe confines of the nest, especially in the thermophilic C. velox. Applying different possible behaviors to solve a unique problem (increased sanitary risk due to intranidal cadavers) might be an adaptive advantage, even if the behaviors differ in terms of cost (Renucci et al. 2010). Additionally, cadaver grooming could be a prophylactic mechanism directed towards all intranidal cadavers, including foreign cadavers which are brought into the nest and serve as a food source (Cerdá and Retana 1997) and might pose a sanitary risk to the colony. Prophylactic grooming has been reported in Acromyrmex echinatior ants which selfgroom before entering the nest chamber containing brood or their mutualistic fungus (Morelos-Juárez et al. 2010) and in Formica selysi ants which allogroom all nestmates reentering the nest (Reber et al. 2011).

Cadavers were transported within the nest, before necrophoresis or deposition onto internal waste dumps occurred. Intranidal transports have also been described in some ant species (e.g. Wilson et al. 1958; Julian and Cahan 1999; Renucci et al. 2010), in honey bees (Visscher 1983) and termites (e.g. Sun et al. 2013) and likely occur when cadavers are brought to more remote areas of the nest. In the field I observed a superficial chamber containing pupal exuviae and cadavers in two colonies (CW pers. obs.). Superficial chambers also exist in the species *Cataglyphis cursor*, in which an individual was also observed removing a *C. cursor* cadaver from the nest (CD pers. obs.). These observations suggest the natural occurrence of intranidal transports and a reduced likelihood that moribund *C. velox* workers leave the nest to die outside, as has been described in three different ant species (Wilson et al. 1958; Heinze and Walter 2010; Bos et al. 2012) and for honeybees (Rueppell et al. 2010).

Cadaver size did not modulate cadaver management behaviors. The only difference observed among cadavers of different size was that the largest and second smallest cadavers were transported by fewer individuals. This differential treatment might result from heterogeneity of the presented cadavers in the quantity of microorganisms they harbored.

Distribution of cadaver-management workload among workers

I found that cadaver transports and groomings were not randomly distributed among workers of the cadaver group (Figure 4), but nestmates were skewed in the performance of these cadaver-management behaviors. Furthermore, task performers differed in the number of cadaver groomings and transports they performed and only a few individuals performed the majority of the work directed towards cadaver during the first experimental step. Of the cadaver-managing workers, 38 % managed cadavers only once across all trials (one grooming only: 39 %; one transport only: 53 %). I conclude that cadaver management labor is divided among nestmates with some individuals having a heavier workload than others, but division of labor is not very strong, as still 37 % of the marked workers performed these tasks and most of them infrequently. Domination of necrophoresis by a few individuals has been demonstrated in ants (Myrmica rubra) and in honeybees, with an extreme specialist bee removing 114 cadavers on 13 days (Trumbo et al 1997; Diez et al. 2011). It is generally assumed that strong division of labor exists for necrophoresis and waste management in honeybees and leaf-cutting ants (Trumbo et al. 1997; Julian and Cahan 1999; Hart and Ratnieks 2001, 2002; Ballari et al. 2007). Even if I include the smallest unmarked workers and assume that none of them performed these behaviors, cadaver-management behaviors would still be performed by 29% of the complete workforce. Comparisons between studies are not straight-forward as the number of necrophoric individuals reported depends on the stimulus level and thereby on the experimental technique (Breed et al. 2002). Up to 30% of Acromymex versicolor ant workers (Julian and Cahan 1999) and 21 – 23 % of honeybees were specialized in necrophoresis and a significant proportion of these bees participated for only 1 day (Trumbo et al. 1997). It is currently unclear how a specialist should be defined and whether individuals performing a task only once should be included (Trumbo et al 1997). Together with these studies on necrophoresis, I suggest that division of labor for cadavermanagement might be less pronounced than is currently assumed. Having numerous nestmates manipulate cadavers might expose them to a low pathogen dose, potentially resulting in immune priming and bestowing a survival benefit upon these individuals ('social immunization', Traniello et al. 2002; Ugelvig and Cremer 2007; Konrad et al 2012). These workers could serve as reserve labor or present an immune barrier in case of an accumulation of cadavers and epidemic within the nest. In the ant Myrmica rubra, workers suffer increased mortality if they were not able to remove their dead (Diez et al. 2014).

Who performs cadaver management?

Task allocation for cadaver-management is poorly understood (Sun and Zhou 2013) and has only been studied for waste management in leaf-cutting ants and necrophoresis. In

honeybees, a genetic component for necrophoresis has been demonstrated (Robinson and Page 1988, 1995), which likely determines olfactoric sensitivity (response thresholds) of workers to necromones (Julian and Cahan 1999). The responsiveness to oleic acid has further found to be caste-specific, with unresponsiveness in the soldier caste in the ant Atta mexicana (López-Riquelme et al. 2006). Cadaver-manipulating workers of the present study might also differ from non-responsive nestmates in a higher sensitivity towards necromones, which might be genetically determined in this polygynous and polyandrous species. Age is a further predictor of necrophoric and waste-management behavior and both tasks are performed by middle-aged individuals (Breed et al. 2002; Mersch et al. 2013; Camargo et al. 2007; Waddington and Hughes 2010) and necrophoric individuals are presumably developmentally advanced in that they start foraging at an earlier age (Trumbo et al. 1997) and possess higher levels of juvenile hormone than bees of the same age performing other age-typical tasks (Huang et al. 1994). In my study, transporters were more likely to forage than nontransporting individuals, a behavior which is typically performed by the oldest workers (Cataglyphis species reviewed in Lenoir et al. 2009). As C. velox foragers leave the confines of the nest, they presumably suffer the highest infection risk in the colony and possess higher levels of active phenoloxidase (Bocher et al. 2007), an important enzyme in individual immune defense (reviewed in Cerenius and Söderhäll 2004). Cadaver groomers and transporters did not differ in size from nestmates belonging to the three biggest size groups and not engaging in cadaver management. Not being able to include the smallest workers of the colony might have masked an effect of body size on cadaver management in this study. In another polymorphic ant with monophasic worker distribution, necrophoric labor was not divided by worker size (Solenopsis invicta, Howard and Tschinkel 1976). An effect of body size on waste management has been demonstrated in leafcutting ants, where waste managers are smaller than foragers (Ballari et al. 2007; Waddington and Hughes 2010). Foraging workers were found to be smaller compared to nonforaging workers, which could be explained by relatively low laboratory temperatures (24.4-29.9°C) compared to what can be found under field conditions (maximal activity temperature 46 °C in the field, reviewed in Cerdá 2001). In the field, the mean size of foraging workers increased with temperature and smaller workers foraged preferentially at lower temperatures (Cerdá and Retana 1997).

Individuals performing one type of cadaver-management behavior were also more likely to perform the other (42% of cadaver managers performed both groomings and transports). I further observed a significant overlap among cadaver transporting and foraging individuals (47% of transporters also foraged). The reported overlap between cadaver-managing individuals and foragers might be increased in my study, given the high percentage of individuals observed feeding in the colony box. Having flexible workers which can switch between cadaver management and other tasks can be advantageous for the colony, if tasks are performed in the same spatial zone and inter-task travel time is thereby reduced or if individuals can transfer previously acquired improvements in one task to another which requires similar motor skills (Wilson 1976). Indeed, both foragers and individuals

transporting nestmate cadavers perform cadaver transports in this species and presumably use the same motor patterns and are exposed to the same sanitary threats. Having the same workers perform both tasks could reduce parasite transmission within the colony. Additionally, an overlap among task groups determines colony level patterns (Wilson 1976; Gordon 1996; Beshers and Fewell 2001) and enables the group to adjust more rapidly to varying environmental conditions, such as the amount of sanitary risk imposed by cadavers (Johnson 2003; Diez et al. 2013a). Waste workers of *Pogonomyrmex barbatus* and necrophoric individuals of *Myrmica rubra* are also able to switch to foraging (Gordon 1989; Diez et al. 2013a) and necrophoric honeybees were observed to further remove debris and perform hygienic behavior (i.e. the removal of diseased brood, Wilson-Rich et al. 2009) at low frequency (Trumbo et al. 1997). In leafcutting ants however, where foragers collect leaf material for their mutualistic fungus, contact with waste workers is avoided and they are even aggressed to avoid contamination (Hart and Ratnieks 2001; Ballari et al. 2007).

Experience as a factor of task allocation

Experience is an important factor which can improve individual and collective performance (e.g. Chittka and Thompson 1997; Langridge et al. 2004; Weidenmüller 2004; Langridge et al. 2008; Weidenmüller et al. 2009). However little is known about an effect of recent experience on collective sanitary behaviors (but see Walker and Hughes 2009; Reber et al. 2011; Westhus et al. 2014). A behavioral modulation through experience depends on the time interval between task performances (Theraulaz et al. 1998). Time intervals which last as long as a night can reduce previously acquired effects (Keasar et al. 1996; Langridge et al. 2004; Westhus et al. 2013). Recurrent task performance of individuals was low in my study with 39% of groomers and 53 % of transporters performing these behaviors only once and only 26% groomers and 15% transporters performing these tasks in two trials on the same day. I was thus unable to satisfactorily address an effect of individual experience on task performance of individual workers. In honeybees and ants, an effect of experience on necrophoresis was not demonstrated, even though specialized necrophoric individuals were more efficient (cadavers removed faster and dropped less) than non-specialists (Trumbo and Robinson 1997; Julian and Cahan 1999), likely due to a greater ability from the outset (Trumbo and Robinson 1997). In the ant Myrmica rubra, necrophoric workers demonstrated spatial memory within a time frame of 7 minutes (Diez et al. 2011). Colony phenotype can also be shaped by experience, through the collective actions of nestmates. Experienced ant workers have been shown to be more efficient in colony emigration (*Temnothorax albipennis*: Langridge et al. 2004; Langridge et al. 2008) and thermal brood relocation (Camponotus *rufipes*: Weidenmüller et al. 2009), leading to a better output of the whole group. Recurrent insertion of nestmate cadavers did neither affect the time delay to cadaver removal, nor the intensity of groomings and transports cadavers received. However, cadavers were groomed by a higher number of individuals with increasing trial, suggesting that the repeated occurrence of cadavers increased the probability of nestmates to perform cadaver grooming and thereby affected individual responsiveness. Rare tasks such as necrophoresis (or even cadaver management) might not provide ample opportunity to improve task performance, as the time interval between the appearances of the task-associated stimuli might be too large for selfreinforcement to last (Trumbo et al. 1997; Diez et al. 2013a). Furthermore, task groups differ in their ability to learn and honeybee pollen foragers demonstrated stronger learning, which is genetically determined and likely due to increased stimuli sensitivity (Latshaw and Smith 2005) and cue biases for learning (reviewed in Papaj and Prokopy 1989). Some motor patterns are more easily modified by experience than others. The restriction of learning to some stimuli or motor patterns in complex and unpredictable situations is assumed to be adaptive, as the costs of learning (time and energy spent, errors) do not arise in more predictable situations where fixed responses are sufficient (reviewed in Papaj and Prokopy 1989). Both cadaver transports and foraging involve transporting cadavers with the mandibles and grooming cadavers is further not expected to differ from self- or allogrooming. Motor skills acquired in these tasks are presumably transferable to cadaver management, so that these behaviors might be less modulated by experience than tasks where the nature of the substrate is not predictable on an evolutionary scale, such as handling live prey (Chittka and Muller 2009).

Theoretical studies proposed that the experience gained from previous performances influences an individual's probability to engage in this task. The likelihood of task performance would increase if previous performance was successful, resulting in a positive feedback factor, and decrease in case of failure or the lack of opportunity (e.g. Oster and Wilson 1978; Plowright and Plowright 1988; Theraulaz et al. 1998; Merkle and Middendorf 2004). In the context of foraging, where success or reward is easily quantified, experiencing success of task performance (or lack thereof) affected task allocation and thereby colony organization of the ant Cerapachys biroi (Ravary et al. 2007). Additionally, the social environment can affect the performance of a task. In constructed colonies of the ant Temnothorax albipennis comprising inexperienced and experienced workers, old experienced individuals were more likely to lead tandem runs than old inexperienced individuals during emigrations (Franklin et al. 2012). Another study examining the performance of hygienic behavior found that non-hygienic bees demonstrated a decreased rate, duration and probability of uncapping and removing dead brood in the presence of hygienic bees (Arathi et al. 2006). Reuniting individuals who had previously encountered 40 nestmate cadavers and nestmates without this experience did not lead to different proportions of individuals engaging in cadaver-management behaviors in three out of four colonies. In the fourth colony (colony B) however, experienced individuals had a higher likelihood to groom and transport cadavers compared to controls. Inter-colony variation is common in social insect studies and might in this case arise through stimulus differences. Did the cadavers of colony B pose a higher sanitary threat, thereby increasing positive feedback ants received when managing them (e.g. removal of microorganisms during grooming or necrophoresis of these cadavers)? Positive feedback is an important factor modulating an individual's propensity to again engage in a task (Plowright and Plowright 1988; Theraulaz et al 1998) and affected allocation of workers to foraging in the ant Cerapachys biroi (Ravary et al. 2007; Robinson et al. 2012). The stimuli triggering cadaver management in my study or the mechanisms underlying increased cadaver management of experienced individuals in colony B remain elusive. Not detecting an effect of experience in colonies A, C and D might be due to a low sample size, the fact that control individuals were not completely naïve or could be explained by only weak modulation of cadaver-management behaviors through experience. It is experimentally difficult to achieve complete naivety in terms of cadavers, if individuals are kept in a typical social environment, where cadavers already occur when young workers eclose. But even though the control group was not completely naive, the cadaver group was exposed to 40 cadavers over a time period of 5 days only, increasing daily cadaver occurrence by 5574%. A previous study on honeybees suggests that learning may not be an important component of necrophoric specialization, given the short tenure of necrophoric bees of 2 - 3 days (Trumbo et al. 1997). Necrophoresis has been demonstrated to be genetically determined with individuals of certain honeybee patrilines having a higher likelihood of task performance (Robinson and Page 1988, 1995), which might constrain a colony's ability to flexibly adapt to changing conditions (Robinson and Page 1995). However, the high percentage of nestmates performing cadaverrelated tasks and the overlap among them suggests that workers performing cadaver management can switch to and stay available for other tasks in the ant C. velox.

