Beyond random and forbidden interactions in plant-pollinator networks : how optimizing energy gain results in morphological matching among subalpine Asteraceae and their flower-visitors

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Contents

General introduction

1

Plants differ widely in the number of species by which they are visited. This generalization degree is a central concept of pollination ecology, since it is assumed to influence species coexistence, community stability (Memmott, Waser & Price 2004; Bascompte & Jordano 2007; Thebault & Fontaine 2010) and ecosystem services (Blüthgen & Klein 2011; Schleuning, Fründ & Garcia 2015). Generalization is assumed to promote high network stability. If a species is lost, links in the network will be maintained by other species with similar interaction partners, decreasing the chance for subsequent secondary extinctions (Dunne, Williams & Martinez 2002; Memmott, Waser & Price 2004). However, a high specialization degree of flower-visitors can be important for pollination, as it increases the likelihood of conspecific pollen transfer among plants, which benefits plant reproduction (Brosi & Briggs 2013). An increasing number of community studies have shown general interaction patterns such as asymmetry (generalist flower-visitors interact with specialist plants and vice versa) and nestedness (a core group of generalists interacts with each other and a few specialists mostly interact with generalists) (e.g. Bascompte *et al.* 2003; Vazquez & Aizen 2004; Bascompte, Jordano & Olesen 2006). Recently there has been an increased interest in how species abundances and their interaction patterns fluctuate through time (Alarcon, Waser & Ollerton 2008; Olesen *et al.* 2008; Petanidou *et al.* 2008; Olesen, Stefanescu & Traveset 2011; CaraDonna *et al.* 2017). However, still little is known about the mechanisms that cause these plant-flower-visitor interaction patterns and their temporal fluctuation, as community studies which incorporate species traits are relatively scarce. Especially, there is a lack of knowledge on how nectar production and flower-visitor foraging efficiency affect these community wide plant-flower-visitor interaction patterns.

It has been argued that species generalization degree and plant-flower-visitor interaction patterns, such as asymmetry and nestedness, largely result from random encounters among individuals of species with different abundances. Abundant plant species will interact more frequently and with more flower-visitor species than rare plant species do and vice versa, which results in an asymmetric and nested community structure (e.g. Dupont, Hansen & Olesen 2003; Vazquez 2005; Vazquez, Chacoff & Cagnolo 2009). In addition, species generalization degree can be determined by species traits which are (partly) determined by phylogenetic relationships among species (Rezende, Jordano & Bascompte 2007) and are often the result of long-term evolutionary processes (Kissling & Schleuning 2015). For example, interactions between species may not occur due to non-overlapping phenologies (Olesen *et al.* 2011). Also, species morphology may constrain the type, number and strength of interactions between plants and flower-visitors, and consequently affect plant-flower-visitor network structures (Santamaria & Rodriguez-Girones 2007; Stang, Klinkhamer & van der Meijden 2007; Stang *et al.* 2009; Ibanez 2012). Morphological traits may act as barriers, restricting access to floral reward to flower-visitors with a certain morphology and/or behaviour. For example, flowers with long corollas exclude animals with short proboscises since these animals are not able to reach the nectar offered by the flowers. Consequently, flowers with long corolla tubes are specialized and visited by animals with a longer proboscis, while animals with a long proboscis can visit a wide diversity of flowers (Stang, Klinkhamer & van der Meijden 2006; Maglianesi *et al.* 2014). Thus, both species phenology and morphology prevent interactions from occurring and these interactions are referred to as forbidden interactions (Olesen *et al.* 2011).

Surprisingly, only few studies have indeed shown that corolla tube length and/or proboscis (or bill) length are associated with species generalization degree (Agosta & Janzen 2005; Stang, Klinkhamer & van der Meijden 2006; Maglianesi *et al.* 2014). Moreover, the influence of other morphological traits and frequency distributions of these traits in the community on species generalization degree and plant-flower-visitor interaction patterns have seldom been investigated. Floral display area can constrain the number of flower-visitors, as a smaller display can make it more difficult to land on a flower and unfold a long proboscis, and therefore to access and extract nectar. Few studies that investigated the effect of display area on the number of flower-visitor species found contrasting results. Although Hegland and Totland (2005) found an effect of floral display area on visitation frequency within a grassland community, Stang, Klinkhamer and van der Meijden (2006) did not find a relationship between display area and their number of flower-visitor species in a Spanish Mediterranean community.

Random encounters and forbidden interactions have often been proposed as main factors explaining plant-flower-visitor interaction patterns. Surprisingly, we lack knowledge on how nectar production and flower-visitor foraging efficiency affects the type and number of interaction partners and consequently plant-flower-visitor network structure. Nectar is one of the most likely traits to be important, as it is one of the major floral rewards for flower-visitors. Although it is known that nectar production affects the type of flower-visitors (Pyke & Waser 1981; Baker & Baker 1983) and their visitation patterns (Leiss & Klinkhamer 2005), it is yet unclear how nectar production affects plant-flower-visitor interaction patterns at the community level. Nectar quality and quantity rarely have been incorporated in community studies. Recently, Carvalheiro *et al.* (2014) have shown that the potential for one plant species to influence another indirectly via shared flower-visitors is greater for plants whose resources (nectar sugar content) were more abundant. In addition resource availability at the community level is shown to affect flower-visitor generalization degree. Tinoco *et al.* (2017) showed that among hummingbirds, especially long billed species were more specialized when the availability of nectar resources at the community level was low. In contrast, Junker *et al.* (2013) showed that the amount of sugar offered per flower was less important for flower visitors' behaviour than other floral traits, including flowering phenology, flower height, nectar-tube depth and floral reflectance.

In some communities, size-matching occurs between the nectar tube depth of flowers and the average proboscis length of their flower-visitors (Stang *et al.* 2009; Sazatornil *et al.* 2016). This size-matching cannot only be explained by morphological constraints imposed by the nectar tube depth on the proboscis length of flower-visitors. For size-matching to occur, flower-visitors with a longer proboscis need to visit flowers with deeper nectar tubes more frequently and avoid shallow-tubed flowers. Size-matching between the nectar tube depth of flowers and the average proboscis length of their flower-visitors has often been explained by these size-constraints in combination with species abundance (Stang *et al.* 2009) or resource competition (Heinrich 1976; Rodriguez-Girones & Santamaria 2005; Rodriguez-Girones & Santamaria 2006). However, aspects related to foraging energetics of flower-visitors, such as the amount of nectar flowers produce, the time it takes flower-visitors to handle flowers and extract nectar (handling time) and their overall net energy gain, are expected to influence flower-visitor flower choice and visitation rate. Consequently these aspects might determine plant-flower-visitor interaction patterns, such as size-matching.

As nectar is one of the major floral rewards for flower-visitors, it is likely that flower-visitors choose flowers based on the amount of nectar or sugar they produce. Since nectar production per flower increases with increasing nectar tube depth (Galetto & Bernardello 2004; Kaczorowski, Gardener & Holtsford 2005; Ornelas *et al.* 2007; Petanidou 2007; Martins & Johnson 2013; Johnson *et al.* 2017), flower-visitors with a longer proboscis might be expected to visit flowers with deeper nectar tubes more frequently. In contrast, flower-visitors with a short proboscis are restricted to shallow flowers, because their short proboscis prevents them to access deeper flowers. This may result in size-matching between the depth of flowers and the average proboscis length of their flower-visitors. So far, nectar reward offered by plants to flower-visitors is mostly studied at the level of individual flowers. However, the attractiveness of plants also depends on the number of open flowers and the clustering of these flowers, and thus the nectar production per inflorescence, plant individual and patch, as this determines the total nectar reward of plants.

The time it takes flower-visitors to handle a flower and extract nectar (handling time) or their foraging efficiency might also affect flower-visitor foraging behaviour in such a way that leads to size-matching between the nectar tube depth of flowers and the average proboscis length of their flower-visitors. In general, it takes flower-visitors more time to handle flowers with deeper nectar tubes, especially if the nectar tube depth exceed the proboscis length (Harder 1983). Therefore, flower-visitors might avoid flowers of which the nectar tube depth exceeds the length of their proboscis. Moreover, although in general, flower-visitors with a longer proboscis handle flowers faster (Inouye 1980; Herrera 1989; Temeles & Roberts 1993; Graham & Jones 1996; Balfour, Garbuzov & Ratnieks 2013), it has been suggested that a long proboscis is of hindrance when foraging of shallow flowers. Thus, flower-visitors with a long proboscis might endure longer handling time, than flower-visitors with a short proboscis, when foraging on shallow flowers (Inouye 1980; Plowright & Plowright 1997; Kunte 2007; Karolyi *et al.* 2013; Bauder *et al.* 2015). Consequently flower-visitors with a long proboscis may visit shallow flowers less frequently or even avoid them.

Optimal foraging theory predicts that flower-visitors should restrict their foraging efforts to those flowers on which they can forage most efficiently and thus those flowers which offer the largest net energy gain (Pyke 1980; Pyke 1984). The net energy gain is determined by the nectar reward offered by flowers, the number of flowers that flower-visitors visit per time unit (visitation rate) and the energy flower-visitors spend while foraging. This visitation rate depends on how long it takes to (i) handle a flower and extract the nectar (handling time) and (ii) to fly from flower to flower (interfloral flight time). Handling time can be affected by floral characteristics, such as nectar volume and sugar concentration, and nectar tube depth (Wolf, Stiles & Hainsworth 1972; Inouye 1980; Harder 1983; Montgomerie 1984; Harder 1986; Hainsworth, Precup & Hamill 1991). In turn, interfloral flight time is determined by the number of open flowers and their clustering (Heinrich & Raven 1972; Inouye 1980). Handling time and interfloral flight time together also determine the energy flower-visitors spend while foraging. If flower-visitors experience a higher net energy gain on flowers with deeper nectar tubes, they are expected to visit these flowers more frequently, which may lead to size-matching between their proboscis length and the average depth of the flowers they visit. However, only few quantitative studies have examined the overall energetics of foraging insects and their flowers in natural communities (but see Harder 1988; May 1988). Therefore, it is unknown how the net energetic gain of flower-visitors is related to the nectar tube depth of flowers (but see May 1988 for butterflies).

Understanding the mechanism that determine species generalization degree and plant-flower-visitor interaction patterns is important since these aspects have important ecological implications. Since most species abundances and their interactions fluctuate from year-to-year (Alarcon, Waser & Ollerton 2008; Olesen *et al.* 2008; Petanidou *et al.* 2008; Olesen, Stefanescu & Traveset 2011; CaraDonna *et al.* 2017), generalization and both interaction asymmetry and nestedness are assumed to prevent species extinctions and increase community stability (Memmott, Waser & Price 2004; Bascompte & Jordano 2007; Thebault & Fontaine 2010; Rohr, Saavedra & Bascompte 2014). In asymmetric and nested plant-flower-visitor networks, if a species is lost, links in the network will be maintained by other species with similar interaction partners, decreasing the chance for subsequent secondary extinctions. However, the reliability of pollinator services not only depends on the number of potential flower-visitor species, but also on the temporal dynamics of these species. Therefore, to ensure pollination, specialized plants might specialize on those flower-visitor species which are less prone to short-term temporal fluctuations.

