



Nesting behaviour and population genetics of solitary bees (Hymenoptera: Anthophila)

Kateřina Černá
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**Nesting behaviour and population genetics
of solitary bees (Hymenoptera: Anthophila)**

Hnízdní chování a populační genetika samotářských včel
(Hymenoptera: Anthophila)

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1 – *Osmia rufa*, © Klára Doležalová

2 – Individually marked female of *Anthophora plumipes*, © Klára Doležalová

3 – *Andrena vaga* with *Stylops* parasite, © Pavel Krásenský ([www. macrophotography.cz](http://www.macrophotography.cz))

4 – Nesting site of *Andrena vaga* with individually marked nests, © Kateřina Černá

5 – *Anthophora plumipes*, © Pavel Krásenský (www. macrophotography.cz)

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Kateřina Černá
Prague, 19th September 2013

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SUMMARY

My thesis is focused on nesting behaviour and population genetics of solitary bees. These two topics, although seemingly unrelated, bring much new information and thus contribute to the better understanding of solitary bee biology that is still insufficiently known.

Although the solitary behaviour represents the necessary original state for the evolution of higher sociality or obligate cleptoparasitism, its role is largely unappreciated. Furthermore, intraspecific cleptoparasitism, which is an alternative and facultative nesting strategy in bees, is a probable antecedent state of obligate cleptoparasitism. Although the obligate cleptoparasitism is a very common strategy in solitary bees, the information about the frequency and the occurrence of intraspecific cleptoparasitism in solitary bees is rare. We studied the nesting behaviour of solitary bees to detect different behavioural patterns that could serve as preadaptations to sociality or cleptoparasitic behaviour and we also focused on the detection and description of intraspecific cleptoparasitism in solitary bees. We chose four model solitary species for these studies – *Andrena vaga* (Andrenidae), *Anthophora plumipes* (Apidae), *Colletes cunicularius* (Colletidae) and *Osmia rufa* (Megachilidae). We described the behaviour of *Andrena vaga* at the nesting site and showed that although the behaviour is individually variable, it is possible to detect several species specific foraging and daily behavioural patterns and interspecific comparison thus should be possible. Further we detected the presence of intraspecific cleptoparasitism in the form of nest usurpations in all the studied species, which implies that intraspecific cleptoparasitism is widely spread in solitary bee species. We further showed that regular nest abandonments and switches are surprisingly frequent in solitary bees and that they may play a crucial role in the evolution of sociality, because they decrease the intraspecific aggressiveness. Behavioural and seasonal data collected during our field observations were further used to describe different factors influencing foraging activity and lifespan in natural populations of solitary bees. We showed that the lifespan is driven both directly by climate and indirectly through climate-dependent changes in activity patterns.

The population genetic studies brought information about the state of populations of solitary bees. Specialisation is a very common strategy in bees, but endangers them by decreasing population size and local gene diversity and by enhancing genetic structure. We studied the population structure of specialised bee *Andrena vaga* in the heterogeneous environment of the Czech Republic and we expected to detect a significant population structure. However, our results confirmed good dispersal ability of this species resulting in generally admixed pattern. Interestingly, two differentiated subpopulations, separated by a wide clinal zone of admixture, were detected within the study area that could reflect a quarterly history of this species. Further, we developed a set of microsatellites for *Anthophora plumipes* and used them to describe the phylogeography of this species in the whole species area and to evaluate the result of its recent introduction in the USA. We detected seven major clades, six in Europe and one in Asia (and in the USA). Genetic distances implied that several of these clades should be considered separated subspecies or even species. We confirmed the Japanese origin of the USA population and stated that the introduction was successful despite the dramatic decrease in genetic variability and N_e in both the source Japanese and the introduced USA population.

Moje disertační práce je zaměřena na studium hnízdního chování a populační genetiky samotářských včel. Tato dvě témata, ačkoli zdánlivě nesouvisející, přinášejí mnoho nových informací a tak napomáhají lépe porozumět biologii samotářských včel, která je stále nedostatečně známá.

Ačkoli samotářské chování představuje původní stav, z něhož se vyvinula všechna vyšší společenská uspořádání, jeho role není dosud plně doceněná. Vnitrodruhový kleptoparazitismus, který představuje alternativní a fakultativní hnízdní strategii včel, je navíc pravděpodobným předchůdcem obligátního kleptoparazitismu. Ačkoli je obligátní kleptoparazitismus u samotářských včel velice častý, informace o četnosti a výskytu vnitrodruhového kleptoparazitismu jsou poměrně vzácné. My jsme studovali hnízdní chování samotářských včel za účelem podchycení vzorců chování, které by mohly sloužit jako preadaptace pro společenské či kleptoparazitické chování, a dále jsme se snažili odhalit přítomnost vnitrodruhového kleptoparazitismu u samotářských včel. Pro studium jsme si vybrali čtyři modelové druhy: *Andrena vaga* (Andrenidae), *Anthophora plumipes* (Apidae), *Colletes cunicularius* (Colletidae) and *Osmia rufa* (Megachilidae). Popsali jsme hnízdní chování druhu *Andrena vaga* a ukázali jsme, že ačkoli je toto chování individuálně variabilní, je možné popsat celou řadu druhově specifických vzorců chování, což umožňuje mezidruhové porovnání. Dále jsme zjistili přítomnost vnitrodruhového kleptoparazitismu ve formě usurpací u všech studovaných druhů, což dokazuje, že kleptoparazitismus je velice častou strategií samotářských včel. Ukázali jsme, že včely překvapivě velice často opouští svá hnízda a zakládají hnízda nová. Takovéto chování může hrát zásadní roli v evoluci sociality, protože vede ke snížení vnitrodruhové agresivity. Data, která jsme získali během terénních pozorování, byla dále použita k popsání různých faktorů, které ovlivňují délku života samotářských včel. Ukázali jsme, že délka života je ovlivňována jednak přímo klimatickými faktory a jednak nepřímo klimaticky podmíněnými změnami v chování.

Populačně genetické studie nám napověděly, v jakém stavu se nacházejí populace samotářských včel. Specializace je velice častou strategií samotářských včel, ale ohrožuje je snížením efektivní velikosti populace a genetické diversity a podporou tvorby populační struktury. V první práci jsme studovali druh *Andrena vaga* v heterogenním prostředí České republiky a očekávali jsme, že zde nalezneme výraznou populační strukturu. Naše výsledky však ukázaly, že tento druh se vyznačuje vysokou schopností disperse, jejímž výsledkem je téměř panmiktická populace. Překvapivě jsme zjistili, že na studovaném území existují dvě částečně oddělené subpopulace, jejichž existence a poloha by mohla odrážet kvartérní historii na studovaném území. Dále jsme vyvinuli mikrosatelity pro druh *Anthophora plumipes* a použili je k popisu populační struktury tohoto druhu na celém území výskytu a k vyhodnocení výsledků nedávné introdukce do USA. Nasbírané populace se štěpily do sedmi hlavních linií, šest z nich se nachází v Evropě a jedna v Asii (a v USA). Na základě genetických vzdáleností jsme zjistili, že řada těchto linií pravděpodobně představuje samostatné poddruhy nebo dokonce druhy. Naše analýzy potvrdily japonský původ americké populace a ukázaly, že introdukce tohoto druhu byla úspěšná navzdory velkému genetickému ochuzení a nízkým efektivním velikostem populace u zdrojové japonské i introdukované americké populace.

AIMS OF THE STUDY AND LIST OF PAPERS

1) Solitary behaviour is the original state for the evolution of higher sociality and obligate cleptoparasitism; however, its role is largely unappreciated. Thus, my first aim was to describe the nesting behaviour of solitary bees and detect different behavioural patterns of solitary bees at the nesting site that could serve as preadaptations to sociality or cleptoparasitic behaviour. Fulfilling of this aim resulted in following papers:

Paper I:

Rezkova, K., Žáková M., Žáková Z. & Straka J. (2012): Analysis of nesting behavior based on daily observation of *Andrena vaga* (Hymenoptera: Andrenidae). *Journal of Insect Behavior* 25: 24-47.

Paper V:

Straka J., Černá K., Macháčková L., Zemenová M. & Keil P. Lifespan in the wild: the role of activity, climate and parasitic infestation in natural populations of bees. Submitted manuscript.

2) Intraspecific cleptoparasitism is a probable antecedent state of obligate cleptoparasitism. Obligate cleptoparasitism is very common in bees, while the information about the intraspecific cleptoparasitism is still insufficient. My second aim was to detect the presence and frequency of intraspecific cleptoparasitism in solitary bees.

The results are described in:

Paper II:

Černá K., Zemenová M., Macháčková L., Kolínová Z. & Straka J. (2013): Neighbourhood society: nesting dynamics, usurpations and social behaviour in solitary bees. *PLoS ONE* 8: 73806.

3) The methods of population genetics are widely used to study ecology, behaviour or kinship relationships in various organisms and they brought several new insights in their biology. The population genetic studies on solitary bees are however rare compared to their diversity. My third aim was to enhance the knowledge about solitary bee genetics and their conservation state by describing their population structure. This aim was fulfilled in following articles:

Paper III:

Černá K., Straka J. & Munclinger P. (2013): Population structure of pioneer specialist solitary bee *Andrena vaga* (Hymenoptera: Andrenidae) in central Europe: the effect of habitat fragmentation or evolutionary history? *Conservation Genetics* 14: 875–883.

Paper IV:

Černá K. & Straka J. (2012): Identification of 37 microsatellite loci for *Anthophora plumipes* (Hymenoptera: Apidae) using next generation sequencing and their utility in related species. *European Journal of Entomology* 109: 155-160.

Paper VI:

Černá K., Munclinger P., Vereecken N. J. & Straka J. Multilocus phylogeography of a widespread Palearctic solitary bee *Anthophora plumipes* (Hymenoptera: Apidae): Mediterranean lineage endemism, island effects and cryptic species. Manuscript based on the poster presentation on the XXIV ICE 2012 in Daegu.

INTRODUCTION

Bees (Apiformes) together with the apoid wasps (Spheciformes) belong to the superfamily Apoidea, suborder Apocrita and order Hymenoptera (Michener 2007). Bees form a monophyletic group consisting of nine principal lineages (families) within Apiformes: Andrenidae (2917 species), Apidae (5749 species), Colletidae (1841 species), Halictidae (4327 species), Megachilidae (4096 species), Melittidae (187 species), Stenotritidae (21 species) and two fossil families Melittosphecidae and Paleomelittidae (Aguilar *et al.* 2013). For the recent comprehensive review on bee phylogeny see (Danforth *et al.* 2013).

My dissertation thesis is focused on solitary bees that include the majority of bee species. Solitary bees also represent the original state in the evolution of bee sociality and obligate cleptoparasitic behaviour (Danforth *et al.* 2013). We have studied nesting behaviour and population genetics of four solitary bee species: *Andrena vaga* Panzer (Andrenidae), *Anthophora plumipes* (Pallas) (Apidae), *Osmia rufa* (L.) (Megachilidae) and *Colletes cunicularius* (L.) (Colletidae). These particular species were chosen for several reasons. Each of them belongs to a different bee family, which is useful for intraspecific comparisons and drawing more general conclusions on solitary bee biology. These species are common spring species in central Europe with plenty of available nesting sites for the field studies. *A. vaga*, *A. plumipes* and *C. cunicularius* nest in the ground, while *O. rufa* nests in pre-existing holes and cavities (Westrich 1990). Although solitary, all of them nests in the aggregations, which makes them interesting models for the study of intraspecific interactions and presence of the cleptoparasitic behaviour.

The introduction to my thesis is divided into two separate parts. The nesting behaviour of solitary bees represents an original stage in the evolution of eusociality from a solitary ancestor as well as in the evolution of obligate cleptoparasitic behaviour from a non-cleptoparasitic solitary antecedent. Therefore, solitary bees should not be neglected in comparative studies focused on the evolution of bee behaviour. For this reason I shortly reviewed the state of knowledge about the nesting biology, social organisation and intraspecific cleptoparasitic behaviour in bees in the first part of this introduction. The second part describes various aspects of bee population genetics with a special attention paid to the conservation genetics, which is recently a highly actual topic. Studies on nesting behaviour and population genetics in solitary bees are still uncommon in recent scientific literature. Therefore, the present thesis aims to contribute to better understanding of these two issues of the solitary bee biology.

Nesting behaviour

COMPONENTS OF NESTING BEHAVIOUR

Nesting behaviour is a broad term that includes many different topics. First, and possibly the largest, category of studies dealing with this topic describes the nest arrangement and larval morphology in bees (e.g. Bohart & Youssef 1976, Eickwort 1977, Rozen *et al.* 2009). Other studies focus on the provisioning behaviour of bees, including daily foraging patterns, number of provisioning flights per day and flight length or the rate of reproduction (e.g. Bennett & Breed 1985, Danforth 1990, Neff & Danforth 1991, Stone 1994, Neff & Simpson 1997, Bischoff *et al.* 2003, Klein *et al.* 2004, Giovanetti & Lasso 2005). Some studies also include information about the time and energy costs of provisioning based on offspring sex ratio or food availability (e.g. Danforth 1990, Al-

cock 1999, Giovanetti & Lasso 2005). However, detailed studies describing the different behavioural patterns of bees at the nesting site are quite unique. This is a shortcoming, since only the detailed knowledge of such behaviour might help us to detect and understand the possible preadaptations of solitary bees to sociality or to adoption of cleptoparasitic behaviour.

As mentioned above, studies on provisioning behaviour in bees are relatively common [reviewed by Eickwort & Ginsberg (1980) or Neff (2008)]. Neff (2008) reviewed available information on the different components of provisioning behaviour in solitary bees and compared various species. Although the interspecies comparison is often complicated (as different scientists use different definitions and methods), he tried to summarize several variables that are commonly used in wider range of studies. The most important parameter is the provisioning rate (the mass of provision brought per certain time unit), because it includes information about the amount of provision necessary for one single cell, individual transport capacity and the number of flights necessary to provision a cell or the number of cells provisioned per day (Neff 2008). Bees provision their nest with a mixture of pollen and nectar and can use just their crop (most members of Hylaeinae or Ceratinini) or crop together with leg or abdominal scopa to carry the pollen and nectar (Michener 2007). There is a trade-off between the number of provisioning flights necessary to complete one cell and the amount of provision brought in one trip. Thus, we should expect that the larger bees should have larger transport capacity (Neff 2008). However, the reality seems to be more complicated. While some studies describe the positive relationship between the body size and transport capacity (Klostermeyer *et al.* 1973), others report no relationship of this kind (Giovanetti & Lasso 2005) or even a negative relationship (Ramalho *et al.* 1998). Neff (2008) summarised results on different studies and concluded that larger species tend to carry larger pollen loads which are however not proportionally larger when the ratio of pollen load mass to body mass is taken into account.

There are big differences in the number of flights necessary to complete provisioning of one cell and it naturally depends on the bee transport capacity (Neff 2008). The minimal number of provisioning flights (two only) is known in the genus *Perdita* Smith (Danforth 1989) and a relatively low number of provisioning flights to complete one cell is common in the whole family Andrenidae (Schönitzer & Klinksik 1990, Bischoff *et al.* 2003, Rezkova *et al.* 2012). On the other hand, species of the family Megachilidae usually perform many short provisioning flights per cell (Frolich & Parker 1983). The number of provisioning flights should also depend on the offspring sex, especially in the species with strong size dimorphism, where males are remarkably smaller than females and thus need less provision for their development. There is a large variation in the offspring production in the nature and the question of optimization of parental investment and the sex allocation is very popular since Fisher's formulation of the sex ratio theory (Fisher 1930). To explain this natural variation, Rosenheim *et al.* (1996) developed a model predicting that greater availability of food resources leads to an increase in the amount of provision per offspring and an increase in the female production, which Kim (1999) proved to be right at least in *Megachile apicalis* Spinola. Danforth (1990), furthermore, showed a strong correlation between the number of provisioning flights and the offspring sex in *Calliopsis persimilis* (Cockerell) and the ability to manipulate the sex allocation according to the actual daily conditions. Maternal control over provided provision and resulting brood size has also been described for *Amegilla dawsoni* (Rayment) (Alcock 1999, Tomkins *et al.* 2001). These studies, thus, show that provisioning

bees are generally able to optimize their foraging and sex allocation strategies according to the actual environmental or physiological conditions.

The provisioning rate further depends on the length of provisioning flights, which varies a lot among different bee species (Neff 2008). Some species are fast with one provisioning flight taking only few minutes (Bohart & Youssef 1976, Schlindwein & Martins 2000), while it often takes more than one hour in the genus *Andrena* Fabricius (Gebhardt & Röhr 1987, Bischoff *et al.* 2003), one of the bee species studied by us. The provisioning flights take less than half an hour in *Anthophora plumipes* (Stone 1994), which we also studied. Surprisingly, Neff (2008) reported that the length of provisioning flight depends neither on the body size nor on the pollen load size as a proportion of body weight. The length and frequency of provisioning flight depends, however, on the amount and availability of food sources (Kim 1999). In addition, Goodell (2003) showed that the lack of food sources prolongs the length of provisioning flights, which increase parasitism rates in unprotected nests.

The number of cells provisioned per one day is a function of provisioning flight length and individual transport capacity. Bees usually provision up to two cells per day (Neff 2008). However, there are many interesting exceptions. Danforth (1990) described that *Calliopsis persimilis* provisions up to six cells per day. On the other hand, bees of the genus *Andrena* belonging to slow provisioners usually need more than one day to complete a single cell (Gebhardt & Röhr 1987, Giovanetti & Lasso 2005). The number of completed cells might be further limited by the speed of egg production, which is likely in above mentioned slow provisioning genus *Andrena* (Neff & Simpson 1997, Bischoff *et al.* 2003).

As mentioned at the beginning of this chapter, the comprehensive studies describing the bee behavioural patterns at the nesting site are uncommon. Because we studied the behaviour of *Andrena vaga* at the nesting site in detail, we searched for the available information about the genus *Andrena*. We were able to find only few studies that describe the nesting behaviour of bees in a sufficient detail (Malyshev 1926, Michener & Rettenmeyer 1956, Gebhardt & Röhr 1987, Schönlitzer & Klinksik 1990 or Bischoff *et al.* 2003). Moreover, we found several more pieces of information scattered across number of other articles. All the collected information on the nesting behaviour in *Andrena* is summarised in our article on the nesting behaviour of *Andrena vaga* (Rezkova *et al.* 2012), where we present various behavioural patterns observed at the nesting site in form of an ethogram and where we describe the changes of the behavioural patterns on a daily scale. This article is a part of my thesis (Paper I). Behavioural data collected during our field observation of *A. vaga*, together with data collected on our second model bee species *Anthophora plumipes*, were further used in our article describing the different factors influencing foraging activity and longevity in natural populations of solitary bees which is also part of this thesis in the form of manuscript (Paper V); by the time of writing this chapter the manuscript is under review.

SOCIAL ORGANISATION IN BEES

Sociality

There are several different types of sociality. Traditionally, we distinguish between four categories: communalism, quasisociality, semisociality and eusociality (Michener 1974). The most important features of eusociality are reproductive division of labour, cooperative brood care and overlapping adult generations (Wilson 1971). Communalism, quasisociality and semisociality possess only some of these characters and thus represent either the (possible) transitional states in evolution towards higher sociality or the terminal stages in the evolution of sociality.

In the communal societies, members of one nest share a common nest entrance, but inside they build and provision their own brood cells (Michener 1974). Communally nesting species can be found within all bee families except for the Stenotritidae family (Michener 1974, Garófalo *et al.* 1992, 1998, Paxton *et al.* 1999, Spessa *et al.* 2000, Kukuk *et al.* 2005). Quasisocial societies are similar to communal [and are often confused with them (Sakagami & Zucchi 1978)] except for the existence of cooperation in cell construction and its provisioning. In this system it is also not predetermined which female will lay her egg in the already prepared cell (Michener 1974). Such societies are however rare in the nature and are known only in family Colletidae and Apidae (Sakagami & Zucchi 1978, Smith & Schwarz 2006) and possibly also in Megachilidae (Michener 1974), though this question is not well resolved yet [see discussion in Sakagami & Zucchi (1978)]. Semisocial communities are characterised by the reproductive division of labour and cooperative brood care, but not by the generation overlap – the queens and workers are usually the sisters (Michener 1974). This type of sociality is relatively common in some eusocial societies during the nest founding period or after the death of the queen and usually represents only a temporary state (Lin & Michener 1972, Landi *et al.* 2003, Bolton *et al.* 2006). Eusocial species are traditionally divided into two further groups: a) primitively eusocial species that are characterised by the three key eusocial characters – reproductive division, overlapping of generations and cooperative brood care, and b) highly eusocial species that include organisms that possess one more feature – the morphological caste differentiation (Wilson 1971, Michener 1974). Eusociality evolved three times independently in carnivorous Aculeate Hymenoptera (one lineage within Vespidae, all Formicidae and one genus in Crabronidae) (Wilson 1971) and four times independently in the two pollen collecting bee families (Schwarz *et al.* 2007, Gibbs *et al.* 2012).

However, the hereinbefore described classification of sociality is inaccurate in many cases and definitely not applicable to all situations, which resulted in formulation of the social continuum theory (Sherman *et al.* 1995) and various discussions and queries (e.g. Crespi & Yanega 1995, Costa & Fitzgerald 1996a, 1996b, Reeve *et al.* 1996). The question of the exact definitions and terminology has not been resolved up to present time (Lacey & Sherman 2005). As the review of different kinds of sociality and their features would require description in an extent far beyond the scope of this thesis, for further reading I recommend the bachelor thesis by Michael Mikát (Mikát 2012) (available at www.aculeataresearch.com, in Czech only) which deals with this topic in detail and which will hopefully soon result in a review article. Hereafter I focus on the description of nesting aggregations as a sort of transitional state between the solitariness and sociality and shared features of solitary and social behaviour.

Nesting aggregation

Nesting aggregation represents a very interesting step between the solitary and social life strategy. Although each female usually nests in a separate nest and thus behaves solitary, the females come in contact very frequently, especially when the aggregations are dense (Alcock 1975). Herein, I shortly summarize the most important factors responsible for the formation and maintaining of the nesting aggregations.

The first factor is the lack of suitable nesting sites, which forces the animals to live together in a small patch of the suitable habitat. The relationship between nesting site choice, environmental conditions and fitness has been shown in many studies (e.g. Michener *et al.* 1958, McCorquodale 1989, Potts & Willmer 1997, Hranitz *et al.* 2009). Philopatry represents the second important factor (Brockmann 1979, Kukuk & Dectelles 1986, Yanega 1990). The places suitable for rearing one generation will be highly probably also suitable for the rearing of subsequent generations (Michener 1974). The defence against parasites and predators is the third factor maintaining the nesting aggregations [for more details see Vulinec (1990)]. There are many species of predators that attack bee nests and cause a density dependent mortality (e.g. Lin & Michener 1972). Such forces should thus counteract the forming of nest aggregation. On the other hand, while the aggregations surely increase the predator and parasite concentration, the probability of destroying the particular nest within the nesting aggregation decreases with the increasing aggregation size resulting in existence of inversely density dependent mortality that seems to be an important force for building and maintaining nesting aggregations (Rosenheim 1990).

The size of the nesting aggregations depends on both biotic and abiotic conditions. It is constant or slowly gradually increasing in optimal and stable conditions (Michener 1974). However, the environmental conditions are often suboptimal, which may result in dramatic interseasonal density changes. Bischoff (2003) observed the population of *Andrena vaga* for four nesting seasons and detected dramatic decrease in population density (down to 10% of original size) in one season due to the unfavourable environmental conditions and high pressure of parasite *Bombylius major* L. (Diptera). Similar population fluctuations were also reported in other studies, e.g. by (Lin & Michener 1972, Franzén & Nilsson 2013).

