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Evolution of sociality and parental care in bees of the genus *Ceratina*

Evoluce sociality a rodičovské péče u včel rodu *Ceratina*

Dissertation

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Prohlášení: Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Mikát

Mgr. Michael Mikát

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Abstract

Small carpenter bees (genus *Ceratina*) are an excellent model taxon for the study of evolution of parental care and origin of eusociality. Prolonged offspring care is typical for this bee genus. Females usually guard their offspring until adulthood and later feed their adult offspring pollen and nectar. Moreover, most of studied species are facultatively eusocial, a trait probably inherited from the common ancestor of the genus.

Although *Ceratina* bees have generally very interesting behavior, detailed studies were performed in only a few species, usually from North America, Japan and Australia. Only anecdotal observations of natural history existed for a few European species, and detailed research has not been performed before my thesis.

The goal of my thesis is to explore the natural history of European species of *Ceratina* and to identify possible costs and benefits of this species' behavioral traits. I focused on following these behavioral traits: social nesting, guarding of offspring until adulthood, and feeding of mature offspring. Through my master project, I discovered biparental care in species *C. nigrolabiata*, therefore the most important goal of my Ph.D. project is the evaluation of costs and benefits of this behavior.

Guarding of offspring by mother significantly influences their survival, because it serves as an effective protection against natural enemies. It was supposed that mothers guard their offspring until adulthood in all *Ceratina* bees. However, we found three species, in which guarding was only a facultative strategy. The mother can continuously guard her offspring, or close and abandon the nest. Guarded nests have a higher number of provisioned brood cells and usually also higher offspring survival rate. A probable benefit of nest abandonment is the possibility of another nesting elsewhere and therefore production of higher number of offspring.

Mothers of *Ceratina* bees usually feed their adult offspring. In North American species *C. calcarata*, dwarf eldest daughters feed their siblings in case of mother's death. Feeding of mature offspring by mother occurs in three studied European species. However, no individual feeds them in case of mother's death.

Eusocial nesting was newly documented in six species. Therefore, eusociality is common under temperate as well as subtropical climates in *Ceratina* bees. Nests with six females were documented in *C. parvula*, which is the highest number of females in one nest ever documented in *Ceratina* bees. Strange eusociality occurs in *C. chalybea*. Eusocial nests contain one old female, up to nine young adults, and new brood cells. These young adults are usually males and a half of them are unrelated to the old female.

Biparental care was not previously known in bees. *Ceratina nigrolabiata* is the first bee species in which biparental care was discovered. A male and female pair was found in almost all nests with provisioned brood cells, but the pairs are not permanent and exchanging of males occurs. The currently present male is usually not related to the offspring, who are fathered by previous partners of the female. However, long term pair stability has benefits for males and also females. If a pair is stable, nest productivity increases and so does the probability of the present male's paternity. We suppose that female mating multiple times paradoxically allows establishment of biparental care. As the female is receptive through the whole nesting season, long-term male survival is selected, therefore the probability of male care development is also increasing.

Keywords: eusociality, parental care, biparental care, parental investments, relatedness *Ceratina,* small carpenter bees, Xylocopinae

Abtrakt

Včely rodu *Ceratina* jsou výbornou modelovou skupinou pro studium evoluce rodičovského chování a eusociality. Je pro ně typická prodloužená rodiřovká péče – matka obvykle hlídá potomstvo do dospělosti a následně ho krmí pylem a nektarem. Většina druhů je fakultativně eusociální a fakultativně eusociální byl pravděpodobně i společný předek celého rodu.
Ačkoliv mají včely rodu *Ceratina* velmi zajímavé chování, podrobné studie byly provedeny jenom na několika druzích. Tyto druhy byly obvykle ze severní Ameriky, Japonska či Austrálie. Od evropských druhů existují jenom ojedinělá pozorování. Podrobný průzkum chování evropských druhů nebyl před mojí disertací proveden.
Cílem práce je prozkoumání prvků chování evropských druhů rodu *Ceratina*. Dále je cílem identifikovat případné zisky a ztráty prvků chování. Zaměřil jsem se na následující vlastnosti: sociální hnízdění, hlídání potomstva do dospělosti a krmení dospělého potomstva. Během mého magisterského studia jsem objevil obourodičovskou péči u druhu *C. nigrolabiata*. Tudíž nejdůležitější cíl mého Ph.D. projektu bylo ohodnocení zisků a ztrát obourodčovského chování.
Hlídání potomstva do dospělosti výrazně zvyšuje jeho přežívání. Důvodem je efektivní obrana proti přirozeným nepřátelům. Dříve se předpokládalo, že matka hlídá potomstvo do dospělosti u všech druhů včel rodu *Ceratina*. Ovšem naše výzkumy ukázaly, že u tří druhů se hlídá matka potomstvo jen u části hnízd. U těchto druhů může matka po dozásobování hnízda buď hlídat potomstvo, nebo hnízdo uzavřít a opustit. Hlídaná hnízda mají vyšší počet zásobovaných komůrek, než hnízda zátkovaná. Pravděpodobnou výhodou opouštění hnízda je možnost dalšího hnízdění, a tedy produkce větší počtu potomků.
Matka včel rodu *Ceratina* obvykle krmí své dospělé potomstvo. U severoamerického druhu *C. calcarata* v případě smrti matky krmí potomstvo trpasličí dcera. Krmení potomstva matkou se vyskytuje u všech tří studovaných evropských druhů, ovšem v případě smrti matky není potomstvo nijak krmeno.
Eusociální hnízdění bylo nově dokumentováno u šesti druhů. Eusocialita u včel rodu *Ceratina* je tudíž hojná i u druhů, které se vyskytují v mírném či subtropickém klimatu. U druhu *C. parvula* bylo nalezeno až šest samic v hnízdě, což je dosud nejvyšší zaznamenaný počet u včel rou *Ceratina*. Velmi neobvyklá eusocialita se vyskytuje u druhu *C. chalybea*. Eusociální hnízda obsahují jednu starou samici, až devět mladých dospělců a nově zazásobované komůrky. Mladí dospělci jsou obvykle samci a asi polovina z nich je nepříbuzná staré samici.
Obourodičovská péče nebyla u včel dosud známa. *Ceratina nigrolabiata* je první druh včely, u které byla obourodičovská péče zaznamenána. Samec a samice se nacházeli společně v téměř všech aktuálně zásobovaných hnízdech. Páry ovšem nejsou stabilní a samci se v průběhu sezony střídají. V důsledku střídání samců samec není obvykle otcem potomstva, protože většina potomků pochází od předchozích partnerů samice. Pokud ovšem samec vydrží v hnízdě dlouho, zvyšuje se míra jeho paternity i celkové množství potomků v hnízdě. Předpokládáme, že vícenásobné paření samice překvapivě podpořilo vznik obourodičovské péče, neboť samice jsou receptivní po celou hnízdní sezonu. Prodloužená receptivita samice selektuje na dlouhověkost samců, a tudíž umožňuje, aby se podíleli na péči.

Klíčová slova: eusocialita, rodičovská péče, obourodičovská péče, rodičovské investice, příbuznost, *Ceratina*, kyjorožky, Xylocopinae

CONTENT

INTRODUCTION

The most fascinating feature of life is its diversity. Organisms differ overwhelmingly in their appearance -- the immense differences in lengths of various organisms spanning multiple orders of magnitude are an excellent example. As Darwin formulated, life is a harsh struggle for survival and reproduction, and only the most successful individuals survive and transmit their genes to new generations. Therefore, it is surprising that high diversity exists. It is clear that one universal way to success cannot exist, because the evolutionary success is conditionally dependent. Some features are useful in one environment, others are successful in another. Moreover, profitability of features can be dependent on their frequency--sometimes a rare strategy is more successful than the common one (Ayala and Campbell, 1974; Gross, 1996). This can help maintain a diversity of strategies even in a homogenous environment.

The most apparent feature of organisms is their morphology. In recent years, a boom of research of genetic features of organisms is also in progress. But probably the most fascinating feature of organisms is their behavior. It is uneasy to study though as it is inherently fluctuating in time and highly variable between individuals. Moreover, behavior is complicated to measure and quantify, and therefore it’s difficult to obtain good data for scientific analyses. In addition, the behavior of organisms can be strongly influenced by the observing researcher. Therefore avoiding experimental artifacts is not easy.

Organisms evolved specific behavior for different purposes: defense, dispersion, foraging for food, and reproduction. However, the most complex behavior is usually present when multiple individuals of the same species interact. It occurs mainly in two situations: mating, where sexual partners interact, and parental care, where a parent interacts with offspring (Alonzo, 2010; Reynolds, 1996; Wong et al., 2013). In addition, some lineages establish societies, where multiple adults care for offspring and have to interact not only with offspring, but also with each other (Wilson, 1971). Repeating interactions between the same individuals select for complex behavior and larger cognitive abilities (Jolly, 1966; Trumbo, 2012). Therefore, sociality correlates with cleverness (Dunbar, 1997). In the case of humans, it is supposed that main evolutionary cause of intelligence is that more clever individuals are better in social games, which leads to higher proportion of acquired resources and better access to mating partners (Dunbar, 2004; Gintis, 2012).

Phylogenetic distribution of complex behavior is very unequal. Animals have more complex behavior than other lineages, probably due to larger body size and ability of movement requiring complex coordination. The most complex behavior and oftentimes strong cognitive abilities are typical for two animal phyla: vertebrates and arthropods. Both groups also convergently evolved similar complex social and parental systems, such as eusociality and biparental care (Cockburn, 2006; Reynolds et al., 2002; Sherman et al., 1995; Trumbo, 2012; Wilson, 1971). However, complex social and parental behavior is also unequally distributed within these two groups, which seems to indicate that some preadaptations for the evolution of complex behavior should exist. I think that the most important is the delay of dispersal from nest. When organisms disperse early, the possibility of interaction between parent and offspring is significantly limited. Moreover, delayed dispersal after reaching adult stage is an important precondition of eusociality (Hatchwell and Komdeur, 2000; Peer and Taborsky, 2007; Schwarz et al., 2011; Trumbo, 2013). Many invertebrates have plankton larvae, which disperse across large distances (Christiansen and Fenchel, 1979). Existence of such larvae prevents these lineages from attaining parental behavior. In case of sponge-dwelling shrimps (*Synalpheus*), only the lineages which lost plankton larvae have evolved eusociality (Duffy and Macdonald, 2010).

Aculeate Hymenoptera is a lineage with extended maternal care connected with nesting behavior (Michener, 2007; Wilson, 1971). This behavior is related to several interesting traits. Aculeate Hymenoptera are extreme K-strategists among invertebrates, a female produces only few offspring through her life, but she provides them with a large amount of care (Michener, 2007; Minckley and Danforth, 2019; Stubblefield and Seger, 1994). Females forage for food long distances from nests (Gathmann and Tscharntke, 2002; Greenleaf et al., 2007), meaning they also have to return to nest in a reasonable amount of time for successful. Therefore, significant cognitive abilities and excellent orientation is necessary for successful nesting (Fauria et al., 2004; Zeil, 1993; Zeil et al., 1996).

Although females perform extensive parental care, the role of males is usually marginalized and reduced to mating (Heinze, 2016; Paxton, 2005; Stubblefield and Seger, 1994). Males of aculeate Hymenoptera are usually short-lived and specialized for interacting with females (Michener, 2007; Schultner et al., 2017; Stubblefield and Seger, 1994). Nesting behavior and parental care are activities reserved for females. Male participation on parental care in Hymenoptera is rare, but exists. There are two main situations where males help care for offspring: male helpers in eusocial societies and biparental care (Brockmann, 1992; Lucas et al., 2011; Suzuki, 2013).

Some lineages of Aculeata evolved multi-female societies, for which is typical an extensive reproductive division of care (Michener, 1974; Wilson, 1971). Therefore, some females give up or delay their reproduction and support reproduction of other group members (Keller and Chapuisat, 2010; Korb and Heinze, 2016; Wilson, 1971). The most known examples are large societies of honeybees or ants. However, many aculeate Hymenoptera which establish simple societies with only two or a few females also exist (Bourke, 1999; Schwarz et al., 2007; Wilson, 1971). These societies are more appropriate for research of social behavior origin (Schwarz et al., 2007; Shell and Rehan, 2017).

Small carpenter bees--genus *Ceratina*, are an excellent model taxon for studies of cooperative behavior. *Ceratina* bees invest in individual offspring more than is usual even in comparison with other aculeate Hymenoptera (Michener, 1990a; Rehan and Richards, 2010a; Sakagami and Maeta, 1977). They are flexible in social life--some species are solitary, other facultatively eusocial (Groom and Rehan, 2018). Moreover males play a surprisingly important role in some species (Mikát et al., 2019b, 2020). Although they show complex and very diverse behavior, *Ceratina* bees have been a relatively neglected group for a long time. Natural history is known only for a few species. In this dissertation, I would like to emphasize knowledge of behavior of *Ceratina* bees, especially of European species of the genus. I hope that this research contributes to knowledge of social, parental, and sexual behavior of animals and its evolution.

CERATINA BIOLOGY

Small carpenter bees (genus *Ceratina*) belong to the family Apidae and subfamily Xylocopinae. The same subfamily contains larger carpenter bees (*Xylocopa*), Allodabine bees (Allodapini) and *Manuelia* (Michener, 2007). Recent phylogenetic analyses show that two wood-nesting bees (*Ctenoplectra* and *Tetrapedia*) are closely related to traditional Xylocopinae, therefore they should be included in this subfamily (Bossert et al., 2019).

Ceratina bees nests in broken twigs with soft pith (Michener, 1990a). Females first excavate a burrow and afterwards provision the constructed brood cells with pollen and nectar (Michener, 2007; Rehan and Richards, 2010a). The nest consists of linearly arranged brood cells separated by partitions made from pith (Michener, 1990a). When provisioning is finished, the mother usually guards the nest until offspring reach adulthood (Mikát et al., 2016; Rehan and Richards, 2010a). This effectively protect offspring against natural enemies (Mikát et al., 2016). When adult offspring emerges, mother feeds them pollen and nectar (Lewis and Richards, 2017; Mikát et al., 2017; Sakagami and Maeta, 1977). Although *Ceratina* bees were traditionally considered as solitary, facultative eusociality occurs in species studied in greater detail (Groom and Rehan, 2018; Michener, 1990a).

Ceratina bees have very interesting biology, however, only a small audience was concentered to this group. This is surprising, because behavioral variability is comparable with halictid bees. Furthermore this group is easier to study, as *Ceratina* bees nest in broken twigs. It is thus very easy to dissect the nests and evaluate their content. We found it is possible to dissect about one hundred nests per one day, therefore a robust dataset for statistical analyses can be collected within a short time. Moreover, experiments with nest observations are simpler by the possibility of easy nest translocation (Mikát et al., 2017).

Similar to almost all bee groups, *Ceratina* biology was studied by Charles Duncan Michener (Michener, 1962, 1990a; Michener and Eickwort, 1966). However, in the second half of the 20th century, the main center of *Ceratina* research was in Japan, where Japanese *Ceratina* were studied mostly by Sakagami and Maeta, but also by other researchers (Maeta et al., 1992; Maeta and Sakagami, 1995; Okazaki, 1992; Sakagami and Maeta, 1989, 1987, 1977; Shiokawa, 1969, 1966). Their works provide a complex insight into nesting and social biology of Japanese species (especially *C. japonica*, *C. flavipes,* and *C. okinawana*). They performed many nests dissections and observations of behavior. From 2008, the leading person of *Ceratina* research worldwide is Sandra M. Rehan. She focuses mainly on the NorthAmerican species *C. calcarata*, which she established as nearly model species. Moreover, she performed multiple experiments on the Australian species *C. australensis* (Dew et al., 2018; Rehan et al., 2010b, 2014) and also some basic characteristics of some tropical species (Rehan et al., 2015, 2009). She also performed multiple phylogenetic analyses of the genus (Rehan et al., 2010a; Rehan and Schwarz, 2014; Rehan et al., 2012, 2013a).

The biology of European *Ceratina* has been poorly studied. Only some old anecdotal observations exist (Daly, 1983; Maeta et al., 1997; Malyshev, 1936). Taxonomy of European *Ceratina* was studied by Howel W. Daly (Daly, 1983), Michael Terzo, and Pierre Rasmont (Rasmont and Terzo, 1996; Terzo, 1998; Terzo and Rasmont, 1997). However, information about nesting biology and offspring care strategy was not precisely evaluated for any species.

NESTING CYCLE

Nests of *Ceratina* bees significantly differ though the nesting cycle. First, a female excavates a burrow in soft pith of a twig (Michener, 2007; Rehan et al., 2010a). Later, the female build the brood cells and provisions them with pollen and nectar. Brood cells are separated by partitions, which are made from the twig's soft pith. *Ceratina* bees do not bring any building material from outside (Malyshev, 1936; Michener, 1990a; Rehan and Richards, 2010a). Brood cells are linearly arranged. The oldest brood cell is innermost, the youngest is outermost (Rehan and Richards, 2010a; Sakagami and Laroca, 1971). After provisioning is finished, the female usually stays in the nest entrance and guards offspring until adulthood (Mikát et al., 2016; Rehan and Richards, 2010a; Sakagami and Maeta, 1977). The female does not perform any foraging activities or forages only little during this period (Mikát et al., 2017). Once the offspring reach adulthood, the young adults crawl up to the nest entrance. They crawl carefully around younger siblings, however, they destroy brood cell partitions in the process. Emergence of young adults leads to resumption of maternal provisioning activity, if the mother is still present (Lewis and Richards, 2017; Mikát et al., 2017; Sakagami and Maeta, 1977). The mother provisions young adults with pollen and nectar and feeds them directly (Lewis and Richards, 2017; Mikát et al., 2017; Sakagami and Maeta, 1977). Both sexes overwinter in adult stage (Daly, 1983; Michener, 1990a; Rehan and Richards, 2010a; Sakagami and Laroca, 1971; Manuscript A). Overwintering can happen in old nests and also newly excavated burrows (Rehan and Richards, 2010a; Sakagami and Laroca, 1971; Sakagami and Maeta, 1987; Shiokawa, 1966; manuscript A). Moreover, I suppose that there exist some exchange of individuals between already existing nests before overwintering (Manuscript B).

NEST TYPE CLASSIFICATION

As *Ceratina* nests dramatically change through nesting cycle, a classification of nest types is necessary for good understanding of *Ceratina* natural history. Different types of nests are appropriate for studying different traits or general biological questions. New founded nests are nests, where burrow is already excavated, but provisioning of brood cells has not started yet. Active brood nests are currently being provisioned. They contain an egg or pollen ball in outermost brood cell. Full brood nests are nests, where provisioning was finished, and young adults are not yet present. Therefore, I classify as full brood nests these with a larva or pupa both in the outermost and innermost brood cell. If an adult already emerged in the inner brood cells, it do not crawl thorough and damage nest partitions. As full-mature brood nests I consider nests, which contain at least one immature offspring, but also contain mature offspring that already crawled through brood cell partitions and is now in contact with the mother. Mature brood nests contain only mature offspring.

There exists some terminological incongruence about classification of nests, where immature and mature offspring are together. These nests are sometimes classified as full brood nests (Lawson et al., 2018; Rehan et al., 2010b; Rehan and Richards, 2010a), or full-mature brood nests (Mikát et al., 2017). I think that the most correct is new category full-mature brood nests because these nests are behaviorally more similar to mature brood nests than full brood nests. Including nests where young adults emerged and crawled through nest partitions as full brood nests is incorrect, because these nests cannot be correctly studied for features usually measured in full brood nests. Young adults crawling through the nest partitions may have destroyed possible empty cells. Moreover, young adults can emigrate from nest, when they crawl through outermost brood cell partition. They also change their position in nest and therefore the nest cannot be correctly analysed for relationship between brood cell position and pattern in maternal investment. These problems are relatively insignificant in case of

American *C. calcarata*, because most of individuals in nest reach adulthood at very similar time. However, there is a larger temporal difference between first and last offspring reaching adulthood in European species. Therefore, I prefer this classification: full-mature brood nests contain some immature and some mature offspring, mature brood nests contain only mature offspring

NEST ARCHITECTURE

Ceratina bees build nests in linear burrows in twigs (Michener, 2007; Sakagami and Laroca, 1971). Therefore, brood cells are linearly arranged: the innermost brood cell contains the oldest offspring, the outermost the youngest offspring (Lawson et al., 2016; Mikát et al., 2017; Sakagami and Laroca, 1971). Moreover, when provisioning begins, an expansion of the burrow is impossible until offspring reach adulthood. *Ceratina* bees are unable carry any external material for nest building and their brood cells are separated by cell partitions made from fillings from the pith wall of the nest (Michener, 1990a; Rehan and Richards, 2010a).

Ceratina nests usually contain between five to ten brood cells on average (Rehan and Richards, 2010a; Sakagami and Laroca, 1971). However, some species, such as *C. cypriaca* or *C. mandibularis*, sometimes produce nests where only one offspring is present (manuscript C). The number of brood cells can vary through nesting season, second brood is usually smaller than first brood (Sakagami and Maeta, 1977; Vickruck et al., 2011; manuscript D). Sociality also can influence the number of brood cells, its effect can be positive, such as in *C. cypriaca*, *C. mandibularis,* and *C. parvula* (manuscript C), but also negative, as in *C. chalybea* (Mikát et al., 2020). However, solitary and social nests were similarly productive in *C. australensis* (Rehan et al., 2010b).

Some *Ceratina* species produce empty cells between brood cells. These cells do not contain any provision or offspring (Batra, 1976; Rehan et al., 2009; Sakagami and Laroca, 1971; Yogi and Khan, 2014). From European species, empty cells are typical for *C. chalybea* (Mikát et al., 2020). Nests of this species usually contain at least one empty cell between brood cells (Mikát et al., 2020). On the other hand, *C. cucurbitina* produce empty cells only in very rare cases (manuscript D). Other European *Ceratina* species have usually intermediate strategy and sometimes produce empty cells (Mikát et al., 2019a; manuscript C and E). Interesting are empty cells in *C. nigrolabiata*, where nests usually contain one or a few empty cells which are much longer than brood cells (manuscript E). Generally, empty cells are supposed to serve as protection against natural enemies (Münster-Swendsen and Calabuig, 2000; Tepedino et al., 1979). We documented different proportions of empty cells between solitary and eusocial nests in *C. chalybea* and *C. mandibularis* (Mikát et al., 2020; manuscript C). The probable reason is that better protected eusocial nests do not need to be protected by empty cells.

NESTING SUBSTRATES

Ceratina bees usually nest in broken twigs with soft pith (Batra, 1976; Michener, 2007; Rehan et al., 2009; Sakagami and Laroca, 1971). They need twigs that are already broken, because they are unable to bite through the woody shell of twigs to create side holes (Daly, 1966; Sakagami and Laroca, 1971). This is a difference from *Xylocopa* and *Manuelia*, which commonly make side holes in the woody sides of twigs (Anzenberger, 1986, 1977; Flores-Prado et al., 2008). *Ceratina* bees almost always excavate their own burrow (Rehan and Richards, 2010a; Sakagami and Laroca, 1971; Shiokawa, 1966), although nesting in preexisting cavities (Daly, 1966; Sakagami and Laroca, 1971) or establishing nest by nest usurpation rarely occurs (Mikát et al., 2016; manuscript E). This is a significant difference from most cavity-nesting bees and wasps, which usually nests in preexisting cavities (Budrienė et al., 2004; Michener, 2007; Morato and Martins, 2006). I suppose that there can be two reasons for nesting in own burrows: first, there is lower probability of molds in new burrows, secondly, since *Ceratina* cannot carry any construction material to the nest, excavating a new burrow provides pith material for constructing the nest partitions (Rehan et al., 2009; Sakagami and Laroca, 1971; Shiokawa, 1966).

Ceratina bees can use a large spectrum of nesting substrates (Shiokawa, 1966). It can be dead stems of herbs, such as *Phoeniculum vulgare* (Mikát et al., 2019a) or *Dipsacus* sp. (Vickruck et al., 2011), shrubs, such as *Rubus* spp. (Daly, 1966; Mikát et al., 2019a; Sakagami and Maeta, 1977; Vickruck et al., 2011), *Sambucus* spp. (Daly, 1966), and *Lantana* spp. (Michener, 1962), and even trees, such as *Rhus* spp., (Vickruck et al., 2011)). From my experience, the most common nesting substrate in the world is *Rubus* spp. In the Meditarrean region, a very common substrate is fennel (*Phoeniculum vulgare*). *Lantana* spp. is a very common substrate in tropical regions. Although *Ceratina* species are usually not specialized to one substrate, substrate preferences vary among species (Vickruck et al., 2011).

PARENTAL FEATURES

PARENTAL INVESTMENT AND PARENTAL CARE

Parental care is defined as any behavior which increases survival chance of offspring (Clutton-Brock, 1991). However, there is terminological incongruence in regards to which traits are classified as parental care and which as other type of parental investment (Smiseth et al., 2012). Therefore, some traits, such as nutrition of gametes or vivipary, are sometimes included in parental care and sometimes not (Gilbert and Manica, 2015; Smiseth et al., 2012). Moreover, parental care in a more strict sense of the word is also very diversified (Gross and Shine, 1981; Smiseth et al., 2012; Trumbo, 2012). Probably the most important forms of parental care are physical offspring protection and provisioning by food (Mas and Kölliker, 2008; Tallamy and Wood, 1986).

The universal benefit of parental care is increase offspring survival (Smiseth et al., 2012; Tallamy and Wood, 1986) on a short-term scale, but it can also influence offspring phenotype and therefore can lead to higher fitness on a long term scale (Alonso-Alvarez and Velando, 2012). However, parental care brings also several drawbacks, which are more diversified and complicated than benefits (Alonso-Alvarez and Velando, 2012; Field et al., 2007; Gross and Sargent, 1985; McNamara and Wolf, 2015; Paez et al., 2004; Trumbo, 2012). Therefore, costs rather than benefits are usually more determining for presence and extent of parental care (Gross, 2005). Direct parental care is time-consuming, meaning that time which parents invest into offspring care cannot be invested in future reproduction (Alonso-Alvarez and Velando, 2012; Stearns, 1992; Tallamy and Denno, 1982). Moreover, parental care can decrease parent survival rate (Ekman and Askenmo, 1986). Parents have to optimize between caring for current offspring and investing in future reproduction (Alonso-Alvarez and Velando, 2012; Stearns, 1992). Moreover, there is a conflict in the amount of care between a parent and offspring. An optimal amount invested by the parent is smaller than is the optimal amount of care for offspring fitness (Seidelmann, 2018; Trivers, 1974).

OFFSPRING PROTECTION

Offspring protection against natural enemies can be an effective way of increasing their survival rate (Buzatto et al., 2007; Kukuk et al., 1998; Thiel, 2000; Zink, 2003). Guarding of offspring is probably the most widespread form of parental care among arthropods (Smiseth et al., 2012). Physical offspring protection can be the most important parental behavior in several groups, e.g. some Hemiptera (Gilbert and Manica, 2015; Zink, 2003) or Opilionida (Machado and Raimundo, 2001). However, guarding of offspring is also commonly performed together with other forms of parental care, especially food provisioning (Tallamy and Wood, 1986; Trumbo, 2012)

Guarding of offspring is a relatively neglected topic in aculeate Hymenoptera, because studies about parental behavior concentrate more on offspring provisioning. An interesting type of guarding occurs in a parasitoid bethylid wasp *Sclerodermus harmandi*, where mother guards the host larva infected with her offspring , protecting them against hyperparasitoids (Hu et al., 2012). Most solitary nesting species abandon their nest after finishing brood cell provisioning (Cowan, 1991; Gess and Gess, 2014; Malyshev, 1936; Michener, 2007). Therefore, specific guarding behavior is not present after provisioning of offspring is finished. More extensive offspring guarding behavior (connected with whole nest protection) occurs in eusocial species (Ayasse and Paxton, 2002).

Ceratina bees have prolonged duration of care of offspring. Females guard offspring until adulthood even in solitary nests (Mikát et al., 2016; Rehan and Richards, 2010a; Sakagami and Laroca, 1971). We documented that this guarding strongly increases offspring survival (Mikát et al., 2016). Mother effectively protects her offspring against natural enemies, such as ants, chalcidoid wasps or other *Ceratina* bees (Mikát et al., 2016). These natural enemies caused high brood mortality in nests where mother was removed (Mikát et al., 2016).

Mother in some *Ceratina* species can crawl through brood cell partitions (Mikát et al., 2016; Rehan and Richards, 2010a; Sakagami and Maeta, 1977). The function of this crawling is cleaning offspring off parasites and excrements (Maeta et al., 1997; Mikát et al., 2016; Rehan and Richards, 2010a). Excrements are usually accumulated in bottom of nest (Sakagami and Maeta, 1977). In *C. cucurbitina*, we documented that nests with removed mother had high parasitism rate of chalcidoid wasps (Mikát et al., 2016). Therefore, we can suppose that mother can remove chalcidoid ectoparasitoids from her offspring, or prevent parasitation through nest wall by her activity.

Crawling through brood cell partitions was documented in only some *Ceratina* species, such as *C. calcarata* (Rehan and Richards, 2010a), *C. japonica* (Sakagami and Maeta, 1977), and *C. cucurbitina* (Mikát et al., 2016). On the other hand, females of any studied species from the subgenus *Euceratina* do not crawl through brood cell partitions (Mikát et al., 2019a, 2016; manuscript E). Species in which crawling through nest is absent have usually more massive nest partitions than species, where females do crawl through the partitions. Therefore, these nests can be less vulnerable in case of mother's death (Mikát et al., 2016). However, species where mother does not crawl through nest partitions can be more vulnerable to several parasitoids, e.g. chalcidoid wasps common in *C. chalybea* nests, even when they are guarded (Mikát et al., 2016)

The architecture of the outermost brood cell varies among species. It can be closed by nest partition, or open without outer partition (Batra, 1976; Mikát et al., 2016; Sakagami and Laroca, 1971). When the partition is open, the mother is in contact with an offspring (Mikát et al., 2016). Open last brood cells occur in almost all guarded nests of *C. chalybea* (Mikát et al., 2016) and most guarded nests of *C. chalcites* (Mikát et al., 2019a). Open last brood cells do not occur in unguarded nests in both species (Mikát et al., 2019a, 2016). Moreover, open last brood cells do not occur in species *C. cucurbitina* (manuscript D) and *C. nigrolabiata* (manuscript E). We suppose that opened outermost brood cell can increase offspring survival when female is present, but an open brood cell is also more vulnerable in case of the mother's death (Mikát et al., 2016).

Solitary aculeate Hymenoptera usually do not perform nest guarding when provisioning is finished, and close their nest (Gess and Gess, 2014; Peterson et al., 2007). The nest closure protects offspring against predators and parasitoids. There are several ways of closing a nest. In ground-nesting species, nest entrance is usually buried after provisioning is finished (Michener, 2007; Neff and Simpson, 1992). Twig nesting species, such as megachilid bees, and sphecid and eumeninae wasps usually create the last partition more massive than the previous ones (Camillo, 2005; Gess and Gess, 2014; Jesus and Garofalo, 2000; Musicante and Salvo, 2010). Pompilid wasp *Dipogon ossarium* include dead ants in the closing plug (Staab et al., 2014). These ants are repellent for potential enemies (Staab et al., 2014).

Before our research started, it was supposed that *Ceratina* bees always guard their offspring until adulthood and therefore do not close and abandon the nest (Rehan and Richards, 2010a). However, we have evidence from several species of the subgenus *Euceratina* that nest can be closed by a filling plug and abandoned. This filling plug is much thicker than a regular nest partition (Mikát et al., 2019a). To this date, we precisely documented the ability of nest closing and abandoning in species *C. chalybea* (Mikát et al., 2016), *C. chalcites* (Mikát et al., 2019a), and *C. nigrolabiata* (manuscript E). These species belong to different species groups in the subgenus *Euceratina* (Daly, 1983; Terzo, 1998), therefore they are not closely related. I suppose that ability of nest plugging and abandoning will occur also in in some other, but not all *Euceratina* species. All studied species with the ability of nest abandonment have nest guarding until offspring adulthood and nest abandoning as facultative strategies. Generally, guarded nests are more valuable than unguarded nests. Guarded nests have on average higher number of brood cells provisioned in all studied species (Mikát et al., 2019a, 2016), (manuscript E). Therefore, I can conclude that closing nest by filling plug can serve as offspring protection, but is less effective than physical nest guarding. The benefit of nest plugging and abandoning is the possibility of second nesting in different place (Mikát et al., 2016). Therefore, nest abandoning is a shift toward more r-strategic production of offspring, with lower investment in individual offspring. Serial nesting is uncommon for *Ceratina*, but widespread in most of solitary aculeate Hymenoptera. Therefore, serial nesting in some *Euceratina* species is a reversion to a common Aculeata trait.

FOOD PROVISIONING

Offspring provisioning with food is one of main form of parental care (Smiseth et al., 2012; Tallamy and Wood, 1986; Trumbo, 2012). Provisioning of offspring with food evolved in many animal groups (Smiseth et al., 2012; Tallamy and Wood, 1986). Offspring in these groups is strongly dependent on parent(s), because they are unable to obtain food independently (Smiseth et al., 2012).

There exist two main types of food provisioning: mass and progressive provisioning (Field, 2005; Smiseth et al., 2012). In case of mass provisioning, parent(s) firstly gather food, lay eggs, and leave offspring to its fate. In case of progressive provisioning, the mother first lays egg and afterwards regularly provisions offspring by food according to its needs (Field, 2005; Trumbo, 2012). Progressive provisioning allows more interaction between parent(s) and offspring, and parent(s) usually provide protection concurrently to food provisioning (Field and Brace, 2004; Schultner et al., 2017). Progressive provisioning also allows the parent(s) to stop investing in dead or unpromising offspring

(Field and Brace, 2004). However, progressive provisioning strategy is more risky in case of the death of parent(s) as offspring are dependent on regular care and without it probably die (Field, 2005).

Ceratina and *Xylocopa* bees combine both types of provisioning. Brood cells are provisioned by mass provisioning (Buchmann and Minckley, 2019; Michener, 2007; Rehan and Richards, 2010a). However, when adults emerge, mother feeds them pollen and nectar (Anzenberger, 1977; Hogendoorn and Velthuis, 1995; Lewis and Richards, 2017; Mikát et al., 2017; Velthuis and Gerling, 1983). When the mother feeds her mature offspring, she feed them directly, therefore by progressive provisioning (Anzenberger, 1977; Mikát et al., 2017; Velthuis and Gerling, 1983). The amount of food supplied to adult offspring is similar to amount of food supplied to brood cells (Hogendoorn and Velthuis, 1995). It is supposed that feeding of mature offspring is key for their survival of winter in temperate climates (Lewis and Richards, 2017; Maeta et al., 1992). However, as this feeding occurs in many species of *Ceratina* and also *Xylocopa*, we can suppose that it can be an ancestral trait of a common ancestor of both genera. Moreover, it occurs also in many subtropical and tropical *Xylocopa* species (Anzenberger, 1977; Velthuis and Gerling, 1983). Therefore, this feeding probably has multiple functions and evolved probably due to different reasons than feeding before winter.

I explored the existence of feeding of mature offspring in several temperate species. Feeding of mature offspring by mother occurred in all studied species: *C. calcarata* (Mikát et al., 2017), *C. cucurbitina*, *C. chalybea,* and *C. nigrolabiata* (manuscript B). In *C. calcarata*, feeding can be performed by dwarf eldest daughter in case of mother's death (Mikát et al., 2017). However, we did not detect this behavior in any of the three studied European species (manuscript B). Therefore, we can pose a question what is the fate of offspring of orphaned or abandoned nests of these species. We suppose that offspring from orphaned and abandoned nests migrate to nests, where mother is still present and feeds the young (manuscript B).

Some organisms, such as birds or aculeate Hymenoptera collect food in distant locations from nest. Most of aculeate Hymenoptera are solitary, therefore nest is unattended at the time when the female is foraging (Gamboa et al., 1978; Soucy and Giray, 2003). When the nest is unattended, it can be vulnerable to an attack of natural enemies (Goodell, 2003; Korb and Heinze, 2016; Seidelmann, 2006). In contrast, females in social colonies can exchange their roles, and at least one can always stand guard on the nest (Gamboa et al., 1978; Litte, 1977; Ohkubo et al., 2018; Soucy and Giray, 2003). Therefore, we can suppose different behavior in solitary and eusocial colonies. Females in eusocial colonies can afford longer foraging trips and spend more time outside the nest. This was shown in several facultative eusocial species. Females in solitary nests perform shorter trips than in social nests, and time spend outside nest is generally longer in social colonies than in solitary nests (Hogendoorn and Velthuis, 1995; Ohkubo et al., 2018). Similarly, in *C. calcarata* the mother spends higher proportion of time outside the nest in nests, where she feeds mature offspring than in nests, where she provisions brood cells, because nests in the phase of cell provisioning are more vulnerable (Mikát et al., 2017). Moreover, we also showed that biparental care can provide similar benefits as eusociality (Mikát et al., 2019b). In *C. nigrolabiata*, the female strongly decreases her foraging activity when the guarding male is removed (Mikát et al., 2019b).

MATERNAL INVESTMENT

The total amount of resources which parents can invest is limited, therefore there exist a trade-off between size and number of offspring (Charnov and Ernest, 2006; Lack, 1947). Usually, investment in individual offspring is optimized (Smith and Fretwell, 1974). As multiple resources are necessary for offspring production, the optimal size of offspring is influenced by availability of these resources (McGinley and Charnov, 1988; Rosenheim et al., 1996). The optimal size of offspring is also influenced by condition of parents (Hendry et al., 2001; Sugiura, 1994). Moreover, optimal size also differs between offspring of different sex (Molumby, 1997; Strohm and Linsenmair, 1997a; Trivers and Willard, 1973).

Mass-provisioning Hymenoptera are an optimal group for studying patterns in maternal investment due to their natural history. As the mother separates food for each offspring to individual brood cell, she can easily distribute her investment (Danforth, 1990; Field, 2005; Johnson, 1988; Seidelmann, 2018; Strohm and Linsenmair, 2000). Resources are clearly separated between offspring, therefore we can easily measure the amount of resources or weight or size of offspring as proxy of investment (Bosch and Vicens, 2002; Kim, 1999; Rehan and Richards, 2010b; Strohm and Linsenmair, 1997a). Moreover, sex of offspring is dependent on the mother's decision. Females store sperm in the spermatheca, and when they release sperm to fertilize the egg, female offspring is the result. Male offspring emerge from unfertilized eggs (Gerber and Klostermeyer, 1970).

Size of offspring is dependent on female condition. Larger females usually produce larger offspring (Maeta et al., 1992; Molumby, 1997; Rehan and Richards, 2010b; Stark, 1992). Moreover, abundance of resources influences investment similarly. When resources are abundant, larger offspring are produced, when resources are scarce, smaller offspring result (Dew et al., 2018; Kim, 1999; Peterson and Roitberg, 2006). Ecological conditions can vary across the distribution range of a species. Therefore, we can suppose variability of size between different parts of the distribution range. We found this variability in species *C. cucurbitina*. Individuals in the center of the distribution range (Italy and Albania) were larger than individuals on the edge of the range (Czech Republic and Crete) (manuscript D).

Females are heavier and therefore costlier in most aculeate Hymenoptera (Helms, 1994; Stubblefield and Seger, 1994), thus the mother optimizes sex ratio of her offspring in relationship to her condition (Seidelmann, 2006). Larger females usually lay more female-biased brood, smaller females lay more male-biased brood (Maeta et al., 1992; Rehan and Richards, 2010b; Strohm and Linsenmair, 1997a; Suguira and Maeta, 1989). Similar effect was observed for density of food resources. When resources are common, the brood is more female-biased, when resources are scarce, the brood is more male-biased (Kim, 1999; Peterson and Roitberg, 2006; Strohm and Linsenmair, 1997b). However, exceptional species with males heavier (and therefore costlier) than females exist. Examples are *Anthidium septemspinosum* (Sugiura, 1994) and *Ceratina chalcites* (Mikát et al., 2019a). In these species, larger females produce more male-biased offspring than smaller females (Mikát et al., 2019a; Sugiura, 1994).

In eusocial Hymenoptera, distribution of resources to offspring is strongly influenced by social environment (Boomsma, 1991; Kapheim et al., 2011; Ratnieks and Wenseleers, 2008). Females that found new social colonies usually produce female-biased first brood, because only females can serve as workers (Packer and Knerer, 1985; Soucy, 2002; Yanega, 1997). Female-biased sex ratio was documented also for facultative eusocial Japanese *Ceratina* (Maeta et al., 1992) Sex ratio investment is influenced by conflict between queen and workers, especially in highly eusocial species (Ratnieks et al., 2006; Trivers and Hare, 1976). Maternal investment can be proximate reason of dimorphism between reproductive dominant and subordinate females (O'Donnell, 1998; Ribeiro et al., 1999; Richards and Packer, 1994). The mother can intentionally malnourish some daughters, enforcing their worker role (Alexander, 1974; Brand and Chapuisat, 2012; Kapheim et al., 2011).

A dwarf eldest daughter is a typical phenomenon of *Ceratina* bees. First brood cell in the nest usually contains female offspring, and this female is smaller than the average female in the nest (Lawson et al., 2018, 2016; Maeta et al., 1992; Mikát et al., 2017; Rehan and Richards, 2010b). The mother intentionally malnourishes this female, enforcing her altruistic role (Maeta et al., 1992; Mikát et al., 2017; Shell and Rehan, 2018). A small female is more willing to take the altruistic role, because of having lower survival rate through winter and lower fecundity (Maeta et al., 1992). This female can feed her siblings, usually in case of maternal death (Lewis and Richards, 2017; Mikát et al., 2017). Feeding of mature offspring by the dwarf sister was documented in several *Ceratina* species, such as *C. calcarata* and *C. japonica* (Lewis and Richards, 2017; Mikát et al., 2017; Sakagami and Maeta, 1977). However, although we found first brood cell with female-biased sex ratio and small females also in the Czech population of *C. cucurbitina* (manuscript D), we excluded presence of altruistic daughter by observations in this species (Manuscript B). We suppose that small-sized daughters in first brood cell can be caused by other factors influencing maternal investment than only intentional malnourishing for altruistic role. Therefore, we claim that a pattern in maternal investment is an insufficient proof for existence of dwarf eldest daughter.

WHO PERFORMS CARE?

Offspring care can be performed by different individuals. Care can be performed by only mother, only father or both parents (Gilbert and Manica, 2015; Goodwin et al., 1998; Reynolds et al., 2002; Trumbo, 2012). Moreover, other individuals than parents can also participate, in combination with one or both parents or alone without parents (Costa, 2006; Wilson, 1971). Non-parental individuals participate in caring for the juveniles in insect societies or cooperative breeding vertebrates (Andersson, 1984; Jennions and Macdonald, 1994; Sherman et al., 1995).

Generally, the most common is maternal care which strongly dominates in arthropods (Gilbert and Manica, 2015; Tallamy and Wood, 1986; Thiel, 2000) and is also very common in vertebrates (Reynolds et al., 2002). Maternal care is the most common type of care in aculeate Hymenoptera (Gilbert and Manica, 2015; Michener, 2007). Usually, females are more willing to invest in care, because they have usually lower costs than males. Males compete more about mating partners, therefore they invest less to care about offspring. (Emlen and Oring, 1977; Gilbert and Manica, 2015; Gonzalez-Voyer et al., 2008; Székely et al., 2000). Females care for unrelated offspring with lower probability than males (Queller, 1997; Trivers, 1972). Moreover, females are usually more physically associated with offspring than males, which disappear after mating, but females have to stay at least to lay eggs or give birth to offspring (Gross and Shine, 1981).

Although females usually invest more in offspring, several groups with exclusive paternal care also exist (Ridley, 1978). The most widespread is paternal care in fishes (Reynolds et al., 2002; Ridley, 1978). Paternal care usually appears in taxa with external fertilization, because males are associated with offspring later than females (Gross and Shine, 1981; Ridley, 1978). Paternal care in arthropods is usually connected with guarding of eggs against natural enemies (Zeh and Smith, 1985). Father can guard offspring, sitting above iy, as in several harvestmen (Opilionida) (Machado and Raimundo, 2001). However, males can also carry their offspring on their body. For example, carrying of eggs on their hemelytra is known from males of true bug family Belostomatidae (Smith, 1997). Moreover, males of sea spiders (Pycnogonida) carry eggs of offspring on their third pair of legs – ovigeres (Arnaud and Bamber, 1988; Tallamy, 2001).

In several species amphisexual care occurs. Care can by performed by either male or female, but not both parents together. Amphisexual care occurs for example in thrip *Bactrothrips brevitubus*, where eggs can by guarded by male or female (Kranz et al., 2002).

OCCURRENCE OF BIPARENTAL CARE

Biparental care is widespread in some vertebrate groups. The most known example are birds, where about 80% of species care for their offspring biparentally (Cockburn, 2006). Moreover, biparental care is widespread also in crocodiles (Reynolds et al., 2002). In fishes, biparental care occurs in 3% of families and is the dominant type of care in several taxa, e.g. cichlid fishes (Klett and Meyer, 2002; Reynolds et al., 2002). Maternal care strongly prevails in mammals, however, biparental care occurs in about 5-9% of species, but is more common in several orders, e.g. primates (Lukas and Clutton-Brock, 2013; Reynolds et al., 2002). Humans are also typical biparental species (Marlowe, 2000).

In insects, biparental care is rare (Gilbert and Manica, 2015; Suzuki, 2013). Cases of biparental care exist in order Thysanoptera (Gilbert and Manica, 2015). It is also documented in several lineages of Blattodea, where *Cryptocercus* is the best documented case (Park and Choe, 2008). *Cryptocercus* cockroaches live in long term pairs and inhabit rotting wood, where they build galleries for offspring (Nalepa and Mullins, 2011; Park et al., 2002). Sister group of *Cryptocercus* are termites (Isoptera), whoseeusociality is apparently derived from biparentality (Engel et al., 2009; Ross et al., 2013). A termite colony is indicated by alate male and female. Reproductives of both sexes are present through whole colony life (Hartke and Baer, 2011; Wilson, 1971).

The highest number of biparental insects are in order Coleoptera (Gilbert and Manica, 2015; Suzuki, 2013). The most explored are burying beetles (*Nicrophorus*), which live on carrions (Benowitz and Moore, 2016; Trumbo, 1991). Passalid beetles are also a large biparental taxon, which contains hundreds of species (Schuster and Schuster, 1997). They live in decaying wood in all tropical regions of the world (Schuster and Schuster, 1997). Biparentality furthermore occurs in several bark beetles (Kirkendall, 1983; Robertson and Roitberg, 1998) and also in some dung beetles (Halffter et al., 2013; Huerta et al., 2003). The male can participate only in preparation of food for offspring by rolling a ball, with later care performed only by the female (González-Vainer, 2015; Sato and Imamori, 1988). However, in several dung beetles, the male also helps the female protect or feed the offspring in later phases (Halffter et al., 2013; Hunt and Simmons, 2002a).

Biparental care occurs also in Hymenoptera, although it is much rarer than maternal care in this order (Gilbert and Manica, 2015). In several sphecid and crabronid wasps, males are associated with nests and can protect them against natural enemies (Hook and Matthews, 1980). However, in-depth observations which can evaluate the male role do not exist for most of these species (Hook and Matthews, 1980). The most explored group are crabronid wasps of the subgenus *Trypargilum* of the genus *Trypoxylon* (Brockmann, 1992; Buschini and Donatti, 2012; Coville and Coville, 1980). The female performs all cell provisioning by spiders and also brings all the mud for construction of the nest (Brockmann, 1992; Buschini et al., 2010). Main male role is nest guarding (Brockmann, 1992; Buschini and Donatti, 2012; Coville and Griswold, 1983) when the female is away on a foraging trip (Brockmann and Grafen, 1989; Buschini and Donatti, 2012). The male can protect the nest against natural enemies, such as Chrysididae or ants (Buschini and Donatti, 2012; Coville et al., 2000). Furthermore, the male can also help with build nest, forming the mud with his mandibles (Brockmann, 1992). In *Tryoxylon superbum*, the male even guard the nest after the female finishes provisioning and leaves (Coville and Griswold, 1984). The male stays in the nest entrance until the offspring pupate (Coville and Griswold, 1984).

There were several examples of male association with nests described in bees (Portman, 2019). However, males do not participate in caring for offspring, or their role is unclear (Mikát and Straka, 2019).

In several species two male forms exist: standard males and macrocephalic males which are usually flightless and strongly philopatric (Danforth, 1991; Kukuk and Schwarz, 1988). Such male forms occur in halictid bee *Lasioglossum* (*Chilalictus*) *erytrurum*. Larger males are macrocephalic and block entrance in communal nests (where live up to 10 females). They can protect the nest against natural enemies (Kukuk and Schwarz, 1988). This system is similar to biparentality, however, (Kukuk and Schwarz, 1988) studied nests in late phase of nesting season, when only pupae and adult females were present in nests. Therefore, males probably care about siblings rather than offspring, although more research and genetical tests are necessary to gain a detailed insight into this system. Another example of bee with dimorphic males is *Perdita portalis* (Danforth, 1991). Macrocephalic males are specialized to philopatric mating. They mate with females inside nests (Danforth, 1991). Macrocephalic males can wait next to a brood cell and mate with the female when she plans to lay egg (Danforth, 1991). However, males of this species do not protect the nest against natural enemies (Danforth, 1991). Macrocephalic males have an interesting mating strategy, however, their behavior was not shown to lead to protection of their offspring (Danforth, 1991; Kukuk and Schwarz, 1988; Mikát and Straka, 2019).

An interesting behavior was observed in colletid bee *Leiproctus muelleri,* where males were observed to guard the nest concurrently with female performing nest provisioning (Houston and Maynard, 2012). However, sample size in this study is very small and male role is insufficiently examined.

We discovered and confirmed the first case of biparental care in bees (Mikát et al., 2019b). In *C. nigrolabiata*, females perform offspring provisioning and males nest guarding (Mikát et al., 2019b). Male-female pairs are established before provisioning season, and almost all provisioned nests contain male-female pair (Mikát et al., 2019b; manuscript E). However, one male does not stay in one nest through whole season, and the nest is guarded sequentially by multiple males through the season (Mikát et al., 2019b). We did not determine reasons why a male disappears from the nest, but there is a strong tendency of males to live in pairs. When one male is removed, he is quickly replaced by another male (Mikát et al., 2019b).

BEHAVIOR OF MALES AND FEMALES IN BIPARENTAL SPECIES

A male and a female cooperate on caring for offspring in species with biparental care. However, there are usually substantial differences between males and females in tasks they perform (Halffter et al., 1996; Trumbo, 2012). Usually males are less active and perform less difficult tasks (González-Vainer, 2015; Hunt and Simmons, 2002a; Royle, 2016). However, males and females have equivalent role in offspring care in some organisms. A typical example are woodlice from the genus *Hemilepistus*, where both male and female collect food, build burrows, and also protect the nest entrance (Linsenmair, 2007, 1985). They alternate in tasks. When one collects food, the other guards the nest entrance (Linsenmair, 2007). The male and female roles are clearly distinct in the biparental bee *C. nigrolabiata*. The female performs nest provisioning, and male nest guarding—he blocks the nest entrance, effectively preventing entry of natural enemies, such as ants (Mikát et al., 2019b).

The evolution of biparental care is influenced by multiple factors: sexual selection, mating behavior, ecological conditions, and also physical and behavioral constrains (Fromhage and Jennions, 2016; Klug et al., 2013; Mock and Fujioka, 1990; Royle et al., 2016). Biparental care is more stable when care of both parents has synergic and not only additional effects (Fromhage and Jennions, 2016).

Existence of biparental care can be influenced by ecological pressures. Some ecological niches increase probability of biparental care. In frogs the tendency to biparentality occurs in species which lay eggs into very small pools, e.g. phytotelmata. The male is responsible for guarding and transporting the offspring, while the female lays trophic eggs, which are an important food supply for offspring (Brown et al., 2010). In insects, biparental taxa commonly live in decaying organic material: wood, excrements or carrions. Biparental care is probably adaptation for better defense of scarce and nutritionally rich source of food (Tallamy and Wood, 1986). Building of nests or galleries is also typical for biparental insects (Suzuki, 2013). In case of *C. nigrolabiata*, emergence of biparental care can be supported by nest architecture. Since *Ceratina* bees make linear nests, one individual is sufficient for nest guarding when the other performs foraging. A linear burrow is not effective for cohabitation of higher number of individuals, therefore a pair of individuals can be the most effective for nest productivity.

Biparental care is influenced by mating behavior (Gonzalez-Voyer et al., 2008; Suzuki, 2013; Zeh and Smith, 1985). Generally, biparental care could arise in situations when opportunities for additional mating are limited and therefore male costs are minimal (Zeh and Smith, 1985). Biparental care can emerge as a by-product of mate guarding. The primary motivation of a male is monopolizing one female, however, he can also perform side behavior, which can help with offspring survival (Kirkendall, 1983; Mikát et al., 2019b; Van Rhijn, 1991). We suppose that mate guarding is the main reason for biparental care in *C. nigrolabiata*. Although the male has only a small benefit from nest guarding (most nests he protects contain unrelated offspring), long-term guarding seems to be the best way of increasing fitness. Mating occurs on nests, and we did not note paternity in any male who guarded the offspring for shorter time than three days. Therefore, we suppose that flitting between nests is not a successful strategy. Moreover, we found that effectivity of guarding per day increases with time of guarding. Therefore, living in a long-term pair is beneficial not only for the female, but also for the male (Mikát et al., 2019b).

There is a conflict between a male and female in pair (Houston et al., 2005; Trumbo, 2012). An important factor influencing parental effort is the chance for future reproduction. High chance for future reproduction reduces parental effort (Tallamy and Brown, 1999; Zeh and Smith, 1985). Although biparental species usually live in monogamous pairs, social monogamy does not mean genetic monogamy. Both parents can have offspring with other partners (Mock and Fujioka, 1990; Reynolds, 1996). Care for foreign offspring can be caused by two main processes: infidelity and turnover of partner. Extra-pair copulation is common in biparental birds during nesting period (Griffith et al., 2002; Møller and Cuervo, 2000). Extra-pair copulation can occur also in some biparental insects (Dillard, 2017; Hunt and Simmons, 2002b). , An important mechanism of decreasing paternity of pair-bonded male in biparental insects is the ability of females to store sperm in spermatheca (Trumbo, 2012). Usually, males have a larger trade-off between care and mating. Any male attempts at extra-pair paternity decrease his effectivity as a parent (Magrath and Komdeur, 2003; Reynolds, 1996). Moreover, when extra-pair paternity is high in whole population, the male has smaller motivation to perform care, because of the higher probability of caring for foreign offspring (Griffith et al., 2002; J Hunt and Simmons, 2002; Kokko, 1999; Møller and Cuervo, 2000).

We found biparental care in bee *Ceratina nigrolabiata*, where male paternity is very low due to fast partner turnover (Mikát et al., 2019b). The male role is nest guarding, which is an easy and low-risk activity (Mikát et al., 2019b). Therefore, we suppose that biparentality is beneficial for males, because they obtain at least some offspring in a situation when performing care has little costs (Mikát et al., 2019b).

EUSOCIALITY

DEFINITION AND OCCURRENCE OF EUSOCIALITY

Insect societies generally fascinated evolutionary biologists. Darwin considered the existence of social insects as an important challenge for his theory (Darwin, 1859). Smith and Szathmary, (1997) included the evolution of eusociality as one of major transitions in evolution of life. Therefore, insect societies are surely one of the most interesting products of evolution.

Eusociality is defined by three originally postulated conditions: reproductive division of labor, cooperative brood care, and generation overlap (Batra, 1966; Michener, 1974; Wilson, 1971). Only societies meeting all three criteria are considered eusocial. If one or more conditions are not fulfilled, the society is described by another term--e.g. semisociality, where generation overlap is absent (Michener, 1974; Wilson, 1971). First researchers, who studied eusociality in detail, considered as eusocial Isoptera and three hymenopteran groups--wasps, bees and ants (Wilson, 1971).

Batra, Wilson, and Michener's classification of societies was originally developed for insects. However, societies with similar organization exist also in vertebrates (Burda et al., 2000; Cockburn, 2006; Jarvis, 1981; Jennions and Macdonald, 1994; Sherman et al., 1995). Vertebratologists developed independent terminology, usually naming societies comparable with eusocial insects as cooperative breeders (Costa and Fitzgerald, 1996; Sherman et al., 1995).

A more detailed insight into natural history discovered some new eusocial lineages of insects, e.g. thrips (Crespi, 1992; Kranz et al., 1999), bark beetles (Kent and Simpson, 1992; Kirkendall et al., 2015; Peer and Taborsky, 2007) or passalid beetles (Dillard, 2019). The discovery of these societies and also comparison between insects and vertebrates challenged the definition of eusociality. Generally, the overwhelming diversity of biological systems at all levels causes difficulties in categorization of life (Dawkins, 2004). Therefore, definitions are usually somewhat vague as it's difficult to find distinct borders between individual categories.

The vague definition of eusociality led to discussions about a new definition in the years 1994-1997. Gadagkar, (1994) considered the presence of altruism a key feature of eusociality. Sherman et al., (1995) concentrated on reproductive skew and existence of variability in reproductive success between members of a society. By their definition, eusocial society is a society, where reproductive skew is present (Sherman et al., 1995). This definition considered as eusocial most of social insects and also cooperative breeding vertebrates (Reeve et al., 1996; Sherman et al., 1995). On the other hand, Crespi and Yanega, (1995) considered as a key factor of eusociality the presence of distinct castes--individuals are irreversibly behaviorally distinct before reaching reproductive maturity. Some individuals are predestinated to be reproductives, others to be helpers (Crespi and Yanega, 1995). This definition does not exclude the possibility of helper reproduction, however, it supposes existence of distinct castes as a condition of eusociality with the worker caste having a smaller ability of reproduction (Crespi and Yanega, 1995). The discussion in 1994-1996 did not provide an sufficient conclusion, causing the definition of eusociality and other types of social organization to remain vague to this date (Dew et al., 2016; Kocher and Paxton, 2014). Here, I prefer and use the original Batra-Wilson-Michener definition of eusociality.

A key feature of eusociality is the reproductive division of labor. However, the presence of reproductive division of labor does not mean exclusive reproduction of one individual (or a pair of individuals). Reproductive subordinate individuals can participate partially on reproduction. The degree of reproductive bias to dominant breeders is called a reproductive skew (Reeve and Keller, 2001; Sherman et al., 1995). There is continuum from societies without shew (where members reproduce similarly) to maximal skew, where only one individual (or a pair) reproduces (Sherman et al., 1995). Exclusive reproduction of one individual is relatively rare (Bourke, 1988; Ratnieks and Wenseleers, 2008). Some, although small, reproductive activity is also performed by workers in highly eusocial colonies, such as honeybees or yellowjackets, where workers produce a part of male offspring (Bourke, 1988; Foster and Ratnieks, 2001; Ratnieks and Wenseleers, 2008). Moreover, multiple reproductive queens can be also present (Hölldobler and Wilson, 1977; Wilson, 1971). Completely sterile workers are rare in eusocial Hymenoptera, they exist for example in some stingless bees or leafcutter ants (*Atta*) (Dijkstra et al., 2005; Tóth et al., 2004).

The boundary between reproductively dominant and subordinate female is weak in small hymenopteran societies. In several groups, such as halictid bees or polistine wasps, differences between queens and workers exists, but workers can mate and have potential to serve as queens in specific situations (Hunt, 2006; Michener, 1990b; Packer and Knerer, 1985; Rehan et al., 2013b). In other groups, such as Stenogastrinae wasps, morphological differences between queens and workers are missing, and their roles are related to their age (Bridge and Field, 2007; Turillazzi, 2013). Although there are slight or no morphological differences between queen and workers, the behavioral difference, especially in reproduction and foraging activity, is usually significant (Kapheim et al., 2016; Michener, 1990b; Sumner et al., 2002).

Obligately eusocial insect species are most popular among eusocial lineages. Obligately eusocial species have the eusocial stage always included in their colony cycle. Their colonies are commonly founded as solitary, however, they are unable produce reproductive individuals without reaching eusocial stage of colony. In contrast with obligately eusocial species, facultatively eusocial species are able to establish eusocial colonies, but they have solitary life as alternative strategy. Solitary and also eusocial strategy can co-occur in one population (Field, 2008; Schwarz et al., 2007). Generally, eusocial insects fascinated scientist, and thus a lot of research about biology of social insects was performed. Most research was concentrated on obligately eusocial groups that establish large societies, such as *Apis*, *Vespula,* and *Bombus*. Well-explored are also some species of ants and termites, groups exclusively consisting of obligate eusocial species with mostly large societies. The best explored hymenopteran groups with small societies are *Polistes* wasps (Jandt et al., 2014; Turillazzi and West-Eberhard, 1996) and *Ropalidia marginata* (Gadagkar, 2009). However, although these species have relatively small societies, they are also obligately eusocial. For evaluation of selective pressures influencing the origin and maintenance of eusociality, facultatively eusocial species are better models because they allow within-species comparison between solitary and eusocial strategy (Chapuisat, 2010; Sakagami and Maeta, 1984; Schwarz et al., 2007; Shell and Rehan, 2017).

Facultatively eusocial societies occur in several hymenopteran taxa. Probably the largest group are sweat bees (Halictidae) (Schwarz et al., 2007). Facultative eusociality is typical also for bees from subfamily Xylocopinae (Hogendoorn and Velthuis, 1999; Rehan et al., 2012; Schwarz et al., 2007) and tribe Euglossini (Freiria et al., 2017; Friedel et al., 2020). Facultative eusociality is also typical for Stenogastrinae wasps (Turillazzi, 2013). Facultative eusociality was documented in crabronid wasps of genera *Microstigmus* (Lucas et al., 2011; Matthews, 1968) and *Spilomena* (Turillazzi et al., 2014). Societies established by species belonging to these groups usually contain only a few individuals (Schwarz et al., 2007; Turillazzi, 2013). These taxa are very useful for studying factors influencing the origin of eusociality or cooperation in general.

Some groups, such as Halictid and Xylocopine bees, contain eusocial and also strictly solitary species. Species with different social strategies are intermixed in phylogenetic tree (Danforth et al., 2003; Groom and Rehan, 2018). It is supposed that eusociality evolved only a few times (twice for Halictidae, once for Xylocopinae and once for corbiculate bees including Eugossini (Bossert et al., 2019; Cardinal and Danforth, 2011; Gibbs et al., 2012; Kocher and Paxton, 2014; Rehan et al., 2012)), but there was frequent eusociality loss within these groups (Danforth et al., 2003; Kocher and Paxton, 2014; Rehan et al., 2012; Wcislo and Danforth, 1997). Moreover, within-species variability between strictly solitary and facultatively eusocial populations can be found (Davison and Field, 2016; Eickwort et al., 1996). This variability can be influenced by climatic conditions (Field et al., 2010) or have a genetic base (Kocher et al., 2018; Soucy and Danforth, 2002).

