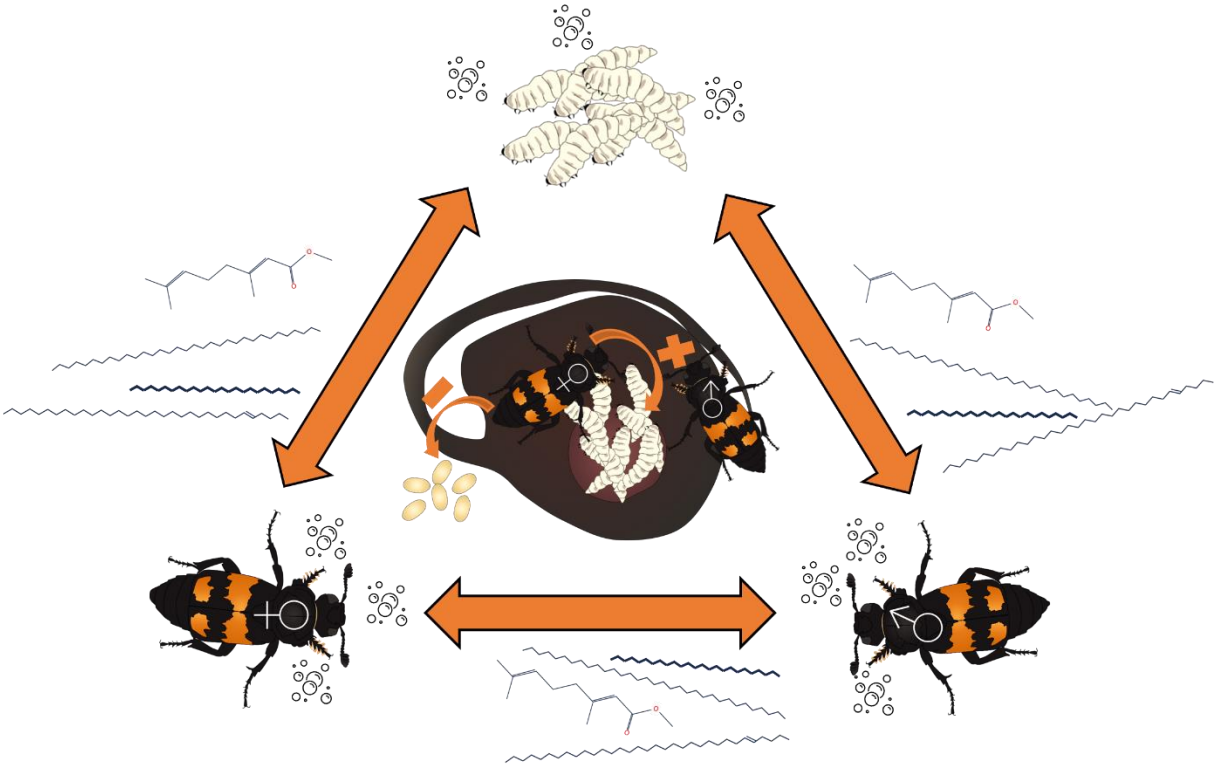
Parent-offspring conflict and communication in burying beetles



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“Like an old radish? You know when you boil a rag? It smells like that.” – Parasite (2019)

"Don't shoot it. We think it's trying to communicate with us." - Evolution (2001)

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Summary

Family life is a cooperative event. However, members of a family are usually not genetically identical which leads to an evolutionary conflict of interest. A conflict has been predicted not only between parents, but also between parents and their offspring, with offspring being selected to demand more investment than is optimal for the parents to provide. Begging signals, directed from offspring to parents, are thought to have evolved as a mechanism for resolving parent-offspring conflict by communicating information about offspring need. While begging signals have received considerable attention, it is still unclear whether a parent-offspring conflict occurs, in whose favour it is resolved, whether the outcome is influenced by internal or social conditions, and which physiological basis underlies conflict resolution. There is a hypothesis suggesting that offspring chemical signals (i.e. begging pheromones) might regulate parental care by affecting maternal hormone levels and future reproduction. However, evidence supporting this hypothesis is limited. To deepen our understanding of parent-offspring interactions and family dynamics, this thesis examines parent-offspring conflict, factors shaping parental investment decisions, offspring chemical signalling, and physiological regulation of care using the burying beetle *Nicrophorus vespilloides* as our model system.

The first part of this thesis involved manipulating the initial brood size of both single and paired female burying beetles. At small brood sizes, we found evidence for a parent-offspring conflict over the production of a second egg clutch. Females benefitted from producing a second clutch, but the current offspring suffered from such a response, as females then often discontinued to care. However, our results suggest that the outcome of the conflict is closer to the offspring optimum. Intriguingly, the presence of a caring male partner shifted the conflict outcome closer to the parental optimum. This finding introduces a novel perspective to parent-offspring conflict theory and highlights an additional factor that may contribute to the evolution of biparental care.

Because of these interesting results, we conducted another study, in which we examined the response of single males towards different initial brood sizes. We found that a surprisingly high number of males stayed and cared for even broods of small sizes, a strategy lying within the interest of the current offspring. Although uniparental male care is rare in nature, our study revealed that males exhibit considerable flexibility, adjusting both the amount

and type of care in response to social and non-social factors. The observed positive correlation between the duration of male care and offspring performance underscores the vital role of paternal care in burying beetles.

The third and fourth parts of this thesis investigated whether offspring emit chemical substances that might affect parental investment and mediate the resolution of parent-offspring conflict. We analysed the cuticular and volatile organic compound (VOC) profiles of larvae during their development. The results showed distinct cuticular and VOC profiles across larval instars. Moreover, the specific scent of second instar larvae increased maternal feeding trips, demonstrating the pivotal role of larval chemical substances in shaping parental behaviour. We further investigated whether the larval scent reflects their nutritional condition. Food-deprived larvae showed changes in both the composition of cuticular profiles and VOCs, which potentially facilitates communication with parents about their nutritional state. Given these findings, burying beetles might be ideal candidates to identify a potential begging pheromone, affecting parental investment.

Previous research showed that a specific maternal hormone and pheromone are indicative of parental investment, as they regulate parental care and future reproduction. In the presence of dependent larvae maternal hormone and pheromone levels are high. This results in temporary infertility in females and sexual abstinence in males, ensuring that both parents care for the current brood. The fourth part of the thesis showed a correlation between maternal hormone levels and larval tactile begging. The fifth chapter explored how age and prior reproductive experience affect maternal hormone and pheromone levels. Age alone did not influence maternal physiology, but pheromone levels increased in older females with reproductive experience. Considering the likelihood that an individual's residual reproductive value diminishes with age and reproductive experience, our findings suggest that older, experienced females may adopt a terminal investment strategy, thereby trying to maintain also male investment in the current brood.

In summary, this thesis significantly advances our understanding of parent-offspring conflict by uncovering novel aspects that influence parental investment. It demonstrates how both female and male care strategies undergo behavioural adjustments in response to changes in the social and non-social environment. Here, a key finding is that chemical profiles related to the offspring's developmental status and nutritional needs are likely to influence parental care

strategies. Importantly, the study reveals that social feedback has a more pronounced effect on altering female physiology than the age or reproductive experience of the females. Overall, my thesis urges further research of social interactions in families to better understand their broader impact on family life across different species.

Zusammenfassung

Familienleben zeichnet sich durch Kooperation zwischen Individuen aus. Allerdings sind Mitglieder einer Familie in der Regel genetisch nicht identisch, was zu einem evolutionären Interessenkonflikt führen kann. Dieser Konflikt betrifft nicht nur die Eltern, sondern auch die Interaktionen zwischen Eltern und ihren Nachkommen. Letztere sind oft evolutionär darauf ausgerichtet, mehr elterliche Investitionen zu fordern, als für die Eltern optimal wäre. Um diesen Konflikt zu lösen, werden Bettelsignale von den Nachkommen an die Eltern gerichtet, um Informationen über den Bedarf der Nachkommen zu kommunizieren. Obwohl zu Bettelsignalen viel geforscht wurde, ist immer noch unklar, ob und in welchem Maße ein Eltern-Kind-Konflikt existiert, wie dieser gelöst wird und ob das Ergebnis des Konflikts von individuellen oder sozialen Bedingungen beeinflusst wird. Eine Hypothese besagt, dass chemische Signale der Nachkommen, insbesondere Bettelpheromone, die elterliche Fürsorge steuern könnten, indem sie den Hormonspiegel der Mutter und damit ihre zukünftige Fortpflanzung beeinflussen. Um das Verständnis für Eltern-Kind-Interaktionen und Familienstrukturen zu vertiefen, untersucht diese Dissertation den Eltern-Kind-Konflikt, die Faktoren, die elterliche Investitionsentscheidungen beeinflussen, chemische Signale der Nachkommen und die hormonelle Regulation der elterlichen Fürsorge am Modellsystem des Totengräberkäfers *Nicrophorus vespilloides*.

Der erste Teil dieser Arbeit manipulierte die Brutgröße am Anfang der elterlichen Pflege zum einen bei einzelnen zum anderen auch bei gepaarten weiblichen Totengräbern. Bei kleinen Brutgrößen zeigten sich Anzeichen eines Eltern-Kind-Konflikts über die Produktion eines zweiten Geleges. Weibchen profitierten von einer zweiten Brut, während der aktuelle Nachwuchs unter der Produktion einer solchen litt, da dafür die Fürsorge in die ursprüngliche Brut oft eingestellt wurde. Interessanterweise verschob die Anwesenheit eines männlichen Partners das Ergebnis des Eltern-Kind-Konflikts näher zum elterlichen Optimum. Diese

Ergebnisse werfen eine neue Perspektive auf die Theorie des Eltern-Kind-Konflikts und betonen einen zusätzlichen Faktor, der zur Evolution der biparentalen Fürsorge beitragen könnte.

Aufgrund dieser spannenden Ergebnisse wurde eine weitere Studie durchgeführt, in der die Reaktionen einzelner männlicher Käfer auf verschiedene Brutgrößen am Anfang der elterlichen Pflege untersucht wurden. Überraschenderweise zeigte sich, dass eine beträchtliche Anzahl von Männchen auch kleine Bruten pflegte, was im Interesse des aktuellen Nachwuchses lag. Obwohl die Pflege durch einzelne Männchen in der Natur selten ist, zeigte diese Studie, dass Männchen eine erhebliche Flexibilität in ihrem Pflegeverhalten aufweisen und sowohl die Menge als auch die Art ihrer Pflege in Reaktion auf soziale und nicht-soziale Faktoren anpassen. Die beobachtete positive Korrelation zwischen der Dauer der männlichen Pflege und der Fitness des Nachwuchses unterstreicht die wichtige Rolle der väterlichen Pflege bei Totengräbern.

Die Teile drei und vier dieser Arbeit untersuchten, ob Nachkommen chemische Substanzen emittieren, die die elterliche Pflege beeinflussen könnten. Hierzu wurden die kutikulären und flüchtigen organischen Verbindungen (VOC) von Larven während ihrer Entwicklung analysiert. Die Ergebnisse zeigten klare Unterschiede in den kutikulären und VOC-Profilen über die Larvenstadien hinweg. Insbesondere der spezifische Duft von Larven im zweiten Stadium führte zu vermehrten Fütterungen durch die Mutter, was die bedeutende Rolle der chemischen Substanzen der Larven bei der Formung des elterlichen Verhaltens unterstreicht. Es wurde weiter untersucht, ob der Larvenduft ihren Ernährungszustand widerspiegelt. Tatsächlich zeigten durch Nahrungsentzug beeinflusste Larven Veränderungen sowohl in der Zusammensetzung der kutikulären Profile als auch in den VOCs, was auf eine mögliche Kommunikation mit den Eltern über den Ernährungszustand der Nachkommen hinweist. In Anbetracht dieser Erkenntnisse könnte der Totengräber ein ideales Modellsystem sein, um potenzielle Bettelpheromone zu identifizieren, die die elterliche Investition beeinflussen.

Frühere Forschungsarbeiten zeigten, dass bestimmte mütterliche Hormone und Pheromone Indikatoren für die elterliche Investition sind, da sie die elterliche Fürsorge und die zukünftige Fortpflanzung regulieren können. In Gegenwart abhängiger Larven sind die Hormon- und Pheromonspiegel der Mutter hoch. Dies führt zu vorübergehender Unfruchtbarkeit bei

Weibchen und sexueller Abstinenz bei Männchen, um sicherzustellen, dass sich beide Eltern um den aktuellen Nachwuchs kümmern. Der vierte Teil der Arbeit zeigte eine Korrelation zwischen den mütterlichen Hormonspiegeln und dem taktilen Bettelverhalten der Larven. Das fünfte Kapitel untersuchte, wie das Alter und die vorherigen reproduktive Erfahrungen von Müttern deren Hormon- und Pheromonspiegel beeinflussen. Das Alter allein hatte keinen Einfluss auf die mütterliche Physiologie, aber die Pheromonspiegel stiegen bei älteren Weibchen mit reproduktiver Erfahrung an. Angesichts der Annahme, dass ältere Individuen mit höherer reproduktiver Erfahrung eine geringere Chance auf weitere Reproduktionen haben, legen unsere Ergebnisse nahe, dass ältere, erfahrene Weibchen möglicherweise die Strategie des „terminal investment“ verfolgen, um auch die väterliche Investition in den aktuellen Nachwuchs zu gewährleisten.

Zusammenfassend trägt diese Dissertation wesentlich zum Verständnis des Eltern-Kind-Konflikts bei, indem sie neue Erkenntnisse zur elterlichen Investition liefert. Sie zeigt, dass chemische Signale oder Hinweise der Nachkommen, die mit deren Entwicklungsstand und mit der Verfügbarkeit von Nahrung zusammenhängen, die elterlichen Strategien beeinflussen können. Wichtig ist dabei die Feststellung, dass soziales Feedback einen stärkeren Einfluss auf die Veränderung der weiblichen Physiologie hat als das Alter oder die reproduktive Erfahrung der Weibchen. Insgesamt ermutigt meine Arbeit dazu, soziale Interaktionen weiter zu erforschen, um ihre breiteren Auswirkungen auf das Familienleben verschiedener Arten besser zu verstehen.

General introduction

Parental care and conflicts between family members

Parental care is defined by Smiseth et al. (2012) as 'any parental trait that enhances the fitness of a parent's offspring, and that is likely to have originated and/or is currently maintained for this function'. Theory suggests that parental care is important for the evolution of family life (Bourke 2011; Royle et al. 2012; Kramer and Meunier 2018). Family-like associations are defined as temporary biological units consisting of one or two parents and their offspring (Kramer and Meunier 2018). Different forms of family associations include uniparental female care (most common), biparental care, and uniparental male care (Clutton-Brock 1991; Furness and Capellini 2019). Theory suggests, that parental care evolves if the benefits are higher compared to the costs of care (Bourke 2011; Alonso-Alvarez and Velando 2012). Parental care during family life is usually divided into pre-hatching care, for example, nest building prior to the presence of offspring, and post-hatching care, such as predator defense, food provision, or protection against pathogens in the presence of offspring (Clutton-Brock 1991; Costa 2006; Cremer et al. 2007; Cotter and Kilner 2010; Balshine 2012; Smiseth et al. 2012; Trumbo 2012b).

In contrast to parental care, parental investment is defined as 'any parental investment by the parents in an individual offspring that increases the offspring's survival and reproductive success at the cost of the parent's ability to invest in other current or future offspring' (Smiseth et al. 2012). The costs of parental investment usually reduce the survival of parents and therefore their chances of future reproductions, as described by the cost-of-reproduction hypothesis (Williams 1966). This is especially true for ageing individuals, which face lower survival probabilities and should increase their investments in their current offspring (Williams 1966; Clutton Brock 1984). Experimental evidence for this hypothesis has been found in many taxa (Pugsek 1981; Cameron et al. 2000; Duffield et al. 2017). Exploring family life in animals offers profound insights into the evolutionary, ecological, and behavioral aspects of social organisms.

Parents need to make careful decisions over their investments, as spent resources cannot be retained for other investments (Trivers 1974). As parents and offspring engage in close interactions they face both overlapping and occasionally conflicting interests (Kilner and Hinde 2012). Conflicting interest between family members about the optimal level of parental

investment is suggested to be driven by the family members' genetic dissimilarities (Kilner and Hinde 2012). Offspring is usually twice as related to itself than to its siblings and demands more care to increase their own fitness. In contrast, parents are selected to provide equal amounts of care to each offspring as they are equally related to each of their progeny (Trivers 1974; Kilner and Hinde 2012). This can result in offspring favoring their own quality and parents favoring the quantity of offspring as well as the offspring' quality, leading to parent-offspring conflicts. A prominent example is the conflict between mothers and their offspring over egg size (Smith and Fretwell 1974; Sargent et al. 1987). Mothers trade off the number of offspring for egg size, as they can either produce many small offspring or fewer, larger offspring (Lack 1947; Smith and Fretwell 1974; Elgar 1990). In fish, for example, females are selected to produce more, but smaller sized eggs, while offspring favor larger egg sizes because larger offspring tend to survive better (Einum and Fleming 1999, 2000). In addition, inter-brood conflicts over parental investments can occur between the current and the future offspring, in contrast to intra-brood conflicts in which members of a current brood dispute over the amount of parental investments (Trivers 1974; Godfray 1995a; Mock and Parker 1997; Parker et al. 2002; Kilner and Hinde 2012; Lessells 2012). Evidence was found for parent-offspring conflict (Janzen and Warner 2009; Kölliker et al. 2015), but the presence and the outcomes of conflicts remain largely unexplored (Kilner and Hinde 2012).

Despite the parent-offspring conflict, males and females also face a sexual conflict over the allocation of resources (Trivers 1972; Chapman et al. 2003; Lessells 2012). Different investment strategies of the sexes might be driven by the strength of sexual selection or their certainty of parentage (Møller 1988; Møller and Birkhead 1993; Westneat and Sherman 1993; Westneat and Sargent 1996; Queller 1997; Royle et al. 2016; Royle 2016). Females, for example, are selected to provide more care than males as females have a higher certainty of their parentage (Trivers 1972; Westneat and Sherman 1993). The sexual conflict over resource allocation leads to a further conflict over mating rates between females and males (Arnqvist 2004; Parker 2006; Alonso-Alvarez and Velando 2012). For females, mating attempts are costly and they therefore favor lower mating rates, whereas males favor high mating rates, as they face lower mating costs (Arnqvist and Rowe 1995; Chapman et al. 1995; Johnstone and Keller 2000; Chapman et al. 2003). This interplay of conflicting interests drives behavioral dynamics within families, but the outcomes of conflicts remain to be investigated.

Parental care strategies and the plasticity of parental care

Parental decisions over their investment are important in family life. Hereby, individuals highly vary in their care strategies (Trivers 1972; Gross and Sargent 1985; Westneat and Sargent 1996; Alonso-Alvarez and Velando 2012). Female care strategies include, for example, the production of additional offspring, caring for the current offspring, abandoning or even terminating their current brood (Székely et al. 1996; Balshine 2012; Trumbo 2012b). Males are unable to enhance the number of offspring alone, but they could decide to care for the current brood or to abandon the offspring to focus their investment on a new reproductive attempt.

A multitude of factors can affect the decision of parents between different investment strategies. Parents adjust their investment, for example, based on social environment like the presence/absence of a breeding partner (Sakaluk et al. 1998; Griggio and Pilastro 2007; Russell et al. 2007; Harrison et al. 2009), the quality of a brood (Wright and Cuthill 1990; Coleman and Fischer 1991; Coleman and Gross 1991; Forsgren et al. 1996; Erikstad KE. et al. 1997; Parejo and Danchin 2006; Magrath et al. 2007), or the competition for resources (Kawecki 1995; Reyes et al. 2016; Venkitachalam et al. 2022). Also, non-social factors can affect the parental investment strategies of males and females, for example, the food availability (Müller, Eggert and Furlkröger 1990; Godfray 1991; Barbasch et al. 2020), the paternity certainty (Møller and Birkhead 1993; Queller 1997; Liker et al. 2015; Royle et al. 2016), or the parental quality. For the latter especially the body size (Lee and Peng 1981; Robertson and Roitberg 1998; Tveraa and Christensen 2002) and the age (Williams 1966; Pugsek 1981, 1983; Clutton Brock 1984; Part et al. 1992; Smith 1993; Mangan 1997; Cameron et al. 2000; Ericsson et al. 2001; Ericsson and Wallin 2001; Isaac and Johnson 2005) of an individual can impact the investment strategies of parents. Most of the previous studies focused on the plasticity of care in females, since females care is most common in nature (Clutton-Brock 1991). However, understanding the plasticity of care behaviors in different family systems could help us to understand the mediation of intra- and interfamilial conflict. Here, especially the signaling and the flow of information between individuals has captured the attention of evolutionary biologists and behavioral ecologists alike as a venue to affect parental investment decisions.

Communication mediates social interactions

In insects, chemical communication is mainly used to mediate social interactions (Wyatt 2008). Insects can communicate their social as well as their mating status, quality, caste, or age, whereby the complexity of chemical compositions increases if more than a single message needs to be transferred between individuals (Symonds and Elgar 2008; Wyatt 2008). Generally, we differentiate between long-range communication e.g., sex pheromones to attract mating partners from a distance (Karlson and Butenandt 1959; Cuvillier-Hot et al. 2001; Jaffe et al. 2007; Haberer et al. 2008) and short-range communication e.g., pheromones on the epicuticle to discriminate sexes (Singer 1998; Howard and Blomquist 2005; Weddle et al. 2012; Buellesbach et al. 2018). Signals on the epicuticle of insect can be cuticular hydrocarbons or lipids which are highly involved in insect' recognition systems (Howard and Blomquist 1982; Lahav et al. 1999; Thomas et al. 1999; Wagner et al. 2000; Howard and Blomquist 2005; Blomquist and Bagnères 2010). Changes in the chemistry of insects can occur due to abiotic and biotic factors (Lockey 1988; Wyatt 2008; Blomquist and Ginzel 2021). In addition, volatile organic compounds are also involved in the chemical communication of insects (Kölliker et al. 2006; Maisonnasse et al. 2010; Noël et al. 2023). Forensic studies have shown that volatile organic compounds are released when macromolecules (proteins, lipids and carbohydrates) are broken down during the decomposition of cadavers (Dent et al. 2004; Statheropoulos et al. 2007; Dekeirsschieter et al. 2009; Brasseur et al. 2012). Studies on insects have shown that forensically important blowflies (Sharma and Tomberlin et al. 2021) and honeybees also produce volatile organic compounds (Le Conte et al. 1994; Traynor et al. 2015).

Effective communication systems are useful in the coordination of parental investment and mating strategies of males and females (Godfray 1991; Kilner and Johnstone 1997; Royle et al. 2002; Bradbury and Vehrencamp 2011; Morales and Velando 2013; Smiseth 2019), as they might help to resolve conflicts arising between family members about resource allocation. This is important since females caring for offspring can sometimes also undergo physiological changes which induce a temporary infertility and helps to focus their investments to the current brood instead of producing additional offspring (Trumbo et al. 1995; Engel et al. 2016), resolving the conflict over parental investments towards the current offspring. A temporary increase in the levels of juvenile hormone III can suppress the Vg biosynthesis (Pinto et al. 2000; Dong et al. 2009) which can impact the ovarian activity for example in ants (Cuvillier-Hot et al.

2004; Brent et al. 2006). The juvenile hormone can also influence the fertility of individuals in termites (Brent et al. 2005; Saiki et al. 2015) or cockroaches (Engelmann 1959; Schal et al. 1997). We know that the chemical profiles of caregivers in subsocial insects can change according to their breeding or mating status (Steiger and Franz et al. 2008; Steiger, Peschke and Müller 2008; Steiger et al. 2009). In a range of eusocial insects, CHCs can act as fecundity cues (Espelie et al. 1994; Ayasse et al. 1995; Liebig et al. 2000; Cuvillier-Hot et al. 2001; Dietemann et al. 2003; Denis et al. 2006; Liebig et al. 2009; Oystaeyen et al. 2014), which can suppress the reproduction of other colony members (Keller and Nonacs 1993; Holman et al. 2010), leading to an increased care for the current brood. In addition, mated females can alter their odor (Johnson and Gibbs 2004; Oppelt and Heinze 2009), which may make them unattractive to other males and suppress mating attempts. Alternatively, mated females can actively alter the composition of their sex pheromones (Ayasse et al. 1999), suppressing the production of attractive substances altogether (Foster 1993). Females can also emit additional, unattractive substances after mating (Tompkins and Hall 1981; Scott and Jackson 1990; Schiestl and Ayasse 2000). The production of so-called 'anti-aphrodisiac' substances can also be observed in males, which they transfer to females after mating in order to reduce females' attractiveness to other males and thus increase their own potential paternity (Happ 1969; Scott 1986; Andersson et al. 2000; Thomas 2011; Peso et al. 2015; Malouines 2017). Regardless of the strategy females choose to alter their odor, becoming unattractive to costly mating attempts may resolve the conflict over parental investment towards the current offspring rather than the production towards further offspring.

Overall there is extensive knowledge available about the chemical communication in adults of eusocial insects (Leonhardt et al. 2016; Schultner et al. 2017; Schultner and Pulliainen 2020). In contrast there is limited knowledge in subsocial species (Steiger 2015; Steiger and Stökl 2017; Nehring and Steiger 2018), not to mention even smaller knowledge of offspring chemistry (see 'The role of offspring cues or signals in insect families'). Nonetheless, offspring signals are of great importance due to their ability to affect caregivers. In eusocial insects, for example, offspring signals can, at least partly, regulate the reproduction of workers (Mas and Kölliker 2011a; Teseo et al. 2013; Ebie et al. 2015; Ulrich et al. 2016). In addition, recent studies have shown that chemical cues from related offspring delay the oviposition in females (Martini et al. 2013; Schoelitsz et al. 2020). Extending our knowledge about offspring chemical profiles

could help to understand the dynamic shifts in conflicts over investments arising from family life.

The role of offspring cues or signals in insect families

Parents typically have the upper hand in conflicts over parental investments due to their physical dominance over their offspring (Alexander 1974; Godfray 1995a). In addition, decisions about parental investments are typically made in advance of offspring presence (Janzen and Warner 2009). Nevertheless, a prior study proposed a hypothetical mechanism suggesting a primer effect of offspring signals on mothers (Mas and Kölliker 2008), which describes an alteration in the physiology of the caregiver, which in turn might also affect their investment decisions in favor of the current offspring and consequently at the expense of future broods. In a later study, Mas and Kölliker indeed found a priming effect of offspring chemistry on the maternal physiology of earwigs mother (Mas and Kölliker 2011a). Similarly, in honeybees, offspring express the pheromone (E)-β-ocimene which has a primer effect on workers as it inhibits their egg production (Maisonnasse et al. 2009). Additionally, offspring signals can help caregivers to focus their investments towards the offspring in biggest need of food provisioning or protection (Le Conte et al. 1994; Smiseth and Moore 2007; He XJ. et al. 2016; Schultner et al. 2017) and with that offspring signals are important factors in the resolution of parent-offspring conflicts over parental investments.

Offspring signals can be visual, chemical, or acoustical (Kilner 1995; Kölliker, Brodie and Moore 2005; Lévy and Keller 2009; Pelletier et al. 2016). Based on the literature, offspring signals can be classified into honest signals, which reflect nutritional needs (Godfray 1991, 1995b; Kilner and Johnstone 1997; Mock et al. 2011), and into quality signals, which reflect the reproductive value of offspring (Kölliker, Brodie and Moore 2005; Mas and Kölliker 2011a). Irrespective of the intention behind offspring signals, they can elicit an increase in the amount and duration of parental investments (Kölliker, Brodie and Moore 2005; Kölliker, Chuckalovcak and Brodie 2005; Mas et al. 2009), which should maximize the fitness of offspring (Godfray 1991; Kilner and Johnstone 1997; Royle et al. 2002; Bradbury and Vehrencamp 2011; Morales and Velando 2013).

As described previously, the knowledge of chemical cues of offspring is limited, although chemical communication is important in insects (see 'Communication mediates social interactions'). In forensics - where the identification of larval instars is important to help determine the age of a decaying body - previous studies showed that larval instars differ in their cuticular hydrocarbon (CHC) and their volatile organic compound (VOC) profiles (Zhu et al. 2006; Roux et al. 2008; Sharma and Drijfhout et al. 2021; Sharma and Tomberlin et al. 2021). A temporal change of VOC profiles during offspring development could also be shown in the honey bee broods (Noël et al. 2023). In honey bees, VOCs further play an important role in the context of brood care, as honey bee workers adjust their food provisioning behavior based on different needs of developmental stages (Le Conte et al. 1994; Maisonnasse et al. 2010; Traynor et al. 2015). In addition, the chemistry of offspring can reflect their health status. For example, in honey bees (*Apis mellifera*), larvae respond to pathogen infections by changing their chemical profile, which influences caregivers investment decisions in hygienic behaviors (Wagoner et al. 2019; Wagoner et al. 2020; Kathe et al. 2021) to protect the colony from disease. In subsocial insects, offspring change their chemical profiles based on their nutritional conditions, for example the CHCs in earwig (Mas et al. 2009) and the VOCs in burrower bugs (Kölliker et al. 2006). However, previous studies in subsocial insect' offspring have not yet identified specific compounds functioning as chemical signals of nutritional need. Understanding offspring' chemical signals or cues can help us to understand their influence on parental investment and physiological adjustments of caregivers. This could in turn shed a new light on the adaptive strategies employed by both parents and offspring in navigating the challenges of family life.

Ecology, behavior, and chemical communication in burying beetles

Approximately 71 burying beetle species can be found in the northern hemisphere (Sikes et al. 2002; Sikes 2005), which live in the woods, grass land or meadows (Nagano and Suzuki 2003). Coexisting *Nicrophorus* species live in different spatio-temporal niches (Pukowski 1933; Wilson and Fudge 1984; Trumbo 1990a; Beninger and Peck 1992; Beninger 1994; Lomolino et al. 1995; Lomolino and Creighton 1996; Scott 1998b), showing remarkably complex family dynamics (Sikes et al. 2002; Costa 2006; Sikes et al. 2016). Additionally, many *Nicrophorus* species were observed to be multivoltine and adults can be kept and bred for 6-8 weeks in the laboratory (Scott 1998b; Hopwood et al. 2014), which makes them a suitable study species to investigate family life.

Burying beetles are mainly resource specialists which use small vertebrate corpses as their food and breeding resources (Pukowski 1933). The beetles locate these carcasses over their volatile organic compounds produced during the decomposition process (Kalinová et al. 2009; Trumbo and Steiger 2020; Trumbo et al. 2021). Competition over carcasses is high since they are scarce and valuable, as they are nutrient rich (Wilson and Knollenberg 1984; Smith and Merrick 2001). In fights between beetles (con- as well as heterospecific), body size usually determines the outcome of competitions (Bartlett and Ashworth 1988; Müller, Eggert and Dressel 1990; Safryn and Scott 2000; Hopwood et al. 2013) and the largest pair of a female and a male will usually monopolize the carcass to reproduce (Otronen 1988; Trumbo 1990a; Steiger et al. 2012; Hopwood et al. 2014; Lee et al. 2014). Beetles discriminate intruders against their mating and breeding partners based on their chemical profiles (Steiger et al. 2007; Haberer et al. 2010; Steiger and Müller 2010; Steiger, Haberer and Müller 2011; Haberer et al. 2014).

Once a suitable carcass is found, it is monopolized, the dominant pair removes fur or feathers from the carcass, rolls it into a ball, and treats it with antimicrobial oral and anal secretions to reduce carcass decay and exposure to other necrophagous animals (Suzuki 2001; Hall et al. 2011; Steiger and Gershman et al. 2011; Arce et al. 2013; Vogel et al. 2017; Duarte et al. 2018; Shukla et al. 2018; Trumbo et al. 2021; Trumbo and Sikes 2021). Around this time, females lay eggs in the surrounding soil from which larvae hatch and crawl to the prepared carcass. To facilitate their offspring's access to the carcass, the parents provide an opening, the so-called feeding cavity, in which larvae aggregate to be provisioned by their parents or to feed themselves (Pukowski 1933). The larval self-feeding sufficiency increases during development

(Eggert et al. 1998; Smiseth et al. 2003) until the third and final larval instar disperses into the soil for pupation (Scott 1998b), usually after the depletion of the resource.

Generally, parental care is induced by the presence of larvae in the feeding cavity using a time-sensitive mechanism (Müller and Eggert 1990). Most commonly, parents care for their offspring together (Wilson and Fudge 1984; Trumbo 1991; Eggert 1992; Eggert and Müller 1997). Biparental care is followed by uniparental female care (Scott and Traniello 1990; Smiseth et al. 2005; Steiger 2013), and lastly male care (Scott 1989; Trumbo and Fernandez 1995; Ward et al. 2009; Luzar et al. 2017). Under biparental care, females mainly provide direct care for the brood (Scott and Traniello 1990; Smiseth and Moore 2004a, 2004b; Smiseth et al. 2005; Walling et al. 2008), whereas males provide more indirect care (Fetherston et al. 1990; Scott and Traniello 1990; Trumbo 1991; Scott 1994; Müller et al. 2007; Trumbo 2007). Direct care describes the food provisioning for offspring, whereas indirect care describes resource maintenance and defense (Moss and Moore 2021). Additionally, males leave the brood earlier than females which care for the offspring till they disperse from the carrion resource (Bartlett 1988; Fetherston et al. 1990; Scott 1998a; Müller et al. 2007; Ward et al. 2009; Royle et al. 2014b; Parker et al. 2015; Ratz and Kremi et al. 2021; Ratz and Monteith et al. 2021). This difference in care between sexes might be driven by a high paternity uncertainty, since females often have stored sperm from previous mating (Müller and Eggert 1989; Müller et al. 2007; House et al. 2008). However, if a female deserts or dies, males increase their effort in offspring provisioning (Bartlett 1988; Scott 1989; Trumbo and Fernandez 1995; Müller et al. 1998; Jenkins et al. 2000; Smiseth et al. 2005; Royle et al. 2014b; Moss and Moore 2021), whereas females do not compensate the loss of a male (Smiseth et al. 2005; Suzuki and Nagano 2009).

Since carrion is scarce and limited, beetles need to make careful decisions over their investments. Parents can adjust the size of their brood to the carcass size by killing surplus freshly hatched first instar larvae (Bartlett 1987; Trumbo 1990b). Conversely, when the brood size is too small to effectively utilize the resource, females may modify their oviposition strategy (Bartlett 1987; Müller, Eggert and Furlkröger 1990; Trumbo and Fernandez 1995; Eggert and Müller 1997). To preserve the carcass for a more suitable and bigger brood, mothers have been observed to kill the initial offspring by purposefully closing the feeding cavity until the second egg clutch has hatched (Eggert and Müller 1997). In contrast, single males, lacking the ability to produce additional offspring, either care for current offspring or abandon the initial brood.

Moreover, females make investment decisions influenced by internal factors for example as they increase their investment in current offspring as they age (Creighton et al. 2009; Trumbo 2009, 2012a).

In addition, offspring is known to affect the investment of their parents (Rauter and Moore 2004; Smiseth and Moore 2004b; Smiseth and Moore 2007; Wang G et al. 2021; Wang W et al. 2021). Larvae develop over three instars (Pukowski 1933) differing in their dependency for parental provisioning (Eggert et al. 1998; Smiseth et al. 2003; Capodeanu-Nägler et al. 2016). They use a tactile begging signal to trigger food provisioning (Rauter and Moore 1999; Smiseth et al. 2003) depending on the proximity of their parents (Smiseth and Moore 2004b) or in response to a volatile substance of the mother (Takata et al. 2019). Tactile begging rates differ with the developmental status of larvae (Smiseth et al. 2003) as well as their nutritional state (Smiseth and Moore 2007). Usually, females are more responsive towards larval begging than males as they show a higher increase in their food provisioning (Smiseth and Moore 2004b; Suzuki and Nagano 2009; Royle et al. 2013; Royle and Hopwood 2017; Capodeanu-Nägler et al. 2018; Moss and Moore 2021).

In addition, previous studies showed that parental care in breeding beetles is affected by a hormonal and pheromonal controlled communication system which is conserved over the genus (Engel et al. 2016; Engel et al. 2019). Usually, during the oviposition period, females and males mate frequently (Müller and Eggert 1989). However, males might mate continuously with females, whereby for females two pairings are enough to fertilize all their eggs (Dressel 1987; Müller and Eggert 1989; House et al. 2008; House et al. 2009) leading to a sexual conflict over mating rates. In addition, higher mating rates can have negative consequences for female care (Head et al. 2014). To resolve this conflict over parental care, breeding females express the pheromone methyl geranate (MG). Usually, MG is expressed in the presence of a male partner (Steiger, Haberer and Müller 2011) and under the presence of nutritional dependent larvae (Engel et al. 2016). Methyl geranate is a volatile pheromone with a known anti-aphrodisiac function (Haberer et al. 2008; Haberer et al. 2010; Engel et al. 2016; Engel et al. 2019). For example, Engel et al. (2016) showed that the production of MG by caring females suppresses male mating attempts and leads to male help during care. Therefore, methyl geranate is suggested to resolve the sexual conflict over mating rates as it helps to coordinate parental investments towards their current offspring (Engel et al. 2016). The coordination of

care in burying beetles is special as the production of the pheromone MG is correlated with the production of juvenile hormone III (Engel et al. 2016). Methyl geranate and juvenile hormone III originate from the same metabolic pathway (Bellés et al. 2005). Levels of JHIII increase as females care for nutritional dependent larvae, and JHIII causes temporary infertility in breeding females (Scott et al. 2001; Scott and Panaitof 2004; Trumbo and Robinson 2008; Engel et al. 2016). This leads to a suppression of egg production in females, and they focus their energy and resources on the care for their current offspring instead of producing additional offspring. Burying beetles are unlike other insects as females maintain high JHIII titers during the duration of parental care (Trumbo et al. 1995; Trumbo 1996). The combination of MG and JHIII regulate parental care in burying beetles and resolves the conflict over mating rate in burying beetles. However, despite for the presence of larvae, carcasses and males, evidence is missing for other social or non-social factors affecting this specific communication system and parental care strategies in burying beetles and therefore their family dynamics.

Aims of this thesis

The aim of this thesis is to shed a new light on the parent-offspring conflict, the involved parent-offspring and parent-parent communication, and their potential impact on conflict resolutions. Therefore, I used the subsocial insect species *Nicrophorus vespilloides* as a model system.

Publication 1 & 2 of this thesis study the effect of initial breeding conditions on parental investment strategies of males and females of different family compositions. Here, I wanted to examine if burying beetles face a parent-offspring conflict (POC), and which party wins the conflict to better understand the dynamics of family life.

Since parents plastically adjust the amount and form of care based on the initial brood size (Publication 1 & 2), this raises the question whether offspring produces chemical signals as a form of social feedback affecting parental care strategies in their favor. In Publication 3, we studied the chemical profiles of the three different larval instars of *N. vespilloides*. Different larval instars show different tactile begging rates to their parents (Smiseth et al. 2003) as they vary in their dependency of parental food provisioning (Pukowski 1933). Understanding offspring chemistry could shed light on the importance of offspring signals in family life. Additionally, Publication 3 established a bioassay testing the females' reaction towards larval

chemistry. The ability to recognize and differentiate offspring of different developmental stages could benefit all members of a family since it might alter the investment decisions of parents and therefore impacts the POC.

We were able to establish that larval instars differ in their CHC and VOC profiles and that females respond to larval chemical substances (Publication 3). However, it remained unclear whether larvae produce a begging signal, and whether such a signal communicates their need or reproductive value (Godfray 1991, 1995b; Mas and Kölliker 2008; Mas et al. 2009). Therefore, in Manuscript 4 we studied the changes in the surface and volatile chemistry of larvae in different nutritional states. Additionally, Manuscript 4 also aims to investigate whether mothers undergo physiological changes when they are confronted with either food deprived or control larvae. Therefore, I analyzed the juvenile hormone III and methyl geranate levels of females as well as the tactile begging rates of larvae in different nutritional conditions. This is based on previous studies showing that females change their JHIII and MG levels in response to needy offspring which affects the parental investments in burying beetles (Engel et al. 2016; Engel et al. 2019) in favor of the current offspring.

Manuscript 4 showed that hungry larvae change their chemical profiles and that mothers respond to increased tactile begging rates of larvae with an increase in their JHIII levels. Based on the impact of a social factor (larvae) on the physiology of females, we wondered if non-social factors might also impact the physiology of burying beetle females. The idea is based on previous studies showing that beetles adjust their behavior as they grew older (Creighton et al. 2009; Trumbo 2009, 2012a). Therefore, Manuscript 5 examines the effect of non-social/internal factors (age & reproductive experience) on the JHIII and MG levels of females. Understanding the effect of other external or internal factors on the physiology of females could help to understand their impact on the investment decisions and life history in burying beetles.

Summaries of the publications and manuscripts of this thesis

Summary publication 1: Parent-offspring conflict and its outcome under uni-and biparental care

Jacqueline Sahm, Madlen A. Prang, Sandra Steiger

Published in Scientific Reports (07 February 2022)

Theory predicts that parent-offspring conflicts occur in families over the optimum level of parental investments. However, only few studies have empirically demonstrated the existence of a conflict and it remains usually unclear whether the conflict is resolved in favor of the offspring or the parents (Kilner and Hinde 2012). In addition, it is unclear whether the outcome of the conflict is different under uniparental versus biparental conditions. To better understand who decides the conflict over investments in their favor, Publication 1 of this thesis investigated the parent-offspring conflict in the burying beetle species *N. vespilloides*.

Burying beetles are ideal to study the parent-offspring conflict as they provide either uniparental female, uniparental male or biparental care on small vertebrate carcasses (Pukowski 1933; Eggert 1992). Examining a species with flexible family constellations can help us to understand the existence of parent-offspring conflicts and their potential resolutions. As carrion resources are scarce and valuable (Wilson and Knollenberg 1984; Smith and Merrick 2001), beetles must make careful decisions over their resource allocation to increase their fitness output. For example, females which are confronted with small broods can decide to resume egg laying (Müller 1987). Increasing the parental fitness outcome via the production of further offspring is suggested to have negative fitness consequences for the present offspring (Eggert and Müller 1997). However, if a conflict over the parental investments really exists, and whether parents or offspring win the dispute remains unclear for burying beetles.

