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Evolution of floral preference and nesting behaviour in osmiine bees

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1. Summary

Bees rely on pollen and nectar as nourishment for their larvae. Their diligence in visiting flowers to collect these floral products makes bees the most ubiquitous pollinators of most flowering plants. Although rarely considered as such, bees are also herbivorous insects that consume large amounts of living plant tissue, namely pollen. As in other herbivorous insects, diet breadth in bees spans a continuum from strong specialisation (oligolecty) on plant species from one or a few related genera, to broad generalisation (polylecty) of host use characterized by pollen collection from a wide taxonomic selection of plant species derived from several families. More importantly, bees vary in their quality as pollinators from indispensable mutualists to antagonistic pollen thieves. Therefore, given the enormous pollen requirements of bees, flowers are expected to balance the need to attract pollinators with the requirement to restrict excessive pollen loss to bees. To achieve protection of their vegetative tissue against herbivorous insects, plants rely on strategies including both morphological and chemical mechanisms. In analogy, plants have evolved various morphological floral traits to conceal their anthers and thus reduce pollen accessibility to bees. Furthermore, increasing evidence suggests that chemical protection of pollen might also play an important role in bee-flower relationships. Consequently, a number of bees have evolved morphological and behavioural adaptations to gain access to concealed pollen. However, the question as to whether bees require physiological adaptations to successfully digest certain pollen types remained largely unexplored.

To tackle this important question, the larval performance of the two closely related and highly pollen-generalist solitary bee species *Osmia*

bicornis and *Osmia cornuta* was assessed when reared on four different pure pollen diets. While *Osmia bicornis* failed to survive on a pollen diet of the viper's bugloss *Echium vulgare* but developed well on a pollen diet of the buttercup *Ranunculus acris*, the reverse held true for *Osmia cornuta*. Both bee species performed well on *Sinapis arvensis* pollen, while neither of the two species managed to develop on *Tanacetum vulgare* pollen. These results strongly suggest that these bees require physiological adaptations for the digestion of certain pollen types. The unsuitability of certain pollen types for many oligolectic and some polylectic bee species further suggests that the presence of protective chemical substances hampers pollen digestion by bees.

It was recently suggested that buttercup (*Ranunculus*) pollen contains high concentrations of ranunculin, the glucosyl hydrate form of the highly reactive and toxic lactone protoanemonin, which causes the toxicity of these plants. To test whether this secondary metabolite was responsible for the unsuitability of *Ranunculus* pollen for larval development of two bee species, bioassays were combined with chemical analyses of *Ranunculus* pollen. The larvae of *Chelostoma rapunculi* and *Heriades truncorum* were respectively reared on *Campanula* and Asteraceae pollen diets obtained from conspecific nests and mixed with ranunculin in five increasing concentrations. Despite the toxic effect of ranunculin on both bee species when present in high concentrations, the concentrations detected in the pollen were found to be too low to kill the bee larvae. Therefore other factors may be responsible for the unsuitability of *Ranunculus* pollen as a food source for the tested bee species.

In addition to pollen nutritional quality, accessibility to pollen in complex flower architectures constrains host plant choice in bees. The recently

formulated ‘constraint hypothesis of host range evolution in bees’ aims to describe the evolutionary mechanisms underlying host choice patterns in bees, but its general validity remains to be tested. Ancestral state reconstruction by phylogenetic inference of floral preferences among species of the *Annosmia-Hoplitis* group revealed that host plant choice in these bees is governed by strong evolutionary constraints. Most oligolectic species in this group exclusively exploit either Boraginaceae or Fabaceae, whereas all polylectic species harvest pollen from both Boraginaceae and Fabaceae. These two plant families are neither closely related nor do they share similar flower morphologies, which implies that similar chemical pollen compositions led to the observed pattern of host choice among bees of the *Annosmia-Hoplitis* group.

Ancestral state reconstruction by phylogenetic inference is not only a powerful tool to unravel the patterns of host plant choice, but also to study the evolution of other biological traits such as nesting behaviour. Applying it to both the genus *Hoplitis* and the *Annosmia-Hoplitis* group revealed that ground nesting in excavated burrows was the ancestral state in these bees. Once the transition from below to above ground nesting was achieved, nesting site preferences strongly diversified. In particular, specialization to nesting in borrows in dead wood and hollow stems has probably promoted dispersal events between the Old and the New World by means of ‘cross ocean rafting’ of whole nests. This dispersal mechanism would explain the exclusive presence of wood nesting *Hoplitis* species in North America.

2. Zusammenfassung

Bienen ernähren sich und ihre Nachkommen von Pollen und Nektar. Ihr sprichwörtlicher Fleiss, mit dem sie von Blüte zu Blüte fliegen um ihre Nahrung zu sammeln, macht sie zu den wichtigsten Bestäubern von Blütenpflanzen. Doch Bienen sind ebenso herbivore Insekten, die grosse Mengen an Pollen, also lebendem Pflanzengewebe, fressen. Ähnlich wie bei anderen herbivoren Insekten reicht der Spezialisierungsgrad bezüglich des konsumierten Pollens von hoch spezialisiert auf Blüten einer einzigen Pflanzengattung oder Pflanzenfamilie (oligolektisch) bis hin zu ausgesprochen unspezifisch, wobei Blüten von einer ganzen Reihe von Pflanzenfamilien als Pollenquellen genutzt werden (polylektisch). Zudem unterscheiden sich Bienen stark in ihrer Bestäubungseffizienz. Einige Bienen sind überaus effiziente Bestäuber und damit oft unverzichtbare Mutualisten der Pflanzen, während andere ausgesprochen ineffizient sind und manchmal gar zu Pollendieben werden können. Deshalb befinden sich Pflanzen im Dilemma einerseits Bienen für die Bestäubung anlocken zu müssen und andererseits ihren Pollen gegen übermässiges Absammeln durch Bienen zu schützen. Eine Vielzahl morphologischer und chemischer Anpassungen ist bekannt, mit denen sich Pflanzen gegen herbivore Insekten zur Wehr setzen. Analog dazu sind zahlreiche Fälle bekannt, wo Pollen durch spezielle Blütenstrukturen vor Bienen geschützt wird. Und tatsächlich weisen jüngste Forschungsergebnisse darauf hin, dass auch chemischer Pollenschutz für gewisse Pflanzen eine wichtige Rolle spielt. Da Bienen eine Reihe morphologischer Anpassungen und Verhaltensmustern entwickelt haben, um sich Zugang zu verstecktem Pollen zu verschaffen, stellt sich die Frage, ob Bienen auch physiologische Anpassungen zur Verdauung von chemisch geschütztem Pollen entwickelt haben.

Um diese Frage zu beantworten, wurde untersucht, ob sich die Larven der zwei sehr nah verwandten und ausgesprochen polylektischen, solitären Bienenarten *Osmia bicornis* und *Osmia cornuta* in ihrer Fähigkeit unterscheiden, sich auf demselben Pollen zu entwickeln. Um dies zu testen wurden Eier der zwei Bienenarten auf vier verschiedene reine Pollenprovisionen umgesetzt, auf denen sich oligolektische Bienenarten zum Teil nicht entwickeln konnten. Es zeigte sich, dass sich die Larven von *Osmia bicornis* problemlos auf Hahnenfuss-Pollen entwickeln konnten, während sie auf Natterkopf-Pollen verendeten. Interessanterweise war dies bei *Osmia cornuta* genau umgekehrt. Zudem konnten sich beide Bienenarten problemlos auf Ackersenf-Pollen entwickeln, währendem sie auf Rainfarn-Pollen verendeten. Diese Resultate sind ein starkes Indiz dafür, dass Bienen physiologische Anpassungen zur Verdauung gewisser Pollensorten benötigen. Das Resultat, dass gewisse Pollensorten den schnellen Tod der Larven vieler oligolektischen und sogar polylektischen Arten verursachen, deutet darauf hin, dass es giftige chemische Substanzen im Pollen haben könnte, die dessen Verdauung verhindern.

Die Resultate früherer Forschungsarbeiten deuten darauf hin, dass Hahnenfuss-Pollen hohe Konzentrationen des Sekundärmetaboliten Ranunculin beinhaltet. Ranunculin ist ein Glucosyl-Hydrat, aus welchem durch Glykolyse das hoch reaktive, giftige Lacton Protoanemonin freigesetzt wird, das wiederum für die Giftigkeit von Hahnenfussgewächsen (*Ranunculus*) verantwortlich ist. Um zu testen, ob dieser Sekundärmetabolit für die unverträglichen Eigenschaften von Hahnenfuss-Pollen verantwortlich ist, wurden ein Biotest und chemische Analysen dieses Pollens durchgeführt. Die Larven der zwei oligolektischen Bienenarten *Chelostoma rapunculi*, ein Glockenblumen-

Spezialist, und *Heriades truncorum*, ein Astern-Spezialist, wurden jeweils auf ihren spezifischen Pollenvorräten aufgezogen, die mit Ranunculin in verschiedenen Konzentrationen vermengt wurden. Dabei stellte sich heraus, dass hohe Ranunculin-Konzentrationen zwar giftig für die untersuchten Bienenlarven sind, die Ranunculin-Konzentration im Hahnenfuss-Pollen jedoch weit unter der toxischen Konzentration liegt. Daraus ist zu schliessen, dass andere, bislang unbekannte Faktoren für die Unverdaulichkeit von Hahnenfuss-Pollen für diese Bienenarten massgebend sind.

Nicht nur die Qualität des Pollens als Larvennahrung, sondern auch der Zugang zum oft in komplexen Blütenstrukturen verborgenen Pollen kann die Auswahl an Wirtspflanzen für Bienen beschränken. Die kürzlich formulierte 'Constraint Hypothese für die Evolution des Wirtspflanzenspektrums bei Bienen' beschreibt die evolutionären Mechanismen, die zu den Mustern der Wirtswahl von Bienen führt. Um die allgemeine Gültigkeit dieser Hypothese zu testen, wurde am Beispiel der *Annosmia-Hoplitis*-Gruppe die Evolution der Blütenpräferenzen anhand einer Phylogenie rekonstruiert. Und tatsächlich konnten die rekonstruierten Muster nur durch das Einwirken starker evolutionärer Constraints erklärt werden. Die meisten oligolektischen Arten dieser Bienengruppe sind entweder auf Boraginaceen-Pollen oder auf Fabaceen-Pollen spezialisiert, während sämtliche polylektische Arten Pollen auf Blüten beider Pflanzenfamilien sammeln. Die Tatsache, dass diese zwei Pflanzenfamilien nur sehr entfernt miteinander verwandt sind und zudem ihren Pollen in höchst unterschiedlichen Blütenstrukturen morphologisch schützen, legt nahe, dass eine ähnliche chemische Zusammensetzung der Polleninhaltstoffe der Präferenz für diese zwei Wirtspflanzen zugrunde liegt.

Die Rekonstruktion ursprünglicher Merkmalszustände anhand einer Phylogenie eignet sich auch zur Entschlüsselung der biogeographischen Verbreitungsgeschichte und der evolutionären Mechanismen der Nistbiologie. So konnten wir zeigen, dass die Gattung *Hoplitis* ursprünglich in der Alten Welt entstanden ist und mehrere Artengruppen unabhängig voneinander Südafrika und die Neue Welt kolonisierten. Zudem zeigte sich, dass in dieser Bienengattung, die sich durch äusserst diverse Nistweisen auszeichnet, nisten in selbst gegrabenen Gängen im Boden ursprünglich ist. Eine Gegenüberstellung der Nistweise und der Biogeographie weist darauf hin, dass Nisten im Totholz die wiederholte Kolonisierung der Neuen Welt entscheidend vereinfachte. Das liegt wahrscheinlich daran, dass die Bienen, noch bevor sie aus den Nestern im Totholz schlüpfen, wie auf einem Floss die Ozeane überqueren können. Diese spezielle Art der Verbreitung könnte auch erklären, warum sich die *Hoplitis*-Fauna der Neuen Welt ausschliesslich aus Totholznisten beschränkt.

3. General introduction

3.1. Bees – an overview

Bees belong to the Hymenoptera, a large insect order that also comprises the ants and the wasps. Almost 20 000 bee species have been described worldwide, which makes them a highly diverse group of organisms (Michener 2007). In contrast to their carnivorous ancestors, the sphecid wasps, bees collect pollen and nectar that they transport to their nests to feed their offspring. Pollen consists of living plant tissue that provides proteins, lipids, sterols and vitamins, and nectar complements these nutrients with sugars and water. All bees directly depend on their host plants, and their proverbial assiduity in visiting flowers makes bees very important pollinators.

The pollen requirement of bees is immense. For instance, some bees need the total pollen content of several hundred flowers for each of their offspring (Müller et al. 2006), and Schlindwein et al. (2005) demonstrated that more than 95% of the total pollen amount produced by a host plant population may be depleted by bees. Their efficiency in pollen removal from flower anthers during a single visit may amount to 70-90% (Thomson 2003). To prevent self-fertilization, many flowers temporally separate the male from the female phase. Hence, pollen-collecting bees often confine their visits to male-phase flowers, which not only inhibits pollination, but also efficiently deteriorates the success of pollination by other flower visitors that are less focused on pollen. The liaison between bees and flowers is therefore far from purely mutualistic but is best regarded as a relationship of mutual exploitation (Westerkamp 1996).

As a consequence, plants are expected to evolve traits to protect their pollen and thus to narrow the spectrum of pollen feeding bees. On the one hand, many flowers restrict access to their pollen by concealing their anthers in specialized flower structures (Müller 1995; Westerkamp 1997; Westerkamp & Classen-Bockhoff 2007). In turn, some bees have evolved specialized morphological or behavioural adaptations to efficiently retrieve the hidden pollen (reviewed in Thorp 2000). On the other hand, growing evidence suggests that plants may also chemically protect their pollen from bees (Guirguis & Brindley 1974; Williams 2003; Praz et al. 2008a) and that bees in turn developed physiological adaptations to successfully digest these pollen types. As a result, bee flower relationships are probably shaped by an evolutionary arms race much like the relationship between plants and herbivorous insects.

3.2. Host plant specializations in bees - an evolutionary constraint

In their natural habitats, bees are often confronted with a staggering array of flowers to choose from. Like their herbivorous counterparts, bees differ widely in the range of host plants they exploit for pollen. Bees that collect pollen on flowers of a few related plant taxa are referred to as oligolectic, whereas polylectic bee species exploit flowers of two or more plant families for pollen (Robertson 1925; Cane & Sipes 2006; Müller & Kuhlmann 2008). Oligolecty and polylecty co-occur in all investigated bee communities and both obviously represent successful evolutionary strategies.

Traditionally, it has been a widely accepted assumption that oligolectic bees have evolved from polylectic ancestors (Michener 1954; Linsley 1958; MacSwain et al. 1973; Iwata 1976; Moldenke 1979; Hurd et al. 1980). However, more recent studies suggest that oligolecty is best considered to be an evolutionary constraint that has been repeatedly overcome in many polylectic bee lineages (Müller 1996a; Larkin et al. 2008; Sedivy et al. 2008). In a study that traced host plant shifts in the osmiine bee genus *Chelostoma*, Sedivy et al. (2008) formulated the 'constraint hypothesis of host range evolution' in bees. This hypothesis suggests that i) incorporations of new hosts are rare events in the evolutionary history of bee lineages, ii) host expansion is only possible if the physiological or neurological constraints imposed by the flowers can be overcome and iii) host shifts among oligoleges are typically proceeded by a period of expanded host range followed by respecialization. However, because this hypothesis was formulated based on patterns of host plant evolution from only a single bee genus (*Chelostoma*), it remains to be tested for its universal validity in other bee groups.

3.3. THE NESTING BEHAVIOUR OF BEES - THE MASTERS OF NEST ARCHITECTURE

All bees build nests in which they amass pollen and nectar to nurture their offspring. While the majority of bees do so in excavated burrows in the ground (Michener 2007) using secretions from the specialized Dufour's gland to line their brood cells with a water-repellent lining (Hefetz 1987), bees of the family Megachilidae use a different strategy. Megachilidae species utilize a variety of foreign material like mud or clay, but also masticated plant material, resin or a variety of flower petals (Westrich, 1989; Banaszak & Romasenko, 2001; Michener, 2007; Müller 2012 and

references therein) to carpenter their brood cells, which allows the construction of above ground nests in a wide range of preexisting niches that vary widely in shape and location. These locations comprise insect borings in dead wood, hollow stems, rock crevices or empty snail shells (Westrich 1989; Müller et al. 1997; Michener 2007). Suitable nesting sites are resources that determine the range of a bee species in both local and global scales. To investigate processes that have governed the evolution of this diversification, and to relate different nesting types to biogeography, we focused on the genus *Hoplitis*, the largest genus of the tribe Osmiini.

3.4. Objectives of this thesis - why Osmiini?

The Osmiini (Hymenoptera: Aculeata: Megachilidae) possess a number of characteristics that make them outstanding model organisms for the study of important biological, ecological and evolutionary traits for bees in general. First of all, in contrast to most other solitary bees that build their nests in excavated burrows in the ground, a number of native osmiine species from a variety of taxa can be reared in artificial nests such as hollow bamboo sticks. This facilitates rearing and allows easy access to the eggs and brood cell provisions for larval feeding experiments. Second, from strict oligolecty to broad polylecty, the host plant spectra of the osmiine bees comprise a wide range of diet breadths. This allows comparative evolutionary and experimental studies of host plant range, physiological adaptations to cope with different pollens, and morphological adaptations to pollen collection on differently shaped flowers. Third, the diversity of nesting biology in the osmiine bees is unmatched by any other bee group. Given a sound phylogenetic framework, the Osmiini, and the genus *Hoplitis* in particular, are highly

suitable representatives for studying the evolution of nesting behaviour in bees. The present thesis takes advantage of these multiple favourable characteristics to approach the following objectives.

The first chapter *(Closely related pollen generalist bees differ in their ability to develop on the same pollen diet: evidence for physiological adaptations to digest pollen)* focuses on the need of bees to physiologically adapt to their pollen diet. The question of whether bees are physiologically adapted to digest unfavourable pollen is investigated by comparing larval performance of two closely related and highly polylectic osmiine bee species on four different pure pollen diets. Since closely related specialist bee species usually have similar pollen preferences, differences in larval performance of closely related polylectic bees on the same pollen diet provides strong evidence for the need of physiological adaptations to digest that pollen.

The second chapter *(Too low to kill: concentration of the secondary metabolite ranunculin in buttercup pollen does not affect bee larval survival)* aims to elucidate the cause of unsuitability of *Ranunculus* pollen for several tested bee species. The impact of different amounts of the secondary metabolite ranunculin, the precursor of toxic protoanemonin present in many buttercup (*Ranunculus*) species, on the larval development of two oligolectic osmiine bee species is tested. These two bee species are specialized on Asteraceae and Campanulaceae, respectively, and their larvae were previously shown to fail to develop on *Ranunculus* pollen (Praz et al. 2008a). To elicit whether ranunculin causes the unsuitability of *Ranunculus* pollen as a food source for the tested bee species, the ranunculin concentration in *Ranunculus* pollen is analysed and compared to the concentrations applied in the biotests.

The third chapter (*Molecular phylogeny of the bee genus* Hoplitis *(Megachilidae: Osmiini) - how does nesting biology affect biogeography?*) shifts the focus from experimental to theoretical evolutionary questions. The main goal is to resolve phylogenetic relationships between species of the osmiine bee genus *Hoplitis* in an attempt to consolidate the current morphology-based systematics with the help of molecular data, or to correct it where necessary. With the help of the resulting phylogenetic tree, this study aims to reconstruct the biogeographic history of the genus and to highlight its intriguing relationship with nesting biology.

The last two chapters focus on the *Annosmia-Hoplitis* group, a species rich and biologically highly diverse clade within the genus *Hoplitis*. Based on a molecular phylogeny and an extensive assessment of the biological peculiarities of 44 species, the fourth chapter (*Host range evolution in a selected group of osmiine bees (Hymenoptera: Megachilidae): the Boraginaceae-Fabaceae paradox*) traces the evolution of host plant choice and tests the universal validity of the previously formulated "constraint hypothesis on host range evolution in bees" (Sedivy et al. 2008). It thereby aims to unravel the seemingly paradoxical evolutionary mechanisms that led to this groups' characteristic affinity to the two remotely related host plant families Boraginaceae and Fabaceae. Furthermore, this study provides insights into the unmatched diversity of morphological and behavioural adaptations that evolved to collect the hidden pollen from flowers with concealed anthers.

Finally, the fifth chapter (*Evolution of nesting behaviour and cleptoparasitism in a selected group of osmiine bees (Hymenoptera: Megachilidae)*) focuses on the evolution of the nesting biology and cleptoparasitism in the *Annosmia-Hoplitis* group. By mapping nesting sites and cleptoparasitic behaviour onto the phylogeny of the *Annosmia-Hoplitis* group presented in the previous chapter, we analyse the evolutionary patterns of nest site selection in this group of bees and ask whether cleptoparasitic *Bytinskia* species have evolved from the same lineage as their hosts.

4. Closely related pollen generalistbees differ in their ability to develop on the same pollen diet: evidence for physiological adaptations to digest pollen[1]

4.1. ABSTRACT

Given the enormous quantitative pollen requirements of bees and their high efficiency in pollen removal, flowers should balance the need to attract bees for pollination on the one hand and to restrict pollen loss to bees on the other hand. Although various morphological flower traits have been identified that reduce excessive pollen losses to bees, the question of whether pollen might also be chemically protected remains largely unexplored. In this study we compared the larval performance of the two very closely related and highly pollen generalist solitary bee species *Osmia bicornis* and *Osmia cornuta* on four different pollen diets. Despite their very large pollen diet breadth, the two bee species showed striking differences in their ability to develop on pollen of the same plant species. *Osmia bicornis* developed well on *Ranunculus* pollen but failed to do so on *Echium* pollen, whereas the reverse held true for *O. cornuta* with the exception of two larvae grown on *Ranunculus* pollen that developed into dwarfish adults. Both bee species performed well on *Sinapis* pollen, while neither of the two species managed to develop on *Tanacetum* pollen. The observed differences in larval survival of these two *Osmia* species when reared on the same pollen diet as well as their failure to develop on *Tanacetum* pollen clearly demonstrate that bees require physiological adaptations to cope with the unfavourable chemical properties of certain pollen. Our results show a remarkable analogy of

[1] Based on Sedivy, C., A. Müller & S. Dorn. 2011. Functional Ecology 25:718-725.

bee-flower relationships with herbivore-plant interactions and possibly indicate that the pollen of certain plant taxa might be chemically protected.

4.2. Introduction

The great majority of flowering plants attract animals to distribute pollen. Bees, which comprise between 20 000 and 30 000 species worldwide, are the primary pollen vectors in most ecosystems (Michener 2007). They differ from all other pollinating animal taxa except for the masarid wasps in one key aspect: pollen and nectar are not only consumed by the adults, but additionally serve as the exclusive food source for their larvae. The pollen of up to several hundred flowers is required to rear one single offspring (Müller et al. 2006), and bees were found to deplete more than 95% of the total pollen amount produced by their hosts (Schlindwein et al. 2005). In addition, bees are highly efficient in pollen collection (Westrich 1989; Müller 1996a), frequently removing 70-90% of all available pollen contained in a flower per visit (Thomson 2003). As bees store pollen immediately after its collection in specialized hairbrushes (scopae) or in the crop, where it is often inaccessible for pollination (Westerkamp 1996; Thomson 2003), pollen carryover curves of bees usually fall off very quickly (Thomson 2003 and references therein). Pollen-collecting bees are therefore often low-efficiency pollinators, which remove much pollen but deposit only little (Thomson & Thomson 1992), or in some cases even none at all, thereby acting as pollen thieves (Hargreaves et al. 2009). In the presence of high-efficiency pollinators, bees with low or no pollination efficiency become functional flower parasites and often considerably decrease pollination success (Wilson & Thomson 1991; Aigner 2001; Thomson 2003). Furthermore, pollen-collecting bees

commute several times a day between nest and host plants, and hence have a limited average foraging range of a few hundred meters only (Zurbuchen et al. 2010), which considerably increases the quantity of pollen withdrawn by bees in the vicinity of their often aggregated nests. Thus, given the high quantitative pollen requirements of bees and their high efficiency in pollen removal, flowers should trade the need to attract bees for pollination against excessive pollen losses to pollen-harvesting flower visitors (Westerkamp 1996).

Various morphological floral traits help to reduce pollen loss by narrowing the spectrum of pollen-feeding flower visitors. Flowers of many plant taxa restrict access to their pollen by concealing it within specialized anthers or flower structures (Vogel 1993; Harder & Barclay 1994; Müller 1995; Westerkamp 1997; Westerkamp & Classen-Bockhoff 2007), from where it can be efficiently harvested only by bees possessing specialized morphological or behavioural adaptations (Thorp 2000; Müller et al. 2006 and references therein). In addition, portioned pollen release over an extended period of time does not only increase the probability of successful pollination by enforcing repeated pollinator visits (Harder & Wilson 1994; Schlindwein et al. 2005), but is expected to limit pollen loss to bees as well (Castellanos et al. 2006). The finding that several pollen specialist and generalist bee species failed to develop on non-host pollen suggests that selection might also act on the nutritional quality or toxicity of pollen to reduce excessive pollen losses to bees (Guirguis & Brindley 1974; Williams 2003; Praz et al. 2008a). In fact, pollen of some plant taxa is of poor nutritional quality due to the lack of essential nutrients or the low protein content (Schmidt et al. 1987, 1995; Roulston & Cane 2000; Génissel et al. 2002; Rasmont et al. 2005), or it contains secondary compounds, which are repellent or toxic to insects (Roulston & Cane 2000; Hargreaves et al. 2009 and references

therein).

Patterns of host plant use by bees, such as the widespread specialization to a few closely related hosts, the occurrence of periods of expanded host range followed by respecialization, or the phylogenetically conserved host associations, display striking similarities to those of herbivorous insects (Janz & Nylin 2008; Sedivy et al. 2008). This indicates that the underlying mechanisms determining host plant use in both bees and herbivores might be based on similar plant characteristics, e.g. plant chemistry. While it is well known that many herbivorous insects are physiologically adapted to cope with the defensive secondary compounds of their hosts (Opitz & Müller 2009), knowledge of whether bees require specialized physiological adaptations to digest the pollen of certain plant taxa is lacking. Due to the high chemical variability of pollen with respect to its content of amino acids, lipids, starch, sterols, vitamins or secondary metabolites (Roulston & Cane 2000 and references therein), we hypothesize that bees need specialized physiological adaptations to cope with the unfavourable chemical properties of some pollen.

To investigate whether bees are physiologically adapted to digest unfavourable pollen, we compared the larval performance of the two very closely related and highly pollen generalist solitary bee species *Osmia bicornis* and *Osmia cornuta* (Megachilidae: Osmiini) (Fig. 1) on four pollen diets containing pure pollen of *Sinapis arvensis* (Brassicaceae), *Ranunculus acris* (Ranunculaceae), *Tanacetum vulgare* (Asteraceae) and *Echium vulgare* (Boraginaceae). Closely related bee species were repeatedly shown to have similar pollen preferences (Sedivy et al. 2008 and references therein) and highly pollen generalist bee species are able to thrive on pollen of a wide array of plant taxa (Westrich 1989). Therefore, differences in larval performance of the two tested *Osmia* species on pollen of the same plant species would provide strong

evidence for the need of physiological adaptations to digest that pollen.

4.3. METHODS

4.3.1. Bee species

The two bee species *O. bicornis* (Linnaeus 1758) and *O. cornuta* (Latreille 1805) are very close relatives. Both are members of the subgenus *Osmia* and within this subgenus belong to the same monophyletic group ('bicornis group'), which comprises about 15 species worldwide (Peters 1978; Michener 2007). *Osmia bicornis* and *O. cornuta* are among the most pronounced pollen generalist solitary bee species in Europe, collecting pollen on at least 18 and 13 plant families, respectively (Westrich 1989). Both species are widespread in the Palaearctic and common in most parts of Central Europe. They nest in a great variety of pre-existing cavities, allowing for artificial breeding in hollow bamboo sticks. The adult females build several brood cells during their lifetime, which lasts for up to 6 weeks (Westrich 1989). Each cell is provisioned with pollen and nectar before a single egg is laid. The hatched larva feeds on the pollen-nectar mixture and develops within a few weeks to the adult insect, which overwinters inside the cell and emerges early in spring.

FIGURE 1: *Osmia bicornis* female enters her nest with a full pollen load (photograph: A. Krebs).

For the present study, cocoons of both *O. bicornis* and *O. cornuta*, originating from one population each (Konstanz, Germany), were transferred to two artificial nesting stands in Zurich containing hollow bamboo sticks. The first stand offered access to a diverse array of flowering plant species (Botanical Garden). The second stand, located inside a large (8 x 10 x 3,5 m) walk-in cage covered with gauze, limited access to a single plant species, *S. arvensis*, planted in 500 pots with two plants each (ETH Campus Hönggerberg).

4.3.2. Origin of pollen

The larvae of *O. bicornis* and *O. cornuta* were reared on four pollen diets containing pollen of a single plant species each, i.e. *S. arvensis*, *R. acris*, *T. vulgare* and *E. vulgare*, as well as on a control pollen diet. To obtain

pollen diets for the experiments, we collected bamboo sticks with freshly completed nests of different bee species (see below), split them longitudinally with a knife and collected the provisions from within the brood cells. Prior to use in the experiments, the provisions were stored at -20 °C.

To obtain pollen diets of *S. arvensis*, we collected brood cell provisions from nests of *O. bicornis* and *O. cornuta* built in the walk-in cage. These brood cell provisions were frozen at -20 °C and subsequently used few days later for the experiments. To obtain pollen diets of the other three plant taxa, we collected brood cell provisions from nests of three solitary bee species, which are strict pollen specialists (Westrich 1989) and which, like *O. bicornis* and *O. cornuta*, belong to the tribe Osmiini: *Chelostoma florisomne* (specialized on *Ranunculus*), *Heriades truncorum* (specialized on Asteraceae) and *Hoplitis adunca* (specialized on *Echium*). These brood cell provisions were collected during the previous season and stored frozen at -20 °C for 6-9 months. The average quantity of nectar sugar in the brood cell provisions of *C. florisomne*, *H. truncorum* and *H. adunca*, which amounts to 54%, 62% and 64% of total dry provision weight, respectively, does not differ substantially from the average quantity of nectar sugar in the provisions of *O. bicornis* and *O. cornuta* (56% and 53%, respectively) (A. Bühler and A. Müller, unpublished data). Hence, we consider the use of brood cell provisions of these three specialist bee species as suitable for our comparative experimental approach.

Nests of *C. florisomne* were collected at Gletterens (western Switzerland), where the main pollen source was *R. acris*. Nests of *H. truncorum* were collected from a fallow at Benken (northern Switzerland), where *T. vulgare* grew at a very high density. Although *H. truncorum* is specialized at the level of plant family, microscopical analyses of pollen

samples from collected brood cell provisions revealed that they all contained < 5% non-*Tanacetum* pollen. Nests of *H. adunca* were collected at several localities in northern Switzerland, where the only available host plant was *E. vulgare*.

To obtain control pollen diets, bees nesting at the Botanical Garden were allowed to collect pollen on the naturally available flower supply. The microscopical analysis of the pollen content of 12 randomly selected brood cell provisions of each species revealed that the pollen collected by *O. bicornis* consisted mainly of pollen of Rosaceae, *Fagus* (Fagaceae) and *Acer* (Aceraceae), while the pollen collected by *O. cornuta* was mainly composed of pollen of several species of Rosaceae.

4.3.3. Egg transfer and larval performance

Rearing of the larvae of *O. bicornis* and *O. cornuta* on the five different pollen diets was conducted in artificial brood cells. Artificial cells were made of small blocks (4 x 2 x 2 cm) of beech wood provided with a drilled burrow (2 cm length, 0,8 cm width) open both at the top and at the front side. The openings were covered with coverslips attached to the block with transparent adhesive tape to permit free viewing into the burrow.

Eggs used for the experiments were carefully detached with a thin spatula from the brood cell provisions in the original nest and transferred to the experimental pollen diet previously placed into the burrow of an artificial cell. Larvae were reared individually in artificial cells. The eggs of both species originated from nests of the same population (Konstanz, Germany). For each bee species, we transferred 30 - 33 eggs onto each of the five pollen diets. As sex could not be determined with certainty a priori, each larva, received the same quantity of pollen diet. To account

for the different body weight of the two species, we provided a quantity of 400 and 600 mg of pollen diet for *O. bicornis* and *O. cornuta*, respectively.

Egg hatching and larval development took place in darkness in the same climate chamber (E7⁄2; Conviron, Winnipeg, Canada) under the following conditions: 25 °C for 16 h followed by a gradual reduction of temperature to 10 °C within 4 h followed by a gradual increase back to 25 °C within another 4 h. Relative humidity was held constant at 70%. The following parameters of larval development were recorded every second day: egg hatching, start of feeding, start of defecation, start of cocoon spinning, completion of cocoon, death. In addition, the larvae were weighed every second day to the nearest 0,1 mg (AB204-S; Mettler Toledo, Greifensee, Switzerland). To prevent mechanical damage to the fragile freshly hatched larvae, weighing started 6 days after hatching. We discontinued weighing as soon as the larvae started to spin a cocoon.

To prevent the spread of diseases, we removed artificial cells with dead eggs or larvae and, upon completion of the cocoons, cleaned the cells from faeces and leftover pollen. Unhatched eggs and larvae that died from external factors, such as foulbrood or mechanical damage, were excluded from all analyses.

Once all larvae had either died or completed their cocoons, conditions in the climate chamber were changed to 26 °C and 60% relative humidity. After 5 months, cocoons were carefully opened with nail scissors to assess survival, imaginal weight and sex.

4.3.4. Data analysis

For survival analyses, we treated all larvae that had completed their cocoon as survivors irrespective of whether they later successfully completed metamorphosis or not. Cocoons were considered completed upon becoming entirely intransparent. Dates of egg hatching, start of feeding, start of defecation, start of cocoon spinning, completion of cocoon and death were determined as the average of the two observation dates between which the respective event occurred.

We used Kaplan-Meier survival statistics to compare larval survival on the different pollen diets following Lee & Wang (2003). The number of days between hatching and completion of the cocoon was considered as 'censored data': individuals that died before the completion of the cocoon represented the exact observations for which the event (death) occurred, while those that completed the cocoon were the censored observations. The latter were considered survivors and thus withdrawn from survival calculations. To test for differences between survival distributions, we applied the log-rank test with Bonferroni correction using the option 'pairwise for each stratum' implemented in SPSS 16.0 (SPSS Inc., Chicago, Illinois, USA) when comparing two groups. We tested differences between larval survival of each bee species on the five pollen diets and compared larval survival of the two species when reared on the same pollen diet. For statistical analyses, SPSS 16.0 for Macintosh OS X was used.

4.4. RESULTS

Of a total of 308 bee eggs transferred, 258 (83,8%) hatched. Eighteen larvae that died from an infection with foulbrood (seven larvae of *O.*

bicornis and five larvae of *O. cornuta* on the *Echium* and six larvae of *O. cornuta* on the control pollen diet) and one larva that died from mechanical damage were excluded from all analyses. Larval survival on the control pollen diet amounted to >90% for both species (Table 1), indicating that both the experimental design and the handling of eggs and larvae had at most a marginal impact on mortality.