Experimental design and its limitations

The occurrence of several cadavers inside the nest, as in the present study, could occur under natural conditions in the case of pathogen transmission from returning foragers to nestmates, colony attack by predators or social parasites, nest damage, flooding and hibernal mortality. I introduced only four cadavers to avoid a sanitary emergency situation, where 'all hands on deck' would be needed and the high task thresholds of usually unresponsive individuals would also be met (Dornhaus 2008, Chittka and Muller 2009). The simple nest design (round petri dish) was chosen to maximize cadaver encounter by incoming, outgoing and intranidal workers and enable filming of the whole nest, but it reduced spatial nest complexity. It rather represented a colony in the early ontogenetic stages, where few workers inhabit a small and simply structured nest. More complexity and nest structure will presumably decrease cadaver encounter rate and thereby increase the division of labor found for cadaver management. The simple nest design could further explain the high number of foragers I observed, even though foragers were defined as ants feeding in the arena and not defined according to their presence in the arena (as has been done for C. velox in Bocher et al. 2007 where lower foraging percentages were reported). When inserting cadavers through the nest cover, I observed several ants inspecting and biting them, which was likely due to colony disturbance and might have resulted in stimulus enhancement (i.e. the proximity of a nestmate to a cadaver attracted the attention of another individual towards it; Leadbeater and Chittka 2007), increasing cadaver encounter rate by nestmates. Disturbed fire ant workers (Solenopsis invicta) have been reported to attack cadavers which would otherwise be removed (Howard and Tschinkel 1976). The behavioral differences I observed among colonies might be common intercolonial variation in behavior (e.g. Breed et al. 2002; Diez et al. 2011), for instance stemming from differential patriline distributions resulting in a different number of individuals with lower response thresholds for task, but it might also be due to heterogeneity of the cadaver stimuli. The cadavers of some colonies could have harboured more microorganisms than those of others, which is expected to be natural situation.

Future research

It would be interesting to investigate whether cadaver-managing individuals show higher investment in physiological immunity as a protection against increased pathogen exposure during task performance or when exiting the nest ('exposure risk hypothesis': Bocher et al. 2007). Increased immune investment of more exposed task groups is suggested by the findings that *C. velox* foragers possess higher levels of active phenoloxidase (Bocher et al. 2007) and waste workers of *Atta sexdens rubropilosa* bigger metapleural glands than workers engaged in less risky tasks (Lacerda et al. 2010). On the other hand, individuals which already possess higher levels of individual immunity might be the ones engaging in cadaver-related tasks. It would further be interesting to know, whether the occurrence of cadaver grooming depends on the diet of the species and is prophylactically directed towards all cadavers, also nutritional ones. It would be interesting to investigate how stimulus quantity, quality (e.g. the virulence of a parasite) or temporal presentation (i.e. periodic or chronic exposure) modulates sanitary response behavior in social insects, a question which has not yet received enough empirical attention. Furthermore, more research is warranted on the actual perception of a disease stimulus by individuals and the transmission of this information within the colony.

CHAPTER 3

Are worker size and phenoloxidase activity of *Cataglyphis velox* workers genetically determined?

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In preparation

TITLE

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ABSTRACT

Polyandry increases the diversity of group members and thereby favors division of labor within the colony. Colonies with increased genetic diversity can also be more resistant towards disease, but the mechanisms underlying increased disease resistance are not well understood. The effects of polyandry on division of labor and disease resistance could be linked if considering physiological disease defense a worker task. In the polymorphic and polyandrous ant *Cataglyphis velox* I analyzed whether there is a direct link between genotype, worker type (young laboratory-born versus old field-derived) and the phenotypic traits worker size and immune investment. As an indication of immune investment I analyzed the level of the enzyme phenoloxidase (PO). I did not find evidence for genetic determination of both worker size and the level of PO, neither for all workers, nor separately in both worker types (young laboratory-born workers versus old field-derived workers). Workers of different size did not differ in their levels of active PO. Interestingly, whereas bigger field-derived workers possessed higher levels of total PO, I could not detect this relationship in young laboratoryborn workers. The age (and potentially the environmental origin) of workers affected the size and immune investment of individuals: laboratory-born workers were smaller and had a reduced variance in size compared to field-derived workers. The older field-derived workers showed higher levels of total PO, but did not differ from the young laboratory-born workers in the variance for this trait and in the level of active PO. The variance in active PO was however lower in old field-derived workers.

INTRODUCTION

The evolution of multiple mating (polyandry, reviewed in e.g. Crozier and Fjerdingstad 2001) resulted in increased genetic diversity within social insect colonies (e.g. Smith et al. 2008; Rueppell et al. 2012). Genetic diversity among nestmates can benefit colonies in (i) giving rise to division of labor; (ii) permitting flexibility and resiliency of the group; (iii) enhancing colony efficiency and productivity (e.g. Fewell and Page 1993; Beshers and Fewell 2001; Crozier and Fjerdingstad 2001; Brown and Schmid-Hempel 2003; Jones et al. 2004; Mattila and Seeley 2007; Oldroyd and Fewell 2007; Jeanson and Weidenmüller 2013) and (iv) enhancing disease resistance (e.g. Baer and Schmid-Hempel 1999, 2001; Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007; Ugelvig et al. 2010). Division of labor, the performance of different tasks such as foraging and brood care by nestmates, is achieved

through self-organization (i.e. without a central leader, Page and Mitchell 1998; Detrain and Deneubourg 2006) and believed to be responsible for the high ecological success of social insects (Oster and Wilson 1978; Hölldobler and Wilson 1990). A worker's genotype has been shown to affect its task preference (e.g. ants: Stuart and Page 1991; Snyder 1992; Blatrix et al. 2000; Julian and Fewell 2004; Waddington et al. 2010; Eyer et al. 2013a; honey bees: Frumhoff and Baker 1988; Robinson and Page 1988, 1989; Oldroyd et al. 1994; Pérez-Sato et al. 2009; social wasps: O'Donnell 1996, 1998), by affecting task-associated response thresholds. Nestmates are assumed to possess different response thresholds for a task (i.e. to respond at different stimulus intensities; response thresholds models reviewed in Beshers and Fewell 2001), which enables a graded colony response (Robinson and Page 1989; Beshers and Fewell 2001; Oldroyd and Fewell 2007). Even if genetically determined, response thresholds are not fixed, but can underlie short-term modulation by recent experience of the individual and environmental conditions individuals are exposed to (e.g. Theraulaz et al. 1998; Weidenmüller 2004; Weidenmüller et al. 2009; Westhus et al. 2013). Genetic diversity can further affect disease dynamics, as it reduces parasite loads and improves disease resistance within the social insect colony (e.g. Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999, 2001; Schmid-Hempel and Crozier 1999; Tarpy 2003; Hughes and Boomsma 2004; Tarpy and Seeley 2006; Seeley and Tarpy 2007). Which proximate mechanisms result in increased disease resistance is not well understood. Compared to colonies with reduced genetic diversity (due to inbreeding), colonies with natural genetic diversity showed improved behavioral anti-parasite defense through higher allogrooming frequencies and earlier hygienic behavior (i.e. the removal of diseased brood from the nest; Ugelvig et al. 2010). Increased genetic diversity did however not lead to higher levels of physiological immune function such as the encapsulation response and the activity of the enzyme phenoloxidase when comparing honeybee larvae among colonies with natural mating numbers (Wilson-Rich et al. 2012).

How tasks are allocated among nestmates is a central question about division of labor (Gordon 1996) and factors demonstrated to affect task allocation are for instance genotype, age, social interactions, rearing conditions, experience and worker size (e.g. Robinson and Page 1988; Hölldobler and Wilson 1990; Pankiw and Page 1999; Beshers and Fewell 2001; Weidenmüller 2004; Ravary et al. 2007; Smith et al. 2008; Weidenmüller et al. 2009; Westhus et al. 2013). Temporal polyethism, in which a sequential order of tasks is correlated with an age-dependent hormonal change, is widespread in social insects. With increasing worker age, individuals switch from intranidal activities such as brood care and cleaning to extranidal tasks such as foraging and nest defense with a higher extrinsic mortality risk (Seeley 1982; reviewed in Robinson 1992; Mersch et al. 2013). Another pattern of division of labor is morphological polyethism, in which different fixed body sizes or shapes of nestmates can be distinguished and predispose individuals to certain task repertoires (Oster and Wilson 1978). In extreme cases, where species have morphological worker castes, the largest workers commonly specialize in foraging and colony defense and the smallest ones in brood care

(Hölldobler and Wilson 1990; Nowbahari et al. 2000; Mertl and Traniello 2009), but task-size matching can also occur in species with unimodal worker size distributions (e.g. Waser 1998). Larger workers of several ant species are better foragers (e.g. Cerdá and Retana 1997; Goulson et al. 2002) and nest defenders (Braendle et al. 2003), show increased longevity (Calabi and Porter 1989; Porter and Tschinkel 1985; but see: Camargo et al. 2007), for instance under starvation (Heinze et al. 2003), but are more expensive to produce compared to smaller workers (Calabi and Porter 1989). Worker polymorphism increases intra-colony diversity, which is believed to result in higher colony efficiency (e.g. Oster and Wilson 1978; Porter and Tschinkel 1985), for instance through better exploitation of external environmental conditions (e.g. Cerdá and Retana 1997). Colony heterogeneity through polymorphism might further be favored by selection as worker size differences can result in varying susceptibility to parasites (Keller 1995; Kermarrec et al. 1990).

Division of labor and individual disease resistance are often studied apart, but can be tightly linked if physiological immune investment is considered a worker task. To counter the selection pressures imposed by a variety of macro- and microparasites (such as helminth worms, fungi, bacteria, viruses and some protozoa, e.g. Schmid-Hempel 1998), social insects evolved anti-parasite defense strategies at the individual as well as at the group level, comprising physiological, behavioral and spatial mechanisms (e.g. Cremer et al. 2007; Wilson-Rich et al. 2009; Evans and Spivak 2010; Stroeymeyt et al. 2014). Behavioral sanitary tasks can be divided among nestmates and task allocation affected by genetic, age and size factors (e.g. Frumhoff and Baker 1988; Robinson and Page 1988; Breed et al. 2002; Ballari et al. 2007; Camargo et al. 2007; Pérez-Sato et al. 2009; Abramowski et al. 2010; Waddington et al. 2010; Mersch et al. 2013), as well as by the social context (e.g. Arathi and Spivak 2001). Sanitary task performance by a subset of workers may limit the exposure of the entire colony to parasites, if few task performers have limited interactions with nestmates (Hart and Ratnieks 2001; Naug and Camazine 2002). Nestmates further differ in their investment into physiological defense mechanisms which serve collective parasite defense. The size of metapleural glands, which' secretions possess antimicrobial activities (reviewed in Yek and Mueller 2010), is positively correlated with worker size (Hughes et al. 2010) and bigger in task groups with higher infection risk (Lacerda et al. 2010). Soldiers of eusocial thrips produce more antifungal secretions than dispersers (Turnbull et al. 2012). At the individual level, nestmates differ in the resources they allocate to individual physiological immune defense, for instance to their encapsulation response (e.g. Doums and Schmid-Hempel 2000; Vitikainen and Sundström 2011), constitutive antibacterial activity (e.g. Armitage et al. 2011) or phenoloxidase activity (e.g. Bocher et al. 2007; Armitage and Boomsma 2010). Spread of diseases which require direct contact between individuals occurs from infectious to susceptible neighbors. If these are neighbors with higher immune investment and increased immune resistance, they could reduce disease transmission within the nest (Cremer et al. 2007; Stroeymeyt et al. 2014). In the ant Camponotus pennsylvanicus, individuals which mounted an immune response could even confer a survival advantage to nestmates at a

secondary immune challenge by transferring antimicrobial compounds in their trophallactic droplet (Hamilton et al. 2011). Increasing age is assumed to correlate with a decline in immune function ('immunosenescence': reviewed for vertebrates and invertebrates in Müller et al. 2013), but empirical evidence is conflicting. Whereas some studies reported a decline in immunocompetence (e.g. encapsulation response and phenoloxidase activity) with age in insects (e.g. Rolff 2001; Doums et al. 2002; Amdam et al. 2005; Moret and Schmid-Hempel 2009), other studies found either no difference or even an increase in immunocompetence with age (Schwarzenbach et al. 2005; Wilson-Rich et al. 2008; Armitage and Boomsma 2010; Roberts and Hughes 2014).

