The role of social and individual pathogen defense in an insect with facultative family life: insights into the early evolution of group living

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SUMMARY

The study of the emergence of group living and its evolution into permanent and complex societal systems is a major topic of interest in evolutionary biology, entailing careful study of the costs and benefits constraining and driving social evolution. One of the most prominent challenges group living species face is an increased risk of pathogen infection. To overcome this threat, individuals in groups can not only increase their investment into personal defenses, such as physiological and behavioral immunity, but also exhibit collective defenses known as social immunity. Recent studies suggest that social immunity plays a key part in the early emergence of sociality – yet little is known about their role in primitive groups, such as facultative family associations. In particular, it remains largely unknown to what degree social immunity in subsocial species is mediated by parental care, or how collective immunity shapes investment into personal immunity in these family groups. In this thesis, I examine these questions in the European earwig *Forficula auricularia*, an insect exhibiting facultative family life.

The first part of this thesis focuses on better understanding the role and effectiveness of social immunity in the European earwig. In **Chapter 1**, I show that social immunity occurs in this species through antimicrobial activity from the feces excreted by both the caring mother and the offspring. In **Chapter 2**, I demonstrate that the feces of offspring provide starvation resistance to siblings if no regular food sources are available, suggesting an additional and non-immune related role of feces production. In **Chapter 3**, I demonstrate that the presence of pathogens does cause offspring to overexpress immunity related genes during family life, but only in absence of a caring mother. Nevertheless, maternal care does not seem to shape offspring immunity long-term: in

Chapter 4, my data reveal that the presence of a pathogen, but not of the mother, affects the immunity of earwig offspring in adulthood. Finally, I address the recent findings of social immunity in subsocial species and their implications on social evolution in **Chapter 5**.

In the second part of this thesis, I focused on advancing our understanding of personal pathogen defenses, a critical component in the role and effectiveness of social immunity in social evolution. In particular, we aimed to highlight the difficulty of correctly assessing personal immune investment by investigating insect immune functions in the light of key physiological constraints. In **Chapter 6**, I showed that larger forceps trade-off with earwig immunity, but only in relatively small earwig males, and only after an immune challenge – overall highlighting the importance of a careful, multipronged approach when measuring immunocompetence. The data presented in **Chapter 7** finally shows that shorter winter length during development increases key parts of adult female immunity, but only if specimen were kept with unfamiliar conspecifics, which emphasizes the role of social stresses in immune investment.

In conclusion, this dissertation emphasizes how the proposed important redefinition of social immunity sets the stage for future inquiries into the role of pathogen pressure in early social evolution. By also highlighting key trade-offs between individual immunity and physical constraints, my work may lay the groundwork for new perspectives and informed investigations into the crucial interplay of personal immunity, social immunity, and the consolidation of facultatively social individuals into interdependent and complex social systems.

GENERAL INTRODUCTION

Early social evolution and the role of pathogens

Maximilian Körner

"Logic clearly dictates that the needs of the many outweigh the needs of the few."

- Mr. Spock, *Star Trek II: The Wrath of Khan* (1982)

SINGLE TO SOCIETY: SOCIAL EVOLUTION

Throughout the history of life on Earth we find evolutionary changes with an impact so profound that they alter habitats and ecosystems on a global scale. These so-called major transitions in evolution represent the formation of increasingly organized collectives by previously individual actors, such as the rise of multicellular organisms from single cells, or the assembly of individuals to form societal systems (Szathmáry and Smith, 1995). These increases in organizational hierarchy can only occur when conflict between individual elements is absent or suppressed. They therefore do not represent some form of inevitable natural progression in complexity, but rather a contingency depending on the selective conditions at any given point in time (Bourke, 2011). As a result, there is no directional bias, no guarantee life can, must, or will evolve towards the next level of organizational complexity. Understanding the processes and conditions enabling the emergence and maintenance of social structures from independent, solitary individuals is one of the central pillars of evolutionary biology.

Sociality can take many forms, ranging from temporary and facultative associations to permanent and obligate societies. These forms are likely to evolve successively, with comparatively simple, subsocial systems transitioning through obligate associations towards highly complex and organized eusocial superorganisms (Bourke and Franks, 1995; Crozier and Pamilo, 1996; Wilson, 1971). The exploration of mechanisms driving these transitions was accompanied by the emergence of *inclusive fitness theory,* which to this day underlies the core framework of sociobiology by explaining how donors of costly interactions with relatives can reap beneficial returns (Hamilton 1964a; Hamilton 1964b; Frank 1998; Marshall 2015). At the heart of

this framework lies the rejection of the notion that animals group up because of benefits to the population or species, and the consideration of fitness benefits provided to each individual in a group as the key drivers of sociality (Williams 1966, Hamilton 1971, Korb & Heinze 2015). This realization was crucial, since we can only begin to explore how sociality first emerged and has evolved if we possess a thorough understanding of the mechanisms that enable the benefits of group living (Kramer and Meunier, 2018). However, surprisingly little is known about the mechanisms that can shape and solidify primitive, facultative subsocial family systems into obligate social groups, and how the social interactions inherent to family life may have shaped the early evolution of sociality.

The emergence of group living

The initial transition from solitary to group living can occur via two different scenarios known as the gregarious and the subsocial pathways (Kramer and Meunier, 2018; Michener, 1969). Gregarious groups form when individuals of the same generation gather, as seen in communally nesting wasps, halictid bees, or shrews (Balloux et al., 1998; Bourke, 2011; J. Costa, 2006; Michener, 1969). The subsocial pathway entails an ongoing association of at least one parent (usually the mother) and members of the next generation, i.e. the offspring (Bourke, 2011; Kramer and Meunier, 2018; Lin and Michener, 1972). While both pathways can lead to the evolution of complex social structures, most animal societies have emerged from the subsocial pathway, possibly due to benefits of the social interactions between the closely related family members (Bourke, 2011; Michener, 1969; Wheeler, 1928; Wilson, 1975). These interactions result in a novel social environment during family life, which has recently been defined as “an

association of one or both caring parents with their offspring after hatching or birth that arose and/or is currently maintained to enhance the fitness of the constituent individuals". Because these social environments represent one of the earliest forms of sociality that can still be found in many contemporary species, they can offer ideal study systems to investigate the initial emergence of sociality (Kölliker, 2007; Kramer and Meunier, 2018).

Benefits and costs of life in a subsocial family system

Understanding the role of family life in the early evolution of sociality requires a thorough grasp on how the associated benefits outweigh the costs of the social system, as is the prerequisite for its emergence and maintenance (Clutton-Brock, 1991; Klug et al., 2012). For group living in general, some benefits are directly tied to simply being among conspecifics and independent of family interactions, such as the "dilution of risk" concept (Foster and Treherne, 1981; Turner and Pitcher, 1986) or the "many eyes theory" (reviewed in Roberts, 1996) which postulate that living in larger groups can reduce the risk of predation by predation by decreasing individual risk to be picked out of the herd or increasing the likelihood that predator is spotted before it can strike, respectively. Similarly, groups can have an advantage through numbers when going after prey (Creel and Creel, 1995; Fanshawe and Fitzgibbon, 1993) or searching for attractive foraging sites (Drent and Swierstra, 1977; Lachlan et al., 1998; Laland and Williams, 1997).

Family life in particular features a number of unique, interaction-based benefits that likely play critical roles in favoring prolonged grouping over rapid dispersal. Traditionally, the majority of these benefits were thought to be derived of the expression of parental care, which can be described as "any parental trait that enhances the fitness of a parent's offspring that is likely to

have originated and/or is currently maintained for this function" (Royle et al., 2012). These traits can include a large array of behaviors with common examples such as food provisioning, nest construction, and the attendance of offspring to fend off predators and pathogens (Clutton-Brock, 1991; Smiseth et al., 2012). These behaviors provide immediate and life-long benefits to offspring, overall improving their odds of survival and increasing their lifetime reproductive success (Alonso-Alvarez and Velando, 2012; Klug and Bonsall, 2014). In addition to benefits derived of parental care, recent studies have highlighted the importance of sibling cooperation during early family life, i.e. interactions between offspring that result in net benefits for the interacting parties (Kramer and Meunier, 2018; West et al., 2007), for example by food sharing (Kramer et al., 2015; Yip and Rayor, 2014).

However, social interactions and the resulting social environment during family life also entail costs for the individual actors. Caring parents often pay for their current reproductive effort with the direct energetic cost of care behaviors, increased predation risk, and reduced mating opportunities, all of which can severely hamper any future reproduction (Alonso-Alvarez and Velando, 2012; Trivers, 1972). Meanwhile, the offspring are subject to the inherent costs of all group living systems, such as increased competition, high risk of inbreeding (if offspring remain in proximity after maturation), cannibalism, and an increased risk of parasite and pathogen spread (Krause and Ruxton, 2002). On top of such direct costs, family life effectuates costly evolutionary conflicts over resource contribution and allocation (Kramer and Meunier, 2018; Parker et al., 2002; West et al., 2002). These conflicts occur among siblings (Mock and Parker, 1997; Roulin and Dreiss, 2012) and parents (Lessells, 2012; Trivers, 1972) but also between generations (parent-offspring conflict; Kilner and Hinde, 2012; Trivers, 1974). Obviously, parental

care can evolve despite these costs, provided that the benefits to family members outweigh the costs through either direct or indirect fitness gains (Hamilton, 1964; Smiseth et al., 2012).

It has been suggested that the aggregation of offspring in space and time (Ruxton and Sherratt, 2006) represents a crucial preadaptation to reduce the costs of parental care by allowing a parent to provide care to a single entity and location (i.e. the brood / nest) as opposed to distributed individuals (Kölliker, 2007; Tallamy and Wood, 1986). This preadaptation can then give rise to an ancestral stage of family life during which key social interactions, like parental care and sibling cooperation, may mutually reinforce themselves, facilitating the evolution of obligate family life and more sophisticated social systems (Kramer and Meunier, 2018; Royle et al., 2012; Wilson, 1975). However, such simple aggregations already entail one of the most important costs to group living, which may directly oppose the evolution of social interactions – namely an increased risk of pathogen and parasite spread (Meunier, 2015; Schmid-Hempel, 1998).

Indeed, infection by parasites and microbial pathogens has been acknowledged as one of the greatest hurdles in the evolution of sociality (Cremer et al., 2007; Hamilton, 1987; Masri and Cremer, 2014; Meunier, 2015; Schmid-Hempel, 1998). Even though much has been learned on how sophisticated groups combat infection risk, the interplay between pathogen pressure and the evolution of social interactions during ancestral group living is rather poorly understood. To thoroughly understand how and why the pressure of pathogens and parasites (hereafter summed up as “pathogens”) influence the evolution of sociality, we must first understand how animals deal with infection risk and pathogens, and why this affects groups differently from individuals.

RESISTING PATHOGEN PRESSURE: EXTERNAL & INTERNAL DEFENSES

Whether in solitary or in a group, the risk of infection is an omnipresent and perpetual hazard for every animal (Lu and St. Leger, 2016). Pathogen infections often have both short- and long-term consequences for the host, such as fitness-detrimental changes to physiology and behavior or increased mortality (Siva-Jothy et al., 2005). Infection always begins with a pathogen reaching a potential host and breaching their defenses. Thus, it is in the best interest of any individual to avoid infection through prophylactic traits such as behavioral immunity and external immune responses. Behavioral immunity can include actively avoiding proximity to high-risk environments, objects or substrates, as well as performing self-medication, e.g. animals surrounding themselves with and/or consuming substances with anti-pathogenic properties (de Roode et al., 2013; de Roode and Lefèvre, 2012; Hart, 2011; Moore, 2002). External immune responses often involve the application of secretions with antimicrobial activity, for example by birds during preening (Bandyopadhyay and Bhattacharyya, 1996; Martín-Vivaldi et al., 2010). However, the majority of physiological pathogen defenses are comprised of internal immune responses that are of critical importance if the external defenses are breached (Cotter and Kilner, 2010a). Internal immunity includes innate mechanisms, for example lysozymes, antimicrobial peptides, phagocytic hemocytes, and the encapsulation of intruders (Cerenius and Söderhäll, 2004; Cotter and Kilner, 2010a; Hillyer, 2016; Lavine and Strand, 2002; Rolff and Reynolds, 2009), as well as adaptive components (in particular in vertebrates) such as lymphocytes targeting specific cuticular antigens of parasites (Janeway et al., 2001). Many of these important mechanisms can be highly complex – thus understanding and quantifying them properly is critical to any effort to understand how animals combat pathogens exposure and infection.

Internal immunity in insects

Given the relative abundance and ease of handling of many invertebrate species, much work has been done to understand invertebrate immunity in general and insect immunity in particular (Gullan and Cranston, 2000; Kurtz and Franz, 2003; Loker et al., 2004). Internal pathogen defense in insects relies on the detection of foreign molecules to deploy immune mechanisms such as the production of antimicrobial peptides, enclosure by hemocytes or melanization, release of reactive oxygen and nitrogen intermediates, and inflammatory cytokines (Bulet et al., 1999; Cotter and Kilner, 2010a; González-Santoyo and Córdoba-Aguilar, 2012; Lavine and Strand, 2002). Pathogens are usually detected via specific receptor proteins that bind to (and thereby identify) characteristic microbial polysaccharides, such as peptidoglycan or lipopolysaccharides from bacterial and β-1,3-mannans from fungal cell walls (Gillespie et al., 1997). These proteins can be found suspended in the hemolymph, but originate from hemocytes and the fat body, the two mainstay organs of insect immunity (Bulet et al., 1999; Dunn et al., 1985; Gillespie et al., 1997). Once a pathogen intrusion has been detected, these two organs can then rapidly synthesize a diverse repertoire of antimicrobial peptides with both specific and overlapping targeting, granting them effective synergies against bacterial and fungal infection (Bulet et al., 2004, 1999; Rolff and Reynolds, 2009).

In addition to the recruitment of antimicrobial peptides, insect hemocytes are key players in many other crucial elements of immunity, such as phagocytosis, encapsulation and nodulation (Adamo, 2012; Lavine and Strand, 2002). Phagocytosis, the ingestion of bacteria or other foreign biotic matter, is mediated by recognition proteins binding to both the foreign cell and the hemocyte (Kanost et al., 2004; Siva-Jothy et al., 2005) but can also target abiotic bodies such as

beads or ink particles (Da Silva et al., 2000; Hernández et al., 1999; Yokoo et al., 1995). Encapsulation and nodulation refer to the binding of hemocytes to larger targets such as parasitoids or bacterial aggregations, respectively, by forming a sheath of hemocytes around the intruder (Lavine and Strand, 2002).

Foreign objects and mechanical injuries not only attract hemocytes but also trigger the process of melanization, i.e. the build-up of a melanin barrier around an intruding body or damaged tissue (González-Santoyo and Córdoba-Aguilar, 2012). In addition to forming a physical barrier, melanin formation catalyzes the production of cytotoxic substances such as phenol and quinone intermediates which can effectively cull intruding microbes (Cerenius and Söderhäll, 2004; Gillespie et al., 2000; Strand and Pech, 1995). While melanization is mediated by several enzymatic and non-enzymatic reactions, one of the most important enzyme cascades involved is the activation of phenoloxidase (or monophenyl L-dopa:oxygen oxidoreductase; EC 1.14.18.1; shortened PO) from its inactive precursor prophenoloxidase (PPO; Söderhäll and Cerenius, 1998). Note, however, that a high concentration of PO does not necessarily imply a strong immune system or a particularly resistant individual, since PO concentration in the hemolymph does not always correlate with pathogen resistance (González-Santoyo and Córdoba-Aguilar, 2012) and the release of cytotoxins through melanization is also associated with a high potential for self-damage (Sadd and Siva-Jothy, 2006).

Individual immunity & group living

Even though vast parts of the individual immune defense occur internally and are heavily affected by resource availability, individual condition, and life history (Lochmiller and Deerenberg, 2000;

Moret and Schmid-Hempel, 2000; Zuk and Stoehr, 2002), they are also subject to strong influence of the immediate surroundings, including the social environment (e.g. a group of conspecifics). This is due to several factors increasing the chance of contracting a pathogen in a group living environment. First, the increased density and/or close proximity to conspecifics, as well as social interactions, may increase the likelihood of coming into contact with an infected individual and contracting the pathogen (Cremer et al., 2007; Hamilton, 1987; Wilson and Cotter, 2008). Second, group members are often more likely to be closely related to each other than to other individuals in the population (especially during family life and in eusocial systems) and thus are very likely to share genetic-based vulnerabilities in terms of immunity, which can greatly facilitate the outbreak of an epidemic (Altizer and Nunn, 2006; Shykoff and Schmid-Hempel, 1991). Finally, the buildup of waste products (i.e. feces) at a stationary food resource or stable nest site represents fertile ground for many pathogens (Weiss, 2006) which is obviously detrimental to fitness (Jackson and Hart, 2009).

Given this risk, it is reasonable to expect a proportional increase in effort to prevent the introduction and/or spread of pathogens in the group, a concept known as density-dependent prophylaxis (also DPP, Wilson and Reeson, 1998). This phenomenon explains how individuals living in groups increase investment into personal immune defense, allowing them to reap the benefits of the group while resisting the increased pathogen pressure discussed above (which in turn can reduce the risk of infection for other group members). This concept has received much empirical support, especially in insects (reviewed in Wilson and Cotter, 2008).

Despite the ample support for density-dependent prophylaxis in invertebrates, a number of studies have highlighted exceptions in social insects where density does not predict individual

immune investment, possibly due to behavioral pathogen defenses or group interactions (reviewed in Elliot and Hart, 2010). For instance, a rapid change in the social environment, such as sudden isolation, can adversely affect pathogen resistance beyond what is predicted by density related immune investment, a phenomenon that is likely caused by a social stress response (Bartolomucci, 2007; Kohlmeier et al., 2016). However, the complicated relationship between stress responses and immunity (Adamo, 2012) remains rather opaque.

It would seem that group density alone is insufficient to properly predict the impact of gregarious behavior on individual immune investments. In addition, the interpretation of sole immunity measurements can be extremely difficult (Adamo, 2004; González-Santoyo and Córdoba-Aguilar, 2012). Thus, assessments of individual pathogen resistance are greatly improved by not only considering social frameworks and interactions, but also feasible trade-offs with behavior (e.g. aggression) and physiology (e.g. body condition, ornaments) which may shape an individuals' investment into personal immunity.

HOW GROUPS AVOID DISASTER: SOCIAL IMMUNITY

Over the last couple of decades, it has been revealed that pathogen defense in groups does not only consist of the sum of individual immune investments, but can also include a communal effort by the surrounding relatives (Cotter and Kilner, 2010a; Cremer et al., 2007). The existence and effectiveness of this *social immunity* has been frequently demonstrated in eusocial insect systems such as termites, ants, and bees (Cotter and Kilner, 2010a; Cremer et al., 2007; Wilson-Rich et al., 2009). In this context, social immunity was initially defined as a functional barrier to pathogen infection forming "a combination of prophylactic and activated responses as well as

behavioral, physiological and spatial mechanisms" (Cremer et al., 2007). Some examples of social immunity can be found in interactions occurring between eusocial workers and their brood. For instance, workers can remove pathogens from the cuticle of nest mates via allo-grooming (Reber et al., 2011). If exposure to a pathogen does occur, infected workers may limit their interactions with the highly valuable brood (Bos et al., 2012; Ugelvig and Cremer, 2007) or even exile themselves from the nest site (Heinze and Walter, 2010; Leclerc and Detrain, 2017; Rueppell et al., 2010) to prevent an epidemic. Several studies in eusocial insects also demonstrate the expression of sanitary behaviors that reduce the spread of pathogens in the nest, for example via removal of cadavers, food waste, and defecation products (Hart et al., 2002; Ulyshen and Shelton, 2012; Visscher, 1983; Zeh et al., 1999) or through the application of antimicrobial secretions to the brood or throughout the nest (Baracchi et al., 2012; López-Uribe et al., 2017; Yek and Mueller, 2011).

Since the coining of the term of social immunity over ten years ago (Cremer et al., 2007), a major proportion of the work investigating the phenomenon has been targeting its occurrence and mechanisms in eusocial insects (Cremer and Sixt, 2009; López-Uribe et al., 2017; Stroeymeyt et al., 2014). Due to the high level of organization in eusocial systems (which as a result are often referred to as "superorganisms"; Wilson and Sober, 1989), social immunity has even be regarded to represent the analogue to an immune system of a multicellular organism, where workers are akin to somatic immunity agents (Cremer et al., 2017). When considering the function of social immunity traits in eusocial species alone, such an approach certainly holds merit, given some examples of convergent evolution between colony-wide responses to infection and those of

metazoic bodies such as the occurrence of “social fever” in honeybee colonies (Starks et al., 2000).

The many insights into social immunity in a eusocial context have greatly advanced our understanding on how these complex structures evolve and are maintained (Cremer et al., 2007; Cremer and Sixt, 2009; Schmid-Hempel, 1998). However, considering social immunity as a derived trait of highly organized social species would overlook the possibility of its occurrence in less derived forms of group living. Since the additional costs imposed by pathogen pressure are not exclusive to eusocial species (Patterson and Ruckstuhl, 2013), communal mechanisms to alleviate these costs may occur in many forms of sociality and could even play a key role in the emergence and maintenance of early, non-derived forms of group living.

Indeed, social immunity has been demonstrated to occur in several non-eusocial group living species, most of which are insects, which display a very high diversity of different social systems (J. Costa, 2006; Wilson, 1971) and often exploit pathogen-rich environments (Siva-Jothy et al., 2005). Many, if not all of these examples bear high resemblance to what has been found in eusocial species. For instance, the subsocial wood-cockroach *Cryptocercus punctulatus* employs nest sanitation by utilizing feces with antimicrobial properties for nest construction and the burial of dead nestmates (Rosengaus et al., 2013). Antimicrobial excretions are also employed by both parents and larvae of the burying beetle *Nicrophorus vespilloides* around the carcass deployed for breeding (Cotter and Kilner, 2010b). Sanitary behaviors that involve preventing a buildup of defecation in the nest (Weiss, 2006) can be found in the cricket *Anurogryllus muticus* (West and Alexander, 1963) and is performed by mothers of the ambrosia beetle *Xyleborinus saxesenii* (Biedermann and Taborsky, 2011). Interactions such as allo-grooming between group

members may also contribute to social immunity in subsocial species (Mas and Kölliker, 2010; Thiel, 1999), but their effects on pathogen spread require further research.

Overall, it is clear that social immunity is not exclusive to eusocial species, but also occurs in subsocial species that share a stable habitat (like a nest or long-term food resource) and exhibit parental and/or sibling care (reviewed in Meunier, 2015). Because subsociality represents a critical first step towards permanent and obligate group living (Clutton-Brock, 1991; Kramer and Meunier, 2018), the occurrence of social immunity in subsocial species suggests that communal immune defense may play a crucial role in the consolidation of sociality from solitary ancestry. However, the majority of our knowledge regarding communal pathogen defense and its interplay with personal immunity is still based on observations and experiments conducted in eusocial species. As a result, the roles of personal and social immunity in the early evolution of sociality remain largely unknown. For example, it is unclear whether groups emerge despite the pressure exerted by pathogens (relying on physiological immunity) and then evolve purposeful social behaviors to reduce infection risk, or whether behaviors exhibited by solitary individuals that also provide pathogen protection to surrounding conspecifics (e.g. sanitary behaviors that reduce predation risk; Vet and Dicke, 1992) facilitate the emergence group living.

To further our understanding in this matter, we must learn how individual and communal pathogen defense interact and shape the social environment in simple social systems such as subsocial groups. In subsocial insects, for example, juveniles are frequently precocial, meaning they can forage for themselves and are less dependent on parental care (J. Costa, 2006; Smiseth et al., 2003). This facultative form of family life is likely to represent an ancestral state of sociality (Kölliker, 2007; Kramer and Meunier, 2018) and therefore offers an ideal model to investigate

how pathogen pressure and social immunity traits interact to shape a social environment that is preferable to swift dispersion.

STUDY ORGANISM

Range & morphology

The European earwig (sometimes called the common earwig) *Forficula auricularia* L. (Dermaptera: Forficulidae) is an insect species that is native to Europe, northern Africa, and western Asia but also occurs invasively in North America, Australia, and New Zealand. Throughout its range, it inhabits arid plains, wooded areas, grasslands, and anthropogenic landscapes such as urban gardens as well as rural fields and orchards (Lamb and Wellington, 1975). European earwigs are nocturnal and omnivorous, foraging at night for a wide variety of food sources such as decaying plant matter, fruit, flower pollen, and live prey such as aphids (J. Costa, 2006; Lamb and Wellington, 1974).

Box 1. Left: Adult earwig female guarding her clutch of eggs. Right: Adult earwig male (center) showing off his forceps in a defensive threat display. Note the sexual dimorphism of the forceps. Adult earwigs reach a length of 12 to 15 mm, with an overall bronze-brown coloration and yellow-ochre colored elytra below the shield-shaped pronotum. Concealed below the elytra are large, ear-shaped hindwings (eponym of "earwig" overall and "*auricularia*" in particular) that are very rarely used for flight. Pictures by Joël Meunier.

Probably the most conspicuous feature of this and other earwig species is the pair of elongated and movable cerci forming a pair of forceps at the end of the abdomen (Langston and Powell, 1975). These forceps are sexually dimorphic (see Box 1) and also vary in function between sexes: female forceps are straight (tweezer-shaped) and mainly serve as weaponry to fend off predators or conspecifics looking to cannibalize brood or offspring (J. Costa, 2006; Moore and Wilson, 1993). Male forceps, on the other hand, are curved (pincer-shaped), larger, and are heavily sclerotized (J. Costa, 2006; Radesäter and Halldórsdóttir, 1993a). In addition to defensive uses, males employ their forceps as a sexual ornament during courtship and as weaponry against contending males to interrupt copulation attempts and in comment fights (Radesäter and Halldórsdóttir, 1993b; Tomkins and Simmons, 1998). Earwigs are hemimetabolous, and the forceps, while present in each of the four larval instars, do not display dimorphic differences until after adults emerge from the fourth instar during early summer.

Maternal care & family life

Adult earwigs start mating promiscuously a few days after they emerge from the fourth and final larval instar. During most of their lives, European earwigs are highly gregarious, frequently resting in large, mixed-sex groups in daytime hideouts (e.g. under bark, in wood or stone cracks, man-made bird shelters; Lamb and Wellington, 1975; Langston and Powell, 1975). In early autumn, however, females cease to aggregate with their conspecifics to seek out a suitable nest site - which usually includes the construction of a burrow - and lay around 40-70 eggs (Radesäter and Halldórsdóttir, 1993b; Ratz et al., 2016). The females then remain with the eggs, performing essential pre-hatching care which entails guarding the nest site from both predators and

conspecifics, cleaning the eggs to remove pathogens (such as fungal spores), application of protective exudates to the egg cuticle and rearrangement of eggs or the nest structure to optimize concealment and microclimate conditions (Boos et al., 2014; J. Costa, 2006; Lamb, 1976a). Interestingly, the mother continues her care after the offspring (called nymphs) hatch from the eggs, forming a subsocial family group; a rare occurrence in insects. For several weeks, females continuously tend to the nymphs by grooming them, guarding the nest, and providing food by moving food items into the nest or through regurgitation (Lamb, 1976a; Mas and Kölliker, 2011; Meunier et al., 2012; Staerkle and Kölliker, 2008). This post-hatching maternal care usually continues until the nymphs have reached the second of their four larval instars (Lamb, 1976b; Wong and Kölliker, 2012).

Despite its extensive nature and possible fitness benefits, post-hatching maternal care in the European earwig is not obligatory for offspring survival and development (Kölliker, 2007; Kölliker and Vancassel, 2007). The precocial nymphs are mobile and able to independently acquire nutrients close to the nest (Lamb, 1976a; Wong and Kölliker, 2012). In addition, earwig nymphs are known to cooperate by sharing food resources, a behavior that increases with groups size, and more importantly, can compensate for low levels of maternal care (Kramer et al., 2015). Resources are shared among nymphs directly through proctodeal trophallaxis (direct gut-to-mouth transfer) or through allo-coprophagy (consumption of feces in the nest). Despite the rather passive nature of allo-coprophagy, in this species it represents a form of sibling cooperation because nymphs' feces production increases in the presence of related, but not unrelated conspecifics (Falk et al., 2014). This socially induced feces excretion within the nest is somewhat surprising, given the costs associated with waste accumulation (Jackson and Hart,

2009; Weiss, 2006, 2003). This conspicuous lack of sanitary behavior may indicate that dwelling in feces may serve functions beyond food sharing, as has been found in subsocial cockroaches and termites (Rosengaus et al., 2013, 1998).

THIS THESIS

Using the subsocial European earwig *Forficula auricularia* as a model, my research was conducted focusing on two major parts, posing five key questions. In the first part, I investigated (1) the occurrence of social immunity during the early, facultative family life of the European earwig (Chapters 1 and 3), (2) whether any such social immunity is mediated by the caring mother, the offspring, or both (Chapters 1, 3, and 4), and (3) whether offspring adapt their personal short- and long-term immune investment depending on the presence of parental care (and potential maternal-mediated social immunity; Chapter 4). In Chapter 2, I follow up on my findings regarding the sanitary behaviors (in an effort to further explain the lack thereof) investigated in Chapter 1. Furthermore, I (4) tackle the important question of how to define social immunity to properly include its occurrences in different types of sociality (Chapter 5). In the second part, I dedicated two chapters to the investigation of individual immunity to better understand how immune investment is affected by physiological constraints. Specifically, I aimed to elucidate (5) how immunity investment in this species may be affected by trade-offs with sexual signals (Chapter 6), and the effects of different climate conditions during rearing (Chapter 7).

Part 1: Occurrence and nature of social immunity in facultative family life

In the first part of my thesis, I investigated the expression of social pathogen defense during the early family life of the European earwig. Moreover, I was interested in whether any social immune defenses are derived from the presence of a caring mother, from cooperative efforts of the young offspring, or both. To further look into the relationship between family life and pathogen pressure, I studied whether the presence of a caring mother during pathogen exposure affects the expression of immunity-related genes in offspring, and whether we find any short- or long-term effects on offspring immunity.

The many studies elucidating the importance of social immunity in highly evolved social systems are crucial to understand the maintenance of sociality but shed little light on the role of social immunity during its early evolution. To mend this lack of knowledge, we must investigate the role of pathogen pressure and social defenses in primitive, non-derived forms of sociality, such as facultative family systems. In Chapter 1, we thus investigated the reported socially induced accumulation of feces (and conspicuous lack of sanitary behavior) during early family life of the European earwig (Falk et al., 2014) to test whether this behavior represents a form of social immunity, similar to what has been described in subsocial cockroaches (Rosengaus et al., 2013) and eusocial termites (Rosengaus et al., 1998). We found that both maternal and nymphal *Forficula auricularia* feces indeed inhibit microbial growth, representing a form of social immunity in a primitive form of group living.

Intrigued by this, we were then interested in whether the socially induced feces production of the nymphs not only represents a cooperative effort to combat pathogens but also

yields nutritional benefits that increase survival resistance. Coprophagy has been demonstrated in a large number of species, including insects, and may be a key factor in facilitating the evolution of permanent groups (Nalepa, 2015; Nalepa et al., 2001). In Chapter 2, I tested whether allo-coprophagy during early family life of the European earwig provides benefits in terms of starvation resistance. We found that consumption of sibling feces kept nymphs alive for longer than having no food, while eating maternal feces did not, indicating that this form of sibling food sharing may be a key player in the consolidation of family life. Furthermore, this allo-coprophagy in a non-derived from of sociality could represent an important first step in the evolution of the crucial hindgut sharing found in some eusocial species.

Interestingly, the feces mediated benefits we found are independent of maternal care. However, due to the facultative nature of earwig family life, offspring are expected to be able to adjust to and survive extreme changes to their social environments, which means that they can develop both with and without their parents. The adaptations that allow this offspring flexibility are generally well studied on a phenotypic level (e.g. Kilner and Johnstone, 1997; Kramer et al., 2015), but surprisingly understudied on a gene expression level (Kronauer and Libbrecht, 2018). In Chapter 3, we addressed this crucial question by means of gene expression analyses of nymphs that were raised either with or without maternal care. Given our previous findings on communal pathogen defense in earwigs, and the importance of pathogen pressure on social evolution in general (Cremer et al., 2007; Jackson and Hart, 2009; Meunier, 2015), these gene expression analyses were also done on nymphs raised in or without the presence of the entomopathogenic fungus *Metarhizium brunneum*. We found that varying the presence of the mother affected gene expression on a larger scale if there is no pathogen present, including the upregulation of

metabolism genes in maternal absence. Conversely, varying the presence of the mother with the pathogen present changed the expression of fewer and completely different genes. Similarly, we found that pathogen presence caused an upregulation of immune related genes only in absence of the mother. Overall, we showed that offspring adjustments to maternal presence involve metabolism changes, but that pathogen pressure seems to play a key role in these adjustments and thus family evolution in general.

Having found evidence for offspring short-term adaptations to pathogen pressure, depending on the presence of a caring mother, we were now interested to see whether these factors shape offspring development in the long term. The importance of post-hatching/birth parental care on offspring immunity has been reported in several vertebrates (Michaut et al., 1981; Saino et al., 1997; von Hoersten et al., 1993), while pathogen prevalence in the habitat can shape individual condition and immune investment (S. C. Cotter et al., 2004; Lindström et al., 2004). In invertebrates, however, the role of parental care in long-term offspring immune investment is poorly understood. In Chapter 4, we therefore studied offspring immunity from the end of family life until adulthood by measuring three important immune factors: phenoloxidase (PO), prophenoloxidase (PPO; always measured in combination with PO), and hemocyte count. Given the importance of pathogen presence to offspring development and immune investment overall, we investigated the simultaneous and interactive effects of early maternal presence, pathogen exposure and developmental stage on offspring immunity. Surprisingly, we found that early maternal presence had no impact on offspring immunity after family life, neither during development nor in adulthood. Pathogen presence, however, increased the hemocyte concentration in adults, while PO / PPO concentration was unaffected at any stage. Both immune

measurements increased during offspring development and were overall higher in females. Our results suggest that long-term benefits of family life in terms of immunity may be mediated by the presence of or interactions with siblings, rather than a caring mother.

While mounting evidence suggests a prevalent role of social immunity by means of family interactions during the early evolution of sociality, literature on the topic has been largely focused on eusocial species (e.g. Cremer et al., 2017; Stroeymeyt et al., 2014). As a result, the general nomenclature and framework of what should or should not be considered as social immunity is somewhat skewed toward permanent and obligatory forms of sociality. In Chapter 5, we reviewed the state of the art concerning the role of communal pathogen defense in social evolution by examining two current frameworks that define what social immunity should entail. We then proposed that a broader definition of social immunity can help to better understand the profound role of pathogens in both the early emergence as well as the maintenance of social systems. To this end, we introduced a novel group living framework for social immunity that includes temporary and/or facultative groups and sets three distinct criteria to keep the definition concise. In brief, we suggested (1) that social immune defense must provide aid to recipient individuals in reducing their infection risk, (2) that donors and at least some recipients must belong to the same group of conspecifics, and finally (3) that the defense mechanism in question must at least partially be selected for due to the pathogen defense it provides to the recipient.

Part 2: The impact of physiological constraints on immune investment

A key pillar of the research on individual and social immune defense is the assumption that we can properly assess the interplay and impact of pathogen pressure and social structure on infection risk and pathogen defense of the individual. However, the implications of pure immune assay results can sometimes be open to interpretation, rendering the estimation of pathogen resistance precarious at best (Owens and Wilson, 1999). This is because immune measurements can be pathogen specific (Adamo, 2004) or may not all equally indicate the ability to fend off infection (González-Santoyo and Córdoba-Aguilar, 2012; Pauwels et al., 2011). To alleviate this difficulty, it is of key importance to not only use multiple immune tests, but also to identify and test physiological constraints that help to interpret the data and shed light on possible explanations for differences in immunity.