Finally, I should note that the females nesting in aggregations come often into contact with other conspecific females and such contacts are not always peaceful. Dense aggregations are known to enhance different kinds of intraspecific cleptoparasitism. I describe this in a further detail in my thesis, and for this reason all four our model species belong to the aggregating ones.

Shared features of solitary and social behaviour

Most of the bee species are solitary and based on known bee phylogeny (Danforth *et al.* 2013), solitariness is clearly the primitive state in bees. Solitary behaviour seems to be in a deep contrast to the sociality. Solitary species always live independently and thereby lack the advantages of sociality (Wilson 1971). This “simplicity” makes them seemingly and unjustifiably less interesting to the researchers. This is probably the main reason why the detailed studies on nesting behaviour are relatively rare in solitary bees (regarding to their known diversity) compared to the number of studies describing social behaviour in various taxa. Here I want to show that the solitary behaviour should be studied more carefully. The knowledge of this phenomenon is crucial for the

understanding of the rise and evolution of sociality and cleptoparasitic behaviour, as the solitariness must have represented the original state for social evolution in all cases. It is the solitary species where we should search for the preadaptations to social as well as obligate cleptoparasitic behaviour. This kind of reasoning is still rather neglected or, at least, underestimated in the contemporary investigation.

There are only few studies that deal with the relationship between the behaviour of solitary bees and their social relatives. Generally, socially polymorphic taxa (such as many species of the family Halictidae or the genus *Ceratina* Latreille), represent ideal models (e.g. Sakagami & Maeta 1984, Eickwort *et al.* 1996, Yanega 1997, Richards 2000, Rehan *et al.* 2009). These studies showed that the sociality might be promoted by specific biotic and abiotic conditions. For example, in *Lasioglossum malachurum* (Kirby) or *Halictus rubicundus* (Christ) the climate seems to be the major factor inducing social behaviour (Eickwort *et al.* 1996, Richards 2000). Colder or generally less favourable climatic conditions do not enable completing more than one nesting cycle, which results in the existence of solitary nesting life cycles. Climatic factors, thus, might be of a specific importance when searching for the reasons of the secondary return to solitariness (Eickwort *et al.* 1996) that occurred several times independently in Halictidae (Danforth *et al.* 2003a). The density of population might be another factor promoting the sociality as showed by Sakagami & Maeta (1984) who experimentally induced social behaviour in *Ceratina* species by dramatic increase in population density.

The studies that would look at the nesting behaviour of obligate solitary bees from the perspective of the evolution of sociality are generally missing. Our aim was to search for the possible preadaptations by studying several species of solitary bees and we have, indeed, revealed some interesting results related to the presence of intraspecific cleptoparasitism and resulting low level of aggressiveness (Černá *et al.* 2013b). This article is a part of presented thesis (Paper II).

INTRASPECIFIC CLEPTOPARASITISM

Bees and wasps, although solitary, often come into contact with conspecific females, especially in dense aggregations (Alcock 1975). Most of these interactions are connected with nests, because nest construction and provisioning represent a big investment of time and energy. When a female uses an already prepared nest structures or provisions of conspecific female, she can spare much energy and increase her fitness rapidly (Field 1992). However, she risks conflict with the nest owner that can be dangerous because of the presence of a very effective defending mechanism – the sting (Kaitala *et al.* 1990). Intraspecific cleptoparasitism is thus a facultative life strategy which is advantageous under specific conditions, environmental or phenotypic, and females do not parasitize for their entire life (Eickwort 1975). There are several factors influencing the adoption of this facultative strategy such as high density of nests at the nesting aggregation (Field 1992) and low number of sufficient places for nesting (Eickwort 1975), temperature (Field 1989), phenotype (Miller & Kurczewski 1973, Wuellner 1999), food availability (Velthuis 1987), age (Malyshev 1936) or seasonality (Miller & Kurczewski 1973).

Opportunities to parasitize often arise suddenly during non-parasitic nesting and potential parasite must choose between two alternative strategies – behave as a cleptoparasite or continue with non-parasitic nesting (Field 1992). Kaitala *et al.* (1990) showed that facultative intraspecific cleptoparasitism is evolutionary stable strategy and they also demonstrated that decision to adopt cleptoparasitic strategy can be mathematically predicted.

Broom *et al.* (2004) further studied the choice of optimal life strategy (including cleptoparasitism) using mathematical modelling. They showed that intraspecific cleptoparasitism without the defence against it evolves when the effective defence against cheating is costly and the evolutionary struggle is long-term. Intriguingly, the resulting intraspecific tolerance is one of the key assumptions in the evolution of any social behaviour (Wilson 1971), which makes intraspecific cleptoparasitism interesting for investigation of the social evolution in bees.

The definition of the word cleptoparasitism differs among various authors. I use this term according to Field (1992) who distinguishes six various kinds of intraspecific cleptoparasitism, division applicable to all Aculeata:

- 1) Theft of provision outside the nest,
- 2) theft of provision from inside nest,
- 3) usurpation of the nest structure after removal of the host cells,
- 4) discarding (parasite uses only the nest cell and discards all the previous provision),
- 5) nest usurpation,
- 6) brood parasitism (replacement of the egg in the fully provisioned cell).

Intraspecific cleptoparasitism is well-known in eusocial species in the sense of intraspecific social parasitism (Kaitala *et al.* 1990, Wossler 2002, Lopez-Vaamonde *et al.* 2004, Michener 2007). Although the mathematical model by Ward & Kukuk (1998) presumes a high probability of intraspecific cleptoparasitism evolution and, similarly, Field (1992) expects the intraspecific cleptoparasitism to be widespread in solitary and communally nesting Aculeata, the existence of such parasitic behaviour has been documented surprisingly rarely. Anyway, all the six types of intraspecific cleptoparasitism do exist in bees. The first two which are typical of carnivorous Aculeata, are very rare in bees because pollen, contrary to caterpillars or spiders, is more difficult to steal. Nevertheless, stealing of pollen provision from conspecific nests was documented in *Xylocopa* Latreille bees (Ben Mordechai *et al.* 1978) and *Apis mellifera* L. was even observed to steal the pollen directly from the legs of some solitary bees (Thorp & Briggs 1980). The other types of intraspecific cleptoparasitism are more common in bees and have been reported in following bee families: Megachilidae [e.g. *Hoplitis anthocopoides* Schenck (Eickwort 1975), *Megachile pyrenaica* Lepeletier (Fabre 1914), *Osmia tricornis* Latreille (Fabre 1914), *Osmia rufa* (Černá *et al.* 2013b) or *Heriades carinata* Cresson (Mathews 1965)], Apidae [e.g. *Xylocopa sulcatipes* Maa (Velthuis 1987), genus *Diadasia* Patton (Eickwort *et al.* 1977, Neff *et al.* 1982), *Centris bicornuta* Mocsáry (Vinson & Frankie 2000) or *Anthophora plumipes* (Černá *et al.* 2013b)], Colletidae [*Colletes cucularius* (Černá *et al.* 2013b) or *Crawfordapis luctuosa* (Smith) (Jang *et al.* 1996)] and Halictidae [*Dieunomia triangulifera* (Vachal) (Wuellner 1999)]. For the summary see also (Weislo 1987, Field 1992). Below I summarise information concerning usurpations and brood parasitism, since these two types of intraspecific cleptoparasitism are the most common ones in solitary bees and we have focused on them in our own investigation.

Nest usurpation

During the nest usurpation, the usurper steals the nest from the original owner, chases him away, finishes the provisioning and finally oviposits and closes the completed cell (Eickwort 1975).

Usurpations often result from the competition for the available nesting opportunities and are thus most common in bees nesting in natural holes, crevices or stems, because these are often irregularly distributed and locally deficient (Eickwort 1975, Eickwort *et al.* 1981, Barthell & Thorp 1995). Usurpations are also common in primitively eusocial species during the nest founding phase. The nest is most vulnerable in the time before the first workers emerge, because the potential usurper gains not only the nest, but also the future manpower of workers and the motivation for theft is thus high (Kaitala *et al.* 1990).

Usurpation is often started by the nest loss. Nestless female can behave as usurpator, which can activate the cascade of subsequent nestless females and new usurpations at the nesting site (Miller & Kurczewski 1973, Field 1992). The tendency to adopt this particular alternative nesting strategy may also be increased in old individuals at the end of the season, when the time becomes the limiting factor (Malyshev 1936). Usurpation can also represent a common nest founding strategy as was described in *Centris bicornuta* (Vinson & Frankie 2000). The big selective effect of usurpations was demonstrated in *Megachile apicalis* Spinola, where it caused the significant body size enlargement, because only the individuals big enough were successful in the nest protection against usurpators (Barthell & Thorp 1995).

We have detected the presence of usurpations in all studied species of solitary bees from different bee families (Černá *et al.* 2013b) and based on this result we consider the presence of usurpations as a widespread phenomenon in solitary bees. However, we also discovered that many situations that look like usurpation at the first sight are not usurpations (in the sense of forced eviction of the previous owner) but occupations of already abandoned nest. We showed that regular nest abandonments and switches are surprisingly frequent in solitary bees and we suggested possible role of the described nest founding dynamics in the evolution of sociality. This cited article is a part of my thesis as Paper II.

Brood parasitism

Brood parasitism is less common type of intraspecific cleptoparasitism than the usurpation. The successful cuckoo bee has to visit an active nest in the short time between the egg oviposition and egg hatching. Good timing is crucial, because eggs laid before the host oviposition are often discovered and discarded by the host female (Field 1992). Parasitic female always destroys (usually discards or eats) the egg of the host to provide enough food for the development of its own larva, because larva of pollen collecting bees is not adapted to kill the host's larva (Field 1989). Parasitic female usually opens the nest, oviposit her own egg, closes the nest and leaves immediately after she discards the host's egg. Only rarely usurper also adds further pollen load before the oviposition and closing the cell (Eickwort 1975).

Intraspecific brood parasitism seems to be rare and was reported in only few bee species, all from the family Megachilidae: *Chalicodoma pyrenaica* (Fabre 1914), *Heriades carinata* (Mathews 1965), *Hoplitis anthocopoides* (Eickwort 1975) and *Osmia tricornis* (Fabre 1914). *Hoplitis anthocopoides* represents an extreme example of

brood parasitism. (Eickwort 1975) described that more than 50% of females behave cleptoparasitically even though they had their own nests and about 15% of the nests were parasitized by the conspecific females. It is similar to the rate of predation and parasitization of brood cells by all other parasites and predators.

Facultative intraspecific brood parasitism, as the most advanced intraspecific cleptoparasitic strategy, probably evolved into the obligate cleptoparasitism in aculeate hymenoptera (Eickwort 1975). Fifteen percent of bees are interspecific obligate cleptoparasites and this behaviour developed independently sixteen times (Michener 2007, Straka & Bogusch 2007). The question is why the intraspecific brood parasitism is so rarely documented. There are three technical problems explaining the possible causes: i) It is difficult to directly observe the nest cells hidden deep in soil or wood, (ii) there are no simple methods for indirect detection of brood parasitism, (iii) brood parasitism might be almost undetectable, considering occurrence of this behaviour in five percent females or less [see Field (1989)].

The detection of brood parasitism thus requires a methodologically specific approach. We have developed a new methodology combining i) the behavioural observation of individually marked females and their nests, ii) the special method for marking of the pollen brought by each female and iii) analysis of maternity to detect alien offspring in the nest using microsatellites. The proof of the brood parasitism would be a detection of an alien offspring in the marked cell content, which should not belong to the egg lying female. We have tested this methodology during our field studies on *Andrena vaga* and *Anthophora plumipes*. The individual marking worked well and among others it helped us to detect the presence of usurpations mentioned above. There was also no problem with the microsatellite analysis as the microsatellites for *A. vaga* and *A. plumipes* worked well during our studies on population genetics (see below). However, we encountered some difficulties with the marking of pollen brought by the provisioning females.

At first we tested two different marking substances during our studies on *Andrena vaga* - marking with vertebrate immunoglobulin G (IgG) proteins described first by Hagler *et al.* (1992) and marking with adhesive fluorescent dyes (powders) detectable in UV light (Foltan & Konvicka 2008). Both of these methods worked, however with quite low efficiency because of low concentration and ambiguity of the mark. That is why we then decided to use a very accurate and sensitive method of elemental marking (using rare or trace elements - lanthanides). This technique is based on marking by rare earth elements and its detection by inductively coupled plasma optical emission spectrometry (ICP-OES). This method works well and we have successfully used it for the laboratory study of the sugar consumption during bumblebee development (Řehoř *et al.* 2013). However, when using this marking method during our field studies we struggled with the problem how to efficiently apply the mark on the bees bringing the pollen. This unfortunately strongly decreased the reliability and effectiveness of this method for our purposes. Further field marking experiments need to be performed to find the optimal way of marker application.

Population genetics

SPECIFIC ASPECTS OF BEE GENETICS

Bees as well as the other groups of Hymenoptera possess many uncommon genetic features that we do not find (or only rarely) in other groups of animals. I do not aim to deal with all bee genetic characteristics in detail, because there are several excellent reviews about this topic (e.g. Crozier 1977, Hedrick & Parker 1997, Pamilo *et al.* 1997), but I only shortly highlight the most important aspects – the haplodiploidy and complementary sex determination (CDS).

There are several genetic systems in insects [for summary see Normark (2003)]. Bees are famous for their arrhenotoky, which means that females are produced sexually from fertilized eggs, while males are produced asexually from unfertilized eggs resulting in haplodiploid character of these organisms. Arrhenotoky has several independent origins and is further known from Nematoda, Thysanoptera (thrips), Rotifera, Arachnida (mites and ticks), Sternorrhyncha (scale insects) or Coleoptera (bark beetles) (Hedrick & Parker 1997, Heimpel & de Boer 2008). There are several possible ways, how to achieve haplodiploidy and CDS represents one of them [for review on different sex determining systems in Hymenoptera see Heimpel & de Boer (2008)]. In CDS sex is determined by genotype at a single gene. Heterozygotes at the sex-determination locus develop into females, while hemizygotes (or homozygotes) develop into males. The CDS is recently known only in 4 from 21 Hymenoptera superfamilies (Apoidea, Vespoidea, Ichneumonoidea and Tenthredinoidea) and based on the phylogenetic distribution it probably represents the original sex determination system in Hymenoptera (Cook & Crozier 1995, Heimpel & de Boer 2008). However, this sex determination system has its drawbacks. In case of decreased overall genetic diversity (and also diversity in sex-determinant locus), which is recently reported in many species (see below), this system produces nonviable or sterile diploid males homozygous at the sex-determination locus [but see Cowan & Stahlhut (2004)], which further endangers the populations by inbreeding depression (Paxton *et al.* 2000, Zayed 2009).

Haplodiploid nature of bees has several important consequences for their genetics, which are different from that of diplodiploids and which have implications to bee conservation (see below). Haplodiploid genetics is in fact very similar to the genetics of X-linked genes of *Drosophila* sex determination system. Hedrick & Parker (1997) reviewed various similarities of these two systems and they pointed out that with equal sex ratios, the effective population size (N_e) for haplodiploids or X-linked genes is only 3/4 that of diploids. Moreover, based on the experimental evidence, the molecular variation in haplodiploids compared to diplodiploids is even less than that predicted from the differences in their effective population size. Packer & Owen (2001) also showed much lower genetic variation in Hymenoptera in their comparative study of Hymenoptera and Lepidoptera. Hedrick & Parker (1997) further showed that the genetic load for X-linked genes is much less than for autosomal genes and similarly it is much less for haplodiploids than for diploids. It is caused by the effect of selection on haplodiploid populations. Selection operates approximately one third faster in haplodiploids or X-linked genes than in diploids for all levels of dominance, because both favourable and deleterious alleles are directly exposed to selection in males for haplodiploids and X-linked genes. This also has consequences for maintaining the genetic variability or for the influence of inbreeding depression (i.e. reduction in fitness of inbred offspring relative to progeny from unrelated parents).

Because the deleterious alleles are purged regularly in haploid males, haplodiploids should suffer less from inbreeding depression [caused by increased homozygosity and related deleterious alleles expression (Hedrick & Parker 1997, Zayed 2009)]. Henter (2003) analysed the data from the literature and he indeed showed that haplodiploids generally suffer less from inbreeding depression than diplodiploids, when they are exposed to the effect of inbreeding. Nevertheless, he [and Zayed (2009)] also showed that the inbreeding depression still occurs in haplodiploids (probably because of the effect of overdominance and CDS or dominance in female limited genes) and haplodiploidy thus does not provide complete protection from the effect of inbreeding depression (Zayed 2009).

Although haplodiploidy brings some disadvantages, it also enables rise and existence of complex sociality, which makes Hymenoptera one of the ecologically most successful insect orders on Earth (Wilson 1971, Wilson & Hölldobler 2005). Although eusociality is also known in diploid species (such as termites or mole rats), the eusocial societies are dominated by species with haplodiploid genetics (Gardner *et al.* 2012). The role of haplodiploidy in the evolution of sociality is popular scientific question since Hamilton's formulation of kin selection theory (Hamilton 1964a, 1964b, 1972) and several different theories and their adjustments have been proposed since that time. Recently, even the possible role of long forgotten group selection theory is revisited (Wilson & Hölldobler 2005, Nowak *et al.* 2010). Unfortunately this topic is far too extensive and is thus not in scope of this short introduction on bee population genetics. Instead, I recommend the following articles that nicely summarise the ideas and progress in the study on the social evolution (Trivers & Hare 1976, Grafen 1986, Wilson & Hölldobler 2005, Nowak *et al.* 2010, Herre & Wcislo 2011, Gardner *et al.* 2012).

CONSERVATION GENETICS

Bee conservation is a very popular topic nowadays. The large number of published studies and reviews report the threat of global loss of bee diversity and discuss the causes and consequences of a potential global pollinator crisis (e.g. National Research Council 2006, Brown & Paxton 2009, Brown 2011), although recently there are indices that the decline has hopefully slowed down (Carvalho *et al.* 2013). However, we still need more studies on ecology [for the review on the role of ecology in bee conservation see Murray *et al.* (2009)], population genetics or behaviour of particular endangered and vulnerable bee species, because this information is crucial for their effective conservation. Hereafter I will shortly mention only a few most important factors that threaten bee populations, although I am aware that there are several others (such as effects of pesticides and other pollutants, invasive species or introduction of new parasites, predators or diseases).

One of the most important environmental factors causing bee decline are habitat degradation and fragmentation caused by extensive agriculture and recent large-scale landscape changes (e.g. Cane 2001, Kremen *et al.* 2002, 2007, Müller *et al.* 2006, Klein *et al.* 2007, Davis *et al.* 2010). Of course, suitable nesting substrates and food sources for bees are invariably patchy and it has been shown that small scale heterogeneity promotes the bee diversity (Schüepp *et al.* 2012). Bees commonly forage for several hundreds of metres [summarised by Gathmann & Tscharnkte (2002)] up to few kilometres in case of bumblebees and honeybees (Steffan-Dewenter & Kuhn 2003, Osborne *et al.* 2008), so patchiness on such scale is definitely not a problem for bees. Many bee conservation managements are thus based on the increasing of heterogeneity in our

rather homogenous agricultural landscape (Schüepp *et al.* 2012, Kennedy *et al.* 2013). On the other hand, large scale habitat fragmentation dramatically reduces not only the number of species and their population densities, but also levels of genetic variation in natural populations, because it creates separated populations from a formerly continuous distribution. This dramatically decreases the overall N_e and thus also genetic variability proportional to N_e (Gilpin 1991). Without frequent migration, these separated populations keep losing their genetic diversity through bottlenecks and drift (Frankham & Briscoe 2010). Franzén & Nilsson (2010, 2013) studied the metapopulation dynamics of solitary bee populations and showed that particular subpopulation sizes fluctuated dramatically and the whole metapopulation showed typical sink-source dynamics, with the distance from the persistent source population and its size being the important predictor of patch occupancy. Moreover, for the survival of the whole metapopulation, large pollen plant populations proved to be essential and more important than the actual bee population size. Moreover, various pollinators respond differently to the landscape changes, which shows the necessity to investigate the entire pollinator community to properly evaluate the true effects of landscape changes on bee communities (Nielsen *et al.* 2012).

Except from the landscape changes, recent global warming and other climate changes belong to another often discussed threatening factors. The warming can influence organismal life histories in various ways which were summarized by many reviews (e.g. Hughes 2000, Walther *et al.* 2002, Parmesan 2006). Regarding the bees, flowers can start blooming earlier and this may influence bee phenology (Bartomeus *et al.* 2011). However, it has also been shown that several plant species are not easily able to alter their phenology according to the climate change, which makes them exceptionally vulnerable in the case of further temperature shifts (Cleland *et al.* 2012). The climatic changes may also play crucial role in the shifts of bee distribution [for many examples see Parmesan (2006)], which are nowadays also common in bees. Bogusch *et al.* (2007) reported 115 new species of Aculeata in the Czech Republic since the year 1989, most of which immigrated from south or southeast Europe. The higher temperature can also directly or indirectly promote many physiological changes such as shift in foraging activity, body size, development time, mortality or lifespan (Straka *et al.* in prep., Radmacher & Strohm 2011). The summary of different effects of elevated temperatures on the physiology of flowering plants and insect pollinators is given by Scaven & Rafferty (2013).

Scientists have traditionally assumed that species are driven to extinction by environmental stochastic factors before genetic factors have enough time to influence them. However, there is growing evidence nowadays that genetic factors themselves play an important role in species decline (Spielman *et al.* 2004, Hanski & Saccheri 2006). Frankham (2005) summarized the role of genetic aspects in species extinction and he especially enhanced the role of inbreeding depression [for a detailed study on inbreeding depression see O’Grady *et al.* (2006)] and loss of genetic diversity. Packer & Owen (2001) and Zayed (2009) reviewed the role of various genetic aspects that are known to be relevant in bee decline and conservation. They showed that bees suffer not only from the genetic features typical for bees (such as haplodiploidy, CSD or lower N_e and genetic diversity of haplodiploids), but also from the effect of inbreeding depression, which is common conservation problem in diploids species (Brook *et al.* 2002). Chapman & Bourke (2001) further showed that sociality could be another threatening factor, because it results in further reducing N_e , increasing population

genetic subdivision and reducing levels of genetic variation relative to solitary species. The knowledge of the background genetics, thus, seems to be of the same importance as the knowledge of the different ecological factors that influence the probability of successful bee conservation.

Conservation genetics in solitary bees

Because my research is focused on solitary bees, I shortly summarize the genetic studies on solitary bees that are relevant for the bee conservation. Solitary bees represent very important element of most functional terrestrial ecosystems and we have evidence that they may be even more efficient pollinators than bumblebees or honey bees (Woodcock *et al.* 2013). Because of the recent environmental changes, many solitary bee populations became more fragmented, which decreases their ability to adapt to environmental changes (Zayed 2009) and also makes them more vulnerable to genetic problems such as increased production of diploid males due to low genetic diversity and N_e (Zayed *et al.* 2004).

Bees feed on pollen and nectar and are often specialized in their food source. Moreover, many bees also need specific nesting conditions. Specialist bees seem to suffer from the negative effects of habitat fragmentation more than generalists (Cane *et al.* 2006, Biesmeijer *et al.* 2006). Several recent studies imply that specialists (food, nesting or both) live in smaller populations where the gene flow barriers given by the lack of suitable habitats or food sources result in a stronger genetic structure and smaller local gene diversity than in generalists (Danforth *et al.* 2003b, Packer *et al.* 2005, Zayed *et al.* 2005, Neumann & Seidelmann 2006, Zayed & Packer 2007, Davis *et al.* 2010).