The hypotheses on the advantage of genetic diversity for the emergence of division of labor and for increased collective disease resistance rely on the assumption that their components such as individual size and immune defense are in part genetically determined. Studies investigating a heritable component of worker size are conflicting, possibly due to differences in sampling (the year of the study, see Bargum et al. 2004) and social context (e.g. Rüppell et al. 2001). A genetic influence on worker size has been reported in several ant species (Fraser et al. 2000; Hughes et al. 2003; Rheindt et al. 2005; Schwander et al. 2005; Jaffé et al. 2007; Evison and Hughes 2011; Huang et al. 2013), but not in *Formica truncorum* (Bargum et al. 2004). In the ant Cataglyphis cursor with continuous worker size distribution, individual size was also not influenced by patriline (Fournier et al. 2008; Eyer et al. 2013a). Worker size determination has long been assumed to result from environmental cues (Hölldobler and Wilson 1990; Wheeler and Nijhout 1984), such as the amount of food available during larval development in holometabolous insects. Worker size has been demonstrated to increase with colony size (e.g. Wood and Tschinkel 1981; Gibson 1989; Tschinkel 1988, 1993, 1998; Hölldobler and Wilson 1990, Clémencet and Doums 2007) and decrease with the presence of multiple queens within the colony (e.g. Schwander et al. 2005; Rüppell et al. 2001). The size of Cataglyphis cursor workers differed between natural habitats (Clémencet and Doums 2007). A genetic basis of disease resistance was demonstrated in several social insect species (e.g. Baer and Schmid-Hempel 1999, 2001; Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007) even though the underlying mechanisms remain unclear in most cases. In leafcutting ant workers, disease resistance varied between patrilines (Hughes and Boomsma 2004), which could relate to a variation in immune response (Armitage and Boomsma 2010). Indeed, an effect of patriline was demonstrated on constitutive antibacterial activity (Armitage et al. 2011) and on the size of the metapleural gland (Hughes et al. 2010; Armitage et al. 2011) in leaf-cutting ants. Heritability of hemolymph phenoloxidase and the ability to encapsulate a foreign body have been demonstrated in non-social insects (e.g. Ryder and Siva-Jothy 2001; Cotter and Wilson 2002; Schwarzenbach et al. 2005). In honeybees however, larvae of different patrilines did not differ in these immune parameters (Wilson-Rich et al. 2012), but diseased larvae showed moderate heritability for the expression of the antimicrobial peptide abaecin (Decanini et al. 2007). The conditional expression of physiological immune parameters is influenced by many environmental factors, such as the presence of nestmates (e.g. Richter et al. 2012; but see: Armitage and Boomsma 2010) or antimicrobial compounds in the nest (Castella et al. 2008; Simone et al. 2009).

We aimed to investigate whether interindividual variation in body size and immunocompetence are based on genetic differences between workers of the ant Cataglyphis velox, Santschi, 1929 (Tinaut 1990). The underlying causes of phenotypic variability among workers are required to understand in what extent traits will adapt to natural selection and changing environmental conditions. I estimated immunocompetence of individuals by measuring the level of the immune enzyme phenoloxidase (both active and total PO). Within young laboratory-born workers of the same age, I was able to control for the confounding factor of individual age. I expected a patriline effect on worker size and immune function, whereby different sibling groups would vary from each other. I assumed lower heritability for the inducible active PO than for the constitutive form of the enzyme (total PO). Foragers of C. velox possess higher levels of active PO (Bocher et al. 2007) and I thus expected higher levels of this enzyme in old field-derived workers. In field-collected leaf-cutting ant colonies (Acromyrmex octospinosus), the level of stored PO decreased over time in the laboratory (Armitage and Boomsma 2010). This finding leads to the assumption of either lower total PO values in young, laboratory-born workers or no difference among both worker types in this trait. Compared to young laboratory-born workers, I expected a higher variation in worker size in old field-derived workers, as they were presumably exposed to more diverse environmental conditions during larval development. A large environmental variation of size in this worker type could hide a genetic effect on worker size and I thus assumed higher worker size heritability values in the young, laboratory-born workers.

Experiments were performed on the polymorphic and polyandrous ant *C. velox*, which offers both genetic and morphological diversity among the workforce. The species reproduces by social hybridogenesis (Eyer et al. 2013b). Within single polygynous colonies, queens combine parthenogenetic reproduction to produce queens (thelytokous parthenogenesis) and males (arrhenotokous parthenogenesis) with sexual reproduction to produce hybrid workers. Nestmate queens are essentially clones and mate with multiple males (generally with 2-5 males) originating from different genetic lineages (Eyer et al. 2013b), thus increasing genetic diversity in the worker force. Colonies are therefore a mix of clones and siblings, ranging in relatedness.

METHODS

Colony collection and rearing

The thermophilic species *C. velox* occurs on the Iberian Peninsula in arid Mediterranean habitats with sparse vegetation (Tinaut 1990). Colonies occupy an underground nest and have

small to medium size, containing from hundreds to a few thousand workers. Workers of *Cataglyphis* species follow the classical age-dependent polyethism, passing from intranidal tasks when young to foraging when older (reviewed in Lenoir et al. 2009). *C. velox* workers are very polymorphic with a continuous worker length range of 4.5-12 mm (Tinaut 1990; Cerdá and Retana 1997), which enables division of labor in foraging and thus a longer daily activity period. Larger workers withstand higher temperatures, achieve higher running speeds and shorter foraging durations than smaller workers and are able to forage at temperatures deadly for other insects. The species scavenges on fresh cadavers of small arthropods and rarely incorporates plant material into its diet (Cerdá and Retana 1997; Cerdá 2001).

Twenty-five mature queenright colonies of *Cataglyphis velox*, Santschi, 1929, were collected from a population in the Sierra Nevada near Grenada, Spain ($37^{\circ}08'$ N, $3^{\circ}29'$ W, 1300 meters altitude) in May 2011 (5 ± 4 queens and 1220 ± 820 workers; mean ± standard deviation). Of these colonies, four with the highest quantity of brood were chosen for this experiment. They were polygynous (six queens in colonies A, B, C and 13 in colony D) and contained 600 to 1900 workers and some brood in the egg and early larval stage at collection. I found no sexual offspring, which *C. velox* colonies typically produce in the Sierra Nevada in July (Eyer et al. 2013b). Complete colonies were brought to the laboratory and reared at a temperature of about 28°C with a 15/9 h day/night light cycle and fed 3 times/week with a mixed diet of live meal worms, frozen crickets, fruit (apple, orange, banana), artificial food (1 L distilled water, 10 g agar, two eggs, 124 g honey, 2 g vitamins (Nature'sPLUS), 2 g salt) and *ad libitum* sugar and water.

Establishment of young workers in the laboratory

To obtain laboratory-born workers of known age, two queenless subcolonies 1 and 2 were generated per colony, each containing 50 intra- and extranidal nestmates of all sizes. These workers originated from the field (field-derived workers). Three times a week, pupae were transferred from the stock colony to subcolony 1, which served as a nursery, until worker emergence. Subcolony 2 contained no brood and served to maintain newly emerged workers of known age (coming from subcolony 1) that were used in the experiment (laboratory-born workers). All field-derived workers of the two subcolonies were color-marked on the thorax (Uni Paint Marker) in order to be able to differentiate them from laboratory-born workers. The first week, newly emerged workers (lighter and unmarked) were transferred from subcolony 1 to 2 when they had achieved walking capacity. For the subsequent weeks, before the transfer was done from 1 to 2, the young workers of known age $(13 \pm 2 \text{ days})$ and the same number of field-derived workers from subcolony 2 were sampled for immune defense and size estimations (see below). To exclude an effect of task on immune investment (Bocher et al. 2007), I tested individuals at an age before they left the nest under laboratory conditions. In C. bicolor, the onset of foraging takes place at about 28 days in the field (Schmid-Hempel and Schmid-Hempel 1984). In order to reduce environmental variation during the experiment, for each subcolony and each week, I performed manipulations to the same number of fieldderived workers and laboratory-born workers. The size of the subcolonies changed due to differing numbers of eclosing workers and the use of laboratory and field-derived workers for experiments. Field-derived workers were replenished by transferring them from the stock colonies into subcolony 1 and from subcolony 1 into subcolony 2. The transfer from subcolony 1 to subcolony 2 took place at least 1 week before immune measurements, comparable to eclosing laboratory workers.

Subcolonies were maintained in the same room under the same environmental conditions as the stock colonies and were kept in plastic boxes ($27 \times 17 \times 11 \text{ cm}$) lined with plaster and coated with fluon. The colony boxes contained a plastered petri-dish nest (diameter 9 cm) darkened with cardboard paper, allowing direct access to hatched workers, and were moistened once a week.

Experiments were performed in July 2011 (2 months after collection, colonies B and C), November 2011 (6 months after collection, colony A) and in April 2012 (11 months after collection, colony D) and lasted about 1 month per colony. Colony D differs from the others in that experiments were performed after hibernation in the laboratory (3 months, temperature: 14.7 ± 1.8 °C, humidity: 58.1 ± 7.5 % humidity, fed about once a week with meal worms, orange, sugar and water). Given the seasonal cycle of C. velox (as has been described in C. iberica: Lenoir et al. 2009), I infer that field-derived workers were at least 8 months old in colonies B and C, 11 months in colony A and 16 months in colony D. In colony A, no young workers eclosed before the experiment started and in colony D (the colony which overwintered), a few workers eclosed the previous summer which were marked with 3 color dots and colored ants were still distinguishable and not used for the experiments the following year. Due to rearing problems in the laboratory, I was not able to include old laboratory-born workers to analyze an effect of age on PO investment. However, since fieldderived workers stayed under controlled laboratory conditions for at least 2 months before the start of the experiment (colony A: 6 months, colony D: 11 months) and were at least 8 months old, they should mainly differ from young laboratory-born workers in their age (and the behavioral and physiological differences associated with it).

Phenoloxidase measurement

The phenoloxidase assay was adapted from Bocher et al. (2007) and Helft et al. (2012), with slight modifications. The level of the active (active PO) and total amount of phenoloxidase (total PO = active PO and stored PPO) was determined for each individual using a spectrophotometer (96-well Microplate reader 680; Bio-Rad, Hercules, CA, USA). After being anaesthetised on ice, workers were decapitated and hemolymph was removed from cutting sites at the head and thorax with a disposable graduated capillary tube (Hirschmann Laborgeräte ringcaps). All manipulations were conducted on ice-blocks to prevent enzyme
activation. Hemolymph was diluted (1:100) in ice-cold sodium cacodilate/CaCl2 buffer (0.01 M Na-Cac, 0.005 M CaCl2, pH = 6.5) and stored at -80°C. Of each individual, 10 μ L of diluted hemolymph was mixed with 5 µL of distilled water for active PO measurement and with 5 μ L of alpha-chymotrypsin (2 mg mL⁻¹ in distilled water; prepared freshly) for total PO measurement, both performed at the same time. Chymotrypsin proteolytically activates stored PO into active PO and thus allows measurement of the total enzymatic activity of the sample (total PO). Samples were incubated for 5 min at room temperature and 17.5 µL of L-DOPA (4 mg mL⁻¹ in distilled water) was added in each well. The mixture absorbance at 490 nm was measured at 30°C every 10 sec for 50 min. The enzymatic activity was determined as the slope of the reaction curve during the linear phase of the reaction (V max) using Microplate Manager 5.2 software (Bio-Rad, Hercules, CA, USA). The enzyme level is proportional to the optical-density increase induced by the transformation of L-DOPA to dopachrome by phenoloxidase (Söderhäll and Cerenius 1998). Within-individual repeatability of enzyme measurements was obtained by analysing intra-well (active PO N = 22; total PO N = 23) and inter-well (active PO N = 36; total PO N = 39) replicates of the same individual. Both active PO (Pearson's product-moment correlation; intra-well; $r^2 = 0.774$; inter-well; $r^2 = 0.899$) and total PO data (Pearson's product-moment correlation; intra-well: $r^2 = 0.683$; inter-well: $r^2 =$ 0.887) showed reasonable repeatability. Negative controls (wells containing only distilled water or only buffer) showed horizontal kinetic curves and minimal activity (0 - 0.2 V max). If the absorbance curve was too irregular, these data points were eliminated. The hemolymph volume collected from each worker varied from 0 to 3.00 µL. As active PO and total PO measures required 0.1 µL each, only active PO was assayed when less than 0.2 µL of hemolymph were collected and samples of less than 0.1 µL were excluded from the PO activity analyses. I favored active over total PO as a previous study found a higher variance for active PO among C. velox workers (Bocher et al. 2007). I obtained active PO values of 341 individuals (180 young laboratory-born workers and 161 old field-derived workers) and total PO values of 310 individuals (171 young laboratory workers and 139 old field-derived workers). Sample size was lower than the total of 425 workers sampled as for many individuals I did either not succeed in collecting enough hemolymph quantity or hemolymph of good quality.

Worker size measurements

The tibia length of the right posterior leg was used as an estimation of individual body size (Bocher et al. 2007). Tibias were photographed with a Sony XCD-SX910CR camera connected to a binocular microscope and tibia length was measured with the ImageJ software (version 1.40, Wayne Rasband, National Institutes of Health, USA).

Genetic analysis

After taking samples for size and immune measurements, individuals were stored in 90% alcohol with 10% Tris-EDTA. In total, 28 queens and 425 workers were genotyped from the four studied colonies, consisting of 229 laboratory-born and 196 field-derived workers (colony A: 65 laboratory-born and 60 field-derived workers; colony B: 57 laboratory-born and 53 field-derived workers; colony C: 78 laboratory-born and 50 field-derived workers; colony D: 29 laboratory-born and 33 field-derived workers; Table 1). Three queens of colony D could not be retrieved from the colony for genotypic analyses as they had presumably already died and were dismembered by the workers (we found not dead queens on the extranidal waste dumps). Ant DNA was extracted from half of the heads through digestion at 55 °C in a 10% Chelex buffer and 15 µL proteinase K at 10 mg/ml for 2 hours. After 3 min of centrifugation at 1400 g, the supernatant was taken and stored at 4 °C before being quickly used in a polymerase chain (PCR) reaction. Ants were genotyped with five microsatellite markers (CC11, CC26, CC58, CC63, CC99) previously developed by Pearcy et al. (2004b) for C. cursor. Each PCR reaction was carried out in a 10 µL volume, containing 1 µL Buffer 10x, 0.15 µL 0.4 mM dNTA, 0.5 units Taq polymerase (Quiagen) and 1µL DNA. The five loci were amplified in two multiplex PCR reactions, using 100 nM of CC26 and CC58 (first set of loci) or 100 nM of CC11, CC63 and CC89 (second set of loci). The reactions were run in a GeneAmp PCR system 9700 Thermal cycler (Applied Biosystems), starting for 10 min at 94°C to denaturate the DNA, followed by ten cycles of: 15 sec at 94 °C (DNA denaturation), 15 sec at 52 °C (primer annealing phase) and 30 sec at 72 °C (DNA elongation). These cycles were followed by 35 cycles of: 15 sec at 89 °C, 15 sec at 52°C and 30 sec at 72°C. The PCRs were completed with a DNA elongation phase at 72 °C for 10 min. The amplified fluorescent products were separated and visualized using an automated ABI Prism 310 sequencer (Applied Biosystems) with ROX 400 size markers and allele sizes estimated with the Genescan 3.2.1 software (Applied Biosystems).

