Evolution of host range in pollen generalist bees
insights from the subgenus Osmia (Megachilidae: Osmiini)

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Evolution of host range in pollen generalist bees - insights from the subgenus *Osmia* (Megachilidae: Osmiini)
Evolution of host range in pollen generalist bees - insights from the subgenus Osmia (Megachilidae: Osmiini)

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1 Summary

Female bees collect large quantities of pollen and nectar for their own nourishment and to provision their offspring with. Accordingly, their close relationship to flowering plants renders bees the most important group of insect pollinators. At the same time, however, bees also compete with the plants for the pollen, which either of them needs for its reproduction. Pollen host ranges of bees vary. Whereas some bees are specialized to plants of one genus or family (oligolecty), others collect pollen from a broader pollen host range (polylecty). Growing evidence suggests that plant traits are the main factors to restrict the host plant range of bees, and that bees have to overcome these constraints in order to broaden of their host range. The aim of this study is to gain a better understanding on the restrictions underlying host plant choice in polylectic bees and on the mechanisms allowing for host range expansion.

To investigate the evolution of host plant choice in polylectic species, the phylogeny of the three closely related Osmia subgenera Osmia, Orientosmia and Monosmia (Megachilidae: Osmiini) was reconstructed based on molecular and morphological data. In combination with microscopical pollen analyses, it revealed that polylecty is the ancestral state of this clade, with oligolecty having evolved twice independently. Several intriguing patterns of host plant choice suggested that floral host ranges of polylectic species are to differing degrees constrained and strongly governed by flower morphology, pollen chemistry or nectar availability. Whereas flower morphology can mechanically restrict the access of flower visitors to the pollen, experimental evidence suggests that plants with freely accessible pollen might exhibit unfavourable pollen properties that impede the development of unspecialized bee larvae in order to minimize pollen loss. We examined larval survival of five solitary bee species on pollen diets of two Fabaceae species, Onobrychis viciifolia and Lotus corniculatus. These plants have their anthers concealed inside their flowers and might therefore not possess unfavourable pollen
properties that additionally restrict the spectrum of pollen harvesting bees. All five bee species successfully developed on *Onobrychis* pollen diet, but larval survival on *Lotus* pollen diet was significantly reduced in two species, indicating that pollen of morphologically complex flowers is not necessarily an easy-to-use nutritional source, and that plants might exhibit multiple traits that challenge the host plant choice of bees.

The second part of this thesis explored mechanisms that might serve bees to broaden their host plant spectrum. Larval rearing experiments with five different populations of the polylectic mason bee species *Osmia cornuta* indicated that intra- and interpopulational variation exists in the ability of this bee to develop on unfavourable *Ranunculus* pollen diet. Of all five populations, few individual survived on pure *Ranunculus* pollen diet, indicating that the physiological ability to cope with the unfavourable properties of *Ranunculus* pollen is broadly existent. Moreover, as several of the *Ranunculus* fed individuals were able to reproduce and to sire viable offspring, this study provided first evidence for variation in the physiological ability of solitary bees to digest non-host pollen. This variation might provide the basis for host expansion and subsequent host shift in response to natural selection. In striking contrast to the detrimental effect of pure *Ranunculus* pollen diet on the great majority of *Osmia cornuta* larvae, admixing up to 50% of *Ranunculus* pollen diet in favourable *Sinapis* pollen diet did neither negatively affect larval survival nor adult body mass. In addition, analyses of female scopal pollen loads of *Osmia cornuta* and three closely related *Osmia* species revealed pollen mixing as common behaviour in these polyleges. Hence, larvae of pollen generalist bees might benefit from the nutrient content of unfavourable pollen without being negatively affected by its unfavourable properties if such pollen is mixed with favourable pollen. Beside physiological mechanisms, polylectic bees might thus also employ behavioural strategies concerning pollen collection, which allow them to broaden their host spectrum to plants with unfavourable pollen properties, albeit only to a certain extent.
2 Zusammenfassung


Um die Evolution der Wirtspflanzenwahl von polylektischen Bienen zu untersuchen, wurde anhand molekularer und morphologischer Daten ein Stammbaum der drei nah verwandten Osmia Untergattungen Osmia, Orientosmia und Monosmia (Megachilidae: Osmiini) erstellt und die Wirtsspektren der einzelnen Arten wurden mittels mikroskopischer Pollenanalysen untersucht. Es konnte gezeigt werden, dass diese Gruppe ursprünglich polylektisch ist und Oligolektie zweimal unabhängig voneinander entstanden ist. Die Pollenspektren der einzelnen Bienenarten wiesen erstaunliche Muster auf, die darauf hindeuten, dass die Wirtspflanzenwahl von polylektischen Arten unterschiedlich stark eingeschränkt ist und sehr stark von Blütenmorphologie, Pollenchemie und Nektarangebot beeinflusst wird.

3 General introduction

With nearly 20 000 species worldwide (Ascher and Pickering 2013), bees comprise a highly diverse and fascinating group of insects. Being derived from within a group of prey-hunting wasps, bees differ from their ancestors in feeding exclusively on plant products such as pollen and nectar (Michener 2007). Hence, their remarkably close relationship to flowering plants and their abundance in most natural ecosystems render bees the most important group of insect pollinators (Buchmann and Ascher 2005; Michener 2007).

Bee-flower relationship

Common history of bees and bee-pollinated plants dates back to the Cretaceous, since when co-evolution has shaped the intimate and very diverse relationship between the two taxa (Danforth et al. 2013). This relationship is unique in that plants rely on bees for their pollinating services, while bees, feeding exclusively on plant products, are in fact herbivores. They collect pollen and nectar, which is stored in their brood cells as larval provision (Westrich 1989). Thus, withdrawing the pollen, which otherwise might have served pollination, bees compete with plants for the same resource that both need for their reproduction. Similar to herbivore-plant interactions, also bee-flower relationships are therefore characterized by adaptations and defence mechanisms on both sides. Employing behavioural as well as morphological adaptations, pollen collection and transport by bees is often highly efficient (reviewed in Thorp 2000), and bees are not necessarily good pollinators (Hargreaves et al. 2009; Westerkamp 1991). Moreover, pollen requirements of bees are enormous (Müller et al. 2006; Schlindwein et al. 2005). Depending on the size of the bee species and the amount of pollen provided per flower, the provision of one single brood cell might require pollen from 7 – 1100 flowers (Müller et al. 2006; Schlindwein et al. 2005).

In order to escape the dilemma of attracting pollinators on the one hand, while restricting pollen loss to bees on the other hand, bee-pollinated plants evolved a
tremendous diversity of morphological and pollen chemical adaptations (Praz et al. 2008c; reviewed in Roulston and Cane 2000). Examples for plants that are almost exclusively bee-pollinated but simultaneously exhibit morphological adaptations against bees can be found in the Fabaceae family (Fægri and van der Pijl 1979). Having their anthers concealed within a “keel” that is formed by the two lowermost petals, visiting bees have to apply force in combination with a complex leg movement pattern in order to trigger the flower mechanism and to collect the pollen (Westerkamp 1997b). As a consequence, a number of bee species is unable to efficiently harvest pollen of these flowers (Westerkamp 1993). In contrast to such morphologically complex flowers, open flowers with easily accessible pollen have experimentally been observed to possess pollen that negatively affects the development of unspecialized bee larvae (Levin and Haydak 1957; Praz et al. 2008c; Reinhard 2011; Sedivy et al. 2011; Williams 2003). If such unfavourable pollen properties are likewise adaptive in terms of restricting the spectrum of pollen feeding visitors, it is expected that morphologically complex flowers do not, or to a smaller extent, exhibit unfavourable pollen properties. On the other hand, as both flower morphological and pollen-chemical constraints have repeatedly been overcome during co-evolution (Sedivy et al. 2008), plants might, just as against herbivores, have evolved multiple defence mechanisms against pollen feeding bees. Assessing the suitability of Fabaceae pollen diets for larval development of unspecialized bees might thus provide new insights into plant-pollinator relationships.

Host spectrum breadth and limitations of host range expansion

In contrast to nectar, which is collected on a wide array of plants, the pollen host spectrum of bees is to a varying extent restricted. Pollen collection in bees constitutes a continuum from specialization to generalization: oligolectic bees (specialists) restrict pollen collection to plants of a single plant genus or family, while polylectic bees (generalists) collect pollen from plants of two to several different plant families (Cane and Sipes 2006; Müller and Kuhlmann 2008). Both
strategies appear to be evolutionarily successful, as oligolecty and polylecty co-occur in all investigated bee faunas and as shifts from oligolecty to polylecty and vice versa repeatedly took place in different bee lineages (Larkin et al. 2008; Michez et al. 2008; Müller 1996; Sedivy et al. 2013; Sedivy et al. 2008; Sipes and Tepedino 2005). In contrast to the long held assumption that polylecty is the ancestral state in bees (reviewed in Müller 1996), growing evidence suggests an oligolectic origin of bees as a whole, especially as the most basal clades appear to be originally oligolectic (Danforth et al. 2013). Plant traits, such as flower morphology or pollen chemistry thereby constitute constraints that set the limits of a species’ host spectrum breadth (Michez et al. 2008; Sedivy et al. 2013; Sedivy et al. 2008). Although these traits can obviously be overcome, the mechanisms underlying host plant shifts in bees are poorly understood. In fact, on-going changes in a bees’ host plant spectrum have never been observed so far. Recent studies on herbivorous insects indicate that substantial variation in the physiological ability to cope with unfavourable non-host plant metabolites might exist within populations of the same species (Matsuki et al. 2011; Piskorski et al. 2011), and anecdotal evidence suggests that similar intra-populational variation might also occur in polylectic bee species (Sedivy et al. 2011). Pre-adaptations concerning the physiological abilities of larvae to develop on non-host diets are one condition for the successful incorporation of a new pollen host, thus, intra- and inter-populational variation in the abilities of bee larvae to develop on non-host pollen diet might possibly indicate an on-going host expansion.

Polylecty is rendered advantageous in terms of reducing the dependency upon a limited spectrum of floral hosts (Eickwort and Ginsberg 1980; Moldenke 1975), but pollen diets from plants with unfavourable pollen properties, such as toxic secondary metabolites or lacking essential nutrients might challenge the physiological abilities of generalist bee larvae (Levin and Haydak 1957; Sedivy et al. 2011; Williams 2003). In contrast to oligoleges, polylectic bee species, however, can provision their offspring with a mixture of pollen from several unrelated plant taxa, and brood cells of the two polylectic species Osmia bicornis and Osmia cornuta indeed often contain more than one pollen type (Budde and Lunau 2007; Tasei
It remains to be investigated, whether pollen mixing in polylectic bees might therefore also be a strategy to mitigate unfavourable pollen properties or to complement missing nutrients. If bee larvae could benefit from the protein content of an unfavourable pollen type that is provided in low concentrations without being negatively affected, pollen mixing might allow a broadened pollen host spectrum without the need to evolve specialized physiological adaptations.

**Thesis outline**

The aim of this PhD study is to gain insights into the factors shaping host plant choice of bees and to assess mechanisms that might allow for an expansion or shift in the pollen host spectrum.

Bees of the three *Osmia* subgenera *Monosmia*, *Orientosmia* and *Osmia* (Hymenoptera, Megachilidae), are used as model organisms to study the patterns of host plant choice in a group of mainly polylectic species. In contrast to previous studies on the evolution of host plant choice in bees, which were conducted on predominantly oligolectic clades (Larkin et al. 2008; Michez et al. 2008; Müller 1996; Sedivy et al. 2013; Sedivy et al. 2008; Sipes and Tepedino 2005), most species of these three *Osmia* subgenera are assumed to be broadly polylectic (Müller 2013; Rust 1974; Westrich 1989). Thus, reconstruction of the phylogenetic relationships in combination with a comprehensive analysis of the pollen host spectra of this group might advance our understanding of the evolution of host plant choice in bees. These *Osmia* species moreover are suitable model organisms for experimental research, as they accept artificial nesting burrows, which facilitates encaged rearing and the manipulation of eggs and brood cell provisions. The latter allows to assess the suitability of pollen from morphologically complex flowers as larval nourishment for unspecialized bees, and to investigate differences between individual bee larvae in their ability to develop on unfavourable pollen diets within and between different populations of the same bee species.
The goal of the first chapter, ‘Phylogeny and floral hosts of a predominantly pollen generalist group of mason bees (Megachilidae, Osmiini)’, is to resolve the phylogenetic relationships within the three Osmia subgenera Monosmia, Orientosmia and Osmia, which most probably form a monophyletic clade (Peters 1978; Praz et al. 2008a), on the basis of molecular data of five genes and morphological data set. In addition, microscopical analyses of the pollen loads of collected female specimens are to provide a comprehensive overview of the pollen spectra of this clade. In combination with the phylogeny, the pollen analyses allow to reconstruct the evolution of host pollen use in this clade, and to elucidate whether the patterns of host plant choice previously observed in oligolectic clades (Larkin et al. 2008; Michez et al. 2008; Sedivy et al. 2013; Sedivy et al. 2008) also hold true for a mainly polylectic clade. As the clade of interest contains at least four species that have successfully been established as commercial pollinators of orchards and several others that have been evaluated for crop pollination in Asia, Europe and North America (Bosch and Kemp 2002; Bosch et al. 2008; Maeta 1978; Torchio 1987), the phylogeny as well as the host plant spectra of this group might also provide important knowledge for the commercial management of these species.

The second chapter, ‘Better safe than sorry? Flowers, which morphologically restrict access to their pollen, exhibit unfavourable pollen properties for bee larval development’, investigates whether pollen diets derived from plants that have their pollen concealed inside complex flower structures permit a successful larval development of both polylectic and oligolectic species that naturally do not collect this pollen. Larval survival, development time and adult body mass of five different megachilid bee species is compared, when being reared on pure pollen diets from two different Fabaceae species, Lotus corniculatus and Onobrychis viciifolia. If unfavourable pollen properties of flowers with easily accessible pollen are adaptive in terms of minimizing pollen loss to bees (Praz et al. 2008c; Sedivy et al. 2011; Williams 2003), pollen from flowers with concealed anthers like the two investigated Fabaceae species is expected to lack such unfavourable properties.
The third and fourth chapter challenge the limitations of the host spectrum breadth of the polylectic bee species *Osmia cornuta*.

In ‘*Intra- and interpopulational variation in the ability of a solitary bee species to develop on non-host pollen: implications for host range expansion*’ it is investigated, whether variation exists within and between different populations of *Osmia cornuta* in the physiological abilities of the larvae to develop on unfavourable pollen diets of *Ranunculus acris* (*Ranunculus*). Such variation is one condition for natural selection to act towards the incorporation of a new pollen host. Larvae of five different European populations of *Osmia cornuta* are reared on pure pollen diets of *Ranunculus* and survival, development time and adult body mass are compared between individuals and populations. In a second step, the fecundity of those individuals that manage to develop to the adult insect is assessed, as only a successful reproduction of the bees surviving on *Ranunculus* diet might allow natural selection to act towards the incorporation of this pollen into the host spectrum of *Osmia cornuta*.

In the last chapter ‘*Pollen mixing in pollen generalist solitary bees: a strategy to complement or mitigate unfavourable pollen properties?*’ the effect of diluting unfavourable *Ranunculus* pollen diet with favourable *Sinapis arvensis* pollen diet is examined by rearing larvae of *Osmia cornuta* on different mixtures of the two diets. If the unfavourable effect of a pure *Ranunculus* pollen diet can be considerably mitigated through providing mixed provisions, pollen mixing in polylectic bees might also constitute a strategy to include otherwise unsuitable plant taxa into their diet, especially if more suitable host plants are rare. To estimate, to which extent such pollen mixing is naturally applied by females of *Osmia cornuta* and three closely related *Osmia* generalists, the frequency of female scopal pollen loads that contain pollen from two or more different plant families is determined.
4 Phylogeny and floral hosts of a predominantly pollen generalist group of mason bees (Megachilidae, Osmiini)

4.1 Abstract

Within the genus *Osmia*, the three subgenera *Osmia*, *Monosmia* and *Orientosmia* form a closely related group of predominantly pollen generalist (“polyleptic”) mason bees. Despite the great scientific and applied interest in several species of this clade, which are promoted commercially for orchard pollination, their phylogenetic relationships remained unresolved so far. We inferred the phylogeny of 21 *Osmia* species belonging to this clade by applying Bayesian and maximum likelihood methods based on five genes and morphology. As our results revealed paraphyly of the largest subgenus, we propose to merge the three subgenera *Osmia*, *Monosmia* and *Orientosmia* into one subgenus *Osmia* comb. nov. Microscopical analysis of female pollen loads revealed that five species are specialized (“oligolectic”) on Fabaceae or Boraginaceae, whereas the remaining species are polylectic, harvesting pollen from up to 19 plant families. Polylecty appears to be the ancestral state with oligolectic lineages having evolved twice independently. Among the polylectic species, several intriguing patterns of host plant use were found, suggesting that host plant choice of these bees is constrained to different degrees and governed by flower morphology, pollen chemistry or nectar availability, thus supporting previous findings on predominantly oligolectic clades of bees.

4.2 Introduction

Among the 19'900 bee species described so far (Ascher and Pickering 2013), only a tiny fraction is commercially used for crop pollination (Bosch and Kemp 2002; Torchio 1987), including several closely related pollen generalist species of the genus *Osmia* (Megachilidae, Osmiini), which are promoted mainly as orchard
pollinators around the world. Within the genus *Osmia*, these commercially managed species belong to the subgenus *Osmia*, which consists of 25 species (Ascher and Pickering 2013) and which forms a monophyletic group with the two species-poor subgenera *Monosmia* and *Orientosmia* (Peters 1978; Praz et al. 2008a). The phylogeny of this group of *Osmia* bees (“*Osmia* s.l. group”) remains largely unresolved, despite the great scientific and applied interest in some of its representatives. Two recent studies including several species of the *Osmia* s.l. group did not satisfactorily uncover their phylogenetic relationships (Bosch et al. 2001; Kwon et al. 2003).

Among the commercial *Osmia* pollinators, *O. cornifrons* has been used to pollinate apple trees in Japan since the 1940s (Kitamura and Maeta 1969). Subsequently, *O. cornuta* and *O. bicornis* have been established for orchard pollination in Europe (Biliński and Teper 2004; Bosch 1994; Krunic and Stanisavljevic 2006), and *O. lignaria* and *O. cornifrons* are now used as pollinators of rosaceous fruit trees in the USA (Abel and Wilson 1998; Bosch and Kemp 2001; Bosch et al. 2006; Torchio 1976; Torchio 1981). Furthermore, *O. ribifloris* has been tested as a potential pollinator of blueberries in the USA (Sampson and Cane 2000; Torchio 1990), and *O. pedicornis*, *O. excavata* and *O. taurus* have been considered for orchard pollination in Asia (Maeta 1978; Wei et al. 2002).

These species promoted for commercial pollination are broadly polylectic, i.e. they harvest pollen on the flowers of two or more different plant families. Apart from these and few further Central European and North American species, for which the pollen hosts are reasonably well known (Bosch and Kemp 2001; Free and Williams 1970; Kraemer and Favi 2005; Márquez et al. 1994; Raw 1974; Rust 1986; Rust 1990; Sheffield et al. 2008; Tasei 1973; Teper and Biliński 2009; Teppner 1996; Torchio 1976; Torchio 1981; Torchio 1990; Vicens et al. 1994; Westrich 1989), the pollen host spectra of the remaining species of the *Osmia* s.l. group have never been analysed in detail. In fact, pollen host preferences as well as the physiological ability to develop on certain pollen diets were found to differ markedly between two
polylectic *Osmia* species within the subgenus *Osmia* (Sedivy et al. 2011; Tasei 1973), suggesting that even pronounced pollen generalist bees exhibit patterns of pollen host use, which may differ from those of related generalist species.

Present knowledge on the host plant choice of bees of the *Osmia* s.l. group suggests that most species are polylectic, while a small proportion of species, including the two species *O. (Osmia) cerinthidis* and *O. (Monosmia) apicata*, are considered oligolectic (Teppner 1996; Westrich 1989), i.e. they restrict pollen collection to plants of a single plant genus or family. The prevalence of polylecty in combination with the occurrence of oligolectic species renders the *Osmia* s.l. group most suitable to address questions about evolutionary transitions between polylecty and oligolecty.

In the present study, we analysed the phylogenetic relationships of the *Osmia* species of the subgenera *Osmia*, *Monosmia* and *Orientosmia* based on five genes and a morphological data set. In addition, we investigated the pollen host spectra of these species by microscopically analysing the pollen loads of collected females. We addressed the following research questions: i) What are the phylogenetic relationships between and within the three subgenera of the *Osmia* s.l. group? ii) What are the pollen hosts of the different species? iii) Are there species-specific patterns of host plant use in the broadly polylectic species? iv) Is oligolecty the ancestral or derived state within this mainly polylectic group of bees?

### 4.3 Methods

#### 4.3.1 Bee species

The subgenus *Osmia* contains 25 described species, 23 of which occur in the Palearctic and two in the Nearctic (Rust 1974; Müller 2013). It is closely related to the exclusively Palearctic subgenera *Monosmia* and *Orientosmia*, which contain one and three species, respectively (Peters 1978; Praz et al. 2008a; Müller 2013). For the present study, we included 17 species of the subgenus *Osmia*, among which *O.*
(Osmia) bicornis and O. (Osmia) lignaria are represented by two subspecies each, and all four species of the subgenera Monosmia and Orientosmia (Table 1). As outgroup, we chose five species of the genus Osmia that represent the five closest related subgenera of the examined group (Praz et al. 2008a). Voucher specimens are deposited in the Entomological Collection of the ETH Zurich.