In the second part of my thesis, I address this issue by investigating the relationships of adult earwig immunity with important life-history factors, such as investment into sexually selected traits. Even though physiologically expensive and conspicuous structures such as ornaments are expected to entail costs reflected in immune investments, several studies in insects show no such trade-off (McCullough and Emlen, 2013; Pomfret and Knell, 2006). In Chapter 6, we tested whether the forceps size of adult male earwigs trades off with immune investment. The length of forceps is positively associated with fighting ability but also number and length of copulations, and the ability to interrupt contesting males' mating attempts (Radesäter and Halldórsdóttir, 1993b; Tomkins and Simmons, 1998). To maximize the interpretability of our immune measurements, we gauged male condition by means of body size and then investigated three immune parameters (PO, PPO, and hemocytes) both before and after

an immune challenge with bacterial cell wall proteins (lipopolysaccharides). We found that weapon size traded off with hemocyte count, but only in low condition males, and only after the immune challenge. Conversely, PO/PPO did not trade off with weapon size. Our results thus highlight the importance of multiple measurements when assessing immunity. Moreover, they reveal a condition-dependent immunity trade-off in a sexually selected trait.

Adaptational challenges do not only include biotic factors of the habitat and social environment, but also climatic affects. Winter has an immense impact on the life history of any creature living in colder climates, especially if offspring are affected by the cold. To avoid adverse conditions, some insects burrow underground to create a microhabitat with a rather constant climate (Danks, 2002). Overwintering success is also tied to winter duration (Colinet et al., 2015; Lee and Dellinger, 1991) which is subject to change in the coming decades due to climate change (Parmesan, 2006; Stålhandske et al., 2015; Vitasse et al., 2017). However, the impact of winter length on adult insect performance is poorly understood. In the 7th and final chapter of my thesis, we investigated whether variations in winter length during rearing affect development of and immunity in adult female earwigs. Given the importance of the social environment in gregarious species, we tested females in familiar, unfamiliar or no groups. We found that females reared during shorter winters hatched earlier and reached adulthood faster. Conversely, females reared under long winter conditions showed higher PPO values, regardless of social environment. However, short winter females had a higher hemocyte count, but only if kept with unfamiliar conspecifics. These results reveal that despite a constant climate, winter duration has life-long effects on immunity and even alters how different social environments affect adult immunity.

CHAPTER 1

Feces production as a form of social immunity in an insect with facultative maternal care

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ABSTRACT

Social animals have the unique capability of mounting social defenses against pathogens. Over the last decades, social immunity has been extensively studied in species with obligatory and permanent forms of social life. However, its occurrence in less derived social systems and thus its role in the early evolution of group-living remains unclear. Here, we investigated whether lining nests with feces is a form of social immunity against microbial growth in the European earwig *Forficula auricularia*, an insect with temporary family life and facultative maternal care. Using a total of 415 inhibition zone assays, we showed that earwig feces inhibit the growth of two GRAM+ bacteria, two fungi, but not of a GRAM- bacteria. These inhibitions did not result from the consumed food or the nesting environment. We then demonstrated that the antimicrobial activity against fungus was higher in offspring than maternal feces, but that this difference was absent against bacteria. Finally, we showed that family interactions inhibited the antibacterial activity of maternal feces against one of the two GRAM+ bacteria, whereas it had no effect on the one of nymphal feces. By contrast, antifungal activities of the feces were independent of mother-offspring interactions. These results demonstrate that social immunity occurs in a species with simple and facultative social life, and thus shed light on the general importance of this process in the evolution of group-living. These results also emphasize that defecation can be under selection for other life-history traits than simple waste disposal.

INTRODUCTION

One of the major costs of group-living is its inherent risk of pathogen infection for group members (Cremer et al., 2007; Masri and Cremer, 2014; Schmid-Hempel, 1998). While solitary species can only use personal immune responses to fight against infections, group-living species also possess the unique capability of mounting collective immune defenses, a phenomenon called social immunity (Cotter and Kilner, 2010a; Cremer et al., 2007). Over the last two decades, a growing number of studies showed that multiple forms of social immunity can be expressed in species with permanent and obligatory social life, such as eusocial insects (reviewed in (Cremer et al., 2007)). These studies were of great interest for the development of research on social immunity in insects, because they demonstrated that the high risks of pathogen infection associated with obligatory and complex forms of social life were likely to select for the emergence of collective defenses against pathogens (Cotter and Kilner, 2010a; Cremer et al., 2007). However, they were of limited relevance to understand whether social immunity only emerged in eusocial systems and therefore represents a secondary trait derived from eusociality, or whether it also occurs in less derived forms of group living and thus possibly plays a central role in the early evolution of group living organisms (Cotter and Kilner, 2010a; Cremer et al., 2007).

One method to address this issue is to investigate the occurrence of social immunity in species with temporary and facultative group-living. This is the case of species with family life, which represents a common form of group-living in insects (Royle et al., 2012; Wong et al., 2013), can be temporary and facultative such as in precocial species (Kölliker, 2007; Smiseth et al., 2003) and is generally considered as a major step in the evolutionary route to eusocial systems (Bourke and Franks, 1995; Royle et al., 2012). In insects, family life is broadly associated with the

expression of care to the eggs and/or juveniles, such as protection against predators, clutch displacement and food provisioning (Trumbo, 2012; Wong et al., 2013). Family life may also include forms of social immunity before egg hatching. For instance, parents groom their eggs to prevent the development of fungal spores in the European earwig *Forficula auricularia* (Boos et al., 2014), apply bacteria with antifungal properties to brood cell prior to oviposition in the European beewolf *Philanthus triangulum* (Kaltenpoth et al., 2005), coat their nest with antimicrobial secretions in the housefly *Musca domestica* (Cardoza et al., 2006) or prophylactically avoid nest sites with high microbial pressure in the burying beetle *Nicrophorus vespilloides* (Rozen et al., 2008). Although prehatching forms of social immunity have been well studied in insects, surprisingly little is known about the nature and occurrence of the post-hatching ones (see e.g. in vertebrates (Gasparini et al., 2006; Jacquin et al., 2012)). Only recent studies showed that parental anal exudates and larval secretions exhibit antimicrobial properties in the burying beetle (Arce et al., 2013; Cotter and Kilner, 2010b; Reavey et al., 2014). In this species, however, larvae feed on the carcass serving as nesting habitat, so that these antimicrobial mechanisms could also reflect evolutionary responses to competition with microbes over food access and/or to the extraordinarily high microbial pressure in this specific habitat.

In this study, we investigated whether social immunity occurs in the form of the production of feces with antimicrobial activity in the European earwig *F. auricularia*, an insect with temporary and facultative family life. In this species, mothers provide care to their offspring in soil burrows for several months, during which all family members - once hatched - line ground and walls with their feces pellets (J. T. Costa, 2006; Falk et al., 2014; Koch and Meunier, 2014;

Kölliker, 2007; J. W. Y. Wong et al., 2014). Earwig maternal care can take multiple forms, such as egg and juveniles (called nymphs) attendance and food provisioning through regurgitation, which have been shown to enhance offspring fitness (Boos et al., 2014; Kölliker, 2007; Meunier and Kölliker, 2013; Staerkle and Kölliker, 2008). Nevertheless, nymph survival does not require maternal care, as nymphs are mobile at hatching and can forage for themselves (Kölliker, 2007; Meunier et al., 2012). Here, we first tested whether (1) earwig feces provides a form of social immunity by inhibiting the development of bacteria and fungus into the nest, and determined whether these effects were independent of the consumed food and nesting material. We then investigated whether (2) antimicrobial activity was stronger in maternal compared to nymphal feces, as expected under the assumption that it reflects a post-hatching form of maternal care. Finally, we tested whether (3) the antimicrobial activity of feces is a socially-mediated trait that is triggered or inhibited by experiencing mother-offspring interactions (Reavey et al., 2014). If antimicrobial properties are induced by mother-offspring interactions, we predict that the feces produced by isolated individuals show lower antimicrobial activities. Conversely, we predict higher antimicrobial activities in feces produced by the isolated individuals if the costs of producing antimicrobial agents in the feces entail a mother-offspring conflict, in which each party tries to reduce its own investment into the production of antimicrobial components while benefiting from that of the other.

MATERIALS AND METHODS

Insect rearing and feces collection

We collected feces pellets in 17 *F. auricularia* families composed of one mother and 36.11 ± 15.8 (mean ± SD) nymphs. These mothers were the first laboratory-born generation of individuals field sampled in 2012 in Dolcedo, Italy, and then maintained under standard laboratory conditions (rearing details in (Meunier et al., 2012)). To determine whether the occurrence of mother-offspring interactions influences the antimicrobial properties of maternal and nymphal feces, the 17 families were randomly distributed among two groups at egg hatching. In the first group, we experimentally prevented mother-offspring interactions by separating mothers from their clutch of nymphs one day after egg hatching (Isolation group, n = 10). By contrast, mothers in the second group were separated from their nymphs ten days after egg hatching (Family group, n = 7). These separations were done by transferring the mother and the clutch of nymphs to two new petri dishes. At day 10, mothers and groups of nymphs from family groups were separated and transferred into two new petri dishes, in which they were maintained until feces collection at day 13 (first developmental instars). This manipulation was also done on the individuals from the isolation groups to standardize the experimental process. The transfer and three day delay between separation and feces collection ensured that the collected feces was relatively fresh and in large enough quantity to conduct the radial diffusion assays.

Individuals received *ad libitum* standardized food (for food composition, see (Meunier et al., 2012)) from day 1 to day 9, and *ad libitum* green-colored pollen (Hochland Bio-Blütenpollen by Hoyer; Food die by DEKO BACK) from day 10 to day 12. Under these conditions, orphaning does not affect nymph quality in terms of developmental time and survival rate (Koch LK and

Meunier J, unpublished data). The use of colored pollen is common in earwig experiments (e.g. (Falk et al., 2014; Meunier and Kölliker, 2012a; Staerkle and Kölliker, 2008)) and was used here to disentangle feces pellets from sand grains in the rearing containers. At day 13, all (colored) feces pellets present in each petri dish were collected using a sterile 10 µl pipette tip. For each petri dish, the total amount of collected pellets was weighed to the nearest 0.1 µg (Pescale), then suspended in 500 µl sterile NaCl solution (0.9%) and finally stored at 4°C. This feces solution was used 2.6 ± 1.5 days (mean ± SD) later to conduct the radial diffusion assays (see below). All petri dishes (diameters 10 and 5 cm before and after separation, respectively) contained humid sand as substrate and a plastic shelter as a nest. They were maintained in a climate chamber at 60% humidity, constant 20°C and 10:14 h light:dark cycle during the course of the experiment.

Radial diffusion assays

We tested the antimicrobial properties of maternal and nymphal feces using a total of 170 radial diffusion assays against two GRAM+ bacteria, one GRAM- bacteria, and two fungi species (see details below). Radial diffusion assays were conducted in petri dishes (diameter 10 cm) filled with PDA (Potato Dextrose Agar, 70139, SIGMAALDRICH) covered with a solution of 109 bacteria or spores/ml. Four samples were tested per plate. To this end, each fourth of a PDA plate received a blank disc (antimicrobial susceptibility test discs, OXOID) in its center, on which 10 µl of feces solution was preliminary applied. The same process was used to conduct a total of 245 controls (49 per microbial species), in which we tested whether growth inhibition could result from the NaCl solution used to dilute the feces (n = 15/species), the food eaten by the tested individuals (10 mg of colored pollen pellets suspended in 1 ml NaCl solution, n = 15/species; 240 mg of standardized food source suspended in 1 ml NaCl solution, n = 4/species) or the sand on which

feces has been released (50 mg of sand suspended in 1 ml NaCl solution; n = 15/species). After inoculation, each plate was incubated at 36°C/24 h for bacteria and at 20°C/48 h or 20°C/72 h for the fungus (for *Saccharomyces cerevisiae* and *Aspergillus niger*, respectively). At the end of the incubation, the zone of clearance (diameter from one edge of the zone of inhibition to the other) was measured three times per sample and then averaged to give one mean value called antimicrobial activity.

The radial diffusion assays were conducted against five microbial species covering a spectrum of groups that have the capability to grow into earwig burrows. First, we used *Staphylococcus aureus* (NCIMB 9518), which is a GRAM+ bacteria known to secrete a range of enzymes and toxins associated with several diseases in vertebrates and invertebrates (Kayser et al., 1998). Second, *Bacillus subtilis* (ATCC 6633) is another GRAM+ bacteria, which is a facultative pathogen commonly found in the soil (Kayser et al., 1998). Third, *Escherichia coli* (ATCC 25922) is a GRAM- bacteria typically found in the intestinal tracts of mammals and insects (Kayser et al., 1998). Fourth, *Saccharomyces cerevisiae* (ATCC 2601) is a fungus known to cause lethal infections in invertebrates (Kayser et al., 1998). Finally, *Aspergillus niger* (wild type strain) is a fungus growing on rotten plant material that can be an opportunistic pathogen (Kayser et al., 1998).

Statistical analyses

We first tested the effect of feces producer (mother or nymphs), family life (isolation or family group) and their interaction on the log-transformed amount of feces produced between day 10 and 13 (i.e. the amount of diluted feces) using a linear model. Because inhibition zones do not follow normal distributions and include a substantial number of zeros across the radial diffusion assays (see results), the significance of the effects of feces producer and family life on

antimicrobial activity were then tested using a series of randomized analysis of variance (randomized ANOVA; (Manly, 1997)). This non-parametric method allows estimating the significance of a factor (i.e. calculate p-values) by running a series of 10,000 ANOVA, in which the response variable (i.e. antimicrobial activity or antimicrobial activity per mg of feces) is permuted across the explanatory factors (i.e. feces producer and family life). Finally, we conducted pairwise comparisons between the antimicrobial activities of the controls (pooled) and the ones of the maternal or the nymphal feces using Mann–Whitney rank tests, in which the significance level $\alpha = 0.05$ was adjusted for multiple testing to $\alpha = 0.025$ using Bonferonni correction. All statistical analyses were conducted using the software R v3.1.1 (http://www.r-project.org). The R script to conduct randomized ANOVA is available on demand.

RESULTS

Each mother produced on average 13.06 ± 2.34 mg (mean ± SE) of feces between day 10 and day 13. This quantity was smaller than the 180.63 ± 18.88 mg of feces produced by the clutch of nymphs during the same period of time (Likelihood Ratio (LR) $\chi 21 = 252.72$, $P < 0.0001$). The total amount of feces produced over three days was independent of family isolation (LR $\chi 21 = 0.97$, $P = 0.324$), or of an interaction between family isolation and feces producer (LR $\chi 21 = 1.12$, $P = 0.290$).

Inhibition zones were found in 25 (73.5%) assays against *B. subtilis*, 10 (29.4%) against *S. aureus*, 19 (55.9%) against *S. cerevisiae*, 17 (50.0%) against *A. niger*, but none (0.0%) against *E. coli*. Maternal feces inhibited the growth of at least one microbial species in 13 (76.5%) of the 17 tested families, while nymphal feces had inhibition effects in every sample from the 17 (100%)

Table 1.1 Influences of feces producer and mother-offspring interactions on antimicrobial activities (a) per full sample and (b) per mg of feces. Feces producers were either the mother or the nymphs. P-values were obtained from randomized ANOVAs, significant P-values depicted in bold.

	B. subtilis	*S. aureus*	*A. niger*	*S. cerevisiae*
(a) Activity per full feces sample				
Feces producer (FP)	P = 0.440	P = 0.098	**P = 0.005**	**P <0.0001**
Mother-offspring interactions (MO)	P = 0.808	P = 0.104	P = 0.342	P = 0.051
FP : MO	P = 0.553	P = 0.934	P = 0.068	P = 0.215
(b) Activity per mg of feces				
Feces producer (FP)	**P <0.0001**	**P = 0.008**	P = 0.095	P = 0.080
Mother-offspring interactions (MO)	P = 0.813	P = 0.575	P = 0.052	P = 0.078
FP : MO	P = 0.812	P = 0.731	**P = 0.037**	P = 0.089

families. None of the controls (NaCl, pollen, standardized food and sand) showed antimicrobial activity in any of the 245 assays (Figure 1.1). The antimicrobial activity of maternal and nymphal feces produced over three days depended on the feces producer and the microbial species, but not on the occurrence of mother-offspring interactions (Table 1.1a, Figure 1.1). Specifically, antimicrobial activities against *A. niger* and *S. cerevisae* were lower in maternal compared to nymphal feces, whereas antimicrobial activities against *B. subtilis* and *S. aureus* were independent of feces producer (Table 1.1a). Except against *E. coli*, each type of feces showed higher antimicrobial activity than the controls (Table 1.2, Figure 1.1). The general antibacterial activity of nymphal feces against *S. aureus* was mostly driven by three points in the data set (Figure 1.1). If these three points were excluded, the resulting mean antibacterial activity of nymphal feces against *S. aureus* would become null and thus smaller than the one of maternal feces (Mann–Whitney test, W = 168, p = 0.008) and similar to the controls (Figure 1.1).

In line with the prediction that antifungal components are more concentrated in maternal than nymphal feces, we found that the antimicrobial activities per mg of feces against *B. subtilis* and *S. aureus* were larger in maternal compared to nymphal feces (Table 1.1b, Figure 1.2). By contrast, feces producer did not influence such activity against *S. cerevisae* (Table 1.1b, Figure 1.2). Overall, the occurrence of mother-offspring interactions did not shape the antimicrobial activities per mg of feces against *B. subtilis*, *S. aureus* and *S. cerevisae* (Table 1.1b, Figure 1.2). However, it interacted with feces producer to shape the antimicrobial activity per mg of feces against *A. niger* (Table 1.1b, Figure 1.2). Specifically, the presence of mother- offspring interactions canceled the antimicrobial activity of maternal feces (Mann–Whitney rank test; W= 52.5, P = 0.040) but had no effect on the one of nymphal feces (Figure 1.2, W= 38, P = 0.807). Note that this interaction was only marginally non-significant when analyzing the overall

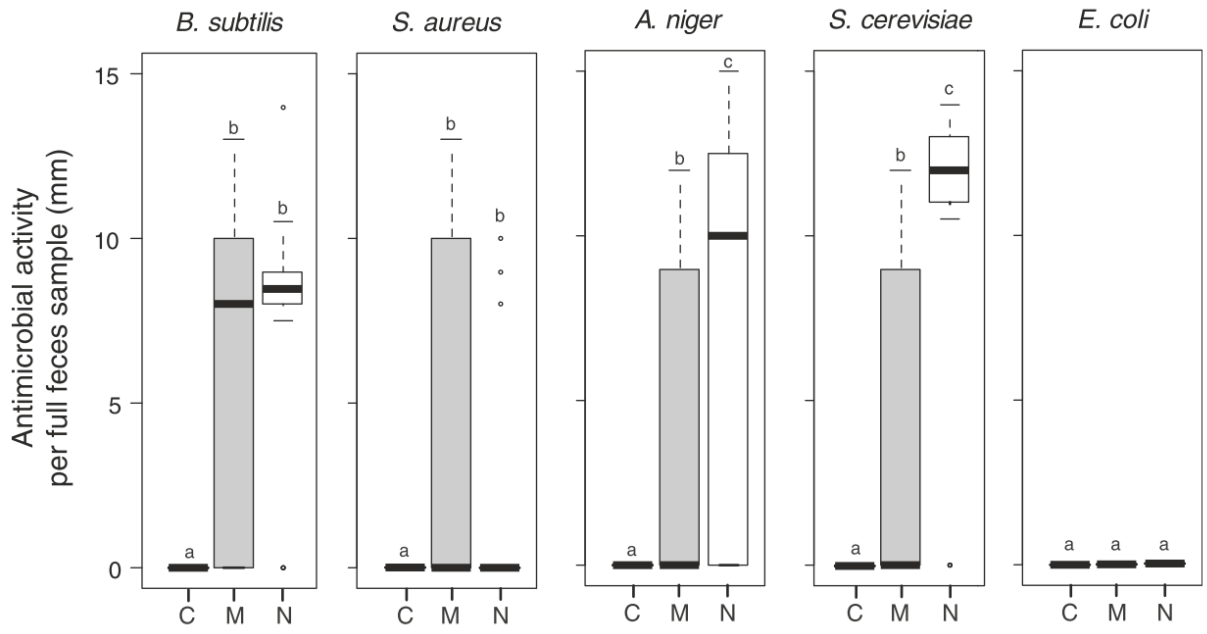

Figure 1.1 Antimicrobial activities of controls (C), maternal (M, grey) and nymphal (N, white) feces. Controls combine assays with NaCl, Pollen, Food and Sand. Boxplots depict median (bold bar) and interquartile range (light bar), with outlying values (circles) and whiskers extending to 1.5 times the interquartile range. Different letters indicate $P < 0.005$.

antimicrobial activity of maternal feces produced over three days (Table 1.1a). There was no family effect on the antimicrobial activities of nymphal and maternal feces (Table 1.3). Across microbial species, antimicrobial activities were comparable (present or absent) between maternal and nymphal feces in 51.4% of the families, a value that was not significantly different from a random distribution (Binomial test against 50%, P = 0.904). Note that the four microbial species (excluding *E. coli*) did not influence the proportion of families with comparable antimicrobial activities between maternal and nymphal feces (i.e. both present plus both absent versus present in only one type; Pearson's Chi-squares test, $\chi^2 = 3.0$, df= 3, P = 0.391).

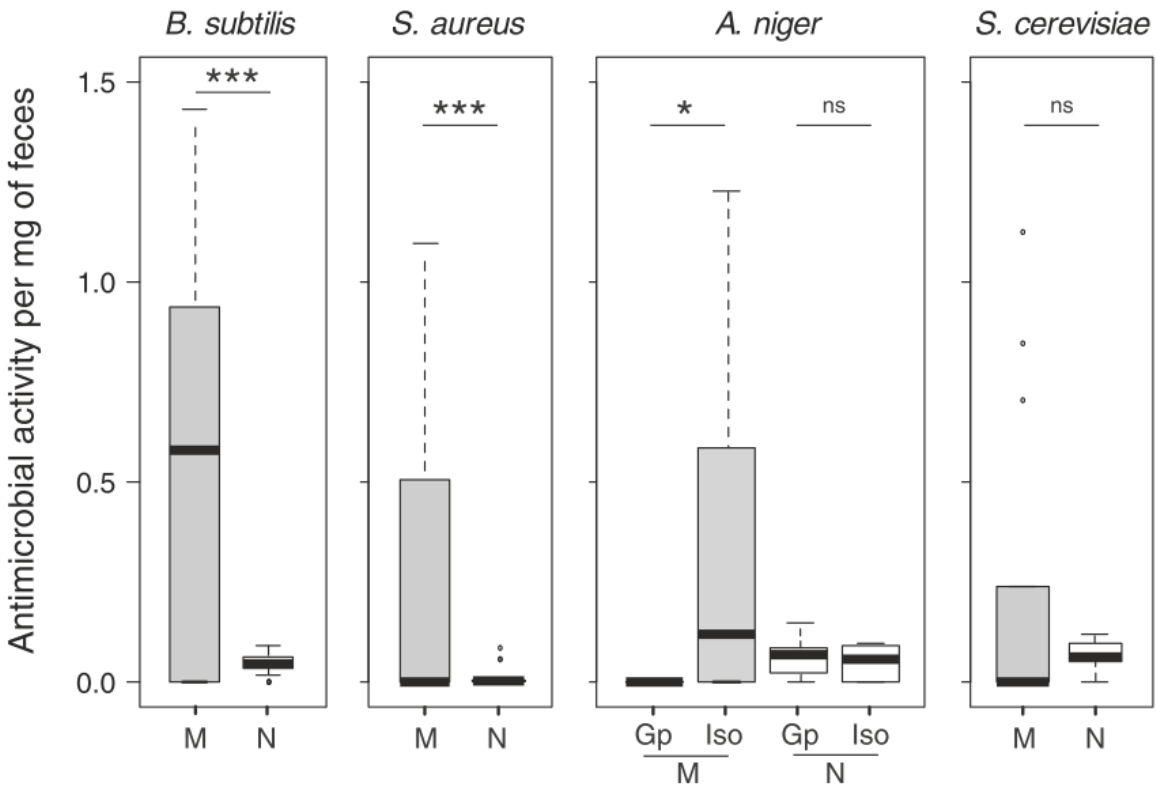

Figure 1.2. Antimicrobial activities per mg of maternal (M, grey) and nymphal (N, white) feces. When reported, feces producers were either maintained in family groups (Gp) or isolated (Iso) before feces collection. Boxplots depict median (bold bar) and interquartile range (light bar), with outlying values (circles) and whiskers extending to 1.5 times the interquartile range. ***P < 0.001; *P < 0.05; nsP > 0.05.

Table 1.2. Comparisons between inhibition zones generated by the controls and the total amount of either maternal or nymphal feces. Statistical values were obtained from Mann–Whitney tests. Significant P-values are in bold. All p-values remain significant after correcting for multiple testing.

	B. subtilis	P value	*S. aureus*	P value	*A. niger*	P value	*S. cerevisiae*	P value
Maternal feces	W = 686	**< 0.0001**	W = 588	**< 0.0001**	W = 539	**<0.0001**	W = 539	**<0.0001**
Nymphal feces	W = 759.5	**< 0.0001**	W = 490	**0.0028**	W = 710.5	**< 0.0001**	W = 759.5	**< 0.0001**

DISCUSSION

Gaining a better understanding of the evolution of the multiple forms of group-living requires insights into the mechanisms that help individuals to limit the inherent risk of infection. Here, we demonstrate that lining nests with feces inhibits microbial development in the European earwig. Specifically, earwig feces showed anti- microbial activities against two GRAM+ bacteria (*B. subtilis* and *S. aureus*) and two fungi (*A. niger* and *S. cerevisiae*). These antimicrobial properties are likely to provide immune benefits to earwig family members, as many microbial entomopathogens have the capability to grow under the underground conditions provided by insect nests (e.g. (Reber and Chapuisat, 2012; Schmid-Hempel, 1998)), several of them are known to frequently attack earwig nests (Ben-Ze'ev, 1986; Hodson et al., 2011; Miller and Zink, 2012), and a recent study showed that even the development of non-entomopathogenic fungus into the nest comes with detrimental effects on earwig fitness (Boos et al., 2014). Together with the fact that earwig nymphs produce more feces when encountering related compared to unrelated conspecific juveniles (Falk et al., 2014), these results thus support that feces production at least partly reflects a kin-triggered form of social immunity.

Table 1.3. Expression of feces antimicrobial activity per family. For each of the five microbial species, we reported the number of family in which an antimicrobial activity was found in both maternal and nymphal feces, in none of them or in either maternal or nymphal feces.

Antimicrobial activity in						
Maternal feces	Nymphal feces	B. subtilis	S.aureus	A.niger	S.cerevisiae	E.coli
Yes	Yes	8	2	3	5	0
No	No	0	9	3	3	17
Yes	No	3	5	2	0	0
No	Yes	6	1	9	9	0

The maintenance of feces in the nest is a poorly studied phenomenon in eusocial insects (Chouvenc, 2013; Rosengaus et al., 1998), in which colony members are generally assumed to evacuate feces into specific nest chambers to prevent microbial development in the colony (reviewed in (Cremer et al., 2007; Weiss, 2006)). This phenomenon has nevertheless been reported in two non-eusocial insects exclusively feeding on their nesting material, the wood cock-roach *Cryptocercus punctulatus* and the burying beetle *N. vespilloides* (Cotter and Kilner, 2010b; Rosengaus et al., 2013; Rozen et al., 2008), for which the use of anal exudates (and their antimicrobial activity) into the nest has been proposed to have at least partially evolved to limit competition with microbes over food access (Cotter and Kilner, 2010b; Otti et al., 2014).

Our study shows that the total amount of feces produced by mothers over three days did not exhibit higher antimicrobial activities than the one produced by nymphs, revealing that feces antimicrobial activity is not a simple form of post-hatching maternal care. Instead, we show that nymphs contributed more to antifungal nest protection than mothers, mostly due to their overall larger production of feces (each mg of nymphal feces exhibited similar antifungal activity than each mg of maternal feces). This higher feces production also allowed nymphs to compensate for

the lower intrinsic antibacterial activity of their feces (activity per mg of feces) against GRAM+ bacteria, thus exhibiting an overall antimicrobial activity comparable to the one of maternal feces. This age-specific effect on the antimicrobial activity per mg of feces suggests differences in composition between nymphal and maternal feces. Feces compositions could differ in terms of quantity and/or quality of residual compounds of their personal immunity, which are known to be present in the feces and to become stronger with aging in other insect species (Beckage, 2008; Dillon and Dillon, 2004; Schmid-Hempel, 2005; Shao et al., 2012; Zerofsky et al., 2005). Another discrepancy in feces composition could result from differing hindgut flora of mothers and nymphs. The insects gut includes a great variety of symbiotic microorganisms that are crucial for growth and protection against infections (Engel and Moran, 2013; Kaltenpoth and Engl, 2014; Kaltenpoth and Steiger, 2014), but that also change with aging (Dillon et al., 2010; Dillon and Dillon, 2004; Kaltenpoth, 2009). Finally, nymphal and maternal feces could vary in terms of chemical products released during defecation. For instance, earwigs possess a pygidial gland on their abdomen that releases chemicals with antimicrobial properties (Gasch et al., 2013). Disentangling among these three non- mutually exclusive hypotheses will be addressed in further studies by investigating the presence of immune components and antimicrobial chemicals inside the feces, as well as by characterizing earwig gut flora.

We found that the antimicrobial activity of maternal feces depended on preliminary interactions with their nymphs. Specifically, family interactions inhibited the antimicrobial activity of maternal feces against *S. cerevisiae*, whereas they had no effect on the one of nymphal feces. This latter result contrasts with the one found in the burying beetle *N. vespilloides*, in which the absence of tending parents lowered the level of antibacterial activity in larvae exudates

(Reavey et al., 2014). In earwigs, our result first reveals that the presence and/or quantity of the compounds mediating the antimicrobial activity of maternal feces against *S. cerevisiae* are socially-dependent. More generally, it suggests that mothers can adapt their investment into such a form of social immunity to the investment expressed by their nymphs. Assuming that investment into social immunity is energetically costly (see e.g. (Cotter et al., 2010)), such maternal strategy could be adaptive and allow mothers to re-allocate their energy into other important life-history traits, such as forms of care and future reproduction (Kölliker, 2007; Meunier et al., 2012). Nevertheless, the effect of family life on feces antimicrobial activities was absent with the four other tested microbial species, indicating that the compounds mediating this activity are fixed during the period of family life. These compounds do not come from the environment, as there was no antimicrobial activity in the food consumed by the individuals and in the sand covering the rearing containers.

A somewhat surprising result of our study was the large number of feces samples with no antimicrobial activity. These negative assays are unlikely to reflect a problem in our methodology, as radial diffusion assay is a standard procedure that has been commonly used to test antimicrobial activities in other insect species (e.g. (Arce et al., 2013; Rosengaus et al., 1998)). They are also unlikely to reflect that feces anti-microbial activity is a family-trait only expressed in a limited number of families, since we showed that the occurrence (or absence) of feces antimicrobial activities was not necessarily the same between nymphs and mothers from the same family. Conversely, our result could reflect a form of specificity in the immune responses mediated by the feces, which is in line with the fact that almost every feces sample inhibited the growth of at least one of the tested microbes. Another explanation could be that feces producers

need some cues to switch on antimicrobial activity in their feces. These cues are unlikely to come from our standardized rearing environment but might reflect that some field sampled mothers have been naturally exposed to pathogens prior to sampling, and that such exposure affected the immunity of their own descendants through transgenerational immune priming (Moret, 2006). However, the occurrence of transgenerational immune priming remains to be tested in *F. auricularia*.

Although earwig feces showed antimicrobial activity against the two tested GRAM+ bacteria, this activity was absent against the GRAM- bacteria *E. coli*. This lack of activity against *E. coli* has been reported in the anti-microbial secretions of other insect, such as the burying beetle *N. vespilloides* [18]. It may reflect either (1) higher physiological costs of mounting antimicrobial protection against GRAM- bacteria (Beckage, 2008), (2) low selection pressure to mount defenses against GRAM- bacteria, e.g. because they are not present in their natural habitat or are important symbiotic organisms in the gut flora (but see (Kaltenpoth, 2009)), or (3) specific resistance of the tested bacterial strain against the antimicrobial compounds present in earwig feces. Further studies should address this issue.

CONCLUSIONS

Overall, we demonstrate that social immunity in the form of lining nest with antimicrobial compounds can emerge and persist in species with primitive forms of group-living. Mounting collective defenses against microbial development could therefore be a widespread phenomenon across social systems and an important one in the early evolution of social life, as it does not require that individuals live in permanent and obligatory groups, and/or that group members compete with microbes for access to nest material as a food source. Interestingly, these

results also emphasize that defecation does not only reflect individual needs of waste disposal but can be under selection for its importance in other crucial life-history traits (Cotter and Kilner, 2010b; Rosengaus et al., 2013; Weiss, 2006).

CHAPTER 2

Growing up with feces: benefits of allo-coprophagy in families of the European earwig

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ABSTRACT

An important issue in the evolution of group living is the risk of pathogen and predator exposure entailed by the inherent accumulation of feces within a nesting site. While many group living species limit this risk by cleaning the nest, others do not, raising questions about the benefits of maintaining feces in the nest and their importance in social evolution. Here we investigated whether one of these benefits could be mediated by coprophagy in families of the European earwig, *Forficula auricularia*. In this insect species, mothers and mobile juveniles (nymphs) line their nests with feces and consume them. In a first experiment, we tested whether access to feces produced by either nymphs or mothers affects nymph survival in both presence and absence of food. The results showed that access to sibling feces, but not mother feces, enhanced offspring survival under food deprivation. Such an effect did not occur when regular food was available. We then conducted a food choice experiment to reveal whether nymphs prefer food to feces, and if they discriminate between feces from their mother, unrelated adult females, unrelated nymphs, or their siblings. We found that offspring generally preferred regular food to feces, but nevertheless always consumed some feces. By contrast, nymphs showed no preference between related sibling or mother feces, and did not discriminate between feces from related and unrelated individuals. Overall, our results suggest that the benefits of coprophagy could favor the maintenance of feces within the nest and promote the evolution of social life.

INTRODUCTION

Although defecation is an essential process to dispose nutritional waste, the accumulation of its product in a nesting site may entail costs for the surrounding individuals that are often thought to hamper the evolution of group living (Weiss, 2006). This is because a wide range of pathogenic bacteria and fungi are known to use feces as a substrate for their development (Bailey, 1955; Bucher, 1957), and because feces releases kairomones that can be used by predators to locate and attack their prey (Agelopoulos et al., 1995; Steidle and Fischer, 2000; Vet and Dicke, 1992). These feces-related risks of infection and predator attacks remain limited in species with low nest fidelity (Quan et al., 2015; Weiss, 2006). However, they can grow dramatically when organismic density is high and/or living space is confined, such as in nest dwelling species (Jackson and Hart, 2009; Schmid-Hempel, 1998; Weiss, 2006). This is why the emergence and maintenance of group living has long been thought to require the expression of sanitation behaviors (Meunier, 2015), such as expelling feces from the nest (Biedermann and Taborsky, 2011; Michener, 1974; Thomson, 1934; Weiss, 2003) or limiting defecation to a single location (Dethier, 1980; Farji-Brener et al., 2016; Georgiev, 2009; Zuri and Terkel, 1998).