SOCIAL BIOLOGY OF *CERATINA*

Most of *Ceratina* nests are solitary, however, the ability of social nesting was detected in most of extensively studied species (Groom and Rehan, 2018; Michener, 1990a; Rehan et al., 2010b; Sakagami and Maeta, 1989, 1987). Studies about sociality in *Ceratina* usually report the presence of multi-female nests (Rehan et al., 2015, 2009; Sakagami and Maeta, 1977). Meeting the three conditions of eusociality is usually based on indirect evidence, such as difference in ovarian development or wing wear (Okazaki, 1987; Rehan et al., 2010b, 2015). If difference in ovarian development and wing wear is reported, fulfilling the classic criteria of eusociality is likely. Moreover, extensive observations of in-nest behavior were performed in Japanese *Ceratina*, which confirm the presence of reproductive division and also cooperative brood care (Sakagami and Maeta, 1987, 1984). The overlap of generations has not been tested, because nests were artificially induced (Sakagami and Maeta, 1987, 1984). Moreover, reproductive division of labor is supported by a genetic study on *C. australensis* (Rehan et al., 2014). Most *Ceratina* species, where multi-female nests were detected, have insufficient information needed for exact classification of the type of sociality. Genetic markers and nest observations will be necessary for better evaluation of sociality. However, information from to this date performed studies supports the view that at least some multi-female nests of the examined species are eusocial. The observed proportion of multi-female nests is low in the studied species. Highest detected proportion of social nests was around 20% in *C. japonica* (Sakagami and Maeta, 1984) and 14% in *C. australensis* (Rehan et al., 2010b). Moreover, the proportion of social nests is extremely low in some species, e.g. less than 1% in *Ceratina flavipes* (Sakagami and Maeta, 1987). As social nests can occur in a very small proportion, assessing social status of some species is difficult. When we detect social nesting, we can consider this species as facultatively eusocial. If the proportion of social nests is high (e.g. 20%), a study of around 20 nests (in active brood or full brood nest phase) can be a sufficient proof of sociality. However, this number of nests is apparently insufficient when the proportion of social nests is low (around 1%). Therefore, examining more than a hundred nests is necessary for a relevant assessment of social strategy. Moreover, social status can vary spatially (Dew et al., 2018; Sakagami and Maeta, 1987), during nesting season (Mikát et al., 2020; Rehan et al., 2010b) or between nesting seasons (Dew et al., 2018; Mikát et al., 2020). Older works commonly examined only a few nests per species in the proper phase (Batra, 1976; Michener and Eickwort, 1966; Sakagami and Laroca, 1971). Therefore, sociality was commonly undetected in some species which are probably facultatively eusocial. Recent studies show that most in-depth investigated species are able to form social nests (Groom and Rehan, 2018; Rehan et al., 2015, 2009). In this dissertation, I present a newly discovered social nesting in six species (*C. chalybea*, *C. bispinosa*, *C. parvula*, *C. mandibularis*, *C. cypriaca,* and *C. chrysomalla*), and I have unpublished data about sociality for some other Palearctic species. Social nesting was not precisely demonstrated in European *Ceratina* to this date, albeit our results support the view that most *Ceratina* species are facultatively social.

Given the scarcity of social nests, we can ask if strictly solitary species exist or if all *Ceratina* species are social, but in some species is sociality extremely rare. I suppose that non-eusocial strategy can occur in some species. North American species, especially *C. calcarata* were very intensively studied in multiple locations, and regular multi-female nests have never been detected (Lawson et al., 2018; Rehan and Richards, 2010a; Vickruck and Richards, 2012). However, a rare type of eusociality occurs in this species: the dwarf daughter can feed her siblings (Mikát et al., 2017). We performed a large sampling of nests of *C. cucurbitina*, and thus we assume this species to be solitary (manuscript D). Similarly, *C. dallatoreana* seems to be solitary as well (Daly, 1966; manuscript C). According to our data, *C. chalcites* is also solitary (Mikát et al., 2019a), however, more nests from different regions should be assessed. Finally, the biparental strategy of *C. nigrolabiata* is probably an alternative to multi-female nesting (Mikát et al., 2019b).

ORIGIN AND MAINTENANCE OF EUSOCIALITY

Although a large amount of research about origins of eusociality was performed, its key factors are still debated (Field and Toyoizumi, 2019; Pernu and Helanterä, 2019; Quiñones and Pen, 2017). Hamilton, (1964) formulated a prediction that altruism should occur, when the recipient's benefit multiplicated by relatedness between donor and recipient is higher than cost to the donor. Hamilton's rule implies that there are two main factors influencing reproductive altruism: relatedness between colony members and ecological factors, which influence costs and benefits (Pernu and Helanterä, 2019; Rehan and Toth, 2015). Moreover, it is clear that eusociality is inequally distributed on the phylogenetic tree of animals (Hughes et al., 2008; Ross et al., 2013). Therefore, natural history traits, which are relatively stable on a long-term scale (such as presence and form of parental care), significantly influence not only costs and benefits, but also the possibility of emergence of cooperative behavior (Bourke, 2014; Korb and Heinze, 2016; Quiñones and Pen, 2017)

STAY OR DISPERSE DECISION

Members of primitive eusocial colonies can decide if they stay in the society or try to reproduce independently (Cahan et al., 2002; Korb and Heinze, 2016). In social Hymenoptera, a female can weight the probability and effectivity of individual nesting against possible benefits of staying in her natal nest. Benefits of staying in the natal nest consist of two parts: indirect fitness gained for related individuals and direct fitness from nest inheritance or own reproduction (Korb and Heinze, 2016; Schwarz et al., 2011).

The decision between staying or dispersing is influenced by nest productivity. When more individuals work, it can be supposed that more offspring will be produced in the nest. However, the increase of productivity itself should be sufficient only in a situation, when increases not only the overall productivity, but also the productivity per individual (Michener, 1964). Empirical studies usually did not show increase in per capita productivity (Michener, 1964; Prager, 2014; Rehan et al., 2014;

Richards, 2011; Soucy and Giray, 2003). The reason can be a difficulty of measuring productivity. When we compared the number of offspring in social vs solitary nests, we have included only successful nests since failed nests are not easy to detect. It can be supposed that solitary nests have a higher probability of total nest failure in comparison to social nests (Leadbeater et al., 2011; Mueller, 1996; Smith et al., 2019; Stark, 1992).

In addition to the options of staying or dispersing, a third possible solution exists: stay for some time, but wait and perform only little work (Hogendoorn and Velthuis, 1995; Richards, 2011; Schwarz et al., 2011). This decision can be analogous to a diapause in solitary organisms: an individual decides not to reproduce immediately and wait to better conditions. It can be nest inheritance in this case. This strategy is relatively common in some simple societies. In Stenogastrinae wasps, subordinate colony members perform work, but adjust their work effort in relationship to the probability of inheritance of breeding position (Field et al., 2006). In *Xylocopa,* the reproductively dominant females perform most of work, including foraging, and subordinate females wait for inheritance of breeding position (Hogendoorn and Velthuis, 1995; Richards, 2011). A similar strategy exists in several allodapine bees (Schwarz et al., 2011). Although a passive female performs no foraging, it can be also beneficial due to help with nest guarding. We found similar situation in *C. chalybea*, where the *s*ociety consists of an old female, which performs all foraging and also reproduction, and young adults, which passively stay in nests (Mikát et al., 2020). However, we suppose that these young adults will reproduce next season.

RELATEDNESS

Relatedness is usually supposed to be an important factor in the evolution of eusociality, and a majority of researchers believe in its importance (Abbot et al., 2011; Bourke, 2011; Pernu and Helanterä, 2019; Strassmann et al., 2011). However, cooperation can also emerge without relatedness, e.g. by reciprocity, as was documented by some studies using mathematical modelling (Nowak, 2006; Nowak et al., 2010). Moreover, simple societies, where evidently unrelated individuals cooperate, exist. These cases are more frequent in vertebrates (Clutton-Brock, 2002), although insect colonies with unrelated helpers were also documented (Queller et al., 2000; Stark, 1992; Vickruck and Richards, 2019).

Single mating is an important precondition for arise of eusociality in eusocial Hymenoptera (Hughes et al., 2008). Single mating of a queen causes high relatedness between colony members (Ratnieks, 1988). Single mating is considered ancestral for all main eusocial hymenopteran taxa (Hughes et al., 2008). Multiple mating emerged in groups with physiologically determined castes, where workers have no opportunity to disperse and have a small ability for reproduction inside the colony (Hughes et al., 2008). Extremely high polyandry is typical for species establishing very large colonies (Boomsma et al., 2009; Kronauer et al., 2007). As cooperation between colony members is necessary in highly eusocial species, benefits of multiple mating prevail, such as higher resistance to diseases due to higher genetic diversity (Boomsma et al., 2009; Palmer and Oldroyd, 2000), possible specialization of workers from different matrilineal lineages to different tasks (Murakami et al., 2000; Oldroyd and Fewell, 2007) or possibility for worker policing (Ratnieks, 1988; van Zweden et al., 2012). However, advantages of multiple mating prevail in societies, where workers are unable to nest independently. Simple Hymenopteran societies have almost always single mating (Hughes et al., 2008).

Although hymenopteran species which form small and simple societies are usually monogamous (Hughes et al., 2008), other factors which decrease relatedness in simple hymenopteran societies also exist. Such a factor can be the fast turnover of a reproductive dominant female (Bolton et al., 2006;

Landi et al., 2003), or reproduction of multiple females in a colony (da Silva et al., 2016; Sherman et al., 1995). Another mechanism which causes a decrease in relatedness is the exchange of colony members between colonies (Brand and Chapuisat, 2016; Soro et al., 2009). Therefore, single-queen single-mated social organization is not necessarily the most primitive state in eusocial Hymenoptera, but probably an important intermediate stage to more complex societies.

In the manuscript Mikát et al., (2020), we document the simple society of *C. chalybea*, where about 50% of helpers are unrelated to the reproductive female. Moreover, females of *C. chalybea* mate multiple times (Mikát et al., 2019b), which can also decrease relatedness in social colonies. The reproductively dominant female performs all food provisioning, but young adults can help with nest protection when the mother is absent from the nest. We suppose that the main benefit for the young adults is feeding by the old female (which occurs simultaneously with provisioning of new cohort of brood cells), while nest protection is a benefit for the old female when she performs a foraging trip or when she dies (Mikát et al., 2020). Therefore, we conclude that mutualistic interactions can play a role in stability of some small insect societies, and relatedness can be sometimes unimportant (Mikát et al., 2020).

CLIMATE

The tendency to eusociality is influenced by climate, usually more social are animals living in warmer and less social in colder climates (Purcell, 2011). The most important factor is increasing seasonality in colder-climate (higher latitude) areas. The survival of social colonies through the winter is difficult. Therefore lot of temperate eusocial species have annual colonies (Greene, 1991; Wilson, 1971). In temperate climate, only a smaller part of year is usually appropriate for activity. More than one generation per year is necessary for establishing eusocial colonies. In high latitudes, there is commonly no time for more than one generation (Chapuisat, 2010; Davison and Field, 2018; Soucy, 2002; Wcislo and Danforth, 1997). For this reason only solitary nesting occurs in these latitudes in some species or populations.

The association between social strategy of species and latitude was well-documented for Halictidae and also Xylocopinae bees (Groom and Rehan, 2018; Purcell, 2011; Wcislo and Danforth, 1997). We explored the situation in subtropical climate (Cyprus), and our results shows that most of local *Ceratina* species are facultatively eusocial (manuscript C). Sociality is associated with length of nesting season. Most of species of Cyprus nest from May to September and therefore are able to rear multiple generations per year (manuscript C). A notable exception is *C. dallatoreana*, which starts hibernating in September. We did not find evidence for social nesting in this species. We suppose that shorter nesting season can be the reason for absence of sociality in this species. However, we've found social nests of *C. chalybea* in the Czech Republic (Mikát et al., 2020), which is almost the northern border of its distribution range of the genus (only one species, *C. cyanea,* reaches farther to north (Terzo and Rasmont, 2011)). Therefore, in some situations social nesting occurs also in high latitudes. In case of *C. chalybea* it can be probably facilitated by the simultaneous provisioning of brood cells and feeding of mature offspring (Mikát et al., 2020), which saves time.

Within-species variability in sociality related to latitude has been documented in several Halicidae bee species (Davison and Field, 2016; Field et al., 2010; Purcell, 2011). However, studies on species belonging to subfamily Xylocopinae, *Ceratina calcarata* (Lawson et al., 2018) and *Ceratina australensis* (Dew et al., 2018) or allodapine bees (Cronin and Schwarz, 2001; Schwarz et al., 2007) did not find social variability related to latitude. We examined the social status of species *C.*

cucurbitina in four European regions and we conclude that this species is solitary in all of these regions. Therefore, we support the view that within-specific social flexibility related to climate does not occur in Xylocopinae bees.

DIRECT FITNESS BENEFITS OF SOCIALITY

Indirect fitness increase, i.e. spreading own alleles via reproducing relatives, is considered the main benefit of eusociality, (Bourke, 2014; Foster et al., 2006; Hamilton, 1964; Strassmann et al., 2011). However, direct fitness benefits can also play role in eusocial societies (Clutton-Brock, 2002; Field et al., 2006; Korb and Heinze, 2016; Leadbeater et al., 2011). There are two main types of direct benefits: a) possibility of nest inheritance and b) partial reproduction as a worker within the nest.

A chance for inheritance of territory is supposed to be an important factor for cooperatively breeding birds (Pen and Weissing, 2000; Ragsdale, 1999; Walters et al., 1988). Population density, and the density of empty territories and their quality, significantly influences the presence of helpers (Komdeur et al., 1995; Pen and Weissing, 2000). A similar situation exists also in some primitively eusocial wasps. Females of Stenogastrinae wasps sometimes adopt nest with unrelated orphaned brood (Field et al., 1998). However, the reason for orphaning is not the nest itself, but the brood. As lifespan of a mature Stenogastrinae wasp is relatively short in comparison with developmental time, an adoption of brood strongly increases the chance that at least one mature female will be present in the colony (Field et al., 1998). Cooperation of unrelated individuals is common in some polistine wasps (e.g. *Polistes dominula*) at the phase of nest founding (Field and Leadbeater, 2016; Queller et al., 2000). Pleometrically founded colonies are consist of a few females, where only one dominates reproduction (Queller et al., 2000). However, this cooperation is advantageous also to subordinate females, because the probability of nest inheritance is higher than the probability of successful solitary nest establishment (Field and Leadbeater, 2016; Leadbeater et al., 2011).

We found eusocial nesting in *C. chalybea*, where direct fitness benefits have to play important role due to low relatedness (Mikát et al., 2020). However, only old females reproduce in these nests. We have not recorded maternity of any young female adult. Also, we have not recorded paternity of a male young adult, therefore mating with old female is not a reason for the young males' presence in the nest. Partial reproduction is not a benefit for young adults (Mikát et al., 2020). Nest inheritance also cannot be the main benefit, because *C. chalybea* usually do not overwinter in old nests (manuscript A) and make new nest next season. We suppose that the main direct benefit for young adults is feeding by the old female, which occurs simultaneously with provisioning of new brood cells.

NATURAL HISTORY

Eusociality is very unequally distributed among lineages of animals (Hughes et al., 2008; Korb and Heinze, 2016; Nowak et al., 2010; Wilson and Hölldobler, 2005). Some natural history traits are important for tendency to evolving eusociality. Probably the most important trait is long parental care connected with offspring philopatry, which generates groups of related individuals (Keller and Chapuisat, 2010; Rehan et al., 2015; Trumbo, 2013). Nesting behavior, with offspring dependent on mother, is probably the main preadaptation of aculeate Hymenoptera to eusociality (Andersson, 1984; Nowak et al., 2010). Moreover, different lineages of Aculeata also differ in tendency for evolving eusociality. Eusociality emerged in lineages which provision offspring by mass provisioning and also progressive provisioning strategy. However, lineages with mass provisioning strategy have often reverted to solitary life, in contrast with progressive provisioners, where reversion to solitary life is unknown (Chenoweth et al., 2007; Gibbs et al., 2012; Groom and Rehan, 2018; Turillazzi, 2013).

An important natural history trait in *Ceratina* bees connected to eusociality is nest reuse. Eusocial nests usually occur in situations where bees perform second nesting in the same twig (Michener, 1990a; Okazaki, 1987; Rehan et al., 2015, 2014). The nest can also be reused from previous season (Sakagami and Maeta, 1984). This trait enables delayed eusociality in species *C. japonica* in cold climate, where is not enough time for more clutches per year (Sakagami and Maeta, 1984). We detected strong association between nest reuse and eusociality in species *C. chalybea* (Mikát et al., 2020).

ROLE OF MALES IN EUSOCIAL SOCIETIES

Eusocial societies can be composed exclusively of females or of individuals of both sexes (Ross et al., 2013). In termites, reproductive castes of both sexes are present in the nest, with workers and solders usually being of both sexes as well (Korb, 2007; Thorne, 1997). However, eusocial hymenopteran societies are usually based on females. In colony-life one or multiple reproductive females (queens) and also subordinates (workers) are females (Bartz, 1982; Wilson, 1971). Hymenopteran eusociality is derived from maternal care, therefore the role of males is marginalized similarly as in solitary species (Ross et al., 2013).

Although the role of males is usually marginal in hymenopteran societies, some exceptions when males can participate in colony life exist. In polistine wasps of the genera *Polistes*, *Mischocyttarus,* and *Ropalidia*, males can help with processing of food, which is collected by workers (Hunt and Noonan, 1979; Kojima, 1993). In bumblebees, males can incubate pupae by heating (Cameron, 1985). Stingless bee males can help with nectar dehydration (Veen et al., 1997). In all these situations is male contribution to colony life very small and has relatively low importance.

More important is the male role in *Microstigmus nigrophthalmus*, where males actively protect nests against natural enemies (Lucas and Field, 2011). About a half of helpers in the colony are males (Lucas and Field, 2011). They can also effectively protect the nest in the absence of females (Lucas and Field, 2011). However, similarly as in other males in hymenopteran societies, they are unable to perform food provisioning (Lucas and Field, 2011).

We described the presence of male helpers in *C. chalybea* (Mikát et al., 2020). The eusocial nests contain up to nine helpers, with most of the helpers being males (Mikát et al., 2020). We suppose that the main motivation of males lies in obtaining food from the queen in the nest. They can help with nest protection against natural enemies in return.

CONCLUSION

Bee genus *Ceratina* is an excellent model group for studying parental behavior. Behavior of *Ceratina* bees is extremely diverse in comparison to other Hymenopterans and other insect groups. Generally, maternal care is prolonged, mother usually guards her offspring until adulthood and after offspring maturation, mother again feeds them with pollen and nectar. Social nests were detected in most of the studied species. Moreover, offspring care traits are often variable within a single species. This variability allows to compare different parental strategies and to identify costs and benefits of these strategies.

Another advantage of *Ceratina* bees as a model group is methodical simplicity of nest research. Nests are present in soft twigs, and the dissection is therefore easier than the excavation of ground-nesting bees or the dissection of nests of *Xylocopa* bees, which are commonly nesting in hard wood. Therefore, it is possible to collect and dissect up to 150 nests per day. Speed of nest processing allows to gather a large sample size for robust analyzes. Moreover, easy nest relocation opens a possibility for manipulative experiments.

Newly discovered biparental behavior in *Ceratina nigrolabiata* is unique. The biparental behavior has not been precisely documented in any bees to this date. As biparental behavior in this species is evidently recently evolved, this species is an immaculate model for studying the emergence of biparentality. Many aspects of their behavior is still unexplored, e.g. degree and mechanism of partner recognition or distribution of offspring of males to different nests.

In my dissertation, I uncover the natural history of several *Ceratina* species. However, social and parental behavior of most species is still unknown. While several phylogenies were published (Rehan et al 2010, 2015), the relationship between some taxa is unclear and this is especially true for European species. Comparative approach, which integrates natural history of multiple species with phylogeny can uncover evolution of social traits. Moreover, genomic and transcriptomic methods which currently undergo a strong progress, can help with the identification of genetic base of social and parental behavior.

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Unpublished manuscripts:

Manuscript A: Mikát, M., Straka J.: Overwintering strategy and longevity of European Small carpenter bees (*Ceratina*). Manuscript, submitted to *Journal of Ethology*

Manuscript B: Mikát., M., Waldhauserová., J., Fraňková, T., Čermáková, K., Brož, V., Zeman Š., Dokulilová, M., and Straka J.: Only mothers feed mature offspring in European *Ceratina* bees. Manuscript, submitted to *Insect Science*

Manuscript C: Mikát., M., Benda, D., Fraňková, T., and Straka J: Evidence of sociality in small Carpenter bees in Cyprus. Manuscript, submitted to *Apidologie*

Manuscript D: Mikát., M., Benda., D., Korittová, C., Mrozková, J., Reiterová, D., Waldhauserová, J., Brož, V., and Straka J., Natural history and maternal investment of *Ceratina cucurbitina*, the most common European small carpenter bee, in different European regions. Manuscrit, submitted to *Journal of Apicultural reseach*

Manuscript E: Mikát., M., Matoušková, E. and Straka J., Nesting of *Ceratina nigrolabiata*, a biparental bee. Manuscript, submitted to *Scientific reports.*

LIST OF ARTICLES AND MANUSCRIPTS

STUDY 1: Mikát, M., Černá, K., Straka, J., 2016. Major benefits of guarding behavior in subsocial bees: implications for social evolution. *Ecol. Evol.* 6, 6784–6797. https://doi.org/10.1002/ece3.2387

STUDY 2: Mikát, M., Franchino, C., Rehan, S.M., 2017. Sociodemographic variation in foraging behavior and the adaptive significance of worker production in the facultatively social small carpenter bee, *Ceratina calcarata*. *Behav. Ecol. Sociobiol*. 71, 135. https://doi.org/10.1007/s00265-017-2365-6

STUDY 3: Mikát, M., Janošík, L., Černá, K., Matoušková, E., Hadrava, J., Bureš, V., Straka, J., 2019b. Polyandrous bee provides extended offspring care biparentally as an alternative to monandry based eusociality. *Proc. Natl. Acad. Sci.* 116, 6238–6243. https://doi.org/10.1073/pnas.1810092116

STUDY 4: Mikát, M., Straka, J., 2019. Reply to Portman: Mate guarding, sib guarding, and biparental care in bees. *Proc. Natl. Acad. Sci.* 116, 9713–9713. https://doi.org/10.1073/pnas.1905235116

STUDY 5: Mikát, M., Benda, D., Straka, J., 2019a. Maternal investment in a bee species with facultative nest guarding and males heavier than females. *Ecol. Entomol*. 44 823-832 https://doi.org/10.1111/een.12768

STUDY 6: Mikát, M., Benda, D., Straka, J., 2020. Unrelated males in colonies of facultatively social bee. bioRxiv. Sumbmitted to *Insectes Sociaux*

STUDY 7 = Manuscrit A: Mikát, M., Straka J.,: Overwintering strategy and longevity of European Small carpenter bees (*Ceratina*). Submitted to *Journal of Ethology*

STUDY 8 = Manuscrit B: Mikát., M., Waldhauserová., J., Fraňková, T., Čermáková, K., Brož, V., Zeman Š., Dokulilová, M., and Straka J.: Only mothers feed mature offspring in European *Ceratina* bees. Submitted to *Insect Science*

STUDY 9 = Manuscrit C: Mikát., M., Benda, D., Fraňková, T., and Straka J: Evidence of sociality in small carpenter bees in Cyprus. Submitted to *Apidologie*

STUDY 10 = Manuscrit D: Mikát., M., Benda., D., Korittová, C., Mrozková, J., Reiterová, D., Waldhauserová, J., Brož, V., and Straka J., Natural history and maternal investment of *Ceratina cucurbitina*, the most common European small carpenter bee, in different European regions. Submitted to *Journal of Apicultural reseach*

STUDY 11 = Manuscrit E: Mikát., M., Matoušková, E. and Straka J., Nesting of *Ceratina nigrolabiata*, a biparental bee. Submitted to *Scientific reports*

STUDY 1

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Major benefits of guarding behavior in subsocial bees: implications for social evolution

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Abstract

Parental care is a behavior that increases the growth and survival of offspring, often at a cost to the parents' own survival and/or future reproduction. In this study, we focused on nest guarding, which is one of the most important types of extended parental care; we studied this behavior in two solitary bee species of the genus *Ceratina* with social ancestors. We performed the experiment of removing the laying female, who usually guards the nest after completing its provisioning, to test the effects of nest guarding on the offspring survival and nest fate. By dissecting natural nests, we found that *Ceratina cucurbitina* females always guarded their offspring until the offspring reached adulthood. In addition, the females of this species were able to crawl across the nest partitions and inspect the offspring in the brood cells. In contrast, several *Ceratina chalybea* females guarded their nests until the offspring reached adulthood, but others closed the nest entrance with a plug and deserted the nest. Nests with a low number of provisioned cells were more likely to be plugged and abandoned than nests with a higher number of cells. The female removal experiment had a significantly negative effect on offspring survival in both species. These nests frequently failed due to the attacks of natural enemies (e.g., ants, chalcidoid wasps, and other competing *Ceratina* bees). Increased offspring survival is the most important benefit of the guarding strategy. The abandonment of a potentially unsuccessful brood might constitute a benefit of the nest plugging behavior. The facultative nest desertion strategy is a derived behavior in the studied bees and constitutes an example of an evolutionary reduction in the extent of parental care.

Introduction

Parental care is a diverse life history trait that includes various types of behaviors (Tallamy and Wood 1986; Trumbo 2012) and that influences other life history characteristics of animals (Gilbert and Manica 2010). The three primary types of parental care are provisioning with food, offspring guarding, and building nests or shelters (Wilson 1971; Thiel 2000; Smiseth et al. 2012).

Parental care is crucial for offspring survival in certain species (Smiseth et al. 2012), while it only increases the fitness of the offspring in others (Martins et al. 1998; Mas and Kölliker 2008). Offspring are usually more dependent if the main form of care is provisioning rather than guarding (Smiseth et al. 2012). The dependency of the offspring on parental care is also affected by the age of the offspring, that is, the younger stages are more parent dependant (Coville and Griswold 1984; Eggert et al. 1998; Smiseth et al. 2003).

One of the most important features that affect the life history of species with respect to parental care is the trade-off between the length of care for the offspring and the number of offspring produced in a lifetime. Longer parental care causes a decrease in the number of offspring over time (Gross 2005; Smiseth et al. 2012; Kölliker et al. 2015). Similarly, when offspring are produced in separate clutches, the parents must decide whether it is more effective to care for the current clutch or to desert that clutch and establish a new one (Olmstead and Wood 1990; Mas and Kölliker 2008).

The aculeate Hymenoptera are one of the most important and most studied groups of invertebrates with parental care (Wilson 1971; Tallamy and Wood 1986; Linksvayer and Wade 2005). In nonkleptoparasitic aculeate Hymenoptera, the parental care typically consists of nest building and provisioning accompanied with nest guarding in certain species (Wilson 1971; Michener 1974). The offspring are provisioned in two primary modes in the aculeate Hymenoptera. The mass provisioners collect and supply a cell with all the necessary food for the young; then, they deposit an egg and close the cell. Typically, the adults do not interact with the offspring; therefore, guarding is not likely to be important for offspring survival (Strohm and Linsenmair 2000; Field 2005). In contrast, the progressive provisioners feed (and guard) their offspring regularly throughout larval development (Field and Brace 2004; Field 2005). Interestingly, there are a few exceptions to this scheme. For example, mothers stay with their offspring until they reach adulthood in certain dung beetles (Trumbo 2012), in eusocial mass-provisioning halictid bees (Michener 1974) and in solitary populations of *Halictus rubicundus* (Eickwort et al. 1996). A remarkable strategy of parental care is also found in the mass-provisioning genus *Ceratina* (Sakagami and Maeta 1977; Rehan and Richards 2010a).

Bees of the cosmopolitan genus *Ceratina* nest in dead stems or sticks with pith (Hogendoorn and Velthuis 1999; Michener 2007) and are either solitary or facultatively eusocial (Sakagami and Maeta 1977; Rehan et al. 2009). The ancestor of this genus (and of the entire Xylocopinae subfamily) was facultatively eusocial (Rehan et al. 2012). *Ceratina* bees guard their nests after the end of provisioning. The nest is guarded by either a single female (mother) or more than one adult female (mother and daughter) until the offspring reach maturity (Sakagami and Laroca 1971; Rehan and Richards 2010a). This guarding behavior is found in all the studied species of *Ceratina* bees (Rehan et al. 2010b). In addition, the guarding behavior is not only a passive strategy in *Ceratina* bees. Females are subsocial and typically crawl through the cell partitions in the nest to examine the offspring (Maeta et al. 1997; Rehan and Richards 2010a).

To test the importance of nest guarding, we performed an experiment where the guarding individual was removed from a nest, and the fate of the offspring in the unguarded nest was monitored. Removal experiments performed in certain invertebrates showed that the absence of the parents could result in an increased mortality of the offspring and a poorer growth (Eggert et al. 1998; Thiel 2000; Kölliker and Vancassel 2007; Werneck et al. 2012).

For the aculeate Hymenoptera, only a few experiments in which the guarding female(s) were removed were performed, primarily with eusocial (Smith et al. 2003) or communal species (Kukuk et al. 1998). Removal experiments were further used to test the role of males in species with biparental care (Coville and Griswold 1984) or in eusocial species with extended male care (Sen and Gadagkar 2006; Lucas and Field 2011). However, no removal experiments have been performed to test the role of the female mother in solitary aculeate Hymenoptera. Moreover, there are observational studies that compared the mortality of brood cells in guarded and naturally orphaned nests, but these studies have an insufficient number of observations and provide ambiguous results (Sakagami and Maeta 1977; Eickwort et al. 1996; Rehan et al. 2009).

The lack of relevant studies that examined the fate of orphaned nests is surprising because nest failure after orphaning is often argued to be one of the most important driving forces in the evolution of sociality. The insurance of offspring survival in the case of the death of the founder represents an important selection pressure for the maintenance of eusociality (Gadagkar 1990; Queller 1994) and for the existence of pleometrotic nest founding (Queller 1994; Queller et al. 2000). The probability of offspring survival after the death of the mother is also considered to affect the benefits obtained from the progressive provisioning (Field 2005).

In this study, we tested the importance of nest guarding for mass-provisioning *Ceratina* bees, which are solitary species with social ancestors. We demonstrated that there are two different strategies to protect the nests, which include an alternative to nest guarding that had not been previously recognized in *Ceratina* bees. We studied these different nesting strategies in two relatively distantly related species: *Ceratina cucurbitina* (Rossi) and *Ceratina chalybea* Chevrier (Fig. 1; Rehan et al. 2010a; Terzo 1998).

Materials and Methods

Study site

The observations and experiments were conducted in the Podyjí National Park, near Znojmo, in the Czech Republic. We received permission to perform the experiments in the National Park (NPP 0781/2011). Most of the data were collected in the locality of the Havranické vřesoviště heathland (48°48′33.595″N, 15°59′35.149″E), but additional data were collected at the Šobes locality (48°49′0.124″N, 15°58′37.708″E). The localities were both open grassy habitats, with solitary trees and shrubs. The study was performed between 2012 and 2014, from the end of May to the beginning of August. The studied species (*C. cucurbitina* and *C. chalybea*) are abundant in the studied localities. They naturally nest in broken twigs and stems of *Rosa canina*, *Centaurea* spp., *Linaria genistifolia*, *Verbascum* spp., and other plants with pith.

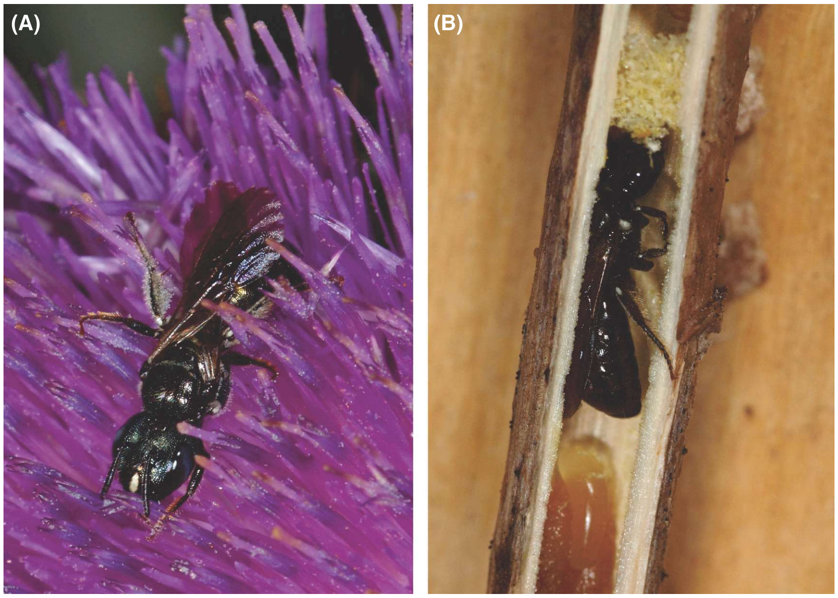

Figure 1. *Ceratina chalybea* collecting pollen from *Onopordum acanthium* (A), and *Ceratina cucurbitina* inside the nest (B). Photograph Lukáš Janošík.

Preparation of nesting opportunities

All the experiments were performed on nests that were artificially prepared as nesting opportunities. Sheaves consisting of 20 cut plant stems were distributed in the study site. Each stem was approximately 30–50 cm long, with an inner pith of more than 3 mm wide in the upper end (Fig. S1). We used stems of *Solidago* spp., *Helianthus tuberosus*, *Echinops sphaerocephalus,* and *Dipsacus sylvestris*. The sheaves were fixed to a rod for stabilization, and they were then fixed to the ground and placed in suitable nesting sites. These nesting opportunities were distributed in the studied localities in April before the beginning of the nesting season. We installed 2600 sheaves, which corresponded to 52,000 nesting opportunities.

Nest dissection

All the nests selected for dissection were collected after 7 p.m. CEST. By collecting the nests early in the evening, we ensured that all the inhabitants had returned to the nest and were inside because *Ceratina* bees are not active at this time (Herrera 1990). The collected nests were carefully opened with a knife in the field laboratory. The original nest structure and the following parameters were recorded in all the analyzed nests: stem substrate species, presence of guarding adult female, the presence and number of living offspring, and the presence of natural enemies. We considered nests as attacked by a focal enemy when we observed such enemy species in the nest at the time of dissection. In the case of nest usurpation, we marked those nests as usurped when discarded pollen or a low number of fresh cells with eggs were observed in the nest.

Description of the guarding strategy

This study was performed in 2012 and 2013. We selected approximately 30 sheaves and dissected all the nests in them every 7–9 days from mid-June to mid-August. In total, we selected 360 sheaves for this experiment; however, not all sheaves contained nests. All the nests from the selected sheaves were dissected. Additional natural nests were collected in 2012; the substrates were *Rubus* spp., *Artemisia* spp., and *Helianthus tuberosus*.

Only nests with full brood and an undisturbed inner structure were used for description of guarding strategy. Nests that contained a larva or a pupa in the outermost cell in the line were considered as full brood nests (Rehan and Richards 2010a,b; Vickruck et al. 2011).

We recorded the presence and position of the guarding female.

We used 60 nests of *C. cucurbitina* (26 in 2012 and 34 in 2013) and 201 nests of *C. chalybea* (14 in 2012 and 187 in 2013) for description of guarding strategy of species.

Comparison of guarding strategies of *C. chalybea*

In *C. chalybea*, we discovered two alternative guarding strategies – nest guarding versus nest abandonment. For comparison of these strategies, we used only data from 2013 (187 nests from 114 sheaves). To compare these strategies, we recorded presence of guarding female, architecture of last brood cell (open vs. closed), length of nest, number of brood cells, number of brood cells with living offspring, and number of brood cells with parasitized offspring.

Phenology of *C. chalybea*

The analysis of the phenology was performed in 2013. For this analysis, 108 sheaves were selected, but only 74 contained *C. chalybea* nests. Each sheaf was regularly controlled between 10 May 2013 and 25 July 2013. The sheaves were controlled every 1–3 days. New nests and the content of older nests were recorded using a pocket flashlight during each control. Confirmed by a later nest dissection, we could reliably observe and determine the content of the first 4 or 5 cm of depth in the nests. The founding date of a *C. chalybea* nest was stated as either a) the date of the first observation of a new nest, where the presence of *C. chalybea* was subsequently recorded, or b) the date of the first observation of a *C. chalybea* female in a nest where another species of insect was the owner of the nest before a *C. chalybea* female was regularly observed. The date of nest plugging was the first date when a plug was observed and the female disappeared. In total, we used 133 observed nests in this analysis, of which 44 were plugged nests.

Removal of the guarding female

This experiment was performed in the nesting seasons of 2013 and 2014 (from the end of June to the beginning of August). The nests and sheaves used for this experiment were different from the nests used for the analysis of the guarding strategy. For this experiment, we selected 500 sheaves in 2013 and 700 sheaves in 2014 for the analysis, but only in some sheaves, we found nests in appropriate stage.

Nests of *C. cucurbitina* or *C. chalybea* with a guarding female at the nest entrance were selected. The guarding female was pulled from the nest. The nest was visually inspected by shining a light inside the nest entrance, and only completely provisioned nests were used for the experiment. A completely provisioned nest of *C. chalybea* was characterized by either a visible partition or the presence of pollen or a larva at the nest entrance. A completely provisioned nest of *C. cucurbitina* was distinguished by a visible partition near the nest entrance.

When a nest was appropriate for this experiment, the guarding female was either removed (two-thirds of the cases) or was returned to the nest to serve as a control (one-third of the cases). To evaluate the influence of nest guarding on the nest success, we performed gradual dissections of all the experimental nests. The dissections were performed at 3, 8, or 20 days after the treatment, using one-third of the nests in each interval. Based on our preliminary results, the interval between the end of provisioning and the emergence of the first adult juveniles took approximately 20 days, which we considered as the approximate duration for the complete provisioning of nests.

The female was removed in 140 nests of *C. chalybea* (72 in 2013 and 68 in 2014) and 208 nests of *C. cucurbitina* (123 in 2013 and 85 in 2014). We used 72 nests of *C. chalybea* (35 in 2013 and 37 in 2014) and 108 nests of *C. cucurbitina* (62 in 2013 and 46 in 2014) as controls. In total, 212 nests from 174 sheaves in *C. chalybea* and 315 nests from 210 sheaves in *C. cucurbitina* were used.

For comparison of mortality in outermost and second outermost cells, we used only nests in which these cells were preserved (nest partitions were not disturbed). Therefore, we used only subset of nests for this analysis: 155 nest from 134 sheaves for *C. chalybea* and 150 nest form 124 sheaves in *C. cucurbitina*.

Comparison between unguarded *C. chalybea* nests and nests with the female removed

We performed an analysis for evaluating the proportion of dead offspring in unguarded *C. chalybea* nests to decide whether it was the cause or the consequence of nest abandonment by the female. For this analysis, we used nests of *C. chalybea* with undisturbed structure from 2013 (50 nests from 45 sheaves) and unguarded full brood nests, which we observed and for which we noted a plug date. The nests were dissected within 30 days from plugging (22 nests from 16 sheaves).

Data analyses

The statistical analyses were conducted in the program R 3.1.0 (R Development Core Team, 2011). When we have more nests from one sheaf, we used mean values per sheaf in all linear models or generalized linear models.

We performed these analyses:

1. Association between guarding and nest architecture of *C. chalybea*: chi-square test.
2. Comparison of nests features between guarded and unguarded *C. chalybea* nests. Explanatory variable was in all cases presence of guarding female. We performed five analyses for different dependent variables. We used linear model for length of nest and length of nests entrance, poison generalized linear model for number of provisioned cells, and number of live offspring and binomial generalized linear model for proportion of cells parasited by chalcidoid wasps. Model equation: response ~ guarding strategy.
3. Comparison of nest founding date between guarded and unguarded *C. chalybea* nests: Binomial generalized linear model. We used guarding as dependent variable and date of nest founding as explanatory variable.

Model equation: guarding strategy ~ date, family = binomial.

4 Influence of female removal to nest fate: We used binomial generalized linear model. We tested these explanatory variables: year, difference between data of removing and dissecting, treatment (removing of female vs. control), and all double interactions. We performed analyses for these dependent variables: at least one live offspring in nest, the presence of ants in nest, the presence of chalcidoid wasp, nest usurpation, live offspring in outermost cell, and live offspring in second outermost cell. Model equation: response ~ (year + date difference + treatment)^2, family = binomial.

5 Comparison of nest features between nests with removed female and plugged nests: We used binomial generalized linear model. We used these explanatory variables: time of nests abandonment and treatment (removing vs. control). We tested these response variables: proportion of live offspring and proportion of chalcidoid wasps. Model equation: response ~ date difference*treatment, family = binomial

Results

Guarding strategy of *C. cucurbitina*

Full brood nests of *C. cucurbitina* were guarded by the mothers in 58 of the 60 examined nests (96.6%). The cell partitions in the nest of this species were relatively more fragile than those in the nests of *C. chalybea*. The adult females were occasionally observed inside the nests around the brood cells as if they were inspecting them (5.1% of the nests with the owner female, 6 of 117 nests). This behavior agrees with the observation that excrements typically accumulate at the bottom of the nest. Moreover, no case of social nesting and male presence in the nests was recorded; however, we observed females feeding adult offspring with pollen.

Guarding strategy of *C. chalybea*

Ceratina chalybea had two alternative types of nest protection (Fig. 2, Table 1). The nest was either guarded by females sitting by the outermost cell or was left unguarded and closed with a plug formed with pith fillings. This nest plug was similar to the material used for the nest partitions, but it was thicker and was approximately 1.5 cm in length. A strong association between the architecture of the outermost cell and the presence of a guarding mother was detected (chi-square test, $\chi^2 = 56.8228$, df = 1, $P < 0.0001$, Table 1). These differences in nest architecture did not reflect the stage of ontogeny of the nests. All the stages of offspring (larva, pupa, or young adult) were found in the outermost cells in both types of architectures. Therefore, the nest plugging represented an alternative guarding strategy in this species.

A mother of *C. chalybea* was never observed inside the nest beyond the outermost cell partition. Excrements of larvae remain inside the brood cells, and they are not removed to the bottom of the nest. The cell partitions remained undisturbed until the offspring matured and were more compact and thicker than the partitions of the *C. cucurbitina* nests. No case of social nesting was recorded, but a male was found in the nest entrance of full brood nests in a few cases (3%, 6 nests of 201).

Differences between guarded and unguarded nests of *C. chalybea*

Guarded nests are more common than unguarded ones (112 guarded nests of 187 nests). Several important characteristics differentiated the guarded and plugged nests (Table 1). For example, the guarded nests were significantly longer (linear model, $F = 24.26$, df = 1, $P < 0.0001$; Table 1) and had a higher number of provisioned cells (Poisson's GLM, deviance = 21.83, df = 1, $P < 0.0001$; Table 1, Fig. 3) than the plugged nests. The guarded nests also had a higher number of live offspring

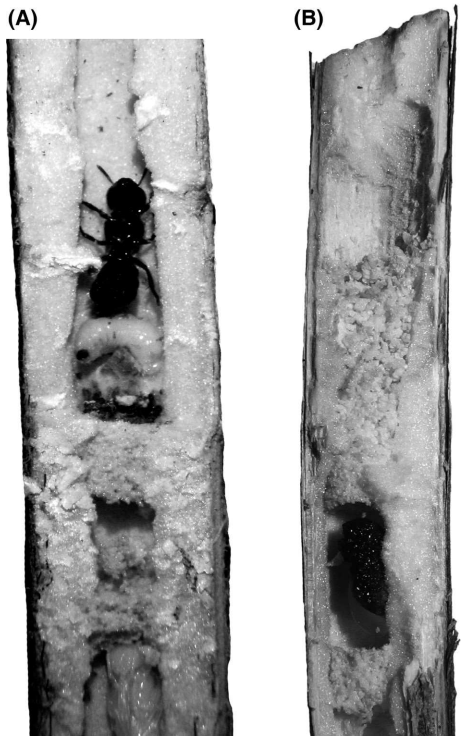

Figure 2. Comparison of the nest architecture of guarded (A) and plugged (B) *Ceratina chalybea* nests.

Table 1. Characteristics of guarded and plugged *Ceratina chalybea* nests (season 2013).

	Unguarded nests	Guarded nests	Together
Total number of nests	75	112	187
Number of nests with an unclosed outermost cell	2	106	108
Number of plugged nests	73	6	79
Chi-square test	χ^2 = 56.8228. df = 1. $P < 0.0001$		
Length of nest (cm)			
Mean	19.18	23.51	21.77
Maximum	31.4	34.7	34.7
Minimum	5.9	8.2	5.9
Standard deviation	5.21	5.66	5.87
Linear model	F = 24.26, df = 1, $P < 0.0001$		
Length of the nest entrance (cm)			
Mean	2.81	4.33	3.72
Maximum	17.8	14.6	17.8
Minimum	0.5	1.0	0.5
Standard deviation	2.50	2.15	2.41
Linear model	F = 17.531, df = 1, $P < 0.0001$		
Number of provisioned cells			
Mean	4.33	6.79	5.80
Maximum	8	11	11
Minimum	1	2	1
Standard deviation	1.56	2.11	2.25
Poisson's GLM family	Deviance = 21.828, residual deviance = 55.391, df = 1 $P < 0.0001$		
Number of cells with live offspring			
Mean	1.29	4.73	3.35
Maximum	8	11	11
Minimum	0	1	0
Standard deviation	1.69	2.410	2.73
Poisson's GLM family	Deviance = 95.487, residual deviance = 143.85, df = 1, $P < 0.0001$		
Proportion of parasitized cells by Chalcidoid wasps			
Mean	0.10	0.07	0.08
Maximum	1.00	0.88	1.00
Minimum	0.00	0.00	0.00
Standard deviation	0.23	0.16	0.19
binomial GLM family	Deviance = 0.0001, residual deviance = 30.9, $P = 0.9928$		

(Poisson's GLM, deviance = 95.45, df = 1, $P < 0.0001$; Table 1, Fig. 3). On the other hand, there is no significant difference in proportion of cells parasitized by chalcidoid wasps (binomial GLM, deviance = 0.0001, df = 1, $P = 0.993$).

Phenology of nest founding and plugging in *C. chalybea*

Ceratina chalybea founded new nests predominantly between May and mid-June; however, a lower frequency of nest founding was observed in the second part of June and throughout July (Fig. S2). The nest plugging began by mid-June and had the maximum occurrence between June 20 and 23. The plugging continued through the last week of June and throughout July with a lower frequency. After mid-June, the numbers of newly founded and plugged nests were similar (Fig. S2).

There was no significant difference between guarded and plugged nest in date of nest founding (binomial GLM, deviance = 0.2226, df = 1, $P = 0.6371$). However, late-founded nest (after June 20) were plugged very rarely.

Effect of removal of the guarding female

When the guarding female was removed, the decrease in offspring survival was highly significant in both species (binomial GLM, *C. cucurbitina*: deviance = 39.18, df = 1, $P < 0.0001$, *C. chalybea*: deviance = 10.92, df = 1, $P = 0.0009$; Table 2 and Fig. 4).

The probability of nest failure increased with the time spent without a guarding female (Fig. 4, Table 2). Twenty days after the experimental treatment, all the offspring died in 21.6% (8 of 37) of the control nests, while this percentage was 81.4% (57 from 70) in the nests where the female *C. cucurbitina* was removed. All the offspring died in 3.9% (1 of 26) of the control nests and in 53.1% (25 of 47) of the nests where the *C. chalybea* female was removed (Fig. 4).

The probability of offspring survival in the outermost cell (unclosed cell with guarding female) was significantly affected by the removal of the female in *C. chalybea* (binomial GLM, deviance = 50.62, df = 1, $P < 0.0001$, Table 2). In contrast, the mortality of the second outermost cell (standard cell without contact between mother and offspring) was not significantly affected by the female removal in *C. chalybea* nests with an undisturbed structure (binomial GLM, deviance = 1.81, df = 1 $P = 0.2456$; Table 2). Conversely, in *C. cucurbitina*, the mortality of the outermost and the second outermost cell was affected by the treatment (Table 2).

Reasons of failure of unguarded nests

The most important natural enemies were the chalcidoid parasitoids, predatory ants, and other bees that usurped the nests (Figs 5 and 6). In general, the occurrence of these enemies was more frequent and had a stronger effect on *C. cucurbitina* than on *C. chalybea*. All natural enemies were more common in nests with guarded female than in control nests both in *C. cucurbitina* and in *C. chalybea* (Fig. 6, Table S1), but differences were not always significant. In *C. cucurbitina*, significant difference

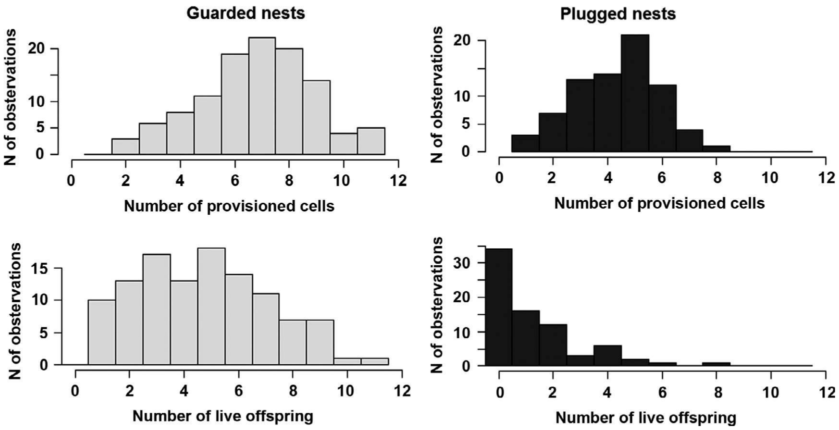

Figure 3. Comparison of guarded and plugged *Ceratina chalybea* nests with regard to the number of provisioned cells and the number of live offspring. Light gray bars represent guarded nests; dark gray bars represent plugged nests.

was in the presence of chalcidoid wasps (binomial GLM, deviance = 10.53, df = 1, $P = 0.0012$; Table 2) and ants (binomial GLM, deviance = 11.30, df = 1, $P = 0.0009$; Table 2). Difference in usurpation frequency was not significant (binomial GLM, deviance = 1.2, df = 1, $P = 0.2741$; Table 2). On the other hand, in *C. chalybea* was significant difference only in nest usurpations (binomial GLM, deviance = 6.18, df = 1, $P = 0.0129$; Table 2), but not in the presence of chalcidoid wasps (binomial GLM, deviance = 0.57, df = 1, $P = 0.4516$; Table 2) and ants (binomial GLM, deviance = 2.17, df = 1, $P = 0.134$; Table 2).

We recorded the presence of other natural enemies; yet, their frequency was too low to be statistically assessed. The numbers of all the recorded natural enemies are shown in Table S1.

Comparison between plugged nests and nest with removed female

We compared the mortality of brood cells between nests with removed females and natural plugged nests in *C. chalybea*, and the results showed that there was marginally significant difference (binomial GLM, deviance = 2.81, df = 1 $P = 0.0932$; Table S2). Difference in proportion of cells parasited by chalcidoid wasps was not significantly differ (binomial GLM, deviance = 1.31, df = 1, $P = 0.2524$).

Discussion

Role of nest guarding for the offspring survival

The guarding of a completely provisioned nest by a parent is obviously highly beneficial for the offspring because it increased the offspring survival in both species of *Ceratina* bees. The primary reasons for the destruction of unguarded nests were the different natural enemies, which were significantly more common in unguarded nests than in control nests of *C. cucurbitina*. In *C. chalybea* nests, the enemies were also more common in nests with the female removed; however, only the usurpation of the nests by other bees had a statistically significant effect. Natural enemies, which cause predation and parasitism pressure, are important drivers in the evolution of extended parental care in general (Wilson 1975). We attribute the observed differences between species to a different nest structure and a different guarding strategy in each species.

The positive effects of nest guarding on offspring survival are known in other nest-making Hymenoptera; however, the current evidence is scarce. In a study by Coville and Griswold (1984), nests with a complete brood that were unguarded by a male of *Trypoxylon superbum* were destroyed by ants; however, the number of unguarded nests was extremely low (only two nests). The survival of the offspring was also significantly reduced after the removal of guarding female(s) in the eusocial *Megalopta genalis* (Smith et al. 2003) and in the communal *Lasioglossum hemichalceum* (Kukuk et al. 1998). The offspring survival was significantly correlated with the presence of a guarding female in the solitary populations of *Halictus rubicundus* (Eickwort et al. 1996). However, this latter study was only observational, and the absence of a female can be the consequence, not the cause, of the offspring death. This outcome is similar to the results of the low offspring survival and other tested features in the plugged *C. chalybea* nests of our study. The three published studies on *Ceratina* bees show ambiguous results. While the study of Sakagami and Maeta (1977) on Japanese *Ceratina* species showed a higher brood cell mortality in orphaned nests, the study of Rehan et al.

Table 2. Effects of the removal of the guarding female on nest survival and natural enemy occurrence. This table shows the results of a binomial GLM. The interaction among factors is marked by "***.**" Significant effects are in bold.

Variable	df	Deviance	Residual Df	Residual deviance	*P*-value
Ceratina cucurbitina, dependent variable: at least one live offspring					
NULL			209	229.79	
Year	1	0.16	208	229.63	0.6855
Date difference	1	22.14	207	207.49	**<0.0001**
Treatment	1	39.18	206	168.31	**<0.0001**
Year *date difference	1	0.79	205	167.52	0.3736
Year*treatment	1	1.70	204	166.45	0.3012
Date difference*treatment	1	0.14	203	166.31	0.7081
Ceratina cucurbitina, dependent variable: presence of chalcidoid wasps					
NULL			209	154.00	
Year	1	0.19	208	153.81	0.6669
Date difference	1	2.03	207	151.28	0.1116
Treatment	1	10.53	206	140.85	**0.0012**
Year*date difference	1	0.63	205	140.22	0.4258
Year*treatment	1	3.66	204	136.94	0.0703
Date difference*treatment	1	2.60	203	134.51	0.1193
C. cucurbitina, dependent variable: presence of ants					
NULL			209	120.64	
Year	1	0.99	208	119.65	0.3200
Date difference	1	2.50	207	117.00	0.1039
Treatment	1	11.30	206	105.97	**0.0009**
Year*date difference	1	1.10	205	104.96	0.3148
Year*treatment	1	0.00	204	104.96	0.9895
Date difference*treatment	1	0.14	203	104.82	0.7112
C. cucurbitina, dependent variable: nest usurped					
NULL			209	77.11	
Year	1	0.46	208	76.65	0.4964
Date difference	1	0.03	207	76.62	0.8741
Treatment	1	1.20	206	75.43	0.2741
Year*date difference	1	2.98	205	72.44	0.0842
Year*treatment	1	0.93	204	71.51	0.3341
Date difference*treatment	1	0.05	203	71.46	0.8204
C. cucurbitina, dependent variable: live offspring in outermost cell					
NULL			123	122.45	
Year	1	1.54	122	120.80	0.1996
Date difference	1	5.30	121	115.78	**0.0249**
Treatment	1	4.00	120	111.77	**0.0453**
Year*date difference	1	1.69	119	110.53	0.2661
Year*treatment	1	0.75	118	109.78	0.3879
Date difference*treatment	1	5.77	117	104.13	**0.0174**
C. cucurbitina, dependent variable: live offspring in second outermost cell					
NULL			123	111.23	
Year	1	3.78	122	107.38	**0.0498**
Date difference	1	21.90	121	86.29	**<0.0001**
Treatment	1	12.97	120	74.06	**0.0004**
Year*date difference	1	0.70	119	73.36	0.4026
Year*treatment	1	0.42	118	72.94	0.5187
Date difference*treatment	1	2.20	117	70.64	0.1292
C. chalybea, dependent variable: at least one live offspring					
NULL			173	151.19	
Year	1	0.07	172	151.12	0.7962
Date difference	1	19.91	171	131.21	**<0.0001**
Treatment	1	10.92	170	120.28	**0.0009**
Year*date difference	1	0.20	169	120.08	0.6570

Table 2. Continued.

Variable	df	Deviance	Residual Df	Residual deviance	*P*-value
Year*treatment	1	0.28	168	119.81	0.5990
Date difference*treatment	1	2.22	167	117.39	0.1196
C. chalybea, dependent variable: presence of chalcidoid wasps					
NULL			173	152.75	
Year	1	0.14	172	152.61	0.7059
Date difference	1	8.82	171	143.74	**0.0029**
Treatment	1	0.57	170	143.18	0.4516
Year*date difference	1	1.12	169	141.22	0.1613
Year*treatment	1	0.38	168	140.83	0.5359
Date difference*treatment	1	0.42	167	140.41	0.5176
C. chalybea, dependent variable: presence of ants					
NULL			173	78.96	
Year	1	4.16	172	74.03	**0.0265**
Date difference	1	2.06	171	71.96	0.1503
Treatment	1	2.17	170	69.31	0.1034
Year*date difference	1	0.67	169	68.62	0.4037
Year*treatment	1	1.20	168	67.59	0.3117
Date difference*treatment	1	0.54	167	67.06	0.4640
C. chalybea, dependent variable: nest usurped					
NULL			173	91.15	
Year	1	2.39	172	88.70	0.1172
Date difference	1	0.65	171	88.05	0.4215
Treatment	1	6.18	170	81.88	**0.0129**
Year*date difference	1	0.04	169	81.83	0.8352
Year*treatment	1	1.86	168	80.47	0.2438
Date difference*treatment	1	0.66	167	79.82	0.4174
C. chalybea, dependent variable: live offspring in outermost cell					
NULL			133	137.47	
Year	1	0.02	132	137.44	0.8828
Date difference	1	0.99	131	136.49	0.3278
Treatment	1	50.62	130	85.87	**<0.0001**
Year*date difference	1	0.18	129	85.69	0.6709
Year*treatment	1	0.37	128	85.31	0.5416
Date difference*treatment	1	1.51	127	83.80	0.2185
C. chalybea, dependent variable: live offspring in second outermost cell					
NULL			133	120.08	
Year	1	14.54	132	105.54	**0.0001**
Date difference	1	3.13	131	101.77	0.0521
Treatment	1	1.81	130	100.42	0.2456
Year*date difference	1	1.99	129	98.59	0.1749
Year*treatment	1	0.02	128	98.55	0.8636
Date difference*treatment	1	2.35	127	96.04	0.1129

(2009) on *C. accusator* found no effects of the female disappearance. However, only a small sample size was used in the latter study. Daly et al. (1967) observed a higher attack of chalcidoid wasps but not Ichneumonidae in orphaned nests than in guarded nests. In addition, all these studies were based on only observations from nest dissections, and they did not register the number of total destroyed nests because they were not considered as *Ceratina* nests. According to the literature, the primary reason for the failure of unguarded nests is the occurrence of ant raids (Coville and Griswold 1984; Kukuk et al. 1998; Smith et al. 2003). Likewise, ants are most likely the primary reason for the nest failure of *Ceratina* bees. Furthermore, the effect of ant assaults was probably underestimated in our data because the ants moved away after their raid and left the nest completely cleaned from debris and cell partitions, which makes their detection difficult.

Although we used nests in artificial nesting opportunities for our experiments, we assume that there are no differences with local natural nests in the general pattern

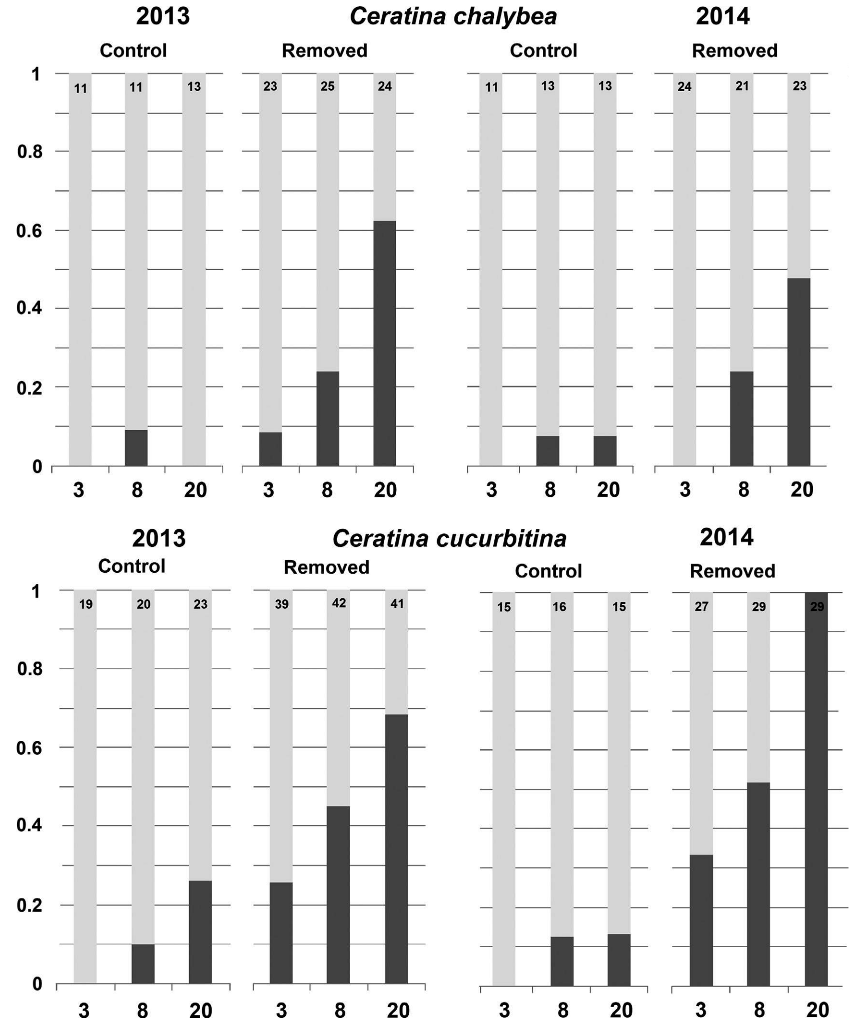

Figure 4. Proportion of survival of control nests and nests with the female removed of *Ceratina chalybea* and *Ceratina cucurbitina*. Light gray columns represent nests with at least one surviving offspring; dark gray columns represent nests with no surviving offspring. The numbers below the columns represent the number of days since the beginning of the treatment to the nest dissection. The numbers on the top of the columns represent the number of nests examined.

and effect of enemies. Our artificial nests were in the immediate vicinity of natural nests, and bees and their enemies were not introduced. The observed enemies were those that usually attack *Ceratina* nests (Daly et al. 1967; Sakagami and Maeta 1977).

Comparison of the guarding strategy in *C. cucurbitina* and *C. chalybea*

The strategy of nest defense is different between *C. cucurbitina* and *C. chalybea*, where guarding was obviously more important for *C. cucurbitina*. Interestingly, *C. cucurbitina* exhibited an obligate nest guarding and subsocial behavior, which consisted of crawling through the nest, as described in detailed studies of other *Ceratina* species (Rehan et al. 2010b). This behavior most likely protects the nest from various natural threats such as parasitism by chalcidoid wasps.