TABLE 1: Egg and larval survival and number of viable adults of the two solitary bee species *Osmia cornuta* and *Osmia bicornis* when reared on freely collected pollen (control) and on four experimental pollen diets.

bee species	pollen diet	no. eggs hatched	surviving larvae			group heterogeneity		no. viable adults
		(unhatched)	no.	%	survival time (d)	p	groups	
O. bicornis	control	31 (0)	28	90.3	39.42 ± 1.56	<0.001	a	28
	Sinapis	27 (5)	25	92.6	47.78 ± 1.68		a	24
	Ranunculus	29 (0)	28	96.6	48.90 ± 1.08		a	28
	Tanacetum	22 (7)	0	0	25.82 ± 2.52		b	0
	Echium	17 (7)	5	29.4	26.59 ± 2.63		b	0
O. cornuta	control	20 (6)	20	100	n.a.*	<0.001	a	17
	Sinapis	28 (3)	27	96.4	40.71 ± 1.26		a	23
	Ranunculus	25 (7)	2	8	16.32 ± 2.27		b	2
	Tanacetum	23 (7)	0	0	18.78 ± 1.61		b	0
	Echium	17 (8)	16	94.1	42.82 ± 1.14		a	12

Survival time gives the Kaplan-Meier survival time in days (mean ± SE) of the larvae on each pollen diet. Group heterogeneity was tested with pairwise log-rank test between all treatments. Diets sharing the same letter did not differ significantly at $P < 0.05$ (post hoc test: pairwise log-rank test using Bonferroni corrections). * Survival time could not be computed as all larvae survived until the cocoon stage (censored data only).

4.4.1. *Osmia bicornis*

The larvae of *O. bicornis* did not perform significantly different on the *Sinapis* and the *Ranunculus* pollen diet (log-rank test, $\chi^2 = 0{,}47$, $P = 0{,}492$; Fig. 2, Table 1) and reached a similar median weight before the onset of cocoon formation of 156 and 154 mg, respectively (Mann-Whitney test, $U = 302{,}5$, $P = 0{,}397$). In contrast, all larvae reared on the

Tanacetum pollen diet died within 10-66 days (median 23 days). Although they constantly fed and defecated, they stayed very small and reached a median weight of only 6,3 mg before they died. None of these larvae started to spin a cocoon. Twelve of the 17 larvae reared on the *Echium* pollen diet died within 16-24 days (median 19 days), reaching a median weight of 20,5 mg before death. Five larvae completed the cocoon and reached a median weight of 114 mg before the onset of cocoon formation. However, none of these five larvae completed metamorphosis to viable adults and all but one died before pupation. Survival did not differ significantly between larvae reared on the *Tanacetum* pollen diet and larvae reared on the *Echium* pollen diet (log-rank test, $\chi^2 = 0,23$, $P = 0,631$; Table 1). However, median larval weight before death was significantly less in larvae reared on the *Tanacetum* pollen diet (6,3 mg) than in larvae reared on the *Echium* pollen diet (20,5 mg) (Mann-Whitney test, $U = 12,5$, $P < 0,001$).

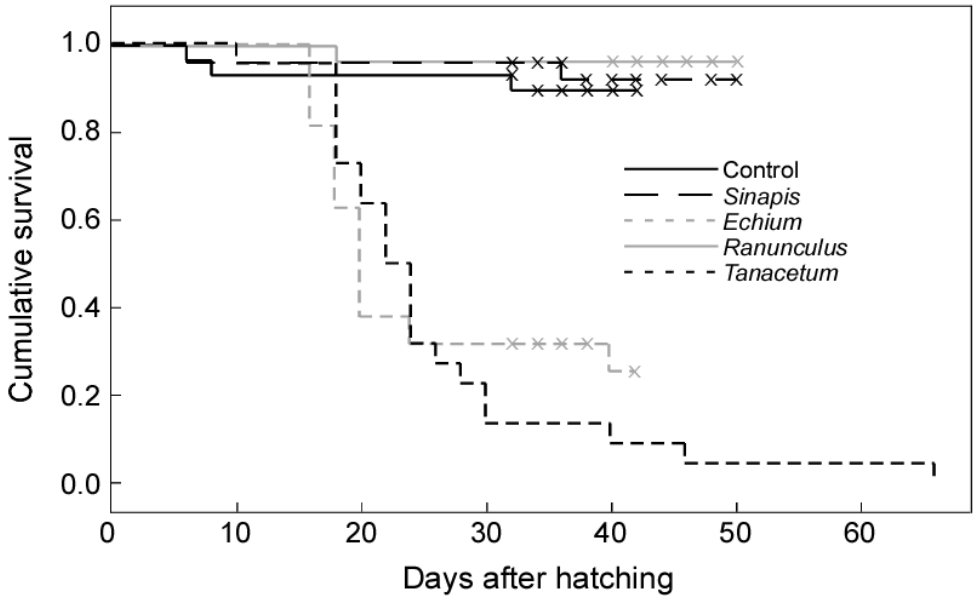


FIGURE 2: Cumulative survival of larvae of *Osmia bicornis* when reared on pollen collected on the naturally available flower supply (control) and on four experimental pollen diets. Crosses indicate individuals that reached the cocoon stage (censored data).

4.4.2. *Osmia cornuta*

The larvae of *O. cornuta* did not perform significantly different on the *Sinapis* and the *Echium* pollen diet (log-rank test, $\chi^2 = 0,12$, P = 0,731; Fig. 3, Table 1) and reached a similar median weight before the onset of cocoon formation of 195 and 190 mg, respectively (Mann-Whitney test, U = 151,0, P = 0,102). In contrast, all larvae reared on the *Tanacetum* pollen diet died within 6-46 days (median 16 days), reaching a median weight of only 7,1 mg before death. All but two larvae reared on the *Ranunculus* pollen diet died within 10-18 days (median 14 days), reaching a median weight of 60 mg before death. The two surviving larvae successfully developed into adult females. However, with a weight of only 68 and 97 mg, these two females were distinctly lighter than average-sized adult females of *O. cornuta*, which typically weigh about 150-200 mg (C. Sedivy, unpublished data). In addition, these two individuals needed 50 and 54 days to complete their cocoon, whereas larvae of *O. cornuta* reared on the *Sinapis* and the *Echium* pollen diet completed their cocoon already after a median of 38 and 33 days, respectively. Survival did not differ significantly between larvae reared on the *Tanacetum* pollen diet and larvae reared on the *Ranunculus* pollen diet (log-rank test, $\chi^2 = 4,27$, P = 0,39 after Bonferroni correction; Table 1). However, median larval weight before death was significantly higher in larvae reared on the *Tanacetum* pollen diet (7,1 mg) than in larvae reared on the *Ranunculus* pollen diet (6,0 mg) (Mann-Whitney test, U = 158,0, P = 0,019).

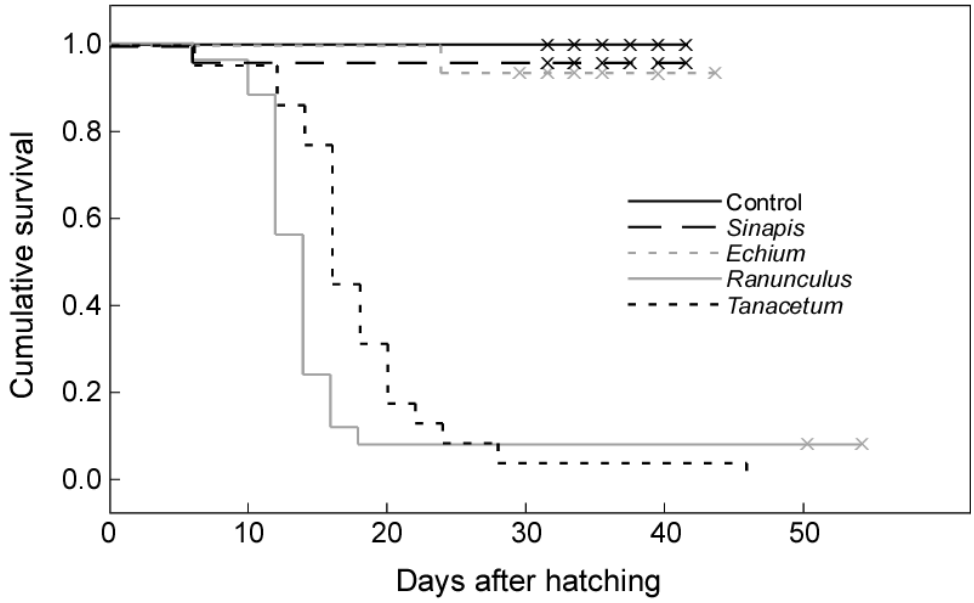


FIGURE 3: Cumulative survival of larvae of *Osmia cornuta* when reared on pollen collected on the naturally available flower supply (control) and on four experimental pollen diets. Crosses indicate individuals that reached the cocoon stage (censored data).

4.4.3. Pollen diets in comparison

Survival of the larvae of *O. bicornis* and *O. cornuta* did not significantly differ on the *Sinapis* pollen diet (log-rank test, $\chi^2 = 0{,}38$, $P = 0{,}539$) and the larvae of both species invariably died when reared on the *Tanacetum* pollen diet. In contrast, larval survival differed significantly between the two bee species when reared on the *Ranunculus* (log-rank test, $\chi^2 = 49{,}70$, $P < 0{,}001$) and the *Echium* pollen diet (log-rank test, $\chi^2 = 14{,}57$, $P < 0{,}001$).

4.5. DISCUSSION

Our comparative experimental approach provided first evidence that larvae of two closely related generalist bee species differ in their physiological ability to digest pollen from the same host plant. *Osmia*

bicornis failed to survive on pollen of *E. vulgare* but developed on pollen of *R. acris*, whereas the reverse held generally true for *O. cornuta*. This difference is striking for highly generalized congeneric bee species and clearly shows that the larvae of *O. bicornis* are physiologically adapted to digest *Ranunculus* pollen, whereas the larvae of *O. cornuta* are adapted to digest *Echium* pollen. Conversely, both *Osmia* species coincided in their inability to develop on pollen of *T. vulgare*, which is an unsuitable pollen source for many unspecialized bee species (Müller & Kuhlmann 2008; Praz et al. 2008a), but not for specialist species (Westrich 1989), again indicating the necessity for physiological adaptations.

Among the *O. cornuta* larvae feeding on *Ranunculus* pollen, two individuals reached the adult stage, whereas all other larvae died. The fact that these two individuals reached only a very low adult weight in spite of their exceedingly long development time adds further evidence for the unfavourable chemical properties of *Ranunculus* pollen. Obviously, these two larvae possessed the physiological machinery to cope with *Ranunculus* pollen, while all other tested larvae of the same population did not. This intrapopulational variation in pollen digestion ability is intriguing as such a variation is the prerequisite for selection acting towards a broader diet by including new pollen hosts. The inclusion of a new pollen host, however, does not only require the overcoming of physiological constraints to successfully utilize the new pollen but also the overcoming of neurological constraints related to the recognition or handling of flowers (Williams 2003; Praz et al. 2008c; Sedivy et al. 2008).

In both bee species tested, larval mortality patterns differed considerably between the pollen diets, indicating that the unfavourable properties of these pollen affected the larvae in different ways. When feeding on *Tanacetum* pollen, all *O. bicornis* and *O. cornuta* larvae remained very small, even those that constantly fed and defecated for more than 2

months and 1 month, respectively. This finding is in line with similar inhibitory effects of Asteraceae pollen on bee larval growth observed previously (Levin & Haydak 1957; Guirguis & Brindley 1974; Williams 2003; Praz et al. 2008a). The mortality pattern on the *Tanacetum* pollen diet is suggestive of either the interference of toxic pollen compounds with nutrient digestion, an insufficient quantity or quality of nutrients in the pollen of *Tanacetum*, such as amino acids or sterols (Pilorget et al. 2010), or of difficulties in extracting essential compounds from the pollen grains. Deficiencies in the content of essential nutrients were also hypothesized by Praz et al. (2008a) to be a possible cause for the failure of three pollen specialist bee species to develop on Asteraceae pollen. In contrast, when feeding on *Ranunculus* pollen, all but the two surviving *O. cornuta* larvae died very soon and attained a significantly lower weight than larvae reared on *Tanacetum* pollen. This finding is compatible with the assumption that *Ranunculus* pollen contains toxic compounds. In fact, anther volatiles of *Ranunculus* were found to be dominated by protoanemonin (Bergström et al. 1995; Jürgens & Dötterl 2004), a potential flower defence compound against destructive feeding by phytophagous insects (Jürgens & Dötterl 2004). Extracts of aerial parts of *Ranunculus sceleratus* containing protoanemonin were indeed found to exhibit insecticidal activity in biotests (Bhattacharya et al. 1993); however, whether and the extent to which protoanemonin affects bee larval development remains to be elucidated. When feeding on *Echium* pollen, the mortality pattern of the *O. bicornis* larvae differed substantially from that on *Tanacetum* pollen and from that of *O. cornuta* on *Ranunculus* pollen. The *Echium*-fed larvae that did not reach the cocoon stage grew normally, attaining a significantly larger weight than larvae reared on *Tanacetum* pollen before they suddenly ceased to grow and died. This mortality pattern might possibly be explained by the

accumulation of toxic pollen compounds, which, after reaching a lethal threshold, caused the sudden death of the larvae. Possible candidates for such toxic pollen compounds in *Echium* pollen are pyrrolizidine alkaloids. These alkaloids, which are contained in high concentrations in the pollen of *Echium* and some Asteraceae species (Boppré et al. 2008), are known to be toxic or deterrent for generalist herbivores (Van Dam et al. 1995; Narberhaus et al. 2005). Although Reinhard et al. (2009) did not find adverse effects of pyrrolizidine alkaloids on adult honeybees when provided in naturally occurring concentrations, the authors hypothesized that these alkaloids, due to their mutagenic effects, might be a threat to the more vulnerable honeybee larvae.

Pollen of different plant taxa is highly variable with respect to its chemical composition (Roulston & Cane 2000 and references therein). Thus, pollen does not appear to be an easy-to-use source of protein for bees. In fact, larval growth and adult life span of bumblebees and honeybees were found to substantially differ among different pollen diets (Schmidt et al. 1987, 1995; Génissel et al. 2002). This finding, however, was generally attributed to quantitative or qualitative differences in the protein content of the tested pollen. To our knowledge, no study has ever shown that bees possess physiological adaptations to digest pollen. The results of our experiments indicate that such adaptations might possibly be widespread in bees, mirroring the situation in herbivore-plant interactions (Opitz & Müller 2009). Interestingly, herbivorous insects may lose the ability to efficiently utilize alternative hosts after having adapted physiologically to the secondary chemistry of their hosts, a phenomenon known as the physiological efficiency hypothesis (Singer 2008 and references therein). Analogously, we hypothesize that the widespread specialization of solitary bees to a restricted number of closely related pollen hosts (Westrich 1989; Michener 2007) may in part

be explained by the inability to digest alternative pollen after the bees' physiology became optimized to cope with the chemistry of a specific pollen host. This hypothesis is in line with the finding that several pollen specialist bee species failed to develop on non-host pollen (Praz et al. 2008a). However, there are also examples of pollen specialist bees, which are able to successfully develop on non-host pollen (Williams 2003; Praz et al. 2008a,c). Possible evidence for physiological adaptations to digest pollen also comes from a recent study on the evolution of host plant choice in bees of the genus *Chelostoma*, which mainly consists of pollen specialist species (Sedivy et al. 2008). The only two pollen generalists that evolved from specialized ancestors broadened their host plant spectrum by incorporating pollen hosts that are the exclusive host plants of closely related pollen specialist species. This suggests that the physiological or neurological capabilities to cope with some of the newly added hosts were inherited from a common ancestor.

Given the evidence that bees need physiological adaptations to digest some pollen, the essential question arises as to whether unfavourable pollen properties have evolved as protection against pollen-collecting bees, whether they are by-products of the plants' physiology serving other primary goals or whether they are a pleiotropic consequence of chemical defence against herbivores in other tissues (Hargreaves et al. 2009). We hypothesize that the high quantitative pollen requirements of bees might have selected for protective properties of the pollen, which serve to filter pollen-consuming floral visitors. This view is supported by the following lines of reasoning: i) As selection has shaped the morphology of flowers to reduce pollen loss to bees (for references, see Introduction), selection may be expected to act on the nutritional quality or toxicity of pollen as well. ii) An increasing number of studies report on the occurrence of secondary compounds in pollen with insecticidal

properties (Detzel & Wink 1993; Jayanth et al. 1993; Datta & Saxena 2001; Pimentel De Carvalho & Message 2004; Hargreaves et al. 2009 and references therein). The extent to which these insecticidal pollen compounds affect bee larval development on the one hand and improve plant fitness on the other hand remains to be elucidated. The findings that the concentration of pyrrolizidine alkaloids in *Senecio jacobaea* (Asteraceae) is distinctly higher in pollen compared to stems and leaves (Budde et al. 2004) and that the pollen of *Lupinus polyphyllus* (Fabaceae) and *Brugmansia aurea* (Solanaceae) contains higher amounts of some alkaloids than leaves or flowers (Detzel & Wink 1993) clearly suggest that some of these insecticidal pollen compounds may indeed exert a protective function. iii) All pollen types experimentally found so far to possess unfavourable properties for bee larval development (several species of Asteroideae and Cichorioideae, *Echium*, *Ranunculus*, *Sinapis*, *Stryphnodendron*; Loper & Berdel 1980; Williams 2003; Pimentel De Carvalho & Message 2004; Praz et al. 2008a; this study) originate from flowers with freely accessible pollen that can easily be harvested by any flower visiting bee. Conversely, we hypothesize that pollen of flowers, which is protected from unspecialized bees within specialized flower structures such as keels, does not possess chemical properties impeding its digestion by unspecialized bees.

In conclusion, the present study provides first evidence that bees need physiological adaptations to cope with the unfavourable chemical composition of some pollen and suggests that the underlying mechanisms causing the observed larval mortality vary among different pollen.

5. Too low to kill: concentration of the secondary metabolite ranunculin in buttercup pollen does not affect bee larval survival[2]

5.1. ABSTRACT

Growing evidence suggests that the freely accessible pollen of some plants is chemically protected against pollen feeding flower visitors. For example, a diet of pollen from buttercup plants (*Ranunculus*) was recently shown to have a deleterious effect on developing larvae of several bee species not specialized on *Ranunculus*. Numerous *Ranunculus* species contain ranunculin, the glucosyl hydrate form of the highly reactive and toxic lactone protoanemonin that underlies the toxicity of these plants. We tested whether the presence of the secondary metabolite ranunculin is responsible for the lethal effects of *R. acris* pollen on the larvae of two bee species that are not *Ranunculus* specialists. To investigate the effect of ranunculin on bee larval development, we added ranunculin to the pollen provisions of the *Campanula* specialist bee *Chel*^{2}*ostoma rapunculi* and the Asteraceae specialist bee *Heriades truncorum* and allowed the larvae to feed on these provisions. Furthermore, we quantified ranunculin in pollen of *R. acris* and in brood cell provisions collected by the *Ranunculus* specialist bee *Chelostoma florisomne*. We demonstrated that although ranunculin was lethal to both tested bee species in high concentrations, the concentration of this secondary metabolite in the pollen of *R. acris* was at least fourfold lower than that tolerated by the larvae of *C. rapunculi* and *H. truncorum* in the

[2]Based on Sedivy, C., R. Piskorski, A. Müller & S. Dorn. 2012. Journal of Chemical Ecology 38:996–1002.

feeding experiments. The ranunculin concentration in the brood cells of *C. florisomne* was on average even twentyfold lower than that in the *Ranunculus* pollen, suggesting that a mechanism different from ranunculin intoxication accounts for the larval mortality reported for bees not specialized on *Ranunculus* pollen.

5.2. INTRODUCTION

Bees, including solitary native species, provide important ecosystem services as pollinators of flowering plants (Kremen et al. 2007). However, they can exact considerable costs on the plants, because they require enormous quantities of pollen to feed their broods (Müller et al., 2006). Flowers are therefore expected to balance the need to attract bees for pollination with the need to restrict extensive pollen losses to bees (Praz et al. 2008a; Sedivy et al. 2011). Various mechanisms have evolved to limit pollen loss by narrowing the spectrum of pollen-collecting flower visitors (Westerkamp 1997; Westerkamp & Classen-Bockhoff, 2007) or by reducing the pollen quantity withdrawn by pollinators per flower visit. Examples of these mechanisms include specialized anthers, pollen-concealing flower structures, and portioned pollen release over extended time periods (Vogel 1993; Harder & Barclay 1994; Müller 1996b; Castellanos et al. 2006).

Growing evidence suggests that some plants that possess freely accessible pollen might also chemically protect their pollen. For example, the pollen of *Stryphnodendron polyphyllum* (Mimosoideae) was found to be poisonous to the larvae of the honeybee (De Carvalho & Message 2004). Similarly, the pollen of *Ranunculus* (Ranunculaceae) did not support larval development of three strict pollen-specialist bees specialized on

Campanula, Echium and Asteraceae, respectively, as well as one highly pollen-generalist bee (Praz et al. 2008a; Sedivy et al. 2011). The larval mortality pattern in these four bee species was characterized by rapid death upon onset of feeding, suggesting that *Ranunculus* pollen may contain secondary metabolites that are toxic to bee larvae. *Ranunculus* pollen is also known to be toxic to adult honeybees, which suffer high rates of mortality when feeding primarily on *Ranunculus* pollen, a phenomenon known as "Bettlacher May sickness" (Morgenthaler & Maurizio 1941).

Fresh plants of the genus *Ranunculus* are well known for their toxic effect on livestock (Kingsbury 1964). This effect arises from high concentrations of the glucoside ranunculin, the precursor of the toxic protoanemonin, present in the plant tissue (Fig. 1) (Benn & Yelland 1968). The content of this secondary metabolite in *Ranunculus* species normally oscillates around 10 mg per g dry weight (d.w.) (Ruijgrok 1966), but can reach nearly 200 mg/g d.w. in *R. cymbalaria* (Bai et al. 1996). Upon infliction of mechanical damage to plant tissue, the non-toxic ranunculin is hydrolyzed by endogenous β-glucosidase, an enzyme stored in the vacuole (Mauch & Staehelin 1989), to yield the highly reactive anhydroaglycone protoanemonin (2,3-dihydro-5-methylidenefuran-2-one), a volatile lactone (Hill & Van Heyningen 1951). When ingested, protoanemonin can cause severe gastric distress in livestock, including irritation of the digestive track, abdominal pain, and diarrhea (Kingsbury 1964). Applied to human skin, protoanemonin may produce erythema and blistering (Benn & Yelland 1968). In addition, protoanemonin has antimicrobial properties (Campbell et al. 1979; Mares 1987; Martin et al. 1990) and exhibits insecticidal effects on fly larvae of *Drosophila melanogaster* (Drosophildae), adult beetles of *Tribolium castaneum*

(Tenebrionidae), and ant workers of *Pheidole pallidula* (Formicidae) (Bhattacharya et al. 1993; Varitimidis et al. 2006).

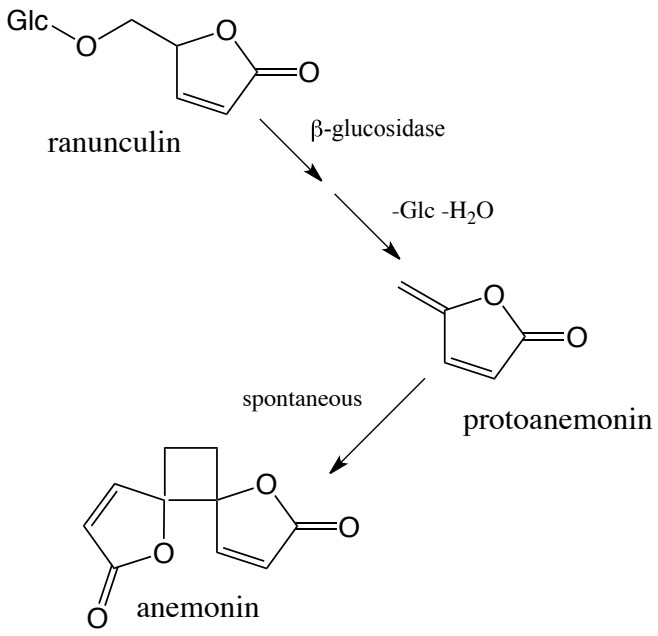


FIGURE 1: The transformation of the glucoside ranunculin to the unstable and toxic lactone protoanemonin in plant tissue and spontaneous transformation to anemonin. Modified after Benn and Yelland (1967).

Some herbivorous insects are able to cope with high concentrations of ranunculin in their diet. The larvae of several leaf and stem mining species of agromyzid flies of the genus *Phytomyza* are specialized on *Ranunculus* and other ranunculin-containing genera of the Ranunculaceae, e.g. *Anemone*, *Clematis*, and *Helleborus* (Spencer 1990). The larvae of *P. ranunculi* and *P. ranunculivora* are often found in leaves of *R. acris* (Pitkin et al. 2010), where they are expected to be exposed to ranunculin concentrations of about 28 mg/g d.w. (Bai et al. 1996). The physiological basis of the ability of these herbivorous insects to tolerate ranunculin and/or protoanemonin remains unknown.

While stems, leaves, and the androecium of *Ranunculus* are known to contain ranunculin in considerable amounts (Ruijgrok 1966; Bonora et al. 1988; Bai et al. 1996), no attempt has been made to quantify ranunculin in *Ranunculus* pollen. The high relative amounts of protoanemonin released from pollen and anthers of some *Ranunculus* species (Bergström et al. 1995) as well as the high concentration of protoanemonin in the androeceum of *R. ficaria*, which was found to be twice as high as the concentration measured in the whole plant (Bonora et al. 1988), may indicate the presence of substantial quantities of ranunculin in the pollen of *Ranunculus*. Furthermore, by thermal desorption of anthers of several Ranunculaceae, Jürgens & Dötterl (2004) detected high relative amounts of protoanemonin in three *Ranunculus* species, and protoanemonin from *Ranunculus* pollen seems to be used in host-plant recognition by the *Ranunculus* specialist bee *Chelostoma florisomne* (Dobson & Peng 1997).

We hypothesized that protoanemonin released from the secondary metabolite ranunculin is responsible for the toxicity of *R. acris* pollen to the larvae of bee species that are not *Ranunculus* specialists. To investigate the effect of ranunculin on bee larval development, we selected two solitary bee species specialized on pollen of plants other than *Ranunculus*. We tested larval performance of *Chelostoma rapunculi*, a *Campanula* pollen specialist, and *Heriades truncorum*, an Asteraceae pollen specialist, on diets consisting of pollen from their natural host plants mixed with ranunculin in various concentrations. In addition, we quantified ranunculin in pollen and flower buds of *R. acris* as well as in brood cell provisions of *Chelostoma florisomne*, which is a pollen-specialist bee species that collects pollen exclusively from *Ranunculus* flowers (Sedivy et al. 2008).

5.3. METHODS

5.3.1. Bee species

To assess the effect of ranunculin on the larval development of solitary bees, we selected two species belonging to the same taxonomic group (Osmiini; Megachilidae) as the *Ranunculus* specialist *Chelostoma florisomne*. These species, *Chelostoma rapunculi* and *Heriades truncorum,* are specialized on *Campanula* (Campanulaceae) and on Asteraceae, respectively (Westrich 1989; Sedivy et al. 2008). Neither of the two species can develop on a *Ranunculus* pollen diet (Praz et al. 2008a). All these bee species nest in pre-existing cavities such as insect-bored holes in dead wood and hollow stems, and they can therefore easily be reared in hollow bamboo stalks. Once provisioning of the brood cells with pollen and nectar is complete, an egg is deposited onto the pollen diet and the female bee closes the cell with a thin wall of clay or resin. Successful larval development ends with spinning a cocoon, in which the bee enters metamorphosis to the adult stage. For the experiments, we used eggs and brood cell provisions from bees nesting in nesting stands on the campus of ETH Zurich.

5.3.2. Bee larval performance

The larvae of *C. rapunculi* and *H. truncorum* were experimentally reared on a *Campanula* and Asteraceae pollen diet, respectively, obtained from conspecific nests and mixed with ranunculin in five increasing concentrations: 0 mg (control), 10 mg, 20 mg, 50 mg and 100 mg per g pollen dry weight (d.w.), henceforth referred to as the control treatment, 10 mg/g treatment, 20 mg/g treatment, 50 mg/g treatment and 100 mg/g

treatment. These concentrations are in line with natural ranunculin concentrations reported for *Ranunculus* plants (Ruijgrok 1966; Bai et al. 1996; Bonora et al. 1988). Pollen dry weight of the brood cell provisions averages approximately 27% in *C. rapunculi* and 33% in *H. truncorum* (A. Bühler and A. Müller unpublished). Ranunculin (> 99% pure) originating from extractions of *Ranunculus* plants was obtained from Michael H. Benn (University of Calgary, Canada). To prepare the experimental pollen diet, ranunculin was ground to fine powder and thoroughly mixed with the brood cell provisions in a mortar. The mixing process was conducted in a very careful and gentle way to prevent destruction of the pollen grains, which might lead to the release of β-glucosidase followed by hydrolysis of ranunculin.

Rearing of the bee larvae was conducted in individual artificial brood cells (for details see Sedivy et al. 2011). Freshly completed bee nests were collected daily from the nesting stands. Each egg was carefully detached with a thin spatula from the brood cell provision and transferred onto 60 mg of the experimental pollen diet previously placed into the artificial cell. Larvae hatched and started feeding between one and three days after transfer onto the experimental pollen diet. Each larva was allowed to feed individually in a single artificial cell to mimic natural conditions. For each bee species and treatment, 24-31 eggs were transferred. Development took place in a climate chamber (E7 / 2; Conviron, Winnipeg, Canada) in darkness at 25 ± 0.5 °C for 16 h followed by a 4 h gradual decrease to 10 ± 0.5 °C, followed by a 4 h gradual increase back to 25 ± 0.5 °C, at a constant 70 ± 0.5 % relative humidity. Egg hatching, initiation of larval feeding, cocoon completion, and incidences of death were recorded every second day. Survival time was considered the time between onset of feeding and either death or

completion of the cocoon. Unhatched eggs were removed from statistical analyses.

5.3.3. Statistical analysis

Kaplan–Meier survival statistics was used to compare larval survival between the different treatments following Lee & Wang (2003). The number of days between hatching and completion of the cocoon was considered as 'censored data'; individuals that died before the completion of the cocoon represented the exact observations for which the event (death) occurred, while those that completed the cocoon were the censored observations. The latter were considered survivors and were therefore withdrawn from survival calculations. To test for differences between survival distributions, the *log-rank test* was applied with Bonferroni correction using the option 'pairwise for each stratum' implemented in the software when comparing two groups. For each bee species, we tested for differences in survival according to pollen-diet treatment, and larval survival of the two species was compared for each ranunculin concentration. For statistical analyses, SPSS 19.0.0 for Macintosh OS X (SPSS Inc., Chicago, Illinois, USA) was used.

5.3.4. Ranunculin recovery

To test whether the ranunculin concentration in the experimental pollen provisions remained stable during the feeding experiments, we added 10 mg/g of ranunculin to brood cell provisions of *Heriades truncorum* and quantified the ranunculin content by LC-MS analysis (see below) immediately after mixing, after 8 days and after 22 days (N = 5 for each time interval). For this experiment, we used exactly the same

methodological procedure including climate chamber conditions as for the bee larval performance experiments.

5.3.5. Plant material

Ranunculin was quantified in pollen and flower buds of *Ranunculus acris* as well as in the brood cell provisions of the *Ranunculus* specialist *Chelostoma florisomne*. At each of eight different locations in Switzerland, which spanned a geographic range of approximately 130 km (comprising locations around Neuchâtel, Solothurn, Aarau, and Zurich), 250 freshly opened flowers of *R. acris* were collected in April 2011. The flowers were bundled and fixed in an upside-down position over a parchment paper cone large enough to collect released pollen. After 24 h the pollen that accumulated at the bottom of the cone was sieved through a 90 µm-pore sieve and stored at -80°C until extraction and analysis. Our pollen collection procedure closely matched the pollen-collecting behaviour of bees, which harvest pollen from dehisced anthers before they deposit it in the brood cells. At each of the eight locations, a single flower bud (close to blooming) was collected from each of five different plants, was immediately frozen in liquid nitrogen, and was stored at -80°C until extraction and analysis. At a large nesting site of *C. florisomne* in the surroundings of Neuchâtel (Gletterens), where *R. acris* was the near exclusive pollen source for this species, we collected eleven freshly completed nests. From each nest, the provision of the outermost (i.e. the most recently completed) brood cell was removed and immediately stored at -80°C until extraction and analysis.

5.3.6. Extractions of plant material

For extraction of ranunculin we basically followed the method described by Bai et al. (1996). All the samples, i.e. pollen, flower buds, and brood cell provisions, were freeze-dried and individually extracted with methanol (3 x 15 ml) by repeatedly and thoroughly grinding in a mortar. To ensure that the mechanical damage inflicted on the plant material did not lead to a significant loss of ranunculin due to the action of β-glucosidase, the grinding was conducted in methanol. To test whether the hard pollen exine was successfully disrupted in order to extract the complete contents of the pollen grains, the ground pollen was examined microscopically. The extracts were filtered through a cotton plug, evaporated to dryness, and stored at -60 °C until LC-MS analysis.

5.3.7. LC-MS analysis

For the LC-MS analysis, the total dried extract was quantitatively dissolved in the mobile phase and, if necessary, an aliquot of the solution was further diluted. High-performance liquid chromatography (HPLC) was performed on an Agilent 1200 HPLC system (Agilent Ltd., Santa Clara, USA) equipped with a binary solvent pump. The separation was performed on a reversed-phase 4,6-mm × 250-mm, 5 µm, Phenomenex ODS Aqua column (Phenomenex, Torrance, USA). An isocratic mode with MeOH/H_2O (0.05% ammonium acetate) (6:4) at a flow rate of 1 ml/min (total run time, 5min) was employed. The sample injection volume was 5 µl.

Mass spectrometry (MS) was performed on an electrospray ionization-quadrupole-time of flight (ESI-Q-TOF) MS system (maXis, Bruker Daltonics, Bexhill-on-Sea, UK). The instrument was operated in a wide-

pass quadrupole mode and the TOF data was collected for m/z 50-1300 with low-collision energy of 8 eV. The optimized ion source and mass analyser conditions were as follows: drying gas, N_2 (99,99%) at 8,0 l/h and temperature of 200 °C; nebulizer pressure 1,6 bar; capillary and endplate voltages 500 V and 4500 V, respectively; TOF tube voltage 9880 V; reflection voltage 2004 V; pusher voltage 1640 V; MCP detector voltage 2927 V. The system was mass calibrated in the positive-ion mode using a methanol solution of sodium formate on the enhanced quadratic algorithmic mode.

The signal of the extracted ion chromatogram at m/z 299.1 ($[M+Na]^+$) was employed for the quantification of ranunculin as sodium adduct. Quantification was performed using a five-point calibration curve obtained with pure ranunculin using the Data Analysis 4.0 and Quant Analysis 2.0 software (Bruker Daltonics, Bexhill-on-Sea, UK).

5.4. RESULTS

5.4.1. Bee larval performance

All larvae of *Chelostoma rapunculi* died within 4-10 days (median, 6 days) when feeding on the 50 mg/g and the 100 mg/g ranunculin treatment (Figure 2a), while 29 % of the larvae survived on the 20 mg/g treatment (Table 1). Larval survival did not significantly differ between the control and the 10 mg/g and 20 mg/g treatments, respectively (log-rank test, $\chi^2 = 5.688$, $P = 0.17$ and $\chi^2 = 3.682$, $P = 0.55$ after Bonferroni correction), but differed significantly between the 10 mg/g and the 20 mg/g treatment (log-rank test, $\chi^2 = 17.998$, $P < 0.001$). The larvae feeding on the control treatment required 20-34 days (median, 28 days) until

completion of the cocoons compared to 34-56 days (median, 42 days) on the 10 mg/g treatment and 32-50 days (median, 44 days) on the 20 mg/g treatment.