By manipulating the initial brood size and the carcass size of single and paired females, Publication 1 showed that many caring females continued to invest in current offspring. In addition, our results showed that the reproductive output of females would have been higher if they had ceased care and opted to produce a second brood instead. However, only some uniparental females responded to small broods with egg laying especially if provided with larger carcasses. If females discontinued care for current offspring this resulted in negative

consequences for the current brood. Together, the results suggest a conflict over resource allocation in burying beetles with offspring having the upper hand. In contrast, biparental caring females laid a second egg clutch more often than uniparental females which led to a higher reproductive output for the former. This suggests that the presence of a male partner alters the conflict outcome towards the parental interests. Potentially, males' presence hinders offspring to interact with females as males also provision offspring. In conclusion, this study could show that females respond plastically to their environment by changing their investment strategy affecting the outcome of the parent-offspring conflict.

Summary publication 2: Brood size, food availability, and body size affects male care decisions and offspring performance

Jacqueline Sahm, Taina Conrad, Larissa Scheu, Sandra Steiger

Published in Ecology and Evolution (08 June 2023)

Parental investment strategies can differ between individuals of the same species, especially between males and females. Different investment strategies of males and females are considered to be driven, for example, by sexual selection or the certainty of parentage (Trivers 1972; Gross and Sargent 1985; Sargent and Gross 1986; Westneat and Sherman 1993; Westneat and Sargent 1996; Alonso-Alvarez and Velando 2012; Pelletier et al. 2016). Publication 1 showed that females of *N. vespilloides* adjust their parental investments based on the given brood and carcass size as well as the presence of males. Until now, we lack knowledge about comparable plastic behavioral responses of males, as previous studies mainly focused on the behavioral plasticity of females (Royle and Hopwood 2017). However, it might be of importance to investigate plastic behavioral responses of males as they also provide care (Clutton-Brock 1991). In addition, a recent study suggested that male investments might influence female' investments and with that the outcome of the parent-offspring conflict over parental investments (Publication 1). Therefore, Publication 2 examines behavioral responses of burying beetle males in *N. vespilloides*.

Burying beetles are ideal to study male care plasticity because if a female deserts the brood or dies, males can compensate for the loss of their partner by increasing their investment

in direct care (Scott 1989; Trumbo and Fernandez 1995; Müller et al. 1998; Smiseth et al. 2005; Royle et al. 2014a; Moss and Moore 2021). Due to the uncertainty of the environment, males - like females – should carefully decide over their investments. Females, for example, frequently respond by producing a second egg clutch if they face small brood sizes caused by hatching failure or predation (Müller 1987; Sahm et al. 2022). Males might be less responsive to environmental factors as male uniparental care is rare in nature and therefore selection might have not favored such a flexibility.

In Publication 2 we manipulated the initial brood size, the resource size, and the quality of each male (here the body size) and observed changes in the care behavior of single males in *N. vespilloides*. We decided to include male size as a factor, because larger males are usually the dominant males which monopolize the carcass and father the highest percentage of the fertilized eggs laid by the females, both of which likely increases their willingness to provide care (Otronen 1988; Royle and Hopwood 2017). Additionally, we examined the downstream consequences of single male care on the offspring performance.

The results of Publication 2 showed that uniparentally caring males provided more indirect care and deserted the offspring less often if they were confronted with larger broods. However, many males also cared for small broods. Therefore, our results suggest that male care can be as plastic as female care in response to brood size (see Publication 1), even if males show different care strategies compared to females. Additionally, our results showed that larger males increased their investment in direct care. This suggests that larger males might have a higher capacity to provide food compared to smaller males, which was also proposed for females (Steiger 2013). Additionally, Publication 2 showed that prolonged male care leads to larger larvae and a higher larval survival rate. Therefore, male care benefits offspring which was also previously shown in other taxa (e.g., in various biparental birds see Bart & Tornes 1989). In conclusion, even as male care is rare in nature, it is a plastic trait comparable to female care with benefits for the offspring. However, it remains unclear if feedback from offspring might be responsible for the behavioral plasticity of caregivers as females and males react sensitively towards brood size.

Summary publication 3: The scent of offspring: chemical profiles of larvae change during development and affect parental behavior in a burying beetle

Jacqueline Sahm, Beatrice Brobeil, Eric Grubmüller, Matthias Schott, Taina Conrad, Johannes Stökl, Sandra Steiger

Submitted in Behavioral Ecology 30th November 2023

Adult insects rely on chemical signals or cues to mediate social interactions (Bradbury and Vehrencamp 2011; Morales and Velando 2013). However, the chemistry of adults can vary with certain abiotic and biotic conditions (Lockey 1988; Sprenger and Menzel 2020; Blomquist and Ginzel 2021). For example, adults change their chemistry with age, nutritional condition, developmental status, sex, or breeding status (Symonds and Elgar 2008; Wyatt 2008; Sprenger and Menzel 2020). Also, in juveniles, chemical profiles change for example based on their developmental stage (Zhu et al. 2006; Roux et al. 2008; Pechal et al. 2014; Sharma and Drijfhout et al. 2021; Zhang et al. 2022), their reproductive value (Kölliker, Chuckalovcak and Brodie 2005; Mas and Kölliker 2011a; Bowers et al. 2018) or their nutritional condition (Mas et al. 2009; Mas and Kölliker 2011a, 2011b). However, overall knowledge about the chemistry of juveniles is lower compared to the chemistry of adults (Symonds and Elgar 2008; Wyatt 2008; Steiger and Stökl 2014; Yew and Chung 2015; Leonhardt et al. 2016; Pasqual et al. 2021; Buchinger and Li 2023). Despite that, offspring signals are of importance in the parental decisions over their investments. For example, in honeybees previous studies showed that workers react towards offspring chemistry by increasing their feeding rates (Le Conte et al. 1994; Le Conte et al. 1995; He XJ. et al. 2016) a reaction that is also shown in subsocial earwigs (Mas and Kölliker 2008; Mas et al. 2009). However, most of the previous studies focused on changes of the entire chemical profile of insect' offspring rather than determining a single substance functioning as a begging pheromone. The exception being honeybees, in which a single substance was determined that functions as begging pheromone and increases the feeding effort in honey bee workers (Traynor et al. 2015; He XJ. et al. 2016). In the context of parental care however it remains unknown if chemical offspring signals exist and if they change with their developmental stage.

Burying beetles are ideal to study the chemical profiles of offspring and the reaction of parents towards offspring chemistry since chemical signals are known to impact multiple social

recognition and communication processes in adults (Steiger et al. 2007; Steiger, Peschke and Müller 2008; Haberer et al. 2010; Steiger and Müller 2010; Steiger, Haberer and Müller 2011). It is known that the presence of offspring affects parental investments in burying beetles in favor of the current offspring (Engel et al. 2016; Sahm et al. 2022; Sahm et al. 2023). Previous studies suggested that chemical signals might be involved in the food solicitation behavior of larvae (Smiseth et al. 2010; Takata et al. 2019), but chemical offspring signals and the reactions of mothers towards offspring chemistry were not yet investigated. Signals of development could be particularly important in burying beetles as the three larval instars of *N. vespilloides* differ in their size, their nutritional dependency from parents and in their tactile begging behavior (Rauter and Moore 1999; Smiseth et al. 2003; Smiseth and Moore 2004b). Therefore, Publication 3 of this thesis studies the existence and potential differences in the larval chemical profiles depending on the developmental status in *N. vespilloides*. Further, Publication 3 tries to identify potential begging signals and lastly establishes a new bioassay testing the female reaction towards larval chemistry.

The data showed that larval instars differ in their surface and volatile chemical profiles. Further, second instar produced the highest amount of octanoic acid methyl ester, acetophenone and methyl geranate (MG; Publication 3). Since second instar larvae also express the highest tactile begging rate (Smiseth et al. 2003) and are fed the most (Fetherston et al. 1990), we suggest that these volatiles might be signals of nutritional need in *N. vespilloides* larvae. We then focused on the pheromone MG due its specific function in burying beetles. MG is produced by caring females to express their temporary infertility to their male partners, leading to a suppression of male' mating attempts and induces male' help in care (Haberer et al. 2010; Engel et al. 2016; Engel et al. 2019). Our data showed that second instar larvae raised by uniparental females produced MG, but under biparental care larvae express even higher levels of MG (Publication 3). Therefore, larvae produce MG in the absence of males in contrast to females (Steiger, Haberer and Müller 2011). We suggest that the increase in larval MG levels under biparental care could hint that MG functions as a begging pheromone directed towards both parents. Lastly, the newly established bioassay (Publication 3) showed that females performed more feeding trips to the odor of the second instar larvae compared to the control odor. This suggests that larval chemical signals or cues might play an important role in mediating parental investment in burying beetles. In conclusion, Publication 3 showed that

burying beetle' offspring express different chemical profiles based on their developmental state and that at least females behaviorally respond based on offspring chemistry. However, the information behind the chemistry of burying beetle larvae remains to be investigated.

Summary manuscript 4: To smell or not to smell: New insights in the chemical profiles of hungry offspring in burying beetles

Jacqueline Sahm, Rosa M. L. P. Staufer, Beatrice Brobeil, Johannes Stökl, Sandra Steiger

Ready to submit

Theory suggests that offspring signals can resolve the outcome of the parent-offspring conflict over parental investment (Trivers 1974). However, the intention behind offspring signals is still discussed among researchers. Offspring signals can be classified into two categories: signals of nutritional need (Godfray 1991, 1995b; Kilner and Johnstone 1997) or signals of reproductive value (Kölliker, Chuckalovcak and Brodie 2005; Mas and Kölliker 2011a). In insects, chemical signals are predominantly involved in communication processes in adults (Wyatt 2008; Steiger and Stökl 2014; Leonhardt et al. 2016). For juveniles, previous studies showed that offspring chemistry can reflect their nutritional conditions and further alters the investment of caregivers either by directly affecting their level of direct care or by manipulating the physiology of caregivers (Le Conte and Hefetz 2008; Mas et al. 2009; Mas and Kölliker 2011a). However, evidence is currently missing if offspring chemistry might reflect nutritional conditions in a parental care system and how mothers react towards needy offspring. Therefore, Manuscript 4 examines the chemical profiles of larvae in different nutritional conditions using the burying beetle *Nicrophorus vespilloides*.

Burying beetles are ideal to study the chemical communication during parental care, as adults use chemical signals to inform about their sex, their breeding and mating status (Steiger and Franz et al. 2008; Steiger, Peschke and Müller 2008; Steiger et al. 2009). Further, burying beetles use a specific hormone and pheromone based communication system to coordinate their mating and care efforts (Engel et al. 2016). Usually, beetles care for nutritionally dependent offspring, whereby offspring dependency of parental feeding changes with larval development (Pukowski 1933; Eggert et al. 1998; Smiseth et al. 2003). A chemical begging

component in the display of nutritional conditions of burying beetle larvae was suggested by earlier studies (Smiseth et al. 2010; Takata et al. 2019). Recently we identified a potential candidate for a begging pheromone in *N. vespilloides* larvae as the second instar expressed the highest levels of MG (Publication 3) and the second instar usually receives the highest feeding rates (Smiseth et al. 2003; Smiseth and Moore 2004b). In addition, Publication 3 showed that mothers react to the chemistry of larvae which also intends for a chemical signal of nutritional need in burying beetles.

To investigate if larvae of *N. vespilloides* change their chemical profiles in response to different nutritional needs, we compared the chemistry of fed and food deprived larvae in Manuscript 4. The data revealed that the surface and the volatile chemical profiles differed between food deprived and fed larvae. This suggests that larvae alter their chemical profiles based on their nutritional conditions. Further, we showed that food deprived larvae express higher amounts of octanoic acid isopropyl ester, but the amount of MG and acetophenone were similar compared to the control larvae (Manuscript 4). Therefore, we suggest that MG might not be a begging signal on its own – as proposed in Publication 3 – but rather a composition of VOCs (maybe also in combination with the CHCs) or the expression of the ester might reflect the nutritional condition of larvae. If the ester functions as a begging pheromone remains to be investigated in a bioassay to see if the ester affects the mothers' behavior.

Since we know that the presence of offspring impacts the investment of parents (see Publication 1, 2 & 3), as well as the physiology of females (Scott and Panaitof 2004; Trumbo and Robinson 2008; Engel et al. 2016) the question arises how larvae of different nutritional conditions affect mothers. Therefore, Manuscript 4 conducted a separate experiment studying the impact of food deprived and control larvae on the juvenile hormone III (JHIII) and methyl geranate (MG) levels of *N. vespilloides* females as common indicators for parental investment (Haberer et al. 2014; Engel et al. 2016). Here, our results show that females responded to increased tactile begging rates of food deprived larvae with an increase in their JHIII titer (MG levels were not affected, Manuscript 4). We suggest that high levels of JHIII may explain the observed increase in parental food provisioning (e.g., Smiseth and Moore 2008). This is based on the fact that JHIII induces temporary infertility in female burying beetles, eliciting a shift in their investment strategy towards care for current offspring rather than generating additional progeny (Trumbo 1997; Trumbo and Rauter 2014; Engel et al. 2016; Trumbo 2019).

Additionally, we suggest that certain chemical substances might exist whose quantities correlate with the rate of larval begging, thereby influencing the elevation of JHIII levels. Further studies might also need to investigate if other factors than social feedback from offspring might impact the JHIII and MG levels and with that the investment decisions of females.

Summary manuscript 5: Maternal hormone levels, pheromones, and terminal investment in breeding burying beetles

Jacqueline Sahm, Cassandra Jackl, Taina Conrad, Sandra Steiger

Ready to submit

Reproductions are costly and lead to reduced somatic states and reduced survival of individuals, which lowers their chances of further reproductive attempts (Williams 1966; Dean 1981; Visser and Lessells 2001). Since spent resources cannot be retained for future reproductive attempts, parents face a conflict with their offspring over their investments (Trivers 1974; Mock and Parker 1997). This is especially true for ageing individuals as older individuals should focus their investments on the current offspring rather than future reproductive attempts due to their reduced survival chances resulting from senescence (Williams 1966; Clutton Brock 1984). Usually, coordination during care helps to reduce the expenditure of personal and shared resources (Godfray 1991; Kilner and Johnstone 1997; Royle et al. 2002; Bradbury and Vehrencamp 2011; Morales and Velando 2013; Smiseth 2019). Previous studies in insects showed that the production of mating signals is increased in older individuals (Sadd et al. 2006; Kuriwada and Kasuya 2011) which could coordinate the investment of parents to focus on the reproductive output in a current breeding attempt. In Manuscript 5 we investigate the impact of age and reproductive experience on the communication system in burying beetles during care.

During biparental care, the significant costs associated with both reproduction and care lead, for example, to a sexual conflict over mating rates in burying beetles. Males benefit from continuously mating (Dressel 1987), whereas higher mating rates can have negative consequences for female care (Head et al. 2014). Burying beetles resolve this conflict using a special communication mechanism. During care females increase their levels of juvenile hormone III which causes them to be temporary infertile (Trumbo 1997; Scott and Panaitof

2004; Engel et al. 2016). In addition, females communicate their temporary infertility to their male partners by emitting the pheromone methyl geranate (MG). MG acts as an anti-aphrodisiac, suppressing male copulation attempts and ensuring male investment in current offspring (Engel et al. 2014; Engel et al. 2016; Engel et al. 2019). In combination MG and JHIII suppress the production of additional offspring and therefore regulate parental investments towards current offspring which resolves their conflict over mating rate. However, despite external factors - such as the presence of larvae, carcasses, and male partners that influence female physiology (Trumbo and Robinson 2008; Haberer et al. 2010; Steiger, Haberer and Müller 2011; Engel et al. 2016) and maternal investment (Sahm et al. 2022) - potential effects of internal factors like age or reproductive experience on the juvenile hormone III and methyl geranate levels of female burying beetles remain to be investigated.

In Manuscript 5 we studied the impact of age and reproductive experience on the physiology of burying beetle females of *N. vespilloides.* The first experiment tested if virgin females of four different age categories vary in their levels of JHIII and MG. The results showed no effect of increasing age on the levels of JHIII and MG produced by females. We suggest that female' burying beetles do not alter their JHIII and MG levels based on their age. In contrast, beetles might adjust their care behavior according to the terminal investment hypothesis (Creighton et al. 2009). Therefore, we propose that females might be able to adjust their care behavior based on social feedback (see Publication 1 -3 & Manuscript 4) independent of their physiology. In an additional experiment we studied whether the JHIII and MG levels of females change as their reproductive experience increases through 2, 3, or 4 reproductions. Here, the results shows that the levels of MG increased with increasing reproductive experience of females, but JHIII levels remained unaffected by the previous reproductive experience of females. The increase in MG could help directing care towards current offspring rather than future offspring (Engel et al. 2016). Since during this second experiment, reproductive experience was coupled to an increase in age, we alternatively suggest, that maybe more than one factor needs to be altered to induce terminal investment in beetles (Farchmin et al. 2020). In addition, our results showed that the JHIII levels are correlated with the clutch size. Therefore, we suggest that females could modify their physiology in response to the anticipated social feedback from their offspring rather than in response to internal factors. Future studies

might shed a new light on which factors might trigger hormonal and pheromonal adjustments in caring burying beetles to better understand the resolution of conflicts during family life.

General discussion

Plasticity of parental investment and the resolution of the parent-offspring conflict in *Nicrophorus vespilloides*

Theory suggests that family life often involves conflicts between all family members over parental investments due to divergent reproductive interests (Trivers 1974; Parker 2006; Hinde et al. 2010; Kilner and Hinde 2012; Kölliker et al. 2015). However, if conflicts occur and whether such conflicts are resolved towards the parents or offspring is still discussed (Kilner and Hinde 2012). This thesis showed that offspring can manipulate females to care for small brood sizes (Sahm et al. 2022). In contrast, our results showed that females would benefit from the production of a second brood – instead of caring for the initial brood - to increase their reproductive output (Sahm et al. 2022). However, only some females produced a second egg clutch in response to insufficient brood sizes - especially on larger carcasses – which had negative consequences for the current offspring (Sahm et al. 2022). We confirm previous studies showing the ability of burying beetle females to produce a second egg clutch (Müller 1987; Trumbo 1990b; Engel et al. 2016). In general, the ability to adjust the brood size to a given resource is well known (Hardy et al. 1992; Zaviezo and Mills 2000; Trumbo et al. 2001; Bezemer and Mills 2003), but the optimal oviposition strategy for parents is not necessarily also optimal for the offspring (Godfray and Parker 1991; Parker et al. 2002; Kölliker et al. 2015). For example, a previous study in turtles showed that mothers and their offspring differ in their optimal egg size, with the egg size that benefits the hatching success of offspring differing from the egg size that is optimal for the mothers (Janzen and Warner 2009). However, in contrast to my thesis, Janzen and Warner (2009) found that the conflict over egg sizes in turtles is resolved towards the maternal optimum. For the first time in burying beetles, my thesis provides evidence for a parent-offspring conflict over the investment of resources in burying beetles, whereby we could show that the conflict is resolved towards the offspring optimum (Sahm et al. 2022).

Behavioral plasticity due to changes in the social environment of individuals (Royle et al. 2014a; Royle and Hopwood 2017) might also drive changes in outcome of family conflicts. This thesis found that females engaged in biparental care (alongside a male partner) tend to lay a second egg clutch more frequently (Sahm et al. 2022). Therefore, we suggest that biparental care might act as a strategy for parents to navigate and resolve conflicts over resource allocation in their own favor. It is plausible that females with a male partner can resume the production of additional offspring, as they can have additional matings and with that additional sperm to fertilize their eggs compared to single females. Evidence for this suggestion was found in *N. vespilloides* by Sakaluk et al. (1998) who showed that the removal of a first clutch in single females resulted in the production of fewer offspring in a replacement brood compared to females with a male partner. Alternatively, males might reduce female' investment in carcass maintenance, which would allow females to invest more into the production of additional offspring. Indeed, male' beetles appear to invest more in carcass maintenance than females (Smiseth and Moore 2004a). In particular, the costs of preparing large carcasses leads to a reduction in the lifespan of males compared to females (Gasperin and Kilner 2015). The cost of preparing larger resources for offspring is also found in other insects, such as, dung beetles, where it leads to a reduction in parental survival (Hunt et al. 2002). Further studies are needed to understand the mechanism based on which a male partner affects female investments and with that the outcome of the parent-offspring conflict over parental investments.

Factors driving plastic responses are often known for females, being responsive especially towards their social environment (Royle et al. 2014a; Royle and Hopwood 2017), but our knowledge of males is limited. Investigating uniparental male, this thesis could show, for the first time in burying beetles, that males provided more indirect care and deserted the offspring less often if they were confronted with larger broods (Sahm et al. 2023). Our result is consistent with previous studies in other taxa showing that the male care strategy depends on the given brood size (Beissinger 1990; Coleman and Fischer 1991; Forsgren et al. 1996; Ward et al. 2009). Additionally, this thesis showed that males provided more direct care on intermediate sized carcasses and males deserted from larger carcasses more often than intermediate carcasses (Sahm et al. 2023). We suggest that males be more likely to invest in broods on intermediate sized carcasses as the latter are less costly to maintain then larger resources. This became evident in previous studies showing that the preparation especially of larger carcass incurs high costs (Xu and Suzuki 2001; Gasperin and Kilner 2015; Ratz and Kremi

et al. 2021). This thesis shows that males, like females, exhibit behavioral plasticity considering their investment strategies in response to external factors, even if their strategies differ (Sahm et al. 2022; Sahm et al. 2023). Further studies are needed to investigate the responsiveness of males and females towards certain factors to understand more about flexible parenting in burying beetles.

Male care strategies in burying beetles were also affected by their body size, as we found that larger males provided more direct care for their offspring compared to smaller males (Sahm et al. 2023). We know from dung beetles, that larger parents can provide more dung for their offspring, which increases offspring fitness (Lee and Peng 1981; Hunt and Simmons 2000). Therefore, it is possible that larger parents are able to provide more food compared to smaller parents (Steiger 2013). Alternatively, larger males may provide more care for their offspring, as their paternity certainty should be higher, as they are the dominant male at the carcass (Eggert 1992). That larger males father up more of the present offspring is a phenomenon also shown in the closely related beetle *Ptomascopus morio*, which does not provide post-hatching parental care (Trumbo et al. 2001; Suzuki et al. 2006). Further studies might investigate which internal or external factors cause larger beetles to provide more care, and to shed more light on the importance of body size in parental investment strategies.

In this thesis, we could show that males which provided care for a longer period, produced larvae of a higher mass and survival rate than males that provided less care (Sahm et al. 2023). Previous studies in other taxa have also shown a positive relationship between the amount of male care and the offspring performance (Bart and Tornes 1989; Gubernick and Teferi 2000). This suggests that – even if male care is rare in nature – males may play a more important role than previously thought. In dung beetles, for example, the assistance of a male partner leads to the production of larger offspring compared to the care of single dung beetle females (Hunt and Simmons 2000). Future studies could investigate the role of male care in other species to understand more about the importance of male care in nature. In conclusion, Publication 1 and 2 of my thesis showed a high plasticity in the female and male care strategies as well as in the resolution of the parent-offspring conflict. My thesis underscores the importance of accounting both male and female contributions in understanding the outcomes of parent-offspring interactions and lays the foundation for understanding how care decisions

may be influenced by both endogenous factors (for example, age, reproductive experience) and social feedback, for example from offspring.

Importance of offspring signals in parent-offspring interactions in *N. vespilloides*

Offspring signals are hypothesized to resolve the conflict over parental investment between parents and their progeny (Trivers 1972, 1974). Previous studies – outside the context of parental care have shown that the chemical profiles of juveniles can vary with certain factors, i.e. their nutritional conditions (Kölliker et al. 2006; Mas et al. 2009), or their health status (Swanson et al. 2009; Kathe et al. 2021). This thesis describes, for the first time in burying beetles (which provide elaborate biparental care for their offspring), the volatile and surface chemistry of *N. vespilloides* larvae in different developmental stages. The results show that the chemical profiles of offspring changes with larval development (Publication 3). Our results are in line with previous studies showing that insect juveniles change their larval chemical profiles in the course of their development (Zhu et al. 2006; Roux et al. 2008; Pechal et al. 2014; Sharma and Drijfhout et al. 2021; Zhang et al. 2022). Generally, chemical profiles of burying beetle larvae (Publication 3) are generally less complex compared to the profiles of adults (Steiger et al. 2007; Keppner et al. 2017). In contrast, in *Tribolium destructor* the chemistry of larvae and adults is similar (Hebanowska et al. 1990). However, since adult chemical signals were shown to coordinate mating and breeding in adults (Steiger et al. 2007; Steiger, Peschke and Müller 2008; Chemnitz et al. 2015; Steiger 2015; Engel et al. 2016; Keppner et al. 2017; Takata et al. 2019), previous studies have indicated that chemical cues of offspring may play an important role in mediating parent–offspring interactions in burying beetles (Smiseth et al. 2010; Mattey et al. 2018). For example, offspring chemistry could help to discriminate against heterospecific larvae (Capodeanu-Nägler et al. 2018; Smith and Belk 2018). This would generally allow parents to allocate their investments to their own rather than unrelated conspecific progeny (Cheney and Seyfarth 1980; Neff and Sherman 2003; Richard and Hunt 2013), which could lead to a higher net benefit of parental investment. Future studies may shed light on a potential role of offspring chemistry in the differentiation between conspecific and heterospecific offspring.

Alternatively, chemical profiles of different larval instars might reflect their dependency of food provisioning by parents. We know from previous studies that different larval instars of burying beetles show different abilities to self-feed (Smiseth et al. 2003). In addition, older

larvae receive lower feeding rates compared to younger larvae (Smiseth and Moore 2008), a phenomenon also shown in honeybees (Traynor et al. 2015). In my thesis we identified a potential candidate for a begging pheromone (Publication 3). This pheromone - methyl geranate - peaked at the second larval instar (Publication 3). The second instar is the one that receives the highest parental feeding rates (Fetherston et al. 1990; Smiseth et al. 2003; Smiseth and Moore 2008). We propose that this may be based on the production of methyl geranate, as second instar larvae produced this pheromone under both uniparental female and biparental care (Publication 3). This makes methyl geranate a good candidate for a signal of offspring nutritional need directed to both parents. Only in honeybees could previous studies identify a single substance that functions as a begging pheromone (Maisonnasse et al. 2009; Maisonnasse et al. 2010). In other subsocial insects, previous studies have shown that caregivers respond to general changes in the chemical profiles of juveniles (Mas and Kölliker 2008; Mas et al. 2009; Mas and Kölliker 2011b), but no single substance has been identified. If the chemistry of burying beetle offspring provides a signal towards their parents and which information is transferred with this signal still needs to be investigated.

In a novel bioassay, my thesis showed, that mothers adjust their parental behavior based on chemical substances released by larvae (Publication 3). Therefore, this thesis provides new insights into the mechanisms by which larvae influence parental behavior in burying beetles. Previous studies in eusocial insects have also shown that chemical signals of offspring can induce different behaviors in parents, such as, hygienic behavior in ants and bee colonies (Masterman et al. 2001; Pull et al. 2018), which protects the colony from pathogens. Outside the context of post-hatching parental care, chemical cues from offspring have been shown to alter the oviposition strategies in female mosquitoes (Schoelitsz et al. 2020). Here, the authors showed that oviposition in females was suppressed in the presence of first and fourth instar larvae (Schoelitsz et al. 2020). Also in ladybirds (A*dalia bupunctata*) chemical cues from related offspring can lead to changes in female oviposition strategy, delaying oviposition (Martini et al. 2013). For burying beetles, questions remain about how offspring chemical signals are perceived and interpreted by caregivers. This is based on previous studies suggesting that the information behind offspring signals can range from their nutritional state (Godfray 1991, 1995b; Kilner and Johnstone 1997), up to their reproductive value (Kölliker, Chuckalovcak and Brodie 2005; Mas and Kölliker 2011a). Future studies are needed to investigate the reaction of burying beetle parents to chemical cues and signals produced by offspring. Understanding the

role of offspring along the function of their chemical signals or cues – produced to affect the investment of caregivers - could help to understand the changes in the outcome of conflicts over parental investment involved in family life.

Examining the intention behind the offspring chemical profiles in burying beetles, my thesis, demonstrates that *Nicrophorus vespilloides* larvae changed their surface and volatile chemical profiles with their nutritional state (Manuscript 4). Previous studies in other subsocial insects also showed that chemical profiles of juveniles can vary with their nutritional conditions and showed that offspring chemistry can affect the feeding behavior of the caregiver (Kölliker et al. 2006; Mas et al. 2009). However, despite in honey bee offspring (Maisonnasse et al. 2010; He XJ. et al. 2016), a single behavior-inducing substance was not described. In my thesis, I compared individual compounds from the volatile profiles of food deprived and fed larvae and found that food deprived larvae expressed higher levels of 2-ethyl-1-hexanol and octanoic acid isopropyl ester compared to control larvae (Manuscript 4). We know that 2-ethyl-1-hexanol can have antifungal properties in plants (Calvo et al. 2020), which may also be the case in burying beetles, as larvae may need to help conserving the resource in which they live (see 'Ecology, behavior, and chemical communication in burying beetles'). However, whether 2-ethyl-1-hexanol could also act as signal of nutritional need, remains to be investigated in a bioassay testing the response of parental beetles to this substance. The function of octanoic acid isopropyl ester is unknown. We therefore propose that this ester may be the first begging pheromone identified in the context of parental care. In addition, we showed that the amount of MG remained constant between the treatment groups, but the Simper-test showed that MG levels led to a separation of the treatment groups (Manuscript 4). Therefore, we still suggest that MG may be a component of the burying beetle begging pheromone as previously suggested (Publication 3). Further studies are needed to test the reaction of females confronted with begging pheromone candidates to shed light on which might elicit parental behaviors.

Finally, we showed that the tactile begging rates of food-deprived larvae were higher than for control larvae (Manuscript 4), which matches previous studies in burying beetles (Smiseth and Moore 2002; Smiseth and Moore 2004b; Smiseth and Moore 2007, 2008) and is consistent with studies in other taxa showing that begging increases with nutritional need (Kacelnik et al. 1995; Leonard and Horn 2001; Mas and Kölliker 2008; Lévy and Keller 2009;

Traynor et al. 2015; Pelletier et al. 2016). We also found that females produced more juvenile hormone III (JHIII) in response to an increase in larval begging, although the amount of methyl geranate (MG) was unaffected (Manuscript 4). JHIII and MG are common indicators of maternal investment in burying beetles (Engel et al. 2016), as previous studies showed that the increase of JHIII causes a shift in the investment decision of females from egg laying to care for current offspring (Panaitof et al. 2004; Engel et al. 2016). Here, results suggest that increased social feedback from offspring seems to be capable of impacting the JHIII levels of females and therefore might also impact the care behavior of mothers, which then in turn could alter the outcome of the parent-offspring conflict. The results for MG levels were puzzling, as MG production in females is known to be influenced by the presence of a male partner (Steiger, Haberer and Müller 2011) as well as the presence of larvae (Engel et al. 2016). We suggest, since we provided a constant number of 10 larvae in our experiment, that females might not have adjusted their MG levels. This is based on a previous study showing that females provided with different numbers of larvae change their physiology in *N. vespilloides* (Engel et al. 2016). Further studies are needed to investigate if different numbers of larvae in different nutritional conditions could impact the maternal physiology (and with that their investment). Overall, this thesis underlines the importance of offspring and their signals towards parents in the regulation of parent-offspring interactions. The question remains if males can also recognize and react to chemical offspring signals or cues, since males - like females - plastically respond towards initial brood sizes (Sahm et al. 2022; Sahm et al. 2023). Understanding if both parents react to offspring signals can help us to understand their impact on the regulation of parental investment in families. However, regulation of family life could also be impacted by other factors rather than social feedback.

Effect of internal factors on the physiology of *N. vespilloides* mothers

The terminal investment hypothesis suggests that ageing individuals should increase their investment into their current reproductive attempts as their chances to survive until a subsequent reproductive event are diminishing low (Williams 1966; Clutton Brock 1984). However, it is unclear if older individuals might also affect their communication during care as they age. In this thesis increasing age had no impact on the levels of JHIII and MG produced by burying beetle females (Manuscript 5). This was surprising, since in burying beetles, previous

studies showed that individuals alter their investment behavior based on their age (Lock JE. et al. 2007; Creighton et al. 2009; Billman et al. 2014) and the physiology of females is usually a good indicator for their investment in offspring (Engel et al. 2016). We suggest that females may not increase their production of JHIII or MG as they age but may dependent on other factors that have been shown to influence their investment strategies (Sahm et al. 2022). For example, Engel et al. (2016) showed that MG levels in females were influenced by the number of larvae present. Alternatively, the physiology of females might affect their levels of JHIII, as JHIII depends on body mass (Trumbo 2018), and since JHIII levels correlate with the levels of MG (Engel et al. 2016), MG levels might also be limited by body size. Studies in other insects, that produce juvenile hormones in relation to their fertility have suggested that the size of the corpora allata may determine JH levels (Khan et al. 1982; Elliott and Stay 2007). Whether this is the case in burying beetles would require further investigation, as a previous study has indeed shown that JHIII is produced in the corpora allata (Scott et al. 2001). However, the most likely interpretation of our results is that age alone might not be sufficient to influence JHIII and MG levels in female burying beetles. This suggestion is based on a behavioral study by Farchmin et al. (2020) where they provided evidence of terminal investment in *N. marginatus* under the condition that beetles were immune-challenged, older, and more experienced. A previous study in birds showed that hormone levels are influences by two factors, as female prolactin levels increased with increasing reproductive experience in older females (Angelier et al. 2007). Similarly, in blackbirds, ageing males increase their prolactin levels as well as their investment in care (Préault et al. 2005). In addition, individuals facing a reduction in their chances of survival, i.e. an immune challenge, have altered their chemical profiles. For example, in the mealworm (*Tenebrio molitor*), immune-challenged males increase their investment in pheromones to become more attractive to females (Nielsen and Holman 2012). The latter case follows the terminal investment hypothesis, but in the form of increased investment in mating effort instead of care. It cannot be excluded that burying beetle females adjust their pheromone production in response to an immune challenge. Further studies are needed to investigate if age in the combination with other factors (i.e., an immune-challenge) might be able to impact the physiology and with that the care decisions of females in burying beetles.

This thesis showed that the levels of MG increased with higher reproductive experience of females, whereas female JHIII levels remained unaffected (Manuscript 5). We suggest that with increasing experience, females may induce the help from their male partner earlier and

therefore increase their levels of MG to suppress mating attempts and to secure male help during care (Engel et al. 2016). Females altering their odor or producing anti-aphrodisiac substances after mating to suppress further male copulation is a phenomenon also observed in other insects (Scott and Jackson 1990; Schiestl and Ayasse 2000; Oppelt and Heinze 2009). Such changes in female chemistry could help to resolve the sexual conflict over mating rates (Chapman et al. 1995; Chapman et al. 2003; Arnqvist 2004; Lessells 2012), a conflict also known to occur in burying beetles (Müller and Eggert 1989; House et al. 2008; Head et al. 2014). Therefore, increasing the level of MG could be a terminal investment strategy as it helps to focus the parental investments of females and males towards the current offspring instead towards the production of additional offspring.

Finally, this thesis found that JHIII levels correlated with the clutch size produced by females (Manuscript 5), which may be an indication for the ability of females to anticipate the upcoming brood. Previous studies in burying beetles could already show that females can anticipate the time frame in which their larvae should reach the resource (Müller and Eggert 1990; Eggert and Müller 2011). In our experiment, we standardized the initial brood size of females to 10 larvae. However, in *Nicrophorus vespilloides*, females can produce clutches varying of 0-45 eggs (Müller and Eggert 1990). Furthermore, we know that at least the level of MG varies with different brood sizes (Engel et al. 2016). We therefore suggest that females may be able to anticipate the number of larvae that should reach the carcass. Since our recent study showed that females react to social feedback by altering their investment patterns (Sahm et al. 2022) we suggest that females may exhibit greater sensitivity to shifts in their social environment than changes in their own somatic state. However, if females can anticipate their brood size and if females adjust their hormone levels accordingly remains to be investigated in the future.

Conclusion

In conclusion, this thesis extends the knowledge about the impact of initial breeding factors (social e.g., the presence/absence of a breeding partner or the brood and non-social factors like their own quality or the food availability) on the parental investment decisions during family life in burying beetles. Publication 1 and Publication 2 showed behavioral plasticity in female'

and male' care strategies in response to the alteration of social factors (e.g., initial brood size, presence/absence of a partner) as well as non-social factors (e.g., resource size, parental quality). Further, this thesis extended the knowledge about changes in the chemical profiles of offspring over their development (Publication 3) as well as in response to food-deprivation (Manuscript 4). In addition, Publication 3 is the first to show that social feedback from offspring in form of chemical signals/cues from their surface can impact the investment strategies of females, whereby this thesis firstly identified a potential begging pheromone in burying beetles. Additionally, females respond to larval tactile begging rates with a change in their physiology which in turn might impact their care strategies (Manuscript 4). Lastly, my thesis found no evidence for an impact of age and only limited effects of reproductive experience on the female' physiology (Manuscript 5). Overall, my thesis indicates that social feedback is more important in altering the investment strategies and in impacting family life compared to internal factors. This work encourages more studies to investigate the broader implications of these findings for evolutionary and ecological processes in diverse species to investigate the existence as well as the outcome of the parent-offspring conflict in family life.

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Declarations of self-contribution

Publication 1: Parent-offspring conflict and its outcome under uni-and biparental care

Concept and study design 0 %, data acquisition 75 %, data analyses and figures 90 %, interpretation of results 50 %, first draft of the manuscript 100 %, revision of the manuscript 50%.

Publication 2: Brood size, food availability, and body size affects male care decisions and offspring performance

Concept and study design 50 %, data acquisition 50 %, data analyses and figures 95 %, interpretation of results 50 %, first draft of the manuscript 100 %, revision of the manuscript 50%.

Publication 3: The scent of offspring: chemical profiles of larvae change during development and affect parental behavior in a burying beetle

Concept and study design 0 %, data acquisition 40 %, data analyses and figures 80 %, interpretation of results 50 %, first draft of the manuscript 95 %, revision of the manuscript 50%.

Manuscript 4: To smell or not to smell: New insights in the chemical profiles of hungry offspring in burying beetles

Concept and study design 0 %, data acquisition 40 %, data analyses and figures 90 %, interpretation of results 50 %, first draft of the manuscript 100 %, revision of the manuscript 90%.

Manuscript 5: Maternal hormone levels, pheromones, and terminal investment in breeding burying beetles

Concept and study design 0 %, data acquisition 75 %, data analyses and figures 95 %, interpretation of results 50 %, first draft of the manuscript 100 %, revision of the manuscript 90%.

Publications and manuscripts

Publication 1: Parent-offspring conflict and its outcome under uni-and biparental care

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Author Contributions

S.S. conceived the study, J.S. and S.S designed the study, J.S. collected the data with help of M.P., J.S. analyzed the data, and J.S. and S.S. wrote the manuscript.

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Abstract

Conflicts over parental investment are predicted to be common among family members, especially between parents and their offspring. Parent-offspring conflict has been studied in many brood-caring organisms, but whether its outcome is closer to the parental or offspring optimum is usually unknown, as is whether the presence of a second parent, a caring male partner, can affect the outcome. Here, we manipulated the initial brood size of single and paired female burying beetles to examine how many offspring are necessary to maintain parental care in the current brood. We found that mothers continued to invest in small broods even if their reproductive output would have been higher if they had discontinued their care and produced a second brood instead. Consequently, our data suggests that the offspring have the upper hand in the conflict. However, our results further show that paired females laid a second egg clutch more often and produced more offspring than single females, suggesting that the presence of a male partner shifts the conflict outcome towards the parental optimum. This latter result not only is a novel aspect of parent-offspring theory, but also represents an additional factor that might explain the evolution of biparental care.

Introduction

Family life involves cooperation and conflicts between family members. One central conflict is the dispute between parents and offspring over parental investment[1–6]. This potential disparity in the optimum level of parental investment arises because of asymmetries in relatedness, i.e., an offspring is more related to itself than to any of its current or potential future siblings, whereas parents are equally related to all their offspring. Consequently, each offspring is selected to demand more investment than parents should provide. Two different sorts of parent-offspring conflicts are known from theory, namely the intra-brood conflict, in which parents and offspring battle over resource allocation among members of the current brood, and the inter-brood conflict, in which disagreement arises concerning resource division between current and future offspring, since investment in current offspring should reduce the amount of resources available for future offspring[1,4,7]. However, a central question is still in whose favor the conflict is resolved.

Although there are few studies that have found evidence for a parent-offspring conflict to occur in nature[8–11], whether the outcome of the conflict is closer to the offspring's or the

parents' optimum is usually unknown[12]. The reason for this lack of information is that the determination of the investment optima for parent and offspring is a difficult task. In some cases, parents might obviously have the upper hand. For example, offspring are not able to influence the amount of nutrition that is provided into their eggs[9]. However, in cases in which parents and offspring interact after birth, offspring might be capable of manipulating parental physiology in such a way that care is prolonged or increased in the current brood at the expense of future reproduction[13,14]. For example, in a variety of mammals, the continued suckling of young causes a temporary infertility in mothers[15–17], and in honeybees, larval begging pheromones have not only been shown to positively affect food provisioning, but also to inhibit egg development in nursing workers[18,19]. Although these examples illustrate that offspring might have the potential to affect the trade-off between investment in current offspring and the parent's expectation of future offspring, it is currently unclear whether they are indeed able to bias the conflict outcome toward their own optimum. A manipulation of parental investment might be achieved by using exaggerated begging signals[1] or, as suggested by Mas and Kölliker ([13]), by solicitation pheromones with a priming effect on maternal physiology.

Surprisingly, at present, we also lack data about whether the outcome of the battle is different under uniparental versus biparental conditions. In nature, the most common form is female uniparental care, but also male and biparental care can occur. There are even species, in which the family composition can vary from brood to brood. Especially in systems in which care is flexible, and in which uniparental care occurs alongside biparental care, offspring might not have the ability to affect the physiology of both parents to the same degree as that of a single parent. For example, in the presence of a helping male, females might reduce their amount of contacts with offspring, resulting in less offspring control over maternal physiology and reproductive behavior.

