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PARENTAL CARE IN A BEETLE SPECIES: ROLES AND INVESTMENTS

(Utódgondozás egy bogárfajnál: Szülői szerepek és befektetések)

Thesis for the Degree of Doctor of Philosophy (PhD)

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I hereby declare that I prepared this thesis within the Doctoral Council of Natural Sciences and Information Technology, Juhász-Nagy Pál Doctoral School, University of Debrecen in order to obtain a PhD Degree in Natural Sciences at the University of Debrecen.

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PARENTAL CARE IN A BEETLE SPECIES: ROLES AND INVESTMENTS

(Utódgondozás egy bogárfajnál: Szülői szerepek és befektetések)

Thesis submitted in partial fulfilment of the requirements
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“It may be argued that to know one kind of beetle is to know them all. But a species is not like a molecule in a cloud of molecules – it is a unique population.”

- E. O. Wilson

PART I

This section encompasses the introductory context and a summary of the scientific topics covered in my thesis. I establish the main inquiries and goals of my research, followed by a brief portrayal of the conducted work, showcasing significant discoveries and outcomes. Finally, I conclude with a comprehensive discussion and summary of my principal findings.

I.1 GENERAL INTRODUCTION

I.1.1. Parental care – Benefits and conflicts

Parental care is a fundamental aspect of animal behaviour that involves the investment of time, energy, and resources in the survival and rearing of offspring (Clutton-Brock, 1991; Klug *et al.*, 2012; Royle *et al.*, 2012). This behaviour is displayed in a variety of ways, such as by protecting, incubating, grooming, feeding, and teaching the young. The primary advantage of parental care is its contribution to increased offspring survival rates (Clutton-Brock, 1991; Roff, 2002; Schrader *et al.*, 2015). Young that are born in a vulnerable state require defence, food, and shelter to ensure their survival. Additionally, parental care can lead to the optimal growth and development of offspring (Schrader *et al.*, 2015; Langenhof & Komdeur, 2018). The main impetus behind providing care is that parental care can improve the reproductive success of parents by increasing the likelihood of gene transmission to future generations, hence it is beneficial for both parents (Clutton-Brock, 1991; Royle *et al.*, 2012). The diversity of such behavioural forms in the animal kingdom can showcase the intricate strategies employed by species. For instance, in the zebra finch (*Taeniopygia guttata*), both parents share caregiving duties simultaneously; they exhibit synchronous care: both the male and female

construct the nest, incubate the eggs, and feed the chicks together (Mariette & Griffith, 2012, 2013; Mainwaring & Griffith, 2013). In sequential biparental care, parents take turns providing care at different stages of the offspring's development. An example is the striped goby cichlid (*Eretmodus cyanosticus*), where the female guards the fertilised eggs and newly-hatched young, and as the offspring grow, the male takes over to care for the fry (Morley & Balshine, 2003). If each parent has specific roles or responsibilities in raising the offspring, it is characterised as differential care. This can be seen in the burying beetle (*Nicrophorus* spp.), where both parents participate in locating and burying a small carcass to serve as food for the developing larvae. The male mostly focuses on defending the territory and maintaining the carcass, while the female primarily cares for the larvae (Scott, 1998; Müller *et al.*, 2007; Trumbo, 2007). A special case is when biparental care involves a clear division of labour, with each parent specializing in different caregiving tasks. For example, in the sex-role-reversed wattled jacana (*Jacana jacana*), the male builds and maintains the nest, incubates the eggs, and cares for the young, while the female defends the nest against predators (Emlen & Wrege, 2004). Conflict of interest is encoded in the system, as parents share the gains but have to bear the costs of caring for offspring individually, particularly in species without the flexibility of decisions during care (Royle *et al.*, 2010).

One common type of conflict between the sexes during parental care is shifting a larger share of the caregiving responsibilities to the other parent, resulting in a conflict over the division of labour and effort (Parker *et al.*, 2002). Conflicts can also arise when parents differ in the amount of resources and effort they invest in caring for the offspring (Clutton-Brock, 1991; Smiseth *et al*., 2012; Royle *et al.*, 2016). Such conflicts can lead to reduced parental investment or desertion, but they can also be resolved through negotiation and compromise between parents (Clutton-Brock, 1991; McNamara *et al.*; 1999; Lessells, 2012; Rosenthal, 2018).

Concisely, parental care is an important aspect of animal behaviour that contributes significantly to the survival and success of offspring. While conflicts between the sexes can arise during parental care, these conflicts are generally resolved through negotiation, compromise, and also by desertion.

I.1.2. Overview of parental care in insects

Parental care is an essential aspect of the survival and success of offspring in many animal species, including insects (Royle *et al.*, 2012; Trumbo, 2012). Insects have evolved a wide variety of parental care strategies that can be classified into two categories: prenatal care and postnatal care (Trumbo, 2012; Wong *et al*., 2013).

Prenatal Care. Parents may invest in their offspring before they are born or hatched by providing them with resources or protection. Some examples of prenatal care include:

- Oviposition site selection: Some beetle and lepidopteran species carefully select where to lay their eggs to ensure that the offspring have access to the resources they need to survive. For example, the leaf beetle *Cassida canaliculata* and the butterfly *Pieris virginiensis* lay their eggs on specific host plants that provide the necessary nutrients and chemical cues for the offspring's development and survival (Heisswolf *et al.*, 2005; Doak *et al.*, 2006).
- Egg guarding: Several species of katydids, treehoppers, and bugs guard their eggs to prevent predation or parasitism. For instance, females of Southeast Asian Treehopper (*Pyrgauchenia tristaniopsis*) and Hibiscus Harlequin Bugs (*Tectocoris diophthalmus*) stand and guard over their eggs until they hatch (Stegmann & Linsenmair, 2002; Jones, 2020).
- Egg transportation: Some bugs and beetles protect their eggs by moving them from natal sites. Females in *Adomerus triguttulus* bugs and Egg Case-

carrying Water Scavenger Beetles (Hydrophilidae: Acidocerinae) can serve as a great example as they carry their eggs on their bodies to protect them from predators and environmental hazards (Nakahira & Kudo, 2008; Short & Garcia, 2014).

- Provisioning: Some species of wasps provide their offspring with food resources before they hatch. For example, digger wasps (e.g., *Ammophila pubescens*) provision their nests with paralysed prey for their offspring to feed on (Field *et al.*, 2007).

Postnatal Care. After offspring are born or hatched, parental care may continue in the form of indirect or direct care. Some examples of postnatal care include:

- Brood defence: Some species of ants and beetles actively defend their offspring against predators or other threats. An example can be the Maritime Earwig (*Anisolabis maritima)* females fight to protect their broods from predators and conspecific competitors (Miller *et al.*, 2011).

- Brood care: Some insects care for their offspring by cleaning, grooming, or moving them to more suitable locations. Burying beetles clean their larvae and move them to areas with more food (see for example Scott, 1998; Smiseth & Moore, 2007).

- Provisioning: Some species of wasps, and beetles provide food or other resources for their offspring after they hatch. For instance, in the social wasp *Ropalidia marginata* and the burying beetle *Nicrophorus vespilloides*, parents provision their nests with food and feed their offspring. (Sen & Gadagkar, 2006; Smiseth & Moore, 2007).

- Cooperative care: Multiple individuals, such as parents or siblings, may assist in the care of the offspring. For example, in some species of ants, workers assist in the care of the queen's offspring (e.g., Lopes *et al.*, 2005).

The act of caring for offspring can be exhibited by females, males, or both, and, as shown above, is sometimes implemented through several types of behaviour within families (Halffter, 1997), genera (Gilbert *et al.*, 2010), and even species (Beal & Tallamy, 2006). Insects that acquired the behaviour of caring for their offspring typically inhabited environments that were unusually harsh or resource-rich, stable and structured, or subject to high levels of predation (Wilson, 2000).

Female-only care occurs across various groups related to the Hexapoda, such as the Arachnida, Isopoda, Chilopoda or Diplopoda (Zeh and Smith, 1985). Biparental care is the most frequent in the order of Coleoptera (Royle *et al.*, 2012; Trumbo, 2012). Although details of biparental care vary among taxa, some common traits can be found. For example, most biparental species make nests underground or in wood burrows (Eickwort, 1981; Brandmayr, 1992). Additionally, males exhibit nest-guarding behaviour against competitors in most species (Tallamy & Wood, 1986; Tallamy, 2001). Food also has been considered to be an important factor for biparental care because some types of food are difficult to process by young, such as rotten wood, or are difficult to defend from competitors without the help of parents, such as carrion and dung (Tallamy & Wood, 1986; Tallamy, 1994). It is noteworthy that biparental Blattodea and Coleoptera species make nests in the food source of the young (e.g., wood-feeding cockroaches) or carry the food to their nests before larval hatching (e.g., dung beetles, dor beetles) (Park & Choe, 2003; Smiseth & Moore, 2007; Hunt & Simmons, 2002a).

Understanding the diversity of parental care strategies in insects can provide insights into the evolution of parental care across different taxa. By examining the

ecological, social, and evolutionary factors that influence parental care, we can gain a better understanding of the underlying mechanisms that drive the evolution of these behaviours. Furthermore, understanding parental care in insects is essential for developing effective conservation strategies for insect species, many of which are threatened by habitat loss, climate change, and other anthropogenic factors (Sánchez-Bayo & Wyckhuys, 2019; Wagner *et al.*, 2021). Here, we aimed to contribute to a more comprehensive picture of offspring care in scarab beetles, as well as the various factors that contribute to its features. To achieve this, the studies presented in my thesis examine the roles and investments of parents during the reproductive season of a beetle species, *Lethrus apterus*, that is unique in several aspects.

I.1.3. The study species

Lethrus apterus (Laxmann, 1770) is among the limited number of beetle species that exhibit biparental care. In contrast to other species within the Geotrupidae family, species of the *Lethrus* genus rely solely on plants as a food source (Nikolajev, 2003). The species is predominantly found in grassland habitats from Hungary to the river Don (Nikolajev, 2003; Král *et al.*, 2013). Adult beetles can range from 2-4 cm in body length and 8.5-14 mm in pronotum width, with a plain black appearance and no flight capability due to the fusion of their elytra (Rosa *et al.*, 2019). Both male and female beetles have robust mandibles used for cutting leaves of various plants for self and offspring nourishment (Nikolajev, 2003). Sexual dimorphism is present, males wear a pair of processes, often referred to as tusks, on the ventral side of their mandibles. The reproductive season lasts from March to late May, with *L. apterus* beetles beginning to search for mates soon after emergence. Typically, males seek and visit tunnels occupied by females, with densities of 10-15 burrows per square meter (Frantsevich *et al.*, 1977; Nikolajev, 2003). Once a pair is formed, resident males vigorously guard the nest against predators, competitors and intruders of the same species (Figure 1). *L. apterus* is a

long-lived insect and is iteroparous, meaning that it can reproduce for two consecutive years, i.e., it is capable of overwintering at least twice.



Figure 1. *Lethrus apterus* male guarding the entrance of the tunnel

Rosa *et al.* (2019) reported that *L. apterus* males exhibit variation in tusk length (Figure 2), with larger males having relatively longer tusks and smaller males having disproportionally smaller tusks compared to body size, while medium-sized males have a steep increase in tusk length with body size. Different-sized males also exhibit variation in fighting and guarding behaviour, with smaller males being less willing to engage in contests with resident males guarding burrows compared to larger males (Rosa *et al.*, 2018).

The tunnels within which *L. apterus* beetles establish their nests are excavated and maintained by the owners throughout the breeding season. The nest construction begins with a slanted entrance channel measuring cc. 20 cm in length, which leads to a vertical main channel. This main channel reaches a depth of 60-100 cm and leads to 5-8 chambers. Each of these chambers terminates in brood

cells where larvae develop separately (pers. obs., Frantsevich *et al.*, 1977; Nikolajev, 2003; Kosztolányi *et al.*, 2015).

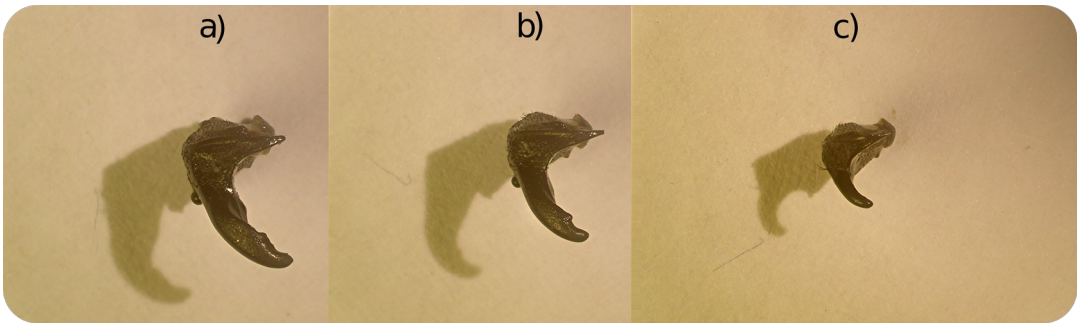


Figure 2. Mandibular processus (“tusk”) of (a) large-, (b) medium-, and (c) small-sized *Lethrus apterus* males.

Previous literature characterised *L. apterus* beetles as a monogamous species with biparental care (Hamilton, 1979). Accordingly, the male *L. apterus* collects and transports leaves into the nest, while the female processes the gathered plant material to form larval food balls inside the nest (e.g., Emich, 1884; Schreiner, 1906; but also see Kosztolányi *et al.*, 2015). The described biparental care in previous studies of *L. apterus* shows similarities to the behaviour observed in *Cephalodesmius* dung beetles (Monteith & Storey, 1981; Dalgleish & Elgar, 2005). Notably, the female *L. apterus* lays a single egg within each leaf-ball, which is then rolled to the end of the brood chambers (Emich, 1884). However, investigations during nest excavations of *L. apterus* have revealed that the egg is positioned behind the leaf-ball, with a delicate layer of soil separating them (pers. obs., see also von Lengerken, 1939). Moreover, recent observations indicate that while females primarily engage in provisioning each brood chamber with plant materials, males' roles may be more focused on defending the nest against intruding males of the same species, as well as potential predators or parasites (Nikolajev, 2003; Kosztolányi *et al.*, 2015; Rosa *et al.*, 2017). These new findings underscore the need to re-evaluate the reproductive behaviour of *L. apterus*, particularly with regard to clarifying the respective roles of both sexes.

I.1.4. Specific background and hypotheses

Insects exhibit a range of complex parental care behavioural forms, demonstrating diverse parental roles and strategies. In onthophagine beetles, the male and the female perform different tasks in the collection of dung for the nourishment of their larvae (Cook, 1990; Hunt & Simmons, 2002a,b). Females typically spend more time within the burrow, constructing dung balls for each larva, while males collect dung above ground. This specialization of tasks is reflected in the different movement and activity patterns of the sexes (Hunt & Simmons, 2002a). Specifically, males tend to travel more frequently between the dung pad and the nest, while females spend proportionally more time underground in the nest compared to males. By hypothesising that **movement patterns of female and male *L. apterus* beetles also reflect the parental roles of the sexes during the breeding season**, we carried out a field study on a natural population of *L. apterus* using pitfall traps to investigate the task division of the sexes **(Study 1)**.

An important aspect of parental care is the conflict of interest between parents over the amount of care provided, despite the shared benefits of care (Parker *et al.*, 2002). Consequently, it is anticipated that each parent aims to minimise their individual parental effort (Webb *et al.*, 1999). Desertion can also be advantageous for individuals, but only if the probability of finding a new partner is high (Queller, 1997; Kokko & Jennions, 2003; Ward *et al.*, 2009). In several species, male desertion tends to occur later in the reproductive cycle, after the young have reached a certain age or developmental stage, and when the benefits of male care are no longer as significant (Gross & Sargent, 1985; Scott, 1990; Jennions & Polakow, 2001; Trumbo, 2007). This timing allows males to maximise their reproductive output while minimising their investment in parental care. For example, in burying beetles (*Nicrophorus orbicollis*), males have been found to desert the nest after the offspring have reached the third instar (Trumbo, 1991; 2007). However, the timing of male desertion can vary depending on the species,

the ecological conditions, and the social context (Kokko & Jennions, 2008). In some cases, males may desert earlier in the reproductive cycle if they face increased competition or reduced future mating opportunities with the current partner, or if they are unable to provide sufficient parental care due to injury or illness (Kokko & Jennions, 2008; Keppner & Steiger, 2021).

Besides, biparental associations suggest that males may benefit from staying with the family and protecting their paternity by keeping rival males away, enhancing their attractiveness and increasing their future mating opportunities (Hunt & Simmons, 2002a; Kvarnemo, 2006; McNamara & Wolf, 2015; Chemnitz *et al.*, 2017; Keppner & Steiger, 2021). In spite of the presence of biparental relationships, discrepancies in parental investment commonly arise among individuals, and parents may exhibit varied responses when their partner's effort decreases (Harrison *et al.*, 2009). In biparental care systems, if a partner becomes handicapped, deserts or dies, the remaining parent may increase its parental investment to some extent (McNamara *et al.*, 2003). Nevertheless, the overall level of care is likely to be lower compared to when both parents contribute fully, potentially resulting in reduced fitness of the offspring (Parker *et al.*, 2002; Musa, 2005). Alternatively, if the parent is already operating at maximum capacity, there may be no room for increased investment, and thus, no change in parental behaviour is expected following mate desertion or disappearance (Smiseth & Moore, 2004).

A common method to investigate the flexibility of parental care decisions and their impact on reproductive success is by conducting the experimental removal of one parent. Burying beetle species, such as *Nicrophorus spp.*, are frequently used in such experiments due to the capability of both male and female parents to perform similar parental behavioural forms within uni- or biparental systems (Eggert & Müller, 1997; Scott, 1998; Müller *et al.*, 2007; Paquet *et al.*, 2017). However, contradictory results have been reported regarding females'

willingness to modify their parental investment after the removal of their mate (Fetherston *et al.*, 1994; Rauter & Moore, 2004; Smiseth *et al.*, 2005). In particular, Fetherston *et al.* (1994) reported increased parental effort by females in response to the removal of their partner in *N. orbicollis*, whereas other studies found no significant changes (Rauter & Moore, 2004; Smiseth *et al.*, 2005). Although several species in the Scarabaeidae and Geotrupidae families exhibit biparental care (Lukas, 2013), parental roles and efforts, and the behavioural repertoire of the sexes, particularly in the latter group, are still relatively understudied. By **characterising the above-ground activities of *L. apterus* beetles' sexes and clarifying the roles of male *L. apterus* during brood care**, we can gain insight into how each parent contributes to offspring care. **Conducting male removal experiments in *L. apterus*** can facilitate **the investigation of the differential contributions of each parent to offspring survival and reproductive success**, as well as define the respective roles of each parent in the breeding process. Furthermore, such experiments can offer valuable insights into the costs and benefits of biparental care and elucidate the evolutionary drivers that have shaped parental behaviour in biparental species (**Study 2**).

In species where males exhibit guarding behaviour, such as post-mating guarding or pair bonding, there is a potential for males to engage in competitive behaviour over access to females. Contest competition can lead to the evolution of traits that enhance a male's fighting ability and success in securing mates (Briffa & Sneddon, 2007; McCullough *et al.*, 2016). As a result, males may develop sexually dimorphic traits such as enlarged body size, weaponry, or elaborate courtship displays. Interestingly, weapons are found to be less expensive to develop and maintain, and may not come at the expense of other traits such as immune function or survival, than previously thought (McCullough & Emlen, 2013; Somjee *et al.*, 2021). This might lead to a great diversity of weapons (McCullough & Emlen, 2013). Furthermore, studies have demonstrated that male-male competition can

drive the rapid evolution of sexually dimorphic traits, as seen in the example of beetles with exaggerated horns or mandibles (Kawano, 2006; Emlen, 2008; Simpson *et al.*, 2011; McCullough & Emlen, 2013; McCullough *et al.*, 2015). Therefore, the presence of intrasexual dimorphism may play a crucial role in the emergence of guarding behaviour and shaping the evolution of reproductive strategies.

Sexually dimorphic beetles often exhibit exaggerated traits such as horns, mandibles, or tibias used for intrasexual aggression and/or mating purposes (Kawano, 2006; Emlen, 2008) and may display intrasexual phenotypic variation (Simpson *et al.*, 2011; McCullough *et al.*, 2015). This variation in body or weapon size is often associated with alternative reproductive tactics (ARTs) due to differences in competitiveness to monopolise mates (Brockmann, 2008). For instance, in onthophagine dung beetles with parental care, males can be grouped into two categories based on their morphology and reproductive behaviour: large (major) and small (minor) males. These males differ in both mating and caregiving tactics (Hunt & Simmons, 2000; Madewell & Moczek, 2006; Simmons & Kotiaho, 2007). Large males invest more in nest-guarding and caregiving, whereas males with no horns tend to perform an alternative, sneaking mating tactic without providing care and are more likely to disperse from one habitat patch to another (Eberhard, 1982, 1987; Moczek & Emlen, 2000; Hunt & Simmons, 2002b; Simmons & Kotiaho, 2007).

Movement patterns can be related to ARTs, as individuals may need to move differently depending on their chosen tactics (Kokko & Jennions, 2008). For example, in species where males exhibit ARTs such as sneaking or satellite behaviour, they may need to move differently to access females and avoid detection by dominant males. This may involve moving more slowly or taking a different path than males using traditional mate-searching behaviour (Moczek & Emlen,

2000; Hunt & Simmons, 2002b). Conversely, males using traditional mate-searching behaviour may need to move more frequently and travel greater distances to find receptive females (Simmons, 2002; Renault, 2020). The study of movement patterns can, therefore, be a crucial component of comprehending the reproductive behavioural forms and strategies of organisms, especially in those species that exhibit alternative reproductive tactics. However, the knowledge regarding the interplay between intrasexual polymorphism and alternative reproductive tactics in beetles remains limited. To address the question of **whether distinct morphotypes of male *L. apterus* exhibit differential reproductive tactics**, we conducted a comprehensive field survey spanning the entire active season (**Study 3**).

In animals, the allocation of limited resources involves a delicate balance between two major components: reproduction and self-maintenance, which includes immunity, especially in iteroparous species (Roff, 2002; Rauw, 2012). The theory of resource allocation posits that there is a trade-off involved when deciding how to invest in these two components (Rolff & Siva-Jothy, 2002; Schwenke *et al.*, 2016). However, the relationship between reproduction and immunocompetence is also influenced by the availability of resources (reviewed in Zera & Harshman, 2001; Harshman & Zera, 2007). In a resource-rich environment, high investment in both self-maintenance and reproductive traits is possible, thus the trade-off might not be apparent (Simmons, 2012; Reavey *et al.*, 2014). Nevertheless, in some cases, trade-offs seem to be genetically hardwired, regardless of resource availability (Stahlschmidt *et al.*, 2013).

Throughout the reproductive season, the allocation of resources towards the production of reproductive tissues, display of courtship and mating behaviour, and provision of parental care present a significant expense for individuals (Bell, 1980; Clark, 2012). Consequently, animals tend to endure a parasite infection rather than engage in active combat against it, ostensibly to enable resource allocation towards reproduction (Hurd, 2001, 2009; reviewed in Martin *et al.*, 2007). Following the

reproductive season, a prioritization of immune up-regulation is expected, which enhances the chances of survival until the subsequent reproductive season (Lochmiller & Deerenberg, 2000; Huyghe *et al.,* 2010). Furthermore, the connection between immunity and reproduction can vary depending on the individual's own condition (Ryder & Siva-Jothy, 2001; Rantala *et al.*, 2003). Individuals in good condition can allocate more resources to both reproduction and self-maintenance, whereas those in poor condition invest less in reproduction and exhibit lower immune competence (Rantala *et al.*, 2003; Sadd *et al.*, 2006; Córdoba-Aguilar *et al.*, 2009b; Krams *et al.*, 2011; Steiger *et al.*, 2012; Barbosa *et al.*, 2016). Additionally, the association between immunocompetence and reproduction may differ between sexes due to sexual dimorphism and life history characteristics influenced by hormones (Boonekamp *et al.*, 2008; Hoffman *et al.*, 2008; Vincent & Gwynne, 2014; Schwenke *et al.*, 2016).

While the intricate relationship between reproduction and immunity is relatively well-understood in vertebrates (e.g., Lochmiller & Deerenberg, 2000; Rolff & Siva-Jothy, 2002; Moore & Hopkins, 2009; O'neal & Ketterson, 2012), less is known about this association in invertebrates, including beetles that exhibit parental care. Although burying beetles, such as *Nicrophorus spp.*, have been extensively studied in this context (e.g., Steiger *et al.*, 2011; 2012; Reavey *et al.*, 2014), other caring beetle species, particularly in natural populations, remain understudied. **To investigate the complex interplay among immunity, reproductive effort, sex, condition** (indicated by body size)**, parasite load, and season,** we performed an encapsulation test and a bacterial growth inhibition assay on the haemolymph of females and males (**Study 4**). This study encompassed both the early and late stages of the reproductive season.

I.2 OBJECTIVES AND MAJOR RESULTS

Here I present the studies included in this thesis, describing the aims, methods, and major results of those. Note that the complete methodology used in the studies, detailed results and their substantive discussion can be found in Part II.

Study 1

Studying sex-specific roles in *Lethrus apterus* during the breeding season via differential movement and activity patterns (Kiss *et al.* 2020)

(i) Objectives

Intersexual differences in mating and care behaviour are often correlated with sexual differences in other traits, such as activity and movement patterns. In *L. apterus*, as presented above, early observations and those from recent decades, about the roles of the sexes and parental division of labour during breeding are contradictory. Therefore, **we aimed to get a more accurate picture of the behaviour of females and males in *L. apterus* during the breeding season**, we carried out a pitfall-trapping experiment on a natural population.

(ii) Methods

We conducted the fieldwork in a hilly meadow near the village of Dorogháza (47°59’29’’N, 19°53’36’’E), in Mátra Mountain. As a study site, we designated an area of 100 m2, where we placed traps 1-1 m apart in a regular square grid. The traps were opened in the daytime, on 16 days between 24th April and 2nd June 2015 (depending on the weather). At the first capture, beetles were marked uniquely using a paint marker, and the width of their pronotum, as an indicator of body size, was measured using a digital calliper. The sex of the individuals was determined by

the presence (or absence) of the “tusk”. We analysed differences between females and males in the number of times an individual was (re)captured, in the time interval between the first and last encounter, and the sum of the distances between successive pitfall traps the individual was caught as a proxy of total distance travelled (Figure 3). In case of the time interval between the first and last encounter and the total travelled distance, we controlled for the number of recaptures.

(iii) Results

We found that females were (re)captured more frequently (Figure 4a), and were resighted for a significantly longer time in the study area than males. We also found a significant difference between females and males in the total distance travelled, as females travelled significantly shorter distances on average than males when controlling for the number of recaptures (Figure 4b).

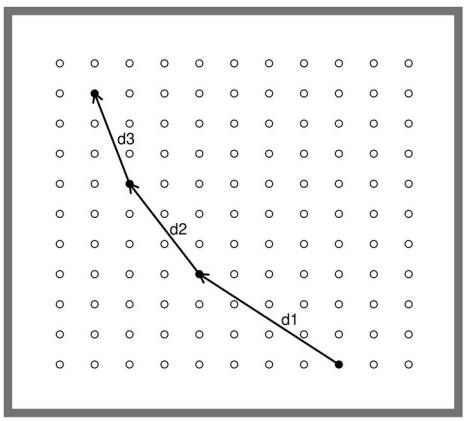


Figure 3. Schematic illustration of the pitfall-traps placed in a square grid and the method for calculating the total travelled distance of a *Lethrus apterus* individual. Arrows represent the distances travelled between two (re)captures, and the total distance travelled by the individual is calculated as d1+d2+d3.