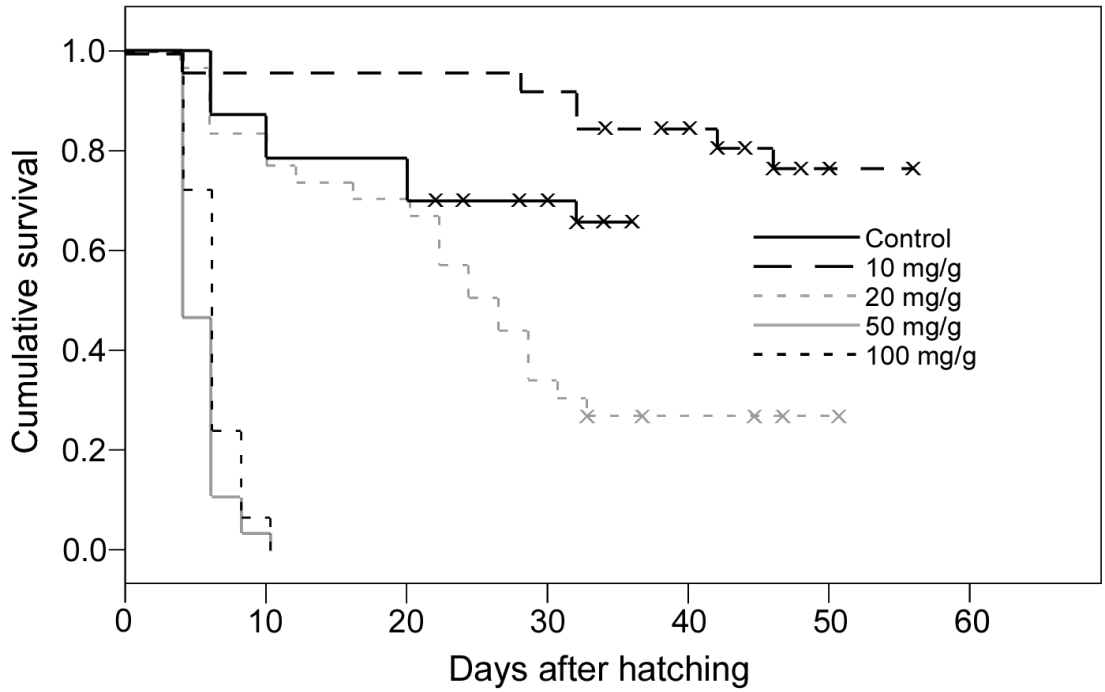
TABLE 1: Larval survival of the two bee species *Chelostoma rapunculi* and *Heriades truncorum* when reared on their host pollen diet mixed with ranunculin

Bee species	Ranunculin concentration (mg/g d.w.)	Hatched eggs (unhatched)	Surviving larvae			Group heterogeneity	
		N	N	%	Survival time (days)[a]	P	Groups
C. rapunculi	0 (control)	23 (1)	15	65.2	26.30 ± 2.35	<0.001	a, b
	10	28 (2)	22	78.6	49.17 ± 2.59		a
	20	31 (0)	9	29.0	26.71 ± 2.99		b
	50	28 (2)	0	0	5.21 ± 0.30		c
	100	30 (1)	0	0	6.07 ± 0.32		c
H. truncorum	0 (control)	29 (1)	23	79.3	35.52 ± 2.39	<0.001	a
	10	25 (5)	21	84.0	39.37 ± 2.34		a
	20	29 (1)	22	75.9	43.93 ± 3.43		a
	50	24 (6)	0	0	6.17 ± 0.34		b
	100	28 (2)	0	0	6.86 ± 0.32		b

[a] Survival time gives the Kaplan-Meier survival time in days (mean ± SEM) of the larvae on each pollen diet. Group heterogeneity was tested with the pairwise log-rank test between all treatments. Diets sharing the same letter did not differ significantly at $P < 0.05$ (post hoc test: pairwise log-rank test using Bonferroni corrections).

All larvae of *Heriades truncorum* died within 4-12 days (median, 6 days) when feeding on the 50 mg/g and 100 mg/g ranunculin treatment (Figure 2b). Larval survival neither differed significantly between the control and the 10 mg/g and 20 mg/g treatments, respectively (log-rank test, $\chi^2 = 0.294$, $P = 0.59$ and $\chi^2 = 0.118$, $P = 0.73$ after Bonferroni correction), nor between the 10 mg/g and 20 mg/g treatment (log-rank test, $\chi^2 = 0.549$, $P = 0.459$; Table 1). The larvae feeding on the control treatment required 30-42 days (median, 34 days) until completion of the cocoons compared to 36-44 days (median, 38 days) on the 10 mg/g treatment and 36-54 days (median, 42 days) on the 20 mg/g treatment.

a)



b)

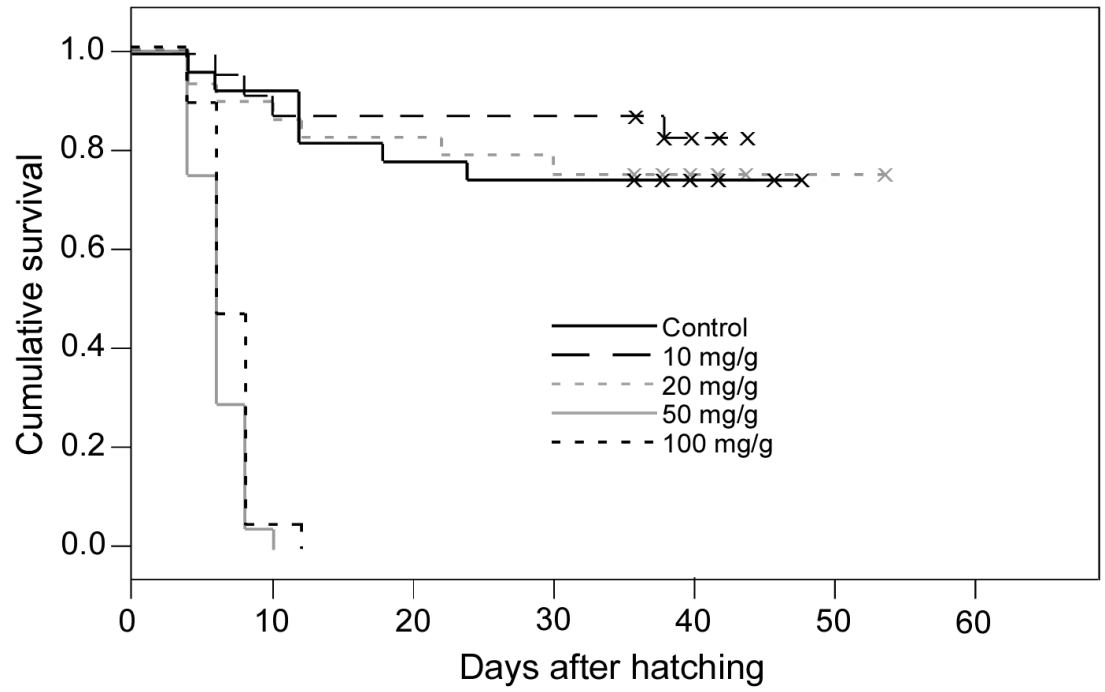


FIGURE 2: Cumulative survival of larvae of bees specialized on plants other than *Ranunculus* when reared on their host pollen diet admixed with different quantities of ranunculin. Crosses indicate the days after hatching at which at least one individual reached the cocoon stage (censored data). (**a**) the *Campanula* specialist *Chelostoma rapunculi*, and (**b**) the Asteraceae specialist *Heriades truncorum*

A comparison between the two bee species tested revealed largely parallel performances across treatments; no significant differences were observed between the survival of the two species in the control, the 10 mg/g, the 50 mg/g or the 100 mg/g treatments (log-rank test, $\chi^2 = 1.48$, P = 0.224; $\chi^2 = 0.995$, P = 0895; $\chi^2 = 3.833$, P = 0.050; $\chi^2 = 3.032$, P = 0.082). In the 20 mg/g treatment survival of *H. truncorum* larvae was greater than that of *C. rapunculi* larvae (log-rank test, $\chi^2 = 11.347$, P < 0.001).

5.4.2. Ranunculin recovery

Recovery rate of ranunculin amounted to 62.5-71.0% (mean, 66.0%, N=5) immediately after its addition to the experimental pollen provision, to 55.4-70.5% (mean, 60.8%, N=5) after 8 days, and to 56.5 – 72.5% (mean, 63.2%, N=5) after 22 days. These results indicate that the concentration of ranunculin mixed to the pollen provisions remained constant over a substantial period of time and that about 60% of the added 10 mg/g ranunculin was biologically available.

5.4.3. Ranunculin content

To quantify the range of ranunculin in the field-collected samples, we assessed its concentration in pollen, flower buds and brood cells (Table 2). Ranunculin concentration in the pollen of *Ranunculus acris* (mean, 0.55 mg/g) was almost forty times lower than that in the flower buds (mean, 19.45 mg/g), but almost twenty times greater than that in the brood cell provisions of *Chelostoma florisomne* (mean, 0.03 mg/g). The maximum concentration of ranunculin found in the pollen of *R. acris* was 1.34 mg/g (Table 2). Neither protoanemonin, the ranunculin anhydroaglycone, nor

anemonin, the product of spontaneous dimerization of protoanemonin, were detected in any of the samples.

TABLE 2: Content of ranunculin quantified by LC-MS in pollen and flower buds of *Ranunculus acris* and in brood cell provisions of the *Ranunculus* specialist bee *Chelostoma florisomne*

Source	N	Mean ± SEM [mg/g]	Range [mg/g]
Pollen	8	0.55 ± 0.18	0.03 – 1.34
Flower buds	8	19.45 ± 5.20	3.72 – 53.93
Brood cell provisions[a]	11	0.03 ± 0.01	0.003 – 0.12

[a] The *Ranunculus* pollen in the brood cell provisions was collected by foraging *C. florisomne* females from *R. acris*, which was the nearly exclusive pollen source for this species at the location where the brood cells were collected. Ranunculin amounts were calculated for pollen dry weight by subtracting average water and nectar contents in the brood cells

5.5. DISCUSSION

Results of the feeding experiments provide clear evidence that the two tested solitary bee species not specialized on *Ranunculus* tolerated the 10 mg/g ranunculin treatment without any measurable effects on larval survival. Based on the ranunculin recovery experiment, the 10 mg/g ranunculin treatment corresponds to an approximate concentration of biologically available ranunculin of at least 5.54 mg/g d.w. However, ranunculin in the pollen of *R. acris* quantified by LC-MS analysis amounted to maximally 1.34 mg/g d.w., a concentration that is at least fourfold lower than that tolerated by *Chelostoma rapunculi* and *Heriades truncorum*. The maximum ranunculin concentration in the brood cell provisions of the *Ranunculus* specialist *Chelostoma florisomne* was one order of magnitude lower (0.12 mg/g d.w.) than in the freshly collected pollen of *R. acris*, although the pollen in the analysed brood cell provisions was derived from this *Ranunculus* species. Ranunculin is lethal when added to the natural pollen provisions of *C. rapunculi* and *H.*

truncorum at very high concentrations (50 mg/g and 100 mg/g treatments). However, these concentrations greatly exceed natural concentrations of ranunculin found in *R. acris* pollen. Hence, the presence of ranunculin cannot explain the mortality of these bees when reared on a *Ranunculus* pollen diet, contrary to hypotheses proposed previously (Praz et al. 2008a; Sedivy et al. 2011).

Survival of *C. rapunculi* larvae feeding on the still high 10 mg/g and 20 mg/g ranunculin treatments was not significantly affected compared to the control treatment. However, mean survival values at the 20 mg/g treatment were low, and difference to the survival at the 10 mg/g treatment was significant, pointing to some adverse effects of the 20 mg/g treatment on larval survival. A sublethal effect (Piskorski et al. 2011a) of both the 20 mg/g and 10 mg/g treatments on *C. rapunculi* was noted as development times of the larvae were prolonged compared to the larvae in the control treatment.

Ranunculin admixed to the pollen provision at 10 mg/g could be recovered at a range of approximately 60%, irrespective of whether the incubation period lasted 0, 8 or 22 days. This finding indicates that a minor proportion of the ranunculin was deactivated during mixing, either through a strong adsorption or a chemical degradation. This result further indicates that the biologically available ranunculin concentration remained constant over long periods after mixing, underlining the validity of the conclusions drawn here.

Two previous studies described protoanemonin, the compound derived from hydrolysis of ranunculin, as the most prominent volatile in the headspace of pollen samples of *R. acris* (Bergström et al. 1995) and after

thermal desorption of anthers of *R. acris* and other Ranunculaceae species (Jürgens & Dötterl 2004). In both cases, only relative amounts were provided and no information was given regarding absolute quantities of this lactone present in a volatile profile poor in other compounds. In another study, in which protoanemonin in different organs of *R. ficaria* was quantified after steam distillation, the androeceum was found to emit almost twice as much protoanemonin as the whole plant (Bonora et al. 1988). In this study, however, ranunculin was not quantified in the pollen itself. Thus, the high amounts of protoanemonin measured in the headspace of the androeceum of *R. ficaria* might have been derived from a high concentration of ranunculin in the anther filaments, or in the anther tissue surrounding the pollen sacs prior to pollen release, rather than directly from the pollen. The low concentrations of ranunculin found in the pollen of *R. acris* in the current study is in line with the trace amounts of protoanemonin recently reported from an analysis of pollen volatiles of *R. bulbosus* (Piskorski et al. 2011b). The ranunculin concentrations we measured in the flower buds of *R. acris*, amounting to up to 54 mg/g d.w., corresponds to published levels of ranunculin in other ranunculin-containing Ranunculaceae species (Ruijgrok 1966; Bai et al. 1996), validating our extraction and quantification methods.

Surprisingly, we found that the level of ranunculin in the pollen provision collected by *C. florisomne* was on average twentyfold lower than in the pure pollen. Pollen in the cell provisions is diluted with nectar admixed by the foraging females at a ratio of approximately 1:1 (based on dry weight; A. Bühler & A. Müller unpublished), explaining some but not all of the discrepancy noted between ranunculin concentrations in the flower pollen and in the bees' brood cell provisions. One process that substantially reduces the ranunculin content in cut *Ranunculus* plants is

drying, which triggers β-glucosidase-mediated autolysis of ranunculin with release of protoanemonin, yielding hay that is non-toxic to livestock (Majak 2001). A similar process can likely be ruled out in the pollen harvested by *C. florisomne*, since the pollen is neither dried nor mechanically damaged during pollen collection, deposition, and storage in the brood cell. We hypothesize that the high sugar concentration originating from the nectar surrounding the pollen grains in the brood cell provisions may lead to an osmotic stress provoking the release of protoanemonin from its precursor protoanemonin, similar to the situation in drying *Ranunculus* plants.

In conclusion, the pollen of *R. acris* contains the secondary metabolite ranunculin in concentrations considerably below the lethal threshold for the tested bee larvae. Thus, we found no evidence that the incapability of several bee species to develop on a *Ranunculus* pollen diet is caused by ranunculin (Praz et al. 2008a; Sedivy et al. 2011). Hence, a different mechanism must underlie larval mortality of bees not specialized on *Ranunculus* pollen, such as the presence of another still unknown toxic pollen compound or the lack of essential nutrients in the pollen, e.g. certain sterols or amino acids.

6. Molecular phylogeny of the bee genus *Hoplitis* (Megachilidae: Osmiini) - how does nesting biology affect biogeography? [3]

6.1. ABSTRACT

The genus *Hoplitis* (Megachilidae: Osmiini) comprises about 360 described species and occurs on all continents except Australia, South America and Antarctica. Using five genes, we inferred the phylogeny of *Hoplitis* including 23 out of the 27 currently recognized subgenera, applying both Bayesian and maximum likelihood methods. Compared to the current morphology-based classification, our phylogeny results in three classificatory changes: first, the subgenera *Alcidamea*, *Cyrtosmia*, *Dasyosmia*, *Megalosmia*, *Monumetha*, and *Prionohoplitis* are merged into one large subgenus ***Alcidamea*** Cresson, 1864, **comb. nov;** second, the subgenera *Annosmia, Bytinskia, Coloplitis,* and *Hoplitis* are merged into one large subgenus ***Hoplitis*** Klug, 1807, comb. nov.; third, the subgenera *Acrosmia*, *Hoplitina*, *Penteriades*, and *Proteriades* are merged into one large subgenus ***Proteriades*** Titus, 1904, **comb. nov.**; We provide evidence that the genus *Hoplitis* has a Palaearctic origin and that colonization events to southern Africa and to the Nearctic, as well as recolonization events from the Nearctic to the Palaearctic occurred. The species of the genus *Hoplitis* exhibit an extraordinary diversity in nesting behaviour, comprising both below and above ground nesting. Parsimony mapping revealed that ground nesting in excavated burrows is the ancestral state among *Hoplitis* bees. We hypothesize that nesting biology

[3]Based on Sedivy, C., S. Dorn & A. Müller. Zoological Journal of the Linnean Society, in press.

strongly affected both range expansion and long-distance dispersal in *Hoplitis*.

6.2. INTRODUCTION

The tribe Osmiini constitutes one of the three large tribes within the bee family Megachilidae along with the Anthidiini and the Megachilini (Michener 2007). It comprises 15 genera and roughly 1'150 species that occur on all continents except Australia, South America and Antarctica (Michener 2007; Praz et al. 2008; Ungricht et al. 2008; Müller 2012; but see Gonzalez & Griswold 2011). Among the osmiine bees, *Hoplitis* Klug is the largest genus containing about 360 described species in 27 subgenera (Ungricht et al. 2009; Müller 2012). While the monophyly of the genus *Hoplitis* is well supported (Praz et al. 2008), subgeneric relationships remain largely unresolved, and the current subgeneric classification of *Hoplitis* (Griswold & Michener 1998; Michener 2007; Müller 2012; Tab. 1) is based on morphological characters, which have not been analyzed by phylogenetic inference.

With 291 described species (81%) in 20 subgenera, *Hoplitis* has its centre of diversity in the Palaearctic, particularly in xeric regions of southern Europe, northern Africa and the Middle East (Müller 2012). 56 species (15%) in ten subgenera occur in North America, 14 species (4%) in one subgenus in southern Africa and one species (< 1%) in India (Michener 2007; Kuhlmann et al. 2011). This distribution suggests a Palaearctic origin of *Hoplitis*, however, phylogenetic evidence for this hypothesis is lacking.

The nesting behaviour of the *Hoplitis* bees is exceptionally diverse (Michener 1968, 2007; Parker 1978; Krombein et al. 1979; Westrich 1989; Müller et al. 1997; Müller 2012). Many species occupy preexisting cavities such as hollow stems, insect borings in dead wood, empty snail shells or stone cracks. Others nest in excavated burrows in the ground or in pithy stems. Some species build exposed nests on the surface of rocks or hide their brood cells in dense vegetation or underneath stones. Moreover, with the subgenus *Bytinskia*, the genus *Hoplitis* comprises the only kleptoparasitic bees among the Osmiini (Mavromoustakis 1954; Warncke 1991a). While nesting in excavated ground burrows is considered the ancestral condition in bees of the family Megachilidae (Litman et al. 2011), the ancestral nesting habit in the genus *Hoplitis* is unknown as are evolutionary patterns of nest site selection.

In the present study, we provide a comprehensive molecular phylogeny of the bee genus *Hoplitis* based on one mitochondrial and four nuclear genes. We use this phylogeny to address the following research questions: i) What are the subgeneric relationships among the *Hoplitis* taxa? ii) What is the biogeographic history of the genus *Hoplitis*? iii) What are the evolutionary patterns of nesting behaviour in the *Hoplitis* bees? iv) How does nesting site selection affect the biogeography of the genus *Hoplitis*?

6.3. METHODS

6.3.1. Taxon sampling

In this study, we refer to the genus *Hoplitis* sensu Michener (2007) with the following alterations proposed by Praz et al. (2008) and Müller (2012): *Stenosmia* is a subgenus of *Hoplitis* rather than a genus of its own,

Micreriades is a subgenus of *its own* instead of a synonym of the subgenus *Alcidamea*, the subgenus *Nasutosmia* belongs to the genus *Osmia* rather than to the genus *Hoplitis*, and the monotypic subgenus *Exanthocopa* is a synonym of the subgenus *Anthocopa*.

TABLE 1: Current and newly proposed subgeneric classification of the bee genus *Hoplitis*. For each subgenus, the number of species described and included in the present study, the distribution and the nesting behaviour are given.

Abbreviations: Afr = Afrotropic, Ori = Oriental, Pal = Palaearctic; 1 = in excavated burrows in the ground, 2 = in insect burrows in the ground, 3 = in depressions or cavities of rocks, 4 = in excavated burrows in pithy stems, 5 = in insect burrows in dead wood or in hollow stems, 6 = in empty snail shells, 7 = cone-like brood cells in dense vegetation or underneath stones, 8 = kleptoparasitic. Underlined = majority of species, parenthesized = very few species. Number of described species based on Müller (2012), data on distribution based on Kuhlmann et al. (2011) and Michener (2007), data on nesting behaviour based on Krombein et al. (1979), Kuhlmann et al. (2011), Michener (2007) and Müller (2012) and references therein.

Current	Proposed new	no	no	Distribution	Nesting
Anthocopa	*Anthocopa*	75	19	Pal, Afr, (Ori)	1, 3, (2), (6)
Stenosmia	*Stenosmia*	9	1	Pal	1
Pentadentosmia	*Pentadentosmia*	22	15	Pal	1
Alcidamea	*Alcidamea*	60	11	Pal, Nea	4 , 5, (7)
Cyrtosmia	*Alcidamea*	1	1	Nea	4, 5
Dasyosmia	*Alcidamea*	2	1	Nea	2, 5
Megalosmia	*Alcidamea*	7	1	Pal	2
Monumetha	*Alcidamea*	6	2	Nea, Pal	5
Prionohoplitis	*Alcidamea*	7	3	Pal	(7)
Annosmia	*Hoplitis*	31	2*	Pal	1
Bytinskia	*Hoplitis*	3	2	Pal	8
Coloplitis	*Hoplitis*	2	-	Pal	?
Hoplitis	*Hoplitis*	51	2*	Pal	3, 1, (5), (6)
Megahoplitis	*Megahoplitis*	1	1	Pal	?
Chlidoplitis	*Chlidoplitis*	7	3	Pal	2
Micreriades	*Micreriades*	9	2	Pal	5
Tkalcua	*Tkalcua*	2	2	Pal	?
Platosmia	*Platosmia*	9	3	Pal	3
Formicapis	*Formicapis*	4	1	Pal, Nea	5
Robertsonella	*Robertsonella*	3	2	Nea	5
Acrosmia	*Proteriades*	5	1	Nea	4, 5
Hoplitina	*Proteriades*	6	2	Nea	5
Penteriades	*Proteriades*	2	1	Nea	5
Proteriades	*Proteriades*	22	3	Nea	5, (2)
Euryparіella	*Euryparіella*	2	-	Pal	?
Jaxartinula	*Jaxartinula*	2	-	Pal	?
Kumobia	*Kumobia*	4	-	Pal	?
incertum sedis	*incertum sedis*	1	-	Pal	?

*represented by only a small fraction of the available species since a comprehensive study of the *Annosmia-Hoplitis* group has recently been conducted (see chapter 7 and 8).

We included 80 *Hoplitis* species in the present study, which represent 23 of the 27 currently recognized subgenera (Michener 2007; Müller 2012) encompassing all Nearctic subgenera (Tab. 1, 2). The four subgenera not included (*Coloplitis*, *Euryparіella*, *Jaxartinula*, and *Kumobia*) comprise merely two to four described species each. For all subgenera that have a range spanning more than one zoogeographic region (*Alcidamea*, *Anthocopa*, *Monumetha*), species from each zoogeographic region were included with the exception of the Indian species *Hoplitis (Anthocopa) matheranensis* (Michener), which is the only *Hoplitis* species known from the Indomalayan region. The *Annosmia-Hoplitis* group, comprising the four subgenera *Annosmia*, *Bytinskia*, *Coloplitis* and *Hoplitis* s. str., is represented by only a small fraction of the available species since another phylogenetic study has recently been conducted that included 44 species of this large species group (see chapter 7). As outgroup, we included 12 species representing all major clades of the tribe Osmiini (Praz et al. 2008) and one representative of the tribe Megachilini (Tab. 2).

TABLE 2: Collection localities, voucher numbers and GenBank accession numbers of the bee species of the genus *Hoplitis* and the outgroup included in the phylogeny. The names of the authors of this study are given with initials. Voucher specimens are deposited in the Entomological Collection of the ETH Zurich. Abbreviations for states: ALG = Algeria, FRA = France, GRE = Greece, ITA = Italy, IRI = Iran, ISR = Israel and Palestine, JOR = Jordan, KGZ = Kyrgyzstan, MAR = Morocco, MGL = Mongolia, OMA = Oman, POR = Portugal, RSA = South Africa, SUI = Switzerland, TUN = Tunisia, TUR = Turkey, UEA = United Arab Emirates, USA = United States of America, UZB = Uzbekistan.

Taxon	Locality	Collector	Voucher Nr.	Genbank Accession Nos.				
				CAD	COI	EF1-alpha	Opsin	NaK
Outgroup								
Ashmeadiella (Ashmeadiella) *aridula*	USA, UT, Garfield Co	T. Griswold	CUIC 1270	HQ995903	Missing	EU851535	EU851641	GU245171
Atoposmia (Eremosmia) *mirifica*	USA, NV, Clark Co	S. Higbee	ETHZ 88	HQ995904	Missing	EU851541	EU851647	HQ995996
Chelostoma (Chelostoma) florisomne	SUI, Chur	E. Steinmann	ETHZ 13	HQ995905	JQ677592	EU851546	EU851652	HQ995997
Haetosmia circumventa	UAE, Sharjah Desert	T. van Harten	ETHZ 97	EU851447	JQ677593	EU851552	EU851658	Missing
Heriades (Heriades) truncorum	SUI, Winterthur	AM	ETHZ 6	EU851448	JQ677594	EU851553	EU851659	Missing
Heriades (Neotrypetes) *crucifer*	USA, AZ, Chiricahua Mts	T. Griswold	CUIC 1149	DQ067194	Missing	EU851555	EU851661	GU245168
Hofferia schmiedeknechti	GRE, Chimara	C. Praz, CS	ETHZ 68	HQ995907	JQ677595	EU851556	EU851662	Missing
Megachile pilidens	SUI, Weiach	AM	ETHZ 12	HQ995885	JQ677596	EU851531	EU851637	HQ995978
Osmia (Osmia) cornuta	SUI, Zürich	AM	ETHZ 2	EU851504	JQ677597	EU851609	EU851714	Missing
Osmia (Osmia) lignaria	USA, UT, Kane Co	T. Griswold	CUIC 1265	HQ995910	Missing	EU851610	EU851715	GU245169
Protosmia (Nanosmia) minutula	SUI, Embd	C. Praz	ETHZ 25	EU851515	JQ677598	EU851620	EU851725	Missing
Protosmia (Protosmia) humeralis	JOR, Wadi Shu'ayb	C. Praz, AM, CS	ETHZ 135	HQ995913	Missing	EU851621	EU851726	HQ996004
Wainia (Caposmia) eremophila	JOR, Wadi al Hasa	C. Praz, AM, CS	ETHZ 125	HQ995916	JQ677599	EU851626	EU851731	Missing
Ingroup								
Hoplitis (Acrosmia) plagiostoma	USA, UT, Cache Co	H. Ikerd	ETHZ 315	JQ677169	JQ677474	JQ677282	JQ660747	JQ677365
Hoplitis (Alcidamea) acuticornis	SUI, Hohtenn	AM	ETHZ 38	JQ677170	JQ677475	JQ677283	JQ660748	JQ677366
Hoplitis (Alcidamea) capsulifer	KGZ. Jalal-abad	L. Pans, R. Best	ETHZ 302	JQ677171	JQ677476	JQ677284	JQ660749	JQ677367

Hoplitis (Alcidamea) freygessneri	MAR, W Quarzazate	AM, CS	ETHZ 183	JQ677173	JQ677478	JQ677286	JQ660751	JQ677369
Hoplitis (Alcidamea) leucomelana	SUI, Hohtenn	AM	ETHZ 16	JQ677174	JQ677479	EU851557	EU851663	JQ677370
Hoplitis (Alcidamea) mitis	SUI, Zeneggen	AM	ETHZ 30	JQ677175	JQ677480	EU851558	EU851664	JQ677371
Hoplitis (Alcidamea) pilosifrons	USA, NY, Thompkins Co	J. Ascher	CUIC 506	EU851454	JQ677481	EU851559	EU851665	Missing
Hoplitis (Alcidamea) praestans	SUI, Kalpetran/VS	AM	ETHZ 74	JQ677176	JQ677482	JQ677287	JQ660752	JQ677372
Hoplitis (Alcidamea) producta	USA, MD Calvert Co	USDA, Hym. group	ETHZ 292	JQ677177	Missing	Missing	JQ660753	Missing
Hoplitis (Alcidamea) tricolor	MAR, N Tazenakh	AM, CS	ETHZ 219	JQ677178	JQ677483	JQ677288	JQ660754	JQ677373
Hoplitis (Alcidamea) tridentata	ITA, Aosta, St-Pierre	C. Praz	ETHZ 15	JQ677179	JQ677484	EU851560	EU851666	JQ677374
Hoplitis (Alcidamea) truncata	USA, MD Calvert Co	USDA, Hym. group	ETHZ 293	JQ677180	JQ677485	JQ677289	JQ660755	JQ677375
Hoplitis (Annosmia) annulata	GRE, Rhodos, Afandou	AM	ETHZ 31	JQ677181	JQ677486	EU851561	EU851667	JQ677376
Hoplitis (Annosmia) bassana	MAR, E Agdz	AM, CS	ETHZ 188	JQ677182	JQ677487	JQ677290	JQ660756	JQ677377
Hoplitis (Anthocopa) batyamae	OMA, Al Kaburah	D. Michez, S. Patiny	ETHZ 246	JQ677195	JQ677501	JQ677302	JQ660768	JQ677390
Hoplitis (Anthocopa) bifoveolata	JOR, Ain Zarqua	AM, CP, CS	ETHZ 170	JQ677196	JQ677502	JQ677303	JQ660769	JQ677391
Hoplitis (Anthocopa) bisulca	GRE, Rhodos, Stegna	AM	ETHZ 32	JQ677197	JQ677503	EU851562	EU851668	JQ677392
Hoplitis (Anthocopa) cristatula	FRA, St. Martin de Crau	F. Amiet	ETHZ 305	JQ677198	JQ677504	JQ677304	JQ660770	JQ677393
Hoplitis (Anthocopa) dalmatica	SUI, Hohtenn	AM	ETHZ 26	JQ677199	JQ677505	JQ677305	JQ660771	JQ677394
Hoplitis (Anthocopa) hemisphaerica	JOR, Wadi Mujib	AM, CP, CS	ETHZ 143	JQ677200	JQ677506	EU851563	EU851669	JQ677395
Hoplitis (Anthocopa) idalia	IRI, Teheran Prov	A. Talebi, CP, CS	ETHZ 321	Missing	JQ677507	JQ677306	JQ660772	JQ677396
Hoplitis (Anthocopa) longispina	TUN	CP	ETHZ 1000	JQ677201	JQ677508	JQ677307	JQ660773	JQ677397
Hoplitis (Anthocopa) mocsaryi	FRA, Alpilles, Aureille	AM	ETHZ 49	JQ677202	JQ677509	JQ677308	JQ660774	JQ677398
Hoplitis (Anthocopa) perezi	GRE, Mt-Parnonas, Aghriani	CP, CS	ETHZ 145	JQ677203	JQ677510	JQ677309	JQ660775	JQ677399
Hoplitis (Anthocopa) pulchella	TUN, N Gafsa	CP	ETHZ 164	JQ677204	JQ677511	JQ677310	JQ660776	JQ677400
Hoplitis (Anthocopa) semirubra	JOR, Ma'in	C. Praz, AM, CS	ETHZ 174	JQ677205	JQ677512	JQ677311	JQ660777	JQ677401
Hoplitis (Anthocopa) similis	RSA, S Nieuwoudtville	M. Kuhlmann	ETHZ 237	JQ677206	JQ677513	JQ677312	JQ660778	JQ677402
Hoplitis (Anthocopa) spec. nov. 1	RSA, Nieuwoudtville	K. Timmermann	ETHZ 108	JQ677192	JQ677498	EU851564	EU851670	JQ677387
Hoplitis (Anthocopa) spec. nov. 2	RSA, Keiskie Mts	M. Kuhlmann	ETHZ 332	JQ677193	JQ677499	JQ677300	JQ660766	JQ677388
Hoplitis (Anthocopa) spec. nov. 3	RSA, Keiskie Mts	M. Kuhlmann	ETHZ 329	JQ677194	JQ677500	JQ677301	JQ660767	JQ677389

Hoplitis (Anthocopa) ursina	ALG, Kenchela Kench	N. Maghni	ETHZ 239	JQ677207	JQ677514	JQ677313	JQ660779	JQ677403
Hoplitis (Anthocopa) villosa	SUI, Säntis, Fälalp	D. Dietiker	ETHZ 17	JQ677208	JQ677515	EU851565	EU851671	JQ677404
Hoplitis (Anthocopa) yermasoyiae	GRE, Aghriani	CP, CS	ETHZ 144	JQ677209	JQ677516	JQ677314	JQ660780	JQ677405
Hoplitis (Bytinskia) erythrogastra	MAR, Tizi-n-Tagergoust	AM, CS	ETHZ 197	JQ677210	JQ677517	JQ677315	JQ660781	JQ677406
Hoplitis (Bytinskia) negevensis	ISR, SW Yotvata	C. Praz, CS	ETHZ 309	JQ677211	JQ677518	JQ677316	JQ660782	JQ677407
Hoplitis (Chlidoplitis) illustris	TUR, Ankara	E. Scheuchl	ETHZ 99	JQ677213	JQ677520	EU851566	EU851672	JQ677408
Hoplitis (Chlidoplitis) spec. nova 1	JOR, Wadi el Hasa	C. Praz, AM, CS	ETHZ 133	JQ677212	JQ677519	EU851567	EU851673	JQ677409
Hoplitis (Chlidoplitis) taenioceras	MAR, N Tazenakht	AM, CS	ETHZ 226	JQ677214	JQ677521	JQ677317	JQ660783	JQ677410
Hoplitis (Cyrtosmia) hypocrita	USA, CA, Tuolumne Co	T. Griswold	ETHZ 94	JQ677215	JQ677522	EU851568	EU851674	JQ677411
Hoplitis (Dasyosmia) biscutellae	USA, CA, Riverside Co	J. Ascher	CUIC 493	EU851464	JQ677523	EU851569	EU851675	Missing
Hoplitis (Formicapis) robusta	SUI, Visperterminen	C. Praz	ETHZ 54	JQ677216	JQ677524	EU851570	EU851676	JQ677412
Hoplitis (Hoplitina) bullifacies	USA, CA San Bernardino Co	T. Griswold	ETHZ 316	JQ677217	JQ677525	JQ677318	JQ660784	JQ677413
Hoplitis (Hoplitina) mojavensis	USA, NV, Clark Co	S. Higbee	ETHZ 102	JQ677218	JQ677526	EU851571	EU851677	JQ677414
Hoplitis (Hoplitis) adunca	ITA, Aosta	AM	ETHZ 9	JQ677219	JQ677527	EU851572	EU851678	HQ996000
Hoplitis (Hoplitis) tenuiserrata	MAR, NW Zagora	AM, CS	ETHZ 190	JQ677247	JQ677556	JQ677344	JQ660813	JQ677440
Hoplitis (Megahoplitis) tigrina	TUR, Ankara	E. Scheuchl	ETHZ 2	JQ677248	JQ677557	EU851573	EU851679	JQ677441
Hoplitis (Megalosmia) princeps	MGL, SSW Baruun-Urt	J. Gelhaus	ETHZ 25	JQ677249	JQ677558	JQ677345	JQ660814	JQ677442
Hoplitis (Micreriades) antalyae	GRE, Rhodos, Afandou	AM	ETHZ 135	JQ677250	JQ677559	EU851574	EU851680	JQ677443
Hoplitis (Micreriades) lebanotica	JOR, Wadi Mujib	C. Praz, AM, CS	ETHZ 147	JQ677251	JQ677560	EU851575	EU851681	JQ677444
Hoplitis (Microhoplitis) spec. Nova	UAE, Wadi Bih	C. Schmid-Egger	ETHZ 297	JQ677252	JQ677561	JQ677346	JQ660815	JQ677445
Hoplitis (Monumetha) albifrons	USA, CA, Contra Costa Co	J. Ascher	CUIC 507	EU851472	JQ677562	EU851577	EU851683	Missing
Hoplitis (Monumetha) tuberculata	SUI, Wasserauen	AM	ETHZ 33	JQ677253	JQ677563	EU851578	EU851684	JQ677446
Hoplitis (Pentadentosmia) cadiza	POR, Mertola	Prosi	ETHZ 252	JQ677254	JQ677564	JQ677347	JQ660816	JQ677447
Hoplitis (Pentadentosmia) enslini	ISR, E Ashalim	C. Praz, CS	ETHZ 325	JQ677255	JQ677565	JQ677348	JQ660817	JQ677448
Hoplitis (Pentadentosmia) gallinula	JOR, Tabaqat Fahl	C. Praz, AM, CS	ETHZ 169	JQ677256	JQ677566	JQ677349	JQ660818	JQ677449
Hoplitis (Pentadentosmia) helouanensis	OMA, Sur	D. Michez, S. Patiny	ETHZ 247	JQ677257	JQ677567	Missing	JQ660819	JQ677450
Hoplitis (Pentadentosmia) karakalensis	IRI, E Kalameh	C. Praz, A. Monfared, CS	ETHZ 299	JQ677258	JQ677568	JQ677350	JQ660820	JQ677451