Although several studies have shown that specialized flower-visitors and flower-visitors with a long proboscis are more prone to extinction (Biesmeijer *et al.* 2006; Burkle, Marlin & Knight 2013), it is yet unclear how short-term temporal fluctuation of flower-visitor species abundance is related to species characteristics. Previous studies which have documented short-term fluctuations in species abundances and plant-flower-visitor interactions (Alarcon, Waser & Ollerton 2008; Olesen *et al.* 2008; Petanidou *et al.* 2008; Burkle & Irwin 2009; Dupont *et al.* 2009; Lazaro, Nielsen & Totland 2010; Olesen, Stefanescu & Traveset 2011; CaraDonna *et al.* 2017), did not document how these fluctuations are related to species specific traits, such as generalization degree (but see Olesen, Stefanescu & Traveset 2011 for butterflies) or proboscis length. Overall, there is still a lack of knowledge about the mechanisms causing fluctuation of species abundances and plant-flower-visitor interactions and how this affects community stability.

The main aim of this study was to investigate whether, in addition to species abundance and morphological traits, flower-visitor foraging efficiency can explain species generalization degree and plant-flower-visitor interaction patterns, such as asymmetry, nestedness and size-matching between the nectar tube depth of flowers and the average proboscis length of their flower-visitors. Further, I used my result to investigate the ways plants can mitigate the risk that in a particular year there are insufficient numbers of flower-visitors to ensure pollination. I specifically hypothesized that plants can either be generalized on flower-visitors which are prone to temporal fluctuations or specialized on flower-visitors which are less prone to temporal fluctuations. These two strategies which will result in equal visitation rates. In other words plants may face a flower-visitor species number (SN) – species predictability (SP) trade-off. To do this, I have studied the interactions of subalpine Asteraceae species, co-occuring in the Colorado Rocky Mountains, and their flower-visitors.

The Asteraceae is one of the most successful and species-rich plant families worldwide (Proctor, Yeo & Lack 1996). Plant species of this family have relatively shallow, easy accessible, tubular flowers which are aggregated in flower heads (capitula). They are visited by a wide diversity of functional groups, including bumblebees, solitary bees, butterflies and flies and therefore are considered to be generalists (Graenicher 1909; Lane 1996; Proctor, Yeo & Lack 1996; Torres & Galetto 2002). Consequently, the Asteraceae often form an important part of the core of plant-flower-visitor networks (Bascompte *et al.* 2003; Memmott, Waser & Price 2004; Gonzalez, Dalsgaard & Olesen 2010). In general, it is assumed that among generalized plant species, plant-flower-visitor interactions are more generalized, more connected, but less structured and thus less predictable. Therefore, studying the interactions among the Asteraceae and their flower-visitors provides the unique opportunity to investigate how different flower-visitor taxa respond to variation in plant traits and how interactions among functionally generalized plant species are structured. Further, the relatively shallow, easy accessible flowers of the Asteraceae do not pose strong morphological constraints on their flower-visitors. Indeed, a large proportion of their potential flower-visitors, mostly bumblebees and butterflies, are expected to have a proboscis long enough to visit all plant species. This makes these plants an ideal study system to investigate the effect of foraging efficiency on plant-flower-visitor interaction patterns.

This thesis consist of seven chapters. In the first experimental chapter, **Chapter 2**, I analyzed the interaction patterns among the Asteraceae and their flower-visitors. I investigated the generalization, connectedness, nestedness and asymmetry of these interactions, the effect of flower density, nectar tube depth and display area on plant species generalization degree and their number of visits, and patterns of size-matching between the nectar tube depth of flowers and the average proboscis length of their flower-visitors. A positive correlation between flower density and plant species generalization degree has often interpreted as the result of random interactions. If interactions indeed occur randomly, not only the number of flower-visitor species, but also the number of visits should increase with increasing flower density. I asked the following questions: (1) Are interactions among generalized plants and their flower-visitors more generalized and connected and less asymmetric and nested? (2) Are plant generalization degree (both the number of flower-visitor species and Shannon diversity index) and the number of flower-visitor visits related to local flower head density, nectar tube depth and display area? And (3) are plant species with flowers with deeper nectar tubes visited by flower-visitors species which have a longer proboscis and how does this degree of size-matching differ among flower-visitor taxa, including bumblebees, solitary bees, butterflies and flies?

It is likely that the nectar reward that flowers offer plays an important role in flower-visitor flower choice and visitation rate. In **Chapter 3** I investigated whether plant species which have flowers with deeper nectar tubes are more rewarding. In this chapter, I analyzed the scaling relationships between nectar tube depth and nectar production of plants at different spatial scales: per single flower, flower head, individual plant and patch. I measured the nectar production rate of single flowers and calculated the nectar production per flower head, individual plant and patch, based on the number of open flowers per flower head and the number of flower heads per plant individual or patch. I addressed the following questions: (1) Do flowers with deeper nectar tubes produce more nectar per single flower and offer more nectar per flower head, individual plant and patch? (2) Are nectar tube depth and nectar production related to the number of open flowers per head and the number of flower heads per individual or patch? Since nectar standing crop is what flower-visitors actually encounter while foraging I also tested (3) whether nectar standing crop is related to nectar production rate.

In **Chapter 4** I investigated how floral and flower-visitor traits determine the time it takes flower-visitors to handle a flower and extract the nectar (handling time) and how this is related to their flower choice. As already mentioned, in general flower-visitors with a long proboscis handle flowers faster (Inouye 1980; Herrera 1989; Temeles & Roberts 1993; Graham & Jones 1996; Balfour, Garbuzov & Ratnieks 2013). However, it has been suggested that flower-visitors with long proboscises endure longer handling time than those with short proboscises when foraging on shallow flowers (Inouye 1980; Plowright & Plowright 1997; Kunte 2007; Karolyi *et al.* 2013; Bauder *et al.* 2015). Consequently, flower-visitors with a long proboscis might avoid shallow flowers or visit them less frequently. In this chapter, I asked the following questions: (1) Do bumblebees, solitary bees, butterflies and flies differ in handling time and is their handling time related to their proboscis length and both nectar tube depth and nectar volume of the flowers they visit? And (2) do insects with a longer proboscis visit more plant species and plant species with flowers with deeper nectar tubes?

Foraging efficiency, rather than handling time, might be more important for flower-visitor flower choice and visitation rate. Foraging efficiency does not only account for the time it takes to handle and extract nectar, but also for the amount of nectar, the number of flowers they visit, flower abundance and clustering and the time it takes to fly from flower-to-flower. The main aim of **Chapter 5** was to investigate whether flower-visitors forage optimally and whether this can explain patterns of size-matching. To this end, I investigated how foraging efficiency of natural foraging bumblebees of *Bombus bifarius* was related to the nectar tube depth of the Asteraceae flowers they forage on. And whether bumblebees visited flowers of plant species on which they had a higher foraging efficiency more frequently. I asked the following questions: (1) is the sugar intake rate of bumblebees, while extracting nectar from a single flower and while foraging on a flower head or in a patch, related to the depth of the flowers they forage on? (2) Is the energetic expenditure of bumblebees related to the depth of the flowers they forage on? (3) Is the (rate of) net energy gain of bumblebees related to the depth of the flowers they forage on? (4) Is the (rate of) net energy gain of bumblebees related to other aspects which determine bumblebee foraging efficiency, including flower head density, sugar content per flower, number of open flowers per head, flight time between heads and the time it takes to visit a flower head? And (5) do bumblebees visit those flowers that have deeper nectar tubes, are more abundant, produce more nectar or provide a larger (rate of) net energy gain more frequently?

In the final experimental chapter, **Chapter 6**, I investigated the temporal, year-to-year fluctuation of flower-visitor species abundance and plant-flower-visitor interactions. To ensure pollination in dynamic and unpredictable environments, in which most flower-visitor species abundances fluctuate from year-to-year, I hypothesize that plant species can either be generalized on flower-visitors which are prone to temporal fluctuations or specialized on flower-visitors which are less prone to temporal fluctuations. These two strategies will result in equal visitation rates. In other words plants will face a flower-visitor species number (SN) – species predictability (SP) trade-off. This hypothesis is based on three assumptions. First of all, the number of flower-visitor species by which a plant is visited, is related to the nectar tube depth of its flowers. In general, plant species with shallow flowers are more generalized (Chapter 2 of this thesis) because flowers with a deep nectar tube can only be visited by flower-visitors with a long proboscis. Secondly, plants species with deeper flowers produce more nectar (Chapter 3 of this thesis). And third, flower-visitors which are less prone to temporal fluctuations are those species which are large, generalized, abundant and/or are social and consequently may have higher energetic requirements. Thus, for plants to be generalized, their flowers need to be relatively shallow. However, these shallow flowers may not produce enough nectar to fulfill the energetic requirements of flower-visitors which are less prone to temporal fluctuations. Therefore, generalized plant species either may not attract flower-visitor species which are less prone to temporal fluctuations, or, if they do attract these flower-visitors, they will not visit them frequently. In turn, plant species which have deep-tubed flowers are able to produce enough nectar to attract flower-visitors which are less prone to temporal fluctuations. However due to their deep nectar tubes, these flowers cannot attract a wide diversity of flower-visitor species and thus will be specialized. To test this hypothesis I asked the following questions: (1) Is the fluctuation of flower-visitor species abundance related to their generalization degree and proboscis length? (2) Does nectar tube depth determine plant generalization degree and how does this relationship fluctuate across years? (3) Do specialist plant species remain more specialized across years? (4) Are specialized plants visited by species that are temporarily more stable? And (5) is flower visitation rate and its fluctuation among years related to plant species generalization degree and its fluctuation among years?

In the last chapter of this thesis, **Chapter 7**, I have summarized my findings and discussed the two main conclusions of my thesis that (i) plant-flower-visitor interaction patterns do not occur randomly, but are determined by nectar production of plants and the energy gain that flower-visitors experience while foraging and (ii) generalizing on flower-visitors which are prone to temporal fluctuations or specializing on flower-visitors which are less prone to temporal fluctuations are two successful strategies to ensure pollination over time and might even be the result of a trade-off.

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2

Interaction patterns among generalized plants species and their flower-visitors: are these interactions less structured?