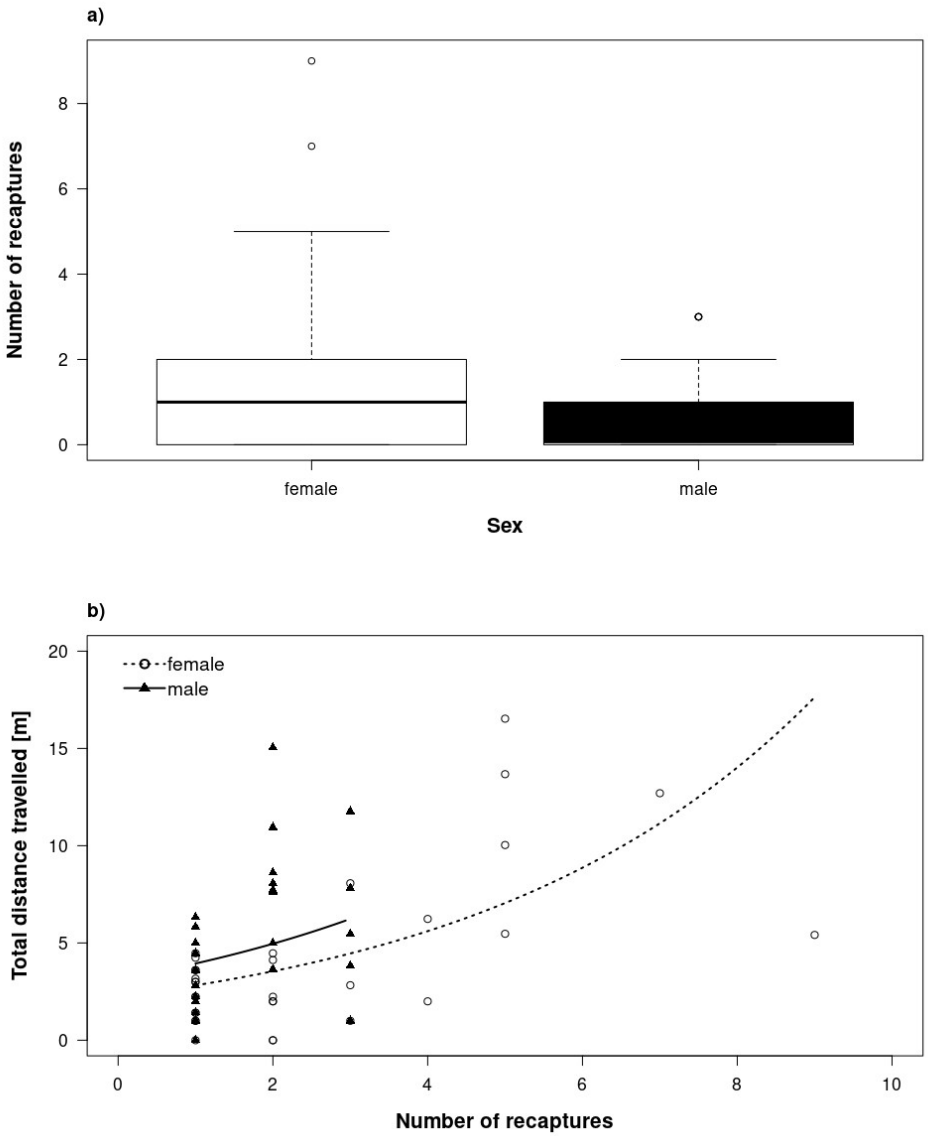


Figure 4. (a) Boxplot diagram illustrating the number of recaptures in female and male *Lethrus apterus* beetles. (b) Relation between the number of recaptures and the total travelled distance in female and male *L. apterus*. Fitted lines are predicted by a linear model. (Figure panels used from Kiss *et al.*, 2020).

Study 2

Investigating the behavioural repertoire and the impact of male removal in *Lethrus apterus* beetles (Kiss *et al.* 2023)

(i) Objectives

Empirical studies on diverse beetle taxa are needed to understand the evolution and maintenance of biparental care. One popular method for studying the flexibility of parental care decisions and their impact on reproductive success is to conduct experiments where one parent is removed. The objective of this study was two-fold. Firstly, **we aimed to describe the activities of male and female *L. apterus* beetles in two natural populations, particularly their above-ground behaviour related to brood care**. We used video recording to observe leaf hoarding, nest guarding, and nest construction or maintenance by the individuals around their nests. We hypothesised that if males are mainly responsible for guarding the nest, their movement would be limited to the entrance and immediate vicinity of their nest, while females would exhibit more diverse behaviour. Secondly, **we conducted an experiment to investigate whether females modify their parental behaviour after the removal of their male partner**. We also studied the effect of male removal on reproductive success.

(ii) Methods

In this two-year investigation at two field sites, we observed the above-ground activities of *L. apterus* beetles in natural populations (first study: 27th April – 22nd May in 2017 near Debrecen, Hungary; second study: 28th April – 14th May in 2018 at Ózd-Susa, Hungary). We marked the nests and their owners individually and recorded their behavioural elements for approximately four hours using an action camera mounted on a tripod near the nest (Figure 5a). The next day, we randomly assigned the nests to control or treatment groups and removed the male from the

treated group. After the treatment, we recorded the activities at the nests for another four hours (Figure 5b,c). We focused on the frequency (and duration) of the following events: being away from the vicinity of the nest (i.e., staying outside of a 10cm radius circle), the number of leaf collecting events (i.e., food provisioning), staying in the vicinity of the nest (i.e., within a circular area with a radius of 10 cm around the nest entrance), being at the entrance of the nest (considered as nest guarding), and the frequency of removing soil from the nest (indicating nest construction or maintenance). At the end of the breeding season in the second study, we excavated all the marked nests to determine their reproductive success. Because new males often replaced resident males during the experiment, furthermore, in some control nests, the resident male deserted by the end of the experiment, we analysed the females' responses to male removal, data from females paired with a resident, new or no male were compared instead of females from control and treatment group (Figure 5b,c).

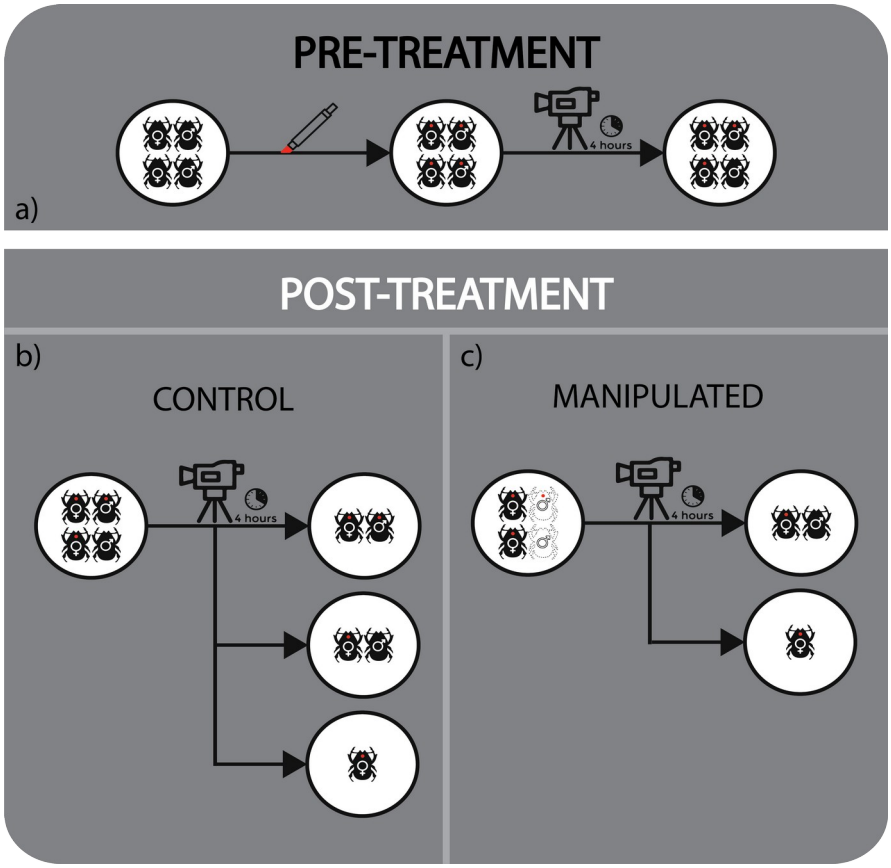


Figure 5. Schematic representation of the experimental design, illustrating marking the individuals, the data collection before (a) and after the treatment (b,c). Beetles with red dots represent marked females and males, and males with dashed outlines indicate instances of experimental male removal in the manipulated group. The observed changes in male presence (resident male, new male, no male) within the experimental groups are also showed.

(iii) Results

Regardless of the treatment, we observed that males frequently abandoned their nests, in both populations, after a few days of staying, leaving the brood and females behind. During the pre-treatment day, we found that in both populations, females had a significantly higher leaf-collecting rate than males and spent more time away from the nest. Conversely, males spent significantly more time in the

vicinity and at the entrance of the nests than females in both populations. Although both sexes seemed to do their share of nest construction, females performed more frequent nest maintenance activities (see Figure 6). Regarding the influence of male partners, we observed no significant distinctions in parental behaviour among females paired with a resident, new, or no males. However, we did observe a higher incidence of empty brood chambers (indicating unsuccessful reproductive events) in nests lacking male residency.

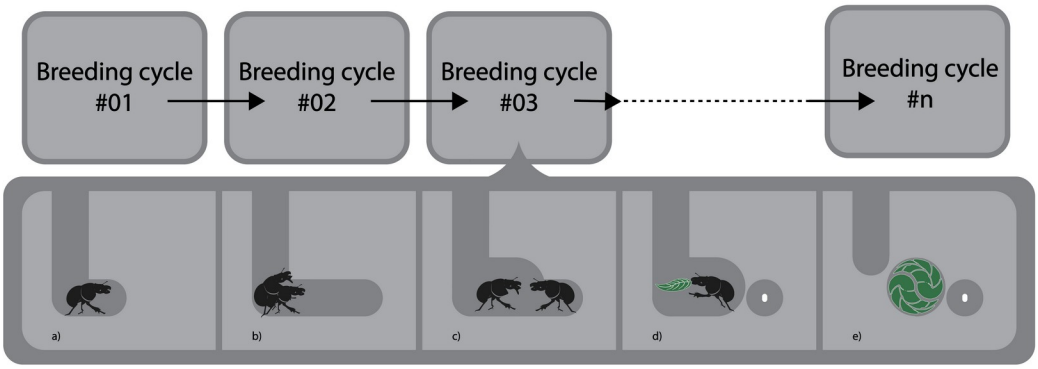


Figure 6. A description of a breeding cycle in the beetle *Lethrus apterus*: Division of underground parental tasks (based on observations and family traits). a) The female excavates a brood chamber that branching off the primary tunnel. b) The male enters the tunnel and engages copulation with the female. c) While the female lays an egg, the male enlarges the brood chamber. d) The female seals the egg cavity with soil and assembles plant material into a leaf ball for the larva. e) Once the leaf ball is prepared, the female seals the brood chamber with soil.

Study 3

Unveiling size-dependent male reproductive tactics in *Lethrus apterus* through movement patterns (Kiss *et al.* 2023)

(i) Objectives

In sexually dimorphic beetle species, where males display exaggerated traits, intrasexual variation of the traits may also be expected. Variation in the size of the traits and thus the existence of different morphotypes is usually linked to alternative reproductive tactics due to differences in competitiveness for mates. Furthermore, as individuals may need to move differently depending on their chosen tactics, investigating movement patterns can be an important aspect of understanding the reproductive behaviour and strategies of individuals, particularly in species with alternative reproductive tactics. *Lethrus apterus* males are found to show variation regarding tusk length and body width ratio (small, medium, and large types). **Our aim was to investigate the mating and foraging behaviour of *L. apterus* through space use of the sexes and to identify potential disparities in reproductive behaviour between male morphotypes**. Throughout a complete active season, we examined the temporal and spatial movement patterns of *L. apterus* beetles.

(ii) Methods

We conducted a capture-mark-resight survey to study the seasonal dispersal and movement behaviour of *L. apterus* beetles near Ózd-Susa, Hungary (17th March – 23rd May 2016). Four line transects, spaced at least four meters apart and totalling 830 meters in length, were walked twice a day over 39 days of fieldwork. By walking along the established transect line and scanning, we could detect individuals within a distance of one meter. Each unmarked beetle was marked after

measuring its pronotum width, and males had their left tusk length measured. We recorded the ID, time, date, and GPS coordinates for each marked or encountered individual. Based on fitted two-breakpoint segmented model (Rosa *et al.*, 2019), we categorised males into three groups (further referred as morphotypes): small (pronotum width ≤ 10.80 mm; n=55), medium (10.80 mm < pronotum width ≤ 12.32 mm; n=357) and large (pronotum width > 12.32 mm; n=424) (Figure 7). To investigate differences in space use and activity patterns between females and different male morphotypes, we analysed the number of times an individual was encountered, the time interval between the first and last encounter, and the distances individuals travelled between two consecutive encounters while controlling for the time of the season.

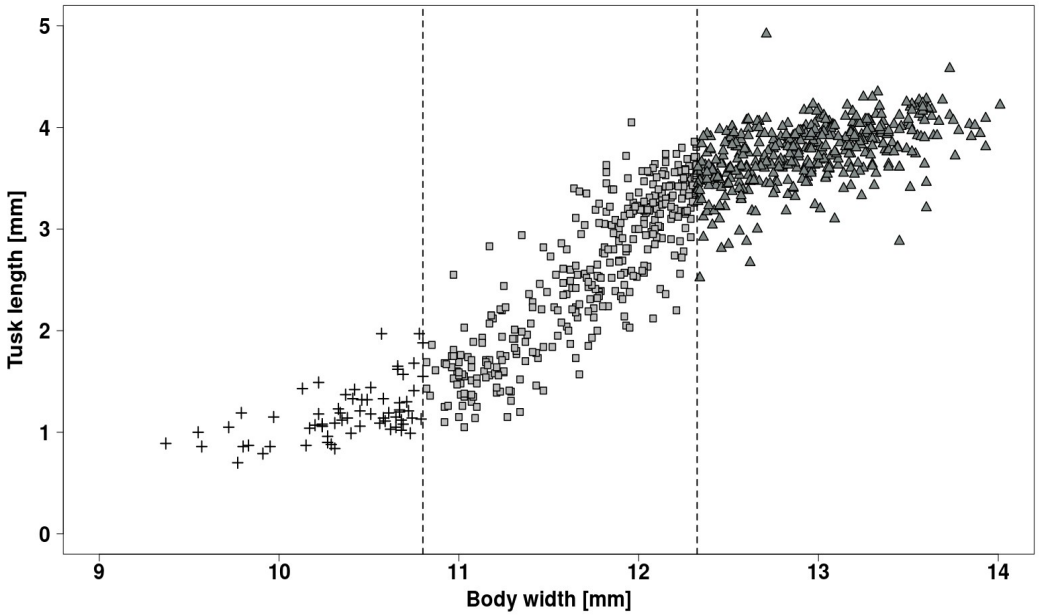


Figure 7. Allometric relationship between tusk length and body width for male *Lethrus apterus.* Vertical dashed lines represent the estimated breakpoints of the segmented model. Different symbols (cross for small-sized, square for medium-sized, and triangle for large-sized males) indicate the distribution of males within these size categories.

(iii) Results

We captured and marked a total of 1624 beetles, 788 females and 836 males, respectively. We found that large- and medium-sized males were resighted more frequently than females and small-sized males. We also found that females were detected for a longer time at the study area than different male morphotypes. However, in the former cases, we got small effect sizes. By analysing the differences in travelled distances between two encounters among females and male morphs, we found that medium males travelled significantly increasing distances as the season progressed. In contrast, females and small males travelled decreasing distances over the season. In case of large males, we found no significant change in travelled distances during the season (Figure 8).

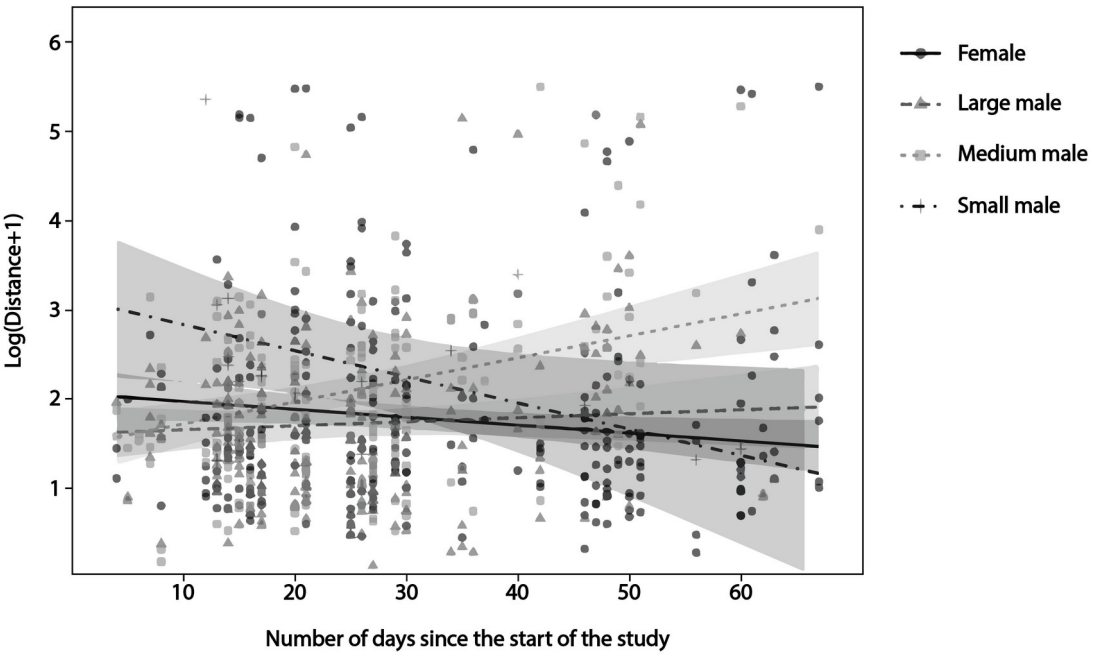


Figure 8. Relationship between the logarithmic travelled distance (in meters) and time in the season between two consecutive encounters in females and the three male morph categories of *L. apterus*. Lines are predicted by fitted linear mixed model. Grey ribbons indicate 95% CI. (Figure used from Kiss *et al.*, 2023, Manuscript).

Study 4

Investigating sex differences of seasonal alterations in immune response and reproductive investment in *Lethrus apterus* beetles (Kiss *et al.* 2020)

(i) Objectives

Both immunocompetence and reproduction are physiologically demanding processes, so trade-offs are expected between these life history traits. Additionally, various factors, including sex, body size, individual condition, seasonal changes, and parasite infection, can affect investments in these traits. **We aimed to explore the relationships among immune response, reproductive effort, sex, condition (indicated by body size), season, and mite load in *Lethrus apterus* beetles**.

(ii) Methods

Beetles were collected in spring of 2017, in Debrecen, Hungary. We measured the pronotum width of the individuals, and the right tusk of males. Since we collected individuals having pronotum width less than 11 mm or 12 mm or more, we categorised females (n=38) and males (n=33) into categories of large or small. The collection took place in late March and early May, i.e., in the early and late stages of the reproductive season. We measured the encapsulation response of the individuals as an indicator of induced cellular and humoral immune response (Figure 9a) and measured the bacterial growth inhibition ability of the haemolymph of the individuals, as an indicator of antimicrobial capacity (Figure 9b). For investigating reproductive investment, we quantified the size of reproductive tissues. The parasite load of the beetles was estimated by the level of mite infection.

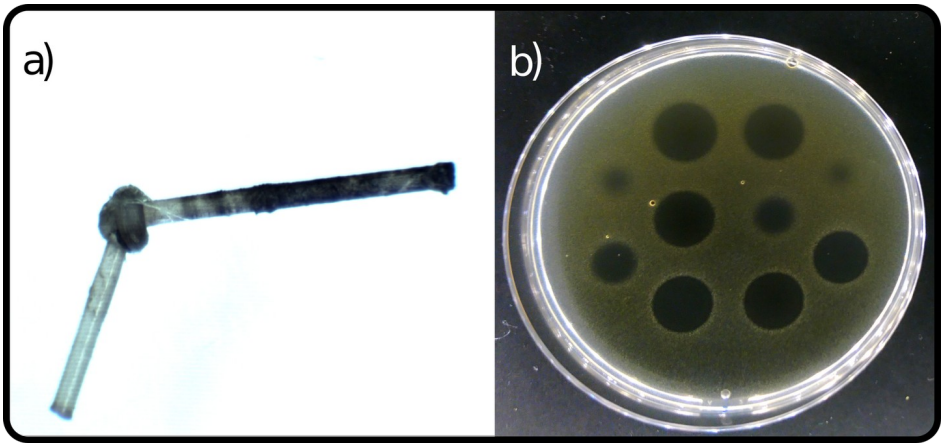


Figure 9. (a) Nylon monofilament implant used for measuring encapsulation response in *Lethrus apterus*. The darker the implant the stronger is the encapsulation reaction. (b) Bacterial inhibition zones by haemolymph samples of *L. apterus* individuals. The larger the zone the stronger is the antimicrobial capacity.

(iii) Results

We found that encapsulation response is size-dependent, large individuals showed significantly higher encapsulation ability than smaller ones (Figure 10a). In case of antimicrobial capacity, we found female bias, females showed stronger antimicrobial capacity (Figure 10b). Furthermore, in individuals with heavy mite load, during the early season, there was a positive relationship between the antimicrobial capacity and reproductive investment, however, during the late season, this relationship turned negative. With regard to reproductive investment, in females, we found that there was a significant positive relationship between total egg size and antimicrobial capacity in the early season, however, this association were eliminated in the late season. In both females and males, we found that body size and season affected reproductive investment. Finally, in males, we found that there was a positive relationship between total testis size and mite load.

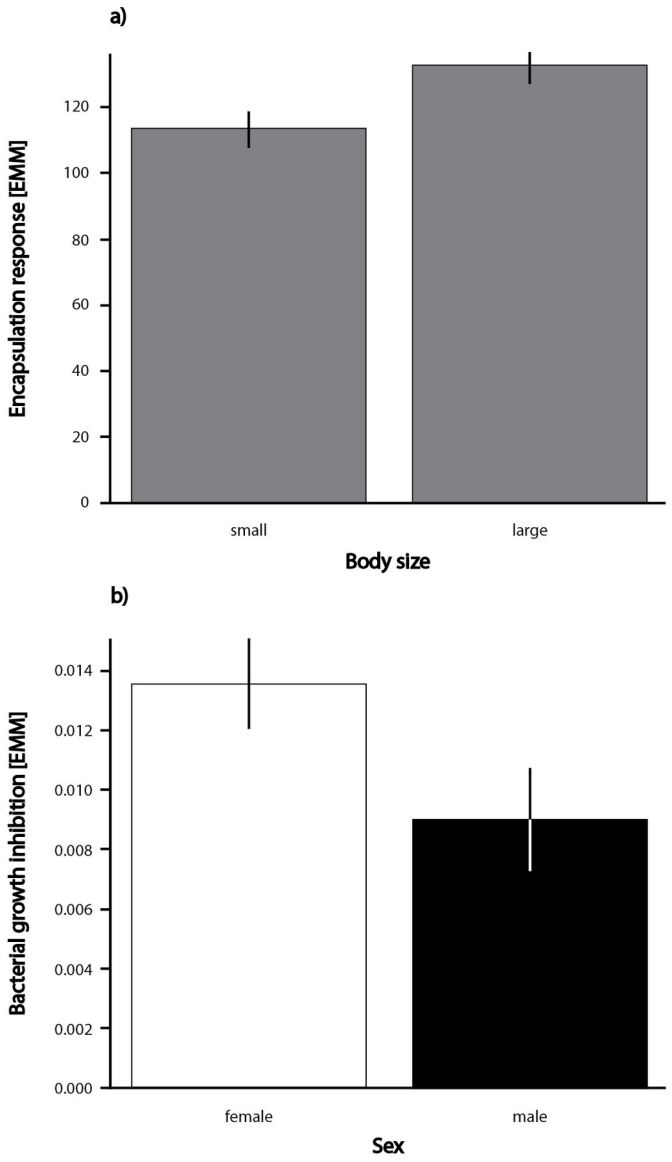


Figure 10. Barplots illustrating the relationship between (a) body size and encapsulation response and (b) sex and bacterial growth inhibition capacity in *Lethrus apterus* beetles. Estimated marginal means (EMMs) are plotted. The error bars represent the SE of EMMs. (Figure panels used from Kiss *et al.*, 2020).

I.3. GENERAL DISCUSSION AND CONCLUSIONS

Beetles are one of the most diverse and species-rich groups of insects, and understanding their parental care behaviour can provide insights into its formation and mechanisms. Additionally, beetles show a diverse array of parental care strategies encompassing uniparental (including maternal and paternal care) as well as biparental care, making them a valuable model organism for investigating the ecological and evolutionary factors that shape parental care (Smiseth *et al.*, 2012; Trumbo, 2012). My thesis is centred on investigating the reproductive behaviour of *Lethrus apterus* and exploring the potential factors that may influence it. Accordingly, my aims were two-fold: Firstly, to determine the roles of the sexes during the reproductive season and, in parallel, to clarify the contradictions between what is described in the early literature and what has been observed in recent years. Secondly, to gain a better understanding of biparental care in scarab beetles and, in turn, its persistence by understanding the various environmental, social and intrinsic physiological factors affecting the species. In my view, this work holds value as most investigations on biparental care in beetles have concentrated on a limited number of species, primarily within the Scarabidae and Silphidae families. To obtain a more comprehensive understanding of the factors influencing the mechanism and maintenance of biparental care, it is essential to investigate species from other families, including the Geotrupidae family.

I.3.1. From biparental to single-parent provisioning

Our studies aimed to investigate the roles of the sexes in relation to the movement and activity patterns of the sexes during the breeding season on a small scale (in an area of 100 m^2) and on a larger scale (covering an area of approximately 10,000

m^2) throughout the active season. In addition, we explored the above-ground behavioural repertoire of the sexes and examined how female behaviour changes in response to the identity of their partner. Our findings indicate that females exhibit greater movement frequency and significantly longer resighting times, but travel shorter distances between captures than males in the case of smaller study area (Study 1). However, these sex differences in the number of recaptures and length of resighting faded when the study was conducted over a large area (Study 3). These findings align with the observations of Kosztolányi *et al.* (2015) that females should play a more active role in collecting plant materials for the offspring, which contradicts earlier notions that males are responsible for collecting leaves to provision the larvae and females deposit and form them into leaf-balls (Emich, 1884; Schreiner, 1906). We also found that females frequently leave the nest to collect leaves and travel to vegetation, supporting the idea that the reflection of parental tasks is evident in movement and activity patterns (Study 2). Our results are consistent with the concept that the leaf-collecting area of females is concentrated around the nest (Nikolajev, 2003), leading us to conclude that females are primarily responsible for provisioning their offspring. Dung and carrion are ephemeral resources, that are subjects of intense competition within and between species (for example, Feer & Pincebourde, 2005; Wall & Lee, 2009). For species such as dung beetles and burying beetles that depend on these resources for provisioning their offspring, involving both parents in provisioning can lead to greater efficiency (Hunt & Simmons, 2000; Palestrini & Rolando, 2001; Capodeanu-Nägler *et al.*, 2016). Moreover, in the case of *Onthophagus* dung beetles, as in early observations of *L. apterus*, task specialisation in offspring provisioning is also documented (Emich 1884; Cook, 1990; Hunt & Simmons, 2002a,b). In contrast, in the case of *L. apterus*, plants and leaf shoots are available in practically unlimited quantities for provisioning, so a single parent should be sufficient to collect and process them for all larvae. Accordingly, our study found

no significant differences in activities related to provisioning behaviour between females with a resident partner, a new male, or alone (Study 2). This also suggests that parental roles are completely separated with task specialisation among the sexes.