In contrast, *C. chalybea* females guard the nest only facultatively and choose between two alternative strategies, that is, they either guard the nest or fill the nest entrance with a plug and desert it. The guarding strategy is closely associated with nest architecture; guarded nests have an unclosed outermost cell in almost all the cases and, therefore, the mother is in physical contact with last offspring. In contrast, unguarded nests have a filling plug in almost all the cases (Table 1, Fig. 2). We exceptionally observed a few guarded nests with a filling plug; however, these nests were probably recently completed, and the females had not finished filling the plug and had not yet left the nest. We also observed a few unguarded nests with an unclosed outermost cell. Such nests were probably abandoned due to the death of the mother.

To the best of our knowledge, the described nest deserting behavior in *C. chalybea* represents the first example of an alternative guarding strategy within the genus *Ceratina*. Although unguarded nests were previously found in other species, these nests were most likely orphaned after the death of the mother (Sakagami and Maeta 1977; Rehan et al. 2009).

Figure 5. Examples of nests that were attacked by natural enemies after removal of female *Ceratina chalybea*: nest attacked by predator(s), most likely ants or Dermaptera (A), nests with cell parasitized by a chalcidoid wasp (B), and nest usurped by another *C. chalybea* female, with the offspring from the first female discarded (C).

We never observed *C. chalybea* females crawling through nest partitions, and the larval excrements were always left in the cells. This pattern shows that females of *C. chalybea*, in contrast to other species, cannot crawl through the nest partitions and, therefore, cannot be in physical contact with the offspring (except for the outermost one). The higher offspring mortality in plugged nests than in guarded nests and the insignificant difference in offspring mortality between plugged nests and nests with a female removed suggest that females may also be effective against enemies. However, removing the guarding female did not have a significant influence on the mortality of the second outermost (closed) cell and on preventing the chalcidoid parasitation.

The guarded nests of *C. chalybea* have a relatively unique nest structure compared to those of other species. While the guarding female of *C. cucurbitina* (and other *Ceratina* species with known nest structure) sits on the nest entrance separated from the outermost offspring by a cell partition, the outermost cell in the guarded nests of *C. chalybea* is open, which enables a direct contact between the mother and the offspring in the outermost cell. However, no contact between the mother and the offspring in the internal cells is possible. For this reason, we consider *C. chalybea* as partially subsocial because of the direct contact of a female with a single offspring in a guarded nest, even though no contact occurs with internally positioned offspring or with offspring in plugged, deserted nests. In general, the offspring in the open cell suffered less mortality than offspring in the internal, closed cells. For instance, it was never parasitized with chalcidoid wasps when the guarding female was present.

In general, the guarding strategy of *C. cucurbitina* is more effective for the offspring survival; however, in the case of the absence of the female, the offspring are more vulnerable than in *C. chalybea*.

Benefits and costs of guarding and deserting nests

The benefit of nest guarding is an apparently high offspring survival. We suppose that the benefit of deserting a nest is the possibility of founding a second nest elsewhere. We did not observe the fate of females that plugged and deserted nests. However, they most likely tried to found new nests. Females that plugged their nests finished them from mid-June to July 2013. In this period, the frequency of new nest founding was similar as frequency of newly plugged nests (Fig. S2). Females that founded nests in July usually had damaged wings (M. Mikát, K. Černá and J. Straka, unpubl. obs.), which indicates that they had already been highly active and had probably founded a nest elsewhere. There was not any significant difference in date of nest founding between guarded and plugged nests, which shows that probably only some females deserted from their first nest and try to found the second nest.

We found that guarded nests had a significantly higher number of provisioned cells and live offspring than plugged nests (Table 1, Fig. 3). The lower number of provisioned cells in plugged nests indicates that *C. chalybea* females deserted less valuable nests with a higher probability. To terminate the investment in an unpromising offspring or clutch is an adaptive behavior known in other animal species (Olmstead and Wood 1990; Manica 2002). However, a question remains to be answered: Can a *C. chalybea* female assess the survival of her offspring and desert nests with higher offspring

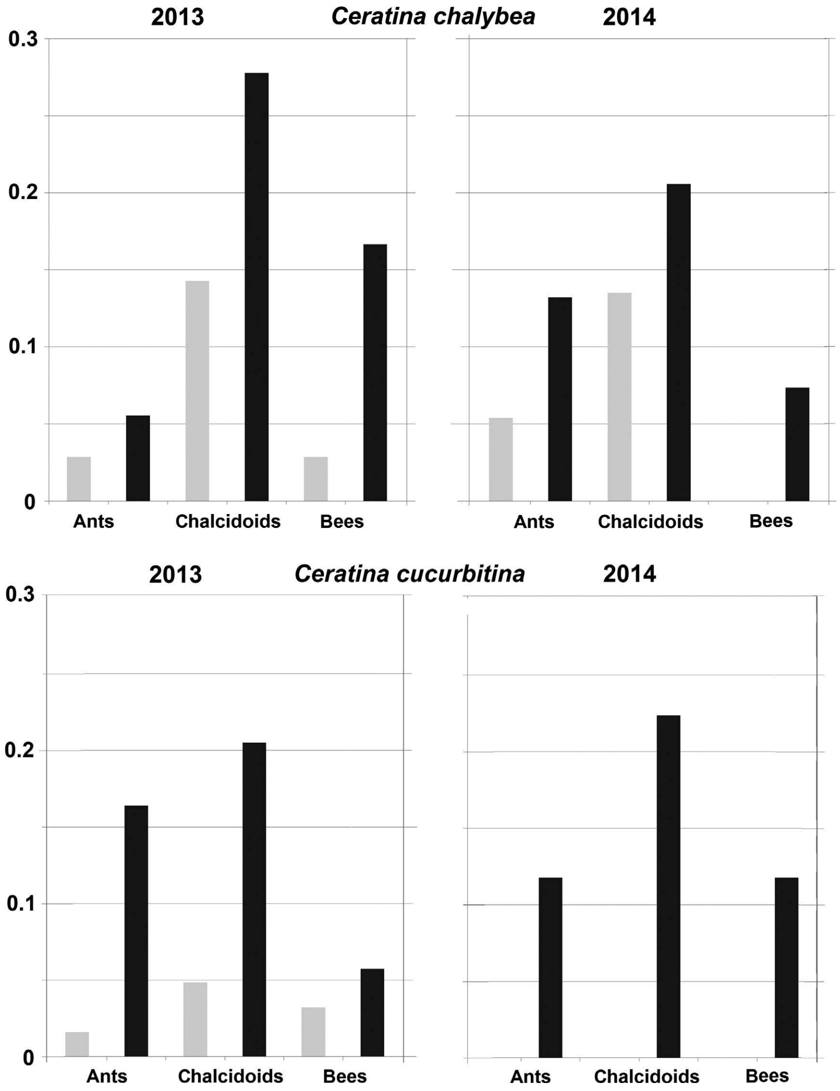

Figure 6. Proportion of *Ceratina chalybea* and *Ceratina cucurbitina* nests attacked by natural enemies. Light gray columns represent the control nests; dark gray columns represent the nests where the female was removed.

mortality or is the higher mortality only the result of the deserting strategy? The nonsignificant differences in offspring mortality and parasitism by chalcidoid wasps between plugged nests and nests with the removed female suggest that a higher mortality in plugged nests in comparison with guarded nests is the result rather than the cause of deserting.

Evolution of the extent of parental care in *Ceratina* bees

Parental care typically tends to increase in complexity (Smiseth et al. 2012; Trumbo 2012). However, *Ceratina* bees are an example of a reduction in the extent of parental care throughout their evolution. Eusociality was lost in certain lineages of the genus *Ceratina* (Rehan et al. 2012) and, at least in *C. chalybea*, the extended maternal care further decreased by the reduction of the nest guarding activities and the loss of the ability to crawl through the cell partitions to inspect the brood.

Data Accessibility

All the primary data were uploaded and are available as online supporting information.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Installing of artificial nesting opportunities in the Havraníky heathland (A).

Figure S2. Phenology of nest founding and nest plugging in *C. chalybea* in season 2013.

Table S1. Recorded natural enemies of *Ceratina* bees in nests with removed female and in control nests.

Table S2. Results of binomial GLM models comparing nests with removed females and plugged nests.

STUDY 2

Mikát, M., Franchino, C., Rehan, S.M., 2017. Sociodemographic variation in foraging behavior and the adaptive significance of worker production in the facultatively social small carpenter bee, *Ceratina calcarata*. *Behavioral Ecolology and Sociobiology* 71, 135. https://doi.org/10.1007/s00265-017-2365-6

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ORIGINAL ARTICLE

Sociodemographic variation in foraging behavior and the adaptive significance of worker production in the facultatively social small carpenter bee, *Ceratina calcarata*

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Abstract
Provisioning for young offspring is an archetypical form of parental investment. *Ceratina calcarata* bees provide extended maternal care to their young and demonstrate an unusual strategy of dual-phase pollen provisioning. Most bees first gather provisions as they establish nests in spring. However, *C. calcarata* mothers will also feed their newly eclosed young a second time, perhaps ensuring their survival during a long winter diapause. Some mothers rear a small, worker-like daughter to assist them during this second provisioning phase. We studied provisioning behavior in *C. calcarata* to examine patterns of maternal investment and foraging dynamics throughout the breeding season. Mothers typically made a high number of short-duration foraging trips each day, whereas late-season females tended to make fewer and longer trips. This difference in foraging duration may indicate a lower risk of brood loss in those nests where mature offspring are present. Nest demographic data revealed that an offspring laid in the first brood cell position is typically female and usually smaller than her siblings. In 29% of the nests, this small daughter was observed to adopt a forager role at maturity and provisioned for her siblings. Dwarf daughters had a higher number of active days and foraging trips per day in orphaned nests than in nests where a mother was present. The foraging behaviors of worker-like daughters were similar in length of foraging trip and handling time to mothers during this second provisioning period. We hypothesize that incipiently social foraging by this smallest daughter may act as a form of insurance against brood loss during occasions when a mother is unable to sufficiently provision for her eclosed offspring during the second phase.

Significance statement
Parental investment in the size and sex of offspring is under strong selection for assured fitness returns. For example, many social insect mothers make an initial investment in small offspring to take on risky foraging behavior while they specialize on future reproduction. Solitary and facultatively social species provide an important baseline to understand the evolution of social complexity from natural variation in maternal care and foraging behavior. Here, we characterize the parental investment strategies of a subsocial small carpenter bee and reveal the potential adaptive significance of prolonged maternal care and worker production in this species. Mothers provide an initial investment that is extended by workers providing alloparental care to siblings. Maternal manipulation of dwarf eldest daughters may serve as an insurance mechanism in the event of maternal mortality to assure the survival of siblings.

Keywords Hymenoptera · Parental investment · Sex allocation · Foraging ecology · Maternal manipulation · Overwintering survival

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Introduction

Offspring provisioning is one of the main forms of parental investment (Tallamy and Wood 1986; Mas and Kölliker 2008). In Hymenoptera, brood provisioning may be an almost constant activity, and one that is highly costly (Willmer and Stone 2004; Morato and Martins 2006). There are two main provisioning strategies in bees: mass provisioning, when all supply of food for young offspring is collected before hatching of egg; and progressive provisioning, in which parents feed offspring directly (Field 2005; Michener 2007). When mass provisioning, a parent can optimize resource allocation among multiple brood, allowing her to decide exactly how much pollen and nectar to distribute to each individual offspring (Michener 1974; Danforth 1990). These brood investments are easily assessed, either through measurement of the provision mass (Johnson 1988; Tomkins et al. 2001; Lawson et al. 2016) or the body mass of a mature offspring (Strohm and Linsenmair 1999; Bosch and Vicens 2002; Rehan and Richards 2010a). As a mother invests more in any individual offspring, she effectively increases that offspring's probability of survival (Cowan 1981; Strohm and Linsenmair 2000). Life-history theory predicts that there is an optimal means of dividing resources among offspring, such that a balance is struck regarding the tradeoff between brood count and brood body size in any given species (Smith and Fretwell 1974).

Mothers may also optimize their brood investment by carefully controlling the allocation of brood sex (Charnov 1982). At a total population level, sex investment is expected to be an equal male to female ratio (Fisher 1930). Most animal species are sexually dimorphic, and optimal brood investment may vary between male and female offspring (Charnov 1982; Fisher 1930). This optimization strategy is especially common in Hymenoptera (Molumby 1997; Rehan and Richards 2010a), in which mothers can directly control which sex they will produce through selective insemination of their eggs (Gerber and Klostermeyer 1970).

In social Hymenoptera, offspring composition and subsequent resource allocation may be strongly influenced by intracolony interactions (Crozier and Pamilo 1996; Bourke 2011). Eusocial species feature a female-biased brood sex ratio due to the fact that female offspring typically develop into sterile or subfertile workers (Wilson 1971; Michener 1974; Schwarz et al. 2007). It is also thought that sex ratio investment in eusocial Hymenoptera is female biased if workers control investment, because workers are more closely related to female than to male siblings (Trivers and Hare 1976). The social roles of individual females in primitive and advanced eusocial species are strongly influenced by nutritional cues during development (Wilson 1971; Michener 1974). It is thus thought that maternal investment, which involves the carefully controlled provisioning of each offspring, likely played an important role in the evolution of sociality (Alexander 1974; Craig 1983), because for subfertile individuals, it can be beneficial to adopt a worker role (West-Eberhard 1975). Dominance hierarchies in social insects are frequently determined by body size; during social interactions, it is normally the smaller individual which assumes a subordinate position (Smith et al. 2009). In temperate species, however, small body size may also reduce an individual's capacity to survive the lengthy winter season (O'Donnell 1998). Temperate *Ceratina* small carpenter bees are normally solitary, but a handful of species are known to also produce a particularly small daughter in the first brood cell position: *C. calcarata* (Johnson 1988; Rehan and Richards 2010a), *Ceratina dupla* (Vickruck 2010), *Ceratina flavipes* (Maeta et al. 1992; Sakagami and Maeta 1977), *Ceratina japonica* (Sakagami and Maeta 1984), and *Ceratina okinawa* (Maeta and Sakagami 1995).

Provisioning behavior in many species of *Ceratina* is atypical among the subsocial and solitary bees, as they perform both mass and progressive provisioning (Sakagami and Maeta 1977; Lewis and Richards 2017). During brood establishment, female *Ceratina* undertake mass provisioning, as may be considered typical of mothers of many bee species (Michener 2007). Later in the season, however, *Ceratina* begin foraging again, progressively provisioning their mature offspring by pollen and probably nectar before the end of the blooming season (Sakagami and Maeta 1977; Lewis and Richards 2017). It is unknown whether this two-period provisioning behavior is universal to *Ceratina*, though it has been documented in temperate species around the globe: *C. japonica*, *C. flavipes* (Sakagami and Maeta 1977), *C. calcarata* (Lewis and Richards 2017), and *C. cucurbitina* (Mikát et al. 2016). Progressive provisioning of mature offspring has also been observed in the closely related genus *Xylocopa* (Richards and Course 2015), but is otherwise uncommon in insects (Wilson 1971; Tallamy and Wood 1986). It is currently thought the second round of feeding helps to ensure that offspring survive a protracted overwintering period before establishing new nests the following spring (Lewis and Richards 2017). Though late-season progressive provisioning in *Ceratina* is normally performed by the mother, this task may also be undertaken by the small, eldest daughter (Sakagami and Maeta 1977; Rehan et al. 2014; Lewis and Richards 2017). It is hypothesized that mothers underfeed their eldest daughter to constrain her development and induce a worker-like role (Maeta et al. 1992; Rehan and Richards 2010a; Lawson et al. 2016); as is seen in the brood manipulation of some primitively eusocial Hymenoptera (Alexander 1974; Kapheim et al. 2011).

The aims of this study are threefold. First, we quantify the foraging and nesting behavior of the North American small carpenter bee species, *C. calcarata*. Second, we describe the prolonged maternal care and worker production in this species. Third, we characterize the role dwarf eldest daughters to serve as alloparental workers and an insurance mechanism in the event of maternal mortality to assure the survival of siblings.

Methods

Study species

The small carpenter bee *C. calcarata* (Robertson 1900) belongs to the family Apidae and subfamily Xylocopinae. This species is broadly distributed across the eastern region of the USA and Canada (Rehan and Sheffield 2011; Shell and Rehan 2016). Once a female establishes her nest within the pithy core of a broken twig, she forages on a wide range of flowering plants (Lawson et al. 2016; McFrederick and Rehan 2016). Active brood nests are always solitary, with only one provisioning female (Rehan and Richards 2010b). Females have two phases of foraging (Lewis and Richards 2017), which are separated by period in which mother guard fully provisioned nest with larvae and pupae (Rehan and Richards 2010b). This species is univoltine and females produce only one nest in their lifetime (Rehan and Richards 2010b; Vickruck et al. 2011). Young individuals of both sexes overwinter then mate and establish new nests in spring (Rehan and Richards 2010b).

Nest establishment and relocation

Nest collections and behavioral observations were performed at the University of New Hampshire in Durham, New Hampshire, USA. In April of 2016, habitable stems were created from raspberry (*Rubus idaeus*) by cutting dry stalks, attaching each to a 40-cm-long bamboo rod, and using this rod to stake the structure in the ground at two fields near this location. *Ceratina* nest establishment was regularly monitored at both farms by visually inspecting the stems at 2-day intervals between May 8 and June 27, and then during weekly intervals between June 27 and July 22. Nest founding was determined by the observation of a small (~ 4 mm), round hole and loose pith in the exposed core of the dead broken stems. Each founded nest was marked with flagging tape and assigned a unique ID, and the date of establishment was recorded.

For observing foraging behavior, we relocated and aggregated nests from original sites to plots with 30 nests. It is known that relocation of nests does not distort female behavior (Lewis and Richards 2017). Relocation was performed in early morning (until 7:00) to ensure that all individuals were inside the nest. We performed primary establishment of foraging plots on May 31; however, we added some later-founded nests after this date to our plots. Therefore, we observed in total 129 nests through the season, however, only some of them (31) for whole season (Table S1).

Nest observations

To begin each observation day, we enclosed each observed nest entrance with a small transparent plastic cup, which prevented bees from departing or returning without first being recorded. When a bee left the nest, she entered the cup and the time was recorded, the cup was then opened to allow the individual to continue foraging. The cup was then placed back over the entrance, such that when the bee returned, she was unable to go inside and therefore she flew around the twig containing her nest. The individual's time of return was recorded and the cup was removed, allowing her to re-enter the nest. On some occasions, an individual returned after only a very brief departure (2–10 s). These observations were removed from the dataset as they were considered highly unlikely to be foraging flights.

From late July onwards, the maturing offspring started to emerge and become active foragers, raising the possibility of multiple bees foraging from a single nest. We thus began recording the age and sex of foraging individuals, and giving each a unique paint mark on the thorax and abdomen. Age was assessed according to wing wear and complexion—mothers have darker wings and extensive wing wear by this point in the season, while the wings of young adults are clear and undamaged (Rehan and Richards 2010b).

We collected behavioral data from approximately 60 nests during each observation day with an average of 10 observation days per nest (range 1–20). Two people (MM and CF) observed the nests together; therefore, each observed 30 nests. Foraging observations were performed 3 or 4 days per week pending suitable conditions. A different set of 60 nests was observed each day, and no nest was observed for two consecutive days. Observations were performed only on sunny or partly cloudy days, as bees are largely inactive during overcast or rainy periods. Nest observations began at 8:00 each day, before females started foraging activity. Observations normally ended at 16:00. Not all bees returned by 16:00; however, in late afternoon, foraging activity strongly decreased between 15:00 and 16:00 with very little foraging activity (7.5% of all departures at this period). Observations were terminated in the event of unexpected rain or overcast skies (usually between 14:00 and 15:45). Length of each foraging trip (difference between time of departure and time of arrival), length of handling time (difference between time of arrival and next departure), and number of foraging flights per day for all mothers and any foraging daughters in each nest were recorded.

Nest dissections

We removed a minimum of 10 randomly selected nests for dissection each week from June 16 to September 15. Additional nests were taken directly from the field at the peak of full brood nest activity (July 20–August 2). Nests were collected before 7:30 or after 19:00 and entrances were sealed with masking tape to ensure that all occupants were present during nest assessments. Nests were briefly stored in a cold room to pacify adult bees and prevent destruction of nest structure; nests were then split longitudinally with a pocket-knife to record developmental stage according to contents (Rehan and Richards 2010b). Newly founded nests contained only a clean pith tunnel without cells, provisions, or partitions; actively brooding nests contained provisions and an egg in the last closed cell; full brood nests contained larvae and pupae but no newly laid eggs; full-mature brood nests contained newly eclosed adults alongside larvae or pupae; and mature brood nests contained only mature bees. Only full brood nests ($N = 100$) were used for analyses of maternal investment, as these nests represented the entirety of any mother's reproductive efforts.

For each nest, we recorded the number and developmental stage of all offspring. Each mother was then measured for head width using a Nikon SMZ800 dissecting scope with mounted Unitron 15854 LED light. Head width was measured as the maximum transectional length between the outer edges of compound eyes viewed face-on. Head width is a reliable proxy of body mass in this species (Rehan and Richards 2010b). We also determined the sex and measured the head width of each juvenile adult offspring. Any offspring collected in egg, larval, or pupal stages were reared to adulthood in lab and assessed for sex and head width upon eclosion. Though some offspring died before eclosing, sex and head width (size) was determined from pupae. We assumed that pupal head width is the same as the head width of resulting adults.

Data analyses

All statistical analyses were performed using R (R Development Core Team 2011). Differences in the observed sex ratio from the expectation from 1:1 were tested by chi square tests. Sexual dimorphism was tested by Wilcoxon rank sum test, because the size distribution of males and females was significantly different from normal (as determined by Shapiro-Wilk tests). Linear mixed models were employed to test factors influencing the length of foraging trips and handling time, with repeated measures on the same nests. Dependent variables were log transformed before analysis. Nest identity and date of observation were included as random factors.

Results

Sex and size of offspring

Of the 761 offspring collected from full brood nests, 401 were male and 360 were female. Although sex ratio was slightly male-biased (53% male), it was not significantly different from the expectation of an equal 1:1 sex ratio (Chi = 2.2089, df = 1, $p = 0.14$). Sex ratio was highly variable between nests. Individual offspring sex was significantly associated with brood cell position. The sex of offspring in the first brood cell was significantly female biased, with males occupying the first brood cell in only 24% (21/86) of nests (Chi square test, $N = 86$, $X^2 = 22.512$, df = 1, $p = 0.00002$). In other brood cells, the sex ratio was not significantly different from 1:1 except for the fourth brood cell position, in which there was a significant male bias (Table S2).

There was a notable difference in body size between male and female offspring as measured by head width: female body size (mean ± SD, 1.99 ± 0.12 mm, $N = 360$) was significantly larger than male body size (1.79 ± 0.09 mm, $N = 401$; Wilcoxon test, $N = 761$, df = 1, W = 114,830, $p < 0.000001$). The mean head width of female offspring in first brood cell position was 1.88 ± 0.10 mm ($N = 65$) and the mean head width of males in first brood cell was 1.70 ± 0.10 mm ($N = 21$). The mean head width for offspring in other cell position than first was 2.02 ± 0.12 mm for females ($N = 295$) and 1.79 ± 0.09 mm for males ($N = 380$). Female offspring in first brood cells were significantly smaller than the average for females in other brood cells from the same nest (Paired t test, $N = 65$, $t = 7.8961$, $p < 0.00001$). The difference in body size between first brood cell males and their male siblings, by comparison, was not significant (Paired t test, $N = 21$, $t = 0.62083$, $p = 0.5425$).

Phenology of foraging behavior

During the cell-provisioning period (May 8 to June 27), mothers were usually active in a high proportion of nests (average 80%; Fig. 1). The highest proportion of active nests was observed in mid-June (June 20), when 100% of the nests were active. During the summer foraging break period (June 28 to July 26), only a small proportion of the nests demonstrated any foraging activity (average 12% of nests). The proportion of nests with actively foraging mothers increased sharply from the end of July through early August, but steadily decreased from August 3 through the end of the season (Fig. 1). In an average day of the mature offspring feeding period, 47% of nests had mother foraging activity. In comparison with mothers, daughters foraged in only 11% of the nests on average in same period. Peak daughter activity was observed on September 1, when offspring were observed foraging in 29% of nests. Young adult bees began leaving their natal nests

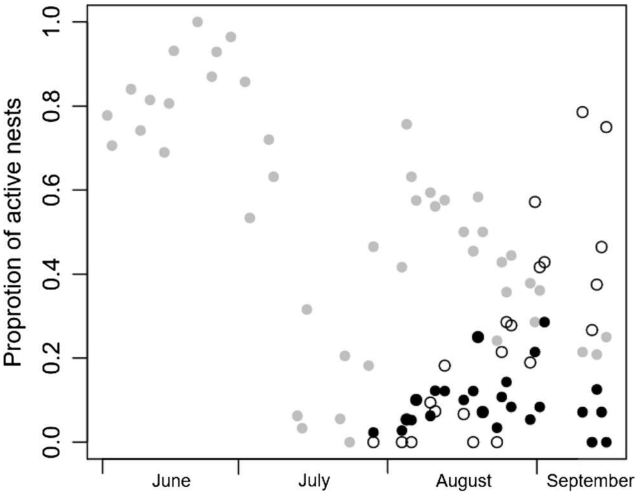

Fig. 1 Season changes in proportion of active nests. Each point represents the daily proportion of nests with foraging activity. Gray symbol–mother, black closed symbols–daughter, black open symbols–leaving young females

without returning at the start of the mature offspring feeding period (July 27), and the proportion of nests abandoned by offspring strongly increased after August 30. Observed offspring nest abandonment reached a peak on September 9, when at least one offspring left 79% of the nests.

During the mature offspring feeding period (July 27–September 1), there was extensive variability in pattern of foraging activity between individual nests. From nests observed for this entire period (*N* = 52), the mother was active at least once in 81% (42/52) of nests, daughters were active in 29% (15/52), and two nests (4%) exhibited foraging behavior by daughters only. In 15% of the nests (8/52), instead of foraging activity, only nest abandonment events were observed during the mature brood feeding period.

Foraging activity of mothers

The highest average maternal foraging activity was observed during the cell-provisioning period (6.12 trips per day; Fig. 2). At this stage, the most active nests had more than 20 foraging trips per day, with the highest recorded number of 35 trips in 1 day. During the summer break period, by contrast, most nests were inactive and those which foraged made few trips per day (0.23 on average, Table 1). Frequency of foraging trips increased again during the mature offspring feeding period, with an average of 1.73 maternal foraging trips per day. Excluding nests without any foraging behavior, the average number of daily foraging trips was 3.54 for active nests. The highest recorded frequency of foraging trips was 15 during this second provisioning period. During the nest abandonment period, the foraging frequency decreased again, sinking to an average of 0.44 foraging trips per day.

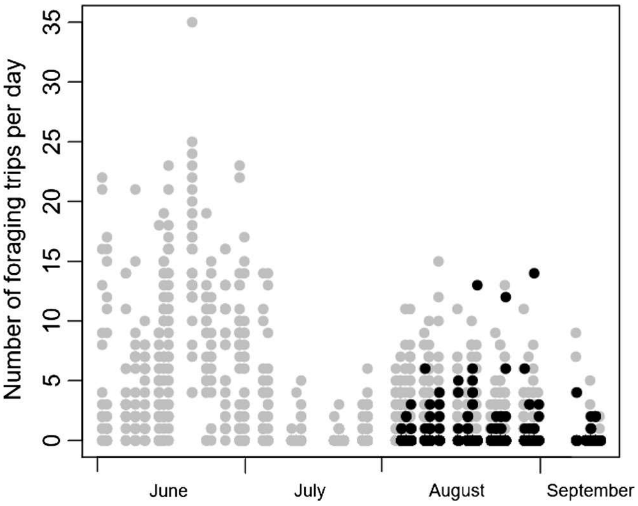

Fig. 2 Number of foraging trips per day for observed mothers (gray) and daughters (black)

The duration of foraging trips was also highly variable, and fluctuated over the course of the season (median 16.88 min, range 0.18–306.8 min). Though most trips were only a few minutes long, there was a highly significant difference between trip duration during the cell-provisioning and mature-offspring feeding periods (mixed linear model, chi = 108.42, df = 1, $p < 0.000001$), with a difference of 23.17 min between periods (95% CI: 16.91–30.62 min). Though fewer in number, foraging trips made during the mature-offspring feeding period generally lasted longer than in the cell-provisioning period (Fig. 3, Table 2). Length of handling time was highly variable (median 11.47 min, range 0.4–279.88 min), but usually lasted only a few minutes. The length of handling time was significantly shorter in the cell-provisioning period than in the mature offspring feeding period (mixed linear model, chi = 11.883, df = 1, $p = 0.00057$), with a difference of 6.11 min (95% CI: 2.45–10.70 min).

Taken together, the number of foraging trips is higher in the cell-provisioning period; however, the length of foraging trips is longer in the mature offspring feeding period. Therefore, this puts into question: in which period do mothers spend more time outside of the nest? Active mothers spent on average 159.0 min per day outside of their nests across the entire active season (range 1.83–597.25 min). In the cell-provisioning period, mothers spent 149.1 min outside the nest on average (median 145.10 min, range 2.61–429.93 min). During the mature-offspring feeding period, however, mothers spent significantly more time outside the nest than during the cell-provisioning period (mean 179.1 min, median 174.96 min, range 5.30–597.25 min; mixed linear model, chi = 4.65, df = 1, $p = 0.0309$), with a difference of 36.55 min (95% CI: 2.81–80.78 min).

Maternal nest orphanage rates also increased throughout the course of the season. Incidence of orphaned nests strongly increased in the first half of August, and more than half of the

Table 1 Number of foraging trips per day of mother and daughter

Observed female	Period	All observational days included			Only days with activity included		
		N	Mean	SD	*N*	Mean	SD
Mother	Cell provisioning	405	6.09	6.12	325	7.59	5.94
	Summer break	185	0.23	0.72	23	1.83	1.15
	Offspring feeding	615	1.73	2.50	300	3.54	2.53
	Leaving	89	0.44	1.44	12	3.25	2.56
	Total	1294	2.79	4.49	660	5.47	4.99
Daughter	Offspring feeding	615	0.26	1.20	56	2.89	2.87
	Leaving	89	0.13	0.57	6	2.00	1.10
	Total	704	0.13	0.85	62	2.81	2.76

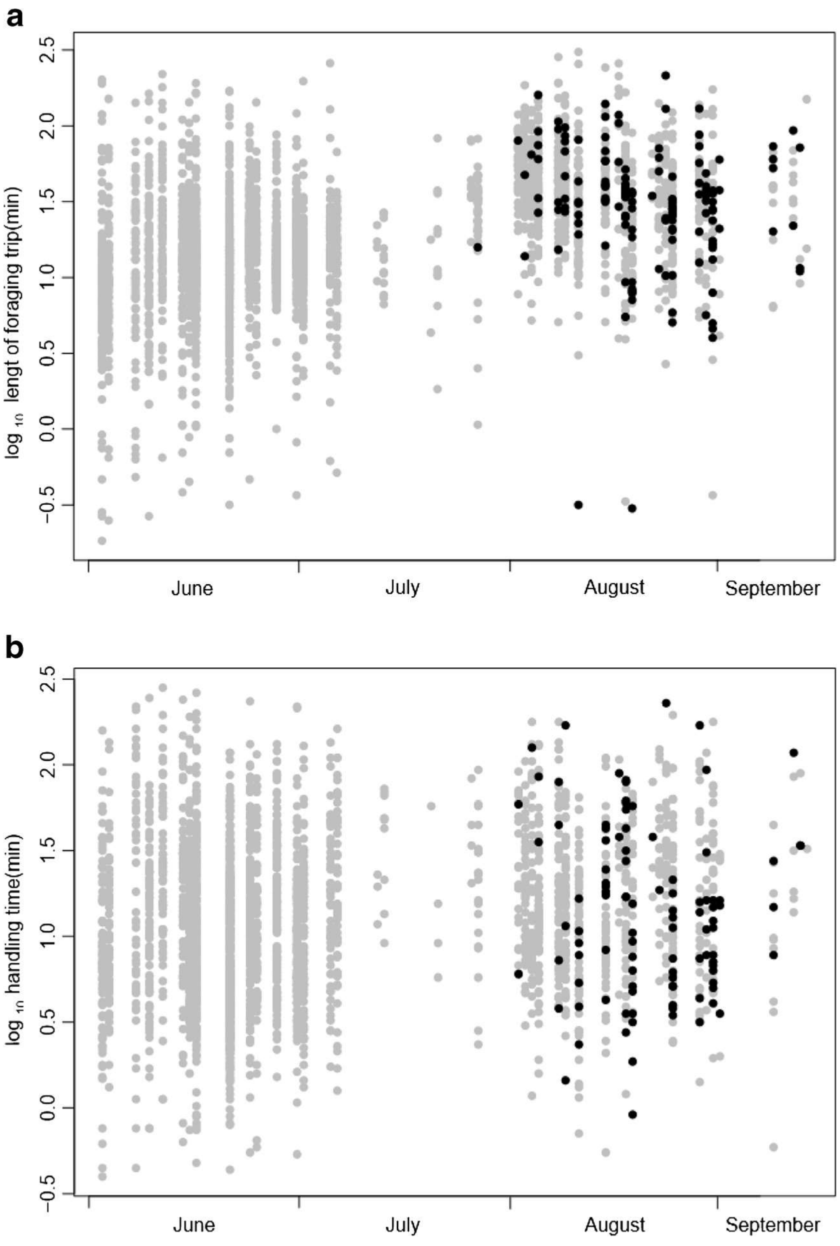

Fig. 3 Length of foraging trips (**a**) and length of handling time (**b**) throughout the season. Both length of foraging trips and handling time are in logarithmic scale; gray points–mother, black points–daughter

Table 2 Quantitative parameters of foraging flight duration and handling times between flights for *Ceratina calcarata*

Focal female	Period	Length of foraging trip (min)				Length of handling time between foraging trips (min)			
		N	Mean	SD	Median	*N*	Mean	SD	Median
Mother	Cell provisioning	2216	18.88	21.08	13.84	1962	19.83	28.31	10.28
	Summer break	37	24.31	20.52	17.27	20	35.78	24.28	28.46
	Mature feeding	885	45.74	35.76	37.42	687	25.34	27.63	15.32
	Leaving	22	40.45	30.82	38.83	16	25.62	26.44	17.15
	All periods	3160	26.61	28.75	16.88	2685	21.39	28.22	11.47
Daughter	Mature feeding	131	38.54	29.71	31.50	94	25.28	36.25	11.33
	Leaving	9	46.24	30.76	52.75	6	39.17	39.75	30.48
	All periods	140	39.03	29.72	31.53	100	26.12	36.41	12.57

remaining nests were orphaned by late August. By the end of the foraging season, in mid-September, nearly all (93%) nests were orphaned.

Foraging activity of dwarf daughters

A daughter was active in 29% of the nests (15/52) that we observed during the whole mature-offspring feeding period. In six of these nests, a dwarf daughter joined her active mother in foraging. In seven nests, the daughter's foraging began following the last day of her mother's foraging. Dwarf daughters were significantly more active, with more foraging trips per day, when operating alone than in those nests in which they were actively foraging with their mother (Poisson generalized linear mixed model, Chi = 5.2129, df = 1, $p = 0.02$). Two nests featured late-season foraging activity only by the daughter, with no contributions from the mother. In two other nests, we observed two different daughters foraging on separate days. In almost all cases, foraging daughters were the smallest female offspring in their nest.

Foraging daughters made an average of 0.26 trips per day during the entire mature-offspring feeding period. However, considering only those nests for which at least one foraging flight was made per day, this average increased to 2.89 trips per day (Table 2). The highest number of daughter foraging trips made on a single day was 14. During the nest abandonment period, there was a decrease of daughter foraging activity to just 0.11 trips per day (Table 1).

Notably, the foraging behavior of active daughters closely mirrored the foraging behavior of maternal foragers in the mature-offspring feeding period (Fig. 3). There was neither difference in length of foraging trips between mothers and daughters (mixed linear model, chi = 0.6309, df = 1, $p = 0.43$), nor was there any difference in the length of handling time between foraging mothers and daughters (mixed linear model, chi = 1.5856, df = 1, $p = 0.21$).

Discussion

Parental investment in the size and sex of offspring is under strong selection for assured fitness returns (Smith and Fretwell 1974). For example, many social insect mothers make an initial investment in small offspring to take on risky foraging behavior while they specialize on future reproduction (Trivers and Hare 1976). Solitary and facultatively social species provide an important baseline to understand the evolution of social complexity from natural variation in maternal care and foraging behavior (Sakagami and Maeta 1977; Schwarz et al. 2007). Here, we characterize the parental investment strategies of a subsocial small carpenter bee and reveal the potential adaptive significance of prolonged maternal care and worker production in this species. Mothers provide an initial investment that is extended by workers providing alloparental care to siblings. Maternal manipulation of dwarf eldest daughters serves as an insurance mechanism in the event of maternal mortality to assure the survival of siblings. Insurance-based mechanisms are key factors that can explain the origin and maintenance of sociality in small insect societies (Field et al. 2000; Shreeves et al. 2003).

Foraging behavior of dwarf daughters

Across all nests, the first brood cell was strongly female biased, and females in this position were generally smaller than other females. The existence of a dwarf eldest daughter has been repeatedly reported for *C. calcarata* across North America (Johnson 1988; Rehan and Richards 2010a; Rehan et al. 2014; Lawson et al. 2016; Lewis and Richards 2017) and in Japanese *Ceratina* species (Sakagami and Maeta 1977, 1984).

We observed a daughter foraging actively in about one quarter of the nests during the mature-offspring feeding period. In general, daughters foraged less frequently than mothers. However, unlike Lewis and Richards (2017), we occasionally observed daughters foraging at the same time as their mothers. As daughter foraging was more common when a mother was absent, we suppose that this behavior may represent a form of insurance in those cases where a mother dies before she is able to feed her mature offspring. The proportion of orphaned nests increased through the mature-offspring feeding period, with dissections indicating extremely high proportions of motherless nests (93%). As such, most mothers probably do not survive until the end of the foraging season in mid-September. The foraging behaviors of active daughters do not appear different from those of foraging mothers, with no significant difference in the duration of foraging trips or handling time between trips. Therefore, it seems foraging daughters may adopt an effective alloparental role, despite clear morphological differences between themselves and their mother, with the head width of mothers 13% larger than their dwarf daughters on average. Daughter foraging may thus be an adaptive solution to situations wherein the death of a mother, which may not be uncommon during the late-season feeding period, would otherwise lead to mature offspring having to undertake risky foraging in order to avoid starvation. When we dissected nests with foraging daughters (n = 8), the forager was the smallest daughter in all instances. Two nests featured two foraging daughters and these individuals were the first and second smallest of the brood. Lewis and Richards (2017) similarly found that foraging was performed by females which were smaller than most other females in nest. Female body size relative to her siblings can strongly influence her probability of assuming a forager role.

Adaptive significance of dwarf daughters

The existence of a dwarf eldest daughter may at first seem paradoxical: during her initial brood provisioning, a mother is presumably in her best physical condition and should therefore be expected to invest in larger offspring (Seidelmann 2006). Her decision to produce significantly smaller offspring instead of normal size offspring may have several explanations, such as foraging more in order to produce larger offspring could lead to higher mortality, or a limitation on the amount of carried food rather than mature oocytes in the early nesting stage (Maeta et al. 1992). However, lower nutritional quality of available pollen is not likely for this polylectic species (Lawson et al. 2016). Under such scenarios, the more beneficial decision might be to invest in the cheaper sex (i.e., male) rather than produce malnourished females (Trivers and Willard 1973; Frank 1987). The persistent production of small females thus suggests the existence of an adaptive social function.

The proximate mechanism for producing a small first daughter is thought to be maternal manipulation of brood provisions (Lawson et al. 2016), as parental manipulation of offspring size is a well-known mechanism for hierarchical stratification of insect societies (Alexander 1974). Further, it may be an optimal investment strategy to produce a dedicated, small, forager-destined female, which costs less than her larger siblings. However, it remains to be determined what ultimate mechanism and possible fitness advantage induces the adoption of foraging and feeding behavior in these small daughters.

One possible explanation for foraging by the smallest daughters is their lower probability of winter survival. Population wide, average female body size is smaller before winter, which suggests a lower survivorship for smaller females in this species (Rehan and Richards 2010b). Foraging by small females also puts them at risk of dying from exhaustion or predation; and it is thus possible that a difference in average female body size before and after winter may be a consequence rather than cause of sibling feeding. If dwarf eldest daughters have low probability to surviving to become future reproductives, then it may be advantageous to help ensure their siblings' overwintering survival and fitness by assisting in feeding. Maeta et al. (1992) experimentally tested cold tolerance in *C. flavipes* and determined a relatively weak influence of body size on offspring survival in cold exposure for both males and females. However, reduced fat stores have been linked to reduced overwinter survivorship in bees and wasps (Strassmann et al. 1984; Toth et al. 2009; Durant et al. 2016, but see Richards and Packer 1996; Weissel et al. 2012). Therefore, it seems that the reduced body size of foraging daughters may already have a negative influence on winter survivorship, which is likely only exacerbated by her increased foraging activity and reduced fat stores.

Size-influenced dominance hierarchies are well documented in several primitive eusocial Hymenoptera (Hogendoorn and Velthuis 1999; Smith et al. 2009). Size-based hierarchies are not always present in *Ceratina* (Rehan et al. 2010), however, with some species demonstrating an atypical division of foraging behavior between a reproductively dominant female and a non-foraging, subordinate female (Rehan et al. 2010); though more common divisions of worker roles are observed as well (Sakagami and Maeta 1984; Maeta and Sakagami 1995). *C. calcarata* is a solitary nesting species in the brood cell provisioning phase (Rehan and Richards 2010b) with social ancestors (Rehan et al. 2012, 2013). As size-dependent aggression is known in *C. calcarata* (Rehan and Richards 2013; Withee and Rehan 2016), it is possible that foraging by small daughters in social nests may be derived from a more typical division of labor.

Daughter foraging occurs in only some nests (29%), and is thus apparently a facultative strategy. As we observed in two nests, foraging behavior may be adopted by at least two different daughters, and is thus not limited to only one female offspring. Also, as females from the first brood cell position were usually, but not always, the smallest female in the nest, eldest female offspring are probably not obligately required to feed their siblings. Production of a small female in the first brood cell may operate as insurance against brood loss in the not uncommon event that a mother dies before the end of the second feeding period.

Conclusion

Foraging behavior in *C. calcarata* is highly flexible and appears dependent on the needs of offspring in the nest. This behavioral plasticity thus appears advantageous to the mother, particularly when considering the small number of brood cells provisioned per day, and increasing likelihood of nest orphanage with seasonal progression. Mother and dwarf daughter foraging behavior during the mature-offspring feeding period is very similar, meaning daughters likely make highly effective alloparents in situations where their services are useful (usually in orphaned nests, but sometimes also in nests where mothers remain present). We propose that foraging by the dwarf daughter is primarily a form of insurance against late-season starvation in the case of maternal mortality. If produced, a dwarf female is usually present in the first brood cell of her nest, but the forager role is not limited to this female, and may be adopted by at least one other small female. It is possible that flexible foraging behavior in an ancestral species allowed for the formation of this unique type of parental care, which may adaptively combine maternal and sibling care to ensure the survival of young adults.

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Compliance with ethical standards

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STUDY 3

Mikát, M., Janošík, L., Černá, K., Matoušková, E., Hadrava, J., Bureš, V., Straka, J., 2019b. Polyandrous bee provides extended offspring care biparentally as an alternative to monandry based eusociality. *Proceedings of National Academy of Sciences of United States of the America* 116, 6238–6243. **https://doi.org/10.1073/pnas.1810092116**

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Polyandrous bee provides extended offspring care biparentally as an alternative to monandry based eusociality

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Parental care behavior evolves to increase the survival of offspring. When offspring care becomes complicated for ecological reasons, cooperation of multiple individuals can be beneficial. There are two types of cooperative care: biparental care and worker (helper)-based care (e.g., eusociality). Although biparental care is common in several groups of vertebrates, it is generally rare in arthropods. Conversely, eusociality is widespread in insects, especially the aculeate Hymenoptera. Here, we present a case of biparental care in bees, in *Ceratina nigrolabiata* (Apidae, Xylocopinae). Similar to eusocial behavior, biparental care leads to greater brood protection in this species. Male guarding increases provisioning of nests because females are liberated from the tradeoff between provisioning and nest protection. The main benefit of parental care for males should be increased paternity. Interestingly though, we found that paternity of offspring by guard males is extraordinarily low (10% of offspring). Generally, we found that nests were not guarded by the same male for the whole provisioning season, meaning that males arrive to nests as stepfathers. However, we show that long-term guarding performed by a single male does increase paternity. We suggest that the multiple-mating strategy of these bees increased the amount of time for interactions between the sexes, and this longer period of potential interaction supported the origin of biparental care. Eusociality based on monandry was thought to be the main type of extended brood protection in bees. We show that biparental care based on polyandry provides an interesting evolutionary alternative.

biparental care | social behavior | paternity | mating frequency | bees

In biparental care, females and males cooperate in the care of offspring (1, 2). Several aspects of offspring care can influence the evolution of biparental care: the synergistic effect of male and female care (3), the inability of one parent to care for offspring effectively (4), or care that has similar costs and benefits for both sexes (5, 6). Biparental care is relatively common among vertebrates: it is the dominant type in birds (1) and frequently occurs in mammal, amphibian, and fish lineages (7). Conversely, biparental care is very rare among arthropods, with only a few known examples, including cockroaches from the genus *Cryptocercus,* as well as burying, passalid, and bark beetles (2, 4, 8). Biparental care has also been documented in crabronid wasps of the genus *Trypoxylon* (subgenus *Trypargillum*) belonging to the aculeate Hymenoptera (9, 10). Generally, in this group of insects, females perform nest provisioning, while males perform nest guarding and help with nest construction (10, 11). Among the aculeate Hymenoptera, male participation in offspring care is rare (2, 12). On the other hand, the occurrence of extensive female care is common, and there have been several origins of eusociality (12–14).

Male and female mating strategies are strongly related to the type of parental care (15, 16). Males, in contrast to females, usually benefit more from an increased number of copulation events than from substantial investment in offspring care (17, 18). Male behavior can be further influenced by female availability. Monopolization of multiple females is usually the most beneficial strategy for males; however, when this is not possible, it is best for males to monopolize at least one female (15). This situation typically occurs in species with low population density (as individuals rarely meet) (19) or with male-biased sex ratios (as most of the females are occupied by other males) (20). Mate guarding of a single female leads to social monogamy. Social monogamy is an association between a single male and a single female, which can last for the whole reproductive life or only temporarily (21). Male participation in offspring care can be a byproduct of mate guarding (19, 22). In this situation, no tradeoff between care and mating opportunities occurs. In the case of social monogamy, investment into parental care is beneficial for the male because he is less likely to gain from interactions with other females (15). It is important to remember that social monogamy does not necessarily mean genetic monogamy: extrapair copulation is not uncommon (21, 23).

Here, we describe biparental care in the solitary apid bee, *Ceratina nigrolabiata*, which represents a case of biparental care in bees. Bees and most other aculeate Hymenoptera have specific natural history traits that can strongly modulate the evolution of parental care, including (*i*) nest-making behavior; (*ii*) haplodiploid sex determination, in which males only genetically participate in female offspring; and (*iii*) a spermatheca, which allows the female to store sperm for months or even years (24). Parallel evolution of biparental care in *C. nigrolabiata* and *Trypoxylon* crabronid wasps is likely, as *Trypoxylon* and *Ceratina*

Significance

Biparental care is a rare strategy of offspring protection in insects. We discovered a case of biparental care in bees, in *Ceratina nigrolabiata*. Benefits of biparental care were identified for both males and females; yet, we found that an extraordinarily high proportion of offspring were unrelated to guard males. This is because females of *C. nigrolabiata* are polyandrous and store sperm. Therefore, the most important benefit for males is increased mating opportunities. We suggest that female polyandry and prolonged female receptivity supported the origin and/or maintenance of biparental care concomitant with extended male lifespan. Biparental care is an alternative method of brood protection to eusociality, which provides similar benefits but arises under different female mating conditions.

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share natural history traits typical of nest-making aculeate Hymenoptera (9–11). As biparental care should increase the fitness of both sexes (2, 6), we tested the benefits of this strategy for males and females separately. We identified the maternity and paternity of brood individuals using microsatellites, and we compared the mating frequency of *C. nigrolabiata* with that of solitary and facultatively eusocial bee species in which single mating is known (14, 25–27). Importantly, the presence of solitary, biparental, and eusocial nesting within the same bee genus allows for comparisons of the existing cooperative strategies.

Results

Presence of a Male–Female Pair in Active Brood Nests. A male–female pair was found in 88% of the active brood nests of *C. nigrolabiata* (404 out of 460; *SI Appendix*, Table S1). In 1% of the active brood nests (5 out of 460; *SI Appendix*, Table S1), one female and multiple males were present. In the remaining nests, there were only adults of a single sex (only a male in 5%: 26 out of 404 nests; only a female in 4%: 18 out of 404 nests; *SI Appendix*, Table S1) or no adults at all (2%: 8 out of 404 nests; *SI Appendix*, Table S1). We did not observe any nest with more than one adult female.

There was a pronounced division of labor between males and females. In all cases, while females were foraging, males remained in their nests (78 nests, each observed for 1.5 h). The male remained inside the nest, near the nest entrance, and when the female departed or arrived, he let her pass (Fig. 1). Ants are natural enemies of *Ceratina* bees (28); therefore, to test whether guard males of *C. nigrolabiata* effectively protect the nest against invaders, we placed an ant at the nest entrance and observed the behavior of the guard male. Guard males prevented the ant from entering the nest in all cases ($n = 41$), and in 61% of cases, males actively pushed the ant out of the nest.

Duration of Male Guarding. Usually, *C. nigrolabiata* males do not stay in the same nest for the entire provisioning season (the provisioning season lasts ∼42 d; see *SI Appendix*). On average, males remain for 7.756 d in a nest (SD = 8.211); therefore, the male inhabitant of the majority of nests changed one or more times during the provisioning season (*SI Appendix*, Figs. S1 and S2). Only in rare cases (11 out of 93) did a single male remain in the same nest for the whole provisioning season. The frequency of male switches significantly influences nesting productivity, measured as the number of offspring in egg and larval stages in an active brood nest. In each year of the study, nesting productivity decreased with increased frequency of male switches [Fig. 2 *A*–*C*; general linearized model (GLM) Poisson; 2013: deviance = 4.97, $P = 0.026$, $n = 27$; 2014: deviance = 5.13, $P = 0.024$, $n = 120$; 2015: deviance = 8.97, $P = 0.003$, $n = 102$].

Experimental Removal of Males from Their Nests. When a *C. nigrolabiata* male was removed from a nest, he was replaced by another male within the first day postremoval in 61% of cases (47 out of 77 nests; *SI Appendix*, Table S2). By the fourth day after removal, the proportion of nests with a guard male did not significantly differ between manipulated and control nests (Fisher's exact test, $P = 0.153$; $n = 76$; *SI Appendix*, Table S2). Removal of the male was not associated with the presence of brood cell damage (Fisher's exact test, $P = 0.694$; $n = 162$); however, it did result in decreased nesting productivity in nests where the couple was successfully reestablished, as measured by the number of eggs (Fig. 2*D*; GLM Poisson, $df = 1$, deviance = 5.09, $P = 0.024$, $n = 73$) and the number of offspring (at or before the third larval stage; Fig. 2*E*; GLM Poisson, $df = 1$, deviance = 5.59, $P = 0.015$, $n = 73$). Interestingly, male removal had a significant influence on female behavior. Females in male-removed nests performed fewer foraging trips [in 1.5 h of observation time post-male removal; Fig. 3*A*; paired Wilcoxon test, V (test criterium of Wilcoxon paired test) = 221, $P = 0.002$; $n = 24$], and they spent less time outside their nests (Fig. 3*B* and Table 1; paired Wilcoxon test, V = 264, $P < 0.001$; $n = 24$).

Experimental Removal of Females from Their Nests. Removal of *C. nigrolabiata* females had a significant effect on the departure of guard males (Fisher's exact test, $P < 0.001$; $n = 94$). When we removed the female from a nest, we checked for the presence of the male the following day: the guard male was present in only 38% of the female-removed nests (18 out of 48), in comparison with 89% of the control nests (41 out of 46). Importantly, female removal resulted in a significantly increased incidence of brood cell damage in the nest (Fisher's exact test, $P < 0.001$; $n = 194$). The presence of natural enemies, such as another *Ceratina* female that had usurped the nest (Fisher's exact test, $P < 0.001$; $n = 194$) or ants (Fisher's exact test, $P = 0.018$; $n = 194$), was also significantly influenced by female removal.

Comparison of the Duration of Foraging Trips Among Species. We compared the average duration of *C. nigrolabiata* foraging trips with that of two sympatric solitary *Ceratina* species. The average duration of a foraging trip was 16.47 min for *C. nigrolabiata* (SD = 10.74 min, $n = 110$ trips of 55 individuals), 10.30 min for *Ceratina chalybea* (SD = 10.01 min, $n = 42$ trips of 21 individuals), and only 4.77 min for *Ceratina cucurbitina* (SD = 7.50 min, $n = 84$ trips of 42 individuals). *C. nigrolabiata* had significantly longer foraging trips than *C. chalybea* (Fig. 3*C*; post hoc Nemenyi test, $P = 0.008$) and *C. cucurbitina* (Fig. 3*C*; post hoc Nemenyi test, $P < 0.001$). Data are summarized in Table 1.

Male Survival During the Nesting Season. The proportion of *C. nigrolabiata* males in the population fluctuated around 54% for the duration of the nesting season [$n = 408$ individuals from all season (May 1 to August 15); fluctuation between 44% and 65%

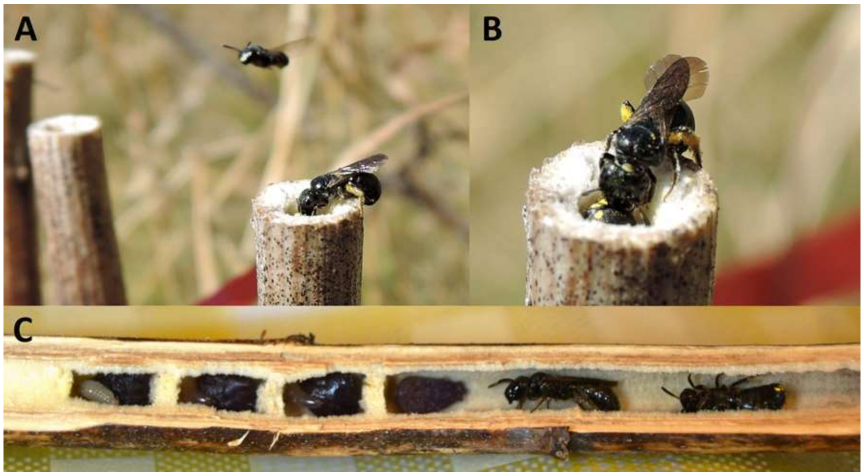

Fig. 1. *C. nigrolabiata* male performs nest guarding; female performs nest provisioning. (*A*) The female arrives at the nest entrance with a pollen load. She uses her fore legs to scratch the metasoma of the male to let her pass. Another male flies around, probably searching for a nest without a guard male. (*B*) Detailed image of the female scratching on the male's metasoma. The male's metasoma is marked yellow. (*C*) Dissected active brood nest with three completed brood cells and one cell currently being provisioned. In *C*, a female (left) and male (right) are present in the burrow.

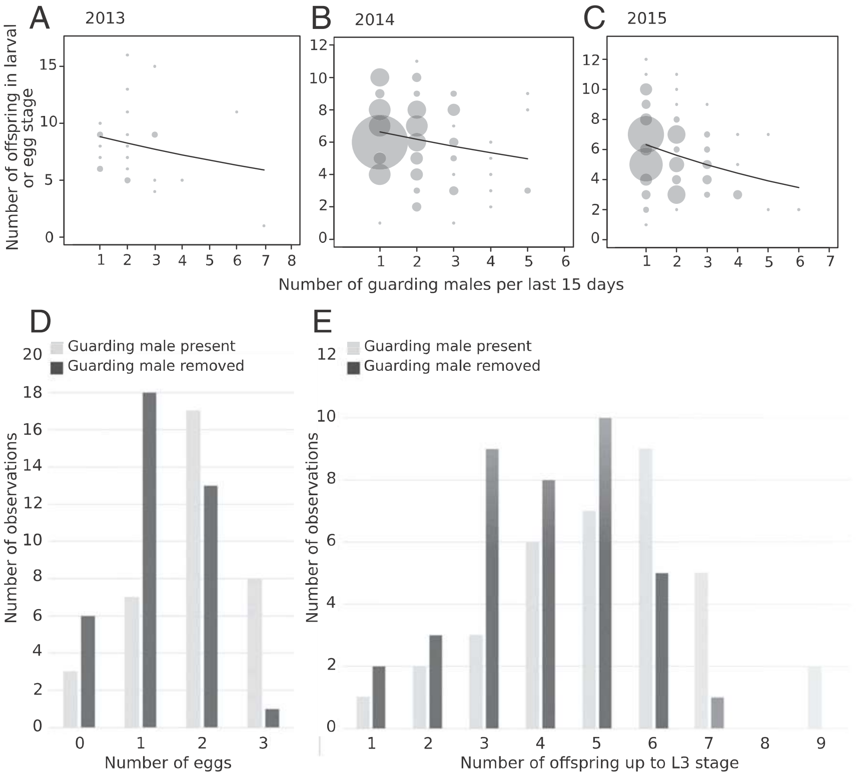

Fig. 2. Effect of male switches on nesting productivity. (*A–C*) Relationship between the number of different guard males in the last 15 d and the number of offspring in egg and larval stages from 2013 (27 nests) (*A*), from 2014 (120 nests) (*B*), and from 2015 (102 nests) (*C*). The relationship is significant for all 3 y (GLM Poisson, 2013: deviance = 4.97, $P = 0.026$; 2014: deviance = 5.1264, $P = 0.023$; 2015: deviance = 8.97, $P = 0.003$). The size of the circle corresponds to the number of observations with the same value. The line indicates the result of the Poisson model. (*D* and *E*) Comparison of nesting productivity between male-removed nests and control nests. (*D*) Number of eggs in the nest. (*E*) Number of offspring up to the larval (L)3 stage in the nest (eggs plus young larvae). We used 38 male-removed nests and 35 control nests from 2014 and 2015. Results were statistically significant for both the number of eggs (GLM Poisson, $df = 1$, deviance = 5.09, $P = 0.024$) and the number of offspring up to the L3 stage (GLM Poisson, $df = 1$, deviance = 5.59, $P = 0.015$).

in different months; *SI Appendix*, Fig. S3]. The proportion of *C. chalybea* males in the population decreased over the nesting season from 48% ($n = 23$ individuals) in the first half of May to 8% ($n = 66$ individuals) in the first half of August. Therefore, few *C. chalybea* males survived for the entire season. In contrast, *C. cucurbitina* males died early. In the first half of May, 56% ($n = 52$) of adults were male; however, the last *C. cucurbitina* male of the parental generation was observed in the second half of June, well before the end of the nesting season.

Mating Frequency and Paternity. At nests, we observed instances of mating throughout the nesting season. *C. nigrolabiata* females predominantly mate multiply: on average, offspring of 3.25 males were found in completely provisioned nests (ranging from 1 to 8, SD = 1.70, $n = 31$ nests). Nests with all offspring belonging to a single father were rare (4 out of 31). Moreover, offspring from different fathers were often intermixed in the nest (*SI Appendix*, Fig. S4). Female multiple mating is also present in two related *Ceratina* species, *C. chalybea* (2.57 males, SD = 0.98, $n = 7$ nests) and *Ceratina cyanea* (2.5 males, SD = 0.70, $n = 2$ nests; Table 1).

Kinship of the guard male to nest offspring was low. At least one offspring of the guard male was detected in 29% of active brood nests ($n = 265$; *SI Appendix*, Fig. S5). On average, active brood nests contained 6.604 offspring, with only 0.638 offspring (10%) fathered by the guard male. However, as hymenopteran males do not contribute genetically to male (haploid) offspring,

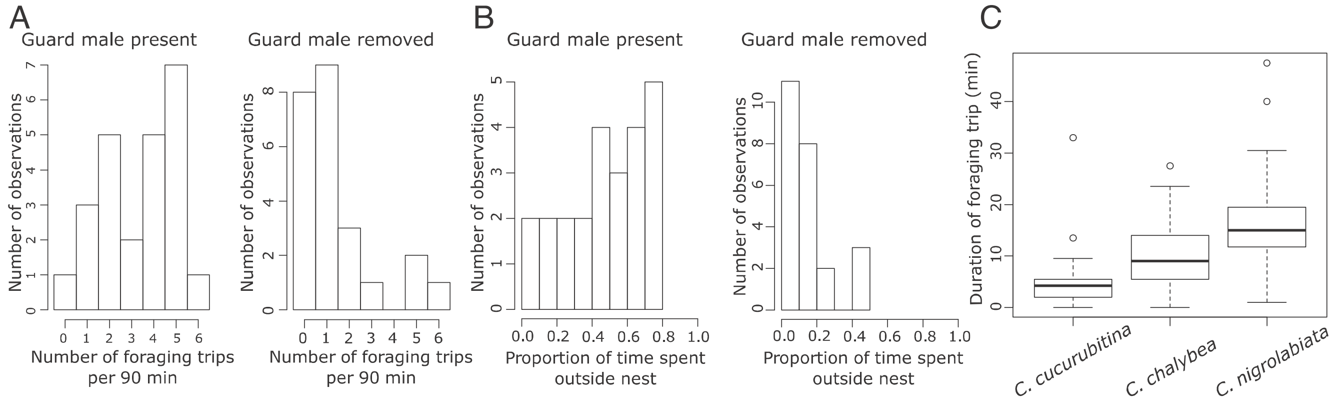

Fig. 3. Effect of male removal on female foraging behavior. (*A*) Histograms of the number of foraging trips comparing male-removed nests and control nests. One treatment and one control nest were observed simultaneously. The difference is statistically significant (paired Wilcoxon test, V = 221, $P = 0.002$; $n = 24$ nest pairs). (*B*) Histograms of the proportion of time spent outside of the nest in foraging activity per one female in male-removed nests and control nests. One treatment and one control nest were observed simultaneously. The difference is statistically significant (paired Wilcoxon test, V = 264, $P < 0.001$; $n = 24$ nest pairs). (*C*) Comparison of the duration of foraging trips between sympatric species of the genus *Ceratina*. The duration of a foraging trip was calculated as the mean of two subsequent trips of one female. We observed 55 nests of *C. nigrolabiata*, 21 nests of *C. chalybea*, and 42 nests of *C. cucurbitina*.

Table 1. Comparison of female mating frequency and duration of foraging trips in four *Ceratina* species

Species	*C. nigrolabiata*	*C. chalybea*	*C. cucurbitina*	*C. cyanea*
Nesting strategy	Biparental	Solitary	Solitary	Solitary
Average mating frequency	3.25 (n = 31)	2.57 (n = 7)	Not available	2.5 (n = 2)
Average duration of foraging trip, min	16.47 (n = 55)	10.30 (n = 21)	4.77 (n = 42)	Not available

n is the number of nests used in the analysis.

the number of female offspring fathered is more important. Guard males, on average, fathered only 17% (169 out of 1005) of female offspring. The maximum number of offspring belonging to the guard male in a nest was seven (*SI Appendix*, Fig. S5).