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Abstract

Plant-flower-visitor networks show general structures, such as asymmetry, nestedness and size-matching between plants and their flower-visitors, which are determined by species abundance and morphological traits. However, interactions among generalized plants, such as the Asteraceae, are assumed to be less structured. Here, we examined the interactions among 16 co-occurring Asteraceae species and their nectar-foraging flower-visitors. We investigated structural features, including network generalization, connectance, asymmetry and nesteness, and analysed how local flower density, nectar tube depth and display area affect both the number of flower-visitor species and visits. In addition, we investigated how size-matching patterns between the nectar tube depth of flowers and the proboscis length of flower-visitors differed among bumblebees, solitary bees, butterflies and flies. Surprisingly, although the Asteraceae were visited by a wide range of flower-visitor taxa, some plant species were extremely specialized. Plant-flower-visitor interactions were highly connected, even and generalized but also asymmetric and nested. Further, the number of flower-visitor species increased with flower head density and display area and decreased with nectar tube depth. In contrast, the number of visits did not increase with flower head density nor nectar tube depth, but only with increasing display area. Interestingly, nectar tube depth and flower head density were negatively correlated and on average, flower-visitor species visited more dense and shallow flowers less frequently. Finally, patterns of size-matching differed among flower-visitor taxa. Overall, our results indicate that even interactions among generalized plant species and their flower-visitors are structured and influenced by plant morphological traits and flower density. Moreover, flower density might not affect plant-flower-visitor interactions by itself but rather through correlation with morphological traits and the frequency distribution of these morphological traits. Consequently, our results highlight the importance of quantitative data analyses, in addition to qualitative data analyses, and trait-abundance correlations to understand plant-flower-visitor network structure.

Key-words: Asteraceae, asymmetry, display area, generalization, nectar tube depth, plant-pollinator community structure, proboscis length, specialization

Introduction

Plants, flower-visitors and their interactions form complex networks in which species differ widely in their generalization degree. While some plant species are visited by numerous flower-visitors, and vice versa, others only have one or a few interaction partners and thus are more specialized. In general, specialized flower-visitors and plants often interact with generalist plants and flower-visitors respectively, resulting in an asymmetric and nested (a core group of generalists interact with each other and a few specialists mostly interact with generalists) community structure (Bascompte *et al.* 2003; Vazquez & Aizen 2004; Bascompte, Jordano & Olesen 2006). Understanding how these structures emerge is important, since they influence species coexistence, community stability (Thebault & Fontaine 2010; Tylianakis *et al.* 2010; Kaiser-Bunbury *et al.* 2017) and ecosystem functions (Blüthgen & Klein 2011; Schleuning, Fründ & Garcia 2015).

Key to understanding plant-pollination network structure is to have insight in the mechanisms that determine why some species are generalized while others are more specialized. It has been suggested that species abundances per se determine their generalization degree and plant-flower-visitor interaction patterns (Dupont, Hansen & Olesen 2003; Vazquez & Aizen 2004; Vazquez *et al.* 2007). This implies that interactions occur based on random encounters among individuals of different species. Consequently, abundant plant- and flower-visitor species will be more generalized as they will interact more frequently and with more species than rare plant- and flower-visitor species. However, other aspects, such as species phenology and traits, may also shape plant-flower-visitor interactions. Species phenology and traits constrain the type, number and strength of interactions between plants and flower-visitors and consequently determine species generalization degree (Stang, Klinkhamer & van der Meijden 2006; Junker *et al.* 2013; Kaiser-Bunbury *et al.* 2014; Renoult *et al.* 2015) and the structure of pollination networks (Rezende, Jordano & Bascompte 2007; Santamaria & Rodriguez-Girones 2007; Stang, Klinkhamer & van der Meijden 2007; Ibanez 2012).

Often phenotypic traits of plants and their flower-visitors correspond. For example, certain type of flower-visitor preference flowers with a particular colour (Renoult *et al.* 2015) or nectar sugar type or -concentration (Baker & Baker 1983). Moreover, the morphology of plants and their flower-visitors often match, which is referred to as size-matching. For example, plants species with flowers with long corollas are often visited by animals with a longer proboscis or bill. This because animals with short proboscis are not able to access the nectar of flowers with long corollas. Consequently, the length of the corolla of flowers and the proboscis (or bill) of flower-visitors are also expected to determine species generalization degree. Plant species with flowers that have short corollas can be visited by a wider variety of flower-visitor species, compared to plant species with flowers that have long corollas. In turn, animals with a long proboscis (or bill) can visit a wide variety of flowers, compared to animals with a short proboscis (or bill) (Agosta & Janzen 2005; Stang, Klinkhamer & van der Meijden 2006; Stang *et al.* 2009; Maglianesi *et al.* 2014). Consequently, morphological constraints also results in an asymmetric and nested community structure (Rezende, Jordano & Bascompte 2007; Santamaria & Rodriguez-Girones 2007; Stang, Klinkhamer & van der

Meijden 2007; Ibanez 2012). Surprisingly, only few studies have indeed shown that corolla tube length and/or proboscis (or bill) length are associated with species generalization degree and mostly among moths (Agosta & Janzen 2005; Johnson *et al.* 2017) and hummingbirds (Maglianesi *et al.* 2014; but see Stang, Klinkhamer & van der Meijden 2006 for a community level study). Moreover, the influence of other morphological traits, such as display area, and their co-variation with flower abundance, on plant-flower-visitor network structure have been rarely considered (but see Junker *et al.* 2013). Display area might constrain the number of flower-visitors, as a smaller display can make it more difficult for large bodied flower-visitors which do not hover, to land on a flower and unfold a long proboscis and therefore to access and extract nectar. Studies that did investigate the effect of display area on the number of flower-visitor species found contrasting results (Hegland & Totland 2005; Stang, Klinkhamer & van der Meijden 2007; Junker *et al.* 2013).

In this study, we used the Asteraceae to investigate how local flower density, nectar tube depth and display area affect the interaction patterns among generalized plants and their flower-visitors. To this end we collected quantitative data on both the number of flower-visitor species and number of flower-visitor visits. The Asteraceae are visited by a wide variety of insects, including bumblebees, solitary bees, butterflies and flies (Graenicher 1909; Lane 1996; Proctor, Yeo & Lack 1996; Torres & Galetto 2002). They have simple, tubular flowers which are aggregated in flower heads (capitula) and easy accessible. We expect that interactions among these plant species and their flower-visitors are more generalized and connected, as flower morphology might not pose strong constraints. Moreover, we expect that these interactions are less structured, specifically less asymmetric and nested, because these structures depend on the presence of specialists plant species, which are mostly plants with complex flowers (Kaiser-Bunbury *et al.* 2014). Furthermore, we investigated how patterns of size-matching differed among flower-visitor taxa, including bumblebees, solitary bees, butterflies and flies, e.g. because these flower-visitor taxa differ in their proboscis length. We asked the following questions: (1) Are interactions among generalized plants and their flower-visitors more generalized and connected and less asymmetric and nested? (2) Are plant generalization degree (both the number of flower-visitor species and Shannon diversity index) and the number of flower-visitor visits related to local flower head density, nectar tube depth and display area? And (3) are plant species with flowers with deeper nectar tubes visited by flower-visitors species which have a longer proboscis and how does this degree of size-matching differ among flower-visitor taxa, including bumblebees, solitary bees, butterflies and flies?

Materials and methods

Plant species and flower-visitor observations

Data were collected in the Colorado Rocky Mountains, USA, in the vicinity of the Rocky Mountain Biological Laboratory, during six weeks in June-August 2011. We included the sixteen most abundant, co-flowering Asteraceae species (Table 1). Plant-flower-visitor interactions were observed in five different meadows, which were approximately 100-200 meters apart. Flower-visitors could move freely between these meadows. The field sites were at approximate 3,000 m elevation, with slopes between 0° and 34°, exposed mostly to the south. Within these meadows, plant-flower-visitor interactions were observed at locations (patches) where the plant species were flowering abundantly. All plant species were observed separately for four hours, by walking through the patch in the meadow were the focal plant species was flowering abundantly. Sampling time was divided into eight sampling intervals of about ½ hour. These sampling intervals were spread over several days, during different times of day (morning and afternoon). All observations took place between 09:00-16:00h at days where there was no strong wind or rain. Flower-visitor species were not caught in order to avoid changing their densities, distributions and behaviour. Instead, the observed flower-visitors were filmed for later identification. These videos were used to determine whether flower-visitors visit flowers for nectar and/or pollen. We only included those flower-visitor species that were foraging for nectar. Flower-visitor species were identified up to species- or morphospecies level. We noted both the flower-visitor species and how many times they visited the plant species (number of visits).

Proboscis length

Proboscis length measurements of solitary bees, butterflies and flies were based on specimens from a reference collection. These specimens were collected in 2009 and 2010, at the same study site as where our study was conducted. Proboscis length of these specimens was measured as described in Stang, Klinkhamer and van der Meijden (2006). For bumblebees, data on the average proboscis lengths were obtained from Macior (1974) (for queens and workers) and Inouye (1976) (for males).

Plant traits

The Asteraceae have relatively small flowers which are aggregated in flower heads (capitula). For most species these heads have two basic types of flowers: tubular, bell-shaped disk flowers in the centre of the head, and strap-shaped ray flowers, in the peripheral, although the heads of some species have only disk or only ray flowers. The studied species belong to the subfamilies Carduoideae (which have only disk flowers) and Asteroideae (which have disk and ray flowers) (Table 1).

We measured the depth of disk flowers since these flowers produce nectar (Mani & Saravanan 1999). The corolla tube of disk flowers consists of two parts: a (very) narrow lower part and an upper wider bell-shaped part. The wider part roughly begins where the stamens insert and ends at the beginning of the corolla lobes, at the base of the deepest cleft in the corolla. It is unlikely that flower-visitors can access the lower part of the corolla tube, since this part is almost filled by the style (Graenicher 1909). Therefore, for each plant species, we measured the depth of the upper wider part of the corolla tube, hereafter referred to as nectar tube (Fig. 1). For each species, we measured the nectar tube depth for at least twenty freshly picked flowers, under a dissecting microscope. Floral display area we defined as the surface area of the flower heads, including the rays for the species that have ray flowers.

In patches where the plants were flowering abundantly and flower-visitors were observed, we counted the number of flower heads per square meter (local flower head density). For most plant species, we calculated the average number of flower heads per square meter, based on ten one square meter plots that were randomly placed within the patch. For *Cirsium sp.2* and *Arnica parryi*, we measured flower head density based on one plot of ten square meter, since these species were flowering at only one location in the meadow.

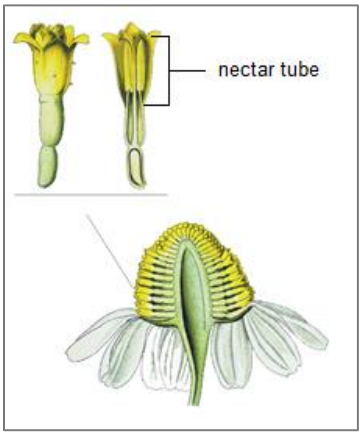

Fig. 1. Morphology of the Asteraceae

Species generalization degree

Species generalization degree was estimated both as the number of interaction partners, e.g. the number of flower-visitor species with which a given plant species interacts or the number of plants species with which a given flower-visitor species interacts, and the Shannon diversity index (Shannon 1948). In contrast to the number of species, the Shannon diversity index takes the number of visits of each flower-visitor species into account. For each plant species, we also calculated the potential number of flower-visitor species, e.g. the number of flower-visitor species that can visit a plant given the nectar tube depth of flowers and proboscis length of the flower-visitors. We assumed that flower-visitors could potentially visit a plant species if their average proboscis length +0.5 mm (natural variation) was equal to or exceeded the nectar tube depth.