4. 3. 2 Molecular data

DNA was extracted from specimens preserved in 96% ethanol and from up to 7 year old pinned specimens using DNeasy Blood and Tissue Kit (Qiagen). One mitochondrial gene and four nuclear genes were amplified by PCR: cytochrome oxidase subunit 1 (COI; 1236 bp), CAD (948 bp), elongation factor1-α (F2-copy) (EF; 1493 bp), long-wavelength rhodopsin (ops; 874 bp) and wingless (wnt; 663 bp). Details regarding the primers selected and the PCR conditions applied are provided in Appendix 1. PCR products were purified using ExoSAP (Thermo Fisher Scientific) and sequenced on an automated 3130xl DNA analyzer (Applied Biosystems) using BigDye technology (Applied Biosystems). The sequences were trimmed and assembled in Sequencher 4.10.1 (Gene Codes Corporation) and visually aligned with MacClade 4.08 (Maddison and Maddison 2005). Reading frame and intron/exon boundaries were determined by comparison with published sequences of Osmia cornuta (CAD, EF, ops) and Osmia lignaria (wnt). To ascertain correct reading frames, the coding sequences were converted to amino acid sequences prior to analysis. All intron sections that could not unambiguously be aligned were excluded. The sequences of all five genes were concatenated to one single matrix comprising 5214 characters. GenBank accession numbers and specimen data are given in Appendix 2.

4. 3. 3 Morphological data

No fresh material for DNA extraction was available for O. (Osmia) kohli and O. (Osmia) longicornis. In order to add these two species to the phylogeny, we collected morphological characters for all 21 ingroup species by external
examination of both females and males under a dissecting microscope. Additionally, we dismembered the male abdomina to analyse the otherwise hidden sterna and genitalia. The search for morphological characters was facilitated by the publications of Peters (1978) and Rust (1974). The morphological analysis resulted in a data matrix containing 38 adult morphological characters (Appendices 3 and 4). We did not code morphological characters for the outgroup species as character homology proved to be impossible to ascertain in many cases.

4. 3. 4 Phylogenetic analyses

We performed phylogenetic analyses applying Bayesian and maximum likelihood methods based on the molecular data matrix alone. In addition, Bayesian analyses were performed based on the ‘total evidence’ data matrix that contained both the molecular and morphological characters. These latter analyses were performed for the 24 species with molecular data as well as for all 26 species included in the present study, treating the molecular partitions of O. kohli and O. longicornis as missing data.

To establish a suitable partitioning regime, a preliminary Bayesian analysis was conducted. Each of the five genes was partitioned into first, second and third codon position (e.g. CAD1, CAD2 and CAD3). The introns of CAD, EF, ops and wnt were combined and treated as one additional partition, resulting in a dataset comprising 16 partitions. We run an analysis in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) for five million generations using a general time-reversible model. The resulting parameter files were examined in Tracer 1.5 (Rambaut and Drummond 2009b) and an appropriate burn-in was discarded. Based on the substitution rates and nucleotide compositions for the 16 partitions, similar partitions were grouped together resulting in the following partitioning regime: partition 1 included COI1, COI3, CAD3, EF3, ops3, wnt3 and the introns (2624 bp); partition 2 included COI2, ops1 and ops2 (866 bp); partition 3 included
CAD1, EF1 and wnt1 (862 bp); and partition 4 included CAD2, EF2 and wnt2 (862 bp).

Applying MrModeltest 2.3 (Nylander 2008), 24 models of nucleotide substitution were tested for each partition and the following models, associated with the lowest Akaike Information Criterion (AIC), were selected: GTR+I+G (partitions 1 and 2), GTR (partition 3), F81 (partition 4). Morphological data were defined as an additional partition, for which the standard model for morphological data implemented in MrBayes was applied.

Bayesian analyses were performed using MrBayes under the partitioning and model regime specified above. Partitions were unlinked to allow all parameter values and overall rate of substitution to differ. Markov Chain Monte Carlo analyses were conducted with one cold and three heated chains. We ran four independent analyses for a total of 120 million generations, sampling trees every 2000 generations. From each run, a burn-in of 10% was discarded as determined in Tracer. The resulting 54000 trees were sampled and combined to a 50% majority rule consensus tree using PAUP*4.0a125 (Swofford 2002). Finally, a maximum clade credibility tree that combined the branch lengths of all the post burn-in trees was generated using TreeAnnotator 1.5.3 (Rambaut and Drummond 2009a).

Maximum-likelihood analyses were performed using RaxML 7.0.4 (Stamatakis et al. 2005). The rapid bootstrapping algorithm with a GTR + CAT approximation was applied to perform 1000 bootstrap replicates. Bootstrap replicates were sampled and combined to produce a 50% majority rule consensus tree in PAUP.

4.3.5 Host plants

To assess the pollen host spectra of the 21 Osmia species, we microscopically analysed the scopal pollen contents of 663 female specimens from museum and private collections following the method described by Sedivy et al. (2013). To account for differences in pollen host use and available plant spectrum within and
between populations of the same species, we aimed to analyse pollen samples from females collected at as many different localities as possible. Due to the rareness of certain species, our goal to analyse 50 pollen loads per species could not be attained for every species.

4.3.6 Ancestral state reconstruction

To reconstruct the evolution of host plant choice, we applied parsimony mapping in MacClade using the topology of the majority rule consensus tree of the Bayesian analysis of the total evidence matrix. In addition, maximum likelihood inference of ancestral character states was conducted for three strongly supported nodes with BayesTraits (Pagel et al. 2004) after the outgroup taxa and the non-nominotypical subspecies of *O. bicornis* and *O. lignaria* had been excluded with Mesquite 2.75 (Maddison and Maddison 2011). Transition rates between the two states "oligolectic" and "polylectic" were constrained to be equal in BayesTraits. We analysed a subset of 1000 randomly chosen trees from the Bayesian analyses of the total evidence matrix each for the 19 ingroup species with molecular data and for all 21 ingroup species, including *O. kohli* and *O. longicornis* for which only morphological data were available. When the latter two species were included, their branch lengths could not satisfactorily be estimated due to the missing molecular data. However, as these species were well nested within the clades for which ancestral state reconstruction was performed, the biased branch lengths are not expected to substantially affect the results. To assess the robustness of the ancestral state reconstructions, we successively constrained the ancestral state of each node to one of the two states by using the “fossil” command in BayesTraits. We compared the average ln-likelihood associated with each state and took a difference of two log units as evidence of a “significantly” more likely ancestral state (Pagel 1999).
4. 4 RESULTS

4. 4. 1 Phylogeny

Bayesian analysis of the molecular data matrix yielded a well resolved phylogeny of the 19 *Osmia* species of the subgenera *Osmia*, *Monosmia* and *Orientosmia* (Fig. 1). Addition of the morphological data set considerably increased the posterior probability values (PP) at three nodes at the base of and within the *bicornis* clade (Fig. 1). Maximum likelihood analysis yielded a less well resolved phylogeny, but there were no topological incongruences compared to the Bayesian analysis (Fig. 1). Bayesian analysis including those two species for which molecular data were missing placed *O. kohli* as sister to *O. tricornis* with maximal support (PP = 100) and *O. longicornis* as sister to *O. cerinthidis* with only weak support (PP = 66) (Appendix 5).

The three subgenera form a maximally supported clade (PP = 100; Fig. 1), as do the two subgenera *Monosmia* and *Orientosmia* (PP = 100). However, monophyly of the subgenus *Osmia* was not confirmed as the clade (*Monosmia* + *Orientosmia*) turned out to be sister to only part of the species of the subgenus *Osmia* (PP = 96-97), rendering the latter subgenus paraphyletic. In addition, *O. (Osmia) ribifloris* appears to be sister of all other species, although with only weak support (PP = 73-75). Based on our phylogeny (Fig. 1), the species other than the basal *O. ribifloris* can be grouped into three morphologically well circumscribed clades (Fig. 1): 1) the *apicata* clade comprising all species that lack clypeal horns and possess extraordinarily elongated mouthparts, which are as long as the whole body when fully extended and still reach beyond the thorax when folded together; 2) the *emarginata* clade comprising all species that have neither clypeal horns nor elongated mouthparts; and 3) the *bicornis* clade comprising all species that have a pair of horns or horn-like structures laterally on the clypeus and possess mouthparts of normal length (Fig 1).
Figure 1. Phylogeny of *Osmia* bees of the subgenera *Osmia*, *Monosmia* and *Orientosmia*. Maximum clade credibility tree based on 54,000 post burn-in trees from four independent Bayesian analyses. Bayesian posterior probabilities are given for the analysis of the molecular data set and for the combined molecular plus morphological data set. As morphological character states were not coded for the outgroup, Bayesian posterior probabilities of the combined data set are only given for the ingroup. Maximum likelihood bootstrap values are given for the analysis of the molecular data set.
Table 1. Pollen host spectrum of 21 *Osmia* species of the subgenera *Osmia*, *Monosmia* and *Orientosmia*. Definitions of bee host ranges after Müller and Kuhlmann (2008).

<table>
<thead>
<tr>
<th>Bee species</th>
<th>n</th>
<th>N</th>
<th>Origin of pollen loads</th>
<th>% pollen grain volume</th>
<th>% pure loads of preferred family</th>
<th>Host range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Osmia</em> (<em>Monosmia</em>) <em>apicata</em></td>
<td>52</td>
<td>34</td>
<td>AM, GR, IL, IR, JO, TR</td>
<td>BOR (<em>Onosma</em>) 82.9, BOR (<em>Echium</em>) 14.4, BOR (<em>Podonosma</em>) 2.5, BOR (indet) 0.3</td>
<td>100</td>
<td>oligolectic on Boraginaceae with strong preference for <em>Onosma</em></td>
</tr>
<tr>
<td><em>Osmia</em> (<em>Osmia</em>) <em>bicornis</em></td>
<td>50</td>
<td>48</td>
<td>AT, CH, DE, ES, FR, LI, GR, IT, IR, KS, MA, NL</td>
<td>RAN (<em>Ranunculus</em>) 22.1, FAG (<em>Quercus</em>) 16.7, CIS 14.1, PAP (<em>Papaver</em>) 10.6, ACE (<em>Acer</em>) 10.4, ROS 4.9, BRA 4.5, BOR (<em>Echium</em>) 3.6, LAM 2.8, CAP (<em>Lonicera</em>) 2.4, SAL (<em>Salix</em>) 1.9, FAB 1.2, JUG (<em>Juglans</em>) 0.7, PLA (<em>Plantago</em>) 0.7, MAL (<em>Tilia</em>) 0.6, MON 0.3, AST 0.2, CAR 0.1, CAM 0.1, unknown 2.2</td>
<td>10</td>
<td>polylectic (19 plant families)</td>
</tr>
<tr>
<td><em>Osmia</em> (<em>Osmia</em>) <em>cerinthidis</em></td>
<td>52</td>
<td>38</td>
<td>AT, CZ, DE, FR, HR, IT, MK, PL, TR, UA</td>
<td>BOR (<em>Cerinthe</em>) 90.5, BOR (<em>Onosma</em>) 2.2, BOR (<em>Echium</em>) 0.1, ROS 4.9, MON 1.4, unknown 0.9</td>
<td>90</td>
<td>oligolectic on Boraginaceae with strong preference for <em>Cerinthe</em></td>
</tr>
<tr>
<td><em>Osmia</em> (<em>Osmia</em>) <em>cornifrons</em></td>
<td>28</td>
<td>21</td>
<td>JP, KR, RU, US</td>
<td>FAB 43.6, ROS 26.3, BRA 13.6, SAL (<em>Salix</em>) 4.2, LAM 4.2, ERI 2.5, AQU (<em>Ilex</em>) 1.3, AMA (<em>Allium</em>) 0.7, RAN (<em>Ranunculus</em>) 0.2, unknown 3.4</td>
<td>18</td>
<td>polylectic (9 plant families)</td>
</tr>
<tr>
<td>Species</td>
<td>Range</td>
<td>Population</td>
<td>Distribution</td>
<td>Forage</td>
<td>Nesting Preference</td>
<td></td>
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<tr>
<td><em>Osmia (Osmia) cornuta</em></td>
<td>CH, DE, DZ, ES, GR, HR, IR, IT, JO, KZ, LI, TR</td>
<td>50 41</td>
<td>ROS 57.5, SAL (<em>Salix</em>) 13.8, PAP (<em>Corydalis</em>) 7.2, ACE (<em>Acer</em>) 7.0, MON 6.7, BRA 2.4, ERI 1.8, RAN (<em>Anemone</em>) 1.7, unknown 2.0</td>
<td>polylectic (8 plant families)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Osmia) emarginata</em></td>
<td>ES, FR, MA, TN</td>
<td>24 17</td>
<td>BOR (<em>Echium</em>) 38.0, CIS 27.7, FAB 26.7, PAP (<em>Papaver</em>) 7.1, LAM 0.6</td>
<td>polylectic (5 plant families)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Osmia) excavata</em></td>
<td>JP</td>
<td>19 15</td>
<td>BRA 67.8, FAB 12.9, CAP (<em>Lonicera</em>) 6.3, MON 4.7, ROS 4.1, RAN (<em>Ranunculus</em>) 0.7, BOR (<em>Echium</em>) 0.1, unknown 3.4</td>
<td>polylectic (7 plant families)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Osmia) fedtschenkoi</em></td>
<td>AF, PK, TJ, UZ</td>
<td>22 12</td>
<td>FAB 77.9, ROS 13.5, BRA 5.1, CAP (<em>Lonicera</em>) 1.5, SAL (<em>Salix</em>) 1.4, unknown 0.6</td>
<td>polylectic (5 plant families) with strong preference for Fabaceae*</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Osmia) kohli</em></td>
<td>IT, MT</td>
<td>33 29</td>
<td>PAP (<em>Papaver</em>) 43.3, FAB 32.3, BOR (<em>Echium</em>) 8.9, RAN (<em>Ranunculus</em>) 4.4, BRA 4.1, CIS 3.3, FAG (<em>Quercus</em>) 1.1, LAM 0.9, JUG (<em>Juglans</em>) 0.5, API 0.4, ROS 0.1, unknown 0.8</td>
<td>polylectic (11 plant families)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Osmia) lignaria</em></td>
<td>CA, US</td>
<td>50 50</td>
<td>SAL (<em>Salix</em>) 27.2, BOR (Hydrophylloideae) 18.0, BOR (<em>Echium</em>) 0.7, FAB 17.1, ROS 16.9, ACE (<em>Acer</em>) 11.0, OLE 1.2, GRO (<em>Ribes</em>) 0.5, AST 0.3, FAG (<em>Quercus</em>) 0.1, unknown 6.9</td>
<td>polylectic (9 plant families)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Collection</td>
<td>Geographic Range</td>
<td>Foraging Habits</td>
<td></td>
<td></td>
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<td>----------------------------------------------</td>
<td>------------</td>
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<td>--------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td><em>Osmia (Osmia) longicornis</em> Morawitz, 1875</td>
<td>3</td>
<td>UZ</td>
<td>ROS 98.1, SAL (Salix) 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Orientosmia) maxillaris</em> Morawitz, 1877</td>
<td>23</td>
<td>KS, KZ, UZ, TM</td>
<td>FAB 100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Orientosmia) maxschwarzi</em> Müller, 2012</td>
<td>6</td>
<td>IR, TR</td>
<td>FAB 99.7, CAM 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Osmia) melanocephala</em> Morawitz, 1875</td>
<td>12</td>
<td>KS, KZ, MN</td>
<td>FAB 73.0, ROS 10.4, MON 9.3, CAP (Lonicera) 2.4, BOR 1.5, unknown 3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Osmia) mustelina</em> Gerstaecker, 1869</td>
<td>50</td>
<td>AT, CH, CZ, DE, FR, GR, IT, IL, JO, TR</td>
<td>FAB 58.4, PAP (Papaver) 16.7, CIS 16.4, BOR (Echium) 4.7, BOR (Anchusa) 0.3, PLA (Plantago) 1.5, ROS 1.1, AST 0.5, RAN 0.1, unknown 0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Osmia) nigrohirta</em> Friese, 1899</td>
<td>27</td>
<td>GR, IR, TR</td>
<td>FAB 51.6, BOR (Echium) 16.3, LAM 12.8, CIS 7.5, AST 3.8, BRA 3.1, ROS 1.4, PLA (Antirrhineae) 1.2, API 0.3, PAP (Papaver) 0.2, unknown 1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Osmia (Osmia) pedicornis</em> Cockerell, 1919</td>
<td>32</td>
<td>CN, JP</td>
<td>FAB 34.1, FAG (Quercus) 23.3, RAN (Ranunculus) 19.1, BRA 12.7, JUG (Juglans) 5.3, ROS 3.4, AST (Cichorioideae) 1.0, unknown 1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1 continued

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Loads</th>
<th>Location</th>
<th>Plant Families</th>
<th>Polylectic (Families)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Osmia (Osmia) ribifloris</em> Cockerell, 1900</td>
<td>50</td>
<td>42</td>
<td>US</td>
<td>ERI (<em>Arbutus, Arctostaphylos, Vaccinium</em>) 50.5, FAB (<em>Cercis, Sophora</em>) 18.1, BER (<em>Berberis, Mahonia</em>) 13.5, GRO (<em>Ribes</em>) 8.0, EBE (<em>Diospyros</em>) 3.3, ROS (<em>Prunus</em>) 2.1, LAM (<em>Salvia</em>) 1.4, ANA (Rhus) 1.4</td>
<td>48 polylectic (8)</td>
</tr>
<tr>
<td><em>Osmia (Orientosmia) scheherezade</em> Peters, 1978</td>
<td>18</td>
<td>10</td>
<td>IR, TR</td>
<td>FAB 100</td>
<td>100 oligolectic on Fabaceae</td>
</tr>
<tr>
<td><em>Osmia (Osmia) taurus</em> Smith, 1873</td>
<td>12</td>
<td>9</td>
<td>JP, RU</td>
<td>JUG (<em>Juglans</em>) 38.1, FAB 21.2, FAG (<em>Quercus</em>) 12.9, ALT (<em>Liquidambar</em>) 9.5, CAP (<em>Lonicera</em>) 5.7, BRA 5.0, ROS 4.8, AST 2.6, RAN (<em>Ranunculus</em>) 0.2</td>
<td>25 polylectic (9)</td>
</tr>
<tr>
<td><em>Osmia (Osmia) tricornis</em> Latreille, 1811</td>
<td>50</td>
<td>41</td>
<td>DZ, ES, IT, MA, TN</td>
<td>CIS 67.1, PAP (<em>Papaver</em>) 14.1, FAB 5.6, BOR (<em>Echium</em>) 3.3, BOR (<em>Anchusa</em>) 0.4, BRA 3.0, RAN (<em>Ranunculus</em>) 1.5, ROS 0.8, LAM 0.8, MON 0.5, PLA (Antirrhineae) 0.3, MIM 0.3, AST 0.1, unknown 2.2</td>
<td>24 polylectic (12)</td>
</tr>
</tbody>
</table>

Notes: n, total number of pollen loads; N, number of pollen loads from different localities. States: AF, Afghanistan; AM, Armenia; AT, Austria; CA, Canada; CH, Switzerland; CN, China; CZ, Czech Republic; DE, Germany; DZ, Algeria; ES, Spain; FR, France; GR, Greece; HR, Hungary; IL, Israel and Palestine; IR, Iran; IT, Italy; JO, Jordan; JP, Japan; KOR, South Korea; KS, Kyrgyzstan; KZ, Kazakhstan; LI, Liechtenstein; MA, Morocco; MK, Macedonia; MN, Mongolia; MT, Malta; NL, Netherlands; PK, Pakistan; PL, Poland; RU, Russia; TJ, Tajikistan; TN, Tunisia; TR, Turkey; UA, Ukraine; US, United States of America; UZ, Uzbekistan. Plant families: ACE, Aceraceae; AMA, Amaryllidaceae; ANA, Anacardiaceae; ALT, Altingiaceae; API, Apiaceae; AQU, Aquifoliaceae; AST, Asteraceae; BER, Berberidaceae; BOR, Boraginaceae; BRA, Brassicaceae; CAM, Campanulaceae; CAP, Caprifoliaceae; CAR, Caryophyllaceae; CIS, Cistaceae; EBE, Ebenaceae; ERI, Ericaceae; FAB, Fabaceae; FAG, Fagaceae; GRO, Grossulariaceae; JUG, Juglandaceae; LAM, Lamiaaceae; MAL, Malvaceae; MIM, Mimosaceae; MON, Monocots; OLE, Oleaceae; PLA, Plantaginaceae; PAP, Papaveraceae; RAN, Ranunculaceae; ROS, Rosaceae; SAL, Salicaceae. * The observed pollen spectrum of *O. fedtschenkoi* might be biased due to a large number of specimens collected at the same locality and date (n = 6).
4. 4. 2 Host plants

Based on the microscopical analysis of the scopal pollen loads, we classified five of the 21 species as oligolectic (Table 1). Three of these pollen specialist species restrict pollen harvesting to Fabaceae and two species are strictly specialized to Boraginaceae exhibiting a clear preference for Onosma and Cerinthe, respectively. Fifteen species were classified as polylectic collecting pollen on 5 to 19 plant families. The host plant spectrum of O. longicornis could not be determined conclusively due to the low number of pollen samples. As one of the three pollen samples contained a mixture of pollen from two plant families, O. longicornis is probably polylectic as are the other members of the bicorinis clade except O. cerinthidis.

Among the polylectic species, several intriguing patterns of pollen host use emerge (Table 1, Fig. 2): 1) Fabaceae play a predominant role as hosts for the species of the emarginata clade and are also regularly exploited by Osmia ribifloris and several species of the bicornis clade; 2) Asteraceae remain almost unexploited; pollen of this family was found in low proportions of mostly below 20% and invariably mixed with other pollen types in only ten pollen loads of six species; 3) O. ribifloris exhibits a distinct preference for plant taxa that bear their anthers inside bell-shaped flowers such as Ericaceae (Arbutus, Arctostaphylos, Vaccinium), Berberidaceae (Berberis, Mahonia), Ebenaceae (Diospyros) and Grossulariaceae (Ribes); 4) Flowers containing no or very little nectar such as Quercus (Fagaceae), Ranunculus (Ranunculaceae), Juglans (Juglandaceae), Cistaceae, Papaver (Papaveraceae) or Liquidambar (Altingiaceae) play an important role as hosts of the clade composed of O. taurus (60.7% pollen from nectarless or -poor flowers), O. kohli (52.6%), O. tricornis (82.7%), O. pedicornis (47.7%) and O. bicornis (64.9%); 5) Two pairs of sister species show very similar host plant preferences: Fabaceae, Cistaceae, Echium (Boraginaceae) and Papaver are the almost exclusive pollen hosts of O. emarginata (99.4% pollen from these four plant taxa) and O. mustelina (96.2%);
Figure 2. (Caption opposite)
Figure 2. (opposite.) Pollen hosts and evolution of host breadth in Osmia bees of the subgenera Osmia, Monosmia and Orientosmia. Parsimony mapping of host breadth is based on the topology of the 50% majority rule consensus tree of 54'000 trees from four independent Bayesian analyses. Outgroup species as well as subspecies were omitted. Oligolectic branches are coloured grey, polylectic branches black, equivocal branches dashed. The pie charts at three well resolved nodes (A-C) give the maximum likelihood probabilities of the two different states, with asterisks indicating that the analyses constraining the more likely state had significantly higher log likelihood values than analyses with the alternative state constrained. The coloured pie charts beside the species names give the proportions of the different pollen hosts collected by each species except O. longicornis (for which only three pollen samples were available) based on microscopical analysis of pollen loads from collected females.