On the other hand, we could expect that specialised species should be well adapted to the sparse and unstable occurrence of their food or suitable nesting places by having either a higher dispersal ability, which could compensate for local extinctions, or a higher effective population size (Peterson & Denno 1998, Exeler *et al.* 2008). Furthermore, specialisation most likely does not threaten easily dispersing species when their food resources are generally sufficient or when the scale of patchiness is negligible (Peterson & Denno 1998). In accordance with this alternative hypothesis, strong gene flow and resulting genetic admixture are often reported in good fliers such as bumblebees (Chapman *et al.* 2003) and also in specialized bee species *Andrena vaga* and *Andrena fuscipes* that are probably well adapted to the natural large scale patchiness of their habitats (Exeler *et al.* 2008, 2010).

We have studied the population structure of *Andrena vaga* in the heterogeneous environment of the Czech Republic (Černá *et al.* 2013a) and our results again confirmed good dispersal ability of this species, although two differentiated subpopulations, separated by a wide clinal zone of admixture, were detected within the study area. This article is part of my thesis (Paper III).

MICROSATELLITES IN SOLITARY BEE RESEARCH

Microsatellites (or simple sequence repeats - SSRs) are short DNA sequences consisting of tandem repeat motifs usually 1-6bp long. The microsatellites belong to the most widely used genetic markers in molecular ecology because they are codominant, highly variable and most importantly because they are neutral. However, their usage has several limitations and drawbacks. The most important one is the need for their de novo development for each species studied, which used to be time consuming and costly (Zane *et al.* 2002). However, nowadays with the fast development of next genera-

tion sequencing methods, microsatellite development is becoming faster, more efficient and cheaper, resulting in increasing number of molecular ecology studies on non-model organisms.

I have resumed all the published articles on microsatellite development and their application for solitary, communal and facultatively eusocial bee species (Table 1).

Msat developed by	Species	Family		Msat application
Paxton <i>et al.</i> (1996)	<i>Andrena carantonica</i>	Andrenidae	c	Paxton <i>et al.</i> (1996, 2000)
Mohra <i>et al.</i> (2000)	<i>Andrena vaga</i>	Andrenidae		Exeler <i>et al.</i> (2008), Černá <i>et al.</i> (2013a)
Danforth <i>et al.</i> (2003b)	<i>Macrotera portalis</i>	Andrenidae	c	Danforth <i>et al.</i> (2003b)
Beveridge & Simmons (2004)	<i>Amegilla dawsoni</i>	Apidae		Beveridge & Simmons (2006)
Černá & Straka (2012)	<i>Anthophora plumipes</i>	Apidae		
Azuma <i>et al.</i> (2005)	<i>Ceratina flavipes</i>	Apidae		
Paxton <i>et al.</i> (2009)	<i>Euglossa annectans</i>	Apidae		Zimmermann <i>et al.</i> (2009), Souza <i>et al.</i> (2010)
López-Urbe <i>et al.</i> (2011)	<i>Eulaema meriana</i>	Apidae		
Souza <i>et al.</i> (2007)	<i>Eulaema nigrita</i>	Apidae		Zimmermann <i>et al.</i> (2009), Souza <i>et al.</i> (2010)
	<i>Euglossa cordata</i>	Apidae		
Langer <i>et al.</i> (2004)	<i>Exoneura nigrescens</i>	Apidae	*	Langer <i>et al.</i> (2006)
	<i>Exoneura robusta</i>	Apidae	*	
Augusto <i>et al.</i> (2012)	<i>Xylocopa frontalis</i>	Apidae		
López-Urbe <i>et al.</i> (2012)	<i>Colletes inaequalis</i>	Colletidae		
Kukuk <i>et al.</i> (2002)	<i>Lasioglossum hemichalceum</i>	Halictidae	c	Kukuk <i>et al.</i> (2005)
Zayed (2006)	<i>Lasioglossum leucozonium</i> ,	Halictidae		Zayed <i>et al.</i> (2007)
	<i>Lasioglossum oenotherae</i>	Halictidae		Zayed & Packer (2007)
Kapheim <i>et al.</i> (2009)	<i>Megalopta genalis</i>	Halictidae	*	Kapheim <i>et al.</i> (2013)
Soro & Paxton (2009)	<i>Halictus rubicundus</i>	Halictidae	*	Soro <i>et al.</i> (2010)
Neumann & Seidelmann (2006)	<i>Osmia rufa</i>	Megachilidae		Neumann & Seidelmann (2006)

Table 1: List of the studies on the microsatellite development and their application in solitary, communal and facultative eusocial species. * - facultatively eusocial species, c – communal species.

The developed microsatellites were used for various purposes. They can be used for the description of population structure of rare, endangered and/or specialised species (Danforth *et al.* 2003b, Beveridge & Simmons 2004, Zayed & Packer 2007, Exeler *et al.* 2010, Černá *et al.* 2013a), useful pollinators (Neumann & Seidelmann 2006) or introduced species (Zayed *et al.* 2007). Microsatellite analysis also showed that the population structure of species with social polymorphism does not depend on the sociality level (Soro *et al.* 2010). The second group of studies is focused on the intranidal relatedness, social structure and mating systems in bees. Zimmermann *et al.* (2009) and Souza *et al.* (2010) studied solitary Euglossini and described single mating and only rare presence of diploid males. Further studies deal with communal or facultative eusocial species. Kapheim *et al.* (2013) described the sociogenetic structure of facultative eusocial *Megalopta genalis* Meade-Waldo. Langer *et al.* (2006) studied the reproductive skew in facultative eusocial *Exoneura robusta* Cockerell and showed that it is negatively correlated with relatedness. Paxton *et al.* (1996, 2000) studied communal species *Andrena carantonica* Pérez (*A. jacobii*, *A. scotica*) and they reported low overall intranidal relatedness but also increased production of diploid males due to presence of intranidal mating and resulting inbreeding (Paxton & Tengö 1996). Finally, Kukuk *et al.* (2005)

studied communal species *Lasioglossum hemichalceum* Cockerell. They compared the brood composition at the end of the nesting season with the situation later after the end of the season. They proved a female biased dispersal of adults at the end of the season, which prevents inbreeding but also decreases the overall intracolony relatedness in the population. Both Kukuk *et al.* (2005) and Paxton *et al.* (1996) thus showed that kin selection probably plays only minor role in the communal societies.

We have developed a set of microsatellites for *Anthophora plumipes* using 454 pyrosequencing during my research (Černá & Straka 2012) and this article is a part of my thesis (Paper IV). We have further successfully developed microsatellite markers for *Ceratina nigrolabiata* Friese, the species we are studying now in detail. We are going to prepare a publication about these microsatellites next year.

The developed microsatellites for *A. plumipes* were used for our preliminary study on phylogeography of this species in the whole distribution range. The study has unfortunately not been finished yet because collection of further material is needed. However, I decided to present at least our preliminary results in my thesis in a form of short manuscript (Paper VI). These data were presented as a poster on XXIV International Congress of Entomology 2012 in Daegu (South Korea).

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SUMMARY OF PAPERS

Paper I:

Analysis of nesting behavior based on daily observation of *Andrena vaga* (Hymenoptera: Andrenidae)

Rezkova, K., Žáková M., Žáková Z. & Straka J. (2012)

Journal of Insect Behavior 25: 24-47

We present a study on nesting behavior of the gregarious solitary bee, *Andrena vaga* Panzer. Based on the daily observation of individually marked females, we constructed an ethogram, determined a sequence of behavioral elements within the provisioning cycle, estimated their length and computed the transition probability between the elements. We confirmed the existence of distinctive pollen and nectar days in *A. vaga* and showed apparent differences in the overall daily provisioning pattern in pollen and nectar days as well as in the probability of transition between some behavioral elements. Bees typically performed one provisioning trip and carried no pollen on nectar days, but they performed up to four pollen-provisioning trips on pollen days. The duration of one pollen trip depended on the number and sequence of the trip in a given day, with the shortest trip usually occurring last in the day.

Paper II:

Neighbourhood society: nesting dynamics, surptations and social behaviour in solitary bees

Černá K., Zemenová M., Macháčková L., Kolínová Z. & Straka J. (2013)

PLoS ONE 8: e73806

Intraspecific cleptoparasitism represents a facultative life strategy advantageous for saving time and energy. However, only a few studies about nesting dynamics have described intraspecific cleptoparasitic behaviour in obligate solitary bees. We focused on nesting dynamics with the characterisation of nest usurpation in four aggregating species belonging to different phylogenetic lineages - *Andrena vaga* (Andrenidae), *Anthophora plumipes* (Apidae), *Colletes cunicularius* (Colletidae) and *Osmia rufa* (Megachilidae). Our study, based on the regular observation of individually marked females, shows that nest owner replacement affect 10-45% of nests across all of the studied species and years. However, 39-90% of these nests had been, in fact, abandoned before owner change and thus true nest usurpations represent only a part of observed nest replacement cases. Females tend to abandon their nests regularly and found new ones when they live long enough, what is in accordance with risk-spreading strategy. We suggest that the original facultative strategy of observed solitary bees is not cleptoparasitism per se but rather reusing of any pre-existing nest (similar to “entering” strategy in apoid wasps). This is supported by gradual increase of nests founded by “entering” during the season with an increase in the number of available nests. Although the common reuse of conspecific nests results in frequent contact between solitary females, and rarely, in the short-term coexistence of two females in one nest, we detected unexpectedly low level of conflict in these neighbourhood societies. We suggest that nesting dy-

namics with regular nest switching and reusing represent a basic driving force to reduce long-term and costly intraspecific aggression, a key factor for the origin and evolution of sociality.

Paper III:

Population structure of pioneer specialist solitary bee *Andrena vaga* (Hymenoptera: Andrenidae) in central Europe: the effect of habitat fragmentation or evolutionary history?

Černá K., Straka J. & Munclinger P. (2013)

Conservation Genetics 14: 875–883

Because patchiness of food sources or nesting opportunities frequently limits gene flow, specialists often exhibit distinct population structures in fragmented habitats. We studied the influence of habitat fragmentation on population structure in the solitary bee *Andrena vaga*, an early spring species that nests exclusively in sandy soil and feeds strictly on willows (*Salix* spp.). Because the homogenous habitat of the German floodplains, where the species was studied previously, resulted in the species' weak population structure, we expected more structured populations in central Europe, where the sandy soils essential for nesting are highly fragmented. We analysed 387 females from 21 localities in the Czech Republic and Slovakia using nine microsatellite loci, and we inferred population structure using landscape genetics and Bayesian clustering methods. Contrary to our expectations, habitat fragmentation did not result in increased genetic isolation at the localities; however, two differentiated groups of localities, separated by a wide clinal zone of admixture, were detected within the study area. The observed pattern suggests that dispersive ability of *A. vaga* compensates the species' dependence on unstable fragmented habitats. We propose that the population structure may mirror a secondary contact formed by the expansion of two populations that had been separated in the past. We emphasise the necessity of knowing the studied species' population history before making conclusions concerning correlations between habitat and population structure, especially in areas of known suture zones created by the secondary contact of populations expanding from separate refugia.

Paper IV:

Identification of 37 microsatellite loci for *Anthophora plumipes* (Hymenoptera: Apidae) using next generation sequencing and their utility in related species

Černá K. & Straka J. (2012)

European Journal of Entomology 109: 155–160

Novel microsatellite markers for the solitary bee, *Anthophora plumipes*, were identified and characterised using 454 GS-FLX Titanium pyrosequencing technology. Thirty seven loci were tested using fluorescently labelled primers on a sample of 20 females from Prague. The number of alleles ranged from 1 to 10 (with a mean of 4 alleles per locus), resulting in an observed heterozygosity ranging from 0.05 to 0.9 and an expected heterozygosity from 0.097 to 0.887. None of the loci showed a significant deviation from the Hardy-Weinberg equilibrium and only two loci showed the significant presence of

null alleles. No linkage between loci was detected. We further provide information on a single multiplex PCR consisting of 11 of the most polymorphic loci. This multiplex approach provides an effective analytical tool for analysing genetic structure and carrying out parental analyses on *Anthophora* populations. Most of the 37 loci tested also showed robust amplification in five other *Anthophora* species (*A. aestivalis*, *A. crinipes*, *A. plagiata*, *A. pubescens* and *A. quadrimaculata*). The result of this study demonstrates that next generation sequencing technology is a valuable method for isolating quality microsatellites in non-model species of solitary bees.

Paper V:

Lifespan in the wild: the role of activity, climate and parasitic infestation in natural populations of bees

Straka J., Černá K., Macháčková L., Zemenová M. & Keil P.

Submitted manuscript

Animal lifespan depends on ecological and physiological constraints. The latter has been studied under controlled conditions but little is known about determinants of lifespan under natural conditions. We studied the factors determining the relationships between length of adult life, foraging activity and environmental conditions (weather) in natural populations of insects on model species of solitary bees *Andrena vaga* and *Anthophora plumipes*. Our research indicates that lifespan is driven both directly by climate and indirectly through climate-dependent activity patterns. We found a negative relationship between proportion of active days and length of life; in contrast, high activity rate within these active days had no negative effect on longevity. Also, individuals activating during warm and/or wet days lived longer, with precipitation being more important determinant of lifespan than temperature. Timing of emergence is another important predictor of length of life - bees that emerged nearer to the end of season (critical time-horizon) lived shorter than bees that emerged earlier. Seasonal timing of emergence was correlated with seasonal temperature and with Strepsiptera endoparasite infestation (*Andrena* only) - bees that emerged nearer to the end of season (critical time-horizon) lived shorter than bees that emerged earlier. Finally, we found that the early emergence of parasitized individuals, not their reduced activity, explains their prolonged lives. We demonstrate that lifespan and activity patterns of wild insect populations are regulated by a tractable interplay of ecological factors which were previously studied only in isolation or in vitro.

Paper VI:

Multilocus phylogeography of a widespread Palearctic solitary bee *Anthophora plumipes* (Hymenoptera: Apidae): Mediterranean lineage endemism, island effects and cryptic species

Černá K., Munclinger P., Vereecken N. J. & Straka J.

Manuscript based on the poster presentation on the XXIV ICE 2012 in Daegu

Anthophora plumipes is a solitary bee species common across the whole Europe, Northern Africa, Middle East and Eastern Asia. *A. plumipes* has recently been introduced to the eastern coast of the USA from Japan and is nowadays a common species there. We

collected samples from 41 localities including samples from USA and Japan. Our aim was to estimate the phylogeographic pattern within species range. We used 11 microsatellite loci and 727-bp sequence of COI for the analysis. We detected 67 COI haplotypes clustering into six major haplotype groups showing strong geographic pattern. Presence of several unique diverged Mediterranean haplotypes indicates high level of Mediterranean endemism. Bayesian clustering analysis of microsatellite data supported the COI groups, however the most likely result of $K=4$ joined the COI groups number I, III and IV together, while it detached the British localities from the continental group (I) to a separate group. We detected low genetic diversity and N_e in British and especially the USA and Japanese localities compared to European ones which can be attributed to bottleneck events during the island colonisation. Japanese and the USA populations are closely related and represent a separate lineage of possible species status.

PAPER I

ANALYSIS OF NESTING BEHAVIOR BASED ON DAILY OBSERVATION
OF *ANDRENA VAGA* (HYMENOPTERA: ANDRENIDAE)

Rezková, K., Žáková M., Žáková Z. & Straka J. (2012)

Journal of Insect Behavior 25: 24-47

Original paper on pages 44-68 available at:

<http://link.springer.com/article/10.1007/s10905-011-9274-8>

PAPER II

NEIGHBOURHOOD SOCIETY: NESTING DYNAMICS, USURPATIONS AND SOCIAL
BEHAVIOUR IN SOLITARY BEES

Černá K., Zemenová M., Macháček L.,
Kolínová Z. & Straka J. (2013)

PLoS ONE 8: e73806

Neighbourhood Society: Nesting Dynamics, Usurpations and Social Behaviour in Solitary Bees

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Abstract

Intraspecific cleptoparasitism represents a facultative strategy advantageous for reducing time and energy costs. However, only a few studies about nesting dynamics have described intraspecific cleptoparasitic behaviour in obligate solitary bees. We focused on nesting dynamics with the characterisation of nest owner replacements and frequency of true usurpation in four aggregating species belonging to different phylogenetic lineages – *Andrena vaga* (Andrenidae), *Anthophora plumipes* (Apidae), *Colletes cunicularius* (Colletidae), and *Osmia rufa* (Megachilidae). Our study, based on the regular observation of individually marked females, shows that nest owner replacement affects 10–45% of nests across all of the studied species and years. However, 39–90% of these nests had been abandoned before owner change and thus true nest usurpations represent only a part of observed nest replacement cases. Females tend to abandon their nests regularly and found new ones when they live long enough, which is in accordance with risk-spreading strategy. We suggest that the original facultative strategy of observed solitary bees during nest founding is not cleptoparasitism per se but rather reuse of any pre-existing nest (similar to “entering” strategy in apoid wasps). This is supported by gradual increase of nests founded by “entering” during the season with an increase in the number of available nests. Although the frequent reuse of conspecific nests results in frequent contact between solitary females, and rarely, in the short-term coexistence of two females in one nest, we detected unexpectedly low level of conflict in these neighbourhood societies. We suggest that nesting dynamics with regular nest switching and reusing reduces long-term and costly intraspecific aggression, a key factor for the origin and evolution of sociality.

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Introduction

Social behaviour is a widely studied phenomenon, fascinating researchers for its cooperative principle, presence of communication, rise of intraspecific tolerance and hidden conflicts [1]. In comparison to social species, the social aspects of obligate solitary species are not commonly studied in detail. Solitary species nesting in aggregations represent one of the exceptions. Hymenoptera often nest in aggregations because places with optimal nesting conditions are limited and/or living in aggregations provide protection from parasites and predators. Here, we do not deal with all the possible factors influencing the rise of aggregations in solitary bees as there are comprehensive reviews on this topic (e.g., [2–6]), but we look at the aggregations as a potential prior state for the evolution of sociality [7]. Although the significance of nesting aggregations in the evolution of bee sociality has been acknowledged in the literature [7], behaviour of their members is usually considered rather simple and social aspects of such behaviour are often neglected: “If a number of organisms are close together, yet do not influence one another, one may speak of the group as an aggregation but not as a society” [8].

Females nesting in aggregations, although solitary, often come into contact with other conspecific females [9]. Most of these

interactions are associated with the nest because constructing and provisioning a nest always requires an investment of time and energy. Such investment is prone to be exploited by cleptoparasitic females, that can either steal the provision from outside or inside of the nest, usurp the nest structure after removing the host cells, discard the provision and use the empty nest cells, usurp the nest including provision or parasitize the brood (= cuckoo behaviour) [10]. When a female uses an already constructed nest structure, supplies or brood cells, she can spare much energy and time and can increase her fitness significantly [10]. However, such a cleptoparasitic female must enter the active nest of conspecific female and she thus risks a conflict with the nest owner, which can be in case of aculeate Hymenoptera very dangerous because of an efficient weapon – the sting [9–11].

Intraspecific cleptoparasitism is closely associated with nest-founding dynamics. Every time a female founds a nest, she can decide either to found and provision a new nest or to parasitize the nest of a conspecific female [12]. Intraspecific cleptoparasitism (sensu Field [10]) thus typically represents a facultative strategy that is advantageous under certain conditions [13]. Ward and Kukuk [14] showed that nest usurpation in the solitary phase of some species of primitively eusocial genus *Lasioglossum*, when the social species does not behaviourally differ from its solitary

relatives, can represent an evolutionarily stable strategy, with different factors triggering the cleptoparasitic behaviour, such as the probability of nest disturbance or death during provisioning, the soil quality or the owner-usurper motivation asymmetry. The probability of adopting a cleptoparasitic strategy might further depend on other factors, such as the availability of suitable nesting places [13], food availability [15], time of the day and temperature [12] or phenotype [16,17].

Brockmann and Dawkins and Brockmann et al. [18,19] studied nest founding strategies in the solitary apoid wasp *Sphex ichneumoneus* in detail. Interestingly, these authors showed that *Sphex ichneumoneus* individually chooses between strategies of “digging” (constructing a completely new nest) and “entering” (inhabiting any existing nest) during each nest founding. Their model implies that usurpations and the incidental cohabiting of one nest by two females (commonly interpreted as nestmate joining) might be only by-products of not being able to distinguish the active nests from abandoned ones when using the “entering” strategy, at least in this species. It represents an interesting addition to the condition-based cleptoparasitic decision making during nest founding described above.

As mentioned above, Field [10] distinguished six types of intraspecific cleptoparasitism that are applicable to all of the aculeate Hymenoptera. Intraspecific cleptoparasitism is common in eusocial bee species during the solitary phase in form of usurpations [15,20,21] or as a form of intraspecific social (brood) parasitism [22,23]. In solitary bee species, nest usurpation with or without the discarding and brood parasitism (cuckoo behaviour) had been documented only in several species from four bee families - the Megachilidae [13,24–27], the Apidae [28–30], the Colletidae [31] and the Halictidae [17] (see also [10,32]).

Although Michener [8] noted that nest usurpations are “not uncommon” for solitary and other bees, this is not supported by published data in our opinion. Therefore, we decided to focus on the nesting dynamics and characterisation of nest usurpations in most of the bee families from the main phylogenetic lineages (Apidae, Andrenidae, Colletidae and Megachilidae) in detail. Because we recognised that true usurpations represent only one of the possible situations that occur during owner change within the nest, we decided to use the more general term nest owner replacement instead of traditionally used term usurpation for any detected ownership change in the nests. The nest owner replacements were detected by individual marking and regular daily monitoring of marked individuals and their nests. The frequent contact given by high frequency of nest owner replacements and low aggressiveness detected among individuals

in these neighbourhood societies show the possible evolutionary significance of intraspecific cleptoparasitism in solitary bees for the evolution of sociality, because described situation within the principle lineages of obligate solitary bees might represent an analogy to the behaviour of solitary species at the beginning of social evolution.

Methods

This study is based on the observations of four univoltine spring species from four bee families (Hymenoptera: Apoidea): *Andrena vaga* (Andrenidae), *Anthophora plumipes* (Apidae), *Colletes cunicularius* (Colletidae) and *Osmia rufa* (Megachilidae). Studied species considerably differ in nest construction. While *A. vaga* and *C. cunicularius* built deep nests in sandy soil, *A. plumipes* dug shallow nests in dry soil protected from the rain (in our case under the staircase) and *O. rufa* nested in pre-existing cavities in the wall, where it brought mud as construction material. All of the species were observed for 1–3 entire nesting seasons in 2007–2010 at different localities in the Czech Republic (Table 1). All the sites are located outside national parks or any other protected areas, no specific permissions were thus required for the behavioural

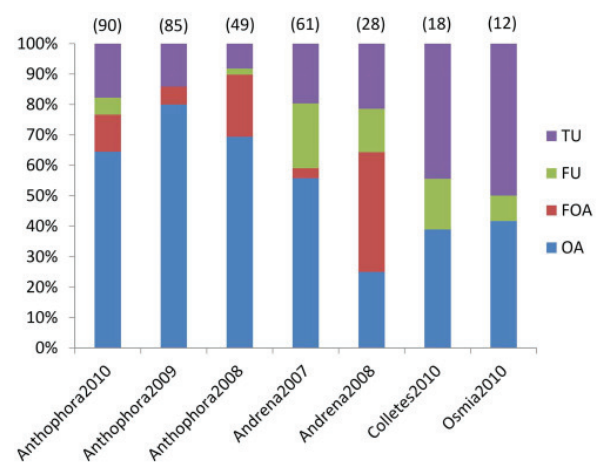


Figure 1. Proportions of different situations during nest owner replacement. The figure shows proportion in different seasons and species. TU – true usurpation, FU – failed usurpation, FOA – failed occupation of abandoned nest, OA – occupation of abandoned nest. doi:10.1371/journal.pone.0073806.g001

Table 1. Summary of the observation period, locality and number of observed females used for behavioural observations in different seasons.