It is justifiable to question what specific actions the males perform in caring for offspring. Our research indicates that in smaller study areas, males were detected less frequently and for shorter periods, suggesting that their primary parental role is nest guarding (Study 1). This is supported by the findings that males spend significantly more time near and at the entrance of the nest compared to females (Study 2). Moreover, according to Study 2, nests deserted by males had a significantly higher count of empty or failed brood chambers, although there was no evidence of a decrease in offspring numbers. By investing heavily in guarding behaviour, males can minimise the probability of females being fertilised by other males, thus increasing their confidence in paternity and ultimately leading to improved reproductive success (Hunt & Simmons, 2002a,b; Simmons & Ridsdill-Smith, 2011; Hopwood *et al.*, 2015). Furthermore, this behaviour provides increased protection for offspring against predators and parasites (Suzuki, 1999; Kvarnemo, 2006; Rodríguez-Muñoz *et al.*, 2011). It is also evident that males frequently abandon their nests after a few days, either voluntarily or due to losing in a fight, resulting in frequent partner changes and short-term mate fidelity. (Study 2). This finding supports that there is intense competition between the males for receptive females (see also Rosa *et al.*, 2018). Additionally, males can potentially increase their reproductive success by engaging in desertion shortly after oviposition in order to seek additional mating opportunities, instead of waiting for their partners to regain receptivity (Queller, 1997; Ward *et al.*, 2009; Kokko & Jennions, 2003; 2008; 2012). In *L. apterus*, the maturation of oocytes does not occur simultaneously, and the large size of matured eggs physically hinders their simultaneous development, indicating that egg-laying takes place one egg at a time

(Nikolajev, 2003). Additionally, females possess underdeveloped spermatheca, which lacks the ability to store sperm or regulate its release during fertilization (López-Guerrero & Halffter, 2000). This may explain why Rosa *et al.* (2019) found no difference in sperm length among different male morphotypes, as there is no possibility of ejaculate mixing for anatomical reasons. Based on this information, each oviposition in *L. apterus* should be preceded by copulation.

Studies on deep burrowing geotrupid beetles, including *Trypocopris vernalis, Typhaeus hiostius, L. apterus,* suggest that in *L. apterus* the breeding season is comprised of multiple, relatively autonomous breeding cycles (Teichert, 1959a,b; Brussaard, 1983; Hanski & Cambefort, 1991). In minotaur beetles (*Typhaeus typhoeus*), the female primarily performs burrowing, while the male assists in brood chamber construction after copulation (Brussaard, 1983). Similarly, in *L. apterus*, while females dominate in nest construction, males also participate (Study 2). Observations of males removing soil from the nest indicate their participation in the excavation of the brood chamber and the cooperative effort between parents to expand the nest. Furthermore, *L. apterus* females paired with new males demonstrated a notably higher rate of soil removal from the nest, which signifies their active preparation for oviposition through the excavation of a new brood chamber. Conversely, females paired with resident males or those without any males displayed diminished investment in nest maintenance, implying that they had already excavated the brood chamber and either completed or prepared for oviposition. (Study 2). Based on the findings, collecting fifty leaves and preparing a leaf-ball for a larva takes at least two days (Frantsevich *et al.*, 1977; Study 1, 2). Thus, considering other nesting phases such as mating, nest construction, breeding cycles are expected to last 3-4 days with sub-events of copulation, brood chamber expansion, oviposition, leaf-ball formation, and sealing the brood chamber with soil (see also Brussaard, 1983; Hanski & Cambefort, 1991; Nikolajev, 2003; Verdú & Galante, 2004).

In summary, this thesis explores the roles of males and females in *L. apterus* by conducting a series of field experiments. Our findings indicate that females are responsible for offspring provisioning, while males primarily engage in nest guarding. This division of labour is evident in the distinct movement and activity patterns observed for each sex. Our results also suggest that parenting roles are highly specialised and separated and that breeding cycles are quite independent of one another. The prevalence of male desertion in search of additional mating partners may select against male care (Queller, 1997; Kokko & Jennions, 2003). Our results contradict those reported in earlier literature on *L. apterus* parenting roles (Emich, 1884; Schreiner, 1906). Since our findings are consistent across three distinct populations with diverse habitats, it is not clear why there are differences between our findings and those of previous researchers. Our study suggests that male care would not be a worthwhile investment if females can provide practically unlimited food resources and males can easily find new mating partners. However, the short-term presence of males in the nest indicates that females are prone to engaging in multiple mating events with different partners throughout a reproductive season. Future research should employ genetic parentage tests to verify this prediction. Additionally, it would be interesting to investigate the impact of resource availability combined with population density on parental roles and mate fidelity.

I.3.2. Size-dependent reproductive tactics of males

The differences in movement and activity patterns of individuals can reflect different parental roles or task specialisation between the sexes, but also alternative reproductive tactics within a sex (Study 3). Thus, Study 3 aimed to investigate the differences in movement and space use of *L. apterus* male morphotypes (i.e., large, medium, and small males). We found that small males (similarly to females) travelled shorter distances and were resighted less frequently than medium and

large males as the season progressed. Morphological differences in male insects are found to be associated with their behavioural differences, particularly in their employed mating strategies (Brockmann, 2008; reviewed in Buzatto *et al.*, 2014). Accordingly, larger males in several species were observed to spend more time and energy on guarding their territory by guarding the entrance of their burrow, leaving it only on rare occasions and on their own accord (Hunt & Simmons, 2002a,b; Rowland & Emlen, 2009; reviewed in Simmons & Ridsdill-Smith, 2011). In contrast, small males were found to be less able to defend a burrow and hence take a resident male's position, which could make them more susceptible to intrasexual interactions with a higher probability of losing contests and moving on compared to larger ones. As a consequence, small males may use context-dependent alternative reproductive tactics, such as sneaking tactics, to avoid competition (Moczek & Emlen, 2000; Rowland & Emlen, 2009; Reynolds & Bryne, 2013). According to Rosa *et al.* (2018), smaller males of *L. apterus* appear to tend to avoid fighting contests in general when acting as intruders. Specifically, the decision to engage in a physical altercation or to disperse and employ sneaking tactics may be contingent upon the presence and size of rival males (Painting & Holwell, 2014). Therefore, small males may gain more benefits by wandering in smaller habitat patches and visiting burrows regularly in search of unguarded females (Harts & Kokko, 2013; Buzatto *et al.*, 2014) or by applying an alternative, sneaking mating tactic to gain access to partners when rivals are more competitive (Moczek & Emlen, 2000; Brockmann, 2008; Reynolds & Byrne, 2013; Painting & Holwell, 2014).

We found that, as the season progressed, there is a significant difference between male morphs of *L. apterus* in the change of travelled distances (Study 3). Specifically, medium males showed an increase in the distance they travelled between two encounters over the season, while small males showed the opposite trend. In the case of large males, no significant relationship was found between the

travelled distance and time in the season. It is possible that, similarly to *Librodor japonicus* beetles (Okada *et al.*, 2008), there are three size-dependent mating tactics in *L. apterus* males. Large males invest more resources in weapons for fighting and guarding and are more successful in monopolizing females and preserving their positions as residents but less agile as they might be more exposed to predation (Hunt & Simmons, 2002a,b; Okada *et al.*, 2008; Rowland & Emlen, 2009; Kojima *et al.*, 2014; reviewed in Renault, 2020). In contrast, for medium-sized males, fighting and maintaining resident positions should be more costly compared to large males, thus, due to their greater mobility, they may reach a higher mating success through dispersing and searching for (unpaired) females (Okada *et al.*, 2008; reviewed in Renault, 2020). Small-sized males, on the other hand, are at a disadvantage in male-male competition, and their size (and probably condition) makes them more susceptible to sneak tactics (Moczek & Emlen, 2000; Hunt & Simmons, 2002b). The findings suggest that small males may use a scramble competition strategy or sneak into nests occupied by larger males and mate with the female (Hunt *et al.*, 1999; Pomfret & Knell, 2008). Furthermore, the long travelled distances by small males at the beginning of the season may demonstrate foraging marches of maturity feeding after emerging to compensate for insufficient larval food supply (Hunt *et al.*, 1999).

In conclusion, our results suggest that male *L. apterus* beetles, based on their movement and activity, adopt size-dependent alternative mating tactics to maximise their reproductive success. These findings are consistent with other studies on polyphenic beetle species, indicating that body size and morphology are closely related to mating behaviour (e.g., Moczek & Emlen, 2000; Brockmann, 2008; Snell-Rood *et al.*, 2010; Simpson *et al.*, 2011). The extent to which various factors influence the phenotypic expression of male tusks in *L. apterus* and the specific selection pressures acting upon them remain unclear and require further investigation. It is plausible that male size, and so tusk size, is not solely

determined by larval diet, indicating the need for additional research to identify other factors that may contribute to variation in body and tusk size.

I.3.3. Investment to reproduction and self-maintenance

Study 4 investigated the complex relationships between immune response, reproduction, sex, size, and seasonality in *L. apterus* beetles. Our study found that both sexes exhibited positive associations between body size and reproductive investment. Furthermore, the results suggest that the encapsulation ability is dependent on body size, which is an indicator of condition and somatic storage capacity (Berger *et al.*, 2008; Prokkola *et al.*, 2013; Beukeboom, 2018), but not antimicrobial capacity. Large or high-quality individuals may possess better resource-gathering and storage capabilities, allowing for uncompromised investment in both immunity and reproduction (Blanckenhorn, 2000; Ardia, 2005; Roff, 2002; Körner *et al.*, 2017). Encapsulation occurs after immune activation, involving cellular and humoral processes, and the amount of melanised cell deposition on the 'parasite' (represented by the nylon monofilament in the study) is generally regarded as the individual's capacity to mount an efficient melanization-encapsulation response (Moret & Schmid-Hempel, 2001; Siva-Jothy *et al.*, 2005; Sadd & Schmid-Hempel, 2008). Therefore, these results indicate that encapsulation ability and reproductive investment in *L. apterus* are condition-dependent, with larger individuals having higher encapsulation ability and disproportionately larger follicle or egg size compared to smaller individuals. After undergoing diapause, individuals resume feeding activities known as maturity feeding upon emerging (see for example Günthart, 1949; Watson & Simmons, 2010; Hiiesaar *et al.*, 2013, 2014). Larger individuals may have access to more resources, including larger quantities of food. This increased resource availability may support the production of larger reproductive tissues, such as larger eggs in females or larger testes in males.

However, we found that antimicrobial capacity is stronger in females (Study 4). The dissimilarities between sexes in terms of their physical size, behaviour, physiology, and overall investment in offspring can potentially lead to variations in their immune response and antimicrobial capacity (Córdoba-Aguilar *et al.*, 2009a; Cotter & Kilner, 2010; Vincent & Gwynne, 2014). Infections that could impede reproductive success have already been reported (Adamo, 1999; Kurtz *et al.*, 2000; Adamo *et al.*, 2001). Roth *et al.* (2011) discovered in the sex-role reversed Broad-nosed Pipefish (*Syngnathus typhle*) that the sex that invests more in offspring demonstrates more robust and targeted immune defences to evade infections that could significantly reduce their reproductive success. The argument is supported by research on beetles that exhibit parental care, such as burying beetles (for example, Jenkins *et al.*, 2000; Smiseth *et al.*, 2005). In *L. apterus*, the female is the primary caregiver, responsible for supplying food for the larvae, making their survival and development heavily reliant on maternal care (Study 1, Study 2). Additionally, nests may contain rich microbiota that poses a risk of infecting the larvae. Females may possess the ability to transport antimicrobial agents into the eggs (Zanchi *et al.*, 2012) as a means of establishing antimicrobial protection. If there is a presence of trans-generational immune priming, females would likely invest more in antimicrobial capacity to ensure an ample supply of antimicrobial agents for the eggs (Moret, 2006; Zanchi *et al.*, 2012). This may also explain the notion that male reproductive investment is not related to antimicrobial capacity. Alternatively, males, because of active mate-searching and frequent mate changes (Study 1, 2, 3), are potentially more exposed to infections and pathogens due to injuries received in fights and increased contact with females (Knell & Webberley, 2004; Fedorka & Zuk, 2005; Bagchi *et al.*, 2021). Exposure to a variety of infectious agents may result in chronically provoked immunity and long-term reduction in antimicrobial capacity (Siva-Jothy *et al.*, 2001; Ardia *et al.*, 2012; Moreno-García *et al.*, 2014; Daisley *et al.*, 2020).

In Study 4, both antimicrobial capacity and reproductive activity showed seasonality during the reproductive season. In females, a positive relationship was observed between reproductive investment and antimicrobial capacity in the early season, but not in the late season. Additionally, a decrease in reproductive investment was observed in large females and males over the course of the season, but not in small females. These findings may be explained by the need to prepare for a long diapause period spent underground, during which beetles can be exposed to parasites or pathogens. Thus, self-maintenance becomes a priority at the end of the breeding season. It is possible that small females do not adequately prepare for diapause, which is not surprising given their lower chances of survival (Ardia, 2005; Renault, 2011). Nonetheless, the absence of disparity in egg production between the early and the late season among small females implies that they might adopt a "best of a bad job" strategy (Renault, 2011). This strategy involves investing a comparable amount of energy into reproduction during the late season, even though it entails the risk of reduced survival during the winter period. From another perspective, smaller females may have a lower likelihood of surviving hibernation. Consequently, in terms of the terminal investment hypothesis, they may prioritise investing in current reproduction, even if it entails the risk of death (Creighton *et al.*, 2009; Duffield *et al.*, 2017).

Interestingly, a seasonal change was also found in the relationship between antimicrobial capacity and reproductive investment, but only under heavy mite load. When the level of parasite burden was low, there was no association between these two factors. The findings indicate that individuals with high levels of mite infection are able to allocate resources towards both immunity and reproduction simultaneously, particularly early in the reproductive season. Conversely, individuals with high antimicrobial capacity exhibited reduced reproductive investment later in the season. In order to combat persistent or additional infections, such as those caused by heavy mite infestations, insects have evolved

mechanisms to induce long-lasting antimicrobial activity, thereby increasing their initial immune response (Haine *et al.*, 2008; Cooper & Eleftherianos, 2017). Additionally, parasites with slower growth rates or higher resource requirements may induce a subsequent reduction in host reproductive effort, coupled with an increase in reproductive investment early in the season (Lefévre *et al.*, 2008; Hurd, 2009). Lastly, individuals harbouring chronic infections may experience advantageous effects by triggering persistent antimicrobial activity, which can indirectly enhance their reproductive output (Hurd, 2009). Our findings further support this notion, as we observed that males infected with mites exhibited larger total follicles compared to those with lower levels of infection.

In summary, our study suggests that the allocation of resources towards immunity and reproduction in *L. apterus* beetles is condition-dependent and influenced by mite infection and the course of the reproductive season. Notably, females exhibit a greater antimicrobial capacity, while encapsulation response does not differ between the sexes. The relationship between immune response and reproduction is dynamic across the season, highlighting the complexity of this association. Regrettably, our study did not control for potential confounding effects of prior pathogen exposure or other ecological factors. Furthermore, the literature currently lacks information on the hormonal mechanisms underlying parental care in *L. apterus* beetles, necessitating further investigation into the relationship between reproductive effort, immunity, and care-related hormones.

I.3.4. Conclusions and future directions

This thesis has provided a more comprehensive understanding of the reproductive biology of *L. apterus*. Despite limitations due to the inability to maintain and breed the species in a laboratory setting, our field experiments have yielded valuable insights into the distinct roles of males and females, the influence of body size on male mating behaviour, and the complex interplay between immunity and

reproduction. These findings can have significant implications for the evolutionary understanding of parental care in beetles, sexual selection, and immune function in insects, and serve as a basis for further investigations in these areas.

As shown in Study 3, male morphs of *L. apterus* differ in space use and movement patterns, implying the existence of alternative mating tactics. As future research, it would be worth exploring what factors influence variation within males in terms of tusk length and body size ratio. Although the amount of larval food determines the size of the *L. apterus* adult (Emich, 1884), it can not be excluded that other factors, whether hormonal or genetic, are involved in the development of morphotypes. Furthermore, it would be valuable to investigate the reproductive success and investment of male morphotypes. Males displaying alternative reproductive tactics often vary in their investment in traits that enhance their mating success or in the level of exposure they face sperm competition. Although small males are at size disadvantage in competing in fight for females (and for their eggs), Rosa *et al.* (2019) suggested the potential for high-risk sperm competition for both large and small males based on the observed canalisation of sperm length. Nevertheless, *L. apterus* females have underdeveloped spermatheca, which allows with a small chance the mixing of ejaculates, and thus sperm competition. Furthermore, when preparing male genitalia, we observed a retractable knobbed structure equipped with brush-like hairs, along with a claw, within the aedagus that maybe used for removing rival sperm from the female tract (Supplementary 1). This implies that due to their limited pre-mating competitiveness, small males might need to allocate greater resources towards enhancing their competitiveness after mating. Considering the canalised sperm length and the potential for rival males to remove sperm, the possibility that small males produce higher quality sperm remains open. To confirm this, an analysis of sperm longevity and sperm

motility (adenosine triphosphate (ATP) content) of different male morphs would be required.

Another direction for future inquiries involves unravelling the relationship between reproductive investment and immunity through the entire active season. Study 4 showed a seasonal variation in antimicrobial capacity, although this relationship might not be linear. To investigate the dynamic nexus between reproduction and immunity, increased sampling through the season would provide a clearer picture. Additionally, in also Study 4, maternal transgenerational immune priming, where antimicrobial agents are transported to the eggs, is assumed as a possible cryptic care behaviour in *L. apterus*. Furthermore, it is conceivable that females may regulate microbial growth and provide a mutualistic microbial community in the brood cell by processing leaves with external antimicrobial secretion before forming a ball. This is supported by Nikolajev (2003) describing that the degradation and preservation ongoing processes in brood cells are different from those in soil and that brood cells have a diverse, peculiar microflora. These findings suggest that the *L. apterus* may be the subject of a number of future studies exploring cryptic maternal care.

I.4. SUMMARY – KEY FINDINGS

- Parenting roles in *L. apterus* beetles are highly specialised and separated, with females responsible for offspring provisioning and males engaging in nest guarding. This division of labour is evident in the distinct movement and activity patterns observed for each sex.
- Male desertion in search of additional mating partners may have selected against male care, as females can provide practically unlimited food resources, and males can easily find new mating partners.
- During the reproductive season, *L. apterus* has a series of short breeding cycles and each egg-laying preceded by a copulation. Short duration of male residence and the frequent male changes at the nests support the idea of sequential polygyny as a characteristic of the mating system in *L. apterus*.
- Male beetles may adopt size-dependent alternative mating tactics to maximise their reproductive success, as the three male morphotypes significantly differ in their movement pattern and space use over the season, indicating that body size and phenotypes are closely related to mating behavioural forms.
- The allocation of resources towards immunity and reproduction in *L. apterus* beetles is condition-dependent and influenced by mite infection and the reproductive season. Females exhibit a greater antimicrobial capacity, while encapsulation response does not differ between the sexes.

I.5. ÖSSZEFOGLALÁS – JELENTŐSEBB EREDMÉNYEK

A szülői gondoskodás sok állatfajnál kulcsfontosságú szerepet játszik az utódok túlélésében és fejlődésében. Az utódgondozás mértéke és típusa fajtól függően széles skálán változhat, melyet számos tényező befolyásolhat, mint például a különböző ökológiai faktorok, életmenet-stratégiák és szociális interakciók. Az utódgondozás működésének és evolúciójának vizsgálata segíthet általánosságban a különböző viselkedési formák kialakulásának és fennmaradásának megértésében, valamint gyakorlati megoldásokat is kínálhat a konzervációbiológia egyes problémáira. Az utódgondozás nem csupán a gerincesek kiváltsága. A bogaraknál, mint az egyik legváltozatosabb és legfajgazdagabb csoportnál, a különböző utódgondozó stratégiák széles skálájával találkozhatunk, legyen szó anyai, apai, vagy kétszülős utódgondozásról. Doktori disszertációmban a nagyfejű csajkó (*Lethrus apterus*) szaporodási viselkedésére és azt potenciálisan formáló tényezők feltárására összpontosítottam. Ennek megfelelően két fő célt fogalmaztam meg: Először is, hogy meghatározzam a nemek szerepét az utódgondozás során és ezzel párhuzamosan tisztázzam a korai irodalom megfigyelései és az utóbbi évek vizsgálatai között felmerült ellentmondásokat. Másodszor, hogy az utódgondozó egyedekre ható különböző környezeti, szociális és fiziológiai tényezők feltárásával közelebb kerülhessünk a kétszülős utódgondozás evolúciójának és fennmaradásának megértéséhez. A lentebb röviden ismertetett négy esettanulmány is ezen célokat szolgálta.

1. vizsgálat. Kiss* és mtsai. (2020) Az ivarok differenciált mozgás- és aktivitás mintázata a szaporodási szezon során a nagyfejű csajkónál. Ecological Entomology, 45: 1504-1508. (*Osztott elsőszerzőség)

A nagyfejű csajkó (*L. apterus*) bogárfaj esetében a szülők együttműködése feladatspecializáción keresztül valósul meg: a hímek őrzik a fészkeket, távol tartva így a predátorokat, rivális hímeket, míg az utódok táplálékául szolgáló leveleket zömében a nőstények gyűjtik. Következtetésképpen a két ivar felszíni mozgásmintázata, aktivitása különbözik a szaporodási időszak során. Ezért természetes nagyfejű csajkó populáció vizsgálatára, Dorogházán, talajcsapda-rendszer felállításával, fogás-jelölés-visszafogás módszert alkalmaztunk. Analíziseink azt mutatják, hogy nőstények nagyobb számban foghatóak vissza, illetve hosszabb ideig láthatóak vissza, mint a hímek. Ellenben a hímek jóval nagyobb távolságokat tesznek meg a teljes szezon során. Eredményeink arra engednek következtetni, hogy a nőstények nagyobb gyakorisággal kisebb távokat tesznek meg a felszínen, amely a fészekben fejlődő utódaik számára történő levélgyűjtéssel hozható kapcsolatba. A hímek által megtett nagy távolságokat magyarázhatja, hogy igen nagy az intraszexuális kompetíció, a hímek komoly párviadalokat folytatnak a nőstényekért, továbbá, a hímek feltehetően igyekeznek minél több nősténnyel párzani. Összességében elmondható, hogy a mozgásmintázatban tapasztalt ivari és léptékbeli különbségek összhangban állnak a feltételezett ivari szerepekkel.

2. vizsgálat. Kiss és mtsai. (2023) Viselkedési repertoár és a hímeltávolítás hatása a nagyfejű csajkónál. Journal of Zoology 320: 202-213.

Az interszexuális konfliktusok az egyedek életmenetével, a szociális környezettel vagy éppen az elérhető források mennyiségével együttesen befolyásolhatják az utódgondozási rendszereket. Jelen tanulmányunkban a kétszülős utódgondozású

nagyfejű csajkó (*L. apterus*) két, természetes populációjának szaporodási és utódgondozási viselkedését figyeltük meg, illetve vizsgáltuk, hogy a nőstények viselkedésében milyen változások következtek be annak függvényében, hogy rezidens hímmel, új partnerrel vagy partner nélkül gondoztak. Továbbá, kíváncsiak voltunk arra, hogy a hímek kiléte/jelenléte (rezidens hím, új hím, nincs hím) hogyan befolyásolja a fészek reproduktív sikerét. Eredményeink alapján a hímek idejük nagy részét a járatban vagy fészek közelében töltik, míg a nőstények nagyobb gyakorisággal hagyják el a fészket és gyűjtenek zöld növényi részeket. Továbbá, habár a nőstények a fészek takarításában dominánsnak mutatkoznak, a hímek is kiveszik ebből a feladatból a részüket. Mindezek mellett a hímek rendszeresen, pár nap után dezertálnak, amely alapján úgy tűnik, hogy a korábbi feltételezésekkel szemben a nagyfejű csajkó jellemző párzási rendszere szekvenális poligínia. Ennek megfelelően, a hímek kiléte/jelenléte nem befolyásolja szignifikánsan a nőstények utódgondozó viselkedését, azonban a fészkek reprodukciós sikerére hathat.

3. vizsgálat. Kiss és mtsai. (2023) Méretfüggő reproduktív hím taktikákról árulkodó mozgásmintázatok a nagyfejű csajkónál. (Kézirat).

Azoknál a bogárfajoknál, ahol igen erős szekunder ivari dimorfizmus tapasztalható, nagymértékű intraszexuális változatosság is várható bizonyos jellegek megjelenésében, a díszesebb/felfegyverzettebb ivar esetében. Megnagyobbodott morfológiai jellegek mérete ivaron belül változatos lehet, méreteloszlások függvényében morfotípusokról beszélhetünk amelyek meglétéhez általában alternatív szaporodási taktikák kapcsolódhatnak. Ennek megfelelően, mivel az eltérő morfotípusba tartozó egyedek eltérő taktikát alkalmaznak, feltételezhetően különböző mozgásmintázatot, területhasználatot is produkálnak. A nagyfejű csajkó hímek esetében három eltérő fenotípusról beszélhetünk a mandibuláris processzusz (“agyar”) és testméret arányának tekintetében (nagy, közepes, kis hím kategóriák).

A célunk az volt, hogy a nagyfejű csajkó párzási és utódgondozási viselkedését tanulmányozzuk az ivarok térhasználatának elemzésével, és azonosítsuk a különböző hím morfotípusok közötti potenciális időbeli és térbeli mozgás-, s így reproduktív viselkedésbeli különbségeket egy teljes aktív szezon során. Ezért fogás-jelölés-visszafogás módszert alkalmaztunk Ózd-Susa településrészen élő, természetes nagyfejű csajkó populáció tanulmányozására nagyjából 1 hektáros területen 2016. március 17. és május 23. között. Összesen 1624 bogarat jelöltünk meg, 788 nőstényt és 836 hímet a vizsgálat során. Eredményeink alapján a nagy és közepes hímeket nagyobb számban lehet visszalátni, mint a kicsi hímeket, illetve nőstényeket. Továbbá, a szezon során a visszalátott egyedek által megtett távolságok elemzésekor azt találtuk, hogy a közepes méretű hímek a szezon előre haladtával, két visszalátás között egyre nagyobb távolságokat tesznek meg, míg a kicsi hímek és nőstények esetében pont fordítva, egyre kisebbek a két visszalátás között megtett távolságok. A nagy hímek esetében nem találtunk szignifikáns változást a megtett távolságokban a szezon előre haladtával. Eredményeink alapján a nagyfejű csajkó hímek méretfüggő alternatív szaporodási taktikákat alkalmazhatnak reprodukciós sikerük maximalizálása érdekében. Ezek az eredmények összhangban állnak a korábbi, bogárfajokon végzett vizsgálatokkal, amelyek alapján a testméret és/vagy morfológia szorosan meghatározhatja a párzási viselkedést. Ezek alapján a nagy, felfegyverzett hímek sikeresebbek a párviadalokban, fészekőrzésben, azonban lomhábbak is, míg a kis hímek számára a besurranó, szatellit taktikák a kifizetődőbbek kis méretükből és fürgeségükből adódóan. Akárhogy is, a hím nagyfejű csajkó fenotípusok és hozzájuk prediktált reproduktív taktikák további, feltáró jellegű kutatásokat igényelnek.