Network parameters

We calculated qualitative and quantitative parameters which are indicative of species- and interaction diversity and network generalization and stability. The qualitative parameters included the number of plant species (P), number of flower-visitor species (A), network size (i.e. total number of potential interactions, $S = P\ x\ A$), total number of interactions recorded (I), network and connectance, $C = I/(P\ x\ A)$, which is the realized proportion of all possible interactions (Jordano 1987). Following Bersier, Banasek-Richter and Cattin (2002) we calculated quantitative parameters including weighted plant (lw_p) and flower-visitor (lw_a) linkage, which is the mean diversity of interaction partners across all species within a guild (plants or animals) (hence, the average plant and flower-visitor species partner diversity), quantitative connectance (C_q), which is the connectance weighted by the quantitative visitation rate of each species and interaction evenness (*IE*), which is a measure of the homogeneity of interaction frequencies across all links in the network (Tylianakis, Tscharntke & Lewis 2007). High values reflect more uniform spread of interactions among the species in the communities. In contrast, IE is low if some species and their links dominate the community while most others are rare. Moreover, we calculated nestedness weighted by sample size (*WNODF*; Weighted Nestedness based on Overlap and Decreasing Fill) (Almeida-Neto & Ulrich 2011) and network specialization (H_2') which is a measure of how selective species are in the network by quantifying how the observed interaction frequencies departs from a theoretical random distribution of interactions driven by species abundances (Blüthgen, Menzel & Blüthgen 2006). It characterizes the distribution of interactions relative to each other, namely their mutual exclusiveness. This metric increases in value when species are highly specialized on specific partners (high partitioning). High specialization indicates high dependency of each species on a few exclusive partners. Low specialization indicates higher functional redundancy. All network parameters were computed in R 3.3.2 (R Development Core Team 2014), using the “networklevel” command in the package bipartite (Dormann, Gruber & Fründ 2008).

Statistical analyses

Statistical analyses were performed, using R 3.3.2 (R Development Core Team 2014). Prior to all analyses, we tested whether the variables were normally distributed, using a Shapiro-Wilk test. To achieve normality, flower head density, floral display area and both plant and flower-visitor generalization degree were $\log_{10}$ transformed prior to all analyses. To analyse whether plant generalization degree (both the number of flower-visitor species and Shannon diversity index) and the number of flower-visitor visits were related to local flower head density, nectar tube depth and display area, we used both single- and multiple ordinary least square (OLS) regression analyses, using backward selection of variables. Moreover, we analysed whether plant species with deeper flowers were visited by flower-visitors with a longer proboscis, using OLS regression analyses. For these analyses, for each plants species, we calculated the average proboscis length of their flower-visitors, divided by the four taxonomic groups: bumblebees, solitary bees, butterflies and flies.

Results

Network structure: generalization, nestedness and asymmetry

Table 1 summarizes the floral traits. Nectar tube depth varied between 1.76 (± 0.08) and 6.83 (± 0.07) mm, display area varied between 10.97 (± 0.46) and 64.09 (± 2.48) mm and local flower head density varied between 4.15 (± 0.59) and 83.55 (± 7.95) heads/m^2. Nectar tube depth decreased with increasing flower head density (r=-0.69, P=0.003). Display area was not correlated with nectar tube depth (r=0.36, P=0.17) nor flower head density (r=-0.15, P=0.57).

In total we observed 64 flower-visitor species, mostly bumblebees, solitary bees, butterflies and flies. Some plant species were also visited by ants, beetles, bugs, hummingbirds and wasps (Fig. 2). The sixteen plant species differed greatly in the number of flower-visitor species visiting them, ranging from 2-29 species. The Asteraceae were most frequently visited by bumblebees, as they made up almost 54% of all visits. The proboscis length of the flower-visitor species varied between 1.40 mm and 19.00 mm.

Interaction network parameters are presented in Table 2. There were almost four times more flower-visitor species (64) than plant species in the community (16). In total, we observed 181 interactions which were 18% of all possible interactions (16*64=1024). The connectance weighted by the number of visits (Cq) was 0.09. Plants shared more flower-visitors than flower-visitors shared plants, as was indicated by the higher linkage of plants (lw_p=9.24 and lw_{fv}=5.83). The interactions in the network were nested (WNODF=14.49; Fig. 2) and uniform distributed across species, as was indicated by the relatively high IE (0.67). Overall, the network can be characterized as generalized, because of the relatively low H_2’ (0.35). Plant species that were more generalized were visited by flower-visitor species that were more specialized. Plant species which were visited by more flower-visitor species were visited by flower-visitor species which visited on average less plant species (r=0.6, P=0.003, n=16; Fig. 3a). In addition, plant species which had a higher Shannon index, and thus a higher diversity of interactions, were visited by flower-visitor species which had a lower Shannon diversity index (r=0.72, P=0.001; Fig. 3b). This indicates that interactions between plants and flower-visitors were asymmetric.

Table 1. Nectar tube depth, flower head density and display area of the Asteraceae species studied. The table gives mean ± s.e.m. and sample size (n). Plant species are named according to the accepted names in The Plant List.

Subfamily	Tribe	Plant species	Nectar tube dept (mm)	Flower head density (number heads/m^2)	Display area (mm^2)
Carduoideae	Cardueae	*Cirsium sp.1*	6.31 ± 0.16 (36)	7.40 ± 2.12 (10)	25.76 ± 0.59 (8)
		Cirsium sp.2	4.33 ± 0.07 (39)	9.65 *	20.89 ± 2.55 (8)
Asteroideae	Senecioneae	*Senecio bigelovii*	4.76 ± 0.06 (24)	18.30 ± 2.90 (10)	13.30 ± 0.41 (8)
		Packera werneriifolia	3.29 ± 0.06 (36)	36.30 ± 5.12 (10)	14.94 ± 0.57 (30)
		Senecio integerrimus	4.19 ± 0.08 (36)	20.70 ± 1.89 (10)	22.38 ± 0.65 (21)
	Astereae	*Erigeron flagellaris*	2.17 ± 0.04 (22)	32.90 ± 3.49 (10)	19.58 ± 0.33 (28)
		Erigeron speciosus	2.60 ± 0.04 (20)	83.55 ± 7.95 (10)	30.44 ± 0.54 (32)
		Heterotheca villosa	3.56 ± 0.03 (22)	55.69 ± 14.64 (10)	27.01 ± 1.18 (12)
		Pyrrocoma crocea	5.23 ± 0.05 (37)	10.91 ± 1.07 (10)	61.07 ± 2.77 (11)
		Solidago multiradiata	1.76 ± 0.08 (20)	47.60 ± 19.81 (10)	10.97 ± 0.46 (10)
	Heliantheae	*Arnica parryi*	4.64 ± 0.05 (40)	6.78 *	11.54 ± 0.43 (16)
		Helianthella quinquenervis	4.13 ± 0.05 (32)	9.10 ± 1.58 (10)	64.09 ± 2.48 (24)
		Heliomeris multiflora	2.33 ± 0.03 (22)	28.10 ± 2.77 (10)	35.60 ± 1.50 (19)
		Rudbeckia occidentalis	4.22 ± 0.06 (43)	4.15 ± 0.59 (10)	40.85 ± 1.95 (13)
		Wyethia amplexicaulis	6.83 ± 0.07 (40)	13.6 ± 1.19 (10)	99.00 ± 3.93 (13)
	Helenieae	*Hymenoxys hoopesii*	3.20 ± 0.04 (38)	21.50 ± 3.48 (10)	74.32 ± 4.11 (11)

* Flower head density was measured based on one plot of ten square meters instead of ten plots of one square meter.

Table 2. Qualitative and quantitative network parameters.

Statistics	**Value**
Qualitative parameters	
Number of plant species (P)	16
Number of flower-visitor species (A)	64
Ratio A/P	4
Number of interactions (I)	181
Network size (S)	1024
Connectance (C)	0.18
Quantitative parameters	
Weighted connectance (C_q)	0.09
Interaction evenness (IE)	0.67
Weighted plant linkage (lw_p)	9.24
Weighted flower-visitor linkage (lw_{fv})	5.83
Network specialization (H_2’)	0.35
Weighted nestedness (WNODF)	14.49

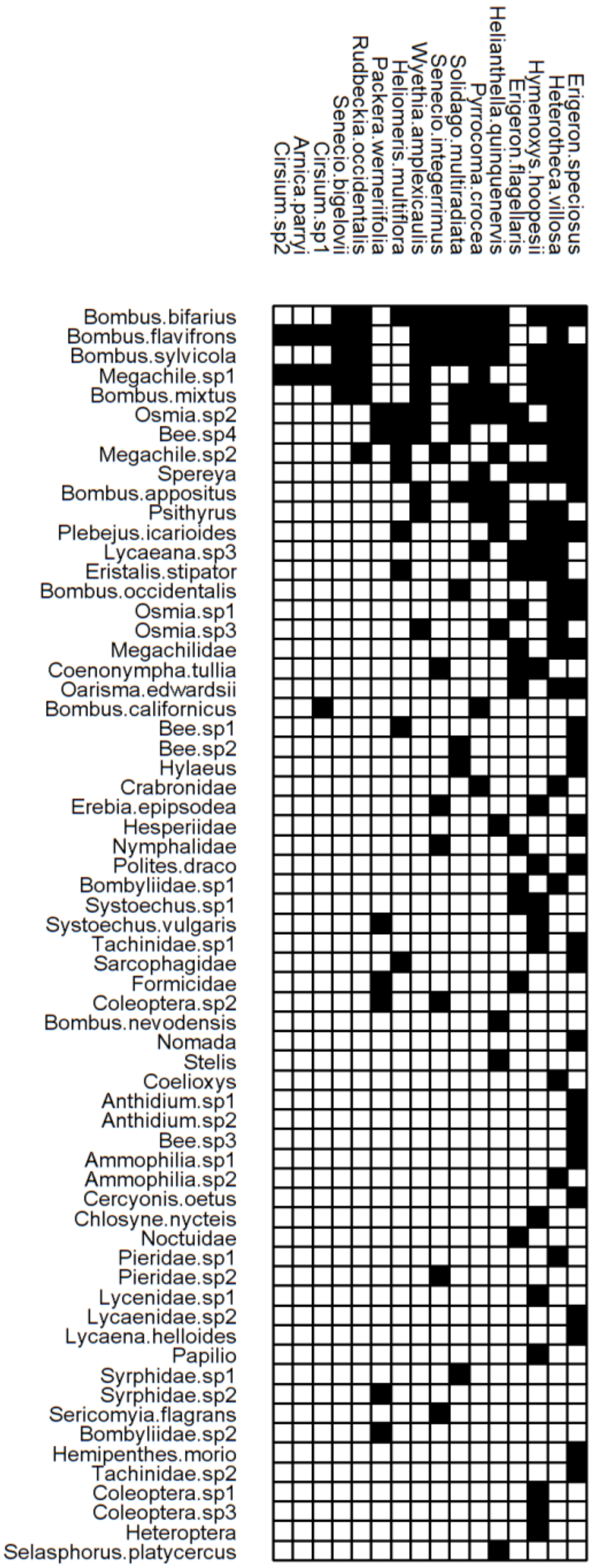

Fig. 2. Qualitative interaction matrix, with the rows representing the plant species, and the columns representing the flower-visitors, identified up to species or morphospecies level.