A growing number of studies have recently shown, however, that keeping feces within a nesting site may provide benefits for group members and could thus promote the evolution of social life. One of these benefits relies on the antimicrobial activities possibly exhibited by feces material. This activity has been demonstrated in multiple species that coat their nest/colony with feces to prevent the growth of pathogens, such as in the dampwood termite *Zootermopsis angusticollis*, the wood cockroach *Cryptocercus punctulatus*, the burying beetle *Nicrophorus*

vespilloides and the European earwig *Forficula auricularia* (Diehl et al., 2015; Reavey et al., 2014; Rosengaus et al., 2013, 1998). A second benefit of keeping feces in a nesting site is that it may foster the consumption of feces produced by conspecifics - a behavior called allo-coprophagy – and thus facilitate the exchange of symbionts between group members. This phenomenon has been well studied in wood-feeding termites, in which allo-coprophagy and proctodeal trophallaxis were shown to mediate the transfer of mutualistic gut bacteria that are essential for digesting specific foods (Cleveland, 1925; Engel and Moran, 2013; Mirabito and Rosengaus, 2016; Nalepa et al., 2001). Finally, a facilitated access to allo-coprophagy may also provide nutritional benefits for group members. Such benefits have been reported in the sub-social German cockroach *Blattella germanica*, in which the consumption of fecal pellets produced by conspecifics increased individuals' resistance against starvation (Kopanic et al., 2001). Coprophagy has also been reported in many solitary species, where it allows for the re-ingestion of otherwise poor food sources (Hirakawa, 2002) or simply supplements the regular diet (Nilsson, 1983).

Interestingly, the benefits of allo-coprophagy could also play a major role in the evolution of family life by mediating the often-overlooked benefits of sibling interactions. For decades, the nature of sibling interactions during family life was typically ranged from the costly sibling rivalry for parental resources to the neutral tolerance between juveniles (Mock and Parker, 1997; Roulin and Dreiss, 2012). Sibling interactions were thus generally considered as a potential inhibitor rather than a promoter of the emergence and maintenance of family life (Royle et al., 2014; Trivers, 1972). However, a growing number of studies have recently indicated that sibling interactions could provide benefits to juveniles through food exchange during family life

(reviewed in Roulin and Dreiss 2012). The consumption of feces during family life could be a key mediator of this food exchange, such as in the European earwig *F. auricularia.* In this species with facultative maternal care (Kölliker, 2007; Meunier and Kölliker, 2012b; Thesing et al., 2015), the presence of nearby related juveniles (called nymphs) during family life has indeed be shown to trigger an increase of feces production by the nymphs, which in turn promotes the transfer of food resources among siblings through allo-coprophagy (Falk et al., 2014; Kramer et al., 2015; Kramer and Meunier, 2016a). Nevertheless, the nutritional and/or non-nutritional benefits of allo-coprophagy for nymphs and thus their possible role in the emergence and maintenance of family life, as well as the mechanisms regulating the expression of allo-coprophagy, remain unknown in this species.

In this study, we investigated the benefits of and the mechanisms regulating allo-coprophagy in nymphs of the European earwig *F. auricularia*. In a first experiment, we tested whether allo-coprophagy enhances nymphs' survival and whether this effect depends on their access to a regular food source. To this end, we manipulated nymph access to nymphal/maternal feces and to a regular food source, and then measured nymph survival rates over 25 days. If access to feces provided nutritional benefits, we expected to find higher survival rates in nymphs that were provided with feces than in nymphs that were not, and that this effect would be stronger in the absence of a regular food source. Conversely, if access to feces provided non-nutritional benefits, we expected nymphs to survive better in the presence of mother and/or nymph feces, independent of the presence of a regular food source. In the second experiment, we tested whether the expression of allo-coprophagy and thus its potential nutritional and non-nutritional benefits in nymphs depends on whether the feces are produced by other nymphs or

by adult mothers, and on the feces producer's relatedness (and/or familiarity). Specifically, we set up a series of paired food-choice tests in which nymphs could choose between regular food and feces produced by an adult female or a nymph, which were either unrelated or related to the focal nymph. If feces were only used as a food source under harsh circumstances, we expected nymphs to prefer the consumption of regular food to any type of feces, as well as to prefer nymphal (i.e. possibly less digested, and hence more nutritious) over maternal feces. Conversely, if coprophagy was also used as a mediator of symbiotic exchanges (i.e. a type of non-nutritional benefit) and these exchanges are family-specific, we expected the nymphs to prefer the consumption of feces from related donors over feces produced by unrelated individuals.

MATERIALS AND METHODS

Animal origin and maintenance

Our two experiments involved a total of 58 clutches produced by a second laboratory-born generation of females field-sampled in 2012 in Dolcedo, Italy (experiment 1: n = 28 females; experiment 2: n = 12 females) and Mainz, Germany (experiment 1: n = 0; experiment 2: n = 18). All these females and progeny were maintained under standard laboratory conditions (detailed in Meunier et al. 2011). Our two experiments started five days after the females' first clutch of eggs hatched and both involved isolated nymphs as feces consumers, as well as groups of nymphs and isolated mothers as feces producers. Five days after egg hatching, five (experiment 1) or six (experiment 2) nymphs per clutch were individually isolated in Petri dishes to be used later as potential feces consumers. Simultaneously, two groups of seven nymphs (experiment 1), or all the remaining nymphs (experiment 2) and each mother (both experiments) were maintained in

separate Petri dishes to produce the feces subsequently offered to the feces consumers. These groups of nymphs and mother donors immediately received an *ad libitum* amount of standard food (experiment 1) or pollen pellets (Hochland Bio-Blütenpollen; experiment 2), both dyed with blue food dye (Deko Back; Reichartshausen, Germany). Pollen pellets were standardized in size and shape using a metal punch-press. The use of food dye increased the visibility of feces for the experimenter, but does not affect other feces properties (Diehl et al., 2015). The standard food was lab-made and mainly included pollen, carrots, cat food and agar (see details in Kramer et al. 2015). All Petri dishes were 5.5 cm in diameter and were furnished either with moist sand (experiment 1) or with a circular sheet of filter paper (Macherey-Nagel GmbH & Co. KG, Düren, Germany) replaced for every test (experiment 2) and used to further increase feces visibility.

Experiment 1: Feces, food deprivation and nymph survival

In this first experiment, we aimed at testing whether access to feces improves nymph survival, and whether this effect depends on the presence of regular food. One day after isolation, each of the five nymphs per clutch were weighed to the nearest 0.001 mg using a microscale (model MYA5; PESCALE, Bisingen, Germany) and then haphazardly attributed to one of the 5 following treatments. Nymphs received either 1) standard food, 2) feces produced by seven of their siblings over the previous days, 3) feces produced by their own mother over the previous days, 4) standard food plus feces produced by seven of their siblings over the previous days or 5) nothing. The treatments were renewed every three days using freshly produced feces and/or standardized food for a total of 25 days, during which we recorded nymph survival. Note that the amount of food/feces provided was the same across treatments and corresponded to the total amount of feces produced by the group of donor nymphs over the three previous days, while

food was provided *ad libitum* Note that the feces provided were never depleted by the focal nymph over three days. To facilitate feces manipulation, each treatment was applied by transferring the isolated nymph into a Petri dish that was either formerly occupied by the corresponding group of nymphs or contained the mother feces or a food source.

Experiment 2: Food choice

In the second experiment, we tested whether nymphs show feeding preferences between feces and standard food, between feces of mothers and nymphs, or between feces of related and unrelated individuals. Note that here, relatedness is confounded with familiarity. Five days after their isolation, each of the six nymphs per clutch were haphazardly assigned to one of the six following food choice setups: 1) food and feces of their own mother, 2) food and feces of their sibling nymphs, 3) feces of sibling nymphs and unrelated nymphs, 4) feces of their own mother and an unrelated mother, 5) feces of their own mother and sibling nymphs, or 6) feces of an unrelated mother and unrelated nymphs. We used the total amount of feces produced by the group of remaining nymphs and/or by the mothers during the four days preceding the tests. The feces and food source provided during the experiments only covered a small fraction of each experimental arena (about 2 to 3 mm^2) and were always provided in a quantity larger than the tested individual could possibly consume. For each test, the substrates were deposited on two opposite sides of the Petri dishes (changed every trial) using a clean metal stick. The focal nymph was placed in the center of the arena using soft steel forceps. We then recorded the time each nymph spent on each type of substrate over 30 minutes using a Sony HDR-CX200E video camera (movies started five minutes after set-up to allow the nymphs to acclimate to the environment). Because earwigs are nocturnal, all filming was done under red light. To confirm that the time

nymphs spent chewing on the substrate reflected food consumption, we also weighed a random subset of 70 nymphs originating from 12 clutches (Mainz population) before and after the tests and correlated this weight change to the time spent chewing the substrates. All videos were analyzed blindly regarding the origin of the assigned feces using the software "The Observer XT11" by Noldus.

Statistical analyses

All statistical analyses were performed using the statistics software R v3.0.3 (http://www.r-project.org/) loaded with the packages *survival*, *MASS* and *car*. Nymph survival (Experiment 1) was tested using a Cox proportional hazards regression model allowing for censored data, i.e. nymphs alive at the end of the experiment. In this model, the nymph weight, the treatment (with 5 levels corresponding to the 5 tested treatments) and their interactions were entered as explanatory variables. To control for the non-independence of the nymphs used in this experiment (5 nymphs per clutch), the clutch of origin of each nymph was entered as a random effect into the model using the *frailty* argument. The significant effect of treatment (see results) was further investigated using model estimates. To correct for multiple testing, the significance level for these pairwise analyses was adjusted using the MFDR (mean false discovery rate) approach to $\alpha_c = 0.033$ according to $\alpha_c = (n + 1)/(n \times 2) \times 0.05$, where n denotes the number of tests (Benjamini and Hochberg, 1995). Note that all the individuals used in experiment 1 came from a single population.

The food choice experiment was analyzed in three steps. In the first step, we conducted a series of six t-tests to determine whether the population of origin determined the proportion

of time a nymph spent chewing one of the two assigned substrates (i.e. time chewing on substrate 1 divided by the total time spent chewing on both substrates combined). Because we found no population effect, we then pooled the populations per type of food choice experiment and conducted a new series of six one-sample t-tests, with which we tested whether the proportion of time a nymph spent chewing on one of the two assigned substrates was significantly different from 0.5. In the last step, we used two one-sample t-tests to analyze whether nymphs still went to the feces side when they faced a choice between food and feces. Specifically, these analyses tested whether the proportion of time a nymph spent chewing on a food substrate was significantly different from 1. These three steps only included the trials during which nymphs were seen chewing on at least one of the two substrates. Finally, we investigated whether the time nymphs were seen on the substrates reflected an actual food intake, i.e. if time spent on a substrate correlated with the absolute weight gained by nymphs during the test, using a linear regression model. We controlled for homoscedasticity and the Gaussian distribution of model residuals in each of these statistical analyses.

RESULTS

Experiment 1: Feces, food deprivation and nymph survival

Overall, 64.3% (90 of 140) of the isolated nymphs died during the 25 days of the experiment. This survival rate depended on the type of feces provided to the nymphs, as well as on the presence of an additional food source (Figure 2.1; Treatment effect: Likelihood ratio (LR) $\chi^2_4 = 98.70$, $P < 0.0001$). Specifically, nymphs with access to nymphal feces survived longer than nymphs with access to maternal feces and longer than nymphs that had access to neither feces nor food source

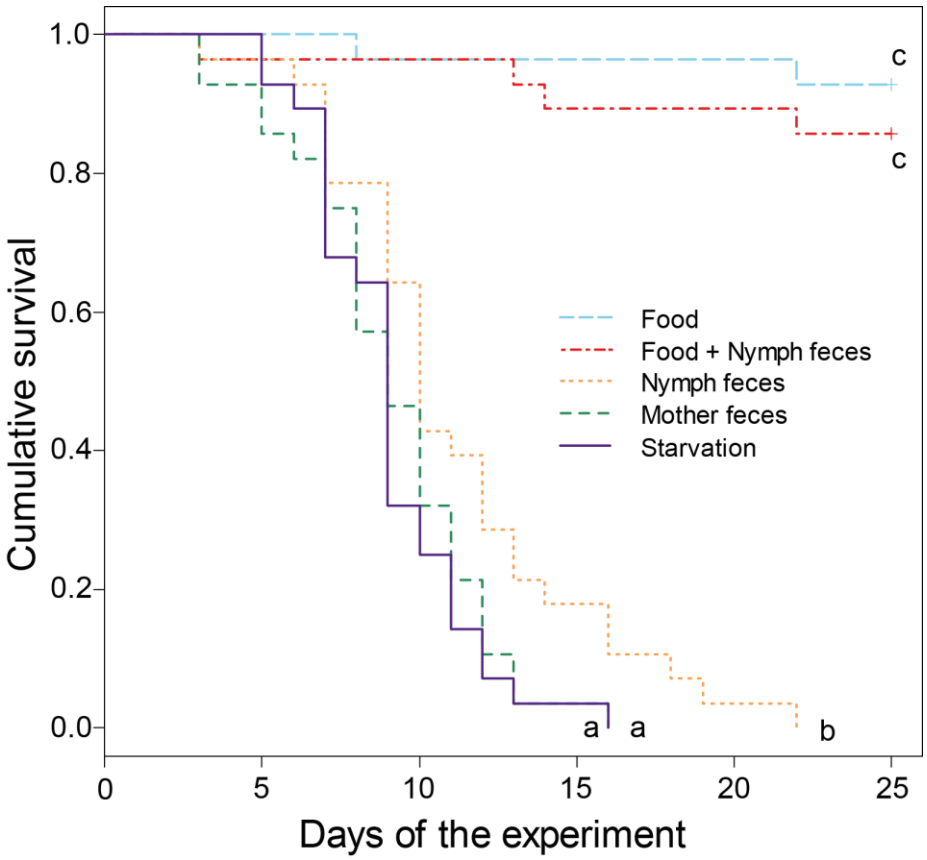

Figure 2.1: Influence of food and feces access on nymph survival. Individual lines represent the cumulative survival rate of nymph that had access to either standard food (Median survival time, LT_{50} ± SE = 49.8 ± 7.7), feces produced by their siblings (LT_{50} = 15.6 ± 0.3), feces produced by their own mother (LT_{50} = 40.7 ± 3.4), standard food plus feces produced by their siblings (LT_{50} = 15.6 ± 0.3) or nothing (LT_{50} = 13.5 ± 0.2). Different letters indicate *P*-value < 0.03 (see Table 2.1).

(Table 2.1). Moreover, the nymphs with access to an additional food source (with or without nymphal feces) survived longer than the nymphs with access to feces only (Table 2.1). By contrast, there was no difference in survival rates between the nymphs that had access to maternal feces and the ones that had access to neither feces nor a food source (Table 2.1). There was also no difference in the survival rates of nymphs provided with regular food or with regular food plus nymph feces (Table 2.1). Finally, heavy nymphs survived longer than light ones (LR χ^2_1 = 9.66, *P* = 0.002; model estimate ± SE = 4797.7 ± 1772.4), an effect that was independent of the treatment (interaction between nymph weight and treatment; LR χ^2_4 = -0.24, *P* = 0.999).

Experiment 2: Food choice

The proportion of nymphs that went at least once on one of the two assigned substrates did not differ between treatments (Fisher exact test, P = 0.699). Across the 30 videos recorded per treatment, nymphs visited at least one food source in 25 (83%) of the tests offering regular food and mother feces, 29 (97%) offering regular food and nymph feces, 27 (90%) offering related and unrelated mother feces, 26 (87%) offering related and unrelated nymphs feces, 26 (87%) offering related mother and nymph feces and finally 27 (90%) offering unrelated mother and nymph feces. In these tests, nymphs spent more time chewing on regular food than on feces produced by related mothers (Table 2.2a) or feces produced by related nymphs (Table 2.2b, Figure 2.2). Nevertheless, during these two food-choice tests, the nymphs still spent a significant amount of

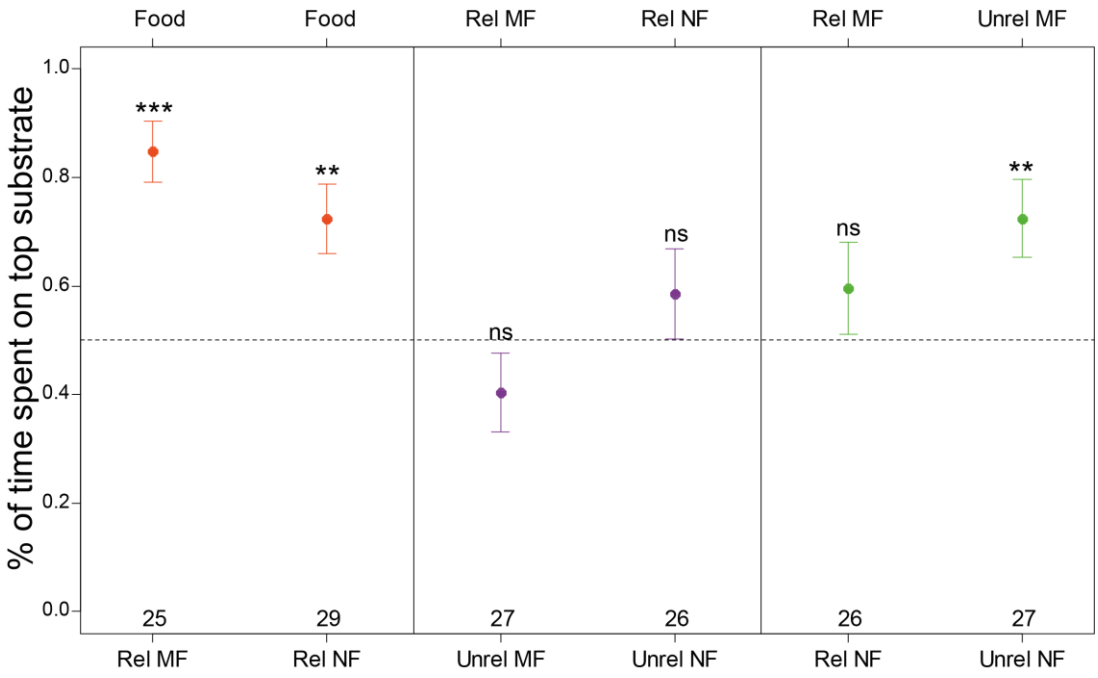

Figure 2.2. **Proportion of time spent by nymphs on the top- compared to the bottom-reported type of substrate in the six food choice trials**. The food choices involved regular food (Food), as well as mother feces (MF) and nymph feces (NF) produced by related (Rel) and unrelated (Unrel) individuals. The number of nymphs that visited one of the two substrates at least once is reported at the bottom of each line. The mean ± standard error is reported. *** $P < 0.001$, ** $P < 0.01$.

time chewing on feces (one sample t-tests against 1; Mother feces: t_{24} = -2.69, *P* = 0.0127; Nymph feces: t_{28}=-4.30, *P* = 0.0002). In the other tests, nymphs made no difference between feces produced by related and unrelated mothers (Table 2.2c) or by related and unrelated nymphs (Table 2.2d, Figure 2.2). Finally, they did not discriminate between the feces produced by related mothers and related nymphs (Table 2.2e), but spent more time on mother than nymph feces when both were produced by unrelated individuals (Table 2.2f, Figure 2.2). Based on the 70 nymphs that had been weighed before and after the food-choice test, we found a positive association between the total time spent chewing the substrates and the absolute weight gained by nymphs (LM, Model estimate $\pm$ SE = 0.0003 $\pm$ 0.0002, t = 2.65, *P* = 0.010).

Table 2.1. Pairwise differences between treatments in the survival experiment (Experiment 1). The reported values are obtained from Coxph model estimates. P-values still significant after MFDR correction are in bold.

	Food	Food + Nymph feces	Nymph feces	Mother feces	Starvation
Food		χ^2_1 = 0.73	χ^2_1 = 30.21	χ^2_1 = 37.77	χ^2_1 = 39.21
Food + Nymph feces	p = 0.390		χ^2_1 = 35.32	χ^2_1 = 44.92	χ^2_1 = 46.94
Nymph feces	***P* < 0.0001**	**p < 0.0001**		χ^2_1 = 5.02	χ^2_1 = 6.28
Mother feces	***P* < 0.0001**	**p < 0.0001**	**p = 0.025**		χ^2_1 = 12.2
Starvation	***P* < 0.0001**	**p < 0.0001**	**p = 0.012**	p = 0.140	

DISCUSSION

The accumulation of feces in a nest is often associated with detrimental effects, such as pathogen spread and growth and attraction of parasites, which all could ultimately hamper the evolution of group living (Sagara et al., 2006; Sato et al., 2003; Schmid-Hempel, 1998; Steidle and Fischer, 2000; Vet and Dicke, 1992; Weiss, 2006). Yet, a few species like the European earwig still maintain

feces in their nest and even consume feces produced by group members (Diehl et al., 2015; Falk et al., 2014; Kramer et al., 2015; Kramer and Meunier, 2016a; Nalepa et al., 2001; Rosengaus et al., 2013). Here, we aimed to improve our understanding of the evolution and maintenance of this behavior in earwigs by investigating the benefits of and the mechanisms regulating allo-coprophagy. Our results demonstrate that access to sibling feces enhanced the survival rate of nymphs in the absence of a regular food source. However, this effect was absent when nymphs had access to maternal feces and disappeared when they also had access to a regular food source. In this experiment, we also showed that nymph weight positively affected their survival, independent of their access to feces and/or a food source. Our food-choice experiments then showed that nymphs always preferred the consumption of food over feces, even if feces consumption still occurred during the tests. These experiments also revealed that nymphs did not discriminate between feces from related and unrelated individuals, or between maternal or nymphal feces produced by related individuals. However, when offered the choice between feces produced by unrelated mothers and nymphs, they preferred the former.

Our data demonstrate that the consumption of nymphal feces significantly delayed death by starvation, a result in line with a nutritional benefit of (allo-)coprophagy. Intraspecific coprophagy for the sake of nutrient intake is well documented in many different taxa including rodents, insects, gastropods, and amphibians (Steinwascher 1978; Stevenson and Dindal 1987; Brendelberger 1997; Takahashi and Sakaguchi 1998; Nalepa et al. 2001). Interestingly, in earwigs, the benefits of coprophagy only occurred when nymphal feces were consumed, while consumption of maternal feces yielded no such effect. On a proximate level, this result may

reflect an age-specific efficiency of the digestive tract in earwigs. The stability of the hindgut fauna is indeed known to become increasingly efficient during insect development (Engel and Moran, 2013), so that feces produced by adults are less likely to contain a large concentration of undigested particles (in other words, they are more likely to exhibit a high nutritional value). Alternatively, this age-specific benefit on nymph survival could rely on differences in the occurrence and/or proportions of various microbial proteins and other components of the hindgut fauna that exhibit specific nutritional values. The consumption of such components through coprophagy is indeed known to provide nutritional benefits in other insect species (Nalepa et al., 2001) and their age-specific variation could thus also explain a delayed death by starvation in nymphs. The efficiency of juveniles and adult digestive tracts, as well as the composition of their hindgut fauna, however, remain to be studied in earwigs.

Despite a general preference for regular food compared to feces, we showed that nymphs always consumed some (maternal or nymphal) feces when they also had access to regular food. This finding indicates that the incentives for coprophagy in earwigs are not limited to the

Table 2.2. Differences in the proportion of time spent by nymphs on each type of substrate in the six types of food choice. Significant *P*-values are in bold.

Food choice test			t-value	df	*P*-value
(a) Regular food	*vs*	Related mother feces	6.14	24	**< 0.0001**
(b) Regular food	*vs*	Related nymph feces	3.49	28	**0.002**
(c) Related mother feces	*vs*	Unrelated mother feces	-1.31	26	0.202
(d) Related nymph feces	*vs*	Unrelated nymph feces	1.04	25	0.309
(e) Related mother feces	*vs*	Related nymph feces	1.12	25	0.272
(f) Unrelated mother feces	*vs*	Unrelated nymph feces	3.10	26	**0.005**

acquirement of nutrients and their aid against starvation. A well-documented mediator of non-nutritional benefits of coprophagy is the transfer of microbes, such as the ones constituting the mutualistic hindgut fauna of an animal (Dillon and Dillon, 2004; Engel and Moran, 2013; Weiss, 2006). In vertebrates, the acquisition of this hindgut fauna through coprophagy has been shown to be of high importance for the development of juveniles in terms of growth and body size, such as in the rat *Rattus norvegicus* (Fitzgerald et al. 1964) and tadpoles of the American bullfrog *Rana catesbeiana* (Steinwascher 1978). Similarly, coprophagy and proctodeal trophallaxis are essential for survival in many xylophagous insects, as it mediates the distribution of crucial cellulose-digesting gut symbionts (Cleveland, 1925). In termites, repeated coprophagy and proctodeal trophallaxis are of special importance because a majority of gut protists is lost during molting and reacquired through coprophagy (Nalepa, 2015). The transfer of immune and/or antimicrobial components could be another, though non-mutually exclusive, non-nutritional benefit for coprophagy. In earwigs, a recent study revealed that feces exhibit antimicrobial properties and that these properties depend on whether it was produced by nymphs or mothers (Diehl et al., 2015). However, whether individuals can acquire these immune components through allo-coprophagy remains to be investigated in earwigs and in insects in general (see (Mirabito and Rosengaus, 2016)).

Our second experiment also revealed that nymphs did not exhibit a preference for the consumption of feces produced by related as compared to unrelated individuals. This suggests that the nutritive and/or non-nutritive benefits of allo-coprophagy do not require a genetic similarity (or familiarity) between donors and recipients. It is nevertheless important to note that this effect might also reflect the fact that, even if clutch joining occurs under natural conditions

(Kölliker and Vancassel, 2007), nymphs mostly encounter feces produced by related individuals within the nest and might therefore not have been able to evolve such a discrimination capability. More generally, further studies should investigate why nymphs exhibited a preference for maternal over nymphal feces when these producers where all unrelated to the consumer.

Across species and taxa, the benefits of family life are typically mediated by one or two caring parents (Royle et al., 2014; Trivers, 1972), while siblings are often thought to only fight and compete over the distribution of resources (Mock and Parker, 1997; Roulin and Dreiss, 2012). However, an increasing number of studies demonstrate that sibling interactions can be mutually beneficial despite the high potential for competition (Kramer and Meunier, 2016a; Roulin et al., 2016, 2012). In earwigs, previous studies demonstrated that nymphs not only share food via allo-coprophagy, but also increase feces production when in contact with related as compared to unrelated nymphs (Falk et al., 2014). Here, we demonstrate that this behavior is (at least partly) a means of helping clutch-members to survive under food deprivation, and also suggest that it could mediate the exchange of microbial fauna between family members. Interestingly, sanitary behavior (such as a feces removal) has been considered to be an important facilitator for the evolution of eusociality (Jackson and Hart, 2009). However, in the early stages of social evolution, such as found in species with facultative family life, the group is maintained for a relatively short time period, so that sanitation may not be a necessity if the nest site can be abandoned before the adverse effects set in. In such a scenario, beneficial effects of feces exchange, such as shared resources and hindgut fauna, could thus be selected for and ultimately promote cooperative behaviors that delay group dispersal and thereby drive the evolution of sociality.

CHAPTER 3

The impact of maternal care on offspring gene expression is lowered by pathogen exposure in an insect with facultative family life

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ABSTRACT

In species with facultative family life, offspring exhibit the unique and ancestral capability to live both with and without their parents, i.e. to adapt to extreme changes in their social environment. While these adaptations are well studied on a phenotypic level, their genetic basis remains surprisingly unknown. Studying how these offspring adapt their gene expression to parental absence is yet of central importance to better understand the genetic basis of why offspring remain in their family units and, more generally, the genetic constraints driving evolutionary transitions between solitary and family life. Here, we investigate how the manipulation of maternal presence changes gene expression in the fat body of juveniles of the European earwig *Forficula auricularia*, an insect with facultative family life. Because parents typically protect their juveniles against pathogens, the above changes in gene expression were also tested in pathogen-free and pathogenic environments. Our results first revealed that variation in maternal presence changes the expression of a set of genes that is completely different and about 10 times larger in juveniles from pathogen-free compared to pathogenic environments. Second, we showed that maternal absence led to the upregulation of metabolism related genes in juveniles, but that pathogenic environments cancelled this effect. Finally, we demonstrated that pathogen presence caused higher expression of immune related genes in juveniles, but only in maternal absence. Overall, this study sheds light on the genetic basis of offspring response to changes in parental presence and reveals that pathogens likely play an important role during family evolution.

INTRODUCTION

Family life, i.e. the association of at least one parent with their offspring, is a taxonomically widespread form of social life in the animal kingdom (Clutton-Brock, 1991; Kramer and Meunier, 2017; Trumbo, 2012; Wong et al., 2013). During this association, parents typically provide care to their juveniles and by doing so, enhance offspring development and survival until adulthood (Klug et al., 2012). For instance, parents can protect juveniles against predators, as well as provide them with food resources that would otherwise be costly or impossible to attain (i.e. Geipel et al., 2013; Staerkle and Kölliker, 2008). Parents can also express forms of social immunity to limit the risks of pathogen infection in their juveniles (Cremer et al., 2007; Van Meyel et al., 2018). These forms involve, for instance, the transfer of antimicrobial agents to offspring via food (e.g. milk in mammals, i.e. Conesa et al., 2008), the grooming and cleaning of offspring to mechanically remove external pathogens (Boos et al., 2014) and/or nest sanitation with the use of self-produced or collected materials with antimicrobial properties (Diehl et al., 2015; Duarte et al., 2015; Van Meyel et al., 2018).

By alleviating or removing unfavorable attributes of the habitat, parents effectively create a microenvironment in which offspring can thrive. As a result, offspring are expected to adapt to the presence and degree of parental care in a similar way as they would to any other environmental factor (Meunier and Kölliker, 2012a; Wong et al., 2013). Empirical evidence has shown that such adaptations occur in terms of phenotypic traits like behavior and energetic investment. For instance, offspring adjust their level of begging in response to the amount of food provisioning in many birds and mammals (Kilner and Johnstone, 1997; Ursino et al., 2011),

larvae of the burying beetle *Nicrophorus vespilloides* decrease their investment in social immunity in the absence of caring parents (Reavey et al., 2014) and nymphs of the European earwig *Forficula auricularia* increase their investment in sibling cooperation when maternal care decreases (Kramer et al., 2015).

While phenotypic responses to variable parental environment have been reported in the offspring of many species and taxa (Kilner and Johnstone, 1997; Kramer et al., 2015; Reavey et al., 2014; Ursino et al., 2011), the genetic basis of this adaptation remains surprisingly unclear. Over the last decade, the gene expression basis of family life has been mostly explored from the parental side and revealed that a number of genes expressed in parents are connected to their levels of investment into care, for example in fishes (Kent and Bell, 2018) and insects (Benowitz et al., 2017; Flynn, 2017; Parker et al., 2015; Roy-zokan et al., 2015). By contrast, little is known on the offspring side, particularly when offspring do not rely on parental care to survive and thus have the capability to live both with and without tending parents (i.e. in precocial, as opposed to altricial species) – a property that likely prevailed in the early evolution of social life (Kramer and Meunier, 2017). Yet, shedding light on how these precocial offspring adapt to the presence/absence of tending parents on a transcriptomic level would offer a unique opportunity to better understand the genetic constraints explaining how family life can evolve from a solitary state, why it can be maintained and/or disrupted over generations, as well as how it can ultimately lead to highly integrated social systems such as eusociality (Kölliker, 2007; Kramer et al., 2017; Kronauer and Libbrecht, 2018). To our knowledge, the few studies exploring offspring gene expression during family life largely focus on an altricial species, i.e. rats, and showed that variation in the level of obligatory parental care induces epigenetic changes in offspring,

translating into an altered defense response and future parental care (Diorio and Meaney, 2007; Meaney, 2001; Weaver et al., 2004). By contrast, how offspring gene expression is associated with variation in the parental environment is still relatively unknown in precocial species (Kronauer and Libbrecht, 2018). Recently, there have been first investigations into the effects of parenting on offspring the burying beetle *Nicrophorus vespilloides*, which also investigated to what degree known immune genes are involved in personal and/or social immunity (Ziadie et al., in press).

Here, we investigated how maternal presence changes offspring gene expression in a precocial insect: the European earwig *Forficula auricularia*. Because protection against pathogens is often considered a major benefit of parental presence (because it is mediated by parental care, Boos et al., 2014; Meunier, 2015; Palmer et al., 2016; Reavey et al., 2014), the above changes in gene expression were also tested in combination with the presence or absence of the common entomopathogenic fungus *Metarhizium brunneum* in the nesting environment. In the European earwig, mothers tend their offspring (called nymphs) for several weeks after egg hatching (Lamb, 1976a; Ratz et al., 2016), during which time they exhibit extensive forms of care such as allo-grooming, protection against nest intruders and food provisioning by regurgitation (Lamb, 1976a; Staerkle and Kölliker, 2008). Mother-offspring interactions have long-term effects in earwig juveniles, as they shape the reproductive strategy of the resulting adult offspring (Meunier and Kölliker, 2012a), as well as the level of care the resulting adult offspring provide to their own descendants (Kölliker et al., 2015; Thesing et al., 2015). Nevertheless, offspring do not require post-hatching maternal care to develop and survive (Kölliker, 2007; Kölliker and Vancassel, 2007; Thesing et al., 2015), mostly because gregarious, mobile nymphs are capable of

foraging on their own (Lamb, 1976a) and can share food resources among their siblings via allo-coprophagy and proctodeal trophallaxis (Falk et al., 2014; Körner et al., 2016; Kramer et al., 2015). Previous studies have shown that earwig's resistance against pathogens heavily relies on social immunity and social interactions. In particular, both adults and juveniles collectively protect their nests against microbes by lining the walls with feces exhibiting antimicrobial properties (Diehl et al., 2015) and mothers increase their investment into egg care when their eggs or nesting area are covered with fungal spores (Boos et al., 2014; Diehl et al., 2018). Note, however, that early maternal presence has no long-term effect on offspring investment into basal immunity (Vogelweith et al., 2017). Finally, the presence and nature of social contacts among earwig adults shape both their levels of basal immunity and their resistance against spores of the entomopathogenic fungus *M. brunneum* (Kohlmeier et al., 2016; Körner et al., 2018).

We conducted a 2x2 full factorial experiment, in which we manipulated the presence of mothers and/or of *M. brunneum* spores in the nesting environment, and then tested the resulting effects on gene expression in the abdominal fat body of the nymphs. The insect fat body is a multifunctional organ, which plays a center role in (i) lipid metabolism and storing/utilizing energy reserves, two key factors in insect growth (Arrese and Soulages, 2010; Canavoso et al., 2001), as well as (ii) the production of antimicrobial peptides and proteins, two crucial parameters in insect immunity (Bulet et al., 1999; Gillespie et al., 1997). If earwig nymphs adapt their metabolism to the social environment created by the mother, we expect nymphs to express genes associated with slower growth as they may favor prolonged family life over quick dispersal in presence of a caring mother. Conversely, if the absence of mothers causes stress for the nymphs, we would expect fat body gene expression to reflect the stress response, for example

in terms of metabolism or immunity (Adamo, 2012; Kohlmeier et al., 2016). If earwig nymphs adapt their immune response to an environment featuring the presence (and absence) of pathogens in the nest, we predict an increased expression of immunity-related genes in nymphs exposed to the pathogen compared to the controls. Finally, if maternal presence facilitates nymphs' pathogen defense, as would be expected if maternal care is a form of social immunity (Cotter and Kilner, 2010a; Meunier, 2015; Van Meyel et al., 2018), we predict that the presence of a mother buffers the effects of pathogens on nymph gene expression.