Hoplitis (Pentadentosmia) laevifrons	ITA, Toscana, Siena	AM	ETHZ 177	JQ677259	JQ677569	JQ677351	JQ660821	JQ677452
Hoplitis (Pentadentosmia) minor	IRI, S Teheran	C. Praz, A. Talebi, CS	ETHZ 300	JQ677260	JQ677570	JQ677352	JQ660822	JQ677453
Hoplitis (Pentadentosmia) minuta	MGL, S Saynshand	J. Halada	ETHZ 234	JQ677261	JQ677571	Missing	JQ660823	Missing
Hoplitis (Pentadentosmia) moricei	MAR	D. Michez	ETHZ 120	JQ677262	JQ677572	EU851580	EU851686	JQ677454
Hoplitis (Pentadentosmia) nitidula	UZB, Karakalpakstan	C. Praz, I. Abdulaev	ETHZ 257	JQ677263	JQ677573	Missing	JQ660824	JQ677455
Hoplitis (Pentadentosmia) quinquespinosa	MAR, W Quarzazate	AM, CS	ETHZ 214	JQ677264	JQ677574	JQ677353	JQ660825	JQ677456
Hoplitis (Pentadentosmia) ruficornis	UZB, Karakalpakstan	C. Praz, I. Abdulaev	ETHZ 243	JQ677265	JQ677575	JQ677354	JQ660826	JQ677457
Hoplitis (Pentadentosmia) rufopicta	UZB, Qarschi Prov	C. Praz, I. Abdulaev	ETHZ 235	JQ677266	JQ677576	JQ677355	JQ660827	JQ677458
Hoplitis (Pentadentosmia) tringa	IRI, Teheran Prov	C. Praz, CS	ETHZ 301	JQ677267	JQ677577	JQ677356	JQ660828	JQ677459
Hoplitis (Pentadentosmia) villiersi	TUN, Nefta	C. Praz	ETHZ 67	JQ677268	JQ677578	EU851581	EU851687	JQ677460
Hoplitis (Penteriades) incanescens	USA, CA, Inyo Co	A. Menke	ETHZ 104	JQ677269	JQ677579	EU851582	EU851688	JQ677461
Hoplitis (Platosmia) africana	MAR, N Tazenakht	AM, CS	ETHZ 220	JQ677270	JQ677580	JQ677357	JQ660829	JQ677462
Hoplitis (Platosmia) incognita	MAR, N Tazenakht	AM, CS	ETHZ 221	JQ677271	JQ677581	JQ677358	JQ660830	JQ677463
Hoplitis (Platosmia) platalea	MAR	D. Michez	ETHZ 129	JQ677272	JQ677582	JQ677359	EU851689	JQ677464
Hoplitis (Prionohoplitis) brachypogon	ITA, Aosta, St-Pierre	C. Praz	ETHZ 42	JQ677273	JQ677583	EU851584	EU851690	JQ677465
Hoplitis (Prionohoplitis) curvipes	ITA, Apulia, S. G. Rotondo	CS	ETHZ 328	JQ677274	JQ677584	JQ677360	JQ660831	JQ677466
Hoplitis (Prionohoplitis) epeoliformis	MAR, NM Zagora	AM, CS	ETHZ 192	JQ677172	JQ677477	JQ677285	JQ660750	JQ677368
Hoplitis (Proteriades) jacintana	USA, CA, San Bernardino Co	M. Irwin	ETHZ 319	JQ677275	JQ677585	JQ677361	JQ660832	JQ677467
Hoplitis (Proteriades) pygmaea	USA, CA, San Bernardino Co	T. Griswold	ETHZ 317	JQ677276	JQ677586	JQ677362	JQ660833	JQ677468
Hoplitis (Proteriades) zuni	USA, UT, Garfield Co	K. Huntzinger	ETHZ 91	JQ677277	JQ677587	EU851585	EU851691	JQ677469
Hoplitis (Robertsonella) nemophilae	USA, Texas, Hidalgo Co	J. L. Neff	ETHZ 283	JQ677278	JQ677588	JQ677363	JQ660834	JQ677470
Hoplitis (Robertsonella) simplex	USA, Texas, Travis Co	J. L. Neff	ETHZ 284	JQ677279	JQ677589	JQ677364	JQ660835	JQ677471
Hoplitis (Stenosmia) minima	TUN, Nefta	C. Praz	ETHZ 159	JQ677280	JQ677590	EU851625	EU851730	JQ677472
Hoplitis (Tkalcua) paralias	TUN, Nefta	C. Praz	ETHZ 137	JQ677281	JQ677591	EU851576	EU851682	JQ677473

6.3.2. DNA sequencing and alignment

We extracted DNA from haploid male specimens conserved in 70% ethanol, but for some species we also used females and pinned specimens not older than three years. We exclusively used the head, while the rest of the body was deposited as voucher in the Entomological Collection of the ETH Zurich. DNeasy Blood & Tissue Kits (Qiagen, Valencia, California, USA) were used for all extractions, and PCR was applied to amplify one mitochondrial (COI, 1185 bp) and four nuclear genes (CAD, 857 bp; EF1-alpha (F2 copy) (EF), 1495 bp; LW-rhodopsin (Opsin), 771 bp; and NaK, 1414 bp). We used internal and PCR primers for sequencing. For details regarding primers and reaction conditions see Table 3. Exo-SAP (Thermo Fisher Scientific, Waltham, Massachusetts, USA) purified PCR products were sequenced on an ABI 3130xl capillary sequencer (Applied Biosystems, Foster City, California, USA) using BigDye technology. Sequences were assembled using Sequencher 4.10.1 (Gene Codes, Ann Arbor, Michigan, USA).

TABLE 3: Primers used and reaction conditions applied for the five genetic markers used in this study.

Primer	Reference	Sequence 5'-3'
CAD		
CADFor5	This study	GCR TAC GAC AAY TGY ATY ACA
CADRev 932	This study	RCT YTC TTG YCT CTG TAT YCT AAC AGC
CADRev1a	Praz et al. 2008b	GCC ATC ACT TCY CCT AYR CTC TTC AT
CAD-MegFor1	Litman et al. 2011	GAR CCY AGY CTC GAT TAY TG
PCR conditions: CAD-MegFor1-CADRev1a: 30" 94°C, 30" 56°C, 45" 72°C		
PCR conditions: CADFor5-CADRev932: 30" 94°C, 30" 56°C, 45" 72°C		
COI		
UEA3	Lunt et al. 1996	TAT AGC ATT CCC ACG AAT AAA TAA
UEA6For	This study	ATT ATT GCW ATY CCW ACW GGW ATT
UEA6	Lunt et al. 1996	TTA ATW CCW GTW GGN CAN GCA ATR ATT AT
UEA10	Lunt et al. 1996	CAA TGC ACT TAT TCT GCC ATA TT

COIFor398	This study	CAA CAT TTA TTT TGA TTT TTT GG

PCR conditions: UEA3-UEA6: 30" 94°C, 30" 55°C, 60" 72°C

PCR conditions: COIFor398-UEA10: 30" 94°C, 30" 55°C, 60" 72°C

EF1-alpha

HaF2For1	Danforth et al. 2004	GGG YAA AGG WTC CAA RTA TGC
Cho10	Danforth et al. 2004	ACR GCV ACK GTY TGH CKC ATG TC
F2Rev1h	This study	AAT CAG CRG CAC CCT TRG GYG G
Exon2Forh	This study	CCR ACY AGA CCY ACV GAC AAA GC
Exon2Rev	Praz et al. 2008b	GGG AAG ACG GAG AGC TTT GT
For4h	This study	AGC TYT RCA AGA RGC TGT HCC

PCR conditions: HaF2For1-F2Rev1h: 30" 94°C, 30" 56°C, 60" 72°C

PCR conditions: For4h-Cho10: 30" 94°C, 30" 56°C, 45" 72°C

PCR conditions: Exon2Forh-Cho10: 30" 94°C, 30" 55°C, 60" 72°C

Opsin

OpsForh	This study	GTA CTY GGA CCT STY TTC TGT
OpsFor5h	This study	GTR CCY GAA GGT AAY ATG AC
OpsRevh	This study	RTA TGG TGT CCA YGC CAT GAA CCA
OpsRev5h	This study	AGC TCK ATA CTT CGG ATG ACT G

PCR conditions: OpsForh-OpsRevh: 30" 94°C, 30" 55°C, 45" 72°C

PCR conditions: OpsFo5rh-OpsRev5h: 30" 94°C, 30" 55°C, 45" 72°C

NaK

NaKFor1	Cardinal et al. 2010	GGY GGT TTC GCS WTG YTG YTG TGG ATC GG
NaKRev1a	Cardinal et al. 2010	CCG ATN ARR AAG ATR TGM GCG TCN AGC CAA TG
NaKFor2	Cardinal et al. 2010	GCS TTC TTC TCB ACS AAC GCC GTY GAR GG
NaKRev2	Cardinal et al. 2010	ACC TTG ATR CCG GCY GAW CGG CAC TTG GC
NaKRevh	This study	GGY GGR TCD ATC ATR GAC ATS AG
NaKForh	This study	CCT YTG CTT CAT CGC GTA CT
NaKRev9	This study	CAG CCT CGA TRA TCT GAT TG
NaKFor6	This study	TTC TYG GTT AYC ATT GGC TYG AC
NaKRev11	This study	GGA ATC TCG CAG ACC TTC TTG T
NaKFor9	This study	CAA TCA GAT YAT CGA GGC TG
NaKRev6	This study	GTC RAG CCA ATG RTA ACC RAG AA

PCR conditions: NaKForh-NaKRev11: 30" 94°C, 30" 55°C, 75" 72°C

PCR conditions: NaKFor9-NaKRevh: 30" 94°C, 30" 55°C, 60" 72°C

PCR conditions: NaKFor1-NaKRev1a: 30" 94°C, 30" 55°C, 75" 72°C

PCR conditions: NaKFor2-NaKRev2: 30" 94°C, 30" 55°C, 75" 72°C

For data alignment we first used the program MAFFT (Katoh et al. 2002) and manually adjusted intron regions using MacClade 4.08 (Maddison & Maddison 2005). Outgroup intron regions and ambiguous ingroup intron

alignments were excluded from all analyses. The sequences of the five genes were concatenated for all phylogenetic analyses resulting in a matrix comprising a total of 5,722 characters of which 1,806 were parsimony informative. GenBank accession numbers are listed in Tab. 3.

6.3.3. Data partitioning and model testing

We partitioned the data into the five gene regions, with each region partitioned into first, second and third positions (e.g. CAD1, CAD2, CAD3). In addition, the introns of CAD, EF and Opsin were combined into one additional partition resulting in a dataset comprising 16 individual partitions. We ran a Bayesian analysis in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2001) for 5 million generations using a GTR model and analysed the resulting parameter files in Tracer 1.4. (Rambout & Drummond 2007). After discarding an appropriate burn-in, we analysed the substitution rates and nucleotide compositions of the 16 partitions in Tracer 1.4. Due to its very strong AT-bias (95.3%), the third codon position of the COI gene was excluded. We grouped similar partitions together resulting in the following final partitioning regime: partition 1 included CAD3, COI1, EF3, NaK3 and the introns; partition 2 included COI2 and Opsin1; partition 3 included CAD1 and Opsin2; partition 4 included EF1 and NaK1; partition 5 included CAD2, EF2 and NaK2. We used MrModelTest 2.3 (Nylander 2004) to select models of sequence evolution based on the Akaike Information Criterion (AIC). After testing 24 models of nucleotide substitution for each partition, we chose the model associated with the lowest AIC value: (GTR+I+G) for partitions 1-4 and (GTR+I) for partition 5.

6.3.4. Phylogenetic analyses

Phylogenetic analyses were performed applying both Bayesian and maximum likelihood methods. For Bayesian analysis we used MrBayes 3.1.2 (Ronquist & Huelsenbeck 2001) under the model and partitioning regime specified above. Partitions were unlinked to allow parameter values and overall rate of substitution to differ. Markov Chain Monte Carlo analyses were run with one cold and three heated chains. We ran 100 million generations sampling trees every 4000 generations. Tracer 1.4 was used to determine an appropriate burn-in after 15 million generations. To produce a 50% majority rule consensus tree reflecting posterior probability values for each node, the resulting 42,500 trees were sampled and combined using PAUP* 4.0a118 for Macintosh (Swofford 2002).

For maximum likelihood analysis we used RAxML 7.0.4 (Stamatakis et al. 2005). The rapid bootstrapping algorithm with a GTR+CAT approximation was applied to perform 1000 bootstrap replicates. To produce a 50% majority rule consensus tree, the bootstrap replicates were sampled and combined in PAUP.

6.3.5. Biogeography

The distribution of the included *Hoplitis* species is based on Michener (2007), Müller (2012) and Kuhlmann et al. (2011, personal communication). The geographic ranges were categorized as i) Palaearctic, ii) Nearctic or iii) Afrotropic (Tab. 1). The ancestral geographic ranges were inferred based on parsimony using MacClade 4.08 (Maddison & Maddison 2005).

6.3.6. Nesting biology

Information about the nesting biology of the *Hoplitis* species were drawn from Müller (2012, and references therein) for Palaearctic species, from Krombein et al. (1979, and references therein) and Michener (2007, and references therein) for Nearctic species, and from Kuhlmann et al. (2011) for Afrotropic species (Tab. 1). In three cases (*H. (Anthocopa) spec. nov. 2*, *H. (Chlidoplitis) illustris* Zanden and *H. (Platosmia) platalea* (Warncke)), we considered information about nesting behaviour of the closely related species *Hoplitis (Anthocopa) conchophila* Kuhlmann, *Hoplitis (Chlidoplitis) heinrichi* Zanden and *Hoplitis (Platosmia) alchata* (Warncke), respectively. The evolution of nesting behaviour was inferred based on parsimony using MacClade 4.08.

6.3.7. Ancestral state reconstruction

To trace the evolution of biogeography and nesting biology, we pruned the outgroup from the majority rule consensus tree of the Bayesian analysis (Fig. 1) before applying parsimony mapping using MacClade 4.08 (Maddison & Maddison 2005). Subsequently, we trimmed the mapped phylogeny by condensing taxa restricted to one zoogeographic region and nesting category to one representative taxon under the maintenance of at least one species per subgenus and of all transitions between different zoogeographical regions and nesting categories (Fig. 2).

6.4. RESULTS

6.4.1. Phylogeny

The trees derived from the Bayesian and the maximum likelihood bootstrap analyses showed no conflicting topology and were therefore combined (Fig. 1). The resulting phylogeny demonstrates monophyly of the genus *Hoplitis* (posterior probability (PP) = 100, bootstrap support (BS) = 100) and the majority of its currently recognized subgenera (PP = 100, BS ≥ 95): *Anthocopa, Pentadentosmia, Chlidoplitis, Micreriades, Tkalcua, Platosmia,* and *Robertsonella*. Monophyly is also probable for the morphologically uniform species of the subgenera *Stenosmia* and *Formicapis*, for which, however, only one species each was included in the present study.

FIGURE 1: Phylogeny of the bee genus *Hoplitis*. Majority rule consensus tree of the 42 500 post burn-in trees from the Bayesian analysis. Bayesian posterior probabilities (above branches) and maximum likelihood bootstrap values (below branches) are shown for all nodes.

Incongruences between the current morphology-based classification and our molecular phylogeny appear in i) the *Alcidamea* group, ii) the *Annosmia-Hoplitis* group, and iii) the *Proteriades* group, which are all

monophyletic (PP ≥ 94, BS ≥ 68; Fig. 1). i) The *Alcidamea* group comprises the species rich subgenus *Alcidamea*, which is polyphyletic with respect to the species poor subgenera *Cyrtosmia*, *Dasyosmia*, *Megalosmia*, *Monumetha,* and *Prionohoplitis*. Although phylogenetic relationships of the basal taxa of the *Alcidamea* group were poorly resolved, a clade comprising *Monumetha* (both Nearctic and Palaearctic), *Cyrtosmia* (Nearctic), all Nearctic and few Palaearctic *Alcidamea* species was strongly supported (PP = 99, BS = 63), with a topology well resolved by both Bayesian and maximum likelihood analysis. ii) The *Annosmia-Hoplitis* group comprises the subgenera *Bytinskia*, *Hoplitis* s. str. and *Annosmia*, the latter of which is polyphyletic due to the basal position of *H. (Annosmia) bassana.* This topology of the *Annosmia-Hoplitis* group is supported by a recent phylogenetic study that included 44 species and demonstrated monophyly of the subgenera *Bytinskia* and *Hoplitis* s. str. (see chapter 7). iii) The *Proteriades* group comprises the species rich subgenus *Proteriades*, which is polyphyletic with respect to the three species poor subgenera *Acrosmia*, *Hoplitina* and *Penteriades*. Furthermore, *Hoplitina* is probably paraphyletic with respect to a clade comprising *H. (Proteriades) zuni* (Parker) and the two species poor subgenera *Acrosmia* and *Penteriades*.

Our phylogeny suggests i) that the subgenus *Anthocopa* is sister group to all other *Hoplitis* species (PP = 93, BS < 50; Fig. 1), ii) that the two subgenera *Stenosmia* and *Pentadentosmia* are sister groups (PP = 100, BS = 78), iii) that the monotypic subgenus *Megahoplitis* is sister to the subgenus *Chlidoplitis* (PP = 90, BS < 50), and iv) that the subgenera *Platosmia* and *Tkalcua* form a monophyletic clade (PP = 97, BS = 86). The position of the subgenus *Micreriades* as the sister of this latter clade is only weekly supported (PP = 70, BS < 50), as is the phylogenetic

relationship between the subgenera *Formicapis* and *Robertsonella* (PP = 69, BS < 50).

6.4.2. Biogeography

Parsimony mapping of the biogeographic distribution of the *Hoplitis* taxa onto the phylogeny reveals a Palaearctic origin of the genus *Hoplitis* (Fig. 2). Within the subgenus *Anthocopa*, two independent colonization events from the Palaearctic to sub-Saharan Africa occurred. In the *Alcidamea* group, one colonization event from the Palaearctic to the Nearctic occurred, followed by two independent recolonizations of the Palaearctic. Furthermore, at least one colonization event from the Palaearctic to the Nearctic occurred in the clade that encompasses the *Proteriades* group and the two subgenera *Robertsonella* and *Formicapis*. One additional colonization event from the Palaearctic to the Nearctic may have occurred in the subgenus *Formicapis*. *Formicapis* consists of four species, which are Palaearctic except for *Hoplitis robusta* (Nylander), which is distributed both in the Palaearctic and the Nearctic. This suggests that *H. robusta* might have colonized the Nearctic from the Palaearctic.

FIGURE 2: Evolution of nesting behaviour and biogeographical patterns in bees of the genus *Hoplitis*. Nesting behaviour (left) and biogeography (right) were mapped onto an 80%-majority rule consensus tree of the Bayesian analysis using the criterion of maximum parsimony. Nesting sites and biogeographic distribution are color-coded as follows. (left side) brown: excavated burrows in the ground; black: preexisting insect burrows in the ground; light blue: small rock cavities; orange: cone-shaped brood cells constructed from leaflets and hidden in dense vegetation or underneath stones; dark green: excavated burrows in pithy stems; light green: insect burrows in dead wood or hollow stems; pink: empty snail shells; red: kleptoparasitic; grey: equivocal. (right side) black: Palaearctic; dark grey: Nearctic. light grey: Afrotropic; Striped lines indicate polymorphic states. Taxa restricted to one nesting category and zoogeographic region were condensed to one representative taxon under the maintenance of at least one species per subgenus and of all transitions between different zoogeographical regions and nesting categories.

6.4.3. Nesting biology

The nesting behaviour of the *Hoplitis* species can be grouped into eight categories (Tab. 1): i) nesting in excavated burrows in the ground, ii) nesting in insect burrows in the ground, iii) nesting in depressions or cavities of rocks, iv) nesting in excavated burrows in pithy stems, v) nesting in insect burrows in dead wood or in hollow stems, vi) nesting in empty snail shells, vii) construction of cone-like brood cells, which are built from leaf fragments imbricately glued together and hidden in dense vegetation or underneath stones, and (viii) kleptoparasitic in nests of species of the subgenus *Annosmia*.

Parsimony mapping of the nesting behaviour of the *Hoplitis* taxa onto the phylogeny revealed that nesting in excavated burrows in the ground is the ancestral state in the genus *Hoplitis* (Fig. 2). Within the most basal subgenus *Anthocopa*, two independent transitions from ground nesting to nesting in rock cavities and one transition from ground nesting to nesting in empty snail shells occurred. In the *Alcidamea* group, nesting in insect burrows in wood or in hollow stems is probably the ancestral state. In this group, nesting in excavated burrows in pithy stems independently evolved three times and both the construction of cone-like brood cells and nesting in insect burrows in the ground evolved once. In the *Annosmia-Hoplitis* group, kleptoparasitic behaviour and nesting in insect burrows in wood or in hollow stems evolved once each. Whether nesting in excavated ground burrows in the subgenus *Annosmia* has been inherited from early ancestors or whether it newly evolved from nesting above ground remains unclear, since the ancestral nesting habit of the basal lineages is equivocal (Fig. 2). In the *Proteriades* group, the great majority of species nests in insect burrows in wood or in hollow stems, rendering

this nesting habit the likely ancestral state. *Hoplitis (Acrosmia) plagiostoma* Michener is the only known species of the *Proteriades* group that additionally excavates its nests in pithy stems. Thus, one transition from nesting in insect burrows in wood or in hollow stems to nesting in excavated burrows in pithy stems occurred in this group. In total, our phylogeny reveals at least 16 evolutionary transitions between different nesting habits (Fig. 2).

6.5. DISCUSSION

6.5.1. Phylogeny

The inferred phylogeny of the genus *Hoplitis* considerably enhances our understanding of subgeneric relationships and provides a sound framework to elucidate biogeographic patterns and the evolution of nesting behaviour in this highly diverse group of osmiine bees. Although the morphology-based subgeneric classification (Griswold & Michener 1998; Michener 2007) was largely confirmed by our molecular phylogeny, we propose three classificatory changes.

First, based on our phylogeny and the morphological investigation of most species of the *Alcidamea* group, we propose to merge all 91 *Hoplitis* species of the subgenera *Alcidamea* (68 species), *Cyrtosmia* (1 species), *Dasyosmia* (2 species), *Megalosmia* (7 species), *Monumetha* (6 species), and *Prionohoplitis* (7 species) into a single subgenus *Alcidamea* for the following reasons: i) Our phylogeny clearly reveals that *Alcidamea* s.str. is not monophyletic. Despite the weakly supported resolution among the basal taxa of the *Alcidamea* group, including species of the subgenera *Alcidamea*, *Prionohoplitis,* and *Megalosmia*, the Holarctic clade

comprising the subgenera *Dasyosmia*, *Monumetha*, *Cyrtosmia* and species of *Alcidamea* is well supported and resolved by both Bayesian and maximum likelihood analysis and clearly turns *Alcidamea* polyphyletic. ii) Neither the Bayesian nor the maximum likelihood analysis support the monophyly of *Prionohoplitis*, but place the included species into three different clades within *Alcidamea* s. str. iii) The subgenus *Alcidamea*, which displays a great morphological variability unmatched by all other *Hoplitis* subgenera, is divided by Michener (2007) into four different groups based on the shape of male tergum 7. However, this character does not divide the species of *Alcidamea* into monophyletic clades, making it unsuitable for classificatory purpose. Our phylogeny supports Warncke (1991b), who merged all the above-mentioned subgenera of the *Alcidamea* group into one subgenus *Alcidamea*. Only few morphological characters unite all species of the *Alcidamea* group, such as the shape of the female clypeus, which is flat and possesses a shiny rim apically, as well as the shapes of male sternites 4 to 7, which are similar in most species. Uniting all six subgenera of the *Alcidamea* group in one subgenus *Alcidamea* results in a morphologically diverse but phylogenetically strongly supported clade.

Second, based on our phylogeny that exactly corresponds to a more comprehensive phylogeny of the *Annosmia-Hoplitis* group (see chapter 7) and on the morphological investigation of most species of this group, we propose to merge all 87 *Hoplitis* species of the subgenera *Annosmia* (31 species), *Bytinskia* (3 species), *Coloplitis* (2 species) and *Hoplitis* (51 species) into a single subgenus *Hoplitis* for the following reasons: i) The basal position of *H. (Annosmia) bassana* renders the subgenus *Annosmia* polyphyletic. Although *H. bassana* differs from the other *Annosmia* species by some minor morphological characters (Warncke 1991c), it is

otherwise very similar to *Annosmia*, which resulted in its inclusion into the subgenus *Annosmia* by Warncke (1991c). Furthermore, *H. bassana* is oligolectic on Boraginaceae as is the majority of species of the *Annosmia-Hoplitis* group (see chapter 7), indicating close relatedness to the other species of this group. ii) The kleptoparasitic species of the subgenus *Bytinskia* evolved from the same lineage as their *Annosmia* hosts (see chapter 8) and, correspondingly, they share most morphological characters with the *Annosmia* species except for the lack of an abdominal scopa in the females and few minor characters, such as the number of mandibular teeth (Michener 2007). iii) The *monstrabilis* clade, which is represented in the present study by *H. tenuiserrata* and which turned out to be sister to all the other species of the subgenus *Hoplitis* (see chapter 7), is morphologically and biologically intermediate between the subgenera *Annosmia* and *Hoplitis*. Whereas the females resemble those of *Annosmia*, the males share a typical morphological character of *Hoplitis* s. str., i.e. a rounded to truncate tergum 7. Furthermore, species of the *monstrabilis* clade nest in excavated burrows in the ground like species of the subgenus *Annosmia*, whereas all the other species of the subgenus *Hoplitis* nest in pre-existing cavities above ground (see chapter 8). iv) Although the two species of the subgenus *Coloplitis* could not be included in our phylogeny due to the lack of fresh material for DNA extraction, their morphology clearly reveals a very close relatedness to *Annosmia*, which was already emphasized by Warncke (1991c). Apart from minor differences in mandible shape and form of sternum 6, *Coloplitis* differs from *Annosmia* merely by the modified proboscis (Griswold & Michener 1998), which is an adaptation to extract pollen from the narrow-tubed flowers of *Heliotropium* (Boraginaceae) (see chapter 7). Similar morphological adaptations to remove pollen from hidden anthers have repeatedly and independently evolved in the

Annosmia-Hoplitis group, e.g. among species of the subgenus *Hoplitis* (see chapter 7), rendering the modified proboscis of *Coloplitis* an inaedequate character to justify its current subgeneric rank. Uniting all four subgenera of the *Annosmia-Hoplitis* group in a single subgenus *Hoplitis* results in a morphologically well characterized, albeit biologically rather heterogeneous taxon. Morphologically, the enlarged subgenus is distinctive due to a conspicuous yellowish membrane below the lateral extremity of the labrum. An alternative solution to eliminate the polyphyly of the subgenus *Annosmia* would have been the establishment of a new subgenus for *H. bassana*, which, however, would result in a monotypic subgenus that appears unjustified due to the morphological similarity of *H. bassana* with *Annosmia*.

Third, based on our phylogeny and the morphological investigation of 25 species of the *Proteriades* group, we propose to merge all 35 *Hoplitis* species of the subgenera *Acrosmia* (5 species), *Hoplitina* (6 species), *Penteriades* (2 species), and *Proteriades* s. str. (22 species) into a single subgenus *Proteriades* for the following reasons: i) Our phylogeny clearly reveals that the subgenus *Proteriades* sensu Michener (2007) is not monophyletic with *Hoplitis (Proteriades) zuni* (Parker) being sister to the clade composed of *Penteriades* and *Acrosmia*. ii) The distinction between *Penteriades* and *Acrosmia* based on the presence of hooked hairs on the female proboscis in the former and the lack of such hairs in the latter (Michener 2007) is not justified because two of the five *Acrosmia* species actually do possess such hooked hairs. In addition, there are no group characters allowing for the separation of *Penteriades* females from *Acrosmia* females (Griswold 1983). Thus, the only characters distinguishing these two subgenera are the modified male antennae in *Acrosmia* and the five-segmented maxillary palpi in *Penteriades*. These

characters, however, do not appear to be sufficient to give each of these two species poor taxa subgeneric status. iii) The monophyly of *Hoplitina* is questionable based both on our phylogeny and the considerable morphological variability among its species (Parker 1976). *Hoplitina* is defined by plesiomorphic characters, such as the moderately long female proboscis lacking hooked hairs, the unmodified male antennae and the lack of a carina on the hind coxa (Michener 2007). However, the shapes of the female clypeus, of the male tergum 7 as well as those of the male sterna 2 and 7 of certain *Hoplitina* species closely resemble the corresponding structures in species of *Penteriades* and *Acrosmia* (Parker 1976). In fact, there are no morphological characters allowing for the separation of females of *H. (Hoplitina) torchioi* (Parker) from females of *Acrosmia* and *Penteriades* (Griswold 1983). By uniting all four subgenera of the *Proteriades* group in a single subgenus *Proteriades*, a morphologically and biologically well-characterized taxon emerges. Its monophyly is strongly supported by both our phylogeny and numerous morphological characters, such as the presence of a median ridge or process on male sternum 6, a mostly bilobed male tergum 7, a basal convexity on the female clypeus in numerous species, the common occurrence of red maculations on the metasoma as well as the presence of hooked hairs on the female proboscis in most species, which are used to extract pollen from narrow-tubed *Cryptantha* flowers (Hurd & Michener 1955).

6.5.2. Biogeography

Our data suggests a Palaearctic origin of the genus *Hoplitis*. This result is supported by the findings that the genus *Haetosmia*, which is exclusively Palaearctic in its distribution, is sister to the clade composed of *Hoplitis*

and the *Osmia* group (Fig. 1), and that approximately 80% of all *Hoplitis* species occur in the Palaearctic (Müller 2012).

The Afrotropic *Hoplitis* species are all members of the subgenus *Anthocopa* (Michener 2007; Kuhlmann et al. 2011). Based on our phylogeny, colonization of sub-Saharan Africa occurred twice independently within *Anthocopa*. The finding that colonization of the sub-Saharan region occurred so rarely in *Hoplitis* in spite of its very high diversity in northern Africa, might reflect the strong dispersion barrier imposed by the Saharan desert, the subtropical savannahs and the tropical forests for osmiine bees in general. These areas are among the most depauperate zoogeographic regions in terms of both bee diversity and abundance (Michener 1979). However, for millions of years Africa has provided a vast and geographically highly diverse connection between the northern and the southern hemisphere. Especially during cool and dry periods that characterized the late Tertiary, large parts of today's subtropical and tropical areas in Central and East Africa were covered by arid grassland (Maley 1996), which may have provided a more suitable habitat for osmiine bees and allowed dispersal to the xeric regions of Southern Africa. After the reestablishment of present climatic conditions, dispersal from the Palaearctic to the Afrotropic might have been largely impeded. This hypothesis is supported by the distribution of one *Anthocopa* species, *Hoplitis reginae* (Cockerell), which occurs in the Democratic Republic of Congo (Cockerell 1932) and might represent a relict of a former pan-African distribution of *Anthocopa*. In fact, a similar biogeographic pattern can be observed in other osmiine taxa (Praz et al. 2008): among the representatives of the *Heriades* group and the genus *Wainia*, which mainly occur in xeric regions of northern and southern Africa, few species also occur in Kenya and the Democratic Republic of Congo, respectively (Michener 2007).

Exchanges between the Palaearctic and the Nearctic were more frequent than exchanges between the Palaearctic and the Afrotropic, a pattern observed for the osmiine bees in general (Praz et al. 2008). Our data reveals at least two colonization events from the Palaearctic to the Nearctic (one in the *Alcidamea* group and at least one in the clade comprising the *Proteriades* group, *Robertsonella* and *Formicapis*) and two colonization events from the Nearctic to the Palaearctic (one within the Nearctic *Alcidamea* clade and one within *Monumetha*). Interestingly, the latter two colonization events pertain to taxa, which range into boreoalpine regions. In *Alcidamea*, the Nearctic species range as far north as Canada (*H. pilosifrons* (Cresson), *H. producta* (Cresson) and *H. truncata* (Cresson)), while the Palaearctic *H. leucomelana* (Kirby) ranges into northern Europe and Northeast Asia. In *Monumetha,* the Nearctic species *H. albifrons* (Kirby) reaches Alaska while the Palaearctic species *H. tuberculata* (Nylander) has a similar northern range as *H. leucomelana*. Similarly, *H. (Formicapis) robusta* (Nylander), which is the only Holarctic *Hoplitis* species, occurs in all boreoalpine regions around the northern hemisphere. The northern distribution of these cold adapted species suggest that dispersal events between the Old to the New World probably occurred across the Bering Strait (Michener 1979; Praz et al. 2008), followed by radiations in the newly colonized areas. Similar biogeographic patterns can be observed in *Osmia* bees of the subgenus *Melanosmia* and in bumblebees (*Bombus*). *Melanosmia* comprises both Palaearctic and Nearctic as well as several Holarctic species (Müller 2012), the latter having a boreoalpine to arctic distribution (Rightmyer et al. 2010). In bumblebees, an estimated number of 21 independent dispersal events between the Old and the New World occurred, mainly among clades containing the most cold adapted species (Hines 2008).

6.5.3. Nesting biology

The nesting biology of bees of the genus *Hoplitis* is extraordinarily diverse encompassing at least eight different nesting habits. Our data strongly suggest that ground nesting in excavated burrows is the ancestral state in *Hoplitis*. This result is supported by the finding that the genus *Haetosmia*, which was recently found to excavate its nests in the ground as well (A. Gotlieb and G. Pisanty, personal communication), is sister to the clade composed of *Hoplitis* and the *Osmia* group (Fig. 1). Nesting in excavated ground burrows is confined to the basal subgenera *Anthocopa, Stenosmia* and *Pentadentosmia,* but also occurs in the more derived subgenus *Annosmia*. Whether ground nesting in this latter group of bees is derived from above ground nesting is unclear since two evolutionary scenarios for the ancestral nesting behaviour in the basal lineages of *Hoplitis* were found to be equally parsimonious. The first scenario suggests that nesting in excavated ground burrows was the ancestral behaviour of the clade comprising all taxa but the subgenera *Anthocopa*, *Pentadentosmia* and *Stenosmia*. This scenario would indicate that no reversal back to ground nesting occurred and that above ground nesting repeatedly evolved from ground nesting, followed by a diversification in the selection of nesting sites. One argument in support of this scenario is that once the transition from nesting in excavated ground burrows to nesting in preexisting cavities above ground has been accomplished, the morphological and behavioural adaptations to efficiently dig nests in the soil, and the cognitive ability to assess crucial aspects of nesting sites, such as soil texture, humidity or temperature, are unlikely to re-evolve (Eickwort et al. 1981; Cane 1991; Neff & Simpson 1991). The second scenario suggests that nesting in insect burrows in wood or in hollow

stems was the ancestral behaviour of the clade comprising all taxa but the subgenera *Anthocopa*, *Pentadentosmia* and *Stenosmia*, followed by a reversal to ground nesting in the ancestor of *Annosmia*. The question, which of these two scenarios applies to the genus *Hoplitis,* must remain unresolved as long as the nesting behaviours of *H. bassana*, *Megahoplitis* and *Tkalcua* are unknown.