4. vizsgálat. Kiss és mtsai. (2020) Szezonális változások az immunitásba és reprodukcióba fektetésben a nagyfejű csajkónál. Journal of Insect Physiology, 121: 104000.

Mind az immunválasz, mind a reprodukció fiziológiai szempontból energiaigényes, költséges folyamatok, így ezen életmenet jellegek között komoly “trade-off” kapcsolat várható. További tényezők, mint az ivarszervek, szaporító képletek mérete, kondíció (vagy testméret), szezonális változások vagy akár a parazita fertőzések befolyásolhatják az önfenntartásba vagy reprodukcióba történő befektetéseket, a közöttük lévő energetikai kapcsolatokat. Célunk ezzel a tanulmánnyal feltárni az asszociációkat az immunkompetencia, a reprodukciós befektetés, a kondíció (testméret), az ivar, a szezonalitás és parazita terhelés között a nagyfejű csajkó bogarak esetében. A vizsgálathoz Debrecen területéről gyűjtöttünk hím és nőstény, kis és nagy méretű egyedeket a szaporodási szezon elején és végén, késő márciusban és május elején. Az egyedek indukált celluláris és humorális immunválaszát fagocitózissal, enkapszulációs teszttel mértünk, illetve az egyedek hemolimfájának baktérium növekedésgátló képességét mérve az antimikrobiális kapacitást határoztuk meg. A reprodukciós befektetés vizsgálatára a reprodukciós szövetek mértünk, hímeknél a teljes hereméretet, nőstényeknél a termelt összpeteméretet. A bogarak parazita terhelését az atkafertőzöttség szintjével határoztuk meg. Megállapítottuk, hogy az enkapszulációs válasz méret-, így potenciálisan kondíciófüggő, a nagyobb egyedek jelentősen nagyobb enkapszulációs képességgel rendelkeznek, mint a kisebbek. Az antimikrobiális kapacitás tekintetében azonban a nőstények dominálnak, erősebb antimikrobiális választ produkálnak. Továbbá, az erősen atkafertőzött egyedeknél a korai szezonban pozitív összefüggés mutatható ki az antimikrobiális kapacitás és reproduktív befektetés között, azonban a szezon végén ez a kapcsolat negatív összefüggést mutat. Reprodukciós befektetéssel kapcsolatban kimutattuk, hogy a

nőstényeknél a korai szezon során pozitív összefüggés van az antimikrobiális kapacitás és reproduktív befektetés között, azonban a szezon végére ez az összefüggés eltűnik. Továbbá, mind a nőstényeknél, mind a hímeknél kimutattuk, hogy a testméretnek hatása van a szezon során a szaporodási befektetés szintjére. Végül, a hímeknél pozitív összefüggést találtunk a teljes hereméret és atka terhelés szintje között. Tanulmányunk alapján az erőforrások elosztása az immunitás és reprodukció esetében nagyban függ a testmérettől, illetve kondíciótól a nagyfejű csajkó esetében, ráadásul ezt tovább befolyásolhatja a parazita fertőzöttség szintje és a szezonalitás. Különösen érdekes, hogy a nőstények nagyobb antimikrobiális kapacitással rendelkeznek, míg az enkapszulációs válasz nem különbözik a nemek között. Összességében, az immunkompetencia és a reprodukció kapcsolata a szezonalitás függvényében dinamikusan változó, így komplex folyamatok sejthetőek a hátterében.

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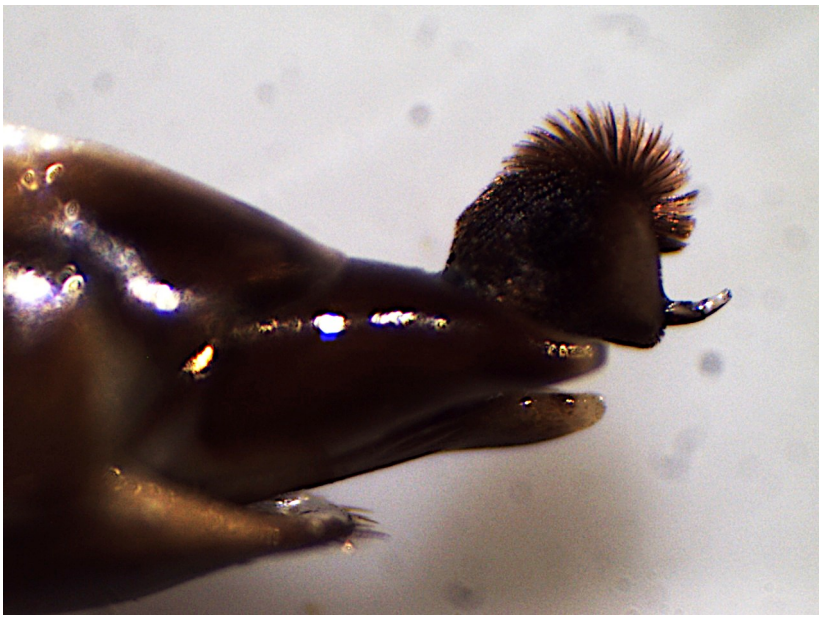
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I.7. SUPPLEMENTARY

Supplementary 1. Apical piece of the aedeagus (parameres) in *Lethrus apterus*



Lateral view of *L. apterus* parameres showing knobbed structure equipped with brush-like hairs, along with a claw, expressed.

PART II

This section comprises the foundational elements of my thesis, encompassing the original publications and submitted manuscripts upon which it is built.

II.1. LIST OF PUBLICATIONS RELATED TO THE THESIS

Kiss, J.*, Németh, Z.*, Kosztolányi, A., Barta, Z. (2020) Differential movement and activity patterns of sexes in a biparental beetle during the reproductive season. *Ecological Entomology* 45: 1504-1508. DOI: https://doi.org/10.1111/een.12920 (*shared first authorship)

Kiss, J., Rácz, R., Váradi, A., Kosztolányi, A., Barta, Z. (2023) Behavioural repertoire and the effect of male removal in a geotrupid beetle with parental care. *Journal of Zoology* 320: 202-213. DOI: https://doi.org/10.1111/jzo.13069

Kiss, J., Rosa, E.M., Rácz, R., Kosztolányi, A., Barta, Z. (2023) Movement reveals size-dependent male reproductive tactics in a geotrupid beetle with parental care. (Manuscript)

Kiss, J., Rádai, Z., Rosa, E.M., Kosztolányi, A., Barta, Z. (2020) Seasonal changes in immune response and reproductive investment in a biparental beetle. *Journal of Insect Physiology* 121: 104000. DOI: https://doi.org/10.1016/j.jinsphys.2019.104000

II.1.1. Personal contributions to publications

	Study 1	Study 2	Study 3	Study 4
Original idea		●	●	●
Study design and data collection		●	●	●
Data analysis	●	●	●	●
Manuscript preparation	●	●	●	●

Study 1

Differential movement and activity patterns of sexes in a biparental beetle during the reproductive season

Kiss, J.*, Németh, Z.*, Kosztolányi, A., Barta, Z. (2020). Ecological Entomology, 45: 1504-1508. DOI: **https://doi.org/10.1111/een.12920** (*shared first authorship)



Ecological Entomology

Ecological Entomology (2020), DOI: 10.1111/een.12920

SHORT COMMUNICATION

Differential movement and activity patterns of sexes in a biparental beetle during the reproductive season

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Abstract. 1. Biparental care is stabilised if parents perform different tasks during care. Specialised parental roles may require different time and energy budgets that in turn are expected to influence the activity and space use of sexes.

2. Here we investigate movement patterns of the biparental *Lethrus apterus* beetle using a grid of pitfall traps in their natural habitat.

3. Sexes of the burrow building *L. apterus* perform different roles during caregiving, as females collect most of the leaves, which serve as food for the offspring while paired males stay mostly in the burrow. We hypothesised that sex differences in mate search and parental activities are reflected in movement patterns.

4. We found that females frequently travelled short distances, whereas males were detected less often but when detected, they travelled significantly longer distances than females.

5. Our results are consistent with the notion that efficient parental food provisioning requires more localised movement and activity patterns. Furthermore, the long distance movements of some males may indicate active mate searching behaviour.

Key words. Biparental care, Geotrupidae, insect, movement activity, sex role.

Introduction

Biparental care can be expected (i) if it is more beneficial for males to stay with their current mate and provide care than to desert and search for new mates, and (ii) if two parents are better than one at improving offspring survival by care (Gilbert & Manica, 2015). Biparental care is often associated with division of labour and task specialisation as the ability of one parent to provide all types of care may be limited (Barta *et al.*, 2014). These sexual differences in care behaviour are frequently correlated with sexual differences in other traits, such as morphology, movement, and activity (Trumbo, 2012; Šklíba *et al.*, 2016).

Intersexual differences are well-studied in dung beetles (*Onthophagus* spp.) where sexes usually differ in body morphology, and take part differently in parental care (Hunt & Simmons, 2002a; Simmons & Ridsdill-Smith, 2011). Typically, males wear horns that are used as weapons during nest guarding against intruders (Simmons & Ridsdill-Smith, 2011). Furthermore, males and females of onthophagine beetles perform different steps of dung collection to provision the larvae and this task specialisation is also reflected in the different above ground movement and activity patterns of the sexes (Hunt & Simmons, 2002a,b): Onthophagine males play an important part by collecting dung above ground thus travel more frequently between the dung pad and the nest, while females spend more time in the nest underground to form dung balls for each larva. Although parental task specialisation and hence sexually different movement and activity patterns are well-studied in dung beetles, the literature about earth-boring beetles that are also known to show biparental care (Lukas, 2013) is scarce.

In the earth-boring beetle *Lethrus apterus* (Laxmann 1770, Coleoptera: Geotrupidae), reproduction lasts from March until June in Hungary. At the beginning of this period, males search for tunnels occupied by females (Frantsevich *et al.*, 1977). After pair formation, the female and the male cooperate in preparing and provisioning a nest underground where the female lays 6–8 eggs and the hatched larvae develop in brood chambers and

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feed on leaf-balls prepared by parents (Emich, 1884). Unlike in dung beetles, Kosztolányi *et al.* (2015) observed that females in this species collect significantly more food for the offspring than males. This study along with Rosa *et al.* (2017) suggests that similarly to other burrow building species (Scott, 1998; Trumbo, 2012), the role of males may be more restricted to the defence of the burrow.

Considering the differences between sexes in mating and care behaviour, different movement patterns and activities of sexes in *L. apterus* are also expected, nevertheless are not yet described. In this study, we investigate the movement of individuals by pitfall traps during the reproductive season with particular attention to potential differences between the sexes. We predict that females, as a result of their more frequent involvement in plant collection around the nest, would be more likely to get recaptured and be seen more frequently and for longer periods of time above ground than nest-guarding males. Longer movements by some males may also be expected if they are not paired with a female or have recently been displaced by a rival male.

Methods

Field procedures

Fieldwork took place in the foothills of the Mátra Mountain near the village of Dorogháza (47°59′29″N, 19°53′36″E), Hungary, between 24 April and 2 June in 2015. We used a square grid of 11×11 pitfall traps covering a square area of 100 m^2 of the field. The traps (400 ml plastic cups) in the grid were 1 m away from each other, as individuals forage about 1 m from their nests (Frantsevich *et al.*, 1977). We used a separate set of 400 ml cups filled with sand to close the traps when they were not in use. As *L. apterus* is diurnal and active only in sunny weather (Frantsevich *et al.*, 1977; and personal observations by JK, AK and ZN), traps were open between 08:00 and 19:00 on 16 days for a total of 88 h (on average 5.5 h a day, as weather permitted). We checked the traps hourly, marked any unmarked individuals caught and noted the date, the position of trap in the grid, and number and IDs of caught individuals. During peak season, between 11–13 May, we recorded newly captured individuals only from the inner 5×5 traps of the grid to reduce capture volume to a manageable level while recaptures were continuously recorded from all traps.

At first capture, we measured the width of the individuals' pronotum with calipers. We determined sex based on the presence or absence of the 'tusks' (ventral mandibular processes). Each beetle was uniquely marked with a paint marker (Marabu Brilliant, Bietigheim-Bissingen, Germany or Marvy Uchida DecoColor, Tokyo, Japan), which made individual identification possible upon later encounters.

Data analyses

All statistical analyses were performed using R (version 3.5.1, R Core Team, 2018). To explore differences between males and females in activity and movement patterns, we investigated the following response variables: (i) the number of times an individual was (re)captured over the study period, (ii) the time interval (in days) between the first and last captures of an individual on the study area, (iii) the total distance an individual travelled during the study calculated as the sum of distances between successive pitfall traps where the individual was caught. Body size may influence movement behaviour (Fountain-Jones *et al.*, 2015); therefore, we controlled in all analyses for individual pronotum width (a good predictor of body size, Rosa *et al.*, 2019). As there is sex difference in pronotum width (Rosa *et al.*, 2019), pronotum width was standardised within sexes.

To test differences between males and females in (i) the number of (re)captures, we used Negative Binomial Generalised Linear Model (further on NBGLM by using 'glm.nb' function in MASS package; Venables & Ripley, 2002) to handle over-dispersed count data. In the full model, we included body size as covariate, sex as factor as well as their interaction as the explanatory terms.

To test whether there are differences between sexes in (ii) the time interval (in days) between the first and last captures, an NBGLM was used. To investigate differences between males and females in (iii) total distance the individuals travelled, we used a linear model where the response variable was log-transformed. In the latter two full models we used sex as factor, body size, number of recaptures as covariates, and all possible two- and three-way interactions (to explore the potential synergistic effects of combined predictors) as explanatory variables.

We conducted backward variable selection by removing the least significant interaction at each step until none met the criterion $P > 0.05$. The significance of retained predictors was tested using 'drop1' function, with likelihood ratio tests in case of NBGLMs, and with F-tests in case of linear models. During the model selection, all interaction terms were dropped from all models (all $P \geq 0.098$). As we expected a priori that main effects may have an effect, they were retained in the final models independently of their significance. Where the number of recaptures was used as an explanatory variable, individuals captured only once were excluded from the analyses.

Results and Discussion

We found that sex had a significant effect on both the number of (re)captures (NBGLM, $\chi^2_1 = 10.974$, $P < 0.001$) and on the time interval between the first and last captures of beetles (NBGLM, $\chi^2_1 = 4.787$, $P = 0.028$), as females were recaptured more frequently and were seen for significantly longer period of time in the season than males (Fig. 1a,b). In the case of total distance travelled (Fig. 2a), we found that sex and number of recaptures had significant effects (linear model, sex: $F_{1,59} = 4.194$, $P = 0.045$; number of recaptures: $F_{1,59} = 19.093$, $P < 0.001$): males travelled significantly greater distances than females did when controlling for number of recaptures (Fig. 2b). Body size had no significant effect on any response variable (all $P \geq 0.203$).

The findings that females were recaptured more frequently, travelled shorter distances and were seen longer during the

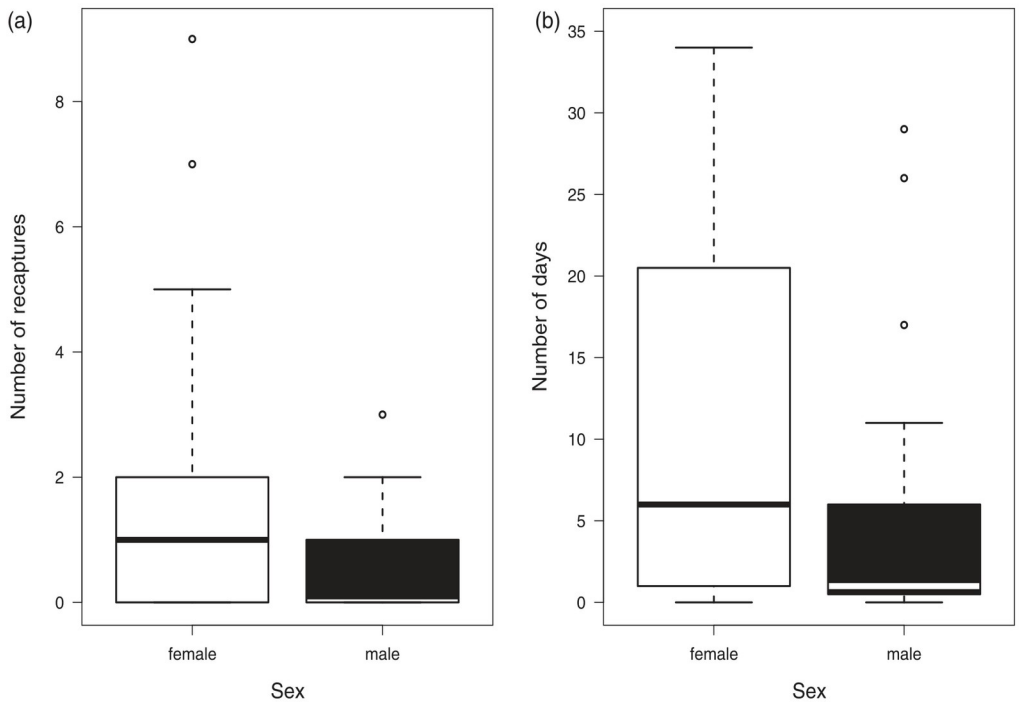


Fig 1. Box plot diagrams of (a) number of (re)captures in female ($n = 58$, white box) and male ($n = 86$, black box) and (b) number of days in female ($n = 32$, white box) and male ($n = 31$, black box) *Lethrus apterus* between the first and last capture on the study area. (Only recaptured individuals are presented on panel (b)). The bottom and top edges of the box indicate the interquartile range (IQR). Bands inside the boxes indicate the median values, whiskers indicate 1.5 times the IQR from the first and third quartiles. Small circles indicate outliers.

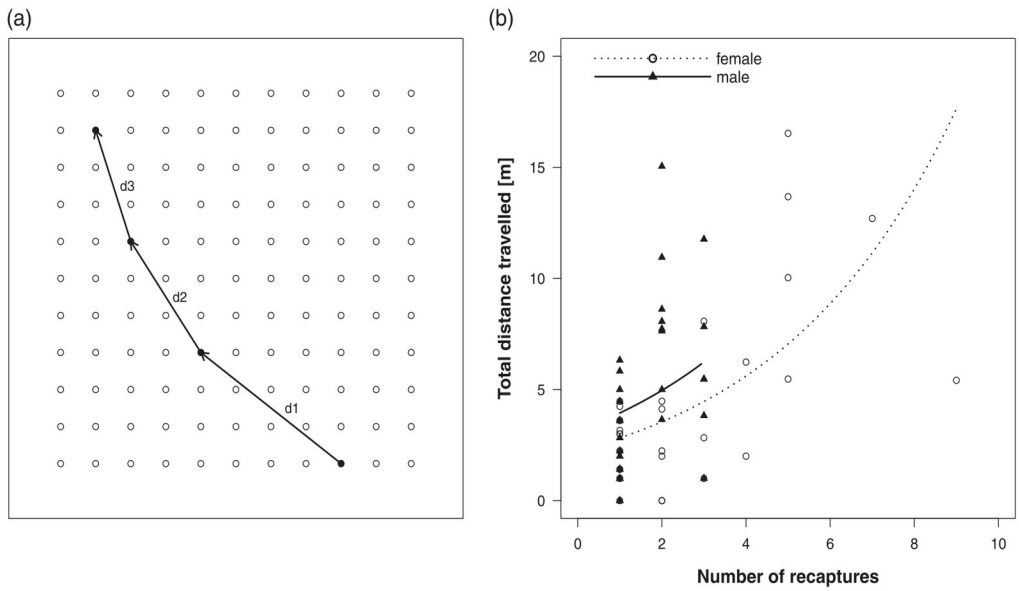


Fig 2. (a) Illustration of calculating travelled distances of *Lethrus apterus* individuals. Here the movement of a male recaptured three times is presented, and the total distance this individual travelled during the study is calculated as d1 + d2 + d3. (b) Relation between number of recaptures and total distance travelled by female ($n = 32$, circle) and male ($n = 31$, black triangle) *L. apterus*. Fitted lines for males (solid) and females (dotted) are predicted by a linear model.

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reproductive season are likely the consequences of females playing a more active role in collecting plant materials for the offspring (Kosztolányi *et al.*, 2015). Each leaf-ball, which is prepared for a larva, is produced from at least two dozen leaves or shoots (personal observation, JK).

Our results that males were seen for shorter periods and less frequently in the study area are consistent with the hypothesis that their main task is to guard the burrow. Consequently, males stay close to or in the entrance of burrows and invest less in food collection. As successful guarding behaviour leads to increased confidence in paternity (mating seems to occur only in or at the entrance of burrows, Frantsevich *et al.*, 1977; and personal observations, JK) and enhanced reproductive success; by high investment in guarding paired males can reduce the chance of cuckoldry (Hunt & Simmons, 2002a,b; Simmons & Ridsdill-Smith, 2011) and offer more protection for the offspring against predators and parasites (Rodríguez-Muñoz *et al.*, 2011). Our finding that males travelled significantly longer distances but were recaptured proportionally less compared to females may be explained by the effort of males actively searching for potential mates. Resident males are expected to have a higher motivation to keep their position in a nest, which is supported by the finding that resident *L. apterus* males usually have a higher success and winning rate in contests compared to the intruders at the burrows (Rosa *et al.*, 2018). Thus, abandoning mate guarding and seeking another partner may only be beneficial for males after losing contest against an intruder male or when the guarded female is resisting to remate (Harts & Kokko, 2013).

In conclusion, our results are consistent with the observation that the female-biased provisioning behaviour in *L. apterus* results in more frequent above ground movements by females. Furthermore, our results support the notion that differences in mate search and care behaviour of the sexes are reflected in different movement and activity patterns.

Acknowledgements

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Author contribution

ZN, AK, and ZB conceived and designed research; ZN performed research; ZB and JK analysed data; and JK wrote the manuscript with input from all authors.

Data availability statement

The data generated and analyzed and complete analysis code (in the form of R script) during the current study are available through GitHub data repository at https://github.com/hannakiss/l.a.movement.pattern.

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Study 2

Behavioural repertoire and the effect of male removal in a geotrupid beetle with parental care

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ORIGINAL RESEARCH

Behavioural repertoire and the effect of male removal in a geotrupid beetle with parental care

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Keywords
beetles; cooperation; nesting behaviour; male removal; parental care; sex roles; *Lethrus apterus*; parental behaviour.

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Abstract

Although biparental care is thought to be rare among insects, it has evolved independently numerous times resulting in diverse forms and varying levels of parental involvement. Several theoretical studies address potential factors promoting biparental care in beetles; however, empirical studies target principally only species of Scarabaeidae and Silphidae families. Here, we investigated the behavioural repertoire of the breeding pairs in two natural populations of a geotrupid beetle *Lethrus apterus* with parental care. Changes in female care as a response to the male's absence were also investigated by experimentally removing males from pairs. We found that males spend the majority of their time at the nest entrance, while females leave their nest and collect leaves at a significantly higher rate than males. We also showed that males often desert their nests and were usually replaced by new males in a short time. The desertion of males, however, does not seem to influence females' behaviour. These findings indicate that *L. apterus* may have a dynamic mating system with typically short-term mate fidelity. Furthermore, our results have advanced our understanding of parental roles and nesting behaviour and contributed to the description of the distinct roles of parents in different phases of nesting events in *L. apterus* beetles.

Introduction

Parental care, defined as any behaviour of the parent that increases its offspring's fitness (Trumbo, 2012), is ubiquitous in the animal kingdom, yet the rich diversity of this behaviour exhibited by insects awaits to be fully explored and understood (Costa, 2006; Cunningham, 2020). As in vertebrates, insect females provide care more frequently than males in cases where care is part of the behavioural repertoire of the species (Kokko & Jennions, 2012; Trumbo, 2012). When male care is present, it is usually performed together with a female resulting in biparental care (Gilbert & Manica, 2015).

An important concept of parental care is that, although the benefits of care are shared between the parents, individuals need to pay the costs of their own parental effort alone resulting in a conflict of interest between the parents over the amount of care (Parker et al., 2002). Due to this conflict, each parent is expected to seek to reduce their personal costs by delegating as many parental duties as possible to their partner. Moreover, by being the first to leave the brood after mating, individuals could not only eliminate all costs of care but gain extra mating opportunities (Webb et al., 1999). Desertion, however, can mainly be beneficial if the chance of finding a new mate is high (Kokko & Jennions, 2008).

The existence of biparental associations implies that in some cases, males can benefit from staying with the family. These benefits may include increased offspring survival (Hunt & Simmons, 2002a), more ensured paternity by keeping rival males away (Kvarnemo, 2006; McNamara & Wolf, 2015; Remeš et al., 2015) and/or by enhanced attractiveness meaning more future mating opportunities (Chemnitz et al., 2017; Keppner & Steiger, 2021).

Even in biparental relationships, individuals often show different levels of parental investment, and parents may respond in different ways to the decrease in their partner's effort (Harrison et al., 2009). In biparental care system, when a partner decides to desert or becomes handicapped or dies, we may expect that its mate will, to some extent, increase its parental investment (McNamara et al., 2003). The overall level of care, however, will likely be lower than it would be if the two parents contribute fully (Houston et al., 2005; McNamara et al., 2003), potentially resulting in reduced offspring fitness (Musa, 2012; Royle et al., 2002). On the other hand, if the parent was already working at its near-maximum capacity, no

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increased investment may be possible. In this case, no change in parental behaviour can be expected after mate desertion/disappearance (Smiseth & Moore, 2004).

A common way to investigate how flexible parental care decisions are, and how those affect reproductive success, is to experimentally remove one of the parents. Burying beetle species are frequent subjects of this kind of experiment (*Nicrophorus spp*, see for example, Pilakouta et al., 2015; Smiseth et al., 2005; Smiseth & Moore, 2004; Suzuki & Nagano, 2009). In this group, parental care is flexible, and both males and females can perform the same parental behaviour within a uni- or biparental system (Eggert & Müller, 1997; Müller et al., 2007; Paquet et al., 2017; Scott, 1998). Interestingly, results about females' willingness to modify their parental investment after the removal of their mate are contradictory (Fetherston et al., 1994, but see also Rauter & Moore, 2004; Smiseth et al., 2005). Like silphid beetles, several species of the Scarabaeidae and Geotrupidae families are also known to show biparental care (Lukas, 2013), but the parental roles and efforts, especially in the latter group, are less explored. To identify the factors that favour the evolution of biparental care in beetles and those that promote the subsequent diversification and maintenance of care, empirical studies on diverse beetle taxa show parental care is needed.