Species	Date	Locality	GPS	N
<i>Andrena vaga</i>	23.3.–25.4.2007	Čelákovice	50°11'0.5"N, 14°46'13.9"E	381
<i>Andrena vaga</i>	28.3.–10.5.2008	Čelákovice	50°11'0.5"N, 14°46'13.9"E	232
<i>Anthophora plumipes</i>	3.4.–2.6.2008	Praha - Strahov	50°4'47.7"N, 14°23'32.7"E	83
<i>Anthophora plumipes</i>	20.4.–28.5.2009	Praha - Strahov	50°4'47.7"N, 14°23'32.7"E	97
<i>Anthophora plumipes</i>	7.4.–9.6.2010	Praha - Strahov	50°4'47.7"N, 14°23'32.7"E	143
<i>Osmia rufa</i>	8.6.–24.6.2010	Praha - Černý Most	50°6'08.3"N, 14°34'14.4"E	26
<i>Colletes cunicularius</i>	30.3.–27.4.2010	Čelákovice	50°10'26.1"N, 14°45'23.1"E	103

N – number of individually marked observed females.
doi:10.1371/journal.pone.0073806.t001

Table 2. Quantification and proportions of different situations during usurpation of the nests in different seasons and species (OA = occupation of abandoned nest, FOA – failed occupation of abandoned nest, TU – true usurpation, FU – failed usurpation, DI – disappear, MO – move, NL – nest-less, NLMO – nest-less for a few days then move, PE – persist in the original nest).

Type of usurpation	Owner after usurpation	<i>Anth</i> 2010	<i>Anth</i> 2009	<i>Anth</i> 2008	<i>Andr</i> 2007	<i>Andr</i> 2008	<i>Colletes</i> 2010	<i>Osmia</i> 2010
OA	DI	22	11	15	16	1	2	5
OA	MO	15	34	10	12	0	5	0
OA	NL	20	8	9	1	3	0	0
OA	NLMO	1	15	0	5	3	0	0
	sumOA	58	68	34	34	7	7	5
FOA	DI	1	1	7	0	1	0	0
FOA	MO	2	4	2	2	1	0	0
FOA	NL	7	0	1	0	7	0	0
FOA	NLMO	1	0	0	0	2	0	0
	sumFOA	11	5	10	2	11	0	0
FU	NL	2	0	0	0	0	2	0
FU	DI	1	0	0	2	0	0	0
FU	MO	0	0	0	3	0	0	0
FU	PE	2	0	1	8	4	0	0
	sumFU	5	0	1	13	4	2	0
TU	DI	7	1	1	2	0	1	4
TU	MO	3	8	3	5	1	2	1
TU	NL	5	2	0	2	2	0	0
TU	PE	1	1	0	3	3	6	2
	sumTU	16	12	4	12	6	9	7
Total nest number		198	234	150	500	286	126	31
Nests with owner change (OA+FOA+TU+FU)		90	85	49	61	28	18	12
= % (of total nest number)		45%	36%	33%	12%	10%	14%	39%
Abandoned nests (moving)		54	137	63	119	53	29	6
= % (of total nest number)		27%	59%	42%	24%	19%	23%	19%
TU nest (% of total nest number)		8%	5%	3%	2%	2%	7%	23%
Nest abandoned prior nest owner replacement (OA+FOA)		77%	86%	90%	59%	64%	39%	42%
Moving due FU+TU (% of abandoned nests)		6%	6%	5%	7%	2%	7%	17%

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observations. The field studies also did not involve endangered or protected species according to Czech or international law.

All of the females and nests on the nesting site were individually marked. We marked females with oil-based markers, using three different colour spots (two thoracic and one abdominal or vice versa) for each female. The nests were marked using pins with a colour combination corresponding to the female owner. We monitored the changes in nest ownership and described the females' activities on the nesting site every day during the season (except for rainy days, when the activity of bees is minimal). When we observed a different female active in the nest (for different activity patterns see [33]), we considered it as the nest owner replacement, and the nest was re-marked by a colour combination corresponding to a new owner.

The raw data that were obtained during the field observations were stored in a relational database, PostgreSQL Database Server 8.3, and we used the SQL language to generate the desired information. During the data analysis, we distinguished the existence of four different states that were hidden in the nest owner replacement situations detected during field observations: 1)

True usurpation (TU) occurred when the host female actively provisioning the nest was replaced by another female that began using this nest. On specific occasions the original nest owner did not leave the nest immediately after the true usurpation resulting in cohabitation of both the usurper and the previous owner in the same nest for limited time (situations TU-PE). Usurper stayed in the nest for at least two days and was observed to provision the nest. 2) Occupation of the abandoned nest (OA) occurred when the last activity of the original female was documented two or more days before a new female occupied the nest. 3) Failed usurpation (FU) occurred when the original owner either continued to live in the original nest or left the nest, but the usurper was not observed occupying the nest the following day(s) after the usurpation attempt. 4) Failed occupation of an abandoned nest (FOA) occurred when a female attempted to occupy an empty nest but was not active inside the nest for more than a few hours and was not observed in the nest in following day(s). However, bees that failed to usurp or occupy the empty nest stayed in the nest longer than during occasional orientation mistakes, which usually last just a few minutes. A one day lack of

Neighbourhood Society in Solitary Bees

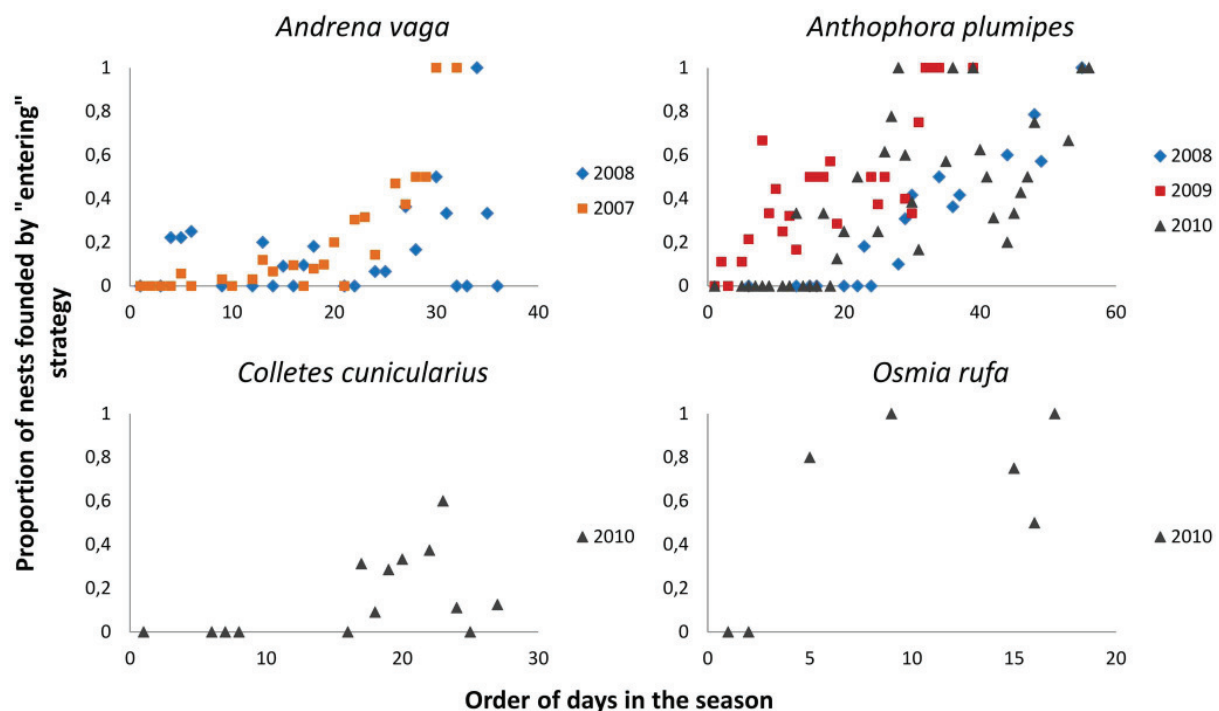


Figure 2. Proportion of nests founded by "entering" strategy each day during the season. The figure shows the results for different species and years.
doi:10.1371/journal.pone.0073806.g002

activity in the nest does not have to mean nest abandonment because the females are not active every day regardless of the weather conditions [33]. For this reason, we used two days as a criterion to state that there was no activity of an individual in the nest.

We further described the fate of the original owners after situations 1–4 to determine how different individuals and species react to threatening situations. The original owner could a) disappear (DI - we did not detect any activity by the original owner at the nesting site afterward), b) move to a new nest (MO), c) be

nest-less (NL - a female was observed for several days at the nesting site but did not found a new nest) and d) persist in the original nest (PE). The former owner of an abandoned nest could further be e) nest-less for more than two days after nest abandonment and then found a new nest (NLMO); such delayed behaviour has not been observed in other situations (a–d).

Because we evaluated the nest-founding strategy in studied species as "digging"/"entering" sensu Brockmann [19], we were further interested in the distribution of the "entering" strategy during the season. We analysed the dependence of the "entering" strategy frequency on the order of days in the season (day 1 = date of first emergence of bees at the beginning of the nesting season) in each species and year using the quasibinomial glm model in the program R 2.14.0 [34]. We further tested correlation between the order of the days and the number of available empty nests (caused by nest abandonment, or death of the owner) using R 2.14.0, because the number of available nests could be an important factor for adopting the "entering" strategy. The correlation between the time and number of empty nests was not tested in *Osmia*, *Colletes* and *Andrena* 2007, because we studied only a part of a large nesting area and we thus did not know the exact numbers of available empty nests.

We found that females often built more than one nest during their life and founding a new nest always resulted in abandoning their old nests (nest was claimed as abandoned the same day, when the original owner founded a new nest elsewhere). The reason for the moving was generally unknown (not caused either by usurpation or by any observed disturbance). To test the hypothesis that females abandon their nests regularly (move) after a specific time, we examined the time intervals that each female spent in each nest and the relationship between the number of nests

Table 3. Results of quasibinomial glm model describing dependency of proportion of founding new nest by "entering" strategy on the order of days within the season and results of correlation of the order of days within the season with the number of available empty nests.

Species	N	Order of day		Available nests	
		F	P	r ²	P
<i>Andrena</i> 2007	26	96.88	<0.001	0.959	<0.001
<i>Andrena</i> 2008	26	1.88	0.1985	NA	NA
<i>Anthophora</i> 2008	18	58.376	<0.001	0.934	<0.001
<i>Anthophora</i> 2009	26	25.845	<0.001	0.985	<0.001
<i>Anthophora</i> 2010	38	15.088	<0.001	0.909	<0.001
<i>Colletes</i> 2010	14	3.1522	0.1012	NA	NA
<i>Osmia</i> 2010	7	4.0307	0.1009	NA	NA

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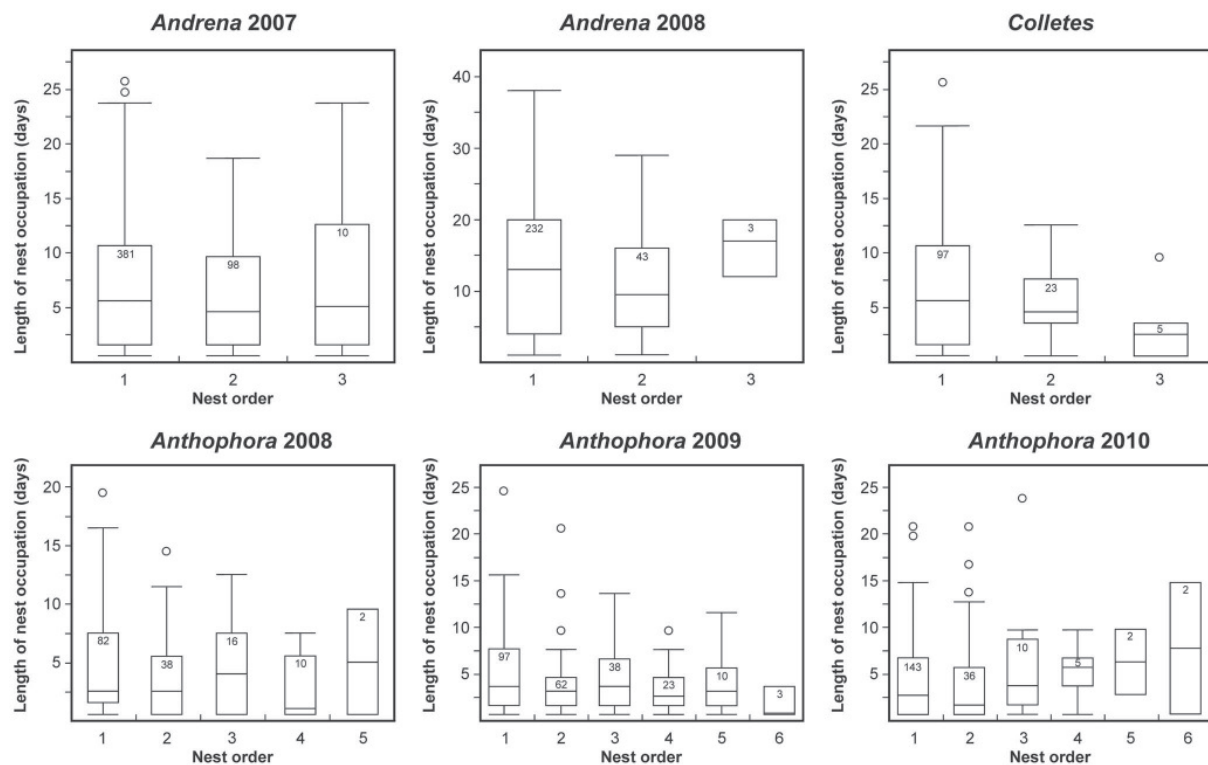


Figure 3. Length of occupation of subsequent nests. The figure shows the results for different species and years. The numbers in the upper part of box-plots indicate the number of cases.
doi:10.1371/journal.pone.0073806.g003

founded by one female and the longevity of the female: 1) We compared the time spent in subsequent nests by all of the females using a one-way ANOVA, and 2) we used a two-tailed standard two-sample t-test to test the hypothesis that females with more than one nest live significantly longer than females with only one nest. We used the program R 2.14.0 [34] and a significance threshold of $\alpha = 0.05$ for both analyses. We omitted the data from *Osmia* in these two analyses because the sample size was not sufficient.

Finally, we quantified all of the cases of observed intraspecific aggressiveness that occurred at the nesting site, usually in close proximity of the nest entrances (such as biting or stinging with audible buzzing) during the observation period for all of the species (across all of the seasons for each species) and compared it with the number of true and failed usurpations, in which we assumed the certain presence of intraspecific contacts between females, and which thus represents the estimate of the minimal expected number of aggressive incidents. Statistical analyses were not performed because of the extremely low number of observed aggressive attacks.

Results and Discussion

Our study shows that nest owner replacements are very common in all of the studied species in all of the observed years. About 10–45% of the nests changed owners during the season (Table 2). However, when the data were analysed in detail, we recognised that most of the nests that changed owners in *Anthophora* and *Andrena* and approximately 40% of the nests that changed owners in *Colletes* and *Osmia* had been abandoned by the female

owners (OA+FOA) before owner change. Only 2–23% of all of the nests were truly usurped (TU) (Fig. 1, Table 2) and true usurpations thus represent rather minor part of observed nest owner replacement situations. In *Andrena*, *Anthophora* and *Osmia*, the nest owner replacement usually occurred quickly during true usurpations, although we observed a few occurrences when both females (usurper and owner) provisioned the nest simultaneously for several days (situation TU-PE in Table 2), which can be interpreted as temporary joining. However, we purposely decided to call such situation TU-PE rather than joining, because we consider this behaviour to be unintentional and accidental contrary to the traditional definition of joining, i.e. a facultative strategy increasing the individuals fitness [7]. Interestingly, the true usurpation resulted in the situation TU-PE in 6 out of 9 cases in *Colletes*. When we analysed the further fate of TU-PE nests and bees in all of the studied species ($N = 16$), we found that although both the original owner and usurper provisioned the nest after usurpation for a few days, the first ($N = 5$), the latter ($N = 6$) or both ($N = 5$) eventually left the nest. We did not recognise any difference between studied species in this event; neither the original owner nor the usurper seems to be benefited. The refugees usually founded a new nest or disappeared in a few days.

Based on the relatively similar character and frequency of different nest owner replacement patterns summarized in Table 2, we assume that the general nest founding strategy of all of the species is in fact very similar, but *Colletes* have remarkably higher incidence of cohabiting of one nest by two females (TU-PE) compared to other species. This result indicates somehow increased tolerance to conspecific females in this species, which

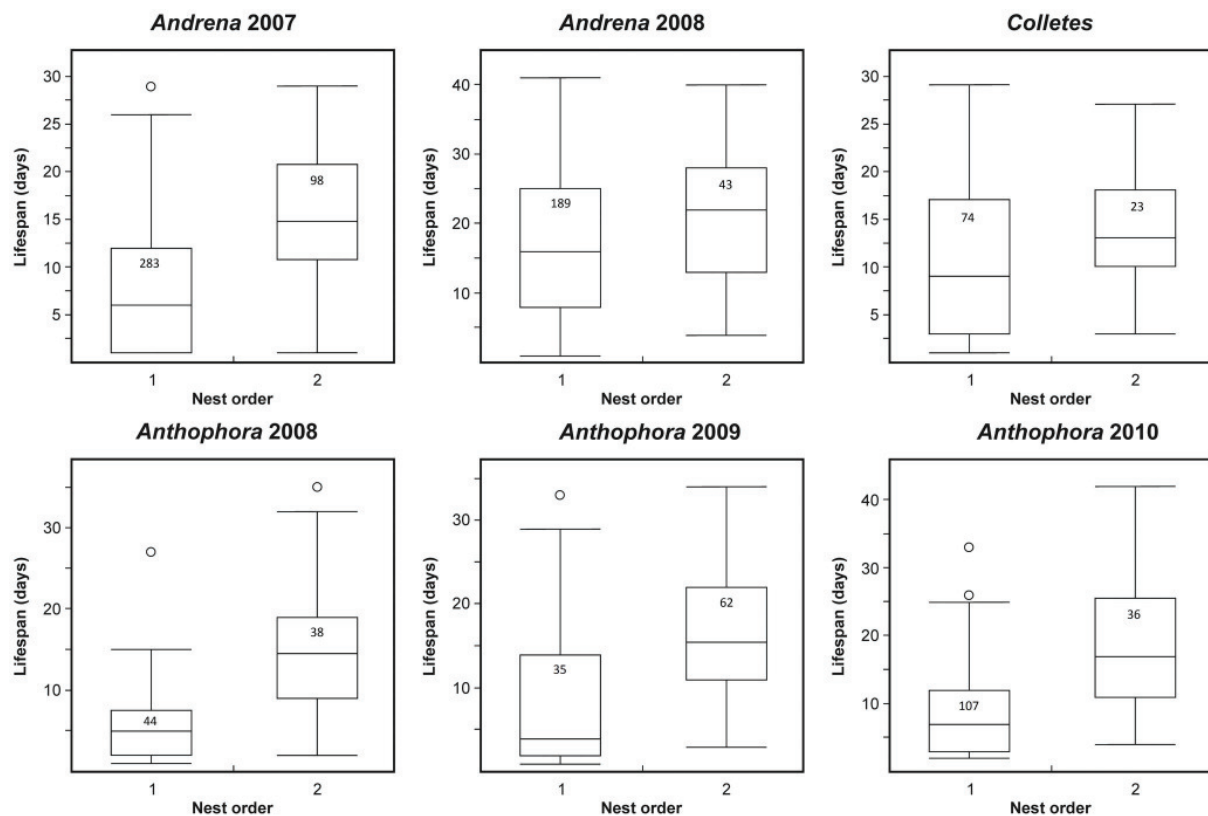


Figure 4. Longevity of females owning one or multiple nests. The figure shows the results for different species and years. 1 = one nest, 2 = multiple nests.

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we have also personally experienced during field observation. Although we are aware of limited number of observations to support this statement, such higher tolerance might generally represent an example of very important intermediate stage for the

evolution of communality and is worth further study (although any sociality is very rare in Colletidae; [35]). Similar accidental joining of a second female to an active nest was also observed in *Sphex ichneumoneus* [18,19]. Neither the analysis of the other types of behaviour of the original nest owner after owner change nor the behaviour of the original host showed any consistent pattern across different season in the studied species, most likely indicating that there is no common nest owner replacement handling strategy and that the behaviour of both the host and the potential usurper most likely depends on the particular circumstances.

We further quantified the general percentage of nest abandonment followed by founding of a new nest for each species and

Table 4. Test of the differences in the length of time spent in subsequent nests (One-way ANOVA) and test of the hypothesis that females with more than one nest live significantly longer than females with only one nest (Two-tailed standard two-sample t-test).

Species	Time in subsequent nests		Longevity and nest count	
	F	P	t	P
<i>Andrena</i> 2007	0.2214	0.6382	−8.4043	0
<i>Andrena</i> 2008	0.2325	0.6300	−3.1206	0.0025
<i>Anthophora</i> 2008	0.5389	0.4640	−5.9029	0
<i>Anthophora</i> 2009	5.61	0.0204	−5.0357	0
<i>Anthophora</i> 2010	2.0849	0.1504	−5.8506	0
<i>Colletes</i> 2010	2.90	0.1001	−1.7866	0.0805
<i>Osmia</i> 2010	NA		NA	

NA – not analysed. For the number of cases in each species and season see Fig. 3 and Fig. 4.

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Table 5. Comparison of number of failed and true usurpations (FU+TU) with the number of observed aggressive incidents in all species across all the years of observation.

Species	FU+TU	Aggressiveness
<i>Anthophora</i> 2008–2010	38	3
<i>Andrena</i> 2007–2008	35	3
<i>Colletes</i> 2010	11	4
<i>Osmia</i> 2010	7	3
Total	91	13

doi:10.1371/journal.pone.0073806.t005

season. In *Andrena*, nest abandonment occurred in 19–24% of all observed nests; in *Anthophora*, this occurred in 27–59% nests; in *Colletes* this occurred in 23% of nests; and in *Osmia* it occurred in 19% of nests (Table 2). Notably, the reason for nest abandonment and subsequent movement appears to be unknown in most cases (the nests that are gradually abandoned during the season, because their owner died, are not included in this analysis). The usurpations, either successful (TU) or unsuccessful (FU), are responsible for, at most, 33% of the detected cases of nest abandonment in *Osmia* and much less in all of the other species (Table 2). The repeated nest abandonment and founding of new nests during the season without any evident reason is reported in many unrelated bee and wasp species [28,29,31,36–40] and seems to be a common feature of solitary bees and wasps. This behaviour might result from an adoption of risk-spreading strategy (bet-hedging) in the places with high parasitic pressure or unstable environmental conditions [39,41,42].