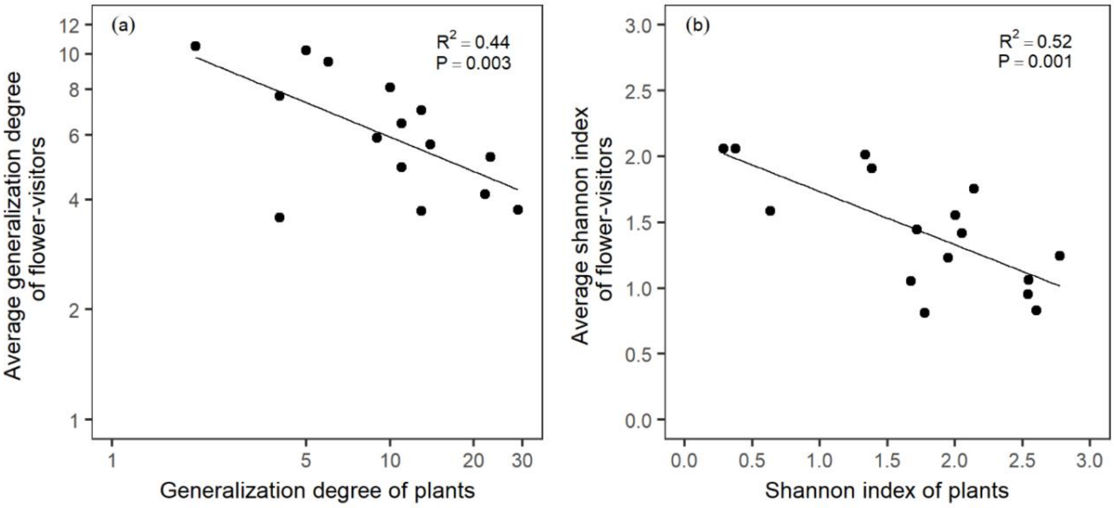

Fig. 3. Generalization degree of the plants in relation to the generalization degree of their flower-visitors, based on (a) the number of interactions and (b) the Shannon diversity index. Each data point corresponds to a single plant species (n=16) and is shown as the mean generalization degree of the flower-visitors observed at that plant.

Floral traits and number of flower-visitors species and -visits

Plant species with a higher flower head density (r=0.61, P=0.006; Fig. 4a), were visited by more flower-visitor species, but did not receive more visits (r=0.20, P=0.23; Fig. 4d). In contrast, plant species with a flowers with larger display were visited by more flower-visitor species (r=0.47 P=0.03; Fig. 4c) and received more visits (r=0.62 P=0.005; Fig. 4f). Plant species with flowers with deeper nectar tubes were visited by fewer species (r=-0.45, P=0.04; Fig. 4b), but did not receive fewer visits (r=-0.25, P=0.17; Fig. 4e). Moreover, the average number of visits per flower-visitor species increased with increasing flower head density (r=0.70, P=0.001) and decreased with increasing nectar tube depth (r=-0.47, P=0.03). The average number of visits per flower-visitor species was not related to the display area of their flower heads (r=-0.06, P=0.83; Fig. 4i). Flower head density, display area and nectar tube depth together explained 82% of the variation in number of flower-visitor species (Table 3).

Nectar tube depth posed a size constraint on the proboscis length of flower-visitors, as interactions occurred mainly between plant- and flower-visitor species that had a proboscis longer than the nectar tube depth of the flowers they visited (Fig. 5). Deeper flowers were visited by a higher proportion of the potential flower-visitor species than shallow flowers, indicated by the steeper slope of the potential number of species compared to observed number of species (b= -8.09, CI= -8.65 - 7.53vs b= -2.27, CI= -5.16 - 0.63; Fig. 6). Plant species were at the most visited by 49% of the potential number of flower-visitor species.

Table 3. Multiple regression with $\log_{10}$ number of observed flower-visitor species as the dependent variable and $\log_{10}$ flower head density (number of flower heads/m^2), nectar tube depth (mm) and $\log_{10}$ display area as independent variables. The explained variance of the presented model is 82% ($R^2=0.82$, F=18.18, DF = 12, P<0.001).

Independent variables	Standardized coefficient	T-value	P-value
Intercept	-0.66	-1.75	0.11
Nectar tube depth	-0.08	-1.96	0.04
Log_{10} flower head density	0.53	3.39	0.003
Log_{10} floral display area	0.85	5.44	<0.001

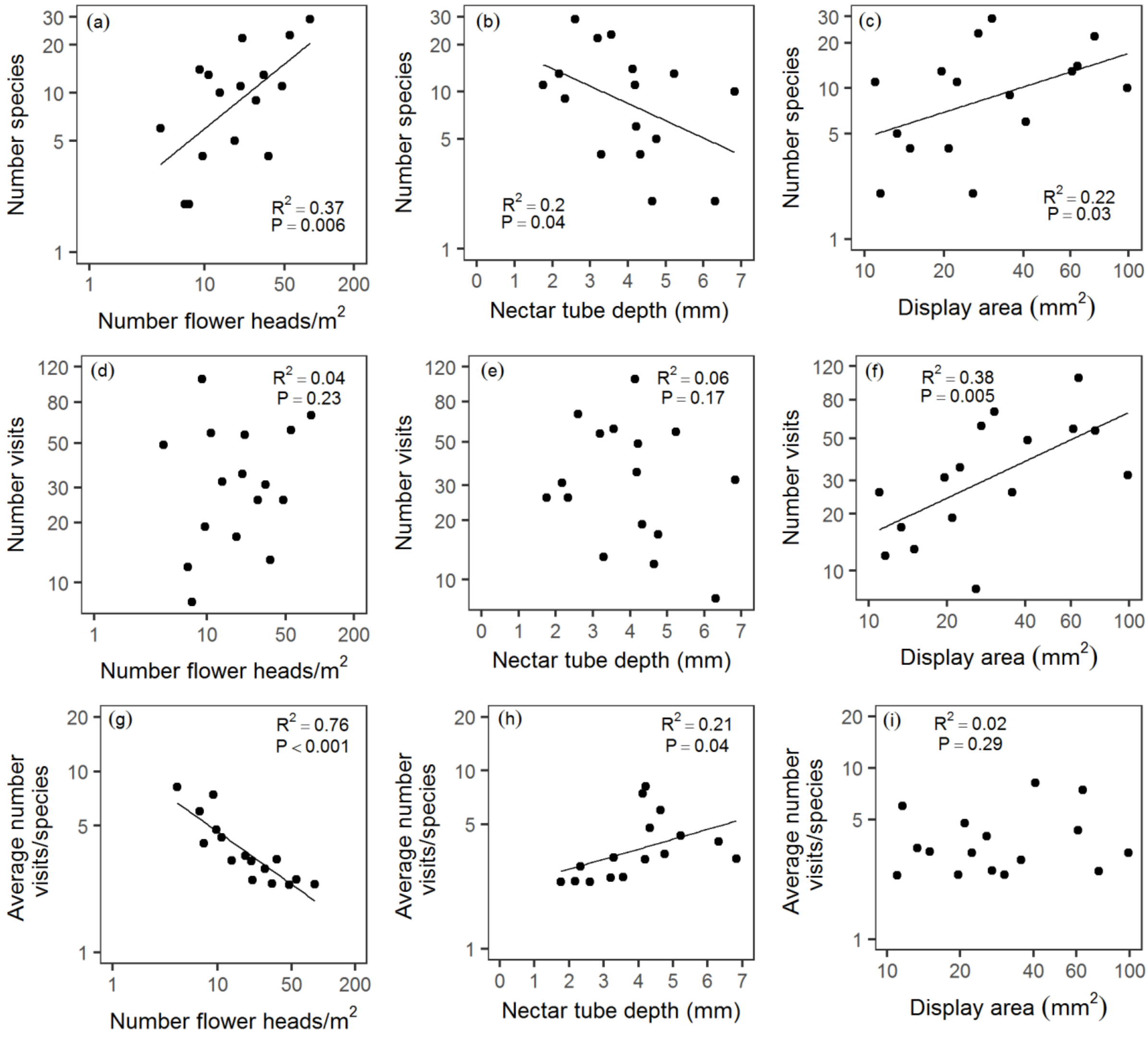

Fig. 4. The observed number of flower-visitor species, number of visits and the average number of visits per flower-visitor species in relation to flower head density (number of flower heads/m^2), nectar tube depth and display area. Each data point corresponds to a single plant species (n=16) and is shown as the total number of flower-visitor species, total number of visits or the mean number of visits of all flower-visitor species observed at that plant species. Number of flower-visitor species, number of visits, flower head density and display area are log$_{10}$ transformed before statistical analysis to achieve normality. The axes of these variables are therefore on a logarithmical scale.

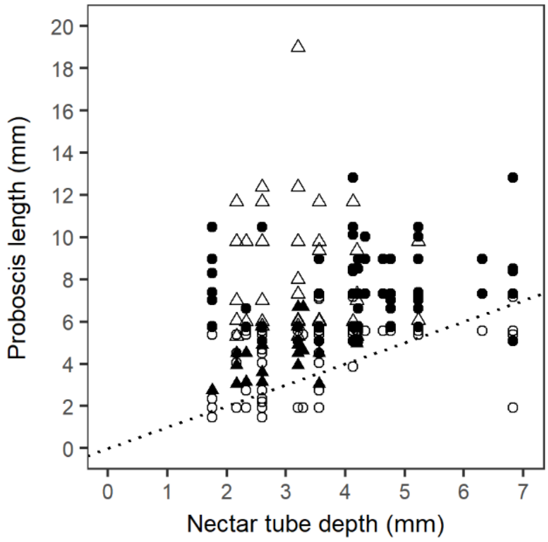

Fig. 5. Observed proboscis lengths in relation to nectar depths of flowers. Each data point represents an interaction between a single plant species and flower-visitor species. Closed circles are bumblebee species, open circles are solitary bee and wasp species, closed triangles are fly species and open triangles are butterfly species. The dotted line (x=y) represents the size threshold, which is the line at which proboscis length equals nectar tube depth. The interactions occur mainly within a triangle above the threshold.