Despite our still incomplete knowledge of the nesting behaviour of bees of the genus *Hoplitis*, we identified at least 16 evolutionary transitions between different nesting habits. Both the considerable evolutionary flexibility and the high diversity of different nesting behaviours in *Hoplitis* appears to be unmatched by any other group of bees.

6.5.4. Impact of nesting biology on biogeography

Comparison of nesting behaviour of the *Hoplitis* bees with their biogeographic distribution reveals two conspicuous patterns. First, almost all taxa that nest in excavated burrows in the ground (i.e., *Annosmia*, *Pentadentosmia*, *Stenosmia,* most *Anthocopa*) have a distinctly southern distribution and occur in desert or mediterranean climates, a pattern that also applies for the ground nesting representatives of the closely related genus *Osmia* (e.g., *Hemiosmia, Ozbekosmia,* some *Tergosmia*). Interestingly, among *Hoplitis (Anthocopa)* and *Osmia (Tergosmia)*, the northernmost species (*villosa* group and *O. tergestensis*, respectively) do not nest in the ground but in preexisting cavities in rocks or between stones (Müller, 2012 and references therein). This apparent incapability of ground nesting osmiine bees to colonize cooler and moister areas might possibly be due to difficulties to protect the brood cell provisions from fungal infection and liquefaction in moist grounds (Litman et al.

2011). In fact, while the abundance of most osmiine bee taxa decreases towards boreal and alpine regions, a pattern observed for most solitary bees (Michener 2007), the ratio of ground nesting osmiine species becomes disproportionately low. Therefore, above ground nesting may have been a precondition among osmiine bees to colonize cool and moist areas.

Second, all dispersal events between the Palaearctic and the Nearctic have taken place among *Hoplitis* lineages nesting in dead wood or hollow stems (i.e., *Alcidamea* group, *Formicapis*, *Monumetha*, *Robertsonella*, *Proteriades* group). This finding strongly supports the hypothesis suggested by Michener (1979, 2007) and Praz et al. (2008) that wood nesting is an important prerequisite for cross-oceanic dispersal in bees. The woody nesting substrate may serve as a raft enabling transport of whole nests across the sea. The colonization of new areas by this mode of dispersal is possible as diapausing bees may endure a journey of close to one year within their nests and as the nests usually contain both males and females, enabling a rapid establishment of a viable population in the newly colonized area. In conclusion, nesting biology may not only constrain a species' potential for range expansion at a local scale but may also greatly affect the potential for long-distance dispersal.

6.6. TAXONOMY

***Hoplitis* subgenus *Alcidamea* Cresson, 1864** comb. nov.
Type species: *Hoplitis producta* (Cresson, 1864)
Monumetha Cresson, 1864, new synonymy
Megalosmia Schmiedeknecht, 1885, new synonymy
Cyrtosmia Michener, 1947, new synonymy

Dasyosmia Michener, 1947, new synonymy

Prionohoplitis Tkalcu, 1993, new synonymy

***Hoplitis* subgenus *Hoplitis* Klug, 1807** comb. nov.

Type species: *Hoplitis adunca* (Panzer, 1798)

Annosmia Warncke, 1991, new synonymy

Bytinskia Mavromoustakis, 1954, new synonymy

Coloplitis Griswold, 1998, new synonymy

***Hoplitis* subgenus *Proteriades* Titus, 1904** comb. nov.

Type species: *Hoplitis semirubra* (Cockerell, 1898)

Hoplitina Cockerell, 1910, new synonymy

Acrosmia Michener, 1947, new synonymy

Penteriades Michener and Sokal, 1957, new synonymy

7. Host range evolution in a selected group of osmiine bees (Hymenoptera: Megachilidae): the Boraginaceae-Fabaceae paradox[4]

7.1. Abstract

Bees are extraordinarily diverse with respect to host plant choice and adaptation. Recent findings suggest that bee host range might be largely governed by evolutionary constraints related to pollen digestion or flower recognition and handling. In this study, we applied phylogenetic inference to address whether such constraints underlie host plant choice in bees of the *Annosmia-Hoplitis* group (Megachilidae) and to what extent these bees have evolved specialized adaptations for pollen collection. We demonstrate that most pollen specialist species exclusively exploit either Boraginaceae or Fabaceae, whereas all pollen generalists harvest pollen from both Boraginaceae and Fabaceae. The counterintuitive affinity towards these two plant families, which are neither closely related nor share similar flower morphologies, demonstrates that pollen host choice is considerably constrained in this group of bees. We hypothesize that this Boraginaceae-Fabaceae paradox might be due to similar secondary metabolites in the pollen of both families, to metabolites that can be detoxified by the same physiological tools or to similar pollen nutrient composition. Contrary to the widely held belief that specialized adaptations for pollen collection are rare among bees, such adaptations are common in the *Annosmia-Hoplitis* bees,

[4]Based on Sedivy, C., S. Dorn, A. Widmer & A. Müller. Biological Journal of the Linnean Society, DOI: 10.1111/j.1095-8312.2012.02013.x.

where they have evolved several times independently to exploit flowers of widely different morphologies.

7.2. INTRODUCTION

During their long evolutionary history, which has largely been shaped by the intimate relationship with flowering plants, bees have conquered all major terrestrial ecosystems and become the most important pollinators of both wild and crop plants (Michener 2007). Bee taxa differ widely in the range of host plants they are capable of exploiting (Cane & Sipes 2006). Specialized bee species collect pollen from the flowers of a single plant genus, subfamily or family ("oligolecty"), whereas generalist bee species exploit flowers of two or more plant families ("polylecty") (Cane & Sipes 2006; Müller & Kuhlmann 2008). While oligolecty is considered the ancestral state in bees (Müller 1996a; Sipes & Tepedino 2005; Danforth et al. 2006; Larkin et al. 2008; Michez et al. 2008; Sedivy et al. 2008; Litman et al. 2011), recent studies on host plant choice in bees of the genus *Chelostoma* suggest that transitions from oligolecty to polylecty were largely impeded by constraints imposed by pollen chemistry and flower morphology (Praz et al. 2008a; Sedivy et al. 2008). This finding led to a new hypothesis suggesting that incorporation of new hosts are rare events in the evolutionary history of bee lineages, that host expansion is only possible if the physiological or neurological constraints imposed by the flowers can be overcome and that host shifts among oligoleges typically proceeded over a period of expanded host range followed by respecialization (Sedivy et al. 2008). This hypothesis postulated for bees closely corresponds to the oscillation hypothesis of host plant range postulated for herbivorous insects (Janz & Nylin 2008), corroborating several studies that report striking similarities in the

patterns of host plant use of bees and herbivores (Sipes & Tepedino 2005; Müller & Kuhlmann 2008; Sedivy et al. 2008; Sedivy et al. 2011). To test whether this "constraint hypothesis on host range evolution in bees" can be generalized beyond the species-poor genus *Chelostoma*, species-rich bee taxa of other genera should be examined.

Bees often show complex morphological and behavioural adaptations to their floral hosts (Thorp 2000). Since anthers or pollen bearing flower structures have to be placed in a suitable position to contact incoming pollinators, pollen cannot be as deeply hidden inside the flower as nectar. Thus, morphological adaptations for nectar uptake are expected to be more common than adaptations for harvesting pollen (Westerkamp 1987). In fact, specialized morphological structures that facilitate pollen uptake are assumed to occur only exceptionally in bees (Westerkamp 1987; Westrich 1989; Wcislo & Cane 1996; Thorp 2000). Bees also have evolved specialized behavioral adaptations to collect pollen such as vibratile pollen harvesting ("buzzing"), which mainly serves to shake pollen out of flowers that conceal their pollen within poricidal anthers or between tightly appressed introrse anthers (Buchmann 1983). While buzzing is widespread in halictid, colletid and apid bees, it has only rarely been observed in andrenid bees and appears to be nearly non-existent among megachilid bees (Buchmann 1983; Teppner 2011). To our knowledge, no study has rigorously investigated the occurrence of specialized adaptations for pollen uptake in any larger taxon of bees. The widely held assumption that such adaptations have only exceptionally evolved in bees might thus be premature.

In the present study, we analysed the evolution of host range in a species-rich taxon of solitary bees, the *Annosmia-Hoplitis* group, which forms a

well supported clade within the genus *Hoplitis* (Megachilidae, Osmiini). Furthermore, we evaluated whether and how often specialized adaptations for pollen collection have evolved in this group of bees. By applying phylogenetic inference, which has repeatedly been shown to be a powerful tool to uncover patterns of host plant choice and to test hypotheses on the evolution of host plant associations (Harvey 1996; Rasmann & Agrawal 2011), we addressed the following research questions:

i) What are the evolutionary patterns of host plant choice in the *Annosmia-Hoplitis* group and to what extent is host expansion governed by evolutionary constraints?

ii) How did host plant choice in the *Annosmia-Hoplitis* group affect the evolution of specialized morphological and behavioural adaptations for pollen uptake?

7.3. METHODS

7.3.1. Bee species

The *Annosmia-Hoplitis* group, which is exclusively Palearctic in its distribution, forms a well supported monophyletic clade within the genus *Hoplitis* (Praz *et al*. 2008b). It consists of four currently recognized subgenera: *Annosmia* (30 described species), *Bytinskia* (3), *Coloplitis* (2) and *Hoplitis* s. str. (50) (Ungricht et al. 2008; Müller 2012). *Bytinskia* is an exception among these subgenera in that its species are cleptoparasites and neither construct nests nor collect pollen (Warncke 1991c). Our extended field work in Europe, North Africa and the Middle East, together with specimens obtained from other collectors, allowed us to include a total of 46 species of the *Annosmia-Hoplitis* group into the

present study (Table 1, 2), among which *H. (Annosmia) aqabaensis* and *H. (Coloplitis) persica* could not be used for the molecular phylogeny due to the lack of fresh material for DNA extraction. For the phylogenetic analyses, 13 species of the genus *Hoplitis* representing all major clades as well as five additional osmiine bee species belonging to genera most closely related to the genus *Hoplitis* (Praz et al. 2008b) were selected as outgroup (Table 1), resulting in a total of 62 taxa including 18 outgroup species.

7.3.2. Molecular phylogeny

DNA sequencing and alignment

When possible, DNA was extracted from fresh haploid male specimens preserved in 100% ethanol, although some females and pinned specimens up to 3 years old were also used. We exclusively extracted DNA from the head, while the rest of the body was deposited as voucher in the Entomological Collection of the ETH Zurich. DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA) was used for all extractions, and polymerase chain reaction (PCR) was applied to amplify one mitochondrial gene and four nuclear genes: COI (1185 bp), CAD (868 bp), EF1-alpha (1495 bp, henceforth EF), LW-rhodopsin (750 bp, henceforth opsin) and NaK (1414 bp). For details regarding selected primers and reaction conditions see Supporting information (Table). Exo-SAP (Thermo Fisher Scientific, Waltham, Massachusetts, USA) purified PCR products were sequenced on an ABI 3130xl capillary sequencer (Applied Biosystems, Foster City, California, USA) using BigDye technology. In some cases, internal primers were used for sequencing

PCR products. Sequences were assembled using Sequencher 4.10.1 (Gene Codes, Ann Arbor, Michigan, USA).

The sequences were aligned with MAFFT (Katoh et al. 2002) and further adjusted by hand with MacClade 4.08 (Maddison & Maddison 2005). All introns of the non-*Hoplitis* outgroup species as well as ambiguous intron alignments of the *Hoplitis* outgroup and ingroup species were excluded. The sequences of the five genes were concatenated for all phylogenetic analyses resulting in a matrix comprising a total of 5712 characters of which 1298 were parsimony-informative: CAD 173 (13%), COI 486 (38%), EF 272 (21%), opsin 194 (15%) and NaK 173 (13%). GenBank accession numbers are listed in Table 1.

TABLE 1: Collection localities, voucher numbers and GenBank accession numbers of the bee species of the *Annosmia-Hoplitis* group included in the phylogeny. The names of the authors of this study are given with initials. Voucher specimens are deposited in the Entomological Collection of the ETH Zurich. Abbreviations for states: ALG = Algeria, CYP = Cyprus, FRA = France, GRE = Greece, ITA = Italy, IRI = Iran, ISR = Israel and Palestine, JOR = Jordan, MAR = Morocco, OMA = Oman, POR = Portugal, SUI = Switzerland, SYR = Syria, TUN = Tunisia, TUR = Turkey, UEA = United Arab Emirates, USA = United States of America.

Taxon	Locality	Collector	Voucher Nr.	Genbank Accession Nos.				
				CAD	COI	EF1-alpha	Opsin	NaK
Outgroup								
Heriades (Heriades) truncorum	SUI, Winterthur	AM	ETHZ 6	EU851448	JQ677594	EU851553	EU851659	Missing
Atoposmia mirifica	USA, NV, Clark Co.	S. Higbee	ETHZ 88	HQ995904	Missing	EU851541	EU851647	HQ995996
Osmia (Osmia) cornuta	SUI, Zürich	AM	ETHZ 2	EU851504	JQ677597	EU851609	EU851714	Missing
Wainia (Caposmia) eremophila	JOR, Wadi al Hasa	C. Praz, AM, CS	ETHZ 125	HQ995916	JQ677599	EU851626	EU851731	Missing
Haetosmia circumventa	UAE, Sharjah Desert	T. van Harten	ETHZ 97	EU851447	JQ677593	EU851552	EU851658	Missing
Hoplitis (Anthocopa) dalmatica	SUI, VS, Hohtenn	AM	ETHZ 26	JQ677199	JQ677505	JQ677305	JQ660771	JQ677394
Hoplitis (Anthocopa) hemisphaerica	JOR, Wadi Mujib	C. Praz, AM, CS	ETHZ 143	JQ677200	JQ677506	EU851563	EU851669	JQ677395
Hoplitis (Pentadentosmia) gallinula	JOR, Tabaqat Fahl	C. Praz, AM, CS	ETHZ 169	JQ677256	JQ677566	JQ677349	JQ660818	JQ677449
Hoplitis (Pentadentosmia) moricei	MAR	D. Michez	ETHZ 120	JQ677262	JQ677572	EU851580	EU851686	JQ677454
Hoplitis (Pentadentosmia) villiersi	TUN, Nefta	C. Praz	ETHZ 67	JQ677268	JQ677578	EU851581	EU851687	JQ677460
Hoplitis (Stenosmia) minima	TUN, Nefta	C. Praz	ETHZ 159	JQ677280	JQ677590	EU851625	EU851730	JQ677472
Hoplitis (Alcidamea) epeoliformis	MAR, Zagora	AM, CS	ETHZ 159	JQ677280	JQ677590	EU851625	EU851730	JQ677472
Hoplitis (Alcidamea) acuticornis	SUI, Hohtenn	AM	ETHZ 38	JQ677170	JQ677475	JQ677283	JQ660748	JQ677366
Hoplitis (Megahoplitis) tigrina	TUR, Ankara	E. Scheuchl	ETHZ 2	JQ677248	JQ677557	EU851573	EU851679	JQ677441
Hoplitis (Chlidoplitis) illustris	TUR, Ankara	E. Scheuchl	ETHZ 99	JQ677213	JQ677520	EU851566	EU851672	JQ677408
Hoplitis (Proteriades) zuni	USA, UT, Garfield Co.	K. Hunzinger	ETHZ 91	JQ677277	JQ677587	EU851585	EU851691	JQ677469
Hoplitis (Platosmia) platalea	MAR	D. Michez	ETHZ 129	JQ677272	JQ677582	JQ677359	EU851689	JQ677464
Hoplitis (Formicapis) robusta	SUI, Visperterminen	C. Praz	ETHZ 54	JQ677216	JQ677524	EU851570	EU851676	JQ677412

Ingroup								
Hoplitis (Annosmia) annulata	GRE, Rhodos	AM	ETHZ 31	JQ677181	JQ677486	EU851561	EU851667	JQ677376
Hoplitis (Annosmia) bassana	MAR, Agdz	AM, CS	ETHZ 188	JQ677182	JQ677487	JQ677290	JQ660756	JQ677377
Hoplitis (Annosmia) christae	ISR, Ein Yahav	C. Praz, CS	ETHZ 311	JQ677183	JQ677488	JQ677291	JQ660757	JQ677378
Hoplitis (Annosmia) hierichonica	JOR, Wadi al Hasa	C. Praz, AM, CS	ETHZ 274	JQ677184	JQ677489	JQ677292	JQ660758	JQ677379
Hoplitis (Annosmia) israelica	MAR, Tiouine	AM, CS	ETHZ 217	JQ677185	JQ677490	JQ677293	JQ660759	JQ677380
Hoplitis (Annosmia) mutica	IRI, Kojeer NP	C. Praz, A. Talebi, CS	ETHZ 294	JQ677186	JQ677491	JQ677294	JQ660760	JQ677381
Hoplitis (Annosmia) nisa	MAR, Agdz	AM, CS	ETHZ 178	JQ677187	JQ677492	JQ677295	JQ660761	JQ677382
Hoplitis (Annosmia) parana	OMA, Yankul	D. Michez, S. Patiny	ETHZ 245	JQ677188	JQ677493	JQ677296	JQ660762	JQ677383
Hoplitis (Annosmia) segura	ISR, Paran	C. Praz, CS	ETHZ 313	JQ677189	JQ677494	JQ677297	JQ660763	JQ677384
Hoplitis (Annosmia) speculum	MAR, Tazenakht	AM, CS	ETHZ 212	JQ677190	JQ677495	JQ677298	JQ660764	JQ677385
Hoplitis (Annosmia) uncaticornis	IRI, Kalus	A. Monfarez, C. Praz, CS	ETHZ 291	Missing	JQ677496	Missing	Missing	Missing
Hoplitis (Annosmia) zonalis	MAR, Tizi-n-Tagergoust	AM, CS	ETHZ 203	JQ677191	JQ677497	JQ677299	JQ660765	JQ677386
Hoplitis (Bytinskia) erythrogastra	MAR, Tizi-n-Tagergoust	AM, CS	ETHZ 197	JQ677210	JQ677517	JQ677315	JQ660781	JQ677406
Hoplitis (Bytinskia) negevensis	ISR, Yotvata	C. Praz, CS	ETHZ 309	JQ677211	JQ677518	JQ677316	JQ660782	JQ677407
Hoplitis (Hoplitis) adunca	ITA, Aosta	AM	ETHZ 9	JQ677219	JQ677527	EU851572	EU851678	HQ996000
Hoplitis (Hoplitis) anthocopoides	POR, Enrrades Furt	AM	ETHZ 253	JQ677220	JQ677528	JQ677319	JQ660785	JQ677415
Hoplitis (Hoplitis) benoisti	MAR, Tizi-n-Tagergoust	AM, CS	ETHZ 222	JQ677221	JQ677529	JQ677320	JQ660786	JQ677416
Hoplitis (Hoplitis) bihamata	ITA, Sardinia, Alghero	N. Vereecken	ETHZ 263	JQ677222	JQ677530	JQ677321	JQ660787	JQ677417
Hoplitis (Hoplitis) brunnescens	MAR, Agdz	AM, CS	ETHZ 191	JQ677223	JQ677531	JQ677322	JQ660788	JQ677418
Hoplitis (Hoplitis) carinata	TUR, Erzurum	J. G. Rozen	ETHZ 249	JQ677224	JQ677532	JQ677323	JQ660789	JQ677419
Hoplitis (Hoplitis) ctenophora	MAR, Tazenakht	AM, CS	ETHZ 215	JQ677225	JQ677533	JQ677324	JQ660790	JQ677420
Hoplitis (Hoplitis) fabrei	GRE, Mt. Kitharion	C. Praz, CS	ETHZ 124	JQ677226	JQ677534	JQ677325	JQ660791	JQ677421
Hoplitis (Hoplitis) fertoni	TUN, Djerba,	H. Schwenninger	ETHZ 320	JQ677227	JQ677535	JQ677326	JQ660792	JQ677422
Hoplitis (Hoplitis) flabellifera	TUR	J. S. Asher, J. G. Rozen	ETHZ 287	JQ677228	JQ677536	JQ677327	JQ660793	Missing
Hoplitis (Hoplitis) hoggara	MAR, Tizi-n-Tagergoust	AM, CS	ETHZ 207	JQ677229	JQ677537	JQ677328	JQ660794	JQ677423

Hoplitis (Hoplitis) holmboei	CYP, Besparmak	AM, CS	ETHZ 334	JQ677230	JQ677538	Missing	JQ660795	Missing
Hoplitis (Hoplitis) homalocera	JOR, Wadi al Hasa	C. Praz, AM, CS	ETHZ 1001	JQ677231	JQ677539	JQ677329	JQ660796	JQ677424
Hoplitis (Hoplitis) insularis	ALG, Ben Badis	S. Aguib	ETHZ 260	JQ677235	JQ677543	Missing	JQ660801	JQ677428
Hoplitis (Hoplitis) jheringi	ITA, L. Trasimeno	D. W. Baldock	ETHZ 1002	Missing	JQ677544	Missing	Missing	Missing
Hoplitis (Hoplitis) lepeletieri	FRA, Bose d'Eina	L. Blondiau	ETHZ 248	JQ677236	JQ677545	JQ677333	JQ660802	JQ677429
Hoplitis (Hoplitis) loti	SUI, Hohtenn	AM	ETHZ 39	JQ677237	JQ677546	JQ677334	JQ660803	JQ677430
Hoplitis (Hoplitis) manicata	GRE, Pavlocastro	C. Praz, CS	ETHZ 130	JQ677238	JQ677547	JQ677335	JQ660804	JQ677431
Hoplitis (Hoplitis) marchali	MAR, Tizi-n-Tagergoust	AM, CS	ETHZ 200	JQ677239	JQ677548	JQ677336	JQ660805	JQ677432
Hoplitis (Hoplitis) monstrabilis	TUR, Erzurum	J. S. Asher, J. G. Rozen	ETHZ 250	JQ677240	JQ677549	JQ677337	JQ660806	JQ677433
Hoplitis (Hoplitis) mucida	MAR, Zagora	AM, CS	ETHZ 171	JQ677241	JQ677550	JQ677338	JQ660807	JQ677434
Hoplitis (Hoplitis) ochraceicornis	MAR, Tizi-n-Tagergoust	AM, CS	ETHZ 259	JQ677242	JQ677551	JQ677339	JQ660808	JQ677435
Hoplitis (Hoplitis) oreades	MAR, Agouim	AM, CS	ETHZ 225	JQ677243	JQ677552	JQ677340	JQ660809	JQ677436
Hoplitis (Hoplitis) pallicornis	JOR, Wadi al Hasa	C. Praz, AM, CS	ETHZ 289	JQ677244	JQ677553	JQ677341	JQ660810	JQ677437
Hoplitis (Hoplitis) pici	GRE, Platania	L. Standfuss	ETHZ 288	JQ677245	JQ677554	JQ677342	JQ660811	JQ677438
Hoplitis (Hoplitis) ravouxi	FRA, La Font del Sastre	L. Blondiau	ETHZ 254	JQ677246	JQ677555	JQ677343	JQ660812	JQ677439
Hoplitis (Hoplitis) species 1	IRI, Kojeer NP	C. Praz, A. Talebi, CS	ETHZ 294	JQ677232	JQ677540	JQ677330	JQ660797	JQ677425
Hoplitis (Hoplitis) species 2	IRI, Kojeer NP	C. Praz, A. Talebi, CS	ETHZ 322	JQ677234	JQ677541	JQ677331	JQ660798	JQ677426
Hoplitis (Hoplitis) species 3	ISR, Ein Yahav	C. Praz, CS	ETHZ 307	JQ677233	JQ677542	JQ677332	JQ660799	JQ677427
Hoplitis (Hoplitis) tenuiserrata	MAR, Tizi-n-Tagergoust	AM, CS	ETHZ 190	JQ677247	JQ677556	JQ677344	JQ660813	JQ677440

TABLE 2: Host plant choice and inferred category of host range in 44 *Hoplitis* species of the subgenera *Annosmia, Coloplitis* and *Hoplitis.*

Species name	n	N	Origin of pollen loads	% pollen grain volume	% pure loads of preferred host	Host range
*Hoplitis (Annosmia) annulata** (Latreille, 1811)	50	34	CYP*, ESP, FRA, GRE*, ITA, MAR, POR, SYR, TUR	BOR (*Echium*) 99.6%, FAB 0.4%	98%	oligolectic on *Echium*
*Hoplitis (Annosmia) aqabaensis** (Warncke, 1991)	11	6	JOR, TUN*	BOR (*Echiochilon*) 100%	100%	oligolectic on *Echiochilon*
*Hoplitis (Annosmia) bassana** (Warncke, 1991)	22	10	ALG, MAR*	BOR (*Trichodesma*) 100%	100%	oligolectic on *Trichodesma*
*Hoplitis ((Annosmia) christae** (Warncke, 1991)	16	6	ISR*	ACA (*Blepharis*) 100%	100%	oligolectic on *Blepharis*
*Hoplitis (Annosmia) hierichonica** (Mavromoustakis, 1949)	50	24	ISR*, JOR*, MAR*, TUN	BOR (*Echium*) 100%	100%	oligolectic on *Echium*
Hoplitis (Annosmia) israelica (Warncke, 1991)	32	23	ISR, JOR, MAR	BOR (*Echium*) 100%	100%	oligolectic on *Echium*
*Hoplitis (Annosmia) mutica** (Warncke, 1991)	7	7	IRI*, TUR	FAB (*Astragalus*) 75%, FAB (indet) 25%	100%	oligolectic on Fabaceae with a strong preference for *Astragalus*
*Hoplitis (Annosmia) nisa** (Warncke, 1991)	42	22	MAR*, TUN*	PLA (Antirrhineae) 100%	100%	oligolectic on Antirrhineae
Hoplitis (Annosmia) parana (Warncke, 1991)	39	14	ISR, OMA	FAB (*Crotalaria*) 91.3%, FAB (Loteae) 8.5%, BRA 0.2%, PLA 0.1%	92%	oligolectic on Fabaceae with a strong preference for *Crotalaria*
*Hoplitis (Annosmia) segura**	34	12	ISR*	FAB (Loteae) 60.3%, BOR	47%	polylectic on Fabaceae, *Echium* and

(Warncke, 1991)				(*Echium*) 26.3%, PLA (Antirrhineae) 12.4%, BRA 1.0%		Antirrhineae
*Hoplitis (Annosmia) speculum** (Benoist, 1934)	29	20	MAR*	PLA (Antirrhineae) 100%	100%	oligolectic on Antirrhineae
*Hoplitis (Annosmia) uncaticornis** (Stanek, 1969)	22	15	IRI*, SYR, TUR	FAB (*Astragalus*) 100%	100%	oligolectic on Fabaceae with a strong or even exlusive preference for *Astragalus*
*Hoplitis (Annosmia) zonalis** (Pérez, 1895)	52	27	ALG, ISR*, MAR*, TUN	BOR (*Echium*) 99.5%, BRA 0.5%	98%	oligolectic on *Echium*
Hoplitis (Coloplitis) persica (Warncke, 1991)	4	1	IRI	BOR (*Heliotropium*) 100%	100%	possibly oligolectic on *Heliotropium*
*Hoplitis (Hoplitis) adunca** (Panzer, 1798)	50	38	AUT, ALG, ARM, SUI*, GER, ESP, FRA, LIE, ITA, MAR, POR, TUR, TUN	BOR (*Echium*) 100%	100%	oligolectic on *Echium*
*Hoplitis (Hoplitis) anthocopoides** (Schenck, 1853)	50	37	AUT, SUI*, CZE, GER, ESP, FRA, ITA, MAR, TUN	BOR (*Echium*) 100%	100%	oligolectic on *Echium*
*Hoplitis (Hoplitis) benoisti** (Alfken, 1935)	57	33	ESP, FRA, MAR*, POR, TUN	BOR (*Echium*) 99.2%, FAB 0.5%, BRA 0.3%	96%	oligolectic on *Echium*
Hoplitis (Hoplitis) bihamata (Costa, 1885)	22	15	FRA, ITA (Corsica and Sardinia)	BOR (*Echium*) 50.7%, LAM (Lamioideae) 41.7%, FAB (indet) 7.6%, FAB (Lot) 0.1%	36%	polylectic on *Echium*, Lamiaceae and Fabaceae
*Hoplitis (Hoplitis) brunnescens** (Benoist, 1950)	30	19	MAR*, TUN	BOR (*Trichodesma*) 61.5%, PLA (Antirrhineae) 21.7%, BOR (*Echium*) 11.5%, FAB (Loteae) 4.1%, AST 0.7%, BRA 0.5%	63%	polylectic on Boraginaceae (mainly *Trichodesma*), Antirrhineae and Fabaceae
Hoplitis (Hoplitis) carinata (Stanek, 1969)	50	20	JOR, SYR, TUR	FAB (Hedysareae) 99.1%, FAB (Loteae) 0.8%, RES 0.1%	98%	polylectic on Fabaceae with a strong or even exclusive preference for Hedysareae

*Hoplitis (Hoplitis) ctenophora** (Benoist, 1934)	33	16	MAR*, TUN*	BOR (*Echium*) 71.6%, FAB (Loteae) 22.2%, FAB (indet) 3.8%, BRA 1.7%, AST 0.7%	68%	polylectic on *Echium* and Fabaceae with a strong preference for *Echium*
*Hoplitis (Hoplitis) fabrei** Zanden, 1987	38	31	GRE*	BOR (*Echium*) 50.4%, CAM 23.7%, FAB (Genisteae) 12.7%, FAB (Fabeae) 3.3%, BOR (*Onosma*) 2.9%, BOR (*Anchusa*) 2.3%, FAB (Trifolieae) 1.6%, LAM 1.6%, FAB (indet) 1%, FAB (Psoraleeae) 0.5%	32%	polylectic on Boraginaceae (mainly *Echium*), Campanulaceae and Fabaceae
*Hoplitis (Hoplitis) fertoni** (Pérez, 1890)	53	25	ALG, ESP, ISR, MAR*, TUN	BOR (*Echium*) 98.1%, FAB (Loteae) 1.2%, FAB (indet) 0.5%, BRA 0.2%	92%	oligolectic on *Echium*
*Hoplitis (Hoplitis) flabellifera** (Morice, 1901)	49	16	ARM, IRI*, ISR, JOR*, SYR, TUR	BOR (*Anchusa*) 67.2%, FAB (indet) 17.6%, FAB (Trifolieae) 7.8%, BOR (*Echium*) 6.8%, BRA 0.6%	63%	polylectic on Boraginaceae (mainly *Anchusa*) and Fabaceae
Hoplitis (Hoplitis) hoggara (Warncke, 1992)	27	7	ALG, MAR, LBA	FAB (Loteae) 94.5%, FAB (indet) 5.3%, AST 0.2%	96%	oligolectic on Fabaceae with a strong preference for Loteae
*Hoplitis (Hoplitis) holmboei** (Mavromoustakis, 1948)	21	13	CYP*, TUR	BOR (*Onosma*) 65.2%, BOR (*Echium*) 24.2%, BOR (*Lithodora*) 7.3%, BOR (indet) 2.2%, unknown 1.1%	95%	oligolectic on Boraginaceae
*Hoplitis (Hoplitis) homalocera** Zanden, 1991	11	7	ISR*, JOR*	BOR (*Podonosma*) 97.4%, BOR (*Echium*) 2.6%	91%	oligolectic on *Podonosma*
*Hoplitis (Hoplitis) insularis** (Schmiedeknecht, 1886)	26	18	ALG, ESP, MAR*, TUN	BOR (*Echium*) 81.7%, FAB (Hedysareae) 10.5%, FAB (Loteae) 5.6%, FAB (Genisteae) 1.8%, AST 0.4%	73%	polylectic on *Echium* and Fabaceae with a strong preference for *Echium*

*Hoplitis (Hoplitis) jheringi** (Ducke, 1898)	12	4	CRO, TUN*	FAB (Loteae) 92.5%, FAB (Fabeae) 5%, FAB (Genisteae) 1.4%, FAB (indet) 1.1%	100%	oligolectic on Fabaceae with a strong preference for Loteae
*Hoplitis (Hoplitis) lepeletieri** (Pérez, 1879)	50	29	SUI*, FRA, ITA	BOR (*Echium*) 100%	100%	
*Hoplitis (Hoplitis) loti** (Morawitz, 1867)	38	36	AUT, SUI*, ITA	FAB (Loteae) 79.7%, CRA 10%, BOR (*Echium*) 6.6%, FAB (indet) 2%, LAM 1.7%	63%	polylectic on Fabaceae, Crassulaceae and *Echium* with a strong preference for Loteae
*Hoplitis (Hoplitis) manicata** (Morice, 1901)	68	33	GRE*, RUM, SRB, TUR	BOR (*Echium*) 99.2%, LAM 0.6%, AST 0.2%	97%	oligolectic on *Echium*
*Hoplitis (Hoplitis) marchali** (Pérez, 1902)	48	24	ESP, MAR*, TUN	BOR (*Echium*) 99.6%, FAB 0.4%	98%	oligolectic on *Echium*
Hoplitis (Hoplitis) monstrabilis Tkalcu, 2000	39	16	TUR	FAB (Hedysareae) 98.9%, FAB (*Astragalus*) 1%, FAB (indet) 0.1%	95%	oligolectic on Hedysareae
*Hoplitis (Hoplitis) mucida** (Dours, 1873)	50	25	ESP, FRA, ITA, ISR, JOR*, MAR, POR, TUN	BOR (*Echium*) 100%	100%	oligolectic on *Echium*
Hoplitis (Hoplitis) ochraceicornis (Ferton, 1902)	7	6	ESP, FRA, POR	BOR (*Echium*) 49.1%, FAB (Genisteae) 18.2%, LAM (Lamioideae) 17.2%, FAB (Loteae) 9.1%, LAM (Nepetoideae) 6.4%	29%	polylectic on *Echium*, Fabaceae and Lamiaceae
*Hoplitis (Hoplitis) oreades** (Benoist, 1934)	39	24	MAR*, TUN	FAB (Loteae) 88.6%, FAB (indet) 10.7%, BRA 0.4%, AST 0.3%	95%	oligolectic on Fabaceae with a strong preference for Loteae
*Hoplitis (Hoplitis) pallicornis** (Friese, 1895)	50	35	CRO, GRE, ITA, ISR, JOR*, SYR, TUR	BOR (*Echium*) 59.9%, CAM 10%, FAB (Trifolieae) 7.7%, FAB (indet) 6.1%, FAB (Loteae) 4.5%, FAB (Fabeae) 4.3%, FAB (Genisteae) 3.7%, HYA (*Muscari*) 1.6%, BOR	52%	polylectic on Boraginaceae (mainly *Echium*), Fabaceae and Campanulaceae

				(*Onosma*) 0.7%, LAM 0.4%, BOR (*Anchusa*) 0.3%, BRA 0.3%, AST 0.2%, unknown 0.3%		
*Hoplitis (Hoplitis) pici** (Friese, 1899)	22	20	CRO, GRE*, TUR	HYA (*Muscari*) 98.5%, LAM 1.4%, AST 0.1%	91%	oligolectic on *Muscari*
*Hoplitis (Hoplitis) ravouxi** (Pérez, 1902)	34	28	AUT, SUI*, GER, ESP, FRA, ITA*, POR	FAB (Loteae) 76.1%, BOR (*Echium*) 13%, FAB (Hedysareae) 5.6%, CRA 5.3%	74%	mesolectic on Fabaceae, *Echium* and Crassulaceae with a strong preference for Loteae
*Hoplitis (Hoplitis) spec. nov. 1**	12	3	IRI*, JOR, SYR	FAB (Hedysareae) 100%	100%	oligolectic on Hedysareae
*Hoplitis (Hoplitis) spec. nov. 2**	17	6	IRI*, TUR	BOR (*Onosma*) 100%	100%	oligolectic on *Onosma*
*Hoplitis (Hoplitis) spec. nov. 3**	11	7	ISR*	BOR (*Trichodesma*) 87.8%, BOR (*Echium*) 12.2%	100%	oligolectic on Boraginaceae with a strong preference for *Trichodesma*
*Hoplitis (Hoplitis) tenuiserrata** (Benoist, 1950)	51	20	MAR*	FAB (Loteae) 98%, BOR (*Echium*) 2%	96%	oligolectic on Fabaceae with a strong or even exclusive preference for Loteae

n = total number of pollen loads, N = number of pollen loads from different localities. Abbreviations for i) states: ALG = Algeria, ARM = Armenia, AUT = Austria, CRO = Croatia, CYP = Cyprus, ESP = Spain, FRA = France, GER = Germany, GRE = Greece, ITA = Italy, IRI = Iran, ISR = Israel and Palestine, JOR = Jordan, LBA = Libya, LIE = Liechtenstein, MAR = Morocco, OMA = Oman, POR = Portugal, SRB = Serbia, SUI = Switzerland, SYR = Syria, TUN = Tunisia, TUR = Turkey; ii) plant families: ACA = Acanthaceae, ASP = Asparagaceae, AST = Asteraceae, BOR = Boraginaceae, BRA = Brassicaceae, CAM = Campanulaceae, CRA = Crassulaceae, FAB = Fabaceae, LAM = Lamiaceae, PLA = Plantaginaceae, RES = Resedaceae. Species for which the host range inferred by microscopical pollen analysis was confirmed by field observations by the authors as well as the states where these observations were made are marked with asterisks.