Brockman et al. and Brockman and Dawkins [18,19] showed that *Sphex ichneumoneus* individually chooses between a strategy of “digging” (constructing a completely new nest) and “entering” (inhabiting any existing nest) during each nest founding. They also noted that the necessary condition for this strategy is the constant supply of empty nests during the entire nesting season caused by the frequent abandonment of the active nest. A similar pattern was also observed in *Crabro monticola* [38], and it corresponds well with the observed behavioural patterns of the bees in our study. Thus, we expect that the original facultative nest founding strategy of all of the observed solitary bee species is using of any pre-existing nest, similar to the “entering” strategy of wasps, rather than cleptoparasitism (here usurpations) per se. From this point of view, the observed true usurpations and occasional coexistence of two females within one nest (TU-PE), described above, represent only by-products of this strategy in which a female does not distinguish between an empty nest and an active nest [18,19].

We examined the distribution of the “entering” strategy during the nesting season of bees because one would expect it to increase with the upcoming end of the season, when the time becomes the limiting factor [43]. We found out that the tendency to found a new nest by utilising any active or abandoned nest increases with the upcoming end of the season in all the species and the relationship is significant in most of them (Fig. 2; Table 3).

We further detected that the number of abandoned empty nests continually increases during the season and the correlation is strong in the seasons and species where the number of abandoned nests was known (correlation coefficients: $P < 0.001$, $r^2 > 0.9$, Table 3). We thus assume that the increasing number of available empty nest during the season might be a key factor that enables the increase in adoption of “entering” strategy. Unfortunately, both factors (order of days and number of empty nests) are so closely correlated, that the glm analyses have not enabled us to distinguish which of these factors is proximate.

Our data suggest that the adoption of “digging” or “entering” strategy is not constant but condition-dependent. As mentioned in the introduction, similar condition-dependent strategy was also reported by other researchers in different species [12,13,15–17]. Notably, and contrary to our results, Brockmann and Dawkins [18] did not find a time-dependent frequency of nests founded by “entering” in *Sphex ichneumoneus*, but they tried to detect it using a different methodology. However, the tendency to enter conspecific nests by old “senile” females at the end of the season was described by Malyshev [44] and Fabre [24] found that the probability of nest usurpation does not decrease with the increasing number of empty nests during the season in *Osmia tricornis*, which is consistent with our results.

Our results showed that regular nest abandonment and nest owner replacement appears to be common in solitary bees; however, this says nothing regarding why the females should leave their nests. We examined the time spent in each nest and the longevity of females and found that 1) the time spent in the nest, regardless of their order, does not significantly differ between the subsequent nests (one-way ANOVA, $P > 0.05$ in all of the studied species except for *Anthophora* in 2009, Table 4, Fig. 3), and 2) the females with more than one nest live significantly longer than females with only one nest (two-tailed standard two-sample t-test, $P < 0.01$ in all of the studied species except for *Colletes*, Table 4, Fig. 4). These two results indicate that when females live long enough, they tend to abandon their nests after some critical time and found a new one. This result might be explained by the concept of a risk-spreading strategy, which states, that genotypes with lower variance in fitness should be favoured at the cost of lower mean fitness in a strongly stochastic environment by means of physiology or behaviour that spreads risk of encountering an unfavourable environment over time or space (see Hopper [42] for examples and details). Neff et al. and Williams et al. [29,45] indeed reported the tendency to construct one-cell nests during heavy rains or attacks from massive amounts of ants in some bee species. The observed pattern of nest abandoning behaviour indicates the likely presence of such risk-spreading strategy in all four studied species, each of which hosts parasites associated to the nesting sites (*Bombus* sp. in *Andrena*, *Colletes* and *Osmia*, *Nomada lathburiana* in *Andrena*, *Melecta albifrons* in *Anthophora*, *Sphcodes albilabris* in *Colletes* and *Stylops ater* in *Andrena*). Although we do not have the precise data on the rate of parasitism in all the species, we know that these parasites can be variably successful in host infestation in different years depending on the particular climatic conditions (unpublished results). Bulmer [46] used mathematical modelling to show that the costs of nest abandonment outweigh the benefits of risk-spreading when considering that the nest must be constructed and defended, but this study did not include nest usurpation, which helps saving some energy and time.

We assume that the increased turnover of nests at the nesting site due to the adoption of the “entering” strategy must have resulted in increase of intraspecific contacts between females. As mentioned, constructing a nest is always a significant investment and therefore should be protected. Contrary to this expectation, the owners obviously do not invest much energy into nest protection, which results in a low level of conflicts between females. We observed only 13 cases of aggressive behaviour, such as pressing bites and attempts to sting, in all of the species during all of the years of observations, compared with 91 cases of true or failed usurpations, which we take as the minimal estimate of cases, where the intraspecific contact between females must have occurred (Table 5). Because we probably strongly underestimated the total number of possible encounters of two females at the nest entrance by reducing it to the number of TU and FU situations, the true ratio of aggressive and nonaggressive encounters is even much lower. Without the individual marking of females, one could say that no interaction among females or interchange of nests occurred at the observed nesting site. We assume that this is the reason why the nest owner replacements and usurpations are rather neglected in solitary bees. Similarly, low levels of aggressiveness at the intraspecific level during female contact has also been reported in other solitary bees [17,31]; and unusual tolerance was also reported in some crabronid wasps, e.g. [16,38,39]. Described presence of intraspecific cleptoparasitism (here usurpations) and observations of low aggressiveness in bees support the mathematical prediction that the tolerance of

cleptoparasitic cheating (= low level of intraspecific aggressiveness even though the usurpations are commonly present) may occur when the necessary protection against conspecific cleptoparasites is long-term and costly [47]. Because contact between female bees in aggregations are obviously very common, and the fighting between them could be very exhausting (such as in *Hoplitis* [13]), this model is likely an analogy to the war of attrition [48]. Bees have an efficient defence mechanism but do not use it, which makes the neighbourhood society peaceful, although there is a relatively high frequency of cleptoparasitism (nest usurpations).

Our study shows a general picture about the nesting behaviour of solitary bees as the possible analogy to the prior behavioural state for the evolution of alternative life-strategies in bees, such as cuckoo behaviour (obligate brood parasitism), communality and indirectly also eusociality. Positive fitness consequences associated with avoiding excavation costs and diluting parasite pressure due to risk-spreading strategy of solitary bees could represent a parallel to the forces driving the evolution of preadaptations important in the evolution of all recent social and cleptoparasitic bee species in the past. Although the described nesting dynamics with common utilising of pre-existing nests seems to be just an opportunistic behaviour, it makes the contacts between conspecific solitary females very frequent. The relatively high number of detected true usurpations in studied species shows that such contacts are not

always amicable. Frequent nest inspections and nest switches may further enhance other cheats like intraspecific cuckoo behaviour, but this behaviour needs to be investigated in more complex studies in the future. On the other hand, usurper accepted by nest owner is practically identical to accepted “joiner” in communal nesting bee [7]. Tolerance of usurpers can thus theoretically represent one of the possible ways towards communality and other types of social behaviour. In fact, our observations of short term coexistence of two females in *C. cunicularius* almost meet a definition of communal nesting [8], which is worth further research.

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Author Contributions

Conceived and designed the experiments: KČ JS. Performed the experiments: KČ LM ZK JS. Analyzed the data: MZ KČ. Wrote the paper: KČ JS.

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PAPER III

POPULATION STRUCTURE OF PIONEER SPECIALIST SOLITARY BEE *ANDRENA VAGA* (HYMENOPTERA: ANDRENIDAE) IN CENTRAL EUROPE: THE EFFECT OF HABITAT FRAGMENTATION OR EVOLUTIONARY HISTORY?

Černá K., Straka J. & Munclinger P. (2013)

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PAPER IV

IDENTIFICATION OF 37 MICROSATELLITE LOCI FOR *ANTHOPHORA PLUMIPES*
(HYMENOPTERA: APIDAE) USING NEXT GENERATION SEQUENCING AND THEIR
UTILITY IN RELATED SPECIES

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<https://www.eje.cz/scripts/viewabstract.php?browsevol=&abstract=1692>

PAPER V

LIFESPAN IN THE WILD: THE ROLE OF ACTIVITY, CLIMATE AND PARASITIC
INFESTATION IN NATURAL POPULATIONS OF BEES

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(submitted manuscript)

Lifespan in the Wild: The Role of Activity, Climate and Parasitic Infestation in Natural Populations of Bees

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Summary

1. Animal lifespan depends on ecological and physiological constraints. The latter has been studied under controlled conditions but little is known about determinants of lifespan under natural conditions.
2. We studied the relationships between length of adult life, magnitude of foraging activity and environmental abiotic conditions in two bee species: a pollen specialist *Andrena vaga* (Andrenidae) and a pollen generalist *Anthophora plumipes* (Apidae). In *A. vaga*, we also studied the influence of the *Stylops* endoparasite on lifespan.
3. Our research indicates that lifespan is driven both directly by climate and indirectly through climate-dependent activity patterns. We found a negative relationship between

proportion of active days and length of life; in contrast, high activity rate within the active days had no negative effect on longevity.

4. Individuals activating during warm and/or wet days lived longer, with precipitation being more important determinant of lifespan than temperature.
5. Timing of emergence was also important predictor of bee lifespan. Individuals that emerged nearer to the end of season (critical time-horizon) lived shorter than earlier emerged individuals. Seasonal emergence timing was correlated with seasonal temperature and with Strepsiptera endoparasite infestation (*Andrena* only). Finally, we found that the early emergence of parasitized individuals, not their reduced activity, explains their prolonged lives.
6. We demonstrate that lifespan and activity patterns of wild populations of insects are regulated by a tractable interplay of ecological factors which were previously studied only in isolation or in vitro.

Key-words: behaviour, foraging, weather, temperature, moisture, critical time-horizon, life-history, Strepsiptera

Introduction

Lifespan has traditionally been considered to depend on ecological and physiological constraints (Stearns 1992). The latter has been intensively studied under controlled conditions, with a special emphasis on metabolic rates (Speakman 2005). Metabolic rates depend on the body temperature and activity of individuals, e.g. (Brown *et al.* 2004), factors that both depend on the ambient temperature in insects (Miquel *et al.* 1976; Sohal, Donato & Biehl 1981). Interestingly, effects of activity and temperature on lifespan have rarely been studied under natural conditions (*in situ*) where they interplay with ecological factors such as predation, diseases, food availability or environmental stochasticity (Stearns 1992; Kawasaki *et al.* 2008; Monaghan *et al.* 2008).

The effect of activity on insect adult lifespan was documented in laboratory studies where flight activity was manipulated in *Drosophila melanogaster* and *Musca domestica* (Sohal & Buchan 1981; Magwere *et al.* 2006). Intensive flight activity in both species causes oxidative damage to some proteins and lipids, resulting in a shorter life (Yan & Sohal 2000; Magwere *et al.* 2006). Although the influence of metabolic stress is obvious, there are some evidences that moderately high activity could have also positive effect on lifespan in some vertebrates (Lee, Hsieh & Paffenbarger Jr 1995; Navarro *et al.* 2004) and insects (Niitepõld & Hanski 2012) trained for regular activity. Metabolic stress from activity can be the reason why long-lived queens of eusocial insects (e.g., ants or termites) drop their wings as early as possible or reduce flying to a minimum (e.g., in bees) (Wilson 1971). In contrast, solitary insects cannot avoid the high flight activity necessary for their own feeding or provisioning of a brood. They can only modulate their behavioural strategy depending on the actual physiological and ecological parameters they experience and optimize this behaviour according to an evolutionary stable strategy (Maynard Smith 1978). Experimental studies show that some

bees are able to optimize their life strategies individually, e.g., to forage with maximum efficiency (Schmid-Hempel, Kacelnik & Houston 1985; Schmid-Hempel 1987).

Although there are *in situ* studies on the effects of climate on insect life histories, e.g., (Vicens & Bosch 2000; Walther *et al.* 2002; Bartomeus *et al.* 2011), these studies fail to address the impact of climate on the actual length of life. In particular, it is known that temperature has a characteristic effect on the survival and development of various insect instars (Dixon *et al.* 2009). For example, survival of adult bees was shown to be negatively affected by the temperature and duration of overwintering of larval stages (Bosch & Kemp 2004). It has also been shown that changes in the temperature-dependent activity of predators can cause a shift in the mortality of foraging insect prey (Logan, Wolesensky & Joern 2006). Finally, Tauber *et al.* (Tauber *et al.* 1998) noted that broad ignorance and underestimation exists regarding the effect of moisture on insect life. We thus expect that climatic conditions are critical *in situ* determinants of lifespan and activity patterns of solitary insects.

Most adult insect individuals are active throughout their lives; they search for resources, usually with the single goal of reproduction. However, not all individuals are fertile, what strongly influence their life traits. Bees of the genus *Andrena* are often parasitized by *Stylops* (Insecta: Strepsiptera), which castrates its hosts by changing their physiology (Brandenburg 1953; Strambi & Girardie 1973). Castration increases the size and/or longevity of the host in some described host-parasite systems (Hurd, Warr & Polwart 2001), which consequently increase the parasite's fitness (Baudoin 1975). In studies on leafhoppers and wasps, stylopized insects (i.e., those parasitized by Strepsiptera) were shown to have longer lives than healthy individuals (Lindberg 1939; Beani 2006; Kathirithamby 2009). The parasite further manipulates behaviour of its hosts. Stylopized *Andrena* bees are manipulated towards precocious nest emergence in the early spring, which most likely increases the chance for effective mating of the parasite (Straka *et al.* 2011). Stylopized bees thus represent unique

model that enables to determine the effect of emergence timing on activity and lifespan. We can expect reduced activity rates in stylopized bees, so that these bees are more likely to survive until the dispersal of the parasite's invasive brood later in season (Straka *et al.* 2011).

Here, we present an empirical exploratory analysis of ecological correlates of the length of active life in natural conditions in adults of two species of solitary bees, an appropriate model group for study of animal lifespan under the natural conditions. The analysis is based on a large dataset that consists of daily observations of hundreds of individual bees of two species across several years. Specifically, we test the prediction that adult bees with high foraging activity should have a shorter lifespan than bees with low activity. We also investigate how the lifespan of solitary bees depends on environmental conditions, such as temperature, sunshine, precipitation and pollen availability, as well as on the proportion of active days, foraging time per day or date of first emergence of a bee. Finally, we hypothesize that individual bees that invest in reproduction (healthy bees) should have shorter lifespans and be more active than sterilized bees (castrated by *Stylops* parasite), as these latter bees do not invest in reproduction.

Materials and methods

Species studied

We studied the above-mentioned phenomena *in situ* in two solitary bee species: *Andrena vaga* Panzer (Andrenidae) and *Anthophora plumipes* (Pallas) (Apidae). These species belong to phylogenetically distant bee lineages (Danforth *et al.* 2006).

Andrena vaga (Fig. 1a) is an oligolectic solitary bee that becomes active between mid March and the beginning of May (Westrich 1990; Rezkova *et al.* 2012). Females (12-15 mm in body length) collect large pollen loads predominantly on *Salix* trees (Bischoff, Feltgen &

Breckner 2003). *Andrena vaga* builds nests in sandy soil in sunny locations and closes the nest entrance by soil every time it leaves (Fig. 1b). *Andrena vaga* are often parasitized by *Stylops ater* (Strepsiptera: Stylopidae) (Reichert 1914). *Stylops* is an endoparasitic insect, which parasitizes species of the genus *Andrena* and is known to manipulate host behaviour (Straka *et al.* 2011).

Anthophora plumipes (Fig. 1c, d) is a solitary bee that becomes active between the end of April and the beginning of June (Westrich 1990). Females (14-15 mm in body length) make short and frequent foraging flights and collect small pollen loads from a wide range of plant species (Westrich 1990). *Anthophora plumipes* builds opened nests in either horizontally or vertically positioned patches of bare soil (Fig. 1d).

The two species differ in the way they regulate body temperature. Although both species are heterotherms, *Andrena* is able to moderately increase body temperature through active muscle work (called endothermic heating or warm-up) (Stone & Willmer 1989). On the other hand, *A. plumipes* is known for its high endothermic warm-up rates (Stone & Willmer 1989; Stone 1993).

Field data collection

Daily observations of *Andrena vaga* females (N = 539) were performed at a nesting site close to the Čelákovice village, Czech Republic (north-eastern periphery of Prague, 50° 10' N, 14° 45' E), between the end of March and the end of April in 2007 and 2008 (see Table S2 for details in supplementary information). The studied nest aggregation is situated on alluvial sand with patches of grass on the margin of a secondary oak and pine forest. Daily observations of *Anthophora plumipes* females (N = 334) were performed at a nesting site in Praha-Strahov, Czech Republic (50° 05' N, 14° 24' E), between early April and early June in 2007, 2008, 2009 and 2010 (see Table S2 for details in supplementary information).

We individually marked all females as they gradually appeared at the nesting sites during the season and checked every *A. vaga* individual for stylopization while marking. Only parasitisation with *Stylops* females which are permanently attached to the host bees was considered. The marking was performed using 6 to 8 different oil-based colours and every specimen received unique combination of three colored spots on scutum and metasomal terga. We followed the observation methods described in Rezkova et al. (Rezkova *et al.* 2012): For each marked female observed on the nesting site we recorded its colour mark, type of activity (leaving nest or returning) and exact time (in hours and minutes). Variability of time (in hours and minutes) spent each day by observation at the field site is considered in analysis of daily activity of bees (Table 1). This method allowed us to monitor bee activities during all the season as well as during the days.

Daily and annual climatic data (Table 1) were acquired from the two local meteorological stations of the Czech Hydrometeorological Institute situated within 10 km from the nesting sites. Pollen grains in the air (per m³) were acquired from three distant places in the Czech Republic (Praha, Plzeň, Brno) to minimize effects of the local position of pollen monitoring station and wind direction.

All data used in this study are accessible for public at www.aculeataresearch.com.

Preparation of data on lifespan and relative activity

Our study is specifically focused on explaining variability of two response variables: (1) *Length of life* (lifespan), which was measured as number of days between the first and last observation of the individual during the season. We are aware that there is a caveat: although *A. vaga* and some other bee species with aggregated nests are known for their philopatry (Rosenheim 1990; Bischoff 2003) we could not rule out that some individuals emerged elsewhere outside of the observed nesting site or that they moved away from the site during

the season. For this reason the observed values of lifespan (and first active date) are used as approximate estimates of real values and should be interpreted as such. (2) The second response variable was *relative activity* (proportion of active days during life) which could be seen as "density" of active days within the whole lifespan. Bees were not activate each day of their life (Minckley *et al.* 1994), thus the active day was defined simply as a day on which a given individual was observed outside of its nest. Although we had the activity data collected at finer (i.e. minutes and hours) temporal scale than days, we decided to aggregate them to days as sampling periods were not exactly constant during each day. However, we still did not discard the finer temporal information entirely and we used it to create variable *relative time of activity during active days* (Table 1) which standardizes the time of daily activity (in minutes) by the total length of daily observation period (in minutes). All variables are listed and summarized in Table 1. We included only individuals recorded with lifespan longer than four days; all of the short-living individuals were discarded from the data.

Statistical analyses

Our main focus was to explain variability in the observed lifespan and relative activity in *Andrena* and *Anthophora* bees. We hypothesized that lifespan can be influenced by environmental conditions, such as temperature; by relative foraging activity, which may also be influenced by environmental conditions (Schmid-Hempel & Wolf 1988); or by the date of first emergence, because individuals that emerge later have a shorter season ahead. In *Andrena*, we also investigated the effect of stylopization on lifespan and relative activity.

To identify which factors might be the most important potential predictors of lifespan in both species (Table 1), we employed regression trees based on binary recursive partitioning (package "tree" in R) (Breiman *et al.* 1984). Regression trees are ideal for such purposes as they are simple, they require no assumptions about the distributions of the data, and they

provide a good picture of data structure (Crawley 2012). We set the within-node deviance to be at least 0.03 times that of the root node (the default is 0.01 in function "tree") so that the tree would not be overly detailed and could be easily plotted.

Following the regression tree analysis, we performed two sets of generalized linear models (GLM; function `glm()` in R) for each of the species. The first set of models used lifespan as a response variable (Gaussian family); the second set of models used the relative activity of individual bees as a response variable (binomial family, logit link). Note that the latter model takes into account the total number of trials (i.e. lifespan) and successful outcomes (i.e. active days) so that 1/2 is treated differently from 10/20 (although the proportion is 0.5 in both cases).

To find a robust minimum adequate model, we used both stepwise backward deletion (predictors are deleted from the full model) and forward addition of predictors (predictors are added to the full model). In both cases, we first fitted the full model with all explanatory variables in their linear and polynomial forms. We used AIC both during the selection process and to compare the resulting models. If the two models were indistinguishable during the stepwise procedure ($\Delta\text{AIC} < 2$), we retained the model with the lower number of predictors. We checked the resulting models to determine whether any of the terms could be replaced by a polynomial or linear form, or by any of the variables that were already deleted. For simplicity, we did not include interactions in the models.

It is sometimes advised to report not only the minimum adequate models but also other candidate models (Burnham and Anderson 2002). However, our best models usually very clearly outperformed all of the other candidate models ($\Delta\text{AIC} > 10$). Also, deletion of the least important variables from the best models did not change the standardized coefficients of the remaining variables substantially. Additionally, the best models were consistent between the two different species and were congruent with results from the regression trees. Finally, the

potentially misleading rate of colinearity between predictors was low (see Table S3 for details in supplementary information). Therefore, we considered our results to be robust (more so given the large n), and we only report the best-fitting minimum adequate models in the results section.

We report the best models by plotting the partial residuals for each variable from the best models in addition to reporting the standardized coefficients for each variable (the "betas" in statistical literature). In addition to the percentage of explained deviance, these results give very detailed insight into the structure of the models and their explanatory power.

Results

Lifespan

Both the regression trees and the best fitting GLMs using lifespan data explained a large proportion of the variability in lifespan within both species (ranging from 50.6 to 72.7%; Figs. 2 and 3), thus indicating that we captured important ecological predictors. In both species, the most important predictors of lifespan were relative activity (higher activity led to shorter life), first active day in the year (the later an individual emerged, the shorter it lived) and precipitation during active days (individuals that were active during wet days lived longer than those that were active during dry days) (Figs. 2, 3). This finding was demonstrated independently by both the regression tree analysis (Fig. 2) and the GLMs (Fig. 3). Both species showed annual differences in lifespan: they lived longer in colder years (Fig. 3).

In *Andrena* bees, we also detected a unimodal effect of temperature during active days, a positive effect of sunshine during active days and a negative effect of pollen availability. Surprisingly, our statistical models did not reveal any evidence for the effect of stylopization on lifespan in *Andrena*, although in the raw data, stylopized bees seemed to live longer

(Wilcoxon rank-sum test, H_a : $\text{long}_{\text{styl}} - \text{long}_{\text{heal}} > 0$, $\alpha = 0.05$; 2007: $Z = 1.9804$, $p = 0.0238$; 2008: $Z = 2.5986$, $p = 0.0047$) and had a lower proportion of active days during their lives (Wilcoxon rank-sum test, H_a : $\text{relact}_{\text{styl}} - \text{relact}_{\text{heal}} < 0$, $\alpha = 0.05$; 2007: $Z = 5.1327$, $p < 10^{-6}$; 2008: $Z = -3.0929$, $p = 0.001$) as compared with healthy individuals. This difference disappeared in the best multiple regression model, when other variables were considered, indicating that stylopization is only an ultimate correlate of lifespan. In *Anthophora* bees, the effect of temperature during active days was positive but weak (Fig. 3). We also detected a weak positive effect of relative activity on lifespan during active days.

Relative activity

Note that, as we demonstrated above, relative activity was an important predictor of lifespan. Therefore, any predictors of relative activity also ultimately influence lifespan. In both species, we found that the most important predictors in the best-fitting GLMs of relative activity (Fig. 4) were the date of the first active day in year (positive effect), the relative time of activity during active days (positive effect) and the precipitation during active days (positive effect) (Fig. 4).