Patriline determination

The absolute number of patrilines within each colony was estimated by comparing worker and mother genotypes inferred from microsatellite analyses. Even though there were multiple queens in each colony, the queens shared the same multilocus genotype within each colony as observed by Eyer et al. (2013b) except for two queens that were homozygote for a locus which was heterozygous for the other queens. This is probably due to the occurrence of recombination events that lead to homozygosity under meiotic thelytoky with central fusion. This mode of thelytoky has been demonstrated for the related species *C. cursor* (Pearcy et al. 2006). From a genetic perspective, colonies could therefore broadly be considered as "quasi"monogynous. All workers examined, except two, are likely the offspring of the mother queens. These two non-natal workers were excluded from subsequent analysis. The fathers' genotypes were inferred from the queen and worker genotypes. When a patriline contained only a single worker and only differed by a single allele from the other fathers (occurred five times), I preferred to be conservative and considered that the different allele originated from a PCR error or mutation. A new patriline was therefore not created for a single worker and the worker assigned to the closest patriline.

In agreement with the mode of reproduction (Pearcy et al. 2004a; Pearcy et al. 2006; Eyer et al. 2013b), the level of worker heterozygosity was very high since all except four workers were heterozygotes at all loci. And in these four workers, only one locus was found in the homozygous state. Out of 29 alleles found over the five loci in the fathers, only one allele in one locus was common with the queens and was found at the heterozygous state in workers so that I could not determine the correct father genotype at this locus. I estimated the allelic frequency in the father gene pool without considering the undetermined genotype at a single locus in a single patriline.

Because of the unusual reproductive system of *C. velox*, with males and females having so different alleles that they could belong to different gene pools (Eyer et al 2013b), it would be incorrect to use the population allelic frequency based on workers to assess the probability of non-detection of a patriline. Given that I observed a total of 49 patrilines, I estimated the allelic frequency only in the male gene pool from the inferred father genotypes. I then estimated the probability that two fathers share the same genotype and would therefore lead to an undetected patriline using the following equation (Boomsma and Ratnieks 1996): $P_{non-det\ ection} = \prod_j \sum_i f_{i,j}^2$ with $f_{i,j}$ being the allelic frequency of the allele *i* at locus j in the fathers gene pool. Over the five loci, I obtained a probability that two fathers share the same genotype of 0.005, which suggests that the variability detected with the five microsatellites was sufficiently high for having only a low proportion of patrilines that could remain undetected.

Statistical analysis

To test whether patrilines were equally distributed among workers, I computed the reproductive skew among (i) all workers, (ii) laboratory-born workers and (ii) field-derived workers using the B index. This index is based on the observed variance in a group corrected by the expected variance from the binomial distribution (Nonacs 2000) and equals zero if patrilines are randomly distributed. I calculated B and tested the null hypothesis that B equals zero by using the program Skew Calculator 2003 (http:// www.eeb.ucla.edu/Faculty/Nonacs). Using two-sided Fisher exact tests, I further determined whether patriline distribution differed among laboratory-born and field-derived workers.

The broad sense heritability ((V_A + V_D) / V_T; V_A = additive genetic variance, V_D = dominance genetic variance, V_T = total phenotypic variance) (Falconer and Mackay 1996) was estimated based on a half-sib design with sires nested within dam, that is patrilines nested within colonies separately for field-derived and laboratory-born workers. I decomposed the total phenotypic variation ($V_T = \sigma_T^2$) into three variance components: inter-colony variation that included both environmental and genetic differences among colonies ($V_C = \sigma_C^2$), interpatriline variation within colonies that included both additive and dominance genetic variance ($V_p = \sigma_p^2$) and intra-patriline variation which represents the uncontrolled variation among workers ($V_e = \sigma_e^2$). The three variance components were estimated using a Linear Mixed-Effects Model (LMM) with patrilines embedded in colony as a random effect and a fixed intercept using Restricted Maximum Likelihood Method (REML) in the package nlme in R (Pinheiro and Bates 2000). The model was run separately for each worker type to estimate the level of broad sense heritability. Because of the haplodiploid sex determination, inter-patriline variation represents ¹/₂ of V_A and ¹/₂ of V_D (Liu and Smith 2000), hence

$$H^2 = \frac{2\sigma_P^2}{\sigma_T^2} .$$

The level of significance of the effect of patriline was tested by comparing the model with and without the effect of patriline using a log likelihood ratio test with a REML (Pinheiro and Bates 2000). To run the LMM I excluded the patrilines represented by single workers (eight patrilines for field-derived workers and seven patrilines for laboratory-born workers). For active PO and total PO analyses, data were log transformed to fulfill the assumption of residual normality and homoscedasticity. Moreover, I first tested an effect of the covariate size as a fixed factor in the LMM. If not significant, size was removed from the model to estimate the variance components.

We also ran a LMM on the full data set comprising both field-derived and laboratory-born workers in order to test for differences between both worker types. The full model included as the fixed effect the worker type (young laboratory-born or old field-derived) and as the random effect patriline embedded in colony. The significance level of the fixed effect was tested by comparing the models with and without the fixed effect using a likelihood ratio test. For this test to be valid, I fitted the two models using the maximum likelihood method (ML) as advised by Pinheiro and Bates (2000). The residuals demonstrated that the variance in worker size was higher in field than in laboratory workers. In order to fulfill the assumption of the model and to test whether the variance in laboratory workers was lower than in fieldderived workers, I also included in the model the possibility of having a different variance in laboratory and field-derived workers using the weight function (weights = varIdent(form=~1|AGE)).

To test for an interaction between the effect of worker type and colony, I removed the effect of patriline (which was not significant) in order to add the possibility that the effect of age

randomly varied within colonies. I thus included age both as fixed effect and as a random effect within colonies.

RESULTS

Differences between young laboratory-born and old field-derived workers

Worker size:

Workers eclosed in the laboratory had a lower size variance than workers eclosed in the field (L-ratio = 24.295, p < 0.001) and were also smaller (L-ratio = 43.747, p < 0.001) (Figure 1). This was true in all colonies (no interaction between worker size and colony; L-ratio < 0.001; p = 0.999).



Figure 1: Comparison of tibia length (mm) among laboratory-born (L) and field-derived (F) workers of colonies A, B, C and D (displayed in the four horizontal panels). The boxplots depict mean, quartiles and range of the data.

Active PO:

Both worker types did not differ in the level of active PO (L-ratio = 0.604; P = 0.437, interaction: L-ratio = 0.820; p = 0.365). Worker size neither affected the level of active PO in all workers (L-ratio = 0.697; p = 0.404), nor in each of the worker types (young laboratory-born: L-ratio = 0.442; p = 0.506; old field-derived: L-ratio = 1.280; p = 0.258) (Figure 2). Young laboratory-born workers had a higher variance in active PO than old field-derived workers (L-ratio = 35.8; p < 0.001, coefficient 0.61 for laboratory workers). This is especially true for colonies B and C (Figure 2). Whereas I determined in young laboratory-born workers $0.962 \pm 1.729 \text{ mOD min}^{-1}$ and in old field-derived workers $0.705 \pm 0.612 \text{ mOD min}^{-1}$ active PO, I obtained in young laboratory-born workers $3.974 \pm 4.395 \text{ mOD min}^{-1}$ and in old field-

derived workers $17.721 \pm 11.932 \text{ mOD min}^{-1}$ total PO. These estimates suggest that most of the enzyme was in the inactive (stored) form in both young laboratory-born and old field-derived workers.



Figure 2: Comparison of the level of active PO (log-transformed) among young laboratory-born (L) and old field-derived (F) workers of colonies A, B, C and D (displayed in the four horizontal panels). The boxplots show mean, quartiles and range of the data.

Total PO:

The level of total PO was not affected by worker size in the laboratory (L-ratio = 0.790; p = 0.374) but was strongly affected by worker size in the field-derived workers (L-ratio = 13.206; p < 0.001). Thus, there was a significant interaction between worker size and type (L-ratio = 5.531; p = 0.018). The level of total PO was higher for old field-derived workers than for young workers born in the laboratory (L-ratio = 104.120; p < 0.001) (Figure 3 and Figure 4). The variance in total PO was not different between old field-derived and young laboratory-born workers (L-ratio = 0.052, p = 0.820).



Figure 3: The level of total PO (log-transformed) is displayed among young laboratory-born (L) and old field-derived (F) workers of colonies A, B, C and D (in the four horizontal panels). The boxplots show mean, quartiles and range of the data.



Figure 4: Relationship between total PO activity (data are log-transformed) and tibia length (in mm) of young laboratory-born workers (filled circles) and old field-derived workers (empty circles) for all workers of the four colonies.

Number of patrilines and patriline distribution

Of the 49 different patrilines detected, 14 were present in colony A, 11 in colony B and 12 in colony C and D each (absolute and effective number of patrilines in Table 1). Patrilines present in a colony were not equitably represented, but some patrilines were present in a higher proportion of workers than others (skew calculation; B values in Table 1; colony A: p = 0; colony B: p = 0; colony C: p = 0; colony D: p = 0.001). The same was true for the

patriline distribution among laboratory-born workers (skew calculation; B values in Table 1; colony A: p = 0; colony B: p = 0; colony C: p = 0) and among field-derived workers (skew calculation; B values in Table 1; colony A: p = 0.006; colony B: p = 0.007; colony C: p = 0.027; colony D: p = 0.011), with the exception of laboratory-born workers in colony D where patrilines were equally represented among workers (p = 0.601). I further found significant differences in patriline distribution comparing laboratory-born and field-derived workers (Fisher's exact tests, Table 1). In colony D, only one of the 12 patrilines was present in both young laboratory-born and old field-derived workers.

Table 1 Sample size and summary statistics of the genetic analysis of young laboratory-born and old field-derived workers and queens of four *C. velox* colonies. For each colony, results for the complete sample (Overall), the young laboratory-born and old field-derived workers are given. The number of queens analyzed (Nq) is followed by the number of workers analyzed (Nw) and the number of patrilines observed for each colony (Np). Those are followed by the effective paternity number (k_e) and the index of skewness (B). The results of an exact test of patriline differences between field and laboratory workers (T_{LF}) are further given per colony. Significance level of B and T_{LF}: * p < 0.05; ** p < 0.01; *** p < 0.001; no asterisk: nonsignificant.

	Overall				Laboratory-born workers				Field-derived workers					
Colony	Nq	Nw	Np	ke	В	Nw	Np	ke	В	Nw	Np	ke	В	$T_{\rm LF}$
А	6	125	14	5.7	0.103 ***	65	8	3.2	0.184 ***	60	14	10.4	0.024 **	<0.0001 ***
В	6	110	11	6.3	0.066 ***	57	8	4.9	0.077 ***	53	10	7.8	0.027 **	0.005 **
С	6	128	12	6.8	0.063 ***	78	9	5.4	0.074 ***	50	12	9.9	0.017 *	0.017 *
D	10	62	12	9	0.001 **	29	9	9.2	-0.005	33	4	3.2	0.061 *	<0.0001 ***

Patriline effect and broad sense heritability

Worker Size:

Most of the worker size variation (87%) was neither explained by colony nor by patriline effects (Table 2), both for field-derived and laboratory-born workers. Accordingly, the broad sense heritability for worker size was low (0.197) in the field-derived workers and nearly zero in the laboratory-born workers (Table 2). In agreement with this low worker size variation among patrilines, in both field-derived and laboratory-born workers, the effect of patriline

was not significant even though the p value was much lower in the field-derived workers (L-ratio = 2.851; p = 0.091) than in the laboratory-born workers (L-ratio < 0.001; p = 0.999), in accordance with a higher level of heritability in the field-derived workers (Table 2).

Table 2 Broad sense heritability (h²) and variance components estimated for old field-derived and young laboratory-born workers for the three parameters worker size, active PO and total PO. The heritability was estimated as twice the variance components due to an effect of patriline in a LMM containing no fixed effect and patriline embedded in colony as a random effect. The number of individuals used for these heritability estimates are given between parentheses below heritability values. The values of the variance components inter-colony variation ($V_c = \sigma_c^2$), inter-patriline variation ($V_p = \sigma_p^2$) and intra-patriline variation ($V_e = \sigma_e^2$) are given. The proportion of variance of the three parameters worker size, active Po and total PO are given between parentheses for each variance component (below variance component estimates). A circle (°) behind a value signifies that the variance could not be properly estimated in the mixed model as there was barely no patriline variation (the real value was < 0.0001 and the confidence intervals were big). The variance level was therefore set to zero.

		Field-deriv	ved workers	s	Laboratory-born workers					
	h^2	σ^2_{c}	σ_{p}^{2}	σ_{e}^{2}	h^2	σ^2_{c}	σ_{p}^{2}	σ_{e}^{2}		
Worker size	0.197	0.024	0.074	0.651	0°	0.054	0°	0.352		
	(187)	(3.2%)	(9.9%)	(86.9%)	(221)	(13.3%)		(86.7%)		
Active PO	0.05	0.028	0.011	0.363	0.12	0.029	0.061	0.939		
	(151)	(7%)	(2.7%)	(90.3%)	(174)	(2.9%)	(5.9%)	(91.3%)		
Total PO	0°	0.284	0°	0.547	0°	0.091	0°	0.562		
	(132)	(34.2%)		(65.8%)	(167)	(13.9%)		(86.1%)		

Active PO:

The effect of patriline on the level of active PO was not significant, both in the young laboratory-born workers (L-ratio = 1.467; p = 0.226) and in the old field-derived workers (L-ratio = 0.335; p = 0.563). Accordingly, the levels of heritability were low (Table 2). The models explained not more than 10% of the variance in the old field-derived and in the young laboratory-born workers (Table2).