Since we are still far away from understanding all the facets of intrafamilial conflicts and how they impact the evolution of family life, we studied parent-offspring conflict by using the burying beetle *Nicrophorus vespilloides* as a model organism. Specifically, by analyzing more than 500 families, we tested (1) whether an interbrood conflict exists, (2) whether the outcome of the interbrood conflict is closer to the parents´ or the offspring optimum and (3) how the outcome is affected by the presence of a male partner. *N. vespilloides* is a particularly valuable study system to address these questions because, first of all, an earlier preliminary study has

provided some indications of an interbrood conflict[20], and secondly, both biparental care and female uniparental care occur in natural populations of this species[21,22].

Burying beetles reproduce on small vertebrate cadavers that serve as a food source for their offspring[23–26]. Upon finding a carcass, parents bury it within the soil, thereby removing fur or feathers, rolling it into a ball-shape, and treating it with antimicrobial secretions to manipulate the microbiome and to reduce decay[27–33]. The parents then cut a feeding cavity into the carcass in which the developing larvae are not only fed by the parents, but can also self-feed from the resource[24–26,34–36]. Larvae are known to beg for food by raising their head toward the parent while waving their legs[36,37]. Both parents respond to begging, but females engage in a higher rate of food provisioning than males[21,38,39]. Since a vertebrate cadaver is a rare but very valuable and highly contested resource, burying beetle parents are expected to make careful decisions about brood sizes to optimize the exploitation of the resource. Due to, for example, egg predation or hatching failure, the initial brood size on a carcass can be small. In such a situation, females have been observed to resume egg laying and produce a second clutch on the same cadaver[40]. However, although investing in a second clutch might enhance the overall reproductive output of the parents, a preliminary study[19] suggests that this has negative effects on the offspring of the first brood, because mothers that resume egg laying have been observed to close the feeding cavity[24]. This helps to preserve the carcass and slows down the deterioration of resource quality[35], but also implies that they discontinue to feed their current offspring. Eggert and Müller ([24]) reported in their review that closing the feeding cavity sometimes even results in the death of the larvae, presumably because they suffocate. Consequently, there might be a parent-offspring conflict over the production of a second clutch and offspring might have evolved mechanisms to inhibit maternal egg production and to promote parental care. In fact, since offspring on a specific carcass are often of mixed paternity the genetic asymmetry within families is stronger leading to higher levels of conflict than in full-sib families[22,41]. However, whether a conflict really exists, and which party wins the dispute under uni- and biparental conditions is currently unclear.

To address these gaps in our understanding of parent-offspring conflict, we conducted two experiments that were specifically designed to test the following predictions. We first of all predicted that females would be able to produce a second clutch on the same cadaver and that the probability to lay eggs would decrease with initial brood size and increase with carcass size. We furthermore predicted that there would be a parent-offspring conflict over the

production of a second clutch. We expected that females that were confronted with a small brood should benefit from producing a second clutch, but offspring should suffer from such a response, as females then close the feeding cavity and discontinue to care. Our experimental design also allowed us to evaluate whether the outcome of the conflict was closer to the parents' or the offspring optimum and whether the outcome differed between uni- and biparental families. Based on the hypothesis of Mas and Kölliker ([13]), we predicted that larvae would be able to influence maternal reproductive physiology and behavior and bias the outcome towards their own interest. However, we also predicted that in the presence of a caring male partner, the outcome would shift in the direction of the parental optimum. Females have been shown to decrease their provisioning rate when caring with a male[38]. Because of the reduced mother-offspring interactions in biparental families, offspring might not have the power to affect their mother's reproductive physiology in such a way as in uniparental families.

Material and methods

Origin and husbandry of the beetles

We used virgin beetles from an outbred laboratory population kept at the University of Bayreuth. The beetles were the 3rd-5th generation of beetles descending from wild-caught beetles collected in a forest in Bayreuth, Germany, during the summer of 2018. Prior to the experiment, the beetles were kept in small plastic boxes (10 x 10 x 6 cm) filled with moist peat. The beetles were maintained in a climate chamber at 20 °C under a dark: light cycle of 16:8 hours and were fed with sliced mealworms twice a week. At the start of the experiment, beetles had either an age of 20 (N = 288) or 30 days (N = 288).

General experimental designs and procedures

To test our predictions in the context of parent-offspring conflict we performed two experiments. Both experiments were conducted in climate chambers at 20 °C. In the first experiment, we used a 6 x 3 x 2 factorial design in which we manipulated the initial number of larvae provided (0, 1, 2, 3, 5, or 10 larvae), the carcass size (mice carcasses of approx. 5 g, 10 g, and 20 g), and the absence or presence of a male partner (i.e., single, and paired females). The treatment, in which parents were initially not provided with any larvae served as a control

group. In this group, no parent-offspring conflict could occur and consequently, larvae could not influence a female's decision to lay a second clutch. For each treatment group, we set up 16 replicates, resulting in an overall sample size of N = 576. Our final sample size was reduced to N = 544, because of the failure of 32 beetles to lay a first egg clutch.

We set up each pair by placing an unrelated virgin male and female in a plastic box quarter-filled with moist peat (10 x 10 x 6 cm). Pairs stayed together for 72 hours to allow repeated mating in order to ensure a sufficient supply of sperm for the fertilization of eggs. In the case of single female trials, we removed the males after the mating period. To test paired females, males were kept with their partner during the entire treatment. After the initial mating period, single and paired females received access to a freshly thawed mouse carcass to initiate breeding. Therefore, a larger amount of moist peat was added to the boxes. Forty-eight hours later, the carcass and beetles were placed into a new, similarly sized plastic box filled with moist peat. This procedure ensured that the eggs were separated from the parents, and that the larvae hatched in isolation. The old container was checked for newly hatched larvae every 4 hours day and night for overall 48 hours. Hatched larvae were pooled together in a Petri dish on a wet filter paper. Larvae were then randomly assigned to the different treatment groups. As parents kill any larvae that arrive on the carcass before their own larvae are expected to hatch[42], we only provided parents with a brood once their larvae had hatched. In this species, parents do not distinguish between unrelated foster larvae and their own offspring[42] making it possible to provide parents with larvae of mixed parentage (see e.g.[43–45]).

After the parents had received a brood of a certain size, we checked the broods every 6 hours for 72 hours, and then we increased the time interval to 8 hours for another 72 hours. After 6 days, we checked the boxes every 12 hours until larvae dispersed from the carrion resource. During the observation periods, we noted whether females had laid a second clutch or not, whether the cavity was open or closed (i.e., whether the females had discontinued to care for broods or not), and whether larvae within a closed feeding cavity were alive or not. Furthermore, as soon as the larvae dispersed for pupation, we recorded the brood size. After dispersal, we transferred the larvae to a new plastic container filled with moist peat for pupation. After eclosion, the number of emerging adults was determined for each brood.

In the second experiment, we provided single females with a freshly thawed mouse carcass of a standardized size (7.5 g – 12.5 g) and small initial broods of varying sizes (0, 1, 2, or

3 larvae). The experimental procedure was similar as described above. However, in our second experiment, we not only counted the number of dispersing larvae, but also determined their mass. We also weighed the females before and after the breeding event to test whether laying a second clutch had a negative effect on the females' weight change during breeding and therefore body condition. In total, we tested 15 single females per treatment (N = 60). Due to the failure of three beetles to a lay a first egg clutch, the final sample size was reduced to N = 57.

Statistical analyses

All data were analyzed and plotted using R version 3.5.1. In a first step, we analyzed which factors influenced a female's decision to lay a second clutch. Therefore, we used the data of our first experiment and conducted a generalized linear model (GLM) fitted with a binomial error structure. As predictors, we included initial brood size (as continuous variable), carcass size, and the presence or absence of a male partner. Since the full-factorial design showed no significant effect of all possible interaction terms between the three factors, we excluded the interaction terms from our analysis.

In a second step, we determined whether there is a parent-offspring conflict over the production of a second clutch, we evaluated the fitness of parents and offspring when females produced a second clutch or not. In our first experiment, we determined whether and how often parents closed the feeding cavity when producing a second clutch and whether the fitness of the current offspring was affected, i.e., whether they died within the closed feeding cavity. To evaluate the fitness of parents, we calculated Wilcoxon rank-sum tests to compare the overall number of dispersing larvae and the number of emerging adults produced by females that laid a second clutch and those that continued to care for their current brood. Using the data from our second experiment, we further investigated the average weight of a larva at dispersal as a fitness parameter for offspring and the and carcass use efficiency as a maternal fitness parameter. Carcass use efficiency, which is calculated by dividing the brood mass at dispersal by the initial carcass mass, indicates how much carrion biomass a female is able to convert into offspring biomass[46–48]. We used Welch's t-tests (because of unequal variances) to compare the mean larval weight and the carcass use efficiency of females that laid a second clutch and those that did not. Furthermore, as an additional fitness indicator for females, we

evaluated their body weight change during breeding. To this end we used a by a two-sample t-test (because of equal variances) in order to compare the weight change of females that laid a second clutch with those that did not.

In a third step, we evaluated whether the outcome of parent-offspring conflict was closer to the parents' or offspring optimum. Therefore, we used the data from our first experiment and determined the smallest initial brood size that was necessary to prevent more than 50 % of the females from laying a second clutch and that triggered them to continue to invest in the current brood. We refer to this brood size as the 'larval tipping point'. Using a Wilcoxon rank-sum tests, we then compared the number of larvae raised at the tipping point with the number of offspring that parents from the control group (treatment in which initial brood size was zero) were able to produce. If the number of larvae that was able to suppress egg laying was significantly smaller than the number of larvae that females of the control group were able to raise, this would indicate that the parent-offspring conflict was resolved closer to the offspring than the parents' optimum. The rationale behind this argument is that the mothers' fitness would have been greater, if they had laid a second clutch instead of continuing to invest into the current brood. In the control, the mothers' decision to lay a second clutch was not influenced by the presence of larvae. We ran the tests separately for single and paired females to evaluate whether the outcome of parent-offspring conflict differed under uni- and biparental conditions. Furthermore, we calculated a binomial GLM to test for an effect of a male partner on the care strategy of females (i.e., whether they closed the feeding cavity or not). Finally, we calculated a quasi-Poisson GLM (to correct for overdispersion) in order to investigate the effects of male presence, initial brood size, and carcass size on the number of dispersed larvae.

Results

Do females lay a second egg clutch, and which factors influence its production?

Females responded to our brood size manipulation by laying a second egg clutch in 236 of 544 trials. As predicted, the egg-laying strategy was affected by the initial brood size and by the carcass size: the beetles' probability of laying eggs decreased with an increasing initial brood size and a decreasing carcass size (Table 1.1; Fig. 1.1A). The presence of a male partner also

affected the egg-laying strategy of *N. vespilloides*. Single females showed a lower probability for laying a second egg clutch than females caring with a male partner (Table 1.1; Fig. 1.1B).

Table 1.1: Summary of models for the effects of initial brood size, carcass size, and the male partner on the probability of producing a second clutch and the number of dispersing larvae. Significant values are in bold.

	Egg-laying probability			Number of dispersing larvae		
Predictors	F	df	p	F	df	p
Initial brood size	98.33	1	**< 0.001**	0.01	1	0.92
Carcass size	25.34	2	**< 0.001**	17.39	2	**< 0.001**
Male partner	34.96	1	**< 0.001**	5.22	1	**0.02**

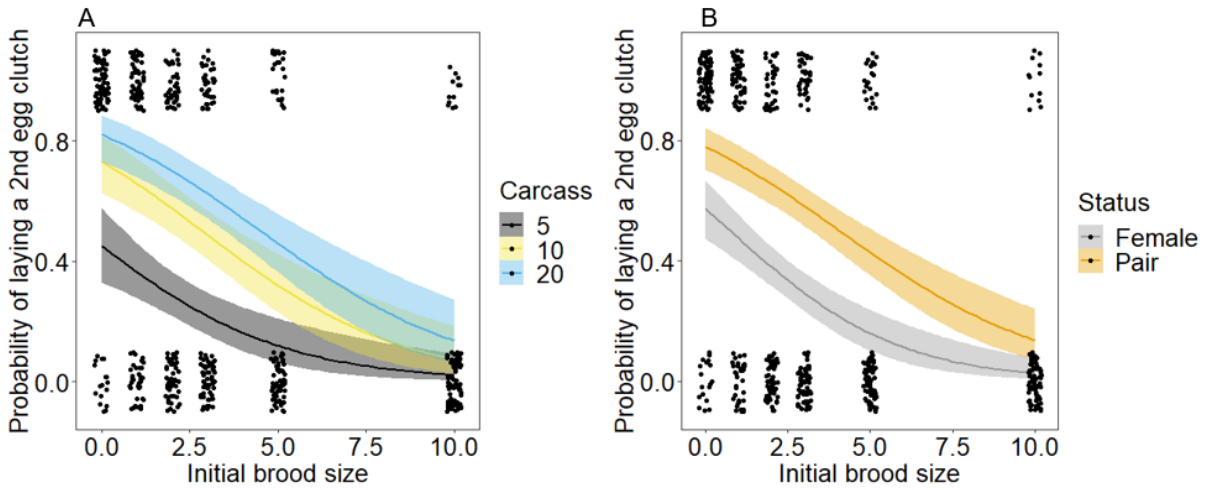

Figure 1.1: *Relationship between the probability of N. vespilloides females to lay a second egg clutch and initial brood size (A) on three different carcass sizes and (B) when breeding with a male partner or alone. The dots represent the original data, the lines represent the calculated regression lines and their respective 95% CI.*

Is there a parent-offspring conflict over the production of a second clutch?

As we predicted, when females responded to small broods by laying a second clutch, they frequently closed the feeding cavity (N = 145). In 37 cases, the entire brood even died. However, whereas the production of a second clutch had a negative impact on offspring fitness, it was beneficial to females, as they were able to produce an overall higher number of dispersing larvae (Wilcoxon rank-sum test; W = 14781, p < 0.001; Fig. 1.2A) and a higher number of offspring surviving to adulthood (Wilcoxon rank-sum test; W = 15096, p < 0.001; Fig 1.2B) than mothers that did not lay a second clutch but that continued to care for the current offspring. The finding of antagonistic fitness effects between parent and offspring was further

substantiated by our second experiment, which considered not only the number of dispersing larvae, but also their mass. Larvae of mothers that did not lay a second clutch but went on to care for their initial brood were characterized by a higher mean mass than the offspring of mothers that produced a second clutch (Welch-two-sample t-test; t = 2.19, df = 46.46, p-value = 0.03; Fig. 1.3A). However, mothers that laid a second clutch showed a higher carcass use efficiency than mothers that did not resume egg laying (Welch-two-sample t-test, t = 2.07, df = 44.39, p = 0.04; Fig. 1.3B). Our second experiment also showed that the production of a second egg clutch did not negatively affect the body weight of the females, as we could not find a difference in the overall weight change during the reproductive event between females that laid a second clutch and those that did not (two-sample t-test, t_{50} = -1.37, p = 0.17; Fig. 1.3C).

When looking at the different female 'strategies' in more detail, we found that, in the majority of cases (405 out of 544; Supporting information Fig. S1.1), active care for the initial brood and egg production were mutually exclusive events: Females either started to produce a second clutch and closed the feeding cavity of the carcass, or they accepted the initial brood without laying a second clutch. Surprisingly, we found that 91 females laid a second clutch but did not close the cavity (Supporting information Fig. S1.1), suggesting that females were able to produce further eggs, while caring for the current brood. However, when we analyzed the total number of dispersed larvae, we found that females that laid eggs but did not close the cavity raised a lower number of offspring than females that closed the cavity and laid a second clutch (Wilcoxon rank sum test, W = 7235, p < 0.001; Supporting information Fig. S1.2A). The same was true for the number of surviving offspring to adulthood (Wilcoxon rank sum test, W = 7366, p < 0.001; Supporting information Fig. S1.2B).

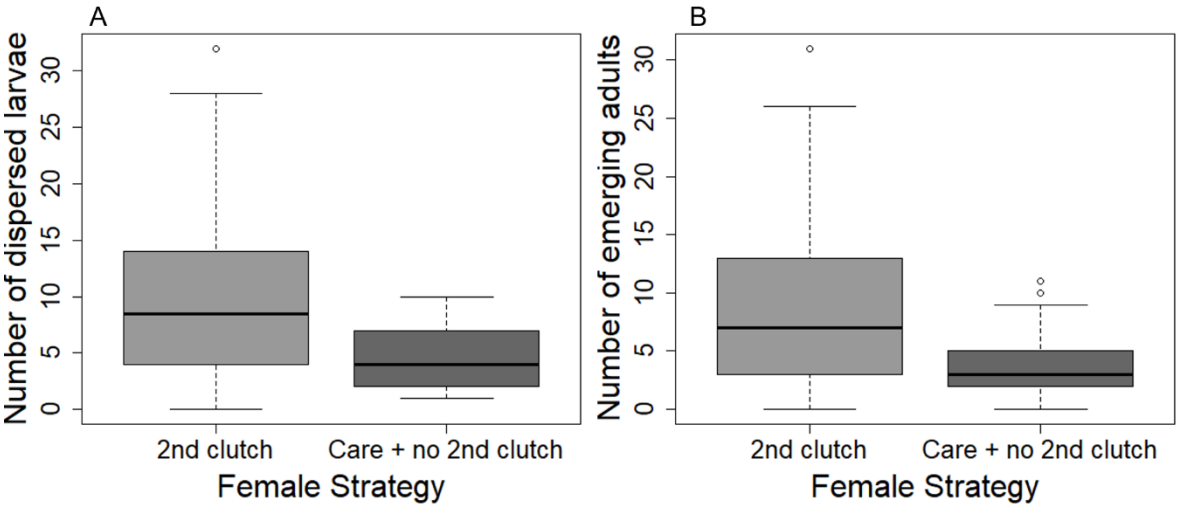

Figure 1.2: (A) Number of dispersed larvae and (B) emerging adults of those females that did not produce a second clutch but continued to care for the initial brood and those females that produced a second clutch.

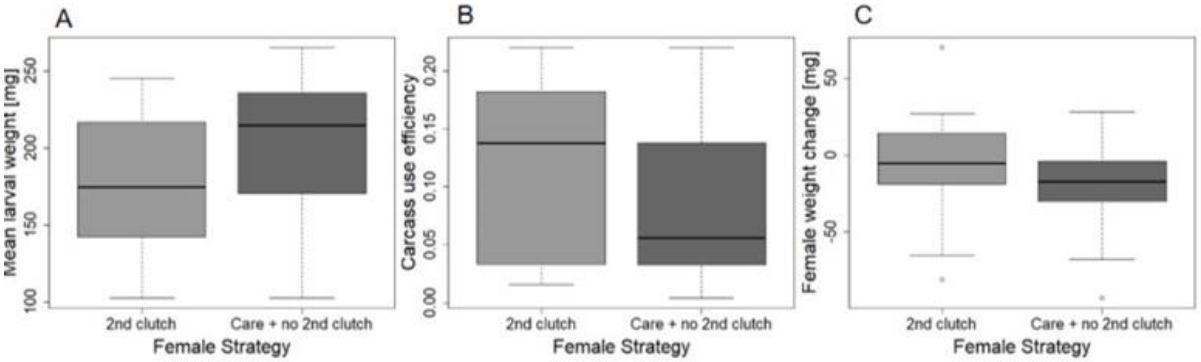

Figure 1.3: (A) Mean larval weight, (B) carcass use efficiency (total brood mass/carcass mass), and (C) body weight change during breeding of those females that did not produce a second clutch but continued to care for the initial brood and those females that produced a second clutch.

Is the conflict outcome closer to the parents' or the offspring optimum and does it differ between uni- and biparental families?

To examine in which direction the parent-offspring conflict is solved in uni- and biparental families, we first determined the 'larval tipping point', from which onward, the majority of females decided to invest in the current brood instead of producing further offspring. We found a tipping point of one larva for single caring females and five larvae for biparental females (Fig. 1.4A). Hence, a larger number of larvae were necessary to suppress egg laying under bi- than uniparental condition. In fact, not only did biparental females show a higher probability of laying a second clutch (Table 1.1, Fig. 1.1B) and of closing the cavity (GLM, $\mathrm{Chi}^2_{1,453}$ = 18.66, p

< 0.001), but these decisions also led to a larger number of dispersing larvae than seen in single females (Table 1.1, Fig. 1.5). However, under both family social conditions parents that did not produce a second clutch at their respective tipping point raised less larvae than parents of the control group (i.e., parents that were initially not provided with any larvae; uniparental: Wilcoxon rank sum test, W = 513, p < 0.0001; biparental: Wilcoxon rank sum test, W = 823.5, p < 0.0001; Fig. 1.4B). Hence, these results suggest that the conflict over the production of a second clutch is resolved closer to the offspring optimum.

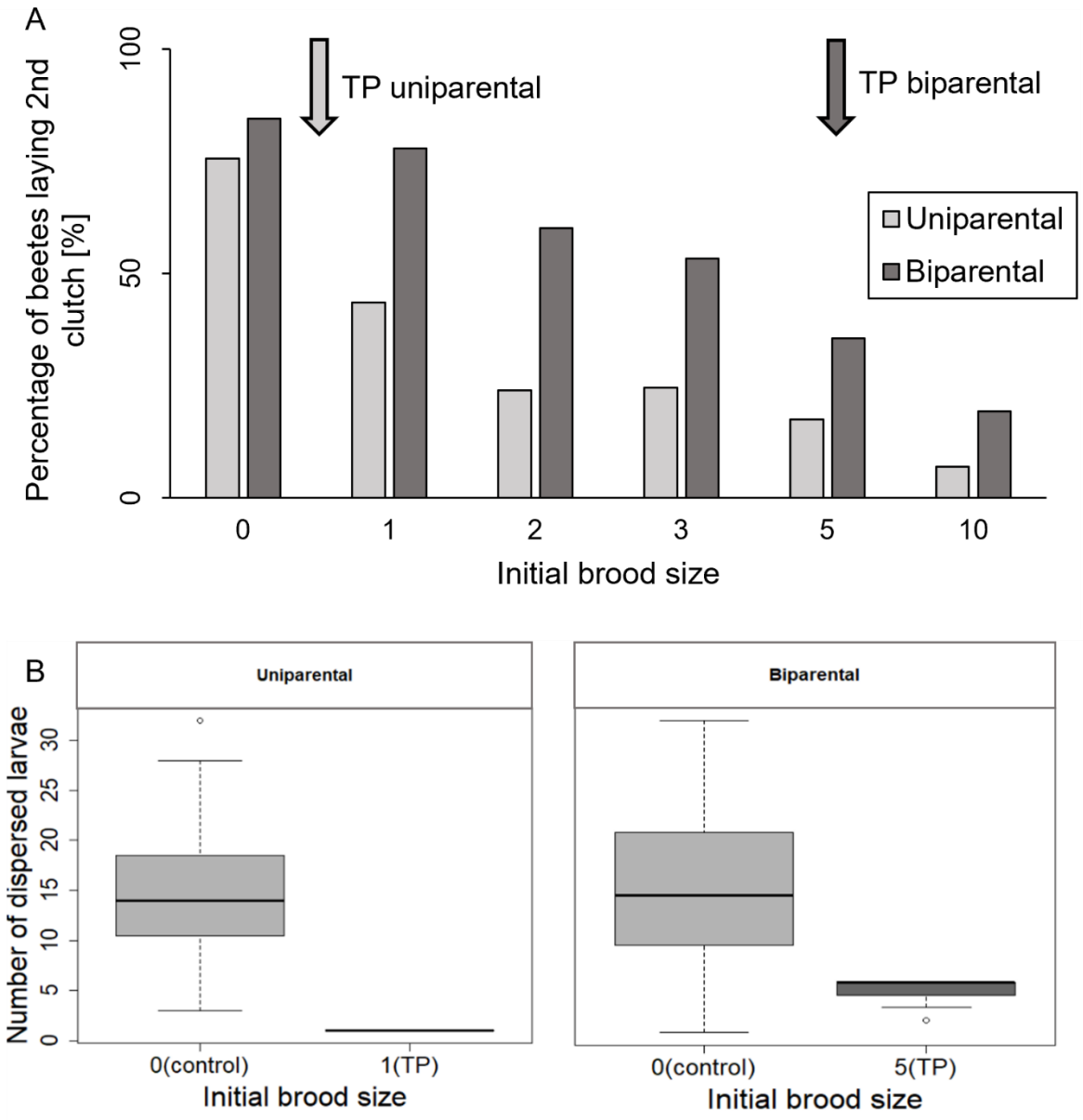

Figure 1.4: (A) Bar plot showing the tipping points of females in uniparental (gray) and biparental condition (dark grey). Arrows in respective colors mark the tipping points (= TPs), i.e., the initial brood size at which less than 50% of females resumed egg laying. Note that at both tipping points, the proportion of females that did not produce a second clutch was significantly smaller than in the respective control groups (i.e. the treatment in which brood size was zero

and females did not receive any larvae; uniparental females: Chi² = 9.70, p = 0.002, biparental females: Chi² = 22.41, p < 0.001). (B) Boxplots showing number of dispersed larvae of females of the control groups and females that did not produce a second clutch at the respective tipping points.

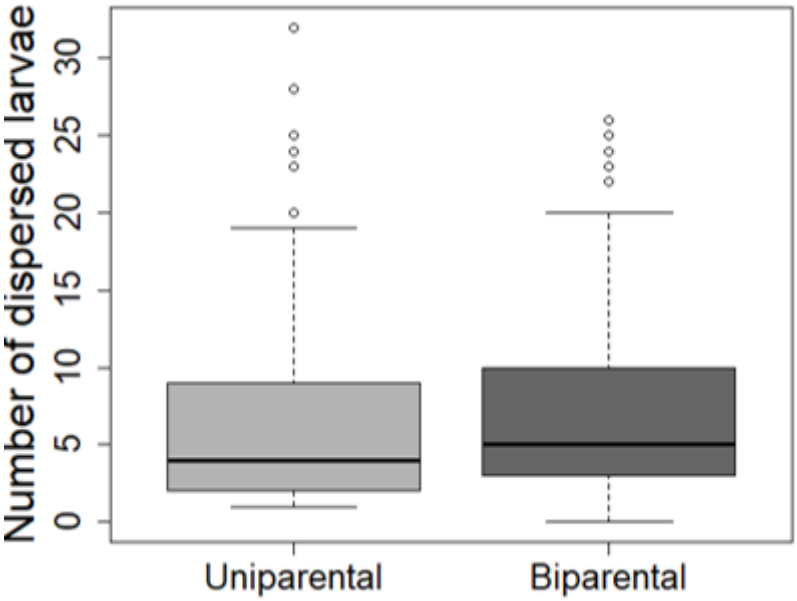

Figure 1.5: Boxplots showing the number of dispersed larvae produced by females under uni- and biparental conditions.

Discussion

Parent-offspring conflict is thought to be a key element of family life. However, empirical evidence is scarce, and it is usually unknown, which party wins the dispute[12]. Here, we found evidence for a parent-offspring conflict over resource allocation between current and future offspring. When confronted with a small brood, females frequently responded by laying a second clutch. However, the investment in a second brood caused a simultaneous increase in parental fitness and a decrease in offspring fitness: Females that stopped investing in their first brood and resumed egg laying had a higher fitness, but the current offspring suffered from discontinued feeding. This suggests a disagreement between parents and offspring over the production of a second brood. Our study furthermore suggests that the outcome of the conflict is closer to the offspring optimum under uniparental care but is shifted in the direction of the parental optimum in the presence of a male partner. Hence, the offspring might be capable of influencing the maternal investment decision, but this capability might be negatively affected by the presence of a second parent.

Vertebrate carrion is a valuable nutrient-rich resource, but corpses suitable for the reproduction of *Nicrophorus* are thought to be rare and difficult to monopolize and hence should be converted efficiently into offspring biomass[24]. When the mortality of eggs or larvae is high, such that fewer offspring are present than the carrion can support, female burying beetles have been observed to produce a second clutch[40,49,50]. We could confirm these previous findings, since nearly half of the female *N. vespilloides* tested responded to our resource and brood size manipulation by laying a second egg clutch. As predicted, we found that the probability of resuming egg laying increased with resource size and decreased with initial brood size. This suggests that females can assess brood and cadaver size and enhance the usage of the valuable resource by producing additional eggs. Indeed, our results revealed that females that responded to a low offspring-carrion ratio by resuming egg laying produced a higher number of dispersing offspring and converted more carrion food into offspring biomass than those that did not.

Optimal clutch size decisions have been studied intensively over the last few decades[51–55]. Many insects oviposit on food patches that represent finite resources and, similar to burying beetles, have been shown to have the ability to adjust the number of eggs laid according to the size and quality of the patch[56–58]. However, when elaborate parental care is involved, the optimal oviposition strategy can differ for parents and offspring, leading to a parent-offspring conflict[11,59,60]. Our results indeed highlight that the production of a second clutch enhanced maternal fitness while simultaneously reducing the fitness of the current offspring. Females that effectively resumed egg laying closed the feeding cavity in which the larvae reside and in several of these cases broods died. Eggert and Müller ([24]) suggested that the larvae suffocate in such a situation, their death being an accidental consequence of the parents' attempt to maintain resource quality for future offspring (see also[35]). Although the initial offspring suffered in our study, mothers produced more dispersing larvae and used the carcass more efficiently compared to mothers who left the feeding cavity open and continued to care for the first brood. Conversely, mothers that invested only in their first brood showed a lower fitness, but their offspring profited from prolonged care, since our second experiment revealed that those larvae had a higher average mass at dispersal. Consequently, we have found evidence for an inter-brood conflict over parental investment. Although the occurrence of an inter-brood conflict has been predicted by theory, it has seldom been shown in natural systems due to the experimental difficulties of demonstrating divergent fitness optima for parents and offspring. A study of

Kölliker et al. ([11]) also found evidence for an inter-brood conflict by examining the trade-off between offspring fitness and a parent's ability to produce a second clutch in earwigs *Forficula auricularia*. Interestingly, in contrast to our study, they found a conflict at the egg stage but not after the nymphs have hatched. Hence, our study contributes to our understanding of family living by empirically showing that parent-offspring conflict over the production of a second brood can also occur after the birth of offspring.

Our fitness analysis indicates that the outcome of the parent-offspring conflict is closer to the offspring optimum, since fewer larvae were necessary to inhibit oviposition than the carcasses would potentially support. In fact, in uniparental families one larva was already enough to suppress egg laying in more than 50% of the females and in biparental families five were required. In both cases, parents raised less larvae than parents from the control group, in which the first brood was removed and the majority of females resumed egg laying. Consequently, females would have benefited from producing a second clutch instead of continuing to care for small broods. The result is especially surprising as parents are thought to be more powerful than offspring and should be able physically to dominate their young[3,61]. But why do parents provide more care than they should? One possible explanation is that the investment optimum of females is closer to the offspring's interest than our fitness analysis suggests. When confronted with a small brood, females can optimize the number of larvae raised and hence the amount of carrion they convert into offspring by laying a second clutch. However, females might use a small brood as an indicator for a harsh environment (e.g. high risk of egg predation), low body condition or lack of sperm and hence, do not anticipate a larger second brood. Furthermore, our analysis only considers reproduction on the current carcass, and not future breeding success on new carcasses. Preserving the carcass for a second brood, which involves combatting microbial competitors and pathogens, might result in high energetic or physiological costs leading to a reduced future reproductive value and a lower lifetime reproductive success. Although this explanation seems plausible, some factors speak against it. First, we found no differences in body mass changes between females that produced a second clutch and those that had not, indicating equal energetic costs of both strategies. Secondly, females can produce up to five replacement clutches, if the first clutches are removed[40], which also argues against high costs.

An alternative explanation for our results might be that manipulative offspring signals are involved. Parent-offspring conflict is thought to drive the evolution of begging signals. Those

signals might honestly reflect the need of the offspring, but also might have the potential of manipulating parents into investing more than they should[3,13,62–66]. Although such manipulative agents have been considered to be evolutionary unstable (see also[67–69] for a debate about queen pheromones either acting as honest signals or manipulative chemicals), theoretical considerations suggest that offspring might indeed produce manipulative signals. Mas and Kölliker ([13]), for example, have proposed a hypothetical mechanism by which offspring-derived primer pheromones influence the hormone system of mothers' thereby suppressing egg production and maintaining parental care in the current brood. Although a tactile begging signal exists in burying beetles[36,70,71], it is currently unknown whether offspring also produce a chemical signal that influences maternal physiology and behavior. We know, however, from a previous study that larvae are able to affect maternal hormone titer and reproductive behavior[49]. Furthermore, burying beetle adults are known to produce a range of chemical signals that serve to coordinate mating and breeding, and hence, larvae probably also communicate chemically[45,49,72–76]. In agreement with our hypothesis, recent experiments indicate that mothers also respond to cues other than tactile begging behavior to adjust their provisioning rate[77,78]. Like us, the authors proposed that chemical cues play an important role in mediating parent-offspring interactions in burying beetles (see also[79]). We advocate that future studies investigate the existence and the effect of potential begging pheromones in parent-offspring associations in burying beetles.

Models of conflict resolution either predict that investment levels lie somewhere between parent and offspring optima or close to the parents' optimum[12], which appears to contradict our findings. However, there are also observations of other family systems, in which offspring seem sometimes to have the upper hand in the battle. For example, there is evidence that during mammalian pregnancy, embryos are capable of influencing maternal blood sugar levels and therefore maternal investment[80]. As a consequence, some mothers suffer from diabetes, but the offspring benefit from gaining more weight. In future work, it will be important to evaluate the outcome of parent-offspring conflict in a range of different species to obtain a more profound picture on how and in which direction the conflict is resolved.

A further key finding of our study is that the outcome of the conflict is shifted slightly towards the parental optimum in the presence of a male partner: more larvae were necessary to suppress the production of a second clutch in biparental than uniparental females. Biparental females were more likely to resume egg laying and, in consequence, produced a

larger number of dispersing larvae than uniparental ones. Since males contribute to offspring feeding, mother-offspring interactions are slightly reduced in the presence of males[38], and hence, offspring might have less opportunity to affect maternal reproductive physiology and behavior than in the absence of males. Interestingly, *N. vespilloides* offspring have been shown to preferentially beg towards females[81]. In view of our findings, such a discrimination between female and male parents should be highly adaptive, since only the interaction with mothers can affect maternal reproductive physiology and ensure that the females do not stop caring and resume egg laying.

Another possibility is that the presence of a male partner triggers a divergence of the maternal interest from the offspring interest rather than causing a shift of the conflict's outcome towards parents. The lack of hatchlings or the presence of only a few of them arriving at the carcass might serve as a cue for unfertilized eggs and sperm depletion, and a resumption of egg production might therefore be more advantageous after additional matings and sperm transfer. (i.e., in the presence of a male). In agreement with this hypothesis, Sakaluk et al. ([82]) found that widowed females were less likely to produce a replacement clutch once the first clutch had been removed[82]. It is also possible that with the male's help in carcass maintenance, males reduce the costs of producing a second clutch for females[83]. However, this explanation only holds when prolonged carcass maintenance entails substantial costs. Fruitful directions for future studies would be to test whether sperm depletion can play a role, and to assess whether prolonged investment in carcass maintenance entails higher costs for uniparental than biparental females.

Regardless of the mechanism behind the shift in the conflict outcome, our findings emphasize that we need to consider number of parents when studying parent-offspring conflict. Furthermore, our study highlights that males benefit from a prolonged association with the family. Previous studies found that offspring thrived equally well when reared by male-female pairs or single females on same sized cadavers in the absence of competitors[84–89]. The question of why males remain with females on the carcass for extended periods remained largely unsolved (but see[90] for possible synergistic effects of biparental care). One factor that might have promoted the evolution of extended male residency is that males benefit from carrion consumption[91]. However, our current study has revealed an additional important aspect that might explain the evolution of biparental care. In a case in which, for example, many eggs fail to hatch the presence of a male ensures the better utilization of the carcass, since

paired females have a higher tendency to produce a second clutch, leading to a larger number of dispersing offspring.

To conclude, our study has found evidence for parent-offspring conflict over the amount of parental investment. Whereas parents benefit from the production of a second clutch when initially confronted with a small brood, their current offspring suffer from this decision. Furthermore, our study indicates that the conflict outcome is closer to the offspring than the parents' optimum. Future studies will help to understand whether offspring-derived chemical signals can influence maternal physiology and behavior and target the trade-off between the care for current offspring and the production of new eggs. Finally, our study advances our understanding of parent-offspring conflict by demonstrating that the presence of a male partner can alter the conflict outcome either because investment levels shift in the direction of the parental optimum or because of an increased deviation between maternal and offspring interests. Such an effect on the conflict outcome could be revealed because biparental care is facultative in burying beetles. It would be interesting to investigate, whether parent-offspring conflict is also affected by the presence of a partner in other family systems with facultative biparental care. We also want to highlight that our results exposed substantial variation in the conflict outcome among families. As previously advocated by Kilner and Hinde ([12]), future studies in the field of parent-offspring conflict not only should focus on population averages but should also acknowledge individual differences. Burying beetles represent an ideal model system for studying such individual variation.

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Supplementary material

Publication 1: Parent-offspring conflict and its outcome under uni-and biparental care

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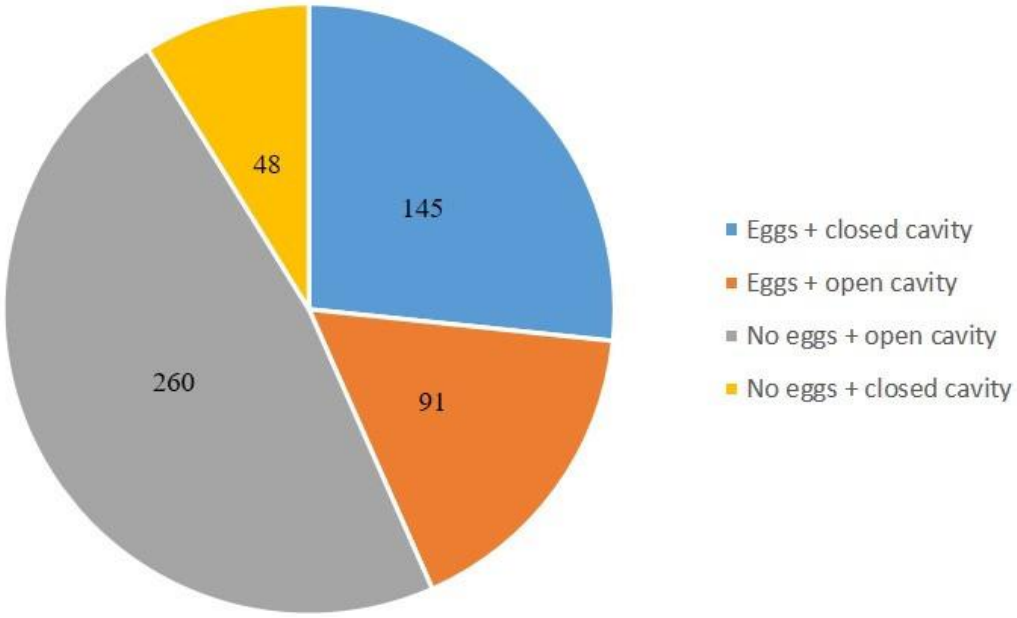

Figure S1.1: Proportions of different strategies used by females when confronted with small broods. Females produced a second clutch or did not and closed the cavity or did not.

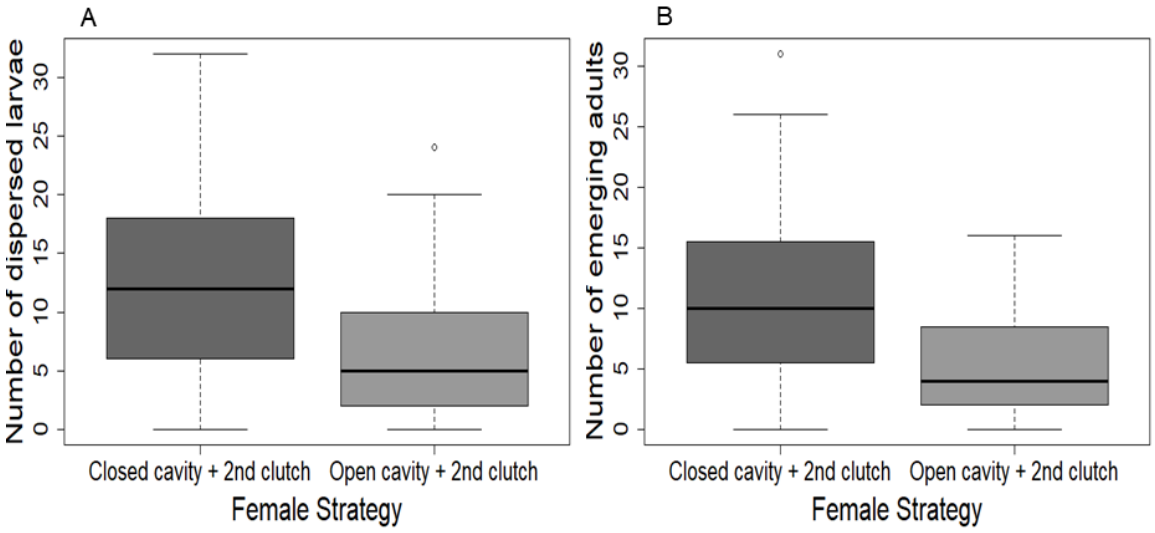

Figure S1.2: (A) Number of dispersed larvae and (B) emerging adults of females that produced a second clutch while leaving the feeding cavity open or closing the cavity.

Publication 2: Brood size, food availability, and body size affects male care decisions and offspring performance

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Author Contributions

J.S. and S.S. conceived and designed the study, J.S. collected the data with help of L.S., J.S. analyzed the data, all authors discussed the results, and J.S. wrote the manuscript with input from T.C. and S.S.

Own contributions

Concept and study design 50 %, data acquisition 50 %, data analyses and figures 95 %, interpretation of results 50 %, first draft of the manuscript 100 %, revision of the manuscript 50%.