We also tested how male fitness is affected by the duration of guarding. Both the number of offspring (Fig. 4; linear model, t = 14.55, $P < 0.001$; $n = 301$) and the number of offspring per day of guarding (*SI Appendix*, Fig. S6) increase with longer durations of guarding. A male gains an average of 0.638 offspring per 7 d of guarding (which is the average duration of guarding). The provisioning season of *C. nigrolabiata* lasts ~42 d; therefore, guarding nests for most of the provisioning season (for example, guarding five nests, each for the average duration) would lead to ~3.19 offspring per male per season.

Discussion

We discovered social monogamy and biparental care in the bee species *C. nigrolabiata*. These strategies were previously unknown in bees. There is a pronounced division of labor between the sexes in this species, similar to another biparental arthropod group, crabronid wasps of the genus *Trypoxylon* (10, 11, 29). While females perform provisioning, males are responsible for nest guarding (Fig. 1). A male–female couple was found in the majority of active brood nests of *C. nigrolabiata* (88%). Nests without an adult couple most commonly contained only male adults, but we also found nests with only female adults and nests without either parent present. Because female-only nests were not the most prevalent, it is likely that active brood nests without a couple were either recently orphaned or deserted by the parents. This suggests that female-only nests are not stable.

The importance of nest and resource defense can select for biparental care (8); in the aculeate Hymenoptera, selective pressures for nest protection are thought to favor eusociality (13). Unguarded nests of *Ceratina* bees are highly vulnerable to natural enemies, especially ants (28); therefore, the benefit of a guard male is most likely similar to the benefit of worker guards in facultatively eusocial nests (30–32). One important benefit of eusocial nesting is nest protection when foraging individuals depart the nest to collect provisions for brood cells (33, 34). Eusocial nesting thus significantly decreases mortality caused by natural enemies (31, 35). We have shown that *C. nigrolabiata* males defend the nest from attacks by enemies such as ants. Interestingly, removal of the guard male did not increase the incidence of brood or cell damage, probably because the removed male was replaced by another male within 1 d in the majority of cases (and maximally within 4 d). By contrast, on a short time scale (hours), we found that absence of the guard male affected female behavior. Male removal resulted in a decreased number of female foraging trips and a decreased amount of time spent outside the nest. In another known biparental hymenopteran, the crabronid wasp, *Trypoxylon vagulum* Richards, females do not perform foraging when the guard male is not present in the nest (29). Similarly, females spend a longer time outside of eusocial nests than solitary nests in the closely related facultatively eusocial bees of the genus *Xylocopa* (30, 36). This difference is also observed in *Ceratina calcarata* Robertson, as females spend more time away from the nests in which they feed adult offspring compared with those in which only vulnerable brood cells are provisioned (37). Likewise, we determined that *C. nigrolabiata* performs significantly longer foraging trips than two sympatric solitary species without biparental care—*C. chalybea* and *C. cucurbitina*. Therefore, biparental care and eusociality, by using two different types of guarding, are strategies that result in similar benefits for foragers, allowing them more time for resource collection thereby increasing nesting productivity.

The presence of a guard male should increase nesting productivity if it allows the female to forage more effectively. Indeed, we have shown that male removal results in altered female foraging behavior as well as decreased nesting productivity. A similar benefit to nesting productivity is known for eusocial nests of *Xylocopa sulcatipes* in comparison with solitary nests (30, 32). Furthermore, effective guarding by males allows females to invest more into female offspring, which contain paternal DNA but are costlier than male offspring (without paternal DNA), as was shown for the biparental crabronid wasp, *Trypoxylon politum* Drury (10). Nesting productivity was also positively influenced by male fidelity in *C. nigrolabiata*; nests with a lower frequency of male switches had a greater number of offspring.

In biparental care, males commonly benefit from caring for their own offspring; therefore, although female infidelity is possible, most of the offspring usually belong to the guard male. In bird and mammal species, more than 90% of the offspring belong to the male who is providing care (21, 23), although some exceptions exist [e.g., the birds *Tachycineta bicolor* (Vieillot) (Hirundinidae) with 31% (38) and *Malurus cyaneus* (Ellis) (Maluridae) with 24% (39) of their own offspring]. In the only other genetically studied biparental hymenopteran, *Trypoxylon albitarse* Fabricius, the guard male fathers 78% of his female partner's offspring (9). Conversely, we found that, for *C. nigrolabiata* males, the fitness benefit from nest protection is generally very low. For *C. nigrolabiata*, only 10% of all offspring (including male offspring, which have no father) and 17% of female offspring were guarded by their own father. Males guarded nests

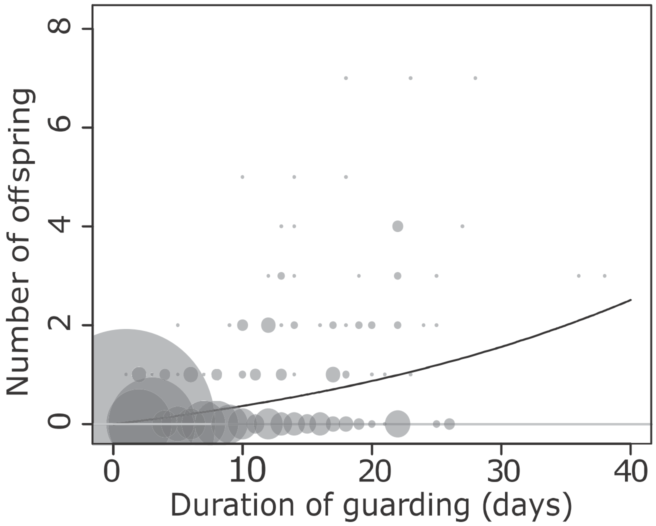

Fig. 4. Influence of the duration of male guarding on the number of his own offspring in the nest. The size of circle corresponds to the number of observations with the same value. The line indicates the result of the log-normal model (n = 301 nests).

with at least one of their own descendants in only 29% of cases. Therefore, *C. nigrolabiata* males regularly guard offspring of alien males or offspring with no paternal DNA. Although male fitness gained by guarding any specific nest is low, the total benefit from guarding behavior per entire season is much higher because males guard several nests sequentially within a season. We calculate that a male gains 3.19 offspring by guarding nests for the entire provisioning season.

As there is little parental fitness benefit from protecting one's own offspring for *C. nigrolabiata* males, we posit that nest guarding behavior (which increases offspring survival via nest protection) is primarily a byproduct of mate guarding. This claim is supported by findings from the female-removal experiment: when we removed the female from a nest, the male usually departed within a few days. Additionally, males do not try to protect offspring without the presence of a female.

In *C. nigrolabiata,* biparental care coexists with high female mating frequency. The mixing of offspring from different fathers in the nest (*SI Appendix*, Fig. S4) indicates that females can store and mix sperm from previous matings in their spermatheca. In most bee species, mating with a single male early in the season is the prevailing mating pattern (25, 26); however, we observed females mating throughout the nesting season in *C. nigrolabiata* and a related species, *C. chalybea*. Interestingly, nests of the related solitary *Ceratina* species, *C. chalybea*, and *C. cyanea*, also have offspring from multiple fathers, suggesting that multiple mating arose before biparental care in the genus *Ceratina*. Generally, it is thought that the multiple mating in females selects against male participation in offspring care (2, 4, 40). Contrary to this assumption, we propose that female multiple mating in *C. nigrolabiata* represents a key preadaptation for biparental care in bees. Bee species with single-mated females usually mate before the provisioning season (26) and have short-lived males (24, 41). For example, there is very low male survival for the duration of the nesting season in *C. calcarata* (42), where 81% of females are singly mated (27). Long-term persistence of mating opportunities selects for prolonged life of males (43, 44), and only a long-living male has the opportunity to participate in offspring care. In accordance with this hypothesis, we detected long-term survival of males throughout the nesting season in *C. nigrolabiata* and, partially, also in *C. chalybea* (*SI Appendix*, Fig. S3).

Biparental care has similar maternal benefits as eusociality. Eusociality originated in the context of monandry, which ensures high relatedness between offspring, encouraging cooperation (14). Indeed, the only eusocial *Ceratina* species with known mating frequency, *Ceratina australensis* (Perkins), is monandrous (31). By contrast, biparental care in *C. nigrolabiata* originated in a situation of multiple mating. Therefore, we suggest that primitive eusociality and biparental care represent two alternative ways to reach the same primary benefits of extended parental care. The evolution of parental care in *Ceratina* is notable for its complicated origin, as the ancestor of the genus *Ceratina* was most likely facultatively eusocial, with obligate solitary behavior being a derived state (45). In *C. nigrolabiata*, we did not find any case of multifemale nesting; however, it is possible that some of the advanced behavioral traits observed in *C. nigrolabiata* are inherited from their eusocial ancestors.

Materials and Methods

Study Species. We used *C. nigrolabiata* Friese (Hymenoptera: Apidae) for most of our analyses. We performed field studies on this species, with the nest being the main unit for almost all of our analyses. A nest usually contains two parents (one male and one female) and juvenile offspring. We compared some traits of *C. nigrolabiata* with those of other sympatric *Ceratina* species: *C. cucurbitina* (Rossi), *C. chalybea* (Chevrier), and *C. cyanea* (Kirby).

Study Site. Research was performed at Havranické Vřesoviště, Podyjí National Park, Czech Republic (48°48′32.2″N 15°59′33.7″E). This locality consists of dry steppe grasslands with heather and scattered shrubs. We conducted our research in 2013, 2014, and 2015. Some additional data are from 2012 and 2016.

Field Experiments.

Installation of nesting opportunities. We used sheaves of cut twigs as artificial nesting opportunities. A sheaf was made from 20 twigs, 30–45 cm in length. For further details about the artificial nesting opportunities, see *SI Appendix*.

Observation of nests and dye marking of males. We marked nests and the males that stayed inside them. We checked all studied nests daily in almost all cases for the duration of the provisioning season. Each unmarked male was marked by a colored dot on the metasomal terga near the apex of the metasoma, visible from the nest entrance. We did not observe any case of a male abandoning the nest immediately after marking. We used five different colors for marking. When we found a marked male, we noted its color. This enabled us to assess the duration of nest guarding by males. We used these data to evaluate the average duration of male guarding and for several other analyses where we correlated the duration of male guarding with other variables. For further details about the marking of males and nests, see *SI Appendix*.

Dissection of nests. Nests selected for dissection were collected after the end of foraging activity (after 6:00 PM; Central European Summer Time). The nest entrance was plugged to ensure that adult individuals were retained inside. Collected nests were stored in a fridge until dissection.

Nests were cut by knife and the following data were recorded: number and sex of adult individuals, length of the nest, length of the entrance burrow (distance between the nest entrance and outer septum), number of provisioned cells, number of live offspring, and presence of natural enemies. Additionally, developmental stage was recorded for each offspring. Details about the developmental stage of offspring are described in *SI Appendix*. We used active brood nests for most of the analyses. An active brood nest was still being provisioned at the time of nest collection and therefore contained an egg in the outermost brood cell (42). We used full brood nests for some of the analyses. These nests contained a larva or pupa in the outermost brood cell. We used data from the dissection of nests to assess the length of the provisioning season and changes to the adult sex ratio during the nesting season; this is described in more detail in *SI Appendix*.

Existence of pairs. In this analysis, we estimated the proportion of nests in which a male and a female were both present. We used all dissected active brood nests (excluding nests from those experiments that involved removal of an adult, male or female). We analyzed 133 nests in 2013, 183 in 2014, and 144 in 2015; therefore, 460 nests were analyzed in total.

Impact of male switches on nesting productivity. We examined the influence of guard male switching frequency on nesting productivity, measured as the number of offspring in the egg or larval stage. Specific details for this analysis are presented in *SI Appendix*.

Removal of Males. We randomly selected some of the nests with both a male and a female present for removal experiments. We performed male removal on some of these nests, while others were retained as controls. We used the same nests to determine the influence of male removal on the presence of brood cell damage, to assess the replacement of removed males, and to examine the impact of male removal on nesting productivity. In a subsequent experiment, we evaluated the effect of male removal on female behavior. Specific experimental details are presented in *SI Appendix*.

Removal of Females. In randomly selected nests (not those used for the male removal experiment), the guard female was removed. Other randomly selected nests were appointed as controls. For each nest in this experiment, we confirmed the presence of both a female and a male before the female removal or control treatment (using the same procedures as the male removal experiments). Specific experimental details are presented in *SI Appendix*.

Comparison of the Duration of Foraging Trips Between Species. The duration of foraging trips in *C. nigrolabiata* and two other sympatric species, *C. chalybea* and *C. cucurbitina*, were observed. On each observational day, all nests present on selected sheaves were observed. Each nest was observed until two intervals between departure and arrival were successfully noted. We calculated the average duration of two foraging trips for each individual. For further details, see *SI Appendix*.

Laboratory Analyses. We developed 10 polymorphic microsatellite loci to genotype 3,547 *C. nigrolabiata* individuals. We calculated the probability of two adults having the same genotype using the Cervus program (46). This probability was 0.00000004. It was also possible to use some of the loci developed for *C. nigrolabiata* for other species: *C. chalybea* and *C. cyanea*. The sex of each genotyped offspring was determined by ploidy. Details about microsatellite locus development, the genotyping process, and sex determination are described in *SI Appendix*.

Computing the relatedness of individuals in the nest.

Maternity. Maternity was tested by manual comparison of the guard female's genetic profile with offspring from the same nest. The guard female was considered to be the mother when all loci agreed or when only one locus disagreed. Individuals that were not offspring of the guard female were found only in rare cases. In these cases, they were always at the bottom of the nest and usually evidently older than the other offspring. These individuals disagreed with the guard female in at least minimally three loci. We suppose that in these cases, the offspring belong to the previous owner of the nest and that this situation arises from nest usurpation with incomplete removal of the usurped female's brood cells. Nonexclusion probability computed by Cervus 3 (46) was 0.055 for the first parent.

Paternity. Paternity of the offspring was tested using two procedures: (*i*) manual comparison—the offspring agreed with the guard male (potential father) in all alleles or disagreed in only one; or (*ii*) Colony software (47). Male offspring (haploid) were not analyzed. The settings used for Colony software and details about the paternity calculations are presented in *SI Appendix*.

Female mating frequency (number of fathers in the nest). We examined how many different fathers had offspring in a nest based on microsatellite analysis. This variable also represents the minimum number of sexual partners of the female. We used Colony software for this analysis. We analyzed mating frequency in 31 nests of *C. nigrolabiata*, 7 nests of *C. chalybea*, and 2 nests of *C. cyanea*. For further details about the calculations of mating frequency, see *SI Appendix*.

Influence of the duration of guarding on male fitness. Selected nests were dissected, and the male–female pair and all offspring were genotyped. Paternity of offspring and relatedness to the guard male were analyzed. We modeled and tested the relationship between the duration of time a male spent guarding a nest and the number of his own offspring in that nest. To test whether the relationship between the duration of male guarding and guard male fitness was statistically significant, a linear model on log-transformed data was used. Assumptions of the model were checked using diagnostic plots. We constructed polynomial models (up to the fifth degree of the polynomial), each of them with and without an intercept. Based on the Akaike information criterion, the best model was determined to be a linear model without an intercept. Statistical analyses were done using R software, version 3.2.5 (48). In total, 301 nests were analyzed (54 in 2013, 171 in 2014, 76 in 2015), containing 2,082 offspring, of which 1,189 were female.

Datasets. The datasets used for this study are provided in Dataset S1.

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STUDY 4

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Author contribution: MM and JS designed research and wrote the paper. MM is corresponding author.

LETTER

REPLY TO PORTMAN:

Mate guarding, sib guarding, and biparental care in bees

Michael Mikát[a,1] and Jakub Straka[a]

The letter from Portman (1) regarding our PNAS article about the biparental bee (2) is an excellent contextual addition to male behavior in bees because the male role in hymenopteran societies is generally overlooked (3) and discussion about it can improve scientific awareness.

Portman (1) creates a summarization of examples of male presence in nests. Although the cited articles are very interesting, most of them are anecdotal observations based on a few observed individuals and without any rigorous samples that would allow statistical analysis. Therefore, it is impossible to conclude with certainty that biparental care is present in the species studied in the original studies. In most of the original papers that Portman (1) cites, different types of male association with nests were observed, and no author referred to the observed behavior as biparental care.

The study on *Lasioglossum erythrurum* (Cockerell) by Kukuk and Schwarz (4) reported a harem system, in which the macrocephalic male controls access to multiple females by nest guarding. Moreover, their paper reported male guarding in the late phase of nesting season when only pupae, adult females, and one guarding male are present in nests. Therefore, male guarding increases survival of pupae in nests, but these pupae can be siblings and not daughters of a guarding male. Male presence in nests of *Lasioglossum zephyrum* (Smith) and *Augochlora pura* (Say) reported by Barrows (5) seems to be accidental male visits of nests, not a regular male presence in nests.

The observation case of *Ceratina smaragdula* (Fabricius) male behavior was not connected to parental care. Males were observed to guard nests with adult offspring, not nests with currently provisioned brood cells (6). We agree that the most likely motivation for males is mate guarding; however, this is not connected to parental care. A male's behavior can help him obtain more fitness but probably has no effect on protection of other individuals because mature offspring are able to protect themselves.

From the publications that Portman (1) mentions, the most promising species in which biparental care can occur is the colletid bee *Leioproctus muelleri* Houston and Maynard. Males of this species were observed to guard nests by more than 1 d during the phase of nesting season when females collected pollen (7). However, further research of this species is necessary to test whether the male helps with offspring protection and whether males obtain direct fitness benefits due to nest guarding.

We present a case of biparental care in bees in which the benefits for males and females are documented and a large sample size is used (2). It is likely that biparental care occurs in other bee species; however, extensive research of natural history and testing the relatedness between family members are necessary for understanding whether the species is truly biparental. We hope that the discussion supports interest in possible male nest behavior in bees and that more complex studies will be performed.

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The authors declare no conflict of interest.

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STUDY 5

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Author contribution: MM designed the research, MM and DB performed the research, MM analysed data, MM wrote the initial draft of manuscript, and JS performed substantial changes in manuscript. MM is corresponding author.

Ecological Entomology

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Maternal investment in a bee species with facultative nest guarding and males heavier than females

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Abstract. 1. Maternal investment can be influenced by several factors, especially maternal quality and possibilities for future reproduction. Mass provisioning Hymenoptera are an excellent group for measuring maternal investment because mothers distribute food sources to each brood cell for each offspring separately. Generally in aculeate Hymenoptera, larger females produce larger offspring and invest more in female offspring than in male offspring.

2. This study investigated patterns of maternal investment in *Ceratina chalcites*, which has an uncommon type of sexual size dimorphism in Hymenoptera: on average, males are heavier than females. It was found that larger females produce a significantly higher proportion of male offspring, as males are the costlier sex in this species.

3. Facultative nest guarding by females was observed. Females can guard offspring until adulthood, as is typical for bees of genus *Ceratina* (34.43% of nests); however, in the majority of cases (65.56% of nests), females plug and abandon the nest. Significant differences were found in the amount of investment between guarded and unguarded nests. Guarded nests had a greater number of provisioned brood cells and a higher proportion of male offspring. It is suggested that mothers have two facultative strategies – either she makes a large investment in the offspring of one nest or she abandons the first nest and carries out a second nesting elsewhere.

Key words. *Ceratina chalcites*, maternal care, sex ratio, sexual dimorphism.

Introduction

Organisms make trade-offs between current and future reproduction (Williams, 1966; Stearns, 1989). In parental care, parents should optimise their investment in offspring (Coleman *et al.*, 1985; Fox & Czesak, 2000; Trumbo, 2012; Kölliker *et al.*, 2015; Seidelmann, 2018). Investment in current offspring is dependent on the parental quality (Molumby, 1997; Nager *et al.*, 1999; Rehan & Richards, 2010a), environmental variables (Strohm & Linsenmair, 1997a; Kim, 1999; Peterson & Roitberg, 2006, 2016; Renauld *et al.*, 2016; Dew *et al.*, 2018; Seidelmann, 2018), and future breeding possibilities (Gross, 2005). Parents may also invest in male and female offspring differently (Trivers & Willard, 1973) depending on the sexual dimorphism of the species (Helms, 1994).

Mass provisioning Aculeate Hymenoptera is an optimal group for studying patterns in maternal investment (Danforth, 1990; Johnson, 1990; Stubblefield & Seger, 1994; Seidelmann, 2018). Although multiple components of maternal investment exist in Aculeate Hymenoptera (Rosenheim *et al.*, 1996; Field *et al.*, 2007), the key component influencing offspring body mass is the food brought to an individual offspring by the mother (Bosch & Vicens, 2002; Seidelmann, 2018). Mothers distribute food to offspring in separate brood cells, and offspring can only feed on the food that has been prepared for their brood cell (Johnson, 1990; Field, 2005; Michener, 2007). Therefore, it is possible to measure the maternal investment by weighing offspring (Tepedino & Torchio, 1982; Bosch & Vicens, 2002) or by using another proxy parameter that is correlated with weight. The most commonly used parameter of size in aculeate Hymenoptera is the head width (Stark, 1992; Strohm & Linsenmair, 1997b; Roulston & Cane, 2000; Bosch & Vicens, 2002; Grüter *et al.*, 2017; Dew *et al.*, 2018). Body mass and head width are also correlated in *Ceratina* bees (Rehan & Richards, 2010a; Rehan *et al.*, 2010).

Studies of mass provisioning Aculeata that have tested the influence of maternal quality on offspring parameters have generally shown that larger mothers produce larger offspring (Molumby, 1997; Strohm & Linsenmair, 1997b; Rehan &

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Richards, 2010a). Maternal size commonly influenced the sex ratio of offspring – larger females produced female-biased offspring and smaller females produced male-biased offspring (Suguira & Maeta, 1989; Molumby, 1997; Strohm & Linsenmair, 1997b; Rehan & Richards, 2010a). In most Aculeata, females are the larger sex and are therefore more expensive than males (Helms, 1994; Stubblefield & Seger, 1994). Studies of species with larger males (which are costlier than females) are generally lacking, with the exception of a study of *Anthidium septemspinosum* (Sugiura, 1994). In this study, larger females produced a male-biased sex ratio, although the sample size was small.

The number of reproductive events per lifetime is another aspect of parental investment. Organisms can reproduce once (semelparous) or multiple times (iteroparous) during their life (Cole, 1954; Stearns, 1976). In the case of semelparity, organisms invest all their energy in one reproduction event and therefore a higher risk of total failure is present (Stearns, 1976; Dangerfield & Telford, 1995). In animals with parental care, semelparity and iteroparity can interact with the total care performance (Tallamy & Schaefer, 1997; Tallamy & Brown, 1999; Zink, 2003). For semelparous caring animals, it is beneficial to care for offspring until the parent's death (Kudô *et al.*, 1992; Tallamy & Brown, 1999); thus there is no pressure to abandon offspring. On the other hand, iteroparous caring animals make a trade-off between present and future reproduction (Tallamy & Brown, 1999). Therefore the abandonment of offspring is beneficial for parents when the benefits of caring for the current offspring are lower than the potential benefits of a new reproductive event (Stegmann & Linsenmair, 2002; Grüebler & Naef-Daenzer, 2010). Parents should desert smaller nests to hedge their bets by investing in new nests to maximise their lifetime reproductive success, while parents of larger nests should guard their initial reproductive investment (Coleman *et al.*, 1985; Armstrong & Robertson, 1988; Olmstead & Wood, 1990; Mikát *et al.*, 2016). In extreme cases, brood destruction can cause a second reproduction event in commonly semelparous species (Futami & Akimoto, 2005).

Nesting animals can perform two types of iteroparity: (i) within nest iteroparity when they have multiple cohorts of offspring in the same nest; and (ii) serial nesting when each cohort of offspring is reared in a different nest (Trumbo, 2013). Serial nesting should constrain the extent of care more significantly because parents must desert older offspring to produce new ones (Trumbo, 2013). Within-nest iteroparity is considered an important condition for the evolution of eusociality (Soucy, 2002; Trumbo, 2013; Rehan *et al.*, 2014).

In aculeate Hymenoptera, most of the solitary species perform serial nesting (Michener, 2007); whereas eusocial species usually perform within nest iteroparity (Trumbo, 2013; Rehan *et al.*, 2014). Even though building a single nest is not common among solitary Aculeata (Peterson *et al.*, 2007), it is the typical strategy for temperate species of the genus *Ceratina*. Mothers usually guard offspring after the completion of brood cell provisioning until offspring reach adulthood (Rehan & Richards, 2010b; Mikát *et al.*, 2016). When offspring develop into adults, mothers feed the adult juveniles pollen and nectar (Sakagami & Maeta, 1977;Lewis & Richards, 2017 ; Mikát *et al.*, 2017) and usually die while feeding the mature offspring (Mikát *et al.*, 2017). Therefore there is no possibility for new reproduction events. In some species it has been shown that, if the mother dies, a sister of the young adults can perform provisioning (Lewis & Richards, 2017; Mikát *et al.*, 2017). This helper sister is usually produced in the first brood cell position and is smaller than other females produced in the same nest (Lawson *et al.*, 2016; Mikát *et al.*, 2017).

Albeit the temperate *Ceratina* usually have a semelparous strategy, facultative iteroparity is known. Within-nest iteroparity relates to the social nesting (Rehan *et al.*, 2010, 2014, 2015) and is present mostly in tropical or subtropical species (Rehan *et al.*, 2012, 2014, 2015). Facultative serial nesting is present in the European species, *Ceratina chalybea* (Mikát *et al.*, 2016). In this species, some females guard their offspring until adulthood; however, other females close their nests using a filling plug and desert the nest after the provisioning is finished (Mikát *et al.*, 2016).

Here, we examined patterns of maternal investment in species *Ceratina chalcites*. This is the largest *Ceratina* species in Europe and probably one of the largest in the world (Terzo, 1998). Similar to *C. chalybea* it belongs to the mostly European subgenus *Euceratina* (Terzo, 1998) and guards its offspring facultatively either until adulthood or only until the end of provisioning. We assessed how maternal investment differs between nests with different guarding strategies. Additionally, in contrast to other *Ceratina* species (Maeta *et al.*, 1992; Rehan & Richards, 2010a), this species has heavier males than females. This uncommon type of sexual dimorphism allowed us to test whether heavier females always invest in females or whether their investment is in the larger, more costly sex. Finally, we examined the level of sociality in *C. chalcites* and interpreted it in the context of *Ceratina* social evolution.

Materials and methods

Study site

Research was performed in Pescariello (Comunne Altamura, Bari Province, Apulia, Italy), in *c.* 4 km surrounding the coordinate 40°53′20.1″N, 16°37′51.3″E. There is an extensive agricultural landscape with fields, pastures, and forests. There are many nest opportunities for *C. chalcites* – mostly near roads, in field margins or in pastures. Fennel (*Foeniculum vulgare*) was the most common nesting substrate. Some nests were present in blackberries (*Rubus* sp.) or other plants with pith.

Nest collection

We used nests from natural nesting opportunities. Nests were collected in the evening, night or early morning (between 18.30 and 06.30 hours Central European Summer Time) to ensure that all inhabitants were inside the nest. Nest entrances were closed to prevent the escape of adult bees. Field research was performed between 16 and 30 June 2017.

Fig. 1. Nest guarding strategies of full brood nests in *Ceratina chalcites*: (a) unguarded nest; (b) guarded nest with closed outermost brood cell; (c) guarded nest with open outermost brood cell. [Colour figure can be viewed at wileyonlinelibrary.com].

Nest dissection

We opened nests using clippers. In each nest we recorded the presence of the mother or other adults, the number of brood cells provisioned, the stage of juvenile offspring and the presence of parasites. We also noted the structure of the last brood cell – whether this cell was open (outermost offspring not separated from mother by partition; Fig. 1c) or closed by a massive filling plug. We recorded the following parameters for each nest: length of nest, length of entrance burrow (from the entrance to the first partition), width of nest, width of pith, and width of twig. For all 99 unguarded nests, we measured the length of a filling plug (last brood cell partition). In 16 randomly selected nests we measured all brood cell partitions. We measured 31 inner brood cell partitions from unguarded nests and 21 from guarded nests, i.e. 52 in total.

In total, we dissected 238 nests of *C. chalcites*. Dissected nests were in different stages. We used full brood nests with an undisturbed structure for almost all analyses. Full brood nests contained a larva or pupa in the last brood cell. Undisturbed nests had preserved partitions between brood cells. We dissected 151 full brood nests. We compared nest parameters between unguarded nests and those guarded by a female. We used 44 active brood nests for the examination of sociality. We considered nests to be active when there was an egg in the last brood cell and the nest was not closed by a filling plug. Ten nests were in a transitional phase between active brood nests and full brood nest stage. They contained an egg in the last brood cell; however, they were closed by the filling plug. We did not include these nests in the analyses.

Examination of sex and size

We reared offspring from selected nests to adulthood. We selected full brood nests with a high proportion of live offspring ($N = 98$ nests). Older offspring (late larval and pupal stages) were reared to adulthood in 1.5-ml vials (Eppendorf, Hamburg, Germany). Younger larvae (with part of the pollen ball remaining) were retained in the original twig until they consumed all pollen. Later, we put these larvae into Eppendorf tubes. We examined the sex and measured the head width of each successfully reared offspring. Head width was measured as the maximum distance between the edges of the head measured across compound eyes. In a subset of individuals, we measured intertegular width and forewing length for evaluation of allometry in sexual dimorphism. Intertegular width was taken to be the shortest distance between inner margins of tegulae. Forewing length was taken to be the distance between the base and the end of a wing. We ripped off the wing before measurement. We measured forewing length only in individuals that had successfully developed wings. We used a binocular magnifier with a scale in the ocular, using 40× magnification for measurement of head width and intertegular width, and 6.67× for measurement of forewing length.

Examination of body mass

We weighed young adults that were reared until adulthood in Eppendorf tubes. When young adults reached adulthood, they were killed by freezing and stored in −20 °C before weighing. We randomly selected 18 males and 19 females to be weighed. We used Scaltec SBC22 (Dania Beach, Florida) for weighing.

Statistical analysis

Statistical analyses were performed using the program R (R Development Core Team, 2011). Sex ratio was tested by χ^2 tests. The difference in size between the first brood cell females and other nest females was tested using a paired *t*-test. The arithmetic mean of head widths of females from the second to the 10th brood cell position from one nest was compared with

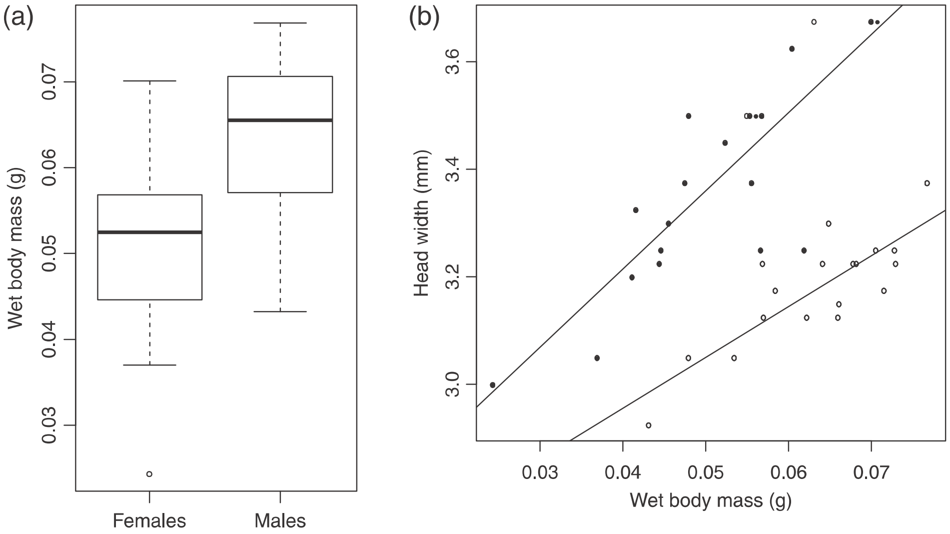

Fig. 2. Sexual dimorphism in *Ceratina chalcites*. (a) Dimorphism in fresh body mass; (b) relationship between head width and fresh body mass. Open circles, males; closed circles, females.

the head width of the female from the first brood cell. Sexual dimorphism was tested using a two-tailed *t*-tests. The correlation between the body and the head width and other morphometric parameters (body mass, forewing length, intertegular span) was tested using a Pearson correlation test. Comparisons between guarded and unguarded nests was tested by linear model except for the comparison of brood cell proportions with live offspring, which was tested using a binomial generalised linear model (GLM). The suitability of models was checked using diagnostic plots.

Results

Sexual dimorphism

Fresh body weight was greater in males ($m = 0.063\,465$ g on average, $N = 18$; Fig. 2a) than in females ($m = 0.050\,680$ g on average, $N = 19$; Fig. 2a) and this difference was statistically significant (*t*-test, $t = -3.93$, d.f. = 34.56, $P < 0.001$). On the other hand, females had a significantly ($t = 13.51$, d.f. = 355.47, $P < 0.001$) larger head width (3.41 mm, $N = 190$) than males (3.21 mm, $N = 188$). Body weight and head width were significantly correlated in both males (correlation test, cor = 0.83, $P < 0.001$, $N = 18$; Fig. 2b) and females (cor = 0.81, $P < 0.001$, $N = 19$; Fig. 2b).

Average forewing length was larger in males (9.62 mm, $N = 47$; Fig. S1) than in females (8.93 mm, $N = 40$). The difference was statistically significant ($t = -6.72$, d.f. = 82.69, $P < 0.001$, $N = 87$). There was a positive correlation between forewing length and head width in males (correlation test, $r = 0.89$, $P < 0.001$, $N = 47$) and also in females (cor = 0.93, $P < 0.001$, $N = 40$).

Average intertegular width was larger in males (2.66 mm, $N = 60$; Fig. S1) than in females (2.54 mm, $N = 48$). The difference was statistically significant (*t*-test, $t = -3.69$, d.f. = 92.63, $P < 0.001$, $N = 108$). There was a positive correlation between intertegular width and head width in males (cor = 0.84, $P < 0.001$, $N = 60$) also in females (cor = 0.92, $P < 0.001$, $N = 48$).

Nest guarding of active brood nests

For active brood nests, only one guarding adult female was present in most cases (88.63%; 39/44 nests). In other active brood nests, three contained an adult female with a male, one contained only an adult male, and one did not contain any adult individual. The number of brood cells in nests was not significantly dependent on the date of nest collecting (linear model, $F = 0.14$, $P = 0.7076$, $N = 44$). No social nests (i.e. with more than one guarding female) were found.

Nest guarding of full brood nests

A guarding female was only present in 34.44% (52/151) of full brood nests. In other nests (65.56%; 99/151), guarding female was not present. In nests with a guarding female, 73.07% (38/52 nests) had opened the last brood cell. In this case, the outermost offspring was not separated from the guarding female by a partition (Figs 2, S1). In nests with the last brood cell opened, a pupa was present in the outermost brood cell in 18 cases, a larva in 11 cases and dead offspring in nine cases. In a minority of nests (14/52 nests), the last brood cell was closed and the offspring was separated from the guarding mother (Fig. 1). In nests with a guarding female, the female was always present in the nest entrance and never observed below brood cell partitions inside brood cells.

Nests without a guarding mother always had the last brood cell closed by a filling plug and the entrance burrow was wider than the regular nest burrow in most cases (Fig. S3).

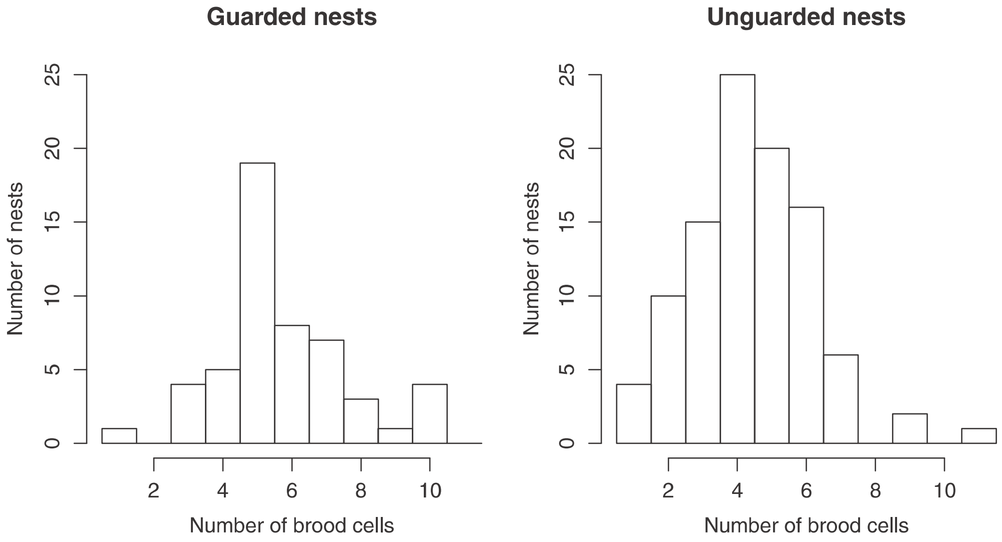

Fig. 3. Histograms of the number of brood cells in guarded and unguarded *Ceratina chalcites* nests. The difference between guarded and unguarded nests is significant (linear model, $F = 18.494$, d.f. $= 1$ $P \leq 0.001$).

The filling plug had an average length of 1.36 cm (range 0.3–5.5 cm, SD = 0.82 cm, $N = 99$; Fig. S4). These filling plugs were substantially thicker than the regular brood cell partitions (mean = 0.44 cm, SD = 0.09 cm, range 0.25–0.6 cm, $N = 31$ partitions from nine nests; Fig. S4). The difference was significant (Wilcoxon test, $W = 3007$, $P < 0.001$). There was some overlap between the length of the regular nest partition and the length of the filling plug which closes the nest. In 8.08% (8/99) of unguarded nests, the filling plug closing the nest was no more than 0.6 cm long (which was the length of the largest detected regular brood cell partition). The length of the nest entrance did not differ between nests with filling plugs shorter than 0.6 cm (mean 2.41 cm, $N = 8$) and longer than 0.6 cm (mean = 3.22, $N = 91$). The difference was statistically insignificant (*t*-test, $t = 1.61$, d.f. = 13.38, $P = 0.1295$).

Old adult males were present in the entrance in 11.11% (11/99) of nests that were not guarded by the mother (one male was present in nine cases and two males were present in two cases).

Full brood nest structure

The average number of brood cells per nest was 4.86 ($N = 151$, range 1–11, SD = 1.93). Empty cells were present only in rare cases (an average of 0.16 empty cells per nest were present, i.e. a total of 17 empty cells in 151 nests). Average nest length and width were 12.41 cm ($N = 151$, range 5.0–22.9, SD = 3.75) and 0.6511 cm (range 0.5–0.9, SD = 0.0857), respectively. When the last brood cell partition was excluded, nest partitions were 0.44 cm thick on average (SD = 0.09 cm, range 0.25–0.6 cm, $N = 52$ partitions). Excrement was found inside each brood cell with a fully grown larva or pupa and not as a huge aggregation of excrement in the innermost brood cell. We did not observe any tracks of a female crawling through nest partitions.

The overall sex ratio of offspring was not significantly different from 1:1 (190 females and 188 males; $\chi^2 = 0.01$, d.f. = 1, $P = 0.9181$); however, the first brood cell position was strongly female-biased (87.5% female; $N = 80$, $\chi^2 = 45$, d.f. = 1, $P < 0.001$). Other brood cell positions had a sex ratio not significantly different from 1:1 or were male-biased (Table S1). Sex ratio in all offspring excluding the first brood cell position was male-biased (59.73% male; $N = 298$, $\chi^2 = 11.28$, d.f. = 1, $P < 0.001$). The head width of females in the first brood cell position was not significantly different from the average head width of females in other positions (paired *t*-test, $t = -0.54$, d.f. = 42, $P = 0.591$).

Comparison between guarded and unguarded nests

Guarded nests had a significantly higher number of provisioned brood cells (linear model, $N = 151$, $F = 18.594$, $P < 0.001$; Table S2; Fig. 3), a higher number of brood cells with live offspring (linear model, $N = 151$, $F = 14.99$, $P < 0.001$; Table S2) and a higher proportion of live brood cells (binomial GLM, $N = 151$, deviance = 4.1126, $P = 0.043$; Table S2). By contrast, there was no significant difference in the length of the nest (linear model, $N = 151$, $F = 2.33$, $P = 0.128$; Table S2) or the length of nest entrance (linear model, $N = 151$, $F = 2.57$, $P = 0.111$; Table S2; Fig. S5).

Sex ratio of offspring substantially differs between guarded and unguarded nests (Fig. 4). Sex ratio was strongly male-biased in guarded nests (70.54% male offspring; $N = 129$, $\chi^2 = 21.78$, $P < 0.001$), but strongly female-biased in unguarded nests (38.95% male offspring; $N = 239$, $\chi^2 = 12.15$, $P < 0.001$). In the first brood cell, the sex ratio was female-biased in both guarded (8% male offspring, $N = 25$; Table S1) and unguarded nests (14.54% male offspring, $N = 55$; Table S1). In guarded nests, sex ratio was significantly male-biased within the two to five brood cell positions (Table S1). In six to 10 brood cell positions, the number of observations was too low to test the significance; however, in guarded nests, only male offspring

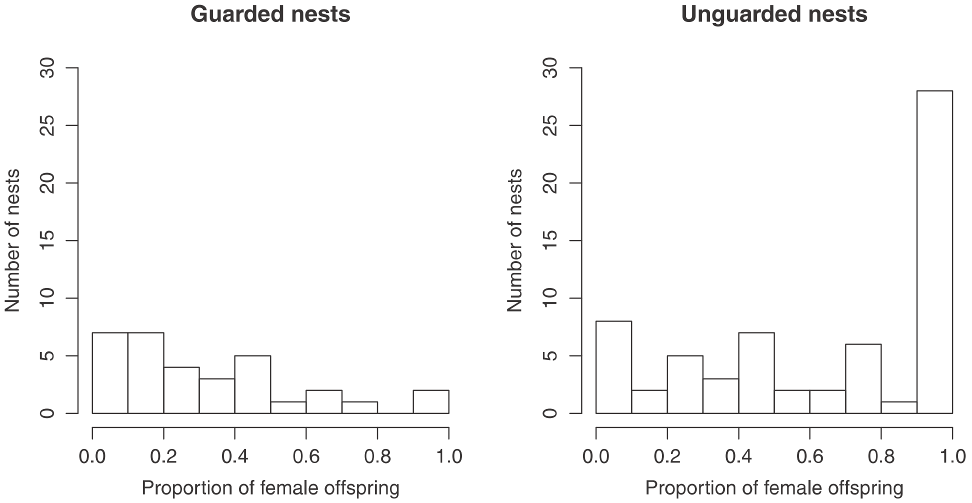

Fig. 4. Histograms of offspring sex ratio in guarded and unguarded *Ceratina chalcites* nests. The difference is significant (binomial generalised linear model, deviance = 35.411, residual deviance = 216.44, $P \leq 0.001$).

were found in these positions. In unguarded nests, the sex ratio was not significantly different from 1:1 in two to six brood cell positions (Table S1). In other brood cell positions of unguarded nests, the number of offspring was too low for statistical testing.

The average head width of female offspring was 3.48 mm in guarded nests and 3.39 mm in unguarded nests. This difference was statistically significant (linear model, $N = 81$ nests, $F = 7.47$, $P = 0.008$; Fig. S6). The average head width of male offspring was 3.22 mm in guarded nests and 3.18 mm in unguarded nests; however, the difference was not statistically significantly (linear model, $N = 66$ nests, $F = 2.64$, $P = 0.109$; Fig. S6).

Relationship between maternal size and investment in guarded nests

Mothers with larger head widths produced a significantly greater number of brood cells (linear model, $N = 46$, $F = 5.60$, $P = 0.022$). There was a positive influence of maternal head width on the proportion of males in the brood (binomial GLM, $N = 31$, deviance = 4.52, residual deviance = 43.71, $P = 0.033$). We also found a positive influence of maternal head width on the average head width of female (linear model, $N = 24$, $F = 11.96$, $P = 0.002$) and male (linear model, $N = 29$, $F = 6.29$, $P = 0.018$) offspring.

Discussion

Sexual dimorphism and maternal investment

Examination of guarded *C. chalcites* nests allowed us to determine the influence of maternal size on offspring investment. We found that larger females produce a larger clutch size and larger offspring of both sexes. Therefore, we can conclude that larger females are generally more successful than smaller females, which is similar to the majority of solitary nesting aculeate Hymenoptera (Cowan, 1981; Kim, 1997; Molumby, 1997; Rehan & Richards, 2010a).

We found a substantial allometry in sexual dimorphism of *C. chalcites*. Males of *C. chalcites* were significantly heavier and had significantly longer forewings and greater intertegular width. Greater forewing length in males than in females was also reported by Daly (1983). However, head width was smaller in males than in females. Body mass, forewing length and intertegular width are significantly correlated with head width, but the relationship is different for males and females (Figs 2b, S4).

Body mass strongly relates to food consumed in aculeate Hymenoptera (Bosch & Vicens, 2002; Macháčková *et al.*, 2018), and therefore we conclude that males are costlier than females. This type of sexual size dimorphism is relatively uncommon in aculeate Hymenoptera – in most species, females are heavier than males (Helms, 1994). Females being heavier than males os also typical for other species of *Ceratina*, i.e. *C. calcarata* (Rehan & Richards, 2010a) and *C. flavipes* (Maeta *et al.*, 1992). Larger females usually produce more female-biased sex ratio than smaller females in species that have larger females than males (Suguira & Maeta, 1989; Molumby, 1997; Strohm & Linsenmair, 1997b; Rehan & Richards, 2010a). Inverse sexual dimorphism in *C. chalcites* allows us to determine in general whether the more capable females invest more resources into females or into the more expensive sex. We found that larger females produce more male-biased brood than smaller females; therefore, they are investing in the more valuable sex. Similarly, males larger than females occur in *A. septemspinosum*, which also has a positive relationship between the female size and the proportion of males in brood (Sugiura, 1994). From our study, we cannot conclude what the benefit of larger male size for males is because we did not perform the test of male mating behaviour. Males being larger than females occurs in the bee genus *Anthidium*, where larger males have a higher ability to defend territory and therefore also a higher mating success (Alcock *et al.*, 1977; Sugiura, 1994).

Comparison of nest guarding and abandoning strategies

We have shown that guarded and unguarded nests are distinct facultative strategies. Guarded nests have the last brood cell frequently opened and therefore the mother is in a direct contact with the outermost offspring (Fig. 1c). As the length of nest entrance did not differ significantly between guarded and unguarded nests, we can suppose that the absence of a mother is not a result of random orphanage but a voluntary decision of the mother. Unguarded nests were closed by a massive filling plug, which was usually much thicker than the standard partition between brood cells (Fig. S2). In 8% of unguarded nests, the filling plug was of a similar size to the standard brood cell partitions. The entrance burrow length of these nests was not significantly different from that of other unguarded nests. We cannot exclude the possibility that some unguarded nests were orphaned and not voluntarily abandoned. However, because the majority (92%) of unguarded nests closed by filling plug were longer than any measured inner brood cell partition and other nests had a short entrance burrow (and therefore seem not to have been abandoned prematurely), we suppose that the frequency of orphaned nests was smaller than 8% and that all, or nearly all, unguarded nests were voluntarily abandoned.

We found a different pattern in maternal investment in guarded and unguarded nests – in particular, sex allocation differs substantially between these strategies. Sex ratio differs between guarded and unguarded nests in two to 10 brood cell positions. Therefore, this suggests that the decision of the mother to guard or not to guard is made early during the nest provisioning and is not a sudden reaction to environmental conditions at the time of nest completion.

Mothers generally invest more in guarded than in unguarded nests. Guarded nests had a higher number of provisioned brood cells, a higher proportion of offspring with the more valuable sex (males) and larger offspring (at least for females). A similar pattern was previously described in *C. chalybea*, in which guarded nests were also more valuable (Mikát *et al.*, 2016). However, in comparison to *C. chalybea*, the difference in the number of surviving offspring between guarded and unguarded nests was weaker. The number of brood cells with live offspring was 3.66 times higher in guarded than in unguarded nests in *C. chalybea* (Mikát *et al.*, 2016), whereas, in *C. chalcites* it was 1.42 times higher in guarded than in unguarded nests. The number of live offspring was influenced by two variables: the number of brood cells provisioned and the brood cell mortality. We found significant differences between guarded and unguarded nests in the number of provisioned brood cells and also brood cell mortality. The number of provisioned brood cells is clearly the result of maternal investment. On the other hand, causality between maternal care and proportion of dead brood cells is unclear. A higher proportion of dead brood cells could also be a result of the maternal abandonment. However, future experiments are necessary to detect the direction of causality.

We found a considerable difference in the sex ratio between guarded nests (70.54% male offspring) and unguarded nests (38.95% male offspring). As we have shown, males are the more costly sex in *C. chalcites* and therefore mothers produce a higher proportion of males in nests with better brood protection.

Organisms with facultative iteroparity can desert their first brood to produce a second brood due to environmental influences (Stegmann & Linsenmair, 2002; Futami & Akimoto, 2005), or they can use abandonment as a strategy for investment in diversification (Hopper, 1999; Zink, 2003). It is evident that *C. chalcites* females frequently desert nests with a high number of viable offspring and that offspring are able to survive without additional care from the mother. These females desert their offspring in 65.56% of cases, which is more common in *C. chalcites* than in *C. chalybea* (40.10%) (Mikát *et al.*, 2016). We suppose that the relatively high offspring survival in *C. chalcites* nests allows the frequent desertion by the female after completion of nest provisioning. However, we do not have direct evidence regarding what mothers do after abandoning the nest. We suppose that the main benefit of desertion is the possibility for serial nesting. We discovered newly founded nests concurrently with recently abandoned nests. The number of brood cells in active brood nests was independent of the date of nest collecting. Therefore, we suppose that new nests were regularly founded throughout the whole observation period. Moreover, in 2015 we observed newly founded *C. chalcites* nests in several localities in southern Italy in the first half of August (M. Mikát, pers. obs.). Therefore, our results support the weak formulation of the semelparity hypothesis that iteroparity reduces the extent of parental care (Stegmann & Linsenmair, 2002).

In most *Ceratina* species that have been studied in-depth behaviourally, offspring are guarded by mothers until adulthood (Rehan & Richards, 2010b; Mikát *et al.*, 2016). Benefits of prolonged maternal care include increased offspring survival (Mikát *et al.*, 2016) and feeding of adult offspring by the mother (Lewis & Richards, 2017; Mikát *et al.*, 2017). To date, facultative nest abandonment has only been described in the closely related species *C. chalybea* (Mikát *et al.*, 2016). The frequency of nest abandonment and founding of new nests was strongly correlated in this species in the late phase of the nesting season (Mikát *et al.*, 2016). Both species belong to the subgenus *Euceratina* (Terzo, 1998). As this subgenus is not a basal group in *Ceratina* phylogeny (Rehan *et al.*, 2010, 2012), we suggest that facultative nest abandonment is a derived state for the subgenus *Euceratina* or a subset of species belonging to this subgenus.

Sociality and extent of parental care in C. chalcites

Ceratina bees are considered to be primarily a eusocial group with multiple reversals to solitary life (Rehan *et al.*, 2012; Groom & Rehan, 2018). We did not find any multi-female nest; therefore, we consider *C. chalcites* to be a solitary species. However, it remains possible that multi-female nests are present later in the season.

Previous work has shown that mothers in some *Ceratina* species continue to feed their adult offspring after reaching adulthood (Sakagami & Maeta, 1977; Lewis & Richards, 2017; Mikát *et al.*, 2017) and that dwarf eldest daughters can perform sibling provisioning in cases of maternal death (Mikát *et al.*, 2017). Specific allocation of food to brood cells by mothers is linked to the provisioning of adult siblings by a sister. In such species, sex ratio is strongly female-biased in the first brood cell position of the nest (Lawson *et al.*, 2016; Mikát *et al.*, 2017), and females in the first brood cell position are usually the smallest females in the nest (Mikát *et al.*, 2017). For *C. chalcites*, we did not perform direct observations of the care provided to mature offspring but we explored patterns in sex ratio and size of provisioned brood. We found that the first brood cell position is extremely female-biased (87.5% of females); however, the first brood cell females were not significantly smaller than other females in the same nest. This is similar to *C. australensis*, in which the first brood cell is female-biased, yet the female in this position is comparable in size to other female offspring (Dew *et al.*, 2018). Direct observations of nests with mature offspring are necessary for the confirmation of the strategy used by *C. chalcites*.

Mothers of *Ceratina* bees typically crawl through nest partitions to clean their offspring, moving excrement and eliminating parasites (Rehan & Richards, 2010b; Mikát *et al.*, 2016). In *C. chalcites*, we never observed mothers inside brood cells. Furthermore, excrement was present in brood cells with old larvae and pupae, not transported to the bottom of the nest. This suggests that the ability to crawl though nest partitions was lost in the common ancestor of *C. chalcites* and related species *C. chalybea*, as *C. chalybea* is also unable to crawl through nest partitions (Mikát *et al.*, 2016).

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Author contributions

MM designed the research, MM and DB performed the research, MM analysed data, MM wrote the initial draft of manuscript, and JS performed substantial changes in manuscript.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. File 'Dataset_Mikat_chalcites' contains two sheets with original data.

Table S1. Sex ratio across brood cell positions in guarded and unguarded nests of *C. chalcites*.

Table S2. Comparison of features between guarded and unguarded nests.

Fig. S1. Sexual dimorphism in *C. chalcites*. (a) Dimorphism in forewing length; (b) relationship between forewing length and head width (open circles, males; closed circles, females); (c) dimorphism in intertegular width; (d) relationship between head width and intertegula width (open circles, males; closed circles, females).

Fig. S2. Examples of guarded nests. The guarding female is not in the photograph in some nests, but it was always present in these nests at the time of nest dissection.

Fig. S3. Examples of unguarded nests. The last brood cell is closed by the filling plug, which is much larger than the regular nest partition.

Fig. S4. Comparison of length of regular partitions in plugged nests, with the length of the filling plug closing the whole nest.

Fig. S5. Comparison of length of nest entrance between guarded and unguarded nests.

Fig. S6. Comparison of average head widths of female and male offspring between guarded and unguarded *C. chalcites* nests. The difference between guarded and unguarded nests was significant for females (linear model, $F = 7.47$, d.f. = 1, $P = 0.008$), but not for males (linear model, $F = 2.63$, d.f. = 1, $P = 0.109$).

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Unrelated males in colonies of facultatively social bee

Short title: Unrelated males in bee colonies

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Abstract

Colonies of social Hymenoptera are usually groups of closely related females, in which the dominant female(s) is specialized for reproduction and subordinate females care for immature offspring. Kin selection is thought to be the main factor that supports social cohesion. We have discovered a simple society of the bee *Ceratina chalybea* with an average of 4.68 colony members that cannot be maintained by kin selection alone. These colonies consisted of old reproductive female, young adults and provisioned brood cells. About half of young adults are unrelated to the old female, and almost all of the young adults are male. The old female provisions new brood cells, while continuing to feed young adult offspring. As young adults do not perform demanding or risky activities, they incur little or no cost, but they do benefit from the food they obtain from the old female.

Introduction

Cooperation between individuals is one of the most interesting biological phenomena. Several mechanisms of cooperation have been proposed [1–4], but it is thought that kin selection is the main mechanism driving the organization of societies [5–7]. This theory claims that the spreading of alleles through one’s own reproduction is equivalent to the spreading of alleles by related individuals [6,8]. The strength of kin selection is strongly influenced by relatedness – any help provided to another individual is only beneficial if the cost of helping that individual is lower than the benefit of the help received multiplied by the relatedness between the individuals [6,9]. Another mechanism of cooperation that has been postulated is based on reciprocity: the cost of helping is compensated by the predicted future benefit [1,3]. Alternatively, cooperation may be a by-product of selfish behaviour [4]. Recently, the plausibility of group selection has been disputed – sacrificing individuals for group benefit can exist as an adaptive feature [5,10,11].

Colony members in almost all social insects are related [7,9,12]. Cooperation of unrelated individuals occurs under specific conditions and some derived states [13,14]. Pleometrosis (nest founding by multiple females) is one of the most common situations in which unrelated members of a colony cooperate [13,15]. In many wasp and bee societies, some proportion of drifting workers have been recorded [14,16]. These workers originate from foreign colonies and are therefore not related to the original nest members [14]. Unlike eusocial insects, unrelated helpers are common in cooperative breeding vertebrates [2].

Societies of the eusocial Hymenoptera are mostly composed of females because the queens and workers are female [17,18] and most males die soon after mating, not participating in the life of the society [18–20]. Exceptions, such as male participation in care, are rare among eusocial societies and male helpers usually have only a minor role in colony life [21–23].

The traditional division of labour in hymenopteran societies is between the queen, who performs most or all egg-laying, and the workers, who are responsible for other tasks, especially food provisioning [18,24]. However, in small societies that are composed of one reproductively dominant female and one, or a few other females, a different type of task division is possible. The dominant female can perform egg-laying as well as food provisioning, with the reproductively subordinate female(s) performing nest guarding and other tasks in the nest [25]. This system of provisioning by the reproductively dominant female is typical for Xylocopinae bees [25,26].

Here, we examined social nests of the small carpenter bee, *Ceratina chalybea.* Small carpenter bees from the genus *Ceratina* make their nests in broken twigs with soft pith [27]. The female excavates a burrow in the pith and later provisions the brood cells [28,29]. After completion of cell provisioning, the female usually guards the offspring until they reach adulthood [29–31]. Subsequently, she provisions newly emerged offspring with pollen and nectar [31–33]. Although most temperate *Ceratina* are solitary [30,34], they belong to the Xylocopinae family, which is ancestrally facultatively social, with obligate solitary nesting being a derived state [35]. Social nests of *Ceratina* contain a few females, usually two but sometimes as many as four [36,37].

Until now, *C. chalybea* was thought to be a solitary bee [30]; however, here we present the first evidence of social nesting in this species. In contrast to most *Ceratina* bees, the females of this species did not obligately guard their offspring until adulthood, rather females had two alternative strategies – either guarding or abandoning the nest after provisioning of brood cells is finished [30]. Clearly, mothers can only perform provisioning of young adults in guarded nests and only these nests can develop into eusocial colonies. Here, we describe the social nests of *C. chalybea.* Moreover, we tested relatednes between colony members. We try examine possible costs and benefits for reproductive dominant and subordinate colony members.

Methods

Study site

We performed field research at the Havranické vřesoviště (coordinates 48°48'32.6"N 15°59'33.6"E) location, near the village of Havraníky, in Podyjí National park. This location is situated in the Southern Moravian region of the Czech Republic. The main experiments were performed in the years 2015 and 2017, but additional data are also presented from the years 2013 and 2018. Office of Podyjí National park permitted this research.

Preparation of nesting opportunities

We studied nests made in artificial nesting opportunities. The nesting opportunities were made from cut twigs with pith, from the following plant species: *Solidago canadensis*, *Helianthus tuberosus*, or *Echinops sphaerocephalus*. Twenty twigs were tied together into a sheaf and fixed to the ground with a bamboo rod. We distributed more than 20,000 nesting opportunities (1000 sheaves) each year. In 2013 and 2015, we collected nests directly from the nesting opportunities. In 2017 and 2018, some nests were collected directly from sheaves at nesting site and other nests were taken from original sheaves and transport to study plot for observation. Nests were collected and dissected after the observation period.

Nest dissection

We collected nests in the evening (after 19:00) to ensure that all inhabitants would be present inside the nest. We stored nests in a fridge between collection and dissection. We opened nests using a knife or clippers, and for each nest we recorded the presence of all adults and non-adult juveniles (eggs, larvae and pupae). For adults, we noted sex and age (parental vs filial generation). The age of adults was easily recognized because adults of the parental generation had extensive wear to their wings. All nests had only one old female. For non-adult juveniles, we recorded the stage and position of its brood cell in the nest. We also recorded the number and position of any empty cells (cells without provisions or offspring, [30]). We measured the length of the nest. We distinguished between new nests and reused nests. Reused nests had adult excrements in the lower portion and unsettled fillings below newly provisioned brood cells (Fig.1). For reused nests, we measured the length of the actively used portion (from the lowest newly provisioned brood cell to the nest entrance). Most of our analyses are based on nests in the late phase of the nesting season, between July 15th and August 15th each year).

Classification of nest stage

We only used active brood nests for our analyses. Active brood nests were an outermost brood cell containing a pollen ball or an egg; therefore, all nests that were currently provisioned. We distinguished two types of nests: solitary nests and social nests. Solitary active brood nests contained only a mother and the provisioned brood cells, no young adults. Social nests usually contained a mother and always had at least one young adult in addition to the provisioned brood cells. All together, between July 15th and August 15th we collected 28 social active brood nests (19 in 2015 and 9 in 2017) and 39 solitary active brood nests (19 in 2015 and 20 in 2017). We also classified some nests as full brood nests. Full brood nests were those in which brood cell provisioning had already been completed (the innermost brood cell contained a larva or pupa). Adult offspring are not yet present in these nests, or if they are present, they have not crawled through the nest partitions.

Observation of social nests

We transported the nests used for observation to special study plots. Transport of these nests was performed in the evening to ensure that all inhabitants were inside the nest. Each study plot contained 24 nets. Here, we only present the results from social *C. chalybea* nests; however, we observed these nests along with nests of other stages and species. Each nest was observed for one observational day, between 8:00 and 16:00 CEST on days with suitable weather. Each plot was observed for the entire time by at least one observer but most of the time there were two observers present. We marked foraging bees with an oil marker (Uni Paint) on the abdomen. We recorded the departure and arrival of foraging bees and noted when adults only departed from a nest (did not return) or newly arrived to a nest. It was necessary to cover the nest entrance with a transparent cup after every arrival of a bee so that the subsequent departure could be observed, since departure is usually very fast. This allowed us to verify whether the bee had been marked previously or if not, to mark it. Nests were dissected after their observational day. We performed this experiment in 2017. We successfully observed 4 social nests of *C. chalybea* per observational day. Two other nests were observed for only a partial day, due to inclement weather conditions.

Analysis of relatedness between individuals in the nest

We previously developed microsatellites for *C. nigrolabiata* [38], which we also used for the analyses of *C. chalybea*. We used the Chelex protocol for DNA isolation. We isolated DNA from the whole body of eggs and larvae, or the abdomen of pupae and adults. We added 4-8 µl proteinase K and 50 µl of 10% Chelex suspension in ddH_20 to dried samples. We mixed the suspension and heated it to 55°C for 45 min, then to 97°C for 8 min in a thermocycler (BioRad). We centrifuged the samples and froze the supernatant for further use.

We used the Type-it Multiplex PCR Master Mix (Quiagen) for multiplex PCR according to the manufacture's protocol. We used primers for ten microsatellite loci ([38], SI Appendix) at a concentration of 0.05 µmol/l. We used the following settings for PCR: 95°C for 15 minutes; 30 cycles of 94°C for 30 s, 60°C for 90 s, 72°C for 60 s; and finally, 60°C for 30 min.