Total PO:

The effect of patriline was not significant, neither in the young laboratory-born (L-ratio < 0.001; p = 0.999) nor in the old field-derived workers (L-ratio < 0.01; p = 0.999). Accordingly, the level of heritability could not be estimated and was considered as zero (Table 2). The models explained no more than 10% of the variance in the field-derived and the laboratory-born workers (Table 2).

DISCUSSION

Phenotypic variation among individuals in a population underlies both genetic and environmental sources, and determination of their respective influence is important to assess the population's potential to adapt to and evolve in heterogeneous environments. In the ant C. velox I investigated interindividual variation in body size and immune investment based on genetic differences between workers. Unexpectedly, the phenotypic traits worker size and level of active and total PO were not explained by genetic variation within both laboratoryborn and field-derived workers (i.e. heritability values close to zero), even though worker age and environmental conditions were controlled for in young laboratory-born workers (Table 2). Foragers of C. velox possess higher levels of active PO (Bocher et al. 2007) and I thus expected higher levels of this enzyme in old field-derived workers. However, both worker types did not differ in the level of active PO and the variance for this trait was lower in old field-derived workers. According to the assumption that the level of stored PO decreases over time in the laboratory (Armitage and Boomsma 2010), I detected higher levels of total PO in field-derived workers than in young workers born in the laboratory. The variance for this trait did not differ among worker types. Workers born in the laboratory were presumably exposed to reduced environmental variation during larval development and accordingly showed reduced variance in size compared to field-derived workers. Compared to field-derived workers, they were also smaller. Interestingly, individuals of different size did not differ in their levels of active PO, but total PO was affected by worker size only in old field-derived workers.

Social structure

As previously described by Eyer et al. (2013b), colonies were polygynous with queens sharing the same multilocus genotype and the heterozygous worker force belonged to several different patrilines (11-14 different patrilines per colony; Table 1). I further detected a significant skew in the distribution of patrilines between young laboratory-born and old field-derived workers, suggesting that either (i) there is a queen turnover and not all queens are equally contributing to egg laying at least temporarily and/or (ii) that sperm are not mixed (sperm mixing seems to be common in some species: e.g. Laidlaw and Page 1984; Holman et

al. 2011; Stürup et al. 2014; but not in others: e.g. Sundström and Boomsma 2000; Wiernasz and Cole 2010) and workers of different patrilines are thus sequentially produced. Both mechanisms would increase genetic relatedness among nestmates of similar age and. The results highlight the need to consider age in genetic studies and hereditary influences in age studies as both can covary.

No effect of patriline on worker size

Similar to other studies (e.g. Bargum et al. 2004; Fournier et al. 2008; Kovacs et al. 2009; Eyer et al. 2013a), I found no heritability in the phenotypic trait worker size, indicating that most of the size variation between workers was due to environmental effects. Moderate heritability of worker size (heritability value = 0.26 ± 0.07) was reported in colonies of doubly-mated queens of the ant Formica selysi (Schwander et al. 2005). In Lasius niger and Leptothorax rugatulus, individual size heritability was demonstrated for sexuals (Rüppell et al. 2001; Fjerdingstad 2005). Heritability estimates are typically context-dependent and independent estimates often do not agree. My relatively small sample size might not provide enough statistical power to detect stronger heritability of worker size in field-derived workers. Additionally, uncontrolled environmental variance could have masked a stronger influence on worker size on C. velox than was found in this study. If worker size is determined by a genotype-environment interaction, providing the same environment might not lead to the phenotypic expression of a patriline effect on size. For instance, if worker size is constrained by food and larval provisioning wasn't adequate, workers could all be of a certain size and no effect of patriline detected. In polygynous C. velox colonies, heritability estimates may have contained maternal effects also. However, these queens shared the same genotype (also described in Ever et al. 2013b) and environment. Heritability estimates can vary between years (e.g. Bargum et al. 2004) and underlie social effects, which can hardly be excluded even under controlled laboratory conditions (Rüppell et al. 2001). The environment of a social insect worker underlies genetic influences as it is shaped by the presence of nestmates (and their genotypes) and individual phenotypes are thus modulated by direct and indirect genetic effects. In honeybees, a colony's genotypic composition affected gene and trait expression of individual bees (Linksvayer et al. 2009; Gempe et al. 2012) and influenced the performance and partitioning of hygienic behavior (i.e. the removal of diseased brood; Arathi and Spivak 2001; Gempe et al. 2012). Even though heredity of queen body size was demonstrated in the ant Leptothorax rugatulus, it was entirely superseded by social influences (Rüppell et al. 2001). The presence of multiple queens can result in smaller and less polymorphic offspring (Rüppell et al. 2001; Schwander et al. 2005). Environmental determination of worker size can be advantageous with respect to division of labor, by permitting colonies to adapt worker size ratios to changing environmental conditions and needs (without being genetically fixed; Passera et al. 1996; McGlynn and Owen 2002; Hughes et al. 2003; Bargum et al. 2004) and by avoiding parasitic worker genotypes specializing in reproduction (Hughes et al. 2003). The growth of ant larvae is affected by nutrition and pheromones (e.g. Hölldobler and Wilson

1990; Wheeler and Nijhout 1984) and soldiers of *Pheidole bicarinata* ants can inhibit larval development into the same caste through a contact pheromone (Wheeler and Nijhout 1984). If a need for soldiers arises in the colony, pheromone levels will decrease and new soldiers develop, allowing colonies to adjust worker size distribution more flexibly and rapidly to current needs than by genetic determination only. Worker size in several species is thus likely determined through interplay of both environmental and genetic factors (Hölldobler and Wilson 1990; Wheeler and Nijhout 1984; Hughes et al. 2003). In the case of *Pheidole bicarinata* larvae, their response towards soldier pheromone is likely affected by genetically determined response thresholds (Hughes et al. 2003), which differ between individuals and will favor certain genotypes to develop into soldier castes (genetic determination of worker morphology in ants: Hughes et al. 2003).

No effect of patriline on phenoloxidase

Genetic diversity reduces parasite loads and increases disease resistance within social insect colonies (e.g. Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999, 2001; Schmid-Hempel and Crozier 1999; Tarpy 2003; Hughes and Boomsma 2004; Tarpy and Seeley 2006; Seeley and Tarpy 2007) but the underlying mechanism remains undetermined (Wilson-Rich et al. 2012). In polyandrous honeybees (with naturally occurring mating numbers), genetic diversity did not increase levels of the physiological immune functions encapsulation response and phenoloxidase activity of larvae and these immune components were further not affected by patriline (Wilson-Rich et al. 2012). Accordingly, I did not find heritability in the level of phenoloxidase, neither for the active enzyme nor the total amount, in the ant C. velox at naturally occurring mating levels. Presumably, disease defense with the help of phenoloxidase is of too vital importance and thereby indispensable for individuals to vary among the worker force based on genetic differences (Wilson-Rich et al. 2012). Indeed, the innate phenoloxidase pathway is a central component of invertebrate disease defense in the hemolymph (Söderhäll and Cerenius 1998). PO activity has been linked for instance to reduced susceptibilty to fungal (e.g. Ochiai and Ashida 1988), bacterial (e.g. Pye 1974) and viral (e.g. Beck and Strand 2007) diseases. Unexpectedly, heritability of hemolymph phenoloxidase and the ability to encapsulate a foreign body have been demonstrated in nonsocial insects (e.g. Ryder and Siva-Jothy 2001; Cotter and Wilson 2002; Schwarzenbach et al. 2005). Hypothetically, the observed genetic variation in these traits arises from antagonistic pleiotropy (i.e. a gene positively affects one trait and negatively another), which is the genetic basis of trade-offs between traits (Roff 1992, as cited in Cotter and Wilson 2002; Cotter et al. 2004). Presumably, different selection pressures of non-social and social insects lead to differences in the heritability of immune traits.

As PO activity involves costly immunopathology (e.g. Sadd and Siva-Jothy 2006), conditional expression depending on the environment or performed task can be advantageous, with individuals performing the riskiest tasks (these are often the oldest workers in colonies

with age polyethism) possessing higher PO levels and thus presumably better protection than their nestmates. Behaviors leading to increased parasite exposure (foraging, frequent nest relocation) correlate with higher levels of active PO (*C. velox*: Bocher et al. 2007) and stored PPO (Scharf et al. 2012). Different immune mechanisms such as behavioral defenses (e.g. hygienic behavior: Spivak and Reuter 2001; Padhila et al. 2013; Ugelvig et al. 2010; allogrooming: Ugelvig et al. 2010) or different physiological defense mechanisms (e.g. antimicrobial peptide abaecin: Decanini et al. 2007; general antibacterial activity: Armitage et al. 2011; metapleural gland size: Hughes et al. 2010; Armitage et al. 2011) seem to underlie stronger genetic modulation and could result in increased disease resistance in genetically diverse colonies. Indeed, *Cardiocondyla obscurior* colonies showed earlier performance of hygienic behavior and higher allogrooming frequencies than inbred colonies with reduced genetic diversity (Ugelvig et al. 2010). Heritability of hygienic behavior in Africanized honeybees was determined to be high (heritability estimate = 0.52), providing potential for selection to act (Padilha et al. 2013).

Difference between young laboratory-born and old field-derived workers

I expected higher heritability estimates from laboratory-born workers than from field-derived workers due to less variable environmental conditions (Falconer and Mackay 1996). However, a comprehensive meta-analysis suggests that there is no significant difference between heritability estimates determined in the laboratory and from natural populations (Weigensberg and Roff 1997). Because of the uncontrolled age (and correlated task) and environmental conditions in old field-derived workers, I further expected a higher variation among colonies or a higher uncontrolled variation of the different traits in this worker type. However, both worker types did not differ in total PO heterogeneity. Unexpectedly, young laboratory-born workers showed higher variance in active PO than field-derived workers of higher age and with a higher uncontrolled variance in age.

According to previous studies, I expected lower levels of active PO in the laboratory-born workers, because (i) they had a lower risk of pathogen exposure and (ii) they were younger than field-derived workers. Behaviors leading to increased parasite exposure (foraging, frequent nest relocation) correlate with higher levels of active PO in *C. velox* (Bocher et al. 2007) and stored PO (Scharf et al. 2012) and increasing age led to higher levels of active PO in *Acromyrmex octospinosus* leaf-cutting ants (Armitage and Boomsma 2010). In bumblebees however, older individuals possessed lower levels of active PO (Moret and Schmid-Hempel 2009), suggesting that an effect of age on active PO investment is not always clear and differs between species. I found no difference between both worker types in the level of active PO, which supports the assumption of Bocher et al. (2007) that it is indeed task performance and not age which results in higher levels of active PO in *C. velox* in foraging ants. Because of the pathological effects of PO activation (e.g. toxic side products harming the host: Sadd and Siva-Jothy 2006), the enzyme should only be activated when necessary, which might explain

the same level of active PO in old field-derived and young laboratory-born workers when tested under laboratory conditions several months after collection (2-11 months). Neither task performance (Bocher et al. 2007) nor age (Armitage and Boomsma 2010; Roberts and Hughes 2014) affected the level of total or stored PO in previous studies. To the contrary, I detected lower levels of total PO in young laboratory-born workers. This finding is in agreement with a study on *Acromyrmex octospinosus* leaf-cutting ants (Armitage and Boomsma 2010), where the level of stored PO was negatively correlated with the duration colonies stayed in the laboratory, even though these colonies were not in a poorer condition. Both results suggest that controlled laboratory conditions decrease the need of individuals to invest into total or stored phenoloxidase, liberating resources for other life-history traits. Differences to previous studies on social insects might further arise from the fact that I tested older individuals (field-derived workers) which overwintered in the field or both in the field and in the laboratory, thus comparing a bigger age span. Previously, social insect workers were either tested in annual colonies (e.g. Doums et al. 2012; Moret and Schmid-Hempel 2009) or up to an age of about 2-4 weeks (e.g. Wilson-Rich et al. 2008; Roberts and Hughes 2014).

Worker size, a trait which can be environmentally determined (e.g. Wood and Tschinkel 1981; Gibson 1989; Tschinkel 1988) but does not vary with age in holometabolous insects (e.g. Hölldobler and Wilson 1990; Nylin and Gotthard 1998), was observed to be lower in laboratory-raised workers and showed higher heterogeneity in field-derived workers. Accordingly, laboratory-born workers of the ant Temnothorax nylanderi were smaller than workers collected in the field (Canovas 2014). Both findings might have resulted from colony disturbance by transferring them to the laboratory. In bumblebees, where worker size gradually increases in annual colonies, a transfer to the laboratory resulted in decreased size of consecutive age groups (Knee and Medler 1965). Wetterer (1999) further observed laboratory Trachymyrmex colonies in declining health producing smaller workers. Even though food was diverse (live meal worms, frozen crickets, apple, orange, banana, a mix of proteins, minerals and vitamins) and provided three times per week in addition to sugar and water available ad libitum, the different diet might not have been sufficient for the production of bigger workers in the laboratory. Additionally, higher temperatures in the laboratory could have accelerated larval development (as has been demonstrated for the ant Solenopsis invicta: Porter 1988) and resulted in smaller eclosing workers.