In *Andrena* bees, relative activity was also higher in the warmer year, 2007, and in bees that became active during days with high pollen availability (Fig. 4). Importantly, stylopized bees had slightly higher relative activity than healthy bees (weak but significant effect; Fig. 4). In *Anthophora* bees, the negative effect of temperature on relative activity was much stronger than in *Andrena* (Fig. 4), and we also found a striking positive effect of sunshine during active days.

First active day

After examination of the above-mentioned results, it was clear that there was one non-climatic variable that had a strong effect on the lifespan: the first active day. Thus, we also checked whether variability in the first active day could be related to some of our seasonal climatic variables or to stylopization (in *Andrena*). In *Andrena* bees, we found a striking difference (by ~15 days) in first active day between healthy and stylopized individuals (Fig. 5). The stylopized bees thus differ from healthy bees mainly by the date of first emergence. *Andrena* bees also became active earlier in the warmer year, 2007 (Fig. 5). Similarly, in *Anthophora* bees, we found that individuals become active earlier in warmer and in wetter years (Fig. 5).

Discussion

As hypothesized earlier, the length of active life should depend on factors such as climatic conditions, food resources, activity regime, predation, and parasites. However, such a correlation has rarely been demonstrated using *in situ* data. Our results show that ecological variables do have a detectable effect on the lifespan of solitary bees, with the prominent variables being moisture (precipitation), magnitude of activity and timing of emergence. Our data led to similar results in both studied species (and also when different analytical techniques were employed), which implies the possible general applicability of our findings.

Activity influences length of life

We had expected relative flight activity to be one of the key factors determining lifespan in the two bee species. The high energetic cost of flight was demonstrated by laboratory experiments (Wolf *et al.* 1989), and the situation in wild populations is unlikely to be different. Accordingly, we confirmed that there is a strong negative effect of proportion of

active days in life (relative activity on the lifespan of both species). We further found that when the bees had a higher relative activity, they were also more active within the active days (relative time of activity). Surprisingly, this higher time of daily activity had either no negative effect on longevity (in *A. vaga*) or even had a positive effect (in *A. plumipes*). The high daily activity thus seems to be sustainable, or even beneficial for longevity; a similar finding has been demonstrated for some mammals (Lee *et al.* 1995; Navarro *et al.* 2004) and recently also for insects (Niitepõld & Hanski 2012) in laboratory experiments. We suggest that experiments manipulating magnitude of activity at short and long temporal scales should be conducted to explore this relationship further.

Climate influences relative activity and lifespan

Relative activity, as one of the main determinants of lifespan was explored in detail in our study. Our analysis shows that the most active individuals (measured by proportion of active days in their life) are those that are active during relatively high-precipitation and low-temperature days. Unsurprisingly, only a minority of individuals activate in such weather conditions. Such bees probably risk a need for time consuming drying and resting. The question is why the bees have fewer inactive days in their life when it is rainy and cold weather and it is probably lower chance to find available pollen. This is not trivial and easy to interpret, because most bees should be active, when the pollen availability is high (Minckley *et al.* 1994) and our results from *A. vaga* do not contradict this relationship. The possible explanations might be connected with our findings, that a) precipitation has positive effect on lifespan, stronger than temperature and b) lifespan is also increased, when *A. vaga* forages under decreased pollen availability of *Salix* trees. However, effect of pollen availability to lifespan needs specific test in the future research, because our data about "pollen availability" are rather rough approximation of this variable.

Another striking result is the unimodal response of lifespan to the temperature during active days in *A. vaga*. According to our knowledge of this species, the most likely interpretation is insufficient thermoregulation of bees exposed to full sun. Females of *A. vaga* cover the nest entrance with sand prior to each departure, which protects the nest from parasites while they are away, but consequently they have to dig inside upon return. The digging can be costly as high temperature on direct sun forces the bee to leave the nesting site after a few minutes of digging and it takes her a few minutes to cool down and continue the digging. Such behaviour may be repeated many times until it finally gets into the nest (Rezkova *et al.* 2012). In contrast, *Anthophora* does not cover its nest entrance and does not show the unimodal response. However, in this species, we detected a trend towards an unusually narrow preference of rather cold temperatures for activity, even though higher temperature seems to increase their longevity. Accordingly, we would expect these bees to be more active on warmer days, but they are not. This discrepancy might be explained by examining the increased activity of *Melecta albifrons* (a cuckoo bee of *A. plumipes*) in higher temperatures (Stone & Willmer 1989); a behavioural pattern typical of other related cuckoo bees (Straka & Bogusch 2007; Rozen, Straka & Rezkova 2009). The risk of parasitism in warmer temperatures might be so high the bees prefer to be inactive and stay in the nest to protect it.

The effect of *Stylops* parasite on lifespan and relative activity

Thus far, little is known on how the *Stylops* parasites influence the lifespan and activity of their hosts. As previously mentioned, according to our raw data, stylopized bees lived longer than healthy ones; however, this effect of stylopization on lifespan disappeared when other predictors were included in the model. The explanation is that the stylopized bees are manipulated by the parasite to emerge earlier in the season and as we showed in our analysis,

the earlier emergence of bees cause their longevity. Early emergence of stylopized bees remains significant even after accounting for other variables, including the presence of the parasite (see also (Straka *et al.* 2011)). Hence, the *Stylops* parasites seem to increase length of life of their hosts indirectly by manipulating their date of emergence, rather than by directly reducing their activity (our original expectation).

Moreover, we found that after the environmental conditions and time of emergence are accounted for, stylopized bees are even relatively slightly more active than healthy bees. In other words, when stylopized and healthy bee emerge at the same day (and other variables will be constant), the bee with parasite will spend more days activating outside the nest than the healthy one. We had expected the opposite trend because parasitized bees are castrated and should not be motivated to provision their brood. The higher relative activity indicates that the castrated bees are manipulated to invest a detectable amount of energy into the growth of the endoparasite and into the development of a number of new invasive larvae; this strategy has also been observed in other host-parasite systems (Hurd *et al.* 2001). On the other hand, the difference in the cost of reproduction (represented by the relative activity of healthy bees) and the cost of parasitization by *Stylops* (represented by the relative activity of stylopized bees) is very small and seems to indicate that the cost of having a parasite is almost as costly as reproduction itself.

Optimization of foraging activity is optimization of lifespan

We have shown that a higher relative activity (the proportion of active days) leads to a shorter lifespan. This trade-off between relative activity and longevity was expected. The question is whether the bees tend to maximize foraging efficiency or food gain (Ydenberg *et al.* 1994). If they maximize gain, then the maximum rate of foraging activity should be maintained at any cost (Werner & Anholt 1993), especially under strong environmental pressure, such as limited

food sources, time or predation. However, we observed that the bee's relative activity is not constant (and thus not maximized). Rather, relative activity gradually increased with increasing pollen availability (data for *A. vaga* only) and, more importantly, with proximity to the end of the season which corresponds to *critical time horizon* (Maynard Smith 1978; Ydenberg *et al.* 1994). Apart from our results there is a little evidence for effect of such a critical time horizon in nature (but see (Javoiš & Tammaru 2004) for a rare study of moths).

Existence of time-dependent strategies in bees can explain why we did not observe any effect of stylopization on longevity of *A. vaga* in the multiple regression model but we did see it in the raw data. Parasitized *A. vaga* emerge early, and thus they are far from critical time horizon. Early emerged bees are less active and live longer lives, than late emerged and more active bees, either parasitized or not. The parasite may increase longevity and decrease activity by the shift in host emergence timing for its own benefits. Because the parasite chooses its host randomly (the transfer occurs with pollen), we are convinced that the emergence of bees is not genetically preprogrammed but that bees are able to change life strategies according to their perception of time (time of emergence). In conclusion, lifespan is regulated by optimization of efficient use of time and activities.

Seasonal effects

In warmer years with warmer early-spring temperatures, animals become active earlier, which is a fact that is frequently discussed in connection to global climate change (Walther *et al.* 2002; Bartomeus *et al.* 2011). Unsurprisingly, our data demonstrated that solitary bees emerge earlier in warmer years.

We observed that both *A. vaga* and *A. plumipes* lived longer in colder years and *A. vaga* had also a generally lower proportion of active days. However, individual bees had a lower proportion of active days and also lived longer when the average temperature during those

active days was higher. This discrepancy in sensitivity to temperature between seasonal and daily data is probably connected to the different effect of temperature on active and inactive bees. Our daily environmental data were collected only for active bees, which have rather endothermic thermoregulation physiology, in contrast to the overall seasonal data, which included numerous records of inactive days for individual bees. Bees that are inactive (i.e., sitting in nests) are physiologically ectothermic. Because the rate of metabolism changes with the ambient temperature in ectotherms, increasing temperatures have a negative effect on the lifespan of inactive bees, as it is known for overwintering bee larvae (Bosch & Kemp 2004).

We also observed a weaker, but still significant, effect of season precipitation on the first date of emergence in *A. plumipes*. Examination of such seasonal responses to moisture has been rather neglected in ecological studies, although these responses have been shown to be important (Tauber *et al.* 1998). Sensitivity of date of emergence to moisture is well known in desert bees (Danforth 1999), and here we also demonstrate this phenomenon in a region with less extreme climate.

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Data Accessibility

All primary data are uploaded as online supporting information.

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Table 1. Overview of variables that were used in *Andrena vaga* (N=539) and *Anthophora plumipes* (N=334) analyses.

Variable name	Description	<i>Andrena</i>			<i>Anthophora</i>		
		Mean	Median	Min-max	Mean	Median	Min-max
Length of life	Number of days between the first and last observation of an individual	16.1	15	4-41	14.01	13	4-41
Number of active days	Number of days, when the bee was observed outside of the nest	6.27	5	2-21	5.48	4	2-29
Relative activity	Number of active days / Length of life	0.41	0.4	0.07-1	0.42	0.4	0.08-1
First active day in year	The day when the animal was first observed being active; counted as number of days since 1 st of January.	98.3	99	84-123	121.3	119	94-157
Health		425 healthy and 114 stylotized individuals			Not applicable.		
Year		Season temperature and season precipitation are coupled in <i>Andrena</i> bees to a single variable			Not applicable.		
Season temperature		Mean temperature in a given year [°C] (1.iii. -31.v.): 2007: 10.2°C; 2008: 8.6°C			Mean temperature in season [°C] (1.iv.-15.vi.): 2007: 15.6 2008: 13.3 2009: 14.6 2010: 12.7		
Season precipitation	Sum precipitation in a given year [mm] (1.iii. -31.v.): 2007: 60.1 2008: 134.0	Sum precipitation in season [mm] (1.iv.-15.vi.): 2007: 70.2 2008: 171.8 2009: 140.0 2010: 169.7			0.18 0.18 0-0.75		
Relative time of activity during active days	Mean proportion of time (within the daily observation period) during which the bee was active and outside of the nest.	12.02 12.14 4.7-17.6			0.32 0.32 0-0.86		
Temperature during active days	Mean daylight temperature during active days [°C]	2.68 1.5 0-12.9			14.33 14.26 9.65-21		
Precipitation during active days	Sum of precipitation during active days [mm]	8.82 8.55 1.1-12.7			8.97 5.9 0-60.7		
Sunshine during active days	Mean sunshine during active days [h]	8.82 9 1-22			6.41 6.55 0-13.5		
Pollen availability during active days [pollen grains in air]	Mean number of pollen grains from willow trees collected in 1 m ³ of air during active days.	Not applicable.			Not applicable.		

Fig. 1. *Andrena vaga* parasitized by *Stylops* (a) and its nest entrance covered by soil (b); *Anthophora plumipes* on a flower (c) and an individually marked female sitting in the nest entrance (d). Photographs credit: P, Krásenský (a, b, c) and K, Doležalová (d).

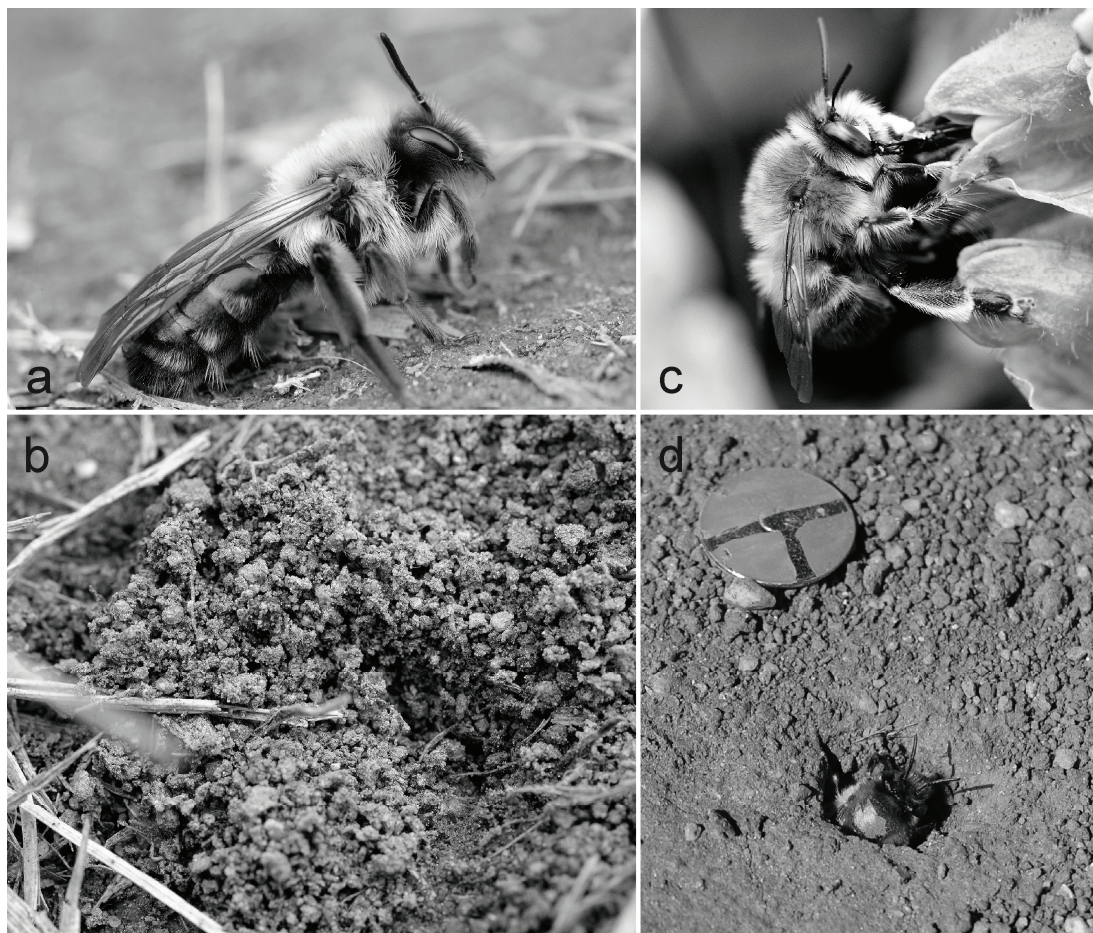
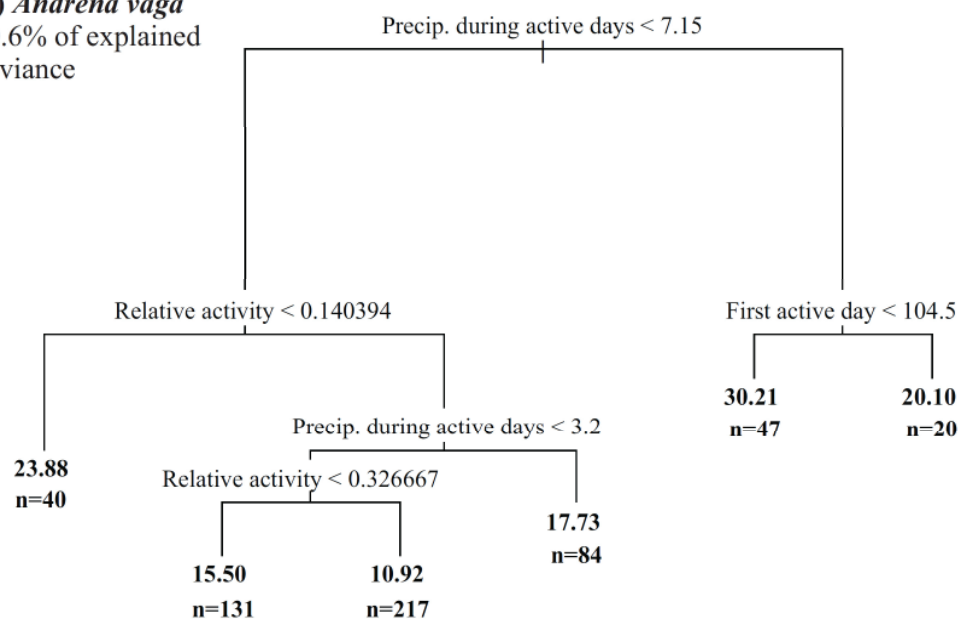


Fig. 2. Regression trees explaining the *lifespan* of the two bee species. The trees are based on binary recursive variance partitioning. As explanatory variables, we used all variables from Table 1. Names of the splitter variables and their split values are indicated at each node. Mean values of lifespan and the number of individual bees (n) in each terminal group are given at each terminal branch. For more details on the predictors see Table 1.

(a) *Andrena vaga*
50.6% of explained deviance



(b) *Anthophora plumipes*
63.4% of explained deviance

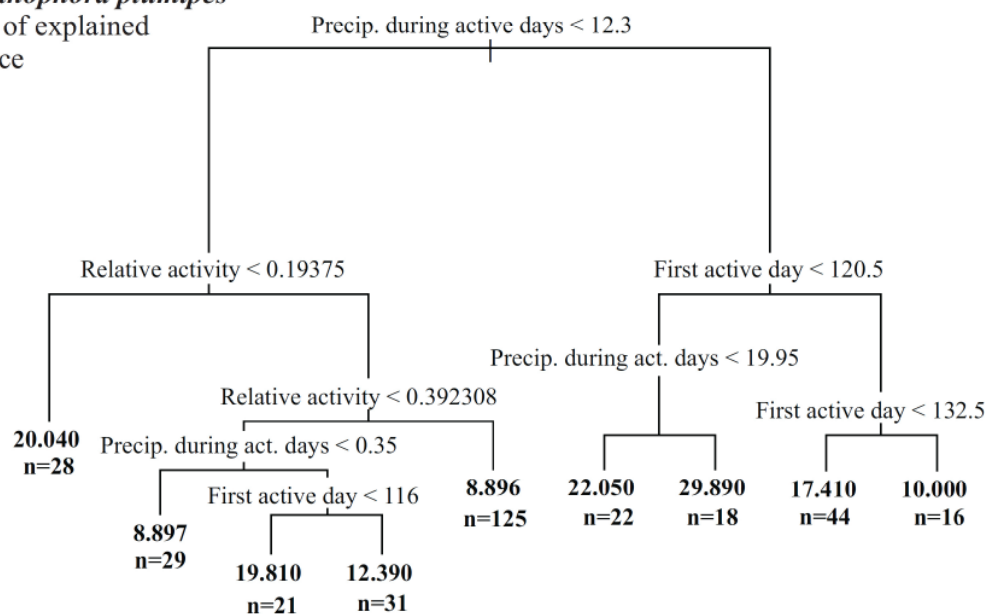
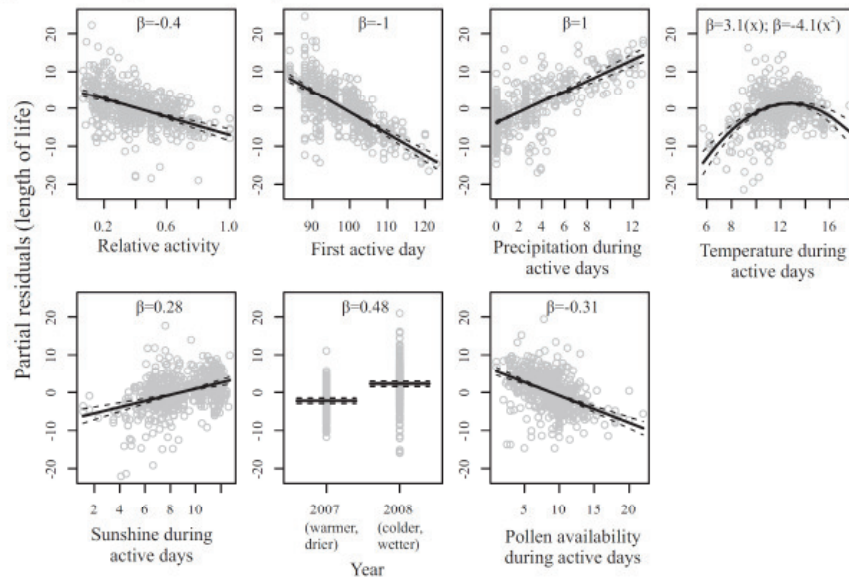


Fig. 3. Partial residual plots of the best fitting generalized linear models (GLM, Gaussian family) explaining *lifespan* in the two species. Partial residuals are those remaining after the effect of all the other variables has been accounted for. Solid lines represent fitted model terms, dashed lines represent standard errors, and grey circles represent partial residuals. The standardized coefficients (β) are provided. The greater the absolute value of β , the more important is the variable in the model (which is roughly equivalent to the steepness of the solid line with steeper lines indicating a stronger effect). For more details on the predictors see Table 1.

(a) *Andrena vaga*: 72.7% of explained deviance



(b) *Anthophora plumipes*: 65% of explained deviance

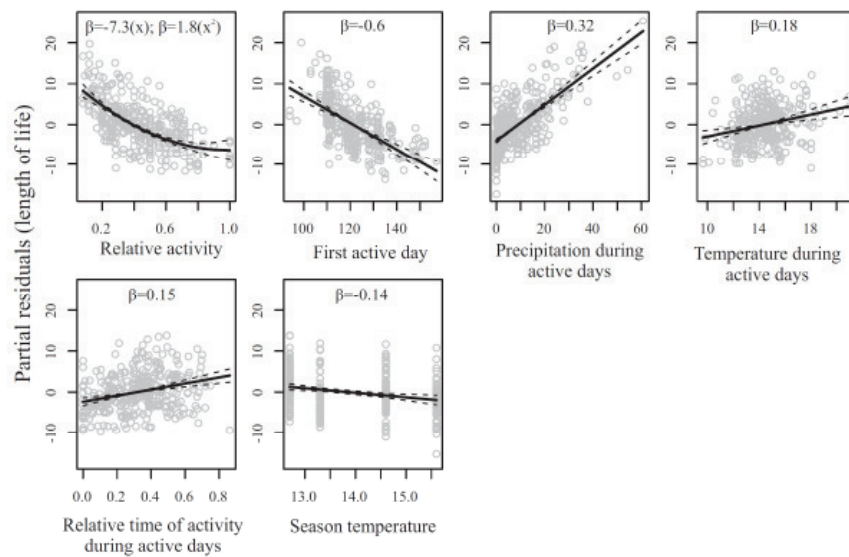
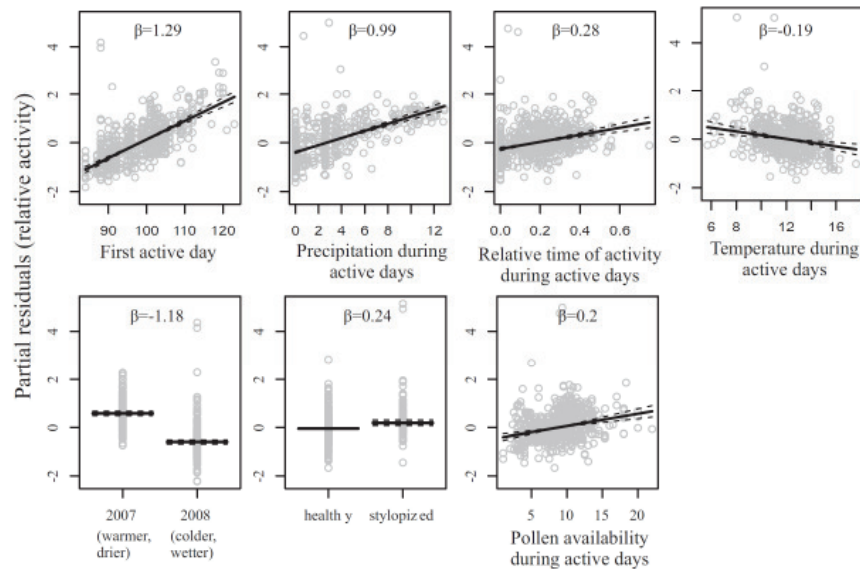


Fig. 4. Partial residual plots of the best fitting generalized linear models (GLM, binomial family) explaining *relative activity* (proportion of active days during the lifespan) in the two species. Partial residuals are those remaining after the effect of all the other variables has been accounted for. Solid lines are fitted model terms, dashed lines are standard errors and grey circles are partial residuals. The standardized coefficients (β) are provided. The greater the absolute value of β , the more important is the variable in the model (which is roughly equivalent to the steepness of the solid line with steeper lines indicating a stronger effect). The y-axis in the plots is in logit scale. For more details on the predictors see Table 1.