Lethrus apterus (Coleoptera: Geotrupidae) is an iteroparous, flightless burrow-digging beetle with predominant female care (Kosztolányi et al., 2015). Unlike other species of Geotrupidae, in *L. apterus* (and in the other members of Lethrinae subfamily), the adults and larvae feed on only plant materials (Nikolajev, 2003). The sexes are dimorphic as only males have ventral mandibular processes, also called tusks. Tusks serve as weapons in intrasexual contests for females (Nikolajev, 2003; Rosa et al., 2018). Adults usually emerge in March and start looking for mates shortly afterwards. Typically, the male searches and visits tunnels occupied by females (Frantsevich et al., 1977). The density of the tunnels can reach up to 10–15 burrows per m^2 (Frantsevich et al., 1977; Nikolajev, 2003). Since mating takes place in the tunnel (Frantsevich et al., 1977; Nikolajev, 2003), our knowledge about the mating behaviour of *L. apterus* is limited. After pair formation, the tunnel functions as a nest which is excavated, expanded and maintained through the breeding season by the owners. In a 60–100 cm deep nest, usually 5–8 *L. apterus* larvae develop separately in their brood chambers (pers. obs., Frantsevich et al., 1977; Kosztolányi et al., 2015; Nikolajev, 2003). According to recent studies about the parental care of *L. apterus*, while females mainly work on provisioning each brood chamber with plant materials, the role of males may be more restricted to the defence of the nest against conspecific intruder males as well as predators or parasites (Kiss et al., 2020; Kosztolányi et al., 2015; Nikolajev, 2003; Rosa et al., 2017). However, the exact composition of male and female behavioural repertoires and time budgets during care are less known. In addition, the results of the recent investigations are inconsistent with earlier literature. *L. apterus* beetles were considered to be monogamous (Hamilton, 1979). Earlier literature also describes that, similarly to *Cephalodesmius* dung beetles with biparental care (Dalgleish & Elgar, 2005; Monteith & Storey, 1981), the male collects and transports leaves into the nest, while the female processes the collected plants and forms balls for the larvae inside the nest (e.g. Emich, 1884; Schreiner, 1906; but see also Kosztolányi et al., 2015). Moreover, it was considered that after forming the leaf balls, the female lays one egg into each leaf ball and then rolls them to the end of the brood chambers (Emich, 1884). However, our experience of nest excavation disproves this, as in all cases we found the egg behind the leaf ball, separated by a thin wall of soil (pers. obs.). These ambivalent characterisations justify a re-examination of the parental care system in *L. apterus*, including the clarification of the roles of the sexes.

In this study, our first aim was to describe the above-ground activities of the sexes in two natural populations of *L. apterus* beetles and thereby clarify the roles of *L. apterus* males during brood care. We video-recorded above-ground behaviour, like leaf hoarding, nest guarding, and nest construction or maintenance of the observed individuals around the nests. If the main role of the males is guarding, then we would expect that their range of movement is restricted to the entrance and close vicinity of their nest, while females would exhibit more diverse behaviour. Our second aim was to test by experimental male removal whether females modify their parental behaviour as a response to the disappearance of their partner. Finally, we investigated the effect of male removal on reproductive success.

Materials and methods

Fieldwork procedures

Our investigation was carried out over 2 years at two field sites. The first field study was performed between 27 April and 22 May in 2017 near Debrecen, Hungary (47°31′28.6″ N 21°34′30.4″ E) on a grassland patch of cc. 1900 m^2. Firstly, we caught females moving on the surface and marked their elytra and pronotum uniquely with a paint marker (Uni Paint, Oil Base Paint Marker PX-20 Medium, Japan or Edding 780 gloss paint marker). After that, if a marked female was observed to collect leaves at least twice and carried those to the same nest, we tagged the nest with a flag, lured the resident male out from the nest with a blade of grass and gently marked its head with a paint marker. We used this method to mark males to avoid desertion as males are more sensitive to handling (JK and MER personal observation). After that, we started recording the activities of the beetles for approximately 4 h (depending on weather conditions; range of 3.47–4.83 h) using an action camera (SJCAM SJ4000 HD Action Cam, 1080P) mounted on a tripod approximately 20 cm from the entrance of the nest. On the following day, we randomly assigned the previously marked nests to either control or treatment group. In the treatment group, we removed the male from the nest and released it 32 ± 1 m (mean ± SD) away from the nest. No male found the way back to the treated nest during our investigation. Males were not removed from the control group, only lured out from the nests to control for the disturbance around the burrow. Immediately after the treatment, we recorded the activities at the nests for another 4 h (also depending on

weather conditions; range of 3.73–4.83 h). The experiment was completed at 35 nests (18 control and 17 treated nests).

The second study was carried out between 28 April and 14 May 2018 at Ózd-Susa, Hungary (48°16′32.8″ N 20°14′46.3″ E). We used the same protocol as above, except that removed males were released further (250 ± 20 m because of the vegetation pattern and topography) and the above-ground activities at the nests were recorded not on the second but on the third day (range of 4.14–4.84 h in case of the pre-treatment videos, and range of 2.72–4.34 h in case of the post-treatment ones). We completed the experiment at 22 (12 control and 10 treated) nests.

At the end of the breeding season, we marked all investigated nests in the Ózd-Susa population with a flag to ease their later localization. Between 23 May and 15 June, we excavated all the marked nests to determine their reproductive success. We recorded the number of offspring as well as the number of empty/unsuccessful brood chambers (i.e. the chambers filled with plant material but contained no living larvae).

In both populations (Debrecen and Ózd-Susa), the resident male was often replaced by a new, unmarked male even during the pre-treatment observations, and new males were often found in the place of the experimentally removed males as well. Furthermore, in some control nests, where we did not expect any partner changes, resident males deserted by the end of the experiment (see Table 1).

Data analyses

Video footages were analysed by the Mwrap event recorder software (Bán et al., 2017). We coded the following events: duration of being away from the vicinity of the nest (i.e. staying outside of a 10 cm radius circle), the number of leaf collecting events (i.e. food provisioning), duration of staying in the vicinity of the nest (i.e. within a circular area with a radius of 10 cm around the nest entrance), duration of being at the entrance of the nest (considered as nest guarding) and the frequency of removing soil from the nest (indicating nest construction or maintenance). All data and statistical analyses were performed in the R statistical environment (version 4.2.2, R Core Team, 2022).

On account of differences in the schedule between the studies, we analysed the data separately for the two populations as well as for the pre- and post-treatment conditions.

Frequency and duration of activities

To investigate the parental roles of the sexes, we used the behavioural data collected only from the pre-treatment (first day) recordings.

We fitted generalized linear mixed effect models (hereafter GLMM(s)), specifically Hurdle negative binomial GLMMs (Panaccio et al., 2021; Zhang, 2019) by using the glmmTMB package (Brooks et al., 2017) to analyse the counted activities, because of their skewed distribution. Hurdle negative binomial models are proposed to handle overdispersion and deal with zero inflation without discriminating between the zeros' origins (i.e. zeros derived due to the absence or no-detection; Zeileis et al., 2008; Zuur et al., 2009). We used the count data of the following activities for each identified beetle per footage: (i) leaving the vicinity of the nest, (ii) being in the vicinity of the nest, (iii) emerging at the entrance of the nest (coming up from the nest), (iv) leaf collection and (v) removing soil from the nest (i.e. nest maintenance). The natural logarithm of the length of the videos (in hours) was included as offsets into the models, which practically means that we compared rates of events instead of their raw count numbers. We used 'sex' as an explanatory variable, and date of recordings and nest ID as random factors in the models.

To analyse differences in the mean duration of the activities, we fitted linear mixed effect models (hereafter LMMs) by using 'glmmTMB' function with gaussian distribution. We calculated the mean duration (in sec) of time spent (i) away from the nest, (ii) in the vicinity of the nest and (iii) at the entrance of the nest (i.e. guarding). We log-transformed these duration values and used them as response variables. Here, 'sex' as an explanatory variable and date of recordings and nest ID as random factors were also used in the models.

Table 1 The numbers of the nests where previously marked resident males, unmarked new males, and no males were found during the pre-treatment (first day in both populations) and post-treatment part (second day in Debrecen and third day in Ózd-Susa population) of the experiments from Debrecen (a) and Ózd-Susa (b). In the last lines, the total number of control and manipulated nests are given

	Control nests		Manipulated nests	
Number of nests	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
(a) Debrecen				
With resident males	14	8	15	-
With no males	0	0	0	3
With new males	4	10	2	14
Total number of nests	18		17	
(b) Ózd-Susa				
With resident Males	9	0	9	-
With no males	0	5	0	3
With new males	3	7	1	7
Total number of nests	12		10	

Table 2 Pre-treatment sex differences in rate (occurence/h) and mean duration (in seconds) of activities related to parental care in two populations, in Debrecen (2017) and Ózd-Susa (2018)

	Debrecen		Ózd-Susa	
Activity trait	χ^2	*P*-value	χ^2	*P*-value
Rate of leaving the vicinity of the nest	132.020	<**0.001**	49.708	<**0.001**
Rate of being in the vicinity of the nest	22.767	<**0.001**	4.6882	0.096
Rate of emerging at the entrance of the nest	27.936	<**0.001**	10.75	**0.005**
Rate of leaf collection	128.64	<**0.001**	48.798	<**0.001**
Rate of nest maintenance	11.468	<**0.001**	9.638	**0.008**
Mean duration of being away from the vicinity of the nest	45.939	<**0.001**	28.995	<**0.001**
Mean duration of staying in the vicinity of the nest	16.418	<**0.001**	9.239	**0.002**
Mean duration of sitting at the entrance of the nest	40.910	<**0.001**	22.197	<**0.001**

Results of the likelihood ratio tests are shown (d.f. = 1, for all cases). Rate variables were investigated using Hurdle negative binomial GLMMs, and duration variables using LMMs. Significant results are highlighted in bold.

Experimental manipulation of parental care

Because male changes occurred also at unmanipulated nests (see Results), instead of comparing the two treatment groups (control vs. manipulated), we used a 'male status' variable, indicating whether a marked resident male, a new, unmarked male, or no male was present beside the female.

To determine the effect of male absence on females' behaviour, we used the counts and the durations (s) of activities (see above) from the post-treatment recordings of females as response variables.

To analyse the rate of the activities, we used negative binomial GLMMs with log(time) offset since there was no excess of zero values in the data of females. For explaining differences in the mean duration of the activities, we used LMMs. In all models, we used 'male status' (marked resident male, new unmarked male, or no male, see above) as a factor, and the date of recordings as a random factor.

Reproductive success

Offspring number and the number of empty/unsuccessful brood chambers were analysed using GLMMs with negative binomial distribution. We used the factor 'male status' (i.e. new unmarked male or no male as the original male was not present at any of the control nests, see Table 1) as an explanatory variable in both models.

The significance of the explanatory variables in each model was tested with a likelihood ratio test using the 'drop1' function in R. To illustrate the effects of the factors on figures, we extracted estimated marginal mean (EMM) effects, along with associated standard errors (SE), using the 'emmeans' function of the emmeans package (version 1.8.4., Lenth, 2023). Log-transformed durations were back-transformed to their original scales before plotting.

Results

Occurrence of male desertion

We found that males routinely deserted the nests in both populations, leaving females and the brood behind, regardless of the treatment (Table 1). Indeed, in the Ózd-Susa population, no resident male was present in any of the studied nests by the third day.

Parental care behaviours

During the pre-treatment days in both populations, females left the vicinity of the nest and collected leaves at a significantly higher rate, and this resulted in spending more time out of the vicinity of the nest than males (Table 2, Figs 1a,d and 2a). Meanwhile, males spent significantly more time in the vicinity and at the entrance of the nests than females in both populations (Table 2, Fig. 2b,c). In addition, females emerged at the nest entrance at a significantly higher rate, and they removed soil from the nest (i.e. nest maintenance) more frequently than males (Table 2, Fig. 1c,e).

Male removal

'Male status' (i.e. marked resident or unmarked new or no male) did not affect the females' parental behaviours overall (Table 3). However, we found that 'male status' affected the nest maintenance rate of females in Debrecen (Table 3). Specifically, females paired with new unmarked males pushed out soil from the nests more frequently than females with the original, marked resident or without a male (Fig. 3). We found no difference related to the 'male status' of the females in the Ózd-Susa population (Table 3).

Reproductive success

We found that 'male status' did not affect the number of offspring (negative binomial GLMM, $\chi^2_1 = 0.117$, $P = 0.733$, Fig. 4). However, we found that 'male status' (i.e. unmarked new male or no male) affected the number of empty/unsuccessful brood chambers (negative binomial GLMM, $\chi^2_1 = 8.670$, $P = 0.003$). Accordingly, the number of unsuccessful brood chambers was significantly higher in nests without male residency (Fig. 4).

Discussion

Our behavioural data confirm the findings of recent studies on parental care in *L. apterus* (Kiss et al., 2020; Kosztolányi

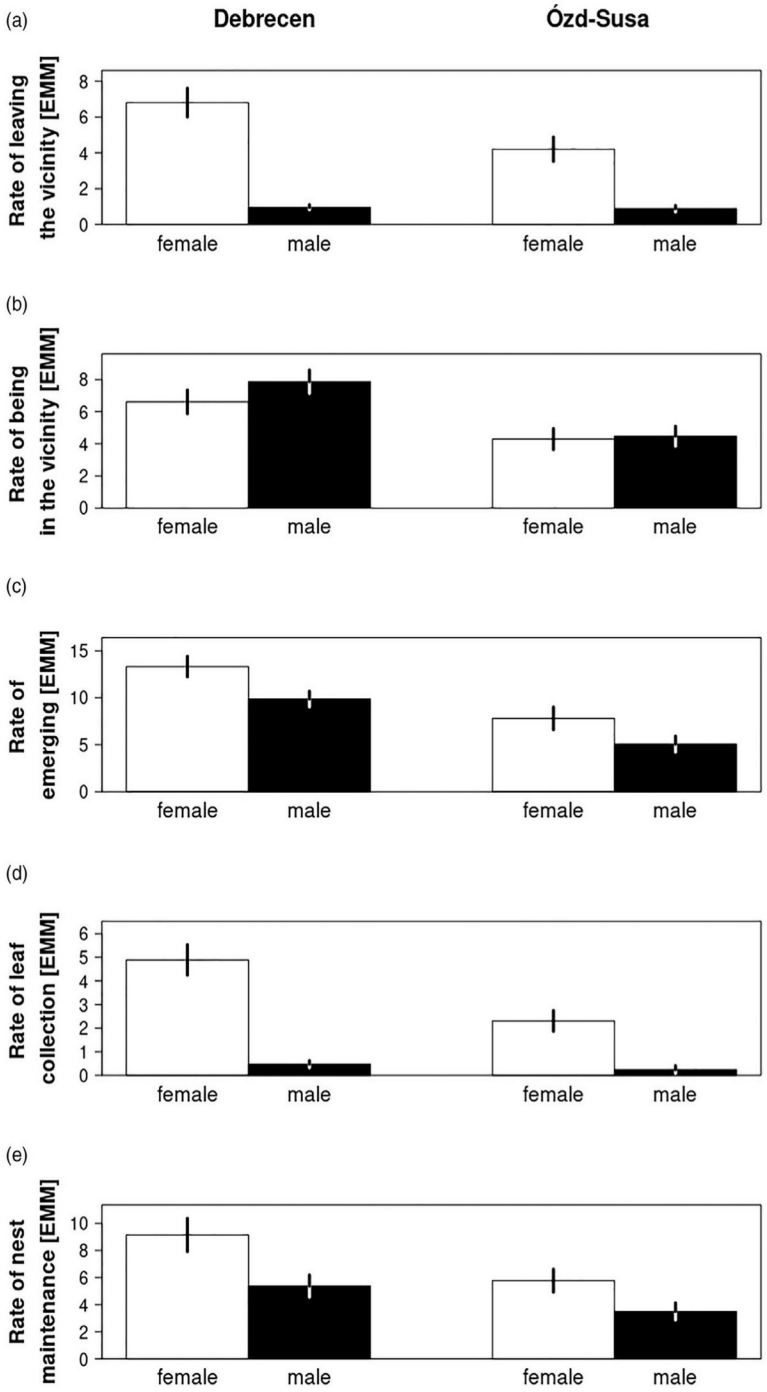


Figure 1 Barplots illustrating females (white box) and males (black box) in rates (counts/h) of activities on the pre-treatment day of the Debrecen (2017) and Ózd-Susa population (2018). Investigated activities were: (a) rate of leaving the vicinity of the nest, (b) rate of being in the vicinity of the nest, (c) rate of emerging at the entrance of the nest, (d) rate of leaf collection and (e) rate of nest maintenance (i.e. pushing out soil from the nest). Estimated marginal means (EMMs) of activity rates are plotted. The error bars represent the SE of EMMs.

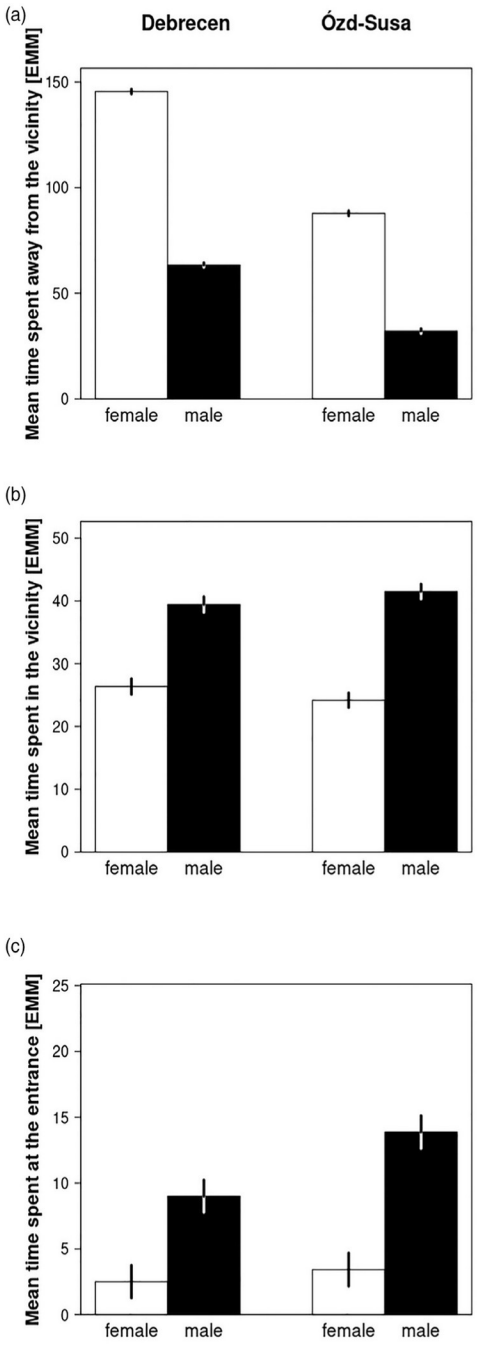


Figure 2 Barplots illustrating differences between females (white box) and males (black box) in the duration (in seconds) of activities on pre-treatment day of the Debrecen (2017) and Ózd-Susa population (2018). Investigated activities were (a) mean time spent away from the vicinity of the nest, (b) mean time spent in the vicinity of the nest and (c) mean time spent at the entrance of the nest. Estimated marginal means (EMMs) of activity rates are plotted. The error bars represent the SE of EMMs transformed back from the original scale.

et al., 2015) suggesting that the primary task of males is nest-guarding, while females provide food for the larvae. Even at nests not manipulated, males deserted after a few days, and new males appeared soon and took over the position of the previous ones at the nests. Furthermore, we found no significant difference in care behaviour between females paired with a resident or a new mate or being without a mate. Females may not modify their care behaviour noticeably depending on the identity of their partner, indicating that there might be a strict division of tasks between the sexes (Barbasch et al., 2021; Henshaw et al., 2019). Our study also demonstrates that the behavioural repertoires of the sexes appear to be very similar in the two populations we studied.

We found that *L. apterus* males desert their nests rather quickly. In fact, in the Ózd-Susa population, no previously marked resident male was found at any of the nests by the third day. Males appeared to leave the nest either on their own accord or because of defeat in a fight (JK, RR and MER personal observation). So, our finding of quick desertion by males may indicate that rival males are more successful in taking over burrows (but see also Rosa et al., 2018) or that the resident male can gain higher fitness by deserting shortly after oviposition (Kokko & Jennions, 2012; Ward et al., 2009). The latter seems more probable because males might be better off searching and fighting for new, additional females with mature eggs than waiting for their partners to be sexually receptive again. Circumstantial evidence suggests that copulation precedes each oviposition. First, the oocytes of the females do not mature simultaneously suggesting that egg-laying occurs egg by egg (Nikolajev, 2003). Second, the egg of *L. apterus* is large compared to the size of the female, physically preventing the simultaneous development of eggs. Finally, females have poorly differentiated spermatheca, which is not expected to provide sperm storage and sperm release regulation during fertilization (López-Guerrero & Halffter, 2000). Consequently, the breeding season in *L. apterus* consists of several, more or less independent breeding cycles with subsequent events like copulation, nest expansion, oviposition, leaf-ball preparation and sealing the brood chamber with soil (Fig. 5; Nikolajev, 2003). Studies in deep burrowing geotrupid beetles (such as *Trypocopris vernalis, Typhaeus spp.)* seem to support the series of independent breeding cycles and the cooperation between the female and male in nest expansion (Brussaard, 1983; Hanski & Cambefort, 1991; Teichert, 1959a, 1959b). According to the study of Brussaard (1983), although the female does most of the burrowing in Typhaeus typhoeus, after copulation, the male also participates in the brood chamber construction. In *L. apterus*, each leaf ball, which is prepared as food for a larva, is produced from approximately 50 leaves or plant shoots (pers. obs., Frantsevich et al., 1977). *Lethrus apterus* beetles are active for an average of 5.5 h a day (Kiss et al., 2020), meaning that, based on our data, females need at least 2 days to collect a ball of leaves (Fig. 1d, 4.89 ± 0.64 (mean $\pm$ SE) leaves were collected per hour in Debrecen, and 2.31 ± 0.44 (mean $\pm$ SE) leaves were collected per hour in Ózd-Susa population). By adding the time required for the other phases of a nesting event, we expect that at least 3–4 days may elapse between two ovipositions (see also Brussaard, 1983; Hanski &

Table 3 Results of the likelihood ratio tests for checking the significance of 'male status' as an explanatory variable (d.f. = 2, for all cases) in fitted models on rate (occurence/h) and duration (in seconds) of activities related to parental care of females in two populations, in Debrecen (2017) and Ózd-Susa (2018)

	Debrecen		Ózd-Susa	
Activity trait	χ^2	*P*-value	χ^2	*P*-value
Rate of leaving the vicinity of the nest	0.248	0.883	2.285	0.130
Rate of being in the vicinity of the nest	1.917	0.383	0.236	0.627
Rate of emerging at the entrance of the nest	1.159	0.560	1.177	0.278
Rate of leaf collection	0.111	0.946	1.514	0.219
Rate of nest maintenance	8.814	**0.012**	0.231	0.631
Mean duration of being away from the vicinity of the nest	1.302	0.522	0.211	0.646
Mean duration of staying in the vicinity of the nest	4.169	0.124	0.024	0.876
Mean duration of sitting at the entrance of the nest	4.024	0.134	0.308	0.579

Rate variables were investigated using negative binomial GLMMs, and duration variables using LMMs. Significant result is highlighted in bold.

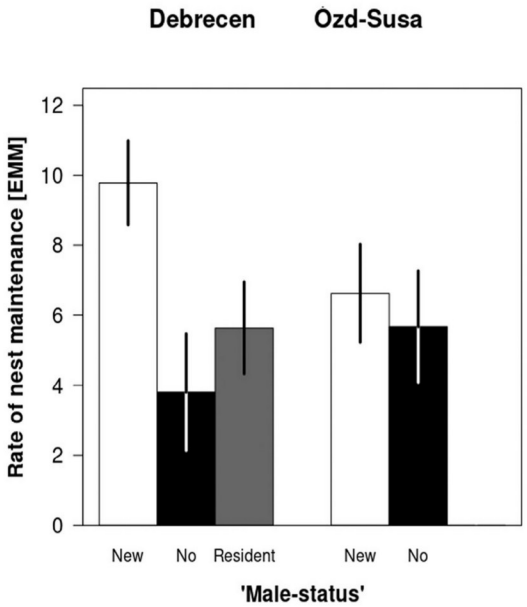


Figure 3 Barplot diagrams illustrating the rate (counts/h) of nest maintenance (i.e. pushing out soil from the nest) in females with different 'male status' (i.e. with new male (white box), no male (black box) or marked resident male (grey box)) from the post-treatment day of the Debrecen (2017) and Ózd-Susa population (2018). In the post-treatment part of the study on the second population, no female was found coupling with a resident male. Estimated marginal means (EMMs) of activity rates are plotted. The error bars represent the SE of EMMs.

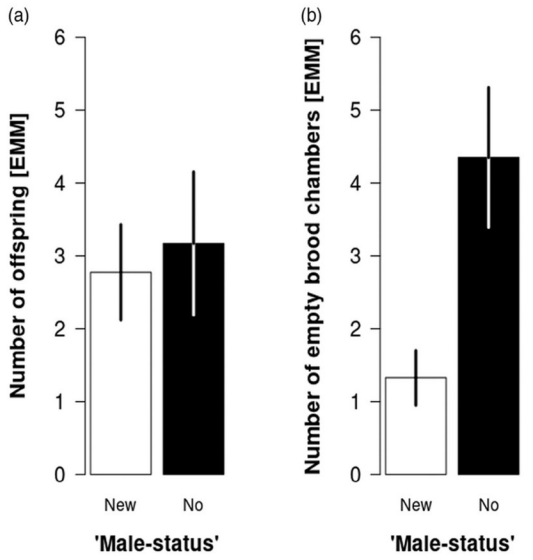


Figure 4 Barplots illustrating (a) number of offspring and (b) number of empty brood chambers in nests of *Lethrus apterus*, with the presence of new unmarked males (white box) or no males (black box). Estimated marginal means (EMMs) are plotted; error bars represent the SE of EMMs.

Cambefort, 1991; Verdú & Galante, 2004). Consequently, males might be better off by deserting soon after the females have laid the egg in favour to pursue other mating opportunities (Kokko & Jennions, 2008; Ward et al., 2009). The high number of intruders (75 fights were recorded on the video footages, in 80% of the nests in the Debrecen population; and 24 fights, in 45.5% of the nests in the Ózd-Susa population) also support our notion that many males are looking for further mating opportunities with other females (Alonzo & Klug, 2012; Kokko & Jennions, 2008; Webb et al., 1999). Nevertheless, the finding that males left the nest after a few days or less implies that females most likely mate with multiple males during a reproductive season, a prediction awaiting a future genetic parentage test.