We mixed 0.8 µl of PCR product with 8.8 µl of formamide and 0.4 µl of marker Liz 500 (Applied Biosystems). We heated the mixture to 95°C for 5 min and let it cool down. Fragmentation analysis was performed on a 16 capillary sequencer at the Laboratory of DNA sequencing at the Faculty of Science, Charles University. We used GeneMarker1.91 software (SoftGenetics, State College, Pennsylvania, USA) for the identification of alleles. Usually, this software correctly identified alleles; however, sometimes manual correction of the size scanner and identification of alleles was necessary.

From the 10 loci we used, 9 provided successful products and all were polymorphic, although polymorphism was highly variable between loci. Loci had between 2 and 33 alleles. Allele frequencies are summarized in [38]. We tested relatedness in 12 social nests. All these nests were collected in 2015. Together, we analyzed 12 old females, 21 non-adult juveniles (15 females and 6 males) and 52 young adults (5 females and 47 males).

Analysis of relatedness between mother and young adults

In social nests, we used two methods to determine if young individuals in the nest (young adults and also non-adult juveniles) are related to the old female. Primarily, we manually compared the genotypes of the old female and offspring to determine their loci compatibility. Male offspring should contain only alleles that the old female has. Female offspring should share at least one identical allele with the old female at each locus. We counted the number of loci that had alleles that were incompatible with the maternal genotype. We also assessed the relatedness of young adults and offspring in Kinship software [39] using the following analysis: Kinship analysis, relatedness option (Pairwise relatedness: Kinship). As offspring could have a coefficient of relatedness as high as 0.5 and unrelated individuals could have a relatedness coefficient as low as 0, we used 0.25 as the cut-off for related individuals. All offspring below this cut-off were considered unrelated to the old female.

Testing the maternity of young adult females

To determine the compatibility of young adult females with possible offspring (non-adult juveniles), we counted the number of incompatible loci – loci in the offspring that only had alleles that could not have been inherited from the young adult. We compared the number of loci with incompatible loci between non-adult juveniles and young adult females with the number of incompatible alleles between non-adult juveniles and old females. We also compared the relatedness calculated by Kinship software between non-adult juveniles and young adult females with the number of incompatible loci between non-adult juveniles and old females.

Testing the paternity of young adult males

We used colony software [40] to test the paternity of young adult males (to the non-adult female offspring) in social nests. Settings in Colony software were: Mating system – Female polygamy, Male polygamy, without inbreeding; Species – Dioecious, Haplodiploid; Length of run – Very long; Analysis method – FL; Likelihood precision – High. For other options, default settings were used. The locus feature was set as all loci codominant. The probability of genotyping error was 0.01; the probability of other errors (for example, mutations) was 0.001 for each locus. The old female was set as the known mother.

Statistics

All statistical analyses were performed in R software [41]. To test the relationship between sociality and nest reuse, we used Fisher's exact test. We tested for differences between social and solitary nests with the year as a covariable (model equations were: feature of nest ~ year*sociality). For length of nest, length of nest entrance, and number of brood cells, a linear model was used. For the proportion of empty cells, a generalized linear model of binomial family was used. It was impossible to test sociality and nest reuse in one model together because both factors were strongly correlated. Therefore, we fitted a primary model with sociality (feature of nest ~ year*sociality) and a secondary model with nest reuse (feature of nest ~ year*nest reuse). We compared the Akaike information criteria (AIC) between models with sociality and models with nest reuse for all four tested features of nests. We compared the relatedness between non-adult juveniles and young adult females with the relatedness between non-adult juveniles and old females. We tested differences in the number of incompatible loci by paired Wilcoxon test. We tested differences in relatedness by paired t-test.

Results

Evidence for social nesting

In the first part of the nesting season (until July 15th), we only observed solitary nests (2013 N=90; 2015 N = 22; 2017 N = 5; 2018 N = 19). None of these nests were reused from the previous season. However, we did find social nests later in the *C. chalybea* nesting season, after the 15th of July. In total, we found 28 social nests. After July 15th, half of the active brood nests we found in 2015 were social nests (19/38, Table 1) and 31.03% (9/29) of the active brood nests we found in 2017 were social nests. In 2013 and 2018, all dissected active brood nests were solitary (N=25, 2013; N=26, 2018). One social full brood nest was found in 2018.

On average, social nests contained 3.68 young adults (4.15 in 2015 and 2.66 in 2017, Table 2), and the maximum number recorded was 9. Most of the young adults were male (89.32%, 92/103). The reproductive female was present in most nests (82.14%, 23/28), but some social nests had been orphaned.

Table 1. The number of solitary and social active brood nests in different years. Only nests collected between 15 July and 15 August are included.

Sociality	Solitary nest			Social nest			
Nest reused?	No	Yes	Together	No	Yes	Together	Total
2013	23	2	25	0	0	0	25
2015	15	4	19	3	16	19	38
2017	15	5	20	2	7	9	29
2018	23	3	26	0	0	0	26
Total	76	14	90	5	23	28	118

Table 2: Number of young adults in *C. chalybea* social nests

Year	2015	2017	Together
N nests analyzed	19	9	28
Average number of young adults	4.16	2.67	3.68
Maximum number of young adults	9	6	9
Minimum number of young adults	2	1	1
SD of young adults	2.09	1.41	2.00
Average number of young adults females	0.26	0.67	0.39
Average number of young adult males	3.89	2.00	3.29
Proportion of males in young adults	0.9367	0.7500	0.8932

Comparison between solitary and social nests

We compared the nest architecture of solitary and social active brood nests. There was a strong association between social nesting and nest reuse: only 15.55% (14/90, Table 1) of solitary nests were reused in comparison to 82.14% (23/28, Table 1) of social nests. This association was significant for both years (Fisher exact test; 2015 – $p = 0.0002$, $N = 38$; 2017 – $p = 0.0140$, $N = 29$).

Solitary and social active brood nests did not differ in total nest length (linear model, $F = 0.81$ $p = 0.3713$, $N = 67$), but they did significantly differ in the length of the actively used portion of the nest (linear model, $F = 17.34$, $p < 0.0001$, $N = 67$). This result held true when we used nest reuse as the explanatory variable instead of sociality (total nest length did not differ – linear model, $F=2.28$, $p = 0.135$, $N = 67$; but the actively used portion of the nest did significantly differ in length – linear model, $F = 43.85$, $p < 0.0001$, $N=67$). When we compared the AIC of both models, nest reuse was better than sociality as an explanatory variable. There are fillings and excrement from previous instances of nesting at the bottom of reused nests; therefore, the length of the effectively used space is shorter (fig. 1).

We also found a difference in the number of brood cells provisioned. Social nests had significantly fewer provisioned brood cells (linear model, F = 7.21 p = 0.0093, N = 67, Table 3). When we tested nest reuse as an explanatory variable instead of sociality, the difference was also significant (linear model, F = 5.25, p = 0.0253, N = 67); however, the model using sociality had a better AIC than the model with nest reuse.

We also found a difference in the presence of empty cells. In almost all cases, brood cells were separated by empty cells in solitary nests, but they were often adjacent in social nests. The proportion of cells that were empty was significantly lower for social nests compared to solitary nests (Binomial glm, deviance = 7.99, residual deviance = 27.73, p = 0.0047, N = 67, Table 3). When we tested nest reuse as the explanatory variable instead of sociality, there was also a significant difference (Binomial glm, deviance 5.97, residual deviance = 29.78, p = 0.0146, N = 67); however, the model using sociality had a better AIC than the model with nest reuse.

Table 3: Comparison of social and solitary active brood nest characteristics. Only nests collected between 15 July and 15 August are included.

Nest Type	Solitary active brood nests			Social active brood nests			All nests
Year	2015	2017	Together	2015	2017	Together	
Number of Nests Analyzed	19	20	39	19	9	28	67
Total Length of Nest – mean	24.53	23.32	23.91	24.31	26.83	25.12	24.41
Length of Nest Used – mean	22.97	20.98	21.95	14.23	17.30	15.21	19.13
Number of Brood Cells – mean	3.58	2.95	3.26	2.21	2.44	2.29	2.85
Proportion of Cells Empty – mean	0.52	0.56	0.54	0.33	0.38	0.34	0.46

Fig. 1: Reused nest of *C. chalybea*. From left to right there are: fillings with excrements of larvae, excrements of young adults, an empty cell, the pollen ball of a brood cell currently being provisioned.

Foraging activity in social nests

We recorded high foraging activity in social nests, which were observed for a full day (mean = 16.5 foraging trips for a day, range = 12–20, N = 4 nests). In all cases, only ole female performed regular foraging activity. We also observed two additional nests per part of day. In one nest, foraging activity was performed by reproductive female and in second nest no activity was recorded. We did not observe any young adults performing foraging activity; however, we did note the emigration of young adults who did not return to the nest (mean = 1.25 for a day and nest, range = 0–2, N = 4 nests). There was also one case of immigration by a young adult (observed entering the nest without having previously departed).

Relatedness in social nests

Relatedness between the old female and non-adult juveniles (eggs and larvae): Software analysis in Kinship software concluded that all non-adult juveniles were related to the old female. However, manual comparison of genotypes showed, that one individual had one locus with one allele that could not have been inherited from the old female. Therefore, non-adult juveniles had all loci compatible with the old female's genotype (95.23%; N = 21, Fig. 2).

Fig. 2: Histograms showing relatedness between the old females and other members of the societies. A) Number of incompatible loci between the old females and non-adult offspring (eggs and larvae), N=21. B) Number of incompatible loci between the old females and young adults, N=52. C) Relatedness between the old females and non-adult offspring (eggs and larvae), calculated using

Kingroup software, N=21. D) Relatedness between the old females and young adults, calculated using Kingroup software, N=52.

Relatedness between the old females and young adults: Young adults were compatible with the old female’s genotype for all loci in 40.39% of cases (21/52, Fig 2); whereas, one locus disagreed with the old female’s genotype in 11.53% of cases (6/52). For the remaining cases (25/52), the genotyped offspring had more than one locus that disagreed with the old female’s genotype. Analysis using Kinship software showed that 55.77% (29/52, Fig 2) of young adults were related to the old female and 44.23% (23/52, Fig 2) were unrelated to the old female. Therefore, we assume that between 44.23% and 59.61% of young adults are unrelated to the old female.

Relatedness between young adult females and non-adult juveniles: Young adult females were only present in 3 out of 12 nests that were genetically analyzed. We compared the relatedness between young adult females and non-adult juveniles with the relatedness between the old female and non-adult juveniles. There were 9 possible pairs of non-adult juveniles and young adult females for evaluation. In 22.22% (2/9) of these possibilities, non-adult juveniles had all loci compatible with maternity of the young adult female; whereas, one locus was incompatible in 33.33% (3/9) of cases and more than one locus was incompatible in 44.44% (4/9) of cases. The genotype of non-adult juvenile offspring was compatible with maternity of the old female in all loci. Therefore, non-adult juveniles have significantly higher compatibility with the genotype of the old female than the young adult females (paired Wilcoxon test, V = 0, p = 0.0206, N=9). Also, we found that the relatedness calculated by Kinship software of non-adult juveniles was higher to old female than young adult females (paired t-test, t = 5.77, df = 8, p = 0.0004, N = 9).

Relatedness between young adult males and non-adult juveniles: We tested the paternity of young adult males using Colony software to determine their relatedness to the non-adult juvenile females.

This analysis showed that none of these offspring (N = 15) were fathered by any of the young adult males. In all cases, the probability of paternity was less than 1%.

Discussion

Sociality in *C. chalybea*

We suggest, that social nests of *C. chalybea* fulfill all three of the conditions for eusociality defined by [24] and [18]: i) reproductive division of labour (only old females reproduce); ii) generation overlap (adults of the parental and filial generations are present); and iii) cooperative brood care (adult members of the colony cooperate in guarding, helping young offspring survive) are all present.

We confirmed the old female’s dominant reproductive role by microsatellite analysis. In all but one case, the genotypes of non-adult juveniles were compatible with maternity of the old female. We suppose that the single locus incompatibility in this one individual is due to a genotyping error or mutation. Young adult females were rarely present in social nests and their maternity is less probable than the maternity of the old female. We also tested that young adult males do not father non-adult juveniles. Therefore, we demonstrated that the old female strongly (probably exclusively) dominates reproduction in social nests and it is highly likely that no other members of the colony reproduce.

The most disputable phenomenon is cooperative brood care. The old female performs all offspring provisioning. We never observed other individuals performing regular foraging activity and we recorded only an occasional emigration or immigration of young adults. We suppose that the presence of young adults is beneficial for nest protection because unprotected nests of *Ceratina* bees [30] and other nest-making social Hymenoptera [42,43] are vulnerable to invasion and destruction.

Social nesting was strongly associated with nest reuse in *C. chalybea*. Nest reuse is generally considered a key factor for the development of sociality in *Ceratina* [36,37,44]. Although nest reuse can be an important factor influencing nest structure in social nests, we showed that sociality itself is a better predictor for the number of brood cells provisioned and the proportion of empty cells in a nest. Therefore, we suggest that at least these aspects of nest use are directly affected by sociality and not only the effects of nest reuse.

Comparison with other insect societies

The social structure of *C. chalybea* is unusual among social insects for several reasons: i) the presence of young adult males, ii) an unusually high proportion of unrelated colony members, and iii) reproductive subordinate individuals perform only nest guarding (not provisioning).

Almost all (89%) members of *C. chalybea* societies are males. This is interesting because in general, males have a very minor role in the Aculeate Hymenoptera [17,19,20]. A few biparental species are known, in particular crabronid wasps from the genus *Trypoxylon* [45,46] and *Ceratina nigrolabiata* [38]. In almost all eusocial species, males are a small minority among the colony members and have a marginal role in comparison to female workers [21,47]. An interesting exception is the crabronid wasp, *Misrostigmus nigrophtalmus*, in which a high proportion of colony members are male, actively participating in nest defense. They are even able to perform this task in the absence of female helpers [22]. But it remains unknown why male participation in eusocial societies is so rare. Phylogenetic constrains might be one explaination. The solitary ancestors of social species have female care without male participation [17,18,20]. Males lack some morphological structures, such as hairs for pollen collection and a sting, which are important for working effectively in eusocial societies [20,48]. Uncommon male behaviours may arise from performing a task or a standard behaviour with a different primary purpose. In our case, it is likely that males can help with nest protection because they primarily block the nest entrance in self-defense. Regardless of how it occurs, this behavior does lead to effective nest guarding.

We determined that about half of the young adults are unrelated to the old female in *C. chalybea* societies. There exist various mechanisms for arising of insects societies composed of unrelated members, such as pleometrosis [13,15], adopting of orphaned brood [49] or exchange of individuals between neighbour colonies [14]. We can exclude the possibility of pleometrosis for *C. chalybea,* because we never found more than one old female in the nest. Adoption of an unrelated brood is possible because nest usurpation and brood removal do occur in *C. chalybea*; however, it is rare and only occurs with orphaned nests [30]. Therefore, incomplete brood removal cannot explain the large proportion of unrelated young adults in nests of *C. chalybea*. Thus, it is very likely that the unrelated individuals in *C. chalybea* nests originate from neighbouring nests. We frequently observed young adults emigrating from and immigrating to nests; therefore, we consider unrelated individuals to be drifting from other nests.

The reproductively dominant (old) female in C. *chalybea* nests performs all foraging and reproduction. Young adults are passive; they do not perform any regular foraging trips. This type of division of labour is generally uncommon in eusocial Hymenoptera [18,24], but it is usual for Xylocopine bees [25,26,37]. In *C. chalybea*, we found the direct opposite of classical queen-worker task division: the *C. chalybea* old reproductive female performs all foraging trips and young adults (reproductive subordinates) only perform guarding. This is different from Allodapine bees, where multiple females commonly perform some foraging [50], and also from east Asian *Ceratina* of the subgenus *Ceratinidia*, where dominance of reproduction is unstable [51,52]. A direct contradiction of the classical queen-worker roles (foraging dominant, passive subordinate) does occur in *Xylocopa* [26,51,53]. However, in *Xylocopa sulcatipes* societies with a larger number of adult members (about 6), foraging is performed by multiple individuals [53]. Therefore, we have probably found a Hymenopteran society with the lowest proportion of foraging individuals.

Benefits for young adults

Subordinate members of insect societies usually benefit from indirect fitness [6,9,11]. However, direct fitness benefits, such as the possibility of inheriting a dominant position [25,26,54] or direct reproduction [55,56], can also be important.

Indirect fitness benefits only occur with non-zero relatedness between the donor and acceptor [6]. However, we have shown that about half of the young adults are unrelated to the old female. Moreover, previous work indicates that *C. chalybea* has a multiple mating strategy [38], which is unusual in simple hymenopteran societies [7]. Drifting individuals and multiple mating generate very low relatedness between colony members. Half of the colony members, those that are unrelated, cannot gain any indirect fitness benefit from helping. Furthermore, the other half of the colony members, those that have non-zero relatedness to the colony's young adults, might only gain a small indirect fitness benefit due to the lower productivity of social nests in comparison to solitary nests.

The possibility for nest inheritance is an important selection factor for the cooperation of unrelated members in small insect societies [54]. Generally, in Xylocopinae bees, nest inheritance is probably a very important driver [25,56]. However, in the case of *C. chalybea* this cannot be an important factor for the retention of sociality, because most of the young adults are male and nest-loyal biparental behaviour is unknown in this species [38]. Additionally, as we did not observe any case of nest reuse from the previous season, therefore we suppose that each female will build new nest next year. For these reasons, we can exclude benefits from nest inheritance as a reason for sociality in the case of *C. chalybea*.

Reproductively subordinate colony members can sometimes directly reproduce in small hymenopteran societies [54,57]. These individuals drifting to foreign nest frequently benefit from direct reproduction and commonly reproduce more than domestic workers [55,58]. However, based on our evidence, it is clear that the old female strongly dominates reproduction in *C. chalybea* societies and reproduction by young adults is negligible or zero. Therefore, direct reproduction cannot be an important motivation for a young adult to stay.

Care of adult offspring is an unusual trait among insects [59], but it is common in Xylocopinae bees [33]. It is likely that young adults remain in their nests because they benefit from the food provided by old female. Long-term cohabitation between an old female and young offspring is a widespread feature in *Ceratina* bees [29]. Many studies of solitary nests have shown that the mother provides pollen and nectar for her young adult offspring [28,31–33]. We argue that social nests arise from nests where mothers feed their adult offspring: first, the mother feeds mature offspring and then she begins to provision new brood cells. However, this strategy can have a significant cost. As provisioning of young adults continues along with providing for new offspring, the mother must divide her resources between the new brood cells and adult offspring; therefore, the amount of food that can be allocated for brood cell provisioning and thus the number of new brood cells is decreased. We observed that the pollen ball in the outermost (open) brood cell, which was currently being provisioned, had an atypical shape in some social nests. We suppose that this pollen ball is partially eaten by young adults. Simultaneous provisioning of brood cells and feeding of young adults has also been documented for *Xylocopa pubescens*. Maternal care of young adults is an important benefit for them [26,60].

Cooperation between organisms is dependent on the costs-benefits ratio [6]. When little cost occurs, little benefit is required to maintain stable cooperation. Young adults of *C. chalybea* do not perform foraging, which is a very risky task for workers in most social insects [18,61]. It is likely that the presence of young adults in *C. chalybea* nests has few costs, because it does not reduce their lifetime reproduction. Females of *C. chalybea* [30] and also other temperate *Ceratina* species do not reproduce before overwintering [29]. Males of temperate *Ceratina* bees survive through the winter and usually mate in the season after overwintering [31,38]. Therefore, remaining in the nest probably has little or no cost to future reproductive success and consequently, only a small amount of benefit is required for young adults to remain.

Benefits for the old female

There was exceptionally low productivity in social nests of *C. chalybea*. In total, there were fewer brood cells provisioned in social nests than in solitary nests. This differs from other social species, where the overall productivity of social nests is either higher [43,62] or at least the same as solitary nests [44,63]. Contrary to workers in large societies, young adults in *C. chalybea* nests did not leave to perform foraging; rather, they stayed inside their nest. However, non-foraging individuals can be beneficial for the society in other ways. It has been shown that the presence of guards in the nest can be effective protection against pollen robbery by conspecific females in *Xylocopa* [26] or nest usurpation [56].

In comparison to solitary nesting, social nesting decreases the risk of total nest destruction [56,64]. In the case of *C. chalybea*, removal of the mother from completely provisioned solitary nests significantly decreases the survival of offspring due to attack by natural enemies [30]. Therefore, the presence of young adults can be a benefit because they are able to protect the younger cohort of offspring. Young adults can serve the nest community through two mechanisms: i) reducing or eliminating the trade-off between nest guarding and offspring provisioning, and ii) at least temporarily, protecting the nest after the death of the mother. It has been shown that social nesting allows for more effective foraging in multiple facultatively eusocial species [26,65]. We did not test the effectiveness of young adult guarding experimentally; however, we did find a difference between solitary and social nests in their architecture. In social nests, empty cells were significantly less frequent than in solitary nests. Empty cells are thought to be an adaptation for protection against parasite attack [66]. Therefore, in social nests, the presence of young adults can protect against attack and the old female are able to reduce the number of empty cells, allowing more space for provisioned offspring. We observed that young adults were present in some *C. chalybea* nests from which the mothers had already vanished. As these young adults are located in the nest entrance, they can protect the brood cells against potential intruders.

It is possible that there was low nesting productivity in social nests because a significant proportion of pollen and nectar was consumed by the young adults and, therefore, could not be used to build brood cells. As about half of the young adults are related to the old female, feeding of young adults alongside brood provisioning can be beneficial for her reproductive success because this supports their survival [32]. From the old female's view, social nesting can be interpreted as maternal care for two cohorts of offspring simultaneously: a new cohort of offspring in the brood cells and an old cohort of young adult offspring. However, it is unclear why the old female tolerates unrelated young adults in the nest. One possibility is that it may be difficult to discriminate between alien and own offspring. In *C. calcarata*, the mother can discriminate between nestmate and non-nestmate young females [67]; however, overall aggression among individuals in mature brood nests is generally low, and when it does occur, it is more often against nestmate than non-nestmate young females [67].

Implications of *C. chalybea* natural history for social evolution

Our observations support the view that benefits for subordinate colony members in small insect societies are not, in many cases, primarily connected to inclusive fitness. It is possible for some females to gain direct fitness benefits, as has been documented in some studies on Xylocopine bees [25,26]. However, in the case of *C. chalybea*, the main benefits are not in the possibility of nest inheritance, but in the extended care of mature offspring. The old female provides pollen and nectar to feed young adults, which helps them survive. The old female tolerates young adults in the nest, because this can provide the benefit of increased nest protection. Therefore, our study supports the importance of mutualistic interactions in the evolution of the early stages of sociality.

As costs to young adults are low, small benefits are sufficient for the maintenance of sociality. We suppose that young adults mainly benefit from the food provided by the old female. Young adults can help with protection against natural enemies; however, their primary motivation for this is probably passive (self-defense). Although the observed society fulfills the definition of eusociality proposed by [18,24],the motivation for the behavior of colony members is mainly selfish. Therefore, the society of *C. chalybea* is something between eusociality and a two-cohort maternal subsociality. Unrelated young adults can be considered parasites, as they take food resources from the old female

Eusociality is ancestral state for all Xylocoinae bees with strict solitarity being a derived strategy [35]. The unusual social organization of *C. chalybea* has some traits in common with typical Xylocopine social organization, especially the presence of unrelated colony members [56,68] and passive reproductively subordinate individuals [25,51]; however, in the quantity of these features, *C. chalybea* is extreme, even among species of the subfamily Xylocopinae. Furthermore, *C. chalybea* society is unique in its inclusion of male colony members.

Here, we have shown that eusociality in bees can be maintained even when the relatedness between colony members is very low and indirect as well as direct fitness benefits (i.e. the possibility of nest inheritance) play small roles. In this case, eusociality is supported by specific natural-history traits (i.e. feeding pollen and nectar to mature offspring and nest reuse). Thus, our results show that good knowledge of natural history is important for interpreting social evolution.

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Data Availability

Dataset is available as SI material of this paper.

Author contribution

MM and JS designed the research; MM, DB and JS performed the research; MM analyzed the data; MM wrote the initial draft of the paper; all autors commented and finalized the paper.

Competing of interests

The authors declare no competing interests.

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Figures

Fig. 1: Reused nest of *C. chalybea*. From left to right there are: fillings with excrements of larvae, excrements of young adults, an empty cell, the pollen ball of a brood cell currently being provisioned.

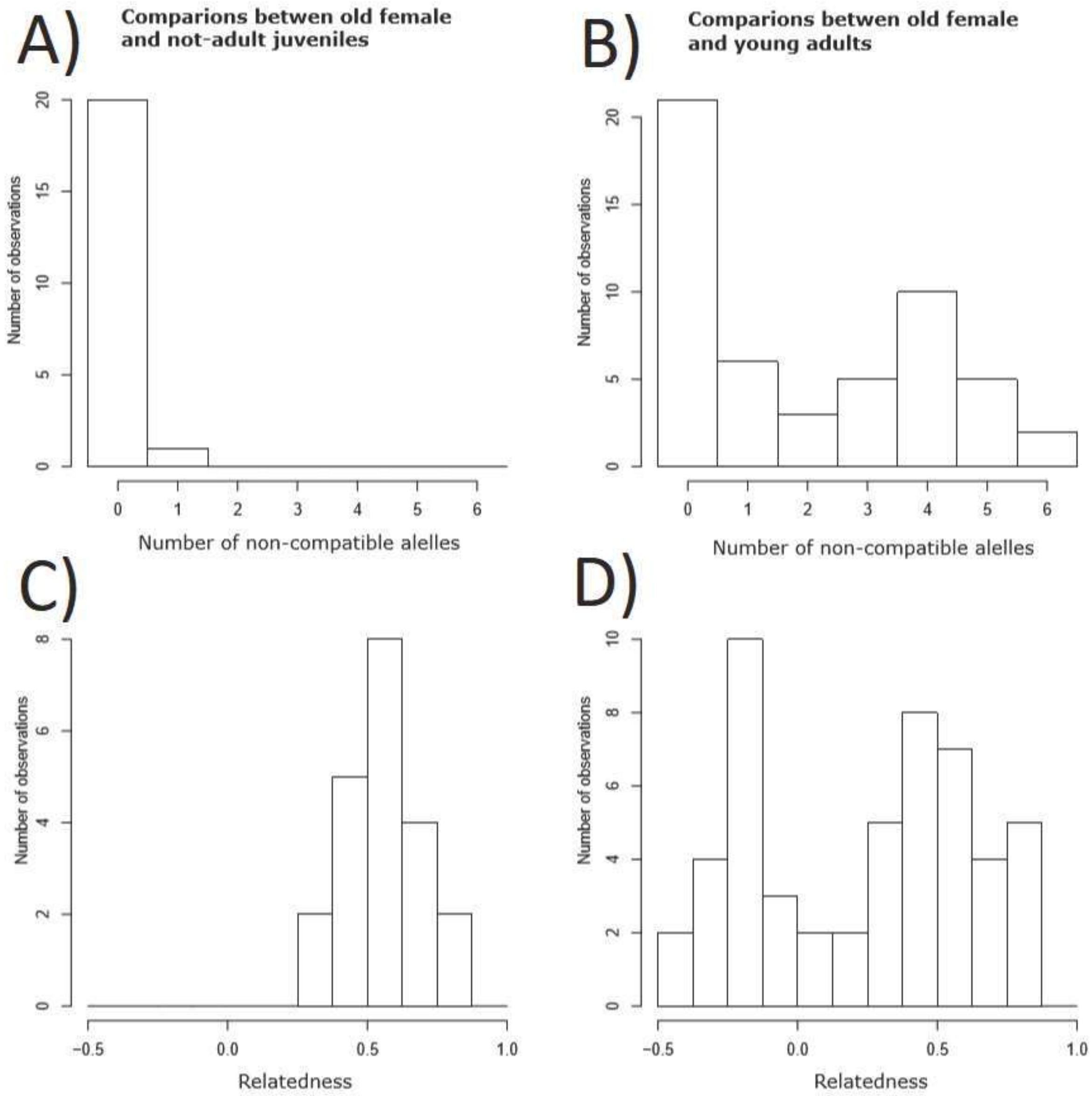

Fig. 2: Histograms showing relatedness between the old females and other members of the societies. A) Number of incompatible loci between the old females and non-adult offspring (eggs and larvae), N=21. B) Number of incompatible loci between the old females and young adults, N=52. C) Relatedness between the old females and non-adult offspring (eggs and larvae), calculated using Kingroup software, N=21. D) Relatedness between the old females and young adults, calculated using Kingroup software, N=52.

STUDY 8

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Abstract:	Aculeate Hymenoptera have two modes of overwintering. In solitary species, adults usually do not enclose before winter and overwinter in their natal nests. In the majority of social species, adults activate before winter and only inseminated females perform overwintering. We examined the winter survival strategy in four species of small carpenter bees (Ceratina) in which both sexes activate before winter and overwinter as adults. All of the species studied are able to use their old nests, nests of other Ceratina species or newly excavated burrows for overwintering. However, the species differ in their strategy. Ceratina cucurbitina usually overwintered in social hibernacula with a large number of individuals, but social hibernation was less often in C. nigrolabiata. On the other hand, individuals of C. chalybea and C. cyanea overwintered predominantly solitary. Interestingly, we detected female individuals that attempted to overwinter for a second time in all species. Surprisingly, males of C. nigrolabiata and C. chalybea are also able to overwinter for a second time in rare cases. These males lived for more than 1.5 years; therefore, they are the longest living bee-males known to date.
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Overwintering strategy and longevity of European Small carpenter bees (*Ceratina*)

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Abstract

Aculeate Hymenoptera have two modes of overwintering. In solitary species, adults usually do not enclose before winter and overwinter in their natal nests. In the majority of social species, adults activate before winter and only inseminated females perform overwintering. We examined the winter survival strategy in four species of small carpenter bees (*Ceratina*) in which both sexes activate before winter and overwinter as adults.

All of the species studied are able to use their old nests, nests of other *Ceratina* species or newly excavated burrows for overwintering. However, the species differ in their strategy. *Ceratina cucurbitina* usually overwintered in social hibernacula with a large number of individuals, but social hibernation was less often in *C. nigrolabiata*. On the other hand, individuals of *C. chalybea* and *C. cyanea* overwintered predominantly solitary.

Interestingly, we detected female individuals that attempted to overwinter for a second time in all species. Surprisingly, males of *C. nigrolabiata* and *C. chalybea* are also able to overwinter for a second time in rare cases. These males lived for more than 1.5 years; therefore, they are the longest living bee-males known to date.

Keywords: hibernation, lifespan, mortality, sociality, hibernaculum, twig-nesting

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Introduction

Organisms are strongly influenced by climate and weather conditions (Addo-Bediako et al., 2000; Dew et al., 2018; Fründ et al., 2013; Skandalis et al., 2011; Straka et al., 2014; Tauber and Tauber, 1981). This is especially true for temperate climates, where large differences between winter and summer temperatures are. As winter temperatures are not suitable for the activity of most temperate insects, most of them survive winter in diapause (Bale and Hayward, 2010; Tauber and Tauber, 1981). Insects have complex life cycles with several developmental stages; however, not all of the stages are able to perform successful overwintering (Bale and Hayward, 2010; Gill et al., 2017; Tauber and Tauber, 1981).

In temperate Aculeate Hymenoptera, there are two main modes of overwintering. Most of the solitary species overwinter in the prepupal, pupal or immature adult stage and do not activate before winter (Fründ et al., 2013; Michener, 2007); therefore, adults of both sexes emerge after winter for the first time. On the other hand, eusocial species and also most of the solitary Halictidae overwinter as adults (Alford, 1969; Gibo, 1980; Greene, 1991; Röseler and Honk, 1990; Sakagami et al., 1984). In these species, mating usually precedes overwintering and males generally die before winter; therefore, only females perform overwintering (Alford, 1969; Greene, 1991; Sakagami et al., 1984; Yanega, 1990).

In temperate Xylocopinae bees (solitary and facultatively eusocial species) adults also mature before winter; however in contrast of most of social species, both sexes can overwinter (Gerling and Hermann, 1978; Rehan and Richards, 2010; Sakagami and Laroca, 1971). Young adults are fed with pollen and nectar by the mother and sometimes by a sister before overwintering (Lewis and Richards, 2017; Mikát et al., 2017; Sakagami and Maeta, 1977). Mating usually occurs after winter (Daly, 1983; Maeta et al., 1992; Michener, 1990; Rehan and Richards, 2010), although facultative pre-hibernation mating has also been documented (Kidokoro et al., 2006). In this study, we examined the overwintering strategy of European species of the genus *Ceratina* which belongs to subfamily Xylocopinae.

We examined the hibernacula of four central-European species of *Ceratina*: *C. cucurbitina*, *C. cyanea*, *C. chalybea*, and *C. nigrolabiata*. We examined the type of shelter in which these bees overwinter (old nests vs. new burrows) and whether they tend to overwinter socially or solitarily. We found some adult mortality during winter in all species. Lastly, we examined if individuals overwinter for the first or the second time.

One of the most important factors for successful winter survival is obtaining an appropriate microhabitat for overwintering (Bogusch et al., 2016; Moore and Lee, 1991; Sinclair, 2015). For example, *Polistes* wasps are able to use a wide diversity of shelters; for example, under bark, in human-made structures, or in old *Dolichovespula* nests (Gibo, 1980). Halictidae overwinter in the ground, some species predominately in natal nests and some in other burrows (Packer, 1983; Packer and Knerer, 1985; Sakagami et al., 1984; Yanega, 1990). Usually, the aculeate Hymenoptera that overwinter in the adult stage use well-protected and unexposed microhabitats for overwintering. However, Xylocopine bees overwinter in burrows in wood or twigs similarly as they do during nesting (Gerling and Hermann, 1978; Kidokoro et al., 2006; Martin, 1991; Rehan and Richards, 2010). In comparison to the underground overwintering species, insects in these habitats are exposed to extreme weather conditions (Dew et al., 2018; Moore and Lee, 1991). *Ceratina* bees can use an old nest or excavate a new burrow for overwintering (Rehan et al., 2010; Rehan and Richards, 2010; Sakagami and Maeta, 1987; Sakagami and Laroca, 1971; Shiokawa, 1966). Alternatively, they can use the preexisting cavities or burrows of other insects (Sakagami and Laroca, 1971). They can either overwinter alone or with other conspecific individuals in one hibernaculum (Kidokoro et al., 2006; Sakagami and Maeta, 1987).

The lifespan of most insects is shorter than one season (Keller and Genoud, 1997). The most notable exceptions from this rule are the queens of highly social species, which usually live longer than one season (Carey, 2001; Keller and Genoud, 1997). Queens of social species spend most of their life in their nest, where they are protected from external mortality factors and bodily damage caused by foraging activity (Keller and Genoud, 1997; Wilson, 1971). In contrast, solitary species and the workers of social Hymenoptera perform intensive foraging activity, which negatively influences their lifespan (Schmid-Hempel and Wolf, 1988; Straka et al., 2014). Therefore, it is not surprising that actively foraging females live for a short time. Some interesting exceptions to these general lifespan characteristics exist in Xylocopine bees (Michener, 1990; Sakagami and Maeta, 1977). Although this group contains solitary and facultatively social species (Michener, 2007; Rehan et al., 2012), some instances of longer-than-one-year lifespans have been documented. Females of *Xylocopa virginiaca* often live two or three seasons (Gerling and Hermann, 1978). Similarly, about 13% of female *C. flavipes* individuals are able to overwinter for a second time (Sakagami et al., 1982). In *C. japonica*, most of the individuals live only one season; however, there are known examples of females that live up to three years (Sakagami and Maeta, 1977). Finally, in North-American *C. calcarata*, some females try to overwinter two times, although a successful nest founding has not yet been documented in the second season (Rehan and Richards, 2010).

Almost all temperate social wasps and most of the social bees have a single annual life cycle – colonies die at the end of the nesting season (Greene, 1991; Wilson, 1971). Clearly, overwintering ecology can influence social evolution. Social wasps of the genus *Vespula* form huge polygynous colonies when they are introduced to an area with a warmer climate than the original one or when they are located near the southern edge of their natural area, which suggests that this is due to the survival of the colonies though the winter (Greene, 1991; Pickett et al., 2001; Ross and Visscher, 1983; Visscher and Vetter, 2003). Furthermore, in Halictidae bees, nest founding by multiple females (pleometrosis) is positively influenced by overwintering in natal nests (Yanega, 1990). The overwintering strategy can also influence the existence of delayed eusociality. Delayed eusociality occurs when a mother overwinters with her daughter and they form an eusocial colony the next year (Plateaux-Quénu, 1992; Sakagami and Maeta, 1989). This trait has been documented in some species of halictid bees (Plateaux-Quénu, 1992; Sakagami et al., 1984) and *Ceratina* bees (Sakagami and Maeta, 1977). The existence of delayed eusociality is dependent on overwintering philopatry and the ability of females to overwinter more than once (Sakagami and Maeta, 1989).

Study area

We performed our research at the Havranické vřesoviště location (48°48'36.4"N 15°59'40.8"E). This locality is in National Park Podyjí/Thayatal in the South Moravian region of the Czech Republic. We performed our data sampling in three different years: 2015 (March 3-4), 2016 (February 13-15) and 2017 (February 23-24). The sampling periods were in the latter part of winter, but they were always before the beginning of the springtime *Ceratina* activity.

Material and Methods

Sampling process

We dissected nests that were made in artificial nesting opportunities, which were installed in the research locality one or more nesting seasons before sampling. The nesting opportunities were made of cut twigs tied into sheaves of 20 twigs and fixed to the ground by a bamboo rod. We collected all of the twigs that could contain *Ceratina* bees (i.e. twigs with holes that were sufficiently large) in selected areas of the research locality. We stored these twigs in the fridge until dissection, which was performed using a knife or clippers. We examined 1365 twigs in 2015, 2342 in 2016, and 2145 in 2017; therefore, in total, we examined 5852 twigs. However, hibernacula were present in only a minority of these twigs. We examined 306 hibernacula with 941 *Ceratina* individuals in 2015, 485 hibernacula with 1952 *Ceratina* individuals in 2016, and 307 hibernacula with 1247 *Ceratina* individuals in 2017. In total, we examined 1098 hibernacula with 4140 *Ceratina* individuals.

We recorded all hibernacula containing at least one *Ceratina* bee and the following parameters were noted for each hibernaculum: number, sex, age, and species of all *Ceratina* bees present; length of the nest, width of the burrow, presence of other animals in the hibernaculum, plant species origin of the twig, and age of the nest. The age of individuals was assessed by observing the amount of wear to the wings – individuals that survived from the previous season had extensively worn and differently colored wings (Rehan and Richards, 2010; Sakagami and Maeta, 1977) (Fig 1).

The age of the nest was assessed based on the presence of fecal pellets on the side of the burrow: old nests had dirty walls, usually with excrements and fillings in the bottom of the nest; whereas, newly excavated burrows had walls that were the same color as the pith of the twig and no residua of excrements (Rehan and Richards, 2010). The species that built the nest was determined primarily by observation of the characteristics of nest structure. The four species of interest produce nests with different length, width, distribution of brood and empty cells, and tightness of partitions (Mikát et al., 2016, MM, unpublished data). However, sometimes the original nest structure was poorly conserved; therefore, in these cases, we assumed that the nest was made by the species with the most individuals present in the nest. When only two individuals of different species were present in an old nest, we assumed that the nest was made by the species that was found closer to the bottom of the nest. Some *Ceratina* individuals were observed in nests built by other hymenopteran species (e.g. crabronid or eumeninae wasps). These nests were classified as foreign nests. We presume that the individuals present in foreign nests and also in newly excavated burrows dispersed from their original nest. We classified foreign nests and newly excavated burrows together as dispersal hibernacula.

Statistics

We used R software for all statistical analyses (R Core Team, 2014). Differences in the number of conspecific individuals in the hibernacula were tested by poisson GLM because the abundances had a poisson distribution. The relationship between the number of individuals in a hibernaculum and the type of hibernaculum (old nests vs other burrows) was also tested by poisson GLM, or with quasipoisson GLM if overdispersion was present. Diagnostic plots for all GLMs were checked. Sex-specific dispersal and sex-specific mortality were tested using chi-square tests.

Results

Hibernation strategy

All of the species of interest can use old nests (made by the same species or by a different species) or newly excavated burrows. In some cases, the old nest was cleared of all residua of nest partitions and evidently enlarged. The species differed in their preferred type of overwintering strategy (Table S1).

Adults of *C. cucurbitina* overwinter in their old nest in the vast majority of cases (86%, 2843/3319; Table S1), but some individuals (13%, 433/3319; Table S1) do overwinter in newly excavated burrows. Only a few individuals overwinter in nests of different species, which mostly are *C. nigrolabiata* nests. *Ceratina nigrolabiata* individuals overwinter in their old nests and newly excavated burrows at similar frequencies to C. *cucurbitina* (44%, 229/517 and 40%, 208/517, respectively; Table S1). A minority of individuals (15%, 80/517; Table S1) overwinter in nests of other species, usually in *C. cucurbitina* nests. Half of the *C. chalybea* individuals overwinter in their own nest, while other individuals overwinter in newly enlarged burrows or in nests of another species – usually in *C. cucurbitina* nests, but also commonly in *C. nigrolabiata* nests. *Ceratina cyanea* usually overwinter in newly excavated burrows; the proportion of individuals that overwinter in their own nest is the lowest among the four *Ceratina* species studied (26%, 27/103; Table S1).

Among the species studied, there was a substantial difference in the number of conspecific individuals residing in the hibernacula (poisson GLM, Deviance=1103.3, residual deviance=2889.5, p=2.2e-16, N=1205, Fig. 2, Fig. 3). The highest average number of individuals per hibernaculum occurred in *C. cucurbitina* (4.9243, Table S2), which was much higher than for all other species: *C. nigrolabiata* (1.7828), *C. chalybea* (1.3673), and *C. cyanea* (1.0957). The largest hibernaculum of *C. cucurbitina* contained 24 individuals; whereas, the maximal number of conspecific individuals per hibernaculum was much lower for the other species: 8 for *C. nigrolabiata*, 4 for *C. chalybea*, and 3 for *C. cyanea* (Fig. 2). The pattern in the aggregation of individuals was species-specific and conserved over the years of this study (Table S2).

The number of overwintering individuals per hibernaculum was dependent on the type of hibernaculum: old *Ceratina* nests contained more conspecific individuals than new burrows in *C. cucurbitina* (quasipoisson GLM, N=674, deviance=689.17, residual deviance=1949.8, p=2.2e-16, N=674) and *C. nigrolabiata* (poisson GLM, N=290, deviance=18.26, residual deviance=174.49, p=1.925e-05, N=290). However, this difference was not significant for the other two species studied, *C. chalybea* (Poisson GLM, N=147, deviance=2.15, residual deviance=48.89, p=0.14, N=147) and *C. cyanea* (Poisson GLM, N=94, deviance=0.007, residual deviance=7.16, p=0.92, N=94).

We frequently found more than one species residing in a hibernaculum. Multi-specific hibernacula were found in 9.10% of cases (100/1098; Table S3). The most common type of multi-specific hibernaculum contained *C. nigrolabiata* and *C. cucurbitina*. Other combinations of multi-specific hibernacula were less frequent; however, we have found most of the possible combinations, including a hibernaculum containing all four species (Table S3). Multi-specific hibernacula most frequently originated from *C. cucurbitina* nests (42%, 42/100): these hibernacula usually contained many *C. cucurbitina* individuals with a few *C. nigrolabiata* individuals residing near the nest entrance. We also commonly found multi-specific hibernacula that were located in new burrows (29%, 29/100) as well as those that originated from *C. nigrolabiata* nests (25%, 25 from 100).

We only found sex-specific dispersal in *C. cucurbitina*. There was a significant difference in the sex ratio of original nests and dispersal hibernacula (Chi square test, Chi=61.69, df=1, p=4.005e-15, N=3319). Sex-specific dispersal was not significant in any of the other species: *C. nigrolabiata* (Chi square test, Chi=0, df=1, p=1, N=517), *C. chalybea* (Chi square test, Chi=0.05, df=1, p=0.82, N=201), or *C. cyanea* (Chi square test, Chi=1.56, df=1, p=0.21, N=103).

Mortality

The proportion of dead individuals differed between species (Table S4, Fig 4): 20% (101/517) in *C. nigrolabiata*, 7% (246/3319) in *C. cucurbitina*, 12% (24/201) in *C. chalybea,* and 10% (10/103) in *C. cyanea*. We found sex-specific mortality in *C. nigrolabiata* (Chi square test, Chi=15.36, df=1, p=8.897e-05, N=517, Fig 4) and *C. chalybea* (Chi square test, Chi=13.02, df=1, p=0.000308, N=201). In both species, there was a significantly higher proportion of dead females than dead males. Moreover, the higher proportion of dead females was evident for both species in all studied years (Table S4). On the other hand, we have not found significant sex-specific mortality in *C. cucurbitina* (Chi square test, Chi=2.19, df=1, p=0.14, N=3319). For *C. cyanea*, the sample size was too small to test the sex-specific mortality.

Repeated overwintering

Although the majority of overwintering individuals were doing so for the first time, we found individuals of all species that were overwintering for the second time (Table S5). These second-time overwintering individuals were more than one year old and were identified by the appearance of their wings (strongly worn, see methods). We found individuals that were overwintering for the second time among the females of all species of interest and among the males of *C. nigrolabiata* and *C. chalybea*. However, the proportion of second-time overwintering individuals was high for *C. chalybea* females (14%, 10/74). The proportion of individuals that were overwintering for the second time was low for males and for all other species (under 2.5%; Table S5).

Discussion

Overwintering strategy

Facultative utilization of old nests and new burrows for overwintering has previously been documented in *Ceratina* bees (Daly, 1983; Kidokoro et al., 2006; Rehan et al., 2010; Rehan and Richards, 2010; Sakagami and Maeta, 1987) and also in *Xylocopa* (Sugiura, 1995). However, this overwintering strategy was unknown for European species. We documented cases of overwintering in old nests as well as emigration to other burrows for all four *Ceratina* species of interest in this study. We found species-specific as well as sex-specific differences in the prevailing habitat chosen for overwintering.

All four *Ceratina* species use the old nests of their own species, newly excavated burrows, and the old nests of different species. Individuals that overwinter in a nest built by their own species can be the individuals that originated in that nest; however, exchange of adults between nests of the same species is possible. Aggressiveness between young adults before overwintering is generally low in *Ceratina* (Rehan and Richards, 2013) and therefore individuals from the original nests probably do not defend their nest against intruders. Exchange of individuals between nests for overwintering has also been documented in *X. virginiaca* (Vickruck and Richards, 2017) and theorized also in *Ceratina* (Sakagami and Laroca, 1971). We were not able to distinguish between individuals that originated in a nest (i.e. those that stayed in their natal nest) and immigrants of the same species to that nest; however, we assume that some proportion of adults originated in a different nest than the one in which they overwintered. Moreover, we often observed hibernacula that contained multi-species (9% of hibernacula were multi-specific). These hibernacula mostly originated from nests of *C. cucurbitina* or *C. nigrolabiata* and contained different combinations of other *Ceratina* species.

The prevailing strategy of hibernation significantly differed among the species but was conserved within species for all three years of the study. Therefore, we suggest that each individual has the capability to overwinter in different locations; however, the tendency towards dispersal from the natal nest is species-specific. Individuals of *C. cucurbitina* usually overwinter in large social hibernacula; whereas, *C. cyanea* bees most often hibernate alone in newly excavated burrows. *Ceratina nigrolabiata* and *C. chalybea* had intermediate strategies. A species-specific tendency to dispersal is also known for some previously studied *Ceratina* species. Similar to *C. cucurbitina*, large assemblages of conspecific adults are typical for *C. flavipes*: for this species, the hibernaculum has an average of approximately three individuals and a maximal number of 33 individuals has been observed (Kidokoro et al., 2006). Conversely, *C. dallatoreana*, like *C. cyanea*, overwinter alone in newly excavated burrows in almost all cases (Daly, 1983).

Sex-specific differences in dispersal distance is known from many animal species (Gros et al., 2008; Lopez-Uribe et al., 2014; Prugnolle and de Meeus, 2002; Yanega, 1990). In *Ceratina* bees, pre-overwintering sex-specific dispersal has been documented in Japanese species (Sakagami and Maeta, 1987). Interestingly, *C. flavipes* females moved away from their original nests to other burrows more often than males did; however, the dispersal pattern was the opposite in *C. japonica* (Sakagami and Maeta, 1987). Furthermore, males always remain in their natal nest, while females sometimes disperse to make new short burrows in *Xylocopa appendiculata circumvolans* (Sugiura, 1995). We only observed a sex-specific dispersal strategy in *C. cucurbitina*: females overwintered in newly excavated burrows more often than males. However, pre-overwintering dispersal was generally low in this species. In the other *Ceratina* species, we found no significant differences between the dispersal of males and females.

Social hibernacula can be an important prerequisite for delayed eusociality (Sakagami and Maeta, 1987, 1989; Sakagami et al., 1984) or pleometrotic nest founding (Packer, 1983; Yanega, 1990). Thus, a tendency towards social hibernation can influence social evolution. Although we observed social hibernacula among all of the species, they were especially common in *C. cucurbitina* and *C. nigrolabiata*. However, no delayed eusociality or pleometrotic nest founding has been observed in studied species (Mikát et al., 2019, 2016). Old nests usually contained the remains of excrements and had dirty walls, so we suppose that they are not suitable for nest reuse, which is necessary for social nesting. Therefore, we suggest that philopatric overwintering is insufficient for establishing multi-female assemblages the following year (nesting season), because females usually emigrate from their natal nests in the spring, as the conditions for nest reuse are not suitable.

Mortality during winter

We did not measure overall mortality during winter because we dissected the nests in February or March. However, as the time of dissection was close to the end of winter, we consider most cases of winter mortality detectable. The mortality observed was moderate (7–20% in dependence to species), but the majority of individuals survived through winter. In contrast, Kidokoro et al. (2006) found much lower winter mortality in *C. flavipes* (under 1%). Lower winter mortality has also been documented for halictids – 9.4% of females in *Lasioglossium duplex* and 4.1% in *Lasioglossium problematicum* (Sakagami et al., 1984).

Ceratina nigrolabiata had the greatest mortality: 20% of the individuals were dead. This species is currently expanding its range in central Europe and was only recently recorded for the first time in the Czech Republic (in 2005) (Straka et al., 2007). It is possible that the climate conditions of the research location are at the limit of *C. nigrolabiata* species niche and that the inhabitable area of this species is constrained by winter survival.

We found sex-specific mortality in *C. nigrolabiata* and *C. chalybea*. In both species, a significantly higher proportion of males than females survived the winter. Mortality of adults can influence adult sex ratio and through this behavior of sexes (Kokko and Jennions, 2008). In this case, it is possible that the differing mortality rates of the sexes supports the strategy of mate guarding during the nesting season that occurs in *C. nigrolabiata* (Mikát et al., 2019).

Repeated overwintering

Previous research has shown that it is relatively common for some individuals to overwinter for a second time in some species of *Ceratina* (Rehan and Richards, 2010; Sakagami et al., 1982; Sakagami and Maeta, 1977), as well as *Xylocopa* (Gerling and Hermann, 1978; Martin, 1991). Indeed, we found females of all four of the species studied that were overwintering for a second time. However, the proportion of females that were overwintering for the second time was generally low (less than 2.5%), except for females of *C. chalybea* (13.51%). Therefore, females of European *Ceratina* species are relatively short-lived in comparison to Asian *Ceratina*, which have been repeatedly documented to overwinter more than once (Sakagami et al., 1982; Sakagami and Maeta, 1977). The proportion of females that overwinter twice is as much as 32% in *C. mesostigmata* (Sakagami and Maeta, 1977). Similarly, in North American *C. calcarata*, a second overwintering has been observed in 6-14% of females (Rehan and Richards, 2010). Furthermore, the evidence to date shows that *Xylcopa* are even longer living: females of *X. virginiaca* commonly survive two to three seasons (Gerling and Hermann, 1978).

An extraordinary finding was the possibility for males of *C. nigrolabiata* and *C. chalybea* to overwinter twice. In the aculeate Hymenoptera, there is a large difference in longevity between the sexes. Females survive for a much longer period than males in most of the species (Alcock, 2013; Koeniger, 2005; Stubblefield and Seger, 1994), although, prolonged lifespan of males occurs in *Cardiocondyla* ants where males remain in natal nests and try to monopolize virgin queens for several months (Heinze, 2016). Although the proportion of *Ceratina* males that were overwintering for the second time is very low (Table S5), the existence of this phenomenon itself is very interesting. Males of the Hymenoptera are generally very short-lived. They usually mate with females at the beginning of the nesting season and die early (Stubblefield and Seger, 1994). For groups in which adults activate before winter, it is usually only the females that overwinter (Alford, 1969; Michener, 2007). *Ceratina* and *Xylocopa* bees are the exceptions to this rule because males can overwinter once and mate with females during the spring (Gerling and Hermann, 1978; Martin, 1991; Mordechai et al., 1978; Rehan et al., 2010; Rehan and Richards, 2010). Studies on the overwintering behavior of *Ceratina* and *Xylocopa* performed to date have not detected the possibility of a second overwintering in males (Gerling and Hermann, 1978; Martin, 1991; Michener, 1990; Rehan and Richards, 2010; Sakagami and Maeta, 1977). In a study of *C. asuncionis*, overwintering of adults of both sexes with worn wings has been documented (Sakagami and Laroca, 1971); however, this species is bivoltine, so it is unclear whether the males of this species with worn wings had survived multiple seasons or just belong to the older generation.

Generally, in *Ceratina* and *Xylocopa* species, the abundance of males strongly decreases over the nesting season (Gerling and Hermann, 1978; Rehan and Richards, 2010). However, in *C. chalybea* and *C. nigrolabiata*, a significant proportion of males survive for the entire nesting season (Mikát et al., 2019). This behavior is very likely due to the prolonged receptivity of females, as males gain fitness by mating with females throughout the nesting season (Mikát et al., 2019). Here, we show that males of *C. nigrolabiata* and *C. chalybea* sometimes attempt to overwinter for a second time, although the proportion of males doing so is very low. From the time that a male matures at the end of July (the period when most *C. nigrolabiata* and *C. chalybea* offspring mature) until March of the second winter, it is around 600 days of mature lifespan, which would make them the longest living males of any bee species. We have no evidence that twice-overwintering males gain extra fitness; however, we assume that for those who overwinter successfully, it is easy to gain fitness because they can mate during a second season. All of the cases we observed, in *C. nigrolabiata* and *C. chalybea*, are likely the longest living adult bee males documented to date.

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Figures:

Fig 1. Two overwintering *C. chalybea* females in an old *C. chalybea* nest. The female that is overwintering for the second time has worn and brownish wings (left). The female that is overwintering for the first time has unworn and grayish wings (right).

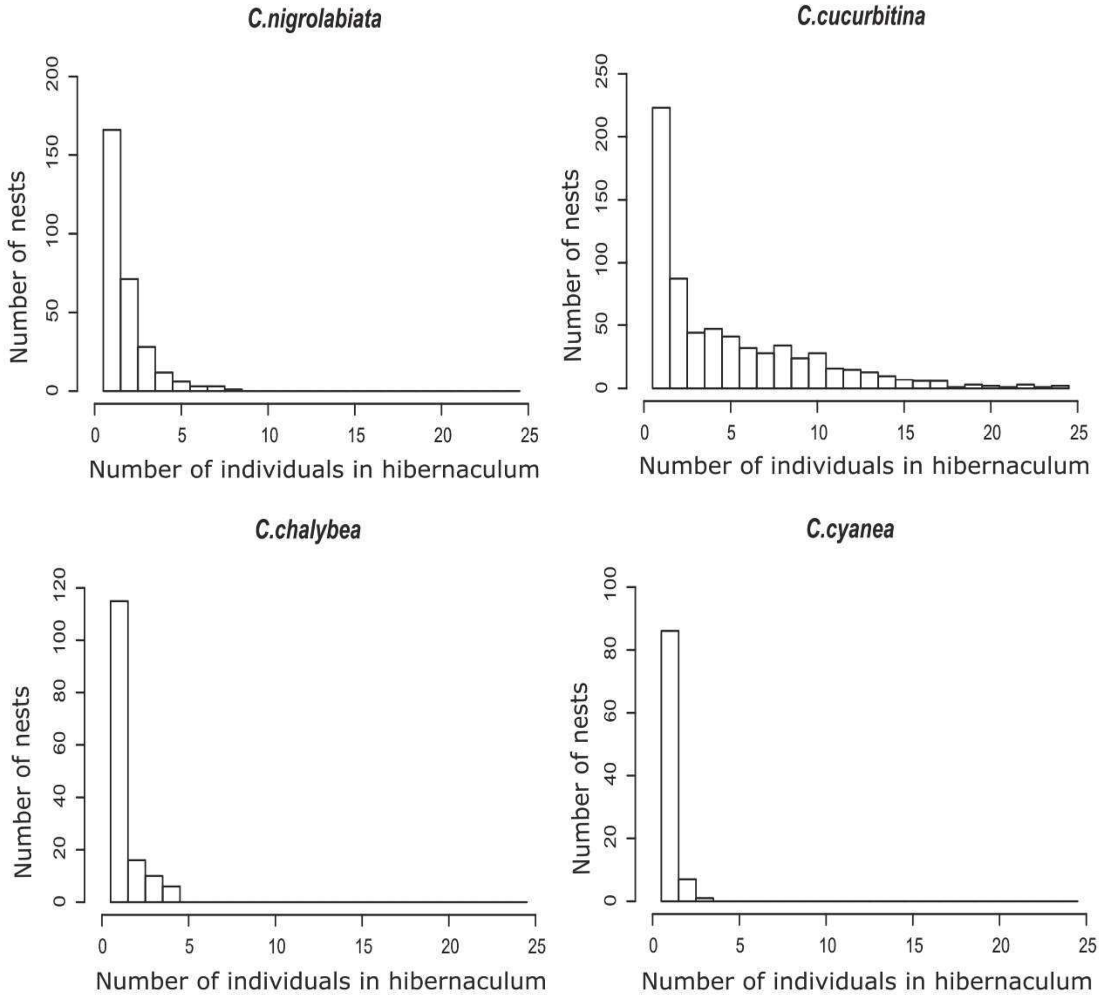

Fig 2. Histogram of hibernaculum size in different *Ceratina* species.

STUDY 7

Mikát., M., Waldhauserová., J., Fraňková, T., Čermáková, K., Brož, V., Zeman Š., Dokulilová, M., and Straka J.: Only mothers feed mature offspring in European *Ceratina* bees.

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Only mothers feed mature offspring in European Ceratina bees

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Keywords:	maternal care, sociality, provisioning, foraging trip, Xylocopinae, Hymenoptera
Abstract:	Parental care directed to adult offspring is an interesting trait in animals. So far it has been documented in Xylocopinae bees (Hymenoptera, Apidae). Moreover, some Ceratina bees (Xylocopinae) are known to feed mature siblings. This is done by the dwarf eldest daughter usually in case of the mother's death. This daughter is intentionally malnourished by her mother and usually originates from the first brood cell. Here, we examined a pattern of care in young adults in three European small carpenter bees: Ceratina (Ceratina) cucurbitina, C. (Euceratina) chalybea, and C. (E.) nigrolabiata. Observations of nest departures and arrivals were performed. Regular foraging trips and feeding of adult offspring by mother were present in all studied species. However, no foraging activity of individuals in nests without a mother was observed. Removal of mothers from nests of C. cucurbitina led to an emigration of young adults from nests. We can conclude that the feeding of siblings by sisters does not occur in those species unlike in the previously studied American species C. calcarata, where the dwarf eldest daughter is present. Young adults from orphaned or abandoned nests probably feed themselves or disperse to another nest where an old female is present.

Only mothers feed mature offspring in European *Ceratina* bees

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Abstract

Parental care directed to adult offspring is an interesting trait in animals. So far it has been documented in Xylocopinae bees (Hymenoptera, Apidae). Moreover, some *Ceratina* bees (Xylocopinae) are known to feed mature siblings. This is done by the dwarf eldest daughter usually in case of the mother's death. This daughter is intentionally malnourished by her mother and usually originates from the first brood cell.

Here, we examined a pattern of care in young adults in three European small carpenter bees: *Ceratina* (*Ceratina*) *cucurbitina*, *C.* (*Euceratina*) *chalybea,* and *C.* (*E.*) *nigrolabiata*. Observations of nest departures and arrivals were performed. Regular foraging trips and feeding of adult offspring by mother were present in all studied species. However, no foraging activity of individuals in nests without a mother was observed. Removal of mothers from nests of *C. cucurbitina* led to an emigration of young adults from nests. We can conclude that the feeding of siblings by sisters does not occur in those species unlike in the previously studied American species *C. calcarata,* where the dwarf eldest daughter is present. Young adults from orphaned or abandoned nests probably feed themselves or disperse to another nest where an old female is present.

Keywords: maternal care, sociality, provisioning, foraging trip, Xylocopinae, Hymenoptera

Introduction

Parental care is an effective way of increasing offspring survival (Eggert et al., 1998; Tallamy & Wood, 1986; Thiel, 2003) and their future fitness (Alonso-Alvarez and Velando, 2012). However, trade-offs exist between the care and future reproduction (Alonso-Alvarez and Velando, 2012; Gross, 2005; Mas and Kölliker, 2008; Tallamy and Denno, 1982; Trumbo, 2012). Therefore, the desertion of offspring can be beneficial for parents that can reproduce again (Gross, 2005; Székely et al., 1996). Animal species with parental care substantially differ in the duration of care invested in their offspring (Tallamy & Wood, 1986; Thiel, 1999). Some species provide only pre-ovipositional care and abandon offspring after food or shelter is provided or guard the egg phase (Gilbert & Manica, 2010; Machado & Raimundo, 2001; Smiseth et al., 2012; Tallamy & Wood, 1986; Trumbo, 2012). On the other hand some animals are able to care for offspring until adulthood is reached (Smiseth et al., 2012; Thiel, 2003).

Several insect lineages perform parental care until offspring adulthood. For example mothers take care of offspring throughout development in the scarab beetle *Kheper nigroaeneus* (Edwards and Aschenborn, 1989) and also in the lace bug *Gargaphia solani* (Tallamy and Denno, 1981). Long-term cohabitation between parents and offspring is commonly associated with sociality and alloparental care (Andersson, 1984; Wilson, 1971). However, the direct care for mature offspring (in imago stage) is generally rare in insects and has been overlooked by leading reviews on insect parental care (Smiseth et al., 2012; Tallamy and Wood, 1986; Trumbo, 2012).