Worker size and phenoloxidase

We observed a correlation between worker size and total PO in old field-derived workers, however not for active PO. This finding is in accordance with a previous study in this species, where the correlation between worker body size and total PO was much stronger than between size and active PO (Bocher et al. 2007). The larger field-derived workers presumably have lower maintenance costs of the same mass of tissue than smaller field-derived workers, leaving more resources for immune investment (Peters 1983; Bocher et al. 2007). Findings of

different studies do not demonstrate this relationship, as bigger *Cataglyphis cursor* workers showed higher levels of active PO (Helft et al. 2012) and larger *Acromyrmex octospinosus* workers did not vary in immune investment from smaller workers (the sampling did however exclude the smallest workers; Armitage and Boomsma 2010). Research on different immune parameters agrees with my findings, as a correlation between worker size and metapleural gland size (Hughes et al 2010), as well as between individual size and the strength of the encapsulation response (Vitikainen and Sundström 2011), have been described.

Interestingly, the link between size and total PO disappeared in the young laboratory-born workers. This suggests that the relationship does not strictly result from a physiological link between worker size and total PO. The mechanisms behind the link between body size and immune investment in old field-derived but not in young laboratory-born workers remain unclear and deserve further empirical attention. Furthermore, comprehensive studies are warranted which compare the heritability of several different immune parameters for a holistic view, using a large sample size and repeating measurements in different habitats and/or over several years. Especially the investigation of social immune defense mechanisms could be promising to disentangle how colony-level genetic diversity increases disease resistance.

7. CONCLUSIONS AND PERSPECTIVES

7.1 What are the benefits of interindividual variation in immune investment?

7.1.1 Behavioral performance of sanitary tasks

Division of labor is considered a key factor of the ecological success of social insects (Oster and Wilson 1978; Wilson 1971) and it arises through variability among nestmates and a certain level of intraindividual consistency in task performance (reviewed in Beshers and Fewell 2001; Jeanson and Weidenmüller 2013). In theoretical studies, interindividual variability benefited groups by permitting increased resilience when faced with external challenges, by decreasing fluctuations in task-associated stimulus levels and allowing more efficient allocation of workers to tasks (reviewed in Jeanson and Weidenmüller 2013). Sanitary division of labor could increase colony survival not only by reducing the number of individuals in direct parasite contact, but also by increasing heterogeneity in nestmate connectivity and thereby limiting parasite transmission within the colony (reviewed in Stroeymeyt et al. 2014). Indeed, leaf-cutting ant workers performing waste management and potentially harbouring parasitic contaminations neither engaged in foraging nor entered chambers with their mutualistic fungus, and were socially avoided and aggressed, likely to minimize disease spread within the nest (Hart and Ratnieks 2001, 2002; Hart et al. 2002; Ballari et al. 2007). The benefits and costs of division of labor and task specialization (i.e. an individual's preferential performance of one task over others) for a colony will strongly depend upon the level of both task specialization and division of labor existing for a specific trait. The performance of sanitary work by task specialists can reduce task switching costs, limit parasite exposure to these individuals and might improve task performance efficiency (e.g. Jeanne 1986; Trumbo and Robinson 1997; Trumbo et al. 1997; Julian and Cahan 1999; but see Dornhaus 2008).

Spatial and temporal heterogeneity in the environment are assumed to be important factors in maintaining interindividual diversity (Nettle 2006; Burns and Dyer 2008). Colony flexibility in the response towards environmental heterogeneity can be achieved through amongnestmate variation in the way a task is performed (i.e. 'behavioral types': Jandt et al. 2014), even in species with low genetic diversity (e.g. *Bombus terrestris*). Nestmate bees (*B. terrestris* and the highly genetically variable *Apis mellifera*) differ in their foraging approaches (e.g. fast and imprecise or slow and precise) which allows colonies to respond more quickly to environmental fluctuations (Chittka et al. 2003; Burns and Dyer 2008; Chittka et al. 2009). Burns and Dyer (2008) suggest that this variability is maintained because it decreases the variation in resource acquisition in the field and they further assume that variation in resource availability will more likely negatively affect a single foraging approach than multiple approaches. Social insects come in contact with a diversity of parasites (e.g.

Hughes et al. 2004), whose occurrence is expected to vary spatially and temporally. Such heterogeneity in parasite occurrence could favour the existence of different behavioral defense approaches within the same workforce.

Empirical work demonstrated benefits to maintaining genetic variation within colonies, as it confers behavioral variability and can thereby improve the colony phenotype (reviewed in Oldroyd and Fewell 2007 and in Jeanson and Weidenmüller 2013). Increased behavioral variability in the workforce may increase the range of worker response behaviors, could improve task allocation and reduce costs associated with task switching (Goldsby et al. 2012; Jeanson and Weidenmüller 2013). Polyandry leads to the presence of several patrilines which can vary in task-associated response thresholds and this variation was demonstrated to improve intranidal thermal homeostasis in honeybees (Jones et al. 2004). According to the response threshold model, interindividual differences in response thresholds allow graded colony responses to increasing stimulus levels and thereby flexible responses towards changing internal and external conditions (reviewed in Beshers and Fewell 2001 and in Oldroyd and Fewell 2007). Both polyandry and polygyny modulate division of sanitary labor in bees and ants, as subfamily origin influences the probability of an individual to engage in a sanitary task (Robinson and Page 1988; Oldroyd et al. 1994; O'Donnell 1998; Waddington et al. 2010; Eyer et al. 2013a; Robinson and Page 1988, 1995; Frumhoff and Baker 1988; Pérez-Sato et al. 2009). These task preferences likely arise through lower response thresholds for sanitary stimuli, since workers performing hygienic behavior have reduced thresholds to take care of diseased brood (Masterman et al. 2001; Gramacho and Spivak 2003). The existence of these low-thresholds individuals is essential, as it permits colonies to eliminate sanitary hazards before parasites can establish within the nest. It has been observed that allogrooming generally occurs immediately upon the detection of a parasite-exposed individual or brood item, before infectious particles can enter the insect body and cause an infection (chapter one and e.g. Ugelvig et al. 2010; Konrad et al. 2012). Individuals with high response thresholds will be unlikely to perform sanitary tasks in healthy colonies and thereby remain available to perform different tasks such as foraging or nest repair. These workers could serve as reserve labor during colony invasion by a parasite, when high levels of task-associated sanitary stimuli will be reached. It is generally assumed that individuals are totipotent and able to switch to other tasks if need arises (Gordon 1996; Beshers and Fewell 2001 and the references therein). Indeed, individuals can even switch between foraging and cadaver management, which has been observed in chapter two and demonstrated for waste workers of Pogonomyrmex barbatus (Gordon 1989) and necrophoric individuals of Myrmica rubra (Diez et al. 2013a). A switch to other tasks will depend on several factors, such as an individual's degree of behavioral plasticity (e.g. Dingemanse and Wolf 2013) and the number of individuals engaged in other tasks (Gordon 1996).

A highly variable workforce further possesses a higher probability to include rare outlier phenotypes, such as necrophoric individuals in honeybees (Robinson and Page 1995), which

can benefit colonies under certain environmental conditions (Jeanson and Weidenmüller 2013). Demographic diversity of Zootermopsis dampwood termites reduced disease susceptibility of individuals compared to demographically homogeneous groups (Rosengaus and Traniello 2001). Genetic diversity can also improve colony resistance to disease (Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999; Schmid-Hempel and Crozier 1999; Baer and Schmid-Hempel 2001; Hughes and Boomsma 2004; Tarpy and Seeley 2006; Seeley and Tarpy 2007; Reber et al. 2008; Ugelvig et al. 2010; but see e.g. Schmidt et al. 2011), but the underlying mechanisms are not yet elucidated. Genetic diversity could increase the efficiency of sanitary task performance and intranidal communication of task needs, since it improved foraging-related signaling in honeybee colonies (Mattila et al. 2008; Mattila and Seeley 2011). Additionally, Cardiocondyla obscurior colonies showed earlier performance of hygienic behavior than inbred colonies with reduced genetic diversity (Ugelvig et al. 2010). Genetic diversity had, however, no impact on short-term task efficiency for necrophoresis in the ant Linepithema humile (Rosset et al. 2005). More research is necessary to understand the relationship between genetic diversity, individual behavioral differences and division of labor to answer questions such as whether the occurrence of different patrilines increases task specialization within the colony (Jeanson and Weidenmüller 2013) and could thereby improve disease resistance.

7.1.2 Physiological immune investment

Individual physiological immune investment typically differs among nestmates, but it is not always clear why variation within the workforce is maintained and why not all individuals show an optimal approach. Why do some individuals invest more in immune defense opposed to others, even though there are costs involved? And why do some individuals invest less even though social insects are assumed to be under high selection pressure imposed by parasites? Interindividual variability in how individuals respond to disease seems to reflect two different types of selection. First, life-history and resource tradeoffs in disease resistance could lead to varying investment among workers, with some individuals maintaining weak immune defenses (low cost but risky) while others invest more heavily (high cost but better protection; Evans and Spivak 2010). Indeed, individuals which perform tasks with a higher likelihood of parasite exposure seem to invest more into physiological defenses (but see: König and Schmid-Hempel 1995; Doums and Schmid-Hempel 2000). Cataglyphis velox foragers possess higher levels of active phenoloxidase (Bocher et al. 2007) and waste workers of Atta sexdens rubropilosa bigger metapleural glands than workers engaged in less risky tasks (Lacerda et al. 2010). A mixed strategy might be more adaptive as colonies whose members invest more strongly into individual immune defense are less productive (Moret and Schmid-Hempel 2004; Evans and Pettis 2005). Second, interindividual variation in disease resistance (e.g. in resistance alleles of patrilines) could be an adaptation to host-parasite coevolution. An increased genetic diversity in offspring through sexual reproduction is believed to be advantageous to hosts under strong pressure from parasites adapted to the most common host genotype ('Red Queen Hypothesis': Ladle 1992 and the references therein). A more diverse workforce might be more likely to successfully recognize and fight different invading parasite strains or species than homogenous nestmates (Evans and Spivak 2010).

7.2 Potential costs of interindividual variation

Whereas the potential benefits of interindividual variation (especially linked to increased genetic diversity) have received research attention, the potential costs and limits of worker variability await further study. The mechanisms underlying worker variability presumably entail different costs at both the individual and the colony level (Auld et al. 2010; Jeanson and Weidenmüller 2013). Experience-based differentiation requires increased resources for neuronal plasticity, especially in mushroom bodies (e.g. Laughlin et al. 1998; Dukas 1999; Farris et al. 2001; Riveros and Gronenberg 2010). Studies on fruit flies with artificially selected enhanced learning abilities demonstrated fitness costs (e.g. reduced larval competitive ability and decreased egg-laying rates) of learning under low food availability (Mery and Kawecki 2003, 2004). Furthermore, gaining experience requires time and errors may occur occasionally (Papaj and Prokopy 1989). Additional genetic material, such as plasticity-regulating loci (Schlichting and Pigliucci 1993), is presumably necessary to encode not only a trait, but also its plasticity (Williams 1966, as cited in Papaj and Prokopy 1989). In colony disease defense, interindividual variability in social interactions can lead to highly connected "super-spreaders", which are able to increase the transmission of disease within the colony (Stroeymeyt et al 2014).

To establish a genetically diverse workforce, a reproductive female either has to share the nest and reproduction with others (polygyny) or has to mate multiply, which often incurs costs (polyandry), such as the risk of injury and disease transmission, an increased risk of predation and increased investment of time and energy (Crozier and Fjerdingstand 2001). According to kin selection theory, increased genetic diversity will decrease relatedness among workers (e.g. Boomsma et al. 1999) and thereby reduce the incentive for cooperation and increase the potential for intranidal conflicts (reviewed in Ratnieks et al. 2006). Reproductive conflicts among workers led to immunosuppression in groups of the ant Diacamma sp. "nilgiri" (Bocher et al. 2007) and could negatively impact non-reproductive division of labor as workers might instead invest into dominance interactions or reproduction. The occurrence of several different subfamilies within one nest could also lead to nepotism (i.e. favoring of one's own kin), where workers use hydrocarbon profile cues to preferentially rear larvae of their own patri- or matriline, if colony costs are not prohibitive. Although evidence for nepotism is generally weak in social insects, workers of the ant *Formica fusca* are able to detect kin relationships and favor their closest kin when rearing brood in polygynous colonies (Hannonen and Sundström 2003). Furthermore, higher genetic diversity might also make colonies more susceptible towards a larger suite of parasites, possibly increasing rates of acquiring infections (van Baalen and Beekman 2006). The potential costs linked to increased genetic diversity can be lower in polygynous colonies if reproductive queens are related and/or if there is a queen turnover (i.e. reduced reproductive lifespan of a single queen; van Baalen and Beekman 2006 and the references therein). In C. velox, nearly all queens are practically clones (chapter three and Eyer et al. 2013b) and multiply mated with males from another genetic lineage, leading to a high level of heterozygosity in the workforce (Eyer et al. 2013b). I further detected a significant skew in the distribution of patrilines between young laboratory-born and old field-derived workers, suggesting that either (i) not all queens are equally contributing to egg laying at least temporarily (queen turnover) and/or (ii) that sperm are not mixed (sperm mixing seems to be common in some species: e.g. Laidlaw and Page 1984; Holman et al. 2011; Stürup et al. 2014; but not in others: e.g. Sundström and Boomsma 2000; Wiernasz and Cole 2010) and workers of different patrilines are thus sequentially produced. Both mechanisms would increase genetic relatedness among nestmates of approximately the same age. Determining the benefits and costs of interindividual variation will be important to address the underlying mechanisms and help to advance questions such as if there is an optimal level of workforce variability, depending on colony size, tasks performed (Jeanson and Weidenmüller 2013) or parasitic pressure. Increased research effort manipulating nestmate variability in immune investment (for instance the demography or the level of individual experience in a sanitary task) is warranted to address colony-level effects of nestmate diversity for disease defense.