MATERIALS & METHODS

Experimental Design

To investigate the effects of mother and/or pathogen presence on gene expression in nymphs' fat body, we used 25 experimental clutches produced by *F. auricularia* females field-sampled in July-August 2015 in Mainz, Germany (49°58'20.5"N 8°11'42.3"E). We reared these females under laboratory conditions until egg production and hatching using a standard protocol (Vogelweith et al., 2017). One day after egg hatching, each experimental clutch was trimmed to 35 nymphs (mean original clutch size ± SE = 49.26 ± 2.1) and then transferred to recipient Petri dishes containing either (i) their own mother and non-contaminated sand (n=7), (ii) their own mother and spore-contaminated sand (n=6), (iii) no mother and non-contaminated sand (n =5) or (iv) no mother and spore-contaminated sand (n=7). All clutches were then maintained under standard conditions (18-20°C, 12:12h, Dark:Light) and provided with an ad libitum amount of standard food (detailed food composition in Kramer et al., 2015), which was changed every three days until the nymphs were used for RNA extraction on day 10. The spore-contaminated and non-

contaminated sands were obtained by preliminary grounding each recipient Petri dish (9 cm diameter) with humid sand and then sprinkling the sand with 100 µl of either a conidiospore solution of *M. brunneum* diluted in 0.05% Tween 80 (107 spores/ml), or a spore-free 0.05% Tween 80 solution, respectively. Note that *M. brunneum* is a natural pathogen of *F. auricularia* and is known to be infectious and lethal to a wide range of insect species, including the European earwig (Diehl et al., 2018; Kohlmeier et al., 2016; Vogelweith et al., 2017).

RNA Extraction & Sequencing

Ten days after we set up the clutches in one of the four experimental treatments, we haphazardly selected one nymph per clutch to extract its fat body and conduct a standardized RNA extraction. At this time, nymphs are still in their first developmental instar (the stage during which family life mainly occurs; Kölliker, 2007) but exhibit greater mobility and independence than immediately after hatching (Körner, pers. obs.). For RNA extraction, each nymph was first removed from its experimental clutch and instantly killed by decapitation; its abdominal fat body was immediately extracted on ice and homogenized in TRIZOL (Invitrogen, ThermoFisher Scientific, Waltham MA, USA) to be briefly stored at -20°C prior to RNA extraction. The extraction process was conducted using the RNAeasy mini extraction kit (QIAGEN) following the corresponding protocol. RNA samples were stored at -80°C after extraction. Library preparation and sequencing of 100bp paired end reads on an Illumina HiSeq 2000/2500 was conducted at BGI Hongkong.

Sample Quality Check and Transcriptome Assembly

The quality of the raw reads was assessed using *FastQC* v.0.11.5 (Babraham Bioinformatics) in conjunction with *MultiQC* v.1.2 (Ewels et al., 2016) followed by removal of Illumina adapter

sequences using *Trimmomatic* v.0.36 (Bolger et al., 2014). We then tested three different *de novo* assembly approaches using two different assemblers: first *Trinity* v.2.4.0 (CITE) with default parameters but minimum contig length set to 300, second *CLC Assembly Cell* v.5.0.4 (QIAGEN) with start positions distance range set to 250-350 and minimum contig length to 300, and third a meta-assembly consisting of the other two, obtained by first removing identical contigs using *cd-hit-est* (Li and Godzik, 2006) and then merging the contigs using *cap3* (Huang and Madan, 1999). We then compared the quality of the three assemblies using *Transrate* v.1.03 (Smith-Unna et al., 2016). We went forward using the results obtained by *CLC Assembly Cell* based on the following *Transrate* result parameters: number of contigs, backmapping rate and number of N's. The raw sequences as well as the contigs assembled by *CLC Assembly Cell* will be made available on NCBI (Project ID: PRJNA477302) upon publication of this manuscript.

Gene Expression Analysis

For the gene expression analysis, the reads were aligned to the assembly contigs and read counts were obtained using *Kallisto* v.0.43.1 (Bray et al., 2016). To test whether mother and/or pathogens presence triggered any changes in gene expression in the nymph fat body, we fitted a generalized linear model (GLM) using the R package *Deseq2* v.1.16.1 (Love et al., 2014). Specifically, we fit the model using a single factor with four levels to account for all combinations of maternal presence and pathogen presence: a) Mother / No Pathogen, b) Mother / Pathogen, c) No Mother / No Pathogen, d) No Mother / Pathogen. We then conducted four pairwise Wald tests to detect any changes in gene expression caused by 1) an effect of maternal presence/absence when pathogens were absent, 2) an effect of maternal presence/absence

when pathogens were present, 3) an effect of pathogen presence/absence when the mother was present and finally, 4) an effect of pathogen presence/absence when the mother was absent. The Wald test was used for significance testing and the resulting false discovery rate (FDR) p-values were adjusted for multiple comparisons using the Benjamini and Hochberg correction (Benjamini and Hochberg, 1995) implemented in *DESeq2*. Genes were considered significantly differentially expressed if FDR-p ≤ 0.05.

Annotation, Enrichment and Pathway Analyses

We annotated all contigs using a *BlastX* (NCBI) search against the non-redundant invertebrate protein database (state June 2017). To obtain gene ontology (GO) terms and Kyoto encyclopedia of genes and genomes (KEGG) pathway IDs corresponding to the differentially expressed contigs, we first used *Transdecoder* v.5.0.2 (Haas et al., 2014) to translate our *de novo* assembled contig nucleotide sequences into amino acid sequences, and used these to conduct an *Interproscan* v.5.26-65 (Finn et al., 2017) search. Enrichment analysis of GO terms was performed using the R package *topGO* v.2.28.0 (Alexa and Rahnenfuhrer, 2016). The list of genes of interest was defined as the set of differentially expressed genes identified previously using *DESeq2*. The p-values for each GO term were obtained by using an exact Fisher test and an FDR correction for multiple testing. Acquisition of KO terms for contigs was followed by the use of the KEGG Mapper (Reconstruct Pathway) to obtain pathways associated with each KO term (http://www.genome.jp/kegg/tool/map_pathway.html; Ogata et al., 1999).

RESULTS

The presence of the mother altered the expression of 102 and 12 genes in the fat body of nymphs reared without and with pathogens, respectively (Figure 3.1a). For the 102 genes differentially expressed in absence of the pathogen, Blast annotations included several metabolism related genes, such as *ethanolamine kinase 1* & *2*, *AAEL003102-PA* (glucuronosyltransferase activity), *hydroxylysine kinase* and *lipid phosphate phosphohydrolase 1-like* (Table 3.1), and the associated GO terms revealed several lipid metabolism-related functions such as transmembrane transport, sulfate transport and assimilation, and phosphatidylinositol phosphorylation (Figure 3.2). Interestingly, 89 of these 102 differentially expressed genes (DEGs) were downregulated in the presence of the mother, whereas only 13 were upregulated. Among the 12 genes differentially expressed in presence of the pathogen, all were upregulated in presence of the mother, and we

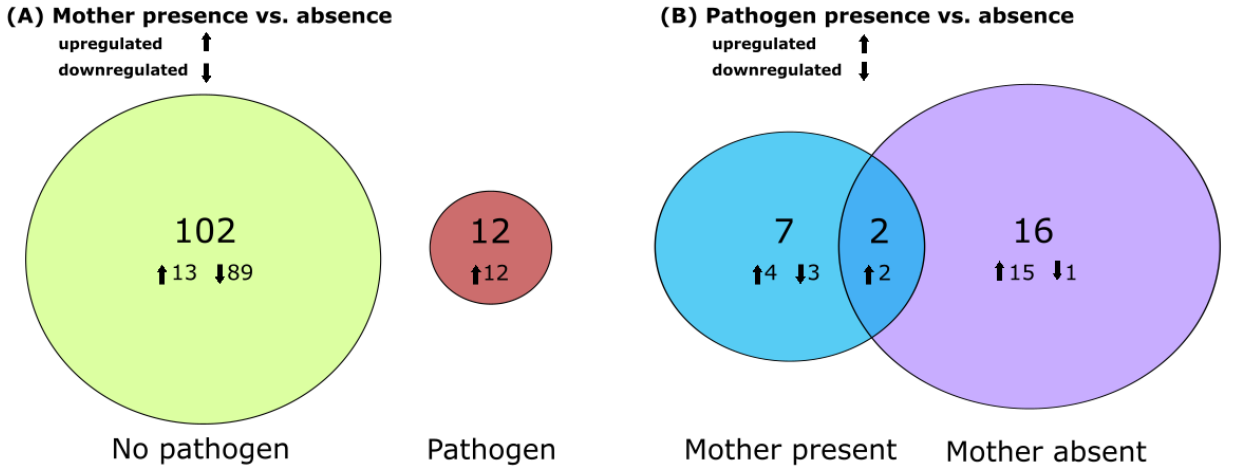

Figure 3.1. Venn-diagrams showing the number of differentially expressed genes in nymph fat body depending on the four combinations of maternal and pathogen presence (Wald test). **A.** DEGs due to variation in maternal presence either in pathogen-free (green) or pathogenic (red) treatments. **B.** DEGs due to variation in pathogen presence when mothers were either present (blue) or absent (purple). The overlap represents DEGs shared between the two treatments. Arrows and adjacent numbers denote subsets of up- and downregulated genes associated with (A) maternal presence and (B) pathogen presence.

found *myrosinase 1-like*, *lysozyme-like* and *chitinase 2*, a gene associated with the synthesis of chitin in cuticle in *Drosophila melanogaster*, along with several genes possibly involved in energy metabolism, such as *family 31 glucosidase*, *maltase A1-like* and *cytochrome b* (Table 3.1). In line with these metabolic genes, the single GO term associated with this DEG list was "carbohydrate metabolic process" (GO:0005975, Figure 3.2). Overall, none of the 102 and 12 genes affected by maternal presence are shared between the pathogen and non-pathogen treatments.

Conversely, the presence of pathogens altered the expression of only 9 and 18 genes in the fat body of nymphs reared with and without a mother, respectively (Figure 3.1b). Among the 9 genes differentially expressed with the mother present, 4 were upregulated in presence of pathogens. Interestingly, we did not identify immunity associated genes among the upregulated contigs, but a gene associated with *myotrophin*, a precursor to *Hugin,* which is known to be involved in larval feeding behavior, larval molting and chitin cuticle buildup (FlyBase FBrf0156956, Meng et al., 2002). There was a single GO term associated with the differentially expressed genes: "modulation by virus of host morphology or physiology" (GO:0019048), upregulated with pathogen presence when the mother was present. On the other hand, among the 18 genes differently expressed without a mother, 15 were upregulated in presence of the pathogen. There are two notable genes in this list of 15 which are known to be linked to immune defense: *pathogenesis-related protein 5*, which is associated with pathogen defense in plants but has been linked to induced immune responses in *Caenorhabditis elegans* and *Tribolium castaneum* (Altincicek et al., 2008; Greenwood et al., 2017; Shatters et al., 2006), and *beta-1,3-glucan-binding protein-like* which can be involved in the recognition of invading microorganisms and activation of the phenoloxidase cascade (Vargas-Albores et al., 1997), an important element

of insect immunity (Gillespie et al., 1997; González-Santoyo and Córdoba-Aguilar, 2012). Overall, 2 of the 9 and 18 genes affected by the presence of pathogens in the environment are shared between the treatments where nymphs were reared with and without a mother (Figure 3.1). The two genes are unannotated and upregulated in the pathogen presence.

DISCUSSION

Offspring living in facultative family systems can receive short- and long-term benefits from parental care but still survive in the absence of a caring parent. In this ancestral form of social life, we therefore expect offspring to adapt their metabolism, energy investment and behavior to attributes of their rearing environment, such as parental care and/or pathogen presence. In our study, we aimed to shed light on the gene expression differences between earwig nymphs reared either with or without their mother, as well as with or without pathogen (and the combination of treatments). We showed that offspring gene expression is affected by both maternal and pathogen presence. Specifically, we found that mother presence/absence has the strongest effect on nymph gene expression, but only if there is no pathogen in the environment. We also revealed that maternal absence leads to upregulation of metabolism related genes, but pathogenic environments seem to nullify this effect. Finally, we showed that pathogen presence induces higher expression of immune related genes, but only in absence of the mother.

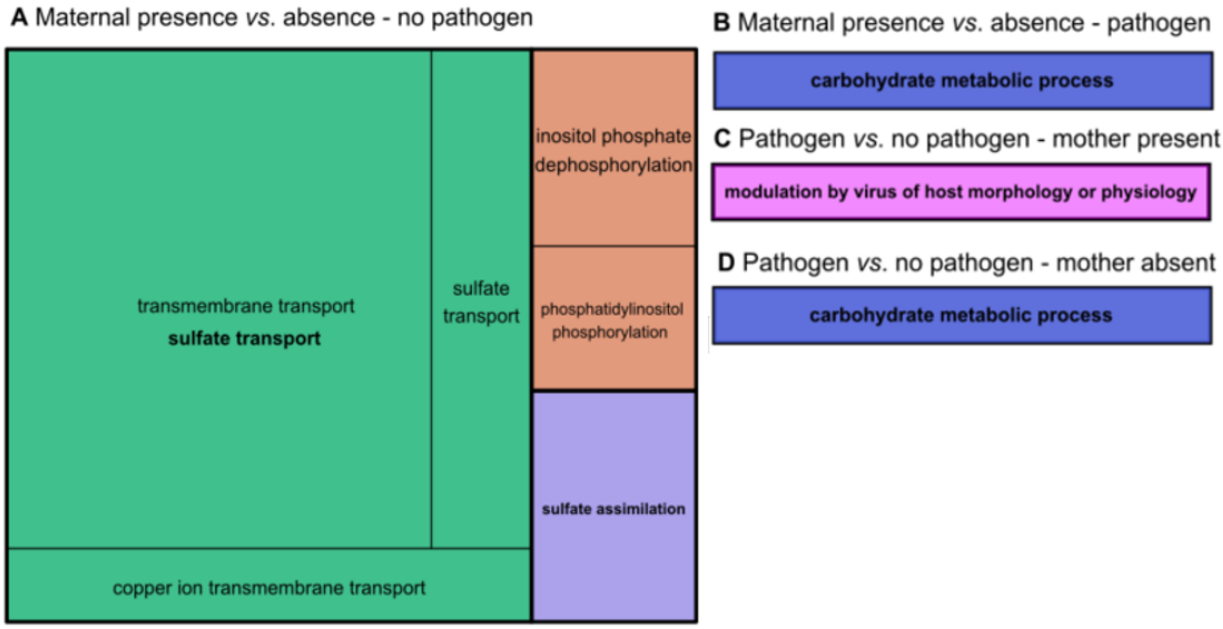

Figure3.2. **A** REVIGO treemap showing significant GO terms ($p<0.05$) in the DEG's between maternal presence and absence in nymphs reared without pathogens. Relative square size is scaled to match degree of overexpression. (REVIGO Gene Ontology treemap http://revigo.irb.hr/; Supek et al. 2011). **B**, **C** and **D** single enriched GO terms of DEG's between **B** maternal presence and absence in nymphs reared with pathogens and **C** pathogen presence and absence in nymphs reared with mother and **D** without mother. Background colors show similarity in function.

Maternal effect

Among the 102 DEGs affected by maternal presence, 89 genes were downregulated in the presence of the mother combined with the absence of the pathogen. Among these 89, we found several annotations associated with phosphate-, glycerophospholipid- and ATP metabolism. Since the fat body plays a central role in lipid metabolism as well as in the storing and utilizing of energy reserves (Arrese and Soulages, 2010; Canavoso et al., 2001), the observed metabolism related gene expression changes are in line with our expectations regarding offspring adaptation to maternal care. Moreover, if downregulation of metabolism related genes is associated slowed nymph development, this change in gene expression could reflect an offspring strategy to longer exploit the social environment generated by their tending parent. This is in line with a general

prediction of the parent-offspring competition over parental care (Trivers, 1974), which posits that offspring are selected to demand more and/or longer care (e.g. here, by slowing down their development) than parents are selected to provide (Trivers, 1974). These genetic changes are also in line with previous phenotypic observations revealing that earwig nymphs adjust their behavior (e.g. begging, sibling food sharing, cannibalism) to the level and/or quality of maternal care (Kramer et al., 2017; Kramer and Meunier, 2016a; J. W. . Wong et al., 2014), develop into adults of greater body size when orphaned (Thesing et al., 2015), as well as aggregate less and scatter within the nest in greater distance upon the removal of the mother (Körner, pers. obs.). Additionally, experimental selection for lower maternal investment into care was shown to induce faster offspring development in earwig nymphs (Kölliker et al., 2015). Hence, this downregulation of fat body metabolism in the presence of a caring mother may represent the first transcriptome evidence for an offspring strategy to delay dispersal from the nest to receive more resources through parental care. Alternatively, metabolism changes may also be caused by social stress due to the orphaning (i.e. sudden isolation, known to affect adults in this species (Kohlmeier et al., 2016). If this hypothesis holds true, we would expect that the gene expression changes involved in a stress response to orphaning would be present regardless of the presence of a pathogen. However, we found that these gene expression changes depended on pathogen presence. This does not support that the response to a social stressor (i.e. orphaning stress) is solely responsible for the observed gene expression patterns.

Somewhat surprisingly, the gene expression changes associated with maternal presence are completely altered with pathogen presence, resulting in 10x fewer and different DEGs. Similarly, the associated gene ontologies are reduced to a single significant GO term

(GO:0005975: carbohydrate metabolic process). While the annotations (and the GO term) still point towards changes in gene expression associated with energy metabolism, such as maltases and glycosidases, all DEGs are upregulated in the presence of the mother (in contrast to pathogen absence). If the nymphs slowed their metabolism in the presence of their mother with no pathogen, this effect is no longer reflected in gene expression with the pathogen around. Instead, the pathogen pressure may incite nymphs to accelerate development to disperse quickly and escape the infected nest. The overexpression of a gene annotated with *Chitinase 2* under pathogen pressure could hint at investment into accelerated growth, since this protein is known to be involved with cuticle formation in *Drosophila melanogaster* and is generally found when organisms reshape chitin armor, i.e. prior to and during molting (Pesch et al., 2017; Sámi et al., 2001). However, *Chitinase 2* is also expressed in entomopathogenic fungi, including *Metarhizium*, upon contact with chitin (Pintö et al., 1997). We thus cannot exclude the possibility that the *Chitinase 2* expression is in fact deriving from the spores *Metarhizium* themselves after they have penetrated the nymph cuticle, provided that the fungal growth entered the fat body. Additionally, increased nymph metabolism may be a stress response to pathogen pressure. In either case, our data show that the presence of a pathogen in the rearing environment appears to override the expression changes associated with maternal presence we find in absence of the pathogen.

Pathogen effect

Manipulating the presence of a pathogen in the absence of the mother revealed 18 differentially expressed genes. Among the 15 DEGs upregulated in pathogen presence, two are known to be linked with immune defense: *Pathogenesis-related proteins* and *beta-1,3-glucan-binding protein-*

like. Across plants and animals, *Pathogenesis-related proteins* represent a long-conserved group of proteins with antimicrobial and antifungal properties such as osmotin and thaumatin and are involved in insect immune defense, for example in the flour beetle *Tribolium castaneum* (Greenwood et al., 2017; Parker et al., 2015; Shatters et al., 2006). Conversely, *beta-1,3-glucan-binding protein-like* is a key recognition protein in the phenoloxidase cascade, which is a major component of insect immune defense against bacteria, viruses and fungi (Gillespie et al., 1997; González-Santoyo and Córdoba-Aguilar, 2012; Pauwels et al., 2011).

Given the importance of the fat body to the insect immune system (Canavoso et al., 2001; Gillespie et al., 1997), we expected to find the upregulation of immunity related genes in pathogen presence. Our results partly support this prediction in earwigs: we show that offspring upregulated the expression of immunity related genes under pathogen presence, but only when their mothers were absent. Parental influence on offspring immunity during parental care has been demonstrated in other species, for example in the mouth-breeding cichlid *Astatotilapia burtoni*, where offspring reared by immune challenged females showed decreased immune gene expression (Keller et al., 2017), or in the burying beetle *Nicrophorus vespilloides*, where the absence of caring parents decreased the expression of antimicrobial activity expressed by the larvae (Reavey et al., 2014). Maternal care is likely to affect pathogen pressure in earwig nests: mothers adapt care behaviors to the presence of pathogens (Diehl et al., 2018) and contribute to feces-mediated social immunity (Diehl et al., 2015). Given that early pathogen exposure can have life-long consequences on immune investment (Vogelweith et al., 2017), the nymphs in our study may increase immune investment when the mother is absent, but favor faster (and parentally aided) development when she is present. In line with this hypothesis, we showed that instead of

overexpressed immune genes, pathogen exposure in maternal presence induced expression of *myotrophin-1-like* in the nymph fat body. Myotrophins are important morphogenetic growth factors, such as the protein *Hugin*, which has been linked to larval molting in *Drosophila melanogaster* (Meng et al., 2002). It is important to note that in our study, any effects of maternal presence are unlikely to be conflated with effects of food access since the nymphs were provided food *ad libitum* and close by. Thus, any gene expression changes are due to the presence of the mother and/or nymph-mother interactions. In the field, orphaning-related gene expression changes may be stronger still, e.g. depending on foraging success of the nymphs and exposure of the nest structure. Overall, our findings suggest that earwig nymphs speed up metabolism and/or growth under pathogen pressure and maternal absence, perhaps to disperse quickly and leave the infested area. Note, however, that any tissue-specific gene expression shifts do not necessarily reflect body-wide expression changes. Future investigation into possible effects of parental environments on offspring metabolism could yield additional insight by including additional tissues, such as muscle tissue (Barazzoni et al., 2005; Bazhan et al., 2017).

CONCLUSIONS

Our study overall provides the first insights on how offspring can adapt to variation in maternal presence/absence on a transcriptomic level in a species with facultative family life. In particular, we revealed that variation in maternal presence changes the expression of 10 times more genes in pathogen-free compared to pathogenic environments and importantly, that these gene expression changes affect an entirely different set of genes. This indicates that pathogenic environment overrides the effects of maternal presence in terms of offspring gene expression,

and thus stresses the central importance of pathogens in the evolution of parental care. We then showed that precocial offspring alter their metabolism in response to maternal presence – even if both offspring and mothers have access to an *ad libitum* food source. This may represent the first transcriptome evidence for an offspring strategy to delay dispersal from the nest and thus to receive more resources through prolonged parental care. Finally, our data show that pathogens caused higher expression of immune related genes in offspring but only in absence of the mother. This suggests that access to social immunity in the form of maternal care may enhance offspring protection against pathogens and therefore allow juveniles to favor investment into development over the activation of their own immune defenses. Overall, this study sheds light on the genetic basis of offspring response to changes in parental presence, and reveals that pathogens likely shaped its use and function during family evolution.

Table 3.1. Selection of differently expressed genes of interest discussed in this paper with the associated Blast annotation, Log fold change, p-value and corresponding Uniprot function.

Compared	Fixed	Blast Annotation	Species (Blast)	LFC	P-value	Uniprot Function	Species (Uniprot)
Mother present *vs.* Mother absent	Control	Ethanolamine kinase 1	*Copidosoma floridanum*	-5.901350858	0.002	Highly specific for ethanolamine phosphorylation	*Drosophila melanogaster*
		Ethanolamine kinase 2	*Tribolium castaneum*	-5.01478676	0.002	Phosphatidylcholine biosynthesis	*Caenorhabditis elegans*
		AAEL003102-PA	*Aedes aegypti*	-4.850008124	0.001	Glucuronosyltransferase activity	*Aedes aegypti*
		Hydroxylysine kinase	*Bactrocera oleae*	-4.653385098	0.003	Kinase activity	*Drosophila ficusphila*
		Lipid phosphate phosphohydrolase 1-like	*Linepithema humile*	-3.786072217	0.01	Phosphatidate phosphatase activity	*Salmo salar*
	Pathogen	Chitinase 2	*Tribolium castaneum*	5.018622444	0.000005	Chitinase activity	*Drosophila melanogaster*
		Family 31 glucosidase	*Pogonomyrmex barbatus*	7.398173464	0.00002	Carbohydrate binding	*Drosophila melanogaster*
		Maltase A1-like	*Amyelois transitella*	7.063138844	0.000001	Catalytic activity	*Agrilus planipennis*
		Cytochrome b	*Occasjapyx japonicus*	7.889323284	0.000001	Metal ion binding	*Drosophila melanogaster*
Pathogen *vs.* control	Mother present	Myotrophin-like (Hugin)	*Hydra vulgaris*	3.841340721	0.009	Larval molting	*Drosophila melanogaster*
	Mother absent	Pathogenesis-related protein 5	*Tribolium castaneum*	5.81993311	0.000003	Partially responsible for acquired pathogen resistance	*Arabidopsis thaliana*
		Beta-1,3-glucan-binding protein-like	*Orussus abietinus*	5.498696727	0.000006	Involved in the recognition of invading micro-organisms. Activates the prophenoloxidase cascade.	*Penaeus vannamei*

CHAPTER 4

Age, pathogen exposure, but not maternal care shape offspring immunity in an insect with facultative family life

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ABSTRACT

To optimize their resistance against pathogen infection, individuals are expected to find the right balance between investing into immune system and limiting the accumulation of toxic immune components into their body. In vertebrates, several factors have been shown to critically affect the direction of this balance in growing offspring, such as the risk of infection, the age-specific condition and/or the access to external help such as parental care. However, the independent and/or interactive effects of these factors on offspring immunity remain poorly studied in insects. Here, we manipulated maternal presence and pathogen exposure in families of the European earwig *Forficula auricularia* to measure whether and how offspring's survival rate and investment into two key immune parameters changed during their development. The pathogen was the entomopathogenic fungus *Metarizium brunneum* and the immune parameters were hemocyte concentration and phenol/pro-phenoloxidase enzyme activity (total-PO). Our results surprisingly showed that maternal presence had no effect on offspring immunity, but reduced offspring survival. The concentration of hemocytes and the total-PO activity increased during development, to be eventually higher in adult females compared to adult males. Finally, pathogen exposure overall increased the concentration of hemocytes – but not the total-PO activity – in adults, while it had no effect on these measures in juveniles. Pathogen exposure also lowered the survival of juveniles during their early development. Our results show that contrary to the risk of infection and the age-specific condition, maternal presence does not shape immune defense in young earwigs. Overall, this indicates that pathogen pressure does not necessarily promote the emergence and maintenance of post-hatching maternal care in insects.

INTRODUCTION

Most living organisms are parasites (Schmid-Hempel, 2011). By altering the growth, fecundity, and survival of their hosts, they represent a strong selective force that drives the evolution of multiple defense in their hosts (Zuk and Stoehr, 2002). To limit the costs of pathogen infections, hosts typically depend on their immune system (Zuk and Stoehr, 2002). In insects, an important part of this defense relies on non-specific and constitutive mechanisms that involve the coordinate action of hemocytes and phenoloxidase (PO) (Siva-Jothy et al., 2005). Hemocytes are immune cells that circulate in the hemolymph and are involved in recognition and encapsulation of pathogens (Lavine and Strand, 2002). Conversely, PO mostly mediates the melanization of foreign objects and operates through the activation of the prophenoloxidase (PPO) cascade, its inactive precursor typically stored in the hemolymph and the hemocytes (Cerenius and Söderhäll, 2004).

Investing into immunity is costly and individuals are thus expected to adjust this investment to their current risk of infection, their general condition and/or their potential access to external help provided by group members (Armitage et al., 2003). Many vertebrates and invertebrates were shown to prophylactically increase their investment into immunity when the risk of infection is high, either due to the presence of pathogens in the environment or a high density of hosts in their vicinity (Barnes and Siva-Jothy, 2000; Sheena C. Cotter et al., 2004; Ruiz-González et al., 2009). For example, populations of the small ground finch *Geospiza fuliginosa* that lived on islands with a high parasite prevalence exhibited higher immune investment compared to birds living under low parasite pressure (Lindström et al., 2004). Animals experiencing favorable conditions either during development and/or adult life are also able to

invest more into energetically costly traits, such as immune defenses (S. C. Cotter et al., 2004; Westneat and Birkhead, 1998). In line with this prediction, large and/or well-nourished individuals are typically known to exhibit higher concentrations of immune components in their blood or hemolymph than small and light ones (S. C. Cotter et al., 2004; Vogelweith et al., 2013b). Finally, how much an individual invests into its immunity may also depend on the help it has received or will receive from others, i.e. on the expression of social immunity (Cotter and Kilner, 2010a; Cremer et al., 2007). Social immunity is a well-studied phenomenon in eusocial insects, where it can take the forms of allo-grooming and hygienic behaviors (Cremer et al., 2007; Meunier, 2015), but is also known to play a central role in simple family units in the form of parental care. The effect of parental care on offspring immunity is well documented in vertebrates, with examples showing that post-hatching parental care enhances the immune response of young barn swallows *Hirundo rustica* (Saino et al., 1997) or that parental deprivation reduces the immunocompetence of juveniles in mice (Michaut et al., 1981) and rats (von Hoersten et al., 1993). Comparatively, the effects of post-hatching parental care on offspring immunity are less clear in invertebrates, with only one study showing that parental deprivation reduces the lytic activity of larval exudate – a mediator of social immunity - in the burying beetle *Nicrophorus vespilloides* (Reavey et al., 2014).

Interestingly, the influence of parental care on offspring immunity may depend on the age of the offspring and their risks of pathogen infection. In many vertebrates and invertebrates, immunocompetence increases during development (DeVeale et al., 2004; Laughton et al., 2011; Pletcher et al., 2002; Trauer and Hilker, 2013). Consequently, the effects of parental care on offspring immunity could be limited to the early stages of development (when parents interact

with their juveniles) and then disappear when these juveniles have developed their own immune defenses. On the other hand, parental care facilitates offspring development with effects often reaching into adulthood, so that immune defenses could still be altered long after parents stopped caring for their offspring. The risk of infection could also determine how much parents invest into the care of their juveniles (Boos et al., 2014) and thus how much the offspring can invest into their own immune defense. For instance, the presence of pathogens in the environment has been shown to increase the expression of parental care in the frog *Hylophorbus rufescens*, as well as in humans, which in turn results in higher survival rates of pathogen-exposed offspring (Bickford, 2004; Quinlan, 2007).

In this study, we investigated the simultaneous and interactive effects of early maternal presence and early exposure to pathogens on offspring immunity during their development in the European earwig *Forficula auricularia*. In this insect species, females provide extensive forms of care to their juveniles (called nymphs) during the two weeks following egg hatching. For instance, they feed the nymphs, groom them and protect them against predators (Kölliker, 2007). Post-hatching maternal care, however, is facultative, as nymphs become mobile quickly, can forage on their own after a few days and are typically capable to develop and survive in the absence of a tending mother (Kölliker, 2007; Meunier and Kölliker, 2012b). Here, we conducted a 2x2 full-factorial experiment in which we manipulated the presence or absence of a mother, as well as the presence or absence of the entomopathogenic fungus *Metarizium brunneum* in the nest during the two first weeks post egg hatching (i.e. the period of family life). We then measured nymph survival and immune defenses at the 2nd, 3rd and 4th developmental instars, as well as in the adults. If maternal care shapes offspring immunity, we predict that maternal

presence improves the short- and long-term survival of offspring reared in a previously contaminated nest. We also expect that maternal presence overall increases the short- and/or long-term capabilities of offspring to invest into their own immune defenses and that such a benefit is stronger when offspring have been reared in contaminated nests. Conversely, if maternal care has limited or no effect on offspring immunity, we predict offspring immune defenses to increase with age and with early pathogen exposure, but these effects to be independent of early maternal presence.

MATERIALS AND METHODS

Insects rearing

Adult *F. auricularia* earwigs were caught in July-August 2015 in Mainz, Germany (49°58'20.5"N 8°11'42.3"E). Immediately after field sampling, earwigs were distributed among plastic containers (37 × 22 × 25 cm) grounded with humid sand. These adults were then allowed to mate freely for four months. Thereafter, all females were removed from their containers to mimic dispersal, a behavior they typically express under natural condition prior to egg laying (Lamb, 1975). The females were isolated in Petri dishes (9 cm diameter) that were furnished with moist sand, maintained under winter conditions (15°C in darkness) and provided with a diet of *ad libitum* standard food (food composition detailed in (Kramer et al., 2015)). Each Petri dish was then checked twice a week for eggs. Food provisioning was stopped when eggs were found, as females typically cease to feed between egg laying and hatching (Kölliker, 2007). At egg hatching, all clutches were transferred to and maintained under summer conditions (18-20°C D:L) until the end of the experiment (conditions detailed in (Ratz et al., 2016)).

Experimental design

A total of 98 clutches were used to measure the effects of early maternal presence and/or early pathogen exposure on two immune parameters on juveniles and young adults. Each clutch was trimmed to 35 nymphs one day after hatching (i.e. 1st instar nymphs) and then transferred to Petri dishes either with (1) their own mother and contaminated sand (n = 25), (2) their own mother and non-contaminated sand (n = 24), (3) no mother and contaminated sand (n = 25) or (4) no mother and non-contaminated sand (n = 24). The contaminated and non-contaminated sands were created by preliminary grounding each recipient Petri dish (9 cm diameter) with humid sand and then sprinkling the sand with either 100 µl of a conidiospore solution of *M. brunneum* diluted in 0.05% Tween (10^7 spores/ml) or with 100 µl of a control spore-free solution of 0.05% Tween, respectively. *M. brunneum* is a common entomophagous fungus in nature which is known to reduce the survival of earwigs and many other group-living insects (Denier and Bulmer, 2015; Kohlmeier et al., 2016; Konrad et al., 2012). On day 14 after egg hatching, all tending mothers were removed from their group of nymphs (when applicable) to mimic natural family dispersal (Meunier and Kölliker, 2012b). Six days later, each group of nymphs was transferred to a large Petri dish (14 cm diameter) grounded with non-contaminated sand and maintained as such until they reach adulthood. Note that adult males and females produced in each family were separated at emergence to ensure virginity and avoid inbreeding at the time of immune measurements (see below) (Meunier and Kölliker, 2013). All animals were provided with an *ad libitum* amount of standard food changed twice a week (detailed food composition in (Kramer et al., 2015)).

We followed offspring survival during their development by counting all group members either five (2nd, 3rd and 4th developmental instar) or ten (adults) days after the first individual of each clutch molted into the next developmental instar. Note that 1st instar nymphs molt into their 2nd instar approximatively 12 days after egg hatching (Meunier et al., 2012; Ratz et al., 2016). The days five and ten were chosen to ensure that (almost) all group members reached the new instar (or adulthood) on the day of counting (see details on developmental times in (Thesing et al., 2015)). After counting, we randomly sampled two nymphs per developmental instar (and one adult male and one adult female per group), weighed these individuals to the nearest 0.001 mg using a microscale (model MYA5; PESCALE, Bisingen, Germany), and used them for immune measurements (see below). Note that these animals were subtracted for the calculation of survival rates.

Measurement of the two immune parameters

We measured two key immune parameters on 2nd, 3rd and 4th instar nymphs, as well as on adult males and females: the total-PO activity and the concentration of circulating hemocytes. Note that earwig individuals cannot be sexed until they reach adulthood. In each of the two nymphs sampled per instar, between 0.2 to 0.5 µl of hemolymph was first extracted with a glass capillary, while 1µl was extracted in each adult male and female (see above). These extracts were immediately diluted in 11 µl (for nymphs) or 25 µl (for adults) of cold sodium cacodylate/$CaCl_2$ buffer (0.01 M sodium cacodylate, 0.005 M CaCl2; pH 6.5) to measure the two immune parameters.

The concentration of hemocytes was measured immediately after hemolymph extraction, using 10 of diluted hemolymph of the nymph and 10 µl of the diluted hemolymph of each male

and female. This counting was done using a Neubauer Improved Haemocytometer and a microscope (magnification x 400), as described in (Vogelweith et al., 2011).

Total-PO activity was spectrophotometrically measured using a standard protocol described in (Vogelweith et al., 2011). Specifically, the diluted hemolymph of one nymph (volume = 1.5 µl) and 16 µl remaining of the diluted hemolymph of each male and female were frozen at -30°C to optimize the measurement of total-PO activity. Each sample of frozen hemolymph was then thawed on ice and centrifuged for 5 minutes at 4°C (4000 × g). Five µl of the resulting supernatant was then added to a microplate well containing 20 µl of PBS and 140 µl of chymotrypsin solution (Sigma C-7762, 0.07 mg/ml of distilled water). A volume of 20 µl of L-dopa solution (Sigma D-9628; 4 mg/ml of distilled water) was then added to each well. The reaction was allowed to proceed for 2h 47min at 30°C in a microplate reader (Thermo scientific Multiskan™ FC Microplate Photometer). Enzyme activity was defined as the slope of the reaction curve during the linear phase of the reaction (Vmax value: change in absorbance units/min) and measured using the R-based program PO-CALC (Kohlmeier et al., 2015). All immune measurements were done blind regarding the early presence of the mother and the early exposure to pathogens.