A mother caring for mature offspring is known in bees of genera *Ceratina* and *Xylocopa* which belong to the subfamily Xylocopinae (Hymenoptera, Apidae). *Ceratina* bees nest in twigs with a pith (Michener, 2007; Sakagami & Laroca, 1971). Mothers excavate a burrow in broken twigs which they later divide into cells (Fig 1, Fig S1). These cells are provisioned using the mass provisioning strategy where each brood cell is provisioned with the amount of food suitable for offspring development into adult stage, and afterwards each brood cell is closed (Michener, 2007; Rehan & Richards, 2010a; Sakagami & Laroca, 1971). With nests provisioned, mothers usually guard their offspring throughout their development until the adult stage (Mikát et al., 2016; Rehan & Richards, 2010a). The whole Xylocopinae subfamily is primarily an eusocial group (Rehan et al., 2012). However, in *Ceratina* and *Xylocopa* bees, social nesting is only a facultative strategy which occurs in a minority of nests (Hogendoorn & Velthuis, 1995; Rehan et al., 2015; Rehan et al, 2009). Although, eusociality was not observed and probably disappeared in several temperate species (Groom & Rehan, 2018; Mikát et al., 2016; Rehan et al., 2012; Rehan & Richards, 2010a), it is possible that some natural history traits in such solitary species can be preserved from their previous social lifestyle.

Mothers of Xylocopinae bees provision adult offspring with pollen and nectar (Anzenberger, 1977; Hogendoorn & Velthuis, 1995; Lewis & Richards, 2017; Mikát et al., 2017; Sakagami & Maeta, 1977; Vickruck & Richards, 2017). Up to this date, feeding of offspring by mother was documented in several *Ceratina* species: *C. japonica* (Sakagami & Maeta, 1977), *C. flavipes* (Maeta et al., 1992), *C. calcarata* (Lewis and Richards, 2017; Mikát et al., 2017), and *C. cucurbitina* (Maeta et al., 1997; Mikát et al., 2016). The duration of the provisioning period of young adults is the same or longer than the period of brood cell provisioning (Mikát et al., 2017). The amount of food which mother collects for adult offspring is similar to the amount of food which is collected for one brood cell (Hogendoorn and Velthuis, 1995; Velthuis and Gerling, 1983). Feeding of mature offspring allows the offspring to obtain enough nourishment for winter survival (Lewis and Richards, 2017) without the risk of individual foraging.

In several *Ceratina* species, feeding of siblings can be performed not only by the mother, but also by a sister (Lewis & Richards, 2017; Mikát et al., 2017; Sakagami & Maeta, 1977). This individual is known as the dwarf eldest daughter (Lawson et al., 2018; Lawson et al., 2016; Mikát et al., 2017; Rehan & Richards, 2010b; Shell & Rehan, 2017). In the first brood cell (the innermost one) a malnourished female is produced (Lawson et al., 2016; Mikát et al., 2017; Rehan & Richards, 2010b).

Malnourished individuals are less aggressive, and it is easier to enforce them to adopt an altruistic role (Lawson et al., 2017). The daughter which performs foraging is usually the smallest female offspring in the nest (Lewis and Richards, 2017; Mikát et al., 2017). The dwarf eldest daughter is an individual that feeds her siblings in the nest where the mother died (Lewis and Richards, 2017; Mikát et al., 2017) and therefore benefits from the inclusive fitness by helping her siblings instead of reproducing herself (Rehan et al., 2014; Shell & Rehan, 2017). To this date, the majority of empirical evidence for the presence and existence of the dwarf eldest daughter is recorded for *C. calcarata* (Lawson et al., 2017; Lewis & Richards, 2017; Mikát et al., 2016; Rehan & Richards, 2010b). The existence of a malnourished daughter in the first brood cell was also recorded in other species in the North American subgenus *Zadontomerus* (Lawson et al., 2018). Moreover, the existence of the dwarf eldest daughter was documented in behavioral observations and is also seen in the pattern of maternal investment in the East Asian subgenus *Ceratinidia* (Sakagami & Maeta, 1977, 1989).

Anecdotal observations of feeding of mature offspring by mother in European *Ceratina* bees exist only for *C. cucurbitina* (Maeta et al., 1997; Mikát et al., 2016). However, no extensive study has been performed to test the observations. Therefore, we decided to study the strategy of feeding the mature offspring in three European species*: C. cucurbitina*, *C. chalybea,* and *C. nigrolabiata*. *Ceratina cucurbitina* (subgenus *Ceratina*) is probably distantly related to the other two species which belong to the subgenus *Euceratina* (Rehan et al., 2010; Terzo, 1998). Studied Japanese and North American species, where the feeding of mature offspring has been documented, belongs to other subgenera.

Neither the presence of dwarf eldest daughter nor the provisioning of siblings has ever been inspected in European species. The dwarf eldest daughter is usually considered to be the insurance strategy in the case of mother's death (Mikát et al., 2017). However, mothers of *C. chalybea* abandon 40% of completely provisioned nests (Mikát et al., 2016). Similar facultative nest abandonment is present also in *C. nigrolabiata* (Mikát and Straka, in prep.). Nests which are abandoned by mother cannot be further provisioned by that mother. Therefore, it is a question if nests abandoned by mother are obligately provisioned by the dwarf eldest daughter, or whether the provisioning of adult offspring is limited to nests guarded by the mother.

Our study had three main questions: 1) Does the mother feed mature offspring in European species? 2) Does the dwarf eldest daughter feed offspring in the three European species? 3) Is there a relationship between the pattern of maternal investment and the presence of an altruistic daughter?

Material and Methods

General design

Our research consists of three experiments: 1) Observation of activity in unmanipulated nests with mature offspring; 2) Experimental removal of mother from nests with mature offspring; 3) Analysis of maternal investment in full brood nests.

Location and time of research

We performed the research near Havraníky village, Southern Moravian Region, Czech Republic. Research was performed in 2017 (between July 30 and August 9) and 2018 (between July 20 and August 9). Observations of nests were performed in location 48°48'34.4"N, 15°59'44.3"E in 2017, and in location 48°48'43.688"N 15°59'41.913"E in 2018. Nests for experiments were collected from artificial nesting opportunities (Fig. S2) within 6 km around mentioned locations.

Comparison between nests with and without mother

We dissected nests and confirmed the presence of mother. For this analysis, we used mature nests (nests where at least one mature offspring was present and crawled through the nest partitions). We calculated the proportion of nests, where mother was present. We compared the number of young adults between nests with mother and without mother. We tested the significance of differences using Wilcoxon test in R software 3.6.1. (R. Core Developmental Team, 2016).

Observation of foraging activity in nests with mother and nests without mother

We established study plots, each of which contained 24 nests. We observed each plot for one day (Fig S3) and noted departures and arrivals of bees. Individual bees were marked for recognition by oil paint marker. We distinguished several categories of activity: old female trip, young adult complete trip (departure and arrival of the same young adult), emigration of a young adult, immigration of a young adult, short visit of a young adult (time between arrival and departure was less than three minutes, usually a couple of seconds, and the individual's abdomen would still be clearly seen in the entrance of a nest). We calculated a number of those activities for each nest per observational day. We also noted when an individual carried a significant amount of pollen on legs. For more details about preparation of study plots and nest observation, see supplementary materials.

All nests were dissected in the evening following the completion of observation. We evaluated the stage in which the nest was in time of dissection (see supplementary materials). Only mature nests (nests where at least one mature offspring crawling through nest partition was; Fig. 1) were used for the evaluation. Presence of mother was noted. For more information about nest dissection, see supplementary materials.

We summarized behavioral data for each nest. We compared this activity among species and between nests with mother and orphaned nests with the potential for sibling feeding (nests where two or more young adults were present and at least one of them was female). We tested the significance of difference by Wilcoxon test, using R software 3.6.1: (R. Core Developmental Team, 2016). We analyzed 48 nests with mother present and 119 nests without mother in *C. cucurbitina*, 74 nests with mother present and 21 nests without mother in *C. chalybea* (and 31 nests with mother present and 61 nests without mother in *C. nigrolabiata*. Number of nests observed during different years (2017 vs 2018) is summarized in Table S.1.

Duration of flight activity of young adults

We calculated the duration of complete trips of young adults as a difference between the time of departure and the time of arrival. We tested the difference in trip duration between young males and young females using Wilcoxon test using R software 3.6.1. (R. Core Developmental Team, 2016).

Analysis of the size of an active daughter

We used nests with mature offspring of *C. cucurbitina* from 2017 for this analysis. We only included nests in which at least one young female was marked, and we had evidence that this female left the nest at least for a short period of time. We compared head width between marked and unmarked females. Head width was measured as the maximum distance between the edges of the head across compound eyes. We used a stereomicroscope fitted with an ocular scale for measuring. We measured 70 unmarked (females for which was no evidence for nest departure) and 45 marked (females which surely at least once departed from nest) females. The difference was tested by the binomial generalized mixed model. Marking status (marked vs unmarked females) was the dependent variable, head width of a female was the explanatory variable, and nest identity was a grouping factor. We performed this analysis using R software 3.6.1. (R. Core Developmental Team, 2016).

Influence of mother removal in mature nests

We tested the influence of mother removal on the number of offspring present in nests of *C. cucurbitina*. We performed this experiment in 4 of our experimental plots. Firstly, there were selected nests with regular foraging activity of the mother. The mother was removed in 15 nests (8 in 2017 and 7 in 2018), 12 nests were retained as controls (4 in 2017 and 8 in 2018). All these nests were dissected after 3 days, and the number of young adult individuals was counted. We tested the difference using Wilcoxon test. We performed this analysis in R software 3.6.1. (R. Core Developmental Team, 2016).

Analysis of presence of dwarf eldest daughter in first brood cell

We used only full brood nests (Fig. 1) for this analysis. These nests were dissected from observation plots and also from other nesting opportunities. Nest partitions between brood cells were preserved still, thus no adult offspring crawled through brood cell partitions. Therefore, it was possible to determine the order of offspring for each individual nest. All young offspring were reared into adulthood. Offspring in prepupal and pupal stages were inserted to 1.5 ml tubes (Eppendorf) and reared until adulthood. Opened nests with offspring in larval stages were closed again and larvae on pollen balls were retained in the nest. Nests were regularly checked, and when offspring reached the pupal stage, they were transferred into 1.5 ml tubes and reared until adulthood. We determined sex and measured head width for each sufficiently reared offspring. Head width was measured with stereomicroscope with a scale in ocular.

We sufficiently reared 1,213 offspring from 169 nests of *C. cucurbitina*, 479 offspring from 123 nests of *C. chalybea,* and 854 offspring from 151 nests of *C. nigrolabiata*. We compared the sex ratio in the first brood cell position with the sex ratio in the whole offspring dataset. We tested the significance of difference by chi-square test. We compared the head width of female in the first brood cell with an average head width of female in other brood cells. We tested the significance of differences with paired t-test. The statistical analyses were performed in R software 3.6.1. (R. Core Developmental Team, 2016).

Results

Nests with mother and without mother

Mother was not present in 78.4% of *C. cucurbitina* mature nests (152/194), 53.8% of *C. chalybea* mature nests (86/160) and 76.7% of *C. nigrolabiata* (102/133) mature nests. Therefore, Mother was not present in a high proportion of mature nests.

Number of young adults between nests with mother and without mother was significantly different in *C. cucurbitina* (Wilcoxon test, N=194, W=1326, p<0.0001, table 1, fig 2), *C. chalybea* (N=160, W=1.9, p<0.0001), and also *C. nigrolabiata* (N=102, W=992, p=0.0012).

Activity of mother

Intensive foraging activity of mother was observed in nests with a mother in all studied species. At least one trip of an old female was observed in 97.9% (47/48) of nests in *C. cucurbitina*, 90.5% (67/74) of nests in *C. chalybea,* and 74.2 % (23/31) of nests in *C. nigrolabiata*, including only nests where mother was found at time of nest dissection. However, in nests where mother was not present at time of nest dissection the mother activity was observed in 8.6% (13/152) of *C. cucurbitina nests*, 8.1% (7/86) of *C. chalybea* nests, and 2.9% (3/102) of *C. nigrolabiata* nests. We therefore suppose that mother disappeared from some nests during the observational day.

The average number of trips performed by an old female per day was 8.35 (maximum: 28, SD=4.8, N=48, Table 2, Table S2, fig. 3) in *C. cucurbitina*, 7.5 (maximum 18, SD=4.8, N=74, Table 2) in *C. chalybea,* and 3.5 (maximum 8, SD=2.5, N=31, Table 2) in *C. nigrolabiata*. Carrying of pollen was observed in all species. However, a mother carrying pollen was observed only in a small proportion of old female foraging trips, notably in 3.3% (13/454) of trips in *C. cucurbitina*, 16.0% (90/563) of trips in *C. chalybea,* and 4.5% (5/112) of trips in *C. nigrolabiata*.

Activity of young adults

We observed young adults leaving or entering nests. This activity was observed in both nests where mother was present and nests without mother (Table 3). Although we were unable to identify the sex of young adults in some cases, the activity of both sexes was frequently present. We observed four types of activity: a complete trip of a young adult, emigration of a young adult, immigration of a young adult and a short visit of a young adult. We observed all these types of activity in all three studied species. However, the average values of those activities were low in comparison to the average number of mother's foraging trips in nests where mother was present (Table 2).

In *C. cucurbitina* and *C. chalybea* trips with departure and arrival, leaving nest without return, and also immigration to the nest without previous departure were significantly more common in nests without mother than in nests with mother (Table 2, Table 4). Abundance of short-term visits was not significantly different between nests with mother and nests without mother in both species (Table 4). In *C. nigrolabiata*, there was not a significant difference in abundance of any type of young adult foraging behavior between nests with mother and nests without mother (Table 4).

Complete trips (departure and arrival) of young adults were observed in all studied species and were performed by both sexes (Tables 2 and 5). The frequency of the complete trips of young adults was low, less than 0.3 trips per day for all species examined (Table 2).

The proportion of trips performed by males was 33.9% (20/59) in *C. cucurbitina* was statistically different from 1:1 (Chi square test, Chi=6.1, p=0.0134). The proportion of trips performed by males was 52.2% (12/23) in *C. chalybea*, the difference was not statistically significant (Chi square test, Chi=0.04, p=0.8348). The proportion of trips performed by males was 53.9% (7/13) in *C. nigrolabiata*, the proportion was not statistically significant (Chi square test, Chi=0.07, p=0.7815).

In almost all cases a young adult performed only one complete trip per day. We observed four young adults that performed two complete trips per day (twice in *C. cucurbitina* female, once in *C. cucurbitina* male, and once in *C. chalybea* male). We did not observe any young adult which performed three or more complete trips per day.

Trips usually had a very short duration, median duration of a trip was few minutes for all species, however, trips which were longer than one hour were also observed (Table 5). The duration of a complete young adult trip did not differ between the sexes in *C. cucurbitina* (Wilcoxon test, N=59, W=316.5, p=0.2346), *C. chalybea* (Wilcoxon test, N=23, W=97, p=0.0584), and *C. nigrolabiata* (Wilcoxon test,,N=13, W=27.5, p=0.3881).

Size of active vs inactive young females of *C. cucurbitina*

Average head width of females that performed flight activity was 1.97 mm (N=45, SD=0.1, range 1.8–2.1 mm, fig 4). Average head width of females for which flight activity was not detected was 1.97 mm (N=70, SD=0.1, range 1.8–2.3 mm). There was no significant difference between females of *C. cucurbitina* in which we recorded flight activity and females in which this activity was not recorded (binomial generalized mixed model, chi=0.3, p=0.5982)

Removing experiments in *C. cucurbitina*

We did not observe any regular foraging activity in nests in which the mother was removed in *C. cucurbitina*, only arrivals or departures without return of young adults were observed (N=12 nests where observation was performed). Nests, where mother was removed, had a significantly lower number of offspring (mean=1.8, N=15, SD=2.0, range 0–7), than control nests (mean=12.1, N=12, SD=7.0, range 1–25; Wilcoxon test, N=25, W=164, p=0.0003, fig 5).

Presence of a dwarf eldest daughter in the first brood cell

In *C. cucurbitina* 51.3% of offspring (622/1213) in full brood nests analyzed were female. In the first brood cell 63.6% (77/121) of offspring were females. The sex ratio in the first brood cell was significantly more female biased than the overall sex ratio (Chi square test, Chi=6.3, p=0.0124). In *C. chalybea*, we found 47.7% (228/478) of females in offspring from all full brood nests analyzed. In the first brood cell, there was 82.4% (52/68) of females. Sex ratio in the first brood cell was significantly more female-biased than the overall offspring sex ratio (Chi square test, Chi=27.3, p=1.768e-07). In *C. nigrolabiata* there was 61.4% (524/854) of females in offspring from all full brood nests analyzed. In the first brood cell there was 52.4% (54/103) of females. The difference was not statistically significant (Chi square test, Chi=3.1, p=0.0799).

In *C. cucurbitina* the average head width of females in the first brood cell was 1.95 mm (N=77, SD=0.09, in all other brood cells it was 1.98 mm (N=545, SD=0.10). The difference was statistically significant (paired t-test, N=76, t=3.2, p=0.0020). In *C. chalybea*, the average head width in the first brood cell was 2.45 mm (N=56, SD=0.10), the average head width in all other brood cells was 2.45 mm (N=152, SD=0.11). The difference was not statistically significant (paired t-test, N=41, t=-0.2, p=0.87). In *C. nigrolabiata*, the average head width in the first brood cell was 1.91 mm (N=54, SD=0.07), in all other brood cells it was 1.91 mm (N=470, SD=0.10). The difference was not statistically significant (paired t-test, N=50, t=-0.8, p=0.46).

Discussion

Foraging activity of mother and feeding adult offspring

Feeding of young adults is an uncommon type of parental care throughout the whole animal kingdom. Young adults are apparently potentially independent, but obtaining food from parents can increase their future fitness. Taking care of young adults in Xylocopinae is a trait parallel to post-fledging care in birds where young adults are also able to fly and live independently, however, care increases their survival and future fitness (Ridley and Raihani, 2007).

In small carpenter bees (*Ceratina*) feeding of adult offspring by a mother has been documented only in species of subgenera *Zadontomerus* (Lewis and Richards, 2017; Mikát et al., 2017) and *Ceratinidia* (Maeta et al., 1992; Sakagami & Maeta, 1977). All species studied in current research feed their adult offspring, and thus this behavior is now documented in species belonging to subgenera *Euceratina* and *Ceratina* s. str. Subgenera, where feeding of mature offspring was confirmed, are not closely related (Rehan et al., 2010, 2012), therefore we can suppose that the feeding of adult individuals by mother is widespread across the genus *Ceratina* and may be even ancestral for the genus.

We observed the foraging activity of mothers in almost all nests where the mother was present. However, mothers carried pollen apparently in a very low proportion of the trips (Table 2). Therefore, females probably carry nectar or a mixture of nectar and pollen in the crop in most occasions. Carrying of pollen may have been undetected in some cases, e.g. when mothers foraged on plants with dark pollen.

The average number of foraging trips per day per female was 8.35 in *C. cucurbitina*, 7.48 in *C. chalybea,* and 3.54 in *C. nigrolabiata* and is comparable to or higher than the number of foraging trips per day in *C. calcarata* mothers which is 3.54 (Mikát et al., 2017), respectively 3.4 (Lewis and Richards, 2017) in a phase of feeding of mature offspring.

Foraging activity of a daughter and feeding adult siblings

Feeding of young adults by a sister (Lewis & Richards, 2017; Mikát et al., 2017; Sakagami & Maeta, 1977) is a unique type of sociality documented in *Ceratina* bees. It usually occurs in the case of mother's death and serves as an insurance for this case (Lewis and Richards, 2017; Mikát et al., 2017; Shell and Rehan, 2017). This trait was documented in the most behaviorally studied species, North American *C. calcarata* (Lewis and Richards, 2017; Mikát et al., 2017) and East Asian *C. japonica* and *C. flavipes* (Sakagami & Maeta, 1977). For other species no behavioral tests or even precise observations, which can test the presence of alloparenal care of mature siblings, have been carried out.

However, we have not observed any case of regular foraging activity of the daughter in species studied here. Although we sometimes observed trips with departure and arrival of a young individual, these trips were very short (a few minutes) in most cases. In almost all cases one individual performed only one trip per day. We observed only four cases of two complete trips per day performed by one individual and no case of more than two trips performed by the same young adult individual. In *C. chalybea* and *C. nigrolabiata*, trips of young males and females were similarly common. In C. *cucurbitina*, there were more common trips of young females than young males, however, males still performed about one third of trips. Duration of young adult complete trips did not differ between males and females in any species. Therefore, the behavior of active young adults is not similar to the behavior of dwarf daughters in *C. calcarata* in which the eldest daughter is active in ca 10% of nests per day on average and performs standard foraging trips (up to 14 trips per day) (Mikát et al., 2017). Foraging trips of the dwarf eldest daughter of *C. calcarata* usually last about 20–35 min, similar duration as the foraging trips of the mother (Lewis and Richards, 2017; Mikát et al., 2017).

When we compared the size of active young females with inactive young females in *C. cucurbitina*, there was no significant difference between these two groups. This result differs from the study of *C. calcarata* where the active dwarf eldest daughters are significantly smaller than inactive females (Lewis and Richards, 2017; Mikát et al., 2017).

Nests with a mother contained a higher number of young adults than nests without a mother in all studied species. This was confirmed by an experimental mother removal from nests of *C. cucurbitina*. Nests, where the mother was removed, contained only 1.8 young adults on average in contrast to control nests, which contained 12.33 young adults on average three days after the treatment. Therefore, we suppose that mother's disappearance led to emigration of young adults from the nest.

Fate of offspring in orphaned and abandoned nests

According to our results, we can conclude that daughters with alloparental behavior do not occur in any of the three studied species. In *C. calcarata*, the dwarf eldest daughter is usually active in the case of mother's death. Therefore, she serves as an insurance (Mikát et al., 2017; Shell and Rehan, 2017). European species probably lack similar.

Ceratina nigrolabiata and *C. chalybea* mothers facultatively abandon their nests after they finish provisioning cells (Mikát et al., 2016) and unpublished data). We were usually unable to distinguish between abandoned nests and orphaned nests because emerging adults disturb nest structure. However, we know from the structure of full brood nests that *C. chalybea* females abandon their nests in 40.1% of cases (Mikát et al., 2016) and *C. nigrolabiata* in 69.66% (62/89, Mikát and Straka, unpublished data, Fig. S1). Therefore, voluntarily abandoned nests were apparently significantly represented in nests without mother. We did not observe regular provisioning by a young female in any nest of *C. chalybea* and *C. nigrolabiata*. Therefore, we can conclude that no individual can feed young adults in these nests. As nest abandonment is a regular strategy of these species, it is clear that foraging in natal nest is not necessary for the survival of offspring in these species.

In nests where young adults are not fed by their mother, three different scenarios are possible for offspring: a) they survive in the nest without feeding before winter; b) they feed themselves directly on flowers; c) they immigrate to a nest where a mother is present and parasite on this female which feeds them. We suppose that winter survival without feeding is not probable because feeding before winter is generally present in temperate Hymenoptera which mature in late summer (Sakagami & Maeta, 1977), and feeding is essential for winter survival in *C. calcarata* (Lewis and Richards, 2017). We repeatedly observed emigration of young adults from nests, and most young adults vanished from nests with removed female of *C. cucurbitina*. Therefore, we suppose that young adults which are hungry try to obtain food elsewhere. We did not focus on observation of feeding of young adults in flowers, therefore we cannot exclude the possibility that they feed themselves. As we observed successful immigrations and also attempts at immigration, we conclude that young adults try to enter foreign nests where mother is still present and let her feed them. Especially in the case of *C. cucurbitina* it is probable that offspring from orphaned nests take advantage of being fed by an unrelated old female. We detected fourteen nests with 16 or more young adults, but the maximal number of brood cells provisioned is 15 at the studied locality (Mikát and Straka, unpublished data, N=320 nests). Testing the relatedness between mother and offspring in nests where old female and young adults are present could be useful for testing this hypothesis.

Maternal investment and predisposition to alloparental care

Helping behavior can be facilitated by malnourishment of some individuals that enforces them to adopt a helper role (Alexander, 1974; Kapheim et al., 2015; Smith et al., 2009). The dwarf eldest daughter syndrome was postulated for *Ceratina*: a female produced in the first brood cell is usually smaller than females in other brood cells (Lawson et al., 2016; Mikát et al., 2017; Rehan & Richards, 2010b). Our studied species differ in the pattern of maternal investment. *Ceratina cucurbitina* exhibited a pattern typical for *Ceratina*. Thus, the first brood cell had a female-biased sex ratio, and females in the first brood cell were on average smaller than other females. *Ceratina chalybea* also had a strongly female-biased sex ratio in the first brood cell, but females in this brood cell did not differ in size from other females. *Ceratina nigrolabiata* exhibited no sex ratio bias in the first brood cell in comparison to all offspring, and the size of female in first brood cell did not significantly differ from other females.

Although *C. cucurbitina* has a pattern of maternal investment typical for the dwarf eldest daughter syndrome, we gathered evidence that alloparental behavior does not occur in this species. Therefore, we suppose that the pattern in maternal investment is not sufficient for proving the presence of the dwarf eldest daughter with alloparental behavior. Maternal investment in Hymenoptera is influenced by multiple factors, e.g. resource availability (Kim, 1999), female condition (Sugiura, 1994), or risk of parasitism (Seidelmann, 2006). Therefore, we can conclude that other factors than deliberate malnourishment can generate common abundance of small females in the first brood cell. Moreover, it is also possible that the presence of this trait is only a retention of the ancestral trait.

Evolution of sociality and parental care

Extensive provisioning of adult offspring is generally widespread in *Xylocopa* (Anzenberger, 1977; Gerling et al., 1983; Hogendoorn & Velthuis, 1995; Richards & Course, 2015) and also in *Ceratina* (Mikát et al., 2017; Sakagami & Maeta, 1977), and here we expand the spectrum of species for which the feeding of mature offspring is documented. Feeding of mature offspring may be a primitive trait for the whole subfamily Xylocopinae, although the main focus was on the temperate species up to this date and thus the study of this trait in tropical species would be useful. However, the main adaptive function of feeding of mature offspring can differ among lineages or climatic areas. For temperate *Ceratina* species it is proposed to be the main function for survival of offspring through winter (Lewis and Richards, 2017; Mikát et al., 2017). We suppose that this is the main function also for our studied species. However, the function of feeding of mature offspring as a behavior increasing the offspring survival through winter is probably evolutionarily derived because the genus originated in tropical Africa (Rehan et al., 2010). Xylocopinae bees are primarily a social lineage (Rehan et al., 2012). An unusual division of labor is present for the most of the multifemale societies in this lineage: reproductively dominant females provision offspring with food while reproductively subordinate females usually serve as rather passive guards (Hogendoorn & Velthuis, 1995; Prager, 2014; Schwarz et al., 2011). Therefore, it is possible that the feeding of mature offspring originated primarily as a result of interactions in social nests.

Larvae of nest-making aculeate Hymenoptera are dependent on food provision from their parents (Tallamy and Wood, 1986; Wilson, 1971). Most of the solitary Aculeata close the nest and abandon their offspring after providing enough provisions (Michener, 2007; Peterson et al., 2007). However, nest guarding through larval development can increase offspring survival (Kukuk et al., 1998; Mikát et al., 2016). Young adults of *Ceratina* are able to fly and therefore are not dependent on being cared for by another individual. Feeding of mature offspring is another way in which parents can increase offspring survival (Lewis and Richards, 2017). However, our results show that the care for young adults is not an obligate strategy in *Ceratina,* and young adults are not fed in some nests. Moreover, the existence of nests with and without adult feeding opens a possibility for migration to different nests in order to be fed by another female.

Disclosure

The authors declare no conflict of interest.

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Tables

Table 1: Number of young adults in nests with and without mother in all three species.

Species	***C. cucurbitina***		***C. chalybea***		***C. nigrolabiata***	
Mother	present	absent	present	absent	present	absent
N nest	42	152	74	86	32	102
Mean number of young adults	9.4	4.1	3.1	1.8	3.2	2.3
SD of number of young adults	5.4	3.1	2.0	1.0	1.4	1.3
Maximal number of young adults	23	18	9	5	7	7

Table 2: Comparison of foraging activity between species and nests with and without mother. From nests without mother only nests with at least two adults and at least one young female at time of nest dissection were included. Given values are average rates per nest and day.

Species	*C. cucurbitina*		*C. chalybea*		*C. nigrolabiata*	
mother present	Yes	No	Yes	No	Yes	No
number of nests observed	48	119	74	21	31	61
old female foraging trips	8.3	0.5	7.5	0.4	3.6	0.0
~ trips with pollen	0.2	0.0	1.2	0.0	0.2	0.0
young adult complete trip	0.1	0.3	0.0	0.3	0.1	0.1
emigration of young adult	0.4	0.7	0.6	1.1	0.7	0.5
immigration of young adult	0.4	0.7	0.3	0.8	0.5	0.4
short visit of young adult	0.2	0.3	0.1	0.2	0.3	0.2

Table 3. Proportion of nests where different types of behavior were present. Each nest was observed for one day. From nests without mother only nests with at least two adults and at least one young female at time of nest dissection were included.

Species	*C. cucurbitina*		*C. chalybea*		*C. nigrolabiata*	
mother present	Yes	No	Yes	No	Yes	No
number of nests observed	48	119	74	21	31	61
old female foraging trips	0.97	0.11	0.91	0.14	0.74	0.01
~ trips with pollen	0.15	0.02	0.41	0.00	0.16	0.00
young adult complete trip	0.06	0.29	0.03	0.23	0.06	0.11
emigration of young adult	0.31	0.50	0.31	0.57	0.48	0.34
immigration of young adult	0.21	0.50	0.20	0.71	0.32	0.33
short visit of young adult	0.17	0.19	0.09	0.19	0.13	0.15

Table 4: Results of Wilcoxon tests comparing abundance of different types of foraging behavior between nests with mother and without mother (W=test criterion of Wilcoxon test, p=p value). Average numbers for nests with mother and without mother are shown in Table 2.

Species	*C. cucurbitina*	*C. chalybea*	*C. nigrolabiata*
N nests	167	95	92
young adult complete trip	W = 3504, **p = 0.0017**	W = 942, **p = 0.0011**	W = 994, p = 0.4412
emigration of young adult	W = 3507, **p = 0.0105**	W = 1006, **p = 0.0171**	W = 811, p = 0.2047
immigration of young adult	W = 3619, **p = 0.0022**	W = 1159, **p<0.0001**	W = 921, p = 0.8134
short visit of young adult	W = 2940, p = 0.6625	W = 855, p = 0.2101	W = 957, p = 0.8806

Table 5: Features of complete young adult trips. Data from all mature nests combined.

Species	*C. cucurbitina*	*C. chalybea*	*C. nigrolabiata*
total number of complete trips of young adults	59	23	13
proportion of trips performed by males	0.3390	0.5217	0.5385
Median duration of a trip (min) – females	1	6	14
Median duration of a trip (min) – males	2.5	1	4
Maximum duration of a trip (min) – females	207	33	166
Maximum duration of a trip (min) – males	75	15	33

Figures

Fig 1: Nesting phases of C. *chalybea*. Upper picture: active brood nest – the outermost brood cell contains an egg – therefore the female will probably continue with provisioning of offspring. Middle picture: full brood nest – the outermost brood cell contains pupa and there are no young adults – therefore partitions between brood cells are still preserved. Mother is not shown in this picture, but she was present in the nest. Only upper part of nest is showed. Bottom picture: mature brood nest – offspring are mature, brood cell partitions are destroyed. Young adults can exchange their positions in the nest. There are excrements of adults at the bottom of the nest. This study examined behavior of individuals in mature brood nests and a pattern of maternal investment in full brood nests.

Fig. 2: Comparison of number of young adults in nests with mother and without mother

Fig. 3: Number of foraging trips per day performed by mothers of different *Ceratina* species.

Fig 4: Comparison of size of active females (which were marked) and females without evidence of activity (unmarked) in *C. cucurbitina*. The difference was not statistically significant.

Fig. 5: Comparison in number of young adults between control nests and nests with removed female in *C. cucurbitina*.

Fig 1: Nesting phases of C. chalybea. Upper picture: active brood nest – the outermost brood cell contains an egg – therefore the female will probably continue with provisioning of offspring. Middle picture: full brood nest – the outermost brood cell contains pupa and there are no young adults – therefore partitions between brood cells are still preserved. Mother is not shown in this picture, but she was present in the nest. Only upper part of nest is showed. Bottom picture: mature brood nest – offspring are mature, brood cell partitions are destroyed. Young adults can exchange their positions in the nest. There are excrements of adults at the bottom of the nest. This study examined behavior of individuals in mature brood nests and a pattern of maternal investment in full brood nests.

513x293mm (144 x 144 DPI)

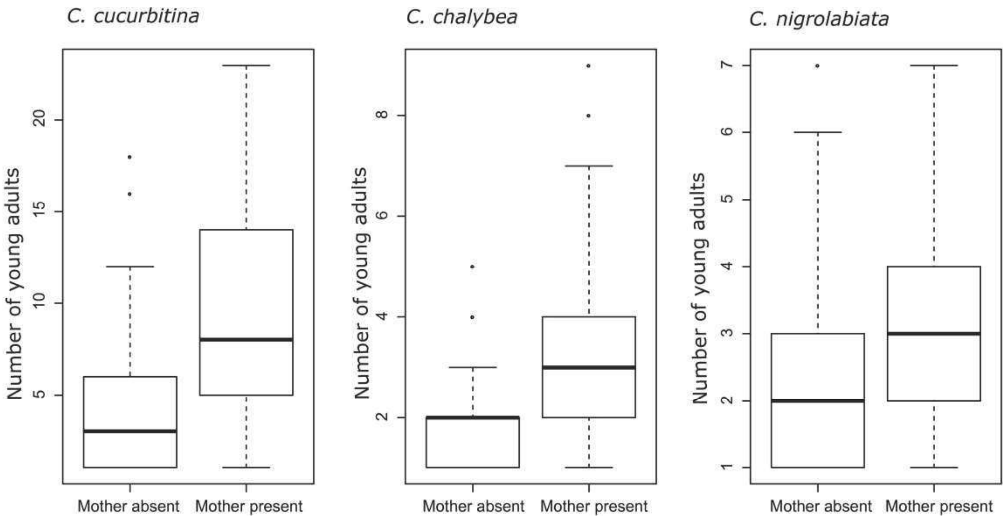

Fig. 2: Comparison of number of young adults in nests with mother and without mother

220x112mm (300 x 300 DPI)

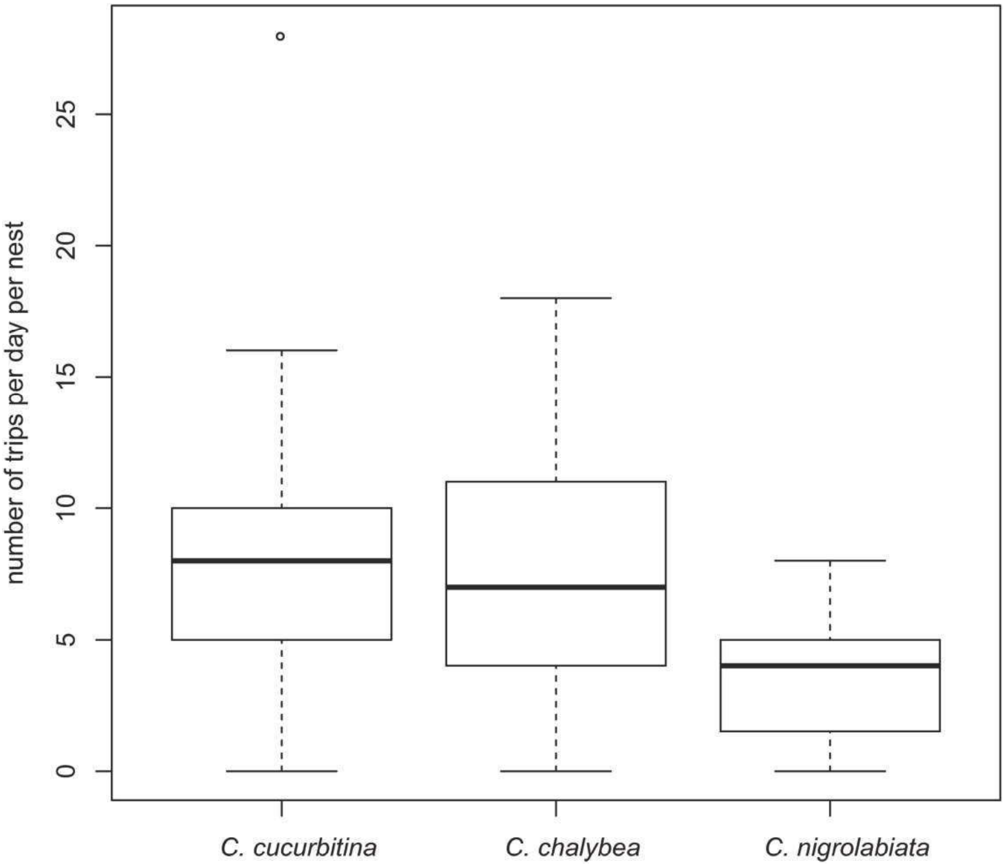

Fig. 3: Number of foraging trips per day performed by mothers of different Ceratina species.

164x141mm (300 x 300 DPI)

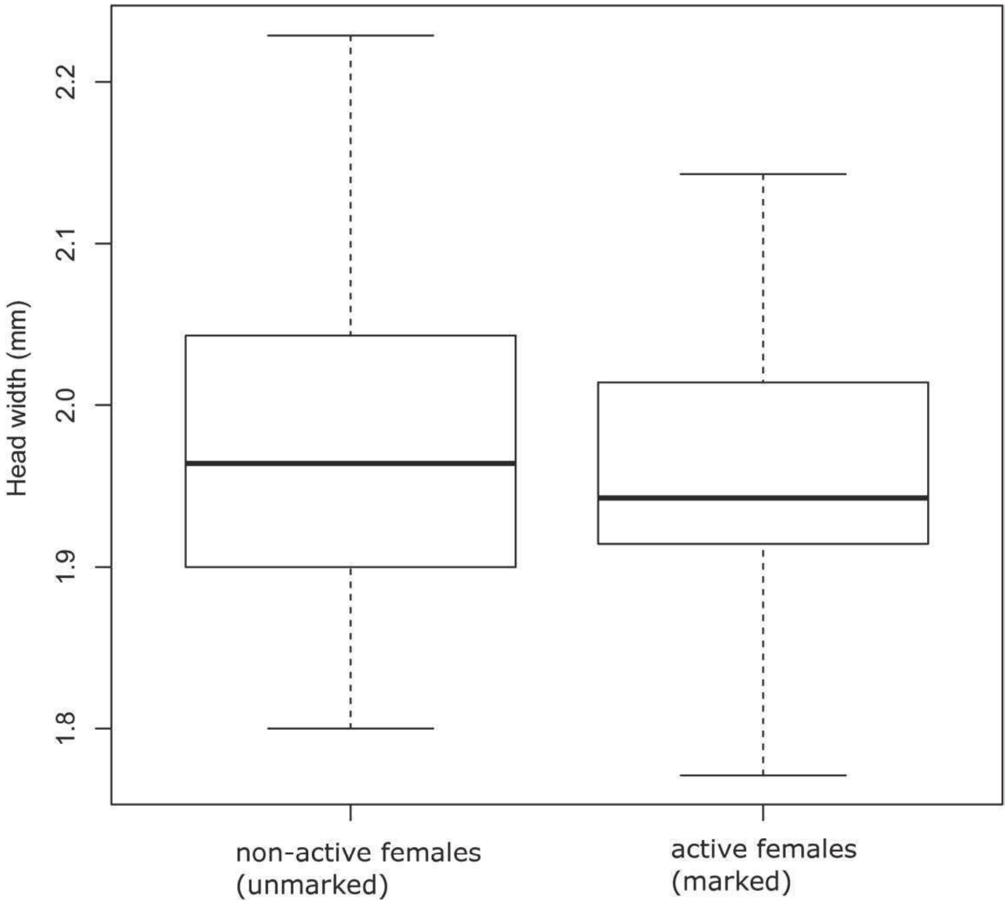

Fig 4: Comparison of size of active females (which were marked) and females without evidence of activity (unmarked) in C. cucurbitina. The difference was not statistically significant.

163x147mm (300 x 300 DPI)

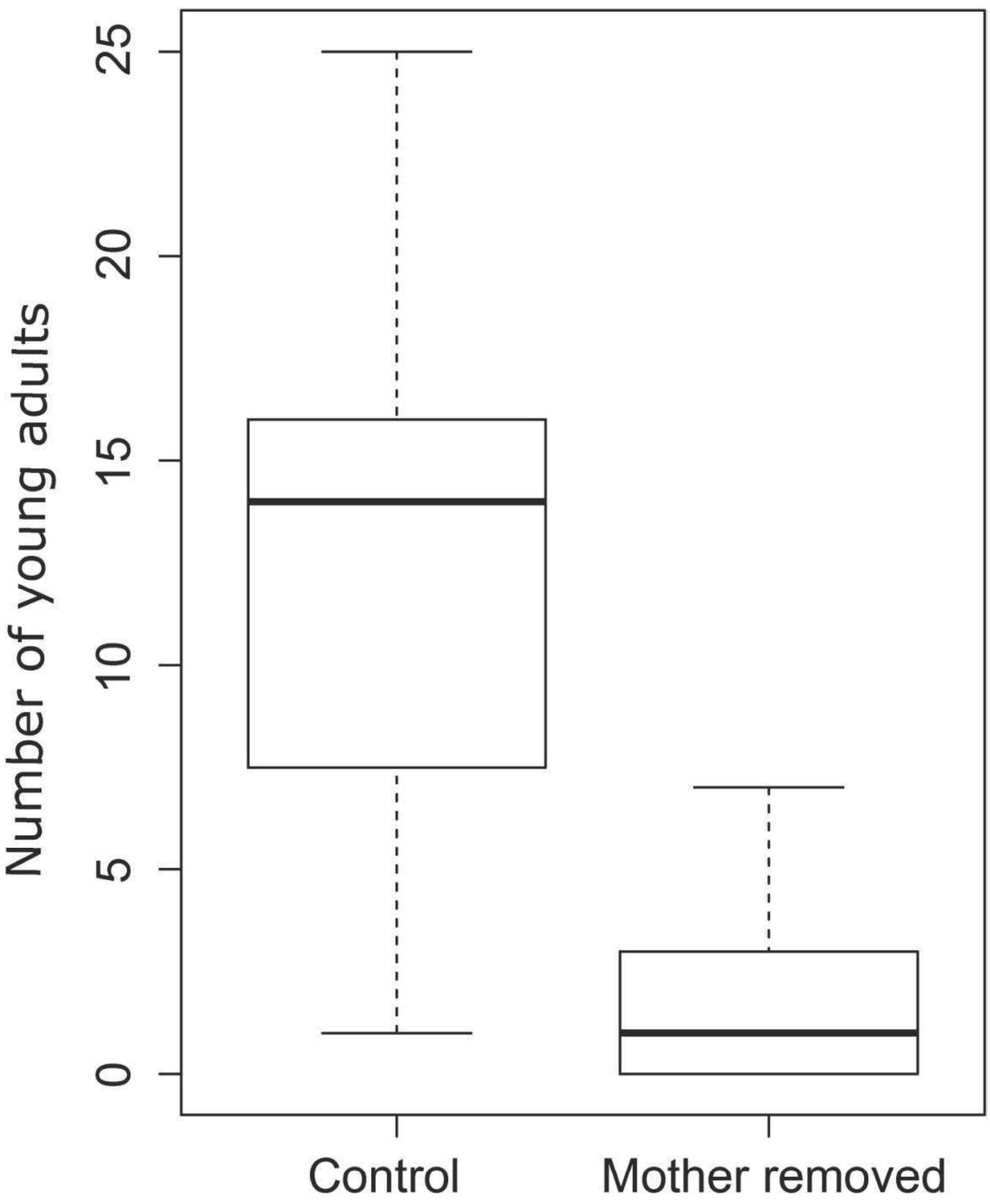

Fig. 5: Comparison in number of young adults between control nests and nests with removed female in C. cucurbitina.

116x140mm (300 x 300 DPI)

Supplementary materials

Table S1: Number of nests analyzed in analysis of foraging activity in different seasons

Species	Mother present	N nests in 2017	N nests in 2018	N nests total
C. cucurbitina	Yes	31	17	48
C. cucurbitina	No	55	64	119
C. chalybea	Yes	15	59	74
C. chalybea	No	4	17	21
C. nigrolabiata	Yes	17	14	31
C. nigrolabiata	No	38	23	61

Table S. 2: Comparison of foraging activity in nests where mother was present between two years. SD=standard deviation

Species	*C. cucurbitina*			*C. chalybea*			*C. nigrolabiata*		
Year	2017	2018	combined	2017	2018	combined	2017	2018	combined
Number of nests observed	31	17	48	15	59	74	17	14	31
average number of old female foraging trips	8.23	8.59	8.35	9.27	7.03	7.49	4.29	2.64	3.55
maximal number of old female foraging trips	16	28	28	15	18	18	8	7	8
SD of old female foraging trips	3.80	6.41	4.82	3.47	5.02	4.81	2.14	2.59	2.46
proportion of nests where old female foraged	1.00	0.94	0.97	1.00	0.88	0.91	0.88	0.57	0.74

Supplementary methods:

Preparation of nesting opportunities: We created artificial nesting opportunities. We used twigs of *Solidago* spp, *Rhus* sp., *Helianthus tuberosus*, and *Echinops sphaerocephalus*. These twigs were cut to a length of around 40 cm. Twenty twigs were tied up together in one sheaf, which was fixed to the ground by a bamboo rod. We installed 2,700 sheaves, which is about 54,000 nesting opportunities. We installed these nesting opportunities to steppe grasslands and heathlands, usually next to shrubs (Fig. S. 2). There are natural nesting opportunities (such as *Rosa* shrubs, or dead stems of *Linaria*, *Centaurea,* or *Verbascum*) and also flowering plants in studied locality, therefore a large population of all studied species is present.

Nest dissection

Nests for dissection were collected after 19:00 CEST to ensure that all nest occupants have returned to their nests. Nest entrances were closed to prevent an escape of bees from nests after nest collection. Nests were stored in a fridge until dissection. Nests were dissected by clippers, and the contents of the nest were analyzed and recorded. Several criteria were recorded: the presence of mother, number of adult offspring, number and the stage of the development of the rest of offspring, presence of nest partitions between brood cells, and the length of the nest. Mothers were distinguished from adult females in the younger generation by the differences in wing wear (mothers do not have complete wing margins, whereas young offspring have complete wing margins (Rehan and Richards, 2010a)).

Here, we present results for full brood nests and mature nests only. Nests with finished provisioning were classified as the full brood nests. Full brood nests contained larva and pupa in the outermost brood cells, but offspring were not in the adult stage or young adults had not crawled through nest partitions. Mature nests contained at least one young adult, which was not separated by cell partitions from the nest entrance. This means that at least one mature offspring crawled through nest partitions to the entrance burrow. Mature nests sometimes also contained immature brood (larvae and pupae).

Preparation of the observation plot:

Sheaves with artificial nesting opportunities were regularly inspected. When we found a hole, we checked if *Ceratina* is present. Sometimes it was not possible to see an abdomen of *Ceratina* in the nest entrance. In these cases we used flashlight to illuminate the inside of the twig, and we were able detect *Ceratina* if it was up to 2 cm from the nest entrance. Nests were marked by ribbons if the presence of *Ceratina* was observed. Species of *Ceratina* were determined.

Nests for observations were randomly selected from sheaves. We took nests from the original sheaf, closed the entrance to the nest and transported nests to the observation plot (Fig. S. 3). We attached nests to the bamboo or wood rod at the observation plot and opened the nest entrance. Transport of nests was undertaken after 19:00 CEST so that all inhabitants were inside nests. There were 24 nests in each observation plot. Observation plots were 1 to 1.5 m long and around 40 cm wide. This size allows comfortable observation of bees. Nests useful for this study (mature nests), and nests in other phases of the nesting cycle including orphaned, abandoned, and also damaged nests were present in study plots. The number of nests per plot in mature nest phase was between 1 and 13.

Nest observations:

We observed nests at least three days after transport to the study plot. We selected 1 to 3 plots for every day of observation. Observation was performed in suitable weather (sunny to partly cloudy, without rain, highest day temperature at least 25°C). From our previous research, we predicted the highest abundance of nests with mother-offspring cohabitation during this period. We began observing nests between 7:20 and 8:20 CEST depending on the weather. Observations started before the first departure of the nest inhabitant that day and ended at 16:00 CEST. Not all bees arrived before this time, however, it is known that *Ceratina* exhibit little activity after 16:00 (Herrera, 1990; Mikát et al., 2017). In total, we observed 221 mature nests of *C. cucurbitina*, 160 of *C. chalybea,* and 133 of *C. nigrolabiata*.

Each observational plot was observed by at least one person for the whole observation period, but usually the plot was observed by two–three people at the same time. Through the observation day, times of departures and arrivals of bees were recorded. As departures of bees were very fast and difficult for visual detection, we used containers for slowing bees, similarly as (Lewis and Richards, 2017; Mikát et al., 2017). Before the observations were started in the morning, lids from the screw-on container were installed. The lid was positioned bellow each nest entrance to ensure that bees were not disturbed from their activities and that departure and arrival was not influenced. After that we awaited the first departure of the nest inhabitant which was recorded. When we observed the bee had returned and entered the nest completely, the time of arrival was noted and the nest was closed with a transparent container which matched the lid and could be safely closed. During the second departure the bee was taken out of the container and carefully marked with an oil marker in a specific color and released to continue in its activity. The age of the bee was checked. The nest was left open without transparent container until the next arrival of the bee. During the day, nests with inhabitants inside were closed with the container and nests with individuals flying around were open to enable the wandering individuals to return to the nest.

We managed to record most of the departures and arrivals of bees. Sex, species, age (old bees, young adults) and the time of arrival or departure was recorded. Active individuals were marked with an oil marker on metasoma for easier recognition of active nest inhabitants.

Supplementary Figures:

Fig. S.1: Full brood nest of *C. nigrolabiata*. The oldest brood cells is on the right (bottom of the nest), the youngest brood cell is on the left. Nest is closed by filling plug and abandoned – mother was not present.

Fig. S.2: Artificial nesting opportunities. Sheaves of 20 twigs were fixed to ground.

STUDY 9

Mikát., M., Benda, D., Fraňková, T., and Straka J: Evidence of sociality in small Carpenter bees in Cyprus.

Submitted to *Apidologie*.

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Author contribution: MM designed research, MM, DB and TF Performed research, MM analyzed data, MM wrote first draft of manuscript, JS performer substantial changes in manuscript, All authors intensively discussed about scientific issues in manuscript and approved final version. MM is corresponding author.

Apidologie

Evidence of sociality in European small Carpenter bees (Ceratina)

--Manuscript Draft--

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Abstract:	Natural history of facultative social taxa is important for understanding the evolution of Eusociality. However, data about the social status of many species are missing. Social status of seven European Ceratina bees in Cyprus was studied. Multifemale nests were found in five species from three different subgenera: C. (Euceratina) mandibularis, C. (E.) cypriaca, C. (E.) chrysomalla, C. (Dalyatina) parvula and C. (Neoceratina) bispinosa. No social nests were found in C. (E.) dallatoreana and C. (N.) schwarzi and also, the sample size was small for the second species. We compared nest productivity between solitary and multifemale nests in C. cypriaca, C. mandibularis and C. parvula. It is apparent that multifemale nests had a higher number of brood cells than solitary nests. Per capita productivity was equal in both nest types. We can conclude that facultative sociality is common in Ceratina bees of subtropical climate and social nesting is the successful strategy.
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Opposed Reviewers:	

Dear Editor,

It is my pleasure to submit an original research manuscript entitled *Evidence of sociality in European small Carpenter bees (Ceratina)* by Michael Mikát, Daniel Benda, Tereza Fraňková and Jakub Straka for your consideration as original article.

Evolution of insect sociality still remains one of the main evolutionary puzzles. Eusucial species with large colonies, such as honeybee, yellowjackets and ants get the most attention. These societies are interesting in the sense of studying cooperation and established societies, however, not in the sense of studying the emergence of eusociality. For this question, groups at the edge of eusociality – typically Euglossini, Halictidae and Xylocopini from bees, are better. These groups contain solitary species and also species with different levels of sociality, therefore comparative analysis in these taxa are optimal. Moreover, many species are interspecifically variable in social behavior. Natural history of the majority of the species belonging to these groups is still unexplored; eventhough comparison of multiple species is crucial for proper understanding of arise of social behavior.

Ceratina bees are excellent model taxon for studying the emergence of eusociality. Although they were primarily considered solitary, there is a growing evidence (lately rewiewed in Groom and Rehan 2018) that most of the species are facultatively social. However, social status is highly correlated with climate – facultatively social are usually tropical species, but solitary are temperate. We analyzed social status of seven Ceratina species inhabiting warm temperate zone – Cyprus island. We detected facultative sociality in five of these species. Moreover, up to four adult females was known to be present in the nest in *Ceratina* societies studied to this date, but we detected also nests with six or five adult females in two species.

Our study shows that facultative eusociality is widespread in South-European *Ceratina* bees. In our manuscript, we present new knowledge about the natural history and social biology of a group, which is essential for understanding of social behavior. Moreover, our conclusions are supported by a strong dataset (based on 1031 nests). Therefore, we hope, that our research will be suitable for *Apidologie*.

Michael Mikát (on behalf of all coauthors)

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Evidence of sociality in European small Carpenter bees (*Ceratina*)

Short title: Sociality in European small carpenter bees

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Abstract

Natural history of facultative social taxa is important for understanding the evolution of Eusociality. However, data about the social status of many species are missing. Social status of seven European *Ceratina* bees in Cyprus was studied. Multifemale nests were found in five species from three different subgenera: *C.* (*Euceratina*) *mandibularis*, *C.* (*E.*) *cypriaca*, *C.* (*E.*) *chrysomalla*, *C.* (*Dalyatina*) *parvula* and *C.* (*Neoceratina*) *bispinosa*. No social nests were found in *C.* (*E.*) *dallatoreana* and *C.* (*N.*) *schwarzi* and also, the sample size was small for the second species. We compared nest productivity between solitary and multifemale nests in *C. cypriaca*, *C. mandibularis* and *C. parvula*. It is apparent that multifemale nests had a higher number of brood cells than solitary nests. Per capita productivity was equal in both nest types. We can conclude that facultative sociality is common in *Ceratina* bees of subtropical climate and social nesting is the successful strategy.

Keywords: facultative social, nesting biology, eusociality, nest productivity, Xylocopinae

Introduction

Biologists were always fascinated by social insects societies (Keller and Chapuisat 2010). Origin of eusociality is one of the key puzzles of evolutionary biology (Wilson and Hölldobler 2005; Keller and Chapuisat 2010; Kennedy et al. 2017) and it was one of the problematic points for Darwin´s original theory. Although social insects are highly studied groups, the research focuses mainly on few species. These species are usually highly social; they are therefore useful for studying many questions concerning cooperation and conflict between individuals, however, they are inappropriate for the study of the origin of eusociality.

With their behavioral variability, facultatively eusocial species are a unique possibility to have a look on both sides of sociality. These species are able to manifest solitary as well as eusocial strategy (Schwarz et al. 2007; Shell and Rehan 2017). The largest lineages in bees are Halictidae, Xylocopinae and Euglossini (Schwarz et al. 2007; Shell and Rehan 2017)

Emergence of social colonies in facultatively eusocial species can be dependent on ecological conditions, e.g. climate (Field et al. 2010; Purcell 2011). Eusocial colonies of facultatively eusocial species consist only of a few females, from two to lower tens (Schwarz et al. 2007; Turillazzi 2013). We are able to compare solitary and social colonies and evaluate the success of these strategies. We might think that social colonies should have higher productivity per one female than solitary colonies. However, productivity of social colonies per one female is often lower than in solitary colonies (Michener 1964; Richards 2011a; Rehan et al. 2014). For a proper assessment of costs and benefits of sociality it is necessary to take into account the probability of nest failure, which is lower in social colonies (Stark 1992; Smith et al. 2007; Leadbeater et al. 2011).

Small societies of facultatively eusocial species are less noticeable than large insect societies. Their frequency can be either very low in population (Sakagami and Maeta 1987) or sociality can occur only in some populations (Field et al. 2010) or during some years (Dew et al. 2018; Mikát et al. 2020). It is therefore not easy to detect facultative eusociality and also some facultatively eusocial groups are traditionally considered as solitary.

Knowledge about the natural history of facultatively eusocial groups is essential for identification of key factors which influence the origin of eusociality. Origin of eusociality can be influenced by ecological pressures and relatedness between individuals (Keller and Chapuisat 2010; Purcell 2011; Kocher and Paxton 2014). However, the evolution of eusociality is strongly influenced by natural history traits of each taxon (Andersson 1984; Ross et al. 2013). For example, facultatively eusocial groups with progressive provisioning such as Stenogastrinae wasps and Allodapine bees have not reversed to strictly solitary behavior (Chenoweth et al. 2007; Turillazzi 2013). On the other hand, mass provisioning groups such as Halictid bees or carpenter bees from genera *Ceratina* or *Xylocopa* frequently reverse to strict solitarity (Rehan et al. 2012; Gibbs et al. 2012; Groom and Rehan 2018). Another example is the architecture of nest in *Xylocopa*. There are different tendencies in groups with linear and branched nests in *Xylocopa*. Species with linear nest building have the tendency to nest eusocialy but species with branched nest building have the tendency to build communal nests (Steen and Schwarz 2000). While the natural history of facultative eusocial taxa is the key knowledge for understanding the social evolution it is still insufficient or unknown for most of taxa. Small carpenter bees (*Ceratina*) are understudied in said aspect and also the comparison with other facultatively eusocial species is scarce.

Small carpenter bees (*Ceratina*) belong to subfamily Xylocopinae in family Apidae and they are an exemplary taxon with the presence of facultative eusociality (Groom and Rehan 2018). Strictly solitary strategy is considered a reversion from facultative eusociality (Rehan et al. 2012). *Ceratina* nest in broken twigs. They build linear nests containing several brood cells (Michener 1990) which are provisioned by mass provisioning (Rehan 2020). When provisioning is finished, mother usually guards her offspring until adulthood (Rehan and Richards 2010; Mikát et al. 2016) and after that feeds mature offspring with pollen and nectar (Mikát et al. 2017; Rehan 2020).

Obligate eusocial *Ceratina* species does not exist. On the other hand, some reversions to strict solitarity is known (Michener 1990; Rehan and Richards 2010; Groom and Rehan 2018). Although most of species are able to develop eusocial colonies, their proportion in population is small - between 1 - 30% (Sakagami and Maeta 1984; Rehan et al. 2010, 2015). Generally, tropical species have a higher tendency to manifest sociality than temperate species (Groom and Rehan 2018).

Social colonies are very small and usually contain only two females (Rehan et al. 2010, 2015; Rehan 2020). Sometimes, there can be up to four residents (Sakagami and Maeta 1984; Michener 1990). More females in colony can lay eggs, though usually one is reproductively dominant (Hogendoorn and Velthuis 1999).

There is a high diversity of *Ceratina* bees in Meditarrean region with 35 species belonging to five subgenera (Terzo 1998; Terzo and Rasmont 2011). However, nesting biology and sociality in most of these species is unknown especially in comparison with North American and Japanese species (Sakagami and Maeta 1977, 1987; Vickruck et al. 2011; Lawson et al. 2018). Goal of our study was to increase our knowledge about sociality of Meditarrean species. We performed a study in Cyprus, because eight *Ceratina* species live there and *C. cucurbitina*, which dominates in most of Meditarrean region, is absent (Terzo and Rasmont 2011; Varnava et al. 2020). We discovered multifemale nests in five species and examined the frequency of multifemale nesting and the number of females in multifemale nests in each species. Moreover, we examined the difference of nest productivity and architecture between solitary and multifemale nests in three species with multifemale nests most commonly present.

Methods

Study area: All samples were collected in Cyprus. Most of the samples were collected around Lefkara village (34.8744575N, 33.3113722E) and between villages Mathiatis and Agios Theodoros. However, other nests were collected in other parts of Cyprus, specifically around Paphos and Limassol. Coordinates of all sampled nests are showed in Online resource 2.

Sampling process: We sampled nests in four periods through nesting season: 25–30 May 2018, 15–28 June 2019, 6–12 August 2019 and 17–22 September 2019. We collected nests from semi-natural nesting opportunities, such as cut twigs next to roads. However, nests were relatively scarce in landscape. Therefore, we cut additional twigs to increasing nesting opportunities. We performed cutting of twigs in March, June and August 2019. The most common nesting substrate was blackberry (*Rubus* spp.), nests were also frequently collected from fennel (*Foeniculum vulgare*) and giant fennel (*Ferula communis*).

Dissecting of nests: We collected nests after 6 p.m. to ensuring that all inhabitants are back from foraging trips and therefore inside nests. Nest entrance was closed by tape at the moment of collection. Nests were opened by clippers. We noted these nest features: number, sex and age of adult individuals, number of brood cells, number and stage of immature offspring, presence of parasites, and nesting substrate. We measured these parameters of nest: length of nest, length of entrance burrow, width of nest, width of pith, width of twig. In total, we sampled 20 nests of *C. bispinosa* Handlirsch, 338 nests of *C. cypriaca* Mavromoustakis, 192 nests of *C. dallatoreana* Friese, 131 nests of *C. chrysomalla* Gerstaecker, 203 nests of *C. mandibularis* Friese*,* 102 nests of *C. parvula* Smith and 45 nests of *C. schwarzi* Kocourek.

There is no existing taxonomic key for species identification of Cyprus *Ceratina*. We used publications from Daly (1983) and Terzo (1998) for species determination. Collected individuals were also compared to determined specimens deposited in Linz museum, Austria.

Collected nests were in different stages: burrows, which contained only adult(s) without any sign of present or past brood cells. However, in case of *C. dallatoreana*, we distinguished burrows with nectar storage on bottom of nest and standart burrows without this storage. Other types of nests were: active brood nests, which contained brood cells and the outermost brood cell contained egg or was recently provisioned with a pollen ball; full brood nest, which contained larva or pupa in the outermost brood cell and all brood cell partitions were preserved; full-mature brood nests, which contained immature stages and also newly enclosed adults which damaged brood cell partitions; and mature brood nests which contained only mature offspring.

Analysis of phenology: Nests which contained at least one adult female were included in this analysis. Therefore, nests with only males, juveniles or parasites were not included in the analysis because the goal of this analysis was to evaluate the behavior of females through the season. We calculated the proportion of different nest stages.

Comparison between solitary and social nests: We included only active brood nests and full brood nests for this analysis. We excluded orphaned nests (nests without a female), because these nests cannot be classified in any cathergory as no female is present there. For most analyses, we analyzed dataset where active brood nests and full brood nests were analyzed together as there was no significant difference in the number of brood cells in most species betveen active brood nests or full brood nests (see results). For analyses calculating with number of brood cells or empty cells we excluded nests parasitized by ichneumonid or *Gasteruption* parasite because these parasites damaged the nest and the number of brood cells was therefore unknown. The statistical analyses were performed in R software 3.6.1. (R. Core Developmental Team, 2016). Chi square tests were used for testing of proportions of social nests in active brood nests vs. full brood nests and between different parts of season. Wilcoxon tests were used for testing effect of sociality to number of brood cells, number of brood cells per one female and length of nests, because data had not normal distribution. Effect of sociality to proportion of empty cells was used by generalized linear model of binomial family.