Ranunculus and Quercus are among the most important pollen hosts of O. bicornis (38.8% pollen from these two plant taxa) and O. pedicornis (42.4%); 6) Several species of the bicornis clade seem to incline towards the use of a main single pollen host, i.e. O. cornuta (57.5% pollen of Rosaceae), O. cornifrons (43.6% pollen of Fabaceae), O. excavata (67.8% pollen of Brassicaceae), O. kohli (43.3% pollen of Papaver) and O. tricornis (67.1% pollen of Cistaceae).

4.4.3 Ancestral state reconstruction

Based on the total evidence phylogeny, parsimony mapping of host plant breadth suggests polylecty to be the ancestral state, and oligolecty to have evolved twice from polylectic ancestors (Fig. 2). Maximum likelihood inference of host plant breadth at the three selected nodes A-C including the two species for which only morphological data were available confirmed this result. The ancestor at all three nodes was most probably polylectic with likelihood probabilities of 99.0% (node A), 79.5% (node B) and 99.2% (node C) and negative log likelihood differences of 3.7, 3.9 and 4.5. These values did only marginally differ for the analysis that excluded the two species with missing molecular data (likelihood probabilities of 99.2% (node A), 79.3% (node B), 99.0% (node C) and negative log likelihood differences of 3.8, 4.0 and 4.6).
4.5 DISCUSSION

The present study provides for the first time a well resolved phylogeny of the commercially managed *Osmia* pollinators and their relatives. It additionally uncovers a number of intriguing patterns of host plant use in this predominantly polylectic group of bees and identifies two evolutionary shifts from polylecty to oligolecty.

4.5.1 Phylogeny

Our phylogeny differs from the current morphology-based subgeneric classification (Michener 2007) in that the subgenus *Osmia* appears to be paraphyletic due to the sister group relationship of (*Monosmia* + *Orientosmia*) with the *emarginata* group. Although the basal position of *O. ribifloris* is only weakly supported, the strongly differing host plant spectrum of this species is in line with its distant position. Given the paraphyly of the subgenus *Osmia* in conjunction with the pronounced morphological resemblance among the species of all three subgenera (Peters 1979; Müller 2012), we propose to merge the three subgenera *Osmia* (25 species), *Monosmia* (1 species) and *Orientosmia* (3 species) into a single subgenus *Osmia* comb. nov.

4.5.2 Patterns of host plant use

Among the most important pollen hosts of bees of the *Osmia* s.l. group are Fabaceae, which are the exclusive hosts of three species of the *apicata* clade, major hosts of all species of the *emarginata* clade and regular hosts of *O. ribifloris* as well as of several species of the *bicornis* clade. The importance of Fabaceae for these bee species is in line with the fact that zygomorphic flowers are common hosts of many higher megachilid lineages such as the *Osmia* s.l. group, whereas the basal representatives of both the Megachilidae and the Osmiini predominantly exploit actinomorphic flowers with well accessible anthers (Litman et al. 2011; A. Müller and C. Sedivy, unpublished). Fabaceae are also among the most important pollen
hosts of the closely related subgenera Pyrosmia, Helicosmia and Melanosmia (Müller 2013), suggesting that the exploitation of Fabaceae by species of the Osmia s.l. group is an ancestral trait. In fact, O. bicornis and O. cornuta were experimentally found to be able to develop on pure pollen diets of two Fabaceae species (M. Haider, S. Dorn and A. Müller, submitted), although both belong to those few species of the O. bicornis group that do not appear to commonly exploit Fabaceae (Table 1).

O. apicata and O. cerinthidis are specialized on Onosma and Cerinthe, respectively, both members of the Boraginaceae. The closest relatives of these two oligolectic bee species are either strictly specialized on Fabaceae or regularly exploit Fabaceae for pollen. This pattern corresponds to the “Boraginaceae-Fabaceae paradox”, which describes the counterintuitive affinity of bees of a clade of Hoplitis species towards both Boraginaceae and Fabaceae, which are neither closely related nor share similar flower morphologies (Sedivy et al. 2013). The affinity of the Hoplitis bees towards these two plant families was hypothesized to be due to similar chemical properties of their pollen, which might require similar physiological adaptations to digest it. In the apicata clade, the considerably elongated mouthparts, which are typical for all its species (Müller 2012), might have contributed to facilitate switches between Fabaceae with long flower tubes (e.g. Astragalus) and Onosma, as such long mouthparts allow to suck deeply hidden nectar during pollen harvesting.

Asteraceae are ubiquitous in most terrestrial habitats and their inflorescences produce considerable amounts of pollen, which is easily harvestable for any flower visitor. However, among the 663 pollen loads of 21 Osmia species analysed in the present study, only ten contained small quantities of Asteraceae pollen, which amazes given the high phylogenetic and morphological diversity of the pollen hosts exploited by these mostly polylectic bees. This observation supports recent findings that the pollen of many Asteraceae taxa possesses chemical properties that interfere with its digestion by the larvae of unspecialized bees and again suggests that bees
need physiological adaptations to overcome these unfavourable properties (Müller and Kuhlmann 2008; Praz et al. 2008c). In fact, the larvae of *O. bicorns* and *O. cornuta* proved to be incapable of developing on a pollen diet composed of pure pollen of *Tanacetum* (Asteroideae) (Sedivy et al. 2011), and pollen of *Taraxacum* (Cichorioideae) was experimentally found to exert lethal effects on developing larvae of *O. lignaria* if provided as sole food (Levin and Haydak 1957; Rust 1990).

The pollen host spectrum of *O. ribifloris* differs strongly from that of all other members of the *Osmia* s.l. group. Species of Ericaceae, Berberidaceae, Ebenaceae or Grossulariaceae, which all have their anthers more or less concealed inside bell-shaped flowers of rather small size, play a predominant role as pollen hosts. As these host plant taxa are not closely related except for Ericaceae and Ebenaceae, which both belong to the Ericales (APG 2009), flower architecture was probably pivotal in shaping the host plant preferences of *O. ribifloris*, supporting previous findings that flower morphology and mode of pollen presentation may play an important role for bees in acquiring new pollen hosts (Müller 1996; Sipes and Tepedino 2005; Sedivy et al. 2008). In fact, females of *O. ribifloris* apply a specialized behaviour when collecting pollen on *Vaccinium* (Torchio 1990). They insert their forelegs into the flowers and rapidly strike the staminal filaments with sufficient force to cause the anthers to vibrate, resulting in pollen release from the poricidal anthers. A similar pollen harvesting technique is applied on flowers of *Berberis* and *Diospyros* (J. Neff, personal communication). Interestingly, females of *O. ribifloris* possess a modified pilosity on the basitarsi of their forelegs composed of apically curved bristles, which probably help to remove pollen from the host flowers, as do similar modified bristles on proboscides or forelegs of other bees that harvest pollen on narrow-tubed flowers (Müller 1995; Sedivy et al. 2013). Another possible adaptation of *O. ribifloris* to its hosts is the particular pilosity of the ventral scopa. Compared to all other species of the *Osmia* s.l. group, this pilosity is relatively sparse and composed of thick hairs, which might facilitate the transport of the large pollen grains of Ericaceae, Berberidaceae and *Diospyros*, similar to the extremely sparse scopal pilosity of some *Hoplitis* and *Tetralonia* species that harvest the giant pollen grains.
of Malvaceae (A. Müller, unpublished) and the stout, unbranched scopal hairs of *Xenoglossa* species that collect the very large pollen grains on *Cucurbita* (Roberts and Vallespir 1978).

In contrast to all other species of the *Osmia* s.l. group, the pollen host spectra of *O. bicornis*, *O. kohli*, *O. pedicornis*, *O. taurus* and *O. tricornis* are dominated by plants that provide no or very little nectar, which supports previous observations on the favoured pollen hosts of *O. bicornis* and *O. tricornis* (Free and Williams 1970; Tasei 1973; Vicens *et al.* 1994). The brood cell provisions of *O. bicornis*, *O. taurus* and *O. tricornis* appear to be relatively dry, which has been attributed to their low nectar content (Maeta 1978; Westrich 1989; Vicens *et al.* 1994). However, sugar content in provisions of *O. bicornis* was not found to significantly differ from that in provisions of *O. cornuta* (A. Bühler and A. Müller, unpublished), a species that usually does not collect pollen on nectarless flowers (Table 1). This suggests that *O. bicornis* and its close relatives must satisfy their nectar needs on other plant taxa than their preferred pollen hosts, as has been assumed previously (Raw 1974). In fact, *O. lignaria* has been hypothesized to compensate for the moderate nectar content of single flowers of *Salix*, one of its preferred pollen hosts (Table 1), by regularly combining visits to both the pollen-rich flowers of *Salix* and the nectar-rich flowers of *Hydrophyllum* on the same foraging bouts (Williams and Tepedino 2003). During nectar uptake on the *Hydrophyllum* flowers, the females also collect their pollen, probably because the simultaneous harvesting of pollen does not induce much additional costs. Correspondingly, the particularly high diversity of pollen hosts of *O. bicornis* encompassing 35% of pollen from plants that produce nectar (Table 1) might partly be due to casual pollen harvesting during flower visits that are primarily intended to collect nectar. Even if the pollen of such nectar hosts does not support larval development when provided as sole food, its unfavourable properties might be compensated or mitigated by the admixture of favourable pollen (M. Eckhardt, M. Haider, S. Dorn and A. Müller, submitted).
Two pairs of polylectic sister species of the *Osmia* s.l. group each show intriguing similarities in host plant choice irrespective of their geographic range. The western Mediterranean *O. emarginata* and the eastern Mediterranean *O. mustelina* almost exclusively exploit hosts from the very same four plant taxa. Similarly, *Ranunculus* and *Quercus* are among the most important pollen sources both of the western Palearctic *O. bicornis* and the eastern Palearctic *O. pedicornis*; these two species are also the only two species of the *Osmia* s.l. group that collect *Ranunculus* pollen in considerable amounts. The larvae of *O. bicornis* were experimentally found to be able to develop on a pure *Ranunculus* pollen diet in contrast to the larvae of *O. cornuta*, where only a small proportion of the tested individuals managed to develop into dwarfish adults (Sedivy *et al.* 2011; Haider *et al.* 2013). This suggests that the physiological ability to digest *Ranunculus* pollen has been newly acquired by the ancestor of *O. bicornis* and *O. pedicornis*. The overlapping host plant spectra as observed for these polylectic sister species are consistent with previous studies on predominantly oligolectic clades, which found that closely related oligoleges often exploit the same host plant taxa and that emerging polyleges usually retain the host used by their ancestors while including new hosts into their diet that are already exploited by closely related species (Larkin *et al.* 2008; Michez *et al.* 2008; Sedivy *et al.* 2008; Sedivy *et al.* 2013). Thus, we hypothesize that also the host plant spectra of broadly polylectic bees are usually conserved to some degree and governed by constraints regarding pollen digestion or flower recognition and handling as is assumed for oligolectic bees (Sedivy *et al.* 2008).

### 4.5.3 Evolution of host range

Polylecty appears to be the ancestral state in the *Osmia* s.l. group with oligolectic species having evolved twice independently from polylectic ancestors, once in the ancestor of the *apicata* clade and once in *O. cerinthidis*. Although the specialization of *O. cerinthidis* might not be complete yet as this species collects pollen to a small extent also from plant families other than Boraginaceae, the strong
preference for *Cerinthe* stands in sharp contrast to the broadly polylectic habit of the other species of the *bicornis* clade and illustrates a transition from polylecty to oligolecty that might even become more pronounced in future. It is tempting to speculate that those polylectic species of the *bicornis* clade that exhibit an affinity towards one single host, i.e. *O. excavata*, *O. cornuta*, *O. cornifrons*, *O. kohli* and *O. tricornis*, might represent a transitional stage between polylecty and oligolecty as has been hypothesized for polylectic species of *Colletes* bees that show a pronounced preference for a single host plant taxon (Müller and Kuhlmann 2008).

In the *Osmia* s.l. group, no switch from oligolecty to polylecty could be observed, which is in contrast to other studies on mostly oligolectic taxa, where shifts from oligolecty to polylecty predominated (Müller 1996; Larkin *et al.* 2008; Michez *et al.* 2008; Sedivy *et al.* 2008; Sedivy *et al.* 2013). Growing evidence suggests that oligolecty is the ancestral state in bees as the most basal clades appear to be highly host specific (reviewed in Danforth *et al.* 2013). As the basal lineages of both the Megachilidae and the Osmiini are also characterized by a narrow host plant range (Sedivy *et al.* 2008; Litman *et al.* 2011), the polylectic ancestry of the *Osmia* s.l. group is consistent with its more recent origin.

### 4.5.4 Conclusions

The present study provides a well resolved phylogeny of a clade of mason bees, which for the first time sheds light on the phylogenetic relationships of those *Osmia* species that have been established as commercial pollinators around the world. Being the first study on the evolution of host plant choice in a group of mainly pollen generalist bees, our results clearly demonstrate that even broadly polylectic bee species may exhibit distinct patterns of host plant use and suggests that flower morphology, pollen chemistry and nectar availability may have played crucial roles in shaping their pollen host preferences.
Appendix 1. Primers and PCR conditions used for amplification of the five genes CAD, COI, Elongation Factor-1α, LW-Rhodopsin and Wingless.

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<td>TGG AAR GAR GTB GAR TAC GAR GTG GTY CG</td>
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<td>CADRev1-Meg</td>
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<td>Praz et al. 2008a</td>
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<td>Litmann et al. 2011</td>
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<td>COIRev2Osm</td>
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# Appendix 2. Specimens used in this study for DNA extraction, with collector, locality information and GenBank accession numbers.

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Appendix 3: Adult morphological characters and character states used in this study. The terminology of bee morphology follows Michener (2007).

**Females and males**

1. Body colour distinctly metallic blue to green (1), black without distinct metallic sheen (0).
2. Proboscis strongly elongated and overlapping the thorax when folded together (1), distinctly shorter (0).
3. Hind coxa ventrally with polished area (1), without polished area (0).
4. Apical zone of metasomal terga with distinct hair bands (2), with indistinct hair bands (1), without hair bands (0).

**Females**

5. Apical zone of clypeus with two projecting tubercles or horns (3), strongly emarginated (2), slightly emarginated (1), unmodified and more or less straight (0).
6. Clypeus medioapically with a bifid projection (3), with a long and more or less parallel-sided projection (2), with a short and triangular to roundish projection (1), without projection (0).
7. Clypeus in the non-modified basal part carinate (1), not carinate (0).
8. Polished apical zone of clypeus lacking or very narrow (0), not carinate (1), carinate (2), with a raised lamella (3).
9. Polished apical zone of clypeus longer than half as clypeal length (2), less than half as long as clypeal length (1), very narrow or lacking (0).
10. Lateral hair brushes at the apex of the clypeus long and narrow and protruding from the horns (3), long and narrow and protruding from the apical margin of the clypeus (2), long and narrow and protruding from below the clypeus (1), short to moderately long and broad and protruding from the apical margin of the clypeus (0).
11. Basal upper margin of mandible less than half as long as apical lower margin (2), about half as long as lower margin (1), more than half as long as lower margin (0).
12. Cutting edge of mandible above the subapical tooth with a short and curved stepped zone (2), with a long and straight stepped zone (1), without a stepped zone (0).
13. Cutting edge of mandible with long and thick bristles in its proximal half (1), without or with much shorter bristles (0).
14. Condylar ridge of mandible not reaching the proximal third of the mandible (1), distinct till near the mandibular base (0).
15. Condylar ridge of mandible distinctly raised proximally (1), not distinctly raised (0).
16. Genal area in its lowermost part with a deep and distinctly edged impression (2), with a rather shallow impression (1), without impression (0).
17. Tibial spur of fore leg not elongated and blunt or with a very short tip (2), weakly elongated and pointed (1), distinctly elongated and pointed (0).
18. Tibial spurs of hind and middle leg rounded (1), acute (0).
19. Colour of metasomal scopa black (2), white (1), yellowish-red (0).
Males

20 Antenna long and extending beyond the thorax (1), shorter and not extending beyond the thorax (0).

21 Antennal segment 3 slightly longer or of the same length as segment 4 (1), shorter than segment 4 (0).

22 Apical margin of clypeus with a broad polished transversal zone (1), without or with a very narrow polished transversal zone (0).

23 Propodeal pit polished or only weakly shagreened (1), densely shagreened and entirely mat (0).

24 Propodeal pit broadly oval to roundish (1), narrowly oval to rectangular (0).

25 Apical margin of tergum 6 slightly emarginated medially (1), not emarginated (0).

26 Apical margin of sternum 2 truncate (1), convex (0).

27 Apical margin of sternum 4 truncate (1), convex (0).

28 Apical margin of sternum 5 emarginate medially (1) not emarginate (0).

29 Sternum 6 with gradulus (1), without gradulus (0).

30 Proximal half of sternum 8 distinctly shorter than distal half (1), of about the same length as distal half (0).

31 Distal part of sternum 8 broad and almost parallel-sided basally and prolonged into a narrow tip apically (1), of other shape (0).

32 Penis valve broadening towards its middle and again narrowing towards the apex (3), gradually narrowing towards the apex (2), parallel-sided in the basal half and distinctly narrowing after the middle (1), parallel-sided over most of its length and narrowing only in the apical fourth (0).

33 Penis valve distinctly longer than gonocoxite (1), as long as gonocoxite (0).

34 Apex of penis valve rather broad and rounded (1), very narrow and pointed (0).

35 Gonocoxite laterally strongly flattened (1), not flattened (0).

36 Gonocoxite subapically with nearly right-angled edge, which is only visible in side view (1), of other shape (0).

37 Apex of gonocoxite curved inwards but not distinctly detached from the subapical part (1), distinctly detached from the subapical part (0).

38 Apex of gonocoxite extended into a long, distinctly stepped and narrow finger (1), of other shape (0).
### Appendix 4. Morphological character matrix. Unknown states are coded as “?”.

<table>
<thead>
<tr>
<th>Species</th>
<th>Character Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Osmia apicata</em></td>
<td>0110000000001000010100001101000010</td>
</tr>
<tr>
<td><em>Osmia bicornis bicornis</em></td>
<td>0000330122000000100010100000000100010001000</td>
</tr>
<tr>
<td><em>Osmia bicornis globosa</em></td>
<td>0000330122000000100010100000000100010001000</td>
</tr>
<tr>
<td><em>Osmia cerinthidis</em></td>
<td>0000310223000001000101000000000100010001000</td>
</tr>
<tr>
<td><em>Osmia cornifrons</em></td>
<td>001310113020010010000001000100010001000010</td>
</tr>
<tr>
<td><em>Osmia cornuta</em></td>
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</tr>
<tr>
<td><em>Osmia emarginata</em></td>
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</tr>
<tr>
<td><em>Osmia excavata</em></td>
<td>0002300223200011001010000001000100010001000000</td>
</tr>
<tr>
<td><em>Osmia fedtschenkoi</em></td>
<td>100220100100011010001010110000100210101000</td>
</tr>
<tr>
<td><em>Osmia kohli</em></td>
<td>000032121210101200010100000100010001000000</td>
</tr>
<tr>
<td><em>Osmia lignaria lignaria</em></td>
<td>100030113201002002101000001003000000000</td>
</tr>
<tr>
<td><em>Osmia lignaria propinqua</em></td>
<td>10003001132010020021010000001003000000000</td>
</tr>
<tr>
<td><em>Osmia longicornis</em></td>
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</tr>
<tr>
<td><em>Osmia maxillaris</em></td>
<td>111200000000010021101001010102010110010000</td>
</tr>
<tr>
<td><em>Osmia maxschwarzii</em></td>
<td>111200000000010020101010101011010010000</td>
</tr>
<tr>
<td><em>Osmia melanocephala</em></td>
<td>00000000001010010001010100000001021001000</td>
</tr>
<tr>
<td><em>Osmia mustelina</em></td>
<td>000000100001010010010101010000001021001000</td>
</tr>
<tr>
<td><em>Osmia nigrohirta</em></td>
<td>000000000000010100100201010000001021001000</td>
</tr>
<tr>
<td><em>Osmia pedicornis</em></td>
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</tr>
<tr>
<td><em>Osmia ribifloris</em></td>
<td>100000100001001012010100000010020100000</td>
</tr>
<tr>
<td><em>Osmia scheherzade</em></td>
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</tr>
<tr>
<td><em>Osmia taurus</em></td>
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</tr>
<tr>
<td><em>Osmia tricornis</em></td>
<td>0003213121010120001010000011000011001000000</td>
</tr>
</tbody>
</table>
Appendix 5. Maximum clade credibility tree for *Osmia* s.l. based on 54,000 post burn-in trees from four independent Bayesian analyses. Bayesian posterior probabilities are given for the analysis of the combined molecular plus morphological data set, including two species (*O. longicornis* and *O. kohli*) of which only morphological data were available.
Better safe than sorry? Flowers, which morphologically restrict access to their pollen, exhibit unfavourable pollen properties for bee larval development

1 Abstract

Empirical evidence suggests that pollen chemistry plays an important role in shaping the pollen host spectra of many bee species. Although the underlying mechanisms are poorly understood, pollen diets of several plant taxa have experimentally been found to impede larval development of unspecialized bees. The pollen of all plant taxa, for which such a detrimental effect on bee larval development has been observed so far, is freely accessible in the flowers and thus easily harvestable for flower visitors, suggesting that this pollen might be chemically protected in order to reduce its loss to pollen feeding animals. In the present study, we tested larval survival of five solitary bee species on pollen diets of the two Fabaceae species *Onobrychis viciifolia* and *Lotus corniculatus*, which have their anthers concealed inside their flowers. As the complex flower morphology of these two species already considerably narrows the spectrum of pollen harvesting bee taxa, we expected bees that usually do not exploit Fabaceae to develop well on *Onobrychis* and *Lotus* pollen diets. All five bee species tested successfully developed on the *Onobrychis* pollen diet, whereas larval survival on the *Lotus* pollen diet was significantly reduced in two species despite the fact that *Lotus* flowers are more difficult to exploit for pollen than *Onobrychis* flowers. We conclude that pollen of morphologically complex flowers with a restricted visitor spectrum is not necessarily an easy-to-use nutritional source.
5.2 Introduction

Bees exploit a large variety of flowering plants for pollen and nectar, which they store in the brood cells of their nests as food for their larvae (Michener 2007; Westrich 1989). Whereas some bee species are strictly specialized on the pollen of a single plant family or genus (“oligolecty”), others are more generalized and collect the pollen of plants from two or more families (“polylecty”) (Cane and Sipes 2006; Müller and Kuhlmann 2008). Recent studies suggest that pollen chemistry contributes to shape the pollen host spectra of bees. Pure pollen diets of *Ranunculus* (Ranunculaceae) and *Asteroideae* (Asteraceae) negatively affected the larval development of all tested bee species that naturally do not collect these pollens (Guirguis and Brindley 1974; Haider et al. 2013; Levin and Haydak 1957; Praz et al. 2008c; Sedivy et al. 2011; Williams 2003), while non-host pollen diets of *Echium* (Boraginaceae) and *Sinapis* (Brassicaceae) prevented the development of some bee species but allowed the development of others (Praz et al. 2008c; Sedivy et al. 2011). Reduced survival of bee larvae on non-host pollen diets might be due either to a strong physiological adaptation of the bees to the pollen chemistry of their specific hosts or to unfavourable chemical properties of the non-host pollen (reviewed in Praz et al. 2008c and Roulston and Cane 2000). In fact, pollen nutritional quality might be poor due to low protein content, lack of essential nutrients or difficult nutrient extractability from within the pollen grains (Herbert et al. 1970; Rasmont et al. 2005; Somerville and Nicol 2006; Weiner et al. 2010; Wille et al. 1985). Furthermore, the pollenkit, a lipid-rich layer coating the pollen grains, might interfere with nutrient assimilation (Williams 2003), or the pollen might contain secondary metabolites that intoxicate the bee larvae (Detzel and Wink 1993; Dobson and Bergström 2000; Kempf et al. 2010; Kevan and Ebert 2005; London-Shafir et al. 2003; Reinhard 2011).