Because the volume of extracted hemolymph and the resulting concentration of hemolymph slightly change between individuals, we standardized the concentration of hemocytes and total-PO activity (immune parameters) per microliter of hemolymph using the following formula: $I \times [(Vh + Vb) / Vh] / Vm$, in which I is the measured immune parameter, Vh is the volume of extracted hemolymph, Vb is the volume of buffer added (i.e. 11 µl for nymphs or

25 µl for adults) and Vm is the volume applied either to the Haemocymeter for hemocyte count (i.e. 10µl) or on the spectrophotometer plate for total-PO measurement (i.e. 5µl).

Statistical analyses

All statistical analyses were conducted using the software R v3.1.2 loaded with the packages *car, lme4, MASS* and *lsmeans*. The survival rate in between each developmental stage (defined here as "age") of offspring (entered using the *cbind* function) was tested using a generalized linear mixed-effects model (GLMM, with binomial error distribution). In this model, the age (second, third and fourth nymphal instars, and adults), early pathogen exposure (presence/absence) and early maternal presence (presence/absence) were entered as explanatory categorical factors,

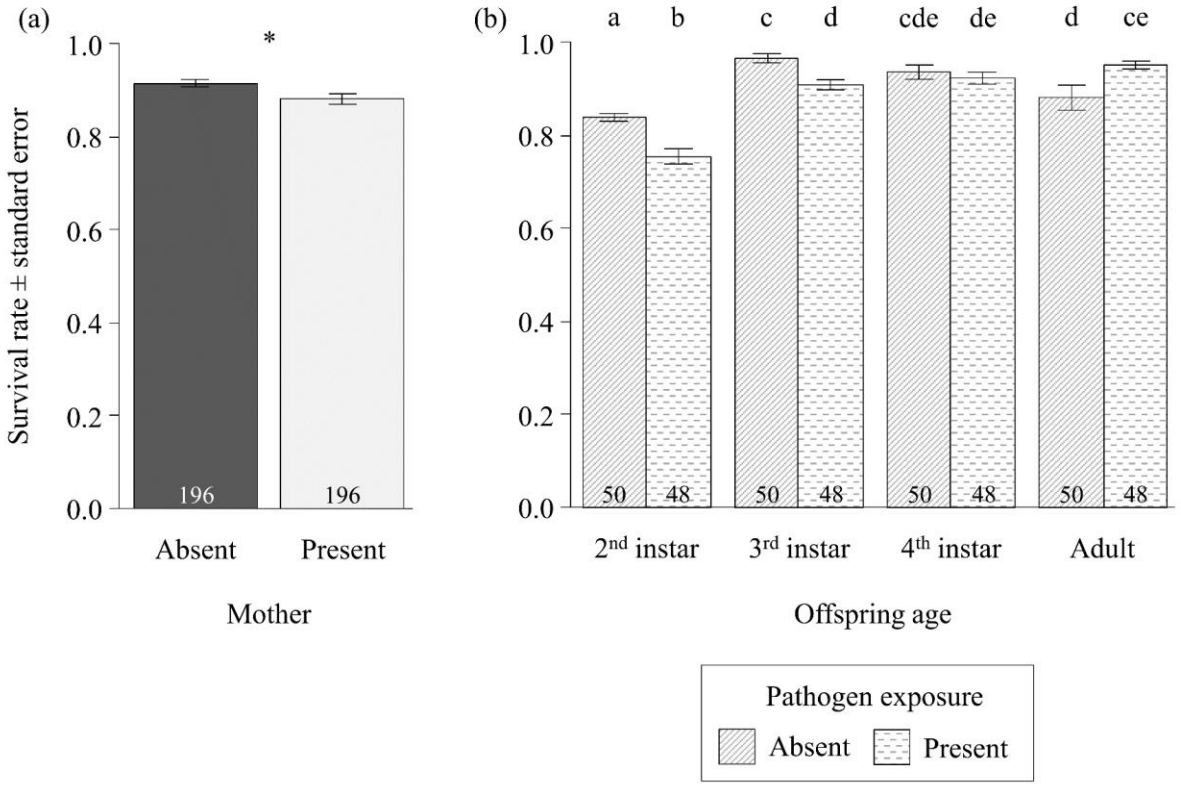

Figure 4.1. Effects of maternal presence, offspring age and pathogen exposure on offspring survival rate. The values are given in function of (a) maternal presence and of (b) the interaction between pathogen exposure and offspring age in between each instar. Sample sizes are provided at the bottom of each bar. Different letters indicate statistically significant differences ($p < 0.05$). * $p < 0.01$.

while the clutch identification (ID) was entered as a random factor to control for the fact that each clutch was used for each age. Because we interested in the survival rate of nymphs until their reach adulthood, adult males and females were pooled as “adults” in this model.

Immune parameters were then analyzed separately for nymphs (for which the sex was unknown) and adults (for which the sex was known). For each nymph and adult data set, hemocyte concentration and total-PO activity were analyzed using two LMMs, in which either the age of the nymphs (second, third and fourth nymphal instars) or the gender of the adults (male or female), early pathogen exposure, early maternal presence and the weight of the measured individual were entered as explanatory factors, whereas the ID was used as a random effect. In nymphs, the weight of each class of age was scaled and centered to correct for the inherent difference in weight between each instar. All models first included all interactions between the explanatory factors and were then simplified stepwise by removing the non-significant interaction terms (all P-values > 0.08). Note that some non-significant interactions are presented here to allow direct comparisons between models, but their removal did not qualitatively change the results.

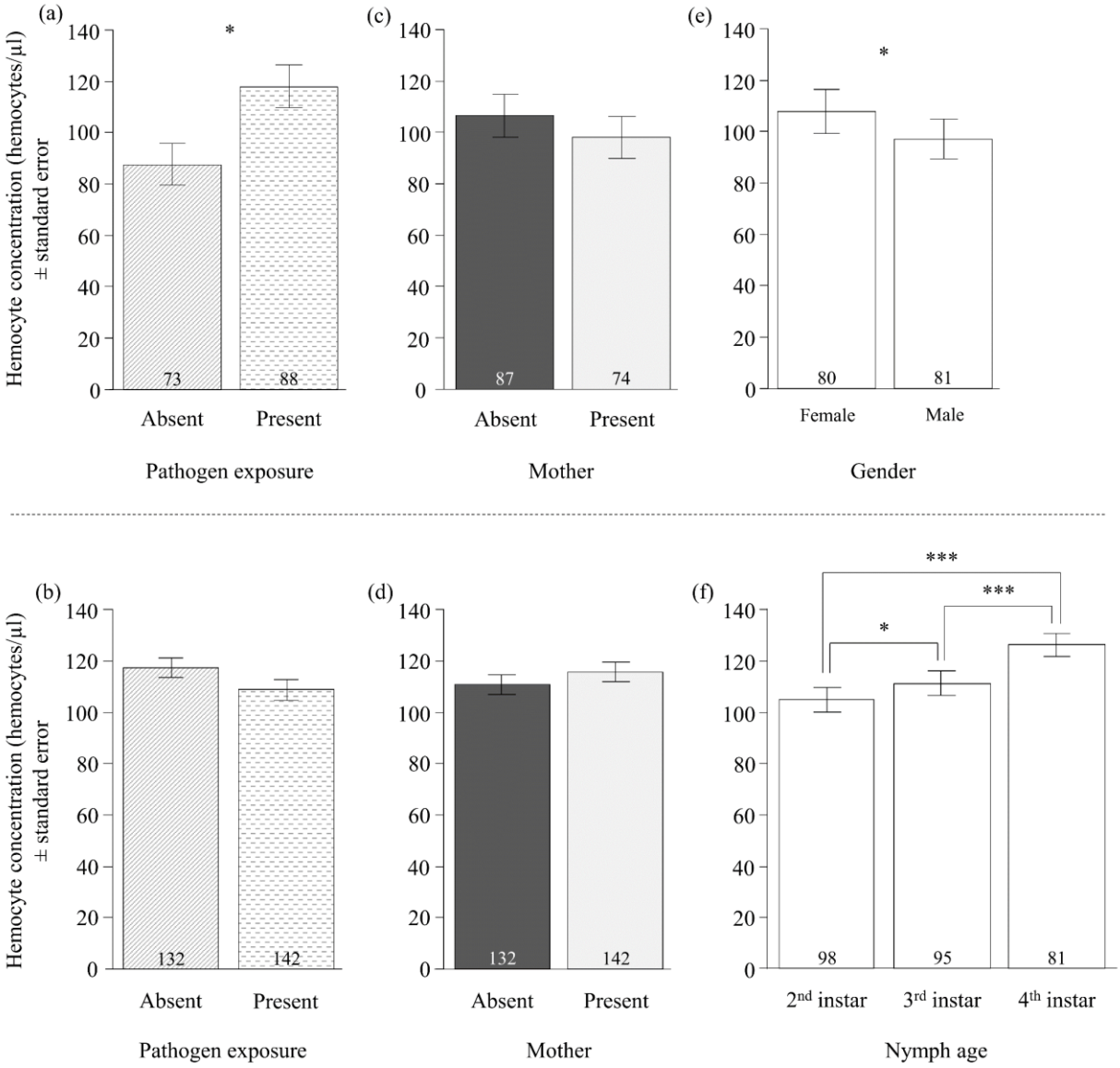

Figure 4.2. Effects of pathogen exposure, maternal presence, and adult gender or nymphal age on hemocyte concentration. The values are given in function of (a, b) pathogen exposure, (c, d) maternal presence and (e, f) gender/ nymph age in adults and nymphs, respectively. White bars represent age/gender, grey hatched/horizontal dotted bars represent the absence/presence of pathogen, and dark/light grey bars represent the absence/presence of mother. Sample sizes are provided at the bottom of each bar. *** $p < 0.0001$.

RESULTS

The presence of a tending mother overall reduced the proportion of offspring that successfully reached adulthood, independent of pathogen exposure and age (Table 4.1; Figure 4.1a). By contrast, offspring survival depended on an interaction between pathogen exposure and age (Table 4.1; Figure 4.1b): the pathogenic fungus *M. brunneum* reduced the survival rate of nymphs between the 2nd and 3rd instar, but did not affect their survival rate between the 3rd and 4th instars and was finally associated with an increased survival rate between the 4th instar and adulthood (Figure 4.1b).

Table 4.1: Effects of age, maternal presence and pathogen exposure on the survival rate of offspring. Significant *p-values* are in bold. Note that non-significant interactions are not reported in this table.

	Survival	
	Chisq	p-value
Age	86.39	**<0.0001**
Maternal presence	4.00	**0.045**
Pathogen exposure	6.21	**0.013**
Age*Pathogen exposure	14.87	**0.002**

Overall, there were contrasting effects of pathogen exposure, maternal presence, body weight, offspring developmental stage and adult gender on hemocyte concentration and total-PO activity in offspring. Specifically, early pathogen exposure increased hemocyte concentration but not total-PO activity in adults (Table 4.2; Figures 4.2a and 4.3a), whereas it did not affect these two immune parameters in nymphs (Table 4.3; Figures 4.2b and 4.3b). Early maternal presence also had no effect on the concentration of hemocytes and on the total-PO activity in

both nymphs and adults (Tables 4.2 and 4.3; Figure 4.2c,d and 4.3c,d). By contrast, the association between body weight and hemocyte concentration was positive in nymphs (Table 4.3; Figure 4.4a; ρ = -0.27; C.I. 95% = [-0.38; -0.16]) but negative in adults (Table 4.2; Figure 4.4b; ρ = 0.27; C.I. 95% = [0.11; 0.41]). There was, however, no association between body weight and total-PO

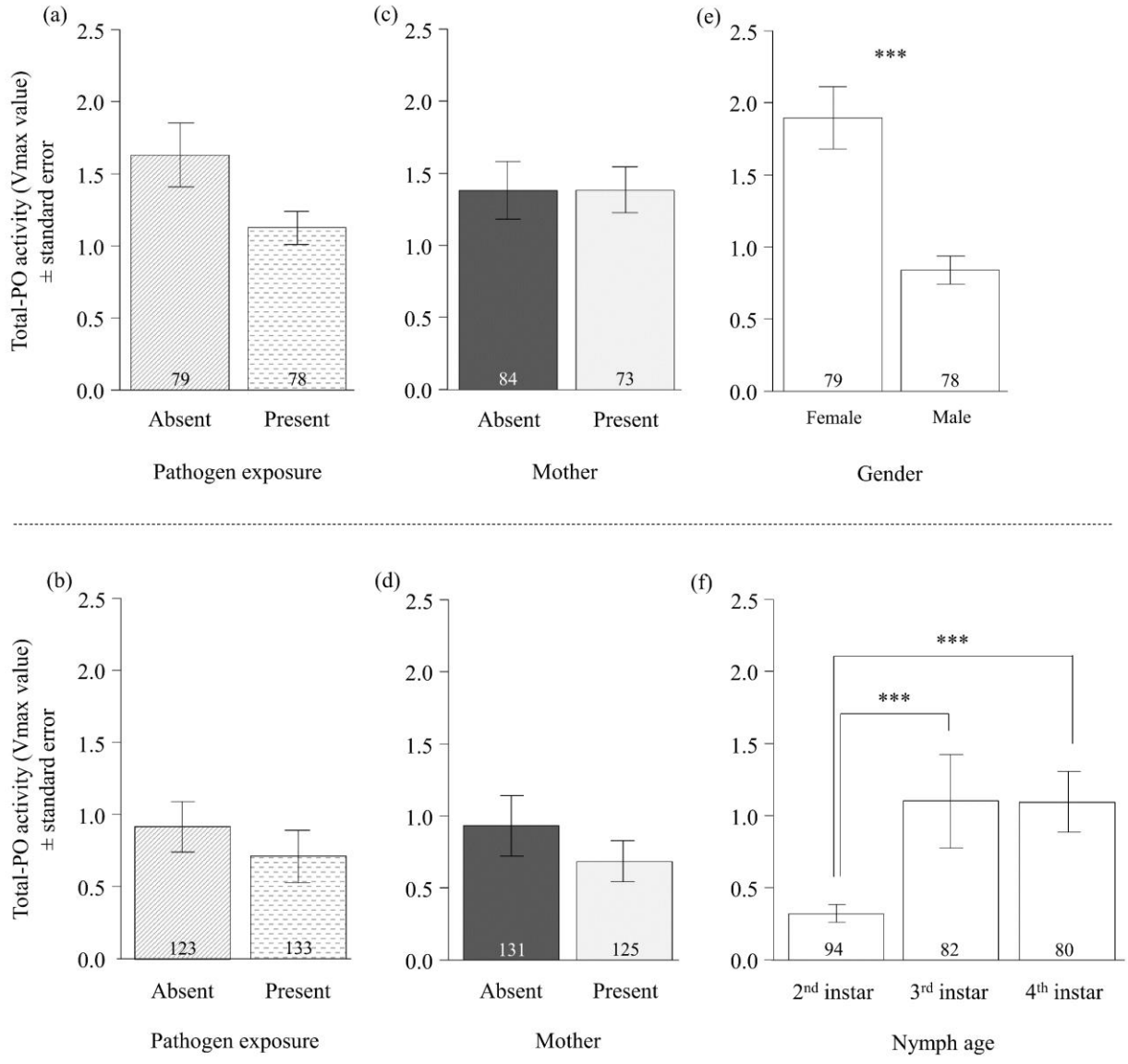

Figure 4.3. Effects of pathogen exposure, maternal presence and adult gender or nymphal age on total-PO activity. The values are given in function of (a, b) pathogen exposure, (c, d) maternal presence and (e, f) gender/ nymph age in adults and nymphs, respectively. Sample sizes are provided at the bottom of each bar. *** $p < 0.0001$.

activity in neither nymphs nor adults (Tables 4.2 and 4.3). Finally, the concentration of hemocytes and the total-PO activity increased between each nymphal instar (Table 4.3; Figures 4.2f and 4.3f) and were higher in adult females compared to adult males (Table 4.2; Figures 4.2e and 4.3e).

DISCUSSION

This study aimed at elucidating the effects of early maternal presence and early exposure to pathogens on the immunity of growing offspring in the European earwig *F. auricularia*. Our results show that the presence of the mother during the first two weeks of life has no effect on the immunity of her offspring at both nymphal and adult stages. By contrast, early pathogen exposure generally increased the concentration of hemocytes – but not the total-PO activity – in adult offspring and had no effect on nymph's immunity. Both hemocyte concentration and total-PO activities increased with offspring development, and these two immune parameters were higher in adult females compared to adult males. Finally, offspring survival was overall lower in clutches that were maintained with a mother or exposed to the pathogen at the beginning of the

Table 4.2: Effects of gender, maternal presence, pathogen exposure and weight on immune parameters in adults. Significant *p-values* are in bold. Note that non-significant interactions are not reported in this table.

	Hemocyte concentration		Total-PO activity	
	Chisq	p-value	Chisq	p-value
Gender	3.97	**0.046**	42.12	**<0.0001**
Maternal presence	0.36	0.547	1.18	0.278
Pathogen exposure	4.99	**0.025**	2.25	0.133
Weight	7.38	**0.006**	0.02	0.900

developmental stages, whereas adult survival was higher when they were early exposed to the pathogen.

Somewhat surprisingly, we found that maternal care has no long-lasting effects on the offspring immunity. This result contrasts with other studies investigating short- or long- term effects of parental care/parental deprivation on offspring immunity in vertebrates (Brown and Shine, 2016; Michaut et al., 1981; Saino et al., 1997; von Hoersten et al., 1993). For instance, nestling immunocompetence increased with parental care 24 hours after an immune challenge in barn swallows *Hirundo rustica* (Saino et al., 1997), and a long-term decrease in immunity has been shown in adult mice early-deprived of their mother (Michaut et al., 1981). The limited effects of maternal presence on offspring immunity reported here therefore reveal that maternal presence does not necessarily shape the immunity of offspring in insects, and more generally

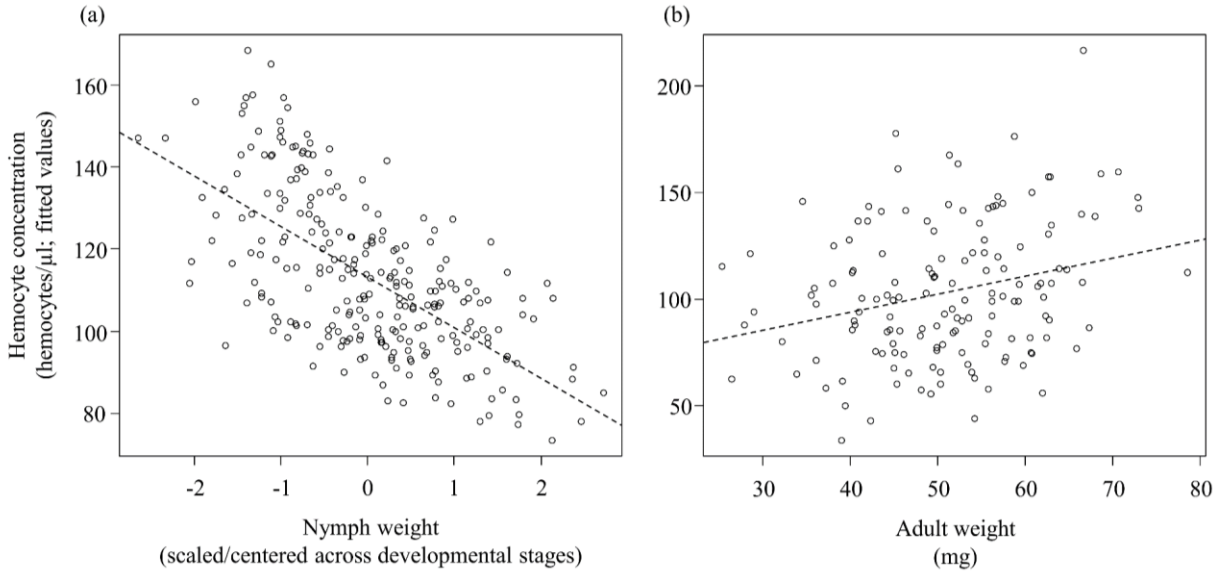

Figure 4.4: Correlation between hemocyte concentration and the weight in (a) nymphs and (b) adults. The hemocyte concentration are fitted values obtained from the LMMs. In nymphs, the weight of each class of age was scaled and centered to correct for the inherent difference in weight between each instar. Each dot represents an individual and the dash line represents correlation between hemocyte concentration and weight.

that pathogens are not a central selective pressure promoting maintenance of post-hatching maternal care in nature. Understanding whether this independence between parental care and offspring immunity is unique to earwig biology (Koch and Meunier, 2014; Kölliker et al., 2015; Thesing et al., 2015) or on the immune system of invertebrates (Beckage, 2008) will require further studies exploring the expression and nature of this link across a larger set of species.

Early exposure to *M. brunneum* did not unmask the effect of maternal care on offspring immunity, which contrasts to a previous result demonstrating that maternal presence improves the survival of eggs exposed to fungal spores in this species (Boos et al., 2014). However, we found an age-specific effect of pathogen exposure on offspring survival, which reflected a reduced survival rate between hatching and 3rd instars, an absence of effect between 3rd and 4th instar and a higher survival rate between 4th instar and adulthood. We propose two non-mutually exclusive scenarios to explain this effect. The first one is that juveniles exhibit relatively weak immune activities, as reported in many vertebrate and invertebrate species (see for instance in reptiles (El Deeb and Saad, 1990) and in the honey bee (Wilson-Rich et al., 2008)), and could thus be more sensitive to pathogen exposure than the older juveniles (Siva-Jothy et al., 2005). In line with this scenario, we found that the levels of total-PO and hemocyte concentrations increased with the developmental stage of the nymphs. The second scenario derives from our experimental design and relies on the fact that the 2nd and 3rd developmental instars were chronologically the first instars emerging after pathogen exposure. This chronology could result in a higher proportion of live spores in the environment of young compared to old offspring and thus in a decreased risk of a novel infection in later instars. These two scenarios predict that the least immunocompetent individuals should be quickly selected against in the pathogen-exposed

compared to the control environment, and that the surviving adult offspring previously exposed to *M. brunneum* should survive better and possess more hemocytes than the control ones – two predictions supported by our results. To disentangle between these two hypotheses, further

Table 4.3: Effects of age, maternal presence, pathogen exposure and weight on immune parameters in nymphs. Significant *p-values* are in bold. Note that non-significant interactions are not reported in this table.

	Hemocyte concentration		Total-PO activity	
	Chisq	p-value	Chisq	p-value
Age	12.65	**0.001**	74.47	**<0.0001**
Maternal presence	0.41	0.520	0.12	0.732
Pathogen exposure	0.17	0.681	0.61	0.436
Weight	18.71	**<0.0001**	0.13	0.721

studies should thus investigate whether offspring exposed to a pathogen either at the beginning of each instar or only at their 1st instar exhibit different or similar survival rates and levels of immunity.

Besides the general increase of offspring's immunity over developmental stages, we found that the association between hemocyte concentration and individual weight was negative in nymphs, but positive in adults. Immunity is generally sustained by either increasing the acquisition of food resources or by reducing energy allocation to other physiological processes such as growth and reproduction (Schmid-Hempel, 2005; Zuk and Stoehr, 2002) (see for examples (Brommer, 2004; Saino et al., 1997; Schmid-Hempel, 2005; Vogelweith et al., 2013a)). However, variation in the amount of resources available to an individual is known to possibly mask investment trade-offs between mutually exclusive functions and even to produce positive associations between these functions at a population level (Koch and Meunier, 2014; Parker and

Begon, 1986; van Noordwijk and de Jong, 1986). The apparent discrepancy between the presence of a trade-off in nymphs and of a positive association in adults therefore suggests that investing into immunity is generally costly in earwigs, but that this cost is masked in adults – possibly due to a higher variation in resource acquisition between adults compared to between nymphs. Investigating variation in foraging strategies and food intake of nymph and adult earwigs, and thus their role in immune investment will be done in the future. This notwithstanding, these results also reveal that maternal presence does not limit the costs of immune investment, further stressing the limited effects of maternal care on offspring immunity.

Independent of body weight, our results finally showed that adult females had more hemocytes and a higher total-PO activity than adult males. This sex-specific in- vestment into immune components is in line with results found across many insect species, such as butterflies (Stoehr, 2007), dragonflies (Rolff, 2002), flies (Mckean and Nunney, 2005) and scorpionflies (Kurtz et al., 2000). In earwigs, males are known to survive only for a single reproductive period (i.e. a few months). By contrast, females' live up to 1.5 years, during which they provide care to their eggs for several months, provide care to the resulting nymphs for several weeks and then often produce a second clutch which they care for during several additional weeks (Meunier et al., 2012; Ratz et al., 2016). Compared to males, females fitness is therefore tightly associated with their capability to survive over several seasons and thus to fight against longer and/or more frequent attacks by pathogens, overall likely explaining their higher investment into immune defense (see also Kohlmeier et al., 2016).

CONCLUSIONS

Our results overall reveal that age, gender, and parasite exposure shape the immune system of the European earwig *F. auricularia*, while the presence of a caring mother did not. Personal immunity and social immunity in the form of maternal care are nevertheless not the only protection against pathogens that can operate within family units (Meunier, 2015). For instance, larvae can participate in social immunity and thus provide immune benefits to their siblings by sanitizing the nest with anal exudates, a phenomenon reported in the buying beetle *N. vespilloides* (Arce et al., 2013) and importantly, in the European earwig *F. auricularia* (Diehl et al., 2015). Our findings therefore reveal that for juveniles, the net benefits of family interactions in terms of protection against pathogen infection are unlikely to come from the mothers, but could instead result from the presence and/or interactions with their siblings. Hence, our study overall calls for further studies investigating the role of sibling behaviors, together with age, gender, and parasite exposure, in the emergence and maintenance of family life in nature.

CHAPTER 5

Social immunity: why we should study its nature, evolution and functions across all social systems

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ABSTRACT

Mounting defences against pathogens is a necessity for all animals. Although these defences have long been known to rely on individual processes such as the immune system, recent studies have emphasized the importance of social defences for group-living hosts. These defences, called social immunity, have been mostly studied in eusocial insects such as bees, termites and ants, and include, for instance, mutual cleaning and waste management. Over the last few years, however, a growing number of works called for a broader exploration of social immunity in non-eusocial species. In this review, we summarise the rationales of this call and examine why it may provide major insights into our current understanding of the role of pathogens in social evolution. We start by presenting the original conceptual framework of social immunity developed in eusocial insects and shed light on its importance in highly derived social systems. We then clarify three major misconceptions possibly fostered by this original framework and demonstrate why they made necessary the shift toward a broader definition of social immunity. Because a broader definition still needs boundaries, we finally present three criteria to discriminate what is a form of social immunity, from what is not. Overall, we argue that studying social immunity across social systems does not only provide novel insights into how pathogens affect the evolution of eusociality, but also of the emergence and maintenance of social life from a solitary state. Moreover, this broader approach offers new scopes to disentangle the common and specific anti-pathogen defences developed by eusocial and non-eusocial hosts, and to better understand the dependent and independent evolutionary drivers of social and individual immunity.

INTRODUCTION

During its life cycle, every animal encounters large numbers of pathogens such as viruses, protozoans, bacteria, helminths and fungi (Lu and St. Leger, 2016). Pathogen infections often have dramatic consequences in a host, ranging from premature death to the modification of a broad set of fitness-related physiological, morphological and behavioural traits (Siva-Jothy et al., 2005). To limit the costs of pathogen infection, hosts have thus developed a multitude of defences encompassed in the term individual immunity (de Roode and Lefèvre, 2012; Schmid-Hempel, 2014; Siva-Jothy et al., 2005). In insects, these defences typically rely on physiological changes limiting pathogen development into the host body (i.e. immune system) (Hillyer, 2016; Siva-Jothy et al., 2005) and on behavioural processes reducing the risk of pathogen exposure and infection, for instance, by prophylactively or therapeutically consuming food sources with anti-pathogenic properties, a process called self-medication (de Roode and Lefèvre, 2012).

Over the last decades, a growing number of studies has revealed that protection against pathogens may not only rely on the defences exhibited by the host itself, but also on defences generated by its surrounding relatives (Cotter and Kilner, 2010a; Cremer et al., 2007; Meunier, 2015). Textbook examples of this *social immunity* typically come from eusocial insects such as bees, ants and termites (Figure 5.1) (Cremer et al., 2007; Meunier, 2015; Schmid-Hempel, 1998; Wilson-Rich et al., 2009). One of these examples is allo-grooming, a behaviour frequently reported in eusocial insects, during which workers groom each other to remove the pathogens present on the cuticle (Reber et al., 2011). Another example encompasses sanitary behaviours, during which workers remove food waste and/or cadavers from their colony to prevent the development of microbial pathogens, as found in many bees, ants and termites (Hart et al., 2002;

Ulyshen and Shelton, 2012; Visscher, 1983; Zeh et al., 1999). Social immunity can also be illustrated by social isolation, during which infected individuals leave their colony (Heinze and Walter, 2010; Rueppell et al., 2010) or reduce contacts to the brood (Bos et al., 2012; Ugelvig and Cremer, 2007) to limit the transfer of pathogens to colony members. Finally, ant and termite workers frequently use self-produced secretions to sanitize the nest walls and/or the brood (Baracchi et al., 2012; López-Uribe et al., 2017; Yek and Mueller, 2011), which is also a common form of social immunity (for an exhaustive list of all the classical forms of social immunity, please refer to (Cremer et al., 2007; Meunier, 2015)).

The discovery of social immunity rapidly led to major advances in our understanding of why and how eusocial insects are efficiently protected against pathogens (Cremer et al., 2007; Cremer and Sixt, 2009; Schmid-Hempel, 1998). It also gave rise to two evolutionary scenarios on the role of social immunity in the evolution of group living. The first scenario posited that social immunity is a phenomenon that has secondarily derived from eusocial systems and thus only plays a role in the consolidation of complex, permanent and obligatory forms of group living exhibiting reproductive division of labour (thereafter called the *eusocial framework*) (Cremer et al., 2017, 2007; Schmid-Hempel, 2017). The other (more recent) scenario postulates that social immunity is an ancestral phenomenon that can be found in many forms of group living and thus, that social immunity also plays a key role in the early emergence and maintenance of group living from a solitary state (thereafter called the *group-living framework*) (Cotter and Kilner, 2010a; Meunier, 2015).

In this study, we review recent empirical data across eusocial and non-eusocial (i.e. group living species that do not exhibit a eusocial organisation) insects to emphasize why it is now time

to study the nature, evolution and functions of social immunity across all social systems. Specifically, we first present the origin and implications of the eusocial framework in our current understanding of anti-pathogen defences in eusocial insects. We then discuss the rationales of the recent call for a switch from a eusocial to a group living framework by shedding light on three major misconceptions that can be fostered by the eusocial framework. In a final part, we stress that understanding social immunity requires boundaries in its definition and thus propose a newly defined group-living framework detailing three criteria that could allow discriminating what is a form of social immunity, from what is not. Overall, we argue that expanding the number of studies on social immunity in a broad taxonomical spectrum of non-eusocial species would provide novel major insights into our general understanding of the common and specific solutions developed by each type of social host to counteract infections and thus, into the role of pathogens in social evolution.

THE EUSOCIAL FRAMEWORK OF SOCIAL IMMUNITY

The eusocial framework of social immunity emerged at the beginning of the 21th century as the result of works conducted by researchers investigating how eusocial insects limit the inherently high risks of pathogen exposure and transmission between colony members (Cremer et al., 2007; Naug and Camazine, 2002; Schmid-Hempel, 1998; Wilson-Rich et al., 2009). The central idea of this framework is that social immunity mimics the individual immunity of multicellular organisms when the unit of selection has shifted from the individual to the colony (Cremer and Sixt, 2009; Masri and Cremer, 2014). In other words, social immunity has “evolved in convergence with individual immunity to protect the entire reproductive entity (i.e. the superorganism, (Kennedy

et al., 2017)) and maximize its fitness" (Cremer et al., 2017). Three examples typically illustrate this parallel between personal and social immunity in eusocial insects. First, wood ants, honeybees and stingless bees collect and incorporate plant resin with antimicrobial properties into their nests to limit the development of microbial pathogens (Christe et al., 2003; Duangphakdee et al., 2009; Simone et al., 2009), a process mimicking individuals' self-medication process to fight an infection (de Roode et al., 2013). Second, honeybee workers can fan their wings simultaneously to increase the temperature of their hive and thereby eliminate heat-sensitive pathogens (Starks et al., 2000), a process mimicking the fever exhibited by a body to fight an infection. Finally, workers of the ant *Lasius neglectus* administer antimicrobial poison inside infected cocoons to prevent pathogen replication and establishment within the colony, just like the individual immune system targets and eliminates infected cells from host body (Pull et al., 2017).

The accumulation of results supporting the parallel between individual and social immunity in eusocial insects rapidly led to the adoption of the eusocial framework by researchers interested in collective defences against pathogens. This adoption then fostered the claim that social immunity is "necessary and essential to eusocial systems" (Cremer et al., 2017) and thus, that social immunity should be considered as a major and unique social parameter once eusociality has emerged (Cremer et al., 2017, 2007, Schmid-Hempel, 2017, 1998).

The limit of the eusocial framework

One pillar of the original eusocial framework is thus that all collective defences against pathogens employed by individuals living in non-eusocial groups are not social immunity, but instead reflect non-derived defences such as communal disease defences and parental care (Cremer et al., 2017;

Schmid-Hempel, 2017). This boundary between eusocial and non-eusocial species rapidly became a major issue in deciphering the common and/or separate evolutionary pathways of collective defences against pathogens across group living species (Cotter and Kilner, 2010a; Meunier, 2015). Moreover, this restriction to eusocial systems opened scope for several important misconceptions concerning the link between social immunity and social evolution. For instance, it might suggest that 1) reproductive division of labour is essential to allow the evolution of social immunity, 2) the presence of social immunity should lower investments into individual immunity in eusocial species and finally, that 3) social immunity does not have counterparts in non-eusocial species (Cremer et al., 2017, 2007; Naug and Camazine, 2002; Schmid-Hempel, 2017, 1998; Wilson-Rich et al., 2009). In the following part, we clarify these three misconceptions using recent empirical findings and demonstrate why they call for considering social immunity as a broader phenomenon that is not exclusive to eusocial species (Cotter and Kilner, 2010a; Meunier, 2015).

On the importance of reproductive division of labour

One misconceptions possibly fostered by the eusocial framework is that the direct fitness costs of performing social immunity are so high for a donor individual that they should prevent the evolution of social immunity in groups where the donors' fitness relies on their own reproduction. In other words, the net benefits of performing social immunity should only be present in groups where donor individuals forego personal reproduction, i.e. in eusocial species with reproductive division of labour (Cremer et al., 2017). The first issue with this prediction is that it neglects that some forms of social immunity are not only unlikely to provide significant

fitness costs to donors (e.g. the use of self-produced secretion to sanitize the nest, the removal of fresh corpses from the nest (Cremer et al., 2007)), but may also provide direct benefits to donor individuals. These direct benefits have been recently revealed in allogrooming, a textbook example of social immunity (Cremer et al., 2007; Meunier, 2015). This behaviour has long been thought to be exclusively costly for donor individuals, because it increases their risk of being exposed to the pathogens present on the recipient individuals. In an elegant study conducted in the ant *Lasius neglectus*, however, Konrad *et al* (Konrad et al., 2012) demonstrated that allogrooming provides direct benefits to both recipients and donors, as it allows donors to prime their own immune system and thus boost their defences against future pathogen exposure. Interestingly, a follow-up study recently showed that these direct benefits are pathogen-specific in that workers immune-primed with one type of pathogen preferentially direct their future allogrooming behaviours toward individuals infected with the same compared to a different pathogen (Konrad et al., 2018).