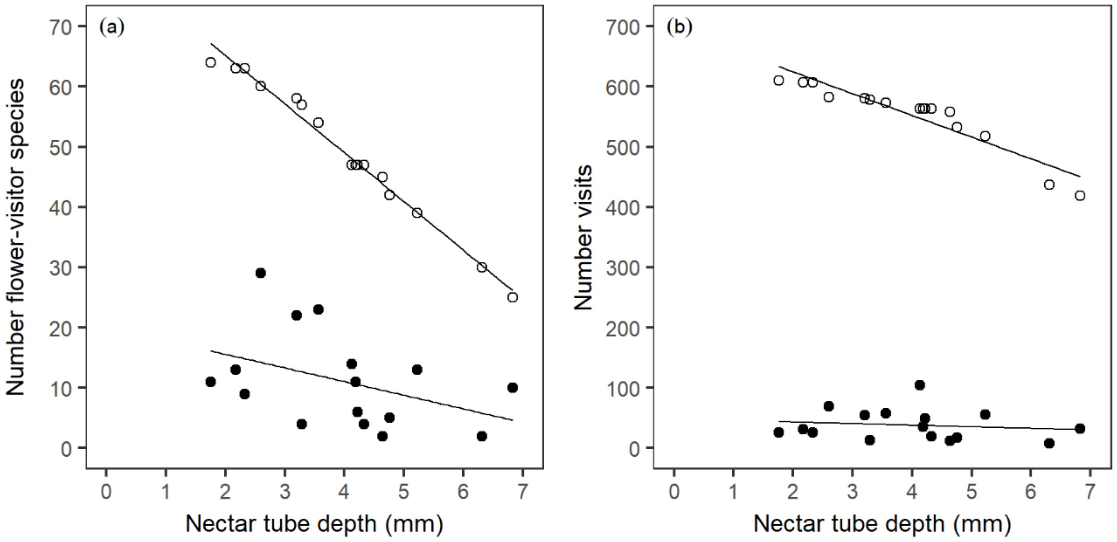

Fig. 6. Observed (filled circles) and potential (open circles) number of (a) flower-visitor species and (b) visits in relation to nectar tube depth of flowers. Each data point corresponds to a single plant species.

Patterns of size-matching

Approximately 60% of the flower-visitor species, which accounted for approximately 80% of the visits, had a relatively long proboscis (>5 mm) (Fig. 7). Plant species with deeper flowers were visited by bumblebees-, solitary bee- and fly species with a longer proboscis, both when accounting for the number of species and the number of visits (Fig. 8a-d,g-h). The degree of size-matching between the nectar tube depth and flower-visitor proboscis length differed among these flower-visitor taxa. In contrast, among butterflies, there was no relationship between nectar tube depth and their average proboscis length (Figs 8e-f). Bumblebees and butterflies had a proboscis long enough to visit all plant species. Therefore, apparently, bumblebee species with a short proboscis avoided flowers with a deeper nectar tube.

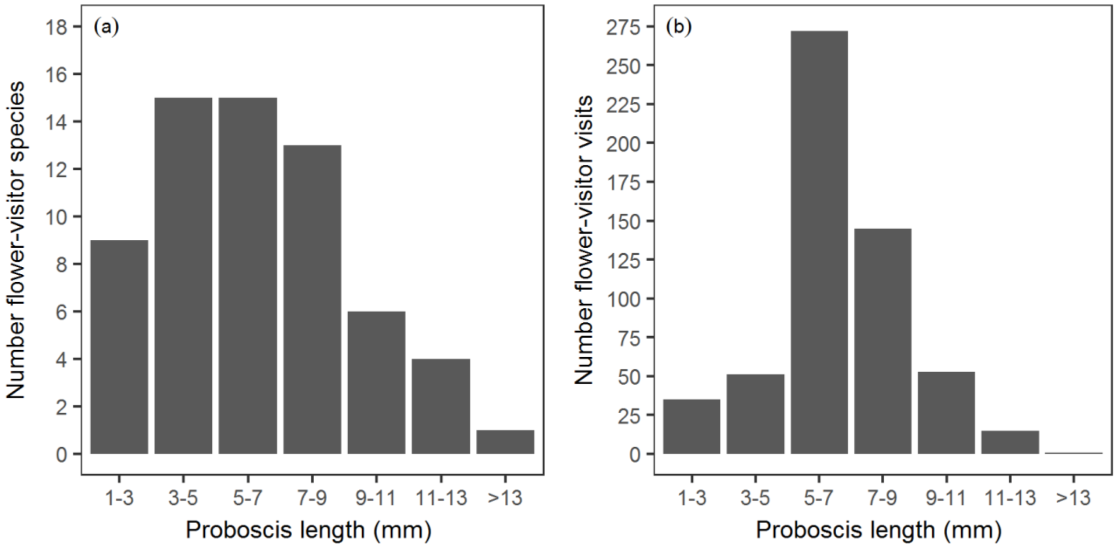

Fig. 7. Distribution of (a) the number of flower-visitor species and (b) the number of visits, based on their proboscis length.

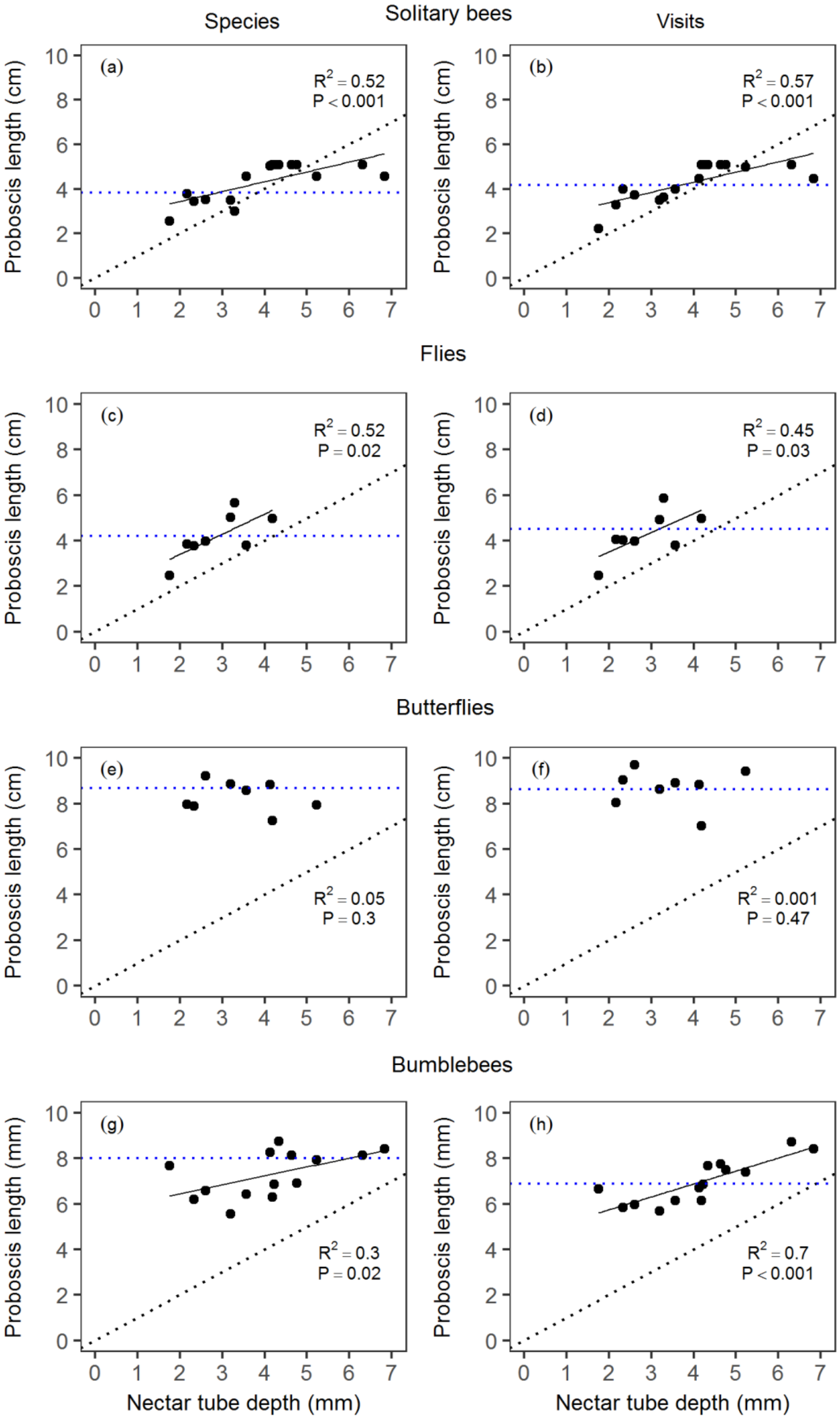

Fig. 8. Observed proboscis lengths in relation to nectar depths of flowers based on the number of (a) species of solitary bees, (b) visits of solitary bees, (c) species of flies, (d) visits of flies, (e) species of butterflies, (f) visits of butterflies, (g) species of bumblebees, and (h) visits of bumblebees. Each data point corresponds to a single plant species (n=16) and is shown as the mean proboscis length of all species observed at that plant. The dotted black line (x=y) represents the expected size threshold and the dotted blue line presents the expected average proboscis length, based on random encounters.

Discussion

Network structure: generalization, nestedness and asymmetry

Although plant species in the Asteraceae family are considered to be generalized, as they are visited by a high diversity of flower-visitor species and taxa (Graenicher 1909; Lane 1996; Proctor, Yeo & Lack 1996), we found that some plant species were highly specialized. We expected the interactions among the Asteraceae and their flower-visitors to be more generalized and connected and less asymmetric and nested, compared to other pollination networks, because the Asteraceae have shallow flowers which are easily accessible. Surprisingly, on average, species of the Asteraceae family were not visited by a higher number of flower-visitor species, compared to other pollination networks (Traveset *et al.* 2016). However, plant-flower-visitor interactions were more even, more connected and more generalized, compared to other pollination networks (Traveset *et al.* 2016). Apparently, these plants share a greater proportion of their flower-visitor species, which indicates plant phylogeny affects flower-visitor visitation patterns (e.g. Rezende, Jordano & Bascompte 2007). Furthermore, in contrast to our expectations, we found comparable levels of asymmetry and nestedness as in complete pollination networks (Traveset *et al.* 2016). This might be explained by the (unexpected) presence of morphological specialized flowers.

Determinants of plant generalization degree

Both nectar tube depth and flower head density determined the number of flower-visitor species by which plants were visited but not the number of visit that plants received. Plant species with a deeper nectar tube had a lower Shannon diversity index and were visited by fewer flower-visitor species, but did not receive fewer visits. On average, flower-visitor species visited plant species with a higher flower head density more frequently. Interestingly, plant species with deeper flowers were also visited by a higher proportion of the potential number of flower-visitor species, based on the floral nectar tube depth and flower-visitor proboscis length. Moreover, plant species with a higher flower head density had a higher Shannon diversity index and were visited by more flower-visitor species, but did not receive more visits per patch. On average, flower-visitor species visited plant species with a higher flower head density less frequently. From this positive relationship between flower density and number of flower-visitor species, which has often been found in plant-flower-visitor networks, it is generally concluded that plant-flower-visitor interactions occur randomly. Also, this relationship has often been explained by the fact that less abundant flower-visitor species have a higher chance of being detected on plant species with a high flower density (Dupont, Hansen & Olesen 2003; Vazquez & Aizen 2004; Vazquez *et al.* 2007) and thus that these results are due to sampling bias. However, our results indicate that plant-flower-visitor interactions do not occur randomly. If plant-flower-visitor interactions would occur at random, then plant species with a higher flower density should also have been visited more frequently. Rather, the flowers of plant species with a higher density of flower heads, were more shallow, and therefore could potentially be visited by more flower-visitor species. This negative correlation between flower head density and nectar tube depth may be an underlying cause for the positive relationship between flower head density and plant generalization degree. Moreover, a relatively high number of flower-visitor species with a short proboscis visited the Asteraceae less frequently and therefore are expected to be less abundant. Due to their short proboscis, they are restricted to plant species with shallow and thus more dense flower heads. This frequency distribution of flower-visitor proboscis length, may also be an underlying cause for the positive relationship between flower head density and plant generalization degree. We rather argue that there is a more ecological explanation for the positive relationship between flower head density and number of flower-visitor species, namely that less abundant flower-visitors may visit abundant plant species more frequently because they provide a more reliable food source (Memmott, Waser & Price 2004).