Data partitioning and model testing

To establish a partitioning regime, a preliminary Bayesian analysis was conducted. Each of the five protein coding genes was partitioned into first, second and third codon position (indicated as e.g. CAD1, CAD2 and CAD3). The introns of CAD, EF and opsin were combined and treated as one additional partition resulting in a dataset containing 16 partitions. An analysis was run in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2001) for 5 million generations using a GTR model, and the resulting parameter files were examined in Tracer 1.4. (Rambout & Drummond 2007). An appropriate burn-in was discarded and the substitution rates and nucleotide compositions for the 16 partitions were determined using Tracer 1.4. Based on these parameters, similar partitions were grouped together resulting in the following partitioning regime: partition 1 included only COI3 (395 bp); partition 2 included CAD3, COI1, EF3, opsin3, NaK3 and the introns (2322 bp); partition 3 included COI2 and opsin1 (588 bp); partition 4 included CAD1, EF1 and opsin2 (828 bp); partition 5 included CAD2 and NaK1 (737 bp); partition 6 included NaK2 (472 bp); partition 7 included EF2 (370 bp).

Models of sequence evolution were selected based on the Akaike Information Criterion (AIC) as implemented in MrModelTest 2.3 (Nylander 2004). After testing 24 models of nucleotide substitution for each partition, we chose the model associated with the lowest AIC value: partition 1 (GTR+G); partition 2-4 (GTR+I+G); partition 5 (GTR+I); partition 6 (F81); partition 7 (HKY).

Phylogenetic analyses

Phylogenetic analyses were performed applying Bayesian, maximum likelihood and parsimony methods. For Bayesian analysis we used MrBayes 3.1.2 (Ronquist & Huelsenbeck 2001) under the model and partitioning regime specified above. Partitions were unlinked to allow parameter values and overall rate of substitution to differ. Markov Chain Monte Carlo analyses were run with one cold and three heated chains. We ran four independent runs of 30 million generations each, resulting in a total of 120 million generations sampling trees every 2000 generations. Tracer 1.4 was used to determine an appropriate burn-in (after 8 million generations) for each individual run. To produce a 50% majority rule consensus tree reflecting posterior probability values for each node, the resulting 88000 trees were sampled and combined using PAUP* 4.0a118 for Macintosh (Swofford 2002). Finally, the program TreeAnnotator 1.5.3 (Rambaut & Drummond 2009) was used to generate a maximum clade credibility tree that combined the branch lengths of all the post burn-in trees.

For maximum likelihood analysis we used RAxML 7.0.4 (Stamatakis et al. 2005). The rapid bootstrapping algorithm with a GTR+CAT approximation was applied to perform 1000 bootstrap replicates. To produce a 50% majority rule consensus tree, the bootstrap replicates were sampled and combined using PAUP.

For parsimony analysis we used PAUP applying the following parameter settings: heuristic search, 10000 bootstrap replicates, 10 random sequence additions with 4 trees held at each step, a maximum of 100 trees retained, TBR branch swapping.

7.3.3. Evolution of host plant choice

Host plants

To identify the pollen hosts of the 44 pollen-collecting *Hoplitis* species included in the present study, we microscopically analysed the scopal pollen contents of 1468 female specimens both from museum and private collections using the method outlined by Westrich & Schmidt (1986). To account for potentially deviating pollen host use by specimens from different populations of the same species, we aimed to analyse pollen samples from females collected at as many different localities as possible. Prior to removing pollen from the abdominal scopae of the female specimens, we estimated the degree to which they were filled. The amount of pollen in the scopae was assigned to five classes ranging from 5/5 (full load) to 1/5 (filled only to one-fifth). The pollen grains were stripped off the scopae with a fine needle and embedded in glycerol gelatin on a slide. When a pollen load was composed of different pollen types, we estimated their percentages by counting the grains along three transects chosen randomly across the cover slip at a magnification of 400×. Pollen types represented by less than 5% of the counted grains were excluded to prevent a potential bias caused by contamination. For pollen loads consisting of two or more different pollen types, we corrected the percentages of the number of pollen grains by their volume. After assigning different weights to scopae according to their degree of filling (full loads were weighted five times more strongly than scopae filled to only one-fifth), we summed up the estimated percentages over all pollen samples for each species. We identified the pollen grains at a magnification of 400× or 1000× based on specific literature (cited in Westrich & Schmidt 1986; Bigazzi & Selvi 1998) and on our own

extensive reference collection. In general, we identified the pollen grains down to tribal or, if possible, to genus level, especially in the family Boraginaceae. Tribal classification of the Fabaceae was based on Lewis et al. (2005). We characterized different degrees of host plant association among species of the *Annosmia-Hoplitis* group according to Müller & Kuhlmann (2008). For simplicity, we do not distinguish between mesolectic and polylectic sensu stricto in this study but refer to both of these categories as polylectic sensu lato (henceforth polylectic).

For 36 species the pollen host range inferred by microscopical pollen analysis was confirmed by field observations in Greece (2006), Jordan (2007), Morocco (2008, 2009), Iran (2009), Israel (2010), Cyprus (2011) and Tunisia (2012).

Ancestral state reconstruction

To reconstruct the evolution of host plant choice within the *Annosmia-Hoplitis* group, we first applied parsimony mapping implemented in MacClade 4.08 (Maddison & Maddison 2005) by using the topology of the majority rule consensus tree from the Bayesian analysis. In addition, we conducted maximum likelihood inference of ancestral character states implemented in BayesTraits (Pagel et al. 2004; Pagel & Meade 2006), which considers branch lengths and phylogenetic uncertainty. To simplify the model for ancestral state reconstruction, transition rates between all states (i.e. pollen hosts) were assumed to be equal using the "RestrictAll" command. We used a subset of 1001 randomly chosen trees from our pool of saved trees from the Bayesian analysis. The outgroup taxa as well as the two cleptoparasitic *Bytinskia* species were excluded using Mesquite for MacOSX (Maddison & Maddison 2007). We

reconstructed the ancestral pollen hosts for all crucial nodes that were strongly supported (all but one with a posterior probability of 100%) using the “AddNode” command.

To assess the robustness of these ancestral state reconstructions, we compared the likelihood values and Bayes factors associated with the three alternative states "oliglectic on Boraginaceae", "oligolectic on Fabaceae" and "polylectic" using the “Fossil” command (Table 3). An improvement in likelihood scores by two negative log units or more, when comparing constraints to alternative states, was taken as evidence for a ‘significantly’ more likely evolutionary explanation (Pagel 1999).

7.3.4. Specialized pollen-collecting behaviour

We investigated the females of all 44 pollen-collecting *Hoplitis* species under a stereomicroscope to detect specialized morphological structures that might facilitate pollen removal from the flowers. The presumed function of these structures was elucidated in the field for six out of eight species, which were found to possess such morphological adaptations. The function of the morphological structures present in the other two species was deduced from the species’ pollen host use as well as from a comparison with the known function of similar structures in unrelated bee species. Furthermore, the pollen harvesting behaviour of 29 additional species was observed in the field to document specialized behavioural adaptations for pollen removal, in particular buzzing.

7.4. RESULTS

7.4.1. Molecular phylogeny

The majority rule consensus tree of the 88 000 trees in the Bayesian analysis resulted in a well-resolved topology (Fig. 1). The topologies resulting from the maximum likelihood and the parsimony analyses were less well-resolved (Supporting information, Fig. S1, S2). However, the comparison between the topologies resulting from the three analytical methods did not reveal a single incongruence, which strongly corroborates the Bayesian topology.

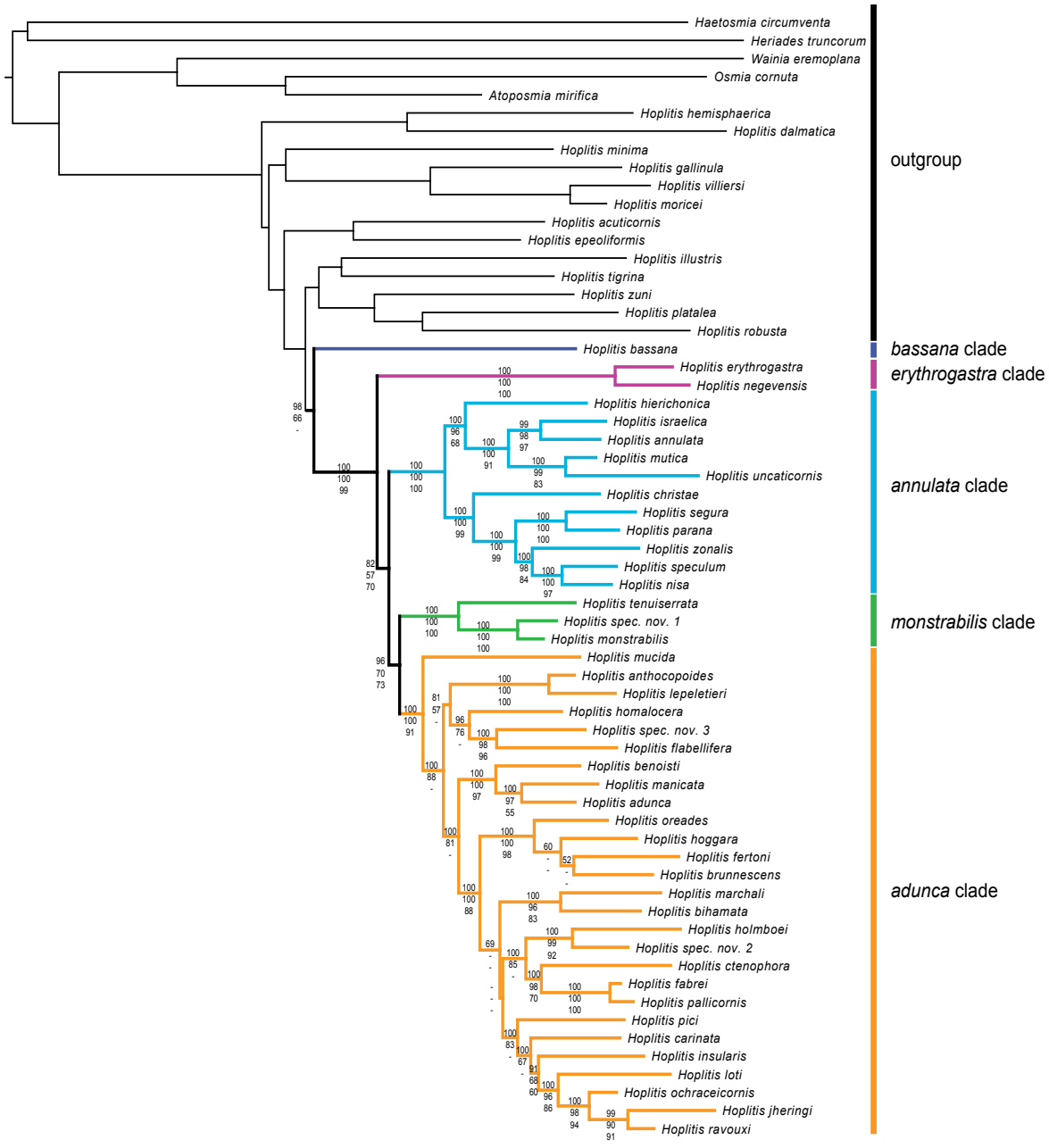


FIGURE 1: Phylogeny of bee species of the *Annosmia-Hoplitis* group. Maximum clade credibility tree based on 880000 post burn-in trees from four independent Bayesian analyses. Bayesian posterior probabilities, maximum-likelihood bootstrap values and parsimony bootstrap values are shown (in this order from top to bottom) for all nodes.

The most basal species of the *Annosmia-Hoplitis* group is *Hoplitis bassana* (Fig. 1). Its position as sister taxon to all other members of the *Annosmia-Hoplitis* group was only weakly supported by maximum likelihood analysis (66% bootstrap support) and not resolved by parsimony. However, the very strong support by Bayesian inference (98% posterior probability), which did not conflict with the topologies resulting from the other analyses, as well as several unique morphological features shared with the other members of this group (Warncke 1991c), indicate that the Bayesian topology is accurate. The remaining species of the *Annosmia-Hoplitis* group can be divided into four main clades, which are all strongly supported by Bayesian analysis, maximum likelihood and parsimony (Fig. 1): i) the *erythrogastra* clade, which comprises the cleptoparasitic species of the subgenus *Bytinskia*; ii) the *annulata* clade, which comprises all species of the subgenus *Annosmia* except *H. bassana*; iii) the *monstrabilis* clade, which comprises several species that are morphologically intermediate between members of the *annulata* clade and those of the *adunca* clade; iv) the *adunca* clade, which comprises the majority of species of the subgenus *Hoplitis*. The latter two clades form a soundly supported monophyletic group comprising all species of the subgenus *Hoplitis*.

7.4.2. Evolution of host plant choice

Host Plants

Based on the microscopical analysis of pollen loads and field observations, we classified 32 of the 44 pollen-collecting species included in the present study as oligolectic (Table 2, Fig. 2). Most of these pollen specialist species restrict pollen harvesting to Boraginaceae

(18 species), among which there are narrow oligoleges specialized to *Echium* (12), *Onosma* (1), *Podonosma* (1), *Trichodesma* (1) and *Echiochilon* (1), and broad oligoleges collecting pollen on *Onosma*, *Echium* and *Lithodora* (1) and *Trichodesma* and *Echium* (1). The other pollen specialists are oligolectic on Fabaceae (10), Antirrhineae (Plantaginaceae) (2), *Blepharis* (Acanthaceae) (1) and *Muscari* (Asparagaceae) (1). Eleven species were classified as polylectic, all collecting pollen on members of both Boraginaceae and Fabaceae. Some of these species additionally exploit Antirrhineae (2), Campanulaceae (2), Lamiaceae (2) and Crassulaceae (2) for pollen. The lack of field observations and the small number of pollen loads available did not allow unambiguous classification of the pollen host range of *H. persica*. It appears, however, that this species has a distinct or even exclusive preference for *Heliotropium* (Boraginaceae).

Ancestral state reconstruction

Nodes A to G: Both parsimony mapping and maximum likelihood inference of ancestral states clearly suggest that the ancestor of the *Annosmia-Hoplitis* group was oligolectic on Boraginaceae (node A; Bayes factor 4.8) as were the ancestors at nodes B to G (Fig. 3, Table 3). Several host shifts away from the exclusive use of Boraginaceae occurred basal to node H: three shifts to Fabaceae, one shift each to *Blepharis* and Antirrhineae and two shifts to polylecty. Interestingly, in both of the latter cases Boraginaceae were maintained as hosts, while Fabaceae (*H. flabellifera*) and Fabaceae and Antirrhineae (*H. segura*) were respectively added to the pollen diet; both of the latter plant taxa are also used as exclusive hosts by related species (Fig. 3). While the great majority of the Boraginaceae oligoleges basal to node H use *Echium* as the exclusive pollen host, *H. homalocera* has specialized on *Podonosma,* and *H.*

flabellifera and *H. spec. nov. 3* additionally exploit *Anchusa* and *Trichodesma*, respectively. Thus, host shifts have taken place between different Boraginaceae taxa, and host broadening through the inclusion of Boraginaceae taxa other than *Echium* has also occurred.

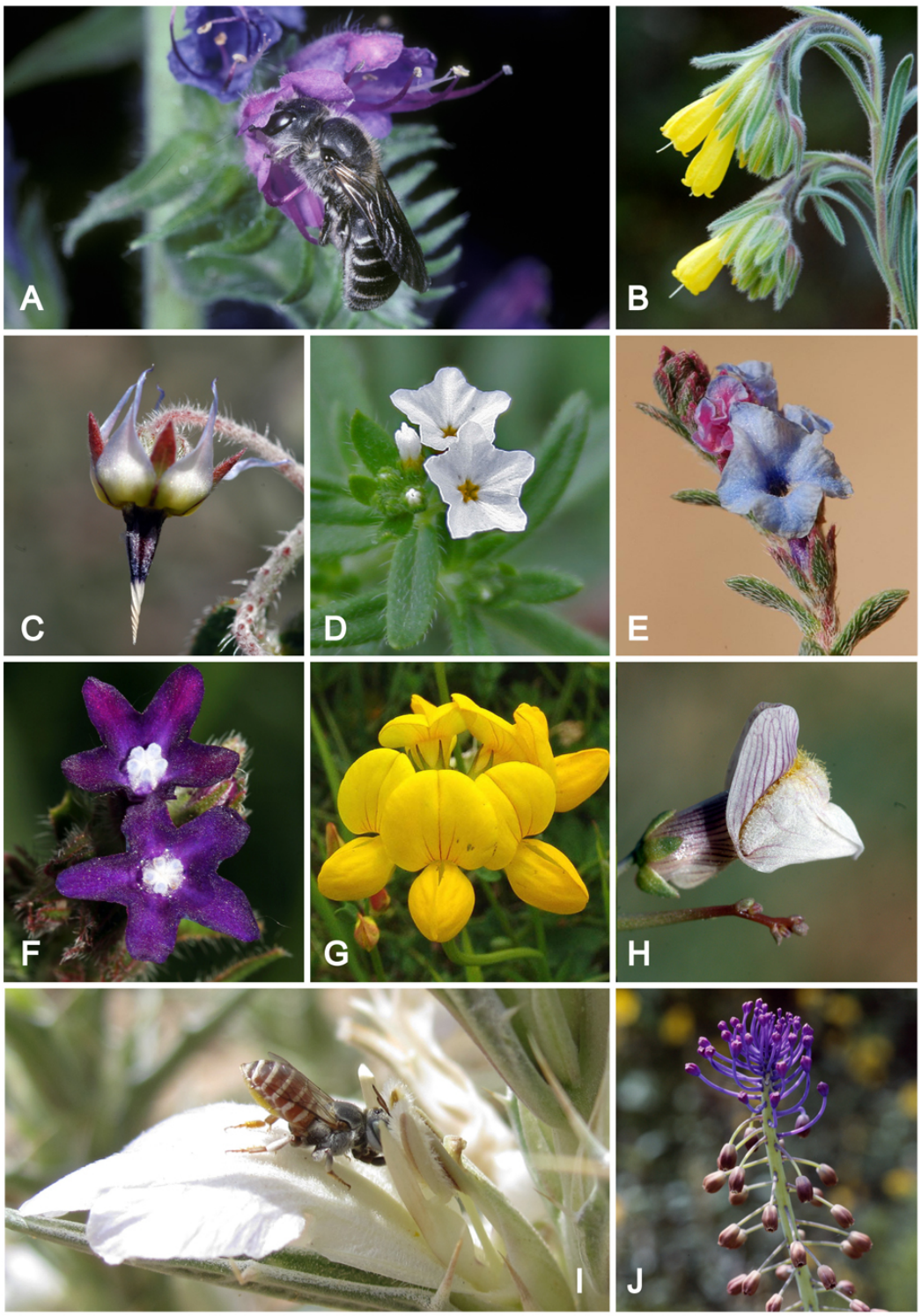


FIGURE 2: Pollen hosts of bee species of the *Annosmia-Hoplitis* group. (A) *Echium vulgare* (Boraginaceae) with *Hoplitis adunca* (photo A. Krebs); (B) *Onosma erecta* (Boraginaceae) (photo Biopix.dk); (C) *Trichodesma* spec. (Boraginaceae) (photo A. Müller); (D) *Heliotropium* spec. (Boraginaceae) (photo J. M. Garg); (E) *Echiochilon fruticosum* (Borageneaceae) (photo M. Chaieb); (F) *Anchusa officinalis* (Boraginaceae) (photo W. Obermayer); (G) *Lotus corniculatus* (Fabaceae) (photo A. Krebs); (H) *Antirrhinum ramosissimum* (Antirrhineae) (photo A. Müller); (I) *Blepharis attenuata* (Acanthaceae) with *Hoplitis christae* (photo C. Sedivy); (J) *Muscari comosum* (Asparagaceae) (photo A. Krebs).

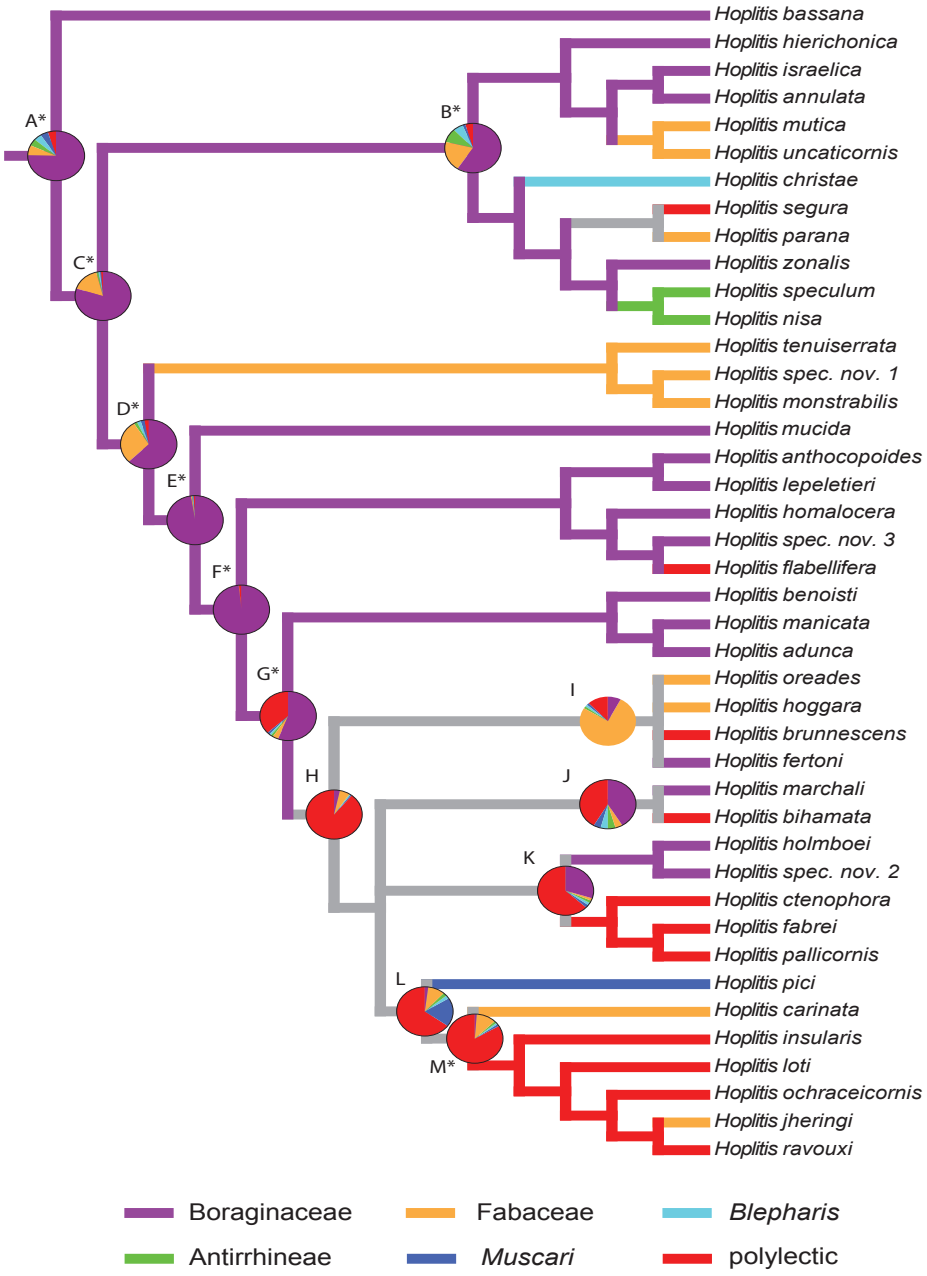


FIGURE 3: Evolution of host plant choice in bee species of the *Annosmia-Hoplitis* group. Majority-rule consensus tree based on 88000 post burn-in trees from four independent Bayesian analyses. Nodes with posterior probabilities lower than 65% were collapsed to polytomies. Outgroup species were omitted as was the *erythrogastra* clade due to its cleptoparasitic behavior. The pollen hosts were mapped onto the tree using the criterion of maximum parsimony. Equivocal branches are colored in grey. The pie charts at 13 well-resolved nodes (A-M) give the probability of the maximum likelihood inference of ancestral pollen hosts. The asterisks indicate that analyses constraining the most likely state had significantly higher log likelihood values than analyses with alternative states constrained.

Nodes H to M: Neither parsimony mapping nor maximum likelihood inference of ancestral states clearly revealed the pollen hosts of the ancestors at nodes H to L (Fig. 3, Table 3). Whereas node M was equivocal under parsimony, maximum likelihood inference favoured polylecty over both oligolecty on Boraginaceae and oligolecty on Fabaceae at node M (Bayes factor 4.6). Therefore, the evolution of host range in the clade unified by node H remains largely unresolved with the number of possible shifts to polylecty ranging between one and four. However, maximum likelihood inference revealed two shifts from polylecty to oligolecty in the ancestor of *H. carinata* and *H. jheringi*, respectively. In addition, Boraginaceae and Fabaceae were revealed to be the most important pollen hosts of the species united by node H. Four species are specialized on Boraginaceae, another four on Fabaceae and nine species are polylectic, exploiting both Boraginaceae and Fabaceae. *Echium* again predominates among the Boraginaceae hosts, while some species show a clear or exclusive preference for other Boraginaceae taxa, such as *Onosma* (*H. holmboei, H. spec. nov. 2*) or *Trichodesma (H. brunnescens)*.

TABLE 3: Results of the maximum likelihood ancestral state reconstruction analyses. Negative log likelihood values at nodes A-M are given when constrained to be oligolectic on Boraginaceae, oligolectic on Fabaceae or polylectic. Bayes factors equal twice the difference between the lowest and the second lowest negative likelihood values.

Nodes	-Ln "fossil"			Bayes Factor
	Boraginaceae	Fabaceae	polylectic	
A	-59.7[a]	-62.1[b]	-62.4	4.8*
B	-59.6[a]	-61.8[b]	-64.0	4.4*
C	-59.6[a]	-61.8[b]	-64.3	4.4*
D	-59.6[a]	-61.8[b]	-64.6	4.4*
E	-59.5[a]	-63.7[b]	-65.3	8.4*
F	-59.5[a]	-66.2	-65.6[b]	12.2*
G	-59.6[a]	-65.9	-64.0[b]	8.8*
H	-60.1[a]	-63.3	-60.3[b]	0.4
I	-60.6	-60.6	-60.6	0.0
J	-60.4[b]	-63.9	-60.0[a]	0.8
K	-60.5[b]	-65.0	-60.0[a]	1
L	-61.1[b]	-63.2	-59.8[a]	2.6
M	-61.9[b]	-62.8	-59.6[a]	4.6*

The lowest and the second lowest negative likelihood values are indicated by superscript (a) and (b), respectively. Bayes factors higher than 4 are considered as strong support, indicated by an asterisk.

7.4.3. Specialized pollen-collecting behaviour

The females of 12 out of the 44 pollen-collecting *Hoplitis* species selected for the present study were found to possess a number of different specialized morphological or behavioural adaptations to collect pollen from their hosts (Fig. 4).

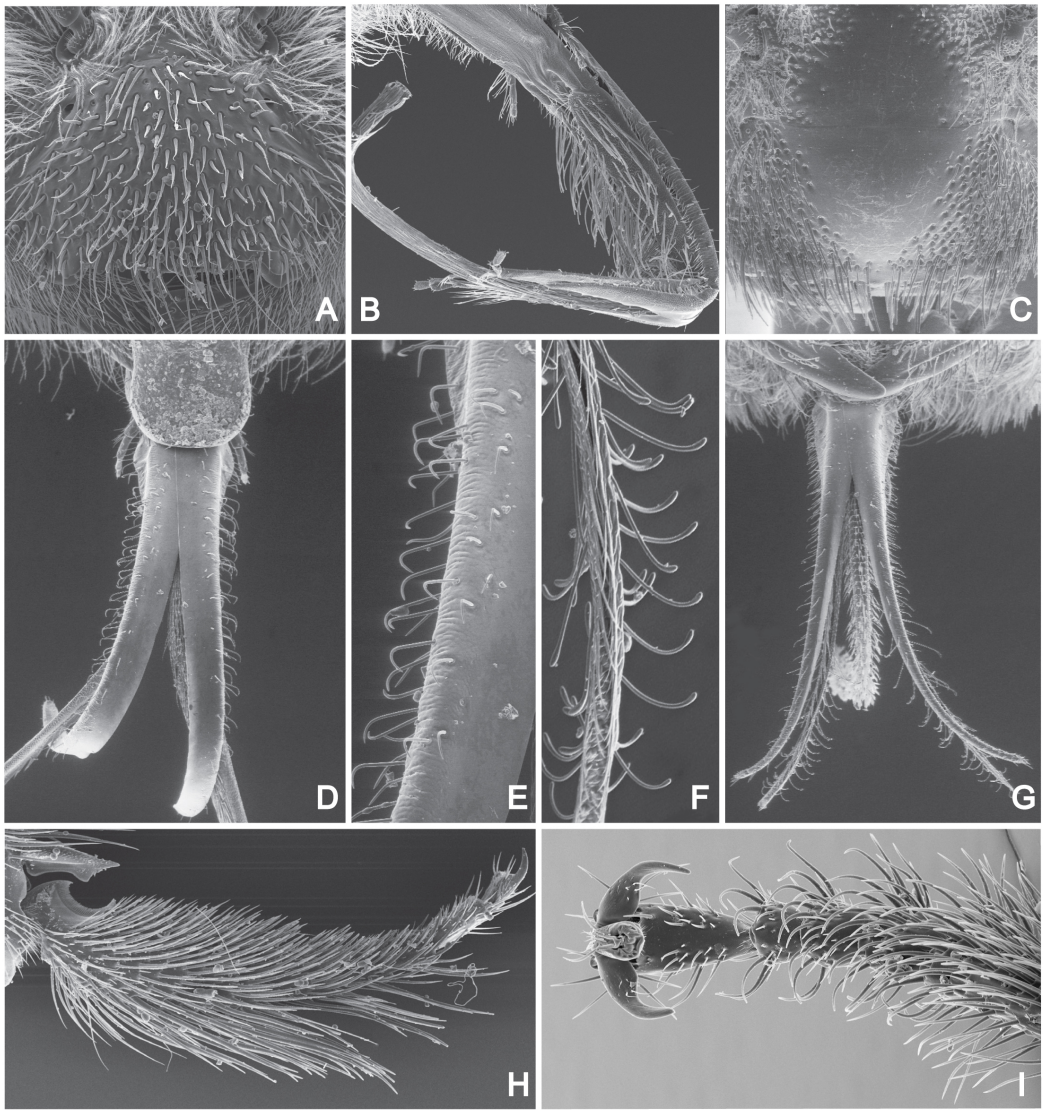


FIGURE 4: Specialized morphological structures for pollen uptake in bee species of the *Annosmia-Hoplitis* group. (A) Stiff and apically twisted bristles on the clypeus of *Hoplitis bihamata*; (B) brush of long and stiff bristles on the ventral side of the labial palpi of *Hoplitis aqabaensis*; (C) convex, hairless and polished clypeus of *Hoplitis nisa*; (D, E) apically hooked bristles on the galeae of *Hoplitis pici* (overview and detail); (F, G) apically curved and capitate bristles on the labial palpi of *Hoplitis persica* (detail and overview); (H) brush of long and stiff bristles on the foreleg basitarsus of *Hoplitis christae*; (I) curved bristles on the foreleg tarsi of *Hoplitis flabellifera.*

In *H. christae,* the lateral side of the basitarsi of the forelegs is equipped with a brush of long and stiff bristles (Fig. 4H). Females visiting the nototribic flowers of *Blepharis attenuata* were observed to harvest pollen, which is hidden between the four tightly appressed and introrse anthers,

by pushing the anthers apart with the head before removing the subsequently exposed pollen with the specialized foreleg pilosity (Fig. 2I). In *H. nisa* and *H. speculum*, the lower median part of the face is distinctly convex, hairless and polished (Fig. 4C). Females of *H. nisa* were observed to gain access to the hidden anthers of the occluded flowers of *Antirrhinum ramosissimum* and *Kickxia aegyptiaca* by pushing the specialized facial area against the upper flower lip, thereby opening the flowers. In *H. flabellifera*, the tarsi of the forelegs are beset with conspicuously curved bristles (Fig. 4I). Females were observed to insert their forelegs into the narrow flower tube of *Anchusa* spec. and to extract the hidden pollen with the specialized bristles by repeatedly and alternately moving the forelegs up and down. In *H. bihamata*, which often collects pollen on Lamiaceae flowers, the clypeus is covered with a conspicuous pilosity composed of stiff and apically twisted bristles (Fig. 4A). Modified bristles of the same type are well known in many bee species that collect pollen on nototribic flowers of Lamiaceae and Antirrhineae (Müller 1996a; b). In *H. pici*, the galeae of the proboscis are beset with apically hooked bristles (Fig. 4D, E). Females were observed to insert their proboscis into the small opening of the urn-shaped flowers of *Muscari comosum* and to extract the pollen with the specialized bristles by repeatedly moving the proboscis back and forth. In *H. aqabaensis,* the ventral side of the first and the base of the second segment of the labial palpi are covered with long and stiff bristles (Fig. 4B). Females were observed to use these specialized bristles to brush the pollen out of the narrow corolla tube of the trumpet shaped flowers of *Echiochilon fruticosum* by repeatedly moving the proboscis up and down. In *H. persica,* which predominantly or exclusively collects pollen on *Heliotropium*, the second segment of the labial palpi is beset with apically curved and capitate bristles (Fig. 4F, G). Very similarly shaped

bristles are developed on the labial palpi of osmiine bees of the genus *Haetosmia,* where they are used to scrape pollen out of the narrow tubed *Heliotropium* flowers (Peters 1974; Müller 2012).