The second issue with this prediction is that it overlooks the central role of kin selection in the evolution of some extreme forms of social immunity and neglects the fact that kin selection also operates in groups of individuals undergoing personal reproduction (Kramer and Meunier, 2016b). This central role can be illustrated by the self-exclusion of infected workers (Figure 5.1), another textbook example of social immunity reported in ants and bees (Heinze and Walter, 2010; Rueppell et al., 2010). This behaviour reflects that infected workers leave their nest to die alone and thereby limit the infection of their colony members. The evolution of such a behaviour typically relies on kin selection, as it becomes adaptive only if it allows the genes of the sacrificed individual to be passed on to the next generation by one or more of the saved group members,

i.e. only if the benefactor and the beneficiaries are genetically related. Based on the same reasoning, such a sacrificial behaviour could *in principle* evolve in subsocial (family) groups if the self-exclusion of infected offspring significantly improves the reproduction of their related siblings. Whether such sacrifices occur in non-eusocial species is, however, unexplored so far. Overall, social immunity is thus not necessarily associated with net fitness costs for donor individuals and it is therefore possible for social immunity to evolve in group-living species without reproductive division of labour.

On the relaxed selection on individual immunity in eusocial species

A second misconception possibly fostered by the eusocial framework is that the emergence of social immunity should relax selection on individual immunity and thus, that individual immunity should be less efficient and/or involve a lower number of genes in eusocial compared to non-eusocial species (Evans et al., 2006; Weinstock et al., 2006). The interest of this prediction resided in the fact that it was relatively easy to test empirically. Unfortunately, the results were at odds with this prediction. On one hand, physiological studies showed that antimicrobial peptides (a component of individual immunity) are more effective in eusocial compared to solitary sister species in bees (Stow et al., 2007) and trips (Turnbull et al., 2011). On the other hand, the recent accumulation of genomic studies comparing insects with different levels of social organization reports no general association between eusociality and the number and/or expression of immune-related genes across ants, bees and termites (reviewed in (Otani et al., 2016)). Hence, shifting the unit of selection from individual to superorganism may not affect the selection pressures exerted on individual immunity (Barribeau et al., 2015). Interestingly, this apparent absence of a general link between individual and social immunities suggests that the emergence

of social immunity does not reduce, but instead complements individual defences against pathogens.

On the absence of social immunity in non-eusocial species

A third misconception possibly fostered by the eusocial framework is that social immunity is exclusive to eusocial species. A recent study, however, revealed that 11 of the 30 anti-pathogen defences found in eusocial insects and classically considered as forms of social immunity (Cremer et al., 2007) can also be found in non-eusocial insects (Meunier, 2015) (see also (Cotter and Kilner, 2010a)). For instance, the use of self-produced components with antimicrobial properties as colony material is not only present in ants and termites (Chouvenc, 2013; Christe et al., 2003), but has been reported in nests of the wood cockroach *Cryptocercus punctulatus*, the European earwig *Forficula auricularia* and the Burying beetle *Nicrophorus vespilloides* (Cotter and Kilner, 2010b; Diehl et al., 2015; Rosengaus et al., 2013). Sanitary behaviours consisting in the removal of waste and feces material from the colony can also be found in several non-eusocial species with high nest fidelity (reviewed in (Weiss, 2006)), such as the subsocial cricket *Anurogryllus muticus* (West and Alexander, 1963). Finally, allogrooming is a behaviour frequently observed in arthropod species where parents remain with juveniles after egg hatching (e.g. (Mas and Kölliker, 2010; Thiel, 1999)), even if its role against pathogen infection needs to be further explored.

Whereas (at least) some forms of social immunity can be present in non-eusocial insects, it is also important to stress that (at least) some forms of social immunity are not present in all eusocial insects. For instance, queens of the pharaoh ant *Monomorium pharaonic* and the wood ant *Formica paralugubris* surprisingly prefer habitats contaminated with a pathogenic fungus to establish their colony (Brütsch et al., 2014; Pontieri et al., 2014), whereas the avoidance of

contaminated areas is classically considered as a form of social immunity in eusocial insects (Cremer et al., 2007). Similarly, experimental exposure to pathogen spores did not trigger higher levels of allogrooming between workers in the ants *Formica selysi* and *Myrmica rubra* (Leclerc and Detrain, 2016; Reber et al., 2011), and co-founding queens of the ant *Lasius niger* perform only very little allogrooming and did not exhibit a better resistance against pathogens when compared to solitary queens (Brütsch et al., 2017). The claim derived from the eusocial framework and stating that social immunity is "necessary and essential to protect the entire reproductive entity and maximize its fitness" (Cremer et al., 2017) should therefore be taken with caution. Arguably, social immunity encompasses a great diversity of forms (Cremer et al., 2007; Meunier, 2015), so that the absence of evidence for one form of social immunity should not be considered as an evidence for the absence of any form of social immunity. Nevertheless, the above findings warn us on the risk to over-interpreting the expression of certain behaviours as social immune responses on the sole basis that they are present in a eusocial species.

WHAT IS AND WHAT IS NOT A FORM OF SOCIAL IMMUNITY?

The shift from a eusocial to a group-living framework has recently generated some confusion on the boundaries of social immunity, which in turn blurred our general view of its nature, evolution and function across species. Here, we clear up this confusion by proposing a newly defined group-living framework detailing three criteria that can be used to determine whether a given defence is a form of social immunity. First, this defence should help recipient individuals to reduce their risks of infection by pathogens, which refers to anything that can produce a disease such as viruses, bacteria, protozoa, prion, fungus and helminths. This encompasses all the potential steps

of an infection, which include direct contact to a pathogen, penetration, development and replication of pathogens into the recipients' body and ultimately infection-derived death of the host (Cremer et al., 2007; Meunier, 2015). The second criterion is that donors and at least some of the recipients should belong both to the same species and to the same social group. This excludes, for instance, all behaviours and collective processes during which individuals from one species provide anti-pathogen defences to individuals from another species, as commonly reported in the context of symbiosis and *cleaning symbiosis* in cleaner fishes (Hopkins et al., 2017). Finally, the third criterion is that the defence should be "at least partly" selected for the anti-pathogen benefits it provides to the recipients. This stresses that social immunity is a target of selection and cannot be a simple by-product of individual immunity. This criterion excludes all individual defences that are either passively enhanced by group living (e.g. herd immunity (Babayan and Schneider, 2012)), selfishly driven by the nearby presence of conspecific individuals (e.g. density-dependent prophylaxis (Wilson and Cotter, 2008)) or that only happens to limit the risk of infection of solitary individuals encountered during a life cycle, such as during mating and/or competitive events. This third criterion also clarifies the rationale to separate the nomenclature between individual and social immunity.

Overall, these three criteria can be fulfilled 1) when group living is permanent, obligatory, temporary and/or facultative and 2) in a broad range of species ranging from insects and arachnids, over birds and fishes, to mammals and social microbes (Cotter and Kilner, 2010a; Meunier, 2015). Importantly, this absence of a dichotomy between eusocial and non-eusocial systems emphasizes that similar selection pressures are likely to have driven the evolution of comparable forms of social immunity across group living species. For instance, the evolution of

the spread of feces with antimicrobial properties on nest walls by eusocial workers in termites (Chouvenc, 2013) is very likely to have evolved under the same selection pressures that the ones selecting for the spread of feces with antimicrobial properties on nest walls by juveniles in family units of burying beetles and earwigs (Diehl et al., 2015; Reavey et al., 2014). To summarize, social immunity can be defined as "any collective or personal mechanism that has emerged and/or is maintained at least partly due to the anti-pathogen defence it provides to other homospecific group members", which is an edited definition of social immunity previously formulated by Meunier (Meunier, 2015).

CONCLUSION

In this review, we emphasized that individuals living either in facultative/temporary groups or in obligatory/permanent colonies can all perform defences against pathogens that may not only help themselves, but also their group members. The presence of these defences in such a large diversity of social systems recently made necessary the shift from a eusocial to a broad conceptual framework of social immunity (Cotter and Kilner, 2010a; Cremer et al., 2017, 2007; Meunier, 2015; Schmid-Hempel, 2017). This shift has generated novel works using the term 'social immunity' in a few subsocial insects such as the European earwig (e.g. (Boos et al., 2014; Diehl et al., 2015; Kohlmeier et al., 2016)) and the burying beetle (e.g. (Duarte et al., 2015; Reavey et al., 2015, 2014)). Here, we claim that it is crucial to expand these first works to a taxonomically broader number of non-eusocial species. The resulting studies would first allow us to disentangle whether the selection pressures favouring the emergence of social immunity have either secondarily evolved to limit the inherently high risk of pathogen exposure in species with an

obligatory and permanent social life (i.e. some forms of social immunity derive from eusociality), or whether they remained constant after the evolutionary shift from solitary to group living (i.e. social immunity is an ancestral process) (Cotter and Kilner, 2010a; Meunier, 2015). Interestingly, it would also allow testing an alternative evolutionary scenario positing that the general risk of pathogen exposure for a solitary individual could have selected for the emergence of group living in order to obtain an additional line of defence such as social immunity (Biedermann and Rohlfs, 2017). Second, a taxonomically broader number of studies on social immunity would allow us exploring the potential trade-off between social and individual immunity across group-living species (Cotter et al., 2010) and thus shed light on the dependent or independent evolutionary drivers of these two lines of anti-pathogen defences across animals. For instance, it would allow us to address questions such as whether certain types of pathogens are more likely to apply selection pressure onto individual instead of social immunity, or whether these two lines of defences necessarily trade-off across social systems (Cotter et al., 2010). Finally, non-eusocial species could offer experimental opportunities that are not available in eusocial species and thus allow exploration of novel factors possibly underlying the expression of social immunity. For instance, a recent study in the European earwig allowed to demonstrate that the recent (but not prolonged!) social isolation of group-living adults induces a stress that specifically lowers their resistance against pathogens, whereas comparing the effects of pathogens on necessarily-newly isolated and non-isolated individuals is often used to test for the occurrence of social immunity in eusocial insects (Kohlmeier et al., 2016). Overall, adopting the group living framework thus opens new perspectives to explore and better understand the common and specific solutions developed by each type of social host to counteract infections and thus, to improve our general

understanding of the role of pathogens in the evolution of all forms of social life. Given the comparatively large amount of works on social immunity in eusocial insects, it is now time to further explore social immunity in a larger and taxonomically broader number of non-eusocial species.

CHAPTER 6

Condition-dependent trade-off between weapon size and immunity in males of the European earwig

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ABSTRACT

Investigating the expression of trade-offs between key life-history functions is central to our understanding of how these functions evolved and are maintained. However, detecting trade-offs can be challenging due to variation in resource availability, which masks trade-offs at the population level. Here, we investigated in the European earwig *Forficula auricularia* whether 1) weapon size trades off with three key immune parameters – hemocyte concentration, phenoloxidase and prophenoloxidase activity - and whether 2) expression and strength of these trade-offs depend on male body condition (body size) and/or changed after an immune challenge. Our results partially confirmed condition dependent trade-offs between weapon size and immunity in male earwigs. Specifically, we found that after an immune challenge, weapon size trades off with hemocyte concentrations in low-condition, but not in good-condition males. Contrastingly, weapon size was independent of pre-challenge hemocyte concentration. We also found no trade-off between weapon size and phenoloxidase activity, independent of body condition and immune challenge. Overall, our study reveals that trade-offs with sexual traits may weaken or disappear in good-condition individuals. Given the importance of weapon size for male reproductive success, our results highlight how low-condition individuals may employ alternative life-history investment strategies to cope with resource limitation.

INTRODUCTION

Optimal allocation of resources into physiological, morphological and behavioural traits is typically known to determine the fitness of an individual (Stearns, 1992). Investing heavily into one life-history trait can, however, reduce the resources available for other processes. This trade-off is common in nature and often affects the intricate balance between survival and reproductive prowess, such as fecundity (Clutton-Brock et al., 1982; Partridge and Farquhar, 1981; Tinkle and Ballinger, 1972) and male weaponry and ornaments. These latter traits are large, extravagant structures that are energetically costly to develop (Emlen et al., 2012; Kodric-Brown et al., 2006) but increase males' reproductive success by enhancing their fighting abilities and/or appeal to female mate choice (Andersson, 1982; Goyens et al., 2016). Males of the stag beetle *Cyclommatus metallifer*, for example, are well known to express extremely large and conspicuous weapons that improve mating success, but come at costs in terms of wing size and flight muscles (Mills et al., 2016).

In nature, the expression of a trade-off between mutually exclusive functions is ultimately governed by the overall available resources an individual can allocate to them (Desmarais and Tessier, 1999; van Noordwijk and de Jong, 1986). Any change in these resources, for instance due to environmental, physiological, and/or genetic variation, is thus expected to mask investment trade-offs or even result in an apparently positive relationship between two specific traits (Parker and Begon, 1986; Smith and Fretwell, 1974; van Noordwijk and de Jong, 1986). Many studies are in line with these predictions. For instance, high-quality females have been shown to exhibit diminished costs of reproduction compared to low-quality ones in two species of ungulates

(Hamel et al., 2009) and males with increased access to resources show a reversed fecundity-longevity trade-off apparently lowering the cost of reproduction in the seed beetle *Callosobruchus maculatus* (Messina and Fry, 2003; Tatar and Carey, 1995). Conversely, several studies report a rather puzzling absence of trade-offs between sexually-selected and non-sexual traits. For example, in the rhinoceros beetle *Trypoxylus dichotomus*, large horns seem to impose no costs on overall growth, mobility, or immunity (McCullough and Emlen, 2013), and in the horned beetle *Euoniticellus intermedius*, where parts of the immune response were shown to positively correlate with male horn length with no sign of a trade-off (Pomfret and Knell, 2006).

The reversal or removal of trade-offs has been suggested for male sexual traits as part of the handicap principle (Zahavi, 1975), which aims to explain how male sexual traits evolved and more importantly, why they - in theory - must be honest, i.e. reflect the condition of the bearer. In a refined model based on the handicap principle, Grafen proposed in 1990 (Grafen, 1990) that marginal costs of advertising sexual traits are higher for individuals in poor condition. This suggests that trade-offs involving sexual traits should be less taxing on other life-history traits in high compared to low-condition males and thus result in an overall positive growth allometry in high condition males only (relative scaling of body parts (Huxley and Tesissier, 1936)). This condition-dependent effect, however, has received mixed support across species (Bonduriansky, 2007), and studies investigating condition-dependence in sexual trait expression rarely include trade-offs with other, non-sexual traits (Cotton et al., 2004). Yet, condition dependency of sexually-selected weapons or ornaments in males has been established in a number of species, such as horn length in the isopod *Deto echinata* (Glazier et al., 2016), eye span in the stalk-eyed fly *Diasemopsis aethiopica* (Knell et al., 1999), or weapon size in the cactus bug *Narnia femorata*

(Miller et al., 2016). To what degree this condition dependency of sexually-selected traits affects any trade-offs with other, non-sexual traits remains largely unknown.

In this study, we investigated whether trade-offs between sexual (forceps length) and life-history (immunity) traits are condition-dependent in males of the European earwig *Forficula auricularia*. In this hemimetabolous insect with a promiscuous mating system (Sandrin et al., 2015), males are well known to wield curved forceps at the end of their abdomen (Figure 6.1). The length of male forceps - which varies at adulthood only - is positively associated with the duration and frequency of copulations, with their general fighting abilities, and with their capability to interrupt the mating attempts of contenders (Radesäter and Halldórsdóttir, 1993b; Tomkins and Simmons, 1998). Male forceps size is a heritable trait (Pike et al., 2017) that can vary dramatically within a population (Tomkins, 1999; Tomkins and Simmons, 1998). During initial development and continuing through adulthood, longer forceps are likely to be more costly than

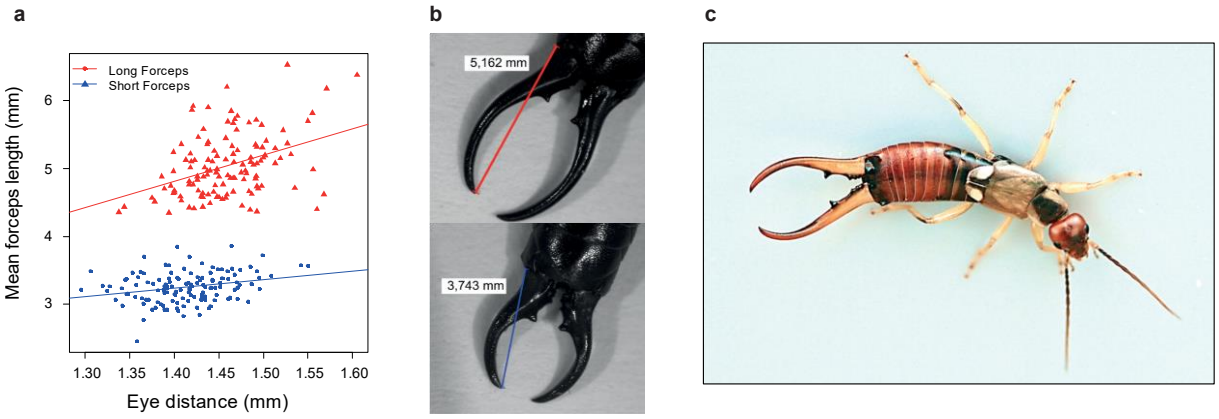

Figure 6.1. (a) Different association between eye distance (proxy for body size) and forceps length for long forceps males (P>0.001) and short forceps males (P=0.041). The difference in correlation was accounted for when calculating the relative forceps length used in the analyses. (b) Illustrative examples of measured long and short forceps. (c) Male specimen of *Forficula auricularia* with long forceps (Photo: Joël Meunier).

smaller forceps for individuals due to their encumbering size and weight (Mills et al., 2016). Here, we tested if forceps length trades off with males' investment into immunity. Immunity is a cornerstone of defence against pathogens and parasites but is also costly and therefore often expected to trade-off with life-history traits (Jacot et al., 2004; Rantala et al., 2007). Specifically, we investigated three key components of males' immune system: phenoloxidase activity (PO), prophenoloxidase activity (PPO; which is measured together with PO as total-PO), and hemocyte concentration (Gillespie et al., 1997; Lavine and Strand, 2002; Strand and Pech, 1995). PO and its inactive precursor, PPO, help individuals resist a large number of pathogens through melanisation and the induction of the release of various cytotoxins (Cerenius and Söderhäll, 2004; Gillespie et al., 2000; Strand and Pech, 1995), while hemocytes are involved in core immune functions such as phagocytosis, nodulation, and encapsulation(Lavine and Strand, 2002). Although PO and total-PO are often expected to correlate, interesting discrepancies have been reported in the literature making the dual measurement worthwhile (Busso et al., 2017). The investments into immune components were measured both before (basal) and 24h after (activated) an immune challenge, since the degree and direction of the expected trade-offs are not necessarily the same during these two physiological stages (Adamo, 2004; Vogelweith et al., 2013b). The immune challenge was done by pricking individuals with a sterile needle either dipped into a control solution (i.e. injury = low immune challenge) or into a lipopolysaccharide solution (LPS = high immune challenge), a component of gram-negative bacteria cell walls that is generally used as a non-pathogenic immune elicitor in insects (Jomori et al., 1990; Moret and Schmid-Hempel, 2000; Sritunyalucksana and Söderhall, 2000). If the condition of a male determines the presence and direction of an investment trade-off between weapon size and immunocompetence in the

direction predicted by Grafen (Grafen, 1990), we expected to detect a trade-off between forceps length and the level of basal immunity and/or immune response in the smallest (i.e. low quality) but not the largest (i.e. high quality) males.

MATERIALS AND METHODS

Insect rearing and definitions of weapon size categories

To investigate condition-dependent trade-offs between forceps length and immunity, we first selected a large number of males exhibiting strong variation in this sexually-selected trait. To this end, we field-caught 1188 males and 1296 females of *F. auricularia* in July-August 2015 from a single natural population located in Mainz, Germany (49°58'20.5"N 8°11'42.3"E). Immediately after field sampling, we distributed these individuals into 36 plastic containers (37 × 22 × 25 cm) grounded with moist sand to homogenize nutrition, habitation and access to mates for the males. Each container received 36 females and 33 males, which were allowed to freely mate for four months. After that time, the 1188 males were sorted to select the top 9% exhibiting the longest forceps and the bottom 9% with the shortest forceps. To this end, we first visually selected a large subset of long and short forceps males and then measured their mean forceps length (as the mean of left and right outer forceps) and eye distance as two proxies of body size (Thesing et al., 2015) and quality (Kramer et al., 2015; Meunier et al., 2012) (detailed selection process in supp. materials). Note that variation in earwig body size reflects both their genetic background and their previous environment (Pike et al., 2017; Wong and Kölliker, 2014). These measurements eventually provided us with a total of 112 “short forceps” males (i.e. the 9.4% of males with the shortest forceps) and 105 “long forceps” males (i.e. the 8.8% of males with the

longest forceps; Figure 6.1). After these morphology measurements, each male was isolated in a Petri dish (5.5 cm diameter) and provided with an *ad libitum* quantity of standard food (composition detailed in Thesing et al., 2015) for 24 hours, before it was used for immune measurements and/or immune challenge (see below). The Petri dishes were furnished with moist sand and maintained under standard laboratory conditions (Koch and Meunier, 2014). All morphometric measurements were done following standard protocols (Kramer et al., 2015; Sandrin et al., 2015; Weiß et al., 2014), in which sizes were taken to the nearest 0.001 mm using the Leica Application Suite 4.5 software (Leica Microsystems, Wetzlar, Germany) on pictures of CO_2 anesthetized males taken under a binocular scope (Leica, MZ 12.5).

Basal immunity and immune challenges

The association between weapon size and immunocompetence was investigated by measuring male investment into three components of their immune system both before (basal) and 24 hours after (activated) an immune challenge. The basal immunity was measured one day after each of the 217 short- and long-forceps males were isolated (note that social isolation does not influence male capacity to fend off pathogens and is thus unlikely to shape their immunocompetence Kohlmeier et al., 2016). At that time, 1 µl of hemolymph was extracted per male (median volume of extraction = 1.0 µl; min = 0.6 µl; max = 2 µl) to measure the number of circulating hemocytes as well as total-PO and PO activities (see below). Just after hemolymph extraction, a random subset of 58 of the short-forceps males and 50 of the long-forceps males (n = 108 total) were immune-challenged by pricking them with a sterile needle that was previously dipped either 1) into a suspension of LPS (diluted in a Ringer solution at 10 mg / ml; n = 29 short- and 27 long-forceps) or 2) into a control solution (100% Ringer; n = 29 short- and 23 long-forceps).

The remaining 109 non-challenged males were used in another experiment (data not shown). All these pricked males were then returned to their Petri dish, where they were given *ad libitum* access to food for 24 hours. The immune response of the 107 surviving males (one long-forceps male died) was finally determined by re-extracting 1 µl of their hemolymph (median = 1.0 µl; min = 0.4 µl; max = 1.4 µl) and measuring hemocyte concentration, as well as PO and total-PO activities (see below).

Measurement of the three immune parameters

The 1 µl of hemolymph per individual to be used for the hemocyte, PO, and total-PO measurements was diluted in 25 µl of cold sodium cacodylate/$CaCl_2$ buffer, of which 10 µl were used immediately to measure hemocyte concentration while 16 µl were frozen to later measure PO and total-PO. If the initial amount of hemolymph was less than 1 µl, we noted the actual amount using a glass capillary and a calliper. The concentration of hemocytes was then measured by visual count using a Neubauer Improved Haemocytometer and a microscope (magnification x 400). The PO and total-PO activities were spectrophotometrically measured using a standard protocol (Vogelweith et al., 2017). In brief, each frozen sample of diluted hemolymph was thawed on ice and centrifuged for 5 minutes at 4°C (4000 $\times$ g), after which 5 µl of the resulting supernatant was added to a microplate well containing 20 µl of PBS, 20 µl of L-dopa solution (Sigma D-9628; 4 mg/ml of distilled water), and either 140 µl of distilled water (PO activity) or 140 µl of chymotrypsin solution (Sigma C-7762, 0.07 mg/ml of distilled water; total-PO activity). The enzymatic reaction was allowed to proceed for 2 hours 47 minutes at 30°C in a microplate reader (Thermo scientific Multiskan™ FC Microplate Photometer). Enzyme activity was defined as the slope of the reaction curve during the linear phase of the reaction (Vmax value: change in

absorbance units/min) and measured using the R-based free program PO-CALC (Kohlmeier et al., 2015). Because the volume of extracted hemolymph and the resulting concentration of hemolymph slightly varied between individuals (see the range of extraction detailed above), we standardized the concentration of hemocytes and total-PO activity (immune parameters) per microliter of hemolymph using the following formula: I x [(Vh + Vb) / Vh] / Vm, in which I is the measured immune parameter, Vh is the volume of extracted hemolymph, Vb is the volume of buffer added, and Vm is the volume applied either to the Haemocymeter for hemocyte count (i.e. 10µl) or on the spectrophotometer plate for total-PO measurement (i.e. 5µl).

Statistical analyses

We first tested whether hemocyte concentration, PO activity, and/or total-PO activity depended on males' body size and forceps length using a series of six general linear models (function *lm* in R). Three models were computed with the immune values taken before the immune challenge, whereas three other models were conducted with the immune values taken after the immune challenge. Note that in the last three models, the type of challenge (control or LPS) was also entered as an explanatory factor, and that we controlled for the values of the considered basal immune trait by entering them as covariate. Each statistical model first included all possible interactions between the explanatory factors (i.e. body size, forceps length and, when available, the type of challenge) and was then simplified stepwise by removing the interaction terms that were not significant (all P-values > 0.08) after which we confirmed best model selection using Akaike Information Criterion (AIC). Note that some non-significant interactions are presented here to facilitate model comparisons, but their removal from the statistical models did not qualitatively change the results. In all six models, forceps length was corrected for body size

within each male category, as these two values are positively associated, but the slope of this association depends on the male size category (Figure 6.1). This correction was done by using the residuals of two general linear models (one for the long- and one for the short-forceps males), in which the forceps length was entered as a response variable and the body size as an explanatory variable. This corrected forceps length thus provided information on whether males had longer or shorter forceps than predicted by their body size within each forceps category, which is exactly the focus of the present study.

To fulfil homoscedasticity and Gaussian distribution of the residuals, all the models were computed using square root-transformed hemocyte concentration and log+0.001-transformed PO and total-PO activities. The statistical analyses were conducted using R v3.1.2 loaded with the packages *car* and *effects*. This latter package was used to plot and interpret the interactions between continuous variables, as it displays the predicted relationship between the response variable and one explanatory variable for different, fixed values of the interacting variable(s) (see Fox, 2003; Kramer and Meunier, 2016a).

RESULTS

Prior to the immune challenge, there was no trade-off between forceps length and the immune defence of males. Specifically, the baseline concentration of hemocytes and the baseline activities of PO and total-PO were independent of male body size (hemocytes: $F_{1,206}$=1.99, P=0.16; PO: $F_{1,196}$=0.41, P=0.525; total-PO: $F_{1,205}$=0.49, P=0.484), as well as of forceps length (hemocytes: $F_{1,206}$=2.69, P=0.103; PO: $F_{1,196}$=1.6, P=0.207; total-PO: $F_{1,205}$=0.01, P=0.914) and of an interaction between these two traits (all P > 0.196).

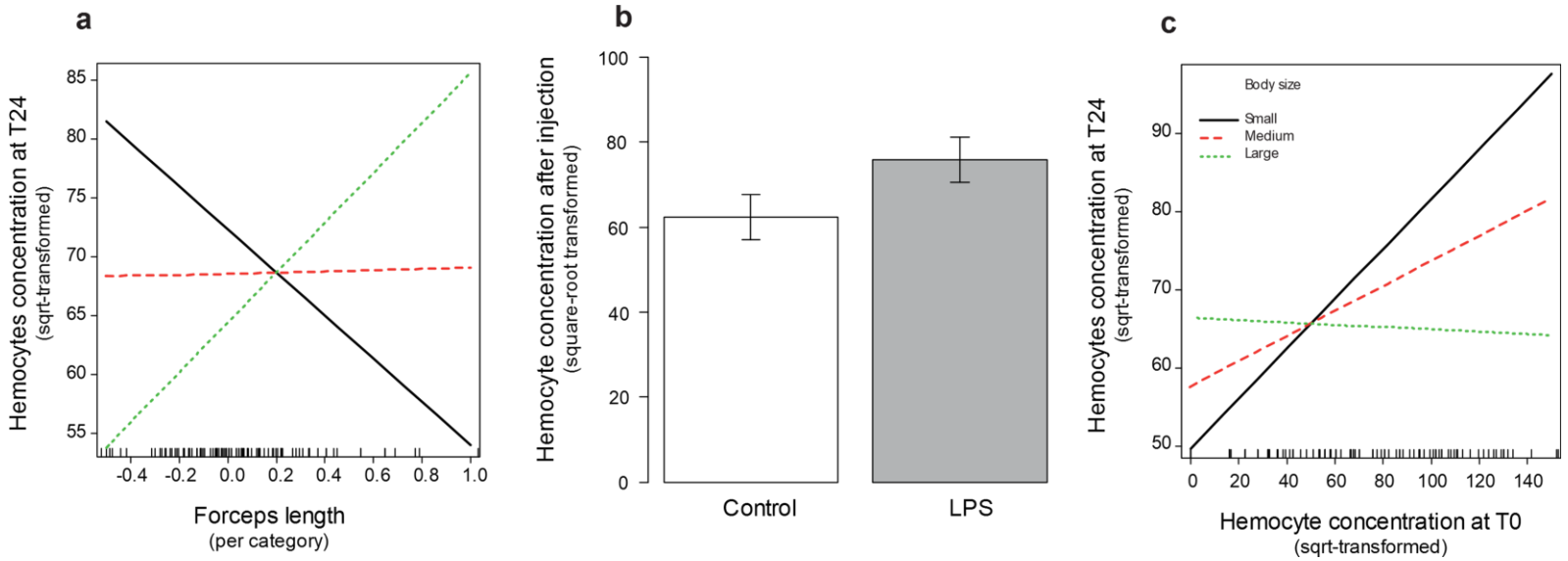

Figure 6.2. Effects of (a) the interaction between body size and the forceps length on hemocyte concentration 24h after the immune challenge, (b) the type of immune challenge, and (c) the interaction between body size and basal hemocyte concentration. In the first and third panel, the straight lines represent males with small body size (1st quartile of the distribution = 1.407), where the dashed lines represent the males with medium body size (median value = 1.437), and the dotted lines represent males with large body size (3rd quartile of the distribution = 1.47). The dashes on the abscissa represent the distribution of males.

Conversely, after the immune challenge, there was a trade-off between forceps length and hemocyte concentration, but its expression depended on males' body size (interaction between body size and forceps length, *P*=0.009; Table 6.1). Specifically, hemocyte concentration traded off with forceps length in the smaller males, whereas hemocyte concentration increased together with forceps length in the largest males (Figure 6.2a). Independent of this effect, post-challenge hemocyte concentration was overall higher in LPS-pricked than in control-pricked males (*P*=0.015, Table 6.1; Figure 6.2b) and the post-challenge and basal levels of hemocyte concentrations were positively correlated in the smaller males only (interaction between body size and basal measurements, *P*=0.004; Table 6.1; Figure 6.2c). Hemocyte concentrations were, however, not shaped by an interaction between the type of immune challenge and body size (Table 6.1). Finally, post-challenge activities of PO and total-PO were overall positively correlated to their baseline activities (all *P*<0.025; Figure 6.3; Table 6.1), but were independent of forceps

Table 6.1. Effects of basal immunity, body size, forceps length, type of pricking (control versus LPS), and their interaction on the hemocyte concentration, PO, and total-PO activities after the injections of LPS or control solutions. The basal immunity corresponds to the value of each immune parameter measured 24h before the pricking. Significant P-values are in bold. These three models first included all possible interactions between the explanatory factors and were then simplified (see Methods).

	Hemocyte number		PO activity		Total-PO activity	
	$F_{(1,98)}$	P	$F_{(1,89)}$	P	$F_{(1,92)}$	P
Basal measurement (Bm)	3.98	**0.049**	5.17	**0.025**	7.07	**0.009**
Body size (BS)	3.16	0.078	0.00	0.951	1.45	0.231
Forceps length (FL)	1.05	0.309	1.34	0.251	0.14	0.709
Type of pricking	6.16	**0.015**	0.14	0.712	0.07	0.795
Body size: Basal measurement	8.49	**0.004**	0.45	0.502	1.01	0.317
Body size: Forceps length	7.21	**0.009**	0.14	0.711	0.18	0.668

length, body size, and the type of immune challenge (all *P*>0.231; Table 6.1). Note that all measures of PO and total-PO activities were independent of hemocyte concentrations (all *P*>0.086; Table 6.2).

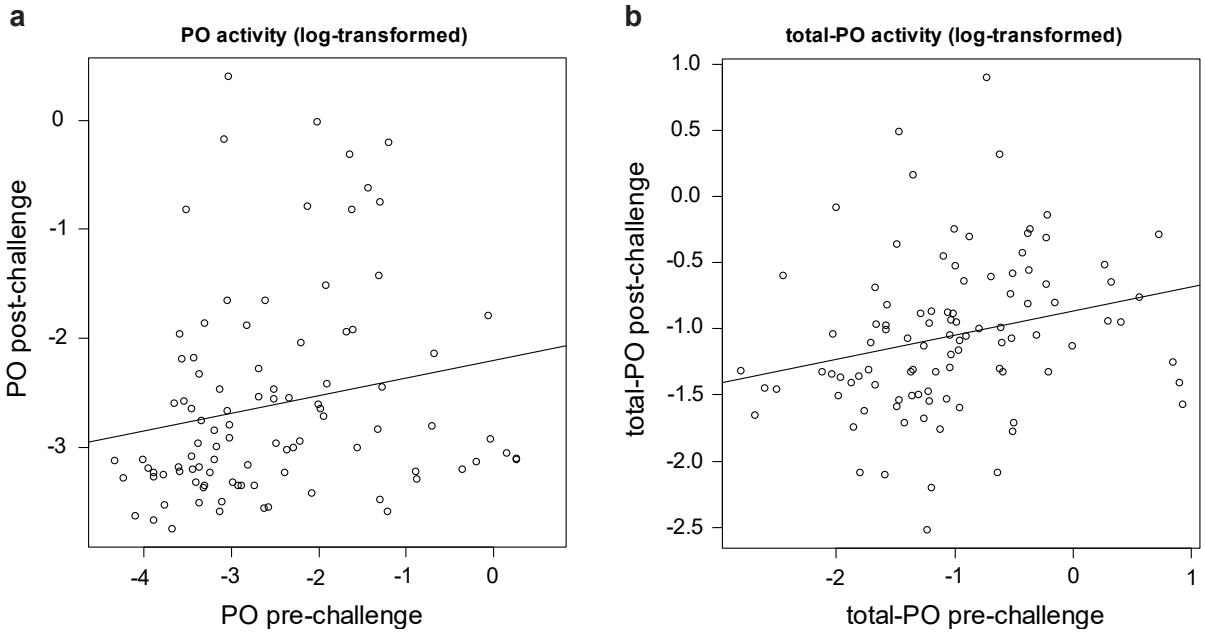

Figure 6.3. Association between the measurements of (a) PO and (b) total-PO activities before and after the immune challenge.