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Abstract

Parental care strategies do not only vary greatly across species, but also within species there can be substantial between- and within-individual variation in parental care behaviour. To better understand the evolution of care strategies, it is crucial to determine how and when parents modify their behaviour in response to internal as well as environmental factors. Here, we investigated the effect of brood size, resource size and an individual's quality on care strategies of uniparental males and examined the downstream consequences on offspring performance in the burying beetle *Nicrophorus vespilloides*. Burying beetles breed on small vertebrate cadavers and, on average, males invest much less in care than females. Nevertheless, we found that uniparentally caring males were responsive to their social and non-social environment and adjusted the amount as well as the type of care to the size of the brood, the size of the cadaver and their own body size. Additionally, we show that the care strategies affected offspring performance. Specifically, males that cared longer had larger and more surviving larvae. Our results add to our understanding of plastic parenting strategies by showing that even the sex that provides less care can evolve a very flexible care behaviour.

Introduction

Parental care, which is a taxonomically widespread strategy, comprises all parental traits that increase the fitness of a parent's offspring (Meunier et al 2022; Trivers 1972; Smiseth et al. 2012; Wong et al. 2013). Parental care can occur before or after the birth of offspring and includes various behaviours, such as the protection of offspring from predators, the maintenance of a favourable microenvironment and offspring provisioning (Balshine 2012; Smiseth et al. 2012; Trumbo 2012) amongst others. Across the animal kingdom, female care is much more widespread than male care (Clutton-Brock 1991) and even in biparental families, males frequently invest less than females and desert the brood earlier or with a higher probability. The reason for this asymmetry is thought to lie in sex differences in the strength of sexual selection, in the association with the embryo, and in the certainty of parentage (Møller 1988; Møller and Birkhead 1993; Westneat and Sherman 1993; Westneat and Sargent 1996; Queller 1997; Liker et al. 2015; Royle et al. 2016). In some species, for example, mothers have a relatively high certainty about their maternity, whereas males – especially in species with

internal fertilization and multiple mating females – are often uncertain about their paternity (Trivers 1972; Westneat and Sherman 1993). Since parental care usually comes with costs to parents and ultimately lowers future reproductive success, offspring desertion can be considered as an investment in re-mating and future young (Székely et al. 1996). The decision how much to invest in a current brood has been predicted and/or empirically shown to depend on a multitude of factors, such as food availability, predation risk, paternity certainty, the value of the brood (e.g., brood size), parental quality (e.g., body size) or the partner's investment decisions (Wright and Cuthill 1990b; Erikstad et al. 1997; Hõrak et al. 1999; Neff 2003; Parejo and Danchin 2006; Magrath et al. 2007; Meunier and Kölliker 2012; Royle et al. 2014; Pilakouta et al. 2015). A range of studies have shown, for example, that parents compensate – at least partially – for the loss of their partner or adjust care behaviour according to brood size (Wright and Cuthill 1990a; Griggio and Pilastro 2007; Harrison et al. 2009; Wang W et al. 2021). Also, attributes of the parent itself, such as body size or condition, have been shown to impact care decisions. For example, in snow petrels (*Pagodroma nivea*), parents in good body conditions guard their chicks longer (Tveraa and Christensen 2002) and in pine engraver beetles (*Ips pini*) larger males leave their brood earlier than smaller ones (Robertson and Roitberg 1998). From an ultimate perspective, parents are expected to modify their care behaviour according to the costs and benefits of care, providing more care when the benefit is higher and providing less care when the costs are higher (Alonso-Alvarez and Velando 2012). However, in families in which males usually provide less care than females, males might not be selected to be as responsive as females to the social or non-social environment they experience. Males might also differ in their care behaviour due to other factors. For example, because males do not have the ability to increase brood size by laying additional eggs, males might abandon small broods more often than females. Although a range of studies have examined female care behaviour after mate loss (Fetherston et al. 1994; Markman et al. 1995; Sakaluk et al. 1998; Sanz et al. 2000; Smiseth et al. 2005; Cantarero et al. 2019; Wang W et al. 2021), only few studies have considered male investment decisions. Thus, it remains unclear how responsive they are towards environmental and internal cues and how they react when confronted with small broods in the absence of their female partner.

We addressed this knowledge gap using the burying beetle *Nicrophorus vespilloides* as a model organism. Burying beetles are an ideal system to examine paternal investment decisions as they provide elaborate biparental care (Wilson and Fudge 1984; Trumbo 1991;

Eggert 1992; Eggert and Müller 1997), uniparental female care (Scott and Traniello 1990; Smiseth et al. 2005; Steiger 2013), as well as uniparental male care (Scott 1989; Trumbo and Fernandez 1995; Ward et al. 2009; Parker et al. 2015; Luzar et al. 2017). *Nicrophorus* beetles use small vertebrate cadavers as a breeding resource (Pukowski 1933; Eggert and Müller 1997; Scott 1998b; Royle et al. 2013). Carrion is a nutrient rich but scarce resource leading to a high competition between con- and allospecific beetles for the monopolization of the resource. In the competition over carrion, the body size of beetles is a good predictor of the conflict outcome with larger individuals usually winning the contests (Otronen 1988; Trumbo 1990; Robertson 1993; Trumbo 1994). During breeding, parents transform the cadaver into an edible nursery for their offspring (Pukowski 1933; Scott 1998b; Trumbo and Robinson 2004; Royle et al. 2013; Duarte et al. 2021). Parents remove fur or feathers, treat the carcass with antimicrobial secretions, and create a feeding cavity, within which larvae aggregate to either feed themselves or to get fed by the parents (Müller et al. 1998; Smiseth and Bu et al. 2003; Smiseth, Darwell and Moore 2003; Shukla et al. 2018). When caring biparentally, females predominantly provide direct care (Scott and Traniello 1990; Smiseth and Moore 2004b; Smiseth et al. 2005; Walling et al. 2008), whereas males often focus on indirect care i.e., carcass maintenance and defence (Fetherston et al. 1990; Trumbo 1991, 2007). Additionally, it is known that males usually desert earlier than females (Bartlett 1988; Fetherston et al. 1990; Scott 1998a; Müller et al. 2007; Ward et al. 2009; Royle et al. 2014; Parker et al. 2015; Ratz et al. 2021). However, if the female deserts or dies, males are able to compensate for the loss of their partner (Bartlett 1988; Scott 1989; Trumbo and Fernandez 1995; Müller et al. 1998; Jenkins et al. 2000; Smiseth et al. 2005). Even though males can adjust their care behaviour based on the presence and absence of a partner, it is still unclear whether males are able to perceive and respond to other environmental factors, such as brood or resource size, or whether they base their care decisions on their own quality. From females we know that they are quite responsive to their breeding environment (Royle and Hopwood 2017). For example, if females face small brood sizes – due to hatching failure or predation - they frequently respond by producing a second egg clutch (Müller 1987; Sahm et al. 2022). They also take into account carcass size and lay additional eggs more frequently when monopolizing larger carcasses (Sahm et al. 2022). Although males are unable to increase the initial brood size, they still might show plastic behavioural responses towards broods of different sizes. Since *N. vespilloides* larvae can self-feed from the carrion resource and can partially survive in the absence of parents

(Capodeanu-Nägler et al. 2016), males might, for example, abandon small broods or spend less time caring for them. Especially on large carcasses, it is also possible that they kill small broods to preserve the carcass and attract a new female via their sex pheromone (Eggert and Müller 1989; Chemnitz et al. 2017). In fact, due to a high paternity uncertainty – as females mate multiple times in burying beetles (Müller and Eggert 1989; Müller et al. 2007; House et al. 2008) - males might be more likely to desert a given breeding attempt rather than care for few offspring. However, up until now empirical studies are missing about the effect of brood and resource size on the care decisions of male burying beetles. Furthermore, it is unclear whether males base their decisions on their own body size. Male body size likely affects the cost-benefit ratio of care and should therefore have an impact on paternal investment decisions.

Our study aimed to tackle the question of whether uniparental male care strategies are influenced by initial brood and carcass size as well as their own quality (i.e., body size) in the burying beetle *Nicrophorus vespilloides*. Additionally, we tested whether the care decisions of males affect offspring performance. Similar to a previous study (Sahm et al. 2022) that focused on female care strategies (under uni- and biparental conditions), we adopted a 5 x 3 factorial design and provided males with 1, 2, 3, 5, or 10 larvae and 5, 10 or 20 g carcasses. We used small brood sizes, because we were especially interested in the response of males when confronted with one or few offspring. Since body size shows high variation between individuals, we used the natural variation in male size instead of manipulating their size in our experiment (Steiger 2013). As response variables, we examined whether males cared for or deserted the brood and the time invested in caring. Furthermore, we evaluated offspring performance by recording the growth and survival of larvae. We predicted that single males would be more likely to stay and invest more time in larger broods and on larger carcasses. Since males of larger size have a higher chance of defending a carcass, and – similar to females (Steiger 2013) – might be able to raise larger larvae or suffer less costs from caring, we predicted that they would desert the brood less frequently or care longer than smaller males. Additionally, we predicted that a male's care strategy should affect offspring performance, with males that invest more time in brood care raising more or heavier offspring.

Material und Methods

Origin and husbandry of burying beetles

This study was conducted using an outbred laboratory population of *Nicrophorus vespilloides* kept at the University of Bayreuth, Germany. Experimental beetles belonged to the 5th generation of *N. vespilloides* descending from wild caught beetles captured in a forest near Bayreuth, Germany, in summer 2018. Beetles were held in small plastic containers (10 x 10 x 6 cm) filled with moist peat. Containers were stored in a climate chamber with a 16:8 dark: light cycle at 20 °C and fed twice a week using sliced mealworms (*Tenebrio molitor*).

Experimental design and procedures

We investigated the effect of initial brood size and carcass size on the behaviour of *N. vespilloides* males using a 5 x 3 factorial design: We manipulated the initial brood size (1, 2, 3, 5 or 10 larvae) as well as the size of a given mouse cadaver (~ 5, 10 or 20 g). We set up 16 pairs of beetles per treatment group using beetles aged between 20- and 30-days leading to a final sample size of 240 pairs. Since 27 replicates failed to produce any eggs, we conducted our analysis with a final sample size of N = 213.

At first, we paired unrelated virgin males and females in plastic containers (9.5 cm x 9.5 cm x 5.5 cm) each filled one-third with moist peat. To ensure sperm supply and egg fertilization, we allowed male beetles to mate multiple times with their female over a 72-hour period. Then we assigned a prior weighted mouse cadaver to each pair (mean ± SD, 5 g: 5.79 g ± 0.96; 10 g: 10.1 g ± 1.27; 20 g: 20.52 g ± 1.38). Since the aim of our study was to analyse the investment behaviour of uniparental males, we removed the female partner after a defined period of egg laying, i.e., 48 hrs after the pairs were provisioned with mice cadavers. To manipulate the initial brood size and to ensure that larvae can hatch in isolation, we separated the male beetles from their eggs, placing them, along with their respective carcass, in a new, equal plastic box filled with moist peat. Over a 48-hour period we checked the old boxes for newly hatched larvae at least every four hours day and night. We pooled synchronously hatching larvae in petri dishes containing a wet paper towel before randomly assigning them to the different treatment groups: Males received either 1, 2, 3, 5 or 10 larvae as initial brood size. Since *N. vespilloides* is

unable to differentiate between their own and unrelated foster offspring based on direct recognition cues (Müller and Eggert 1990), we were able to provide males with larvae of mixed parentage. Burying beetles frequently kill larvae that arrive sooner on the carcass than their own larvae would (Müller and Eggert 1990), therefore, we only provided males with an initial brood once their own larvae had hatched. The manipulation of brood size and the use of larvae of mixed parentage is a well-established protocol in burying beetles (Rauter and Moore 1999; Oldekop et al. 2007; Engel et al. 2016; Sahm et al. 2022).

To analyse how males respond to the different-sized broods, we checked the respective containers every six hours over a 72-hour period following the assignment of larvae to the males and subsequently every eight hours for another three-day period. Finally, we observed the treatments every twelve hours until the larvae dispersed from the carrion for pupation. During each observation, we recorded whether the male was off or on the carcass and whether he was at or inside the feeding cavity. Similar to Moss and Moore (2021), we categorized all instances, in which the male associated with the larvae and therefore was at or inside the feeding cavity as direct care and all instances in which males occurred at the carcass without contact to the larvae as indirect care. If the male left the carcass for more than 12 consecutive hours before the larvae dispersed from the carcass, we defined his behaviour as offspring desertion. As soon as larvae dispersed, we determined the pronotum width of males as a measure of body size. To investigate how male behaviour and size affects the survival and fitness of offspring we counted and weighed the dispersing larvae before we placed them in a new box containing moist peat, allowing them to pupate. Lastly, we recorded the number of eclosed adults.

Statistical analysis

All data were analysed and plotted using R version 3.6.1. We first examined whether initial brood size, carcass size, the interaction between initial brood and carcass size and a male's body size affected the care decisions of *N. vespilloides* males. Initial brood size and body size were entered as continuous variables and carcass size as a category. As response variables, we used (1) the decision of males to either desert or care for a given brood, (2) the duration of care, (3) the absolute amount of direct care (the number of observations in which the male was

found at or in the feeding cavity), and (4) the absolute amount of indirect care (the number of observations in which the male was found at the carcass but not at or in the feeding cavity). The variable 1 were fitted to a generalized linear model (GLM) with a binomial distribution, and 2-4 were fitted to a GLM with a Poisson distribution. We also tested whether there is an association between offspring development time (i.e., the time between larval arrival on the carcass and dispersal) and care duration using a GLM with a Poisson distribution.

For our analyses of offspring performance, we tested the effect of initial brood size, carcass size and male size on the average weight of dispersing larvae using a GLM with a Gaussian distribution, and on the larval survival rate till dispersal as well as from dispersal to eclosion using quasi binomial GLMs. Again, the interaction between carcass size and initial brood size was included in the models. Additionally, we calculated GLMs to investigate if male care duration, the absolute amount of direct care and the absolute amount of indirect care affected the average larval weight, the survival rate of larvae till dispersal and the larval survival rate till adulthood. Since the total number of observations per brood depended on the time of offspring dispersal and therefore varied between broods, we additionally calculated the relative amount of direct/indirect care by dividing their amounts by the sum of all observations. In separate GLMs we analysed the effect of the relative amount of direct/indirect care on the average larval weight and the survival rate of larvae till dispersal and from dispersal to eclosion. The analyses involving the duration and amount of care were conducted in separate GLMs because of a collinearity between these predictor variables (as well as between them and male body size). However, to examine whether any effects of the duration and amount of care on offspring performance depended on initial brood or carcass size, we re-ran all the models and included brood size, carcass size and the interaction between brood and carcass size as fixed effects.

All F-, χ^2- and p-values provided in the text and the tables were obtained using the "Anova" function of the R package '*car*' (Fox and Weisberg 2017). In addition, we calculated R^2 for generalized linear models in the R package '*rsq*' (Zhang 2018). We furthermore performed post hoc tests using the "emmeans" or the "emtrends" (for comparisons of slopes) function in the '*emmeans*'-package (Lenth 2019), if carcass size or the interaction between carcass size and initial brood size showed significant effects in our models. P-values were adjusted for multiple comparisons using the Tukey-method.

Results

The impact of initial brood, carcass, and body size on male care decisions

From the 213 males, only 4 were never observed on the carcass after providing them with larvae. Most males (N = 141) remained with the brood until larval dispersal and 71 males engaged in parenting but deserted the brood earlier. The probability of offspring desertion decreased with increasing initial brood size (Table 2.1, Fig. 2.1A). The highest percentage of offspring desertion was found with 1 larva as initial brood (48.8 %), while the lowest desertion of larvae was observed when males obtained 10 larvae to care for (15 %). Further, the frequency of offspring desertion was affected by carcass size (Table 2.1; Fig. 2.1B). We found that 26.15 % of males abandoned their offspring at 5 g, 18.92 % of males deserted at 10 g and 37.84 % left their offspring at 20 g carcasses (Table S2.1). Neither the male size nor an interaction between carcass and initial brood size showed an effect on male desertion (Table 2.1).

Table 2.1: Summary of the model on the effects of brood size, carcass size, male pronotum size and the interaction between carcass size and brood size on the probability of offspring desertion ($R^2 = 0.09$) and the care duration ($R^2 = 0.02$). Significant values are in bold.

Predictors	Offspring desertion			Care duration		
	Chi^2	df	p	Chi^2	df	p
Initial brood size	4.04	1	**0.04**	0.51	1	0.47
Carcass size	9.73	2	**0.008**	25.76	2	**<0.001**
Male size	0.25	1	0.62	28.15	1	**<0.001**
Carcass size x brood size	3.75	2	0.15	13.22	2	**0.001**

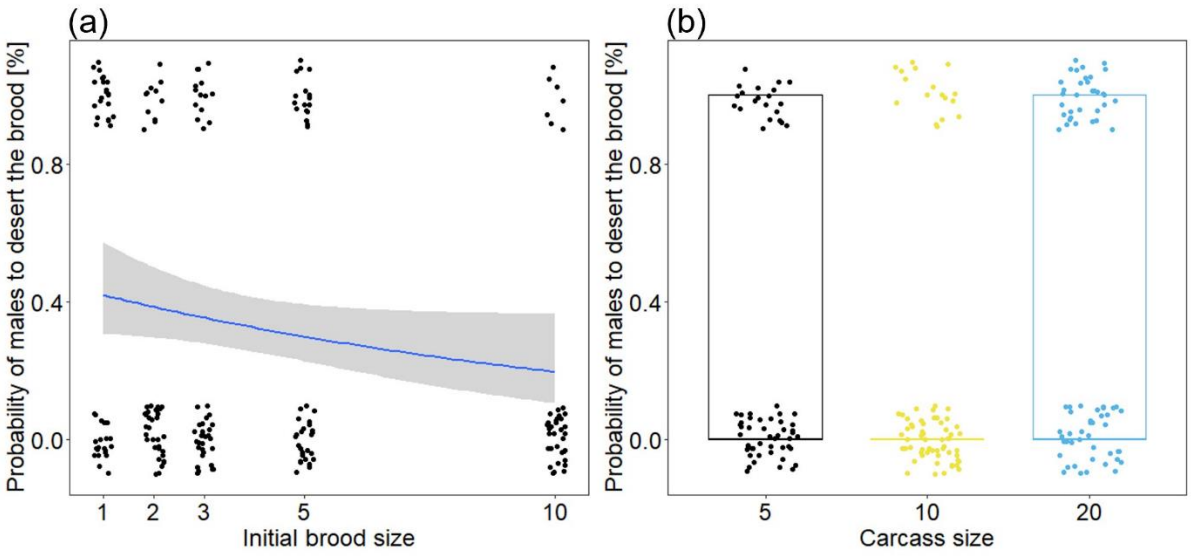

Figure 2.1: Relationship between the probability of N. vespilloides males to desert their given brood and (a) the initial brood size and (b) the carcass size. The dots represent the original data, the lines represent the calculated regression lines and their respective 95% CI.

The duration of male care was not affected by the initial brood size, but carcass size showed an effect (Table 2.1). More importantly, the interaction between carcass and initial brood size was significant (Table 2.1). On 5 g carcasses, care duration slightly increased with increasing initial brood size, on 10 g carcasses it remained constant across brood sizes and on 20 g carcasses, the time males spent with the brood decreased with increasing brood size (Fig. 2.2A). Slopes differed significantly between 5 and 10 g carcasses and between 5 and 20 g carcasses (Table S2.2). Male size affected care duration (Table 2.1), with larger males caring longer than smaller ones (Fig. 2.2B). We also found an association between offspring development time and care duration, with males remaining longer on the carcass when offspring dispersed later (GLM, $Chi^2_{1,212} = 1242.7$, $p < 0.001$).

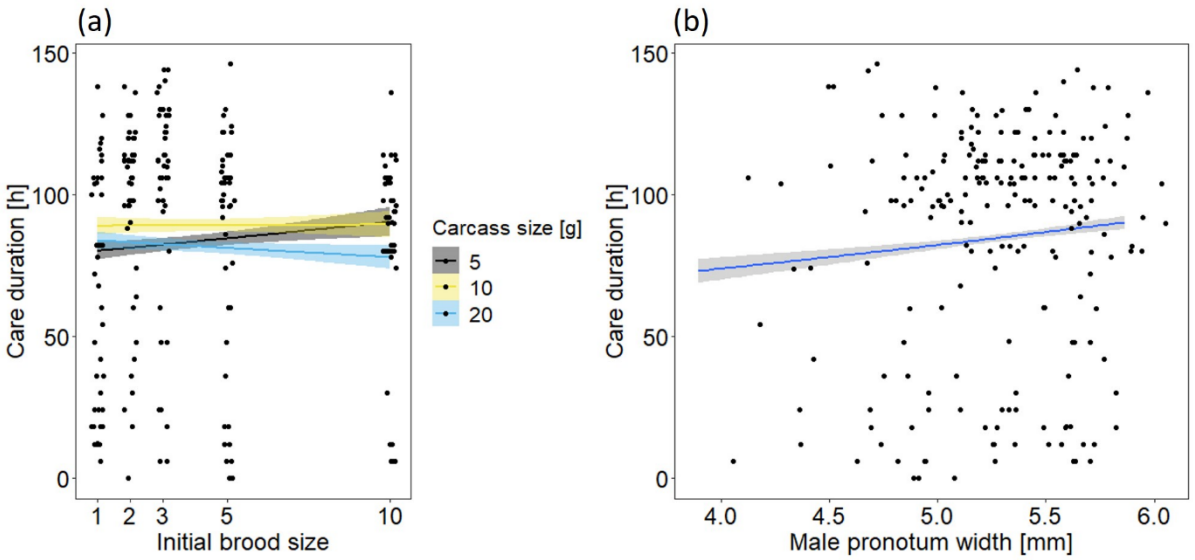

Figure 2.2: Relationship between the care duration of males and (a) the initial brood size on three different carcass sizes and (b) the size of males. The lines show the calculated regressions and their 95% CI, while the dots show the original data.

The amount of direct as well as indirect care was affected by the initial brood size. Surprisingly, males provided less direct care and more indirect care with increasing brood size (Table 2.2, Fig. 2.3A, 2.3B). The size of the carcass had only an effect on the amount of direct care (Table 2.2), with males showing more direct care with intermediate and large carcass size than on small carcasses (Table S2.3, Fig. 2.3C). There was no interaction effect between initial brood size and carcass size on both types of care (Table 2.2). Larger males provided direct care more often than smaller males (Table 2.2; Fig. 2.3D). Body size had no effect on the amount of indirect care (Table 2.2).

Table 2.2: Summary of the model on the effects of initial brood size, carcass size, male pronotum size and the interaction between carcass size and brood size on the amount of direct care (R^2 = 0.07) and indirect care (R^2 = 0.06) of males. Significant values are in bold.

Predictors	Direct care			Indirect care		
	Chi^2	df	p	Chi^2	df	p
Initial brood size	6.19	1	**0.01**	17.83	1	**<0.001**
Carcass size	20.94	2	**<0.001**	0.99	2	0.61
Male size	10.03	1	**0.001**	0.01	1	0.92
Carcass size x brood size	5.25	2	0.07	2.65	2	0.26

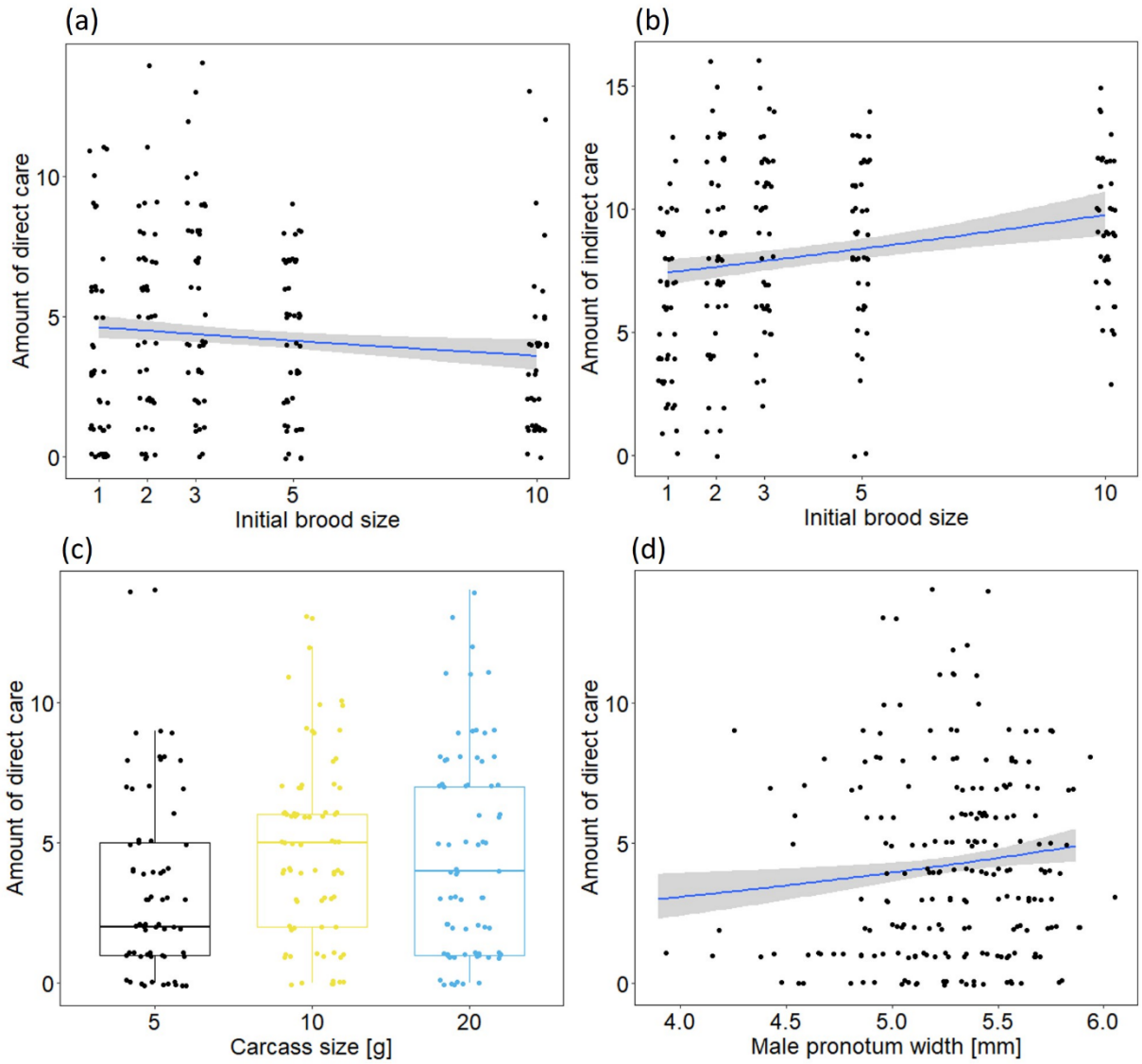

Figure 2.3: Relationship between the amount of (a) direct care and (b) indirect care with the initial brood size. (c) shows the relationship between the amount of direct care and the carcass size, and (d) the male size. The amount of direct care is the number of observations in which the male was found at or in the feeding cavity. The amount of indirect care is the number of observations in which the male was found at the carcass but not at or in the feeding cavity. The lines show the calculated regressions and their 95% CI, while the dots show the original data.

Offspring performance

We found that the average weight of the dispersing larvae decreased with increasing brood size (Table 2.3). This effect, however, depended on carcass size (Table 2.3). On 5 g carcasses, the average weight of dispersing larvae decreased with increasing initial brood size, whereas the average larval weight slightly increased on 10 and 20 g carcasses (Fig. 2.4A). Slopes differed significantly between 5 and 10 g carcasses and between 5 and 20 g carcasses (Table S2.4). Male

size showed no effect on the mean larval weight at dispersal (Table 2.3). We found that with increasing male care duration (GLM, $F_{1,191}$ = 7.49, p = 0.007, Fig. 2.4B) and with a higher absolute amount of direct care (GLM, $F_{1,191}$ = 9.94, p = 0.002, Fig. 2.4C), the dispersing larvae showed a higher average weight. Looking at the relative amount of direct care, we also found a positive effect on average larval weight (GLM, $F_{1,191}$ = 5.85, p = 0.02). Neither the absolute (GLM, $F_{1,191}$ = 2.1, p = 0.15) nor the relative amount of indirect care (GLM, $F_{1,191}$ = 0.53, p = 0.47) showed an effect on the average larval weight.

Table 2.3: Summary of the model on the effects of initial brood size, carcass size, male pronotum size and the interaction between carcass size and brood size on the average larval weight at dispersal (R^2 = 0.27) and the larval survival until dispersal (R^2 = 0.06). Significant values are in bold.

Predictors	Average larval weight			Survival until dispersal		
	F	df	p	F	df	p
Initial brood size	8.01	1	**0.005**	6.21	1	**0.01**
Carcass size	1.32	2	0.27	0.46	2	0.63
Male size	0.06	1	0.8	7.83	1	**0.006**
Carcass size x brood size	28.17	2	**<0.001**	0.17	2	0.84

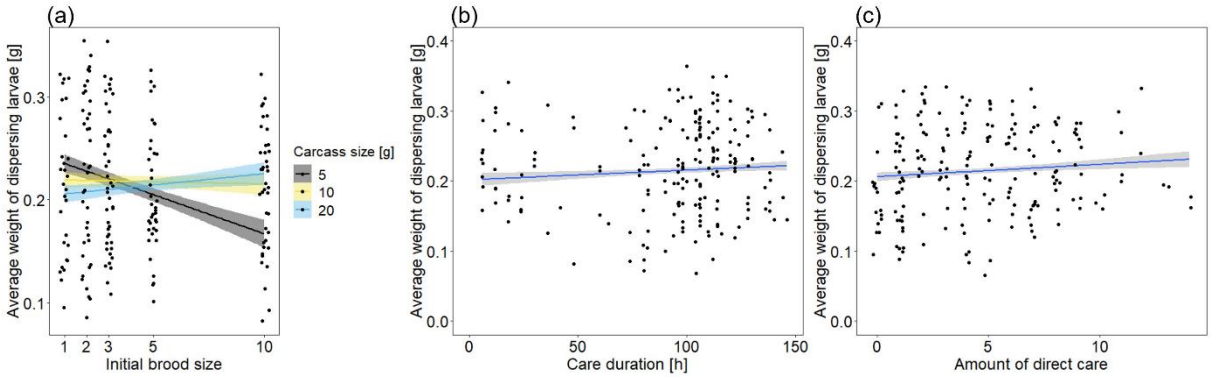

Figure 2.4: Relationship between the average weight of dispersing larvae and (a) the initial brood size on three different carcass sizes, (b) the care duration of males, and (c) the amount of direct care. The amount of direct care is the number of observations in which the male was found at or in the feeding cavity. Shown are the calculated regression lines and their respective 95% CI. The dots represent the original data.

Larval survival rate until dispersal (Table 2.3, Fig. 2.5A) and from dispersal to eclosion (Table S2.5, Fig. S2.1A) was higher with increasing brood size. Carcass size had no effect on the survival rate till dispersal (Table 2.3) and from dispersal to eclosion (Table S2.5). We found no effect of the interaction between the initial brood and carcass size on survival rate until dispersal (Table 2.3) and from dispersal to eclosion (Table S2.5). Larval survival rate until

dispersal was higher if the caring male was larger (Table 2.3, Fig. 2.5B). Male size, however, did not affect larval survival rate from dispersal to eclosion (Table S2.5).

The duration of male care had a positive effect on the survival rate of larvae until dispersal (GLM, $F_{1,212} = 39.63$, $p < 0.001$, Fig. 2.5C) and from dispersal to eclosion (GLM, $F_{1,156} = 24.44$, $p < 0.001$, Fig. S2.1B). The absolute amount of direct as well as indirect care had a positive effect on larval survival until dispersal (direct: GLM, $F_{1,212} = 22.17$, $p < 0.001$, Fig. 2.5D; indirect: GLM, $F_{1,212} = 38.78$, $p < 0.001$, Fig. 2.5E) and from dispersal to eclosion (direct: GLM, $F_{1,156} = 7.87$, $p = 0.006$, Fig. S2.1C; indirect: GLM, $F_{1,156} = 39.21$, $p < 0.001$, Fig. S2.1D). Further, the relative amount of direct care had a positive effect on the larval survival until dispersal (GLM, $F_{1,212} = 7.55$, $P = 0.006$) but no effect on the survival rate from dispersal to eclosion (GLM, $F_{1,156} = 0.85$, $p = 0.36$). In contrast, the relative amount of indirect care had neither an effect on the survival rate until dispersal (GLM, $F_{1,212} = 3.33$, $p = 0.07$) nor from dispersal to eclosion (GLM, $F_{1,156} = 2.63$, $p = 0.11$).

When we included brood and carcass size in all the models that tested for effects of duration or amount of care on offspring performance, all the significant effects remained with one exception: the effect of the relative amount of direct care on average larval weight was not significant anymore.

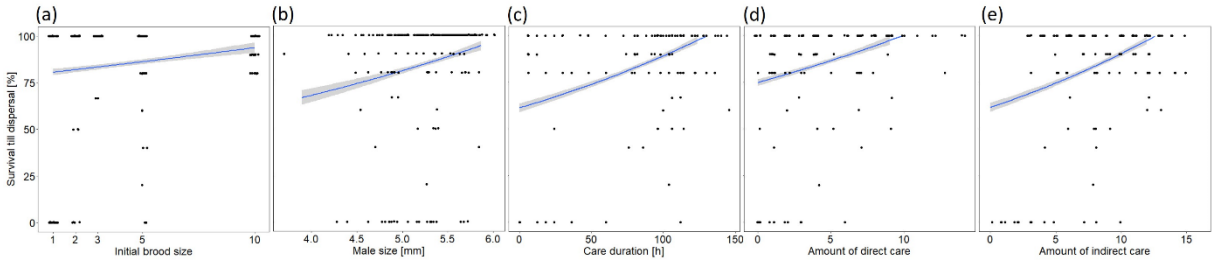

Figure 2.5: Relationship between the larval survival rate until dispersal and (a) the initial brood size, (b) the size of males, (c) the duration of care, (d) the amount of direct care by males, and (e) the amount of indirect care from males. The amount of direct care is the number of observations in which the male was found at or in the feeding cavity. The amount of indirect care is the number of observations in which the male was found at the carcass but not at or in the feeding cavity. Shown are the calculated regression lines and their respective 95% CI. The dots represent the original data.

Discussion

In this study, we investigated factors influencing the care strategies of uniparental males and their downstream consequences on offspring performance. By analysing more than 200 burying beetle families, we found that initial brood size, carcass size and male body size affected the amount and type of care males provided. Furthermore, we found that males that cared longer and showed a higher amount of direct care raised more and heavier larvae. Thus, our study shows that uniparental males are plastic in their care strategies, adjusting the amount and type of care behaviour to the value of the brood and the resource as well as their own quality, and these adjustments appear to have consequences for offspring survival and fitness.

Our first main finding was that a surprisingly high number of males stayed and cared for a given brood until larval dispersal and this despite the small initial brood sizes used in the experiment. Even when only one larva arrived at the carcass, about half of the males decided to provide care and did not desert the brood. The reason for this is likely the low abundance of suitable breeding resources in nature. Small vertebrate cadavers are nutrient rich but ephemeral and unpredictably distributed resources. The probability to find a new cadaver and monopolize it is low, which makes staying on an already found breeding resource and raising a given brood likely a beneficial strategy. Moreover, previous studies revealed that males also benefit personally from remaining with the brood because they themselves can feed from the carrion resource. This, in turn increases their attractiveness to females as they are able to produce a higher quantity of their sex pheromone after having reared a brood (Chemnitz et al. 2017; Keppner and Steiger 2021).

Further we found that brood size affected male care strategies. Males deserted a brood less frequently and increased their amount of indirect care with increasing brood size. This suggests that uniparental *N. vespilloides* males - similar to females (Sahm et al. 2022) - can estimate the value of the brood and make decisions based on it. Our result is in line with a previous study which also showed that uniparental males abandoned smaller broods more frequently than larger broods (Ward et al. 2009). In general, our result adds to growing evidence that males are sensitive to social cues and invest according to the size and therefore the value of a brood. A brood-size dependent male care strategy has, for example, also been found in snail kites (Beissinger 1990), in bluegill sunfish (Coleman et al. 1985; Coleman and

Fischer 1991) or in sand gobies (Forsgren et al. 1996). Surprisingly, although we found that males increased their amount of indirect care with increasing brood size, brood size had a negative effect on the amount of direct care. We would have expected that uniparental males - similar to uniparental females or biparental males – spent more time provisioning larvae when brood size increased (Rauter and Moore 2004; Smiseth and Moore 2004a; Smiseth et al. 2007; Wang G et al. 2021; Wang W et al. 2021). Also, in many birds increasing brood size usually results in an increased provisioning rate by parents (Neuenschwander 2003; Ardia 2007; García-Navas and Sanz 2010). One explanation for our result might be that we did not distinguish if males provisioned themselves or their offspring when they visited the feeding cavity (scored as direct care). To save more food for their offspring, it is possible that uniparental males reduce the amount of carrion consumed by themselves with increasing brood size by visiting the feeding cavity less often.

Our next significant finding was that male care strategies were affected by carcass size. Males deserted more frequently from large carcasses than from intermediate sized carcasses. They also provided more direct care on intermediate carcasses than on small carcasses. A likely explanation for these findings is that tending broods on intermediate sized carcasses results in the best cost-benefit ratio of care. Small carcasses have less food available and can lead to a low-quality brood. Large carcasses, on the other hand, might be very costly to maintain and defend, making it unprofitable to raise broods of small sizes. That larger carcasses are more costly to prepare was also suggested in previous studies (Xu and Suzuki 2001; de Gasperin and Kilner 2015; Ratz et al. 2021). For example, de Gasperin and Kilner (2015) found that the preparation of larger carcasses resulted in a reduced lifespan of male beetles. In general, our results highlight that males are able to evaluate resource size and are consistent with the results of previous studies that examined male care behaviour under biparental care. Bartlett (1988), Kishida and Suzuki (2010) and Ratz et al. (2021) for example, found that males are sensitive to carcass size and leave the brood earlier as carcass mass decreases. Males of other species are also known to monitor resource availability (e.g., Barbasch et al. 2020) or other non-social environmental factors (e.g., Green and McCormick 2004) and adjust care behaviour accordingly. For example, male glass frogs increase both the frequency and the amount of time spent incubating eggs when humidity is declining (Delia et al. 2013).

Another key finding of our study was that male care strategies depended on a male's body size, albeit the effects were relatively small. Larger males cared longer and provided more direct care than smaller males. This observation is consistent with the idea that larger males suffer lower costs from maintaining the carcass and caring for the brood or have a greater benefit from doing so. A previous study on females found that larger females were able to raise heavier larvae, likely because they have a greater capacity to feed the offspring (Steiger 2013). Likewise, larger males might be able to ingest, process and regurgitate a higher amount of food, leading to a higher larval mass and making it more profitable to defend the brood for a longer time than smaller males. Although we could not find any effect of male body size on offspring mass, we found a positive effect on larval survival until dispersal, a result that is in line with our hypothesis. However, based on our study it is impossible to disentangle cause and consequences. Males might stay longer because they have a higher reproductive output, but it is also possible that the higher reproductive output is the consequence of their prolonged stay. Furthermore, there are also alternative explanations for our results. Larger males might have a higher chance to defend the brood and resource from intruders and therefore larger males might tend to stay longer with their brood. This seems likely, as previous studies in burying beetles found that larger males are indeed predominantly the winners in competitions over resources (Bartlett and Ashworth 1988; Otronen 1988; Luzar et al. 2017). We also need to consider the possibility that larger males remained longer and showed a higher presence in the feeding cavity (scored as direct care) because larger males need to consume more carrion food to replenish their energy reserves. In fact, Pilakouta et al. (2016) showed that larger parental beetles spent more time feeding from the carcass and gained more weight during the breeding event than smaller ones. Whether the prolonged stay of larger males is due to selfish reasons or for the benefit of the larvae needs to be evaluated in future studies. Interestingly, a study of Smith et al. (2014) found an opposite effect of male body size on residency time, with smaller males remaining longer with the brood than larger ones. The seemingly contradictory result might simply be explained by species differences in the effect of body size on care duration or residency time: the study of Smith et al. (2014) focused on *N. orbicollis*, a species of larger average body size than *N. vespilloides*. However, it is also possible that the effect varies between populations and depends on the local intensity of inter- and intraspecific competition for carrion resources and the availability of mating partners. Recent studies found that even populations in close proximity can differ in their mean body size and breeding strategy, likely

caused by differences in population densities and community structure (Sun et al. 2020). In general, if male size determines the ability to secure further mating or breeding opportunities, the importance of an individual's size for paternal care decisions might vary according to the abundance of potential mates or breeding resources (see also Robertson and Roitberg 1998).

Concerning offspring performance under uniparental male care, we found that the interaction between initial brood and carcass size showed a significant effect on offspring mass. On small carcasses brood size had a negative impact on larval mass, whereas on larger carcasses brood size had a positive effect. This result is in line with a study of Schrader et al. (2015), which found that brood size had a beneficial effect on larval mass at lower larval densities (i.e. number of larvae per gram carcass) but a detrimental effect on larval mass at higher larval densities. Schrader et al. (2015) argued that this likely reflects a density dependent shift from sibling cooperation to competition. If the number of larvae per gram carcass is very high, the larvae inevitably compete for food, because there is a limited amount of food available for each larva. If larval density is low, they benefit from having siblings because collectively they are more efficient in utilising the resource (Prang et al. 2022). However, currently there is only mixed evidence for sibling cooperation in *N. vespilloides*. A study of Magneville et al. (2018), for example, did not find any positive effect of brood size on offspring performance and Prang et al. (2022) only found signs of sibling cooperation when larvae developed on a parentally unprepared carcass. Interestingly, the study of Schrader et al. (2015) found a positive effect of brood size on offspring mass only in the absence of parents, but not under biparental care. Here, we revealed a positive effect of brood size on offspring mass under uniparental male care. Moreover, not only offspring mass but also offspring survival increased with brood size. However, based on our data we currently cannot say whether the positive effect of brood size on offspring performance is caused by sibling cooperation. It is also possible that males invest more in care when confronted with larger broods. In fact, although our own study did not find any positive effect of brood size on care duration or the amount of direct care, we found that males were less likely to desert larger broods and showed more indirect care. It is also possible that males actively cannibalized small unrentable broods, as we had some broods with no surviving larvae. Irrespective of the underlying mechanisms – which must be studied in more detail - our result shows that under male care, offspring benefit from having siblings when the carcass is large enough. This result has important wider implications for our understanding of the evolution of family life. When it comes to sibling interactions past studies have

predominantly focused on sibling competition (Kramer and Meunier 2018). However, siblings can profit from each other either because they actively cooperate, for example, by sharing food (Falk et al. 2014) or cleaning each other (Roulin et al. 2016) or because larger broods are able to extract a higher per-capita investment from their parents than smaller ones.