Interestingly, all plant species known so far to exhibit pollen properties that negatively affect bee larval survival (several species of Asteroideae and Cichorioideae, *Echium, Ranunculus, Sarcobatus, Sinapis, Stryphnodendron*) have
open flowers with freely accessible pollen that can easily be collected by any flower visitor (Levin and Haydak 1957; Loper and Berdel 1980; Pimentel de Carvalho and Message 2004; Praz et al. 2008c; Reinhard 2011; Sedivy et al. 2011; Williams 2003). Since the quantitative pollen requirements of bees are enormous (Müller et al. 2006; Schlindwein et al. 2005), plants with freely accessible pollen are at risk that a large proportion of their pollen does not serve pollination but instead ends up in the brood cells of bees, including highly unspecialized ones. In these plants, unfavourable pollen properties might thus serve to reduce excessive pollen loss by narrowing the spectrum of pollen harvesting bees (Praz et al. 2008c). In contrast, complex flowers that hide their pollen inside specialized floral structures limit pollen loss by restricting access to those bee taxa that are behaviourally or morphologically adapted to exploit them and serve as more reliable pollinators (Harder and Barclay 1994; Müller 1995; Vogel 1993; Westerkamp 1997a; Westerkamp and Classen-Bockhoff 2005). Examples for such morphologically complex flowers are species of the Fabaceae, which conceal their anthers in a “keel” that is formed by the two lowermost petals and which are nearly exclusively visited by bees. In order to collect pollen from these flowers, bees have to apply force in combination with a specialized leg movement pattern (Westerkamp 1997b). As a consequence, unspecialized bee taxa are not able to efficiently harvest pollen on these flowers (Westerkamp 1993). If the unfavourable chemical pollen properties of flowers with freely accessible pollen are indeed adaptive in terms of reducing pollen loss to bees, flowers of the Fabaceae would not, or to a lesser extent, be expected to chemically protect their pollen because the spectrum of pollen harvesting bees is already considerably limited to those taxa that are able to efficiently operate and to pollinate them.

In the present study, we investigated whether the larvae of five species of solitary bees, which naturally do not collect pollen on Fabaceae, are able to successfully develop on pure pollen diets of the two Fabaceae species *Onobrychis viciifolia* and *Lotus corniculatus*, which differ in their floral complexity. We hypothesized that neither of the two pollen diets impedes larval development of the
five unspecialized bee species, or if it does, that larval survival is higher on the pollen diet of the more complex *Lotus* flowers.

5.3 METHODS

5.3.1 Study system

Bee species

We chose five bee species belonging to the tribe Osmiini (Apoidea: Megachilidae) to test for their ability to develop on pollen diets of two Fabaceae species, *Lotus corniculatus* Linnaeus and *Onobrychis viciifolia* Scopoli. All five bee species are widespread and common throughout Europe. Three of them are strictly oligolectic (Westrich 1989): *Chelostoma florisomne* (Linnaeus 1758) and *Chelostoma rapunciuli* (Lepeletier 1841) exclusively collect pollen on *Ranunculus* spp. (Ranunculaceae) and *Campanula* spp. (Campanulaceae), respectively, while *Heriades truncorum* (Linnaeus 1758) is specialized on Asteraceae with a clear preference for the subfamily Asteroideae. The two other species, *Osmia bicornis* (Linnaeus 1758) and *Osmia cornuta* (Latreille 1805), are broadly polylectic. Pollen analysis of the scopal content of 50 collected females each recovered pollen hosts belonging to 17 and 8 different plant families, respectively (M. Haider, S. Dorn, C. Sedivy and A. Müller, unpublished). Whereas no Fabaceae pollen was recorded in the scopal pollen loads of *O. cornuta*, a very small proportion (1.2%) of Fabaceae pollen was found in the pollen loads of *O. bicornis*, indicating that Fabaceae are occasionally used as pollen hosts by this species. All five bee species nest in a variety of pre-existing cavities such as insect borings in dead wood or hollow plant stalks. The females build several brood cells per nest, which are provisioned with a mixture of pollen and nectar before a single egg is laid on top of each provision. The hatched larva feeds on the pollen-nectar provision and spins a cocoon after having consumed the entire provision. *O. bicornis* and *O. cornuta* complete their development already in autumn and overwinter as imagines within their brood cells. The other three species overwinter as pupae (*C. florisomne*) or larvae (*C. rapunciuli*).
and *H. truncorum*) and complete metamorphosis to the adult insect in the following spring or early summer.

**Plant species**

*Lotus corniculatus* (hereafter *Lotus*) is a perennial herb growing in heaths and grasslands across Europe, Asia and North Africa (Jones and Turkington 1986). *Lotus* flowers exhibit secondary pollen presentation and they operate in the “piston mechanism” (Westerkamp 1997b): the upper rim of the keel is connate except for a small opening at the keel tip and visiting bees exert pressure on both keel and wings, which leads to a downward movement of the keel. Thereby a small dose of pollen is released through the opening at the keel tip. To harvest larger amounts of pollen during a single visit, bees have to repeatedly move the wing-keel complex downwards in a pump-like action.

*Onobrychis viciifolia* (hereafter *Onobrychis*) is a perennial herb, which is native to southern Central Asia and has been introduced as forage legume to Europe and North America, where it is now widespread in temperate regions (Hayot Carbonero *et al.* 2011). *Onobrychis* flowers do not exhibit secondary pollen presentation and they operate in the “valvular mechanism” (Westerkamp 1997b): the upper rim of the keel is not connate and when the keel is moved downwards through pressure exerted by the visiting bees, it opens along its total length making the anthers accessible as long as the pressure is maintained.

Pollen collection from *Lotus* flowers thus requires more sophisticated movement patterns by the bees than from *Onobrychis* flowers, which is exemplified by the fact that honeybees and the polylectic *O. bicorns* are able to collect pollen from *Onobrychis* flowers, but appear to be unable to efficiently exploit *Lotus* flowers for pollen (M. Haider and A. Müller, personal observation).
5.3.2 Experimental design

Experiments with *C. rapunculi*, *H. truncorum* and *O. bicornis* were conducted in the years 2010 and 2011, those with *C. florisomne* and *O. cornuta* in the year 2012. Pollen of the two Fabaceae species was collected in 2010 and 2011.

Experimental pollen diets

Potted plants of *Lotus* and *Onobrychis* were placed in two strictly separated compartments of a large walk-in cage (12 x 8 x 3.5 m) at the Experimental Research Station of the ETH Zürich at Eschikon. The walk-in cage was covered with gauze and provided with hollow bamboo stalks as bee nesting sites. In each compartment about 150 individuals of *Megachile rotundata* (Fabricius 1784) (Apoidea: Megachilidae) were released in order to allow them to collect the Fabaceae pollen that was later used in the experiments. *M. rotundata* is a polylectic bee species with a preference for Fabaceae (Westrich 1989). It is native to Europe, but due to its rarity in its native range cocoons of this species were imported from a commercial bee breeder (JWM Leafcutter Inc) in the USA. Completed nests of *M. rotundata* were taken to the laboratory, carefully opened and the pollen/nectar provisions withdrawn. All *Lotus* and *Onobrychis* provisions collected at one time were carefully mixed in one petri dish each. As control pollen diets we used for each of the five tested bee species brood cell provisions collected by the female bees under natural conditions. Completed nests of the five species were collected at previously established nest aggregations in and around Zürich (Switzerland). Nests were taken to the laboratory and carefully opened. Eggs were removed from the brood cell provisions with a thin pair of tweezers and the provisions withdrawn. All pollen provisions were stored at -20°C for at least 24 h up to one year before use in the experiments. In contrast to the three oligolectic species, for which the composition of the cell provisions was known due to their strict pollen specialization, composition of the control pollen diets for the broadly polylectic species *O. bicornis* and *O. cornuta* was unknown. Depending on body size of the imagines, the following amount of pollen provision was provided for each larva: 90 mg for
Due to a shortage of *Lotus* pollen in 2012, larvae of *O. cornuta* received only 300 mg of pollen diet despite the slightly larger body size of *O. cornuta* compared to *O. bicornis*. Preliminary experiments indicated that this amount is adequate for successful larval development.

**Egg transfer**

For each bee species, we removed maximally three eggs per nest, which were transferred to one artificial brood cell each containing a pollen diet of *Lotus*, *Onobrychis* and the control pollen diet, respectively. Artificial brood cells were made of blocks of beech wood (4 x 2 x 2 cm for the two larger species *O. bicornis* and *O. cornuta*; 2 x 2 x 1 cm for the smaller species *C. florisomne*, *C. rapunculi* and *H. truncorum*) provided with a drilled burrow (2 cm length, 0.8 cm diameter and 1.3 cm length, 0.4 cm diameter, respectively) open both at the front and on the top. These openings were covered with coverslips attached to the block with transparent adhesive tape to permit free viewing into the burrow. As the pollen provisions collected by *M. rotundata* were relatively fluid, artificial brood cells containing the *Lotus* and *Onobrychis* pollen diets were incubated for 12 hours in a climate chamber (see below) before egg transfer to obtain a firmer consistency comparable to the control pollen diets.

**Larval development**

After egg transfer, the artificial brood cells were incubated in constant darkness within a climate chamber (E7/2; Conviron, Winnipeg, Canada) under the following conditions: 25°C for 16 hours followed by a gradual reduction of temperature to 10°C within four hours followed by a gradual increase back to 25°C within another four hours. Relative humidity was constantly held at 60%. Larval development was checked every second day and the following developmental stages were recorded: 1) egg hatching, 2) feeding without defecating, 3) feeding and defecating, 4) start of cocoon spinning, 5) completion of cocoon. A cocoon was considered completed upon becoming intransparent or as soon as the larva had
emptied its gut and stopped moving. Brood cells were kept in the climate chamber until autumn and then stored at 4°C in constant darkness for overwintering. Individuals of *O. bicornis* were sexed and weighed to the nearest 0.1 mg (AB204; Mettler Toledo, Switzerland) the following spring after metamorphosis to the adult stage had been completed. Individuals of *C. florisomne* and *O. cornuta* were sexed and weighed as black diapausing pupae or as fully developed imagines, respectively, already in fall before hibernation. Due to an infestation with *Melittobia acasta* (Hymenoptera: Eulophidae) in one year, all diapausing individuals of *C. rapunculi* and *H. truncorum* were killed and their adult mass could not be assessed.

5. 3. 3 *Data analysis*

Eggs that did not hatch and larvae that had undoubtedly died from external factors such as mechanical damage, attack by parasitic wasps or chalkbrood were excluded from all analyses. Survival of larvae on the different pollen diets was analysed using Kaplan-Meier survival statistics. The number of days between hatching and completion of the cocoon were considered as “censored data”: individuals that died before the completion of the cocoon represented the exact observations for which the event (death) occurred. Those that completed the cocoon and entered diapause, i.e. the survivors, were considered the censored observations and thus withdrawn from survival calculations. Differences between survival distributions were analysed with pairwise log-rank tests implemented in SPSS Statistics 20.0.0 and controlled with False Discovery Rate (FDR) correction (Benjamini and Hochberg 1995). Differences in development time (time between egg hatching and completion of the cocoon) and adult body mass were analysed with Kruskal-Wallis one-way analysis of variance, followed by pairwise Mann-Whitney U tests and FDR correction. Adult body mass was analysed separately for males and females because females of *O. bicornis* and *O. cornuta* are distinctly larger than males. Due to the low number of females that reached the adult stage, adult body mass was statistically explored for males only. Statistical analyses were conducted with SPSS Statistics 20.0.0 for Macintosh OS X.
5.4 Results

A total of 469 transferred eggs hatched. Four larvae died of chalkbrood, six of parasitism by the eulophid *Melittobia acasta*, two were killed by unidentified parasitic wasps, four died of mechanical damage due to handling and four escaped from the brood cells. These larvae were excluded from all analyses.

*Celostoma florisomne*

Larval survival of the *Ranunculus* specialist *C. florisomne* differed significantly between the pollen diets (Kaplan-Meier analysis, log-rank test: $\chi^2 = 22.018$, df = 2, $P < 0.001$; Table 1, Fig. 1a). Survival of larvae reared on the *Lotus* pollen diet was significantly reduced compared with larvae reared on both the *Onobrychis* pollen diet and the control pollen diet (pairwise log-rank tests: *Lotus* - *Onobrychis*: $P = 0.001$; *Lotus* - control: $P < 0.001$). No significant difference in the survival between larvae reared on the *Onobrychis* pollen diet and the control pollen diet was found (pairwise log-rank test: $P = 0.280$). Larval development time did not differ significantly between larvae reared on the different pollen diets (Kruskal-Wallis test: $P = 0.560$; Table 1, Fig. 2). It ranged on all three tested pollen diets from 28 to 56 days (median: 40). Male adult body mass differed significantly between the pollen diets (Kruskal-Walis test: $P < 0.001$; Fig. 3a). Males reared on the *Lotus* pollen diet had a significantly lower body mass than those reared on the *Onobrychis* pollen diet and the control pollen diet (Mann-Whitney U tests: *Lotus* - *Onobrychis*: $U = 74.0$; $P < 0.001$; *Lotus* - control: $U = 106.0$; $P < 0.001$). Males reared on the *Onobrychis* pollen diet had a significantly lower body mass than those reared on the control pollen diet (Mann-Whitney U test: $U = 281.5$; $P < 0.001$).
Table 1. Larval survival and development time of five species of solitary bees reared on a pollen diet of *Lotus corniculatus* and *Onobrychis viciifolia* and on a control pollen diet.

<table>
<thead>
<tr>
<th>Bee species</th>
<th>Pollen diet</th>
<th>No. eggs hatched</th>
<th>Surviving larvae</th>
<th>Group heterogeneity</th>
<th>Development time [d]</th>
<th>Group heterogeneity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>p</td>
<td>groups</td>
<td>Median</td>
</tr>
<tr>
<td><em>C. florisomne</em></td>
<td><em>Lotus</em></td>
<td>27</td>
<td>10</td>
<td>37</td>
<td>&lt; 0.001</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td><em>Onobrychis</em></td>
<td>30</td>
<td>24</td>
<td>80</td>
<td>b</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td><em>Control</em></td>
<td>29</td>
<td>27</td>
<td>89.7</td>
<td>b</td>
<td>40</td>
</tr>
<tr>
<td><em>C. rapunculi</em></td>
<td><em>Lotus</em></td>
<td>43</td>
<td>6</td>
<td>14</td>
<td>&lt; 0.001</td>
<td>a</td>
</tr>
<tr>
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<td><em>Onobrychis</em></td>
<td>40</td>
<td>24</td>
<td>60</td>
<td>b</td>
<td>41</td>
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<tr>
<td></td>
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<td>47</td>
<td>31</td>
<td>66</td>
<td>b</td>
<td>30</td>
</tr>
<tr>
<td><em>H. truncorum</em></td>
<td><em>Lotus</em></td>
<td>33</td>
<td>18</td>
<td>54.5</td>
<td>0.057</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td><em>Onobrychis</em></td>
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<td>26</td>
<td>76.5</td>
<td>a</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td><em>Control</em></td>
<td>34</td>
<td>30</td>
<td>88.2</td>
<td>a</td>
<td>32</td>
</tr>
<tr>
<td><em>O. bicornis</em></td>
<td><em>Lotus</em></td>
<td>21</td>
<td>21</td>
<td>100</td>
<td>0.029</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td><em>Onobrychis</em></td>
<td>19</td>
<td>19</td>
<td>100</td>
<td>a</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td><em>Control</em></td>
<td>24</td>
<td>20</td>
<td>83.3</td>
<td>a</td>
<td>31</td>
</tr>
<tr>
<td><em>O. cornuta</em></td>
<td><em>Lotus</em></td>
<td>14</td>
<td>14</td>
<td>100</td>
<td>0.208</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td><em>Onobrychis</em></td>
<td>30</td>
<td>30</td>
<td>100</td>
<td>a</td>
<td>26</td>
</tr>
<tr>
<td></td>
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<td>23</td>
<td>22</td>
<td>95.7</td>
<td>a</td>
<td>24</td>
</tr>
</tbody>
</table>

Notes: Differences in the survival of each bee species on the three different pollen diets were tested using Kaplan-Meier analysis (post hoc test: pairwise log-rank tests with False Discovery Rate control). Development time represents the number of days between hatching and completion of the cocoon (begin of diapause) of all survivors. Differences in the development time of each bee species on the three different pollen diets were tested by a Kruskal-Wallis test followed by pairwise Mann-Whitney U tests and False Discovery Rate control. Group heterogeneity: for each bee species, pollen diets sharing the same letter did not differ significantly at \( P < 0.05 \).
Figure 1. Cumulative survival of larvae of five solitary bee species when reared on a pollen diet of *Lotus corniculatus* and *Onobrychis viciifolia* and on a control pollen diet that was collected by each species itself. a) *Chelostoma florisomne*, b) *Chelostoma rapunculi*, c) *Heriades truncorum* d) *Osmia bicornis* and e) *Osmia cornuta*. Crosses indicate individuals that reached the cocoon stage (censored data).
**Chelostoma rapunculi**

Larval survival of the *Campanula* specialist *C. rapunculi* differed significantly between the pollen diets (Kaplan-Meier analysis, log-rank test: \( \chi^2 = 23.717 \), df = 2, \( P < 0.001 \); Table 1, Fig. 1b). Survival of larvae reared on the *Lotus* pollen diet was significantly reduced compared with larvae reared on both the *Onobrychis* pollen diet and the control pollen diet (Kaplan-Meier analysis, pairwise log-rank tests: *Lotus* - *Onobrychis*: \( P < 0.001 \); *Lotus* - control: \( P = 0.002 \)). No significant difference in the survival between larvae reared on the *Onobrychis* pollen diet and the control pollen diet was found (pairwise log-rank test: \( P = 0.661 \)). Larval development time differed significantly between larvae reared on the different pollen diets (Kruskal-Wallis test: \( p < 0.001 \); Table 1, Fig. 2). Development time was significantly prolonged on the *Onobrychis* pollen diet (28-52 d, median: 41) compared to the *Lotus* pollen diet (34-42 d, median: 36) (Mann-Whitney U test: \( U = 114.5 \), \( P = 0.025 \)) and the control pollen diet (24-40 d, median: 30) (\( U = 702.5 \), \( P < 0.001 \)), and it was significantly longer on the *Lotus* pollen diet than on the control pollen diet (\( U = 167.5 \), \( P = 0.001 \)).

**Heriades truncorum**

Larval survival of the Asteraceae specialist *H. truncorum* did not differ significantly between the pollen diets (Kaplan-Meier Analysis: \( \chi^2 = 5.718 \), df = 2, \( P = 0.057 \); Table 1), but it tended to be worse on the *Lotus* pollen diet (Fig. 1c). Larval development time differed significantly between larvae reared on the different pollen diets (Kruskal-Wallis test: \( p < 0.001 \); Table 1, Fig. 2). Development time was significantly prolonged on the *Onobrychis* pollen diet (32-52 d, median: 36) compared to the control pollen diet (28-38 d, median: 32) (Mann-Whitney U test: \( U = 677.5 \), \( P < 0.001 \)). Development time on the *Lotus* pollen diet (30-52 d, median: 35) did not differ significantly from the two other pollen diets after FDR correction (*Lotus* - *Onobrychis*: \( U = 677.5 \), \( P = 0.047 \); *Lotus* - control: \( U = 363.5 \), \( P = 0.039 \)).
Figure 2. Development time of larvae of five solitary bees species that successfully reached the cocoon stage when reared on a pollen diet of *Lotus corniculatus* and *Onobrychis vicifolia* and on a control pollen diet that was collected by each species itself. Significant differences are indicated as * (p < 0.05), ** (p < 0.01) and *** (p < 0.001) after FDR correction (Kruskal-Wallis test followed by pairwise Mann-Whitney U tests).