DISCUSSION

We investigated whether the forceps size of field-sampled earwig males traded off with their immunocompetence and whether the expression and direction of this trade-off depended on male body size. Our data partially confirm our predictions: we showed that male forceps size traded off with hemocyte concentration in small males after an immune challenge (independent of the type of immune challenge). In large males, however, post-challenge hemocyte concentration did not trade-off but instead increased with forceps size. Our results also reported

an absence of trade-off or of a positive association between forceps length and the basal concentration of hemocytes, as well as between forceps length and either the basal or the post-immune challenge levels of PO and total-PO activities.

Our results show a condition-dependent trade-off between forceps size and the post-challenge concentration of hemocytes in earwig males. This result is in line with the trade-off between sexual traits and immunocompetence reported in numerous species ranging from vertebrates (barn swallows *Hirundo rustica*; Saino et al., 1996) to invertebrates (crickets *Gryllus campestris*; Jacot et al., 2004; wolf spiders *Hygrolycosa rubrofasciata* Ahtiainen et al., 2005). Here, however, our findings reveal that even if investment into sexual ornamentation and weaponry is a costly affair, this cost is significant (in terms of immune function) only for males exhibiting an overall small body size. Knowing the general benefits of exhibiting long forceps in earwig males' mating success (Radesäter and Halldórsdóttir, 1993b; Tomkins and Simmons, 1998), these results suggest that the emergence of alternative mating strategies limiting the

Table 6.2. Spearman correlation tests among hemocyte concentration, PO, and total-PO activities measured a) before and b) after pricking. Significant P-values are in bold.

a) Measurements before immune challenge	Hemocyte concentration	PO activity	Total-PO activity
Hemocyte concentration	n = 210	rs = 0.06	rs = 0.12
PO activity	P = 0.412	n = 200	rs = 0.81
Total-PO activity	P = 0.086	**P < 0.0001**	n = 209
b) Measurements after immunechallenge	Hemocyte concentration	PO activity	Total-PO activity
Hemocyte concentration	n = 107	rs = -0.15	rs = -0.12
PO activity	P = 0.134	n = 101	rs = 0.67
Total-PO activity	P = 0.278	**P < 0.0001**	n = 101

importance of forceps length could be favoured in small males (Gross, 1996). In line with this hypothesis, male earwigs with short forceps have been shown to sneak copulations more frequently than better armed males (Tomkins and Brown, 2004). Alternatively, small males may still opt for reduced investment into immunocompetence to increase forceps growth and therefore better compete with larger conspecifics (Kirkwood and Rose, 1991; Vinogradov, 1998) when the risks of pathogen infections are low in the population, or when high levels of competition facilitate a high-risk/high-reward mating strategy. Disentangling these different evolutionary scenarios would require further investigations into the importance of pathogen pressure on forceps development in young males, as well as their expression of alternative mating strategies. Note, however, that a study recently revealed that forceps length is highly heritable in this species (Pike et al., 2017), suggesting that short forceps males might use an alternative mating strategy to reach a similar fitness as long forceps males.

The condition-dependent trade-off between forceps length and hemocyte concentration only appeared after an immune challenge, regardless of challenge type (pricking or control). This is somewhat surprising, since the basal number of circulating hemocytes is traditionally assumed to reflect an individual's ability to mount an immune response (Lavine and Strand, 2002; Lawniczak et al., 2007). Nevertheless, our results may reveal that upregulating immune capacity in response to a threat could be more cost efficient than maintaining a constantly high level of immunity. This has been previously proposed in larvae of the moth *Eupoecilia ambiguella* (Vogelweith et al., 2013b), where body size is positively associated with hemocyte concentration after, but not before an immune challenge.

Although the LPS-pricked males showed an overall higher hemocyte concentration than control-pricked males, the type of immune challenge had no effect on PO and PPO. These results confirm that the wounding itself is sufficient to trigger an immune reaction in terms of hemocyte concentration, a phenomenon reported in several other insects (Kanost et al., 2004; Korner and Schmid-Hempel, 2004; Vogelweith et al., 2013b), but insufficient to trigger an upregulation of PO/PPO. This latter absence of effect was surprising, as the concentration we used is relatively high (e.g. 20x higher than what was required to elicit an immune response in the bumblebee *Bombus terrestris*; Moret and Schmid-Hempel, 2000*,* an insect with a comparable body weight). Nevertheless, it illustrates that various immunity pathways may react differently to immune challenges (Lavine and Strand, 2002; Pauwels et al., 2011; Vogelweith et al., 2013b).

Similarly, the condition-dependent trade-off between forceps length and immunocompetence affected the hemocyte concentration, but not the PO/PPO activity. While immune parameters do not always represent pathogen resistance equally (Adamo, 2004; Lavine and Strand, 2002; Pauwels et al., 2011), past studies have reported associations of the PO/PPO enzyme cascade with pathogen response (Gillespie et al., 1997) and with individual condition (Barnes and Siva-Jothy, 2000; González-Santoyo and Córdoba-Aguilar, 2012). Interpreting the immune function of high or low levels of PO/PPO activities can, however, be difficult. This is because reaction products of PO activation include several proteases and oxygen radicals that can actually harm the host as well as the intruder, meaning that an overexpression can prove costly and even detrimental through autoreactive damage (González-Santoyo and Córdoba-Aguilar, 2012; Sadd and Siva-Jothy, 2006). As a result, having a more capable, stronger immune system may not automatically imply higher cytotoxic responses, like those induced by the PO

cascade. Showing that males of different size and/or quality exhibit an equal response in the PO/PPO enzyme cascade could thus actually reflect the better condition of the larger individuals, since they only have to pay for a relatively meek immune response in comparison to their smaller conspecifics.

While we did not detect any correlation between body or forceps size and baseline hemocyte concentration, we found that the association between pre- and post-challenge hemocyte concentration depended on the size of the males. In small males, the post-challenge concentration of hemocytes was higher when the basal concentration of hemocytes was also high (and vice versa). This means that small males fit our initial expectations of basal hemocytes indicating an individual's ability to mount an immune response (Lavine and Strand, 2002; Lawniczak et al., 2007). In large males, however, the recruitment of hemocytes after an immune challenge appears to be independent of their basal concentration of hemocytes. Overall, these results are in line with the assumption that body size is a reliable indicator of individual quality (Blanckenhorn, 2000; Koch and Meunier, 2014; Kramer et al., 2015; Meunier et al., 2012; Ratz et al., 2016; Roff, 1992) and hint at a condition-dependent change in immune investment.

Overall, our results reveal that being a short- or a long-forceps male (regardless of the traits that may covary with forceps length under natural conditions) has important implications in terms of immunity in male earwigs, mostly regarding hemocyte concentration. By using field-sampled individuals that may have experienced natural events specific to their forceps length, we also showed that trade-offs between crucial traits such as sexually-selected weapons/ornaments and immunocompetence can be condition-dependent within the same sex, population, and habitat. While condition dependency of sexually-selected traits has been

demonstrated before, how their apparently variable costs affect and trade-off with other important traits was largely unknown. This is, however, of special importance since the cost of ornaments and therefore the associated trade-offs with other life-history traits are often thought to be crucial for the evolution of honest signalling (Emlen, 2001; Rands et al., 2011; Zahavi, 1975). Finally, our data show that sexual traits like weaponry can be of higher priority than immunocompetence in poor condition individuals, indicating a high-risk/high-reward strategy, while good condition individuals can equally invest in both traits to strike an even balance between attractiveness and survival.

CHAPTER 7

Extended winters entail long-term costs for insect offspring reared in an overwintering burrow

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ABSTRACT

Winter imposes an ecological challenge to animals living in colder climates, especially if these adverse conditions coincide with reproduction and offspring rearing. To overcome this challenge, some insects burrow in the soil to protect adults, larvae, or eggs from negative effects of winter. However, whether this protection is effective against any long-term consequences of changes in winter duration is unclear. Here, we investigated the long-term effects of winter length variation on eggs of the European earwig *Forficula auricularia*. In this insect, females construct and maintain a burrow between late autumn and spring, in which they provide extensive forms of care to their eggs and then juveniles. We experimentally maintained earwig females under two winter durations of either four or six weeks and examined the resulting effects in terms of 1) hatching date, 2) developmental time of juveniles until adulthood, 3) adult mass at emergence, and 4) investment of adult offspring females in three key immune parameters: hemocyte concentration, phenoloxidase, and prophenoloxidase activities. Because earwigs' resistance against pathogens relies on their social environment, effects of winter length on immunity were tested on females exposed to different social environments: with familiar conspecifics, unfamiliar conspecifics, or in isolation. Our results reveal that after the winter treatments, eggs reared in short winters hatched earlier and the emerging juveniles reached adulthood faster than juveniles from eggs exposed to long winters. We also showed that prophenoloxidase was 30% higher in females from the long compared to short winter treatment, regardless of social environment. Finally, we found that hemocyte counts where twice as high in short compared to long winter females, but only with unfamiliar conspecifics. Overall, our study reveals that maintaining and caring for eggs in a burrow does not prevent the costs associated with increased winter duration.

INTRODUCTION

Winter can be a major challenge to all animals living in temperate and cold climates. Both the severity and duration of winter alter many life history traits, such as size and time of first reproduction (Altizer et al., 2006; Fretwell, 1972), as well as affect the mobility and metabolism of individuals from many species and taxa (Adamczewski et al., 1993; Bale, 1987; Danks, 2000; Lee Jr., 1991). Moreover, winter often reduces the availability of resources for overwintering individuals, generally resulting in food deprivation, desiccation, and increased mortality (Danks, 2000; Lee and Dellinger, 1991; Sperry and Weatherhead, 2012; Williams et al., 2015).

Ectotherms are often considered particularly sensitive to changes in temperature during winter because they cannot regulate their body temperature physiologically (Huey, 1976). While this lack of regulation often explains the severely limited geographic range of many vertebrate ectotherms (Buckley et al., 2012), invertebrate ectotherms – and insects in particular – can be found in surprisingly many cold climates (Downes, 1965; Turnock and Fields, 2005), indicating that they have evolved adaptations to endure and thrive in the cold (Lee and Dellinger, 1991). These adaptations are generally divided into physiological mechanisms that allow them to either avoid or tolerate freezing (Bale, 1987; Lee and Dellinger, 1991; Sinclair, 2015; Zachariassen, 1985), and into behavioral strategies involving seeking out protective microhabitats such as shelter below tree bark, rocks, or in constructed burrows (Baer and Schmid-Hempel, 2005; Baust, 1976; Danks, 2002; Gehrken, 1984; Sinclair, 2001).

Burrowing into the soil and remaining inactive to avoid sub-zero temperatures has been well-studied in a number of insect species (Danks, 2002), such as carabid and crysomelid beetles

(Costanzo et al., 1997; Montero and Lietti, 1998) and noctuid moth larvae (O'Brien and Kurczewski, 1982; Young and Price, 1977). While these strategies are often accompanied by pre-programmed or environmentally queued states of energy conservation involving metabolic changes such as dormancy, quiescence or diapause (Hahn and Denlinger, 2011; Koštál, 2006), some species remain surprisingly active in their burrow, such as the European earwig which continually provides maternal care (Lamb, 1976a). In these species, such an activity is expected to be costly to the fitness of an individual (e.g. due to cannibalistic consumption of eggs or increased mortality) and thus lead to important trade-offs between the benefits of protection from cold and energy conservation on one hand, and the ability to perform complex behaviors such as parental care toward overwintering eggs on the other hand (Clarke et al., 2013; Danks, 2002; Koch and Meunier, 2014; Ruf et al., 2012).

The success of ectotherm overwintering, however, is not only tied to the degree of winter cold, but also to its duration (Colinet et al., 2015; Lee and Dellinger, 1991). In species that cease feeding during overwintering, and thus determine their investment into cryoprotective and/or energy reserves at the beginning of winter, longer winters can lead to energy depletion and lower winter survival rates (Han and Bauce, 1998; Morris and Fulton, 1970). Furthermore, prolonged cold can lead to an accumulation of chilling injuries, which can further decrease survival either on their own or in interaction with energy depletion (Koštál, 2006; Sinclair, 2015). Finally, since individual metabolism and performance depend on the thermal conditions experienced during development, the duration of cold periods is expected to shape adult performance of offspring reared during winter (Le Lann et al., 2011).

The duration of winter length is often a flexible parameter, which varies not only between geographic locales, but also from year to year (Bonan, 2015). Interestingly, the accelerating global climate change reported over the last decades is a novel major cause of changes in mean temperature and season duration across the planet, which may profoundly shape the phenology and abundance of many plant and animal species (Butler and Tran, 2017; Estrella and Menzel, 2013; Parmesan, 2006; Princé and Zuckerberg, 2015; Ramseyer et al., 2009; Stålhandske et al., 2015; Vitasse et al., 2017; Williams et al., 2017).

In this study, we investigate whether winter duration has long-term effects on offspring of the European earwig, *Forficula auricularia*, a hemimetabolous cosmopolitan insect exhibiting pre- and post-hatching maternal care (Kölliker, 2007; Meunier et al., 2012). In this species, females provide pre-hatching care by constructing a burrow in late autumn or early winter to lay their eggs and overwinter with the clutch until hatching occurs in early spring (Koch and Meunier, 2014; Lamb, 1976a). Over the course of winter, females cease to feed but continuously groom their eggs to prevent the growth of pathogenic fungi, a behavior crucial to reproductive success (Boos et al., 2014; Diehl et al., 2018). This species is native to Eurasia but has reached nearly worldwide distribution as an invasive species, for example in North America and the Falkland Islands (Langston and Powell, 1975; Maczey et al., 2016). This widespread occurrence suggests that the species is able to adapt to a variety of winter conditions, but little is known about the effects of winter phenology. Additionally, seasonality is expected to affect reproduction in this species, as females can produce a second clutch in spring and the likelihood of producing a second clutch is generally higher in populations from warmer climates (Meunier et al., 2012; Ratz et al., 2016).

In our experimental setup, earwig clutches were experimentally kept under winter conditions (5°C) for either 33 or 47 days to reveal whether winter duration has any subsequent effect on important life history traits in the offspring upon reaching adulthood. The difference in cold period length represents recorded phenological shifts in the study animals' home range between 1950 and 2001 (Linderholm, 2006). Specifically, we were interested in whether winter length determines 1) the hatching date of the eggs, 2) the developmental speed of the juveniles (called nymphs) into adults, 3) the mass of the adults, and 4) the immune-competence of the resulting adult offspring. Because a recent study revealed that resistance against pathogens of earwig adults at least partly relies on their social environment (Kohlmeier et al., 2016), exploring the effects of winter length on earwig immunity required us to control for their social environment. To this end, the adult females were exposed to different social environments consisting of females being maintained in groups with either constant or changing group members, or suddenly isolated after long-term group living. Note that we chose to only investigate females because immune expression in males of this species may be affected by their tendency to engage in combat, as well as by reported trade-offs with their conspicuous weaponry (Körner et al., 2017). Female immune-competence was measured using three key components of insect immunity: phenoloxidase activity (PO), prophenoloxidase activity (PPO; which is measured together with PO as total-PO), and hemocyte concentration (Gillespie et al., 1997; Körner et al., 2017; Lavine and Strand, 2002; Strand and Pech, 1995). In insect immunity, PO mediates the melanization of foreign objects and the release of cytotoxic agents through the activation of PPO, its inactive precursor mostly stored in the hemolymph and the hemocytes

(Cerenius and Söderhäll, 2004). Hemocytes are immune cells suspended in the hemolymph that are involved in recognizing and encapsulating pathogens (Lavine and Strand, 2002).

We expected that long winters would slow down egg development, as low-temperature conditions can arrest development in insect eggs (Tatar and Yin, 2001). Phenological shifts during early life-stages may not necessarily carry over to later stages (Salis et al., 2017), so we predicted no difference in developmental time from nymph to adult, which typically takes two months but can vary slightly between populations (see Ratz et al. 2016). While insect immunity is known to vary with season and temperature and can directly trade-off with cold responses, these relationships seem largely species dependent, and how these trade-offs affect developing insects in the long-term is yet unclear (Ferguson and Sinclair, 2017; Linderman et al., 2012). Given the importance of early development for life history traits, we expected adults' immune competence to reflect any development trade-offs compensating for the phenological shift between the two winter treatments. Finally, in line with previously shown immune-sensitivity to changes in the social environment (Kohlmeier et al., 2016), we expected adults to reflect challenged immunity when living in changing group compositions compared to constant groups.

MATERIALS AND METHODS

Animal origin & winter treatments

We caught *F. auricularia* adults in July-August 2015 in Mainz, Germany (49°58'20.5"N 8°11'42.3"E). Immediately after field sampling, we distributed these field-caught individuals among 36 plastic terraria (37 × 22 × 25 cm), which were grounded with moist sand and cardboard shelters and kept at 18°-20°C dark:light. These animals were allowed to mate freely from August

15th to November 12th. After that time, each female (approx. 1500 individuals) was isolated in a Petri dish (ø 9 cm) to mimic natural dispersion and encourage egg production (Lamb 1975). The Petri dishes were setup with moist sand and maintained in constant darkness. Each female was provided with an *ad libitum* amount of food (artificial diet mainly consisting of pollen, cat food, wheat germ, and agar; see details in Kramer et al., 2015), renewed once per week. Immediately after isolation, temperatures were sequentially decreased to initiate winter conditions: 7 days at 15°C, 7 days at 10°C, 7 days at 8°C and finally, 33 days at 5°C. At this point, we haphazardly selected half (~650) of the females ("short winter") to immediately enter the sequential warm-up phase (7 days at 8°C, 7 days at 10°C, and then maintained at 15°C until egg hatching), while the other half ("long winter") spent an additional 14 days at 5°C prior to entering the warm-up phase (for an exact timetable see Figure 7.1). From the day of egg laying, each clutch was monitored daily to check for emerged offspring. Egg hatching is generally well synchronized within a clutch and all offspring typically emerge within a day (Meunier and Kölliker, 2013). The hatching date for each clutch was noted. Following egg hatching, females and their offspring were moved to a climate chamber, where they were subjected to standard summer conditions (18-20°C D:L) and provided with *ad libitum* food (conditions detailed in Kramer et al. 2015). Upon reaching adulthood, males and females were separated to prevent uncontrolled sib-mating (Meunier and Kölliker, 2013). Note that because females cease feeding during egg care (Meunier et al., 2012), the food present in each Petri dish at egg laying was removed and no fresh food was provided until egg hatching.

Experimental setup

We investigated the effects of varying winter length on offspring development and several life history traits of adult female earwigs. We first measured the hatching date and development time of juveniles into adults. To this end, we recorded the dates of egg hatching and the number of days between egg hatching and the final molt into adulthood in 177 haphazardly selected unrelated females (short winter n=90, long winter n=87). We then manipulated the social environment of the adult females to test whether winter length shaped socially induced changes in their immune competence. To this end, we haphazardly selected a subset of 123 unrelated females (short winter n=65, long winter n=58, all unrelated) two weeks after their final molt. Each of these females (hereafter called "focal females") were isolated for two weeks, then transferred to a new Petri dish with a group of three unfamiliar and unrelated females (hereafter called "non-focal females").

The two-week timespan was chosen to allow the non-focal females to acclimate to their new group environment. Just before joining their conspecifics, each focal female was weighed to the nearest 0.001 mg using a microbalance (model MYA5; PESCALE, Bisingen, Germany). Conversely, the non-focal females were haphazardly selected at the same time as the focal females, and were of the same developmental age - i.e. had been adults for about the same time as the focals (varying by 3 days max.). All non-focal females were marked by removing one of their two elytra (wing cover) tips under brief CO_2-anaesthetization, which made it easy to distinguish non-focals from the focal females (elytra tips bear a clear, white spot in this species). Within a non-focal group, we always cut the same elytra but alternated the elytra side (left or right) between each replicate.

The goal of introducing our focal females to non-focals was to obtain the four following treatments: 1) familiar group unmanipulated, 2) familiar group manipulated, 3) changing group, and 4) sudden isolation. In the "familiar group unmanipulated" treatment, focal females were left with the initial group of non-focals and underwent no manipulations whatsoever until the measurements at the end of the experiment. In the "familiar group manipulated" treatment, focal females were manipulated (removed and re-introduced) after days 7, 14, and 21 to encounter the same group of non-focal females. In the "changing group" treatment, focal females were manipulated after days 7, 14, and 21 to encounter a new, unfamiliar group of three non-focal females. Finally, in the "sudden isolation" treatment, focal females were manipulated to encounter a new, unfamiliar group at day 7 and 14, and were then maintained in isolation from day 21 onward. For treatments "group change", "familiar group manipulated" and "sudden isolation", each new group encounter was done by moving the focal female into a new Petri dish, limiting her access to the new environment under a plastic containment for 10 minutes, subsequently adding the three non-focal females to the Petri dish, and finally removing the plastic containment. Finally, twenty-four hours after the last group encounter (or isolation), we measured the investment into three key immune parameters (see below) in each of the focal females.

Immunity assays

We measured three key immunity parameters in 112 females (6 females died during the experiment, 5 yielded insufficient hemolymph amounts) after the 22 days of social treatments: the Phenoloxidase activity (PO), Phenol- and Prophenoloxidase activity (total-PO), and the hemocyte concentration. The measurements were done following a standard protocol

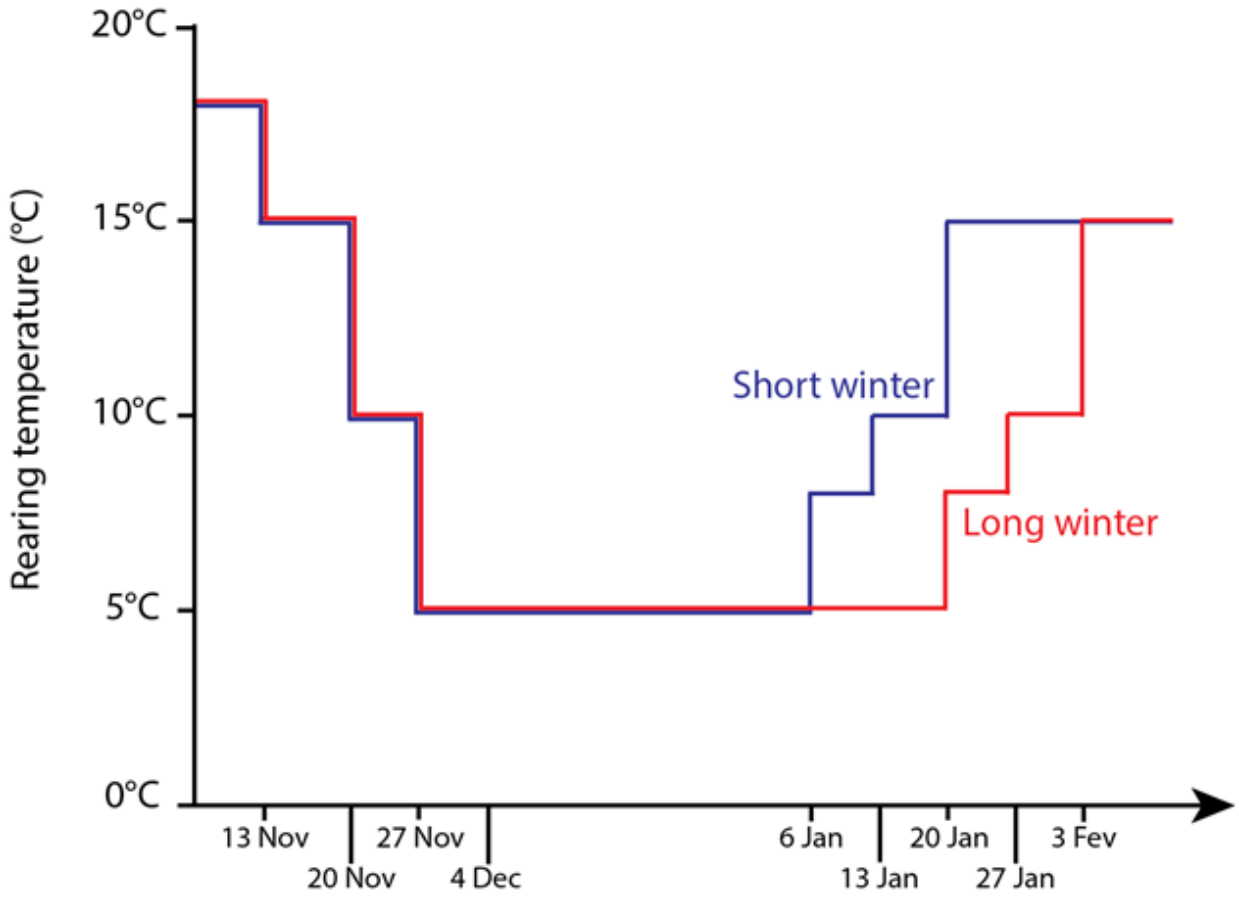

Figure 7.1. Detailed dates of temperature changes and durations of both the short winter (blue) and the long (red) winter treatments.

developed in earwigs (Vogelweith et al., 2017). In brief, we extracted hemolymph from each focal individual by piercing the soft cuticle between the 5th and 6th dorsal armor segments with a sterilized needle. We then used a chilled sterile capillary to extract 1 µl of hemolymph per individual from the wound, which was immediately diluted in 25 µl of cold sodium cacodylate/$CaCl_2$ buffer. Of this mixture, 10 µl were used right away to count the circulating haemocytes using a Neubauer Improved Haemocytometer and a microscope (magnification x 400), while the remaining 16 µl were frozen to later measure PO and total-PO. The PO and total-PO enzyme activities were spectrophotometrically measured using a standard protocol (Körner et al., 2017). Specifically, the frozen hemolymph-buffer sample was thawed on ice and

centrifuged for 5 minutes at 4°C (4000 × g) before pipetting 5 µl of the resulting supernatant to a microplate well containing 20 µl of PBS, 20 µl of L-dopa solution (Sigma D-9628; 4 mg/ml of distilled water), and either 140 µl of distilled water (PO activity) or 140 µl of chymotrypsin solution (Sigma C-7762, 0.07 mg/ml of distilled water; total-PO activity). The enzymatic reaction was then photometrically recorded for 2h 47min at 30°C in a microplate reader (Thermo scientific Multiskan™ FC Microplate Photometer). Enzymatic activity was defined as the slope of the reaction curve during the linear phase of the reaction (Vmax value: change in absorbance units/min) and measured using the free R-based program PO-CALC (Kohlmeier et al., 2015). To control for the effect of body size on immune functions, each focal female was weighed to the nearest 0.001 g using a microbalance (MYA5; PESCALE Bisingen, Germany) prior to hemolymph extraction.

Statistical analyses

We conducted all statistical analyses using the software R v3.4.3 loaded with the packages *car*, *multcomp*, and *emmeans*. We first tested whether our manipulation of winter length caused a difference in hatching date by comparing the days of egg hatching from the long and short winter treatments. Note that these days were relative to the first hatching date, i.e. values of 5 and 25 correspond to clutches that hatched 5 and 25 days after the first clutch that hatched in the entire experiment, respectively. We then employed two additional *t*-tests to compare the development time from hatching to adulthood as well as the female mass at the start of the experiment between the winter treatments.

The immune measurements were analyzed using three general linear models (GLM), in which the response variable was one of the three immune parameters (hemocyte concentration, PO activity, or total-PO activity), the two explanatory factors were the social treatments and winter treatments (short or long winter) and female mass at hemolymph extraction was entered as a covariate to control for any effects of female mass on their immunity. When significant, the interactions between treatments were further investigated using post-hoc least-squares means corrected for multiple testing (Tukey method; *emmeans* package). To fulfil homoscedasticity and Gaussian distribution of the model residuals, all models were computed using the square root-transformed hemocyte concentration and log+0.001-transformed PO and total-PO activities.

Table 7.1. Effects of winter treatment, social treatment, and their interaction on the hemocyte concentration, PO activity and total-PO activity. Note that female mass was added as a covariate to control for any effects on immunity. Significant P-values are in bold.

	Hemocyte concentration			PO activity			Total-PO activity		
	df	F	P	df	F	P	df	F	P
Female mass	1,103	0.56	0.455	1,82	3.32	0.072	1,86	0.20	0.657
Winter length	1,103	0.49	0.486	1,82	2.58	0.112	1,86	10.00	**0.002**
Social treatment	3,103	2.77	**0.046**	3,82	0.89	0.452	3,86	0.52	0.668
Winter length × Social treatment	3,103	4.48	**0.005**	3,82	1.22	0.308	3,86	1.00	0.397

RESULTS

Life history traits

Overall, manipulating winter duration shaped egg and juvenile development, as well as adult female mass. In particular, short winters were associated with both earlier egg hatching (t_{162} = 19.38, P < 0.001; Figure 7.2a) and shorter development time from egg hatching to adult emergence (t_{162}= 3.70, P < 0.001; Figure 7.2b). Additionally, short winter females were heavier than long winter females (t_{175} = -2.89, P = 0.0043; Figure 7.2c).

Immunity assays

Females from the short winter treatment showed a lower total-PO activity than females from the long winter treatments, independent of the social treatment (Table 7.1, Figure 7.3). By contrast, the effect of winter length on the number of circulating hemocytes in the females' hemolymph

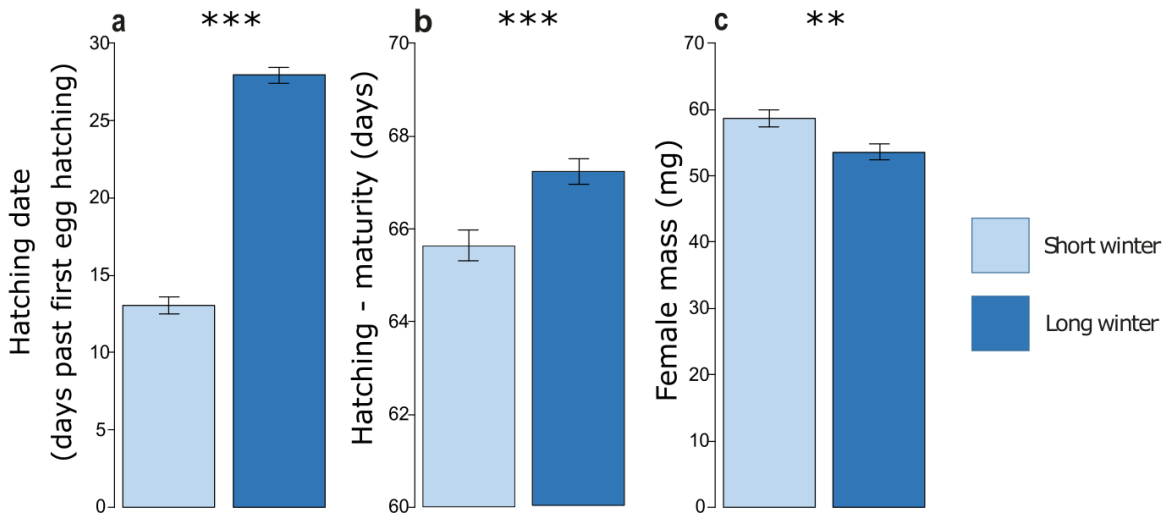

Figure7.2: Effect of winter length on a) hatching date (the number of days a clutch hatched past the overall first egg hatching regardless of treatment), b) development time from hatching to maturity and c) female mass after the winter treatments at the beginning of the social treatments. Stars indicate significance, errors bars depict the standard error (SE).

depended on the social treatment (significant interactions shown in Table 7.1). Specifically, females from the short winter treatment showed a higher hemocyte count than females from the long winter treatment only when they were confronted with a changing group composition (Figure 7.4; Tukey post-hoc test, P = 0.017; Table 7.2). Finally, there were no effects of either winter length or social treatments on the PO activity of females (Table 7.1).

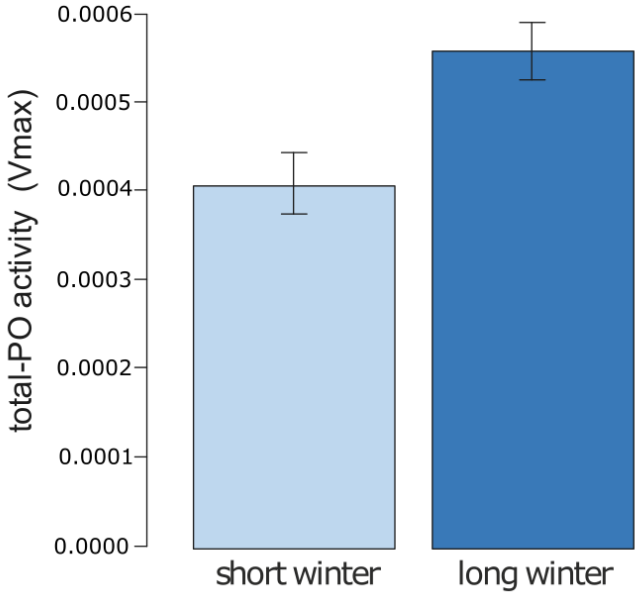

Figure 7.3: Effect of winter treatments on the total-PO activity of females. Values shown represent the reaction speed (Vmax) of the combined Phenoloxidase- and Prophenoloxidase in the individuals' hemolymph. Error bars depict the standard error (SE).

DISCUSSION

In our study, we investigated the effects of variation in winter length during early development on hatching date, development time, mass, and adult immunity in lab-reared female offspring of the European earwig. We report that longer winters cause a clear shift in both hatching date and development time: juveniles from the long winter treatment hatched later and took more time than short winter individuals from their first developmental instar to adulthood. Moreover, adult

offspring females were heavier after the short compared to long winter treatment. We also found an interaction between the winter length and social environment on adult immunity. Specifically, females reared in the short winter treatment had a higher hemocyte count than long winter females, but only when the females encountered a changing group composition. On the other hand, females from the long winter treatment exhibited an overall higher combined phenoloxidase– and prophenoloxidase activity (total-PO) than females from the short winter treatment. We detected no differences in phenoloxidase activity (PO).

In line with our predictions, female offspring in the long winter treatment hatched later than those in the short winter treatment. In this species, egg development seems to be arrested during low temperature conditions and then advances quickly once temperatures increase (M. Körner, pers. observation). This delay in development could either represent a direct

Table 7.2. Full summary depicting statistical differences in hemocyte count between all levels of both the winter treatments ("SW" = short winter; "LW" = long winter) and the social treatments using post-hoc least-squares means corrected for multiple testing (Tukey method). Significant P-Values are in bold.

	Familiar group unmanipulated (LW)	Familiar group unmanipulated (SW)	Sudden isolation (LW)	Sudden isolation (SW)	Familiar group manipulated (LW)	Familiar group manipulated (SW)	Changing group (LW)
Changing group (SW)	p=0.103	**p=0.005**	p=0.857	p=0.636	p=0.886	p=0.243	**p=0.017**
Changing group (LW)	p=0.999	p=0.999	p=0.463	p=0.632	p=0.503	p=0.944	
Familiar group manipulated (SW)	p=0.999	p=0.752	p=0.979	p=0.998	p=0.982		
Familiar group manipulated (LW)	p=0.850	p=0.269	p=1.000	p=0.999			
Sudden isolation (SW)	p=0.935	p=0.363	p=0.999				
Sudden isolation (LW)	P=0.834	p=0.226					

consequence of suboptimal thermal conditions (i.e. Marshall and Sinclair, 2015) or an adaptation to avoid unfavorable environmental conditions (Hodek and Hodková, 1988; Tauber and Tauber, 1976). By reducing or arresting developmental speed, the individual eggs may retain more energy not just for survival but also for the crucial post-hatching growth phase. Conversely, early emergence in low temperatures would likely result in high mortality and stunted development (Colinet et al., 2015). In many insect species overwintering in a dormant or quiescent state, such periods of energy conservation and diapause are often regulated by photoperiods (Koštál, 2006; Schebeck et al., 2017). In our study, the developing earwig eggs were maintained in constant darkness, reflecting their subterranean microhabitats in nature (Lamb, 1976a). Thus, it seems that mainly temperature is responsible for triggering the arrest and continuation of development in overwintering eggs of the European earwig, suggesting that global warming could play a major role in the biology of this species (and its reproductive cycles) over the next decades.