We found that *L. apterus* males spend much time at the entrance of the nest (most likely to keep intruders and predators away), while the females provision the brood cells with plant materials collected and carried from the surrounding

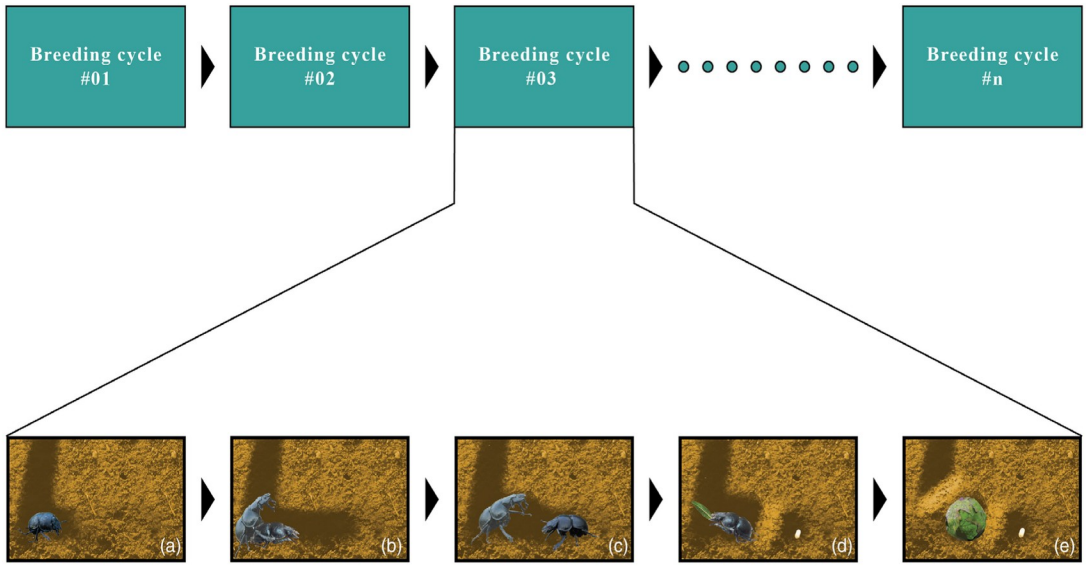


Figure 5 Breeding cycle of *Lethrus apterus* beetles based on observations and the family characteristics. The five panels illustrate the supposed main subsequent events of a cycle: (a) The female excavates the brood chamber branching off the main tunnel. (b) The male joins in the tunnel, the pair copulate. (c) While the female lays the egg, the male expands and widens the brood chamber. (d) The female seals the egg cavity with soil and collects plant material to form a leaf ball for the larva. (e) The brood chamber is sealed with soil after the leaf ball is prepared by the female.

vegetation. Although females seemed to be predominant in nest construction, males also took their share. Removal of soil from the nest by males may indicate involvement in brood chamber excavation, which would also confirm the idea of cooperation between the parents in nest expansion. In onthophagine beetles, sexes cooperate in dung collection and brood ball construction for the larvae (Hunt & Simmons, 2002a; Simmons & Ridsdill-Smith, 2011), as males help in collecting and transporting dung from the dungpad to the nest, while females spend more time to process dung and form balls for the larvae inside the nest (Hunt & Simmons, 2002a). The notion that in *Ontophagus* species, both sexes participate in providing food for the larvae, but not in *L. apterus*, can be explained by the limited and ephemeral availability of dung as a source compared to more plentiful plant materials (Hanski & Cambefort, 1991; Simmons & Ridsdill-Smith, 2011; Verdú & Galante, 2004). Additionally, in species with intense male–male competition, as in the case of *L. apterus* beetles (Nikolajev, 2003; Rosa et al., 2018), vigorous nest defence behaviour can result in a correlated decline in the amount of other care activities that males could provide (Hunt & Simmons, 2002b; Schrader et al., 2020).

Although the same trend was observed in the activity pattern of the sexes in both populations, the individuals from the Ózd-Susa population seemed to be less active. While the sample sizes in the Ózd-Susa population were lower, these subtle differences might be explained also by behavioural variation among years or populations due to different weather and other ecological conditions (Ratz et al., 2016; Wong et al., 2013). For example, insects may avoid adverse temperatures by behavioural mechanisms, for example, searching for and staying in optimal microclimate and reducing activity during adverse periods (Gullan & Cranston, 2014; Smolka et al., 2012). Furthermore, temperature and humidity can affect the breeding activities of the individuals as well as the amount of energy and time invested in nest construction (Holley & Andrew, 2018; Macagno et al., 2016; Mamantov & Sheldon, 2021; Sowig, 1996).

We found that females in *L. apterus* did not differ in the level of their parental investment when their partners deserted or were experimentally removed. However, the majority of the females did not remain alone for long after their partners were gone. In several burying beetle species, partners flexibly share the tasks of caring for offspring (Benowitz et al., 2016; Rauter & Moore, 2004; Smiseth et al., 2005). Thus, parents can cooperate in performing the same tasks and compensate for the partner's effort in case of mate removal (Creighton et al., 2015; Fetherston et al., 1994; Smiseth et al., 2005; Suzuki & Nagano, 2009). On the contrary, in *L. apterus*, the roles of the sexes seem to be more fixed, with females providing most of the care for the offspring and males guarding the entrance of the tunnels. Moreover, it is likely that males desert before the leaf balls are prepared for their offspring by their mates, in which case, females should provide all provisioning as males do not contribute significantly to the well-being of the offspring. Consequently, females may not be expected to alter their parental behaviour in case of male desertion. Nonetheless, further studies, such as female removal experiments, are required to ascertain whether the males do care for offspring in the absence of the females or not.

Based on the observed frequent mate changes in *L. apterus*, each laid egg might be fertilized by a different male, which is supported by studies on the female reproductive system of *Lethrus* species (López-Guerrero & Halffter, 2000; Nikolajev, 2003), and males may desert the female soon after successful fertilization (i.e. males may perform 'pre-oviposition' guarding). Our result, that females paired with new partners removed soil from the nest at a significantly higher rate, might indicate their preparation for oviposition preceded by building a new brood chamber. Conversely, the finding that females paired with resident males (or no males) invested less in nest maintenance might indicate that they had already excavated the brood chamber and prepared for or completed oviposition (or have not mated yet, in the case of lone females). On the other hand, in the Ózd-Susa population, we found no significant difference between females with new or no partners in nest maintenance after the treatment. However, trends were similar to those of the Debrecen population and the lack of significant effect might be traced back to the lower sample size in Ózd-Susa population. It is also possible that since we observed the nests 1 day after the treatment in the Ózd-Susa population, the females with new partners might have been close to the end of brood chamber excavation by then. Nevertheless, the details of the underground behaviour of *L. apterus* beetles are still largely unknown.

Our results showed that the absence or presence of the male partner had no significant effect on the offspring number but did affect the number of unsuccessful chambers. Specifically, nests of the Ózd-Susa population, where no males were found on the post-treatment day, had a significantly higher number of unsuccessful chambers. The lack of mate guarding may have contributed to the reduced survival of offspring (Gilbert et al., 2010; Requena et al., 2009). By guarding, the males can keep not only rivals but also parasites and predators away from the nest (Suzuki, 2013). It has been reported in several insect species with parental care, that conspecific intruders, but in some cases the parent, tend to kill resident larvae to gain reproductive benefits (Huerta et al., 2003; King & Fashing, 2007; Koulianos & Schwarz, 2000; Trumbo, 2006). Nevertheless, we have no information about ovicide or larvicide behaviour in *L. apterus* beetles. Overall, despite this, we have no information about the females' parental efforts nor the actions between the end of the experiment and the nest excavation. Thus, we do not consider these results to be a good proxy for explaining differences in reproductive success in the case of male presence or absence.

In conclusion, we show for the first time that *L. apterus* beetles appear to have a particularly dynamic mating system with typically short-term mate fidelity, while the roles of the sexes are rather stable. Females seemed to be solely responsible for provisioning, and they did not change their behaviour dramatically after the experimental removal of their partner. Males spent most of their time guarding the nest and fighting with rival males, but it *remai*ns unclear whether this behaviour is beneficial solely for the male or also for the offspring. Overall, our results s*u*ggest that contrary to previous characterization as a monogamous system (see Hamilton, 1979), *L. apterus* may have a sequential polygamy mating system. Nevertheless, it will be of high interest to examine the underground activities of the sexes to get a clearer picture of care behaviour in *L. apterus* beetles.

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Study 3

Movement pattern reveals size dependent male reproductive tactics in a geotrupid beetle with parental care

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Movement pattern reveals size-dependent male reproductive tactics in a geotrupid beetle with parental care

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Abstract

Differences between males and females in reproductive behaviour can also be manifested in their movement patterns. Similarly, when males exhibit polyphenism, morphs may utilize distinct reproductive strategies and consequently display differences in their movement patterns as well.

Lethrus apterus is a sexually dimorphic beetle species with parental care. Males wear "tusks" (ventral mandibular processes), which are used as weapons during fights between males. Sexes perform different roles during reproduction, as males are predominant in mate-searching and nest-guarding, while females are primarily responsible for offspring provisioning. Furthermore, males are polyphenic regarding tusk length to body size ratio.

To see how reproductive strategies translate to movement patterns, we investigate the sexes' movement in *L. apterus*, over the whole breeding season using data from resightings of individually marked individuals.

We found that there are significant differences between the females and the different male morphs in travelled distances and temporal changes of movements as well: Travelled distances between two encounters decreased over the season in females and small males, but increased in medium males. Meanwhile, we found no significant change in the movement of large males during the season.

While our findings for males, which include more frequent and longer distance movements, support the idea of active male mate-searching, they also support the idea of sequential polygyny as a characteristic of the mating system of *L. apterus*. Our finding that males' movement patterns are dependent on body size also suggests that different-sized males, i.e., morphotypes, may exhibit size-dependent reproductive and movement strategies.

Keywords: beetle, capture-recapture, mate search, parental care, reproductive tactic

Introduction

Searching for resources such as food, mates, and shelter has a significant impact on an animal's fitness (Bell, 1991). Although both males and females may search for mates, according to empirical and theoretical studies, usually males are predominant in active mate-searching (Kokko & Mappes, 2005; Kokko & Jennions, 2009). This phenomenon can be seen as an outcome of the different amount of energy each sex invest into the offspring (Framenau, 2005; Kokko & Mappes, 2005). Males generally are the searching sex because they can benefit more from additional mating efforts (Clutton-Brock & Parker, 1992; Kokko & Jennions, 2008).

While staying with a receptive female and guarding her after mating may not always be advantageous for males, certain conditions such as small female home ranges, breeding synchrony, high risk of sperm competition, or need for defense and caregiving may motivate males to establish a pair bond instead of seeking additional mates. (reviewed in Kvarnemo, 2018). In species where guarding behavior is present, males may engage in contest competition over access to females, leading to the evolution of traits that enhance fighting ability and result in sexual dimorphism. (Briffa & Sneddon, 2007; McCullough *et al.*, 2016).

In conspicuously sexually dimorphic beetle species, males often show exaggerated traits such as horns, mandibles, or tibias used for intrasexual aggression and/or mating purposes (Kawano, 2006; Emlen, 2008), and may display intrasexual polyphenism (Simpson *et al.*, 2011; McCullough *et al.*, 2015). This variation in body or weapon size is often associated with alternative reproductive tactics (ARTs) due to differences in competitiveness to monopolize mates. (reviewed in Brockmann, 2008). For instance, in onthophagine dung beetles with parental care, males can be grouped into two categories regarding their morphology and reproductive behaviour; large (major) and small (minor) males differ in both mating and caregiving tactics (see, for example, Hunt & Simmons, 2001; Madewell & Moczek, 2006; Simmons & Kotiaho, 2007). Large males invest more in nest-guarding and caregiving, whereas males with no horns tend to perform an alternative, sneaking mating tactic without providing care, and are more likely to disperse from one habitat patch to another (Eberhard, 1982, 1987; Moczek & Emlen, 2000; Hunt & Simmons, 2002b; Simmons & Kotiaho, 2007).

Movement patterns can be related to ARTs, as individuals may need to move differently depending on their tactics (Kokko & Jennions, 2008). For example, in species where males exhibit ARTs such as sneaking or satellite behaviour, they may need to move differently to access females and avoid detection by dominant males. This may involve moving more slowly, taking a different path than males using traditional mate-searching behaviours (Moczek & Emlen, 2000; Hunt & Simmons, 2002b). Conversely, males using traditional mate-searching behaviours may need to move more frequently and travel greater distances to find receptive females (reviewed in Renault, 2020). Therefore, movement patterns can be an important aspect of deciphering the reproductive behaviours and strategies of individuals, particularly in species with ARTs (Kokko & Jennions, 2008).

Of geotrupid beetles with parental care, in the *Lethrus apterus* (Coleoptera: Geotrupidae), males have pronounced mandibular processes (also called "tusks"), which are used as weapons during fight contests and nest-guarding (Nikolajev, 2003; Rosa *et al.*, 2017). Rosa *et al.* (2019) reported that *L. apterus* males show a polyphenic tendency in respect to tusk length: large males have longer tusks, while small ones have smaller tusks relatively to their

body size, and there is a group of medium-sized males where the tusk length increases linearly with body size. Different sized males also differ in fighting and guarding behaviour: smaller males are less willing to engage in a contest with a resident male guarding a burrow compared to larger ones (Rosa *et al.*, 2018). However, alternative reproductive tactics related to the different male morphs in *L. apterus* are unknown, and this is also true for the Geotrupidae family as a whole.

Here, we investigated movement patterns by collecting data on spatial and temporal extent of space use in *L. apterus* during a whole active season. *L. apterus* is an iteroparous, flightless beetle which is active between March and June in Hungary. After emerging, at the beginning of the season, both males and females stay in shallow burrows until the start of the mating season (Frantsevich *et al.*, 1977; Nikolajev, 2003). Later, when the maximum daytime temperature reaches 15-22 °C (referred to as the "time of apple tree blossom"), the males start to prowl and visit tunnels occupied by females (Frantsevich *et al.*, 1977; Nikolajev, 2003). *L. apterus* males engage in intense fighting contests during the reproductive season to monopolise females, the resident males fiercely guard the entrance of the tunnels against conspecific competitors and intruders (Nikolajev, 2003; Kosztolányi *et al.*, 2015; Rosa *et al.*, 2017; 2018; Kiss *et al.*, 2020a; 2023). After mating, the female begin to expand the tunnel with the help of the male, which then functions as a nest (Nikolajev, 2003; Kiss *et al.*, 2023). The female lays 6-8 eggs consecutively, and the hatched larvae develop in separated brood chambers and feed on a leaf ball prepared for each offspring separately before hatching (Emich, 1884). It is primarily the females' task to search for and collect the shoots and leaves from the vegetation in the vicinity of the nest for preparing leaf-balls (Kosztolányi *et al.*, 2015; Rosa *et al.*, 2017; Kiss *et al.*, 2020a). In contrast to earlier literature, which described the *L. apterus* as a monogamous species (Hamilton, 1979), according to the results of Kiss *et al.* (2023), males often desert their partners a few days after copulation, presumably leaving them soon after oviposition.

Our aims in this study are (i) to investigate space use in relation to mating and foraging behaviour of the sexes, and (ii) to detect potential differences in reproductive behaviour between male morphs. We conducted a field survey during the whole active season to test the following predictions: (i) Males will be more likely to get resighted, travel longer distances and be seen more frequently but for a shorter period of time above ground than females as a result of active mate-searching (and frequent desertion) of males and smaller home ranges of females; and (ii) there will be spatial and temporal differences in movement behaviour between male morphs due to differences in competitive ability and thus different movement tactics. We expect that large males will move on smaller areas, travelling shorter distances, as they can easily monopolise nests and thus females due to their size and competitive abilities. Medium-sized males are expected to travel increasingly larger distances as the number of females available for them presumably decreases in time. Smaller males, due to their size, may be restrained and use the sneaker tactic, wandering in a small area, visiting numerous nests and searching for unguarded females.

Activity and movement patterns of *L. apterus* beetles have already been studied by Kiss *et al.* (2020a); however, that work mainly focused on the reflection of task specialisation in movement patterns of the sexes. Accordingly, the investigation was limited to the reproductive season (between 24th April and 2nd June), and it was carried out in a smaller area (100 m^2) using pitfall traps. However, by monitoring a considerably larger area throughout the whole active season (from mid-March to late May), we had the opportunity here to track the movement of beetles from early spring emergence through the breeding season to their preparation for diapausing. Moreover, since we

assume that male morphs adopt different reproductive tactics which may appear in movement behaviour differences, a larger scale in time and space of studying is needed to reveal this.

Material and methods

Field procedures

To investigate seasonal dispersal and movement behaviour of *L. apterus* beetles, a capture-mark-resight survey along line transects was conducted in 2016 between 17th March and 23th May near Ózd-Susa, Hungary (48°16'33.3"N, 20°14'46.6"E). As part of the survey, we traversed four predetermined line transects spaced a minimum four meters apart. By walking along transect line, we were able to spot individuals within a distance of 1 meter. Each of them was 830 meters long, encompassing an estimated area of 1 hectare. The four transect walks took about three to four hours to finish (depending on the number of individuals sighted). If weather permitted, we sampled the transects twice a day.

In case of encountering an unmarked individual we measured the width of the individuals' pronotum, and, in case of males, the length of the left tusk with calipers to the nearest 0.01 mm. We determined sex based on the presence or absence of tusks. Each beetle was then uniquely marked with a paint marker (Uni Paint, Oil Base Paint Marker PX-20 Medium, red, gold, silver, or green, Japan or Edding 780 gloss paint marker, gold, silver, red, Germany), which allowed subsequent individual identification. Individuals were always released at the same location where they had been captured. Date, time and GPS location of the capture were also recorded. In the case of encountering an already marked individual, we recorded its ID, the time and date and the GPS coordinates of the location where the individual was found. The weather conditions allowed for a total of 39 days of field work over the 68 day long study during which we marked a total of 1624 individuals (788 females and 836 males).

Data analyses

GPS coordinates of the locations where individuals were encountered (i.e., captured/resighted) were georeferenced (for accurate and efficient processing of spatial data) and distances between consecutive encounter locations for each individual were calculated by using QGIS software (version 3.18.3-Münich, QGIS Development Team, 2021). All statistical analyses were performed using R (version 3.6.3, R Core Team, 2020).

To find differences in movement patterns over the season between females and male morphs, we investigated the following response variables: (I) the number of occasions an individual was encountered, (II) the time interval (in days) between the first and last encounter, and (III) the travelled distance between two encounters estimated by the distance (in meters) between the consecutive encounter locations of an individual (i.e., distance travelled per resighting). In case of (II) and (III), only individuals were included in the data analyses that were resight at least once.

We categorized the encountered males into three morphs to investigate potential differences in movement and activity between males of different sizes and to compare them with females'. To do so, we fitted a two-breakpoint segmented model based on the study of Rosa *et al.* (2019). As a result, we differentiated between large (pronotum width > 12.32 mm; n=424), medium (12.32 ≤ pronotum width > 10.80; n=357) and small (pronotum width ≤ 10.68; n=55) males. Then we used "sex-size" as a categorical variable with three levels of males (as explained above) and a level of female.

To test whether there is a difference between females and male morphs in the (I) number of encounters and the (II) time interval (in days) between the first and last encounter, we used Negative Binomial Generalized Linear Models because of the skewed distribution of the data (further on NBGLM by using "glm.nb" function in MASS package; Venables & Ripley, 2002). In the full models "sex-size" as factor was the explanatory term. In case of (II), we controlled for the “number of encounters” and used it as covariate.

To test whether there is a difference between females and male morphs in (III) the distance travelled between two encounters we used linear mixed model (further on LMM by using "lmer" function in lme4 package; Bates *et al.*, 2015) where the response variable was log-transformed to ensure non-skewed distribution of residuals. The movement activities of individuals can vary during the season according to different phenological phases and inter- and intrasexual behavioural differences (Bots *et al.*, 2009). Since we were also interested in exploring the temporal movement patterns, we controlled for time of season. I.e., we calculated the number of days between the start of the study and date of encounter and the number of days between two encounters. We used the following explanatory terms in the full model: "time of season" (i.e., number of elapsed days since the start of the study), "time since the previous encounter" (in days), "sex-size" as factor, and 3- and all 2-way interactions. The ID of the individual was used as a random factor.

We conducted backward variable selection by removing the least significant term (according to p-values) at each step until none met the criterion $P > 0.05$. Likelihood ratio tests were used with "drop1" function in R to test the significance of variables in fitted models. We used "emtrends", “emmeans”, and “pairs” functions of the emmeans package (1.5.5 version, Lenth, 2021) as post-hoc tests and to investigate interaction.

Because our sample sizes were relatively high, some significant effects may have low biological relevance, therefore we calculated partial and semi-partial R^2 values as indicators of effect sizes (Bodner, 2017). We calculated Kullbach-Leibler-divergence based partial R^2 values for independent variables of NBGLMs using "rsq.partial" function of "rsq" package (Zhang, 2018), and determined semi-partial R^2 values (both marginal and conditional R^2) values for LMMs using "partR2" package (Stoffel *et al.*, 2021). R^2 values of >0.02, >0.13 and >0.26 representing small, medium, and large effects, respectively (Cohen, 1992).

Results

Number of encounters

We captured and marked a total of 1624 beetles, 788 females and 836 males. 20.6% (n=162) of the females and 26% (104 large, 99 medium, and 14 small sized) of males were resighted at least once. We found that "sex-size" had a significant effect on number of encounters (NBGLM, $\chi^2_1=14.249$, P=0.003, Table 1a). Large and medium males were resighted more frequently than females over the season , but we found no significant difference between the females and small males, nor between different male morphs in number of encounters (Figure 1a, Table 1b). However, ”sex-size” showed a relatively small effect size (partial $R^2=0.014$).

Time interval between the first and last encounter

In case of female-male comparison, we found that "sex-size" had an effect on the time interval between the first and last encounter during the season (NBGLM, $\chi^2_1=8.072$, P=0.045), so females were detected for more days in the study area than different male morphs (Figure 1b, Table 2a), but the difference was not significant and here we also got small

effect size (partial R^2=0.019, Table 2b). "Number of encounters" showed a significant positive effect (NBGLM, χ^2_1=27.411, P<0.001; partial R^2=0.060).

Table 1. Results of **a)** post-hoc test and **b)** comparisons for analysing differences between females and male morphotypes of *Lethrus apterus* in number of encounters. We calculated **a)** the estimated marginal means (EMMs) of number of encounters for the levels of "sex-size" (female, large male, medium male, small male) and **b)** compared the EMMs with one another.

a)

"Sex-size"	**EMMs**	**SE**	**df**	**z**
Female	0.330	0.029	Inf	-12.485
Large male	0.533	0.059	Inf	-5.705
Medium male	0.489	0.059	Inf	-5.890
Small male	0.328	0.100	Inf	-3.652

b)

Comparison	**Contrast (EMMs)**	**SE**	**df**	**z**
Female – Large male	0.620	0.088	Inf	-3.378
Female - medium male	0.675	0.102	Inf	-2.609
Female – small male	1.005	0.319	Inf	0.015
Large male – medium male	1.090	1.179	Inf	0.523
Large male – small male	1.622	0.526	Inf	1.491
Medium male – small male	1.488	0.489	Inf	1.211

Table 2. Results of **a)** post-hoc test and **b)** comparisons for analysing differences between females and male morphotypes of *Lethrus apterus* in number of days between the first and last encounter. We calculated **a)** the estimated marginal means (EMMs) of number of days for the levels of "sex-size" (female, large male, medium male, small male) and **b)** compared the EMMs with one another.

a)

"Sex-size"	**EMMs**	**SE**	**df**	**z**	**P**
Female	10.78	0.731	Inf	35.079	<0.001
Large male	8.75	0.753	Inf	25.215	<0.001
Medium male	9.10	0.796	Inf	25.268	<0.001
Small male	5.94	1.437	Inf	7.360	<0.001

b)

Comparison	Contrast (EMMs)	SE	df	z	P
Female – Large male	1.232	0.135	Inf	1.895	0.230
Female - medium male	1.184	0.131	Inf	1.529	0.420
Female – small male	1.816	0.456	Inf	2.374	0.082
Large male – medium male	0.961	0.118	Inf	-0.320	0.989
Large male – small male	1.474	0.379	Inf	1.509	0.432
Medium male – small male	1.533	0.395	Inf	1.661	0.345

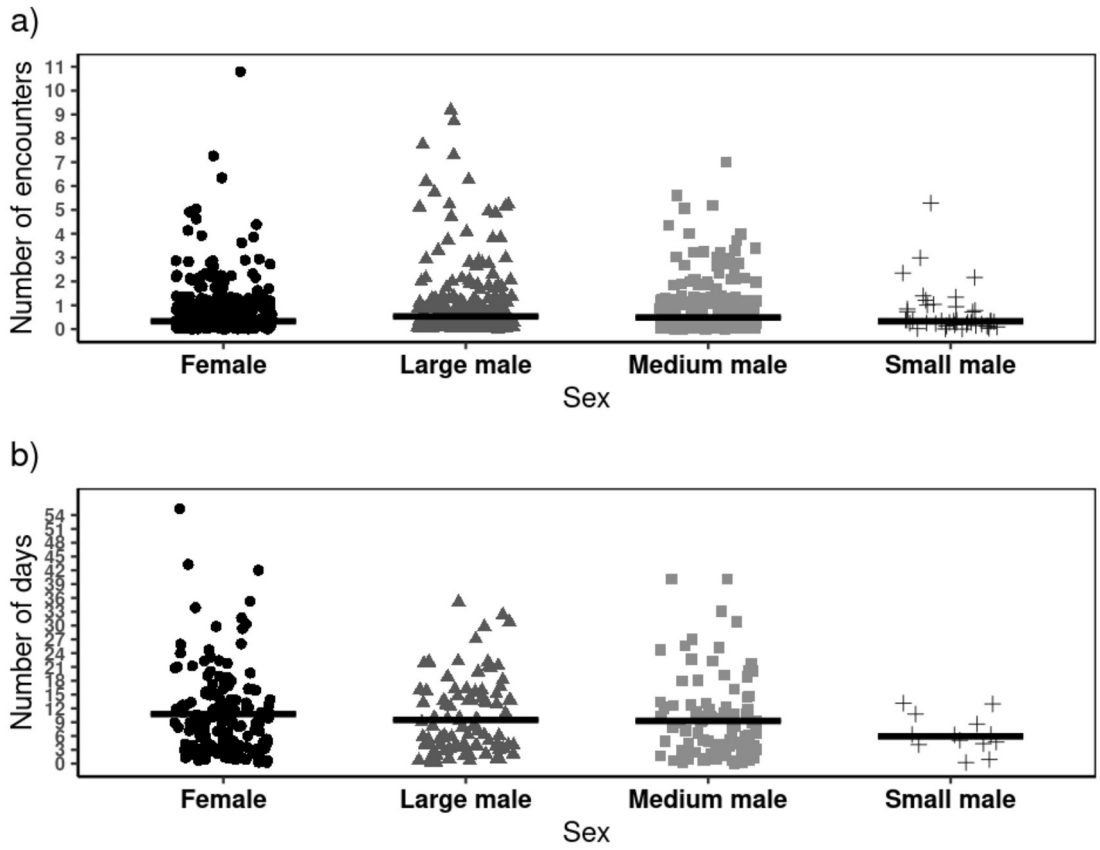


Figure 1. Jitter plots of **a)** the number of encounters and **b)** the number of days between the first and last encounter on the study area in female (black circle) and large- (dark gray triangle), medium- (medium gray square), and small male (dark grey cross) *L. apterus* beetles. Bands indicate mean value.

Distance travelled between two encounters

By analysing differences in the travelled distance between two encounters among female and male morphs, we found that interaction between "sex-size" and "time of season" was significant (LMM, χ^2_1=27.368, P<0.001; marginal R^2/conditional R^2=0.035/0.155). In medium males there was a positive relationship between the travelled distance and "time in season" (post-hoc test, b=0.025, t_{577}=3.949, P<0.001), whereas in females and small males, there was a negative relationship (post-hoc test, females: b=-0.009, t_{524}=2.236, P=0.026; small males: b=-0.029, t_{419}=2.100, P=0.037). We did not find a significant relationship in large males (post-hoc test, b=0.004, t_{549}=0.802, P=0.4229) (Figure 2). "Time since the previous encounter" also had an effect on distances travelled (LMM, χ^2_1=40.731, P<0.001; marginal R^2/conditional R^2=0.052/0.172), i.e., the more days passed between two encounters, the greater the distance travelled by beetles.