Results

Phenology

***C. cypriaca*:** Proportion of active brood nests and full brood nest is relatively stable throughout nesting season. The most different month was September, when proportion of active brood nests was lowest and proportion of mature brood nests the highest (Fig. S 1, Online resource 1).

***C. dallatoreana*:** The proportion of active brood nests was high in May (40%, 8/20) and June (54%, 15/28), it decreased in August and no active brood nests were found in September. Main peak of full brood nests was in June and August. Proportion of burrows without nectar storage was stable throughout the season. However, proportion of burrows with nectar storage was highest in September (77%, 54/70), and relatively common in August and May. No burrows with nectar storage were found in June.

C. chrysomalla: Proportion of active brood nests was relatively stable between May and August. However, no active brood nests were found in September. The highest proportion of full brood nests was in June and September.

C. *mandibularis*: Proportion of active brood nests and full brood nests was stable through nesting season. Mature brood nests and full-mature brood nests occurred from June, but their proportion was stable between June and September.

C. parvula: Active brood nests were very common in May (58%, 7/12) and August (68%, 15/22), and their proportion was the lowest in September (10%, 2/21). Full brood nests occurred from June to September and their proportion was highest in September.

C. bispinosa and ***C. schwarzi***: Not enough data.

Number of brood cells provisioned

Average number of brood cells provisioned was 4.11 (N=9, SD=3.25, range 1-8) for *C. bispinosa*, 1.67 (N=129, SD=1.41, range 1-9) for *C. cypriaca*, 3.42 (N=49, SD=2.17, range 1-11) for *C. dallatoreana*, 4.49 (N=63, SD=2.44, range 1-11) for *C. chrysomalla*, 2.79 (N=97, SD=1.96, range 1-11) for *C. mandibularis*, 5.11 (N=65, SD=3.66, range 1-20) for *C. parvula* and 2.9 (N=10, SD=1.60, range 1-5) for *C. schwarzi*. There was no significant difference in the number of brood cell provisioned between active brood nests and full brood nests for most species (Table 1). Significant difference in brood cells provisioned between active brood nests and full brood nests was in *C. cypriaca*, where active brood nests had a higher number of brood cells provisioned (2.50, N=40, SD=2.11, range 1-9) than full brood nests (1.30, N=89, SD=0.66, range 1-4).

Nest orphanage

Full brood nests were usually guarded by female, however, we found nests without female in some species. The proportion of full brood nests without female was 0% (N=6) in *C. bispinosa*, 2.75% (3/109, Table 2) in *C. cypriaca*, 25% (7/28) in *C. dallatoreana*, 26.44% (8/30) in *C. chrysomalla*, 6.25% (4/64) in *C. mandibularis*, 3.25% (1/28) in *C. parvula* and 0% (N=7) in *C. schwarzi*.

Evidence for social nesting

C. bispinosa: Proportion of multifemale nests was 27.27% (3/11, Table 2). Other nests were solitary. Two multifemale nests contained two females, one contained three females and two males. Multifemale nests were found in May, June and September.

C. cypriaca: Proportion of multifemale nests was 6.21% (10/161). Proportion of solitary nests was 91.92% (148/161). In 1.23% (2/161) of nests one male and one female were present. In 0.62% (1/161) of nests one alive and one dead female were present. There was no significant difference in the proportion of social nests between active brood nests and full brood nests (Chi square test, Chi = 2.20, df = 1, p = 0.1376, Table 3). Nine multifemale nests contained two females, one contained two females and one male. Multifemale nests were found in June, August and September.

C. dallatoreana: We found only solitary nests (N=65)

C. chrysomalla: Proportion of multifemale nests was 3.07% (2/65). Other nests were solitary. Both multifemale nests contained two females.

C. mandibularis: Proportion of multifemale nests was 18.01% (20/111). Proportion of solitary nests was 79.27% (88/111). Male and female was present in 1.80% (2/111) of nests and one alive and one dead female was present in 0.90% (1/111). There was a significant difference in proportion of multifemale nests between active brood nests and full brood nests (Chi square test, Chi=17.57, df=1, p=2.759e-05). In active brood nests, proportion of multifemale nests was 35.29% (18/51) and in full brood nests it was only 3.33% (2/60). Most of the multifemale nests (70% 14/20) contained two females. However, we also found one nest which contained three females, four nests which contained four females and one nest which contained five females. Multifemale nests were found in all studied periods, proportion of social nests did not significantly differ between periods (Chi square test, Chi=0.51, df=3, p=0.9177).

C. parvula: Proportion of multifemale nests was 6.15% (4/65). Other nests were solitary. Two multifemale nests contained two females, one contained four females and one contained six females. Multifemale nests were found in August and September.

C. schwarzi: We found only solitary nests (N=14).

Differences between solitary and multifemale nests

Number of brood cells: Number of brood cells provisioned was significantly higher in multifemale nests in all three species with sufficient number of nests (Wilcoxon test: *C. mandibularis*: W=1273.5, p=4.068e-06, *C. cypriaca*: W = 879, p=8.603e-05, *C. parvula*: W = 243, p=0.0009, Fig. 1). For *C. mandibularis*, number of brood cells provisioned was 2.31 (N=77, SD=1.54, range 1-11) in solitary and 4.65 (N=20, SD=2.3, range 1-8) in multifemale nests. For *C.*

cypriaca, number of brood cells provisioned was 1.59 (N=120, SD=1.38, range 1-9) on average in solitary and 2.77 (N=9, SD=1.39, range 1-6) in multifemale nests. For *C. parvula*, number of brood cells provisioned was 4.85 (N=61, SD=2.64, range 1-13) in solitary and 15.25 (N=4, SD=3.40, range 12-20) in multifemale nests. However, number of brood cells per one female (per capita productivity) was not significantly different between solitary and social nests in any species with sufficient sample size (Wilcoxon test, *C. mandibularis*: W=735.5, p=0.7556, *C. cypriaca*: W=607, p = 0.4319, *C. parvula*: W=134.5, p=0.7412).

Length of nest: Social nests were significantly longer than solitary nests in *C. mandibularis* (Wilcoxon test, W=1147.5, p=0.03469) and *C. parvula* (Wilcoxon test, W=212, p=0.01451), but not in *C. cypriaca* (Wilcoxon test, W=937, p=0.1605). In *C. mandibularis*, solitary nests were 10.96 cm long on average (N=88, SD=5.86, range=2.0–27.6) and social nests 13.51 cm long on average (N=20, SD=4.96, range=5.5–21.4). In *C. cypriaca*, solitary nests were 9.67 cm long (SD=4.07, N=148, range=3.10-28.60) and multifemale nests 12.64 cm long (N=10, SD=6.15, range=5.7-23.4). In *C. parvula*, solitary nests were 6.56 cm long (N=61, SD=2.98, range=2.00-16.7) and multifemale nests 12.78 cm long (N=4, SD=6.21, range=6.3-21.2).

Proportion of empty cells: Proportion of empty cells was higher in solitary nests (29%, N=77 nests, SD=0.24) than in multifemale nests (17%, N=20 nests, SD=0.18) in *C. mandibularis*. The difference was statistically significant (GLM binomial, deviance 13.53, df=94, residual deviance 94.11, p=0.0002). However, this difference was not significant for *C. cypriaca* (GLM binomial deviance 0.59, df=126, residual deviance=105.54, p=0.4408) or *C. parvula* (GLM binomial, deviance=1.05, df=63, residual deviance=18.75, p=0.30).

Branched nest architecture

In *C. cypriaca*, we found one nest (out of 338 nests) with two branches within the nest. This nest was solitary and contained brood cells in both branches. It was present in the stem of *Ferula* with 2.5 cm in diameter. *C. cypricata* nests were frequently found in *Ferula* – proportion of nests in *Ferula* was 68.64% (232/338). Although Ferula stems had 1.75 cm in diameter on average, we did not encounter more branched nests.

Discussion

Presence of sociality

Although *Ceratina* bees were traditionally considered as solitary and most of nests are solitary, the possibility of multifemale nesting was detected in multiple studied species (Michener 1974, 1990, 2007; Sakagami and Maeta 1977; Rehan et al. 2010). The most recent work reviewing the presence of sociality in the genus *Ceratina* (Groom and Rehan 2018) states that the ability of social nesting is present in 26 out of 34 studied species. However, European species studied to this date are considered to be solitary or biparental (Mikát et al. 2016, 2019a, b). The only exception is *C. chalybea*, where unusual sociality with males as helpers occur (Mikát et al. 2020). Social biology of southern European species was mostly unexplored to this date. We examined social strategy in seven species and we detected social nests in five of them: *C. mandibualis*, *C. cypriaca*, *C. chrysomalla*, *C. parvula* and *C. bispinosa*. We have not detected social nests in two species: *C. dallatoreana* and *C. schwarzi*. However, number of nests analyzed is too low to interpret the social status in *C. schwarzi*. For *C. dallatoreana*, number of examined nests (56) can be insufficient if sociality is very rare, though, Daly (1966) also reported *C. dallatoreana* as solitary species. In our opinion, *C. dallatoreana* is probably solitary, although more data from multiple regions could be useful.

Ceratina bees have been intensively studied mainly in temperate regions, where solitary species prevail (Lawson et al. 2018; Groom and Rehan 2018) but data from tropics showed that there strongly prevails facultatively social species (Rehan et al. 2009, 2015; Groom and Rehan 2018). Cyprus, where we performed our research, lays in subtropical zone, and we showed that facultatively social species prevail there as well. Similarly, Japanese species which live in warm-temperate or even subtropical climate are also facultatively eusocial (Sakagami and Maeta 1977, 1987). Generally, the possibility for multiple brood per year is supposed to be an important factor for social nesting (Field et al. 2010; Purcell 2011). *Ceratina* bees have long nesting season in Cyprus. Three species, *C. mandibularis*, *C. parvula* and *C. cypricaca* had active brood nests even in late September and in all these species multifemale nests were documented. On the other hand, we have not observed any active brood nests in September in *C. dallatoreana* which had only solitary nests in our observation and in *C. chrysomalla*, which has a low proportion of social nests. Subtropical species *C. okinawana*, in which multifemale nests occur, has three peaks of reproduction per year (Okazaki 1987). Therefore, season in subtropical climate seems to be sufficiently long for multiple broods per year.

Features of social nests

Number of individuals: *Ceratina* bees build linear nests, therefore females need to pass each other when one of them leaves or enters the nest. Linear nests do not allow effective avoidance of higher number of females, therefore number of individuals per nests is constrained by nest architecture in *Ceratina* (Rehan et al. 2009). Two females in *Ceratina* nests perform effective division of labor. One performs foraging, the other performs guarding of nest (Sakagami and Maeta 1984; Hogendoorn and Velthuis 1999; Rehan et al. 2010). However, more females probably cannot work effectively, because only one brood cell is provisioned at a time.

Social *Ceratina* nests usually contained only two females and can contain up to four females (Sakagami and Maeta 1984; Rehan et al. 2010). Although two-female nests were the most common social nests in all social species in this study, we repeatedly detected nests with more individuals. We found even six females in a nest of *C. parvula* , five females in another one and commonly four females in nests of *C. mandibularis*. Therefore, these species are able to build unusually large societies in comparison to other *Ceratina* bees.

Males: In rare cases, we found adult males present in active brood nests and full brood nests. We observed male-female pair in *C. mandibularis* and *C. cypariaca*. Moreover, we found adult males in multifemale nests in *C. cypriaca* and *C. bispinosa*. These males can be accidental visitors and also male helpers which philopatrically stay in the nest from previous nest clutch. We suppose that none of studied species have regular biparental strategy, which is known from *C. nigrolabiata* (Mikát et al. 2019b). Also, male behavior is different than in *C. chalybea*, where young males prevails in social nest (Mikát et al. 2020). However, larger datasets, nest observation and genetic tests are necessary for uncovering the role of males in these nests.

Nest productivity: Social nesting should be beneficial, if nesting productivity per one individual increases. However, empiric studies usually show a decreasing productivity per one individual (Michener 1964; Richards 2011b; Prager 2014). Studies performed on *Ceratina* bees up to this date showed different results. Per capita productivity between solitary and social nests do not differ in *C. japonica* (Sakagami and Maeta 1984), but significantly decreases in social nests of *C. australensis* (Rehan et al. 2014; Dew et al. 2018) and *C. chalybea* (Mikát et al. 2020). We found that social nests are more productive than solitary in three species with enough data. However, productivity per capita did not differ between solitary and multifemale nests. As per capita productivity is the same for both strategies, their coexistence is not surprising. Moreover, social nesting can be beneficial when the number of provisioned brood cells per female is the same in solitary and also social nests, because social nests have a higher colony survival (Hogendoorn and Zammit 2001; Smith et al. 2007). In *Ceratina* bees destruction of orphaned nests is highly probable (Mikát et al. 2016), and social nests have logically smaller probability of nest orphanage.

Nest architecture: Social nests were generally longer than solitary nests, although the difference was significant only for *C. parvula* and *C. mandibularis* and not for *C. cypriaca*. As multifemale nests have higher productivity, it makes sense that they need more space for brood cells. There can be two reasons for this difference: higher probability of establishing social nests in larger burrows or enlarging the burrow after the first brood clutch.

We found that empty cells are more often in solitary nests than in social nests in *C. mandibularis*. Empty cells are considered to be a protection against natural enemies (Tepedino et al. 1979; Münster-Swendsen and Calabuig 2000) and more effectively guarded multifemale nests probably do not need this protection. However, we have not found a difference in empty cells proportion in *C. cypriaca* nor *C. parvula*. Empty cells are scarce in both species therefore they probably rely on other types of nest protection.

Nesting biology of *Ceratina* in Cyprus

Nest guarding: Although *Ceratina* bees usually guard their nest until adulthood, facultative nest abandonment has been documented in two European *Ceratina* species (Mikát et al. 2016, 2019a). We found only a few full brood nests without any female in the most of currently studied species. About 25% of full brood nests without female were found in *C. chrysomalla* and *C. dallatoreana*. However, we have not found a clear distinction in nest architecture between nest with mother and without mother in these species, on the contrary the situation in *C. chalybea* and *C. chalcites*, where abandoned nests are closed by filling plug that is much thicker than the usual nest partition (Mikát et al. 2016, 2019a). Therefore, we suppose that female always tries to guard her offspring until adulthood in all species here and she does not perform facultative nest abandonments. However, further research of guarding strategy is necessary for confirmation of obligate nest guarding for *C. dallatoreana* and *C. chrysomalla*.

Branched nests: Branched nests are common in *Xylocopa* bees (Gerling et al. 1983; Steen and Schwarz 2000). However, it has not been documented for *Ceratina* bees yet (Eardley and Daly 2007; Vickruck et al. 2011). *Ceratina* bees usually nest in relatively narrow twigs, where there is not enough space for a branched nest. However, *C. cypriaca* commonly uses broken *Ferula* stalks with about 2 cm in diameter, where there can be enough space for a branched nests. We documented only one branched nest. This situation can be either a mistake or a regular strategy of species. But this phenomenon deserves future research due to a possible influence of nesting architecture on social organization (Steen and Schwarz 2000).

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Author contribution

Michael Mikát designed research, Michael Mikát, Daniel Benda and Tereza Fraňková Performed research, Michael Mikát analyzed data, Michael Mikát wrote first draft of manuscript, Jakub Straka performer substantial changes in manuscript, All authors intensively discussed about scientific issues in manuscript and approved final version.

Data availability

All data are denerated for this study is showed in spreadsheet Online resource 2

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Tables

Table 1: Number of brood cells provisioned in active brood nests and full brood nests in different species. Only non-orphaned nests are included. Difference in number of brood cells between active brood nests and full brood nests was tested by Wilcoxon test.

Species	Number of brood cells			Wilcoxon test		Nests analyzed
	Active brood nests	Full brood nests	Both types together	W	p	
C. bispinosa	4.4	3.75	4.11	10	1	9
C. cypriaca	2.5	1.30	1.67	2352	**0.0003**	129
C. dallatoreana	3.21	3.93	3.42	198	0.2136	49
C. chrysomalla	4.14	5.19	4.49	318.5	0.0729	63
C. mandibularis	3.11	2.5	2.79	1407	0.0854	97
C. parvula	5.66	5.11	5.43	510	0.9786	65
C. schwarzi	2.86	3	2.9	11	0.8963	10

Table 2: Number of nests with different social status. Only active brood nests and full brood nests were included. Solitary nests contained only one female, multifemale nests contained at least two females (and sometimes also males). Orphaned nests contained no female, rarely contain male or dead female. Male + female nests contained one male and one female. Dead + solitary nests contained one living and one dead female.

Species	**solitary**	**multifemale**	**orphaned**	**Male and female**	**Dead and solitary**	**Nests analyzed**
C. bispinosa	8	3				11
C. cypriaca	148	10	3	2	1	164
C. dallatoreana	56		9			65
C. chrysomalla	63	2	12			77
C. mandibularis	88	20	4	2	1	115
C. parvula	61	4	2			67
C. schwarzi	14					14

Table 3: Number of solitary and multifemale nests in active brood nests and full brood nests. Numbers of nests with other social status was not shown.

	Active brood nests		**Full brood nests**	
Species	**solitary**	**social**	**solitary**	**social**
C. bispinosa	3	2	5	1
C. cypriaca	47	6	101	4
C. dallatoreana	35	0	21	0
C. chrysomalla	42	1	21	1
C. mandibularis	31	18	57	2
C. parvula	35	3	26	1
C. schwarzi	7	0	7	0

Figures

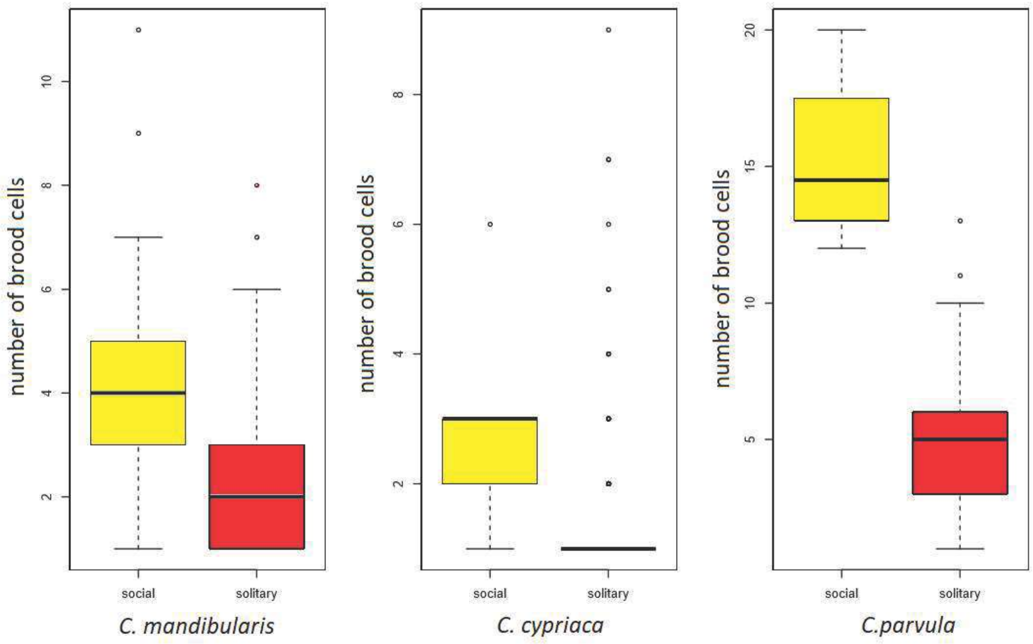

Fig. 1: Comparison of productivity of solitary and social nests

Evidence of sociality in European small Carpenter bees (*Ceratina*)

Short title: Sociality in European small carpenter bees

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Supplementary materials

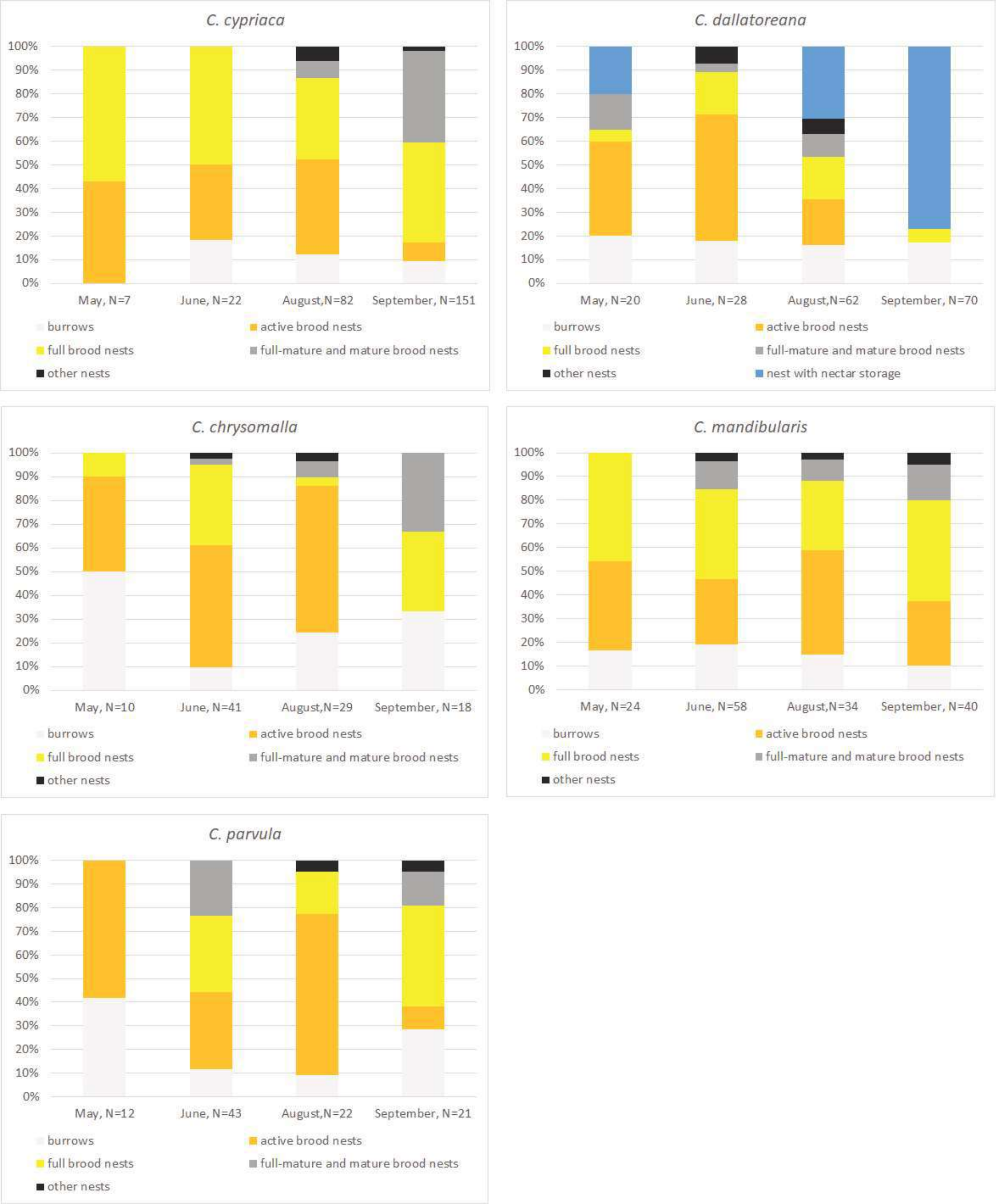

Fig S. 1: Phenology of *Ceratina* species in Cyprus

STUDY 10

Mikát., M., Benda., D., Korittová, C., Mrozková, J., Reiterová, D., Waldhauserová, J., Brož, V., and Straka J., Natural history and maternal investment of *Ceratina cucurbitina*, the most common European small carpenter bee, in different European regions.

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Natural history and maternal investment of Ceratina cucurbitina, the most common European small carpenter bee, in different European regions

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Natural history and maternal investment of *Ceratina cucurbitina*, the most common European small carpenter bee, in different European regions

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Abstract

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Ceratina cucurbitina is the most common small carpenter bee in Europe. We examined social status of nests and maternal investment in four European locations: Czech Republic, Italy, Albania, and Crete. This species is solitary in all studied locations. We found evidence for nest reuse and multiple nests per year founding, however, this behavior is relatively uncommon. The average number of brood cells provisioned was 8.75, however, this value was significantly lower in Crete than in other regions. Sex of offspring related to brood cell position; the inner brood cells tended to be female-biased and outer brood cells male-biased. Despite that we did not find evidence for typical dwarf eldest daughter pattern of maternal investment in most studied regions.

Generally, conditions for *C. cucurbitina* reproduction seem to be better in the center of the range (Italy, Albania) than on the margins (Czech Republic, Crete). Higher number of brood cell provisioned and/or larger size of offspring was found in central regions than in marginal regions.

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Introduction

Knowledge of the natural history of species is necessary for understanding of evolution of behavioral and ecological traits (Agrawal 2017) and also effective nature conservation (Greene 2005, Able 2016). Natural history of most species is still unexplored, therefore discovering new facts about most species of some lineage is useful (Greene 2005, Trumbo 2012). As the detailed studies about natural history are time-consuming, most of them are performed only at a single locality. However, many organisms inhabit an area with a wide range of ecological conditions (Vannote and Sweeney 1980, Brown *et al.* 1996, Rasmont *et al.* 2015). Therefore, natural history can substantially differ between different parts of distributional areal (Foster 1999, Brockmann 2004, Field *et al.* 2010). Studies which explore natural history in multiple parts of a distribution range of species are useful for understanding the complexity of a species' natural histories and possible variability influenced by different ecological conditions.

Climatic conditions strongly influence the survival and fitness of organisms. Species have to adapt to different climatic and ecological conditions in different parts of their range. For example, higher temperature in warmer areas and lower latitude allows longer active season. When reproductive season is longer, multiple generations per year are possible (Brockmann 2004, Zeuss *et al.* 2017). Body mass can also variate with latitude. One mechanism is Bergmann's rule—larger body mass is correlated with lower temperature, because larger individuals can thermoregulate better. However, the converse Bergmann rule postulates that larger organisms are present in lower latitudes because longer season allows more time for development (Blanckenhorn and Demont 2004, Meiri 2011). Furthermore, offspring body mass can also be influenced by resource availability (Rosenheim *et al.* 1996, Kim 1999), which can variate with ecological conditions in various ways.

Another important trait which covariates with latitude is the tendency to social behavior (Purcell 2011, Schürch *et al.* 2016, Groom and Rehan 2018). Temperate species with large ranges commonly differ in the number of generations between northern and southern populations. Less brood cohorts occur in colder climates (northern parts of ranges in north hemisphere) than under warmer climate (Richards 2000, Davison and Field 2016). The ability to rear at least two broods in one season is a necessary condition for "non-delayed eusociality" (Sakagami and Maeta 1989). Interspecific social polymorphism depending on climate is widespread in halictid bees (Field *et al.* 2010, Purcell 2011). However, it has not been found in xylocopine bees. Apparent association between sociality and latitude has not been found in *Ceratina calcarata* (Lawson *et al.* 2018) and *C. australensis* (Dew *et al.* 2018), or any allodapine bees (Cronin and Schwarz 2001, Schwarz *et al.* 2007).

Organisms have to solve the trade-off between investing in the amount or the size of offspring (Stearns 1989, Strohm and Linsenmair 2000). This trade-off is modulated by ecological conditions. It is possible to invest more in offspring individuals if conditions are good (Rosenheim *et al.* 1996). Mass provisioning aculeate Hymenoptera are a good group for studying pattern in maternal investment. Mother provisions the brood cells, which are separated by nest partitions— thus each offspring can feed only on the food provided in its broods cell (Michener 2007). Therefore, body size of offspring is strongly determined by the amount of provision prepared by mother (Strohm and Linsenmair 2000, Bosch and Vicens 2002, Lawson *et al.* 2017). Moreover, the sex of offspring is also determined by mother's decision, depending on whether she releases sperm from spermatheca or not. Offspring will be female in the former case, male in the latter (Gerber and Klostermeyer 1970, Page *et al.* 2002). It is known that maternal investment is determined by the size of the female. A larger females produces larger offspring (Stark 1992, Molumby 1997). Resource availability also plays a role—the more resources are available, the larger the size of offspring (Rosenheim *et al.* 1996, Kim 1999, Bosch 2008).

Maternal investment can vary not only spatially, but also temporally. It can be modulated according to changes in environmental conditions, especially temporal variability of resources (Kim 1999, Paini and Bailey 2002). Moreover, the conditions of the mother change through the nesting season. Females are more worn and tired in later phases of nesting season than in the beginning (Sugiura 1994, Seidelmann 2006). In many mass provisioning aculeate Hymenoptera it is easy to determine the relative age of offspring. The eldest brood cell is the innermost in the nest and the youngest is outermost, the brood cells producing a linear sequence (Kim 1992, Michener 2007, Rehan and

Richards 2010). Therefore, brood cell position usually correlates with the size of offspring and/or sex ratio (Coville and Griswold 1984, Suguira and Maeta 1989, Stark 1992).

Ceratina bees are an interesting group due to the very diverse social and parental behavior. The ancestor of the whole genus (and related genera which belong to the subfamily Xylocopinae) was likely facultatively eusocial, and thus the obligate solitarity is a secondary state (Rehan *et al.* 2012). *Ceratina* females perform two phases of offspring provisioning—provisioning of brood cells and feeding mature offspring (Mikát *et al.* 2017). Between these phases of offspring provisioning, the mother usually sits in nest entrance and guards the immature offspring against natural enemies (Sakagami and Maeta 1977, Rehan and Richards 2010, Mikát *et al.* 2016).

Although *Ceratina* bees have extremely interesting social evolution, good data about natural history exist only for a few species, mostly from Japan, North America or Australia (Sakagami and Maeta 1977, 1987, Rehan and Richards 2010, Rehan, Richards, *et al.* 2010, Lawson *et al.* 2018), from more than 200 species described in the world (Michener 2007).

Ceratina cucurbitina is the most commonly collected *Ceratina* species in southern Europe and the whole Mediterranean region (Daly 1983, Terzo and Ortiz-Sánchez 2004, Terzo and Rasmont 2011, Özbek and Terzo 2016). *Ceratina cucurbitina* is also interesting due to its phylogenetic position. It is the only European species from the subgenus *Ceratina*, however, the rest belonging to the subgenus *Euceratina* (Terzo 1998). Phylogenetic position of the subgenus *Ceratina* is closer to the stem of the phylogenetic tree of the whole genus, and thus the subgenus *Ceratina* may be paraphyletic (Rehan, Chapman, *et al.* 2010, Rehan *et al.* 2012). Although the knowledge of natural history of this species can be important due to possible ecological importance of the species and also interpretations of social evolution of the genus, information about the natural history of the species is only partial. (Daly 1983) examined patterns of hibernation, (Maeta *et al.* 1997a, 1997b) behavior in nest and (Mikát *et al.* 2016) studied importance of nest guarding to offspring survival. However, a study examining patterns of maternal investment and possible sociality in a large dataset has not been performed to this date.

This study has two main goals: 1) assess nesting strategy and sociality of *C. cucurbitina* and 2) evaluate geographic variations in nesting strategy and sociality.

Material and Methods

Studied localities

We performed our research in four areas: Czech Republic, Italy, Albania, and the Greek island Crete (fig S1). In the Czech Republic, we performed research in Southern Moravian region in Podyjí National Park. We obtained data from 2012, 2013, 2014, 2015, 2017, and 2018. In Italy, we performed research in several localities across the middle and south Italy (Regions: Lazio, Puglia, Basilitata, Campania, Molisse, Abruzzo, fig S2). We collected data in 2013, 2014, 2015, and 2017. In Albania, we collected data in several localities across the country in 2018 (Fig S3). In Crete, we collected data in several localities in the north coast in 2018 (Fig S4).

Dissection of nests

We collected nests in evenings (after 17:30 of local time) to ensure that all individuals were inside the nest. In the Czech Republic, we collected nests from artificial nesting opportunities, which we installed in the localities before the nesting season (Mikát *et al.* 2016), in other locations we collected natural nests from broken or cut twigs next to road margins and hedges (Fig S5). We opened nests with clippers or knife and noted the nest structure. We determined the amount and sex of adult individuals, number of brood cells, number of empty cells and stage of juvenile offspring. We distinguished adults of parental and filial generation by wing wear. Wings were apparently worn in adults of parental generation. Moreover, we noted if mother was present in the nest entrance, or inside brood cells. We measured the length of the nest, the length of the nest entrance (distance between the nest entrance and first brood cell partition), width of the twig, width of pith, and width of nest.

We evaluated the nest stage. Supposed newly founded nests were those which contained a new burrow and female without any provisions. As active brood nests we considered nests which had at least one brood cell and the outermost brood cell was open and with pollen, or was closed, but contained an egg. As full brood nests were considered nests, which contained larva or pupa in the outermost brood cell and which did not contain any adult offspring, or if adult offspring was present, it did not pass though nest partitions (Fig S6). As mature-full brood nests were classified nests, which contained a mixture of adults and immature stages. Adults crawled through the nest partitions and destroyed the original nest structure. As mature brood nests were classified nests which contained only mature adults.

Phenology

We calculated the proportion of different nest stages in different periods through the nesting season. Nests, where all offspring were dead, and also those with newly excavated burrow by young adults were excluded from analysis. Moreover, we counted proportion of nests where old female was present for each period.

We analyzed phenology in the Czech Republic (Podyjí National Park) and southern Italy. For analysis of phenology in the Czech Republic, we combined data from 2012, 2013, and 2015 (in 2017 and 2018 were selectively collected some nest stages). 372 nests are included in this analysis. From analysis of phenology in southern Italy, we combined data from 2014, 2015, and 2017 from several localities. We included 569 nests in this analysis. More details about phenology analysis are presented in SI materials.

Analysis of sociality

We used active brood nests (nests where mother provisions brood cells) with at least one living female for this analysis. We determined the number, sex, and age of adults in nest entrances. We analyzed 108 nests from Czech Republic, 77 from Italy, 118 from Albania, and 29 from Crete. In total we analyzed 332 nests.

Analysis of full brood nest structure: Full brood nests which contained guarding mother were included in this analysis. We included 322 nests from Czech Republic, 147 from Italy, 97 from Albania and 31 from Crete in this analysis. Together, we analyzed 597 full brood nests. Brood cells were not counted in 10 nests which were parasitized, and the parasites disturbed the brood cell partitions (e.g.

Ichneumonidae). In Czech Republic and Italy, we compared nests from main peak of nesting (early summer, June-July) with second peak of nesting (late summer, August-September).

Offspring sex and size

We used only full brood nests for this analysis. We selected nests where most offspring individuals were in advanced developmental stage and therefore with high probability of survival till adulthood. We noted the nest code and brood cell position (1st is innermost brood cell, the highest number is outermost brood cell) for each individual. From these nests, we moved all offspring into Eppendorf 1.5 ml tubes and reared them there to adulthood. For increasing probability of survival, we inserted a piece of toilet paper in the tube which contained a larva. Piece of toilet paper prevent attaching of larva to wall of tube. We identified sex of all successfully reared offspring. Moreover, we measured the width of head of offspring and also mother under stereomicroscope with the scale in the ocular.

We measured 1,213 offspring from 169 nests from Czech Republic, 316 offspring from 42 nests from Italy, 676 offspring from 80 nets from Albania, and 99 offspring from 24 nests from Crete.

Statistics

We used software R for statistical analyses (R. Core Developmental Team 2016). Differences in the number of brood cells and the size of individuals between regions were tested by TukeyHSD tests applied to Anova models. We tested difference from the overall sex ratio of 1:1 by chi-square test. Moreover, we tested difference of sex ratio for different brood cell position by chi-square tests, as null hypothesis we considered the overall sex ratio in focal location. The influence of brood cell position to size of offspring was tested by linear mixed models. We used package “nlme” for this test, nest identity was used as random factor.

Results

Phenology in the Czech Republic: Founding of new nests begins in May. Brood cell provisioning begins in the second half of June. Active brood nests strongly dominated in this period and the first half of July (Fig. 1). First full brood nests occured in the first half of July, and full brood nests dominated in second half of July. In the first half of August dominated full-mature brood nests. From second half of August mature brood nests. Although active brood nests were rare after a half of July, and full brood nests after the end of July, both types sometimes occurred in August and September.

Phenology in Italy: In May, most of the nests were in new founded nest stage, however some brood nests also occurred. In June, full brood nests were the most common, however active brood nests and full-mature brood nests also frequently occurred. In August and September, mostly mature brood nests occurred (about 70% of nests, Fig. 2), however, all stages, including active brood nests and full brood nests, were present.

Nest orphanage: Mother was not present in 2.06% active brood nests in total (9/341). Proportion of orphaned nests in different regions was: 0.84% (1/120) in Albania, 2.53% (2/79) in Italy, 3.33% (1/31) in Crete and 2.7% (3/111) in the Czech Republic. Dead mother was observed in 0.58% (2/341) of active brood nests (one case was observed in Albania and one case in Crete). Proportion of active brood nests, where a living mother was present was 97.36% (332/341)

We found missing mothers in 6.64% (41/654) of full brood nests. Proportion of orphaned nests in different regions was: 8.49% (9/122) in Albania, 8.70% (14/166) in Italy, 8.82% (3/34) in Crete and 4.45% (15/342) in the Czech Republic. All these nests were without any plug at the end of the nest and thus the last brood cell partition had the same appearance as other brood cell partitions. Dead mother was present in 2.45% (16/654) of full brood nests. Proportion of nests with dead mother in different regions was: 5.35% (6/112) in Albania, 3.01% (5/166) in Italy, 0% in Crete and 1.46% (5/342) in the Czech Republic. In Albania and Italy, most of the dead females were parasitized by a conopid fly. Proportion of full brood nests, where a living mother was present was 91.28% (597/654).

Sociality: We found two females in 0.3% (1/332) of active brood nests. The only record of a nest with two females was from locality in Boge in Albania. Nests with two females contained only one egg. Nests were not reused and both females had worn wings. In Albania, proportion of multifemale nests was 0.85% (1/118). No social nest was found in the Czech Republic (N = 108), Italy (N = 77) and Crete (N = 29). No social nest was fond in the second cohort of active brood nests either (active brood nests collected in August and September, N = 37, 13 from the Czech Republic and 24 from Italy). We have not found any male in active brood nest.

Full brood nest structure: Brood cells are separated by fragile partitions. Female is able crawl through nest partitions – we found mothers inside brood cells in 6.36% of nests (38/597). Excrements of larvae were usually aggregated at the bottom of the nest. The outermost brood cell was always closed by a partition.

The average number of brood cells provisioned was 8.75 (N = 587). The maximal number of brood cells provisioned was 18, however, nests with more than 15 brood cells were very rare (3/586). There was a significant variance in number of brood cells between regions. In the peak of the presence of full brood nests (June and July), the highest average number of brood cells provisioned was in Albania (10.53, N = 92, Table 1), then in Italy (9.05, N = 112) and the Czech Republic (9.06, N = 302) and the lowest number of brood cell provisioned was found in Crete (5.26, N = 31). Although the number of brood cells was significantly higher in Albania than in the Czech Republic (TukeyHSD test, $p < 0.0001$) and Italy (TukeyHSD test, $p = 0.0001$), the overall pattern was similar in these three sites (Fig. 3). On the other hand, there was a substantially lower number of brood cells provisioned in Crete. In Crete, the maximal number of brood cells in full brood nest was very low (7), however, we found some larger active brood nests with up to 9 brood cells. There was a commonly observed pattern with substantially different offspring age in neighboring brood cells in Crete.

We compared a number of brood cells between nests in the peak time of the season (June and July) and late full brood nests (August and September). Average number of brood cells provisioned was low in late phase of nesting season in the Czech Republic (6.42, N = 19) and also in Italy (4.40, N = 31). The difference between early and late summer nests was statistically significant for the Czech Republic (linear model, F = 20.36 $p < 0.0001$, fig. 4) and also for Italy (linear model, F = 91.40, $p < 0.0001$, fig. 4).

Empty cells were very rare, they did not occur in most of the nests. Average number of empty cells per nest was 0.068 (N = 581).

Average length of early summer nest was 13.60 cm (N = 581, range 3.7–28.0 cm). Average length of nests differs between regions. The longest nests were in Albania (15.7, N = 88, Table 1), middle-length nests in the Czech Republic (13.6, N = 302) and Italy (13.4, N = 111) and the shortest were in Crete (11.1, N = 31). Late summer nests were shorter than early summer nests. Nests late in the summer had an average length of 11.7 (N = 30) in the Czech Republic and 11.3 (N = 19) in Italy. Differences in the nest length between early and late summer nests were statistically significant in the Czech Republic (linear model, F = 6.35 p = 0.0122) and in Italy (linear model, F = 5.68, p = 0.0185).

Size of offspring and mother: Mother's head width was 1.991 mm (N = 315) on average. Largest mothers occur in Italy with 2.063 mm (N = 42) head width on average, next are the mothers of Albania (2.004 mm, N = 80), and the smallest were in the Czech Republic (1.971, N = 169) and Crete (1.968, N = 24). There was a significant variance between regions (Anova, F = 8.71, $p < 0.0001$, Table 2, Fig. 5). TukeyHSD post-hoc pair comparisons showed a significantly bigger size of mothers in Italy in comparison to other three regions (Italy-Albania p = 0.0250, Italy-Crete p = 0.0041 and Italy – Czech Republic $p < 0.0001$). Difference between Albania, Czech Republic and Crete was insignificant.

Average head width of a female offspring was 2.014 mm (N = 1,141). The largest were female offspring in Italy (2.073 mm, N = 158), then in Albania (2.068 mm, N = 317), the Czech Republic (1.979 mm, N = 622) and the smallest were in Crete (1.890, N = 55). There was a significant variance between regions (Anova, F = 88.75, $p < 0.0001$). TukeyHSD post-hoc comparisons showed that there was a significant variance between almost all pairs of regions. Only difference between Italy and Albania was insignificant.

The average head width of a male offspring was 1.862 mm (N = 1163). The largest male offspring were in Albania (1.911mm, N = 359), then in Italy (1.876 mm, N = 158), and the smallest were in the Czech Republic (1.832 mm, N = 591) and Crete (1.813 mm, N = 55). The difference between regions was significant (Anova, F = 40.15, $p < 0.0001$). TukeyHSD post-hoc comparisons showed that there was a significant variance between almost all of regions. Only difference between Crete and Czech Republic was insignificant.

Sex ratio: Sex ratio of offspring was not significantly different from 1:1 in all studied regions: Albania (317 females, 359 males, Chi square test, Chi= 2.61, p = 0.1062), Crete (44 females, 55 males, Chi square test, Chi= 1.22, p = 0.2689), Czech Republic (622 females, 591 males, Chi square test, Chi= 0.79, p = 0.3734) and Italy (158 females, 158 males, Chi square test, Chi = 0, p = 1).

Brood cell position and sex and size of offspring

Sex ratio variates with a brood cell position in the nest. Generally, lower brood cell positions (especially the first brood cell position) tended to be female-biased and upper brood cell positions tended to be male-biased. Significantly female-biased brood cell positions were these: 3rd in Albania (Chi square test, Chi = 6.10, p = 0.0136, N = 66), 1st in Crete (Chi square test, Chi = 5.36, p = 0.0206, N = 23) and 1st in the Czech Republic (Chi square test, Chi = 6.25, p = 0.0124, N = 121). Male biased was the 9th brood cell position in Albania (Chi square test, Chi= 5.64, p = 0.0175, N = 44), the 11th in Albania (Chi square test, Chi = 9.39, p = 0.0021, N = 30), the 12th in Albania (Chi square test, Chi = 15.39, $p < 0.0001$, N = 24), the 9th in the Czech Republic (Chi square test, Chi = 6.06, p = 0.0139, N = 82), the 10th in the Czech Republic (Chi square test, Chi = 9.50, p = 0.0021, N = 50), 11th in the Czech Republic (Chi square test, Chi = 8.34, p = 0.0039) and the 10th in Italy (Chi square test, Chi = 4.03, p

= 0.0448). Other brood cell positions had the sex ratio not significantly different from the ratio in the whole population (Table S1).

Generally, we found a negative relationship between the offspring size and a brood cell position with the smallest being the uppermost individuals. In female offspring, size significantly decreased with a brood cell position in Albania (Linear mixed model, F = 12.16, $p < 0.0001$, table S2) and Crete (Linear mixed model, F = 9.07, p = 0.006), but a significant relationship was not found in the Czech Republic (Linear mixed model, F = 0.15, p = 0.6968) and surprisingly positive relationship was documented in Italy (Linear mixed model, F = 5.72, p = 0.0184). In males, offspring size significantly decreased with brood cell position in Albania (Linear mixed model, F = 38.93, $p <0001$), the Czech Republic (Linear mixed model, F = 4.02 p = 0.0455) and Italy (Linear mixed model, F = 6.91, p = 0.0097) and this relationship was insignificant in Crete (Linear mixed model, F = 2.38, p = 0.1327). Although offspring were generally smaller in inner than outer brood cells, the largest offspring were usually not in the first brood cell but between the 2nd and the 5th brood cell position (Table S1)

Size of a female offspring in the first brood cell was significantly smaller than other females in a nest in the Czech Republic (paired t-test, t = 3.20, N = 75, p = 0.0020), Italy (paired t-test, t = 5.56, N = 22, $p < 0.0001$), but not in Albania (paired t-test, t = 0.42, N = 34, p = 0.6763). In Crete, females in first brood cell were larger than average females in other brood cell positions in the nest (paired t-test, t = -3.12, N = 14, p = 0.0082).

Discussion

Sociality

Genus *Ceratina* is primarily a eusocial lineage, therefore strict solitarity has to be the secondary state (Rehan *et al.* 2012). However, all known social species so far are social only facultatively – they are able to found both solitary and social nests (Sakagami and Maeta 1984, Rehan *et al.* 2009, 2015, Rehan, Richards, *et al.* 2010). In some species, proportion of social nests is low e.g. less than 1% in *C. flavipes* and social nests occur only in some regions (Sakagami and Maeta 1987). It is a difficult to disprove the ability of founding social nests in some species. Large datasets are necessary for evaluation of the sociality status. We analyzed a large number of active brood nests (N= 322) from multiple parts of the region and found only one multifemale nest in Albania. We suppose that this nest was not a social nest, but only temporily visited by a second female in the nest owned by the first female. That nest was not reused, on the contrary with most multifemale *Ceratina* nest known from other species (Sakagami and Maeta 1984, 1987, Rehan, Richards, *et al.* 2010). As we supposed that one multifemale nest is rather an error than a facultative strategy, we consider *C. cucurbitina* a solitary species.

The essential trait for eusociality to unfold is an ability of a single female to subsequently provision multiple clutches. (Wilson 1971, Schwarz *et al.* 2007, Rehan, Richards, *et al.* 2010). We suppose that females of *C. cucurbitina* are able to found multiple nests per year, because we found active brood nests and full brood nests in the late phases of nesting season. However, the prevalence of these nests was low. We suppose that most of the females die while they provision mature offspring of their first nests. All active brood nests from August and September were solitary, therefore we suppose that founding multiple nests is not connected to sociality in case of *C. cucurbitina*.

Ceratina bees are more often social in tropical than in temperate regions (Groom and Rehan 2018). However, the influence of climate on sociality was documented only in interspecific level (Groom and Rehan 2018). Influence of climate to within-species variability in sociality was not documented in two well-explored species: *C. calcarata* (Lawson *et al.* 2018) and *C. australensis* (Dew *et al.* 2018). We also confirm that no social polymorphism is associated with climate in *C. cucurbitina*. We suppose, that *Ceratina* bees have different response to climate than halictid bees, for which iwithin-species sociality is modulated by climate and is well documented (Field *et al.* 2010, Purcell 2011).

We have not found any male in active brood nests and males die early in the nesting season (Mikát, Janošík, *et al.* 2019). Therefore, we exclude the possibility of male participation on care in this species as it was discovered recently for *C. nigrolabiata* (Mikát, Janošík, *et al.* 2019).

Guarding of full brood nests in *C. cucurbitina*

Ceratina cucurbitina always tends to guard her offspring until adulthood and female frequently crawls though nest the partitions and inspects brood cells (Fig S7). We suppose that studied nests where we have not found a mother were accidentally orphaned, not voluntary abandoned. We did not observe any evidence of an active closing of the nest in a nest where the mother was missing, as it is known in *C. chalybea* (Mikát *et al.* 2016) and *C. chalcites* (Mikát, Benda, *et al.* 2019). Continuous nest guarding is an effective protection against natural enemies (Mikát *et al.* 2016). The nest guarding and the ability of brood cell inspection is a usual strategy in genus *Ceratina* (Rehan and Richards 2010, Mikát *et al.* 2016), however, it has not been found in species which belong to the most common European subgenus *Euceratina* (Mikát *et al.* 2016, Mikát, Benda, *et al.* 2019). *Ceratina cucurbitina* as a member of subgenus *Ceratina* has a typical strategy for the genus.

Maternal investment and phenomenon of dwarf eldest daughter

A phenomenon of a dwarf eldest daughter has been postulated for well-explored North-American species *C. calcarata.* First brood cell should usually contain a female that is smaller than other females produced in the nest (Rehan and Richards 2010, Lawson *et al.* 2016, 2018, Mikát *et al.* 2017). A function of this female is to feed younger siblings with pollen and nectar, especially in the case of mother's death (Lewis and Richards 2017, Mikát *et al.* 2017). Moreover, a sister which can feed young siblings has also been detected in the East-Asian species belonging to subgenus *Ceratinidia* (Sakagami and Maeta 1977). Therefore, it is possible to hypothesize that a dwarf eldest daughter phenomenon is widespread or universal across the whole genus.

However, pattern of a first brood cell in *C. cucurbitina* was female biased only in the Czech Republic and Crete and it was relatively slightly biased (74% of females in Crete, 64% in the Czech Republic). We confirmed that a female in the first brood cell position is significantly smaller than other average females in the Czech Republic and this works also for Italy, but not for Albania. Moreover, female in the first brood cell was larger than the average females in Crete. Therefore, the pattern of maternal investment typical for a dwarf eldest daughter was found in the Czech Republic, but not in other regions. However, behavioral observations of *C. cucurbitina* performed in the Czech Republic do not support presence of a dwarf eldest daughter (Mikát et al, unpublished data).

Generally, inner brood cells were often female biased and outer brood cells male biased. Size of offspring of both sexes usually decreased with brood cell position, but the largest offspring were not present in the first brood cells, but usually between the second and fifth brood cells. We suppose that observed pattern in maternal investment is rather a result of optimization of maternal investment according to local conditions and not of the female malnourishment in order to enforce her to take up an altruistic role. We think that other mechanisms, such as decreasing foraging effectivity connected with a risk of brood cell parasitism (Seidelmann 2006, Peterson and Roitberg 2015) and a different abundance of food resources through the nesting season (Alcock *et al.* 2005) should shape the pattern in maternal investment in *C. cucurbitina*. Therefore, we claim that pattern in investment resembling the dwarf eldest daughter phenomenon can be generated also by other factors than directed malnourishment and the finding of this pattern is not a sufficient evidence for the presence of a foraging dwarf eldest daughter. As a pattern of maternal investment should be influenced by multiple factors at the same time, future research is necessary to determine the key factors influencing a maternal investment in this species.

Differences in maternal investment between regions

Number of brood cells provisioned was substantially smaller in Crete in comparison to other studied regions and significantly higher in Albania than in Italy and the Czech Republic. However, the difference between Albania vs Italy and Czech Republic in absolute numbers was relatively small. We suppose that the number of brood cells provisioned is influenced by precipitation that is connected to the abundance of floral resources. In Crete, there is the driest climate among the studied localities and therefore floral resources are scarcer than in other regions. In Crete, we repeatedly observed offspring in strongly different developmental stages in neighboring brood cells in a nest. We suppose that female interrupted brood cell provisioning in days when food resources were rare.

We found a significant variance among regions in the head width of mothers, female offspring and also male offspring. Although the pattern within these three groups was different, generally the bees in Italy and Albania were larger than bees in the Czech Republic and Crete. The smallest bees occur in Crete. Observed pattern fits neither Bergman rule nor Converse Bergmann rule (Blanckenhorn and Demont 2004, Shelomi 2012) as the largest bees are in the middle latitudes and the smallest in extreme latitudes. The largest size in the center of the areal was documented in several organisms – (Virgós *et al.* 2011, DeGregorio *et al.* 2018). Larger individual size in the center of the areal makes sense, because there should be better ecological conditions in the center than on the margin of areal (Pironon *et al.* 2017). However, the larger individuals are in the center of the areal detected only in some studies, no pattern or an opposite pattern was also detected (Pironon *et al.* 2017). We suggest, that the largest bees are in Italy and Albania, because there is the most comfortable environment for this species. Albania and Italy are in the center of *C. cucurbitina* areal, the Czech Republic is in the northern border and Crete is in the Southern border (Terzo and Rasmont 2011). Therefore, we can suppose that in the center of areal should be better conditions than on the northern and southern margins. Resource availability is possibly the most important factor influencing size in Aculeate Hymenoptera. When resources are abundant, mother produces larger offspring than in case of scarce resources (Kim 1999, Peterson and Roitberg 2006, Bosch 2008). .

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Disclosure statement

The authors declare no conflict of interests.

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Tables

Table 1: Characteristics of full brood nests of *C. cucurbitina*. Nests from June and July are from main peak of full brood nests, nests from August and September are probably nests from second nesting.

Country	Albania	Crete	Italy		Czech Republic	
Month	VI+VII	VI+VII	VI+VII	VIII+IX	VI+VII	VIII+IX
Nest analyzed	92	31	112	31	302	19

Average number of brood cells	10.61	5.26	9.05	4.40	9.06	6.42
Range of brood cell number	1-16	3-7	3-18	1-10	1-15	2-12
Standard deviation of brood cell number	2.68	1.19	2.42	2.09	2.46	2.71
Average number of empty cells	0.04	0.00	0.01	0.03	0.11	0.11
Average length of nest	15.7	11.1	13.4	11.3	13.6	11.7
Range of length of nest	6.5-28	6.8-19.9	6.2-26.1	4.8-19.8	3.7-26.3	6.2-19.2
Standard deviation of length of nest	3.9	3.1	4.5	3.9	3.3	3.4
Proportion of mothers inspecting brood cells	0.0795	0.0323	0.0541	0.0333	0.0795	0.0000

Table 2: Characteristics of mother and offspring in full brood nests of *C. cucurbitina*. Number of nests examined is the same as the number of measured mothers. Only nests from early summer (June and July) peak of full brood nests are included.

	Albania	**Crete**	**Czech Rep.**	**Italy**	**Together**
N of measured mothers	80	24	169	42	315
average head width (mm)	2.004	1.968	1.971	2.063	1.991
N of measured female offspring	317	44	622	158	1141
average head width (mm)	2.068	1.890	1.979	2.073	2.014
N of measured male offspring	359	55	591	158	1163
average head width (mm)	1.911	1.813	1.832	1.876	1.862
proportion of males	0.5311	0.5556	0.4872	0.5000	0.5048

Figures

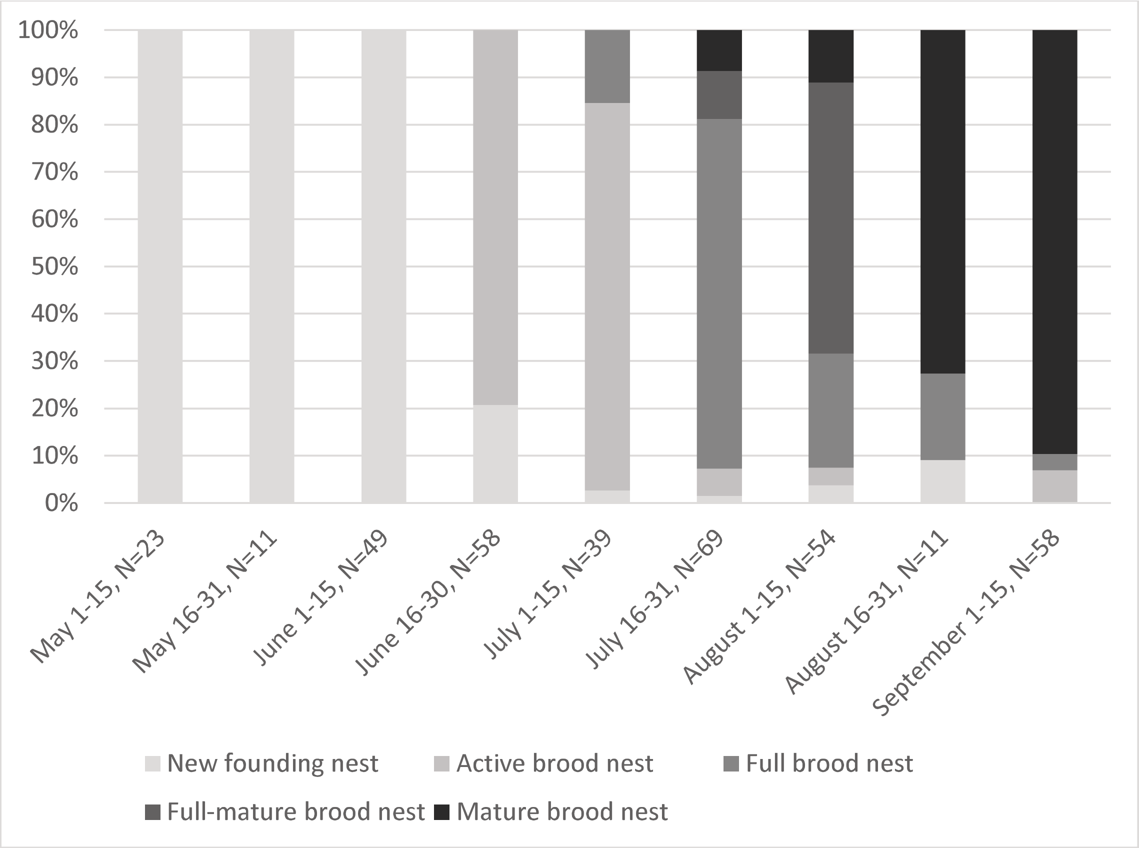

Fig 1: Proportion of different nest stages through the nesting season in the Czech Republic.

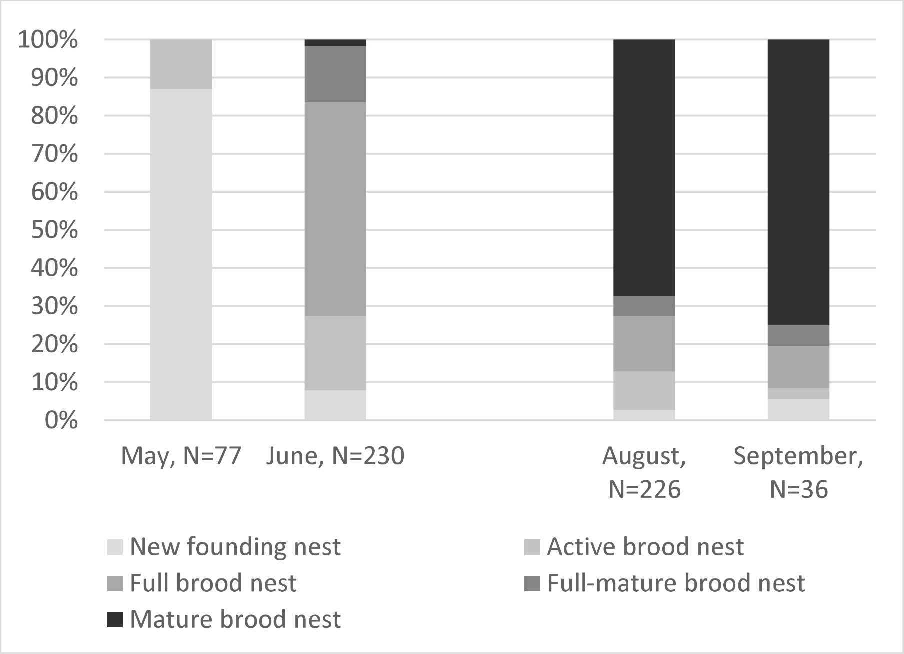

Fig 2: Proportion of different nest stages through the nesting season in Italy.

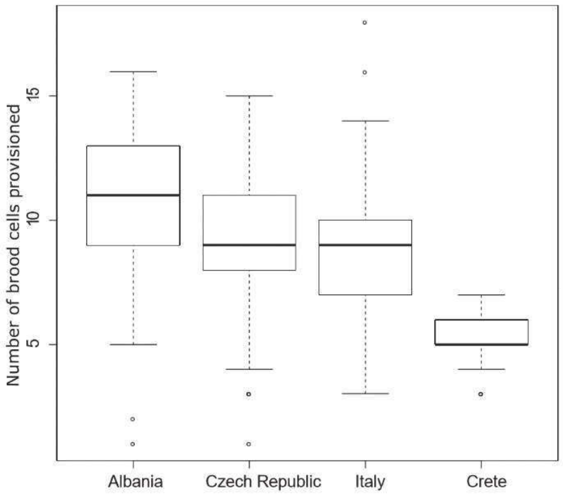

Fig. 3: Number of brood cells provisioned in full brood nests in early summer (June and July) season in different regions

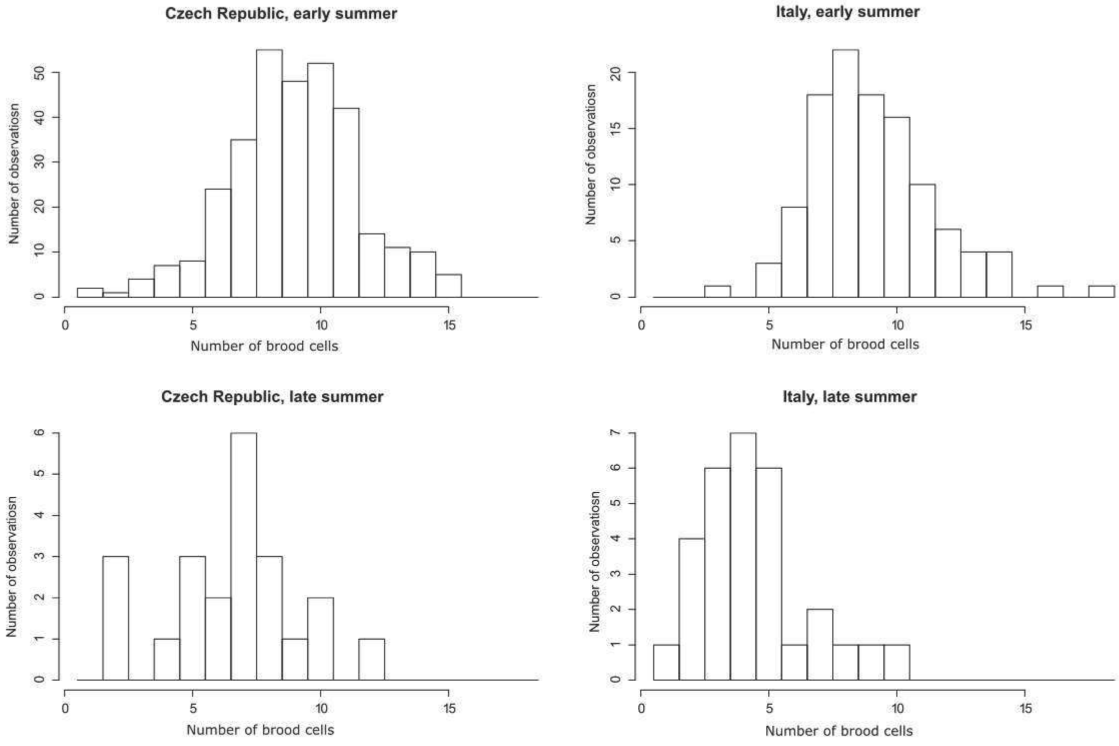

Fig 4: Number of brood cells provisioned in full brood nests – comparison between early summer (June and July) and late summer (August and September) nesting season

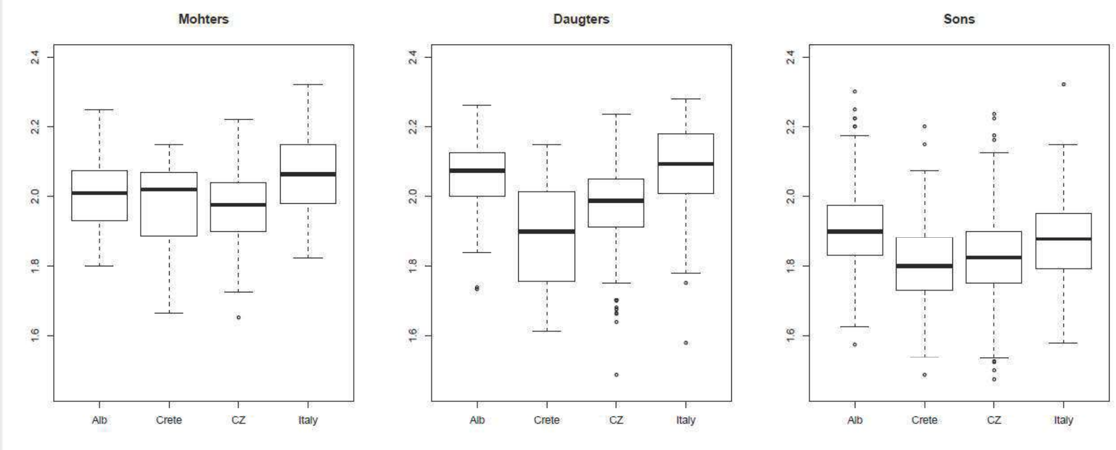

Fig. 5: Head width of mothers, offspring of female sex and offspring of male sex in different regions.