(a) *Andrena vaga*: 68% of explained deviance



(b) *Anthophora plumipes*: 34.1% of explained deviance

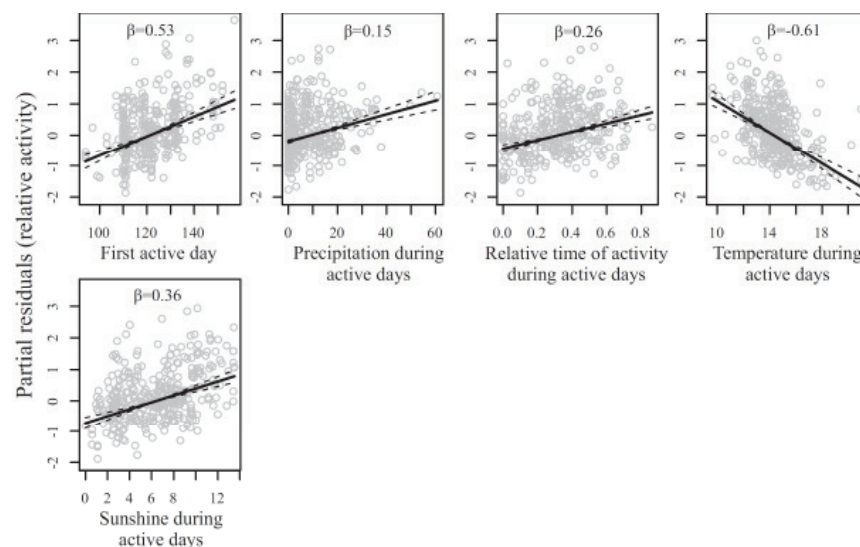
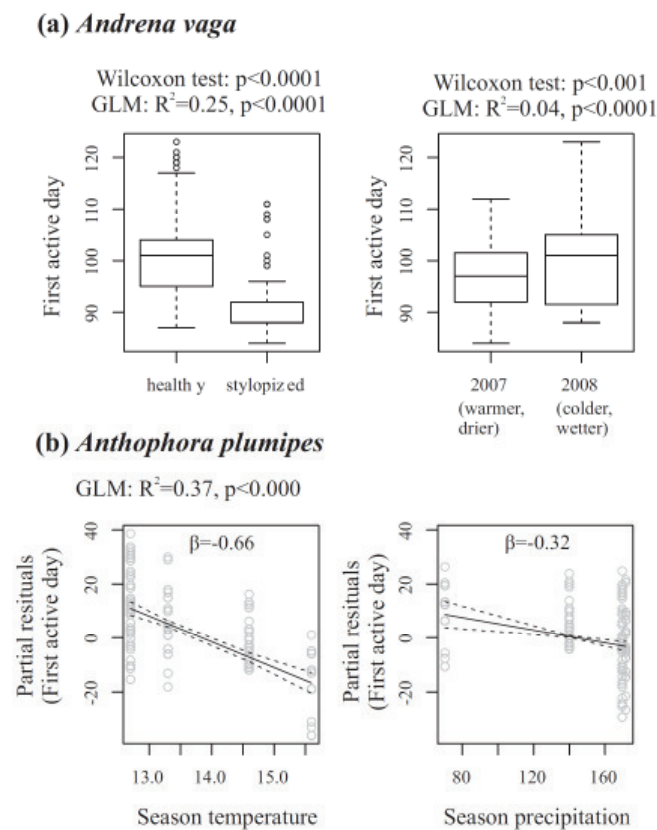


Fig. 5. Variables that correlate with the *first active day*. (a) The relationship between stylopization and first active day, and between season and first active day, in *Andrena* bees (boxplots show medians, quartiles and outliers). (b) Partial residuals of the generalized linear model explaining first active day in *Anthophora* bees.



Supplementary Information**Table S2.** Summary of dates and frequency of daily field observations of studied bees.

	beginning date (M/D)	end date (M/D)	season length (# of days)	observation (# of days)	females observed >4 active days	total females marked
<i>Andrena</i>						
2007	3.25	4.28	35	31	272	455
2008	3.28	5.10	44	35	267	283
<i>Anthophora</i>						
2007	4.4	5.24	51	19	59	177
2008	4.9	6.2	55	23	66	123
2009	4.20	5.28	39	26	91	133
2010	4.13	6.9	58	52	118	207

Table S3. Pearson's correlation coefficients between continuous variables used in this study. Values greater than 0.3 or lower than -0.3 are indicated by bold letters. For variable description see Table 1.

a) <i>Anthophora</i>										
Activity (act. days)	# of active days	Temperature (act. days)	Precipitation (act. days)	Sunshine (act. days)	Season temperature	Season precipitation	First active day	Lifespan		
# of active days									0.38	
Temperature (act. days)	-0.16									
Precipitation (act. days)	-0.03	-0.04								
Sunshine (act. days)	0.01	0.37	-0.25							
Season temperature	-0.11	0.14	-0.19	0.38						
Season precipitation	0.16	-0.16	0.18	-0.21	-0.91					
First active day	-0.26	0.34	0.17	-0.37	-0.59	0.47				
Lifespan	0.17	0.03	0.13	-0.02	0.11	-0.1	-0.37			
Relative activity	0.32	-0.22	-0.11	0.01	-0.25	0.3	0.09	-0.34		
b) <i>Andrena</i>										
Activity (act. days)	# of active days	Temperature (act. days)	Precipitation (act. days)	Sunshine (act. days)	Pollen availability	Season temperature	Season precipitation	First active day	Lifespan	
# of active days									0.47	
Temperature (act. days)	0.04									
Precipitation (act. days)	0.13	-0.18								
Sunshine (act. days)	-0.09	0.62	-0.5							
Pollen availability	-0.3	0.14	-0.26	0.17						
Season temperature	-0.29	0.55	-0.54	0.71	0.36					
Season precipitation	0.22	-0.55	0.54	-0.71	-0.36	-1				
First active day	0.18	0.21	0.18	0.03	-0.42	-0.2	0.2			
Lifespan	0.14	-0.14	0.18	-0.24	-0.29	-0.42	0.42	-0.3		
Relative activity	0.44	0.21	-0.01	0.16	-0.14	0.07	-0.07	0.53	-0.24	

PAPER VI

MULTILOCUS PHYLOGEOGRAPHY OF A WIDESPREAD PALEARCTIC SOLITARY BEE *ANTHOPHORA PLUMIPES* (HYMENOPTERA: APIDAE): MEDITERRANEAN LINEAGE ENDEMISM, ISLAND EFFECTS AND CRYPTIC SPECIES

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manuscript based on:

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Author's note:

Presented manuscript is based on the presentation of our preliminary results at the XXIV International Congress of Entomology 2012 in Daegu (the original poster also attached). We decided to summarise our actual results on *A. plumipes* phylogeography in the form of this short manuscript as a part of my Ph.D. thesis. Any comments to this manuscript are very welcomed.

Multilocus phylogeography of a widespread Palearctic solitary bee *Anthophora plumipes* (Hymenoptera: Apidae): Mediterranean lineage endemism, island effects and cryptic species

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Abstract:

Anthophora plumipes is a solitary bee species common across the whole Europe, Northern Africa, Middle East and Eastern Asia. *A. plumipes* has recently been introduced to the eastern coast of the USA from Japan and is nowadays a common species there. We collected samples from 41 localities including samples from USA and Japan. Our aim was to estimate the phylogeographic pattern within species range. We used 11 microsatellite loci and 727-bp sequence of COI for the analysis. We detected 67 COI haplotypes clustering into six major haplotype groups showing strong geographic pattern. Presence of several unique diverged Mediterranean haplotypes indicates high level of Mediterranean endemism. Bayesian clustering analysis of microsatellite data supported the COI groups, however the most likely result of K=4 joined the COI groups number I, III and IV together, while it detached the British localities from the continental group (I) to a separate group. We detected low genetic diversity and N_e in British and especially the USA and Japanese localities compared to European ones which can be attributed to bottleneck events during the island colonisation. Japanese and the USA populations are closely related and represent a separate lineage of possible species status.

Introduction:

Although the phylogeographic studies on various insect taxa are relatively common, they are surprisingly rare in bees (e. g. Garnery et al. 1992, Soucy and Danforth 2002, Dick et al. 2004) and nearly missing in solitary bees except for few studies on population structure on rather local scale (e. g. (Neumann and Seidelmann 2006, Beveridge and Simmons 2006, Exeler et al. 2010). *Anthophora plumipes* (Pallas) seems to be a suitable model for such study. It is a common polylectic early spring solitary bee widespread across the Europe, North Africa, Middle East and East Asia (Ascher and Pickering 2012). It also inhabit Mediterranean region, one of the biodiversity hotspots, famous for its large species diversity and high level of endemism (Blondel and Blondel 2010) as well as for the presence of several quarterly refugia (Stewart et al. 2009). The wide distribution of this species (as well as the existence of several differently coloured morphs) led also to several independent species descriptions, resulting in more than 20 different synonymous names for this species (Ascher and Pickering 2012).. Furthermore, since 56 adults of *A. plumipes* were introduced to Maryland (USA) from Japan (Sugata-Cho, Matsue) in 1989 (Batra 1994) as new potential pollinators, *A. plumipes* provides a suitable model for evaluating genetic effects of a recent introduction. We have obtained material of *A. plumipes* from the USA as well as from the most parts of its areal range (444 individuals from 41 localities, Fig. 1, Table 1) to complete following aims:

- 1) To disentangle the general phylogeographic pattern of *A. plumipes* within its areal range.
- 2) To evaluate the effects of recent introduction into the USA on *A. plumipes* genetic variability.

Methods:

A. plumipes was collected at 41 nesting localities in 2011-2012 (Table 1). We preferentially collected females; however, males were also included at localities where we have not

managed to collect sufficient number of females..

Individuals were genotyped at 11 novel microsatellite loci (anth75, anth76, anth52, anth8, anth47, anth33, anth45, anth64, anth51, anth61, anth1; (Černá and Straka 2012)) and a 727-bp long part of COI mtDNA was sequenced using either primers Pat and Ron (Simon et al. 1994) or primers developed in our previous study on bees (Habermannová et al. 2013). COI sequences were aligned using the ClustalW (Thompson et al. 1994) implemented in BIOEDIT 7.1.3.0 (Hall 1999) and sequences were collapsed to unique haplotypes using an online version of FaBox 1.41 (Villesen 2007).

The alignment was visualised using haplotype network constructed in the Network 4.6.1.0 (fluxus-engineering.com). We used median-joining algorithm (Bandelt et al. 1999) with null parameter epsilon, the MP option was chosen to simplify the network (Polzin and Daneshmand 2003). The matrix of genetic distances between haplotypes was calculated by BIOEDIT 7.1.3.0 (Hall 1999) using model F84 (Felsenstein 1984).

Bayesian and Maximum likelihood (ML) methods were utilized for phylogenetic analysis using the MrBayes (Huelsenbeck and Ronquist 2001) and PhyML (Guindon and Gascuel 2003) programs respectively. We used five *Anthophora*, one *Amegilla* and one *Habropoda* species as outgroups. The best DNA substitution model was estimated in jModelTest 2 (Posada 2008) using the AIC value (Akaike 1974) corrected for sample size (Sugiura 1978, Hurvich and Tsai 1989). The Bayesian analysis consisted of four simultaneous chain runs of 15 million generations, saving every thousandth tree. Convergence of chains was inspected using the program Tracer (Rambaut and Drummond 2007). The first 25% of trees were discarded as burn-in. For ML analyses 500 bootstrap replicates were performed for calculating the branch support values.

Basic population genetic characteristic, such as the mean number of alleles or expected heterozygosity, were calculated for localities with 15 or more females collected using

GenAlEx (Peakall and Smouse 2006). Population structure was examined using the Structure 2.3.4 program (Pritchard et al. 2000). Since Structure enables combination of diploid and haploid data we analysed male and female genotypes together. Admixture correlated model was chosen and 20 000 steps of burn-in of repetitions were followed by 200 000 repetitions. We performed 10 run for every K (expected number of populations) from 1 to 10. (Previous shorter runs with $K > 10$ showed very low similarity and posterior probability.) . The averages of individual and population assignments were used to produce the final graphs using the Distruct program (Rosenberg et al. 2002). Delta K method (Evanno et al. 2005) and comparison of similarities of parallel runs for different K as described in (Ehrich et al. 2007) was used to estimate the most likely K value.

We estimated the effective population size (N_e) in all the populations with more than 15 females collected using the LDNE program (Waples and Do 2008). We restricted the analyses to alleles with frequency higher than 0.01 and 0.05 respectively and used both JackKnife and Parametric methods to estimate the confidence intervals. Because of haplodiploid nature of bees, resulting effective population size is probably overestimated; however, it may still be useful for relative comparison of localities.

Results and discussion:

We have detected 67 unique COI haplotypes. The haplotype network clustered the haplotypes into six major haplotype groups, that we numbered I – VI and that clearly exhibited a strong geographic pattern (Table 1 and Fig. 1). Haplotypes from the USA and Japan fell into the same haplotype group (VI), which confirms Japanese origin of the USA population. Most of the samples collected in continental Europe clustered into one haplotype group (I) which exhibits partial geographical substructuring corresponding to Central and Western Europe (Fig. 2). Most of the haplotypes however occurred in both subclusters with

only 3 haplotypes unique to Central European subcluster and none unique for Central European subcluster. In the Mediterranean area we detected 17 unique haplotypes organised in four clusters (II, III, IV and V) which indicates high level of Mediterranean endemism in *A. plumipes* (Fig. 1, Fig. 2). Interestingly, mainland Greece localities possessed typical continental haplotypes belonging to group I (Fig. 1), which may indicate, that the species expanded into the Eastern Europe from the Balkan peninsula (and possible position of one refugium). *A. plumipes* belongs to partial heterotherms and generally tolerates much colder climate than is usual in bees (Stone and Willmer 1989) and thus we could expect not only the presence Mediterranean refugia, but also the cryptic Northern refugia, which has have not been known until recently (for summary see (Stewart et al. 2009)).

The mean genetic distances between the haplotype groups (Table 2) were calculated to discriminate the level of taxonomic relatedness in the studied species (Hebert et al. 2003). Although intragroup distances were small (0.005 on average), differences between main groups were surprisingly large (Table 2), with the largest distance detected among VI group (USA + Japan) and all other European groups (avg. distance 7.4 %). While the COI distances within species usually do not exceed 2-3 % of pairwise differences (Hebert et al. 2004), the mean percentage of sequence divergences of COI sequence between congeneric species is regularly 8% or more (Hebert et al. 2003). The detected divergence thus implies that the Japanese (and USA) and European populations may represent two distinct species. Japanese population is called *Anthophora plumipes* ssp. *villosula* Smith, 1854 (Batra 1994) and this may represent a valid name of this presumably separate species. Moreover, the Mediterranean haplotype groups might also represent separate subspecies, as their mutual genetic distances and distances from mainland European populations were 0.03 or higher (Table 2).

The best DNA substitution model found by program jModelTest 2 for our data was TIM3 + I + G. However, as this model is not implemented in MrBayes program for Bayesian

analysis, the second suggested DNA substitution model (GTR + Γ + I) was used instead. Mean log likelihood of resulting trees from Bayesian analysis was -3418.582. Best ML tree found in analysis showed -3195.7870 log likelihood. The resulting phylogenetic tree corresponds well to the pattern of haplotype network and distinguished six major clades that match the detected haplotype groups (Fig. 3). The USA+JP haplotypes (group VI) were found to be a sister lineage to all European haplotypes. This is not surprising, when we take into account geographic as well as genetic distance between the populations. The statistical support of the crown branches that correspond to the haplotype groups was generally high (Fig. 3); however, the exact topology of clades was unfortunately not supported sufficiently to draw any other detailed conclusions about relationship of mainland European populations.

In Structure, the Evanno *et al.* (2005) method and similarity values inferred the optimal number of clusters $K=4$, which corresponded to following haplotype groups: 1) I (except for British localities) + III + IV 2) I (only Great Britain), 3) V + II and 4) VI (Fig. 4). However, for $K=6$, the clusters corresponded well to the clusters in the haplotype network and phylogenetic tree, although there were some differences (Fig. 4). While the Structure consistently divided the WE group into continental and British, this pattern was not detected in the COI analyses. *A plumipes* is common in England, but is missing in Scotland and Ireland (Roberts 2010), which, together with the Structure clustering, might imply relatively recent colonization of the Great Britain from the Western Europe. This hypothesis is further supported by the markedly decreased N_e and genetic diversity in British populations (see below), which could result from founder effect during colonization. The other difference is clustering of Greek groups II and III. Group III clusters with European group I, while group II seems to cluster with Israel group IV. However, the sample size of Greek and Israel populations is generally small and needs to be increased before we can draw any detailed conclusions from their position.

LDNE showed large differences between continental and island populations. All the island localities (British and Japanese) and introduced USA population exhibited low levels of genetic diversity and N_e compared to continental populations (Tab. 3). This together with the star pattern of haplotype network in these areas (Fig. 2) indicates past bottleneck event followed by rapid population expansion as the consequence of islands colonisations. The USA population was introduced from Japan and we thus expected to find a noticeable decrease in genetic diversity and N_e in USA population compared to the Japanese population, as it was described in other recently introduced species (Zayed et al. 2007). However, our analysis showed that both the source Japanese populations and the USA population share low levels of genetic diversity and N_e (Table 3). It should be noted that we collected sufficient sample just from one Japanese locality and hence we cannot completely exclude the possibility of a local effect. Further, we showed that the USA + Japan populations probably represent a distinct species. The suboptimal performance of microsatellites developed for *A. plumipes* could thus represent other possible explanation of low genetic variability. However, we have not detected the presence of null alleles when analysing haploid males from these populations, which makes this possibility unlikely. The more probable explanation is that the low genetic diversity of Japanese population is a consequence of a bottleneck event, such as a founder effect during the colonization of Japanese islands from the continental Asia.

The production of nonviable or sterile diploid males homozygous at the sex-determination locus is a common problem in bee species with dramatically reduced genetic variability (Paxton et al. 2000, Zayed et al. 2007, Zayed 2009). (Zayed et al. 2007) reported, that about 30% of males of *Lasioglossum leucozonium*, which were recently introduced to USA and which also suffered similar drastic reduction of genetic diversity and N_e , were diploid. Interestingly, we have not detected presence of any diploid in our sample of 40 males, which deserves further investigation in the future.

Conclusion:

Both mitochondrial and nuclear markers inferred similar phylogeographic pattern in *A. plumipes*. Most of the continental European localities form a single cluster, with tendency to separate the Western Europe or Great Britain from the rest of the localities. Mediterranean localities showed high level of endemism. American and Japanese localities formed a separate clade probably representing a cryptic species which supports the Japanese origin of the USA population. However, the introduced USA population does not show lowered genetic variability when compared to the Japanese source population.

In future we would like to concentrate on the following topics: 1) to add more gens to the analysis to improve phylogenetic signal and reconstruct phylogeography of this species, 2) to describe the pattern of Mediterranean endemism of *A. plumipes* in detail, 3) to localize the glacial refugia of the species, 4) to collect and analyse more samples from continental eastern Asia enabling to solve the question of the extreme loss of genetic diversity and N_e in Japanese populations, and finally 5) to collect more samples in the USA and focus on the detection of diploid males.

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Table 1: Localities, numbers of analysed males (M) and females (F) for microsatellites (Msat) and Cytochrome Oxidase I (COI) and assignments to haplotype groups (HG).

Code	State	GPS N (°)	GPS E/W (°)	Msat (F)	Msat (M)	COI (F+M)	HG
LI	Austria	48.220125	14.270411E	8	2	3	I
RZ	Austria	48.783041	15.987323E	1	1	2	I
BE	Belgium	50.840090	4.392774E	6	2	7	I
BERO	Belgium	50.162805	5.225310E	1	0	1	I
BR	Belgium	50.840090	4.392774E	3	10	15	I
MN	Belgium	50.462121	3.954778E	5	3	10	I
HA	Czech Rep.	48.815293	16.002886E	1	3	5	I
HV	Czech Rep.	49.637431	16.910184E	3	0	3	I
LD	Czech Rep.	48.809003	16.807895E	12	0	10	I
MO	Czech Rep.	49.723017	17.080155E	20	15	27	I
OP	Czech Rep.	49.940314	17.897853E	20	0	0	I
PHA	Czech Rep.	50.079816	14.392088E	19	0	16	I
OD	Denmark	55.395814	10.390014E	2	0	2	I
FR	France	49.546935	0.9477961	8	2	8	I
VI	France	50.623696	3.110378E	6	12	16	I
DE	Germany	47.809895	7.628806E	19	0	0	I
KO	Germany	47.661021	9.168491E	6	0	5	I
BU	Hungary	47.465781	18.925617E	11	4	5	I
KE	Hungary	46.754542	19.474315E	4	7	10	I
RO	Romania	44.758766	21.791835E	0	2	1	I
DM	Great Britain	52.604117	0.368591E	20	0	20	I
EN	Great Britain	52.228241	0.900042W	20	7	25	I
NR	Great Britain	52.633334	1.416676E	0	8	7	I
SH	Great Britain	52.712480	2.751749W	0	4	3	I
IT	Italy	44.491505	8.057709E	4	1	3	I
AK	Greece	37.650000	22.840910E	0	5	5	I
MID	Greece	37.883333	22.866667E	1	2	1	I
MY	Greece	37.734167	22.758333E	0	2	2	I
OL	Greece	37.636667	21.637000E	0	8	5	I
KA	Greece	35.025558	24.772374E	0	8	3	II
PT	Greece	38.022943	24.518909E	1	5	5	III
IS	Israel	32.250845	34.862122E	3	3	4	IV
MP	France	43.616371	3.866224E	0	4	3	V
AR	Spain	41.037542	0.559956W	2	2	3	V
CA	Spain	40.974765	0.831897W	0	1	4	V
MA	Spain	40.418332	3.711427W	13	4	16	V
VH	Spain	41.354329	1.034416W	2	11	5	V
KT	Japan	35.680377	134.979057E	1	5	5	VI
KY	Japan	35.025731	135.780501E	18	19	32	VI
MW	Japan	33.612332	130.505905E	1	0	1	VI
USA	USA	37.221785	77.289169W	23	18	34	VI
	Sum			264	180	332	

Table 2: Matrix of mean genetic distances (GD) between six detected haplotype groups. The minimal and maximal values for intra- and intergroup comparisons are in bold.