Four out of the 34 *Hoplitis* species for which pollen harvesting behaviour was observed were found to collect pollen by buzzing the flowers of their boraginaceous hosts: *H. holmboei* and *H. spec. nov. 2* on *Onosma fruticosa* and *Onosma spec.*, respectively, *H. homalocera* on *Podonosma orientalis* and *H. spec. nov. 3* on *Trichodesma ehrenbergii*. Based on the phylogeny (Fig. 1), buzzing behaviour has independently evolved at least twice in the *Annosmia-Hoplitis* group, once in the clade comprising *H. holmboei* and *H. spec. nov. 2* and once in the clade comprising *H. homalocera*, *H. spec. nov. 3* and *H. flabellifera*.

7.5. DISCUSSION

We constructed a well supported phylogeny of 44 bee species of the *Annosmia-Hoplitis* group using sequence data from five separate gene coding regions to trace the evolution of host plant choice and specialized pollen-collecting behaviour. Results suggest that host plant choice is governed by strong evolutionary constraints as was postulated by the recently formulated constraint hypothesis of bee host range evolution (Sedivy et al. 2008). Furthermore, our findings show that specialized adaptations for pollen uptake from flowers of widely different architectures have independently and repeatedly evolved, which is likely due to the predominance of plant taxa with complex floral morphologies among the hosts of the *Annosmia-Hoplitis* bees.

7.5.1. Evolution of host plant choice

Host plants

We found that Boraginaceae species are the primary hosts of the *Annosmia-Hoplitis* group and that Fabaceae species are common secondary hosts. Boraginaceae are a particularly diverse plant family with regard to flower architecture and mode of pollen presentation, and *Annosmia-Hoplitis* bees exploit flowers with widely variable flower morphologies within this plant family (Fig. 2). Exploited Boraginaceae genera possess either i) freely accessible anthers in open flowers (*Echium*), ii) anthers hidden in narrow corolla tubes (*Anchusa*, *Echiochilon*, *Heliotropium, Lithodora*) or iii) streukegel blossoms, in which introrse anthers form a pollen containing cone around the style (*Onosma*, *Podonosma*, *Trichodesma*). Several broadly oligolectic or polylectic species were found to simultaneously exploit two or three Boraginaceae genera with considerably differing flower morphologies. These species combine pollen hosts of the flower types i) and ii), i) and iii) or i) and ii) and iii). The Fabaceae are the second most important host plants of the *Annosmia-Hoplitis* group. In contrast to the Boraginaceae hosts, the flower morphology of the Fabaceae hosts is distinctly less variable. All exploited Fabaceae taxa have typical keel blossoms that completely hide the anthers inside the lowermost petal. All the other important host plant taxa of the *Annosmia-Hoplitis* group also possess flowers that conceal their anthers, i.e. in occluded flowers (Antirrhineae), below the upper lip (*Blepharis*, Lamiaceae) or in a narrow corolla (*Muscari*). Thus, apart from *Echium*, the host plant spectrum of bees of the *Annosmia-Hoplitis* group is dominated by plant taxa with hidden anthers, from which pollen is difficult to remove.

Patterns of host-range evolution

Oligolecty is the ancestral state in the *Annosmia-Hoplitis* group. This finding contrasts with the traditional view that polylecty is ancestral in bees and that oligolecty subsequently evolved to either reduce interspecific competition for pollen (Linsley 1958; Michener 1979) or to increase foraging efficiency (Lovell 1913; 1914). It is, however, in line with several recent studies that identified the basal species of different bee taxa as oligolectic (Müller 1996a; Sipes & Tepedino 2005; Larkin et al. 2008; Michez et al. 2008; Sedivy et al. 2008; Litman et al. 2011).

Polylecty in the *Annosmia-Hoplitis* group evolved at least three times independently from oligolecty, twice basal to node H and at least once apical to node G (if the ancestor at node H was polylectic), or several times apical to node G (if the ancestor at node H was not polylectic). Host broadening followed two distinct patterns. First, the two polylectic species *H. segura* and *H. flabellifera* broadened their diet under maintenance of the exclusive pollen host of their closest relatives, i.e. Boraginaceae. Second, the pollen hosts newly incorporated into the diets of these two polyleges, i.e. Fabaceae and Antirrhineae, are the exclusive hosts of closely related species. Both patterns also hold true for the polylectic *Hoplitis* species united by node H, irrespective of whether the ancestor at node H was polylectic or oligolectic on Boraginaceae. These two patterns of host broadening documented in the present study seem to be widespread in bees. Maintenance of the original host was also recorded in polylectic bee species of the Anthidiini (Müller 1996a), *Diadasia* (Sipes & Tepedino 2005) and *Chelostoma* (Sedivy et al. 2008), and incorporation of new pollen hosts already used by related species was also found in the latter two bee genera (Sipes & Tepedino 2005; Sedivy et

al. 2008). Our study also shows that the incorporation of entirely new pollen hosts, such as *Blepharis*, *Muscari* or Lamiaceae, has occasionally occurred in the *Annosmia-Hoplitis* group.

Oligolecty in the *Annosmia-Hoplitis* group evolved at least twice from polylecty. *H. carinata* and *H. jheringi* respecialized to the exclusive use of Fabaceae from polylectic ancestors that exploited both Boraginaceae and Fabaceae. These are clear cases of respecialization after a phase of expanded host range as was postulated by the constraint hypothesis of host-range evolution in bees (Sedivy et al. 2008). We therefore suggest that all the oligolectic *Annosmia* and *Hoplitis* species basal to node H, which are dependent on plant taxa other than Boraginaceae, specialized to their current hosts after a shorter or longer phase of expanded host range, i.e. polylecty.

Evolutionary constraints

Both a pronounced fidelity to Boraginaceae species and a distinct affinity to Fabaceae species as alternative hosts suggest that evolutionary constraints underlie the patterns of host plant choice in bees of the *Annosmia-Hoplitis* group. Boraginaceae species play exclusive or important roles as pollen hosts for 30 out of the 44 pollen-collecting *Hoplitis* species, indicating that host shifts away from Boraginaceae might be considerably constrained. Neurological constraints related to the recognition or handling of the Boraginaceae flowers are probably less important than physiological constraints related to pollen digestion. First, flowers of several *Echium* species possess a specific scent bouquet that differs from that of other Boraginaceae such as *Anchusa officinalis* (Burger et al. 2010a,b; 2012), which suggests that olfaction might

decisively contribute to the recognition of the different Boraginaceae hosts by the *Hoplitis* bees. Second, the Boraginaceae hosts belong to three morphologically different flower types, each necessitating different behaviours for pollen extraction. Nonetheless, bees of the *Annosmia-Hoplitis* group repeatedly included such flowers into their host plant spectrum and evolved a variety of morphological and behavioural adaptations to exploit them. Thus, neurological constraints fail to fully explain the conserved host plant choice in the *Annosmia-Hoplitis* group. Instead, we suggest that physiological constraints underlie the pronounced fidelity to the Boraginaceae. The pollen of *Echium vulgare* and *E. plantagineum* contains particularly high concentrations of pyrrolizidine alkaloids (Boppré et al. 2008), which are known to be toxic or deterrent for non-specialized herbivores (Van Dam et al. 1995; Narberhaus et al. 2005). Since most other Boraginaceae genera exploited by the *Hoplitis* bees for pollen are known to contain pyrrolizidine alkaloids in their vegetative parts (El-Shazly et al. 1998; El-Shazly et al. 2003; Souza et al. 2005), these toxic secondary metabolites might also occur in their pollen. We therefore hypothesize that the *Hoplitis* bees have physiologically adapted to tolerate the specific secondary chemistry of the Boraginaceae pollen, which in turn has lowered their capability to exploit alternative hosts, a phenomenon that is well known for herbivorous insects (Strauss & Zangerl 2002; Singer 2008; Becerra et al. 2009; Futuyma & Agrawal 2009).

The "Boraginaceae-Fabaceae paradox"

Given the evolutionary scenario detailed above, the biased affinity of the *Annosmia-Hoplitis* bees to Fabaceae species as alternative hosts calls for an explanation. Ten out of the 44 pollen-collecting *Hoplitis* species are

strictly dependent on Fabaceae and all eleven polylectic species exploit Fabaceae beside Boraginaceae. This striking affinity towards both Fabaceae and Boraginaceae is not confined to bees of the *Annosmia-Hoplitis* group, it appears to be a widespread and common phenomenon among osmiine bees. Based on our current knowledge of host plant choice in the Palearctic osmiine bees, several clades contain both Boraginaceae and Fabaceae specialists and all 48 polylectic Palearctic osmiine species that collect pollen on Boraginaceae also exploit Fabaceae (compiled from Müller 2012). The explicit choice of both the Boraginaceae and the Fabaceae appears to be paradoxical as these two plant families are neither closely related nor share similar flower morphologies. In fact, Boraginaceae and Fabaceae are phylogenetically distant families belonging to the Asterids and Rosids, respectively (Judd et al. 2008), with their common ancestor dating back to 120 Myr (Wikström et al. 2001). Furthermore, Boraginaceae and Fabaceae strongly differ in their mode of pollen presentation and in their flower morphology. Therefore, bees require different pollen-harvesting techniques to exploit the flowers of these two plant taxa. Also, it is unlikely that the often syntopic occurrence of Fabaceae with Boraginaceae in semidesert or rocky habitats, where the *Annosmia-Hoplitis* group is most diverse, enforced the distinct affinity to Fabaceae. Several other plant taxa are also common in these habitats, including members of the Asteraceae, Brassicaceae and Zygophyllaceae (S. Sedivy & A. Müller unpublished data), and these are not or only exceptionally exploited by *Annosmia-Hoplitis* bees. Thus, we suggest that physiological constraints related to the pollen chemistry are most likely to explain the Boraginaceae-Fabaceae paradox similar to herbivorous insects where host plant chemistry often plays a significant role in the evolution of host shifts (Beccera 1997). Three different mechanisms might underlie the

Boraginaceae-Fabaceae paradox. First, the physiological tool required to successfully digest Fabaceae pollen may have been inherited from an early common ancestor specialized on Fabaceae. Unfortunately, it is not clear whether this mechanism underlies the Boraginaceae-Fabaceae paradox because the phylogenetic position of the *Annosmia-Hoplitis* group within the genus *Hoplitis* remains unresolved (Praz et al. 2008b). Second, the pollen of Boraginaceae and Fabaceae may contain similar secondary metabolites or metabolites that can be detoxified using the same physiological tools. Interestingly, the Fabaceae genus *Crotalaria*, which is the main host of *H. parana*, contains pyrrolizidine alkaloids in its vegetative parts (Fletcher et al. 2009) as do most Boraginaceae genera exploited by *Hoplitis* (see above), suggesting that similar plant defence compounds may indeed represent a common constraint imposed in parallel by at least some of the Fabaceae and Boraginaceae hosts. Third, the pollen of Boraginaceae and Fabaceae may possess similar essential nutrients the bees are dependent on. Preliminary data indicates that most larvae of the *Echium* specialist *Hoplitis adunca* are able to develop to the cocoon stage on a Fabaceae pollen diet, whereas they are unable to develop on a *Campanula* pollen diet (M. Haider, C. Sedivy, S. Dorn & A. Müller unpublished data). This is surprising given that *Campanula* pollen proved to be a suitable pollen diet for the larvae of multiple oligolectic bee species specialized on plants other than *Campanula* (Praz et al. 2008a,c). It suggests that the pollen of Boraginaceae and Fabaceae might indeed share some unique qualitative or quantitative compositions of essential nutrients, which *Annosmia-Hoplitis* bees require for their successful development.

7.5.2. Specialized pollen-collecting behaviour

Twelve of the 44 investigated pollen-collecting species of the *Annosmia-Hoplitis* group possess specialized morphological or behavioural adaptations for pollen uptake. This high proportion might be due to the fact that the host plant spectrum of the *Annosmia-Hoplitis* bees is dominated by plant taxa that conceal their pollen within the flowers and indicates that such adaptations, which are usually considered to be very rare among bees (Westerkamp 1987; Westrich 1989; Wcislo & Cane 1996; Thorp 2000), might be more common than previously assumed.

Specialized morphological pollen harvesting devices among *Annosmia-Hoplitis* bees are used to exploit three different floral types, i.e. i) narrow-tubed flowers, in which the anthers are hidden inside a narrow corolla, ii) nototribic flowers, in which the raised position of the anthers renders efficient pollen collection difficult, and iii) occluded flowers, which have to be opened to gain access to the anthers. Specialized bristles on forelegs or probosces that are used to remove pollen from narrow-tubed flowers have independently evolved four times in the *Annosmia-Hoplitis* group. Similar adaptations to extract pollen from narrow flower tubes are also known from few species of other bee taxa (Parker & Tepedino 1982; Müller 1995; Müller & Kuhlmann 2003; Neff 2004; Milet-Pinheiro & Schlindwein 2010) as are specialized adaptations to exploit nototribic flowers (Müller 1996a,b; Rightmyer et al. 2011). In all species possessing this latter type of adaptation, a specialized pilosity located either on the face or on the thorax is used to comb the pollen directly out of the raised anthers of nototribic flowers. While the specialization reported for *H. bihamata* in the present study conforms to this type of adaptation, we are not aware of an adaptation similar to that recorded for *H. christae*, which

uses specialized hairbrushes of the forelegs to wipe pollen out of the nototribic flowers of its exclusive host. Furthermore, although the occluded flowers of the Antirrhineae are well known to be visited only by bees strong enough to gain access to the hidden anthers (Vargas et al. 2010), morphological adaptations to open such flowers, such as those documented in this study for *H. nisa* and *H. speculum,* have not been previously recorded in bees.

Four species of the *Annosmia-Hoplitis* group apply buzzing to shake pollen out of their host flowers, which are typical streukegel blossoms (Teppner 2011). Among bees of the Megachilidae, which is the third most speciose bee family (Michener 2007), buzzing behaviour has been reported so far only for two *Megachile* and one *Hoplitis* species (Neff & Simpson 1988; Dukas & Dafni 1990; Müller et al. 1997). Our finding that buzzing has evolved at least twice independently within the *Annosmia-Hoplitis* group indicates that this behaviour might be more widespread among the megachilid bees than hitherto assumed.

7.5.3. Conclusions

We found that host plant choice in bees of the *Annosmia-Hoplitis* group is considerably constrained probably due to physiological limitations, which corroborates the general validity of the constraint hypothesis of host range evolution in bees (Sedivy et al. 2008). We speculate that physiological adaptations to the pollen chemistry of the Boraginaceae are associated with preadaptation to use Fabaceae pollen, which might be due to similar secondary metabolites in the pollen of both families, to secondary metabolites that the bees can detoxify by using the same physiological tool or to similar qualitative or quantitative compositions of

primary metabolites the bees rely on. Interestingly, the Boraginaceae-Fabaceae paradox is not restricted to the *Annosmia-Hoplitis* group but appears to be a widespread phenomenon in osmiine bees and possibly other bee taxa as well. This calls for further comprehensive studies combining phylogenetic inference and extensive host plant data with pollen chemical analyses and feeding experiments. The unexpectedly high number of *Annosmia-Hoplitis* species possessing specialized adaptations for pollen uptake challenges the widely held assumption that such specializations have only exceptionally evolved in bees and make further studies combining careful morphological analysis with field observations highly desirable.

TABLE S1: Primers used and reaction conditions applied for the five genetic markers used in this study.

Primer	Reference	Sequence 5'-3'
CAD		
CADFor5	This study	GCR TAC GAC AAY TGY ATY ACA
CADRev 932	This study	RCT YTC TTG YCT CTG TAT YCT AAC AGC
CADRev1a	Praz et al. 2008b	GCC ATC ACT TCY CCT AYR CTC TTC AT
CAD-MegFor1	Litman et al. 2011	GAR CCY AGY CTC GAT TAY TG
PCR conditions: CAD-MegFor1-CADRev1a: 30" 94°C, 30" 56°C, 45" 72°C		
PCR conditions: CADFor5-CADRev932: 30" 94°C, 30" 56°C, 45" 72°C		
COI		
UEA3	Lunt et al. 1996	TAT AGC ATT CCC ACG AAT AAA TAA
UEA6For	This study	ATT ATT GCW ATY CCW ACW GGW ATT
UEA6	Lunt et al. 1996	TTA ATW CCW GTW GGN CAN GCA ATR ATT AT
UEA10	Lunt et al. 1996	CAA TGC ACT TAT TCT GCC ATA TT
COIFor398	This study	CAA CAT TTA TTT TGA TTT TTT GG
PCR conditions: UEA3-UEA6: 30" 94°C, 30" 55°C, 60" 72°C		
PCR conditions: COIFor398-UEA10: 30" 94°C, 30" 55°C, 60" 72°C		
EF1-alpha		
HaF2For1	Danforth et al. 2004	GGG YAA AGG WTC CAA RTA TGC
Cho10	Danforth et al. 2004	ACR GCV ACK GTY TGH CKC ATG TC
F2Rev1h	This study	AAT CAG CRG CAC CCT TRG GYG G
Exon2Forh	This study	CCR ACY AGA CCY ACV GAC AAA GC
Exon2Rev	Praz et al. 2008b	GGG AAG ACG GAG AGC TTT GT
For4h	This study	AGC TYT RCA AGA RGC TGT HCC
PCR conditions: HaF2For1-F2Rev1h: 30" 94°C, 30" 56°C, 60" 72°C		
PCR conditions: For4h-Cho10: 30" 94°C, 30" 56°C, 45" 72°C		
PCR conditions: Exon2Forh-Cho10: 30" 94°C, 30" 55°C, 60" 72°C		
Opsin		
OpsForh	This study	GTA CTY GGA CCT STY TTC TGT
OpsFor5h	This study	GTR CCY GAA GGT AAY ATG AC
OpsRevh	This study	RTA TGG TGT CCA YGC CAT GAA CCA
OpsRev5h	This study	AGC TCK ATA CTT CGG ATG ACT G
PCR conditions: OpsForh-OpsRevh: 30" 94°C, 30" 55°C, 45" 72°C		
PCR conditions: OpsFo5rh-OpsRev5h: 30" 94°C, 30" 55°C, 45" 72°C		
NaK		
NaKFor1	Cardinal et al. 2010	GGY GGT TTC GCS WTG YTG YTG TGG ATC GG
NaKRev1a	Cardinal et al. 2010	CCG ATN ARR AAG ATR TGM GCG TCN AGC CAA TG
NaKFor2	Cardinal et al. 2010	GCS TTC TTC TCB ACS AAC GCC GTY GAR GG
NaKRev2	Cardinal et al. 2010	ACC TTG ATR CCG GCY GAW CGG CAC TTG GC
NaKRevh	This study	GGY GGR TCD ATC ATR GAC ATS AG

NaKForh	This study	CCT YTG CTT CAT CGC GTA CT
NaKRev9	This study	CAG CCT CGA TRA TCT GAT TG
NaKFor6	This study	TTC TYG GTT AYC ATT GGC TYG AC
NaKRev11	This study	GGA ATC TCG CAG ACC TTC TTG T
NaKFor9	This study	CAA TCA GAT YAT CGA GGC TG
NaKRev6	This study	GTC RAG CCA ATG RTA ACC RAG AA

PCR conditions: NaKForh-NaKRev11: 30" 94°C, 30" 55°C, 75" 72°C

PCR conditions: NaKFor9-NaKRevh: 30" 94°C, 30" 55°C, 60" 72°C

PCR conditions: NaKFor1-NaKRev1a: 30" 94°C, 30" 55°C, 75" 72°C

PCR conditions: NaKFor2-NaKRev2: 30" 94°C, 30" 55°C, 75" 72°C

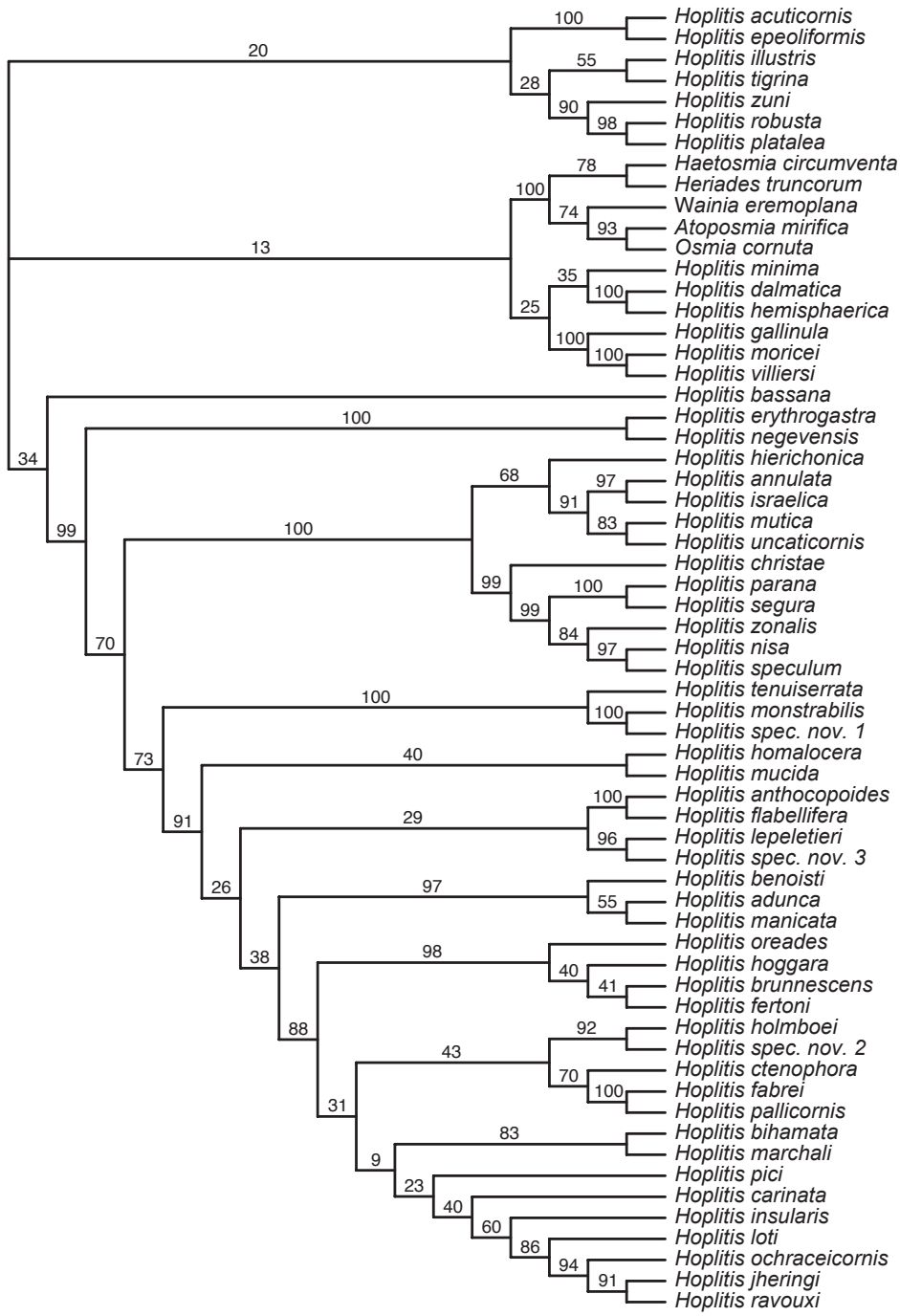


FIGURE S1: Phylogeny of bee species of the *Annosmia-Hoplitis* group. Parsimony bootstrap consensus tree with bootstrap values.

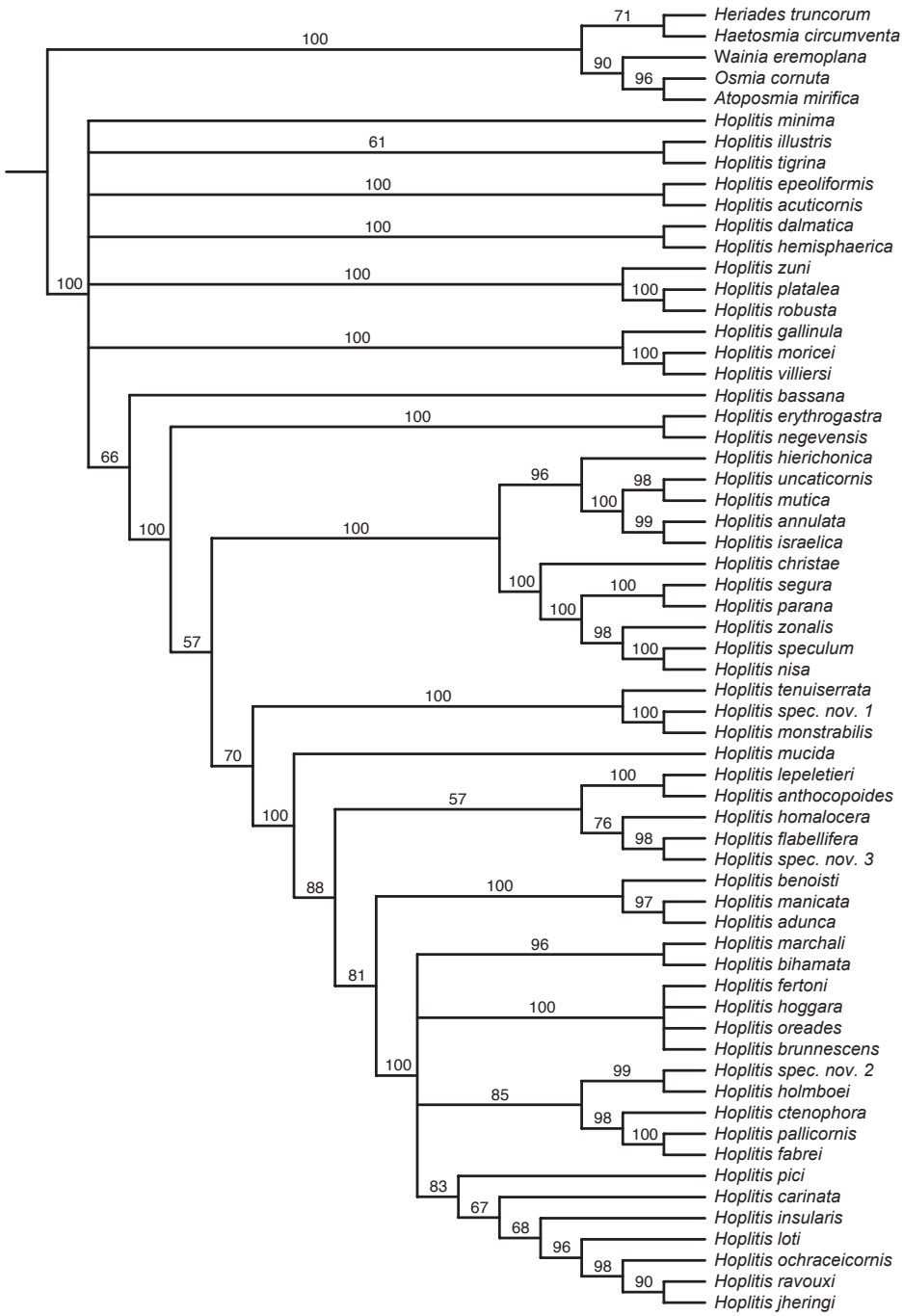


FIGURE S2: Phylogeny of bee species of the *Annosmia-Hoplitis* group. Maximum likelihood tree with bootstrap values. All nodes with bootstrap values of less then 50% were collapsed.

8. Evolution of nesting behaviour and cleptoparasitism in a selected group of osmiine bees (Hymenoptera: Megachilidae)[5]

8.1. Abstract

The construction of nests to rear offspring is restricted to vertebrates and few insect taxa, such as termites, wasps and bees. Among bees, species of the family Megachilidae are characterized by a particularly high diversity in nest construction behaviour. While many megachilid bees nest in excavated burrows in the ground, others place their brood cells in a variety of above ground cavities or attach them to the surface of a substrate, and yet others have adopted a kleptoparasitic habit. Evolutionary transitions between the different nesting sites and between conventional nesting and kleptoparasitism in bees are poorly understood. In this study, we traced the evolution of nesting site selection and kleptoparasitism in the *Annosmia-Hoplitis* group (Osmiini), which displays an exceptionally high diversity in nesting behaviour. We found that the evolution of nesting behaviour proceeded unidirectionally from nesting in excavated burrows in the ground to nesting in rock depressions and cavities, followed by the colonization of snail shells and insect borings in dead wood or hollow stems. Kleptoparasitism evolved once and the kleptoparasitic species have derived from the same lineage as their hosts.

[5]Based on Sedivy, C., S. Dorn & A. Müller. Biological Journal of the Linnean Society, in press.

8.2. INTRODUCTION

Several vertebrate and insect taxa construct nests to protect and rear offspring. In insects, nest construction has been reported so far only for five orders, among which Isoptera (termites) and aculeate Hymenoptera (wasps and bees) contain the great majority of nest building species (Gullan & Cranston 2000). Bees possess particularly elaborate and diverse nest construction behaviours, differing widely in nest architecture and the selection of both nest building materials and nesting sites (Westrich 1989; Michener 2007). The majority of bees build their nests in burrows that are excavated by the females in the ground (Michener 2007). Nesting in excavated ground burrows occurs in all bee families as well as in the majority of sphecoid wasps, from which the bees arose, and is therefore probably the ancestral nesting habit in bees (Eickwort et al. 1981; Engel 2001; Michener 2007; Litman et al. 2011). While nesting in excavated ground burrows is the ancestral condition in bees of the family Megachilidae as well (Litman et al. 2011), many megachilids nest above ground and place their brood cells in a variety of preexisting cavities such as insect borings in dead wood, hollow stems, rock crevices or empty snail shells (Westrich 1989; Michener 2007). Others attach their nests to the surface of a substrate, or build them within burrows gnawed into pithy stems or rotten wood. In fact, the three most speciose tribes among the megachilid bees, i.e. Anthidiini, Megachilini and Osmiini, all comprise both below ground and above ground nesting species (Michener 2007), indicating that either numerous independent and unidirectional shifts to above ground nesting from below ground nesting ancestors have occurred, or, alternatively, that repeated shifts in both directions took place. Since a sound phylogenetic framework is still lacking for most megachilid bee taxa and because the nesting behaviour of many groups has not been

described so far, it is unclear whether and how often reversals from above ground to below ground nesting have occurred during the evolutionary history of the megachilid bees.

Numerous bee lineages have adopted a kleptoparasitic way of life (Rozen 1991; Michener 1978, 2007; Rozen & Kamel 2007; Danforth et al. 2008; Cardinal et al. 2010; Ramirez et al. 2010). These kleptoparasitic bees ("cuckoo bees") neither build their own nests nor collect pollen, but instead enter the nests of pollen collecting bees to lay their eggs in either closed or still open brood cells (Westrich 1989). Usually, the kleptoparasitic bee larva kills the host larva and feeds on the provisions collected by the adult host bee. Kleptoparasitism in bees has independently evolved many times in four of the seven bee families (Michener 2007; Danforth et al. 2008; Cardinal et al. 2010). Kleptoparasitic bees always attack other bee species and often belong to the same lineage as their hosts, a phenomenon known as Emery's rule (Wilson 1971), which states that parasites and their hosts share common ancestry and hence are closely related to each other (Emery 1909). Although Emery's rule was formulated for social parasitism in the Hymenoptera, it also holds true for a number of kleptoparasitic bee taxa. Exceptions are the cleptoparasitic species of the subfamily Nomadinae, which attack unrelated bee taxa (Michener 2007). Among the megachilid bees, kleptoparasites are represented with species rich lineages in the tribes Dioxyini, Anthidiini and Megachilini, whereas the only kleptoparasites within the tribe Osmiini are few *Hoplitis* species of the subgenus *Bytinskia*, which attack congeneric species of the subgenus *Annosmia* (Mavromoustakis 1954; Warncke 1991a; Michener 2007). Although the *Bytinskia* species resemble their hosts morphologically,

suggesting a close relatedness, their specific phylogenetic position remains unclear.

In the present study, we traced the evolution of nesting site selection and kleptoparasitism in the *Annosmia-Hoplitis* group (Megachilidae, Osmiini). This group of solitary bees contains species that nest below and above ground (Westrich 1989; Rozen et al. 2009; Le Goff 2010), that have adopted a kleptoparasitic habit, i.e. species of the subgenus *Bytinskia*, and that are hosts of these kleptoparasites (Mavromoustakis 1954; Warncke 1991a; Michener 2007). By mapping nesting sites and kleptoparasitic behaviour onto a well supported molecular phylogeny of the *Annosmia-Hoplitis* group (see chapter 7), we addressed the following research questions: i) What are the evolutionary patterns of nest site selection in this group of bees? ii) Did the kleptoparasitic *Bytinskia* species evolve from the same lineage as their hosts?

8.3. METHODS

The *Annosmia-Hoplitis* group, which is exclusively Palearctic in its distribution and forms a well supported monophyletic clade within the genus *Hoplitis* (Praz et al. 2008; see chapter 6), consists of four currently recognized subgenera: *Annosmia* (30 described species), *Bytinskia* (3), *Coloplitis* (2) and *Hoplitis* s. str. (50) (Ungricht et al. 2008; Müller 2012). *Bytinskia* is an exception among these subgenera in that its species are kleptoparasites (Warncke 1991b). A recent molecular phylogenetic study that included species of all subgenera except *Coloplitis* revealed that the most basal species of the *Annosmia-Hoplitis* group is *Hoplitis bassana* (see chapter 7), which was treated by Warncke (1991b) as a member of the subgenus *Annosmia*. The remaining species of the *Annosmia-Hoplitis*

group can be divided into four main clades: i) the *erythrogastra* clade, which comprises the species of the subgenus *Bytinskia*; ii) the *annulata* clade, which comprises all species of the subgenus *Annosmia* except *H. bassana*; iii) the *monstrabilis* clade, which comprises several species that are morphologically intermediate between members of the *annulata* clade and those of the *adunca* clade; and iv) the *adunca* clade, which comprises the majority of species of the subgenus *Hoplitis*. The latter two clades form a monophyletic group comprising all species of the subgenus *Hoplitis*.

8.3.1. Nesting behaviour

To document the nesting behaviour of the *Annosmia-Hoplitis* species, we performed field excursions to Greece (2006), Jordan (2007), Morocco (2008, 2009), Iran (2009), Israel (2010), Cyprus (2011) and Tunisia (2012). In addition, we conducted a comprehensive literature survey on nesting sites, nest building material and kleptoparasitic behaviour. In total, we gathered data on the nesting behaviour of 28 *Annosmia-Hoplitis* species belonging to all subgenera except *Coloplitis* (Table 1).

8.3.2. Ancestral state reconstruction

To trace the evolution of nesting site selection and kleptoparasitism, we first applied parsimony mapping implemented in MacClade 4.08 (Maddison & Maddison 2005) by using a well resolved molecular phylogeny, which has recently been inferred for 44 species of the *Annosmia-Hoplitis* group based on sequence data from five separate gene-coding regions (see chapter 7). Data on nesting sites and kleptoparasitic behaviour was grouped into five categories (see Results)

and mapped onto the majority rule consensus tree, which is based on 88 000 post burn-in trees from four independent Bayesian analyses (for details, see chapter 7).

In addition, we inferred the ancestral nesting behaviour at the most basal nodes by applying Markov chain Monte Carlo (MCMC) inference using the “AddMRCA” command in BayesTraits (Pagel et al. 2004; Pagel & Meade 2006). Since the high number of estimated rates due to the grouping into five nesting categories led to highly unstable harmonic means, data on nesting sites and kleptoparasitic behaviour was reduced to three categories (see Results). We used a subset of 1 000 randomly chosen trees from our pool of saved trees from the Bayesian analysis. The outgroup taxa were excluded using Mesquite for MacOSX (Maddison & Maddison 2007). We used a gamma prior with its mean and variance seeded from uniform distributions on the interval 0 to 10 (command: rjhp gamma 0 10 0 10) and set the deviation of the normal distribution to 6, which led to the suggested acceptance rates of roughly 20% (Pagel & Meade 2006). To assess the robustness of these ancestral state reconstructions, we compared Bayes factors and average harmonic mean values after 10 000 000 iterations associated with the two alternative states "nesting in excavated ground burrows" and "nesting in preexisting cavities above ground" using the “Fossil” command. An improvement of average harmonic mean values by two negative log units or more, when comparing constraints to alternative states, was taken as evidence for a ‘significantly’ more likely evolutionary explanation (Pagel 1999).