Although flower depth and animal proboscis- or bill length are generally assumed to influence species generalization (Herrera 1996; Agosta & Janzen 2005; Stang, Klinkhamer & van der Meijden 2006; Stang *et al.* 2009; Maglianesi *et al.* 2014; Johnson *et al.* 2017), this is mostly found among hummingbird and hawkmoth pollinated plants. Torres and Galetto (2002), did not find a significant correlation between nectar tube depth and the number of flower-visitor species among South American Asteraceae species. While Torres and Galetto (2002) measured the whole corolla tube length, we measured the functional length of the nectar tube, which is the part of the corolla tube in which flower-visitors can insert their proboscis. This highlights the importance of measuring the functional size of traits when relating morphological traits to flower-visitor visitation patterns.

In contrast to flower head density and nectar tube depth, both the number of flower-visitor species and visit increased with increasing flower display area. Previous studies have shown that among the Asteraceae, ray flowers play an important role in attracting flower-visitors (Stuessy, Spooner & Evans 1986; Andersson 2008). However, Pohl, Van Wyk and Campbell (2011) have shown that manipulating the size of ray flowers did not affect flower visitation patterns. Therefore, other aspects, e.g. flower-visitor foraging efficiency, may also affect flower-visitor flower selection and consequently plant generalization degree. For example, a larger display might make it easier for insects to land and to unfold a long proboscis and in turn to access and extract nectar. Moreover, flowers with larger displays might offer higher rewards, either because they produce more nectar per flower or have more open flowers per flower head. Especially for flower-visitors with a long proboscis, which have higher energetic requirements (Heinrich 1983), this would make flowers with larger displays more attractive. Consequently, the importance of display area for flower-visitor flower choice may depend on the type of flower-visitors and plants in the community. This may explain why previous studies, which examined different plant-flower-visitor communities, found contrasting results (Hegland & Totland 2005; Stang, Klinkhamer & van der Meijden 2006).

Patterns of size-matching among different flower-visitor taxa

Size-matching between the depth of flowers and the proboscis length of flower-visitors was inconsistent among flower-visitor taxa. Flowers with deeper tubes were, on average, visited by bumblebees, solitary bees and flies with a longer proboscis. We did not find this relationship among butterflies. It is surprising that even among bumblebees we found this pattern, since bumblebees had a proboscis long enough to visit all plant species. Consequently, this pattern suggests that shallow flowers were selectively avoided by species with a long proboscis, while flowers with deeper nectar tubes were selectively avoided by species with a short proboscis. This indicates that not only size constraints play a role in flower-visitor foraging choice. Although flower density might make flowers more attractive, flower head density can neither explain this preference for deep flowers because flowers with deeper tubes were less dense. Rather, patterns of size-matching may results because flower-visitors avoid resource competition (Rodriguez-Girones & Santamaria 2006). Moreover, the preference for deep flowers might also result from increased foraging efficiency of flower-visitors when they forage on flower which match the length of their proboscis, as (1) they can handle these flowers more quickly (Plowright & Plowright 1997) and (2) in some plant-flower-visitor communities, deeper flowers are more rewarding (Galetto & Bernardello 2004; Kaczorowski, Gardener & Holtsford 2005; Fenster *et al.* 2006; Ornelas *et al.* 2007; Gomez *et al.* 2008; Martins & Johnson 2013; Carvalheiro *et al.* 2014). These differences in foraging efficiency might result in competitive niche differentiation between species with longer and shorter proboscises. Like bumblebees, butterflies had a proboscis long enough to visit all plant species studied. Butterflies may select different flowers, based on sugar concentration (Watt, Hoch & Mills 1974; Pyke & Waser 1981) or display area, compared to bumblebees and solitary bees. Overall, incorporating traits which are related to flower-visitor foraging efficiency, such as nectar production, in community studies will be an important next step to better understand plant-flower-visitor network structure.

Further, patterns of size-matching differed among flower-visitor taxa. In accordance with a Spanish community, among flies, the degree of size-matching was independent of the nectar tube depth (Stang *et al.* 2009). However, among bumblebees, there was a closer matching between the nectar tube depth and proboscis length on flowers with deeper nectar tubes, while among solitary bees, there was actually the closest matching among medium-sized flowers. This pattern can be explained by the frequency distributions of flower-visitor proboscis lengths. Bumblebee species had a relatively long proboscis, compared to the depth of the flowers, while solitary bees did not. Moreover, among solitary bees, species with a medium-size proboscis length most frequently visited the plants.

Overall, we have shown that even among generalized plants, plant-flower-visitor interactions are structured and predictable. Flower-visitors did not forage randomly, as only the number of flower-visitor species, but not the number of visits was related to flower head density. Rather, plant generalization degree was determined by nectar tube depth and display area, the negative correlation between nectar tube depth and flower head density and flower-visitor proboscis length frequency distributions. Our results highlight the importance of quantitative data analyses, rather than qualitative data analyses and trait-abundance correlations to understand pollination community structure.

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3

Scaling relationships between flower size and nectar reward at the scale of single flowers, flower heads, individual plants and patches among subalpine Asteraceae

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Abstract

Rewards offered by plants to flower-visitors are mostly studied at the level of individual flowers. However, the foraging efficiency of flower-visitors, and therefore the attractiveness of plants, is also determined by rewards on larger spatial scales: the inflorescence, individual plant and the local distribution of individual plants. Here, we investigated the scaling relationships between nectar tube depth and nectar rewards at different spatial scales among fourteen co-occurring Asteraceae species. To this end, we measured nectar production and nectar standing crop of single flowers and calculated the amount of sugar produced per flower head, individual plant and patch of one square meter, based on the number of open flowers per flower head and the number of flower heads per plant individual and patch. For all plant species, the nectar production rate was measured both in 2011 and 2013. We found that plant species with deeper flowers produced more nectar per flower, both in volume and sugar content. In contrast, sugar concentration was not related to nectar tube depth. Deeper flowers also offered more sugar per flower head, individual plant and patch. Specifically, sugar content per flower and flower head increased allometrically with increasing nectar tube depth, with a scaling factor of two and three respectively. These scaling factors were similar in both years. Moreover, nectar volume and sugar content of flowers that were bagged for 24-hours were positively correlated with nectar volume and sugar content of the nectar standing crop. Sugar concentration of bagged flowers was not related to the sugar concentration of the nectar standing crop. Overall, our results provide more insight in how nectar tube depth is related to nectar reward of plants at different spatial scales, which is important for understanding how nectar rewards may act as an underlying factor promoting flower-visitor selection for deeper flowers.

Keywords: Asteraceae, allometry, daily inflorescence size, floral traits, nectar standing crop, nectar production rate, nectar tube depth

Introduction

Nectar is one of the major floral rewards for flower-visitors. In some flowers nectar is easily accessible while in others it is placed at the bottom of a (long) nectar tube and difficult to access. The depth of this tube is an important floral trait since it determines both the nectar reward (Galetto & Bernardello 2004; Kaczorowski, Gardener & Holtsford 2005; Ornelas *et al.* 2007; Martins & Johnson 2013; Johnson *et al.* 2017) and size (Barrow & Pickard 1984; Peat, Tucker & Goulson 2005; Stang *et al.* 2009) and number of the flower-visitors (Torres & Galetto 2002; Agosta & Janzen 2005; Borrell 2005; Stang, Klinkhamer & van der Meijden 2006; Johnson *et al.* 2017). Flowers with deeper nectar tubes are visited by flower-visitors with a longer proboscis (Barrow & Pickard 1984; Stang *et al.* 2009), as the nectar tube acts as a barrier, limiting the access to flower-visitors which have a proboscis that exceeds the length of the nectar tube (Corbet 2000; Stang, Klinkhamer & van der Meijden 2007). It has been argued that flowers with deep nectar tubes produce more nectar either because flowers with deeper tubes are able to hold more nectar or have larger nectaries (Petanidou, Goethals & Smets 2000; Galetto & Bernardello 2004) or in order to attract these long-tongued flower-visitors (Kaczorowski, Gardener & Holtsford 2005; Ornelas *et al.* 2007; Martins & Johnson 2013; Johnson *et al.* 2017), which can potentially visit a wide variety of flowers (Borrell 2005).

Nectar reward offered by plants to flower-visitors is mostly studied at the level of individual flowers. However, the attractiveness of plants also depends on the number of open flowers and the clustering of these flowers, as this determines the total nectar reward and foraging efficiency of flower-visitors. The number of open flowers increases the total nectar reward and clustering reduces the time that flower-visitors have to flight between flowers and therefore the energy they spend while foraging. Dense clustering, such as in flower heads of the Asteraceae, makes it even possible for insects to walk between flowers (Heinrich 1972), which reduces their energetic expenditure immense, as walking can require about 100 times less energy per second than flight at the same thoracic temperature (Heinrich & Raven 1972). Therefore, by increasing the number of open flowers and clustering these flowers in dense inflorescences, even shallow tubed flowers with low nectar amounts might be attractive for large flower-visitors. Although it has been argued that there might be a trade-off between flower size and number of open flowers (Harder & Cruzan 1990; Worley *et al.* 2000; Sargent *et al.* 2007; Kettle *et al.* 2011), the scaling relationships between nectar production and number of open flowers have rarely been investigated (Harder & Cruzan 1990).

In this study we investigated whether plant species with deeper flowers are more rewarding. To this end, we analyzed the scaling relationships between nectar tube depth and the nectar production at different spatial scales: per single flower, flower head, individual plant and patch among fourteen co-occurring Asteraceae species in the Colorado Rocky Mountains. The Asteraceae is one of the most successful and species-rich plant families worldwide (Proctor, Yeo & Lack 1996), which provides the (unique) opportunity of having a great diversity of species occurring at the same meadow. Species that belong to the Asteraceae family have tiny flowers which are aggregated in flower heads and visited by a wide diversity of flower-visitors (Graenicher 1909; Lane 1996; Torres & Galetto 2002).