**Osmia bicornis**

Larval survival of the pollen generalist *O. bicornis* differed significantly between the pollen diets (Kaplan-Meier-Analysis, log-rank test: $\chi^2 = 7.100$, df = 2, P = 0.029; Table 1, Fig. 1d), but post-hoc analyses did not detect significant differences between larvae reared on the Fabaceae pollen diets and the control pollen diet (pairwise log-rank tests: control - *Onobrychis*: P = 0.066; control - *Lotus*: P = 0.053). Larval development time differed significantly between larvae reared on the different pollen diets (Kruskal-Wallis test: P < 0.001; Table 1, Fig. 2). Development time was significantly shorter on the *Lotus* pollen diet (22-32 d, median: 26) than on both the *Onobrychis* pollen diet (24-36 d, median: 28) and the control pollen diet (22-38 d, median: 31) (Mann-Whitney U tests: *Lotus* - *Onobrychis*: U = 309.5, P = 0.002; *Lotus* - control: U = 35.0, P < 0.001), and it was significantly shorter on the *Onobrychis* pollen diet than on the control pollen diet (U = 104.5, P = 0.015). Male adult body mass did not differ significantly between the pollen diets (Kruskal-Wallis test: P = 0.155; Fig. 3b).
Figure 3. Adult body mass of a) Chelostoma florisomne, b) Osmia bicornis and c) Osmia cornuta when reared on a pollen diet of Lotus corniculatus and Onobrychis viciifolia and on a control pollen diet that was collected by the bee species itself.

Osmia cornuta

Larval survival of the pollen generalist O. cornuta did not differ significantly between the pollen diets (Kaplan-Meier-Analysis, log-rank test: $\chi^2 = 3.143$, df = 2, $P = 0.208$; Table 1, Fig. 1e). Larval development time differed significantly between larvae reared on the three pollen diets (Kruskal-Wallis test: $p < 0.001$; Table 1, Fig. 2). Development time was significantly prolonged on the Lotus pollen diet (26-
42 d, median: 29) compared with both the Onobrychis pollen diet (24–32 d, median: 26) and the control pollen diet (20–32 d, median: 24) (Mann-Whitney U tests: Lotus - Onobrychis: U = 65.0, P < 0.001; Lotus - control: U = 274.0, P < 0.001), and it was significantly longer on the Onobrychis pollen diet than on the control pollen diet (U = 490.0, P = 0.002). Male adult body mass differed significantly between the pollen diets (Kruskal-Wallis test: P < 0.001; Fig. 3c). Males reared on the Lotus pollen diet had a significantly higher body mass than those reared on the Onobrychis pollen diet and the control pollen diet (Mann-Whitney-U tests: Lotus - Onobrychis: U = 20.0; P = 0.006; Lotus - control: U = 112.0, P < 0.001). Males reared on the Onobrychis pollen diet had a significantly higher body mass than those reared on the control pollen diet (Mann-Whitney-U test: U = 273.0, P < 0.001).

5.5 DISCUSSION

The two Fabaceae pollen diets differed in their suitability as larval food for the tested bee species. All five bee species successfully developed on the Onobrychis pollen diet, whereas larval performance on the Lotus pollen diet differed among bee species: larval survival was equally successful as on the control pollen diet in the polylectic Osmia bicornis and O. cornuta, but significantly reduced in the oligolectic Chelostoma florisomne and C. rapunculi. The oligolectic Heriades truncorum tended to perform worse on the Lotus pollen diet as well, but this result was marginally not significant. Thus, our initial expectation that the pollen of Fabaceae does not exhibit unfavourable properties, which impede larval development of bees that naturally do not collect this pollen, was confirmed for one Fabaceae species. However, it was not supported for the second Fabaceae species, indicating a more complex pattern of interactions between plants with hidden pollen and pollen feeding flower visitors.

One possible explanation for the reduced survival of the oligolectic bee species on the Lotus pollen diet might be a strong physiological dependency of the specialists upon the pollen chemistry of their specific hosts, which might have led to constraints regarding the utilization of alternative pollen. Such constraints might be
due to physiochemical gut conditions (e.g. pH, redox potential), which are regulated by the insect and which might be influenced by food constituents other than nutrients and allelochemicals. As a consequence, the same food might undergo different biochemical transformations in the gut of different species, leading to differences in nutrient availability, allelochemical activity and pathogen infectivity (Appel 1993; Berenbaum 1980 and references therein). Alternatively, the oligolectic bee species might depend upon certain pollen constituents of their specific hosts and therefore do worse on the non-host *Lotus* pollen. In fact, secondary plant compounds are known to be of nutritional value for some specialized herbivores (Bernays and Woodhead 1982; Kato 1978; Neville and Luckey 1971; Rosenthal 1983; Rosenthal et al. 1982; Slansky 1992 and references therein). However, the successful development of all three oligolectic bee species on the *Onobrychis* pollen diet renders a strong physiological dependency upon the chemistry of their specific pollen hosts rather unlikely.

Another possibility is that the increased larval mortality of the three oligolectic bee species on the *Lotus* pollen diet (although marginally not significant in *H. truncorum*) is attributable to unfavourable properties of the *Lotus* pollen. First, *Lotus* pollen might be nutritionally deficient due to low protein content or lack of essential compounds such as certain amino acids or sterols. However, pollen of Fabaceae including *Lotus* has a relatively high protein content and contains all amino acids that are regarded essential for the honey bee (Hanley et al. 2008; Roulston et al. 2000; Somerville and Nicol 2006; Wille et al. 1985). Moreover, deficiencies in nutrient content would presumably also have affected the larvae of the two polylectic species, which was not the case. Males of *O. cornuta* even gained more weight when reared on the *Lotus* pollen diet compared with both the control and the *Onobrychis* pollen diet, indicating a good nutritional quality of *Lotus* pollen.

Second, the inner layer of the pollen grain wall, the intine, might resist digestion by the bees and thus inhibit or limit nutrient extraction from the pollen protoplasm (Dobson and Peng 1997; Suárez-Cervera et al. 1994). If the chemical composition of the *Lotus* pollen intine renders the efficient extraction of nutrients
difficult, the surviving bees would have been expected to exhibit a prolonged larval development time in compensation for the reduced nutrient extractability. However, compared to the *Onobrychis* pollen diet, development time on the *Lotus* pollen diet was for none of the three oligolectic species prolonged.

Third, the pollenkit, an oily substance coating the exine of the pollen grains, might be difficult to digest (Peng et al. 1985) and therefore interfere with nutrient assimilation by the bee larvae, as was suggested to be the reason for the failure of *Osmia lignaria* to develop on Asteraceae pollen (Williams 2003). However, in contrast to Asteraceae pollen, which is often covered by a thick layer of pollenkit, the pollen grains of *Lotus* do not appear to possess similarly large amounts of pollenkit (A. Müller, personal observation), rendering interference of the pollenkit with the digestion of the *Lotus* pollen diet questionable.

Fourth, *Lotus* pollen might contain secondary compounds that are toxic to the developing bee larvae. A growing number of studies report on the occurrence of secondary metabolites in pollen, sometimes in considerable quantities (Detzel and Wink 1993; Dobson and Bergström 2000; Kempf et al. 2010; London-Shafir et al. 2003; Reinhard 2011). While the concentration of such secondary compounds in pollen might be too low to negatively affect pollen-feeding bees (Sedivy et al. 2012), the cyanogenic glycoside amygdalin in pollen of *Prunus dulcis* negatively affected survival of adult honey bees at natural concentrations (Kevan and Ebert 2005), and pyrrolizidine alkaloids in pollen of *Echium vulgare* were lethal to honeybee larvae at concentrations that correspond to the natural alkaloid concentration in *Echium* pollen (Reinhard 2011). *Lotus* is polymorphic regarding absence or presence of toxic cyanogenic glycosides in its leaf tissue (Jones 1962). Whether cyanogenic glycosides are also present in pollen of cyanogenic *Lotus* plants has, to our knowledge, not yet been examined. However, in *Prunus dulcis*, a member of the Rosaceae, which are characterized by cyanogenic glycosides in their vegetative parts (Conn 1969), the cyanogenic glycoside amygdalin has been detected in considerable concentrations also in the pollen (London-Shafir et al. 2003). This finding as well as
the occurrence of quinolizidine alkaloids in the pollen of another Fabaceae species with morphologically complex flowers (Lupinus polyphyllus) (Detzel and Wink 1993) render the existence of plant allelochemicals in Lotus pollen possible. Interestingly, the larvae of all three oligoleptic bee species that managed to develop on the Lotus pollen diet stayed relatively small and started to spin their cocoon long before they had consumed the entire provision. This observation might possibly be due to the accumulation of toxic substances in the larval bodies reaching a near lethal threshold or by the feeding deterrent properties of cyanogenic glycosides (Nahrstedt 1985). In fact, the premature termination of feeding might also explain why the larvae of C. florisomne did not exhibit a prolonged development time on the Lotus pollen diet despite their significantly reduced adult mass, and why Lotus reared larvae of C. rapunculi and H. truncorum exhibited a shorter development time (although marginally not significant in H. truncorum) than the Onobrychis reared larvae despite their reduced survival.

If the pollen of Lotus indeed contains plant allelochemicals, the successful development of the two polylectic Osmia species on the Lotus pollen diet calls for an explanation. Differences in the survival between generalists and specialists on the same diet might be due to a broader array of detoxification tools owned by the generalists compared to more specialized detoxification tools owned by the specialists, as has been shown for some herbivorous insects (Gleadow and Woodrow 2002; Krieger et al. 1971; Li et al. 2003; Ramsey et al. 2010). Indeed, generalist herbivores are able to cope with certain plant secondary compounds that they have never encountered before (Fox et al. 1997; Matsuki et al. 2011; Piskorski et al. 2011), and the ability to digest unfavourable non-host pollen exists in different populations of O. cornuta (Haider et al. in press). However, in a study that compared larval performance of a generalist and a specialist Osmia bee species on non-host pollen, larvae of the specialist performed better on the novel pollen than larvae of the generalist, suggesting that specialization does not necessarily reduce the ability of a bee species to use non-host pollen (Williams 2003). Alternatively, the successful development of O. bicornis and O. cornuta on the Lotus pollen diet might result
from the possession of specific adaptations to detoxify cyanogenic glycosides. *O. cornuta* exhibits a strong preference for Rosaceae as pollen hosts and also *O. bicorne* often exploits Rosaceae (Tasei 1973; M. Haider, S. Dorn, C. Sedivy and A. Müller, unpublished), rendering both species highly suitable for pollination of orchards (Bosch 1994; Gruber *et al.* 2011; Krunic and Stanisavljevic 2006). Many Rosaceae contain cyanogenic glycosides in their vegetative parts (Conn 1969), and in almond (*Prunus dulcis*) these glycosides also occur in the pollen (London-Shafir *et al.* 2003). Interestingly, *O. cornuta* is commercially used for almond pollination and larval survival on almond pollen provisions is high (Bosch 1994; Torchio *et al.* 1987), indicating that *O. cornuta* larvae can develop well on a pollen diet that comprises cyanogenic glycosides in concentrations that were found to negatively affect the survival of adult honey bees (Kevan and Ebert 2005).

The morphology of *Lotus* flowers, which requires the application of force and a particular movement pattern of the bee in order to efficiently harvest pollen (Westerkamp 1997b), renders pollen collection difficult. In fact, the spectrum of bee taxa that possess behavioural adaptations to collect pollen on *Lotus* and other Fabaceae taxa is considerably restricted and many bee taxa are unable to efficiently exploit these flowers for pollen and nectar (Westerkamp 1993, 1997b; Westrich 1989). Nevertheless, the present study clearly suggests that the pollen of *Lotus* possesses unfavourable properties for bee larval development. These properties might contribute to further narrow the already limited spectrum of flower visitors or, alternatively, to deter pollen-feeding animals other than bees.

In conclusion, the results of the present study partly contradict the hypothesis that flowers with hidden anthers do not possess unfavourable pollen for bee larval development. In fact, their interactions with pollen feeding insects appear to be more complex. Whereas pollen of *Onobrychis* supports development of bee species not specialized on Fabaceae, pollen of *Lotus* apparently exhibits unfavourable properties despite the fact that it is more difficult for bees to remove pollen from the *Lotus* flowers than from the *Onobrychis* flowers.
6 Intra- and interpopulational variation in the ability of a solitary bee species to develop on non-host pollen: implications for host range expansion

6.1 Abstract

1. Pollen host choice in bees is in many cases highly conserved, which might partly be due to physiological limitations of bee larvae to digest non-host pollen. These limitations need to be overcome in order to incorporate new pollen hosts, however, the mechanisms underlying such host expansion are poorly understood.

2. In this study, we examined intra- and interpopulational variation in the ability of larvae of the solitary bee species *Osmia cornuta* (Megachilidae) to develop on a non-host pollen diet of *Ranunculus acris* (Ranunculaceae) by comparing larval performance within and between five geographically distant European populations.

3. The majority of bee larvae from all tested populations died when reared on the *Ranunculus* pollen diet. Between 10% and 43.5% of all larvae per population reached the cocoon stage and 48% of these emerged as viable adults from the cocoons, indicating that the physiological ability to cope with the unfavourable properties of *Ranunculus* pollen exists in each population.

4. The bee larvae of one population exhibited significantly reduced survival on the *Ranunculus* pollen diet compared to three of the four other populations.

5. Although bees that successfully developed on the *Ranunculus* pollen diet showed a distinctly prolonged development time, exhibited higher mortality during diapause and reached a considerably lower adult weight compared to individuals fed the control pollen diet, several of the *Ranunculus* fed individuals were able to reproduce and to sire viable offspring.

6. This study provides the first evidence for both intra- and interpopulational variation in the physiological ability of solitary bees to digest non-host pollen. This variation might enable host expansion and subsequent host shifts in response to natural selection.
6.2 Introduction

Bees collect large quantities of pollen and nectar for their own nourishment and as food for their offspring (Michener 2007). The plants exploited by bees are remarkably diverse and host plant spectra differ widely between bee taxa (Westrich 1989). According to the breadth of their host plant spectrum, bees are classified as pollen specialists, which restrict pollen collection to one plant family, subfamily or genus (“oligolecty”), or as pollen generalists, which harvest pollen from plants of two or more different plant families (“polylecty”) (Robertson 1925; Cane and Sipes 2006; Müller and Kuhlmann 2008).

Recent studies suggest that many polylectic bee lineages are derived from oligolecic ancestors (Müller 1996; Sipes and Tepedino 2005; Danforth et al. 2006b; Larkin et al. 2008; Michez et al. 2008), that host shifts among oligoleges are probably always preceeded by a shorter or longer period of polylecty (Sedivy et al. 2008) and that closely related generalist bee species may exploit different hosts (Sedivy et al. 2011). In all these cases, bees have incorporated new hosts into their diet range. However, the mechanisms underlying such host expansion in bees are poorly understood. It has been shown that bees usually broaden their diet range while continuing to utilize the original host and that host shifts frequently occur to plants that are already exploited by close relatives (Müller 1996; Sipes and Tepedino 2005; Sedivy et al. 2008). These findings suggest that certain plant traits impose constraints on the bees, which render host expansion difficult. In fact, host plant choice seems to be highly conserved in bees and incorporation of new hosts appears to occur much less often than previously thought (Sedivy et al. 2008). Constraints that impede the easy and rapid acquisition of new pollen hosts may be due to neurological limitations related to the recognition or handling of flowers (Praz et al. 2008b) or to physiological limitations related to pollen digestion, including lack of essential nutrients (Levin and Haydak 1957; Suárez-Cervera et al. 1994; Williams 2003; Praz et al. 2008c). Thus, bees have to overcome these constraints in order to
broaden their pollen diet, which then allows for subsequent respecialization, i.e. host plant shift.

A possible mechanism underlying host expansion and subsequent host shift is genetic variation in the ability of individuals of the same population to successfully overcome constraints imposed by non-hosts. Such intrapopulational variation has recently been reported for herbivorous insects. Larval survival of the oriental fruit moth (*Grapholita molesta*) that typically feeds on a wide range of rosacean fruit trees varied considerably between individuals reared on a diet supplemented with juglone, the main defence compound of the non-host plant walnut, which belongs to the phylogenetically distant family of Juglandaceae (Piskorski *et al.* 2011). Similarly, larval survival of the highly generalist gypsy moth (*Lymantria dispar*) varied considerably between individuals fed leaves of *Eucalyptus*, a plant genus that does not naturally occur in the distribution range of this Palearctic moth species and that contains secondary metabolites, which are unfamiliar to *L. dispar* (Matsuki *et al.* 2011). In both herbivorous species, the ability to develop on a chemically and phylogenetically unfamiliar host is present in some individuals even though the moths do not exploit or never have encountered the plant species. Anecdotal evidence suggests that similar intrapopulational variation in the ability to successfully use certain plant hosts might also exist in bees. When reared on a diet of buttercup (*Ranunculus*) pollen, all larvae of the pollen generalist mason bee species *Osmia cornuta* of a south German population died except for two females, which developed into dwarf-sized adults (Sedivy *et al.* 2011). Interestingly, the same bee species has been reported to collect *Ranunculus* pollen in Spain and in northern Italy (Márquez *et al.* 1994; Nepi *et al.* 1997), and *O. bicornis*, a close relative of *O. cornuta*, is known to frequently collect pollen of *Ranunculus* and to successfully develop on it (Westrich 1989; Vicens *et al.* 1993; Sedivy *et al.* 2011).

Genetic variation among individuals in their ability to successfully exploit a certain plant is a condition for natural selection to act towards the incorporation of that plant into the normal diet of a population. Correspondingly, the proportion of
individuals that are able to overcome constraints imposed by potential hosts is expected to differ among populations that are under differing selection regimes. In fact, interpopulational variation in host plant use or performance is well known among herbivorous insects (Jaenike 1990 and references therein), but to our knowledge such interpopulational variation has not been reported for bees so far.

In the present study, we analysed intra- und interpopulational variation in the ability of larvae of Osmia cornuta (Latreille 1805) to develop on a pollen diet of Ranunculus acris L. by comparing larval performance within and between five geographically distant European populations. We hypothesized that i) some individuals in every population are able to overcome the unfavourable properties of a Ranunculus pollen diet and that ii) differences in the performance of larvae reared on a Ranunculus pollen diet exist between the five populations.

6.3 METHODS

6.3.1 Bee species

Osmia cornuta (Latreille 1805) (Apoidea: Megachilidae) is a common solitary bee species in Central Europe and is widespread throughout the Palearctic (Peters 1978; Westrich 1989; Fig. 1). Its distribution ranges from northern Africa in the south to northern Germany in the north, and from Portugal in the west to Turkmenistan in the east (Peters 1978). The species is subdivided into three subspecies and the nominate form, O. c. cornuta, occurs in Central Europe (Peters 1978). It mainly inhabits warmer areas below 500 m and has an early seasonal flight period that usually lasts from the beginning of March until the beginning of May (Westrich 1989). O. cornuta is broadly polylectic and collects pollen from plants belonging to many different families (Westrich 1989, Márquez et al. 1994). Although small quantities of Ranunculus pollen have been found in brood cell provisions of O. cornuta in southern Europe (Márquez et al. 1994; Nepi et al. 1997), Ranunculus is not a regular pollen host in Central Europe as suggested by field observations and
the almost complete absence of *Ranunculus* pollen in the scopal pollen loads of collected females (M. Haider, S. Dorn, C. Sedivy and A. Müller unpublished). *O. cornuta* nests in a great variety of pre-existing cavities and willingly accepts hollow bamboo sticks as nesting site. Female bees build several brood cells per nest, which are provisioned with a mixture of pollen and nectar before a single egg is laid on top of each provision. The hatched larva feeds on the pollen-nectar provision and completes its development by autumn, after which the fully developed adult overwinters in its cocoon within the brood cell.

![Figure 1. Copulating pair of Osmia cornuta (picture: A. Krebs).](image)

6. 3. 2 *Bee Populations*

To test for intra- and interpopulational differences in the capability of *O. cornuta* to develop on a pollen diet of *Ranunculus*, we selected five populations of the same subspecies, *O. c. cornuta*, originating from Belgrade (Serbia), Bologna (Italy), Erfurt (Germany), Konstanz (Germany) and Troyes (France) (Fig. 2). The
distance between these five populations ranges from 380 km to 1300 km. Regular gene flow between the five populations is unlikely as all populations are separated from each other by the Alps or other mountain ranges, which are unsuitable habitats for the thermophilous *O. cornuta*.

**Figure 2.** Origin of the five tested European populations of *Osmia cornuta*. A = Belgrade, Serbia; B = Bologna, Italy; C = Erfurt, Germany; D = Konstanz, Germany; E = Troyes, France (map modified from wikipedia.org).

### 6.3.3 Origin of the pollen diet

Larvae of *O. cornuta* were reared on a pollen diet of *Ranunculus acris* L. (Ranunculaceae) collected by females of the *Ranunculus* specialist *Chelostoma florisomne* (Apoidea: Megachilidae) at Gletterens in western Switzerland. Reed stems containing brood cells of *C. florisomne* were collected and taken to the laboratory, where they were carefully opened longitudinally with a knife and the
pollen/nectar provisions withdrawn. As a control, we used a pollen diet of *Sinapis arvensis* (Brassicaceae) collected by the females of *O. cornuta* in a large walk-in-cage (see below). Species of Brassicaceae are known to be regular pollen hosts of *O. cornuta* (Westrich 1989; Márquez *et al.* 1994; M. Haider, S. Dorn, C. Sedivy and A. Müller unpublished). Prior to use in the experiments, all freshly collected provisions were stored at -20°C for at least 24 hours. In addition, we also used provisions that were collected during the previous season and stored at -20°C for up to one year.