	I	II	III	IV	V	VI
VI	0.0771	0.0770	0.0680	0.0688	0.0767	0.0017
V	0.0432	0.0542	0.0395	0.0476	0.0084	
IV	0.0332	0.0445	0.0363	0.0048		
III	0.0351	0.0414	0.0037			
II	0.0430	0.0028				
I	0.0084					

Mean intragroup GD = 0.005

Table 3: Number of alleles (Na), expected heterozygosity (He) and effective population size (Ne) of large continental (MO, OP, DE) and island populations (DM, EN, KY, USA) with sufficient female sample size ($N_{\text{fem}} > 15$).

Loc.	State	Nfem	Na	He	Freq.	Ne	Confidence intervals		
							Parametric	Jackknife	
MO	Czech Republic	20	6.6	0.695	0.05	97.4	36.8	∞	38.6
OP	Czech Republic	20	6.7	0.662	0.01	94.9	39.6	∞	45.0
					0.05	24.4	15.6	45.3	15.9
DE	Germany	19	6.2	0.588	0.01	33.7	21.0	69.4	20.7
					0.05	316.0	42.2	∞	39.4
DM	Great Britain	20	2.5	0.292	0.01	163.7	43.8	∞	39.8
					0.05	9.9	2.8	47.4	2.0
EN	Great Britain	20	3.3	0.360	0.01	12.8	3.7	69.1	2.8
					0.05	6.6	2.8	14.3	2.2
KY	Japan	18	2.4	0.226	0.01	11.3	5.4	26.2	3.4
					0.05	1.4	0.9	2.2	0.8
USA	USA	23	2.8	0.248	0.01	1.7	1.1	2.9	1.0
					0.05	1.6	1.0	2.6	0.7
					0.01	1.4	1.0	1.9	0.9
									2.2

Fig. 1: Map of sampled localities. Colours indicate presence of haplotypes of the six major haplotype groups.



Fig. 2: Haplotype network based on mitochondrial COI sequences consisting of six major haplotype groups. Colours used for the haplotype groups are the same as in the Fig. 1 and Fig 3. The length of branches responds to the genetic distance between the haplotypes.

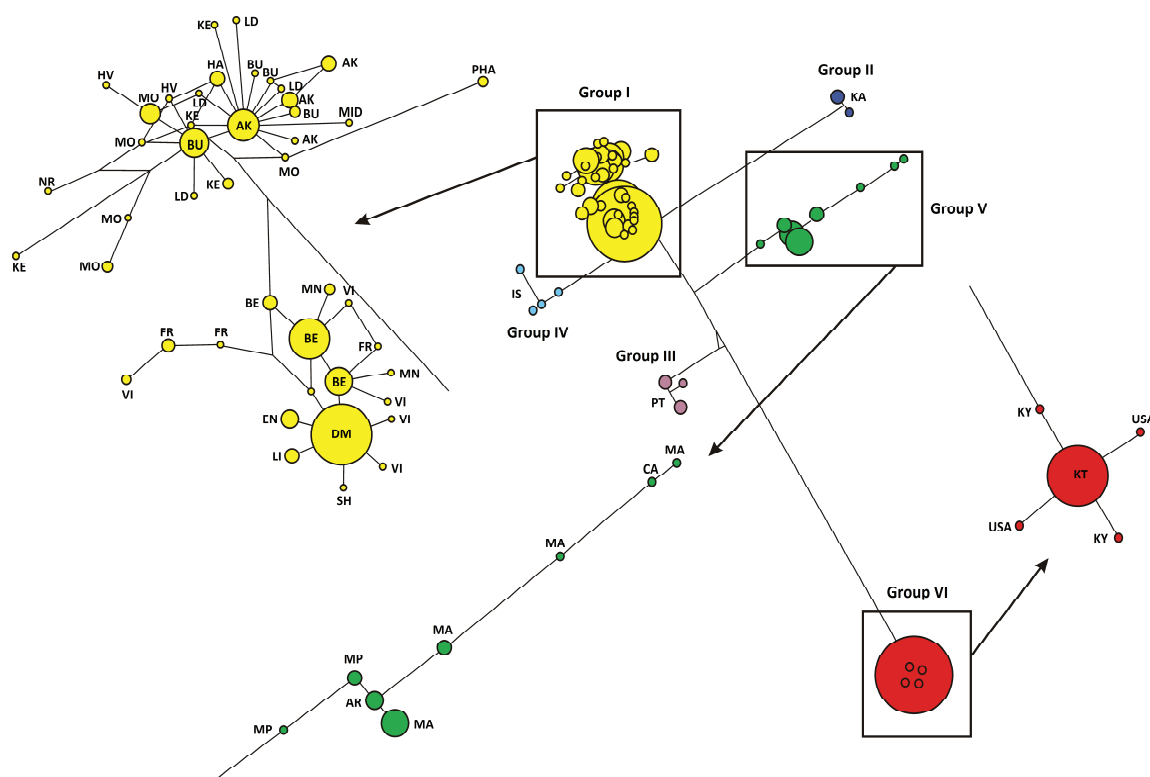


Fig. 3: Phylogenetic tree resulting from the Bayesian analysis with branch lengths. Bayesian posterior probabilities/ the maximum likelihood bootstrap values are given. Clades with posterior probabilities below 0.50 are collapsed. Bootstrap values below 50 are replaced by an asterisk (*). Tree branches from maximum likelihood analysis incongruent with presented Bayesian tree are indicated by dash (-). Haplotype groups are indicated on the right.

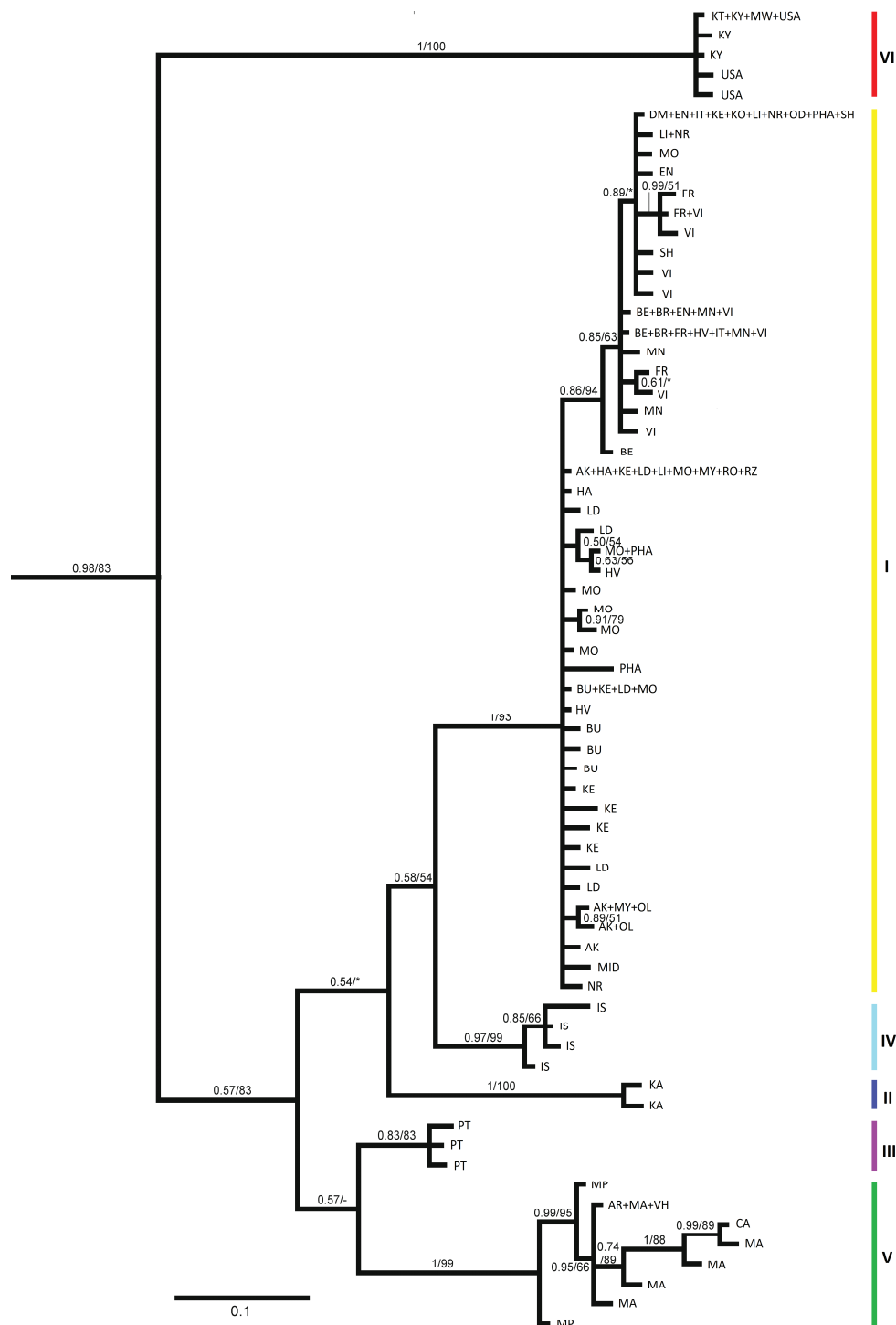
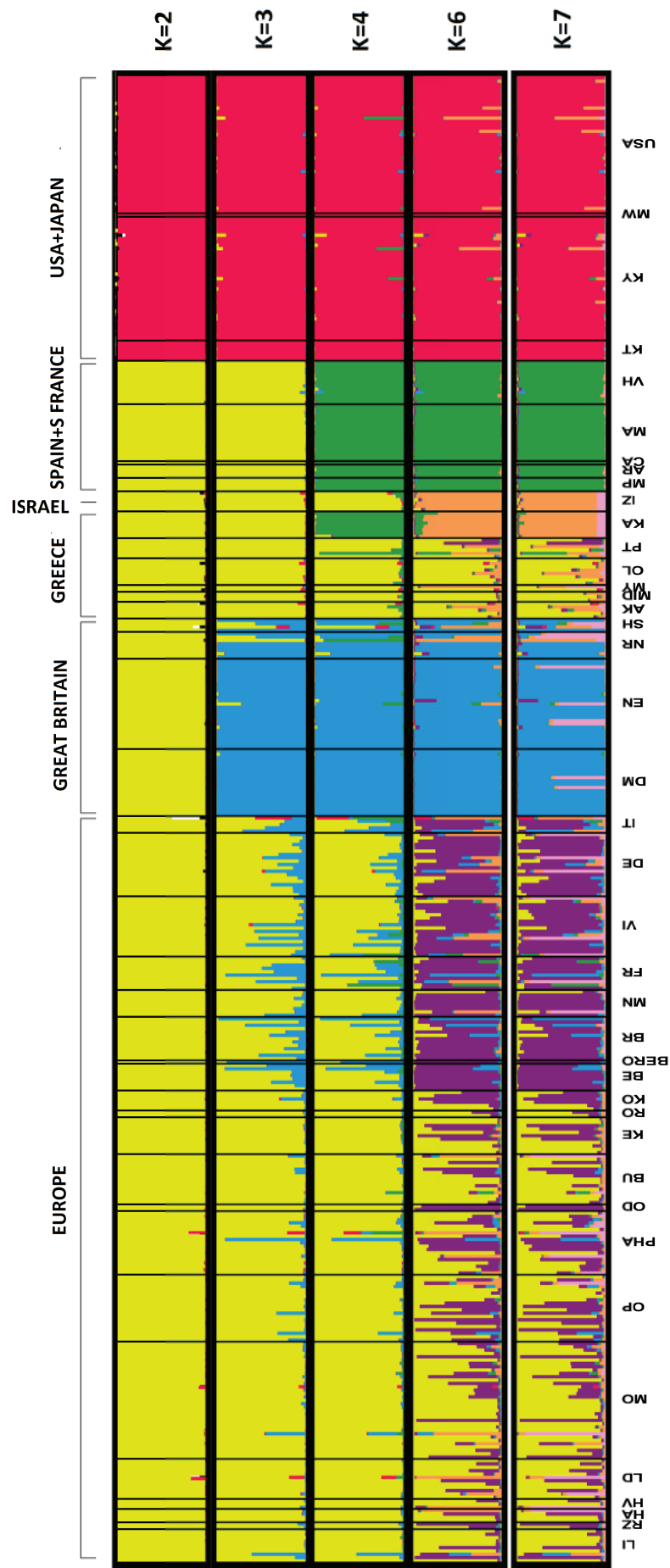


Fig. 4: Structure clusters for K from 2 to 7.



CONCLUSION AND FUTURE PROSPECTS

My study aimed to fulfil three goals – 1) describe the nesting behaviour of solitary bees and find possible behavioural patterns of solitary bees at the nesting site that could serve as preadaptations to the sociality or cleptoparasitic behaviour, 2) detect the presence and frequency of intraspecific cleptoparasitism in solitary bees and 3) enhance the knowledge about solitary bee genetics and their conservation state by describing their population structure.

The first aim was fulfilled by the description of behaviour of *Andrena vaga* at the nesting site, which resulted in detailed ethogram and characterising of species specific foraging and daily behavioural patterns. We found that bee behaviour can be rigorously described, although the individual behavioural variability is large. This makes interspecific comparison of bee species possible in future research. We are planning to perform such interspecific comparative behavioural analysis for our model species. Obtained seasonal data on nesting behaviour of *Andrena vaga* and *Anthophora plumipes* further enabled us to determine environmental and biotic factors that influence the lifespan of solitary bees in the wild.

Following the research on basic behavioural patterns of some bees, we detected and studied intraspecific cleptoparasitism in the form of nest usurpations in bees from four different bee families; intraspecific cleptoparasitism thus probably represents a widespread alternative nesting strategy in solitary bees. We further showed that regular nest abandonments and switches are surprisingly more frequent in solitary bees than formerly expected. We suggested that described nest founding dynamics may play a crucial role in the early evolution of sociality, because it results in the substantial decrease of intraspecific aggressiveness. We will continue to study intraspecific cleptoparasitism with the effort to detect a presence and frequency of brood parasitism in solitary bees by means of our novel methodology combining the marking of pollen brought by provisioning females using lanthanides, continuous behavioural observation of individually marked bees and their nests and analysis of maternity. We believe that brood parasitism is more common in solitary bees than known and expected, similarly to the surprisingly high detected frequency of nest usurpations.

Finally, we fulfilled the third aim by the description of population structure of two solitary bee species *Andrena vaga* and *Anthophora plumipes*. We showed that *Andrena vaga*, although strongly specialised in food and nesting requirements, is well adapted to the patchy distribution of suitable nesting habitats and contrary to expectations does not show the signs of increased population structure even in very heterogeneous environment of central Europe. However, we detected the presence of two genetically distinct groups that could result from the secondary contact of populations separated in the past. Except for the genetic differentiation, these two groups differ also in the presence of parasite *Stylops ater*. We are going to study the cause of described population structure in detail by collecting more samples of *A. vaga* from Western and Eastern Europe. We will also test the extent to which the distribution and the population structure of *Stylops* parasite mirror the patterns detected in its host bee *A. vaga*. Considering *Anthophora plumipes*, the other bee species studied by us, the first preliminary phylogeographic study in its wide distribution range revealed presence of six distinct populations. Some of them could be classified as subspecies or even separate

species based on genetic distances. Increased population differentiation was detected especially in Mediterranean region. This species was recently introduced to the USA from Japan, which we confirmed in our analysis, and this introduction seems to be very successful in spite of a severe decrease of genetic diversity and N_e of both the source Japanese population and the resulting USA population. We are planning to improve our sampling of this species especially in the Mediterranean region and Asia before we complete this study so we can better answer all the questions that have arisen during the preparation of our preliminary study (such as detected presence of high Mediterranean endemism or causes of decrease in genetic variability of Japanese population).

Population genetics of common Palearctic solitary bee
Anthophora plumipes (Hymenoptera: Anthophoridae)
in whole species areal and result of its recent introduction in the USA



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Introduction:

Anthophora plumipes (syn. *A. pilipes*) is a common polylectic early spring solitary bee spread across the Europe, North Africa, Middle East and East Asia (www.discoverlife.org). 56 adults of *A. plumipes* were introduced to the Maryland (USA) from Japan (Sugata-Cho, Matsue) in 1989 (Batra, 1994). We have obtained material of *A. plumipes* from the USA as well as from the most parts of its areal range (444 individuals from 41 localities, Fig. 1) to complete following aims:

- 1) Describe the general phylogeographic pattern of *A. plumipes* within its areal range.
- 2) Evaluate the result of recent introduction of *A. plumipes* in the USA and confirm Japan origin of this population.



Fig. 1: Map of sampled localities. Colours illustrate geographical distribution of major haplotype groups.

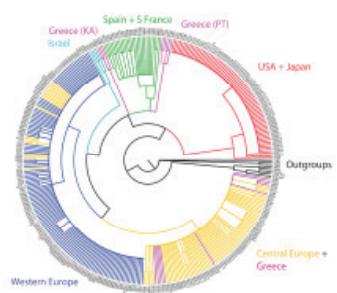


Fig. 3: NJ tree based on mitochondrial DNA (COI) sequence. Colours correspond to geographic origin

Locality	State	Na	Nfem	Freq.	Ne	Confidence intervals		
						Parametric	Jackknife	
MO	Czech Republic	6.7	20	0.05	97.4	36.8	Infinite	38.6
				0.01	94.9	39.6	Infinite	45.0
OP	Czech Republic	6.6	19.6	0.05	24.4	15.6	45.3	15.9
				0.01	33.7	21.0	69.4	20.7
DE	Germany	6.2	19	0.05	316.0	42.2	Infinite	39.4
				0.01	163.7	43.8	Infinite	39.8
DM	Great Britain	2.5	20	0.05	9.9	2.8	47.4	2.0
				0.01	12.8	3.7	69.1	2.8
EN	Great Britain	3.3	20	0.05	6.6	2.8	14.3	2.2
				0.01	11.3	5.4	26.2	3.4
KY	Japan	2.4	18	0.05	1.4	0.9	2.2	0.8
				0.01	1.7	1.1	2.9	1.0
USA	USA	2.8	23	0.05	1.6	1.0	2.6	0.7
				0.01	1.4	1.0	1.9	0.9

Tab. 1: Estimates of effective population size (Ne) based on microsatellite analysis. Na - mean number of alleles per loci, Nfem - number of analysed females (mean per loci)

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Methods:

* We used 11 novel microsatellite loci (anth75, anth76, anth52, anth8, anth47, anth33, anth45, anth64, anth51, anth61, anth1; Cerna & Straka, 2012) + 1131-bp sequence of COI mtDNA using primers Pat + Ron (Simon et al. 1994). COI sequences were aligned using a Clustal W alignment (Higgins et al. 1994) implemented in BIOEDIT 7.1.3.0 (Hall 1999).
* Network 4.6.1.0 (fluxus-engineering.com) was used to identify unique haplotypes and construct a haplotype network. We used median-joining algorithm (Bandelt et al. 1999) with null parameter epsilon and MP option to clean up network (Polzin & Daneschmand 2003).
* Neighbor-joining (N-J) tree and N-J bootstrap tree of COI sequences were conducted using PAUP* (Swofford 1999). Branch support consist of 10,000 bootstrap replicates. We used *Amegilla quadrijasciata*, *Habropoda laboriosa*, *Anthophora pubescens* and three Spanish *Anthophora* spp. as outgroups for the analysis.
* Population structure was examined using program Structure 2.3.4 (Pritchard et al. 2000) using microsatellites. The number of clusters (K) was varied between K = 1 and K = 10 (previous shorter runs with K > 10 showed very low similarity and posterior probability). We used admixture correlated model, burn-in of 20 000 repetitions, followed by 200 000 repetitions. Each value of K was run ten times, we used the averages of individual and population assignments of these runs to produce the final graphs using program Distruct (Rosenberg et al. 2002) and method of Evano et al. (2005) to estimate the optimal K.
* We estimated the effective population size (Ne) in all the populations with more than 15 females collected using program LD NE (Waples & Do 2008). We used microsatellite alleles with frequency higher than 0.01/0.05 respectively and used both default methods (Jackknife and Parametric) to estimate confidence intervals. Because of haplo-diploid nature of bees, the effective size is probably overestimated, however, it may be still useful for relative interpopulation comparison.

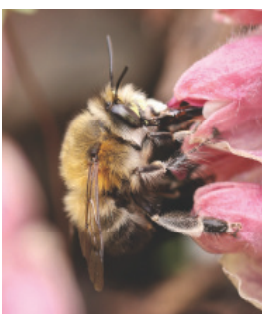


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Results:

We detected 67 haplotypes in seven major haplotype groups (Fig. 2). Central Europe and Western Europe haplotype groups are often of mixed origin indicating presence of migrants. Presence of several unique Mediterranean haplotypes indicates high level of Mediterranean endemism.

The NJ tree showed seven major clades consensual to the haplotype clusters (Fig. 3).

The Structure analysis inferred clustering to four groups (K=4). However, for K=6 the inferred clusters correspond best to clusters within haplotype network and NJ tree (Fig. 4).

LDNE showed dramatic decrease in Ne of British and especially USA + Japan populations compared to continental populations (Tab. 1). These values of Ne together with the decreased genetic variability and star pattern of haplotype network indicates past bottleneck event (founder effect) followed by rapid population expansion.

All the analyses confirm Japan origin of the USA population. Both population shares extremely low genetic variability on both nuclear and mitochondrial level (Fig. 2, Tab. 1).

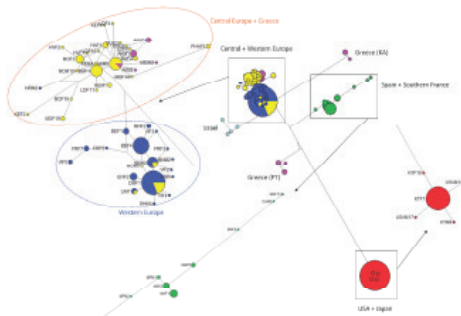


Fig. 2: Haplotype network based on mitochondrial COI sequences, colours demonstrate geographic origin of haplotypes: red—USA + Japan, violet—Greece, blue—Western Europe, green—Spain + Southern France, yellow—Central Europe, light blue—Israel.

However, the USA population does not show pattern of further recent bottleneck event compared to maternal Japan population. The introduction was highly successful, this species is common in the Eastern coast of the USA.

The origin of Japan population and the cause of drastic loss of genetic variability in this population is interesting topic for future research.

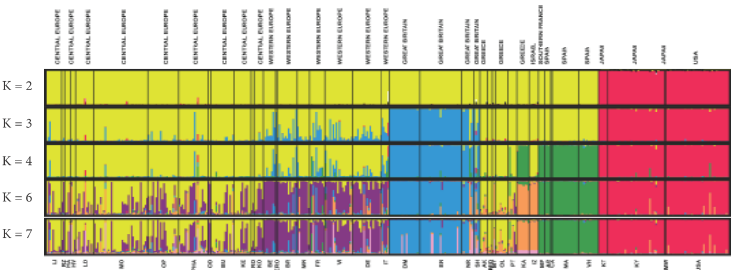


Fig. 4: Population structure based on microsatellite analysis using Structure. We show 5 graphs with highest similarity between runs (>0.9), K - number of clusters

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