7.3 The modulation of sanitary division of labor and the costs and benefits of helping

Sanitary division of labor (i.e. individuals perform different sanitary tasks from the group repertoire) is considered a group-level trait and its expression (i.e. strength) will presumably depend on the investigated species, colony size, colony genetic and demographic diversity, on the focal task, individual experience and on the task-associated stimulus level (i.e. parasitic pressure). Increased division of labor is expected in bigger colonies (Bourke 1999; Thomas and Elgar 2003; Jeanson et al. 2007; Holbrook et al. 2011) and for more virulent parasites, which pose a higher sanitary risk. On the other hand, highly virulent parasites or high parasite abundance might lead to a weaker division of labor. "All hands on deck" could be needed to deal with the sanitary hazard, including task performance of individuals with high response thresholds for this task. The number of task-performing individuals (i.e. low or strong division of labor for this task) and their identity (e.g. high versus low social connectivity, high versus low activity level, naïve versus experienced) will affect intra-colony parasitic spread (Stroeymeyt et al. 2014). Relatively weak division of labor seems to occur for cadaver management in *Cataglyphis velox* and a significant percentage of cadaver transporters were also observed foraging (chapter two). Presumably, the sanitary risk arising due to the management of nestmate cadavers is either limited or also occurs for foragers scavenging on fresh cadavers of other arthropods. The parallel performance of both tasks might thus not increase intranidal disease transmission in this species. At low exposure risk, a weak division of sanitary labor and thereby task performance by a higher percentage of nestmates might be expected if exposure results in immune priming and confers survival benefits upon a secondary parasite contact (e.g. Konrad et al. 2012). A strong division of sanitary labor would be expressed in a 'sanitary caste', whose existence has not yet been reported in social insects. If collective sanitary defense is efficient against the establishment of parasites within the nest, the costs of colony-level physiological immune investment can be reduced (Evans et al. 2006; Simone et al. 2009).

The performance of sanitary tasks could affect an individual's cuticular hydrocarbon profile, either through direct parasite effects or through the activation of the immune system (e.g. Richard et al. 2008), which could increase agonistic interactions (e.g. Hart and Ratnieks 2001; Richard et al. 2008) or decreasing social interactions and thereby reinforcing division of sanitary labor.

The performance of behavioral sanitary tasks can represent a double-edge sword both at the level of the task performer and the colony. Parasite-exposed and immune-stimulated individuals are groomed by nestmates which increases survival of the exposed individual through the removal of parasitic stages (e.g. Drees et al. 1992; Rosengaus et al. 1998b; Hughes et al. 2002; Little et al. 2006; Aubert and Richard 2008; Yanagawa et al. 2008; Walker and Hughes 2009; Reber et al. 2011; Tragust et al. 2013a). But at the same time, performing sanitary care increases the risk of disease uptake by the caregiver (e.g. Kramm et al. 1982; Cremer et al. 2007) and could facilitate parasitic spread within the colony (Schmid-Hempel 1998; Hughes et al. 2002; Konrad et al. 2012; reviewed in Stroeymeyt et al. 2014). In the leaf-cutting ant Atta sexdens rubropilosa, media workers who attempted to pile and cover Metarhizium anisopliae conidiospores had a higher mortality than nestmates with less contact (Jaccoud et al. 1999). Chapter one and other studies using Metarhizium anisopliae point however to a low risk of disease contraction among nestmates of different developmental stages (adults: Rosengaus et al. 1998; Hughes et al. 2002; brood: Ugelvig et al. 2010; Tragust et al. 2013a). A caregiver might further have to deal with costs associated with higher physiological immune investment to limit the increased risk of infection (e.g. Sadd and Siva-Jothy 2006; Lacerda et al. 2010; Konrad et al. 2012). These costs suggest that providing sanitary care requires altruistic acts of the task-performing nestmate. But performing sanitary tasks can increase inclusive fitness of the caregiver, if copies of its genes are passed on to the next generation (Hamilton's Kin selection theory: Hamilton 1964; Foster et al. 2006). Through priming of the physiological immune system, the caregiver can even receive survival benefits upon a future exposure with the same parasite (e.g. Konrad et al. 2012). A naïve individual might thus only limit its contact to parasites and exposed or infected nestmates at high parasite infectivity or quantity (Diehl-Fleig and Lucchese 1991; Milner and Staples 1996; Mburu et al. 2009) or if the individual itself is highly susceptible or valuable (Ugelvig and Cremer 2007). It is still unknown under which conditions an individual should either avoid a sanitary hazard or increase immune defense investment and research investigating the cost-benefit ratio of caregivers as well as parasite transmission within colonies, should receive more attention (Cremer et al. 2007; Ugelvig and Cremer 2007; Stroeymeyt et al. 2014).

7.4 Who is expected to invest more heavily into immune defense?

As the performance of parasite defense can be costly, individuals with a lower residual value for the colony, such as old workers, are generally expected to perform these tasks ('residual value theory' e.g. Kolmes and Fergusson-Kolmes 1989; Bot et al. 2001). Indeed, directionality in the mechanisms of task allocation has been detected with workers switching to more dangerous tasks with age (Schmid-Hempel and Schmid-Hempel 1993; Tripet and Nonacs 2004). In chapter two, cadaver transporters showed significant overlap with foraging individuals, which might signify that cadaver transporters belong indeed to the oldest workers of the colony or that age polyethism is less pronounced in C. velox. Irregularities in the agepolyethism schedule have been described for the closely related Cataglyphis cursor (Retana and Cerdá 1990, 1991). Honeybees performing hygienic behavior and necrophoresis, as well as leaf-cutting ants performing waste-management, are older than nurses but younger than foragers (Arathi et al. 2000; Breed et al. 2002; Mersch et al. 2013; Camargo et al. 2007; Waddington and Hughes 2010). Having individuals of medium age perform sanitary tasks could be advantageous for the colony, as these individuals do not risk transmitting disease to the brood, but are young enough to be able to profit from an eventual immune protection acquired during task performance. In the case of sanitary brood care (chapter one), nurses seem most likely to perform the task within the nest as they will be the first to notice the sanitary threat and possess the highest probability to respond towards (non-sanitary) brood stimuli. Older worker cohorts typically have reduced contact with the brood. Rapid detection of diseased brood is especially important in ants, where brood is typically stacked facilitating cross-infection (Ugelvig et al. 2010; Tragust et al. 2013b). If the physiological status of an individual interacts with its expression of sanitary tasks, one would expect workers performing sanitary tasks (and having the highest risk of parasite exposure) to show increased physiological immune investment (Bocher et al. 2007; Lacerda et al. 2010; but see: König and Schmid-Hempel 1995; Doums and Schmid-Hempel 2000).

Morphology could be another factor of sanitary task allocation, as leaf-cutting ant waste managers were smaller than foragers (e.g. Ballari et al. 2007). Smaller individuals are less costly for the colony to produce (on an individual basis; e.g. Calabi and Porter 1989) and might thus be expected to have a higher probability of sanitary task performance, if task performance is not constrained by worker morphology. Social insects are associated with a variety of symbiotic microorganisms, which can for instance serve nutritional or disease defense purposes (e.g. Currie et al. 1999; Hughes et al. 2008). Nestmates may vary in the abundance of symbiotic partners on or within their bodies, which would lead to variability among nestmates in their contribution to colony disease defense. Major workers of the leaf-cutting ants *Acromyrmex* have a higher cuticular abundance of a mutualistic bacterium (an

actinomycete which produces antibiotics and suppresses the growth of *Escovopsis*, a specialized fungal parasite of their mutualistic fungus garden) than minor workers. Major workers are also more present in parts of their fungus gardens where *Escovopsis* is most abundant (Poulsen et al. 2002). In social insect colonies, it is common to find inactive workers (e.g. Cole 1986; Jandt and Dornhaus 2009) who might invest more resources into physiological immune defense since they spend less energy on the performance of behavioral tasks. They could thus serve as immune barriers against disease spread (Bocher 2007; Robinson 2009; Stroeymeyt et al. 2014). In the ant *C. velox* however, inactive workers did not differ in the level of phenoloxidase from active workers (Bocher 2007).

Having been previously infected by a parasite primes the physiological immune system and thereby confers a survival advantage upon a secondary challenge (Konrad et al. 2012; reviewed in Masri and Cremer 2014). Either an infection or a previous exposure to a parasite could also affect sanitary response thresholds, a question which has to my knowledge not yet been investigated. If a previous infection would lower response thresholds for sanitary tasks, individuals with increased immune protection (immune-primed individuals) will be more likely to perform the riskiest tasks in the colony, conferring increased disease protection to the colony. Nestmates differ in their responsiveness towards sanitary stimuli (e.g. Masterman et al. 2001; Gramacho and Spivak 2003; López-Riquelme et al. 2006) and studies on honeybees suggested that individuals with the lowest response thresholds for disease brood are indeed the ones who are more likely to perform hygienic behavior (Masterman et al. 2001; Gramacho and Spivak 2003). Furthermore, the proportion of foraging honeybees infected with Nosema apis is higher under harsher environmental conditions (Woyciechowski and Kozlowski 1998). Response thresholds can be genetically determined (e.g. Pankiw and Page 1999; Kryger et al. 2000; Masterman et al. 2001; Jones et al 2004; Scheiner and Arnold 2010) and further underlie plasticity due to factors such as age, nutrition, rearing conditions; recent experience of the individual and rate of stimulus increase (Plowright and Plowright 1988; Theraulaz et al. 1998; Pankiw and Page 1999; Pankiw et al. 2001; Weidenmüller 2004; Yousif 2005; Weidenmüller et al. 2009; Westhus et al. 2013). Previous infection or exposure to parasites might be another source of response threshold variation for sanitary tasks.

7.5 Nature versus nurture: phenotypic plasticity in immune defense mechanisms

The relative contribution of genetic and environmental effects on traits (such as individual size) varies among species and ranges from mainly environmentally determined phenotypes to nearly complete genetic control (see for a review on genetic *versus* environmental determination of social insect caste: Schwander et al. 2010). Individual phenotypes are determined by complex interactions of genotypic and environmental effects, which modulate gene expression patterns (Smith et al. 2008b). Especially during the last years, permitted by the development of new molecular genetic tools, differences in gene expression patterns

among task groups have been detected in several species but most of the genes underlying heritable effects of social organization still need to be identified (Smith et al. 2008b). Smith et al. (2008b) even assume that the epigenetic differences detected among task groups could only be 'the tip of the iceberg' and that more discoveries of heritable effects on components of social organization are expected.

Disease resistance is an important life-history trait and its efficiency closely related to individual fitness (Cotter et al. 2004). It is generally assumed that natural selection decreases additive genetic variation in traits determining individual fitness and that fitness-increasing alleles become fixed. Secondly, high environmental and non-additive genetic contributions to phenotypic variation may be responsible for a low heritability of fitness-related traits (Merilä and Sheldon 2000 and the references therein). Unexpectedly, studies on non-social insects have demonstrated significant levels of additive genetic variation in immune components such phenoloxidase and antibacterial activity (Kurtz and Sauer 1999; Ryder and Siva-Jothy 2001; Cotter and Wilson 2002; Cotter et al. 2004; Schwarzenbach et al. 2005). Hypothetically, the observed genetic variation in these traits arises from antagonistic pleiotropy (i.e. a gene positively affects one trait and negatively another), which is the genetic basis of trade-offs between traits (Roff 1992, as cited in Cotter and Wilson 2002; Cotter et al. 2004). Increased physiological immune investment involves costs in form of resources and the risk of immunopathology (e.g. Moret and Schmid-Hempel 2000; Sadd and Siva-Jothy 2006). Trade-offs exist across several insect taxa both between immune defense and other life-history traits (e.g. reproduction, longevity, competitive ability: Sheldon and Verhulst 1996; Kraaijeveld and Godfray 1997; Moret and Schmid-Hempel 2000; Armitage et al. 2003; reviewed in Schmid-Hempel 2003) and between different components of physiological immune defense (e.g. phenoloxidase and antibacterial activity: Moret and Schmidt-Hempel 2001; Moret and Siva-Jothy 2003; Cotter et al. 2004; Ruiz-González et al. 2009). Maximum immune defense is thus not optimal immune defense (Zuk and Stoehr 2002) and can only be bought at the expense of other important traits, a possible reason why additive genetic variation in immune components could be maintained (Cotter et al. 2004).

Selection pressures vary across species and differ between non-social and social species, potentially leading to differences in the heritability of immune traits. Once workers were limited to serve mostly as non-reproductive helpers in social insects, natural selection could shape their traits to increase colony fitness (Smith et al. 2008b). In social insects, disease transmission is assumed to be increased due to the high density of closely related and interacting individuals and disease defense is typically performed by (mostly) sterile individuals (Cremer et al. 2007 and the references therein). For these workers, inclusive fitness gains are expected to outweigh individual defense (Cotter et al. 2013). Collective immune mechanisms might decrease the need to invest into physiological immune defense (e.g. Evans et al. 2006; Castella et al. 2008; Simone et al. 2009), which comprises not only costs at the individual but also at the colony level. Honey- and bumblebee colonies whose

members invested more strongly into individual immune defense produced fewer offspring (Moret and Schmid-Hempel 2004; Evans and Pettis 2005). In chapter 3, patrilines of the ant *C. velox* did not significantly vary in the level of phenoloxidase and thus showed no heritability for this trait. Accordingly, patriline differences in the level of phenoloxidase or in the capacity to encapsulate a foreign body were not detected among honeybee larvae (Wilson-Rich et al. 2012). Environmental determination and thus condition-dependence of immune investment might confer increased disease defense only to individuals performing the riskiest tasks within the colony and thereby limit costs of immune investment at the colony level. Furthermore, condition-dependent immune expression might provide colonies with a higher flexibility under rapidly changing environmental conditions. If for instance behavioral immune defense is modulated by individual experience or the rate of stimulus increase, as has been demonstrated for thermoregulating bumblebees (Westhus et al. 2013), colonies might be better able to limit the spread of a parasite within the nest.