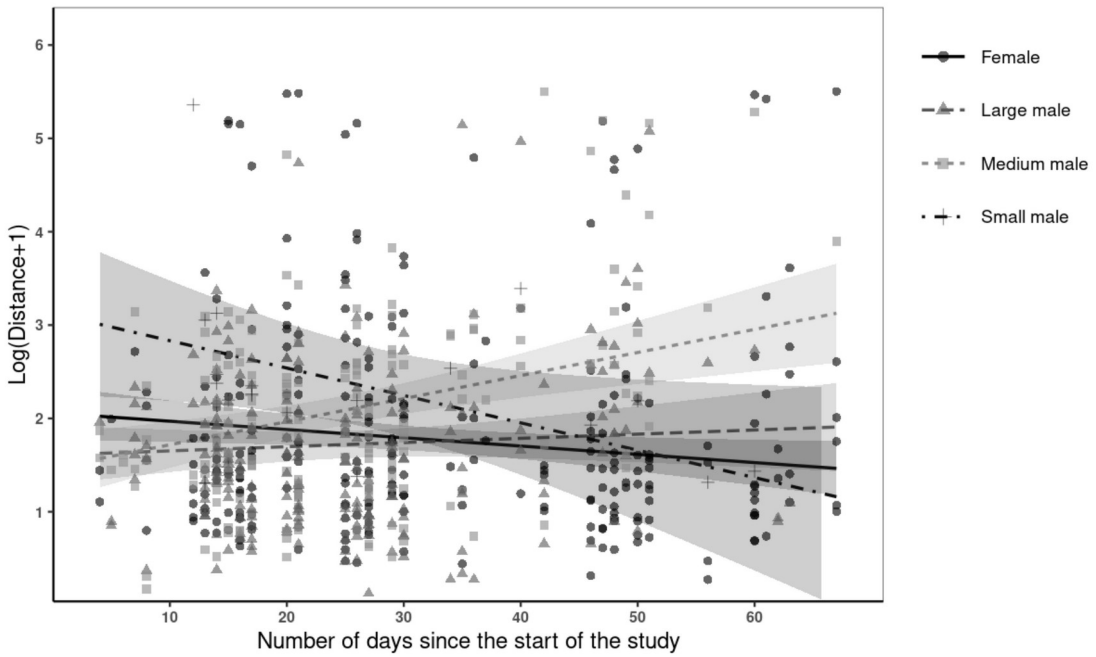


Figure 2. Relationship between the time in the season and logarithmic travelled distance (in meters) between two consecutive encounters in females and the three male morph categories of *L. apterus*. Lines are predicted by fitted linear mixed model. Grey ribbons indicate 95% CI.

Discussion

In agreement with our predictions, we found that males tended to be resighted more times but were seen for a shorter period of time in the studied area than females, but not small ones. Additionally, in terms of travelled distances, medium-sized males increased the travelled distance between two encounters as the season progressed while small-

sized males and females decreased. Besides, there was no significant relationship between the travelled distance and time in the season in large males.

The findings that females tended to be resighted less frequently but were seen longer during the season and that the distance between two encounters shortened as the season progressed corroborate the recent observations on the reproductive behaviour of *L. apterus* females. Females search and collect plants for the larvae in a smaller area around the nest during the breeding season (Frantsevich *et al.*, 1977; Kiss *et al.*, 2020a). They undertake approximately twelve foraging marches a day in order to form a leaf ball in each brood chamber, where a larva will develop in the nest underground (Frantsevich *et al.*, 1977; Kiss *et al.*, 2023). Furthermore, they can quickly and straightly return to the nest underground when they are frightened or disturbed during plant searching (Frantsevich *et al.*, 1977). It follows that the targeted movements of females and time-consuming food provision in small areas may have resulted in a low resighting number but still a long time presence of females. Our finding that females' travelled distances were shrinking over time suggests that with early-season swarming, females may search for suitable places for burrowing. Later, as the breeding season comes, and the food supply becomes more plentiful, their movements may become more localized to the vicinity of the nest. In contrast to dung beetles or burying beetles, in *L. apterus*, feeding and reproduction are not linked to limited, ephemeral resources such as dung or carrion.

L. apterus males were seen for a shorter period of time compared to females; however, they were resighted more times than females in the study area, which contradicts the results of a previous study (Kiss *et al.*, 2020a). This is likely due to differences in the study design, particularly differences in the size of the study area (100 m^2 *versus* 10000 m^2) and in the duration of the study (breeding season *versus* whole active season). Still, statistically significant differences between the sexes were present in this study, in the case of the number of encounters and time interval between the first and last encounters, the effect sizes were very small. These negligible effect size values may, in theory, belie the practical impact of sex as an explanatory variable. The fact that a significant value was obtained with a small effect size is due to the large number of elements. Besides this, we do not refute that sexes can be encountered with different rates or can be seen for different durations in an area, but we would not draw any major conclusions here.

Our results that males tended to be more resighted, seen for a shorter period of time in the study area than females, and they were travelling longer distances between encounters as the season progressed, support the observations that males are the sex which actively search for mates (Frantsevich *et al.*, 1977; Nikolajev, 2003). Unlike other beetle species with parental care, the presence of *L. apterus* females is not indicated by resources such as dung piles or carcasses (for example, Hunt *et al.*, 1999; Komdeur *et al.*, 2013; Pilakouta *et al.*, 2016), so it should take much effort for males to search for partners. Socially monogamous snapping shrimps (*Alpheus angulosus*), similarly to *L. apterus*, live in burrows and show predominant female care (Mathews, 2002a, 2002b, 2007). In snapping shrimps, it is also the male who actively searches for mates, and after pair formation, males guard the female and the burrow and do their share of burrow construction. However, male snapping shrimps usually leave their partners after a while and search for additional unpaired receptive females (Mathews, 2002b, 2007), so it might be better to call them sequentially polygamous. As a consequence of active mate search in males in this species should travel ever longer distances to find a new partner during the reproductive season (Heuring & Hughes, 2020). The similarities with snapping shrimps raise the possibility that *L. apterus* might as well be a sequentially polygamous species, i.e., individuals form pair-bond with different individuals sequentially in the same season, and it probably has a temporary mate-guarding type of mating system.

Our results indicate that movement and activity are influenced by body size in males, as small males were resighted less and travelled shorter distances as the season progressed, compared to medium and large males. Morphological differences within males are usually related to behavioural differences derived from their status (Moczek & Emlen, 2000; Brockmann, 2008). Larger males may aim to preserve their positions as residents by spending more energy on guarding the entrance of the burrow, leaving it only on rare occasions and on their own accord (Hunt & Simmons, 2002a,b; Rowland & Emlen, 2009; Simmons & Ridsdill-Smith, 2011). Meanwhile, smaller males may be less able to defend a burrow or take a resident male's position as an intruder after a successful fight (Moczek & Emlen, 2000; Brockmann, 2008; Rowland & Emlen, 2009). Therefore, smaller males possibly more exposed to costly or high-risk intrasexual interactions with higher probability to lose contests and move on compared to larger ones. In *L. apterus*, smaller males as intruders appear to avoid fight contests in general (Rosa *et al.*, 2018), which suggests that smaller males may not invest much in intrasexual competition or may use context-dependent alternative reproductive tactics, i.e., decision to stay and fight or to disperse and sneak depends on the presence and size of a rival male (Painting & Holwell, 2014). If this holds, then smaller males may gain more benefits by wandering in a smaller habitat patch and visiting burrows regularly in search of unguarded females (Harts & Kokko, 2013) or may apply an alternative, sneaking mating tactic to gain access to partners when rivals are more competitive (Moczek & Emlen, 2000; Brockmann, 2008; Painting & Holwell, 2014).

We found that male morph categories of *L. apterus* differed in the change in travelled distances between two encounters as the season progressed. Specifically, medium-sized males showed an increase in the distance they travelled between two encounters over the season, while in small-sized ones the opposite trend was found. In the case of large males, there was no significant relationship between the travelled distance and time in the season. However, no significant difference was found in the number of encounters and the number of days between the first and last encounter among the male morphs (Table 1b, Table 2b). Okada *et al.* (2008) found that males of the *Librodor japonicus* beetle with different sizes do not differ in frequency of dispersing or in the number of leaving habitat patches. However, they showed that *L. japonicus* males have size-dependent alternative mating behaviours, as small males usually adopt sneaking tactics, medium-sized males are apt to disperse earlier from the habitat patch to avoid intrasexual competition while large males invest more in guarding. Based on the spatial and temporal differences in movements in male morph categories, it is possible that, similarly to *L. japonicus* beetles, there are size-dependent alternative mating tactics in *L. apterus* males. In many beetles, large males invest more resources in weapons for fighting and guarding (Emlen, 2008). Also, large males with large horns or tusks are more successful in monopolising females and preserve their positions as residents but less agile and more exposed to predation (Hunt & Simmons, 2002a,b; Kojima *et al.*, 2014). Therefore, fighting, staying and guarding might be more advantageous for large-sized males than dispersing and travelling long distances (Hunt & Simmons, 2002a,b; Okada *et al.*, 2008; Rowland & Emlen, 2009; reviewed in Renault, 2020). In contrast, medium-sized males should pay a higher cost for fighting and maintaining their positions against other males, so they can raise their probability of mating success with dispersing and searching for unpaired females (Okada *et al.*, 2008; reviewed in Renault, 2020). It would explain the finding that medium-sized males showed an increase in travelled distances over the season. Last but not least, because of their size (and thus probably their condition), small-sized males are at an immense disadvantage in male-male competition. The finding that small males travel longer but over time shortening distances (compared to medium- and large-sized males) may suggest the use of sneak tactics. Minor males

might stay and wait for the chance to sneak into the nests occupied by a larger male, and mate with the female (Moczek & Emlen, 2000; Hunt & Simmons 2002b). It is also conceivable that small males demonstrate a scramble competition strategy; they stay and circuit in a smaller area and visit the nests in search of an unpaired female (Hunt *et al.*, 1999; Pomfret & Knell, 2008; Chao *et al.*, 2012). Furthermore, the result that small-sized males travelled longer distances at the beginning of the season may indicate foraging marches after emerging to compensate for the insufficient larval food supply. In scarab beetles, the size of the individual is primarily determined by the amount of food provisioned during larval development (Hanski & Cambefort, 2014). It is possible that small-sized males, in lack of energy reserves, need to consume larger amount of food at the beginning of the season during maturation feeding to provide the energy needed for gamete production and to become sexually receptive (Rasmussen, 1994; Hunt *et al.*, 1999). As a result, they may spend more time crawling on the surface in search of fresh plant shoots. Overall, even though we showed a size- and morph-dependent movement behaviour in *L. apterus* males, which support our prediction of potential alternative mating tactics, additional research is needed to fully characterize the alternative tactics of this species.

In conclusion, our results confirm the observation that male is the mate-searching sex in *L. apterus* beetles. We also showed that the movement behaviour of the sexes change over time, and depend on the different phenophases and differences in reproductive behaviour between the sexes. Furthermore, the differences between the three male morph categories (large-, medium- and small-sized males) in travelled distances and movement patterns suggest that each male morph may use different mating and reproductive tactics.

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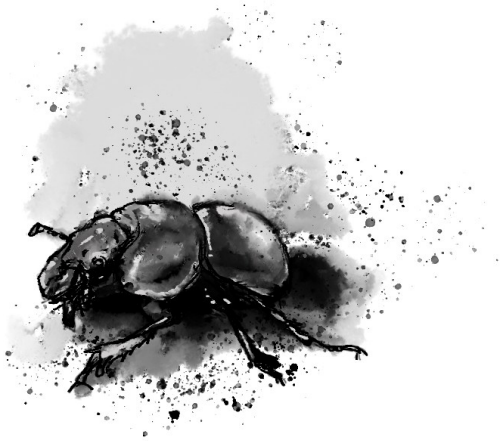
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Study 4

Seasonal changes in immune response and reproductive investment in a biparental beetle

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Seasonal changes in immune response and reproductive investment in a biparental beetle

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ABSTRACT

Immunity and reproduction are physiologically demanding processes, therefore trade-offs are expected between these life history traits. Furthermore, investments in these traits are also known to be affected by factors such as sex, body size, individual condition, seasonal changes and parasite infection. The relationship between immunity and reproduction and the effect of other factors on this relationship were investigated in many species, but there are a small number of studies on these patterns in biparental invertebrates. *Lethrus apterus* is an iteroparous biparental beetle with predominant female care in respect of collecting and processing food for larvae. Males guard the nest built underground and also their mate. Here we investigate how sex, body size, time within the reproductive season and parasite load may influence the relationship between immunocompetence and reproductive investment in this species. In beetles from a natural population we quantified immune response by measuring the encapsulation response, antimicrobial activity of hemolymph, the investment into reproductive tissues by measuring the size of testis follicles in males and total egg size in females, and parasite load by counting the number of mites on the beetles. We found that the encapsulation response is condition-dependent, as large individuals showed significantly higher encapsulation ability than small ones. Antimicrobial capacity was significantly higher in females than in males. In case of antimicrobial activity there was also a seasonal change in the relationship between immunity and reproductive investment, but only under heavy mite load. Reproductive investment was influenced by the interaction between body size and season (in females) and by body size and season (in males). Furthermore in females the interaction between antimicrobial activity and season indicated that reproductive investment increased with antimicrobial activity early in the reproductive season. By investigating the relationship between immunity and reproductive investment in a natural population of a biparental beetle species, we can conclude that investments into these important life history traits are governed by complex interactions between physiological and environmental factors. Our results are discussed in the context of life history evolution, highlighting the role of the assessed factors in shaping trade-offs themselves (in invertebrates).

1. Introduction

Animals in nature face complicated situations where they have to make decisions on how to allocate the usually limited resources between demanding functions of the organism. Two of the main contestants for the limited resources are reproduction and self-maintenance. A major component of the latter is immunity, especially in long lived animals (Flajnik and Du Pasquier, 2004; Buchmann, 2014). The resource allocation theory predicts a negative relationship (trade-off) between the investment into these two components (Rolff and Siva-Jothy, 2002), with an extent depending on the individual's condition (Zera and Harshman, 2001; Harshman and Zera, 2007; Simmons, 2012; Krams et al., 2015; Reavey et al., 2014).

Animals are expected to invest more in immunity when parasite burdens are high (Lindström et al., 2004). However, during the reproductive season, growing reproductive tissues, courtship and mating behaviour, and parental care can also pose high costs on individuals, and iteroparous animals usually opt to tolerate a parasite infection during the reproductive period rather than fighting against it (Kortet et al., 2003; reviewed in Martin et al., 2008). When the reproductive

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season ends, immune upregulation is frequently prioritized in order to increase the likelihood of survival until the next reproductive season (Huyghe et al., 2010). Additionally, the relationship between immunity and reproduction can differ among individuals as a result of differences in their condition (Ryder and Siva-Jothy, 2001; Rantala et al., 2003). For instance, it has been found that good individual condition, as indicated by large body or ornament size, release of high level of pheromones or loud and long acoustic signals may allow higher investment into both reproduction and self-maintenance (Barbosa et al., 2016; Córdoba-Aguilar et al., 2009; Jacot et al., 2004; Kelly and Jennions, 2009; Krams et al., 2011a,b; Rantala et al., 2003; Sadd et al., 2006; Steiger et al., 2012). Individuals of lower condition, on the other hand, often invest less into reproduction and also show lower immune competence (Simmons and Zuk, 1992; Rantala and Kortet, 2004). Furthermore, the association between immunocompetence and reproduction might also be shaped differently in the sexes, due to sexual differences in life history characteristics (Rolff, 2002; Roth et al., 2011), including hormone-driven sexual differences (Boonekamp et al., 2008), and sexual dimorphism (Hoffman et al., 2008; Vincent and Gwynne, 2014).

The production of gametes constrains effective reproductive success in females much more strongly than in males, and increased longevity generally helps females to increase their fitness by increasing the number of reproductive bouts. Enhanced investment into immunity would be expected to contribute to higher lifetime reproductive success by increasing survival, and hence the number of viable offspring, and/or by providing parental care sufficiently. On the other hand, males usually benefit from increasing mating rates, thus males' fitness can be maximalized by expenditure of immediately available resources on mating to fertilize as many eggs as possible (Rolff, 2002). Also, secondary sexual dimorphism may complicate the association between immunity and reproduction further in invertebrate species where males develop exaggerated traits, such as appendages that are used as weapons in male-male contests (Emlen, 2008). Investing into weaponry usually results in increased mating success, but at the same time is also expected to lead to less efficient immunity due to the energetic conflict in investing limited resources either to immunity or to weaponry. It also should be noted that differences in immunocompetence between the sexes are largely determined by differences in life history and resource allocation patterns and not by sex per se, which notion is supported by empirical research of Roth et al. (2011).

Details of this complex relationship between reproduction and immunity is relatively well understood in vertebrates (e.g. Lochmiller and Deerenberg, 2000; Rolff and Siva-Jothy, 2002; Lutton and Callard, 2006; Moore and Hopkins, 2009; O'neal and Ketterson, 2012), but much less is known about this association in invertebrates, including beetles. Apart from studies on burying beetles (*Nicrophorus sp.;* for example Steiger et al., 2011; 2012), other beetle species showing parental care seem to be understudied from this aspect, especially in natural populations.

Here we examined the relationship between immune response and reproductive investment over the reproductive season in males and females of *Lethrus apterus* (Coleoptera: Geotrupidae), an iteroparous beetle species with biparental care (Wilson, 1971; Clutton-Brock, 1991). Adult *L. apterus* beetles show a great variation in body size as their body length varies between 12 and 25 mm (Nikolaev, 2003), and can live up to several years, thus they may reproduce more than once during their lifetime (Nikolaev, 2003). After the adults have emerged from underground in spring, in early reproductive season males and females starts to form pairs, and then the pair digs a 50–90 cm deep vertical underground tunnel ending in six to eight brood chambers containing one egg each (Kosztolányi et al., 2015). During the reproductive season, between April and June (Emich, 1884), these chambers are filled up with plant materials which serve as the sole food source for the larvae during their development (Nikolaev, 2003; Kosztolányi et al., 2015; Rosa et al., 2017). Sexes show dimorphism: males are larger and only males have two mandibular processes ("tusks") that may serve as weapons in contests or as ornaments (Nikolaev, 2003; Rosa et al., 2017; Rosa et al., 2019). The sexes also differ in their role in parental care as females are predominantly responsible for collecting and preparing food for the larvae (Kosztolányi et al., 2015; Rosa et al., 2017), thereupon their presence is essential for the survival of the offspring, while males guard the nests against intruders (Rosa et al., 2018).

In this study, we investigated the associations between immunocompetence, reproductive investment, condition (body size), sex, season and parasite load. Immune response was estimated by measuring the level of encapsulation and the ability of bacterium growth inhibition of the hemolymph (BGI thereafter). Encapsulation involves both induced cellular and humoral immune responses (Nappi et al., 2004; Sideri et al., 2008; Rádai et al., 2018), while BGI ability reflects antimicrobial capacity (Nakatogawa et al., 2009; Shia et al., 2009; Castella et al., 2010; Rádai et al. 2019). As an indicator of reproductive investment, we measured the extent of reproductive tissues. Furthermore, as mite infection of different levels has often been observed in *L. apterus* (J.K.; pers. obs.), we also considered the effect of natural mite infection on immune response and reproductive investment.

We predicted that (i) if both immunity and reproductive investment are condition dependent, then individuals with larger body size and, thus presumably larger somatic stores, can allocate relatively more into these traits, compared to smaller ones, and therefore expecting no trade-off between these traits in large individuals. We also predicted (ii) a female-biased immunocompetence, since, compared to males, females may gain higher fitness benefits by investing in self-maintenance to provide food for their larvae. We presumed that (iii) there is a seasonal change in allocation for both immunity and reproductive investment: nearer to the end of the breeding season, the individuals may show higher-level of immunocompetence due to investing more to self-preservation to survive till the next reproductive season. Finally, (iv) in heavily mite infected individuals we expected to see a steeper negative slope of immunity-reproduction trade-off.

2. Methods

2.1. Collecting beetles

Beetles were collected near Debrecen (47°31′28.6″N 21°34′30.4″E), Hungary. The study site was a grass patch of 11,600 m^2 surrounded by industrial facilities. As *L. apterus* is a protected species in Hungary, and because of the invasive procedures of our study, instead of sampling beetles from the whole range of size distribution, we minimized the number of individuals used by collecting only the top and the bottom of the size distribution (Rosa et al., 2019). We measured the maximum pronotum width of the beetles with digital callipers (Workzone GT-DC-02) to the nearest 0.01 mm (as an indicator of body size) and the length of the right mandibular processus of the males. Based on their pronotum size, we differentiated between large (pronotum width $\geq$13 mm; 10 individuals in early and 9 in late season) and small (pronotum width $\leq$11 mm; 7 in early and 7 in late season) males and large (pronotum width $\geq$12 mm; 9 in early and 12 in late season) and small (pronotum width $\leq$11 mm; 9 in early and 8 in late season) females. The collection took place between 28th and 30th March (18 females and 17 males; hereafter referred to as 'early in the reproductive season') and between 2nd and 10th May (20 females and 16 males; hereafter referred to as 'late in the season'). In total 71 individuals were collected. After collection, individuals were kept separated in 50 mL tubes and transferred to the laboratory within 1 h where they were kept on 6 °C (Ahtiainen et al., 2005) till measuring encapsulation response.

2.2. Encapsulation response

To measure the encapsulation ability, we anaesthetized the beetles

4–6 h after collection by chilling them on ice for approximately 10 min, until they did not move actively (Xia et al., 2013). We wiped off the abdomen with cotton swabs sodden with 70% (v/v) ethanol. The individuals were pierced between the 9th and 10th sternits with a hypodermic needle (Medicor Neomed, 21G 1 1/4″ 0.8 × 30 mm) under a magnifier, and a 3 mm long, sterilized nylon monofilament (Carp Zoom Picker High Quality Picker Line, 0.14 mm; rubbed with sandpaper) were inserted into the hemocoel of the beetles. To ensure accurate and consistent size, the monofilaments were cut and knotted at one end under a dissecting microscope. Hemolymph loss during implantation was minimal. Following the implantation, the beetles were kept alive individually in 50 mL Falcon-tubes containing wet filter-paper. The tubes were placed in plastic containers filled with moist soil to avoid desiccation and were kept in dark on room-temperature. After 12 h of inserting the implants, we anaesthetized the individuals again and removed the monofilament in a non-destructive manner by grabbing it by the knot. The removed monofilaments were placed in Eppendorf-tubes, filled with 70% (v/v) ethanol and stored in −20 °C. Later each implant was photographed under a stereomicroscope (Euromex StereoBlue SB.1903) from two different angles using a DC.5000C - Euromex CMEX-5 USB camera with the ImageFocus4 analysing software (version 2.8). As the implants were bended at the knot, they could be placed in two opposite directions on the object disk, and thus the turning angle between the two photographs was ca. 180°. We repeated the photography of the implants, so on each filament four photos were taken. The images were analyzed using the imageJ software (version 1.50d; Rueden et al., 2016). To estimate encapsulation ability we measured the average grey values obtained over the part of the monofilament which was submerged into the abdomen of the beetle and over an area of similar size of the background (Euromex Black-and-white object disk, 94 mm) and then subtracted the two values. Repeatability estimates were calculated using the rpt function from the R package rptR (version 0.9.21; Stoffel et al., 2017). Encapsulation measurements were highly repeatable through measurements ($R = 0.977$, $P < 0.001$). Therefore, we used the mean value of the four measurements per implant as an estimate of an individual's encapsulation ability in the analyses.

2.3. Hemolymph sampling for bacterial growth inhibition (BGI) assay

At the time we removed the monofilament from beetle's abdomen, we took 10 μL hemolymph with glass capillary tubes from each anaesthetized beetle (Hirschmann Laborgeräte, sodium-heparinized minicaps, 100 μL) by cutting off the front right tibia and pressing gently the beetle's abdomen (Grégoire and Goffinet, 1975). The samples were added to 40 μL ice-cold phosphate-buffered saline (PBS 10x, pH = 7.4) and were frozen at −70 °C until analysis. After the hemolymph was sampled, the beetles were kept anaesthetized on 6 °C till dissection (see below).

To measure BGI we applied the method described in Castella et al. (2010). BGI of hemolymph was investigated using inhibition zone assay under a sterile, ventilated hood. For this assay we prepared agar plates in petri dishes from 10 mL of 1% agar solution (1 g bacto-tryptone, 1 g NaCl, 0.5 g yeast extract and 1 g agar in 100 mL distilled water) with approximately 10^6 cells mL^{-1} of *Micrococcus luteus* always from the same culture line. On each plate, 2–2 μL of twice-diluted hemolymph samples from 5 individuals were applied on randomized places within each dish creating 10 inhibition zones. Samples were spaced evenly from each other. This procedure was repeated two more times resulting 6 inhibition zones in 3 plates per individual. The plates were incubated on 30 °C for 24 h, then photographed using a cellphone with high-definition camera (Sony Xperia X Compact, 23 MP, f/2.0, 24 mm, 1/2.3″, PDAF). On these photos the area of the clear zones indicates the ability of hemolymph to inhibit the bacterial growth; the larger the zones are, the larger the inhibition ability is. We measured the area of these inhibition zones and the diameter of Petri-dishes with the ImageJ software (Rueden et al. 2016). We divided the inhibition zone sizes by dish size to control for potential deviation coming from slight variation in photographing distances. We calculated the repeatability of inhibition zone size measurements using the rpt function where Petri-dish ID and individual ID were used as random factors: Petri-dish ID was only slightly repeatable ($R = 0.022$, $P < 0.001$), but the individual ID was highly repeatable ($R = 0.922$, $P < 0.001$). That is Petri-dishes were more or less indistinguishable from each other because the variance between Petri-dishes was small. On the other hand, the inhibition zones from the same individuals were highly similar. During the analyses we used the mean BGI values calculated from sizes of 6 inhibition zones (2 zones per 3 Petri-dishes) per individuals.

2.4. Estimating reproductive investment

The reproductive investment of the beetles were measured by the extent of reproductive tissues: the total egg size produced by females and total follicle (i.e. testes) size of males. The abdomen of the individuals was detached 6 h after the hemolymph sampling, and cut open by the sternal region using microscissors under an Euromex StereoBlue SB.1903 stereomicroscope. In females, all eggs were removed from the ovarioles in Ringer solution (NaCl 7.5 g, KCl 0.35 g, $CaCl_2$ 0.2 g; Ephrussi and Beadle, 1936), whereas in males the whole testicles were removed and resolved into follicles. The eggs and the follicles were photographed with the same microscope and camera as the monofilament implants (see above). The total area of eggs and follicles were measured using ImageJ software (Rueden et al., 2016). Pixel units were converted to mm^2 by determining pixel size using images of millimetre paper that were taken with the same settings. For testing repeatability of measurements, we randomly choose 10 females and 10 males and remeasured their eggs and follicles once more. The measuring of the area of eggs ($R > 0.999$, $P < 0.001$) and follicles ($R > 0.999$, $P < 0.001$) were highly repeatable.

2.5. Measuring mite infection

The level of mite infection was determined by counting the number of mites on the abdomen under a stereomicroscope after removing the elytra.