Supplementary materials

Supplementary methods

Sources of dissected nests in the Czech Republic: We randomly selected *C. cucurbitina* nests for dissections in years 2012, 2013 and 2015. Moreover, in 2013 and 2014, we performed the experiment to test the influence of mother removal on nest survival (Mikát *et al.* 2016). Here, we used nests where mother was not removed and served as control for several analyses. In 2017 and 2018, we preferentially collected full brood nests, because nests in this phase are most informative for the assessment of species strategy (e.g. pattern in maternal investment). We checked the presence of the mother in nest using a flashlight in the field. Subsequently, we pushed mother by a straw, and collected those nests where the mother did not crawl further down to the nest (when mother did not crawl further down the nest is probably in a full brood nest stage). This method allows to predominately collect nests in a full brood nest stage, but sometimes also nests from other phases were collected.

Most of the analyzed nests come from artificial nesting opportunities. Most of these artificial nesting opportunities were stems of *Solidago* spp. *C. cucurbitina* commonly uses not only sheaves installed in focal year, but also stems from previous year. However, in 2012 and 2013 we did not obtain enough nests from these artificial nesting opportunities, therefore, we used nests from natural nesting opportunities. These nests were collected in circle which radius spread around 20 km from main locality.

Sources of dissected nests in Italy, Albiania and Crete: We collected nests from natural and seminatural nesting opportunities. Most collected nests originated from twigs cut by human on the edge of roads, dirt roads, or pasture edges. Some nests were collected from twigs broken by natural processes. Nests were present in dead twigs with soft pith in all cases. The most common nesting substrate were twigs of *Rubus* sp. in all three areas. *Phoeniceum vulgare* was the second most important substrate in Italy and Crete. *Sambucus ebulus* was an important substrate in one locality in

Albania. The minority of nests collected were in other substrates, e.g. several dead stems of Apiaceae and Asteraceae.

Localities differed in abundance of proper nesting substrate. In several localities (e.g. Santa Marinella, Pescariello and Cassino in Italy, Skadar in Albania) *C. cucurbitina* nests were very common and research was more limited by nest processing than nest collecting. However, in some other localities nests were less abundant and the number of dissected nests was limited by their availability.

Sources of nests for analyses:

Phenology: We performed this analysis only for the Czech Republic and Italy, because in other areas we did not select samples throughout the whole season. We combined data from several years because we obtained more complete coverage of season with this combination. We are aware that each season is different; however, our main goal was primarily to find the main pattern in phenology (e.g. presence of bivoltinism).

We used only the nests which were randomly sampled to this analysis. Therefore, we used data from 2012, 2013 and 2015 from the Czech Republic and we excluded nests used as control in removing experiment (Mikát *et al.* 2016) from this analysis. We did not use nests from 2017 and 2018, because we preferentially dissected full brood nests. We used data from all collected nests from Italy for this analysis.

Presence of sociality: We used all collected active brood nests in this analysis.

Full brood nest structure: We used full brood nests from all areas and years in this analysis. We excluded nests where mother died or disappeared, because death of mother can stop nest provisioning prematurely and therefore these nests are not suitable for the assessment of natural history of species. Moreover, offspring die in almost all orphaned nests (Mikát *et al.* 2016). We also excluded nests where parasite was present and destroyed more brood cells because it was usually impossible to determine number of brood cells, which was destroyed.

Offspring sex and size: In the Czech Republic, we used nests from 2017 and 2018 only for this analysis. In Italy, we used nests from 2017 which were collected in locality Pescariello, city Altamura only to this analysis (around coordinates 40°54'12.1"N 16°38'10.4"E). In Albania and Crete we used full brood nests across all sampled localities.

Supplementary tables

Table: S1: Sex ratio in different brood cells in different regions

Albania

Brood cell position	N of females	N of males	proportion of females	Chi square test
1	37	37	0.5	Chi = 0.15, p = 0.6998
2	41	29	0.586	Chi = 3.01, p = 0.0826
3	42	24	0.636	Chi = 6.10, p = 0.0136
4	39	29	0.574	Chi = 2.31, p = 0.1289

5	30	37	0.448	Chi = 0.04, p = 0.8391
6	39	26	0.6	Chi = 3.57, p = 0.0587
7	27	33	0.45	Chi = 0.02, p = 0.8833
8	24	25	0.49	Chi = 0.02, p = 0.8932
9	12	32	0.273	Chi = 5.64, p = 0.0175
10	15	23	0.395	Chi = 0.53, p = 0.4684
11	5	25	0.167	Chi = 9.39, p = 0.0022
12	1	23	0.042	Chi = 15.39, p = 8.7e-05
13	3	12	0.2	Chi = 3.26, p = 0.0712
14	2	2	0.5	
15		1	0	
16		1	0	
Total	317	359		Chi = 2.61, p = 0.1062

Crete

Brood cell position	N of females	N of males	proportion of females	Chi square test
1	17	6	0.739	Chi = 5.36, p = 0.0206
2	11	9	0.55	Chi = 0.38, p = 0.5367
3	5	14	0.263	Chi = 1.48, p = 0.2245
4	5	9	0.357	Chi = 0.11, p = 0.7423
5	5	9	0.357	Chi = 0.11, p = 0.7423
6	1	5	0.167	
7		3	0	
Total	44	55		Chi = 1.22, p = 0.2689

Czech Republic

Brood cell position	N of females	N of males	proportion of females	Chi square test
1	77	44	0.636	Chi = 6.25, p = 0.0124
2	76	55	0.58	Chi = 1.89, p = 0.1694
3	79	57	0.581	Chi = 2.01, p = 0.1565
4	75	59	0.56	Chi = 0.88, p = 0.3470
5	72	69	0.511	Chi = 0.00, p = 1.0000
6	72	58	0.554	Chi = 0.67, p = 0.4248
7	59	59	0.5	Chi = 0.03, p = 0.8661
8	46	61	0.43	Chi = 2.38, p = 0.1229
9	30	52	0.366	Chi = 6.06, p = 0.0138
10	14	36	0.28	Chi = 9.50, p = 0.0021
11	8	25	0.242	Chi = 8.34, p = 0.0039
12	7	8	0.467	Chi = 0.01, p = 0.9241
13	5	5	0.5	
14	2	2	0.5	
15		1	0	
Total	622	591		Chi = 0.79, p = 0.3734

Italy

Brood cell position	N of females	N of males	proportion of females	Chi square test
1	23	18	0.561	Chi = 0.32, p = 0.5695
2	23	14	0.622	Chi = 1.50, p = 0.22
3	23	12	0.657	Chi = 2.52, p = 0.1125
4	21	17	0.553	Chi = 0.19, p = 0.6589
5	17	21	0.447	Chi = 0.19, p = 0.6589
6	16	19	0.457	Chi = 0.09, p = 0.7619
7	13	18	0.419	Chi = 0.45, p = 0.5036
8	12	8	0.6	Chi = 0.41, p = 0.5242
9	6	8	0.429	Chi = 0.29, p = 0.593
10	3	12	0.2	Chi = 4.03, p = 0.0448
11	1	6	0.143	
12		3	0	
13		2	0	
Total	158	158		Chi = 0, p = 1

Table S2: Average head widths (mm) of offspring in relationship with brood cell position in different regions. Sample sizes are in table S1.

	Females				Males			
	Albania	Crete	Czech Republic	Italy	Albania	Crete	Czech Republic	Italy
1	2.054	1.914	1.951	2.034	1.886	1.850	1.795	1.871
2	2.076	1.849	1.976	2.068	1.927	1.881	1.845	1.911
3	2.088	1.960	1.986	2.051	1.967	1.759	1.857	1.891
4	2.072	1.818	1.999	2.111	1.961	1.856	1.842	1.877
5	2.069	1.915	1.978	2.058	1.961	1.795	1.850	1.904
6	2.083	1.825	1.965	2.091	1.932	1.800	1.839	1.869
7	2.065		1.993	2.067	1.917	1.742	1.816	1.892
8	2.065		2.002	2.087	1.906		1.837	1.852
9	2.074		2.011	2.134	1.914		1.823	1.874
10	2.008		1.978	2.203	1.882		1.826	1.819
11	2.040		1.908	1.965	1.874		1.812	1.850
12	2.250		1.950		1.820		1.766	1.817
13	1.937		1.875		1.846		1.798	1.836
14	2.017		2.013		1.874		1.688	
15					1.825		1.775	
16					1.785			

Fig S. 1: Map of regions, where nests were sampled

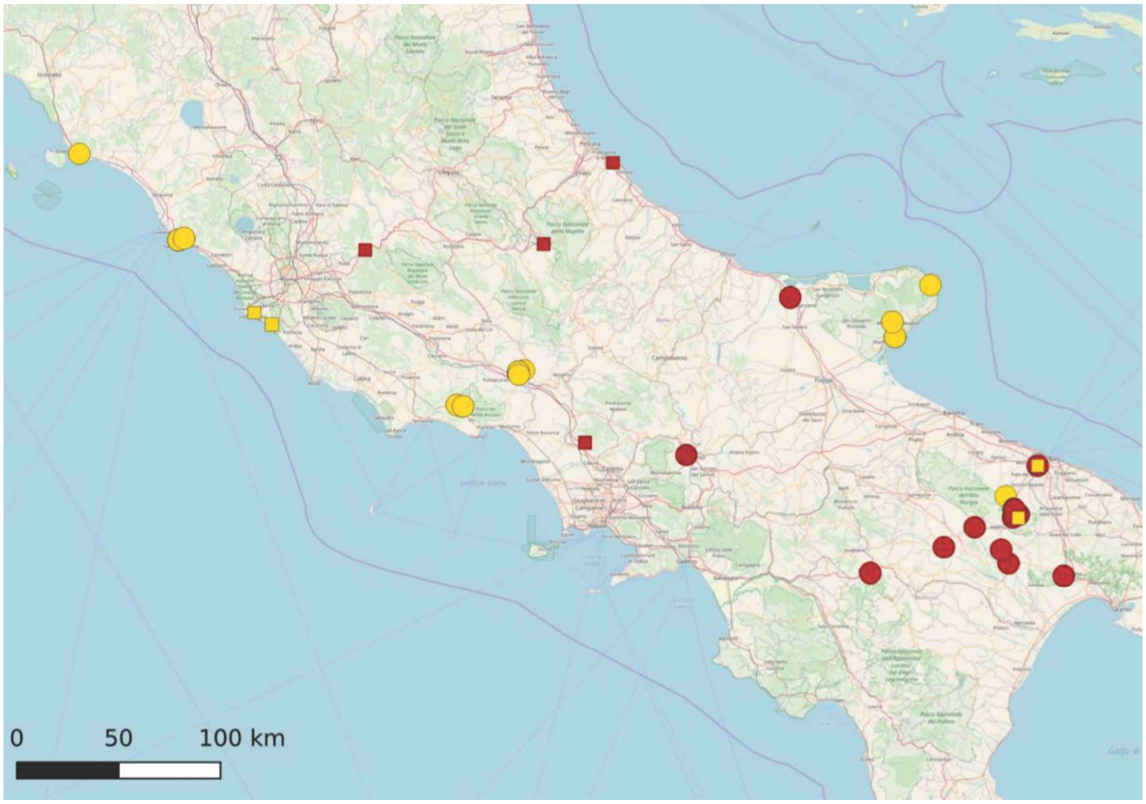

Fig S. 2: Map of locations in Italy, where nests were sampled. Red circles – locations where active or full brood nests were sampled in early summer. Yellow squares – locations where full brood nests were sampled in late summer. Red squares – locations sampled in early summer, where only new founding nests were sampled. Yellow squares – locations sampled in late summer, where only full-mature and mature brood nests were sampled. Nests from locations marked by circled were included in analysis of sociality or full brood nest structure, nest from all locations were used in phenology analysis.

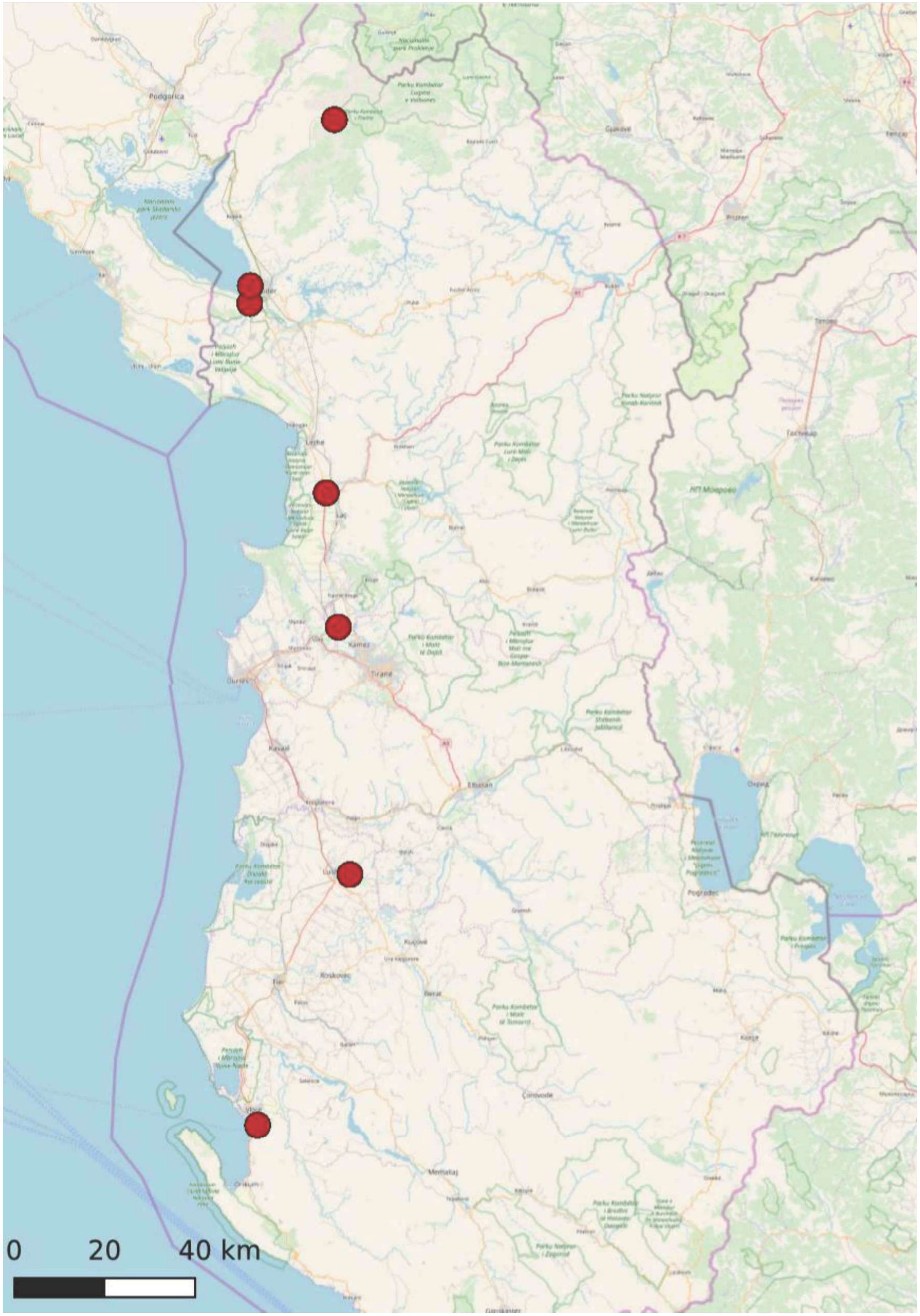

Fig. S. 3: Locations in Albania, where nests were sampled

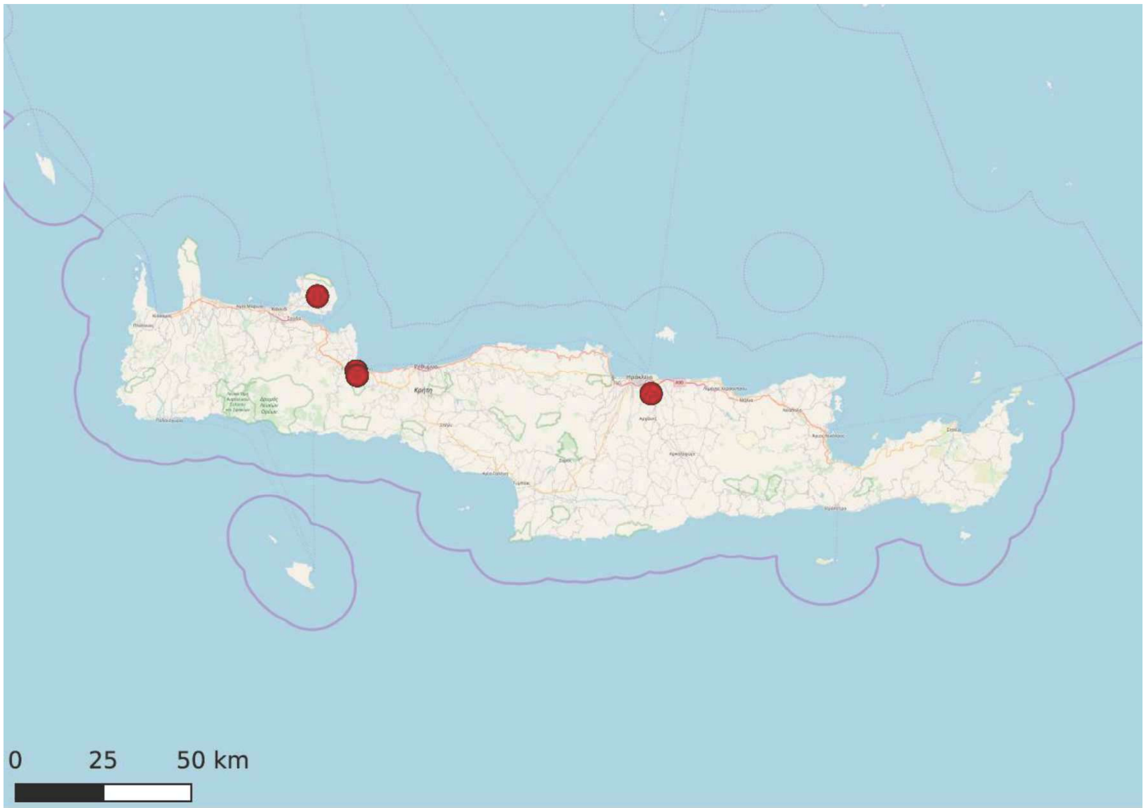

Fig S.4: Locations in Crete, where nests were sampled

Fig. S. 5: Typical habitat for *C. cucurbitina* – *Blackberry* (*Rubus* sp.) shrub next to path, twigs are regularly cut or broke by human activity (Lushnje, Albania).

Fig S. 6: Full brood nest of *C. cucurbitina*. Partitions between brood cells are only partially preserved, because mother crawled through partitions. Excrements are aggregated at the bottom of nest. Empty cells are not present.

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Nesting of *Ceratina nigrolabiata*, a biparental bee

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Abstract

Biparental care is very rare in insects, and it was well-documented in only one bee species to this date--*Ceratina nigrolabiata*. Here, we describe the nesting cycle of *C. nigrolabiata*. Pairs of *C. nigrolabiata* are established before female starts offspring provisioning. After provisioning is finished, the male abandons the nest, and so does the female in most cases. Males present in completely provisioned nests are probably mainly temporary visitors. The female can perform long-time offspring guarding, but only 22% of completely provisioned nests are guarded by a female. Most nests (54%) are closed and abandoned, when provisioning is completed, and other (24%) are orphaned before provisioning is finished. Guarded nests have statistically higher number of brood cells provisioned than unguarded nests. Mortality of offspring is caused mainly by interspecific usurpation or parasitism by Ichneumonidae or *Gasteruption*.

Introduction

Parental care is a very effective way of increasing offspring survival [1–4]. However, it has also significant costs, because care is time-consuming and causes considerable damage to the parent [1,5]. Moreover, there is strong trade-off between investing in care or in future reproduction [1,6]. Therefore, mating strategies substantially interact with patterns in parental care [7–9].

Parental care is highly diverse, organisms differ in the length of performed care, in the amount of care invested, and in the form of performed care [3,10]. Moreover, care can by performed by only male, only female or both parents [11,12]. Even in the case of biparental care, the amount and form of care invested usually differs between sexes [13,14].

In arthropods which care about offspring, maternal care is the most common [3,11,15,16]. Paternal or biparental care is generally rare in Arthropods. The well-studied examples of biparental care in insects are cockroaches of the genus *Cryptocercus*, and burying, passalid, and bark beetles [8,10]. In other arthropods, biparental care is known from a few species of isopods, amphipods and semi-terrestrial crayfish [15], among them, biparental care is best documented in woodlice of the genus *Hemilepistus* [17].

For acuelate Hymenoptera intensive parental care is typical and usually consist of nest-making and provisioning of offspring by food [3,18]. Maternal care is the most common, however eusociality also evolved several times and is widespread in some lineages [18–20]. Male participation in parental care is very rare in Aculeata. Males can perform little care in several eusocial societies, such as bumblebees or polistinae wasps, however, their role is usually small [21–23]. Males have a significant role in several species of crabronid wasps of the genus *Trypoxylon,* where biparental care is documented [24,25].

Ceratina nigrolabiata has very unusual patterns of care. This species is the only known biparental bee [26]. The female performs brood provisioning, and the male performs nest guarding [26]. Although the female has a invests more in care, the male's presence increases nesting productivity [26]. Generally, all small carpenter bees of the genus *Ceratina* nest in broken twigs with soft pith [18,27]. In the beginning of season, a female excavates a burrow [28]. Later, she provisions brood cells; brood cells are linearly arranged, innermost brood cell contains the oldest offspring and the outermost contains youngest offspring [27,28]. After finishing provisioning, the mother usually guards her offspring until adulthood [28,29]. When offspring matures, mother feeds them pollen and nectar [30]. *Ceratina* species of the same subgenus as *C. nigrolabiata* (*C. chalybea* and *C. chalcites*) perform facultative nest guarding [29,31]. A female can guard her nest until adulthood and feed her offspring or the close nest by a filling plug and abandon it [29,31].

In our previous paper [26] we reported the presence of biparental care in *C. nigrolabiata* and evaluated benefits of males and females through the phases of provisioning of brood cells. Here we describe the natural history of *C. nigrolabiata* through the whole nesting cycle. We evaluate patterns of contribution of parents in different phases of the nesting cycle. Moreover, we compare maternal investment between different strategies--guarded and abandoned nests.

Results

Phenology

Ceratina nigrolabiata excavate new nests mainly in May and June, however, some newly excavated nests were also recorded later in the season (Fig. 1). Active brood nests occurred from half of June and appeared in high proportion through whole July. First full brood nests first occurred at the end of June, but the main peak of full brood nests was in July. Full brood nests were also frequent in August. Full-mature and mature brood nests occurred from end of July, and they were very frequent through August. Other types of nests occurred mainly in the beginning and at the end of season. In the beginning of season occurred mainly old hibernacula or adults *C. nigrolabiata* visiting nests of other *Ceratina*. In the late phases of season occurred abandoned nests with only parasites and newly excavated burrows for hibernation.

Type of nest founding

We found two types of newly founded nests. Newly excavated nests, which were built by excavating pith from a twig. Discarded nests are the other type. These nests were built from previous nest of *Ceratina* (probably other *C. nigrolabiata* in most cases) by discarding a part of or all original offspring (Fig. S1, Fig. S2). Pollen provisions of the previous nest owner were usually moved to the sides of nest (Fig. S1). From newly founded nests, 82.69% (86/104) were newly excavated and 17.30% (18/104) were discarded nests. When we counted only nests founded after half of June, the proportion of discarded nests was 22.78% (18/79). From active brood nests, 4.66% (29/622) had apparent relics of usurpation and discarding.

Presence of parents

Newly excavated nests: In newly founded nests, only male was present in 53.48% of nests (46/86, Table 1), only female was present in 10.46% of nests (9/86) and male and female together were present in 36.04% (31/86) nests. Newly founded nests were on average 5.47 cm long (SD=4.68, range 1-22.1, N=86). Nests with only male were on average 3.82 cm long (SD=3.26, range 1.2-16.7, N=46), nests with only female were on average 5.73 cm long (SD=4.72, range 1-14.1, N=9), nests with both male and female were on average 7.85 cm long (SD=5.49, range 1.9-22.1, N=31). Nests with both parents were significantly longer than nests with only a male (Tukey HSD test on logarithmic data, difference=0.6743, p=0.0003), but not significantly longer than nests with only a female (Tukey HSD test on logarithmic data, difference 0.4427 p=0.2256).

Discarded nests: We observed nests of *C. nigrolabiata*, where nest partitions were destroyed and pollen from brood cells was placed on side of the nest. We suppose that original offspring were discarded out the nest (and on several occasions, we observed discarding of offspring out of the nest). In 72.22% (13/18) of discarded nests one male and one female were present. Female and two males were present in two nests, only a male was present in one nest, only a female was present in one nest and no adult was found in one nest.

Active brood nests: We found male-female pair in 84.72% of nests (527/622), female and two males were found in 1.29% of nests (8/622), female and three males were found in 0.16% (1/622) of nests, no adult was present in 1.76% (11/622) of nests, only male was in 5.6% (35/622) and only female in 6.43% (40/622) of nests.

Full brood nests: Most of full brood nests (73.51%, 493/672) were not guarded by any parent (Table 1). When a full brood nest was guarded, then usually by a female (15.18%, 102/672). Only male was present in 4.31% (29/672) and male and female were present in 7.14% (48/672). Males were significantly more often present in nests, where female was also present, than in nests without a female (Chi square test, Chi=81.06, df=1, p<2.2e-16).

Full-mature brood nests: Most of full-mature brood nests were not guarded by any adult of parental generation. No parent was present in 57.67% of nests (102/177). If guarding adult was present, it was usually a female in 27.11% (48/177). A pair of male and female was present in 10.73% (19/177) and only male was present in 4.51% (8/177). Interestingly, an old female was present more often in full-mature brood nests than in full brood nests (Chi-square test, Chi=16.96, df=1, p=3.826e-05).

Full brood nests: Usually, a nest was not guarded by any adult of parental generation (80%, 68/85). Nests guarded only by male and only by female were present in the same proportion 9.51% (8/85). In one case a nest guarded by male and female together was present.

Duration of actual male presence

The male found at time of nest dissection remained in active brood nest for 10.69 days on average (N=308, SD=7.53, range 1-38). In full brood nests, the male found at time of nest dissection remained there for 4.93 days on average (N=30, SD=6.73, range 1-25). However, difference between nests with male and female couple and nests where male was present alone was significant (poisson GLM, p=2.2e-16, N=30, deviance=77.64, residual deviance=131.57). Males remained for 7.55 days on average (SD = 7.68, N=18, range 1-25) in nests, where female was also present, but only 1 day on average (N=12, SD=0) in nests where only male was present. In full-mature brood nests, male stayed 3.33 days on average (N=6, SD=2.5, range 1-8) at the time of nest dissection. Sample size is too small for testing difference between nests where female was present and absent, but we observed both males which stayed in a nest one day and males which stayed multiple days.

Structure of full brood nest

Full brood nests contained on average 7.59 brood cells (range 1-21, SD=3.76, N=566). Empty cells were relatively scarce, but usually present. There were 1.33 (range 0-8, SD=1.33, N=530) empty cells per nest on average. Therefore, brood cells were usually adjacent. However, when an empty cell was present, it was usually much longer than a brood cell (Fig. 2). Length of nest was 15.12 cm (N=670, SD=4.08) on average and entrance burrow was 3.63 cm long (N = 657, SD = 3.63) on average.

We distinguished three types of full brood nests: 1) guarded nests, 2) plugged nests, 3) orphaned nests. In guarded nests, old female was present. Last brood cell was always closed by nest partition. Other two nest types, plugged or orphaned nests, were without presence of an old female. Plugged nests had last brood cell closed by a filling plug. Filling plug was much thicker than the regular nest partition (Fig. 2) Filling plug was 1.41 cm long (N = 307, SD = 0.87, range 0.2-8.0) on average. Moreover, plugged nests had usually modified nest entrance. All pith between nest entrance and filling plug was excavated. In orphaned nests, last brood cell was closed by regular partition, which was not thicker than regular brood cell partition. Sometimes, last brood cell was partially provisioned by pollen and was not closed by brood cell partition. This type of brood cell did not contain living offspring.

Comparison between different full brood nest strategies

The most common were plugged full brood nests (53.42%, 359/672). The proportion of orphaned (24.25%, 163/672) and guarded (22.32%, 150/672) nests was similar. The number of brood cells was 9.85 on average in guarded nests (SD=4.14), 6.68 (SD=3.53) for orphaned nests and 7.06 (SD = 3.30) for plugged nests (Table 2). There was significant difference in number of brood cells between types of nests (Anova, N=566, F=33.14, p=2.482e-14). However, post hoc tests show that guarded nests are different from plugged (Tukey HSD test, p = 0.0000) and orphaned nests (Tukey HSD test, p = 0.0000), but there is no difference between plugged and orphaned nest (Tukey HSD test, p = 0.5695). Length of nest was not significantly different between nest types (Anova, N=666, F=0.71 p=0.4916). However, length of entrance burrow significantly differs between nest types (Anova, F=309.73, p<2.2e-16) and Tukey HSD tests shows significant difference between all three nest types. Longest was nest entrance in orphaned nests (mean 7.93 cm, N=157, SD=4.67), after it in guarded nests (mean 4.00 cm, N=148, SD=3.76) and then the shortest in plugged nests (mean 1.56 cm, N=352, SD=1.21, Table 2).

There was distinct difference in proportion of full brood nest strategies through the season. Proportion of plugged nests was the highest in the beginning of full brood nest season (beginning of July) and later decreased. On the other hand, proportion of guarded and orphaned full brood nests increased from beginning of July to August (Fig. 3).

Proportion of nests attacked by ichneumonid or *Gasteruption* parasites was 16.66% (25/125) for guarded nests, 11.04% (18/163) for orphaned nests and 17.27% (62/359) for plugged nests. Presence of parasite which consumed multiple brood cells (Ichneumonidae or *Gasteruption*) did not significantly differ between nest types (Chi square test, Chi=3.45, df=2, p=0.1776, N=672). When we excluded nests attacked by an ichneumonid or *Gasteruption* parasite, proportion of dead brood cells was 83.41% (N=127 nests) for guarded nests, 77.92% (N=141 nests) for orphaned nests and 82.74% (N=298 nests) for plugged nests. Difference was not statistically significant (binomial glm, N=565, deviance=1.40, residual deviance=209.12, p=0.4973)

Developmental stage diversity in active brood nests

Ceratina nigrolabiata have very fast development in comparison to rate of provisioning. In 5.14% (32/622) of active brood nests was at least one offspring in adult stage. Active brood nests, where adult was present in the innermost brood cell, had large number of brood cell provisioned (mean 13.71, SD 2.59, range 6-19, N=29) in comparison to nests, where younger stages were present at the bottom of nest. Nests with pupa at the bottom had 10.8 brood cells on average (SD-2.84, range 5-19, N=144). Nests with larva at the bottom had 5.69 brood cells on average (SD = 2.25, range 1-15, N=268). Nest with egg at the bottom had 2.2 brood cells on average (SD = 1.15, range 1-6, N=83).

Paternity of nests with small number of offspring

Guarding male was usually not the father of offspring in young provisioned nests. Guarding male was the father of 6.25% (10/160) of all offspring and 9.9% (10/101) of female offspring in nests with 1-3 offspring (N=70 nests). No offspring was fathered by guarding male in nests with only one offspring (N = 17). Proportion of offspring guarded by own father was 6.2% (2/32) in nests with two offspring and 7.2% (8/111) in nests with three offspring

Parasites

The most common nest parasites were Ichneumonidae and *Gasteruption*. Both destroyed multiple brood cells and commonly destroyed a large part of a nest. We found an icheumonid parasite in 6.81% (125/1836) of nests and *Gasteruption* in 2.83% (52/1836) of nests. In 38 cases, we were unable to determine if parasite is an ichneumonid or *Gasteruption*. When we suppose the same proportion of both parasites in determined and undetermined larvae, we can assume that 8.26% of nests were parasitized by Ichneumonidae and 3.44% by *Gasteruption*. Usually, there was only one larva of these parasites per one nest. We observed 8 cases of two Ichneumonidae larvae in one nest, 2 cases of two *Gasteruption* larvae in one nest and 2 nests where *Gasteruption* and ichneumonid larvae was present together.

Proportions of attacked nests differed between nesting phases. No new founding nests were attacked, as there is no food for the parasite. In active brood nests, only 2.74% were parasited by Ichneumonidae and only 1.43% by *Gasteruption*. In full brood nests, 11.68% were parasitized by

Ichneumonidae and 4.83% by *Gasteruption*. Complete number of parasitized nests is summarized in Table 3.

Other parasites were very rare. We found chalcidoid wasps in 0.44% (8/1836), Malachidae beetles in 0.16% (3/1836) and conopid flies in 0.05% (1/1836) of nests.

Discussion

Role of males

Biparental care is an uncommon type of parental care in insects [8,11] and from bees it is confirmed only in *C. nigrolabiata* [26]. There is extensive division of labor between males and females in *C. nigrolabiata*. Female does all nest provisioning, but male participates in nest guarding [26]. From other Hymenoptera, biparentality is well documented in several species of the genus *Trypoxylon* [25,32,33]. Males in Hymenoptera are usually short-lived and die shortly after mating [18,34], therefore, there is a little possibility for performing any care. However, some male participation on care is known also in other Hymenoptera species [8,35].

Males of *C. nigrolabiata* were present in nests before provisioning started. They were present in newly excavated and discarded nests. New founded nests with male-female pair were more common than nests with only female. Male can help female with nest excavation by throwing filling from nest or by discarding offspring of previous nest owner. Therefore, males have partial role with nest building, similarly with males in crabronid wasps from genus *Trypoxylon,* where males help with smoothing of mud using their mandibles [24].

Although a male is commonly present in the nest before provisioning starts, he is usually not the father of offspring which female laid immediately after she start provisioning. We found out that the guard male was the father of only 6.25% offspring in nests with 1-3 provisioned brood cells. This proportion is even smaller than average proportion of offspring guarded by own father which was 10% [26]. Therefore, it is evident that the female mates before provisioning season and the male came to nest primarily as stepfather.

Our results show that males are present in nests where receptive females are also present. They are often in newly founded nest and in almost all active brood nests. However, they scarcely occurred in full brood nests or mature brood nests. Moreover, when the female is removed from active brood nests, the guarding male usually disappears after few days [26]. Therefore, main male motivation is mate-guarding behavior, not direct offspring care. Males in full brood nests and mature brood nests stay only one or a few days. Therefore, they cannot be fathers of any offspring in the nest. We suppose that two motivations for male presence are possible: a) mating with newly emerging young females and b) staying overnight in the burrow in case of single males.

Although male primary motivation is mate-guarding, our previous study shows that male is beneficial for nest productivity [26]. We suppose that presence of male in nest is useful as protection of nest when female is on a foraging trip. In active brood nests is male present in vast majority of nests and when male is not present, female foraging activity strongly decreases [26].

Generally, behavior of males and females of *C. nigrolabiata* is similar to biparental species of genus *Trypoxylon*. In *Trypoxylon*, females also perform all nest provisioning and males stay at the nest entrance and protect the nest against natural enemies [24,33]. Biparental care is supposed to be a by-product of mate-guarding in both groups [26,32]. Some differences between *Trypoxylon* and *Ceratina* exist. In *Trypoxylon*, males stay in the nest entrance head out [33,36], which allows them to guard more actively than *C. nigrolabiata* males, which block nest entrance by metasoma. Moreover, *Trypoxylon* males usually spent the night outside the nest [24,33], but males of *C. nigrolabiata* do not leave nest at night. Generally, we can consider *C. nigrolabiata* and *Trypoxylon* as taxa which convergently developed very similar biparental behavior. Moreover, it is possible that similar behavior occurs also in colletid bee *Leiproctus muelleri*. In this speces, males perform nest guarding when female provisions nest [37]. However, more detailed research is necessary for evaluation of male role in this species. Behavior of males is different in all other hymenopteran groups, in which they assist with caring for offspring. In small eusocial colonies of *Microstigmus nigrophalmus*, males help with nest protection, however there are more males in nest and they don't sit in the entrance, but patrol across the whole nest [23]. Male participation on care was detected in some polistine wasps or bumble bees and stingless bees, but role of males is only small and males help with thermoregulation or food processing [21,22,38,39]. Macrocephalic males were documented in *Lasioglossum* (*Chilalictus*) *erytrurum*, which can guard nest against ants [40]. However, these males were observed in late phase of nesting, when no brood was produced. Therefore, they probably guard their siblings, not offspring.

Alternative nesting strategies

Parental care is costly and reduces future reproduction [1,6,41]. Therefore, animals optimize time when they leave their offspring [41,42]. Most non-eusocial nest-making Hymenoptera abandon nest after provisioning is finished [18,43,44], although guarding of nest can substantially increase offspring survival [29,40]. However, guarding of nest by female until offspring adulthood is typical for *Ceratina* bees [28,29].

Our results show that *C. nigrolabiata* has alternative nesting strategies. Some females are trying to guard nest until adulthood of offspring. However, most females plug nests by a filling plug and abandon it. This facultative behavior was already documented in *C. chalybea* and *C. chalcites* [29,31]. We suppose that females, which abandoned their nest, build a second nest elsewhere. We do not have direct evidence for this statement, but we found newly founded nests and active brood nests also in late phases of nesting season (Fig. 1). Moreover, almost all full brood nests were plugged in early phases of nesting season, but guarded nests prevailed in late phases of nesting season. Therefore, we suppose that females usually abandon their early nest(s) and guard their last nest. Females can probably abandon their nest, when there is enough time for second nesting. It corresponds with the semelparity hypothesis. It means that opportunities for reproduction can reduce the extent of parental care [45,46].

Abandonment of larger brood by mother is generally less probable than abandonment of smaller brood [2,47]. We found out that guarded nests have significantly higher number of brood cells provisioned than abandoned nests. However, we have not detected direct effect of guarding on offspring survival. There was no difference in proportion of nests attacked by an ichneumonid or *Gasteruption* parasite between guarded and abandoned nests. Moreover, there was also no difference in proportion of dead brood cells. However, the most important cause of brood destruction in case of female removal in *Ceratina* bees is usurpation by other *Ceratina* or nest destruction by ants [26,29]. This type of attack lead to destruction of whole or a significant part of nests, but we were unable to detect such effect by simple comparison of different nest types. Therefore, long-term observations of nest mortality are necessary for comparison of success of guarding and abandoning strategies.

Guarded and plugged nests differs in number of brood cells provisioned, overall difference in nest architecture was small between nest types. In C. chalybea and mostly also in C. chalcites, last brood cell was open in guarded nests [26,29]. However, last brood cell is closed in both nest types in *C. nigrolabiata*. Moreover, last nest partition can be enlarged to filling plug also in some guarded nests (Fig. 2).

Although most of nests without mother are voluntarily abandoned, we detected high proportion of nests (22%) which seems to be orphaned. This is an important difference from *C. chalybea* and *C. chalcites*, where orphaned full brood nests are extremely rare or completely missing [29,31].

Rate of development

Ceratina nigrolabiata have excessively fast development in comparison to the duration of provisioning period of the nest. Therefore, the largest active brood nests contain already adult offspring at the bottom. Active brood nests with adults at the bottom contained on average more offspring than full brood nests. Moreover, nest with the largest number of brood cells provisioned (23) were not full brood nest but active brood nest with adults at the bottom.

High rate of offspring development lead to less risk of nest abandonment by mother. Adult offspring crawl though nest partitions to the top. They can protect immature siblings against potential intruders soon after mother emigration.

High rate of offspring development complicates determining the average number of offspring in complete nests. The reason is that larger nests are in the stage of full brood nest for a shorter time, which is only stage when counting of total number brood cells provisioned is possible. When offspring crawl though uppermost brood cell partition, they can emigrate from natal nest. Therefore, average number of brood cells provisioned can be underestimated due to lower probability of detection of a large nest. Moreover, proportion of guarded nests can be also underestimated, because these nests are larger (and therefore less detectable) on average than plugged or abandoned nests.

Natural enemies

Ceratina bees are attacked by a wide spectrum of natural enemies. However, the influence of parasitism is usually low due to effective nest protection and short time of larval development [48]. The most common parasites, which we observed, were ichneuomids and *Gasteruption*. Both parasites have similar effect on nests. Their predacious larvae are much larger than *Ceratina,* and they destroy several brood cells (Fig. S3). Number of broods destructed by one ichneuomonid or one *Gasteruption* is probably about four, but it is difficult to count them as partitions are damaged.

We suppose that the most relevant stage for assessing parasitation is full brood nests. Earlier nest stages had not sufficient time to be parasited. On the other hand, both parasites and *Ceratina* offspring can emigrate from later nest stages, thus full-mature brood nests and mature brood nests aren't suitable for assessing parasitation. As about 12% of full brood nests were parasitized by Ichneumonidae and 5% by *Gasteruption*, we think that these parasites cause substantial brood loss in this species. On the other hand, other brood parasites were very rare and they probably do not affect *C. nigrolabiata* population substantially.

Nest usurpation plays an important role in *C. nigrolabiata* strategy. From new founded nests, 18.2 % were established by usurpation. Moreover, removing experiments show that usurpation by other *Ceratina* bee is the most important reason of failure of nest with removed female (Mikát et al., 2019), and most of these usurpers are conspecific individuals of *C. nigrolabiata*. Therefore, interspecific competition plays apparently important role in *C. nigrolabiata*. However, it is a question, why some females frequently abandon nests. Frequency of unguarded nests is even larger than in related species *C. chalybea* and *C. chalcites* [29,31]. In plugged nests is the nest entrance usually excavated, and therefore its usurpation by other *Ceratina* is prevented. It is impossible to guard the nest effectively and therefore plugged nests are probably unattractive for nest usurpation.

Methods

Location

We performed research in Podyjí National Park and surrounding areas (South Moravian Region, Czech Republic), mostly in Havraníky heathland (around coordinates 48°48'32.867"N 15°59'34.963"E). We performed research in 2013–2018.

General design

We dissected nests from artificial nesting opportunities. Some of these nests were used also for other experiments (partially published in [26]), but here we present different aspects of *C. nigrolabiata* biology than in our previous paper.

Preparation of nesting opportunities

We studied *C. nigrolabiata* nests from artificial nesting opportunities. We used twigs of *Solidago canadesnis*, *Echinops spareocephalus*, *Helianthus tuberosus,* and *Tanacetum vulgare*. We cut twigs to 30–50 cm long fragments. Twenty of these fragments were tied together into one sheaf. Each sheaf was fixed by a bamboo rod to ground. The sheaves were installed before nesting season (April or early May). We established about 1000 sheaves, which corresponds to 20,000 nesting opportunities each year. Therefore, we established around 120,000 nesting opportunities during the whole research.

Nest dissection

Nests were collected in evening (after 18 h CEST) to ensure that all inhabitants were present inside the nest. Nests were stored in a refrigerator between time of collection and dissection. Each nest was open by a knife or clippers. Following parameters were noted for each nest: length of nest, length of nest entrance, number and stage of immature individuals, number and sex of adult individuals, presence of parasites. Position in a nest was noted for each individual. We also noted the presence of nest partitions, which separated brood cells. We specifically noted presence of a filling plug (enlarged last brood cell partition, which is usually about 1 cm long).

We classified nests to categories. Earliest occurred new founded nets, which contained burrows with only adult individual(s) and no pollen ball or provisioned brood cells. We divided such nests to two sub-categories. Newly excavated nests, which were newly established, and discarded nests, which were established in twigs that already housed another *Ceratina* nest. In other words, the nest was usurped. Active brood nests contained a pollen ball in the outermost currently provisioned brood cell or an egg in the outermost closed brood cell. Moreover, these nests were not closed by a filling plug. Full brood nests contained a larva or pupa in the outermost brood cell, and the partition of the outermost brood cell was still undisturbed. If young adults were present in the nest, they did not crawl through the outermost partition. Full-mature brood nests had disturbed the outermost brood cell partition (mature adults probably crawled out through this partition) and contained at least one immature offspring. Mature brood nests contained only mature offspring and no juveniles. Some nests did not fit any of these categories. These nests can be old burrows used for hibernation, nests partially destroyed by ants or other non-*Ceratina* natural enemy coming through the nest entrance, nest or burrows of other bees or wasps accidentally visited by *C. nigrolabiata*.

We analyzed 1,836 nests of *C. nigrolabiata*: 86 nests were newly excavated, 622 were active brood nests (460 of them were already used for same analyses published in [26]), 672 were full brood nests, 177 were full-mature brood nests, 85 were mature brood nests, 18 were discarded nests, and 176 nests were impossible to classify as any standard category.

Analysis of phenology: We calculated proportion of types of nests through different parts of nesting season. We excluded nests dissected in September (N=11), due to the small number of nests dissected in this period from analysis. Therefore, we included 1825 nests.

Analysis of nest structure: We calculated number of provisioned cells, empty cells, and living offspring for full brood nests and active brood nests. Number of provisioned cells was possible to calculate only in nests which were not influenced by an ichneumonid or *Gasteruption* parasite, or only slightly influenced. These parasites destroyed multiple brood cells, and it was impossible to determine the precise number of brood cells destroyed. The number of empty cells was possible to be calculated in nests which were not parasited by *Gasteruption* or Ichneumonidae, and if any adult offspring destroyed partitions in the bottom part of the nest. When adult offspring destroyed brood cell partition of at least one brood cell, it was impossible to determine if an empty cell were destroyed or not.

Full brood nests were classified in three categories: guarded, plugged, and orphaned. Guarded full brood nests contained an old adult female in nest entrance. Plugged nests were not guarded by a female and were closed by a filling plug (nest partition on average 1.33 cm thick, much more than regular brood cell partition). Orphaned nests were not guarded by a female and had the same size of the last brood cell partition as the other partitions in that nest. Sometimes, last brood cell was opened, without living offspring and only partially provisioned by pollen in orphaned nests. Adult males can be present in nest entrance of all types of these nests. We did not use the presence of male as a factor for nest classification. The statistical analyses were performed in R software 3.6.1. [49]

Duration of guarding of last male: We measured how long was nest guarded by a male which was present at time of nest dissection. We performed this analysis in years 2013–2016. In selected nests, we daily checked for presence of guarding male. When we found an unmarked male, we marked him with an oil dye. When we found marked male, we noted his color. Therefore, we were able to determine, how long is the male present in the nest. For detailed description of this method, see [26]. We examined this feature in 308 active brood nests, 30 full brood nests and 6 full-mature brood nests.

Paternity in small nests: For this analysis a subset of nests analyzed by previous study was used [26]. Procedures of DNA isolation, microsatellite genotyping, and paternity analysis are described in [26]. Nests were selected for analysis according to these characteristics: 1) Nests had between one to three provisioned cells with offspring, 2) guarding parent pair was present, 3) all offspring in the nest was offspring of guarding female. 17 nests with one offspring, 16 nests with two offspring, and 37 with three offspring were included in this analysis. In these nests, we tested paternity of the guarding male.

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Author contribution

MM and JS designed research, MM, EM and JS Performed research, MM analyzed data, MM wrote first draft of manuscript, JS performed substantial changes in manuscript. All authors approved final version.

Competing interests

The authors declare no competing interests.

Data availability

All Relevant data are attached in XLS file as supplementary material.

Tables

Table 1: Presence of individuals of parental generation in different nest stages. FBN: full brood nest. M=male, F=female

Nest stage	No adult	M	MM	F	MF	MMF	MMMF	All nests
Newly excavated nest		46		9	31			86
Discarded nest	1	1		1	13	2		18
Active brood nest	11	35		40	527	8	1	622
Full brood nest	494	29	1	102	48			672
FBN~guarded	0	0		101	49			150
FBN~orphaned	139	23	1	0	0			163
FBN~plugged	353	6		0	0			359
Full-mature brood nest	102	8		48	19			177
Mature brood nests	68	8		8	1			85

Table 2: Comparison between guarded, orphaned, and plugged full brood nests.

Nest type	guarded	orphaned	plugged	all nests
Number of nests analyzed	150	163	359	672
Proportion of nests	0.22	0.24	0.54	1
Brood cells in nests				
Mean	9.85	6.70	7.07	7.6
Range	1 - 21	1 - 16	1 - 18	1 – 21
Standard deviation	4.14	3.53	3.31	3.76
Empty cells				
Mean	1.16	0.46	1.84	1.34
Maximum z N_FC	0 - 6	0 - 5	0 - 8	0 - 8
Standard deviation	1.19	0.88	1.33	1.34
Number of live offspring				
Mean	7.52	4.98	5.31	5.72
Range	0 - 21	0 - 16	0 - 17	0 - 21
Standard deviation	4.19	3.32	3.32	3.66
Length of nest				
Mean	14.79	15.1	15.28	15.12
Range	4.4 - 23.4	4.2 - 26.9	4.3 - 30.4	4.2 - 30.4
Standard deviation	3.54	3.8	4.41	4.09
Length of nest entrance				
Mean	4	7.94	1.56	3.64
Range	0.5 - 17.5	0.17 - 21.3	0.4 - 15	21.3
Standard deviation	3.68	4.67	1.22	3.97

Table 3: Numbers of nests attacked by different groups of parasites. Ichneumonidae or *Gasteruption* young larvae were undistinguished.

Parasite	Ichneumonidae		*Gasteruption*		Ichneumonidae and *Gasteruption*	Not identified	Chalcidoid wasps		Conopidae	Malachidae	Unattacked nests	Total nests
Number per nest	**1**	**2**	**1**	**2**	**1+1**	**1**	**1**	**2**	**1**	**1**	**NA**	**NA**
Newly founded nest	0	0	0	0	0	0	0	0	0	0	86	86
Discarded nest	0	0	0	0	0	0	0	0	0	0	18	18
Active brood nest	10	0	6	0	0	6	2	0	0	0	598	622
Full brood nest	58	5	24	2	0	22	4	2	0	2	553	672
Full-mature brood nest	15	1	1	0	1	5	0	0	0	0	154	177
Mature brood nest	7	1	3	0	1	0	0	0	0	0	73	85
Other burrows	25	1	14	0	0	5	0	0	1	1	129	176
Total number	**115**	**8**	**48**	**2**	**2**	**38**	**6**	**2**	**1**	**3**	**1611**	**1836**

Figures

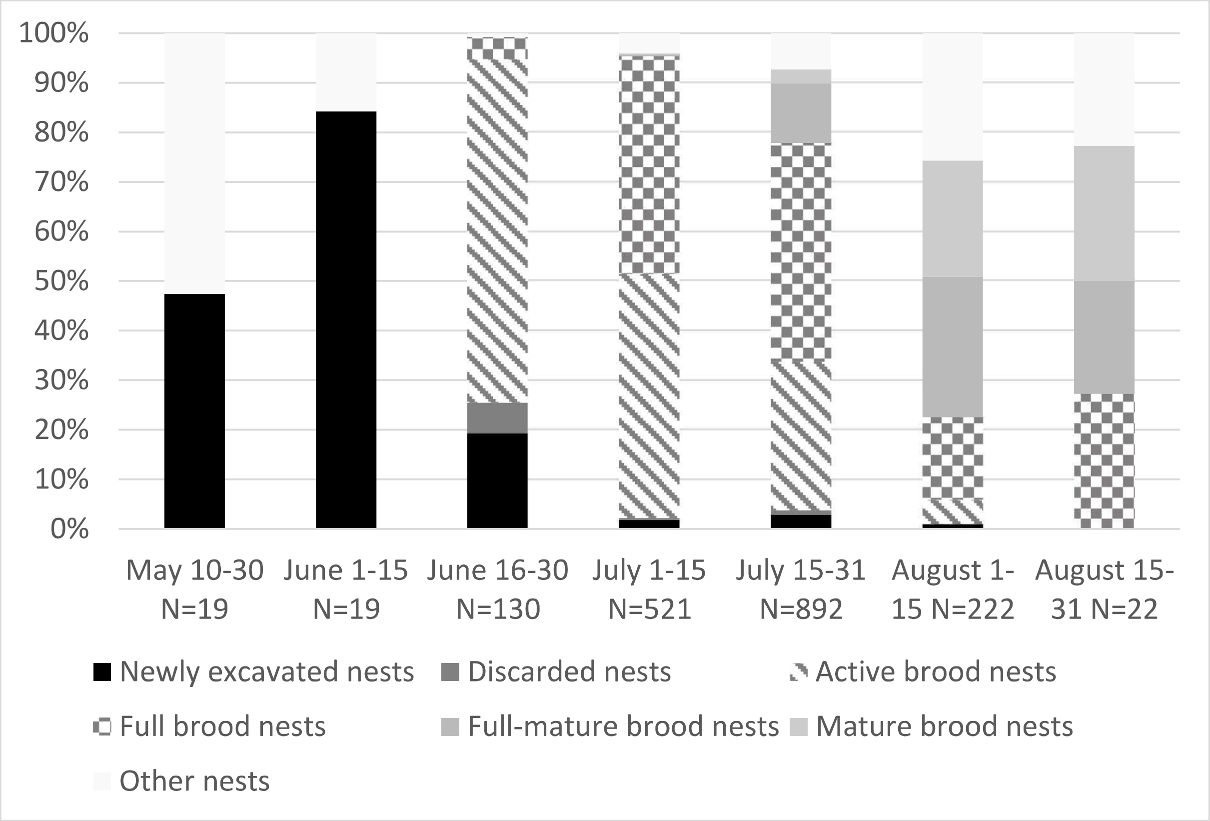

Fig. 1: Phenology of *C. nigrolabiata* through nesting season.

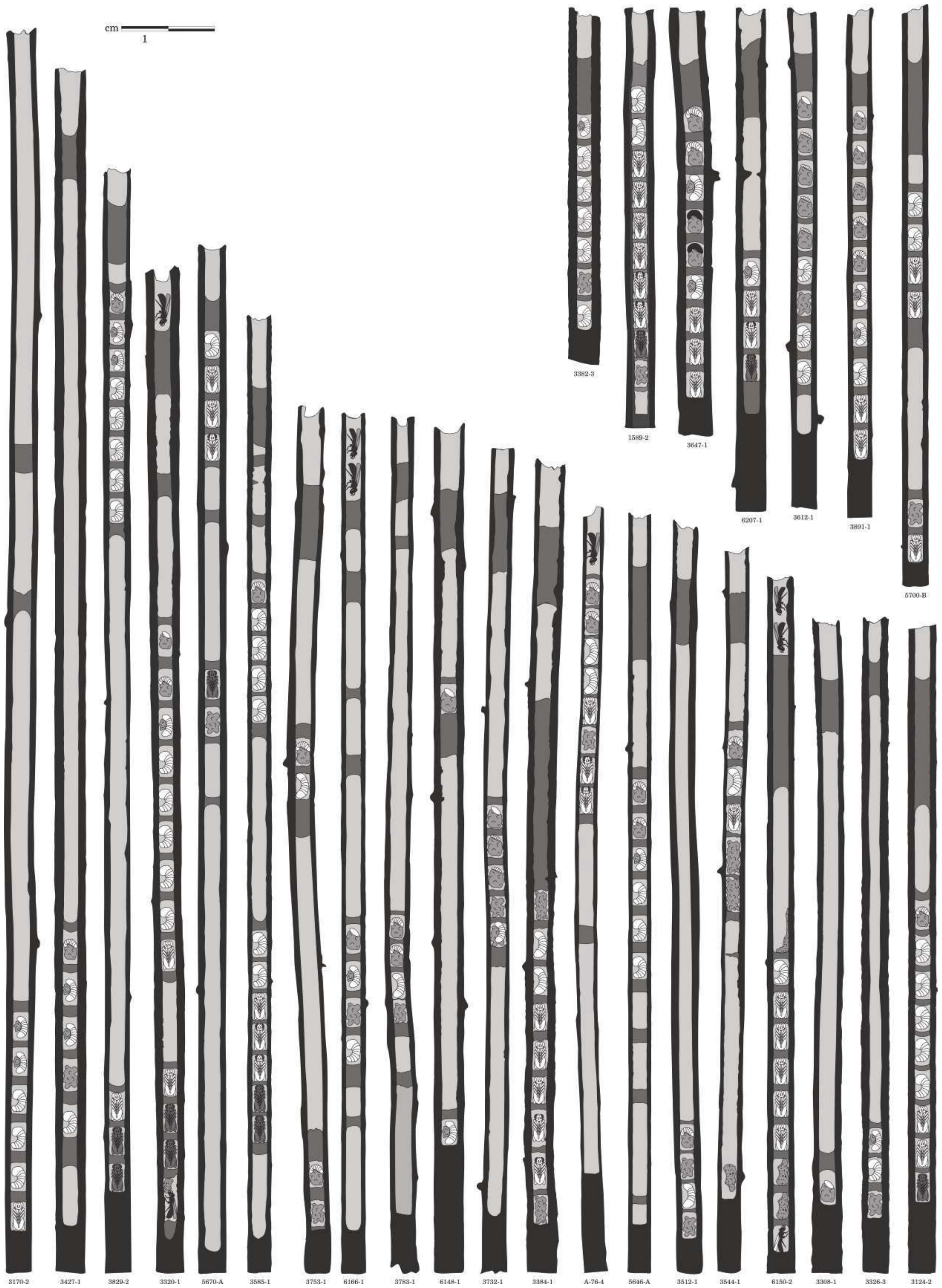

Fig. 2: Examples of nests structure of full brood nests of *C. nigrolabiata*

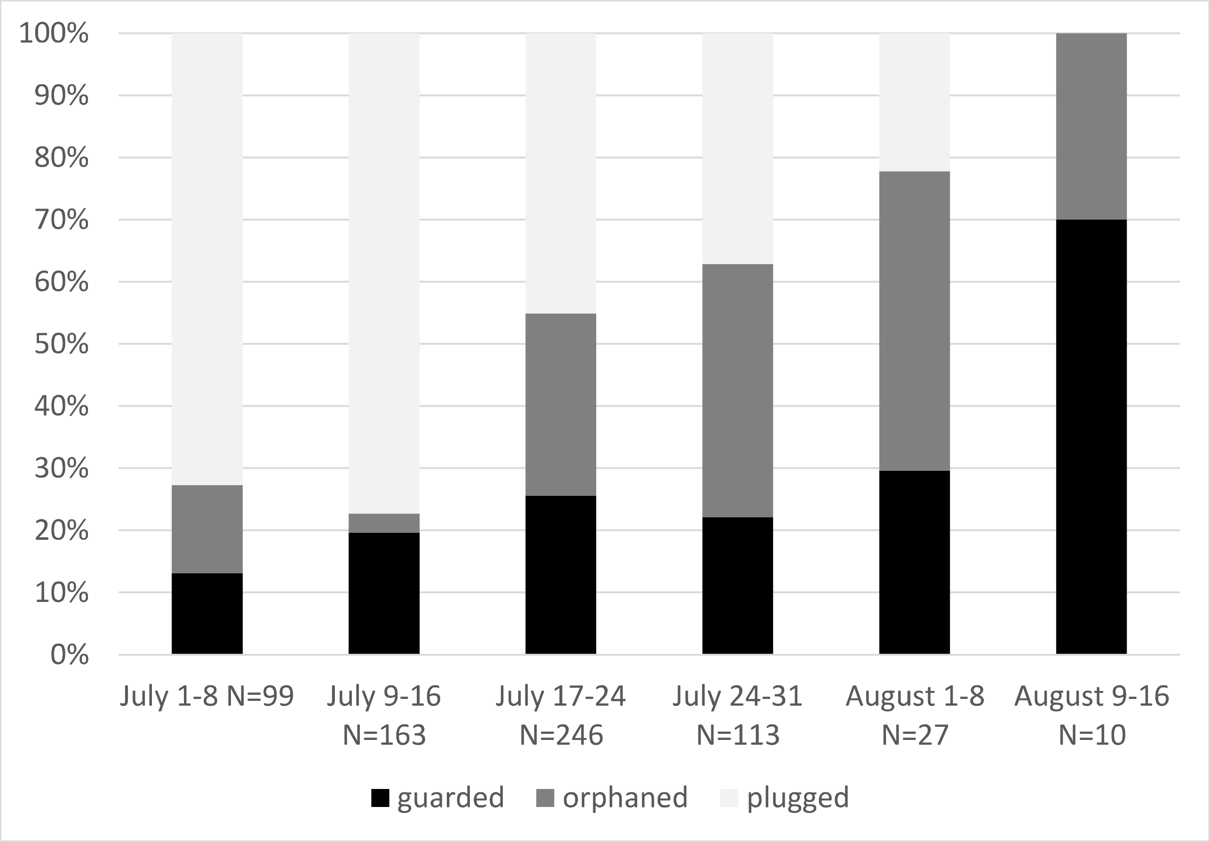

Fig. 3: Proportion of types of full brood nests through the season