6.3.4 Experimental setup

Experiments with the populations of Erfurt and Konstanz were conducted during spring 2010. Experiments with the populations of Belgrade, Bologna, Troyes and a second part of the Konstanz population were conducted during spring 2011. About 100 female and 100 male cocoons from each population (one male and one female cocoon per nest) were overwintered in a climate chamber (E7 ⁄ 2; Conviron, Winnipeg, Canada) at -3°C from December until end of April, resulting in a delay of emergence of about two months. This emergence delay was necessary as the normal flight period of *O. cornuta* usually overlaps only marginally with that of *C. florisomne*, which is active from May to June. After overwintering, the cocoons were placed in artificial nesting stands inside walk-in cages (10 x 8 x 3.5 m) covered with gauze. For each population a separate walk-in cage was used. Each cage was provided with about 800 *Sinapis arvensis* plants in 400 pots as pollen and nectar sources and with hollow bamboo stalks and moist soil as nesting resources. The cocoons of the Konstanz population were placed in an artificial nesting stand outside the cages at the Experimental Research Station of the ETH Zürich at Eschikon and the emerged bees were allowed to collect pollen and nectar freely. The delayed emergence prevented these bees from coming into contact with the local population of *O. cornuta*. Of each population, we transferred between 60 and 80 eggs onto each of the two pollen diets, except for the Troyes population, of which fewer eggs were transferred due to an unexpectedly short flight period of this
population. Two eggs per female were removed from the nests, one of which was later placed on a *Ranunculus* pollen diet and one on a control pollen diet. As the females often build more than one nest during their flight period, females of all populations were individually marked with numbered opalite plates to avoid the use of more than two eggs per individual bee in our experiments. The eggs were carefully transferred with a thin pair of tweezers onto the pollen diets previously placed in artificial brood cells. These artificial cells were made of small blocks of beech wood (4 x 2 x 2 cm) provided with a drilled burrow (length 2 cm, width 0.8 cm), open both at the top and at the front side. These openings were covered with coverslips attached to the block with transparent adhesive tape to permit free viewing into the burrow. Based on the average weight of the provisions from ten female brood cells, we provided 600 ± 5 mg of pollen diet per artificial brood cell. As eggs could not be sexed, each cell was provided with the same quantity of pollen diet despite the fact that under natural conditions male cells receive less food than female cells, which is explained by the distinctly smaller size of adult males (Westrich 1989). This experimental artefact did not appear to bias our results as the male larvae did never consume the entire cell provision in contrast to the female larvae that usually consumed the entire provision before they started to spin the cocoon.

6. 3. 5 Larval development

After egg transfer, the artificial brood cells were incubated in constant darkness within a climate chamber (E7 / 2; Conviron, Winnipeg, Canada) under the following conditions: 25°C for 16 hours followed by a gradual reduction of temperature to 10°C within four hours followed by a gradual increase back to 25°C within another four hours. Relative humidity was constantly held at 60%. Larval development was checked every second day and the following developmental stages were recorded: 1) egg hatching, 2) feeding without defecating, 3) feeding and defecating, 4) start of spinning silk, 5) completion of cocoon. After the cocoons had been completed, cells were kept in the climate chamber until autumn to allow for
the development of the bees to the adult stage. In autumn, all cocoons were weighed with the adult bees inside (AB204; Mettler Toledo, Switzerland) and afterwards stored at 4°C in constant darkness for overwintering. In early spring, the sex of the bees was determined through a small hole cut at the anterior end of the cocoon with a pair of small scissors. Males are easily distinguished from females by their white facial pilosity (Fig. 1). Sexing was done in early spring only, as opening the cocoons already in autumn would have caused high mortality of the overwintering bees.

6. 3. 6 Reproductive capability of the Ranunculus fed individuals

The ability to reproduce was tested for all individuals of the Bologna, Belgrade, Konstanz and Troyes populations that successfully developed on the Ranunculus pollen diet in 2011. In contrast to 2010 when 6 females developed into viable adults, no females, but only males emerged from the cocoons in 2012, which might have been due to the production of predominantly male eggs by the nesting O. cornuta females triggered by a temporary pollen-shortage phase in the walk-in cages in 2011 (see Strohm and Linsenmair 1997; Kim 1999; Bosch 2008). Therefore, the reproductive ability could only be tested for 18 males. These males were allowed to fly within a large walk-in cage together with 18 females that had developed on a Sinapis arvensis pollen diet. Completed nest were collected to determine the ploidy of the larvae. As in the Hymenoptera unfertilized eggs develop into haploid males, whereas fertilized eggs develop into diploid individuals that are generally females (Elias et al. 2009), production of diploid progeny by the Sinapis fed females would unambiguously indicate that their eggs had been fertilized by the sperm of the Ranunculus fed males. For the ploidy analyses, two post-defecating larvae were sampled per nest, including the innermost larva, which is usually female, and the outermost larva, which is usually male. Ploidy was determined using flow cytometry following the protocol provided by Ruf et al. (2013), using the head of the larvae for the extraction of cell material. For extraction and staining of cell material, we used CyStain® PI absolute T (Partec GmbH, Münster, Germany). Flow cytometric analyses were performed using a BD FACSCalibur™ multicolour flow cytometer.
(Becton, Dickinson and Company, Franklin Lakes, NJ, USA) equipped with a blue argon laser (excitation: 488 nm) and a band pass filter of 585 nm to detect PI fluorescence. For each sample, 15 000 nuclei were measured in an FL2-W/FL2-A gated region containing haploid, diploid and tetraploid cells, using the software CellQuest™ Pro (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The ploidy of the larvae was determined using flow cytometric DNA histograms of the head of adult *O. cornuta* males and females as a reference.

6. 3. 7 Data analysis

Eggs that did not hatch and larvae that undoubtedly died from external factors, such as mite attack or mechanical damage during handling, were excluded from all analyses. For analysis of survival, all larvae that completed their cocoons were counted as survivors, irrespective of whether they later successfully completed metamorphosis. Cocoons were regarded as completed when they became intransparent. Survival of larvae on the different pollen diets was analysed using Kaplan-Meier survival statistics (Lee and Wang 2003). The number of days between hatching and completion of the cocoon was considered to be “censored data”: individuals that died before the completion of the cocoon represented the exact observations for which the event (death) occurred. Those that completed the cocoon, i.e. the survivors, were considered the censored observations and thus withdrawn from survival calculations. Differences between survival distributions were analysed with log-rank tests, using the option “pairwise for each stratum” implemented in SPSS Statistics 19.0 and controlled with false discovery rate (FDR) correction (Benjamini and Hochberg 1995). Development time (time between egg hatching and completion of the cocoon) and adult weight (including cocoon) were analysed for those bees that successfully reached the adult stage because sex determination was not possible in the larval stage. Differences in the development time were analysed by applying a generalized linear model using pollen diet, population and sex as fixed factors. Differences in the adult weight were analysed for males only by applying a generalized linear model using pollen diet and
population as fixed factors. The number of *Ranunculus* reared females that completed metamorphosis was too low to apply statistical tests. Significant differences between the populations were identified using Bonferroni post-hoc tests. Statistical analyses were conducted with SPSS Statistics 19.0 for Macintosh OS X.

6. 4 RESULTS

A total of 504 transferred eggs hatched (Table 1). Three larvae that died from mechanical damage and one larva that died from an infection with mites were excluded from all analyses. Larval survival on the control (*Sinapis*) pollen diet was > 93% for each of the five populations (Table 1) and 95% of the survivors on the control pollen diet developed into viable adults, indicating that our rearing method had at most a marginal effect on larval performance.

![Figure 3](image-url) Cumulative survival of the larvae of *Osmia cornuta* from five geographically distant European populations reared a) on a control pollen diet of *Sinapis arvensis* and b) on a pollen diet of *Ranunculus acris*. Crosses indicate individuals that reached the cocoon stage (censored data). Different letters indicate significant differences (p < 0.05) between the populations (Kaplan-Meier analysis).
Table 1. Larval survival, development time and number of viable adults of individuals derived from five different populations of *Osmia cornuta* reared on a control pollen diet of *Sinapis arvensis* and on a pollen diet of *Ranunculus acris*.

<table>
<thead>
<tr>
<th>Pollen diet</th>
<th>Population</th>
<th>No. eggs hatched</th>
<th>Surviving larvae</th>
<th>Group heterogeneity</th>
<th>Development time [days]</th>
<th>No. viable adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>p</td>
<td>groups</td>
<td>Median</td>
</tr>
<tr>
<td><em>Sinapis</em></td>
<td>Belgrade</td>
<td>32</td>
<td>30</td>
<td>93.8</td>
<td>0.94</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Bologna</td>
<td>62</td>
<td>60</td>
<td>96.8</td>
<td>a</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Erfurt</td>
<td>65</td>
<td>64</td>
<td>98.5</td>
<td>a</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Konstanz</td>
<td>54</td>
<td>53</td>
<td>98.1</td>
<td>a</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Troyes</td>
<td>20</td>
<td>19</td>
<td>95</td>
<td>a</td>
<td>34</td>
</tr>
<tr>
<td><em>Ranunculus</em></td>
<td>Belgrade</td>
<td>49</td>
<td>12</td>
<td>24.5</td>
<td>0.004</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Bologna</td>
<td>62</td>
<td>14</td>
<td>22.6</td>
<td>ab</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Erfurt</td>
<td>80</td>
<td>8</td>
<td>10</td>
<td>b</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Konstanz</td>
<td>54</td>
<td>17</td>
<td>31.5</td>
<td>a</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Troyes</td>
<td>23</td>
<td>10</td>
<td>43.5</td>
<td>a</td>
<td>46</td>
</tr>
</tbody>
</table>

Notes: Development time represents the number of days between hatching and completion of the cocoon of all bees that developed into viable adults. Differences in the survival of the populations were tested using Kaplan-Meier analysis. Group heterogeneity: populations sharing the same letter did not differ significantly at $P < 0.05$ (post hoc test: pairwise log-rank tests with False Discovery Rate control). $m = \text{males, } f = \text{females.}$
6. 4. 1 Larval survival

Larval survival on the *Ranunculus* pollen diet was significantly reduced in all five populations (Kaplan-Meier analysis, log-rank tests; Belgrade: $\chi^2 = 32.331$, $P < 0.001$; Bologna: $\chi^2 = 70.097$, $P < 0.001$; Erfurt: $\chi^2 = 112.365$, $P < 0.001$; Konstanz: $\chi^2 = 54.597$, $P < 0.001$; Troyes: $\chi^2 = 11.407$, $P = 0.001$; Fig. 3). The majority of all larvae died on the *Ranunculus* pollen diet, however, some individuals from every population reached the cocoon stage (Table 1). The percentage of these survivors ranged from 10.0% among larvae of the Erfurt population to 43.5% among larvae of the Troyes population. 47.5% of all survivors successfully developed into viable adults (Table 1).

Larval survival on the *Ranunculus* pollen diet was significantly different between the populations (Kaplan-Meier analysis, log-rank test, $\chi^2 = 15.272$, $P < 0.004$). This difference was due to a significantly reduced survival of larvae of the Erfurt population compared to larvae of all other populations except the Bologna population (Kaplan-Meier analysis, pairwise log-rank tests; Erfurt-Troyes: $\chi^2 = 8.598$, $P = 0.003$; Erfurt-Konstanz: $\chi^2 = 9.639$, $P = 0.002$; Erfurt-Belgrade: $\chi^2 = 8.971$, $P = 0.003$; Erfurt-Bologna: $\chi^2 = 0.717$, $P = 0.397$; Table 1; Fig. 3). Larval survival did not differ significantly between the other four populations (results of statistical tests not shown).

6. 4. 2 Development time

Larval development time did not differ between males and females reared on the same pollen diet (Wald $\chi^2 = 0.949$, df = 1, $P = 0.330$). Development time on the *Ranunculus* pollen diet was significantly prolonged in larvae of all five populations (32-54 days, median: 42) compared to development time on the control pollen diet (26-44 days, median: 34) (Wald $\chi^2 = 131.582$, df = 1, $P < 0.001$; Fig. 4).

Development time on the *Ranunculus* pollen diet differed significantly between larvae derived from the different populations (Wald $\chi^2 = 15.922$, df = 4,
P = 0.003): development time of larvae of the Belgrade population was significantly prolonged compared to that of larvae of the Erfurt (Bonferroni post-hoc test: P = 0.046) and the Konstanz (Bonferroni post-hoc test: P = 0.015) populations. Development time did not differ significantly between all the other populations (results of statistical tests not shown). There was no significant interaction between population and pollen diet (Wald $\chi^2 = 4.179$, df = 4, P = 0.382) nor between pollen diet and sex (Wald $\chi^2 = 0.949$, df = 1, P = 0.330).

6.4.3 Adult weight

Adult weight of males reared on the *Ranunculus* pollen diet was significantly lower in all populations (46.5 - 98.1 mg, median: 67.8 mg) compared to those reared on the control pollen diet (48.4 - 178.5 mg, median: 110.4 mg) (Wald $\chi^2 = 73.034$, df = 1, P < 0.001; Fig. 5).

Adult male weight did not differ significantly between populations (Wald $\chi^2 = 1.901$, df = 4, P = 0.754), however, there was a significant interaction between population and pollen diet (Wald $\chi^2 = 11.383$, df = 4, P = 0.023). This interaction was due to the presence of significant interpopulational differences in the weight of individuals reared on the control pollen diet and the absence of such interpopulational differences between individuals reared on the *Ranunculus* pollen diet. *Sinapis* fed males of the Erfurt population were significantly heavier than those of the Belgrade and Bologna populations (Bonferroni post-hoc tests: Erfurt - Belgrade: P < 0.001; Erfurt - Bologna: P = 0.019) and *Sinapis* fed males of the Konstanz population were significantly heavier than those of the Belgrade population (Bonferroni post-hoc test: P = 0.016).
Figure 4. Development time of larvae of *Osmia cornuta* that successfully reached the adult stage when reared on a control pollen diet of *Sinapis arvensis* (Belgrade: n = 30, Bologna: n = 55, Erfurt: n = 62, Konstanz: n = 50, Troyes: n = 17) and on a pollen diet of *Ranunculus acris* (Belgrade: n = 5, Bologna: n = 8, Erfurt: n = 4, Konstanz: n = 8, Troyes: n = 4).

Figure 5. Adult weight (with cocoon) of males of *Osmia cornuta* from five different European populations reared on a control pollen diet of *Sinapis arvensis* (Belgrade: n = 28, Bologna: n = 42, Erfurt: n = 43, Konstanz: n = 15, Troyes: n = 10) and on a pollen diet of *Ranunculus acris* (Belgrade: n = 5, Bologna: n = 8, Erfurt: n = 3, Konstanz: n = 3, Troyes: n = 4).
6.4.4 Reproductive capability of Ranunculus fed individuals

To test their reproductive capability, males of *O. cornuta* that successfully developed on a *Ranunculus* pollen diet were allowed to mate with females reared on a *Sinapis* pollen diet, and 52 offspring larvae from 26 nests were analysed for their ploidy. Diploid individuals were found in 19 nests, clearly indicating that at least some of the *Ranunculus* fed males produced fertile sperm.

6.5 DISCUSSION

The results of this study clearly demonstrate that the ability to develop on a pollen diet of *Ranunculus acri*s differs within and between populations of the solitary bee species *Osmia cornuta*. In each of the five tested populations, a small proportion of the *Ranunculus* fed individuals reached the adult stage, indicating that the physiological ability to cope with the unfavourable properties of *Ranunculus* pollen exists in each population. Such intrapopulational variation in larval performance on non-host diets has not been previously recorded in bees. It was recently observed, however, in herbivorous insects. In two generalist moth species and a generalist beetle species, the physiological ability to develop on an unfamiliar host was found to vary considerably between larvae of the same population (Fox et al. 1997; Matsuki et al. 2011; Piskorski et al. 2011). In contrast, specialized leaf beetles of the genus *Ophraella* exhibited only little genetic variation influencing larval survival, oviposition and feeding responses on unfamiliar hosts (Futuyma et al. 1995). Differences in the ability of generalist versus specialist herbivores to cope with unfamiliar hosts might be due to a broader range of non-specific detoxifying tools used by generalists compared to specific tools required by the specialists to cope with the secondary chemistry of their exclusive hosts (Krieger et al. 1971; Li et al. 2003; Ramsey et al. 2010). Correspondingly, the experimental finding that all larvae of two pollen specialist bee species quickly died on a *Ranunculus* pollen diet (Praz et al. 2008c) suggests that the larvae of the generalist *O. cornuta* might possess
non-specific physiological tools that enable at least some individuals to successfully develop on a *Ranunculus* pollen diet.

Individuals of *O. cornuta* that developed on the *Ranunculus* pollen diet showed a distinctly prolonged development time, exhibited higher mortality during diapause and reached a considerably lower adult weight compared to individuals fed the control pollen diet. This finding adds further evidence in support of the unfavourable properties of *R. acris* pollen for bee larval development (Praz et al. 2008c; Sedivy et al. 2011). The mechanism underlying the adverse effect of *Ranunculus* pollen on larval development of unspecialized bee species is still unknown (Sedivy et al. 2012). Its unfavourable properties might result from deficiencies or imbalances in nutrient content, from structural properties that render extraction of pollen nutrients difficult, from interference of the pollenkit with nutrient assimilation or from toxic secondary pollen compounds (see Praz et al. 2008c and Roulston and Cane 2000 for a discussion of possible unfavourable pollen traits). Pollen of *Ranunculus* has an intermediate protein content as has the pollen of *Sinapis* (Wille et al. 1985, Roulston et al. 2000) and it contains all the amino acids known to be essential for the honeybee, albeit in rather low concentrations (McLellan 1977; Szczęsna 2006). Therefore, the content of both proteins and essential amino acids in the pollen of *Ranunculus* appears to be sufficient for a successful development. We cannot exclude, however, that other essential nutrients are lacking in *Ranunculus* pollen, such as sterols, which insects need for the synthesis of certain hormones, such as ecdysone (Svoboda et al. 1978; Rasmont et al. 2005). The fact that 80% of the larvae of *O. cornuta* already died within 14 days after onset of feeding, that the larvae turned conspicuously green shortly before they died and that the surviving larvae, irrespective of whether female or male, did not consume the entire cell provisions before they started to spin a cocoon might possibly indicate the presence of an unknown toxic compound in the pollen of *Ranunculus*. 
While it is well known that small adult body size considerably reduces bee reproductive fitness (Kim 1997; Seidelmann et al. 2010; but see Bosch and Vicens 2006), the Ranunculus fed adult males of O. cornuta were able to reproduce and to sire diploid offspring. Provided that Ranunculus fed adult females of O. cornuta are fertile as well, intrapopulational variation in the ability of O. cornuta to digest R. acris pollen might enable the incorporation of R. acris into the normal pollen host spectrum of O. cornuta, if the exploitation of R. acris flowers provides a fitness benefit within a particular population. The exploitation of R. acris might be favoured by a lack of sufficient pollen from other sources due to natural fluctuations in local flower abundances or due to anthropogenic habitat alteration, which may alter insect-host associations (Tabashnik 1983; Singer et al. 1993; Singer et al. 2008). Furthermore, it might be selected for by a large increase in the abundance of R. acris flowers in the bees' habitats due to fertilization, which favours the nitrophilous R. acris (Dorioz et al. 1987; Rudmann-Maurer et al. 2008), or by a closer phenological matching between the flowering period of R. acris and the flight period of O. cornuta due to climate change, which might cause advances in the phenology of both bees and their host plants (Hegland et al. 2009; Bartomeus et al. 2009; Forrest and Thomson 2011). Interestingly, climate change was recently found to lead to locally asynchronous advances in the phenologies of bees and their host flowers, such that the bees flight period advanced less quickly than the hosts' flowering time (Forrest and Thomson 2011; but see Bartomeus et al. 2011). An earlier advance of the flowering period of R. acris relative to the flight period of O. cornuta would extend the temporal overlap in phenology between O. cornuta and R. acris, which is currently very brief in many regions of Central Europe, and thus might enable natural selection to act towards the incorporation of R. acris into the diet of O. cornuta.

Beside intrapopulational variation, our study also revealed variation between populations in the performance of O. cornuta larvae on Ranunculus pollen. While interpopulational variation in larval performance on an unfamiliar host has been shown in a generalist beetle herbivore (Fox et al. 1997), our study provides the first
documentation of such variation in the ability of a solitary bee to digest pollen of a particular non-host. Larvae of the Erfurt population exhibited significantly reduced survival on the *Ranunculus* pollen diet compared to larvae of all other populations except the Bologna population. We hypothesize that the absence of *R. acri*s in the surroundings of Erfurt, which is due to a very dry climate in this region (M. Möhler, personal communication), might possibly account for this low larval survival rate. Similarly, in herbivorous insects, interpopulational variation in both larval performance and female host plant preference are usually explained by differences in the local host plant spectrum (Jaenike 1990; Keeler and Chew 2008; Downey and Nice 2011).

Our study focused on the performance of *O. cornuta* larvae on an unfamiliar pollen host. Incorporation of a new pollen host, however, does not only require physiological adaptations of the bee larvae to cope with the chemistry of the new pollen, but also neurological adaptations of the mother bee to recognize and exploit the host flowers. In fact, female host plant preference and larval performance are not necessarily correlated in herbivorous insects (Mayhew 1997, 2001) or in bees (Praz et al. 2008b) as is suggested by the “preference-performance hypothesis” or “optimal oviposition theory” (Jaenike 1978). Females of the pollen specialist solitary bee species *Heriades truncorum* refrained from collecting pollen on the flowers of two non-hosts although pollen of both non-host species were experimentally found to support larval development (Praz et al. 2008b). In that study, however, floral morphology of the non-hosts differed considerably from that of the exclusive host, suggesting that the *Heriades* females were not able to detect or collect the non-host pollen. In contrast, the flowers of *Ranunculus* have freely accessible anthers and strongly resemble the flowers of Rosaceae, which is the preferred host plant taxon of *O. cornuta* (Tasei 1973; Márquez et al. 1994). Moreover, *O. cornuta*, a highly polylectic species, collects pollen from host species that differ widely in flower morphology (Westrich 1989) and therefore likely possesses a broader repertoire of pollen collection behaviours than the oligolectic *H. truncorum*. Harvesting pollen of *Ranunculus* flowers should therefore not pose a major challenge for *O. cornuta*, and
increased co-occurrence of *R. acris* with *O. cornuta* is expected to result in quick acceptance of the *Ranunculus* flowers by pollen collecting females of *O. cornuta* if their exploitation is favoured by natural selection.