2.6. Statistical analyses

Statistical analyses were performed using R (version 3.5.1, R Core Team 2018). Body size was used as a two-level factor (small and large, see above) in the analyses. In order to remove the strong correlation between reproductive investment and body size, and also to make reproductive investment of the sexes measured on different scales comparable, we calculated the within-size normalised reproductive investment by normalising total egg size within females and total follicle size within males (i.e. for each sex we subtracted the within-size-category mean value from the individual measurements and divided by the within-size standard deviation), and used as an independent variable in our models explaining immunocompetence. Hereafter we refer to this explanatory variable as normalised reproductive investment. Sex and time of the season were also two-level factors (female and male; early and late). We also normalised the logarithm of mite numbers plus one over the whole sample to handle its skewed distribution.

To investigate encapsulation and BGI abilities of individuals, we used two linear model sets (one for encapsulation and one for BGI ability) with the following explanatory variables: (i) body size, (ii) sex, (iii) season, (iv) the normalised mite numbers, and (v) normalised reproductive investment and all two- and three-way interactions between the explanatory variables. We also investigated how the absolute reproductive investment within males (total follicle size) and within females (total egg size) was influenced by (i) body size, (ii) season, (iii) number of mites, (iv) normalised BGI values, (v) normalised encapsulation values and their all possible two-way interactions by using

linear models. BGI and encapsulation values were normalised over the whole sample. Low sample sizes prevented us to test any higher level interaction in these models. Note that in the latter analyses we used the total egg and follicle sizes, and not the normalised values as dependent variables. As body size and size of reproductive tissues are usually positively correlated, to investigate relative reproductive investment of the individuals, we calculated relative total egg size in females and relative total follicle size in males by dividing the measurements by pronotum width and repeating the analyses by using them as dependent variables.

For selecting the best models explaining the variance in our response variables, model reductions were accomplished by variable elimination based on p-values ('drop1' function in R). Non-significant interactions were removed before testing main effects, however, because we expected a priory that main effects may have an effect, they were retained in the final models independently of their significance. Significance of variables in the final model was tested using the 'drop1' function. We used the 'emmeans', 'pairs' and 'emtrends' functions of the emmeans package (1.3.0 version, Lenth et al., 2019) to extract estimates for the marginal mean (EMM) effects, and their corresponding standard error (SE), test statistic, and p-value.

3. Results

3.1. 1. Encapsulation

In the fitted model only body size had a significant effect ($F_{1,65}$ = 5.333, P = 0.024, all P ≥ 0.184 for sex, season, normalised mite numbers and normalised reproductive investment): large individuals had significantly higher encapsulation ability compared to small ones (Fig. 1).

3.2. Bacterial growth inhibition

We found that females had significantly higher BGI values than males ($F_{1,61}$ = 5.299, P = 0.024, Fig. 2). The three-way interaction between season, mite load and the normalised reproductive investment was also significant ($F_{1,61}$ = 9.087, P = 0.004): BGI values were affected by normalised reproductive investment when beetles were heavily infected with mites (Fig. 3, Table 1), and its direction changed with season, in early reproductive season the relationship between normalised reproductive investment and BGI was positive, whereas it was negative late in the season. Unlike in case of encapsulation, body size had only a marginal effect on BGI ($F_{1,61}$ = 3.650, P = 0.061).

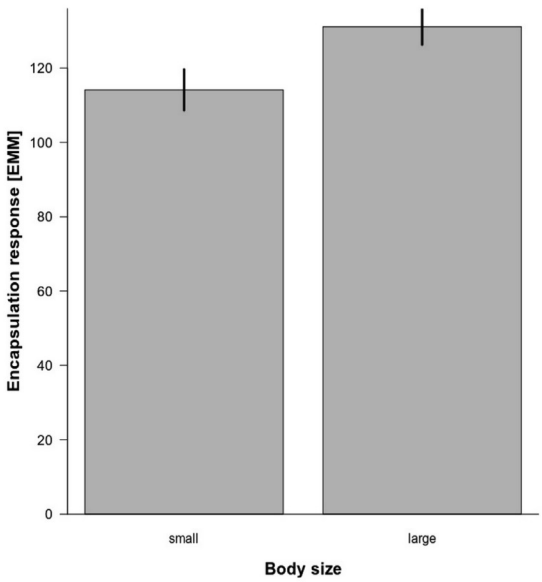


Fig. 1. Relationship between body size and encapsulation response in *L. apterus* beetles. Estimated marginal means (EMMs) of encapsulation ability are plotted. The error bars represent the SE of EMMs.

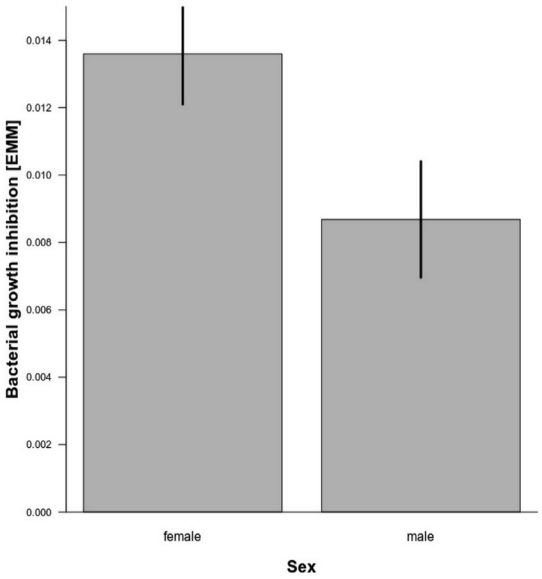


Fig. 2. Differences between females and males in BGI capacity of hemolymph in *L. apterus*. Estimated marginal means (EMMs) of BGI values are plotted. The error bars represent the SE of EMM's.

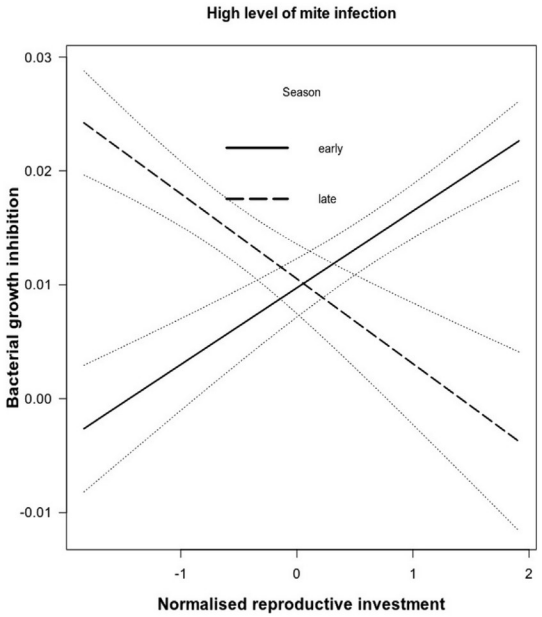


Fig. 3. Relationship between normalised reproductive investment and BGI capacity of hemolymph - predicted by fitted linear model - in early (solid line) and late (dashed line) of reproductive season in *L. aperus* beetles having high level of mite infection (estimated at the 75% percentile of mite infection). Dotted lines indicate SE from fitted linear model.

Table 1
Result of post-hoc comparison for analyzing the interaction between season, mite load and normalised reproductive investment that had a significant effect on BGI ability of *L. apterus* beetles. We calculated the estimated marginal slopes of normalised reproductive investment for the levels of season (early and late) and for the 25%, 50% and 75% percentiles (low, medium, high, respectively) of the normalised logarithm of mite numbers. Results were averaged over the level of sex.

Season	Normalised logaritm of mite numbers	Slope of normalised reproductive investment	SE	Df	t ratio	P value
Early	Low	−0.003	0.004	61	0.771	0.444
Late	Low	0.000	0.002	61	0.069	0.946
Early	Medium	0.003	0.002	61	1.491	0.141
Late	Medium	−0.004	0.002	61	2.212	0.031
Early	High	0.007	0.002	61	3.234	0.002
Late	High	−0.007	0.003	61	2.488	0.016

3.3. Reproductive investment

The interaction between body size and season was significant in the model on total egg size ($F_{1,27}$ = 5.016, P = 0.034): large females produced significantly larger total egg size than small ones in early (post hoc comparison: b = -28.96, t_{27} = 5.127, P < 0.001, Fig. 4) but not in late (post-hoc comparison: b = -11.08, t_{27} = 2.319, P = 0.119) season, and among large females, but not among small females, total egg size decreased with the progression in the season (post-hoc comparison, large females: b = 16.81, t_{27} = 3.460, P = 0.009; small females: b = -1.08, t_{27} = 0.190, P = 0.998; Fig. 5). Furthermore, the interaction between season and BGI ability was also significant ($F_{1,27}$ = 7.333, P = 0.012): total egg size increased with BGI capacity early in the reproductive season, whereas it was not affected by BGI late in the season (post-hoc comparison; early in the season: b = 6.67, t_{27} = 2.403, P = 0.023; late in the season: b = -3.00, t_{27} = 1.358, P = 0.186, Fig. 5). Neither encapsulation ability nor mite load had significant effect on female reproductive investment (encapsulation: $F_{1,27}$ = 1.372, P = 0.252, mite load: $F_{1,27}$ = 0.032, P = 0.860). For relative female reproductive investment, the same variables were retained in the final models as in case of absolute female reproductive investment, and the results were qualitatively the same (details are in Table 2).

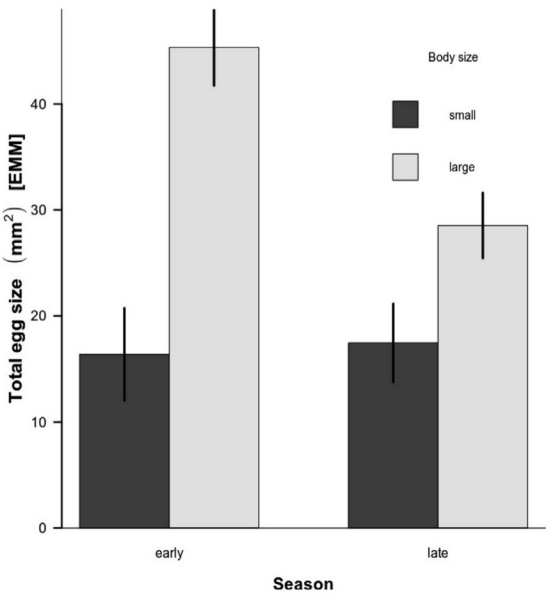


Fig. 4. Relationship between body size and total egg size in early and late of the reproductive season in *L. apterus* females. Estimated marginal means (EMMs) on females' total egg size are plotted. The error bars represent the SE of EMMs.

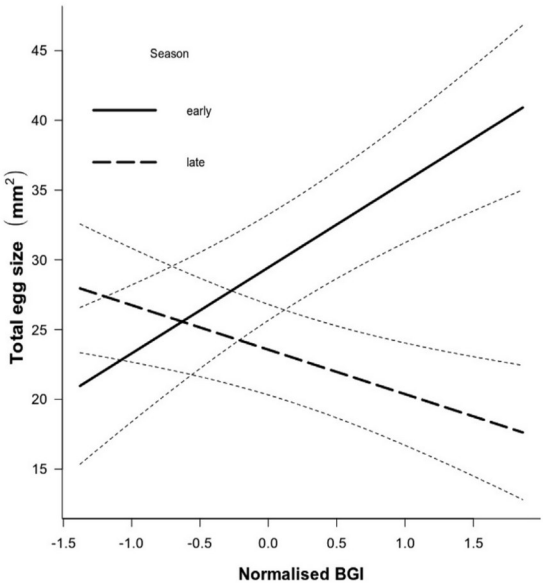


Fig. 5. Relationship between total egg size and normalised values of BGI ability - predicted by fitted linear model - in early (solid line) and late (dashed line) reproductive season of *L. aperus* beetles. Dotted lines indicate SE from fitted linear model.

Table 2
Results of post-hoc comparisons for analyzing the interactions between body size and season and between season and normalised BGI that had a significant effect on relative reproductive investment of females in *L. apterus*. (a) We calculated the estimated marginal means (EMMs) of body size (small and large) for the levels of season (early and late) and compare the EMMs with one another. (b) We calculated the estimated marginal slopes of normalised BGI for the levels of season (early and late).

(a)

Comparison	Contrast (EMMs)	SE	Df	t ratio	P value
Small, early – Large, early	−2.158	0.485	27	4.452	< 0.001
Small, early – Small, late	−0.124	0.488	27	0.254	0.994
Large, early – Large, late	1.449	0.417	27	3.479	0.009
Small, late – Large, late	−0.584	0.410	27	1.426	0.495

(b)

Season	**Slope of normalised BGI**	**SE**	**Df**	**t ratio**	**P value**
Early	0.604	0.238	27	2.538	0.017
Late	−0.273	0.190	27	1.441	0.161

Total follicle size was influenced by body size and season. Bigger males had significantly larger total follicle size than smaller ones ($F_{1,27}$ = 53.164, P < 0.001, Fig. 6a). The total follicle size was larger at the beginning of the reproductive season ($F_{1,27}$ = 28.367, P < 0.001, Fig. 6b). Mite load only had a marginal effect on total follicle size ($F_{1,27}$ = 4.103, P = 0.053, Fig. 7). Neither encapsulation ability nor BGI ability had significant effect on male reproductive investment (encapsulation: $F_{1,27}$ = 0.018, P = 0.893, BGI: $F_{1,27}$ = 0.259, P = 0.614). Similarly, relative reproductive investment was influenced by body size (larger males have larger relative testis size;

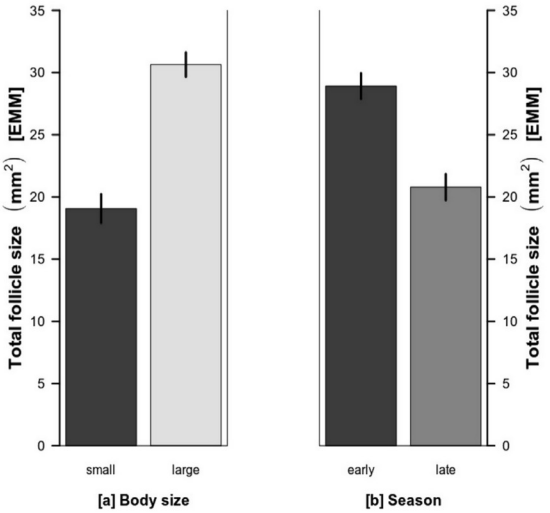


Fig. 6. Relationship between absolute total follicle size as an indicator of male reproductive investment and (a) body size and (b) season in *L. apterus* males. Estimated marginal means (EMMs) of males' total follicle size are plotted. The error bars represent the SE of EMMs.

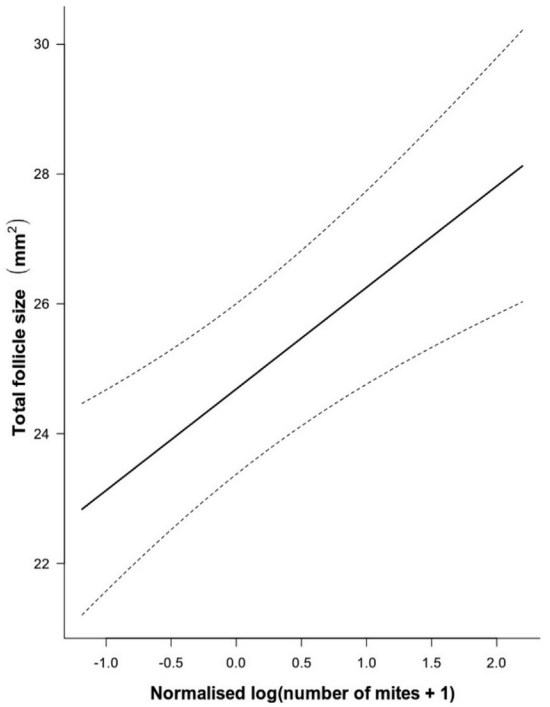


Fig. 7. Relationship between total follicle size and normalised logarithm of mite numbers as predicted by fitted model (solid line) in *L. apterus* beetles. Dashed lines indicate SE from fitted linear model.

$F_{1,27} = 27.899$, $P < 0.001$) and season (relative testis size was smaller late in the season; $F_{1,27} = 42.714$, $P < 0.001$), and mite load did not have a significant effect ($F_{1,27} = 3.420$, $P = 0.075$).

4. Discussion

Our results suggest a complex relationship between immunity and reproduction in *Lethrus apterus* beetles. We found that encapsulation ability depends on body size, an indicator of the capacity of somatic stores and condition. We did not find any difference between the sexes in regards to encapsulation response, but females showed higher level of bacterium growth inhibition (BGI) capacity than males. We also found a seasonal change in allocation into both immunity and reproductive investment, but this was only apparent under heavy mite load, as in early season normalised reproductive investment and BGI ability showed a positive relationship, but late in the season this association turned to negative. In case of reproductive investment, we found that in both males and females there was a positive relationship between reproductive investment and body size and seasonal change was also found in investment to reproduction. Furthermore, in females, there was a positive relationship between BGI capacity and reproductive investment in early season, but not in late season.

These results might suggest that both encapsulation ability and reproductive investment in *L. apterus* are condition dependent, as larger individuals had larger total follicle or egg size and also showed higher encapsulation ability compared to smaller ones. Large body size may hold larger nutrient storage and indicate better condition (Berger et al., 2008; Beukeboom, 2018), as large / good quality individuals might be better at gathering and storing resources, enabling uncompromised investment into both reproduction and immunity (Blanckenhorn, 2000; Hendry et al., 2001; Roff, 2002). Interestingly, though, we observed no difference between small and large individuals in BGI capacity. Notably, encapsulation takes place after immune activation, comprising cellular and humoral processes as well, and the extent of melanized cell deposition on the 'parasite' (simulated by the nylon monofilament in our study) is generally interpreted as the individual's capacity to mount an efficient immune response (Moret and Schmid-Hempel, 2001; Schmid-Hempel, 2003; Sadd and Schmid-Hempel, 2008). On the other hand, BGI ability, an indicator of antimicrobial capacity, is realised in larger part by the antimicrobial peptides (AMP's) and lyzozymes available in hemolymph, hence informs us about the momentarily available antimicrobial capacity to break down cell walls which is hypothesised to bear maintenance costs. Considering these mechanistic differences between the two assessed immune measures, it seems likely that mounting an immune response of substantial deployment costs (i.e. encapsulation) is more firmly dependent on individual condition (i.e. on size in our case, Rantala et al., 2003; Rantala et al., 2004; Rádai et al., 2018) than the maintenance of deployable AMP's and lyzozymes.

In accordance with our prediction, we found that antimicrobial capacity is female-biased. Differences between sexes in size, behaviour, physiology and overall investment into offspring might contribute to differences in immune capacity (Córdoba-Aguilar et al., 2009b; Cotter and Kilner, 2010; Aisenberg and Peretti, 2011; Vincent and Gwynne, 2014). Roth et al. (2011) found that life history and resource allocation differences between females and males constrain considerable differences in immunocompetence of sexes, and the sex which invests more into the offspring, shows more active and specific immune defence, possibly to avoid infections that would substantially reduce reproductive success (Adamo, 1999; Kurtz et al., 2000; Adamo et al., 2001). Studies on beetles with parental care (such as burying beetles) also seem to support this hypothesis (Jenkins et al., 2000; Smiseth et al. 2005; Boncoraglio and Kilner, 2012). In *L. apterus* it is the female who invests more in providing food for the larvae, thus the survival and development of the larvae chiefly depend on maternal care and maternal survival. It is also worth noting that females collect leaves and lay eggs underground in the nests, while males spend much of the time at the entrance of the burrows (Kosztolányi et al., 2015; Rosa et al., 2017), i.e. sexes are likely exposed to different types and abundances of microbes (Cotter and Kilner, 2010; Jacobs et al. 2016). Furthermore, it is reasonable to assume that females should invest more into antimicrobial capacity. The nest might be rich in pathogens as it is a moist place underground in the soil. As eggs are developing underground, we would expect that females may transport antimicrobial agents into the eggs (Zanchi et al., 2012) to establish their protection. If such *trans-*

generational immune priming exists, then females should invest more into antimicrobial capacity to warrant the proper amount of antimicrobial agents for transportation into the eggs (Zanchi et al., 2012). Furthermore, immune challenged females enhance immunity of their offspring by increasing the amount of antimicrobial components transported into their eggs (Moret, 2006) that is hypothesized to be associated with induced production of antimicrobial molecules by females. In lack of information about juvenile hormone (JH) production in sexes of *L. apterus*, it is pure speculation that lower antimicrobial capacity of males is a consequence of male-biased JH production that suppresses humoral immunity (Rolff and Siva-Jothy, 2002; Contreras-Garduño et al., 2009). However, this could also explain our result that males with large follicles might be susceptible to mite infection, since JH promotes the development of follicles (Soller et al., 1999), but suppresses defence mechanisms against mites and against pathogens transmitted by mites as well (González-Tokman et al., 2011; Ilvonen and Suhonen, 2016). Also, large males with larger testes might be more successful in mate choice and mating (Rosa et al., 2018), so they might be more exposed to mite infection due to increased number of contact with other males and females (i.e. due to fights and searching for females; Knell and Webberley, 2004).

The amount of reproductive tissues decreased over the season in both sexes, although in females this decrease was only observed among large individuals. This finding can be explained by the preparation for the long diapause period spent underground. For small females, there was no difference between early and late egg-production. Large females with good condition may be able to store more mature eggs because of their greater abdomen volume and / or by carrying more resources they may allow to mature eggs at faster rates (see Berger et al., 2008) and at the right time start to prepeare for diapausing. Whereas, small females with less resources and smaller abdomen volume may attempt to use a kind of 'best of bad job' tactic by investing the same amount of energy into reproduction in late season as well, and thus taking a risk of decreasing survival over the winter by not preparing sufficiently for diapausing, which is not unexpected as their chance of survival is probably lower anyway (Renault et al., 2003; Renault, 2011).

We also found that the association between reproductive investment and antimicrobial capacity in *L. apterus* changed over the reproductive season. However, this was apparent only in individuals heavily infected by mites. Under this condition, there was a strong positive relationship between induced antimicrobial capacity and reproductive investment in early season, and a negative relationship late in the season. This suggests that individuals with high level of mite infection are able to manage investment into both induced immunity and reproduction at the same time in early reproductive season. Meanwhile, in late of the season, beetles with high antimicrobial capacity showed significantly lower reproductive investment. For managing persistent or additional infections as in case of heavy mite infection, long-lasting antimicrobial activity has been evolved for protection (Haine et al., 2008), so that immune response is initially higher in heavily infected individuals. Furthermore, parasites that have relatively slower growth or their reproduction requires more resources may induce later decrease in reproductive effort of the hosts, which can be accompanied by increased reproductive effort in early reproductive season (Hurd, 2009). Anyway, individuals with long-term infections may acquire adaptive benefits by inducing long-lasting antimicrobial activity (Jacot et al., 2005; Kaunisto and Suhonen, 2012) and increasing reproductive output by altering the timing of reproduction (Hurd, 2009; Vézilier et al., 2015).

However, as we used field-collected individuals for our study, we had therefore no information about previous pathogen exposures or latent infections of the individuals, those potential confounding effects on immunity and on other traits that were not controlled for, moreover, any other biotic and abiotic ecological factors, that the individuals could have been exposed to before collection, were unknown. As individuals were collected from a quite isolated population (see in Methods), we can expect that previous challenges affected uniformly the sampled beetles.

Except small females there was a decline in size of reproductive tissues in both sexes over the reproductive season, suggesting that late season individuals usually invest less into reproduction probably as part of their preparation for the long diapausa until the next spring. Individuals heavily parasitized by mites may gain from an increased investment into reproduction in early season as their heavy mite infection might seriously lessen their chance to survive the winter. Late in the season, however, we found that heavily infected individuals invested less in reproduction which finding might be explained by that heavily infected individuals finish reproduction earlier than the less infected ones and hence their reproductive organs were more regressed at the time of sampling.

Our study showed that relationship between immune and reproductive traits can be shaped by several factors, nevertheless it is advised to investigate the effect of interactions between those factors. As we still know little about complex host-parasite interactions, especially in beetles with biparental care, it will be of major interest to study how other life history traits can be affected and how relationships between those traits can be shaped by induced immunity detectable under long-term parasitism. Furthermore, as the hormonal background of parental care in beetles is still unknown and to get a clearer picture about reproduction-immunity relationship during caregiving, there is a need for research, in particular, into exploration of the relationship between reproductive effort and immunity shaped by hormonal compounds related to caregiving behaviour.

CRediT authorship contribution statement

Johanna Kiss: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Zoltán Rádai:** Methodology, Validation, Resources, Investigation, Writing - review & editing. **Márta Erzsébet Rosa:** Validation, Investigation, Resources, Writing - review & editing. **András Kosztolányi:** Formal analysis, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Zoltán Barta:** Conceptualization, Formal analysis, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2019.104000.

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MTMT ID: 10057375

List of publications related to the dissertation

Foreign language scientific articles in international journals (3)

1. **Kiss, J.**, Rosa, M. E., Rácz, R., Kosztolányi, A., Barta, Z.: Behavioural repertoire and the effect of male removal in a geotrupid beetle with parental care.
   J. Zool. [Epub ahead of print], 1-12, 2023. ISSN: 0952-8369.
   DOI: http://dx.doi.org/10.1111/jzo.13069
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2. **Kiss, J.**, Németh, Z., Kosztolányi, A., Barta, Z.: Differential movement and activity patterns of sexes in a biparental beetle during the reproductive season.
   Ecol. Entomol. 45 (6), 1504-1508, 2020. ISSN: 0307-6946.
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3. **Kiss, J.**, Rádai, Z., Rosa, M. E., Kosztolányi, A., Barta, Z.: Seasonal changes in immune response and reproductive investment in a biparental beetle.
   J. Insect Physiol. 121 (1), 1-9, 2020. ISSN: 0022-1910.
   DOI: http://dx.doi.org/10.1016/j.jinsphys.2019.104000
   IF: 2.354

List of other publications

Foreign language scientific articles in international journals (5)

4. Rádai, Z., **Kiss, J.**, Nagy, N. A., Somogyi, A. Á., Fülöp, A., Tóth, Z., Alexáné Babits, M., Németh, Z.: State and physiology behind personality in arthropods: a review.
   Behav. Ecol. Sociobiol. 76, 1-20, 2022. ISSN: 0340-5443.
   DOI: http://dx.doi.org/10.1007/s00265-022-03259-6
   IF: 2.944 (2021)

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5. Nagy, N. A., Németh, Z., Juhász, E., Póliska, S., Rácz, R., **Kiss, J.**, Kosztolányi, A., Barta, Z.: Inotocin, a potential modulator of reproductive behaviours in a biparental beetle, Lethrus apterus.
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   Sci. Rep. 11, 1-10, 2021. EISSN: 2045-2322.
   DOI: http://dx.doi.org/10.1038/s41598-021-97415-z
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7. Rádai, Z., **Kiss, J.**, Babczyńska, A., Kardos, G., Báthori, F., Samu, F., Barta, Z.: Consequences of rapid development owing to cohort splitting: just how costly is it to hurry?
   J. Exp. Biol. 223 (6), 1-15, 2020. ISSN: 0022-0949.
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8. Rosa, M. E., **Kiss, J.**, Barta, Z., Kosztolányi, A.: Size-dependent investment in tusk length, testis size and sperm length in a biparental geotrupid beetle.
   J. Zool. 309 (2), 106-113, 2019. ISSN: 0952-8369.
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Total IF of journals (all publications): 22,797
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