Another key finding of our study was that offspring raised by larger males showed a higher survival rate until dispersal. We suggest two non-exclusive hypotheses to explain this result. First, larger males might have a higher efficiency to allocate food to their offspring, as they might be able to predigest a higher amount of food in a specific time. This idea has also been proposed by Steiger (2013), who found that larger burying beetle females raised heavier offspring. In our current study, we found that larger males cared longer and showed a higher amount of direct care. Hence, a second possibility is that the higher survival rate is simply the consequence of the higher amount of time invested in care. That larger males are better fathers has also been found in other species. For example, in biparental dung and tenebrionid beetles, females assisted by larger males produced heavier offspring (Hunt and Simmons 2000; Heg and Rasa 2004) and in a sand goby, larger males lost less eggs during egg guarding than smaller ones (Lindström and Hellström 1993; Hunt and Simmons 1998).

Finally, our study showed that an increased amount of direct and indirect care and an increased care duration of uniparental males correlated positively with larval mass and survival. This result indicates that the males do not only remain with the carrion resource due to personal benefits and confirms that the variables we measured indeed reflects parental care, i.e., parental traits that enhance offspring fitness. That offspring performance is positively linked to the amount of male care has also been shown in other taxa. For example, in a Neotropical glass frog, early male removal resulted in a higher embryo mortality due to dehydration than later removals. Also in a range of biparental birds (see Bart and Tornes 1989 and references therein) and in the biparental California mouse, *Peromyscus californicus* (Gubernick and Teferi 2000), male removal reduced offspring survival.

It has been widely accepted that parenting strategies can be complex and flexibly adjusted to the social and non-social environment as well as to the caregiver's own quality or condition (Royle et al. 2014; Royle and Hopwood 2017). However, how responsive parents are, depends on how strong selection has acted on behavioural plasticity in the past. Furthermore, a high

responsiveness towards the social environment might limit the plasticity of care behaviour towards the non-social environment and vice versa (Royle et al. 2014). Here we found that even though male uniparental care is thought to be rare in nature, burying beetle males are very responsive and adjust their care strategies to both their social (offspring) as well as their non-social (carrion resource) environment. They even base their decisions on their own body size. Earlier studies also found that males are plastic in their response, providing more care in the absence than in the presence of females (Bartlett 1988; Smiseth et al. 2005; Royle et al. 2014; Moss and Moore 2021). These results, together with the previous finding that male uniparental care is as effective as female uniparental care (Parker et al. 2015), might indicate that male uniparental care occurs more frequently than previously thought. However, there are also signs that males are not as responsive as females to offspring need (Suzuki and Nagano 2009; Royle and Hopwood 2017; Moss and Moore 2021), suggesting that selection on plasticity acts much stronger on the primary caregiver, likely because higher offspring contact can promote the evolution of fine-tuned parent-offspring communication. In general, who cares and to which degree is very flexible in burying beetles, but which factors promote or impede flexible parenting strategies in caring species is largely unknown and requires further research. We especially need more studies quantifying the level of responsiveness exhibited by both male and female parents. To this end, the removal of the female parent or the primary caregiver – as done in our study – could be a valuable tool to reveal how responsive the secondary caregiver is towards environmental or intrinsic cues. This is because the presence of the primary caregiver likely masks or limits the displayed responsiveness of the second parent.

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Supplementary material

Publication 2: Brood size, food availability, and body size affects male care decisions and offspring performance

Jacqueline Sahm, Taina Conrad, Larissa Scheu, Sandra Steiger

Table S2.1: Post hoc pairwise comparisons for the effect of carcass size (5 g, 10 g, 20 g) on the probability of offspring desertion.

response	predictor	estimate (±SE)	z-ratio	p-value
offspring desertion	carcass size			
	10 vs 20	-1.18 (0.37)	-3.17	**0.004**
	10 vs 5	-0.56 (0.39)	-1.42	0.33
	20 vs 5	0.63 (0.36)	1.73	0.2

Table S2.2: Post hoc pairwise comparisons for the interaction effect of carcass size (5 g, 10 g, 20 g) and initial brood size on care duration (i.e., level-wise comparisons of slopes).

response	predictor	estimate (±SE)	z-ratio	p-value
care duration	carcass size x brood size			
	10 vs 20	0.01 (0.006)	1.26	0.42
	10 vs 5	-0.01 (0.06)	-2.44	**0.04**
	20 vs 5	-0.02 (0.06)	-3.59	**0.001**

Table S2.3: Post hoc pairwise comparisons for the effect of carcass size (5 g, 10 g, 20 g) on the amount of direct care.

response	predictor	estimate (±SE)	z-ratio	p-value
direct care	carcass size			
	10 vs 20	0.02 (0.08)	0.25	0.97
	10 vs 5	0.36 (0.09)	4.19	**<0.001**
	20 vs 5	0.34 (0.09)	3.93	**<0.001**

Table S2.4: Post hoc pairwise comparisons for the interaction effect of carcass size (5 g, 10 g, 20 g) and initial brood size on average larval weight (i.e., level-wise comparisons of slopes).

response	predictor	estimate (±SE)	z-ratio	p-value
average larval weight	carcass size x brood size			
	10 vs 20	-0.002 (0.001)	-2.07	0.1
	10 vs 5	0.007 (0.001)	5.32	**<0.001**
	20 vs 5	0.01 (0.001)	7.36	**<0.001**

Table S2.5: Summary of the GLM predictors affecting the survival of larvae from dispersal to eclosion (R^2 = 0.19). Significant values are in bold.

Predictors	Survival to eclosion		
	F	df	p
Initial brood size	10.02	1	**0.002**
Carcass size	2.86	2	0.06
Male size	1.71	1	0.19
Carcass size x brood size	0.51	2	0.6

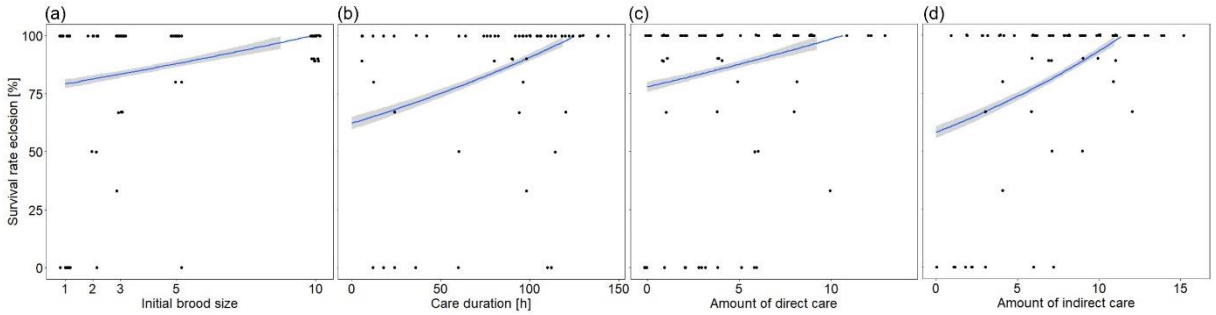

Figure S2.1: Relationship between the larval survival rate from dispersal to eclosion and (a) the initial brood size, (b) the care duration, (c) the amount of direct care and (d) the amount of indirect care. The amount of direct care is the number of observations in which the male was found at or in the feeding cavity. The amount of indirect care is the number of observations in which the male was found at the carcass but not at or in the feeding cavity. The dots represent the original data and we calculated regression lines and their respective 95% CI.

Publication 3: The scent of offspring: chemical profiles of larvae change during development and affect parental behavior in a burying beetle

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Author Contributions

S.S. conceived the study, J.Sahm and S.S. designed the study, J.Sahm collected the data with help of E.G. B.B., J.Sahm, E.M.G., T.C., M.S. and J.Stökl analysed the data, and J.Sahm, M.S., J.Stökl and S.S. wrote the manuscript. All authors read and approved the final manuscript.

Own contributions

Concept and study design 0 %, data acquisition 40 %, data analyses and figures 80 %, interpretation of results 50 %, first draft of the manuscript 95 %, revision of the manuscript 50%.

Abstract

Chemical cues and signals, especially in insects, play a pivotal role in mediating interactions between individuals. Past studies have largely focused on adult semiochemicals and have neglected those of juvenile stages. Especially in the context of parental care, larval odor might have a profound impact on parenting behavior, guiding parents in how much resources they should allocate to the different developmental stages. However, whether ontogenetic changes occur in subsocial species and whether larval-emitted scents influence parent-offspring interactions is largely unknown. Using three different sampling techniques, we analyzed the cuticular and VOC profile of the three larval instars of the burying beetle *Nicrophorus vespilloides*, which is known for its elaborate parental care. We found distinct differences in the cuticular and VOC profiles across the three larval stages. Second instar larvae, which receive more frequent feedings from parents than the other larval stages, released greater amounts of acetophenone, methyl geranate, and octanoic acid isopropyl ester than the first and third instar. Additionally, using a newly developed bioassay with automated video tracking, we found that adding the odor of second instar larvae to first instar larvae increased the number of maternal feeding trips. Our results suggest that the odor produced by larvae plays an important role in mediating parent-offspring interactions. Given these findings, burying beetles might emerge as a promising candidate for identifying a potential begging pheromone.

Introduction

Parental care occurs in many taxa and can be provided pre- or postnatally. Forms of care can range from protecting offspring from predators to providing food for the young (Clutton-Brock 1991; Balshine 2012; Smiseth et al. 2012; Trumbo 2012). Parental care increases offspring fitness (Trivers 1972; Smiseth et al. 2012; Wong et al. 2013) but also includes costs for parents in form of used resources, time, and energy, and ultimately reduces their residual reproductive value (Alonso-Alvarez and Velando 2012). To maximize the net benefit of care, recognition and communication between family members is essential (Ingold 1973; Hepper 1986; Waldman 1987, 1988; Clutton-Brock 1991; Bradbury and Vehrencamp 2011; Royle et al. 2012; Schultner et al. 2017; Steiger and Stökl 2017). For example, the ability to recognize offspring helps caring individuals to allocate resources towards their own instead of heterospecific or unrelated conspecific offspring (Kaplan et al. 1978; Cheney and Seyfarth 1980; Mateo 2002; Neff and

Sherman 2003; Richard and Hunt 2013). While numerous species rely on location or temporal cues for offspring recognition, previous studies have demonstrated that many species dealing with brood parasitism possess the ability to distinguish between their own and heterospecific offspring using direct cues (Briskie et al. 1992; Lotem et al. 1992; Lyon 2003; Suzuki and Nagano 2006; Wang et al. 2008; Smith and Belk 2018). Furthermore, recognizing the nutritional state, age, or the developmental stage of offspring could be of critical benefit to both caregivers and offspring, as these attributes can determine the need for parental protection or food provisioning (Le Conte et al. 1994; Smiseth et al. 2007; Smiseth and Moore 2007; Traynor et al. 2015; He XJ. et al. 2016; Schultner et al. 2017). Parents might extract information about condition, age, or development stage by assessing the size of offspring or using other visual, chemical or acoustic cues (Kilner 1995; Kölliker et al. 2005; Lévy and Keller 2009; Pelletier et al. 2016). However, in a range of parenting species offspring are known to actively produce begging signals that reflect offspring need or quality and that influence the amount or duration of care (Kilner and Johnstone 1997; Royle et al. 2002; Kölliker et al. 2005; Mas et al. 2009; Mock et al. 2011). Given that the degree to which offspring rely on parental care depends on their developmental stage, the intensity or frequency of such begging signals typically changes as offspring grow (Davies 1976; Hirose and Balsam 1995; Smiseth et al. 2003; Jaeggi et al. 2008).

When it comes to insects, chemical cues and signals are the most widespread means of mediating interactions between individuals. Interestingly, although chemically mediated interactions have been intensively studied in several insect orders, these studies usually focused on adults and have largely ignored juvenile stages (Symonds and Elgar 2008; Wyatt 2008; Steiger and Stökl 2014; Oi et al. 2015; Yew and Chung 2015; Leonhardt et al. 2016; Pasqual et al. 2021; Buchinger and Li 2023). Currently, there are just a few studies available that examined chemical substances released by juveniles and even fewer that investigated whether there are qualitative or quantitative differences between development stages. For example, in a forensically important blowfly species, recent research has found differences in the composition of cuticular hydrocarbon (CHC) profiles (Sharma and Drijfhout et al. 2021) as well as the emission of volatile organic compounds (VOCs) (Sharma and Tomberlin et al. 2021) between different larval instars. A study of honey bee (*Apis mellifera*) broods also found a temporal change in the VOC profile during development (Noël et al. 2023). In this context, it has already been established that honey bee workers can discern the age of larvae based on VOCs, as evidenced by their different responses to the odours of young versus old larvae (Le

Conte et al. 1994; Maisonnasse et al. 2010; Traynor et al. 2015). Contrary to our extensive knowledge about chemically mediated recognition and communication in eusocial insects (Leonhardt et al. 2016; Schultner et al. 2017; Schultner and Pulliainen 2020), our understanding of these processes in subsocial species that provide post-hatching care remains limited (Steiger and Stökl 2017; Nehring and Steiger 2018). Also here, studies have typically focused on adults (Steiger 2015; Steiger and Stökl 2017) and to the best of our knowledge there is no study that analysed how chemical profiles change during development. There are, however, studies suggesting the existence of chemical begging signals. Research has shown that food deprivation has an effect on the quantity of specific cuticular hydrocarbons in earwig nymphs (Mas et al. 2009) and on VOC profiles in burrower bugs (Kölliker et al. 2006). In order to deepen our understanding of chemical substances released during offspring growth and to establish a foundation for future investigations into the chemistry of parent-offspring interactions, we conducted an in-depth study on the chemical profiles of different larval stages using the burying beetle *Nicrophorus vespilloides*, known for its elaborate pre- and post-hatching care, as a model system. To this end, we analysed larval cuticular lipid profiles using solvent extraction and VOC profiles using active and passive headspace techniques. In addition, we established a suitable bioassay to show that larval odour affects parental behaviour.

Burying beetles provide elaborate biparental care for their offspring using small vertebrate carcasses as a breeding resource (Pukowski 1933; Eggert and Müller 1997; Scott 1998; Royle et al. 2013). Monopolized carcasses are transformed into a ball-like shape, whilst removing fur or feathers, and treating the carcass with anti-microbial secretions to prevent decomposition (Suzuki 2001; Cotter et al. 2010; Arce et al. 2013; Vogel et al. 2017; Shukla et al. 2018; Miller et al. 2019). Additionally, beetles cut a hole into the prepared carcass, in which larvae aggregate either to be provisioned by their parents or feed themselves (Eggert and Müller 1997; Eggert et al. 1998; Scott 1998; Smiseth et al. 2003; Royle et al. 2013; Trumbo 2017). Food provisioning appears to be primarily triggered by larval tactile begging (Rauter and Moore 1999; Smiseth et al. 2003). Hereby, larval begging increases with hunger (Smiseth and Moore 2007) and proximity to parents (Smiseth and Moore 2004). *Nicrophorus* larvae pass through three instars during development (Pukowski 1933), with each instar exhibiting differences in size, begging rate, and dependency on parental food provisioning (Eggert et al. 1998; Smiseth et al. 2003). In particular, it is known that 2nd instar larvae show the highest tactile begging rate and are the most frequently fed among the three instars (Smiseth et al.

2003). However, it is currently unknown whether larvae produce chemical cues or signals that reflect their developmental stage and therefore their dependency on parental food provisioning.

Previous studies showed that chemical cues and signals play an important role in *Nicrophorus* beetles. For instance, they use VOCs emitted by decaying carcasses to locate their breeding resources over long distances (Kalinová et al. 2009; Trumbo and Steiger 2020). Furthermore, VOCs and cuticular lipids are used by adults to identify sex, previous mating partners, and their breeding partners (Steiger et al. 2007; Steiger, Peschke and Müller 2008; Steiger et al. 2009; Haberer et al. 2010, 2014; Chemnitz et al. 2015; Keppner et al. 2017) and therefore play an important role in social interactions. Moreover, brood caring females produce a volatile (methyl geranate) that reflects their hormonal state and acts as an anti-aphrodisiac to males (Engel et al. 2016; Engel et al. 2019). It has also been suggested that breeding beetles emit chemical stimuli that triggers begging behavior in larvae (Smiseth et al. 2010; Takata et al. 2019). In the case of the offspring, there are certain hints that parental beetles differentiate between development stages of larvae. For example, Takata et al. (2013) showed that brood regulation mostly concerns newly hatched larvae reaching the carcass. Furthermore, Engel et al (2016) showed that when females were regularly provided with 1st instar larvae, females continued to care for their given offspring instead of producing future offspring. But when faced with 3rd instar larvae, females resumed egg laying (Engel et al. 2016). Additionally, studies have also found corresponding effects of the larval stage on maternal juvenile hormone titres (Scott and Panaitof 2004; Trumbo and Robinson 2008). There are also some indications that parents base care decisions on chemical cues produced by larvae. Mattey et al. (2018) showed that females provide different amounts of care for inbred and outbred larvae. They hypothesize that inbred larvae produce a signal based on which females recognize their poor genetic condition and suggest that this signal is of chemical nature (Mattey et al. 2018). Furthermore, various studies found that parents can evaluate brood size. They resume egg laying when brood size is very low (Sahm et al. 2022) or cull some offspring when there are more larvae than the resource can support (Bartlett 1987; Trumbo and Fernandez 1995; Smith et al. 2015). It is possible that parents use the amount of VOCs released by larvae for such decisions. Moreover, it is also known that parents of some *Nicrophorus* species are able to discriminate between own and heterospecific larvae, a behaviour likely mediated by chemical cues (Capodeanu-Nägler et al. 2018; Smith and Belk 2018). Hence, investigating the production of VOCs and cuticular lipids by

Nicrophorus larvae, and determining whether they reflect their developmental stages, could be of key importance to better understand parent-offspring interactions during family life.

To examine the chemical profile of *N. vespilloides* larvae, we collected VOCs and cuticular lipids from all three instars. We predict that the chemical profiles of the 1st and 2nd instar differ from that of the 3rd instar, given that the first two stages are much more dependent on parental provisioning than the latter. Furthermore, if larvae express a chemical begging signal, we predict that 2nd instar larvae differ in their chemistry as they are fed more frequently by the parents and also show a higher tactile begging behaviour compared to the other two instars (Eggert et al. 1998; Smiseth et al. 2003). In the passive headspace samples, we detected methyl geranate (MG), but it was unclear if larvae themselves produce MG or whether it is a residual from females who are known to produce MG in the presence of larvae. To verify that larvae actively produce MG, we exploited the fact that females only produce MG when caring for offspring with a male partner (Steiger et al. 2011; Engel et al. 2016). Hence, we additionally analysed the MG emission of larvae raised either uni- (single females) or biparentally (female and male). Finally, we tested whether parental feeding rate is affected by the odour of larvae.

Material and methods

Beetle origin and husbandry

We used larvae of *Nicrophorus vespilloides* beetles that originated from outbred populations kept in our laboratory at the University of Bayreuth, Germany. Beetles descended from wild-caught beetles captured near Bayreuth, Germany and were kept in small plastic boxes (10 x 10 x 6 cm) filled with moist peat and stored in a climate chamber at 20 °C under a 16:8 h light: dark cycle. Beetles were fed with sliced mealworms (*Tenebrio molitor*) twice a week.

Solvent extractions

To produce larvae for the solvent extraction (and the active headspace), we randomly paired unrelated, virgin males and females in plastic boxes (10 x 10 x 6 cm) half-filled with moist peat. Pairs were given access to a weighted mouse carcass of approx. 20 g. After 48 hours we separated the eggs form their parents, by transferring beetles and carcasses into a new, similar

sized box filled with moist peat. 24 hours later, we checked the boxes containing the eggs for hatching larvae every hour. We analysed the chemistry of all three different larval instars. Larvae of the first 1st instar were either 0 hours (newly hatched) or 6 hours old, larvae of the 2nd instar were 24 hours old, and larvae of the 3rd instar were either 48 or 72 hours old (larval mass, mean ± SD, L1: 3.28 mg ± 1.02; L2: 17.22 g ± 3.25; L3: 132.8 g ± 58.95). Therefore, newly hatched larvae were either directly subjected to chemical analysis or larvae were assigned to their own parents for 6, 24, 48, and 72 hours before they were analysed.

To extract the cuticular lipids from the surface of the larvae, we adjusted our approach based on size and weight differences among the instars. We pooled five larvae from the 1st instar, three larvae from the 2nd instar, and used a single larva for each of the 3rd instars, placing them in a 1.5 ml glass vial for the extraction. Before conducting solvent extractions, we collected the active headspace of the larvae (see below). The larvae were then freeze-killed at -20 °C. Following this, we added *n*-hexane (Rotisolv, Carl Roth, Karlsruhe, Germany) as a solvent to dissolve the cuticular substances on their surface. Due to size differences of larval instars, we added 300 µl *n*-hexane to the 1st and 2nd instar larvae for 5 min and we added 1000 µl *n*-hexane to the 3rd instar larvae for 3 min. Afterwards the extracts were transferred into new, 1.5 ml glass vials and the larvae were discarded. We evaporated the extracts under a gentle nitrogen stream to a volume of approx. 50 µl. Then we added 1 µl *n*-hexane containing 20 ng Eicosane (Sigma-Aldrich, St. Louis, USA) as internal standard and auto-injected 1 µl of each extract splitless into the GC-MS (Shimadzu GC2030 gas-chromatograph connected to a Shimadzu QP2020NX mass-spectrometer; Shimadzu, Duisburg, Germany). The GC contained a non-polar capillary column (SH-Rxi-5Sil MS, length = 30 m, inner diameter = 0.25 mm, film thickness = 0.25 µm, Shimadzu, Duisburg, Germany) and the oven temperature was raised from 40 °C to 300 °C at a rate of 5 °C/min and finally held for 20 min. We used helium as a carrier gas (linear velocity = 50 cm/s). *n*-alkanes were identified through a comparison of their mass spectra and retention indices with a reference mixture of alkanes (Sigma-Aldrich, St. Louis, USA). Other CHCs were identified by interpretation of the MS spectrum and comparison of the retention index with the literature (Carlson et al. 1998). Other compounds were identified by comparing their mass spectra and linear retention indices with the NIST database. We characterized the positions of the double-bonds in mono- and diunsaturated compounds by analyzing samples derivatized with dimethyl disulfide samples (Carlson et al. 1989) in the GC-MS system as described above. Additionally, we compared the retention indices of the

unsaturated substances of the larvae with those identified previously in adults (Steiger et al. 2007).

Active headspace analysis

Generally, headspace describes the gas phase, for example around an object, in our case around the larvae. For the active headspace we pumped the gas phase actively into our collective medium, whereas for the passive headspace (see below) we collected the headspace without using an active force, but by placing a fibre above the larvae to collect the VOCs via diffusion.

For the collection of the active headspace of larval instars, we used the same larvae as for the solvent extractions. Each sample was placed in a silanized glass jar (inner diameter = 3 cm) containing a wet filter paper to prevent larval desiccation. The glass jar was connected to a 'headspace filter' and a membrane pump as well as an activated charcoal filter to clean incoming air (50 mg; Supelco, PA, USA). Headspace filters consisted of a 2 cm long glass tube (inner diameter = 2 mm) enclosed on both ends with silanized glass wool (Sigma-Aldrich, Supelco, St. Louis, USA) and contained 3 mg of Carbotrap® B and 3 mg Tenax® (both Sigma-Aldrich, Supelco, St. Louis, USA). Before usage filters were conditioned using a Clean Cube (SIM, V1.0, Oberhausen, Germany). Headspace analyses were conducted in a climate chamber at 20 °C. Here, larvae were placed inside the glass jars for 20 min to accumulate their volatiles before we sucked air through the jar using the pump (~200 ml/min) to collect volatiles for 5 min. Afterwards headspace filters were stored at –20°C in a freezer till further analysis. Prior to analysis, we added 20 ng of methyl undecanoate (Sigma-Aldrich, St. Louis, USA) dissolved in 1 µl of *n*-hexane (Rotisolv, Carl Roth, Karlsruhe, Germany) as internal standard. Headspace filters were desorbed (300 °C for 8 min) using a thermal desorption system (TD-30R, Shimadzu, Duisburg, Germany) connected to a GC-MS system as described above. The oven temperature was raised from 50 °C to 200 °C at a rate of 5 °C/min before raised to 280 °C at a rate of 15 °C/min, which was then held for 10 min. Helium was used as carrier gas (linear velocity = 36.3 cm/sec). Prior to the comparison of the active headspace chemistry of larval instars we removed siloxanes and other contaminations from our analyses. Given the absence of discernible variations in the chemistry between larval instars (see results), we opted against conducting further in-depth characterizations of the substances detected.

Passive headspace analysis

To generate larvae for passive headspace sampling, beetle pairs were given access to a mouse carcass (~ 8-12 g), and we checked the boxes after 72 hours for hatching larvae every two hours. We collected either five 1st instar larvae (newly hatched larvae), five 2nd instar larvae (24 hours old), or five 3rd instar larvae (48 hours old) in 4 ml glass vials.

Furthermore, we collected passive headspace samples from 2nd instar larvae raised under uni- or biparental care for 24 hours to specifically investigate their MG production. We focused on 2nd instar larvae as they showed the highest amount of MG (see results). Previous studies showed that females caring for 2nd instar larvae produced the highest amount of MG if their male partner was present (Steiger et al. 2011; Engel et al. 2016). Under uniparental care, females produce no or only trace amounts of MG. Hence, collecting larval headspace volatiles under uni- and biparental care allows us to investigate if MG is produced by the larvae or whether it is just transferred from biparental caring females to the larvae. We randomly set up 20 unrelated pairs of males and females of *N. vespilloides* beetles in plastic boxes (10 x 10 x 6 cm) half-filled with moist peat and provided them with a carcass of approx. 10 g. 48 hours after beetles had access to the carcass, males were removed in half of the boxes to create uniparental caring females. About 24 hours later, we checked the boxes for larval hatching every two hours. After a parental care period of 24 hours, we collected five 2nd instar larvae from each family and placed them separately in a 4 ml glass vial.

We collected the passive headspace of each sample described above using SPME (= Solid-Phase Microextraction). At first, we created an opening in the lid of the 4 ml glass vials containing the collected larvae of each instar/treatment using an injection needle. Here, we inserted a SPME fibre (PDMS/DVB, 65 µm, fused silica, 24Ga, Supelco, Bellefonte, USA) which was used to collect larval volatiles for 30 min. SPME fibres were desorbed in the injector of the GC, which was coupled to the MS, for 5 min at 250 °C. The GC contained a non-polar column (like the column used in 'Solvent extractions'). Starting at 50 °C (held for 2 min) we raised the oven temperature of the GC to 280 °C with a rate of 5°C/min to separate the volatiles. Helium was used as carrier gas (linear velocity = 40 cm/s). Afterwards, SPME fibres were conditioned in the GC-MS injection port at 250 °C for 30 minutes before being used again. Further, a calibration curve was created by applying varying amount of synthetic methyl geranate (5-200

ng/µl) to a filter paper inside a 4 ml glass vial and sampling the methyl geranate with SPME fibres for 15 minutes inside a fume hood. The identification of the substances was achieved by comparing their mass spectra and linear retention indices with those of synthetic reference compounds.

Arena experiments

To test whether parental feeding rate is affected by the odour of larvae, we established a behavioural choice assay that allowed us to measure the response of caring females using automated video tracking. To this end, we exploited the fact that females feed their larvae also outside of the carrion resource; specifically, they move from the cadaver to a different site and regurgitate food to them (J. K. Müller, personal communication). A validation experiment served to test the suitability of the choice assay and a subsequent experiment to test the response of females to the surface extracts of larvae.

For both experiments, we paired unrelated virgin males and females in plastic boxes (10 x 10 x 6 cm) half-filled with moist coconut coir and provided each pair with a mouse carcass (8.5 g – 12.5 g). After 48 hours, we separated the eggs from the parents by transferring the females and carcasses into a new, similarly sized box filled with moist peat. The males were removed at this time point as our aim was to focus on female behaviour. 24 hours later, we checked the boxes containing the eggs for hatching larvae every hour. Once the larvae had hatched, we transferred the corresponding mothers along with her carrion resource into an arena, which was designed to offer the females a binary choice. The arena consisted of a rectangular plastic box (12 x 12 x 6 cm) filled with a thin layer of moistened plaster with three round, shallow impressions of different sizes (Fig. S3.1). A larger one in the corner of the arena, in which a medium petri dish (94 mm diameter, 16 mm height) was placed, and two smaller ones in close proximity, matching two smaller petri dishes (35 mm diameter, 10 mm height). Both impressions containing the smaller petri dishes were at the same distance and angle from the larger petri dish. Females, along with their carrion resource, were consistently placed in the larger petri dish, while larvae were positioned in the smaller ones. To prevent the larvae from escaping, the inner walls of the smaller petri dishes were treated with Antlock (Antstore, Berlin). Additionally, a damp piece of paper towel was placed inside to maintain humidity for the larvae. Each arena was sealed with an anti-reflective glass pane that had been sprayed with antifog

(Cressi, Barcelona, Spain). This design not only prevented the females from escaping but also facilitated video tracking. All arena experiments were conducted in a dark climate chamber under red light and at 20 °C.

To validate the suitability of the arena assay, we tested the females' response to 1 versus 10 1st instar larvae. Larvae were randomly drawn from a pool of newly hatched larvae and transferred to the two petri dishes. We then counted the number of visits of the females at the two petri dishes for 8 hours using video recordings and an automated analysis technique (see details below). As we expected, females spent more time at the petri dish with 10 larvae rather than 1 larva (see results), we therefore performed a subsequent experiment. We tested the females' response to the surface extract of larvae compared to a control. To obtain the larval extract, we used 2nd instar larvae, as they are fed most frequently by the parents. The larvae had been reared biparentally for 24 h. For each septum, we extracted a batch of 20 larvae in 700 µl *n*-pentane (Rotisolv, Carl Roth, Karlsruhe, Germany) for 3 min. Each larval extract was evaporated to approximately 10 µl and applied to the septum. Because the 2nd instar larvae used for extraction were raised on a carrion resource and thus had been in contact with carrion substances, we prepared an extract of carrion odours as the control. For this, we rubbed both the in- and outside of a parentally prepared cadaver with five filter paper pieces (area of 1 cm^2), extracted them using 5 ml *n*-pentane for 3 min and applied 10 µl to a silicone GC septum (Septa-N8, diameter = 1.3 mm, Macherey-Nagel, Dören, Germany). Using a silicone septum offers an advantage in that it provides a constant emission of substances over a longer time period compared to the use of filter paper, for instance (Engel et al. 2016). For the experiment, one of the petri dishes was then equipped with a silicone septum soaked with larval extract, while the other had a septum soaked with the control extract. Furthermore, we placed two first instar larvae in each of the petri dishes to provide an additional stimulus for the females and to give them the opportunity to feed the larvae. Again, we counted the number of visits of the females at the two petri dishes for 8 hours using video recordings and an automated analysis technique (see details below).

Video analysis

For the video recordings, HD TVI mini cameras (BSC TVI 2811, 2.8 – 12 mm, Eutin, Germany) were used, with a frame rate of 25 and a resolution of 960 x 576 pixel. The cameras were

connected to a recording device (LUPUS - LE918 4K 8 Channel NVR, LUPUS-Electronics GmbH, Landau, Germany) Analyses of the recorded videos utilized a custom-built Python script (Version 4.3). The Python script enables the user to manually select round regions of interest (ROIs) from the first frame of the video. The software then isolates these ROIs (here the two petri dishes; Fig. S3.1), frame by frame, and conducts a comparison between successive frames. In instances where no motion occurs, ROIs remain black; however, movement is represented by the conversion of the moving pixels to white. The script assesses motion by measuring the proportion of white pixels relative to the total ROI area, which gives a percentage of the area that was active over time.

The resulting patterns of activity in the video data, denoted by spikes, were indicative of movement. To differentiate between the movement of the females and the larvae, we established a specific activity threshold. Activities above this threshold were attributed to the beetles, while those below it were ascribed to the larvae. To determine these thresholds, we analyzed the plots alongside the actual behavior of the beetles as observed in the videos. This analysis indicated that a threshold of 65 was appropriate for the validation experiment, which involved comparing ten larvae to one. For the subsequent experiment that included two larvae and additional chemical cues, a lower threshold of 30 proved to be sufficient (Fig. S3.2). To establish the number of female visits per petri dish, the peaks with a maximum above the respective threshold in all plots of a recording were determined. Note that the automatic video tracking technique cannot detect whether a female visit actually involves feeding or not.

Statistics

All statistical analyses were performed in R (version 4.2.2, R Core Team). When analyzing the chemical profiles of the different larval instars, we always used the relative amounts of the substances. We removed all substances from the dataset which represented less than 0.5% of the total peak area before we standardized each profile to 100%. We identified 42 cuticular substances in the surface extracts (N_{oh} = 17, N_{6h} = 8, N_{24h} = 22, N_{48h} = 25, N_{72h} = 20; summary in Table S3.1) and 45 volatile substances in the active headspace samples (N_{oh} = 16, N_{6h} = 10, N_{24h} = 18, N_{48h} = 22, N_{72h} = 16). For our passive headspace samples of the three instars, we focused on ten most prominent volatile organic compounds (=VOCs) (N_{oh} = 18, N_{24h} = 16, N_{48h} = 20;

summary in Table S3.2). For the VOCs, we additionally calculated the absolute amount of each substance per larva prior to the analysis.

To determine if larval instars can be separated based on cuticular lipids or VOCs deriving from active or passive headspace, we calculated three PERMANOVAs (=Permutational analysis of variance; 'adonis2()' command in the 'vegan' package) as well as pairwise PERMANOVAs ('pairwise.adonis()' command in the 'pairwiseAdonis' package; Bonferroni-corrected p-values) based on Bray-Curtis-dissimilarities. Additionally, we visualized the data using nMDS (= non-Metrical Multidimensional Scaling) plots based on Bray-Curtis-dissimilarities in the R-package vegan and heatmaps ('heatmap.2()' command in the R-package gplots. Finally, we calculated SIMPER tests ('simper()' command) for each dataset. SIMPER tests show the contribution of each substance to the differentiation between larval instars. For the substances contributing most to the differentiation of larval instars, we further tested whether their amount differed significantly between larval instars using Kruskal-Wallis tests followed by pairwise Wilcoxon tests with Bonferroni correction. Lastly, we analysed the difference in the amount of MG between uni- and biparental raised 2nd instar larvae using a Wilcoxon rank sum test (N = 10 each).

For the arena experiments we compared the number of visits of females (a) between the petri dish containing 1 larva versus the petri dish containing 10 larvae (N_{1vs10} = 17) and (b) between the extracts of L2 larvae and the extracts of the carrion ($N_{L2vsCarrion}$ = 12) using paired Wilcoxon tests.

Results

Cuticular lipids of the three larval instars

The solvent extractions revealed 42 substances of *N. vespilloides* larvae from different instars (Table S3.1). We found differences in the cuticular lipid profile between larval instars (PERMANOVA, F = 27.18, P = 0.001; Fig. 3.1A). Thereby all instars differed from each other (for each pairwise PERMANOVA, F > 11.9, P < 0.01). SIMPER tests showed that diMeC$_{27}$ (SIMPER test; 0.71) contributed highly to the separation of the 1st instar larvae from the 2nd and 3rd instar larvae (Fig. 3.2A). Furthermore, between the 1st and 2nd instar predominantly 6,9-C$_{25}$diene (SIMPER test; 0.66) and 2,4-diMeC$_7$ (SIMPER test; 0.69) separated the instars, whereas between

the 1st and the 3rd instar 3-MeC$_{23}$ (SIMPER test; 0.67) and 7-C$_{25}$ene (SIMPER test; 0.69) contributed most to the separation (Fig. 3.2A). The substances contributing most to the differentiation of the 2nd and 3rd instar were C$_{14}$ene (SIMPER test; 0.66), 7-C$_{25}$ene (SIMPER test; 0,69), and 6,9-C$_{25}$diene (SIMPER test; 0.71; Fig. 3.2A). Analysing the substances separately we found that larval instars differed in their produced levels of 3-MeC$_{23}$ (Kruskal-Wallis test, Chi^2 = 22.75, P < 0.001; Fig. 3.3A), diMeC$_{27}$ (Kruskal-Wallis test, Chi^2 = 25.33, P < 0.001; Fig. 3.3B), 6,9-C$_{25}$diene (Kruskal-Wallis test, Chi^2 = 6.33, P = 0.04; Fig. 3.3C), 7-C$_{25}$ene (Kruskal-Wallis test, Chi^2 = 25.51, P < 0.001; Fig. 3.3D), 2,4-diMeC$_7$ (Kruskal-Wallis test, Chi^2 = 21.74, P < 0.001; Fig. 3.3E), and C$_{14}$ene (Kruskal-Wallis test, Chi^2 = 55.79, P < 0.001; Fig. 3.3F). Larvae of the 1st instar showed higher relative amounts of 3-MeC$_{23}$ and diMeC$_{27}$ compared to the other instars (pairwise Wilcoxon test, P < 0.01). For the 2nd instar, we found higher amounts of 7-C$_{25}$ene, 2,4-diMeC$_7$, and C$_{14}$ene compared to the 1st instar (pairwise Wilcoxon test, P < 0.02), and higher amounts of 3-MeC$_{23}$ and 6,9-C$_{25}$diene compared to the 3rd instar (pairwise Wilcoxon test, P < 0.02). Lastly, 3rd instar larvae showed higher amounts of 7-C$_{25}$ene, 2,4-diMeC$_7$ and C$_{14}$ene compared to the 1st instar (pairwise Wilcoxon test, P < 0.001) and higher relative amounts of C$_{14}$ene compared to the 2nd instar (pairwise Wilcoxon test, P < 0.001).

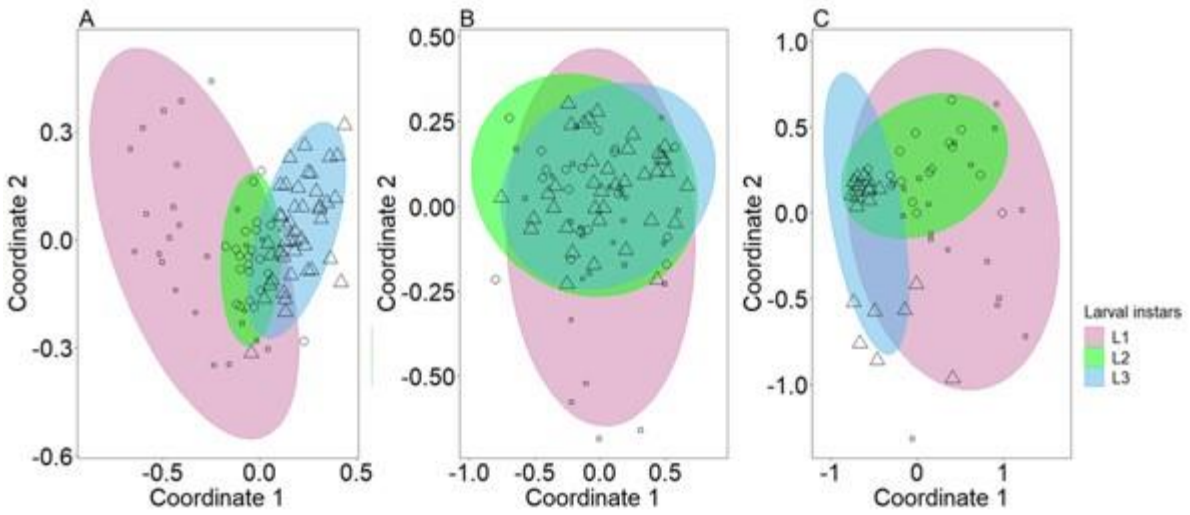

Figure 3.1: NMDS ordination based on Bray-Curtis dissimilarities of A) the chemical profile of the solvent extractions from three larval instars (N = 92), B) the chemical profile of the active headspace analysis from three larval instars (N = 82), and C) the chemical profile of the passive headspace analysis from three larval instars of N. vespilloides (N = 54). Each symbol represents the chemical profile of one sample. Confidence ellipses denote 95% confidence areas around the group centroid (pink = first instar, green = second instar, blue = third instar).

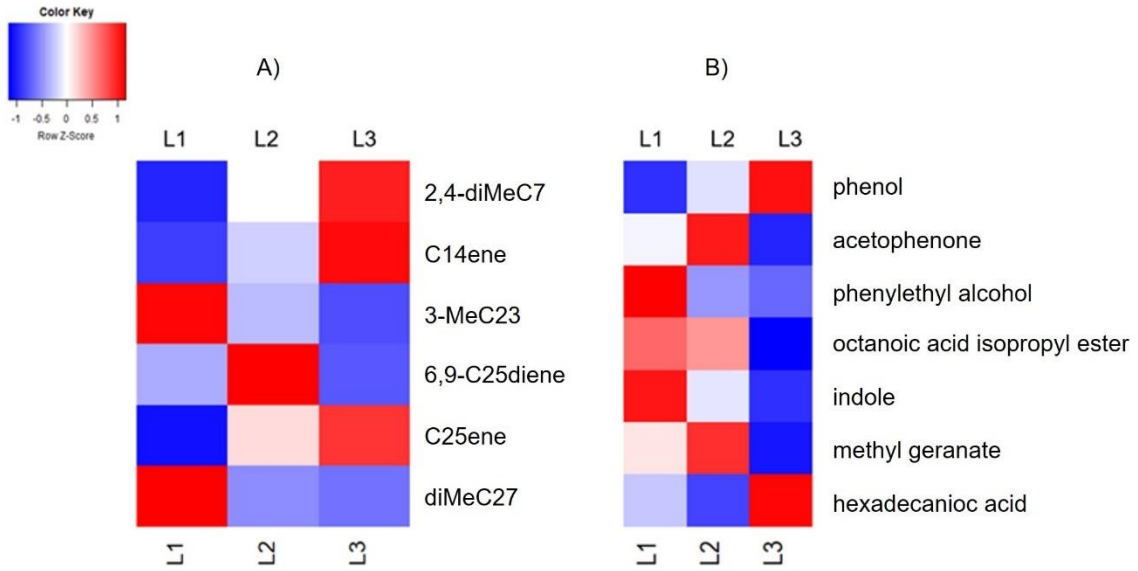

Figure 3.2: Heatmap of the relative amount of A) the six substances found in the surface extractions that contributes most to the differentiation of larval instars based on Simper-tests (see results) and B) the seven substances of the passive headspace analysis showing significant differences between larval instars (see results). The colour of the squares reflects the row z-score and therefore, the deviation from the mean quantity of each substance across all larval instars: red shades denote values above the mean (higher quantity), blue shades indicate values below the mean (lower quantity), and white represents values near the mean.