In conclusion, the present study provides first evidence for both intra- und interpopulational variation in the physiological ability of solitary bees to digest non-host pollen. This variation might provide a basis for natural selection to act in favour of host expansion and subsequent host shifts.
Pollen mixing in pollen generalist solitary bees: a strategy to complement or mitigate unfavourable pollen properties?

7.1 Abstract

Generalist herbivorous insects, which feed on plant tissue that is nutritionally heterogeneous or varies in its content of secondary metabolites, often apply dietary mixing to improve nutrient balance or to reduce exposure to harmful secondary metabolites. Pollen is similarly heterogeneous as plant tissue in its content of primary and secondary metabolites, suggesting that providing their offspring with mixed pollen diets might be a promising strategy for pollen generalist bees to complement nutrient imbalances or to mitigate harmful secondary metabolites of unfavourable pollen. In the present study, we compared larval performance of the pollen generalist solitary bee species *Osmia cornuta* (Megachilidae) on five experimental pollen diets that consisted of different proportions of unfavourable pollen diet of *Ranunculus acris* (Ranunculaceae) and favourable pollen diet of *Sinapis arvensis* (Brassicaceae). In addition, we microscopically analyzed the pollen contained in the scopal brushes of field-collected females of *O. cornuta* and three closely related species to elucidate to which degree these pollen generalist bees mix pollen of different hosts in their brood cells. In striking contrast to a pure *Ranunculus* pollen diet, which had a lethal effect on most developing larvae of *O. cornuta*, larval survival, larval development time and adult body mass of both males and females remained nearly unaffected by the admixture of up to 50% of *Ranunculus* pollen diet to the larval food. Between 42% and 66% of all female scopal pollen loads analyzed contained mixtures of pollen from two to six plant families, indicating that pollen mixing is a common behaviour in *O. cornuta* and the three related bee species. The present study provides first evidence that the larvae of pollen generalist bees can benefit from the nutrient content of unfavourable pollen without being negatively affected by its unfavourable chemical properties if such pollen is mixed with favourable pollen. We conclude that the widespread pollen
mixing by females of pollen generalist bees should also be considered as a possible strategy to exploit flowers with unfavourable pollen and to optimize larval food quality.

7. 2 INTRODUCTION

Pollen is not an easy-to-use nutrient source for pollen feeding flower visitors such as bees. Its collection is labour intensive due to the frequently small pollen quantities contained in a single flower (Müller et al. 2006), its removal from the flowers often requires specialized morphological or behavioural adaptations (Thorp 2000), and its nutrient content differs greatly among plant taxa (Roulston and Cane 2000). In addition, pollen might exhibit unfavourable chemical properties that reduce its suitability as nutrient source for pollen feeders because i) its nutrients might be difficult to extract from within the protoplasm (Suárez-Cervera et al. 1994; Dobson and Peng 1997), ii) it might be low in protein content or lack essential nutrients (Herbert et al. 1970; Wille et al. 1985; Rasmont et al. 2005; Somerville and Nicol 2006; Weiner et al. 2010), iii) its pollenkit layer might interfere with nutrient assimilation (Peng et al. 1985; Williams 2003), or iv) it might contain harmful secondary metabolites such as alkaloids, lactones, diterpenes or cyanogenic glycosides (Detzel and Wink 1993; London-Shafir et al. 2003; Gunduz et al. 2008; Kempf et al. 2010; Roelants et al. 2010; Sedivy et al. 2012). While the concentration of secondary metabolites in pollen was too low to negatively affect the larvae of two solitary bee species (Sedivy et al. 2012), secondary metabolites in pollen of Piptadenia stipulacea (Mimosaceae), Prunus dulcis (Rosaceae), Ricinus communis (Euphorbiaceae) and Zigadenus venenosus (Melanthiaceae) negatively affected survival of adult honey bees (Hitchcock 1959; Kevan and Ebert 2005; de Mesquita et al. 2010; de Assis Junior et al. 2011), and pyrrolizidine alkaloids in pollen of Echium vulgare were lethal to honeybee larvae at concentrations that correspond to the natural alkaloid concentration in Echium pollen (Reinhard 2011). Unfavourable chemical pollen properties might also underlie the failure of several pollen specialist
and pollen generalist bee species to develop on non-host pollen (Praz et al. 2008c; Sedivy et al. 2011; Haider et al. 2013). Thus, increasing evidence suggests that pollen chemistry is much more important in shaping pollinator-flower relationships than hitherto thought.

Bees differ widely in their selection of pollen hosts. In contrast to pollen specialist (“oligolectic”) species that exclusively collect pollen on flowers belonging to a single plant genus or family, pollen generalist (“polylectic”) species are behaviourally and physiologically able to exploit flowers of two to many plant families (Cane and Sipes 2006; Müller and Kuhlmann 2008). Due to the enormous pollen quantities bees need for their own reproduction (Schlindwein et al. 2005; Müller et al. 2006; Larsson and Franzen 2007; Cane et al. 2011; Schäffler and Dötterl 2011), polylecty is considered advantageous in reducing dependency upon a restricted number of pollen hosts (Moldenke 1975; Eickwort and Ginsberg 1980). In contrast to oligolectic bee species, females of polylectic species can provide their progeny with a mixture of pollen from widely different and unrelated plant taxa, what they indeed often do (Westrich 1989; Williams and Tepedino 2003; Budde and Lunau 2007). Thus, polylecty at the individual level might possibly represent a strategy to benefit from particular nutrients of unfavourable pollen (e.g. proteins) by complementing its nutrient deficiencies or mitigating its harmful secondary metabolites with favourable pollen. Pollen mixing in polylectic bees has to our knowledge never been hypothesized to be adaptive in this regard. In contrast, dietary mixing in generalist herbivorous insects has received much attention in the past decades and was repeatedly found to positively affect the herbivores' performance (Bernays et al. 1994; Hägele and Rowell-Rahier 1999; Behmer et al. 2001, 2002; Singer et al. 2002; Pöykkö and Hyvärinen 2003; Unsicker et al. 2008; Johns et al. 2009; but see Bernays and Minkenberg 1997).

In the present study, we test the hypothesis that pollen mixing might be a strategy of polylectic bees to complement or mitigate unfavourable pollen properties by comparing larval performance of the pollen generalist solitary bee *Osmia cornuta*
(Megachilidae) on experimental pollen provisions that contain different proportions of unfavourable buttercup (*Ranunculus*) pollen diet. Although the underlying mechanism remains unclear (Sedivy et al. 2012), a pure diet of *Ranunculus* pollen adversely affects larval development of *O. cornuta* in that it is either lethal or results in dwarfish adults (Sedivy et al. 2011; Haider et al. 2013). In addition, we microscopically analyzed the pollen contained in the scopal brushes of field-collected females of *O. cornuta* and three closely related species to elucidate to which degree these polylectic bees mix pollen of different hosts in their brood cells under natural conditions. We hypothesized i) that larval survival and adult body mass of *O. cornuta* increase with decreasing proportions of *Ranunculus* pollen in the experimental diet, and ii) that the females of the four polylectic bee species regularly harvest pollen from several hosts on a single foraging bout, which eventually results in mixed pollen diets for the developing bee larvae.

### 7.3 METHODS

**7.3.1 Bee species**

*Osmia cornuta* (Latreille 1805), which is widespread in the Palaearctic and common in the warmer parts of Central Europe, is active from mid March to end of April (Westrich 1989). The females nest in a great variety of pre-existing cavities, where they build several brood cells during their lifetime, which lasts for up to six weeks. Each cell is provisioned with a mixture of pollen and nectar before a single egg is laid on top of the provision. The hatched larva feeds on the pollen-nectar provision and develops within a few weeks to the adult bee, which overwinters inside the brood cell within its cocoon and emerges the following spring. *O. cornuta* is a pronounced pollen generalist bee and collects pollen on many different plant families (Westrich 1989; Màrquez et al. 1994). Pollen analysis of scopal contents of collected females revealed that Rosaceae are the preferred hosts and that, among others, *Salix* (Salicaceae), *Corydalis* (Papaveraceae) and *Acer* (Aceraceae) are also
frequently exploited for pollen (M. Haider, S. Dorn, C. Sedivy and A. Müller, unpublished data).

For the present study, 150 female and male cocoons of *O. cornuta* originating from Konstanz (Germany) were transferred to two walk-in cages (8 x 10 x 3.5 m) at the experimental research station of ETH Zurich at Eschikon. Each walk-in cage contained two artificial nesting stands provided with hollow bamboo sticks as nesting sites and about 450 flowering plants of *Sinapis arvensis* (Brassicaceae) as pollen and nectar sources. Pollen of *S. arvensis* is readily collected by females of *O. cornuta* and supports larval development (Sedivy *et al.* 2011).

7.3.2 Experimental pollen diets

Larvae of *O. cornuta* were reared on five pollen diets that contained different ratios of *Ranunculus acris* pollen diet (R) to *Sinapis arvensis* pollen diet (S) determined by weight (AB204-S; Mettler Toledo, Greifensee, Switzerland): 0% R to 100% S; 25% R to 75% S; 50% R to 50% S; 75% R to 25% S; and 100% R to 0% S. The pollen mixtures were generated by thoroughly but carefully mixing pollen diets of *R. acris* and *S. arvensis* in petri dishes with a spoon until a homogeneous mixture was obtained. The initial amount of pollen diet was 200 mg per brood cell; shortly before the pollen diet had been completely consumed by the feeding larva, an additional 400 mg of pollen diet was provided.

To obtain pollen diets of *S. arvensis*, we collected brood cell provisions of nests of *O. cornuta* built in the walk-in cages. To obtain pollen diets of *R. acris*, we collected brood cell provisions of nests of the *Ranunculus* specialist bee *Chelostoma florisomne* (Megachilidae) at Gletterens (Western Switzerland), where *R. acris* was the near exclusive pollen host. Since water and nectar sugar content in the brood cells of *O. cornuta* and *C. florisomne* do not differ significantly (A. Bühler and A. Müller, unpublished data), our results are not expected to be biased due to different nectar amounts in the provisions of the two bee species. All cell provisions
were collected in spring 2012 and kept frozen at -20°C for at least 24 hours till the onset of the experiments, which were conducted in spring and summer 2012.

7.3.3 Egg transfer and larval performance

All eggs used for the experiments originated from *O. cornuta* females that nested in the walk-in cages. For each of the five pollen diets between 33 and 37 eggs were transferred. The eggs were removed from the brood cell provisions with feather tweezers and carefully transferred to artificial brood cells containing the experimental pollen diet. These brood cells were made of small blocks (4 x 4 x 2.2 cm) of beech wood provided with a drilled burrow (2 cm length, 0.8 cm width) open both at the top and the front side. The openings were covered with coverslips attached to the block with transparent adhesive tape to allow free viewing into the burrow. We usually transferred five eggs per original nest, i.e. one egg to each of the five different pollen diets. As the females often build more than one nest during their flight period, all females were individually marked with numbered opalite plates to avoid the use of more than five eggs per individual bee.

After egg transfer, the artificial brood cells were incubated in constant darkness within a climate chamber (E7/2; Conviron, Winnipeg, Canada) under the following conditions: 25°C for 16 hours followed by a gradual reduction of temperature to 10°C within four hours followed by a gradual increase back to 25°C within another four hours. Relative humidity was constantly held at 60%. Larval development was checked every second day and the following developmental stages were recorded: 1) egg hatching, 2) feeding without defecating, 3) feeding and defecating, 4) start of cocoon spinning, 5) completion of cocoon. After the cocoons had been completed, the brood cells were kept in the climate chamber until autumn to allow for the development of the bees to the adult stage. In autumn, the imagines were sexed through a small hole cut at the anterior end of the cocoon and weighed including their cocoons to the nearest 0.1 mg (AB204; Mettler Toledo, Greifensee, Switzerland).
7.3.4 Composition of female pollen loads

We microscopically analyzed the pollen contained in the metasomal scopae of 50 females of *O. cornuta*. In addition, we analyzed 50 pollen loads each of *O. bicornis* (Linnaeus 1758), *O. lignaria* Say 1837 and *O. tricornis* Latreille 1811. The latter three bee species are pronounced pollen generalists as *O. cornuta* and also belong to the *Osmia bicornis* group, which is a monophyletic clade comprising about 15 species worldwide (Peters 1978, Michener 2007, M. Haider, S. Dorn, C. Sedivy and A. Müller, unpublished data). The pollen grains were stripped off the scopae with a fine needle and embedded in glycerine gelatine on a slide. We identified the pollen grains to family level and determined the number of different pollen types by counting the grains along two lines chosen randomly across the cover slip at a magnification of 400x. Pollen types represented by less than 5% of the counted grains were excluded to prevent potential bias caused by contamination.

7.3.5 Data analysis

Unhatched eggs and larvae that died from external factors, such as mechanical damage during handling, were excluded from all analyses. Survival of larvae on the different pollen diets was analyzed using Kaplan-Meier survival statistics (Lee and Wang 2003). All larvae that completed their cocoons were considered as survivors, irrespective of whether they later successfully completed metamorphosis or not. Cocoons were regarded as completed when they became intransparent. The number of days between hatching and completion of the cocoon was considered to be “censored data”. Individuals that died before the completion of the cocoon represented the exact observations for which the event (death) occurred, those that completed the cocoon, i.e. the survivors, represented the censored observations. Differences between survival distributions were analyzed with log-rank tests, using the option “pairwise over strata” implemented in SPSS Statistics 20.0.0 and controlled with false discovery rate (FDR) correction (Benjamini and Hochberg 1995). Due to the pronounced sex dimorphism in size, differences in larval development time (number of days between egg hatching and completion of the
cocoon) and adult body mass were analyzed separately for males and females with Kruskal-Wallis one-way analysis of variance. Pairwise comparisons were performed with Mann-Whitney U tests and controlled with FDR correction. Statistical analyses were conducted with SPSS Statistics 20.0.0 for Macintosh OS X.

7.4 RESULTS

A total of 155 transferred eggs of *Osmia cornuta* hatched. One larva that died from mechanical damage was excluded from all analyses. Larval survival on the pure *Sinapis arvensis* pollen diet (0% R) amounted to 96.7% (Table 1), indicating that our methodology had a negligible effect on larval mortality.

<table>
<thead>
<tr>
<th>Pollen diet</th>
<th>No. eggs hatched</th>
<th>No. surviving larvae</th>
<th>Larval development time [d ± SE]</th>
<th>Group heterogeneity</th>
<th>No. viable adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% R : 100% S</td>
<td>30</td>
<td>29 (96.7%)</td>
<td>32.3 ± 0.4</td>
<td>&lt; 0.001</td>
<td>a 26 (86.7%)</td>
</tr>
<tr>
<td>25% R : 75% S</td>
<td>31</td>
<td>30 (96.8%)</td>
<td>34.5 ± 0.7</td>
<td>a</td>
<td>28 (90.3%)</td>
</tr>
<tr>
<td>50% R : 50% S</td>
<td>30</td>
<td>30 (100%)</td>
<td>33.7 ± 0.4</td>
<td>a</td>
<td>30 (100%)</td>
</tr>
<tr>
<td>75% R : 25% S</td>
<td>30</td>
<td>22 (73.3%)</td>
<td>36.5 ± 0.5</td>
<td>b</td>
<td>18 (60.0%)</td>
</tr>
<tr>
<td>100% R : 0% S</td>
<td>34</td>
<td>6 (17.6%)</td>
<td>40.7 ± 1.9</td>
<td>c</td>
<td>4 (11.8%)</td>
</tr>
</tbody>
</table>

Notes: Differences in the survival on the five different pollen diets were tested using Kaplan-Meier analysis (post hoc test: pairwise log-rank tests with False Discovery Rate control). Group heterogeneity: pollen diets sharing the same letter did not differ significantly at $P < 0.05$. 

Table 1. Larval survival, larval development time and number of viable adults of the pollen generalist bee species *Osmia cornuta* when reared on five different pollen diets composed of different mixtures of non-host pollen diet of *Ranunculus acris* (R) and host pollen diet of *Sinapis arvensis* (S).
Figure 1. Cumulative survival of larvae of *Osmia cornuta* when reared on five different pollen diets composed of different mixtures of non-host pollen diet of *Ranunculus acris* (R) and host pollen diet of *Sinapis arvensis* (S). Bars indicate individuals that reached the cocoon stage (censored data).

7. 4. 1 Larval survival

Larval survival of *O. cornuta* differed significantly between the pollen diets (Kaplan-Meier analysis: $\chi^2 = 117.84$, df = 4, $P < 0.001$; Fig. 1, Table 1). Larvae reared on 0% R, 25% R and 50% R all performed significantly better than larvae reared on 75% R (Kaplan-Meier analysis: 0% R: $\chi^2 = 6.10$, $P = 0.014$; 25% R: $\chi^2 = 6.33$, $P = 0.012$; 50% R: $\chi^2 = 9.10$, $P = 0.003$) and larvae reared on 100% R (Kaplan-Meier analysis: 0% R: $\chi^2 = 37.11$, $P < 0.001$; 25% R: $\chi^2 = 40.61$, $P < 0.001$; 50% R: $\chi^2 = 42.74$, $P < 0.001$). Larvae reared on 75% R performed significantly better than larvae reared on 100% R (Kaplan-Meier analysis: $\chi^2 = 23.93$, $P < 0.001$). Larval survival did not differ between 0% R and 25% R (Kaplan-Meier analysis: $\chi^2 = 0.001$, $P = 0.981$), between 0% R and 50% R (Kaplan-Meier analysis: $\chi^2 = 1.00$, $P = 0.317$) and between 25% R and 50% R (Kaplan-Meier analysis: $\chi^2 = 0.97$, $P = 0.325$).

Among the 117 individuals that reached the cocoon stage, 11 later died as larva ($n = 8$), pupa ($n = 2$) or imago ($n = 1$) within the cocoon. These individuals were excluded from the analysis of larval development time and adult body mass.
Figure 2. Larval development time of males and females of *Osmia cornuta* when reared on five different pollen diets composed of different mixtures of non-host pollen diet of *Ranunculus acris* (R) and host pollen diet of *Sinapis arvensis*. No females reached the adult stage when reared on a 100% *Ranunculus* pollen diet.

7.4.2 Larval development time

Larval development time of males differed significantly between the pollen diets (Kruskal-Wallis test: *P* < 0.001; Fig. 2). It was significantly shorter on 0% R (median: 32 d) than on all other pollen diets except for 50% R (median: 33 d), and significantly longer on 100% R (median: 41 d) than on all other pollen diets except for 75% R (median: 35 d) (Mann-Whitney U tests: 0% R vs. 25% R: *P* = 0.002; 0% R vs. 50% R: *P* = 0.057; 0% R vs. 75% R: *P* = 0.003; 0% R vs. 100% R: *P* = 0.001; 25% R
vs. 100% R: \( P = 0.014 \); 50% R vs. 100% R: \( P = 0.005 \); 75% R vs. 100% R: \( P = 0.109 \).

Male development time did not differ among pollen diets containing 25% R (median: 34 d), 50% R and 75% R (Mann-Whitney U tests: 25% R vs. 50% R: \( P = 0.143 \); 25% R vs. 75% R: \( P = 0.237 \); 50% R vs. 75% R: \( P = 0.05 \)).

Larval development time of females differed significantly between the pollen diets (Kruskal-Wallis test: \( P = 0.039 \); Fig. 2). It was significantly shorter on 0% R (median: 34 d) than on 75% R (median: 38 d) (Mann-Whitney U test: \( P = 0.006 \)). Female development time did not differ among pollen diets containing 0% R, 25% R (median: 34 d) and 50% R (median: 35 d) (Mann-Whitney U tests: 0% R vs. 25% R: \( P = 0.53 \); 0% R vs. 50% R: \( P = 0.232 \); 25% R vs. 50% R: \( P = 0.833 \)), nor between pollen diets containing 25% R and 75% R (Mann-Whitney U test: \( P = 0.32 \)) and pollen diets containing 50% R and 75% R (Mann-Whitney U test: \( P = 0.062 \)). Larval development time of females reared on 100% R could not be compared with that of females reared on the other pollen diets as no females reached the cocoon stage on 100% R.

7.4.3 Adult body mass

Adult body mass of males differed significantly between the pollen diets (Kruskal-Wallis test: \( P = 0.001 \); Fig. 3). It was significantly lower on 100% R (median: 71.3 mg) than on 0% R (median: 119.7 mg), 25% R (median: 114.9 mg) and 50% R (median: 123.3 mg), and significantly lower on 75% R (median: 109.4 mg) than on 50% R (Mann-Whitney U tests: 0% R vs. 100% R: \( P < 0.001 \); 25% R vs. 100% R: \( P = 0.001 \); 50% R vs. 100% R: \( P < 0.001 \); 50% R vs. 75% R: \( P = 0.005 \)). Male adult body mass did not differ among pollen diets containing 0% R, 25% R and 50% R (Mann-Whitney U tests: 0% R vs. 25% R: \( P = 0.363 \); 0% R vs. 50% R: \( P = 0.302 \); 25% R vs. 50% R: \( P = 0.120 \)), nor between pollen diets containing 25% R and 75% R (Mann-Whitney U test: \( P = 0.132 \)). Differences in male adult body mass were not significant after FDR correction between pollen diets
containing 0% R and 75% R (Mann-Whitney U test: P = 0.034) and between pollen diets containing 75% R and 100% R (Mann-Whitney U test: P = 0.038).

Figures 3. Adult body mass of males and females of *Osmia cornuta* when reared on five different pollen diets composed of different mixtures of non-host pollen diet of *Ranunculus acris* (R) and host pollen diet of *Sinapis arvensis*. No females reached the adult stage when reared on a 100% *Ranunculus* pollen diet.