According to expectations based on the trait individual size (Bargum et al. 2004), arguments both against (see above) and for a heritable component to immune investment exist. If interindividual variation in immune investment positively affects the efficiency of division of sanitary labor in that individuals with a higher protection perform the riskier tasks (as suggested by the findings of Bocher et al. 2007 and Lacerda et al. 2010, but see: König and Schmid-Hempel 1995; Doums and Schmid-Hempel 2000) or efficiently hinder disease spread as immune barriers, then selection on worker immune investment should be diversifying. A diversifying selection would lead to heritability of immune investment and workers of genetically more diverse colonies would be expected to show a higher immune trait variance (which was not demonstrated in honeybee larvae: Wilson-Rich et al. 2012). More research is needed in this field to determine the relative importance of both environmental and genetic effects on immune investment and to be able to address the question why colonies of higher genetic diversity are more resistant towards disease. Social immune defense behaviors seem a likely candidate (as in Ugelvig et al. 2010), since behavior is expected to show stronger plasticity than other immune defense barriers (Hughes and Cremer 2007) and heritability for hygienic behavior has been demonstrated in africanized honeybees (Padilha et al. 2013).

7.6 When is experience-modulated behavioral plasticity expected to occur?

Experience-dependent behavioral modulation is assumed to occur in all aspects of life and even short-lived fruit flies learn in feeding, predator avoidance, aggression and sexual behaviors (reviewed in Dukas 2008). The modification of behavior due to learning could in some cases increase fitness, as it increases foraging success in bees and wasps (e.g. Dukas and Visscher 1994; O'Donnell and Jeanne 1992) and led to a higher growth rate in grasshoppers (Dukas and Bernays 2000). The estimation of fitness benefits is not trivial because it is not clear how time or energy (in which costs or benefits of learning are often measured) relate to fitness (Papaj and Prokopy 1989). To be able to satisfactorily answer whether insects should

learn a certain task requires quantification of the fitness cost – benefit ratio of learning under natural conditions (Dukas 2008). Natural selection could alter learning, as variation in learning has a heritable component (e.g. Brandes 1988, 1991) and because differences in learning ability could be associated with fitness differences (e.g. Dukas and Bernays 2000).

The neural economy hypothesis assumes that high environmental complexity does not allow programming all recognition and response behaviors into the DNA or nervous system, due to physiological constraints (reviewed in Papaj and Prokopy 1989). If information storage is physiologically constrained, learning could be more advantageous than genetically fixed behavior if it is more economical in terms of the amount of useful information stored (Papaj and Prokopy 1989). Indeed, only a limited amount of information might be storable and interference could occur between acquired experiences (e.g. Lewis 1986; Chittka and Muller 2009). The environmental unpredictability hypothesis, another hypothesis to explain the evolution of learning, assumes that the high unpredictability of the environment within an individual's lifetime or over successive generations does not allow natural selection to program all appropriate recognition and response behaviors (reviewed in Papaj and Prokopy 1989). It is expected that experience-dependent behavioral modulation more likely occurs in moderately unpredictable environments and less likely occurs in either extremely predictable or in extremely unpredictable environments than fixed behavior (reviewed in Papaj and Prokopy 1989). Behavioral avoidance of parasites could be learned in response to selection from relatively predictable and high-cost parasites. Insects might rely on innate mechanisms to detect parasites but parasites could vary in spatial or temporal occurrence. If an abundance of parasites is positively correlated with a certain space or time, insects might learn these parameters to improve parasite avoidance.

Task groups can differ in their capacity for learning which might result in a differential degree of experience-induced plasticity for different tasks. Honeybee pollen foragers for instance were better learners than foragers with nectar preference, which might result from a general higher sensitivity to environmental foraging-associated stimuli of these bees (Latshaw and Smith 2005). Furthermore, individuals show cue biases for learning, which means that they learn certain stimuli faster than others. Honeybees learn odors similar to flower scents more rapidly than other odors (reviewed in Papaj and Prokopy 1989). Additionally, some motor patterns are more susceptible to modification by experience than others (Papaj and Prokopy 1989). Restricting experience-dependent plasticity to certain stimuli or motor patterns is assumed to be adaptive, as it could confine learning to situations, which are too unpredictable or complex for pre-programmed responses (Papaj and Prokopy 1989).

Temporary limitation in acquired experience effects (or short-term memory limits: Menzel 1983) might be adaptive as it could allow higher flexibility towards changing environmental conditions and only allow the persistence of behavioral modulation, which is still advantageous under the current conditions. The persistence of acquired experience-effects depends on the time delay between task performances (e.g. Westhus et al. 2013) and acquired

experience may vanish over night (e.g. Keasar et al. 1996; Westhus et al. 2013). Sanitary stimuli which occur only infrequently are presumably unlikely to lead to long-term behavioral modification, protecting against costs associated with learning in terms of neuronal resources (e.g. Laughlin et al. 1998; Farris et al. 2001; Riveros and Gronenberg 2010), time required and occasional mistakes. For rare events, the infrequent benefits gained by experience-dependent behavioral modulation might not outweigh the costs. In the ant *Myrmica rubra*, short-term spatial memory of necrophoric ants lasted only 7 minutes, presumably leading to a dispersion of cadavers in the field (Diez et al. 2011).

Research on how either repeated exposure to sanitary hazards or repeated performance of sanitary tasks affects individual response behavior was only lately initiated (e.g. Trumbo and Robinson 1997; Walker and Hughes 2009; Diez et al. 2011; Reber et al. 2011), even though social insects are presumably repeatedly exposed to sanitary threats. This research field might lead to exciting insights on an important mechanism of interindividual variation and deepen understanding of host-parasite interactions. Many questions await further study. It remains unexplored whether experience in sanitary task performance can result in task specialization and thus division of labor for these defenses. In chapter two, the repeated exposure to cadavers or performance of cadaver management increased the number of individuals engaging in cadaver grooming behavior and affected task allocation in only one out of four colonies. Inter-colony differences could arise through many factors, such as the sanitary stimulus presented. Of course this does not mean that other parameters of cadaver management are not affected by experience, simply that my experimental protocol was not able to reveal it. In chapter one, ant workers trained to both sham- and fungus-treated larvae increased larval grooming duration and thereby increased the removal of conidiospores by trained ants. If these experienced individuals were more likely to respond towards a sanitary threat within the colony, they could become task specialists. If experienced individuals specialize on the sanitary task, parasite spread might be reduced though behavioral compartmentalisation and higher task efficiency of experienced individuals. Increased efficiency of task performance could be achieved through lowered response thresholds (thus responding earlier towards a sanitary threat, at lower parasite abundance), a higher response probability, a reduced delay to response, an increasing response duration or improved motoric capacities (enabling the removal of more parasites in the same amount of time). Individuals specialized on necrophoric behavior removed cadavers faster and more successfully than less experienced individuals (Apis mellifera: Trumbo and Robinson 1997; Acromyrmex versicolor: Julian and Cahan 1999), but an effect of experience was not demonstrated (Trumbo and Robinson 1997). Feedback processes are important for self-reinforcement to occur (Plowright and Plowright 1988; Theraulaz et al 1998) and the successful performance of foraging resulted in lasting division of labor between successful and unsuccessful Cerapachys biroi workers (Ravary et al. 2007). It remains to be investigated what provides negative or positive feedback (What are the possible reinforcers?) in the context of disease defense. Do workers assess their own efficiency in performing the task, e.g. by sensing the decreasing number of

fungal conidiospores on exposed nestmates? Does increased effectiveness in conidiospore removal affect future responses of these ants? Research effort is still necessary to address whether experience affects the allocation of sanitary labor and leads to sanitary task specialization. It further remains elusive whether specialists of different sanitary tasks are really more efficient. It would be interesting to analyze if and how stimulus quantity, quality (e.g. the virulence of a parasite) or temporal presentation (i.e. periodic or chronic exposure) affect experience-dependent modulation of sanitary response behaviors.

7.7 Empirical difficulties to analyze proximate mechanisms contributing to interindividual variation in immune defense

Interindividual differences among social insect nestmates have long been recognized and regained interest during the last years in studies on behavioral syndrome (reviewed in Jandt et al. 2014). In social insects, studies on interindividual variability are complex in that selection acts both at the individual and at the colony level and worker variability can be caused by an interplay of non-mutually exclusive mechanisms (Jeanson and Weidenmüller 2013). Especially in species with temporal polyethism, an individual's age, physiology, the performed task and the level of acquired experience can strongly covary and their relative importance on individual response behavior and responsiveness are difficult to disentangle. Patriline and age of the individual might also be correlated, as some patrilines could be older than others (chapter 3), requiring studies on either age or patriline effects to also investigate the other factor. Furthermore, genetic factors can influence differential sensitivity towards environmental factors (e.g. by determining response thresholds; e.g. Masterman et al. 2001; Smith et al. 2008a; Scheiner and Arnold 2010) and influence an individual's rate of aging (e.g. Calderone and Page 1988) or its capacity to learn (Brandes 1988, 1991). Many genes are pleiotropic (i.e. one gene influences multiple phenotypic traits) and traits polygenically influenced (West-Eberhard 1989 and the references therein; Butcher et al. 2006), adding further complexity. Studies investigating task allocation to sanitary work thus need meticulous protocols which try to disentangle the multitude of potential mechanisms.

Uncertainties about the definition of task specialists in the current literature could complicate comparisons and generalizations between tasks and among species. Whereas in some publications task specialization is described as a concept "in which a small fraction of same-age workers perform the majority of work of certain tasks" (Julian and Cahan 1999), others describe specialization as a high concentration of individual "work effort in a single task" (Dornhaus 2008). Task specialization seems to be rather a statistical concept, "reflecting an individual's tendency to perform particular tasks more often than others" (Duarte et al. 2011), and ranges from short-term behavioral differences to fixed morphological variation in large insect societies (Robinson 1992; Duarte et al. 2011). The work by Trumbo et al. (1997) on necrophoric honeybees is often cited as an example of strong specialization on a sanitary behavior. But already these authors discuss the difficulties of classifying an individual as a

task specialist due to an unclear definition, which needs to include an appropriate timescale of specialization and a minimal number of task performances (a certain performance threshold) (Trumbo et al. 1997). In chapter 2, I did not analyze the complete behavioral profile of workers because of low brood availability and thus do not know whether some individuals specialized on cadaver grooming and transports. The full behavioral profile of workers is necessary to be able to identify them as task specialists because frequent task performers could instead be highly active 'elite' individuals, which perform every task in high frequency (Plowright and Plowright 1988; Trumbo et al. 1997).

Especially in social insects, the social environment strongly influences individual response behavior and even gene expression (e.g. Calderone and Page 1992; Arathi and Spivak 2001; Arathi et al. 2006; Gempe et al. 2012), complicating its study within the social context. A trait might not only underlie direct genetic effects (i.e. genetic origin), but trait differences among patrilines might also arise through indirect genetic effects (i.e. environmental effect), where the genes of nestmates modulate the phenotype of the focal individual. If nurses favour one patriline over others, larvae of the preferred patriline might grow faster (Bargum et al. 2004 and the references therein). I have thus trained *Platythyrea punctata* workers individually (chapter one) to investigate an effect of individual experience on task performance. However, to analyze how colonies allocate workers to sanitary tasks, manipulations within the colony context are necessary and I have thus studied cadaver management within the nest. In this social environment however, the relative contribution of self-reinforcement might have been hindered by social interactions and their effect on division of labor (Jeanson et al. 2008).

Investigating interindividual differences in disease defense is further complicated by a potential infection of the focal worker. Behavioral modifications of infected individuals increase distance to valuable colony members and reduce social interactions. Exposed or infected individuals have been shown to stay away from the brood chamber (Ugelvig and Cremer 2007; Bos et al. 2012), to decrease queen attendance (Wang and Moeller 1970), interact less with nestmates (e.g. Bos et al. 2012) or to precociously initiate foraging (e.g. Wang and Moeller 1970; Goblirsch et al. 2013). In some species, moribund individuals altogether leave the nest (e.g. Heinze and Walter 2010; Rüppell et al. 2010; Bos et al. 2012). Behavioral modifications might stem from parasitic manipulation, disease side-effects and behavioral changes of the focal individual or its nestmates (Ugelvig and Cremer 2007; Stroeymeyt et al. 2014). Not only infection, but already an immune response without parasitic exposure can lead to reduced learning abilities in honeybees (e.g. Mallon et al. 2003; Iqbal and Mueller 2007) and in bumblebees (e.g. Alghamdi et al. 2008). An alternative for experimental work would be to use a disease proxy, which triggers a sanitary behavior in the same way as a real parasite, but does not result in infection. Finding a suitable proxy which triggers the same (so far unknown) feedback mechanisms, a prerequisite for selfreinforcement to occur (Plowright and Plowright 1988; Theraulaz et al. 1998), is not trivial. Methods estimating physiological immune investment further harbour weaknesses. If the ability of an individual to resist infection is qualified using a specific parasite, the obtained results might be specific to the parasite studied and the efficacy of the immune system will be only indirectly measured. Other factors such as behavioral mechanisms might confound the results (Cotter et al. 2004). Due to the amount of work involved, studies examining immune investment tended to concentrate on just one or two components of the physiological immune system, rather than a comprehensive suite of immune traits. However, only a holistic approach will advance the field of ecological immunology.

Repeated exposure to a parasite can have multiple effects on the host, influencing parameters of individual responsiveness and sanitary task performance, as well as social interactions, spatial location or immune investment of the individual. Monitoring all of these parameters and manipulating one without the others seems a challenging task, potentially leading to the non-detection of weak effects. Increased research effort in the study of experience effects on immune defense and an increased support of publications containing negative results are required for a holistic view on if and how phenotypic plasticity in immune defense underlies individual experience.

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10. AFFIDAVIT

I hereby confirm that I prepared this thesis independently, by exclusive reliance on the literature and tools indicated therein.

This thesis has not been submitted to any other examination authority and it has not been published.

Paris, 05.09.2014

Claudia Westhus