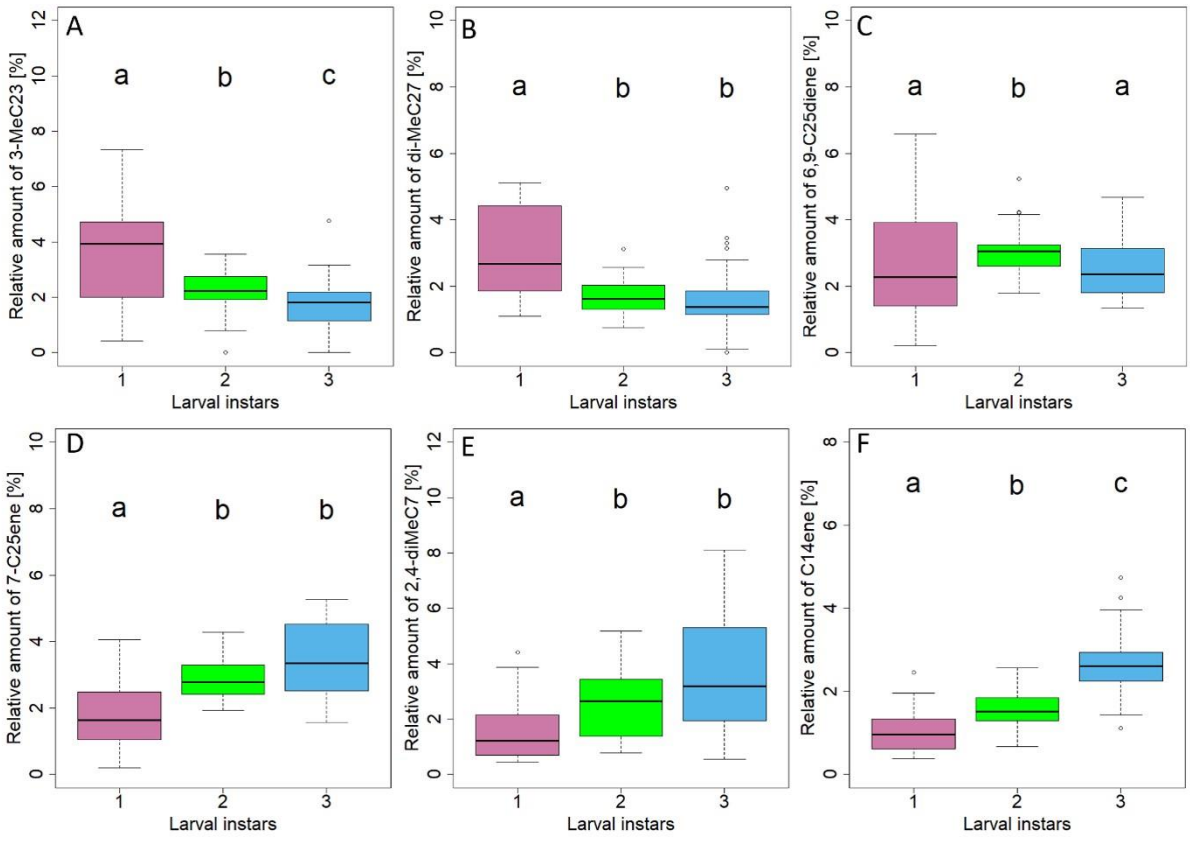

Figure 3.3: Boxplots showing the six surface substances which showed the highest contribution (based on Simper-tests) in the differentiation between larval instars. Shown are the relative amount of A) 3-MeC23, B) diMeC27, C) 6,9-C25diene, D) 7-C25ene, E) 2,4-diMeC7 and F) C14ene for the three larval instars of N. vespilloides. Different letters indicate significant differences after Bonferroni correction.

VOCs of the three larval instars

In our active headspace samples of larvae, we found 45 substances. However, our analysis revealed no differences between larval instars based on their active headspace chemistry (PERMANOVA, F = 1.94, P = 0.07; Fig. 3.1B).

For analysing the passive headspace of larval instars, we focused on ten substances (Table S3.2). Our analyses revealed an overall difference between larval instars based on their passive headspace chemistry (PERMANOVA; F = 12.83, P = 0.001; Fig. 3.1C). Pairwise comparisons showed that all three instars differed from each other in the relative amount of VOCs produced (pairwise PERMANOVA, F > 4.7, P < 0.02). SIMPER tests showed that the instars were predominantly separated based on methyl geranate (SIMPER test; > 0.5) and indole (SIMPER test; > 0.5; Fig. 3.2B). In addition, the 1st and 2nd instars are separated by phenol (SIMPER test; 0.30) and the 2nd and 3rd instars by hexadecanoic acid (SIMPER test; > 0.65; Fig. 3.2B). Testing the substances separately, we found that larval instars differed in their produced levels of acetophenone (Kruskal-Wallis test, Chi^2 = 17.11, P < 0.001; Fig. 3.4A), methyl geranate (Kruskal-Wallis test, Chi^2 = 33.53, P < 0.001; Fig. 3.4B), octanoic acid isopropyl ester (Kruskal-Wallis test, Chi^2 = 23.02, P < 0.001; Fig. 3.4C), indole (Kruskal-Wallis test, Chi^2 = 25.22, P < 0.001; Fig. 3.4D), phenethyl alcohol (Kruskal-Wallis test, Chi^2 = 19.45, P < 0.001; Fig. 3.4E), phenol (Kruskal-Wallis test, Chi^2 = 19.00, P < 0.001; Fig. 3.4F), and hexadecanoic acid (Kruskal-Wallis test, Chi^2 = 20.71, P < 0.001; Fig. 3.4G). 1st and 2nd instar larvae produced relative higher levels of methyl geranate, octanoic acid isopropyl ester, indole, and phenethyl alcohol compared to 3rd instar larvae (pairwise Wilcoxon test, P < 0.05). Furthermore, 2nd instars produced more acetophenone than 3rd instars (pairwise Wilcoxon test, P < 0.001) and 3rd instars produced a higher amount of hexadecanoic acid and phenol than larvae from other instars (pairwise Wilcoxon test, P < 0.02). For the relative amounts of 2-ethyl-1-hexanol, ethyl caprylate, and quinoline, no differences were found between larval instars (pairwise Kruskal-Wallis test, P > 0.07). Interestingly, even when we calculated the absolute amount of substances emitted per larva, we found that the 2nd instar larvae produced a higher amount of acetophenone (pairwise

Wilcoxon test, P = 0.004), methyl geranate (pairwise Wilcoxon test, P < 0.001) and octanoic acid isopropyl ester (pairwise Wilcoxon test, P < 0.001) than the larger 3rd instar larvae, and they also emitted a higher amount than the 1st instar larvae.

We detected methyl geranate in the headspace of uniparentally raised 2nd instar larvae, indicating that the larvae produce methyl geranate themselves. However, the amount of methyl geranate measured was higher under biparental than uniparental care (Mann-Whitney U test, W = 77, P = 0.04; Fig. 3.5).

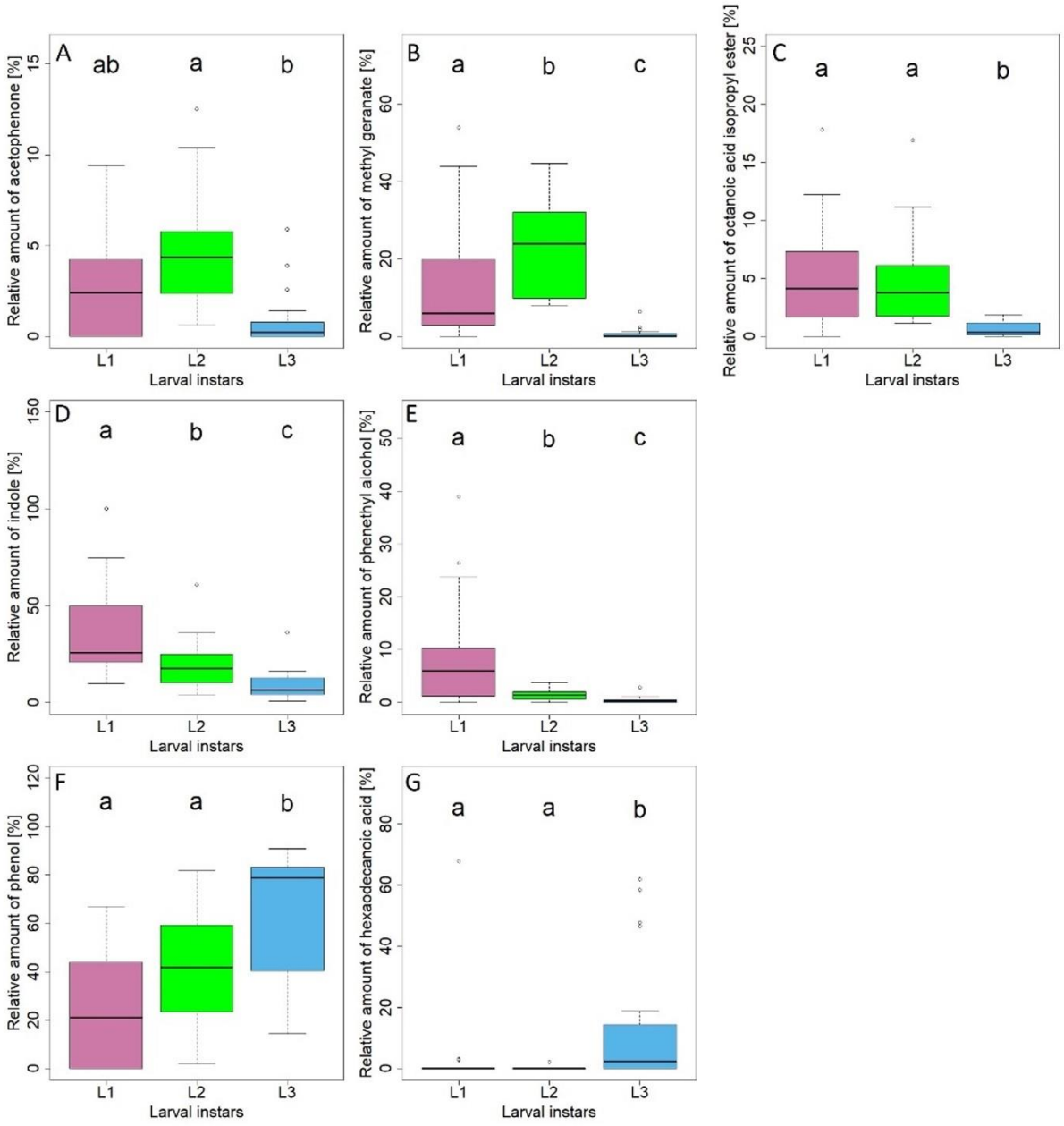

Figure 3.4: Boxplots showing the seven passive headspace substances differing between larval instars. Shown are the relative amount of A) acetophenone, B) methyl geranate, C) octanoic acid

isopropyl ester, D) indole, E) phenethyl alcohol, F) phenol and G) hexadecanoic acid for the three larval instars of N. vespilloides. Different letters indicate significant differences after Bonferroni correction.

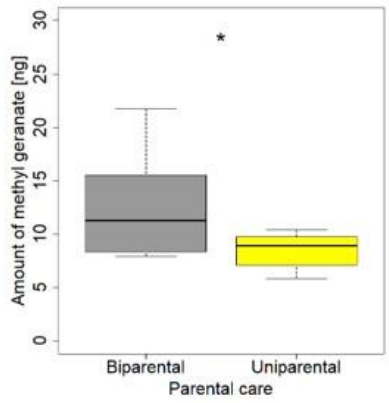

Figure 3.5: Boxplots showing the absolute amount of methyl geranate produced by larvae raised either under uni- or biparental care. Values represent the absolute amount of MG collected from five larvae. Asterisk indicates the level of significance (= P < 0.05).*

Arena experiments

We found that females visited petri dishes with 10 larvae more often than petri dishes with 1 larva (paired Wilcoxon test, V = 6, P < 0.001; Fig. 3.6A). Females also preferred petri dishes containing extracts of L2 larvae to those containing carrion extracts (paired Wilcoxon test, V = 6, P = 0.01; Fig. 3.6B).

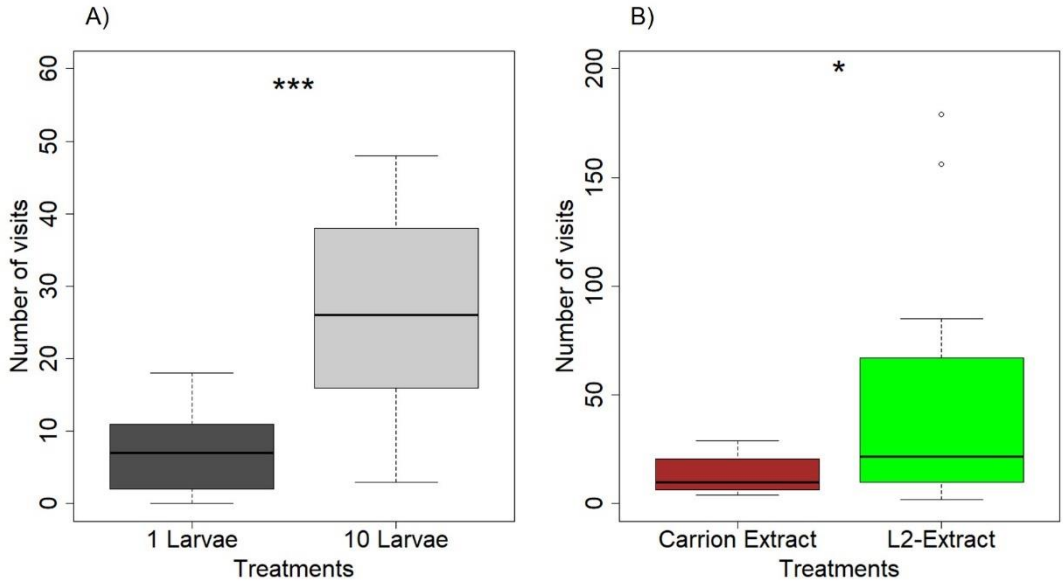

Figure 3.6: Boxplots showing the number of female visits over an 8 hour period to petri dishes containing A) 1 larva or 10 larvae (N = 17) and petri dishes containing B) 2 larvae and either carrion or L2 extracts (N = 12). Asterisks indicate the level of significance (= P < 0.05; *** = P < 0.001).*

Discussion

Our chemical analyses revealed that 1) burying beetle larvae produce a diverse set of cuticular lipids and VOCs, 2) both the composition of the cuticular profile and the VOC profile differ between the three larval instars, 3) larvae produce methyl geranate (MG), the same volatile as breeding mothers, and 4) second instar larvae, which are known to be fed more frequently by the parents than the other two instars, emit higher amounts of acetophenone, MG and octanoic acid isopropyl ester than the first and third instar. We were also able to establish a suitable bioassay using automated video tracking to better understand the role of larval odour in parent-offspring interactions. We found that adding the odour of second instar larvae to first instar larvae increased the number of maternal feeding trips. Taken together, our results suggest that chemical cues (or even signals) produced by larvae play an important role in mediating parent-offspring interactions in burying beetles.

In the solvent extract of larvae, we detected 42 substances, mostly cuticular hydrocarbons. Although this suggests a less complex profile compared to adults, which exhibit over 90 substances (Steiger et al. 2007; Keppner et al. 2017), it is possible that lower quantities in larvae resulted in some substances being below our detection threshold. While we found no

qualitative differences in the cuticular profile across the three instars, we did notice quantitative variations. The 1st instar larvae had higher amounts of 3-MeC_{23} and diMeC_{27}, while the 2nd instar larvae produced more 6,9-C_{25}diene, and the 3rd instar larvae had a higher concentration of C_{14}ene than the other two instars. Interestingly, changes in the CHC profile during development have also been observed in other necrophagous insects. For instance, in several forensically important species of the fly families Calliphoridae and Sarcophagidae, different larval development stages differ in their CHC composition (Zhu et al. 2006; Roux et al. 2008; Pechal et al. 2014; Sharma and Drijfhout et al. 2021; Zhang et al. 2022). Studies revealing age-dependent changes in CHC composition are, however, not limited to carrion insects; such changes have also been observed in Lepidoptera larvae, for instance (de Renoables and Blomquist 1983). Given that all these studied species lack parental care, the observed changes in CHCs might not have a communicative function in parent-offspring interactions, suggesting the influence of other factors. Sharma et al. (2021) and Zhu et al. (2006) found age-related shifts from shorter to longer chained CHCs in blowfly larvae and interpreted these shifts as a potential adaptation that prepares older larvae for survival in drier environments. This could also be true for burying beetle offspring, as third instar larvae leave the carcass to pupate in the drier soil. However, our data do not show an ontogenetic shift from shorter to longer chained CHCs, suggesting that other factors may be influencing the CHC profile. Although we cannot currently rule out that the observed changes in CHC profiles are merely metabolic byproducts, it is possible that these changes serve a communicative function. Particularly when considering that cuticular lipids play a fundamental role in guiding interactions among burying beetle adults (Steiger et al. 2007; Steiger and Franz et al. 2008; Steiger et al. 2009; Keppner et al. 2017), it seems reasonable that larvae might use them to communicate their developmental stage or age to their parents.

We did not only find differences in CHC compositions, but the VOC profile also differed between the three larval instars. The differences were only detected when analysing the passive headspace samples but not the active ones, a finding that underlines the value of implementing diverse sampling methods to obtain a more detailed picture of VOCs produced by insects. A likely reason for the difference between the sample methods could be that the different absorbents used vary in their efficiency in absorbing larval volatiles. Like the cuticular profile, the VOC profile did not show any qualitative differences, only quantitative ones. Of particular interest was, that the second instar larvae emitted higher amounts of acetophenone,

methyl geranate, and octanoic acid isopropyl ester compared to the other two instars. Given that parental food provisioning typically peaks during the second instar (Smiseth et al. 2003), these VOCs could potentially act as begging pheromones, enhancing the effectiveness of the tactile begging behavior. That VOCs can vary with age and function as potential begging pheromones, mediating interactions between larvae and caregivers, was also shown in previous studies in honey bees (Traynor et al. 2015; He XJ. et al. 2016; Noël et al. 2023). Specifically, (E)-*β*-ocimene, emitted in higher quantities by younger larvae compared to older ones, influences worker foraging (Traynor et al. 2015), and its production increases when larvae are food-deprived (He XJ. et al. 2016).

We found that *N. vespilloides* larvae produce MG, the same substance as that produced by caring mothers. Maternal MG has been shown to play a key role in regulating mating and care behaviour in burying beetles (Engel et al. 2016; Royle 2016; Engel et al. 2019). During the time of intensive brood care, when parents are tending to young larvae, females do not produce any further eggs (Engel et al. 2016; Sahm et al. 2022). During this time, they emit MG, which reliably reflects their reproductive state and functions as an anti-aphrodisiac inhibiting male mating behaviour (Engel et al. 2016). Interestingly, it is the interaction with the young larvae that triggers maternal MG emission and prevents females from producing further eggs. This is evident from the fact that removing the brood or replacing it with older, third instar larvae result in the cessation of MG emission and a resumption of egg laying as long as sufficient carrion resources are available. It is known from (E)-*β*-ocimene in honey bees that it does not only regulate worker provisioning behaviour but also inhibits egg production (Maisonnasse et al. 2009). Hence, it is possible that larval MG also has such a dual function, acting as a begging signal and preventing mothers from allocating resources into egg production (Steiger and Stökl 2018). In fact, the concept of such begging pheromones with both releaser effects on behaviour and primer effects on maternal reproductive physiology was anticipated earlier by Mas and Kölliker (2008), suggesting their prevalence in brood-caring insects. However, an alternative possibility is that larvae emit MG to enhance its anti-aphrodisiac effect, since it is in the larval interest that both parents care for them and are not distracted by matings. This hypothesis could also explain the observed higher MG emission from larvae raised in biparental conditions compared to those raised by females alone. Future studies are needed to unravel the function of larval MG.

It is certainly unlikely that all the VOCs we have identified are involved in parent-offspring interactions. One or several substances might mediate interactions between larvae, for example, serve as an aggregation pheromone. This could aid newly hatched larvae in locating the carrion resource more easily, fostering communal feeding for the brood's benefit (Schrader et al. 2015; Prang et al. 2022). Larval aggregation pheromones have been found, for example, in flies (Mast et al. 2014), moths (Jumean Z et al. 2005; Díaz-Siefer et al. 2021), bugs (Chen and Liang 2015), or locusts (Torto et al. 1996; Wertheim et al. 2005). It is also likely that some of the substances found in the larval headspace have no communicative function. Substances like phenol, acetophenone, phenylethyl alcohol, indole and hexadecenoic acid have also been detected in the secretions of adults and might be released by larvae due to their antimicrobial properties (Degenkolb et al. 2011; Haberer et al. 2014).

Finally, we were able to establish a suitable bioassay by exploiting the fact that females also feed their larvae outside the carrion resource. This off-nest feeding allowed us to implement a binary choice test coupled with automatic video tracking. We found that females visited two larvae supplemented with larval extract more frequently than those supplemented with the control extract. Thus, our bioassay demonstrates that females respond to larval-derived odours and supports our notion that interactions between parents and offspring are driven, in part, by chemical cues or signals. However, based on this current data, we cannot say which of the chemical components have a communicative function, nor what kind of information the mothers are extracting. It is possible that they simply use chemical compounds to estimate the number of larvae. If the parents prefer to feed larger broods and utilize these chemical cues to assess brood size, this could explain the observed differences in visiting rates in our bioassay. However, it is also possible that they use them to assess age, nutritional state or other qualitative aspects of larvae. Given these possibilities and considering our data alongside the theory on the evolution of begging signals, we believe that burying beetles represent promising candidates for identifying a potential begging pheromone that influences parental investment.

In conclusion, our results highlight the importance of studying the scent of juvenile stages, thereby considering both cuticular lipids as well as more volatile substances. The strong focus on the chemistry of adults in the last decades has hampered our understanding of the role of larval semiochemicals. Through our research, we have demonstrated that the composition of chemical profiles undergoes developmental shifts, suggesting that such

ontogenetic changes are likely to be widespread across insects. Moreover, our study has successfully established the importance of larval-derived odours in mediating parent-offspring interactions in burying beetles. We hope that these findings will encourage future studies to test the factors that drive the age-related chemical plasticity, as well as to test the significance of single compounds or mixtures emitted by larvae. Offspring semiochemicals are likely to be heavily involved in the regulation of family life.

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Supplementary material

Publication 3: The scent of offspring: chemical profiles of larvae change during development and affect parental behavior in a burying beetle

Jacqueline Sahm, Beatrice Brobeil, Eric Grubmüller, Taina Conrad, Matthias Schott, Johannes Stökl, Sandra Steiger

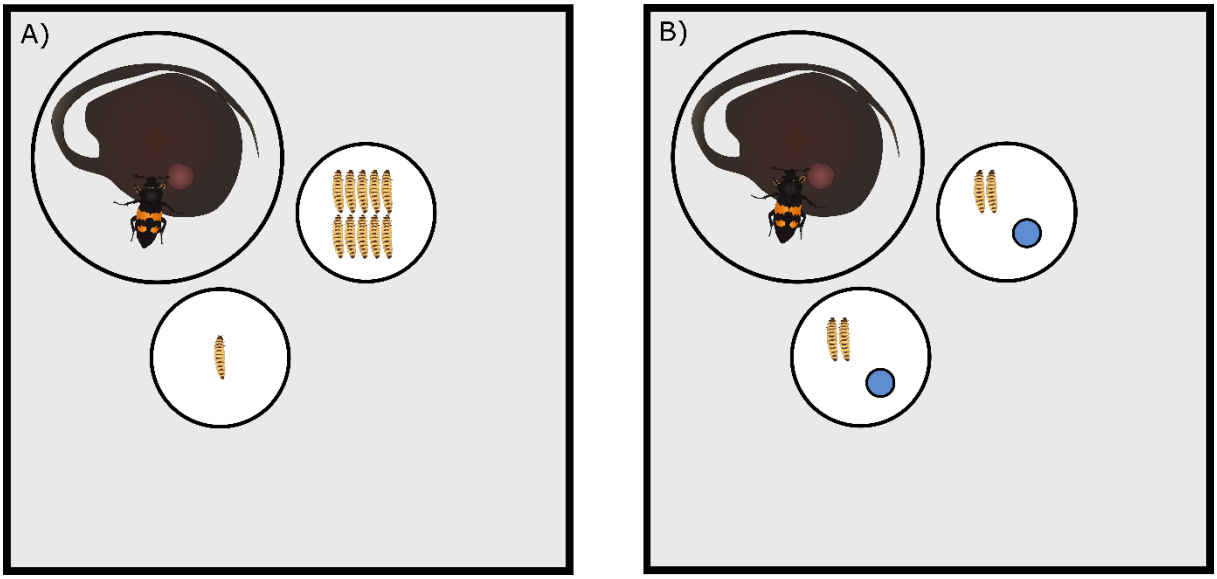


Figure S3.1: Illustration of the arena design. A) shows the set up to validate our arena experiment by placing either 1 larva or 10 larvae in a small petri dish for a female to choose from. In B) we added 2 larvae per petri dish and a septum containing either the carrion extract as a control or the extract of larvae from the 2nd instar.

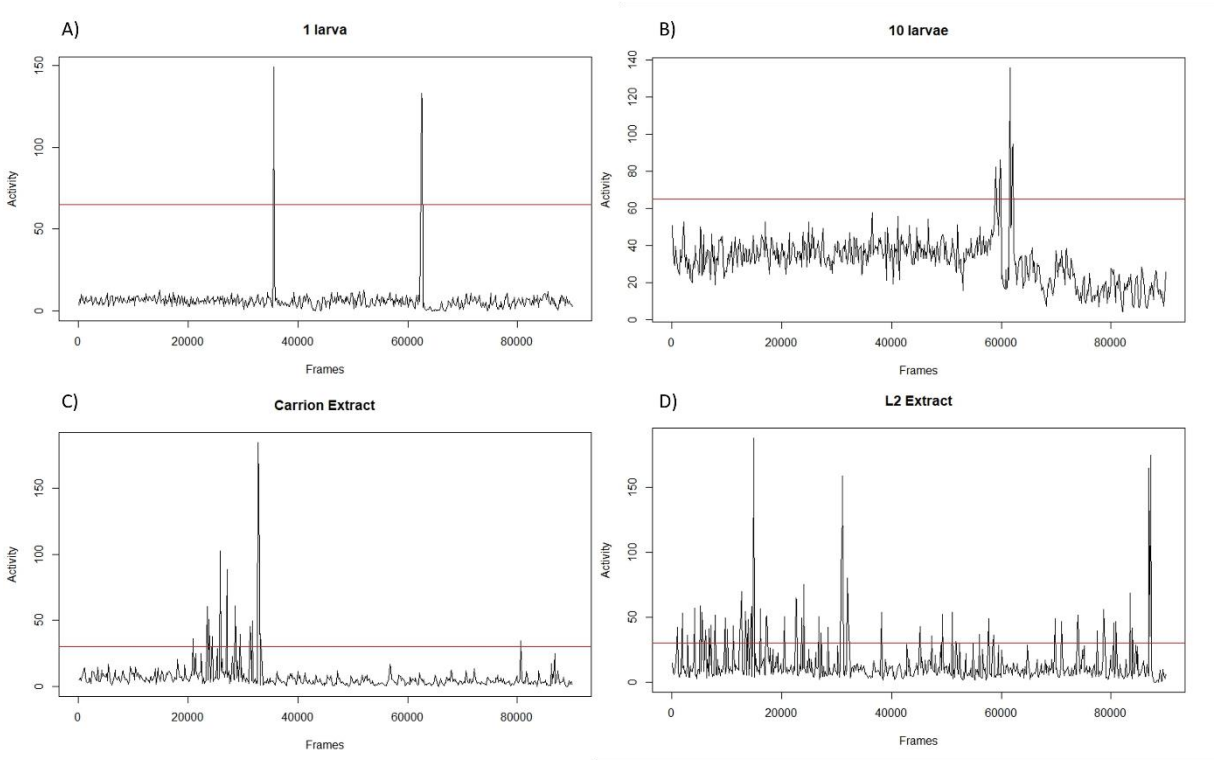


Figure S3.2: Plots show the patterns of activity in form of spikes which indicate the movement of females. A) and B) represent the activity patterns of the validation trials where females had the choice between 1 larva and 10 larvae, respectively. C) and D) show the activity pattern of females choosing between carrion extracts and L2 extracts, respectively. Red lines represent the thresholds defined for the activity of females. In A) and B) an activity value over 65 and in C) and D) an activity higher than 30 were defined as a response from the female to the treatments.

Table S3.1: Mean and standard deviations of the relative contribution (%) of specific substances found in the surface extracts of *N. vespilloides* larvae from three different larval instars separated by GC (mean relative peak area). Mass spectra of unidentified substances (marked with a *) are provided at the end of the supplement.

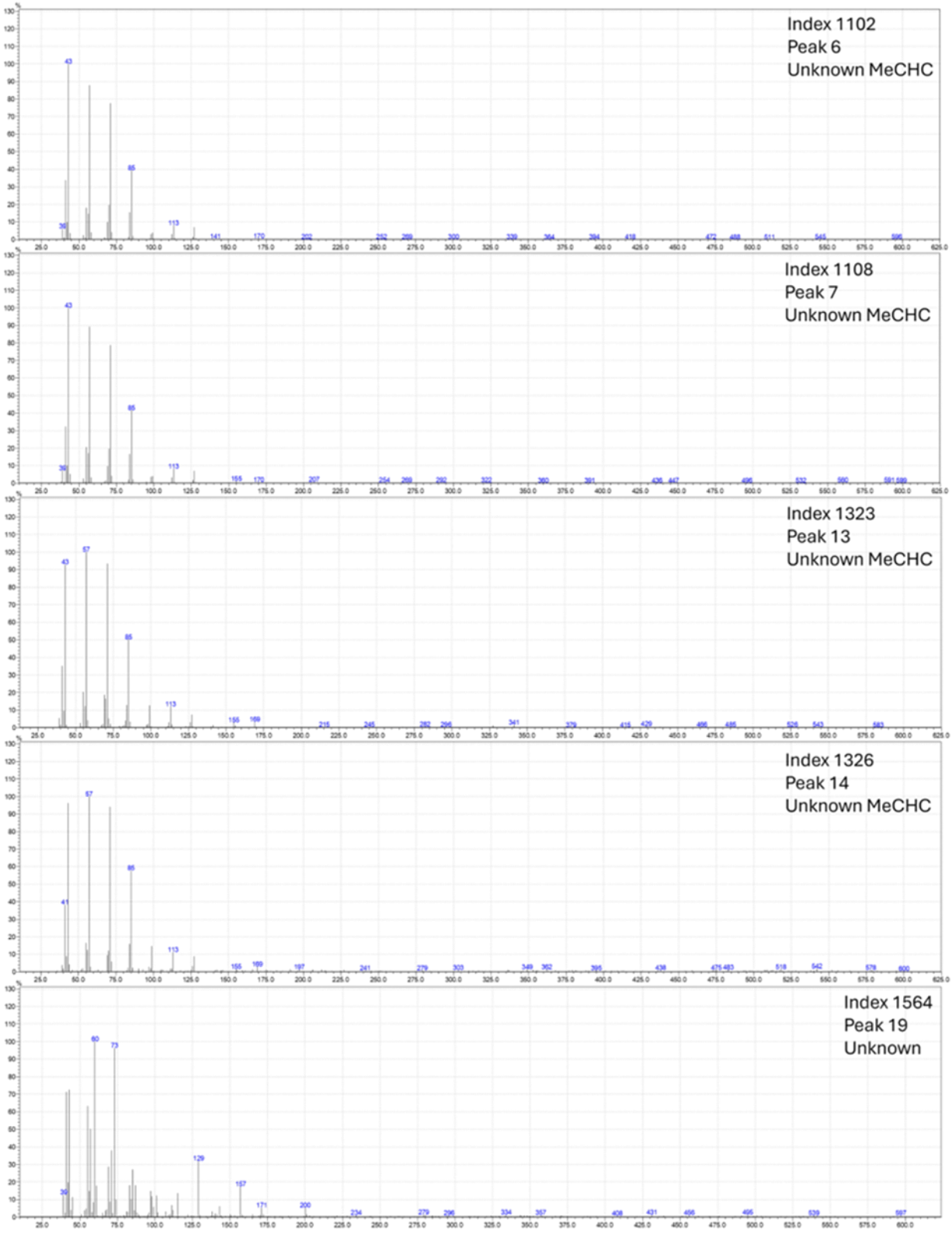
Peak number	Retention time	Retention index	Substance	1st instar	2nd instar	3rd instar
1	4.90	813	2,3,5-triMeC$_6$	0.21 ± 0.17	0.38 ± 0.22	0.51 ± 0.32
2	5.07	819	2,4-diMeC$_7$	1.61 ± 1.11	2.64 ± 1.31	3.75 ± 2.05
3	6.09	861	4-MeC$_8$	0.41 ± 0.28	0.66 ± 0.29	0.96 ± 0.44
4	11.79	1057	4-MeC$_{10}$	0.82 ± 0.53	1.32 ± 0.50	1.99 ± 0.79
5	11.96	1061	3-MeC$_{10}$	0.15 ± 0.15	0.28 ± 0.22	0.42 ± 0.17
6	13.10	1102	unknown MeCHC*	0.37 ± 0.26	0.55 ± 0.25	0.89 ± 0.35
7	13.27	1108	unknown MeCHC*	0.11 ± 0.12	0.18 ± 0.12	0.36 ± 0.16
8	15.56	1190	C$_{12}$ene	0.60 ± 0.37	0.97 ± 0.38	1.49 ± 0.54
9	17.37	1273	nonanoic acid	0.15 ± 0.29	0.94 ± 0.73	0.97 ± 0.62
10	18.23	1279	3-MeC$_{12}$	0.44 ± 0.35	0.67 ± 0.28	1.14 ± 0.38
11	18.84	1295	Indole	0.19 ± 0.23	0.43 ± 0.21	0.60 ± 0.28
12	19.07	1300	*n*-C$_{13}$	0.13 ± 0.21	0.33 ± 0.19	0.51 ± 0.26
13	19.30	1323	unknown MeCHC*	0.12 ± 0.23	0.33 ± 0.21	0.52 ± 0.26
14	19.43	1326	unknown MeCHC*	0.25 ± 0.23	0.44 ± 0.17	0.65 ± 0.22
15	20.99	1390	C$_{14}$ene	1.05 ± 0.53	1.57 ± 0.44	2.64 ± 0.72
16	23.78	1500	*n*-C$_{15}$	0.09 ± 0.14	0.10 ± 0.13	0.38 ± 0.14
17	23.45	1512	dimethyl butylphenol	2.11 ± 1.21	3.10 ± 0.82	5.56 ± 1.36
18	24.84	1549	MeC$_{15}$	0.09 ± 0.17	0.20 ± 0.22	0.45 ± 0.36
19	25.05	1564	Unknown*	0.12 ± 0.20	0.23 ± 0.22	0.42 ± 0.23
20	25.86	1592	C$_{16}$ene	0.89 ± 0.48	1.30 ± 0.32	2.22 ± 0.55
21	30.24	1792	C$_{18}$ene	0.49 ± 0.27	0.72 ± 0.20	1.32 ± 0.41
22	34.21	2007	C$_{20}$ene	0.14 ± 0.19	0.14 ± 0.19	0.63 ± 0.37
23	36.25	2100	*n*-C$_{21}$	7.31 ± 3.51	4.57 ± 1.31	2.02 ± 1.41
24	38.01	2200	*n*-C$_{22}$	1.41 ± 0.40	0.77 ± 0.44	0.33 ± 0.61
25	39.71	2300	*n*-C$_{23}$	23.96 ± 8.57	17.82 ± 4.36	11.68 ± 2.83
26	40.91	2369	3-MeC$_{23}$	3.65 ± 1.70	2.23 ± 0.83	1.75 ± 0.82
27	41.34	2400	*n*-C$_{24}$	0.75 ± 0.54	0.74 ± 0.39	0.61 ± 0.33
28	41.51	2406	diMeC$_{23}$	2.05 ± 1.42	1.91 ± 1.03	1.07 ± 0.62
29	41.89	2430	dimethyl benzylphenol	0.12 ± 0.24	0.47 ± 0.60	0.63 ± 0.75
30	42.27	2457	4-MeC$_{24}$	1.17 ± 0.55	0.70 ± 0.39	0.56 ± 0.28
31	42.34	2469	6,9-C$_{25}$diene	2.62 ± 1.74	3.10 ± 0.77	2.50 ± 0.84
32	42.44	2481	9-C$_{25}$ene	14.07 ± 7.75	20.31 ± 3.59	17.16 ± 3.54
33	42.56	2490	7-C$_{25}$ene	1.88 ± 1.07	2.88 ± 0.64	3.48 ± 1.06
34	42.91	2500	*n*-C$_{25}$	5.80 ± 1.94	7.57 ± 1.82	6.92 ± 3.23
35	44.03	2571	3-MeC$_{25}$	7.25 ± 2.35	5.42 ± 1.72	5.96 ± 2.21
36	44.56	2605	3,7/3,9-diMeC$_{27}$	4.34 ± 4.53	5.92 ± 1.34	4.97 ± 1.45
37	45.37	2671	6,9-C$_{27}$diene	1.95 ± 1.23	0.42 ± 0.36	1.04 ± 0.43

38	45.46	2675	9-C_{27}ene	6.40 ± 3.82	3.19 ± 1.04	5.15 ± 2.24
39	45.88	2700	*n*-C_{27}	0.30 ± 0.35	0.35 ± 1.01	0.32 ± 0.32
40	46.22	2734	unknown amide	0.66 ± 1.09	2.11 ± 1.59	3.58 ± 1.87
41	46.93	2773	3-MeC_{27}	0.67 ± 0.53	0.38 ± 0.65	0.28 ± 0.48
42	47.41	2805	diMeC_{27}	3.09 ± 1.28	1.68 ± 0.54	1.60 ± 0.88

Table S3.2: Mean and standard deviations of the relative contribution (%) of specific volatiles found in the passive headspace of *N. vespilloides* larvae from three different larval instars separated by GC (mean relative peak area).

Substances	1st instar	2nd instar	3rd instar
phenol	25.20 ± 22.51	41.14 ± 21.74	65.50 ± 23.63
2-ethyl-1-hexanol	1.38 ± 2.59	1.02 ± 1.21	4.79 ± 13.95
acetophenone	2.67 ± 2.56	4.85 ± 3.19	0.87 ± 1.51
phenethyl alcohol	9.13 ± 10.39	1.45 ± 1.04	0.45 ± 0.66
ethyl caprylate	1.58 ± 2.04	2.11 ± 1.04	3.71 ± 6.69
octanoic acid isopropyl ester	5.37 ± 4.79	4.81 ± 4.07	0.63 ± 0.61
quinoline	0.48 ± 0.79	0.91 ± 0.79	0.65 ± 0.75
indole	36.10 ± 23.86	20.22 ± 13.65	9.18 ± 7.75
methyl geranate	13.99 ± 16.54	23.35 ± 12.11	0.68 ± 1.46
hexadecanoic acid	4.10 ± 15.47	0.13 ± 0.52	13.53 ± 20.75

Mass spectra of unidentified substances form Table S1



Manuscript 4: To smell or not to smell: New insights in the chemical profiles of hungry offspring in burying beetles

Jacqueline Sahm, Rosa M. L. P. Staufer, Beatrice Brobeil, Johannes Stökl, Sandra Steiger

Ready to submit

Author Contributions

S.S. conceived the study, J.Sahm and S.S designed the study, J.Sahm collected the data with help of R. M. L. P. S. and B. B., J.Sahm and J. Stökl analyzed the data, and J.Sahm, J. Stökl and S.S. wrote the manuscript.

Own contributions

Concept and study design 0 %, data acquisition 40 %, data analyses and figures 90 %, interpretation of results 50 %, first draft of the manuscript 100 %, revision of the manuscript 90%.

Abstract

To enhance parent and offspring fitness, optimizing resource allocation requires parents to discern offspring needs. In response to starvation offspring may produce solicitation signals leading to an increased provisioning behavior of caregiving individuals and affecting maternal reproductive physiology. In insects, chemical cues play a pivotal role in mediating interactions. Here we investigate the chemistry of *Nicrophorus vespilloides* larvae, a burying beetle providing elaborate parental care, under varying nutritional conditions. Additionally, we investigated the physiological reactions of caring females to larvae of different nutritional states. We collected the surface chemicals and volatile organic compounds (VOCs) of fed or food deprived larvae using three different sampling methods. We found that the surface as well as the volatile chemistry of larvae differed with nutritional conditions. Our results suggest that food deprived larvae can potentially signal their needs to parents by altering their chemistry. Here, caring females showed no alteration in their production of methyl geranate although tactile larval begging was increased in food deprived larvae compared to control larvae. However, with increasing tactile begging females responded with an increase in their JHIII production. Therefore, our data suggests that females do adjust their physiology based on the nutritional state of offspring. Future studies are needed to investigate the behavioral responses of parental individuals to chemical cues and signals produced by offspring in different nutritional states and therefore their impact on family dynamics.