Adult body mass of females differed significantly between the pollen diets (Kruskal-Wallis test: P = 0.021; Fig. 3). It was significantly lower on 75% R (median: 161.6 mg) than on 0% R (median: 175.9 mg) and on 50% R (median: 168.4 mg) (Mann-Whitney U tests: 0% R vs. 75% R: P = 0.006; 50% R vs. 75% R: P = 0.012). Female adult body mass did not differ among pollen diets containing 0% R, 25% R
(median: 163.1 mg) and 50% R (Mann-Whitney U tests: 0% R vs. 25% R: P = 0.268; 0% R vs. 50% R: P = 0.694; 25% R vs. 50% R: P = 0.171), nor between pollen diets containing 25% R and 75% R (Mann-Whitney U test: P = 0.661). Adult body mass of females reared on 100% R could not be compared with that of females reared on the other pollen diets as no females reached the adult stage on 100% R.

7.4.4 Composition of female pollen loads

In *O. cornuta*, 52% of the pollen loads analyzed consisted of a single pollen type, while 48% were mixed and contained pollen of two to five plant families. The proportion of mixed pollen loads was 56% in *O. bicornis* (two to six plant families), 42% in *O. lignaria* (two to three plant families) and 66% in *O. tricornis* (two to four plant families).

7.5 DISCUSSION

The present study shows that larval survival of the pollen generalist solitary bee species *Osmia cornuta* is not affected by the admixture of up to 50% of unfavourable *Ranunculus acris* pollen diet to the larval provisions, which strikingly contrasts with the lethal effect exhibited by the pure *Ranunculus* pollen diet on most developing *O. cornuta* larvae. Similarly, larval development time and adult body mass of both *O. cornuta* males and females did not differ among provisions containing 0%, 25% and 50% of *Ranunculus* pollen diet, except for male larvae that reached the cocoon stage slightly faster when reared on a 0% *Ranunculus* pollen diet than on a 25% *Ranunculus* pollen diet. These findings strongly suggest that the larvae of *O. cornuta* can benefit from the nutrient content of *Ranunculus* pollen without being negatively affected by its unfavourable chemical properties as long as the proportion of this pollen in the larval diet does not exceed 50%.

Whereas a beneficial effect of pollen mixing on larval performance in polylectic solitary bees has never been reported before, dietary mixing was found to
positively affect performance of generalist herbivorous insects in many studies (Behmer 2009 and references therein). Better performance of generalist herbivores on mixed compared to uniform diets was found to be due to nutrient complementation (Behmer et al. 2001; Unsicker et al. 2008), to mitigation of harmful secondary metabolites (Singer et al. 2002) or to a combination of both (Bernays et al. 1994), whereas in other studies the positive effects of nutrient complementation and toxin mitigation proved to be difficult to disentangle (Hägele and Rahier 1999; Behmer et al. 2002). We suggest that toxin mitigation might be the more likely explanation for the better performance of the larvae of *O. cornuta* on mixed pollen diets than nutrient complementation. In contrast to the larvae that were reared on the three provisions containing the lowest amounts of *Ranunculus* pollen diets, the larvae that successfully developed on the two provisions containing the highest amounts of *Ranunculus* pollen diets consumed considerably less of the provided pollen diet, which most probably contributed to their low adult body mass. This observation suggests that *Ranunculus* pollen is not nutritionally deficient, otherwise the larvae would have been expected to consume the entire provisions to compensate for the lacking nutrients. In fact, pollen of *Ranunculus* has a similar protein content as pollen of *Sinapis* (Wille et al. 1985; Roulston, Cane and Buchmann 2000; Somerville and Nicol 2006; Budde and Lunau 2007), and it contains all the amino acids considered to be essential for honeybee development (McLellan 1977; Szczesna 2006). Instead, we hypothesize that the *O. cornuta* larvae discontinued feeding prematurely to avoid intoxication by potential secondary metabolites in the *Ranunculus* pollen. Dilution of these metabolites below a detrimental threshold might have contributed to the better larval performance on diets with admixed *Sinapis* pollen. This hypothesis is supported by the finding that most *O. cornuta* larvae reared on a pure *Ranunculus* pollen diet either died during early development (<15 days) or survived and reached the cocoon stage. This finding is in line with the increased susceptibility of first instar larvae of the moth *Spodoptera eridania* towards plant allelochemicals (Johnson and Bentley 1988).
In the present study, we found that 48% of the pollen loads of field-collected females of *O. cornuta* and 42% to 66% of those of the three closely related species were mixtures of pollen from two to six plant families. As each single brood cell of these pollen generalist bee species is provisioned with pollen of about 20 to 40 foraging bouts (Raw 1972, Tasei 1973, Maddocks and Paulus 1987), a substantial proportion of their larval provisions is expected to consist of pollen mixtures. In fact, 13 out of 31 brood cells of *O. cornuta* and 27 out of 52 brood cells of *O. bicornis* contained pollen mixtures of two to four pollen types (Tasei 1973), and all 76 larval provisions analyzed for *O. bicornis* were mixtures of at least three pollen types (Budde and Lunau 2007). Bumblebees, which are usually similarly pronounced pollen generalists as species of the *Osmia bicornis* group, frequently exploit flowers of several plant species during a single foraging bout (Goulson 2003). Between 34% and 63% of all pollen loads analyzed for *Bombus lucorum* and *B. pascuorum*, respectively, were mixtures of two to eight pollen types (Free 1970), foragers of *B. pascuorum* frequently switched pollen hosts and collected significant amounts of pollen from up to three plant species during a single foraging bout (Leonhardt and Blüthgen 2012), and the analysis of the larval meconia of three *Bombus* species revealed that 62% to 96% of all individuals had devoured pollen of two to five plant taxa during their development (Brian 1951). These findings indicate that the larvae of polyleptic bee species often consume mixed pollen diets.

Pollen collection from several unrelated plant taxa during the same or consecutive foraging bouts, which results in mixed pollen diets, might be advantageous for polylectic bees simply because such a behavior allows the simultaneous exploitation of a multitude of valuable flower resources, increasing the quantity of pollen and nectar harvested per unit of time (Williams and Tepedino 2003). However, the results of our experiments suggest that the females of polylectic bee species might adopt pollen mixing also as a strategy to exploit flowers with unfavourable pollen, particularly under food shortage. Such a strategy seems to be highly advantageous as it broadens the pollen host spectrum to plant taxa possessing pollen that does not support larval development when provided as sole food because
its digestion requires specialized physiological adaptations. Larvae of polylectic bee species that feed on pollen mixtures might thus benefit from the nutrient content of a particular pollen without being negatively affected by its unfavourable properties. If females of pollen generalist bee species were indeed able to optimize the pollen composition in the larval food provisions according to the flower supply and the physiological demands of their progeny, we would expect that their cell provisions regularly contain small to intermediate amounts of unfavourable pollen. Anecdotal evidence exists that the provisions of polylectic bees may indeed contain unfavourable pollen admixed to favourable pollen. Unfavourable pollen of *Ranunculus* was found partly mixed with other pollen in brood cells of *O. cornuta* (Tasei 1973; Márquez et al. 1994; Nepi et al. 1997), and *Echium* pollen, which does not support larval development of *O. bicornis* (Sedivy et al. 2011), was recorded mixed with other pollen in six out of 50 pollen loads of *O. bicornis* (M. Haider, S. Dorn, C. Sedivy and A. Müller, unpublished data). Similarly, the broadly polylectic *Osmia lignaria* occasionally exploits *Taraxacum* (Asteraceae) for pollen (Torchio 1976, 1982; Sheffield et al. 2008), although this pollen was experimentally found to have detrimental to lethal effects on developing larvae if provided as a pure diet (Levin and Haydak 1957; Rust 1990). Larval provisions of 16 out of 31 brood cells of *O. lignaria* contained 1% to 100%, on average 29.6% of unfavourable *Taraxacum* pollen, and faeces of 11 out of 31 larvae contained 2% to 25%, on average 7.2% of *Taraxacum* pollen (Torchio 1976).

In conclusion, the present study provides first evidence that the larvae of solitary bees can benefit from the nutrient content of unfavourable pollen if such pollen is mixed by the mother bees with favourable pollen in the brood cells. Therefore, polylecty in bees might possibly also have evolved as a strategy to complement nutritional imbalances or to mitigate harmful secondary metabolites of unfavourable pollen, thus contributing to reduce the bees' dependency upon hosts with favourable pollen. However, whether the females of polylectic bee species are able to conduct targeted pollen mixing to optimize food quality for their progeny remains to be investigated.
8 General Discussion

Focusing on the group of mainly polylectic bees of the subgenus *Osmia*, the present study explored the complex relationships between flowering plants and their bee pollinators. It used molecular genetic tools to investigate species relationships, and microscopic analysis of pollen loads and bee rearing experiments to assess the factors shaping the host plant associations of these bees. Pollen host choice appeared to be even in polylectic species strongly restricted by plant traits, but experimental evidence suggests that bees might employ different mechanisms to overcome these constraints and thus to expand their floral host range.

The phylogeny of the three closely related *Osmia* subgenera *Osmia*, *Orientosmia* and *Monosmia*, was reconstructed based on molecular and morphological data. Microscopical analyses of the scopal pollen loads of female specimens revealed that five species of this clade are pollen specialists, while all others are pollen generalists. In agreement with its more recent origin (Litman et al. 2011), polylecty appeared to be the ancestral state of the examined group, with oligolectic lineages having evolved twice independently. The patterns of host plant use suggested that pollen host choice of polylectic species is strongly constrained by plant traits such as flower morphology, pollen chemistry or nectar availability (section 4). These floral constraints might even be amplified, if plants employ multiple strategies to restrict pollen loss to bees. Rearing experiments indicated that in addition to a morphologically hindered access of bees to the anthers, pure pollen diet of *Lotus corniculatus* (Fabaceae) might possess unfavourable properties that impede development of unspecialized bee larvae (section 5).

At the same time, bees acquired physiological and behavioural traits, which might allow them to overcome such constraints. First, substantial intra- and interpopulational differences in the larval survival on unfavourable *Ranunculus* pollen diet showed that in several populations of the polylectic bee species *Osmia cornuta* the physiological adaptations needed to cope with unfavourable *Ranunculus*
pollen diet exist. At least some of the bees that successfully developed on *Ranunculus* diet were fertile and sired viable offspring, indicating that one condition for natural selection to act towards host expansion is fulfilled (section 6). And second, polylectic bees might employ pollen mixing as a strategy to mitigate unfavourable pollen properties. Up to 50% of unfavourable *Ranunculus* pollen admixed to favourable *Sinapis* pollen diet neither negatively affected bee larval survival nor adult body mass. Analysis of female scopal pollen loads indicated that pollen mixing is a common behaviour of polylectic *Osmia* species. Thus, beside physiological traits, also neurological traits concerning pollen collection might enable polylectic bees to broaden their pollen range to plants with unfavourable pollen properties, albeit only to a certain extent (section 8).

*Host plant choice of polyleges is strongly governed by plant traits*

Our understanding of host plant choice in bees was substantially advanced by recent phylogenetic studies on the evolution of host plant choice in different bee lineages (Danforth et al. 2006b; Larkin et al. 2008; Litman et al. 2011; Michez et al. 2008; Müller 1996; Sedivy et al. 2013; Sedivy et al. 2008; Sipes and Tepedino 2005), by bee rearing experiments (Praz et al. 2008c; Reinhard 2011; Sedivy et al. 2011; Williams 2003) and by behavioural studies concerning female pollen collection (Praz et al. 2008b; Williams 2003; Williams and Tepedino 2003). These studies led to two main conclusions. First, in contrast to the long held assumption that polylecty is the ancestral state in bees (reviewed in Müller 1996), the origin of bees as a whole is now considered oligolectic, especially as the most basal clades appear to be originally oligolectic (Danforth et al. 2013). And second, not interspecific competition, but plant traits such as floral shape, colour and morphology as well as pollen chemistry are suggested to be the main factors influencing host plant choice in bees (Michez et al. 2008; Müller 1996; Sedivy et al. 2008). As a consequence of the constraints imposed by these plant traits, bees might need physiological adaptations regarding pollen digestion (Praz et al. 2008c; Sedivy et al. 2011) or neurological adaptations regarding flower recognition and handling (Praz et al. 2008b) in order
to successfully exploit a certain plant taxon for pollen. Accordingly, the constraint hypothesis of host plant use in bees (Sedivy et al. 2008) suggests that i) incorporation of new pollen hosts only rarely occurs during evolution, ii) an expansion of the host spectrum is only possible, if the physiological or neurological constraints imposed by flowers are overcome and iii) host shifts among oligoleges are preceded by a period of host expansion, followed by re-specialization.

These findings and postulates on the evolution of host plant choice in bees predominantly derive from studies upon oligolectic species (but see Sedivy et al. 2011). In contrast, the present study focused on polylectic species. Its results illustrate several striking similarities between host plant choice of polylectic and oligolectic species. Although polylectic bees might, unlike their oligolectic relatives, collect pollen from plants of many different plant families, pollen host ranges of polylectic species are restricted as well (Westrich 1989). One of the clearest examples for the limits of host plant choice in polyleges might be the nearly complete absence of Asteraceae among the pollen hosts of the broadly polylectic species of the subgenus Osmia, which stands in contrast to the morphological and phylogenetical diversity of pollen hosts used in this clade on the one hand, and the prevalence of Asteraceae in many habitats on the other hand. This pattern corroborates previous reports, which suggest that the pollen of Asteraceae might exhibit unfavourable properties due to low protein content or due to secondary plant metabolites, as a consequence of which bees require physiological adaptations in order to successfully use Asteraceae as pollen hosts (Müller and Kuhlmann 2008; Praz et al. 2008c).

Pollen analyses also revealed, that not only oligoleges, but also closely related polyleges often exhibit strikingly similar pollen preferences, suggesting that closely related polylectic species might possesses a physiological (or behavioural) repertoire of adaptations inherited from common ancestor. In turn, related species lacking certain adaptations might not be able to exploit the same plant taxa, as clearly illustrated by the use of Ranunculus pollen in bees of the subgenus Osmia: Whereas
Ranunculus is a major host of the two sister species *O. bicornis* and *O. pedicornis*, it is at the most marginally exploited by *O. cornuta*. In fact, larvae of *O. cornuta* are in contrast to *O. bicornis* usually not able to develop on a pure Ranunculus pollen diet (Sedivy *et al.* 2011, this thesis section 6) and thus seem to lack the physiological adaptations acquired by the ancestor of *O. bicornis* and *O. pedicornis*. Likewise, physiological adaptations in bees of the subgenus Osmia to digest Fabaceae pollen might explain why larvae of both, *O. bicornis* and *O. cornuta*, were in contrast to larvae of two other megachilid species able to successfully develop on pure Lotus pollen diets. As Fabaceae are one of the main host plant taxa of this group of Osmia bees, possibly also *O. bicornis* and *O. cornuta* possess the physiological machinery to cope with Fabaceae pollen, although they do not commonly collect it.

We showed that beside chemical pollen properties also flower morphology strongly impacts the floral host choice of polylectic species. In the extreme, these constraints might lead to a re-specialization (Sedivy *et al.* 2008). The observed host range of the oligolectic *O. cerinthidis* might state a good example for a potentially recent re-specialization, as this species is derived from within a group of broadly polylectic species and as its specialization appears to be not complete yet. In fact, also several pollen generalist Osmia species seem to incline towards the use of a single main host, indicating that selection pressure might possibly also lead in these species towards a narrowing of the pollen host spectra.

Thus, results of the present study suggest that plant traits likewise influence floral host choice of polylectic and of oligolectic species, and that the borders of polylecty and oligolecty merge seamlessly.

**Bees might overcome constraints imposed by plants**

Although transitions from polylecty to oligolecty repeatedly occurred (Michez *et al.* 2008; Sedivy *et al.* 2013; Sipes and Tepedino 2005, this study section 4), evolutionary shifts in the opposite direction, i.e. from oligolecty to polylecty, have more often been demonstrated so far (Larkin *et al.* 2008; Michez *et al.* 2008; Müller 1996; Sedivy *et al.* 2013; Sedivy *et al.* 2008), and also host shifts of oligolectic species
are probably always preceded by a period of host expansion (Sedivy et al. 2008), indicating that host range expansion in bees must frequently occur despite the antagonistic constraints imposed by plants. So far, host range expansions have only been detected in retrospect, by means of species-level phylogenies in combination with host plant analyses (see literature cited at the beginning of this paragraph). The results on the broadly polylectic species *O. cornuta* provide, for the first time, experimental evidence that enough intra- and interpopulational variation in larval physiological abilities might exists, to provide a basis for natural selection to act towards host range expansion. Moreover, the host plant spectra of the two sister species *O. bicornis* and *O. pedicornis* suggest that an ancestor of these species must likewise have included *Ranunculus* in its diet. Bees, however, not only need to overcome physiological but also neurological constraints regarding flower recognition and handling (Praz et al. 2008b; Sedivy et al. 2008) in order to broaden their host ranges. As flower morphology, shape and colour of *Ranunculus* are not different from other plants within the host range of *O. cornuta*, flower recognition and handling alone should not pose any problem for the polylectic *O. cornuta*. In fact, *Ranunculus* pollen has been detected in pollen provisions of *O. cornuta* in Italy and Spain (Márquez et al. 1994; Nepi et al. 1997). Preliminary behavioural experiments with a German population suggest that *O. cornuta* females of that population refrain from collecting *Ranunculus* pollen even in the absence of other pollen sources, but that exceptions from that rule exist (S. Wolf, M. Haider, S. Dorn and A. Müller, unpublished data). Thus, females of *O. cornuta* seem to perceive the unfavourable properties of *Ranunculus* pollen (Burger et al. 2012; Dobson 1988; Piskorski et al. 2011) and therefore generally refrain from collecting it. However, similar to the variation in the physiological abilities of *O. cornuta* larvae to successfully develop on *Ranunculus* pollen diet, variation might also exist in the pollen collection behaviour of adult *O. cornuta* females.

Another, additional path for host expansion in polylectic bees is suggested by our finding that admixing up to 50% unfavourable *Ranunculus* pollen diet to favourable *Sinapis* pollen diet does neither negatively affect larval survival, nor adult
body mass of *O. cornuta*. In fact, female pollen generalist bees frequently do also naturally collect pollen of several unrelated plant families during a single foraging bout (Brian 1951; Free 1970; Goulson 2003; Leonhardt and Blüthgen 2012; this study, section 7) and accordingly also their larval provisions regularly contain pollen mixtures (Budde and Lunau 2007; Tasei 1973). Thus, pollen mixing of polylectic bees parallels the dietary mixing of generalist herbivorous insects (reviewed in Behmer 2009) and might serve as a strategy to mitigate the effect of toxic substances or to complement lacking nutrients. Using this strategy, polylectic bees like *Osmia cornuta* might be able to broaden their pollen diet, albeit to a limited extent. Especially if other pollen sources are rare, they might profit from the protein content of unfavourable pollen without the need to evolve specialized adaptations. In turn, as this strategy almost calls for mistakes regarding over-provisioning with unfavourable pollen, it might increase the selection pressure acting upon physiological adaptation and thus increase the chance of completely adopting such plant taxa as new pollen hosts.

*An “arms race“*

So far, protective chemical pollen properties have been reported from flowers with easily accessible pollen on the one hand (see discussion in chapter 3), and complex flower morphologies, which restrict access of bees to the pollen, have been described on the other hand (reviewed in Praz et al. 2008c). According to their complex flower morphology, the keel flowers of Fabaceae have been described as “bee flowers with adaptations against bees“ (Westerkamp 1997b). Results of the present study however suggest that this perspective is oversimplified. Fabaceae flowers might, in addition to their morphologically complex flower architecture, employ unfavourable pollen properties in order to further narrow the spectrum of pollen feeding visitors. Likewise also flowers of other plant taxa possibly posses more adaptations against pollen feeding visitors than realised so far. Considering the versatile adaptations evolved by bees in order to overcome antagonistic plant traits (Müller 1995; Neff 2004; Praz et al. 2008c; this study sections 6 and 7; Thorp
1979; Thorp 2000) in combination with Van Valens (1973) “Red Queen Hypothesis”, which states that interacting species must keep evolving in order to persist, it seems not surprising that plant species might in fact employ multiple strategies in order to minimize pollen loss and thus to succeed in the competition for pollen.

Outlook and future research

For host plant recognition, oligoleptic species might rely on a restricted array of cues of their respective host plants, and simple presence or absence of these cues might strongly contribute to their pollen host choice (Burger et al. 2010; Burger et al. 2012; Dobson 1988). In contrast, polylectic species have to choose the “right” flowers from within an even greater and more diverse pool of potential pollen sources in order to provide their offspring with suitable pollen diet. The factors that determine such pollen choices, especially of polylectic species, are poorly understood. To gain a better understanding future studies could investigate how female generalist solitary bees react to flowers that are evolutionary new to them, i.e. that they can never have encountered before, due to non-overlapping geographical ranges or due to diverging activity periods. Does pollen collection behaviour on such plant taxa correlate to larval performance? Can polylectic bees likewise discriminate between pollen that is nutritionally poor and pollen that contains toxic secondary metabolites?

Furthermore, do polylectic bees indeed employ pollen mixing as a strategy to mitigate unfavourable pollen properties, if e.g. the collection of unfavourable pollen is favoured due to higher availability of these flowers, due to larger amounts of pollen, due to a shorter distance to the nesting stand, or due to easier pollen collection on the flowers?

In addition, future studies should pay attention to what extent nectar quality and quantity affects pollen choice of polylectic species, as pollen collection of several
species of the subgenus *Osmia* appeared to be strongly biased towards the collection of pollen from flowers that provide little or no nectar.

To gain insights in the factors shaping pollen host choice in bees, there is no way around chemical pollen analyses. In contrast to leaf tissue, little is known about secondary metabolites in pollen so far (but see Detzel and Wink 1993; Dobson and Bergström 2000; London-Shafir *et al.* 2003; Reinhard 2011; Sedivy *et al.* 2012). Similar to correlations between the pollen host ranges of certain bee species and the floral morphology of their host plants, extensive analyses of secondary metabolites in pollen might reveal new aspects of pollen host choice in polylectic species.
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