TABLE 1: Nesting behaviour of bee species of the *Annosmia-Hoplitis* group.

Species name	Nesting site	Nesting material	References
Hoplitis (Annosmia) annulata (Latreille, 1811)	in excavated burrows in rather hard soil; the nests are 2-8cm deep and contain 1-4 cells at the end of the main burrow or of side burrows	brood cell partitions and nest plug built from mud; the cells are excavated from the ground, their walls are smoothed and hardened on the inside but apparently not newly constructed	Ferton (1892), Le Goff, (2010), Mavromoustakis (1957)
Hoplitis (Annosmia) christae (Warncke, 1991)	in excavated burrows in the soil	-	Warncke (1991c)
Hoplitis (Annosmia) hierichonica (Mavromoustakis, 1949)	in excavated burrows in rather hard soil; the single nest found so far was 2-3cm deep and contained 3 cells, of which two were linearly arranged at the end of the main burrow and one at the end of a short side burrow	brood cell partitions built from mud; the cells are excavated from the ground, their walls are smoothed and hardened on the inside	C. Sedivy, C. Praz & A. Müller (personal observation)
Hoplitis (Annosmia) speculum (Benoist, 1934)	in excavated burrows in hard soil	-	A. Müller (personal observation)
Hoplitis (Annosmia) tkalcuella Le Goff, 2003	in excavated burrows in rather hard soil; the entrance burrow ramifies after a length of ca. 1cm into four branches each containing 1-3 linearly arranged cells	brood cell partitions and nest plug built from mud	Le Goff (2003a, 2010)
Hoplitis (Annosmia) zonalis (Pérez, 1895)	in excavated burrows in hard-packed sandy soil; the nests are 4-5cm deep containing 1-2 cells at the end of the main burrow or of very short lateral burrows	brood cell partitions built from mud; the cells are excavated from the ground, their walls are smoothed and hardened on the inside but apparently not newly constructed	Mavromoustakis (1954), C. Sedivy & C. Praz (personal observation)
Hoplitis (Bytinskia)	cleptoparasitic in nests of *Hoplitis (Annosmia) zonalis*		Mavromoustakis (1954), A.

erythrogastra (Mavromoustakis, 1954)			Müller (personal observation)
Hoplitis (Bytinskia) negevensis (Warncke, 1991)	cleptoparasitic in nests of *Hoplitis (Annosmia) christae*		Warncke (1991a), C. Sedivy & C. Praz (personal observation)
Hoplitis (Bytinskia) parasitica (Warncke, 1991)	cleptoparasitic in nests of *Hoplitis (Annosmia) eremophila* and probably other *Annosmia* species		Warncke (1991a)
Hoplitis (Hoplitis) adunca (Panzer, 1798)	in insect burrows in dead wood or in hollow stems, sometimes in wall crevices or in abandoned cells and burrows of aboveground or belowground nesting aculeate Hymenoptera	nest plug, cell partitions and varying parts of the cell walls, sometimes entire cells, built from mud without addition of pebbles	Banaszak & Romasenko (2001), Benoist (1931), **Bosch, Vicens & Blas** (1993), Brechtel (1986), Friese (1923), Grandi (1961), Le Goff (2004), Malyshev (1937), Müller, Krebs & Amiet (1997), Popovici-Baznosanu (1909), Westrich (1989)
Hoplitis (Hoplitis) anthocopoides (Schenck, 1853)	in depressions, cracks or surface irregularities of rocks where the brood cells are more or less exposed; in small rock cavities containing 1-3 hidden cells; nests contain 1-33, mostly 3-8 cells	brood cells and nest plug built from mud and pebbles; exposed cells finally covered by a continuous layer of mud and small pebbles	Banaszak & Romasenko (2001), Benoist (1931), Bonelli (1971), Eickwort (1973, 1975b), Friese (1923), Gerstaecker (1869), Le Goff (2004), Müller, Krebs & Amiet (1997), Westrich (1989)
Hoplitis (Hoplitis) benoisti (Alfken, 1935)	exposed on rock surface; in small rock cavities containing 1-3 hidden cells or exceptionally in insect burrows in the ground	brood cells and nest plug built from mud without addition of pebbles	Benoist (1931), Le Goff (2004, personal communication)
Hoplitis (Hoplitis) bihamata (Costa, 1885)	in small rock cavities containing 1 brood cell	brood cells built from mud intermixed with small pebbles	Benoist (1931)

Hoplitis (Hoplitis) fabrei (Zanden, 1987)	in small rock cavities containing 2 brood cells	-	G. Le Goff & N. Vereeken (personal communication)
Hoplitis (Hoplitis) fertoni (Pérez, 1890)	in empty snail shells containing 1-8 brood cells	brood cell partitions and nest plug built from mud, sometimes intermixed with small pebbles	Ferton (1890, 1908), Le Goff (2003b), Sedivy, Praz & Müller (personal observation)
Hoplitis (Hoplitis) homalocera Zanden, 1991	-	females collect mud on the ground for nest construction	G. Pisanty (personal communication)
Hoplitis (Hoplitis) lapidaria (Morawitz, 1877)	in rock fissures	-	Morawitz (1877)
Hoplitis (Hoplitis) lepeletieri (Pérez, 1879)	in depressions, cracks or surface irregularities of rocks where the brood cells are more or less exposed; nests contain 2-8 cells	brood cells built from mud and pebbles; exposed cells finally covered by a continuous layer of mud and small pebbles	Banaszak & Romasenko (2001), Benoist (1931), Bonelli (1969), Ferton (1901), Friese (1923), Gogala (1999), Westrich (1989), A. Müller & C. Sedivy (personal observation)
Hoplitis (Hoplitis) loti (Morawitz, 1867)	in depressions, cracks or surface irregularities of rocks where the brood cells are more or less exposed; sometimes in abandoned cells of *Hoplitis lepeletieri* or in small rock cavities where the cells are hidden; nests contain mostly 3-4 cells	brood cells and nest plug built from mud and pebbles; exposed cells finally covered by a continuous layer of mud and small pebbles	Banaszak & Romasenko (2001), Benoist (1931), Blüthgen (1920), Friese (1895, 1923), Micheli (1931), Morawitz (1867), Westrich (1989), A. Müller & C. Sedivy (personal observation)
Hoplitis (Hoplitis) manicata (Morice, 1901)	in hollow plant stems	nest plug, cell partitions and large parts of the cell walls, sometimes entire cells, built from mud without addition of pebbles	G. Le Goff (personal communication)

Hoplitis (Hoplitis) marchali (Pérez, 1902)	in small rock cavities containing 1-2 brood cells	brood cells and nest plug built from mud without addition of pebbles	Le Goff (2005, personal communication)
Hoplitis (Hoplitis) monstrabilis Tkalcu, 2000	in excavated burrows in hard soil; the nests are very shallow, some cells are only 2-2.5cm below the soil surface; nests contain 5-8 cells attached to the main burrow or arranged in a linear series	brood cell partitions built from mud	Rozen *et al.* (2009)
Hoplitis (Hoplitis) mucida (Dours, 1873)	in small rock cavities containing 1 cell	brood cells and nest plug built from mud without addition of pebbles	Le Goff (2005, personal communication)
Hoplitis (Hoplitis) ochraceicornis (Ferton, 1902)	in small rock cavities	brood cells built from mud	Benoist (1931), Ferton (1902)
Hoplitis (Hoplitis) oreades (Benoist, 1934)	-	females collect mud on the ground for nest construction	A. Müller (personal observation)
Hoplitis (Hoplitis) pallicornis (Friese, 1895)	in small rock cavities	brood cells built from mud and small pebbles	Gogala (1999)
Hoplitis (Hoplitis) pici (Friese, 1899)	exposed on rock surface	brood cells built from mud	P. Hartmann (personal communication)
Hoplitis (Hoplitis) ravouxi (Pérez, 1902)	in depressions or surface irregularities of rocks where the brood cells are more or less exposed; in small rock cavities where the cells are hidden; nests contain 1-8 cells	brood cells and nest plug built from mud and pebbles; exposed cells finally covered by a continuous layer of mud and small pebbles	Benoist (1931), Blüthgen (1920), Stöckhert (1933), Westrich (1989), A. Müller (personal observation)

8.4. RESULTS

8.4.1. Nesting behaviour

The nesting behaviour of the 28 species of the *Annosmia-Hoplitis* group is highly diverse (Table 1) and can be grouped into five categories (Fig. 1): i) nesting in excavated ground burrows (members of the *annulata* clade and the *monstrabilis* clade), ii) nesting in depressions or cavities of rocks (members of the *adunca* clade except for the three species listed below), iii) nesting in insect burrows in dead wood or in hollow stems (*Hoplitis adunca* and *Hoplitis manicata*), iv) nesting in empty snail shells (*Hoplitis fertoni*), and v) kleptoparasitic in nests of species of the *annulata* clade (members of the *erythrogastra* clade). Although nesting sites considerably vary among the species of the *Annosmia-Hoplitis* group, the material used for nest construction is always mud, often combined with small pebbles (Table 1).

TABLE 2. Results of the Bayesian ancestral state reconstruction of nesting behaviour. Average harmonic mean values after 10 000 000 iterations are given for nodes A-E when constrained to be "nesting in excavated ground burrows" or "nesting in preexisting cavities above ground". Bayes factors equal twice the difference between the two harmonic mean values.

Nodes	Harmonic mean "fossil"		Bayes factor
	Excavated ground burrows	Preexisting cavities above ground	
A	10.6	13.2	5.2*
B	10.2	13.4	6.4*
C	10.5	13.3	5.6*
D	10.8	13.3	5.0*
E	13.4	10.9	5.0*

Bayes factors higher than 4 are considered as strong support (indicated by an asterisk).

FIGURE 1: Nests of bee species of the *Annosmia-Hoplitis* group. (A) Female of *Hoplitis annulata* leaving her nest in the ground (photo N. Vereecken); (B) brood cell of *Hoplitis monstrabilis* excavated in the ground (note the thick cell wall, photo J. Rozen); (C) female of *Hoplitis ravouxi* entering a small hole in a rock containing a single hidden brood cell still being provisioned (photo W. Loederbusch); (D) exposed brood cell of *Hoplitis loti* built from mud and pebbles (photo A. Müller); (E) old nest of *Hoplitis lepeletieri* constructed in a rock depression; (F) multi-celled nest of *Hoplitis fertoni* built in an empty snail shell of *Otala lactea* (note that the cell walls are entirely constructed from mud, photo G. Le Goff); (G) nest of *Hoplitis adunca* in a hollow stem containing several brood cells with feeding larvae and an empty vestibular cell just behind the entrance plug (photo A. Krebs).

8.4.2 Ancestral state reconstruction

Parsimony mapping of the five categories of nesting behaviour onto the phylogeny of the *Annosmia-Hoplitis* group (Fig. 2) suggests that the common ancestor of the clade comprising the *annulata*, *monstrabilis* and *adunca* clades nested in excavated burrows in the ground, and that one transition occurred each from i) nesting in excavated ground burrows to nesting in rock depressions or rock cavities (in the ancestor of the *adunca* clade), ii) nesting in rocks to nesting in insect burrows in dead wood or hollow stems (in the ancestor of *H. adunca* and *H. manicata*) and iii) from nesting in rocks to nesting in empty snail shells (in *H. fertoni*). Furthermore, kleptoparasitism evolved only once in the *Annosmia-Hoplitis* group and the two kleptoparasitic species *H. erythrogastra* and *H. negevensis* evolved from the same lineage as their hosts, which are *H. (Annosmia) zonalis* and *H. (Annosmia) christae*, respectively (Fig. 2, Table 1).

Parsimony mapping did not allow uncovering the ancestral nesting behaviour at nodes A and B (Fig. 2), since the nesting biology of the most basal species *H. bassana* is unknown. MCMC inference, for which the nesting categories ii) to iv) (see above) were summarized to "nesting in preexisting cavities above ground" (see Material and methods), clearly suggests, however, that nesting in excavated ground burrows is the ancestral state at nodes A (Bayes factor (BF) = 5.2; Table 2) and B (BF = 6.4). Furthermore, the results of the MCMC inference are in line with the findings of the parsimony mapping, indicating that the ancestral nesting behaviour was nesting in excavated ground burrows at node C (BF = 5.6) and D (BF = 5.0) and nesting in preexisting cavities above ground at node E (BF = 5.0).

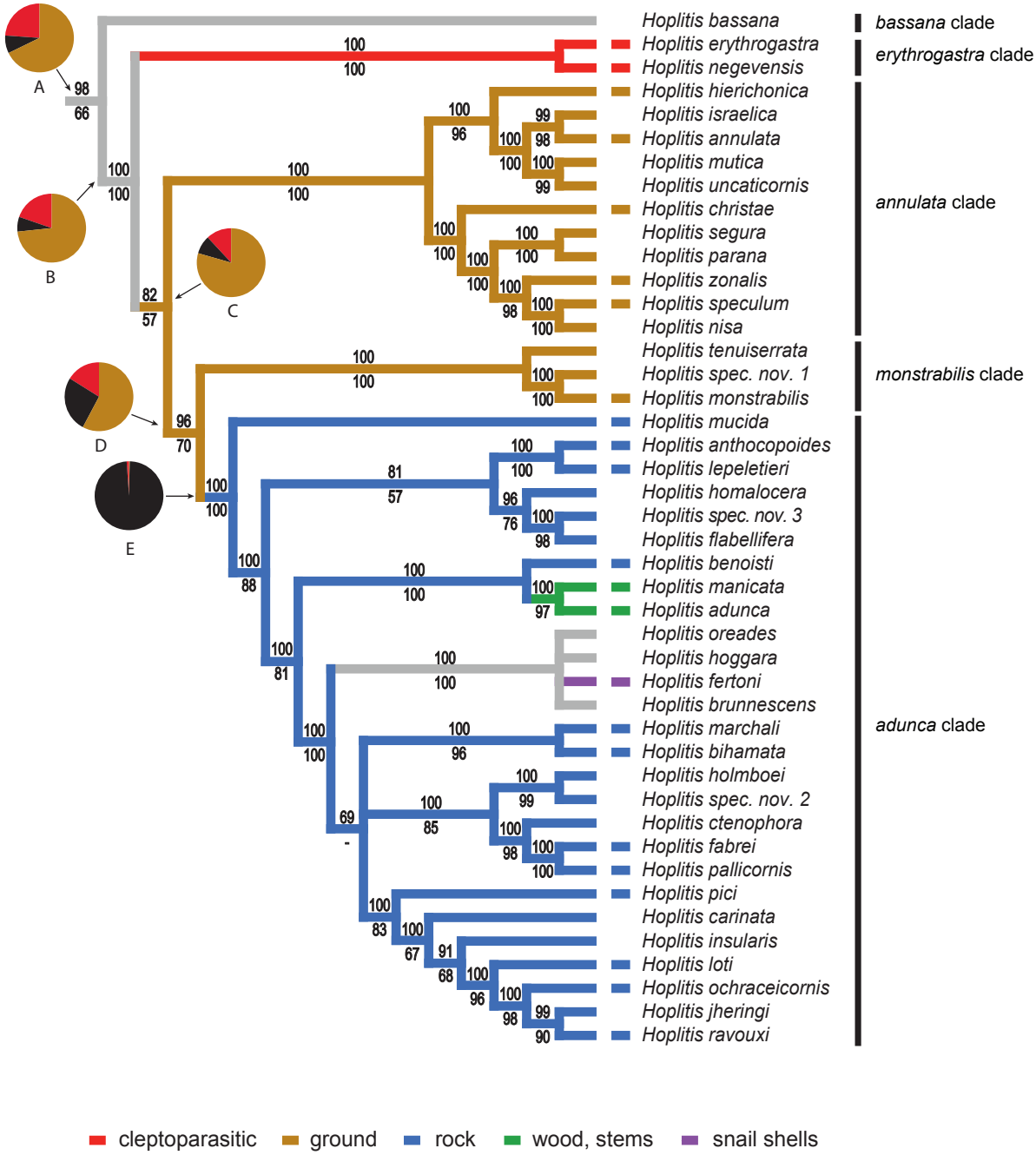


FIGURE 2: Evolution of nesting behaviour in bee species of the *Annosmia-Hoplitis* group. Five categories of nesting behaviour were mapped onto the majority rule consensus tree of a Bayesian analysis (see chapter 7) using the criterion of maximum parsimony. Equivocal branches are coloured in grey. Results of the Bayesian reconstruction of ancestral nesting behaviour at the five basal nodes are shown by pie charts. For this analysis, the three nesting categories "rock", "wood, stems" and "snail shells" were summarized to the category "nesting in preexisting cavities above ground" (black). Bayesian posterior probabilities (above) and maximum-likelihood bootstrap values (below) are shown for all nodes. Nodes with posterior probabilities lower than 65% were collapsed to polytomies.

8.5. Discussion

The observed patterns of nesting site selection and kleptoparasitic behaviour in bees of the *Annosmia-Hoplitis* group reveal a number of evolutionary transitions between different nesting sites and uncover the phylogenetic relationships between the kleptoparasitic *Bytinskia* species and their *Annosmia* hosts.

As the nesting behaviour of the most basal taxon, *Hoplitis bassana*, is unknown, it is not possible to determine the ancestral nesting habit of the *Annosmia-Hoplitis* group by parsimony mapping. However, Bayesian ancestral state reconstruction strongly indicates that ground nesting in excavated burrows is ancestral in this group of bees. Our results reveal one transition from below ground nesting to above ground nesting, one transition from nesting in rocks to nesting in dead wood or stems and one transition from nesting in rocks to nesting in snail shells. The evolution of nesting site selection in the *Annosmia-Hoplitis* group therefore appears to have been unidirectional from below ground to above ground nesting, followed by a diversification in the selected nesting sites after above ground nesting has been achieved. Once the transition from nesting in excavated ground burrows to nesting in preexisting cavities above ground has been accomplished, both morphological and behavioural adaptations required to dig into the soil and the cognitive ability to assess crucial aspects of nesting sites (e.g. soil texture, humidity or temperature) might be subsequently lost (Eickwort et al. 1981; Cane 1991; Neff & Simpson 1991). Once lost, it is unlikely that these complex behavioural and cognitive capabilities can be easily regained. It is therefore not surprising that no reversal from above ground to below ground nesting has occurred

in the *Annosmia-Hoplitis* group, and we hypothesize that such reversals are rare evolutionary events in bees in general.

In contrast to the high diversity of selected nesting sites, the material used for nest construction is strongly conserved in the *Annosmia-Hoplitis* group. All species exclusively employ mud, sometimes in combination with small pebbles. The walls of the brood cells of *Hoplitis annulata* and *Hoplitis zonalis*, both members of the more basal *annulata* clade, are only smoothed and sometimes hardened on the inside but not built from mud (Le Goff 2010; C. Sedivy & C. Praz, personal observation). In contrast, the cells of *Hoplitis monstrabilis*, a member of the more derived *monstrabilis* clade, exhibit a 1-2mm thick cell wall composed of compact soil, which might represent a special coating constructed by the female bee from mud imported into the burrow (Rozen et al. 2009; see also Fig. 1B). It is possible that the capability to build whole brood cells from mud below ground was the key evolutionary achievement that subsequently allowed members of the *adunca* clade to construct their brood cells in differently shaped above ground cavities, and thus to save the labour intensive digging of burrows into the ground. Indeed, the brood cell walls of many species of the *adunca* clade, including those that nest in empty snail shells or in hollow stems, are partly or entirely built from mud (Westrich 1989; Le Goff 2003b; see also Fig. 1F). However, until the brood cell construction in species of the *monstrabilis* clade has been thoroughly studied, this evolutionary scenario remains speculative.

The phylogeny of the *Annosmia-Hoplitis* group placed the kleptoparasitic *Bytinskia* species basal to the *annulata* clade, which contains their hosts. Hence, kleptoparasitism in the *Annosmia-Hoplitis* group evolved in concordance with Emery's rule, which states that parasites and their hosts

share the same common ancestor (Wilson 1971). Except that they lack a scopa, *Bytinskia* species share all key morphological characteristics with the species of the *annulata* clade, which supports their placement in the same lineage as their hosts.

Intraspecific parasitism (usurpation) by which females usurp the nests of conspecifics and lay their eggs in the foreign brood cells is assumed to be an important behavioural prerequisite to promote the evolution of interspecific parasitism (kleptoparasitism) (Eickwort 1975a). This behaviour was observed in several megachilid bee species (Fabre 1914; Custer & Hicks 1927; Matthews 1965; Raw 1972; Eickwort 1975a; McCorquodale & Owen 1994) and, interestingly, it is especially pronounced in *Hoplitis anthocopoides* (Eickwort 1975a), a member of the *Annosmia-Hoplitis* group. In the studied nest aggregation, 11 out of 20 females were observed to usurp conspecifics. This behaviour ranged from occasional fights for an unfinished brood cell to the reopening of a foreign cell and the replacement of the host egg or young larva with the egg of the usurping female, followed by resealing of the brood cell. As frequency-dependent selection is expected to favour usurpation only as long as the proportion of usurping females is not too high within a population (Field 1992), kleptoparasitic species should mainly evolve when usurping individuals begin to lay their eggs into the nests of another species (Eickwort 1975a). The more closely the host species corresponds to the evolving kleptoparasite with respect to nesting site, nest architecture and pollen source, the more likely it is to become a regular host. In the *Annosmia-Hoplitis* group, closely related species that possess the same nesting habits and pollen hosts often occur syntopically (A. Müller & C. Sedivy, personal observation), and conditions for the evolution of kleptoparasites may therefore be favourable in this group.

9. General Discussion

Taking advantage of the high biological and ecological diversity of the bee tribe Osmiini, this thesis tackles a number of crucial questions regarding the evolutionary history and ecological implications of bee-flower relationships. The main results of both experimental and evolutionary studies presented here indicate that pollen host choice is far from accidental and that pollen is not an easy-to-use food source for bees. Instead, plants seem to protect their pollen both morphologically and chemically to prevent excessive loss of this valuable plant tissue to bees. In their remarkable evolutionary plasticity, many bees have in turn developed a staggering array of morphological, behavioural and physiological adaptations to circumvent these protective properties. Bee-flower relationships therefore largely resemble herbivore-plant interactions and can be regarded as the result of an evolutionary arms race rather than mutualistic coevolution as was traditionally assumed.

First, we demonstrated that bees require physiological adaptations to successfully digest certain pollens. The two closely related, polylectic mason bee species *Osmia bicornis* and *Osmia cornuta* were experimentally grown on four different pollen diets and were shown to largely differ in their ability to develop on the same pollen. While *Osmia bicornis* failed to survive on pollen of the viper's bugloss *Echium vulgare* but developed on pollen of the buttercup *Ranunculus acris*, the reverse held true for *Osmia cornuta*. In both bee species tested, larval mortality patterns differed considerably between the pollen diets, indicating that the unfavourable properties of these pollens affected the larvae in different ways, and that toxic secondary metabolites might be responsible for the unsuitability of these pollens for the tested bee species.

The subsequent aim was to demonstrate that the secondary metabolite ranunculin, a substance in buttercup pollen that releases the highly reactive protoanemonin upon contact with the enzyme β-glucosidase, is toxic for non-specialized bees when present in natural concentrations. Ranunculin was experimentally admixed to the natural pollen diet of the oligolectic osmiine bee species *Chelostoma ranunculi* and *Heriades truncorum* in different concentrations to test the resistance of their larvae to this substance. The larvae of these two bee species, which were previously shown to fail to develop on a *Ranunculus* pollen diet (Praz et al. 2008a), died at high ranunculin concentrations while they survived at low concentrations. Analysis of naturally occurring ranunculin concentrations in buttercup pollen revealed that it was at least fourfold lower than the ranunculin concentration tolerated by the tested bee species, and was hence too low to cause larval mortality.

The investigation of phylogenetic relationships within the genus *Hoplitis* led to the proposal of three systematic changes. A Palaearctic origin for the genus *Hoplitis* was revealed, with several independent colonization events of southern Africa and of the Nearctic, and two re-colonization events from the Nearctic to the Palaearctic. The highly diverse nesting biology was classified into eight different categories of nesting behaviour. Parsimony mapping of nesting behaviour revealed that ground nesting in excavated burrows was the ancestral state as was shown to be the case for Megachilidae (Litman et al. 2011). We further argue that nesting in dead wood was a key innovation that allowed repeated colonization events between the Nearctic and the Palaearctic regions. Dead wood provides dry shelter in moist environments and can act as a 'raft' to transport whole nests across oceans (Michener 1979; Praz et al. 2008b).

Finally, the last two chapters focus on the species rich and highly diverse *Annosmia-Hoplitis* group in a phylogenetic context. Tracing the evolution of floral preferences of 44 species revealed that host plant choice is governed by strong evolutionary constraints and represents a process that is far from accidental, as was previously proposed by the 'constraint hypothesis of host range evolution in bees' (Sedivy et al. 2008). The intriguing affinity of these bees to both Boraginaceae and Fabaceae, two very distantly related plant families, as either exclusive or combined pollen hosts, represents a seemingly paradoxical host choice pattern that repeatedly and independently evolved in different groups of osmiine bees. The bees of the *Annosmia-Hoplitis* group display an unusual affinity to plant taxa with flowers that conceal their pollen. This led to the evolution of a variety of different specialized morphological and behavioural adaptations for pollen uptake, indicating that physiological constraints related to pollen chemistry rather than behavioural constraints related to flower handling governed host shifts in the *Annosmia-Hoplitis* group.

Mapping of the nesting behaviour onto the phylogeny of the *Annosmia-Hoplitis* group again revealed that nesting in excavated burrows in the ground was the ancestral state in these bees, a pattern that seems to hold true for bees in general (Michener 2007). Once the transition from below ground to above ground nesting was achieved, specialization on different types of nesting sites occurred, including nesting in rock crevices, dead wood or even empty snail shells. Furthermore, the *Annosmia-Hoplitis* group comprises the only cleptoparasitic osmiine bee taxon, the subgenus *Bytinskia* that parasitizes members of its sister taxon *Annosmia*. Hence, this study confirmed Emery's rule, which states that parasites and their hosts usually share the same common ancestor (Wilson 1971).

9.1. PATTERNS OF HOST PLANT CHOICE IN BEES - A COMPARISON

Since Robertson (1925) first recognized oligolecty and polylecty as two alternative strategies for bees to make the most rewarding pollen choice from the dazzling array of different flowers they are confronted with, researchers have speculated about the evolutionary mechanisms governing the diet breadth in bees (Michener 1954; Linsley 1958; Thorp 1969; Michener 1979, Wcislo & Cane 1996). Hypotheses regarding this intriguing aspect of bee biology were not rigorously tested until 1996, when Müller combined microscopic pollen analyses with novel phylogenetic methods for palaearctic anthidiine bees. Since then, several studies have used this approach to trace the evolution of host plant choice in different model bee taxa (Michez et al. 2004, 2008; Sipes & Tepedino 2005; Larkin et al. 2008; Sedivy et al. 2008; this thesis chapter four). However, it was Müller (1996) who first provided evidence for two important findings that had a pioneering impact on understanding the evolution of host plant choice in bees. First, oligolecty appears to be the ancestral state in bees, which was later corroborated by evidence from the genera *Andrena*, *Chelostoma* and the *Annosmia-Hoplitis* group (Larkin et al. 2008; Sedivy et al. 2008; this thesis chapter four), from the bee family Megachilidae (Litman et al. 2011) and for bees in general (Danforth et al. 2006; Michez et al. 2008). Second, he was the first to propose that oligolecty is best considered an evolutionary constraint that has been repeatedly overcome by many polylectic bee taxa. The constraints acting on host range in bees may be classified into two types. First, physiological constraints are related to the fact that bees require physiological adaptations for pollen digestion. Evidence comes from rearing experiments conducted with both oligolectic (Williams 2003;

Praz et al. 2008a) and polylectic (Williams 2003; this thesis chapter one) osmiine bees. Second, neurological constraints are related to the recognition or handling of flowers. The solitary bee *Heriades truncorum* refused to harvest pollen on *Campanula* and *Echium* in the absence of its specific host, the Asteraceae, although both types of non-host pollen support larval development (Praz et al. 2008c). The first study that specifically explored the impact of these evolutionary constraints in a phylogenetic context in bees of the genus *Chelostoma* resulted in the formulation of the 'constraint hypothesis of host range evolution in bees' (Sedivy et al. 2008). This hypothesis suggests that i) incorporations of new hosts are rare events in the evolutionary history of bee lineages, ii) host expansion is only possible if the physiological or neurological constraints imposed by the flowers can be overcome and iii) host shifts among oligoleges typically proceeded by a period of expanded host range followed by respecialization. In chapter four of this thesis, the general validity of this hypothesis is corroborated using the example of the *Annosmia-Hoplitis* group, a species rich taxon within the genus *Hoplitis*.

9.2. *CHELOSTOMA* VERSUS *ANNOSMIA-HOPLITIS* GROUP - A COMPARISON

As discussed in chapter four, the evolutionary patterns of host plant choice in the *Annosmia-Hoplitis* group strongly concur with the implications proposed by the constraint hypothesis based on the host choice patterns in *Chelostoma*. Despite these analogies, there are also a number of intriguing differences between the two groups. First, *Chelostoma* species exploit flowers that belong to eight different plant orders distributed among all major angiosperm lineages. Despite this complete disregard for host plant systematics, both visual appearance (e.g. coloration, radial symmetry) and the open pollen presentation are

strikingly similar across most exploited host plants. Analogously, bees of the genera *Macrotera* and *Diadasia* (Danforth 1996; Sipes & Tepedino 2005) as well as of the tribes Fideliini and Lithurgini (Litman et al. 2011) confine pollen collection mainly to large flowers with radial symmetry and well-exposed stamens of remotely related plant families (e.g Cactaceae, Convolvulaceae, Malvaceae, Onagraceae). As a consequence, *Chelostoma* species lack specific morphological adaptation for pollen uptake. This pattern indicates that neurological constraints related to the recognition or handling of flowers predominantly affected host shifts in *Chelostoma*.

In strong contrast, although comprising about twice as many species as the genus *Chelostoma* (Müller 2012), bees of the *Annosmia-Hoplitis* group exploit pollen of only six plant orders. Furthermore, they exhibit a striking preference for host plants with diverse morphological obstacles for pollen collection, especially among different genera of their preferred host plant family, the Boraginaceae (see figure 3 in chapter four). To overcome these obstacles, these bees have acquired an unmatched array of specialized pollen harvesting devices (see figure 4 in chapter four). Also, even host plant shifts to remotely related plant families occurred without exception to flowers with concealed pollen, predominantly to Fabaceae. Contrary to *Chelostoma*, constraints related to physiological adaptations for pollen digestion may underlie host shifts in bees of the *Annosmia-Hoplitis* group.

Another major difference between the two groups is the proportion of unique host shifts. In *Chelostoma*, all host shifts occurred only once with the exclusive exception of two independent shifts to the Asteraceae. In contrast, among the species of the *Annosmia-Hoplitis* group, all but three host shifts involved both the Boraginaceae and the Fabaceae. This finding might be an indication that preadaptation to successfully utilize Fabaceae

pollen, induced by physiological adaptation to digest Boraginaceae pollen, could have been a main factor leading to the repeated host shifts to Fabaceae.

Despite the validity of the general predictions of the 'constraint hypothesis' for the evolutionary patterns of host plant shifts in both the genus *Chelostoma* and the *Annosmia-Hoplitis* group, the more subtle differences only become evident when comparing different groups of bees. Therefore, further such studies are needed to enhance our understanding of the different constraints that appear to play a key role in the evolution of bee-flower relationships.

9.3. Pollen protection from bees - an evolutionary scenario

9.3.1. Morphological protection

Flowers are the interface that plants use to perform sexual reproduction via insects and other mobile animals. To attract these pollinators, most flowers offer sugar as bait and advertise their offering with conspicuous colours and exquisite scents. Under optimal conditions for the plant, the nectaries, the anthers and the style are arranged in a way that, during nectar consumption, pollen is deposited on the visitors' body and carried on to the style of the next conspecific flower. Depending on the size and shape of the flower visitor, the elements of the interface require a different arrangement for optimal functionality. This system has been highly successful and has promoted the evolution of the breath taking diversity and beauty of today's flowers.

In contrast to most other pollinators, bees not only consume nectar but also efficiently collect large amounts of pollen, which they transport to their nests where it is lost to the plants for sexual reproduction (Müller et al. 2006; Michener 2007; introduction in chapter one). Plants should therefore trade the need to attract bees for pollination against excessive pollen losses to pollen harvesting flower visitors (Westerkamp 1996). Various morphological floral traits help to reduce pollen loss by narrowing the spectrum of pollen feeding flower visitors (chapter four). Examples for typically bee-pollinated plants that hide their pollen are many members of the Antirrhineae, Fabaceae, Lamiaceae and many Boraginaceae (see Figure 2 in chapter four).

The evolutionary processes leading to such protective floral morphologies are straightforward. A mutation leading to even a slight change in size, shape or arrangement of petals, anthers and style may prevent excessive pollen collection by some bees that are no longer able to reach the pollen or significantly increase pollination success by those who still can. The plant individual holding the new mutation has an increased reproductive success leading to higher fitness and allowing the new trait to quickly spread within the population.

9.3.2. Chemical protection

However, many plants have flowers with openly accessible anthers such as the Asteraceae, Rosaceae and many Ranunculaceae. In order to prevent excessive pollen loss, these plants are expected to protect their pollen chemically against bees (Praz et al. 2008a; discussion in chapter 1). Indeed, all pollen types experimentally found to possess unfavorable properties for bee larval development originate from flowers with freely

accessible pollen that can easily be harvested by bees (Loper & Berdel 1980; Williams 2003; Pimentel De Carvalho & Message 2004; Praz et al. 2008a; discussion in chapter 1). An evolutionary scenario of the development of a chemical protection against bees is, however, more complex than for a morphological protection. A mutation leading to the incorporation of toxic secondary compounds into the pollen only acts on the bee larva that consumes it when the damage to the plant (the removal of the pollen by the bee) is already done. Although the toxins in the pollen might kill the larva, the individual plant does not experience a direct selective advantage over conspecifics. The selective advantage would arise only if the pollen-collecting bee had a mechanism to assess the pollen quality before collecting it, ether during the process of host recognition or host exception, and could avoid unsuitable pollen. In this case, the plant would have to signal the toxicity of its pollen, and the bee would have to perceive this signal accurately and quickly enough to avoid collection of the pollen.

Adult female bees not only consume nectar but also pollen. In the honeybee *Apis mellifera*, the protein content of the consumed pollen is the main factor that influences oogenesis in queenless worker bees (Human et al. 2007). However, both the regularity and the amount of pollen consumption in female solitary bees remain unknown. A constant assessment of pollen quality by the pollen-collecting bees may lead to an avoidance of toxic pollen and thus promote the evolution of chemically protected pollen.

9.4. OUTLOOK AND FUTURE RESEARCH

This study revealed that even the highly polylectic solitary bee species *Osmia bicornis* requires physiological adaptations to digest certain pollen types. Polylectic bees are often confronted with a staggering display of different flowers from which they have to make the most rewarding choice. Host recognition in oligolectic bees is genetically determined (Praz et al. 2008c) and both olfactory and visual cues are important for host localization (Burger et al. 2010a,b; Milet-Pinheiro et al. 2012). However, little is known so far about host finding, assessment of pollen quality for larval development or the decision processes for host choice in polylectic bees. What are the key factors that determine the selection of a pollen host? Could polylecty represent a strategy to make the protein of toxic pollen accessible by blending it with other non-toxic pollen, thereby diluting concentrations of secondary metabolites to non-toxic concentrations? Answering these questions would further increase our understanding of bee-flower relationships and in particular shed light into the intreaguing role of bees as herbivorous insects.

10. Literature

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12. Curriculum vitae

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