Introduction

In family life parents and offspring dispute over resource allocations. We differentiate between the inter-brood conflict over parental investments between current and future offspring and the intra-brood conflict, where members of a current brood dispute over the resources allocated by the parents (Godfray 1995a; Mock and Parker 1997; Parker et al. 2002; Kilner and Hinde 2012; Lessells 2012). Here, offspring signals could enable parents to concentrate their investments for example towards needy offspring. Indeed, a range of taxa show offspring food solicitation signals, which can be acoustical, visual, or chemical in nature and are known to elicit food provisioning responses from caregivers (Le Conte et al. 1994; Weary and Fraser 1995; Kilner and Johnstone 1997; Pankiw et al. 1998; Manser and Avey 2000; Smiseth and Moore 2007; Mas et al. 2009; Traynor et al. 2015; He XJ. et al. 2016). For example, food deprived bird

chicks increase their begging rate and parents react by increasing their investment in food provisioning (Mondloch 1995; Bowers et al. 2018). Such food solicitation signals are considered honest as they incur costs and rely on the need of the signaler (Godfray 1991, 1995b; Kilner 1997; Kilner and Johnstone 1997; Saino et al. 2000; Mock et al. 2011). Other studies, however, suggest that offspring produce signals based on their quality reflecting their reproductive value (Kölliker et al. 2005; Mas et al. 2009; Mas and Kölliker 2011b; Bowers et al. 2018). Unfortunately, our knowledge about the chemical communication in subsocial insect juveniles and the effect of food deprivation on such signals is small. A recent study investigating chemical profiles of juveniles in a subsocial beetle providing elaborate biparental care (*Nicrophorus vespilloides*) presented a potential begging pheromone (Sahm et al. submitted to BE). Studies in other subsocial insects showed that the cuticular hydrocarbon profiles (earwig nymphs; (Mas et al. 2009) or the volatile profiles (burrower bug nymphs; (Kölliker et al. 2006) differ with nutritional conditions of juveniles and elicit feeding responses in caregivers.

Theory suggests that offspring signals resolve the parent-offspring conflicts over parental investment in no one's favor (Trivers 1974). However, Mas and Kölliker (2008) suggested that offspring signals might have releaser effects on the behavior of mothers as well as a primer effects on the physiology of mothers. In a later study, Mas and Kölliker (2009) showed that offspring signals affect the care behavior of females (releaser effect) in earwigs. Additionally, offspring signals also directly affected the reproductive physiology of mothers (primer effects) (Mas and Kölliker 2011a). Also in honey bees (*Apis mellifera*), previous studies showed that offspring signals have a primer effect on the physiology of workers bees (offspring signals induce a suppression in worker' egg development) as well as a releaser effect on their behavior as they induce an increase in the feeding rate of workers (Le Conte et al. 1994; Le Conte et al. 1995; Le Conte and Hefetz 2008; Maisonnasse et al. 2009; Maisonnasse et al. 2010). Changing both the care behavior and the physiology of mothers, leads to an increased investment into current instead of future offspring, which can resolve the parent-offspring conflict towards the offspring's favor. Studies in mammals also showed that offspring can influence parental care by triggering a hormone induced temporary infertility in mothers (Beach 1976; Konner and Worthman 1980). For example, in lactating mammals, the expression of the hormone prolactin, which suppresses ovulation, allows females to prioritize investments in the current offspring (Hamada et al. 1980; Dorrington and Gore-Langton 1981). Therefore, the presence of offspring can alter the resource allocation of mothers towards their current

offspring. To shed light on the potential importance of signals of need in parent-offspring interactions, we study the chemical profiles of larvae in different nutritional conditions using the burying beetle *Nicrophorus vespilloides* as model system.

Burying beetles utilize small vertebrate carcasses and provide elaborate biparental care for their offspring (Pukowski 1933; Eggert and Müller 1997; Scott 1998; Royle et al. 2013). After monopolizing a carcass, a female and male beetle remove fur or feathers and roll it into a ball-like shape while smearing antimicrobial secretions on the surface to prevent microbial decay (Suzuki 2001; Cotter et al. 2010; Arce et al. 2013; Vogel et al. 2017; Shukla et al. 2018; Miller et al. 2019). During preparations, females lay eggs in the surrounding soil, and hatching larvae then aggregate inside a parentally prepared feeding cavity to be fed by parents or to provision themselves (Eggert and Müller 1997; Eggert et al. 1998; Scott 1998; Smiseth et al. 2003; Smiseth and Moore 2004; Royle et al. 2013; Trumbo 2017). Generally, in burying beetles, biparental care is regulated by a specific mechanism. Females that monopolize a carcass and care for nutritionally dependent larvae, produce high levels of juvenile hormone III (JHIII) (Engel et al. 2016). The production of JHIII elicit a temporary infertility of females, which is signaled to the male partners via the emission of methyl geranate (MG) (Engel et al. 2016; Engel et al. 2019). MG expression leads to the suppression of mating attempts and induces paternal help in care (Engel et al. 2016). Generally, the levels of JHIII and MG produced by females are common indicators for their investments in care (Haberer et al. 2010, 2014; Engel et al. 2016). In addition, parental care is affected by tactile larval begging (Rauter and Moore 1999; Smiseth et al. 2003). Tactile larval begging alters with the nutritional state of larvae (Smiseth and Moore 2007; Smiseth and Parker 2008) as well as with the presence of their parents at the feeding cavity (Smiseth and Moore 2004). A previous study showed that females prefer to feed hungry larvae (Smiseth and Moore 2008), which suggests that the tactile begging of burying beetle larvae is an honest signal. We also know, that *N. vespilloides* develop over 3 larval instars, differing in their dependency on parental food provisioning (Pukowski 1933) and their begging frequency (Smiseth et al. 2003). The question whether burying beetle larvae also influence family dynamics by producing chemical signals and cues is still discussed. It could be shown that parents act on a time-sensitive mechanism to discriminate between their own and foreign larvae in a brood (Müller and Eggert 1990), but it is still possible that larvae express their presence or nutritional conditions chemically. For example, there are hints for chemical signals reflecting offspring quality, since females care more for inbred than for outbred larvae (Mattey

et al. 2018). Additionally, a recent study showed that larvae change their chemistry based on their developmental status, whereby second instar larvae showed the highest levels of the potential begging pheromone candidate MG (Sahm et al.; submitted in BE). In a novel bioassay, this study also showed that females alter their care decisions based on larval feedback (Sahm et al. submitted to BE). However, until now it is unclear if chemical signals and cues exist that communicate the nutritional state of larvae in burying beetles and therefore may alter the investments of parents in care.

To answer this question, we used three different sampling methods to collect the surface and volatile chemistry of fed or food deprived larvae. If larvae produce a chemical signal reflecting their nutritional condition, food deprived larvae may express different chemical profiles compared to fed larvae. In a separate experiment we altered the nutritional state of larvae and investigated their tactile begging behavior as well as their impact on the JHIII and MG production of breeding females. We expect to confirm a higher tactile begging rate in food deprived second instar larvae compared to fed larvae as previously shown by Smiseth et al. (2003). Additionally, if changes in the nutritional state and therefore in larval begging affects females, we expect that females caring for food deprived larvae express higher levels of JHIII and MG in accordance with increasing investment in needy offspring. This is based on the finding of Engel et al. (2016) showing that female confronted with nutritionally dependent larvae express higher levels of JHIII and MG.

Material and methods

Beetle origin and husbandry

Nicrophorus vespilloides larvae used in the experiments belonged to the outbred population kept in our laboratory and derived from wild-caught beetles captured in Bayreuth, Germany. Throughout the experiment, beetles were kept in small plastic containers (10 x 10 x 6 cm) filled with moist peat and fed twice a week. We kept the beetles in a climate chamber under a 16:8 h dark: light cycle at 20 °C. Beetles were fed with sliced mealworms (*Tenebrio molitor*) twice a week.

Solvent extractions

To investigate potential changes in the volatile and surface chemistry produced by *N. vespilloides* larvae in different nutritional conditions we firstly set up virgin females with unrelated males of *N. vespilloides* in a small plastic container (10 x 10 x 6 cm) half filled with moist peat. Beetle pairs had access to a mouse carcass of ~25 g. After 48 h of egg-laying we placed beetles and carcasses in a new, similar sized box with moist peat. About 72 h after beetles had access to a carcass, we checked the old boxes for hatching larvae every 2 h. We assigned at least six hatched larvae to their respective parents to prevent possible effect of mixed parentage on the larval chemistry. After 24 h of parental care, three larvae stayed with their parents on the carcass while three larvae were deprived of food (Ø = 3.33 h). Therefore, larvae were placed inside a new, similar sized plastic box half-filled with moist peat to prevent larval desiccation. We decided to examine 2nd instar larvae as they show the highest level of tactile begging behavior (Smiseth et al. 2003) and 2nd instar larvae expressed potential chemical begging signals (Sahm et al. submitted in BE). After in average 3.33 h, we weighted the larvae of both treatments before we placed them inside the 'headspace instrument' to analyze their active volatile chemistry (description below). Afterwards, we placed the larvae inside a 1.5 ml glass vial and stored them in a -20 °C freezer before extracting the surface chemicals.

The freeze-killed larvae were covered in 500 µl *n*-hexane (Rotisolv, Carl Roth, Karlsruhe, Germany) as a solvent to extract the cuticular substances from the larval surface. After 3 min we transferred the extract to a new glass vial and discarded the larvae. Then, we evaporated the extracts to a volume of approx. 20 µl, before we added 20 ng eicosane (Sigma-Aldrich, St. Louis, USA) dissolved in 1 µl *n*-hexane as an internal standard to each sample. Then we auto-injected 1 µl of each sample splitless in a GC-MS (Shimadzu GC2030 gas-chromatograph connected to a Shimadzu QP2020NX mass-spectrometer; Shimadzu, Duisburg, Germany). The oven temperature of the GC was raised from 40 °C to 300 °C with a rate of 5 °C per minute and then hold for 20 min. Helium was used as carrier gas (linear velocity = 50.0 cm/sec). The GC contained a non-polar capillary column (SH-Rxi-5Sil MS, length = 30 m, inner diameter = 0.25 mm, film thickness = 0.25 µm, Shimadzu, Duisburg, Germany). Final samples sizes were: $N_{control}$ = 45 and $N_{food\ deprived}$ = 46. We removed all contaminations (e.g. siloxanes) and toxic substances (e.g. phenol) from the analysis. Then we identified the compounds in the surface extracts as described previously (Sahm et al. submitted in BE).

Active headspace sampling

To collect the active headspace of larvae in different nutritional conditions, we placed the larvae of different treatments inside a silanized glass jar (inner diameter = 3 cm; for the construction of our headspace instrument see Sahm et al. submitted to BE). We accumulated the amount of larval volatiles inside the glass jars by leaving the larvae in the instrument for 20 min before we created an airflow for 5 min using a membrane pump (~200 ml/min). Headspace filters containing larval volatiles were stored at -20 °C. Prior analyses, we added 20 ng methyl undecanoate (Sigma-Aldrich, St. Louis, USA) dissolved in 1 µl *n*-hexane as an external standard to an empty thermal desorption filter. All filters were analyzed in a thermal desorption system (TD-30R, Shimadzu, Duisburg, Germany) connected to a GC-MS at a temperature of 300 °C for 8 min before the substances got transferred into the GC-MS (system described above). The oven temperature inside the GC was raised from 50 °C to 200 °C at a rate of 5 °C/min. Without a holding period we further raised the temperature to 280 °C with 15 °C/min and held the final temperature for 10 min. The used carrier gas was helium at a linear velocity of 50.3 cm/sec. We ended up with $N_{control}$ = 41 and $N_{food\ deprived}$ = 43. Prior comparing the active headspace profile of larvae of different treatments, we removed any siloxanes and other contaminations from our analyses. We opted against in-depth characterizations of the detected substances as active headspace profile did not differ between treatments (see results).

Passive headspace analysis

For the collection of the passive larval headspace, we provided beetles with a mouse carcass (~ 8-12 g) and placed them in plastic boxes (10 x 10 x 6 cm) half-filled with moist peat. After 72 h, we checked the boxes for hatching larvae every two hours. After 17 h of care, larvae usually reach the second instar. Here, we deprived larvae from food for 3 h ($N_{food\ deprived}$ = 19). Therefore, we carefully transferred the larvae onto a small petri dish containing a moistened paper towel and placed them inside a dark climate cabinet at 20 °C for 2.5 h. Including the sampling time of 30 min (see below), larvae were food deprived for 3 h in total. Larvae of the remaining boxes stayed 3 h with their parents as a control group ($N_{control}$ = 22) prior to the analysis.

For the sampling of the passive headspace using SPME (= Solid Phase Microextraction) we placed five 2nd instar larvae per sample in a 4 ml glass vial. Then we pierced the lid of the vials with an injection needle, inserted a SPME fibre (PDMS/DVB, 65 µm, fused silica, 24Ga, Supelco, Bellefonte, USA) and collected larval volatiles for 30 min. Desorption and analysis of the volatiles followed the protocol described previously (Sahm et al. submitted in BE). The SPME fibres were conditioned before the analysis using the GC-MS injection port at 250 °C for 30 min. We identified the volatiles by comparing their mass spectra and linear retention indices with those of synthetic reference compounds.

Larval tactile begging and female' reaction to larvae in different nutritional conditions

To estimate the tactile larval begging rate and the subsequent effect on the physiology of females, we firstly provided beetle pairs with a mouse carcass (~ 8-12 g) in plastic boxes (10 x 10 x 6 cm) half-filled with moist peat. 72 h after the set-up we checked for hatching larvae every 2 h day and night. Hatching larvae were pooled inside a petri dish containing a moist tissue. Additionally, beetles and carcass were transferred to a new, similar sized plastic box. Then, we randomly assigned ten larvae to each beetle pair which larvae already hatched, which is a common procedure in burying beetles (Smiseth and Parker 2008; Steiger 2013; Sahm et al. 2022). The boxes were then stored in a dark climate chamber at 20 °C. After 17 h of parental care, larvae of the food deprived treatment were placed in a petri dish containing a moist tissue for 3 h. Larvae of the control treatment stayed with their parents for 3 h.

20 h after the set ups we observed the tactile begging behavior in both treatments. To this end, we counted and then discarded the larvae which females cared for and exchanged them with either 1) food deprived larvae from a petri dish or 2) control larvae from another box. To observe the begging behavior of larvae we widened the feeding cavities to improve our ability to monitor larval activities. Afterwards we allowed beetles and larvae to acclimatize to their new environment for 15 min. Observation took place in a climate chamber at 20 °C under red light. The tactile begging rate was observed over instantaneous scan sampling in one-minute intervals for 30 min (Rozen et al. 2008; Smiseth and Morgan 2009; Capodeanu-Nägler et al. 2018; Takata et al. 2019). We were able to observe four containers simultaneously. Afterwards boxes were placed in a dark climate cabinet at 20 °C.

To investigate the impact of larvae with different nutritional conditions on their mothers, we additionally measured the levels of methyl geranate (= MG) and juvenile hormone III (= JHIII) produced by females 3.25 h after observing the begging rates of larvae (this equals 24 h after parental care started). We chose this time point because Engel et al. (2016) found that JHIII and MG production were highest in females caring for 24 h old larvae. Firstly, we conducted active headspace sampling of females to analyze their MG levels (see 'Active headspace sampling') but instead of larvae we placed each female singly inside a glass jar to collect female volatiles ($N_{control}$=28 and $N_{food\ deprived}$=25). Secondly, we collected the hemolymph of females to quantify their JHIII levels (see below).

Juvenile hormone III of females

To analyse the amount of JHIII produced by each female, we first pierced the intersegmental membrane on the ventral side of females using an injection needle (Braun, Sterican, Ø = 0.60 x 25 mm) before we collected the excreting haemolymph using a calibrated 10 µl glass capillary (BLAUBRAND® intraMARK Micropipettes). The collected haemolymph was measured using a slide gauge and then transferred into a conic 1.5 ml glass vial. We then added 300 µl *n*-hexane (Carl ROTH, ROTISOLV® ≥ 99 %, Pestilyse®) and 5 µl *n*-hexane containing 100 ng of vernolic acid methyl ester (Sigma-Aldrich, Supelco, St. Louis, USA) as an internal standard to each sample. Then we vortexed the solution for 30 s to separate the clear hexane phase containing JHIII from the unwanted parts of the haemolymph. Afterwards, we transferred the clear hexane phase into a new conic glass vial before placing the samples in a freezer (-20 °C). Prior to the analysis, we evaporated each sample to 2-3 µl using a nitrogen stream. 1 µl of each sample was then manually injected into the GC-MS (GC2030 and QP2020NX MS, Shimadzu). The GC contained a non-polar capillary column (see 'Solvent extractions'). The temperature in the GC-oven was raised from 150 °C to 300 °C with a rate of 10 °C/min. Helium was used as carrier gas (linear velocity = 50 cm/sec). For JHIII we analyzed $N_{control}$=19 and $N_{food\ deprived}$=20.

Statistical analysis

We performed all statistical analyses in R (version 4.2.2, R Core Team). Priorly, we calculated the relative amounts of each substance for the surface, active and passive chemical profiles of the larvae. Additionally, we excluded substances from the datasets which represented less the 0.5 % of each of the total profiles. Afterwards we standardized each profile to 100 %. We identified 33 cuticular substances in the solvent extractions, ($N_{control}$ = 45 and $N_{food\ deprived}$ = 46; Table S4.1), 44 volatile substances in the active headspace samples ($N_{control}$ = 41 and $N_{food\ deprived}$ = 43), and ten volatile organic compounds for the passive headspace samples ($N_{control}$ = 22, $N_{food\ deprived}$ = 19; Table S4.2).

To investigate if food deprivation alters the cuticular lipids as well as the VOCs deriving from the active or passive headspace, we calculated three PERMANOVAs (=Permutational analysis of variance). Furthermore, we used nMDS plots (=non-Metrical Multidimensional Scaling) based on Bray-Curtis-dissimilarities to visualise the data. Additionally, we calculated SIMPER tests for each dataset to analyse which substances contributed most to the separation of the treatment groups. Afterwards, we calculated which treatment group showed significantly different levels of single substances using Kruskal-Wallis tests.

For the response of females to larvae with different nutritional states we calculated the begging rate by dividing the number of begging events by the observation time (30 min; calculated by brood). For the absolute amount of JHIII of females we divided the peak area of JHIII by the peak area of the internal standard and multiplying it with the amount of the internal standard (100 ng). The MG amount was calculated accordingly (amount of the internal standard methyl undecanoate = 20 ng). Additionally, we calculated the JHIII titer per female by dividing the JHIII amount by the hemolymph volume of the respective female. Using generalized linear models (GLMs) fitted with Gaussian error structures we than investigate the influence of larval begging rate and the treatment group as well as their interaction on the female' levels of (a) JHIII ($N_{control}$ = 19, $N_{food\ deprived}$ = 20) and (b) MG ($N_{control}$ = 28, $N_{food\ deprived}$ = 25).

Results

Cuticular lipids of larvae in different nutritional conditions

The solvent extractions of fed and food deprived larvae revealed 33 substances (Table S4.1). Our analysis showed that larvae of different nutritional conditions differ in their surface chemistry (PERMANOVA; F = 3.54, p = 0.004; Fig. 4.1A). SIMPER tests showed that larvae of different nutritional conditions were predominantly separated by x-MeC_{15} (SIMPER test; 0.7), x.x-diMeC_{25} (SIMPER test; 0.67), 3-MeC_{25} (SIMPER test; 0.62), C_{25} (SIMPER test; 0.58), C_{25}diene (SIMPER test, 0.53) and C_{23} (SIMPER test, 0.46; see also Fig. 4.2A). Comparing the amount of each substance directly using Kruskal-Wallis test, food deprived larvae showed a higher level of C_{25}diene compared to fed larvae (Kruskal-Wallis test, Chi^2 = 4.82, p = 0.03; Fig: 4.3A). In contrast, fed larvae produced higher levels of MeC_{15} (Kruskal-Wallis test, Chi^2 = 6.28, p = 0.01; Fig: 4.3B) and 3-MeC_{25} (Kruskal-Wallis test, Chi^2 = 3.90, p = 0.048; Fig: 4.3C) compared to food deprived larvae.

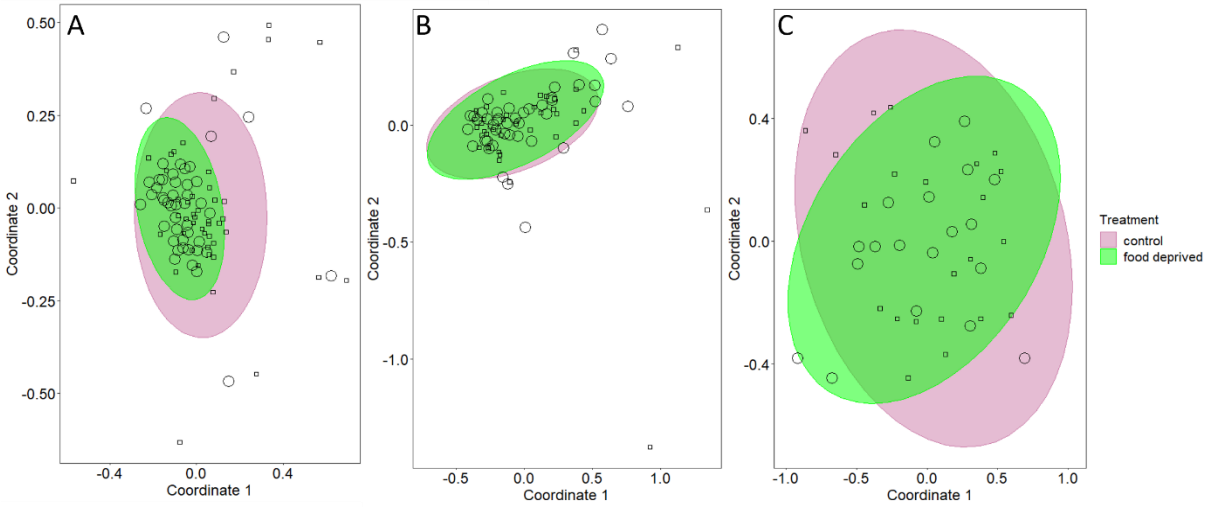


Figure 4.1: *NMDS ordination based on Bray-Curtis dissimilarities from fed and food deprived larvae of N. vespilloides based on A) their surface chemistry ($N_{control}$ = 45; $N_{food\ deprived}$ = 46), B) their active headspace chemistry ($N_{control}$ = 41; $N_{food\ deprived}$ = 43), and C) their passive headspace chemistry ($N_{control}$ = 22; $N_{food\ deprived}$ = 19). Each form represents the chemical profile of one sample. The ellipses denote 95% the confidence areas around the group centroid.*

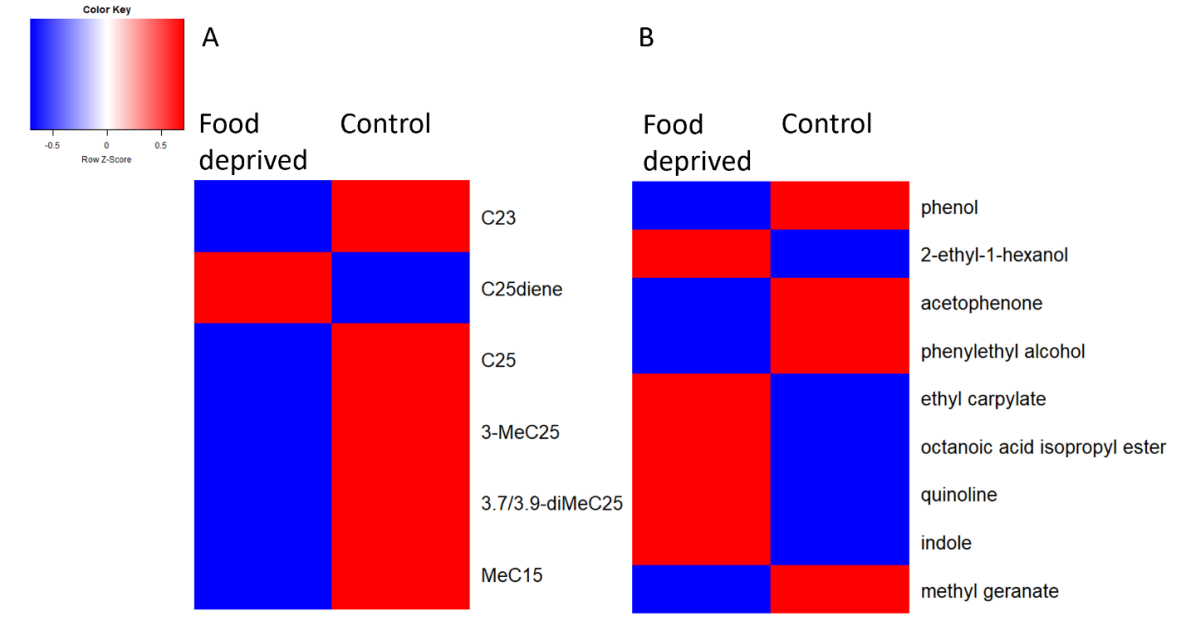


Figure 4.2: Heatmap based on the Bray-Curtis dissimilarities of A) the six surface substances that contributed most to the separation of larva in different nutritional needs (SIMPER tests; see results) and B) the ten VOCs found in the passive headspace of food deprived and fed larvae (see results).

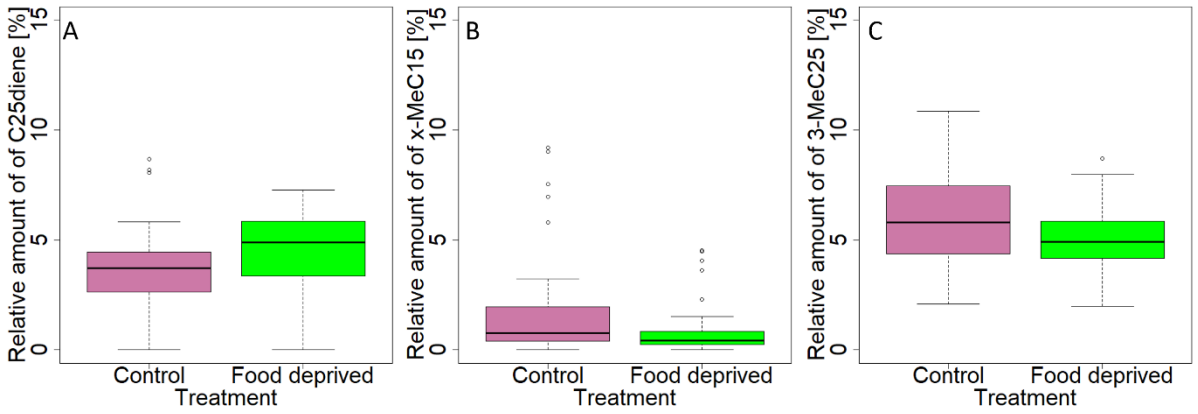


Figure 4.3: Boxplots showing the relative amount of A) C_{25}diene, B) MeC15, and C) 3-MeC_{25} being the surface substances separating fed (control) and food deprived larvae (based on Simper-tests; see results).

VOCs of larvae in different nutritional conditions

Our data for the active headspace samples of larvae in different nutritional conditions revealed 44 substances. The relative amounts of headspace substances showed no differences between nutritional conditions of larvae (PERMANOVA, F = 0.69, p = 0.51; Fig. 4.1B).

For the passive headspace of larvae in different nutritional needs we focused on ten substances (Table S4.2). The data revealed a difference in the passive headspace chemistry based on different nutritional conditions of larvae (PERMANOVA; F = 3.69, p = 0.02; Fig. 4.1C). SIMPER tests revealed that food deprived and fed larvae were predominantly separated by methyl geranate (SIMPER test; > 0.7), indole (SIMPER test; > 0.5) and phenol (SIMPER test; > 0.3; Fig. 4.2B). When comparing the amount of each substance directly using Kruskal-Wallis test, we found that food deprived larvae produced higher levels of 2-ethyl-1-hexanol (Kruskal-Wallis test, Chi^2 = 5.48, p = 0.02; Fig. 4.4A) and octanoic acid isopropyl ester (Kruskal-Wallis test, Chi^2 = 13.98, p < 0.001; Fig. 4.4B) compared to fed larvae. In contrast, control larvae produced higher levels of phenethyl alcohol (Kruskal-Wallis test, Chi^2 = 19.79, p < 0.001; Fig. 4.4C) than food deprived larvae. The relative amounts of acetophenone, methyl geranate, indole, phenol, hexadecanoic acid, ethyl caprylate, quinoline showed no differences between larvae of different nutritional conditions (Kruskal-Wallis test, p > 0.07).

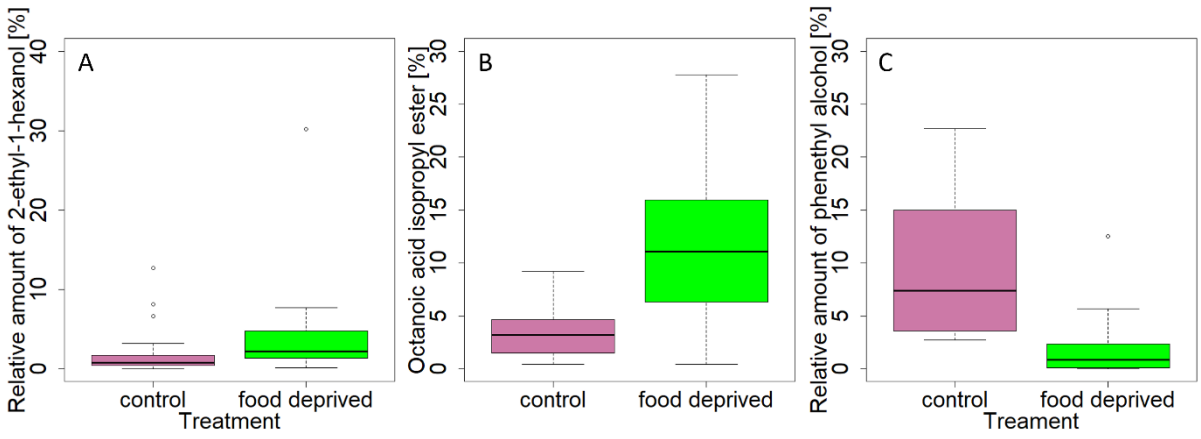


Figure 4.4: *Boxplots show the relative amount of A) 2-ethyl-1-hexanol, B) octanoic acid isopropyl ester, C) phenethyl alcohol in fed (control) and food deprived larvae in N. vespilloides.*

Offspring tactile begging and the physiological reaction of mothers

To investigate if larvae affect the physiology of females, we confronted females with either food deprived or fed larvae. We found that food deprived larvae showed an increased tactile begging rate compared to fed larvae (GLM; F1,46 = 10.15, p < 0.01; Fig. 4.5A) which is supporting previous studies (Smiseth and Moore 2004; Smiseth and Moore 2007). Furthermore, our analysis revealed that females expressed higher JHIII titer if they were confronted with larvae

expressing a higher begging rate (GLM, F1,31 = 5.77, p = 0.02; Fig. 4.5B). In contrast MG levels were not altered by larvae begging rates (GLM, F1,23 = 1.2, p = 0.29).

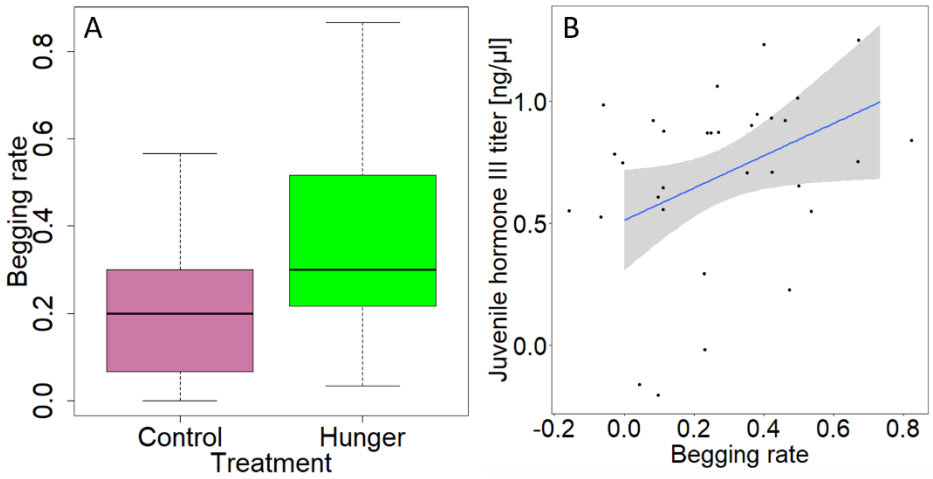


Figure 4.5: *A) shows the tactile begging rates observed from food deprived and control N. vespilloides larvae. B) shows the relationship between the juvenile hormone III titer produced by caring females and the begging rate of larvae they were confronted with ($N_{control}$ = 19, $N_{food\ deprived}$ = 20).*

Discussion

In this study we examined the influence of food availability on the chemistry of burying beetle offspring. Although, we found no differences in the active headspace chemistry, the solvent as well as passive headspace chemistry differed between fed and food deprived larvae. Therefore, our study provides evidence that burying beetle larvae change their chemical profile based on their nutritional conditions. We need to note that based on our data we do not know whether parents perceive these chemical profiles of offspring. The response of parents towards offspring chemistry therefore remains to be investigated. If parents would react towards offspring chemistry with an increase in their feeding rate, we might suggest them being of nutritional need. However, our study could show that caring females increased their JHIII levels in response to increased tactile larval begging. This is cohesive with findings of previous studies in burying beetles, showing that nutritionally dependent larvae can manipulate the investment of care giving individuals (Rauter and Moore 1999; Smiseth et al. 2003; Smiseth and Moore 2008).

We found over 30 substances on the surface of larvae, which is surprising since larvae are not fully sclerotized compared to adults (Pukowski 1933). Generally, cuticular lipids and hydrocarbons are used in various recognition processes in eusocial as well as subsocial insects (Dietemann et al. 2003; Wyatt 2008; Smith et al. 2009; Traynor et al. 2015; Nehring and Steiger 2018; Steiger and Stökl 2018). For example, subsocial insects use CHCs to recognize mating or breeding partners (Steiger et al. 2007; Steiger et al. 2008; Steiger et al. 2009) and eusocial insects allocate their tasks via chemical signals (Le Conte and Hefetz 2008; Ma et al. 2019). While previous studies have mainly focused on adults (Wyatt 2008; Schultner and Pulliainen 2020), there are advantages to extending our knowledge of offspring chemistry since offspring signals can impact the investment of parents (Trivers 1974). Although, so far, evidence is missing if burying beetles react towards offspring chemical cues of nutritional need, we were able to show in a recent study, that females alter their investment strategy based on larval feedback (Sahm et al. submitted to BE). In earwigs, a previous studies showed that mothers indeed react towards chemical signals of offspring by increasing their food provisioning rates (Mas et al. 2009). Also in bumblebees, a previous study by den Boer and Duchateau (2006) showed that workers increased their feedings if confronted with larval extracts of food deprived larvae. Such a strategic resource allocation based on offspring signals can ultimately enhance the fitness of caregivers and resolve the parent-offspring conflict over resource allocation (Godfray 1995a; Mock and Parker 1997; Parker et al. 2002; Kilner and Hinde 2012) as it enables parents to prioritize the offspring in highest need of provisioning. Therefore, investigating chemical substances produced by larvae is important as offspring play a significant role in parent-offspring interactions.

Our first key finding is that larvae of different nutritional needs express quantitative different cuticular profiles. Our results align with previous studies showing that insect offspring alter their chemistry in response to food deprivation (Le Conte et al. 1995; Kölliker et al. 2005; den Boer and Duchateau, M. J. H. M. 2006; Mas et al. 2009). For example, Mas et al. (2009) showed that earwig nymphs in low food conditions expressed certain cuticular substances in a lower quantity compared to nymphs of higher food conditions (Mas et al. 2009). Therefore, our study suggests that food deprived burying beetle larvae can vary their cuticular chemical profile based on their nutritional conditions. Alternatively, larvae of different nutritional conditions might differ in their chemical profiles simply based on the fact, that their chemistry are metabolic by-products. If food deprived larvae reduce or alter their metabolism, they might

change their CHC profiles based on the amount of food present or absent. This suggestion is based on the fact that in adult insects, CHCs are known to be originated from the fatty acid metabolism (Blomquist and Bagnères 2010; Chung and Carroll 2015). Furthermore, chemical profiles of adult insects are highly plastic and vary with different abiotic and biotic conditions (Lockey 1988; Sprenger and Menzel 2020; Blomquist and Ginzel 2021), for example the diet of adults is known to affect their chemistry (e.g., Liang and Silverman 2000).

Our next key finding is that food deprived larvae express quantitatively different volatile profiles compared to fed larvae. This aligns with the results of previous studies showing that volatiles of insect offspring can change with nutritional conditions (Kölliker et al. 2006; He XJ. et al. 2016) leading to an increased provisioning from caregivers. Future studies are needed to investigate how burying beetle parents react towards the different VOCs expressed by larvae. Therefore, as an alternative explanation we want to note that we collected the control larvae directly from the feeding cavity, whereas the food deprived larvae were merely placed in soil. This could have an impact on the chemistry of the larvae since the carrion itself emits several volatiles (Kalinová et al. 2009; Trumbo and Steiger 2020), which could be missing on the volatile profiles of food deprived larvae. Alternatively, the chemistry of food deprived larvae might differ from fed larvae because they have no contact with their parents. This is based on the finding of previous studies which showed that chemical substances of breeding adults are known to affect the tactile begging of larvae (Smiseth et al. 2010; Takata et al. 2019) a mechanism which could also affect chemical begging in burying beetles.

Looking at single substances investigated in our study we found that food deprived larvae emit higher levels of octanoic acid isopropyl ester and 2-ethyl-1-hexanol compared to the control larvae. In accordance with our discussion about the changes in the cuticular chemistry of needy larvae, changing the expression of octanoic acid isopropyl ester and 2-ethyl-1-hexanol hints that offspring can signal their nutritional condition to their parents. An alternative explanation for the different amounts of the ester in fed and food deprived larvae can be that esters can function as aggregation pheromones (Hallett RH. et al. 1995; Morin et al. 1996; Duthie B et al. 2003; Rochat et al. 2004; Jumean Z et al. 2005). The function as aggregation pheromone is also likely the case for burying beetle offspring, as larvae generally aggregate in the feeding cavity of the carcass (Pukowski 1933), but this needs to be investigated further.

In contrast, little is known about 2-ethyl-1-hexanol, except that Haberer et al. (2014) found it in the headspace of breeding burying beetles. Previous studies on plants have shown antifungal properties of 2-ethyl-1-hexanol (Calvo et al. 2020; Wang et al. 2022). This is also likely for burying beetles as they provide antimicrobial secretions to slow down the deterioration of their resource (Vogel et al. 2017; Shukla et al. 2018; Miller et al. 2019). However, the increased expression of 2-ethyl-1-hexanol in food deprived larvae is puzzling since they were starved away from the carcass in our study. Therefore, we suggest that 2-ethyl-1-hexanol might be a component of a begging pheromone, but the exact function remains to be investigated.

Additionally, our study showed that control larvae produced higher levels of phenethyl alcohol compared to food deprived larvae. I general, phenylethyl alcohol is mostly known to reduce bacterial and fungal growth, and thus could help to preserve the food resource (Lester 1965; Lingappa BT. et al. 1969; Corre et al. 1990; Mo and Sung 2007). In burying beetles, previous studies have shown small amounts of phenylethyl alcohol in the headspace of adult beetles (Degenkolb et al. 2011; Haberer et al. 2014). It is plausible that fed larvae exhibit elevated phenylethyl alcohol levels compared to their food deprived counterparts, as they remain on the carcass with their parents and benefit from helping to preserve the carcass. Alternatively, control offspring might signal their better nutritional conditions and with that a higher reproductive value. Previous studies showed that offspring in better nutritional conditions change their chemical profiles (Kölliker et al. 2005; Mas and Kölliker 2011a) with caregivers preferably feeding offspring in better conditions (Mas and Kölliker 2011b). If burying beetles signal their nutritional need or their reproductive value remains to be investigated.

Our next key finding is that we found no impact of nutritional state on the levels of methyl geranate (=MG) emitted by larvae. This was surprising, as we suggested in a previous study that MG could act as potential begging pheromone as it was expressed at the highest level in second instar larvae (Sahm et al. submitted to BE). However, if we consider the results of our Simper-test we found that MG is responsible for the separation between larvae of different nutritional conditions (see results). Therefore, even as we were not able to show a difference in the relative amounts of MG between treatments, MG could still be a component of the begging pheromone of burying beetle larvae. All in all, we suggest that maybe a composition of larval VOCs (with/without changing surface profiles), might communicate the nutritional conditions of larvae. This is based on previous studies showing that signals that consist of more than on

component are widespread among animals (Rowe 1999; Kilner 2002; Partan and Marler 2005; Jacob et al. 2011). However, since control and food deprived larvae could differ in their target of signaling, it is possible that larvae express different volatiles depending on their proximity to parents as well as their nutritional conditions. Indeed, previous studies investigating burying beetle larvae showed that the proximity of parents (Smiseth and Moore 2008) and the nutritional need of larvae (Smiseth and Moore 2004; Smiseth and Moore 2007) both led to an increase in larval begging. Further, a previous study in *N. quadripunctatus* showed that females produce a volatile pheromone that induces larval begging (Takata et al. 2019). If such a volatile exists in *N. vespilloides*, then the food deprived larvae of this study would lack this trigger from their mothers to increase their chemical begging compared to fed larvae. Since previous studies showed that larvae beg more to females than to males (Suzuki 2015; Paquet et al. 2018) we suggest that also in other burying beetle species females might produce a volatile to trigger larval begging.

Lastly, we conducted a separate experiment to investigate the response of females to fed or food deprived larvae. We found that food deprived larvae increase their tactile begging rates compared to fed larvae, a result in line with previous studies (Smiseth and Moore 2004; Smiseth and Moore 2007). In response to increased tactile begging rates, we found that females produced higher levels of JHIII, but MG levels were not affected. Generally, JHIII causes a temporary infertility in female burying beetles, resulting in an increased investment in their current offspring rather than producing additional offspring (Trumbo 1997; Trumbo and Rauter 2014; Engel et al. 2016; Trumbo 2019). Increased levels of JHIII could potentially explain why females increase in their food provisioning in response to increased tactile larval begging (Smiseth and Moore 2008). We want to note the possibility that there could be certain chemical substances whose quantities correlate with the changes in the tactile begging rate of larvae and therefore could also affect the rise in JHIII levels. This assumption is based on previous studies which showed that larval VOCs can affect the physiology and care of mothers (Kölliker et al. 2006; Traynor et al. 2015; He XJ. et al. 2016). For example, in honeybees, (E)-β-ocimene regulates the provisioning behavior and inhibits the egg production of workers (Maisonnasse et al. 2009). That offspring begging pheromones can have both a releaser effect on the behavior and a primer effect on the reproductive physiology of mothers was anticipated first by Mas and Kölliker (2008). Therefore, further knowledge about chemical offspring begging signals could

help us to better understand their function for the parent-offspring communication and therefore in the family life of insects.