Our data show that individuals reared during a longer winter not only hatched later, but also needed longer to reach adulthood. Such a long-term effect of temperature change and/or phenological shifts on offspring development is not ubiquitous among insects overwintering during developmental stages. For example, studies on tettigoniid species report an adaptive increase in developmental speed following longer cold periods (Ingrisch, 1985) and a recent study in the winter moth *Operophtera brumata* reports that shifts in early development can be at least partially compensated in subsequent development stages (Salis et al., 2017). Interestingly, such adaptations are typically associated with important fitness costs. For instance, an accelerated development due to shorter and/or milder winters can reduce adult body mass in the glassworm *Chaoborus crystallinus* (Büns and Ratte., 1991) and to reduce adult body size and fecundity in the

Rocky Mountain butterfly *Parnassius smintheus* (Matter et al., 2011). Contrary to these findings, our results indicate an overall positive effect of shorter winter periods, as a shorter juvenile phase likely results in decreased predation, parasite pressure as well as overall mortality (Williams, 1999). This calls for further empirical studies exploring the nature and importance of these apparent benefits on long-term reproductive success in earwigs.

We found that short winter females exhibited higher hemocyte counts than females from the long winter treatment, but only in social environments with changing group members. Given the potential threat of infection through new group members, the increased hemocyte count likely reflects an increased ability to react to immune challenges (Lavine and Strand, 2002; Poyet et al., 2013; Vogelweith et al., 2013b). Higher investment into immune traits is often linked to favorable conditions during development (Vogelweith et al., 2017), and hemocyte performance has in turn been positively associated with insect overwinter survival (Krams et al., 2011) further indicating a positive long-term effect of shorter winters. Interestingly, such positive effects appear to be masked under any other social treatment: neither constant group members nor the sudden loss of the social environment affected the circulating hemocyte count. The disruption of social bonds therefore appears to have a primary role in females' investment into hemocyte number, which emphasizes the importance of (disrupted) social environments in individual defenses against pathogens in social insects (Kohlmeier et al., 2016).

Long winter females showed an overall higher total-PO activity, independent of social treatment. The expression of PO/total-PO is notoriously difficult to interpret, as high levels of expression are not only harmful to infectious agents, but also to the host itself (González-Santoyo and Córdoba-Aguilar, 2012; Sadd and Siva-Jothy, 2006). In our laboratory setup, we can safely

exclude the possibility of additional pathogen pressure in the long winter treatment. Our results therefore suggest that the overall higher expression of total-PO reflects the higher capability of short winter compared to long winter females to deal with the associated costs. More generally, the apparent discrepancy between our results on hemocyte concentration, total-PO, and PO

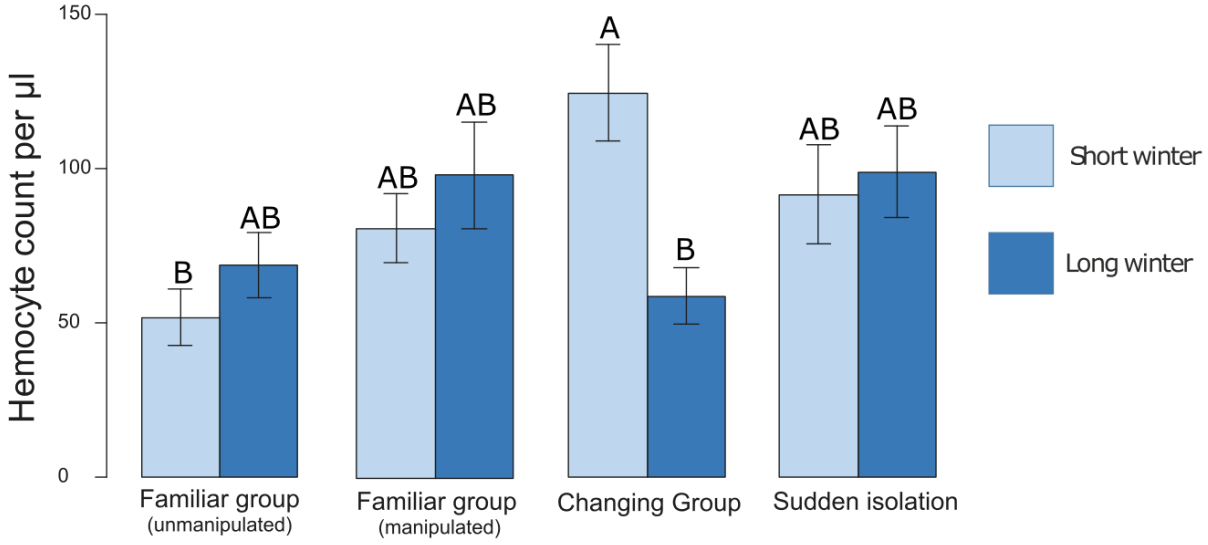

Figure 7.4: Showing the effect of an interaction between winter length and social treatment on female hemocyte count. Different letters indicate significance. Errors bars depict the standard error (SE). For all pairwise comparisons see Table S1.

activities emphasizes that variation in winter duration entails an important stress on female offspring, which later shapes their investment into some components of their immune system (Adamo, 2012) that do not necessarily correlate (i.e. Körner et al., 2017). Note that even if the three measures of potential immunity we present do not always reflect an individuals' ability to fight off an infection (González-Santoyo and Córdoba-Aguilar, 2012; Laughton et al., 2017), they nevertheless demonstrate that variation in winter length during egg development entails long-term physiological consequences in the resulting adults.

While this study does not specifically identify which mechanisms underlie the reported effects of winter duration in earwigs, our results suggest either a) a disruption of key processes during embryonal gestation in the eggs or b) an adaptation to natural phenological variations with life-long consequences for life history traits. In support of the first hypothesis, the conditions of winter dormancy have been identified as critical for both adult and developing individuals in several species, including burrowing ones (Costanzo et al., 1997; Han and Bauce, 1998; Matter et al., 2011). However, given the cosmopolitan distribution of the European earwig and its population-dependent ability to produce a second clutch during spring (Meunier et al., 2012; Ratz et al., 2016), it is also possible that mid-development adaptations to changed climatic conditions could be triggered by either the guarding mother or processes in the eggs themselves. Future studies comparing the egg development on a biochemical and transcriptional level in eggs from different climate zones and populations may help to disentangle these two hypotheses.

CONCLUSION

Overall, we demonstrate that rearing offspring under variable cold periods during winter can entail long-term consequences not only in terms of development and growth, but also in terms of immune responses. In particular, our results indicate that prolonged overwintering during early development results in offspring showing slower development and reduced socially-induced immune capacity, but higher overall levels of costly constitutive cytotoxic immune activity. While this may indicate a generally lower offspring quality caused by a prolonged egg phase, whether these effects represent hampered development or are in fact an adaptation to harsher conditions remains unknown. More generally, our study underlines the importance of investigating the effects of climate variations on the gestation and development of overwintering insect species in a time of climate change.

PERSPECTIVES

The role of pathogen pressure in shaping social systems

Maximilian Körner

"Every individual matters. Every individual has a role to play.
Every individual makes a difference." – Jane Goodall, 1999

Pathogens represent a powerful selective force on all animal life, shaping evolution of their behavioral, sexual and life history traits (Schmid-Hempel, 2003; Wilson and Cotter, 2008). To combat infection, animals employ varied and versatile behavioral and physiological immune mechanisms (de Roode and Lefèvre, 2012; Lu and St. Leger, 2016; Siva-Jothy et al., 2005), which often require considerable energy investment (S. C. Cotter et al., 2004; Lochmiller and Deerenberg, 2000). The costs of investing into an immune defense are often proportional to current infection risk (e.g. Lindström et al., 2004) and are thus expected to constrain the evolution of any strategy or behavior that entails increased pathogen pressure – an effect that has been of particular interest to investigative efforts into the evolution of sociality. Social life is thought to greatly facilitate pathogen spread through close proximity (and often relatedness) to - and interactions with – multiple cohabiting conspecifics (Altizer and Nunn, 2006; Hamilton, 1987; Shykoff and Schmid-Hempel, 1991; Wilson and Cotter, 2008). Since sociality can be found across nearly all animal taxa (J. Costa, 2006; Wilson, 1971), many studies have strived to elucidate how individuals in groups overcome inherent infection risks while still receiving benefits from the social environment, a feat considered paramount in the evolution and maintenance of sociality (Cremer et al., 2007; Hamilton, 1987; Masri and Cremer, 2014; Meunier, 2015; Schmid-Hempel, 1998).

Literature of the last two decades highlights two major mechanisms at the heart of pathogen resistance in groups. First, individuals in groups compensate inherent infection risks by increasing investment into their personal immunity, a crucial life history trait of all animals (i.e. density dependent prophylaxis; Wilson and Cotter, 2008; Wilson and Reeson, 1998). However, notable exceptions have been reported in species exhibiting extremely high densities in their

nests (i.e. eusocial species; Pie et al., 2005). This discrepancy is due to the phenomenon of social immunity, the second mechanism of pathogen defense in groups. While initially defined as a phenomenon in eusocial systems that provides colony-level pathogen protection (Cremer et al., 2007; Cremer and Sixt, 2009) social immunity has since been found in a growing number of species, including several subsocial ones (Duarte et al., 2015; Reavey et al., 2014; Rosengaus et al., 2013, Chapter 1), which has inspired an overall shift in perspective regarding the occurrence and importance of collective immune defenses in non-eusocial species, and the early evolution of sociality (Chapter 5).

In this thesis, we investigated pathogen defense in a primitive social system, the European earwig *Forficula auricularia*, with two primary goals in mind. The first objective was to reveal whether and how social immunity occurs in this species, and how it impacts the expression of personal immunity. Because this endeavor relies considerably on the ability to correctly assess the investment into and the effectiveness of personal immunity, the second objective was to better understand how personal immunity is affected by physical constraints, such as environmental conditions and investment into sexually selected traits. We aimed to provide results that help us understand the role of collective immunity during family life, an important model to investigate the early evolution of sociality (Kramer and Meunier, 2018; Royle et al., 2012), to what degree social immunity depends on and derives of parental care in this setting, and how it interplays with individual immunity to fend off pathogens and enable group living as a feasible strategy.

Intriguingly, our investigations into the occurrence of social immunity were in part informed by the apparent and surprising lack of sanitary behaviors reported in the European

earwig (Falk et al., 2014). The behavior of socially induced defecation resembles a form of social immunity reported in sub- and eusocial species, namely the application of antimicrobial exudates on the surrounding nesting area or habitat (Rosengaus et al., 2013, 1998). In this thesis, we showed that this is indeed also present in the European earwig *F. auricularia* (Chapter 1). Specifically, we showed that feces of both the mother and nymphs inhibited the growth of two fungi and two GRAM+ bacteria. We also found that antifungal activity was higher in offspring feces compared to maternal feces, and that interactions between mother and offspring only affected the inhibitory effects of maternal feces against one GRAM+ bacteria. Interestingly, we also showed that feces produced by the nymphs (but not maternal feces) aid sibling survival under starvation (Chapter 2), indicating that pathogen defense may not be the sole or original driver of the selection for this feces sharing behavior.

The social immunity mechanism we found is exhibited during family life, the benefits of which are typically expected to be mediated by parental care (Alonso-Alvarez and Velando, 2012; Kölliker, 2007; Lin and Michener, 1972). However, based on our measurements of offspring gene expression during early family life, we demonstrated that this is not necessarily the case for social immunity in the European earwig. In particular, we first showed that most changes in offspring gene expression occurred when varying the presence of the mother in a pathogen-free environment, and that these changes may revolve around shifts in the expression of metabolic genes (Chapter 3). Furthermore, we found that completely different (and fewer) genes were affected by varying maternal presence under pathogen exposure. Finally, transcriptomic differences between the presence and absence of the pathogen involved the upregulation of immune genes when the pathogen was present, but only in absence of the mother. These

findings indicate short-term adjustment in the nymphs driven by interactive effects of maternal and pathogen presence, but do not inform us about long-term consequences. Thus, our next investigation looked into nymph development and immune investment across their entire lifespan, and whether these traits are affected by maternal and/or pathogen presence during family life. Somewhat surprisingly, we found no long-term effect of maternal presence on offspring immunity (Chapter 4). However, pathogen presence during family life resulted in higher hemocyte count - a key part of the insect immune system (Lavine and Strand, 2002) - in adults, even though there was no effect during development. These findings overall show that regardless of the infection risk during family life, offspring immune investment is unaffected by maternal presence once family life has ended.

Since personal immune investments are not only shaped by the social environment (Altizer et al., 2006; Piesk et al., 2013; Zuk and Stoehr, 2002), we conducted experiments aimed at exploring the existence and importance of trade-offs between key life history traits and adaptations, such as sexually selected traits and the impact of variation in climatic conditions, on personal immunity measurements. We found that individual elements of earwig immune responses are affected differently by such physical constraints. In particular, we found that hemocytes, but not phenoloxidase, trade-off with the sexually selected forceps size in earwig males. Interestingly, this trade-off depends on the physical condition of the earwigs and is only revealed in certain components of the immune system, and only after an immune challenge (Chapter 6). Similarly, when varying climatic conditions during development, our data shows that shorter winter length appears to increase adult female hemocyte count, but only if they were kept with unfamiliar conspecifics. In addition, longer winters were linked to delayed hatching and

adulthood as well as higher prophenoloxidase values, regardless of social environment (Chapter 7).

In the final chapter of this thesis, I aim to explore the implications of my results in light of past endeavors investigating the role of social and individual immunity in the consolidation and maintenance of social life. By combining the state of the art of the literature with reasoning derived from the data presented, I hope to create a synthesis that may serve to inform future studies into social immunity in particular and social evolution in general.

PATHWAYS TO SOCIAL IMMUNITY

By examining communal pathogen defense in a species that exhibits a primitive form of group living, my data further emphasize that social immunity is not limited to advanced eusocial systems (Chapter 1). Along with other studies (e.g. Duarte et al., 2015; Palmer et al., 2016; Reavey et al., 2014), this work is part of a general shift in the perception of social immunity and in its role in the early evolution of sociality. Initially, social immunity was defined as a derived phenomenon occurring in eusocial insect societies, where it has been established as a key factor in maintaining increasingly complex social systems facing equally increasing risks of infection (Cremer et al., 2017, 2007; Masri and Cremer, 2014). However, communal immune defenses have since been shown to occur in a number of non-eusocial species, including subsocial systems, indicating that social immunity may represent an ancestral trait directly involved in the initial emergence and maintenance of group living (Chapter 5). The traditional importance conferred to infection risk in the context of sociality thus begs the question at what point social immunity mechanisms defenses emerged during social evolution.

The studies presented in the first part of this thesis show that social immunity also occurs during family life of the European earwig, a model which strongly resembles ancestral social systems (Kramer and Meunier, 2018). Intriguingly, this form of communal pathogen defense is not exclusively driven by parental care, which has long been considered a central pillar of social evolution (Royle et al., 2012; Tallamy, 1984), but is expressed – in sum to an even greater degree – by the offspring (Chapter 1). Unlike past studies in the burying beetle *Nicrophorus vespilloides,* which found larval contributions to social immune defense to diminish in parental absence (Reavey et al., 2014), our findings reveal that offspring-mediated social immunity can occur independently of interactions with caring parents (Chapter 1). Furthermore, even though earwig offspring appear to adapt their metabolism and immune gene expression to maternal presence during early family life (Chapter 3), long-term immune investment is unaffected (Chapter 4), further indicating that the net benefits of family interactions in terms of pathogen defense are not mediated by parental care.

In light of these findings, we cannot discount the possibility that the traits contriving social immunity in this species may have been selected for prior to the rise of parental care, preceding the consolidation of sociality. To explore this line of reasoning, I present two main scenarios that represent different points of origin for social immunity traits during the early evolution of sociality. These scenarios mainly differ in how dealing with the costs of disease and parasitism can either constrain or drive social evolution (Hock and Fefferman, 2012).

(1) Social life preceding social immunity mechanisms

In the first and arguably more traditional scenario, the inherently high risk of infection for grouping individuals that may constrain the evolution of sociality is only compensated for by increased personal immune defense and self-serving behavioral immunity during the initial emergence of sociality (Boomsma et al., 2005; Schmid-Hempel, 1998; Wilson and Reeson, 1998). Once the consolidation of sociality has been allowed by allocating resources into personal immunity, an increasing group size, group duration, or complexity of social organization can then give rise to behavioral and/or physiological mechanisms selected for because of their social immunity capacities (Cremer et al., 2007; Meunier, 2015, Chapter 5). In line with this scenario, social immunity is often thought more likely to occur with increasing group size and social complexity (Turnbull et al., 2011; Wilson and Holldobler, 2005), as has been demonstrated when comparing bee species of different levels of sociality (Stow et al., 2007). Complex nests and social structures, staples of increasing levels of social organization, are likely prerequisites of specialized social immunity mechanisms such as modified social interaction including self-exclusion from the group (Heinze and Walter, 2010), reduction of social interactions by infected workers (Bos et al., 2012), specific compartmentalization of the nest (Naug and Camazine, 2002; Pie et al., 2004), and avoiding garbage disposal workers (Hart and Ratnieks, 2001). While we cannot exclude the possibility of such behaviors originating in a solitary context, they seem less likely to be selected for or stem from any selfish behavior in solitary species, rather than evolve in a complex social environment. Overall, this scenario may best fit the evolution of derived/extreme forms of social immunity that occur in more derived and complex social systems (not implying exclusivity), rather than forms of social immunity that occur in less derived groups.

(2) Social life facilitated by pre-adapted social immunity mechanisms

In the second scenario, the omnipresent risk of infection does not constrain but facilitate the formation of groups. This occurs because of behaviors and mechanisms expressed by solitary individuals that were originally selected for selfish reasons, but also reduce pathogens in the immediate surroundings, potentially benefiting conspecifics (Cremer et al., 2007). For example, behavioral immunity such as the sanitary removal of feces (Tanaka and Kasuya, 2011) or the mechanical removal of pathogens (Leung et al., 2001) in solitary species may greatly benefit conspecifics and thus favor aggregative behavior. Such "pre-adapted" mechanisms may also have been selected for reasons unrelated to infection risk, such as the removal of feces to avoid predation or as a modified burrowing behavior (Vet and Dicke, 1992; West and Alexander, 1963). This reduced pathogen prevalence around an individual expressing such traits directly help to avoid infection and allow individuals to reduce investment into their personal immunity (reducing or removing the need for density dependent prophylaxis; Pie et al., 2005) and thus give rise to positive selection of gregarious behaviors as a direct result of pathogen pressure, rather than despite it.

Perhaps to an even greater degree, pathogen pressure may facilitate group consolidation in species that live solitarily but temporarily aggregate (e.g. for mating; Aluja, 1994; Emlen, 1976) or prepare a nest area to shelter eggs (Chapter 7, Danks, 2002; Lamb, 1976). For instance, offspring hatched in a nest location picked for low pathogen prevalence may be much less likely to disperse – an important prerequisite for sociality. Similarly, pre-hatching care behaviors beyond nest construction, such as grooming of eggs to ward off fungal spores (Boos et al., 2014), likely represent an ideal starting point for the evolution of continued, post-hatching parental

care. Generally, this second scenario may explain the evolution of some social immunity mechanisms occurring in non-derived, subsocial systems. It highlights that such "pre-adapted" mechanisms preceding sociality may pave the way for group consolidation. Perhaps it can also help to shift the spotlight towards social immunity mechanisms as key mediators of the initial formation of groups and their consolidation as subsocial systems.

Understanding the evolutionary past of social immunity

To understand which of the proposed scenarios is in line with any given social immunity mechanism is to learn when during the species' evolution the trait initially emerged, which in turn may yield key insights into the selective pressures leading to its evolution. In order to look into the evolutionary past of any trait in any species, we must identify the genetic base for that trait through gene expression analysis. Once a gene or a group of genes has been associated with a social immunity trait, its (their) age can be determined in several ways. Perhaps the most straightforward method is to look into sequence divergence from closely related species that live solitarily or do not express the social immunity trait in question (Capra et al., 2013). In conjunction with knowledge about species phylogeny, limited conclusions can be drawn as to when a trait may have emerged in a species. A more accurate result may be achieved by determining the mutation rate of the gene(s) in question by analyzing the ratio of substitution rates (dN/dS), which compares the rate of mutation of gene A with a conserved gene B. This process has been established as state-of-the-art to determine gene age within species or investigate convergent evolution (e.g. Finck et al., 2016; Romiguier et al., 2017).

There are some notable difficulties to consider, however. Some social immunity traits of interest may not solely serve pathogen defense, rendering the experimental identification of their genetic basis difficult if secondary purposes cannot be reliably excluded. The work presented here may represent such an issue since the feces-mediated antimicrobial defense of the European earwig also serves to increase starvation resistance among siblings (Chapter 1, Chapter 2). Perhaps more importantly, however, the trait of interest may not even derive from gene expression patterns of the species in question, but could be based on microbes of mutualistic nature, i.e. microbiota. Indeed, bacterial proteins and/or competitive interactions are a likely explanation for the mechanisms we describe in Chapter 1 and may be of tremendous importance not only to social immunity in earwigs, but to the evolution of sociality overall. Genetic, transcriptomic and mutation rate analyses must thus be designed with diverse and impactful microbiota in mind.

Beyond parental care: mutualists as drivers of group consolidation

One of the central aims of this work is to elucidate if and how social immunity occurs in a system with facultative family life and parental care, and how individual and communal defenses interact to defeat the constant threat of microbial antagonists. Parental care is a key element of social evolution, and likely represents an important factor in the evolution and expression of collective immune defenses in subsocial species. In the burying beetle *Nicrophorus vespilloides,* both larvae and parents exude antimicrobial compounds (Arce, 2013; Rozen et al., 2008) but the degree of parental efforts to ward off pathogens appears to decide how much the offspring are willing or able to contribute (Reavey et al., 2014). In the subsocial short-tailed cricket *Anurogryllus muticus*, sanitary behaviors during family life appear to be exclusively mediated by the caring female (West

and Alexander, 1963). Above, I highlighted how grooming of eggs by caring parents may translate to parental care in the form of allo-grooming of offspring, a possible social immunity behavior (Chapter 5) that can be readily observed in the European earwig (Mas and Kölliker, 2010). I also demonstrated how the presence of the earwig mothers can shape the expression of immunity genes in her offspring (Chapter 3).

However, social immunity in subsocial species is not necessarily tied to parental care, and there likely exist group defenses important to initial group formation that are not mediated by or originating from caring parents. For example, antimicrobial linings in nests of the wood cockroach *Cryptocercus punctulatus* are produced by both parents and older offspring (Rosengaus et al., 1998). Furthermore, data I present in this work provide evidence for social immunity occurring independently of parental influence (Chapter 1). Intriguingly, these fecal pathogen defenses may share the same underlying mechanism, namely mutualistic gut bacteria, indicating that indiscriminate defense against microbes may not at all be in the best interest of potential hosts (Dillon and Dillon, 2004; Engel and Moran, 2013).

Despite the early discovery of bacterial abundance and its apparent heritability in insects (e.g. *Wolbachia* in 1924; Hertig and Wolbach, 1924), the extent of the evolutionary interplay between host and microbe has long been underappreciated. For example, gut microbiota and similar endosymbionts suspected to be maintained to aid in digestion (e.g. breaking down cellulose in termite guts) are now known to also have extensive impacts on their host's fitness by means of affecting behavior and reproduction (Rolff and Reynolds, 2009; Rosengaus et al., 2011). In addition, endosymbionts often help to protect their hosts from pathogens, either passively by occupying a niche potential pathogens may settle (Dillon and Dillon, 2004), or actively by

mediating host defenses or mounting measures of their own (Daw and Falkiner, 1996; Kitano and Oda, 2006; Ryu et al., 2008). As a result, recent years have seen more and more studies highlighting the potentially extreme importance of endosymbiotic and mutualistic microbes as a key benefit and major promotors of the evolution of group living (Biedermann and Rohlfs, 2017; Lombardo, 2008; Nalepa, 2015).

For instance, trophic interactions are thought to play a pivotal role in the evolution of aggregative behaviors in the German cockroach *Blattella germanica* (Kopanic et al., 2001). Gut microbes can also alter pheromones implicated in nestmate recognition in eusocial species (reviewed in Lizé et al., 2013), and kin recognition affecting aggressive behavior in the pre-social wasp *Goniozus legneri* (Lizé et al., 2012), emphasizing the potential role of host-microbe interactions in laying crucial groundwork for the emergence of sociality. Further down the line, consolidation of sociality through parental retention of offspring and limited offspring dispersal may be driven by the exchange of symbionts critical to digestion via coprophagy and trophallaxis (Nalepa et al., 2001), provided these symbionts cannot be obtained from the environment. In line with this, mutualistic host-microbe relationships requiring not a single, but repeated inoculations would greatly increase selective pressure towards permanent social structures (Lombardo, 2008).

In Chapter 1, I show evidence for a broad-spectrum pathogen defense exhibited through the feces of adult and juvenile European earwigs. This defense may very likely be mediated by mutualistic gut bacteria, and these bacteria could thus represent a central pillar of maintained subsociality in this species. Assuming that these endosymbionts are transferred vertically from mother to egg (which is not unlikely given the facultative nature of family life in this species), a

symbiosis of this kind may precede social behaviors such as parental care, family life and social aggregation. It may thus represent a "pre-adapted" collective immunity trait that may have greatly facilitated the rise of group living in this species. Note again that the benefits of such a host-microbe interaction may not have originated as pathogen defense, but through the enhanced value of allo-coprophagy we observed in Chapter 2.

We have only just begun to shed light on the many interactions of microbiota with their hosts, yet their role in social immunity specifically and social evolution in general cannot be overstated. Going forward, I believe it to be crucial to consider and control for microbial effects on their host's immunity and behavior to the greatest possible degree in order to discover how deep the link between micro- and macroorganisms can go. Furthermore, it may be of great interest to gauge the evolutionary age of endosymbiotic relationships by using phylogenies and suitable molecular clocks to properly assess the evolutionary impact of any given strain of symbiotic microbes (Kuo and Ochman, 2009). To this end, we must first get a grasp on the diversity of microbial mutualists of the host in question and learn the nature and mechanisms of their interactions.

Identification of mutualistic microbiota has been reliably performed via 16S rRNA sequencing in a number of species (e.g. Dillon et al., 2010; Jakubowska et al., 2013; Rosengaus et al., 2011) and can be considered an obligatory first step in unveiling host-microbe interactions. From there, the relationship between microbiota and host sociality can be elucidated by 1) investigating horizontal microbiota spread through group interactions, and 2) identifying microbial manipulation of host behavior or physiology (i.e. pheromones, cuticular hydrocarbons) by e.g. artificially depriving hosts of their mutualists, and finally 3) assessing to what degree

mutualist compete with or help defend against pathogenic microbes, and vice versa. The microbial species that such an analysis reveals as potentially (or definitely) relevant to social evolution may not be constrained to a single host species, so particularly impactful strains should be investigated in other species as well. It is important to keep in mind, however, that microbial mutualists may be pathogenic to their symbiotic host depending on host size, fitness, or behavior (Nishiguchi et al., 2008) or to other hosts entirely (Webb et al., 2006). Additionally, commensal bacteria can profoundly shape their hosts' immune system (Feldhaar and Gross, 2008; Ivanov and Honda, 2012). In order to properly understand the host-mutualist interactions and their impact on social behaviors and pathogen defenses, it is of key importance to get reliable and comprehensive insight into individual immunity.

Personal immunity: foundation of a social world?

Social immunity has been shown to be a major factor in the maintenance of sociality, and may be no less important in its initial emergence (Cremer et al., 2007; Meunier, 2015, Chapter 5). While communal immune defenses can provide highly effective prophylactic and curative measures against pathogens, personal immunity remains a crucial and ultimate line of defense for individuals in groups or otherwise. Investment into personal immune defense is determined by a variety of factors, including pathogen prevalence (which in turn may be shaped by collective defenses; Chapter 1, Chapter 4), parental care (Chapter 3), age (Chapter 4), investment into sexually selected traits (Chapter 6), climate conditions, and social environment (Chapter 7). As mentioned above, personal immunity is also closely tied to microbiota, featuring intricate interactions that can be costly (Krams et al., 2017), reciprocal (Hooper et al., 2012; Maynard et al., 2012) or downright pathogenic (Nishiguchi et al., 2008).

Exploring the possible origins of social immunity traits, we have postulated two scenarios that describe two distinct ways, in which pathogen pressure may inhibit or facilitate the emergence of sociality. Yet in both of these scenarios, personal immunity and the associated costs play a pivotal role by either 1) overcoming the inherent infection risks associated with sociality (costly) or 2) offering respite from pathogen pressure due to pre-adapted traits that reduce pathogen prevalence and thus the required investment into individual immunity (beneficial). Investigations into the origins of social life and the surrounding mechanisms are therefore infeasible without taking into account the associated costs and benefits to individual immunity. Care must be taken, however, to consider and/or challenge several elements of the immune system in order to detect possible trade-offs and their consequences when gauging the investment of any individual into personal immunity (Chapter 6, Chapter 7). Ideally, immune investment should be assessed by measuring multiple components (e.g. phenoloxidase and hemocytes), before and after an immune challenge (e.g. pathogen exposure, mechanical wounding) and in conjunction with gene expression analyses. In summary, investment into personal immunity and the resulting effectiveness of an immune response need to remain at the heart and center of any future investigation into the interplay between social immunity, pathogen pressure, and their combined effects on social life.

CONCLUSION

The central goal of my work was to investigate the occurrence and nature of social immunity in an insect with facultative family life to gain insight into the early stages of social evolution, and how they were shaped by pathogen pressure. My findings not only confirm what has been found

in other subsocial models – that social immunity is expressed and of profound importance in non-derived social systems – but also demonstrate that social immunity is not exclusively mediated by caring parents, which are traditionally considered to be the key promoter of benefits in subsocial groups. In addition, I show for the first time how precocial offspring adjust to maternal absence in terms of gene expression, and that this adjustment is significantly altered when facing a lethal pathogen, further emphasizing the key importance of microbial defenses in simple social systems. Consequentially, I postulate a widened view on social immunity away from the original eusocial framework in order to encompass all forms of social life that express collective immune defenses. Finally, I effectively demonstrate the intricacies of evaluating individual immune responses using trade-offs and variations in environmental conditions, a critical step towards understanding how subsocial group living, social immunity, and pathogen pressure interact and evolve towards the countless forms of social life we find in the world.

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CURRICULUM VITAE

PERSONAL DETAILS

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EDUCATION

Since October 2014:	Graduate student at the Institute of Organismic and Molecular Evolution, University of Mainz, Germany Topic: "The role of social and individual pathogen defense in an insect with facultative family life: insights into the early evolution of group living." Expected time of completion: December 2018 Supervisors: Susanne Foitzik & Joël Meunier
September 2014:	Master of Science (Biology) at the Institute of Organismic and Molecular Evolution, University of Mainz, Germany Topic: "The role of frass sharing in the early development and pathogen defense of the European earwig." Supervisor: Joël Meunier
June 2012:	Bachelor of Science (Biology) at the Department of Animal Ecology, University of Göttingen, Germany Topic: "Analyzing prey of Lithobius species using molecular gut content analysis." Supervisors: Kerstin Heidemann & Bernhard Eitzinger
June 2005:	University entrance qualification (Abitur) at the Gymnasium Kaiser-Friedrich-Ufer in Hamburg, Germany

LABORATORY AND TECHNICAL EXPERIENCE

Laboratory:	Tissue-specific RNA extraction, DNA extraction (phenol-chloroform) and purification, Cuticular hydrocarbon extraction, Hemolymph extraction, Hemocyte quantification, Primer design, PCR, Gradient PCR, Gel electrophoreses, Microsatellite analysis, DNA sequencing (Sanger), Rearing and care of insects, Growth and quantification of entomopathogenic fungi (*M.brunneum*)
Programs & Software:	R (statistics & graphics), Bash (Unix shell), BLAST (NCBI), Bioedit, MEGA, Structure, Primer Premier, CLC asssembly cell, Trinity, Kallisto, DEseq2, Transrate, FastQC, Trimmomatic, topGO, Inkscape, Microsoft Office suite

PRESENTATIONS & OUTREACH

Invited talk “Growing up with feces: waste mediated starvation resistance & social immunity in an insect with facultative maternal care” at the 11th European Congress of Entomology in Naples, Italy, July 2018

Poster presentation “Winter length matters. Long-term effects of prolonged winter conditions during rearing of the European earwig” at the 16th congress of the European Society for Evolutionary Biology in Groningen, Netherlands, Aug 2017

Invited talk “Getting sick of your group: Investigating the role of immunity in the early evolution of sociality” at the “Evolutionary Biology and Genetics Colloquium”, University of Potsdam, Germany, May 2017

Spotlight “Fecal Feasts Bring Earwig Families Together”, written by Erica Tennenhouse and published in Discover, Aug. 2016

Talk “Can Males afford honesty? On forceps size & immunocompetence in earwigs” at the 16th congress of the International Society for Behavioral Ecology in Exeter, UK, Jul 2016

Poster presentation “Family in feces: The benefits of allo-coprophagy between sibling offspring of the European earwig” at the 15th congress of the European Society for Evolutionary Biology in Lausanne, Switzerland, Aug 2015

Talk “Disgusting or refreshing? The role of feces in the family life of the European earwig” at the 11th Ecology and Behavior Meeting in Toulouse, France, May 2015

PUBLICATIONS

9. Körner M, Vogelweith F, Foitzik S and Meunier J. The impact of maternal care on offspring gene expression is lowered by pathogen exposure in an insect with facultative family life. In prep.

8. Van Meyel S, Körner M, and Meunier J. 2018. Social immunity: why we should study its nature, evolution and functions across all social systems. *Current Opinion in Insect Science*, 28:1-7

7. Körner M, Foitzik S and Meunier J. 2018. Shorter winters during egg development provide developmental and immune benefits in earwigs. *Journal of Thermal Biology*, 74:116-122

6. Körner M, Vogelweith F, Foitzik S, Meunier J. 2017. Condition-dependent trade-off between weapon size and immunity in males of the European Earwig. *Scientific Reports*, 7 (1): 7988.

5. Kramer J, Körner M, Diehl JMC, Scheiner C, Yüksel-Dadak A, Christl T, Kohlmeier P, Meunier J. 2017. When earwig mothers do not care to share: parent-offspring competition and the evolution of family life. *Functional Ecology*, 31: 2098-2107.

4. Vogelweith F*, Körner M*, Foitzik S und Meunier J. 2017. Age, pathogen exposure, but not maternal care shape offspring immunity in an insect with facultative family life. *BMC Evolutionary Biology*, 17: 69. (*Authors contributed equally)

3. Körner M, Diehl JMC and Meunier J. 2016. Growing up with feces: benefits of allo-coprophagy in families of the European earwig. *Behavioral Ecology*, 27: 1775-1781.

2. Diehl J, Körner M, Pietsch M and Meunier J (2015). Feces production as a form of social immunity in an insect with facultative maternal care. *BMC Evolutionary Biology*, 15, 40.

1. Eitzinger B, Micic A, Körner M, Traugott M, Scheu S (2013) Unveiling soil food web links: new PCR assays for detection of prey DNA in the gut of soil arthropod predators. *Soil Biology and Biochemistry*, 57, 943-945

TEACHING AND SUPERVISION

Diploma thesis co-supervision: Julia Meyer, Sep 2015, Thesis: Feces and pathogen defense in the European earwig, Forficula auricularia. Department of Ecology and Social Evolution, University of Mainz, Germany

Teaching assistant (HiWi), Winter 2013, Statistical course (R) teaching aid during practical “Evolution, Ökologie & Verhalten der Tiere II” Department of Ecology and Social Evolution, University of Mainz, Germany

Research assistant (HiWi), Summer 2014, Field work, Winkler Extractor sample collection & identification, J.F. Blumenbach Institute of Zoology and Anthropology, Göttingen, Germany