In conclusion, our study shows that burying beetle offspring respond to food deprivation by altering their surface and volatile chemistry in accordance with their tactile begging behavior. This confirms that the chemistry of burying beetle' offspring is condition dependent and reflects the nutritional state of larvae. Additionally, our study underlines that changes in care in response to larval begging might be determined by changes in maternal juvenile hormone III levels. It is possible that females use the larval chemical profiles in their parental investment decisions. Future studies should investigate parental responses to larval chemistry to understand its specific function in the family life of *Nicrophorus*. Overall, our study provides evidence that even in less socially complex societies, offspring produces a variety of chemical signals and cues based on their state which could impact the conflict over resource allocation between parents and their offspring. Therefore, our study highlights the importance of chemical signals and cues from offspring in parent-offspring communication during family life.

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Supplementary material

Manuscript 4: To smell or not to smell: New insights in the chemistry of hungry offspring in burying beetles

Jacqueline Sahm, Rosa M. L. P. Staufer, Beatrice Brobeil, Johannes Stökl, Sandra Steiger

Table S4.1: Mean and standard deviations of the relative amounts (%) of each substance found in the solvent extracts of *N. vespilloides* larvae varying in their nutritional conditions (mean relative peak area).

Peak number	Retention time	Substance	Food deprived	Control
1	4.90	unknown MeCHC	0.23±0.15	0.18±0.13
2	5.07	unknown MeCHC	1.64±1.03	1.17±0.81
3	6.09	4-MeC_8	0.37±0.24	0.27±0.2
4	11.79	4-MeC_{10}	0.78±0.7	0.71±0.89
5	11.96	unknown MeCHC	0.16±0.15	0.14±0.17
6	13.10	unknown MeCHC	0.34±0.28	0.34±0.48
7	13.27	unknown MeCHC	0.13±0.14	0.14±0.18
8	15.56	C_{12}ene	0.56±0.5	0.59±0.88
9	18.23	3-MeC_{12}	0.54±0.45	0.63±0.69
10	18.84	unknown MeCHC	0.35±0.26	0.44±0.42
11	20.99	C_{14}ene	1.38±0.93	1.73±1.47
12	23.78	C_{15}	0.34±0.18	0.39±0.26
13	24.84	MeC_{15}	0.85±1.11	1.73±2.27
14	25.86	C_{16}ene	1.96±0.75	2.15±1.05
15	30.24	C_{18}ene	1.35±0.43	1.4±0.45
16	34.21	C_{20}ene	0.66±0.24	0.71±0.41
17	36.25	C_{21}	6.29±6.32	8.42±9.74
18	38.01	C_{22}	0.78±0.26	0.79±0.24
19	39.71	C_{23}	20.94±6.42	22.26±9.06
20	40.91	3-MeC_{23}	1.87±0.65	1.97±0.84
21	41.34	C_{24}	0.74±0.34	0.75±0.39
22	41.51	diMeC_{23}	1.37±0.7	1.27±0.74
23	42.27	4-MeC_{24}	1.12±1.75	1.2±1.4
24	42.34	C_{25}diene	4.6±3.37	3.86±3.25
25	42.44	C_{25}ene	27.09±6.61	21.59±7.11
26	42.91	C_{25}	6.85±1.54	7.3±2.68
27	44.03	3-MeC_{25}	5.02±1.46	5.94±2.21
28	44.56	3,7-diMeC_{27} and 3,9-dimethylC_{27}	5.64±1.62	5.97±2.05
29	45.37	C_{27}diene	0.68±0.21	0.66±0.28
30	45.46	C_{27}ene	2.88±0.79	3.09±0.82
31	45.88	C_{27}	0.21±0.15	0.32±0.27
32	46.93	3-MeC_{27}	0.85±1.76	0.36±0.42
33	47.41	diMeC_{27}	1.4±0.42	1.55±0.8

Table S4.2: Mean and standard deviations of the relative amount (%) of each volatile found in the passive headspace of *N. vespilloides* larvae of different nutritional conditions (mean relative peak area).

Substances	Control	Food deprived
phenol	32.7±17.38	30.57±18.34
2-ethyl-1-hexanol	2.07±3.07	4.25±6.45
acetophenone	6.61±4.91	6.44±4.59
phentylethyl alcohol	11.22±10.9	2.05±3
ethyl carpylate	1.96±1.4	3.72±3.01
octanoic acid isopropyl ester	3.44±2.11	11.47±7.15
quinoline	0.88±0.65	1.2±1.01
indole	22.83±12.95	24.29±11.41
methyl geranate	18.29±10.91	16.01±9.28

Manuscript 5: Maternal hormone levels, pheromones, and terminal investment in breeding burying beetles

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Ready to submit

Author Contributions

S.S. conceived the study, J.S. and S.S designed the study, J.S. collected the data with help of C.J., J.S. analyzed the data, and J.S., T.C. and S.S. wrote the manuscript.

Own contributions

Concept and study design 0 %, data acquisition 75 %, data analyses and figures 95 %, interpretation of results 50 %, first draft of the manuscript 100 %, revision of the manuscript 90%.

Abstract

Ageing individuals face a diminishing residual reproductive value as their lifespan and fecundity reduces. This leads to the terminal investment hypothesis suggesting that older individuals which struggle to survive, should increase their investments into current offspring. The coordination of investments can be achieved over the hormonal states in females, which lead to a temporary infertility and help females to focus on care. In mammals, hormonal changes occur if individuals age. However, evidence is missing in insects that maternal hormones are affected by age or reproductive experience. To shed light on this relationship we conducted two experiments to estimate the effects of age (in 4 different age classes of virgin females) and reproductive experience (after 2, 3 or 4 reproductions) on female physiology. We used the burying beetle species *N. vespilloides* which performs elaborate biparental care on a limited carrion resource. In burying beetles, maternal physiology changes based on the presence of nutritionally dependent larvae, which leads to a temporary infertility of females. Additionally, females express methyl geranate which helps to coordinate parental investments by inducing male help in care. Our results show that age had no effect on JHIII and MG levels. Therefore, we suggest that females do not adjust their physiology according to the terminal investment hypothesis. However, MG levels increased with reproductive experience, although JHIII did not change, indicating a cost with each reproduction followed by the expression of a higher levels of the female's anti-aphrodisiac to increase male help in care. Further, research should explore how different levels of JHIII and MG effect on care levels and therefore family life.

Introduction

Mating and parental care entails costs such as increased mortality and reduced chances for future reproductions (Dean 1981; Chapman et al. 1998; Visser and Lessells 2001; Hunt et al. 2002; Yoccoz et al. 2002; Yanagi and Miyatake 2003; Gilbert et al. 2010; Smith et al. 2015). Therefore, males and females face a decision regarding the allocation of their resources between either their current offspring or future offspring (interbrood conflict), whereby resources invested in one cannot be retained for the other (Trivers 1974; Mock and Parker 1997). As individuals grow older their decisions to reproduce are influenced by their diminishing residual reproductive value as their lifespan and fecundity reduces (Newton et al. 1981; Gustafsson and Sutherland 1988; Gustafsson and Pärt 1990; Westendorp and Kirkwood 1998).

This leads to the terminal investment hypothesis which suggests that as individuals age and are therefore confronted with increased threats to their survival, individuals should allocate a greater share of their energy and resources towards their current reproductive attempt (Williams 1966; Clutton Brock 1984). Experimental evidence for the terminal investment hypothesis has been shown in various species including birds (Pugsek 1981, 1983; Part et al. 1992; Smith 1993), apes, and other mammals (Cameron et al. 2000; Ericsson et al. 2001; Ericsson and Wallin 2001; Isaac and Johnson 2005). Evidence of terminal investment has also been shown in insects (Poizat et al. 1999; Duffield et al. 2017; Farchmin et al. 2020). Considering parental care, Part et al. (1992) found that in the collared flycatcher older females increased their feeding rates whereby suffering substantial weight loss and surviving at a lower rate compared to younger females. Other studies investing ageing individuals showed that they produce more sex pheromones in response to a survival threat to increase their mating success (Polak and Starmer 1998; Sadd et al. 2006; Krams et al. 2011; Kuriwada and Kasuya 2011). For example, Duffield et al. (2018) showed that in crickets older males increased their calling effort if they were immune challenged which supports the terminal investment hypothesis in the context of mating effort. Such a coordination of parental care or mating efforts can help to resolve the parent-offspring conflict over resource allocation towards the current offspring.

The coordination of mating as well as parental efforts can also be achieved by altering hormonal states in females. In the context of family life, previous studies showed that caring females experience periods of hormone induced temporary infertility (Beach 1976; Konner and Worthman 1980; Trumbo 2012b; Trumbo and Rauter 2014). In mammals, for example, lactation supports offspring provisioning and is associated with the hormone prolactin, which suppresses ovulation, allowing females to prioritize investment in current offspring rather than producing additional offspring (Hamada et al. 1980; Dorrington and Gore-Langton 1981). Therefore, the change of hormonal states in females can change the investment of parents and therefore resolves the conflict over parental investments towards the current offspring. Such hormonal changes in females can be induced by the social feedback from offspring (Mas and Kölliker 2011; Engel et al. 2016). Despite hormonal changes, males as well as females can also express anti-aphrodisiac substances to coordinate mating and parental care. Previous studies showed that anti-aphrodisiac substances can suppress mating attempts and focuses the partner to invest in current offspring (e.g., Schlechter-Helas et al. 2011; Engel et al. 2016). However, as individuals undergo changes in their investment decisions with age, they might

also adjust their physiology. For example, in birds, Angelier et al. (2007) showed that the levels of prolactin expressed are correlated with the reproductive experience of females. Until now, studies on hormonal changes with age and reproductive experience are limited. In this study, our focus was to explore whether age or reproductive experience of females influences the hormonal and pheromonal changes and consequently their investment decisions in the burying beetle *Nicrophorus vespilloides*.

Since in many *Nicrophorus* species, adults can be kept and bred over weeks in the laboratory (Scott 1998; Hopwood et al. 2014) they are a suitable study system to investigate the effect of age and experience on parental and mating efforts. Further, burying beetles provide elaborate biparental care for their offspring (Pukowski 1933; Eggert and Müller 1997; Scott 1998; Royle et al. 2013). A beetle pair monopolizes a small vertebrate carcass, transforms it into a ball like shape, and preserves the carrion by spreading antimicrobial secretions over the surface (Pukowski 1933; Scott 1998; Trumbo and Robinson 2004; Royle et al. 2013; Vogel et al. 2017; Shukla et al. 2018; Duarte et al. 2021). Larvae aggregate inside a feeding cavity, where they either self-feed form the carcass or parents regurgitate food to larvae in response to tactile larval begging (Müller et al. 1998; Smiseth and Bu et al. 2003; Smiseth, Darwell and Moore 2003). In general, females provision more than males which focus on carcass maintenance and defense but commonly males desert the brood earlier than females (Fetherston et al. 1990; Scott and Traniello 1990; Trumbo 1991; Smiseth and Moore 2004; Smiseth et al. 2005; Müller et al. 2007; Walling et al. 2008; Royle et al. 2014; Ratz et al. 2021). Due to the considerable costs associated with both reproduction and parental care for both sexes, a conflict arises regarding the allocation of resources – invest either towards current offspring or focus on one's own survival and the chance of future reproductions (Creighton et al. 2009; Ward et al. 2009; Cotter et al. 2011; Smith et al. 2015).

Biparental care is coordinated by a hormonal induced infertility of females via juvenile hormone III (Trumbo 1997; Panaitof et al. 2004; Scott and Panaitof 2004; Engel et al. 2016). Juvenile hormone III (= JHIII) leads to the allocation of female' resources towards their current rather than future offspring (Scott and Panaitof 2004; Trumbo and Rauter 2014) and the production is affected by external factors such as the presence of provisioning-dependent larvae or the monopolization of a carcass (Trumbo et al. 1995; Trumbo 1997; Scott et al. 2001; Engel et al. 2016). Furthermore, burying beetle' females communicate their temporary infertility to their male partner by emitting the pheromone methyl geranate (=MG). MG

functions as an anti-aphrodisiac, suppressing male copulation attempts and ensuring male investment in current offspring (Engel et al. 2014; Engel et al. 2016; Engel et al. 2019). The production of MG is higher in the presence of a male partner rather than in single females, which produce only trace amounts of MG (Steiger et al. 2011). While it is well established that external factors like the presence of larvae, carcasses, and male partners can influence the female 'physiology (Steiger et al. 2011; Engel et al. 2016) as well as parental investments (Sahm et al. 2022), it remains unclear whether internal factors like the age or reproductive experience have an impact on the female's JHIII and MG production and their behavior. Prior research provided evidence of changing investment patterns in burying beetles as they get experienced (Creighton et al. 2009; Trumbo 2009; Billman et al. 2014; Farchmin et al. 2020). However, those studies primarily focused on the effect of age and reproductive experience on the reproductive output and parental efforts of burying beetles, leaving effects on the regulative aspect of the parental care system relatively unexplored.

To investigate how age and previous reproductive experience affect breeding *N. vespilloides* females, we conducted two experiments. In the first experiment, we compared copulation rates, clutch sizes, and the levels of JHIII and MG produced by breeding females of different ages (20, 35, 50 and 65 days). In burying beetles, parental investment in current offspring is usually connected to the production of JHIII and MG production (Engel et al. 2016). Given that older individuals are expected to reduce their investment in future reproductions and prioritize current offspring, we predict that ageing females should produce more JHIII and MG in accordance with the terminal investment hypothesis (Williams 1966). In our second experiment we investigated the consistency of MG emissions in a group of females whose reproductive experiences were altered. Here, we predict repeatable among-individual variation in MG levels. Additionally, we investigated the effect of previous reproductive experience on JHIII and MG production in different groups of females. Three groups of females bred either 2, 3 or 4 times before we analyzed their JHIII and MG production. We predict that females will exhibit increased JHIII and MG levels with increasing reproductive experience. This expectation stems from the understanding that reproduction and parental care entails costs, resulting in a decline in a female's reproductive value with each additional reproduction (Dean 1981; Chapman et al. 1998; Yoccoz et al. 2002; Weladji et al. 2010; Smith et al. 2015) which should lead to a shift of investments to current rather than future offspring in burying beetles.

Material and Methods

Husbandry of beetles

We investigated outbred beetles originated from wild caught burying beetles (*Nicrophorus vespilloides)* sampled near Bayreuth, Germany. Beetles were kept singly in plastic boxes (12 x 9 x 4.5 cm) containing moist peat and placed in a climate chamber under a 16:8 h dark: light cycle at 20 °C. Keeping beetles singly prevent an exchange of substances, thus, enabling us to access their individual chemistry and secures control over the mating status of beetles. Beetles were fed twice a week with sliced mealworms (*Tenebrio molitor*) throughout the experiment.

General experimental procedure

In two experiments we investigated the effect of female age, and their prior reproductive experience on clutch sizes and female levels of juvenile hormone III (= JHIII) and methyl geranate (= MG) in biparental caring females of *N. vespilloides*.

For both experiments, we firstly haphazardly paired unrelated females and males in plastic boxes (12 x 9 x 4.5 cm) lined with moist paper tissues. We measured each of the beetle's pronotum width using a slide gauge before observing the copulation rate of beetle pairs. The paper tissues allowed us to observe successful copulations performed by each pair in the first experiment. To avoid disturbances during mating, copulation rate was not measured again in the second experiment. During the observation, beetle pairs acclimatized 10 min on the paper tissues before we recorded the number of successful copulations during a 30-min observation period. We were able to simultaneously observe 4 boxes every 2 h.

After observing the copulations (or directly following the setup of beetle pairs in the second experiment), we placed beetles in a similar sized plastic boxes half-filled with moist peat and provided a priorly weighted mouse carcass (= 10 ± 2.5 g). 48 h later, we moved the beetles and carcass to a new box filled with moist peat to isolate the eggs and allow the larvae to hatch separately. We counted the eggs laid in the 'old' boxes to determine each pair's clutch size. 24 h later (72 h after beetles had access to a carcass) we checked the 'old' boxes for hatched larvae every 2 h. Hatching larvae were pooled in a petri dish lined with a moist tissue to prevent larval desiccation. We randomly assigned 10 larvae of mixed parentage to each beetle pair but only if their own larvae had already hatched. This is a common procedure in burying beetles (Rauter

and Moore 1999; Oldekop et al. 2007; Engel et al. 2016; Sahm et al. 2022), as *Nicrophorus vespilloides* beetles cannot distinguish between own and unrelated larvae once their own larvae have hatched (Müller and Eggert 1990). After 24 h of parental care, we sampled the emitted volatiles of females via headspace sampling (see below). Then we removed the hemolymph of females to assess their JHIII levels (see below) before freeze-killing all beetles at -20 °C. We collected MG and JHIII after 24 h of parental care since Engel et al. (2016) found the highest JHIII and MG production at this point of parental care in *N. vespilloides*.

Effect of age on copulation rate, clutch size, and JHIII levels of breeding females

The first experiment focused on virgin females and investigated the effect of female age on their copulation rate, clutch size, and their production of JHIII and MG. Therefore, a new, virgin female was haphazardly paired with a virgin male following the 'general experimental procedure'. We set up 40 pairs in four different age categories: 20, 35, 50 or 65 days. This experiment was conducted in two follow-up generations. Due to hatching failures, we reached the following sample sizes for each age category: $N_{20\ days}$ = 22, $N_{35\ days}$ = 18, $N_{50\ days}$ = 26, $N_{65\ days}$ = 25.

Effect of previous reproductions on the clutch size and JHIII levels of breeding females

The primary objective of this setup was to investigate the consistency of the MG production and the clutch size within females. To assess consistency of MG levels and clutch size in females, we conducted four successive trials (once after 20, 35, 50, and 65 days), each involving a new male and a fresh carcass (see 'general experimental procedure'), separated by 15-day intervals. New males were chosen to randomize possible effects of their breeding partner on the physiology of females. Following each of these four reproductive cycles we counted the number of eggs laid by females and measured their MG levels (N = 11, due to hatching failures and the death of females; details below). Unfortunately, measuring the repeatability of JHIII is impossible since the collection of hemolymph for analysis is lethal for the females.

To investigate the influence of reproductive experience on the levels of JHIII, we conducted an additional analysis involving the three different groups of females (= 2, 3 and 4 reproductions; N = 11). Initially, in each of these three groups, the 20-day old females were

paired with a male partner and provided with a carcass. Subsequently, at the age of 35, all three groups of females were paired again. After this second reproductive cycle, we collected the hemolymph of one of the groups ($N_{35\ days}$ = 11). The remaining two groups were paired again at age 50 with hemolymph collected again from only one group to estimate their JHIII levels after three reproductions ($N_{50\ days}$ = 11). Lastly, the remaining group of females was paired again at age 65 and we measured their JHIII levels after this fourth reproduction ($N_{65\ days}$ = 11). All pairings followed the 'General experimental procedure' outlined earlier, while the analysis of the JHIII is detailed below.

Headspace sampling

To analyze the amount of methyl geranate (=MG) produced by breeding females, we collected their emitted volatiles via headspace sampling. Headspace samples were collected in a climate chamber at 20 °C. Each female was individually placed in a silanized glass jar (3 cm inner diameter). The construction of the headspace instrument and the headspace filter used in this experiment followed the directions of previous studies (Sahm et al. submitted to BE; Manuscript 4). Prior to the sampling process, volatiles of females were accumulated for 20 min before we created an airflow through the instrument for 5 min using the membrane pump (~200 ml/min). The headspace filters, containing the absorbed volatiles, were then stored in a freezer at -20 °C. Before analysis, we added 1 µl of methyl undecanoate (= 20 ng; Sigma-Aldrich, St. Louis, USA) dissolved in *n*-hexane (Carl ROTH, ROTISOLV® ≥ 99 %; Karlsruhe, Germany) as internal standard. The volatiles were desorbed at a temperature of 300 °C for 8 min using a thermal desorber (Shimadzu, TD-30R) coupled to GC-MS (Shimadzu GC2030 gas-chromatograph connected to a Shimadzu QP2020NX mass-spectrometer; Shimadzu, Duisburg, Germany). The GC was equipped with a non-polar capillary column (SH-Rxi-5Sil MS, length = 30 m, inner diameter = 0.25 mm, film thickness = 0.25 µm, Shimadzu, Duisburg, Germany). The oven temperature within the GC was programmed to raise from 50 °C to 200 °C at a rate of 5 °C/min. Subsequently, the temperature was raised to 280 °C at a rate of 15 °C/min and held at this level for 10 min. Helium served as the carrier gas (linear velocity = 36.3 cm/sec).

Juvenile hormone III of females

To quantify the amount of JHIII produced by females, we pierced the intersegmental membrane on the ventral side of females with an injection needle (Braun, Sterican, Ø = 0.60 x 25 mm) and collected the excreting hemolymph with a calibrated 10 µl glass capillary (BLAUBRAND® intraMARK Micropipettes). The collected hemolymph was measured using a slide gauge and transferred into a conical 1.5 ml glass vial. Subsequently, 100 µl of *n*-hexane (Carl ROTH, ROTISOLV® ≥ 99 %, Pestilyse®) containing 100 ng of vernolic acid methyl ester (Sigma-Aldrich, Supelco, St. Louis, USA) as an internal standard was added to the hemolymph. The solution was vortexed for 30 s, effectively separating the clear hexane phase containing JHIII from undesired components of the hemolymph. The hexane phase was transferred into a new conical glass vial, and all samples were stored in a freezer (-20 °C). Before analysis, we evaporated each sample to reduce the volume to 2-3 µl using a nitrogen stream. Subsequently, 1 µl of each sample was manually injected into the GC-MS (C2030 and QP2020NX MS, Shimadzu). The GC was equipped with a non-polar capillary column (description above). The temperature within the GC-oven was programmed to rise from 150 °C to 300 °C at a rate of 10 °C/min, with a final hold period of 20 min. Helium served as carrier gas (linear velocity = 50 cm/sec).

Statistical analyses

We analyzed and plotted all data using R version 4.2.2. In the first experiment, we investigated the impact of female age several key factors, including the copulation rate, clutch size, JHIII levels and MG levels. The copulation rate was calculated by dividing the number of successful copulations by the observation time (30 min). Further, we calculated the JHIII levels by dividing the peak area of JHIII by the peak area of the internal standard and multiplying it by the known quantity of the internal standard (100 ng). The calculation of MG levels followed the same procedure (quantity of the internal standard = 20 ng). Prior to the analyses we $\log_{10}$-transformed the amount of JHIII and MG. We used generalized linear models (GLMs) fitted with Gaussian-error structures to investigate the influence of female size and age on the copulation rate. Furthermore, we used (GLMs) fitted with Gaussian-error structures to analyze the impact of female size, age, clutch size, and the copulation rate on (a) the amount of JHIII and (b) the amount of MG amounts produced by the females.

In the second experiment, we used linear mixed effect models (LMEs; R-command = lmer; package = "lme4") to assess the repeatability of (a) the MG levels and (b) the clutch size of females across different reproduction cycles. In these LMEs, the sample-ID of females was included as random factor. We estimated the repeatability of MG levels and clutch size using the *lmm* method (using the rptGaussian() function from the "rptR" and "statmod" packages). Additionally, we investigated the effect of the number of previous reproductions, female pronotum size, and the clutch size on the JHIII amount, produced by different females using a gaussian GLM. Please note, that due to the lethal nature of the hemolymph extraction for the evaluation of the JHIII levels, assessing repeatability was not feasible.

All reported F- and p-values were generated using the Anova()-function of the "car" package, following the methodology outlined by Foy and Weisberg (Fox and Weisberg 2017).

Results

Effect of age on copulation rate, clutch size, MG, and JHIII levels of breeding females

Female age had generally no effect on the amount of JHIII (GLM, $F_{3,73} = 0.70$, $p = 0.55$; Fig 5.1A), or the amount of MG produced (GLM, $F_{3,76} = 0.21$, $p = 0.89$, Fig. 5.1B), nor on the clutch size (GLM, $F_{3,78} = 1.6$, $p = 0.2$) or on the copulation rate (GLM, $F_{3,78} = 0.53$, $p = 0.66$). Analyzing the impact of female pronotum size, we found that bigger females showed higher copulation rates than smaller females (GLM, $F_{1,78} = 6.68$, $p = 0.01$; Fig. 5.2). However, female pronotum size had no effect on the amounts of JHIII (GLM, $F_{1,73} = 0.02$, $p = 0.88$) or MG (GLM, $F_{1,76} = 0.87$, $p = 0.35$). Furthermore, when exploring the influence of the copulation rate on the female physiology, we found no effect on the amount of JHIII (GLM, $F_{1,73} = 2.39$, $p = 0.13$) or the amount of MG (GLM, $F_{1,76} = 0.006$, $p = 0.94$). Finally, regarding the effect of clutch size, our analysis revealed no effect on the amount of JHIII (GLM, $F_{1,73} = 0.04$, $p = 0.84$) and MG (GLM, $F_{1,76} = 0.008$, $p = 0.93$).

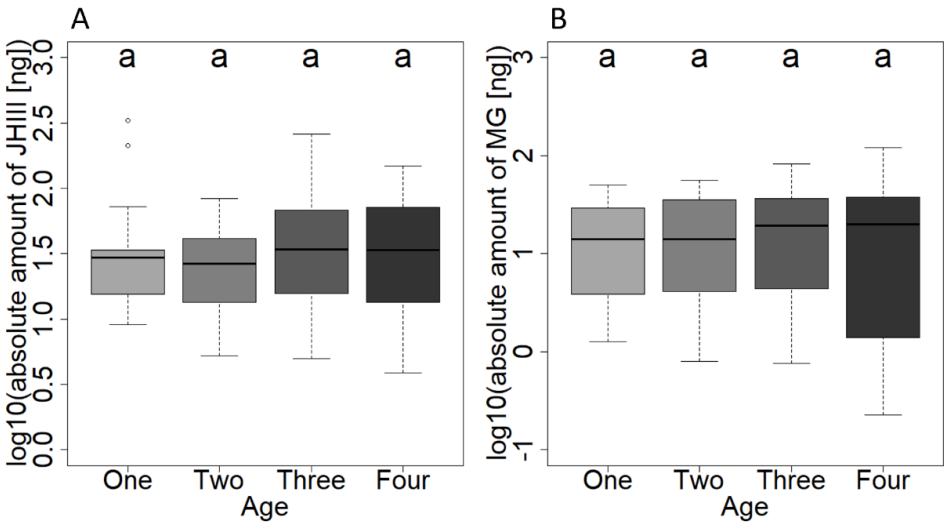

Figure 5.1: Boxplots show the A) log_{10}-transformed amount of juvenile hormone III and B) log_{10}-transformed amount of methyl geranate of females (N = 11) of different ages.

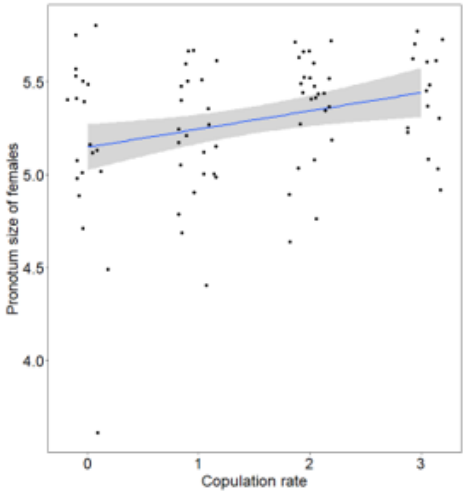

Figure 5.2: Relationship between the pronotum size of N. vespilloides females and the number of copulations observed in 30 minutes (N = 83). The dots represent the original data, the lines represent the calculated regression lines and their respective 95% CI.

Effect of previous reproductions on the clutch size, MG, and JHIII levels of breeding females

In examining the repeatability of MG levels produced by females, and measuring it after each of the four reproductions, we found an increase in the amount of MG for individual females with increasing reproductive experience (aov-command, F_3 = 3.57, p = 0.03; Fig. 5.3B). In addition, our analyses reveal that MG levels of females are repeatable (repeatability for sample-ID R = 0.52, p = 0.002). The clutch size of females was affected by the number of previous

reproductions (aov-command, F_3 = 3.07, p = 0.04; Fig. 5.3A), but our analyses revealed that clutch size of females were not repeatable (repeatability for sample-ID, R = 0.14, p = 0.2).

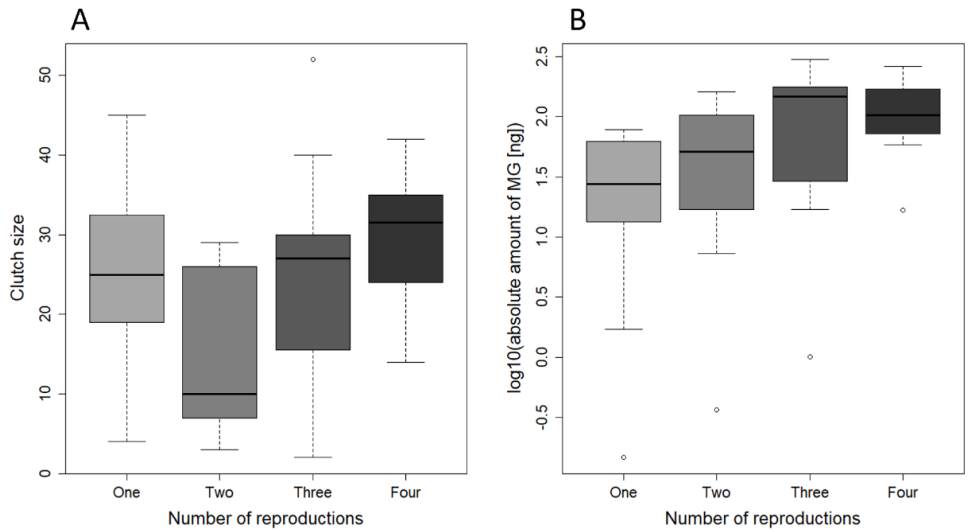

Figure 5.3: Boxplots show the A) Clutch size by the same group of females over four reproductive bouts and B) shows the log_{10}-transformed amounts of methyl geranate produced by the same group of females (N = 11) after different numbers of reproductions.

Further, we found an effect of the number of previous reproductions on the clutch size (GLM, $F_{3,93}$ = 2.7, p = 0.05; Fig. 5.4A). However, previous reproduction showed no effect on female JHIII levels (GLM, $F_{2,23}$ = 2.64, p = 0.09, Fig. 5.5A). Due to a GC malfunction baseline JHIII levels of females (after 1 reproduction) are missing. Similarly, when considering the pronotum size of females, we found no effect on clutch size (GLM, $F_{1,93}$ = 0.05, p = 0.82), or the JHIII levels of females (GLM, $F_{1,23}$ = 0.03, p = 0.87). In a final analysis, we found females who laid larger clutch sizes produced higher JHIII levels (GLM, $F_{1,23}$ = 6.27, p = 0.02, Fig. 5.4B).

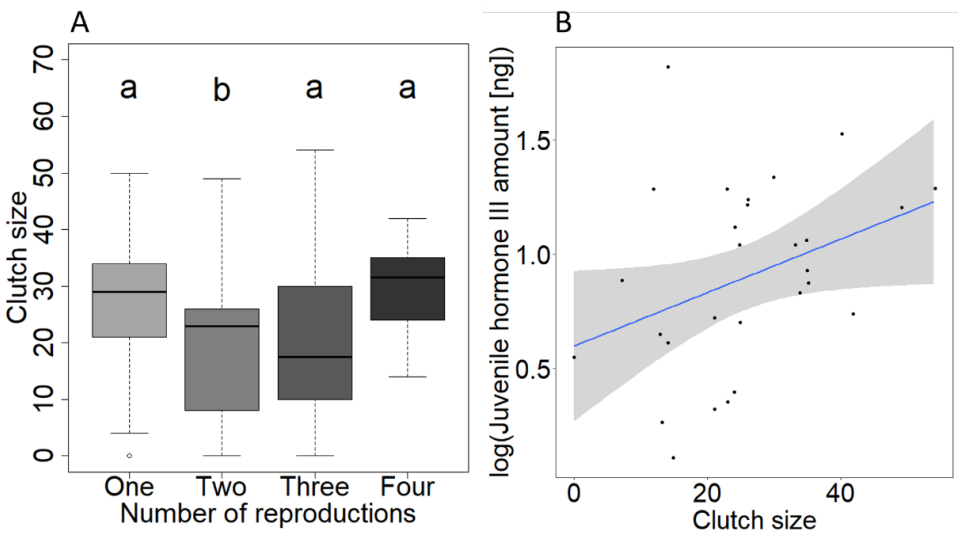

Figure 5.4: A) boxplots show the clutch size of females after 1-4 reproductions. B) shows the relationship between the log_{10}-transformed amount of juvenile hormone III produced by N. vespilloides females and their clutch size (N = 28). The dots represent the original data, the lines represent the calculated regression lines and their respective 95 CI.

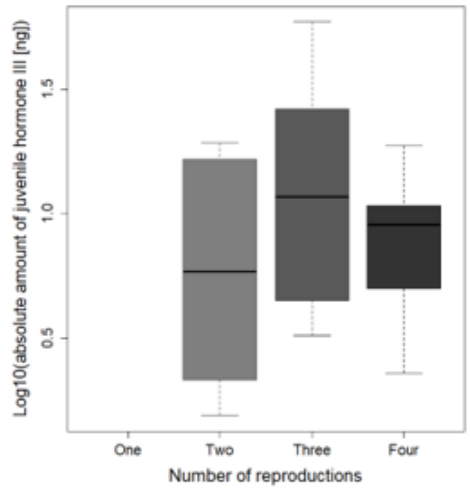

Figure 5.5: Boxplots show the log_{10}-transformed amount of juvenile hormone levels produced by females (N = 11) after either 2, 3 or 4 reproductions.

Discussion

Here we studied the effect of age and previous reproductive experience on the JHIII and MG levels of breeding *N. vespilloides* females. Age of females showed no effect on their JHIII and MG levels, but MG levels increased with increasing reproductive experience of females. The latter follows the terminal investment hypothesis (Williams 1966; Clutton Brock 1984) as higher levels of MG in experienced females helps them to focus their investments – and the

investments of their partners - towards care for current offspring. Therefore, our study extends the knowledge about the impact of internal factors like age or previous reproductive experience on the physiology of burying beetle females which could help understanding their plasticity in care (Sahm et al. 2022).

Our first key finding shows that caring *N. vespilloides* females of increasing age showed similar levels of JHIII and MG. This result contradicts our prediction, as we expected higher JHIII and MG levels in ageing females. This was based on previous studies which showed that older individuals focus their investments towards their current rather than future offspring (Part et al. 1992; Smith 1993; Creighton et al. 2009; Duffield et al. 2018; Farchmin et al. 2020) following the terminal investment hypothesis (Williams 1966; Clutton Brock 1984). Based on our result we suggest that *N. vespilloides* females do not adjust their JHIII and MG levels based on age. Maybe external factors play a bigger role in female' investment decisions than internal factors. For example, carcass monopolization, nutritional dependent larvae, and the presence of males are known to alter the female' physiology (Steiger et al. 2011; Engel et al. 2016) as well as parental investment decisions (Sahm et al. 2022; Sahm et al. 2023). Especially feedback from offspring in form of chemical or tactile signals might be of importance in females' investment decisions (Sahm et al. submitted in BE; Manuscript 4; Smiseth and Moore 2002; Smiseth, Darwell and Moore 2003; Smiseth and Moore 2004b). However, in this study, we provided females with a constant number of 10 larvae. Based on the results of a previous study, 10 larvae are enough to trigger a physiological response in females (Engel et al. 2016). Engel et al. (2016) however, showed that females confronted with different numbers of larvae, increased their emission of MG with increasing number of larvae present. Since we did not increase the number of larvae in our experiment, females might not respond with an increase in their JHIII and MG levels as they age. Another possible explanation could be that JHIII is simply not an indicator of terminal investment decisions. Instead burying beetles might adjust their investment decisions as they age but not their physiology. For example, older females invest more into current offspring by producing larger broods or by changing their care behavior whereby investing less into their own somatic state (Creighton et al. 2009; Ward et al. 2009; Cotter et al. 2011; Smith et al. 2015). Another possible explanation could be that in burying beetles age alone is not enough to induce terminal investment decisions. For example, in crickets age in combination with an immune challenge led to an increase in the calling efforts of males following the terminal investment hypothesis (Duffield et al. 2018). Also in burying

beetles, a recent study by Farchmin et al. (2020) showed that terminal investment only occurs in older, immune-challenged breeders with reproductive experience. Therefore, future studies might need to examine a combination of factors to study terminal investment in ageing burying beetles.

Our study found no effect of age on copulation rate. The first contradicts previous findings in males, showing that older males mate at a higher rate than younger ones (Benowitz et al. 2013). We need to note that the young males investigated by Benowitz et al. (2013) were between 11-14 days old which could mean that maybe young males were immature. In contrast, our young females ranged between 20-35 days ensuring that they reached sexually maturity. However, since one or two matings are sufficient for females to fertilize all their eggs, they might not increase their copulation rate as they age to increase their insemination (Dressel 1987; Müller and Eggert 1989; House et al. 2008; House et al. 2009). We suggest that females might rather adjust their oviposition or their care behavior rather than their mating effort. Here, we found no effect of age on the clutch size produced by females. That older individuals should invest more into the production of a larger egg clutch was predicted based on the terminal investment hypothesis (Williams 1966; Dean 1981; Chapman et al. 1998; Yoccoz et al. 2002; Weladji et al. 2010; Smith et al. 2015). Our results do not indicate that females adjust their clutch size based on age. However, we suggest that *N. vespilloides* females might invest more into current eggs by increasing the mass of eggs laid. This is based on previous findings by Trumbo (2012a) showing that ageing *N. orbicollis* females invested more in current offspring by laying larger eggs. Further studies could examine if older females of other burying beetle species also invest more in current offspring by laying larger eggs.

In addition, our results showed that larger females copulated more often than smaller females. Larger females might have a higher energetic capacity (Steiger 2013) and might copulate more often as they can store more eggs which need to be fertilized.

Our next key finding shows that MG levels increases with a higher reproductive experience of females. This partly fits our prediction that higher experience would lead to higher MG levels due to the cost of previous reproductions. Higher levels of MG could help to focus the remaining energy and resources to the current rather than future offspring. This is based on the fact that high levels of MG suppress male mating and induce male investments for the current breeding attempt (Engel et al. 2016). Therefore, an increase in MG levels with

reproductive experience can also resolve the conflict over mating rates between males and females (Müller and Eggert 1989; House et al. 2008; Head et al. 2014). As discussed previously, one factor, i.e. age, seems to be not enough to affect the mothers' physiology, but age combined with reproductive experience could lead to terminal investment. As previously described, Farchmin et al. (2020) showed that females of *Nicrophorus marginatus* only increased their investment in the current brood if they were older, experienced, and immune-challenged. In our study, females that produced higher amounts of MG were not only more experienced but also older. Therefore, we suggest that more than one factor is needed to trigger terminal investment in the physiology of *N. vespilloides*.

In contrast, JHIII levels were not affected by the number of previous reproductions of females. However, since baseline values for JHIII are missing in this experiment due to a GC malfunction (baseline = JHIII of females after 1 reproduction), it is difficult to come to interpret these results.

Our study found that JHIII titers were higher in females which produced larger clutches. We suggest that females might anticipate a larger brood. Clutch sizes in *Nicrophorus* can vary between 0 and 45 eggs (Müller et al. 1990; Steiger et al. 2007) depending on the carcass size (Müller et al. 1990; Scott 1997). Since we standardized the brood size to 10 larvae per females in our experiments, females which laid larger egg clutches might had anticipated more offspring than we provided them. Therefore, females might have increased their JHIII titer in advance of the upcoming care for their current brood. It is possible that female anticipate their upcoming investments as they are also able to estimate the timing of larval hatching (Müller and Eggert 1990). Further studies would be needed to specify if females can adjust their physiology in anticipation of their offspring. Another possible explanation for our results could be that females might be able to express higher JHIII titers if they are in better conditions. This is based on a previous study which showed that females in better condition - for example of larger body size - are known to lay a larger number of eggs (Steiger 2013).

In conclusion, our study shows no effects of age and limited effects of reproductive experience on the JHIII and MG levels of breeding *N. vespilloides* females. However, we presented the possibility that more than one factor triggers terminal investment in the physiology of burying beetles. Understanding the influences of internal factors on the maternal physiology are of interests for divers animal taxa next to burying beetles, since hormonal

changes of a mother can be found in other animal taxa leading to investment in current offspring (McNeilly 1979; Hamada et al. 1980; Konner and Worthman 1980; Dorrington and Gore-Langton 1981; McNeilly 2001). Such hormonal changes can impact the parent-offspring conflict over parental investments and mating rates. Further studies are needed to investigate if the change of more than one internal factor might lead to changes in the maternal hormone and pheromone levels of burying beetles. Lastly, we suggest that social feedback might be of greater importance rather than the age or experience of individuals in changing their physiology and therefore their investment behavior.

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Statutory declarations - (Eidesstattliche) Versicherungen und Erklärungen

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Hiermit erkläre ich mein Einverständnis, dass die elektronische Fassung meiner Dissertation unter Wahrung meiner Urheberrechte und des Datenschutzes einer gesonderten Überprüfung unterzogen werden kann.

(§ 8 Satz 2 Nr. 8 Promotionsordnung für die Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth)

Hiermit erkläre ich mein Einverständnis, dass bei Verdacht wissenschaftlichen Fehlverhaltens Ermittlungen durch universitätsinterne Organe der wissenschaftlichen Selbstkontrolle stattfinden können.

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Ort und Datum

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Unterschrift (Jacqueline Sahm)