Single flowers produce minute amounts of nectar (Pleasants 1981; Pohl, Van Wyk & Campbell 2011), which is assumed to make clustering of flowers into inflorescences crucial to attract flower-visitors (Heinrich & Raven 1972). The tubular flowers of the Asteraceae differ in size but resemble each other in morphological structure and nectar organization (Mani & Saravanan 1999). These aspects make the Asteraceae an ideal plant family to investigate the scaling relationships among nectar tube depth, nectar production and the number of open flowers. However, comparative studies at the family level (within one community), especially on floral nectar production of single flowers, are rare. Due to their minute amounts of nectar, most studies measure the nectar production of the flower heads (Pohl, Van Wyk & Campbell 2011). Although they are often included in community studies, only Torres and Galetto (2002) performed comparative analyses on the nectar production of the South American Asteraceae. They analysed the correlation between tube depth and nectar sugar composition, rather than nectar volume and sugar content. Here we address the following questions: (1) Do flowers with deeper nectar tubes produce more nectar per single flower and offer more nectar per flower head, individual plant and patch? (2) Is the nectar tube depth and nectar production related to the number of open flowers per head and the number of flower heads per individual and patch? Since nectar standing crop is what flower-visitors actually encounter while foraging we also tested (3) whether nectar standing crop is related to nectar production rate.

Materials and methods

Study site and plant species

Data were collected in the vicinity of the Rocky Mountain Biological Laboratory, Gunnison County, Colorado, USA in 2011 and 2013. In total, fourteen Asteraceae species were sampled which belonged to the subfamilies Carduoideae and Asteroideae (Table 1). The Asteraceae have tiny flowers (flowers) which are aggregated in flower heads (capitula). The heads of most species have two basic types of flowers: tubular, bell-shaped disk flowers, which are found in the center of the head, and strap-shaped ray flowers, which are found in the peripheral, although the heads of some species have only disk or only ray flowers. The studied plant species either had disk and ray flowers or only disk flowers (Fig. 1).

Nectar tube

For the studied Asteraceae, the corolla tube of the disk flowers consists of two parts: a (very) narrow lower part and an upper wider bell-shaped part. The wider part roughly begins where the stamens insert and ends at the beginning of the corolla lobes, at the base of the deepest cleft in the corolla. Nectar is produced at the base of the narrow part of the corolla tube and migrates up this part into the wider upper part of the corolla tube. The lower part of the corolla tube is almost filled by the style. Consequently, the lower part only contains traces of nectar and it is unlikely that flower-visitors can access this part of the corolla tube (Graenicher 1909). Therefore, for each plant species, we measured the depth and width of the upper wider part of the corolla tube, hereafter referred to as nectar tube. Consequently, the width of the nectar tube is not a direct indication for the width of the nectary. We measured the depth and width of the nectar tube for at least eighteen freshly picked flowers, to the nearest 0.01 mm, under a dissecting microscope. The width of the nectar tube was measured at the base of the nectar tube. In addition, we measured the diameter of the style.

Nectar production

We measured the nectar production of disk flowers that were bagged for 24-hours as an estimate for the nectar production rate (NPR). Ray flowers are often sterile and do not produce nectar (Mani & Saravanan 1999). Flowers were bagged between 8 and 9 AM and collected 24-hours later. We used bags made of fine gauze that do not change the temperature within the bag and thus will not influence the nectar concentration (Corbet 2003). Each year, on average, 43 disk flowers were sampled from thirteen flower heads, for each plant species. We collected the nectar from flowers in the most inner ring of the flower head, which were those flowers which were most recently opened. In addition, in 2013, nectar standing crop (NSC) was measured for eight of the fourteen species, also during the early morning. NPR gives an indication of the total nectar amount that flowers produce. In contrast to NPR, NSC is affected by flower-visitor visitation and is what flower-visitors actually encounter in the field.

Nectar was extracted with microcapillary tubes, ranging in size between 0.2, 0.25, 0.5 and 1.0 μL. Capillary size depended on the quantity of the nectar produced by the flowers and the nectar tube width. With the microcapillary tubes we probed the base of the nectar tube until no more nectar could be removed and then used digital callipers to measure the distance the nectar had migrated up the microcapillary tubes. Nectar volume was determined by converting the distance measured to volumes (μL). Nectar sugar concentration (expressed in % w/w in a solution) was measured with a hand-held refractometer. When nectar samples of a single flower were too small for sugar concentration measurements, nectar samples of flowers from the same head were pooled to obtain a single concentration measurement. Sugar content (mg) was quantified as the product of the nectar sugar concentration, which was first converted from wt/wt to wt/vol, multiplied with the nectar volume, as suggested by Bolten *et al.* (1979). In 2011, nectar production rate was measured in the lab under a dissecting microscope, within an hour after collection of the bagged heads. In 2013, both nectar standing crop and nectar production rate were measured in the field.

Based on the nectar volume after bagging for 24-hours, we calculated the height the nectar rises in the nectar tube, using the following formula:

$$h = v/\pi r^2 - \pi s^2 \tag{1}$$

where h is the height the nectar rises in the nectar tube, v is the nectar volume, r is the radius of the nectar tube and s is the radius of the style.

Number of flowers per head and number heads per individual and patch

For each species we counted the number of open disk flowers per head, the number of open heads per individual and the number of open heads per patch of one square meter. For most plant species, we calculated the average number of flower heads per square meter, based on ten one square meter plots that were randomly placed within the patch. For the *Cirsium sp.2* and *Arnica parryi*, we measured flower head density based on one plot of ten square meter, since these species were flowering at only one location in the meadow.

Statistical analyses

Statistical analyses were performed using R version 3.3.2 (Team 2014). Prior to all analyses, all variables were $\log_{10}$ transformed to determine the scaling coefficients. To analyse whether nectar tube depth was and to nectar volume, concentration and sugar content we used both Pearson correlation and single ordinary least square (OLS) regression analyses. The relationship between nectar tube depth and both nectar volume- and sugar content per single flower were established using regression of actual values as well as standardized linear phylogenetically independent contrasts (PICs). Phylogenetically independent contrasts were calculated using the package “ape” in R. A phylogeny for the studied species was based on existing tree published by Zanne *et al.* (2014). Analyses of covariance (ANCOVA) were performed, including year as a fixed factor and nectar volume- or sugar content of a single flower, to test the effect of year on these relationships. Further, we analysed whether nectar standing crop and nectar production rate were related, using Spearman correlations analyses. Even after $\log_{10}$ transformations nectar standing crop parameters were not normally distributed.

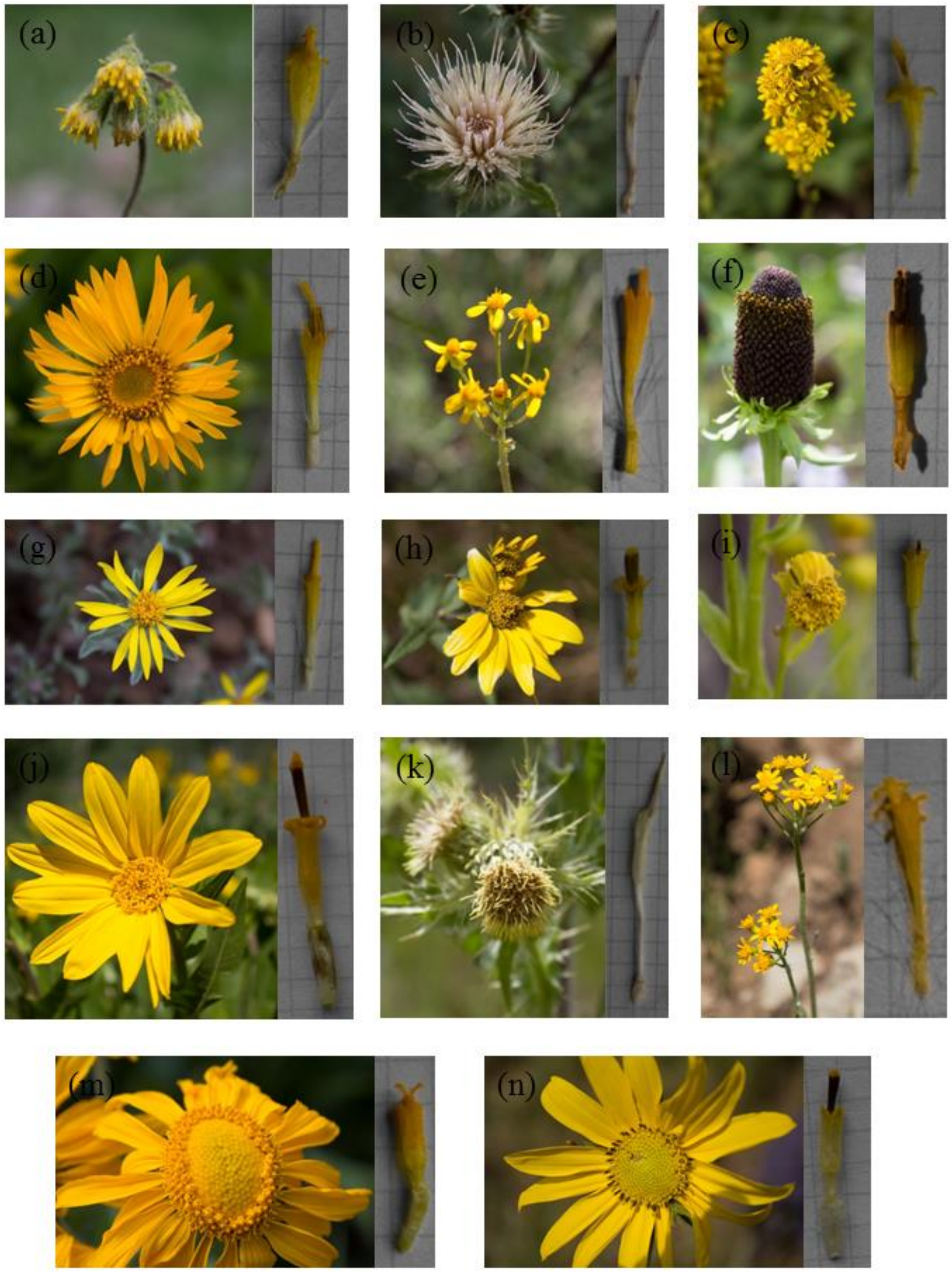

Fig. 1. The species of Asteraceae. (a) *A. parryi* (b) *Cirsium sp.2* (c) *S. multiradiata* (d) *P. crocea* (e) *S. integerrimus* (f) *R. occidentalis* (g) *H. villosa*. (h) *H. multiflora* (i) *S. bigelovii* (j) *W. amplexicaulis* (k) *Cirsium sp.1* (l) *P. werneriifolia* (m) *H. hoopesii* and (n) *H. quinquenervis*.

Results

Nectar tube depth and nectar production

The studied species had relatively shallow flowers, as nectar tube depth varied between 1.76 to 6.83 mm (Table 1). The nectar tube of deeper flowers was also wider (r=0.74, P=0.002). Bagged flowers produced minute amounts of nectar which was relatively concentrated and varied greatly among species (Fig. 2). As a consequence of the minute amounts of nectar, the nectar tube was never filled for more than 11%. Across plant species, nectar volume (r=0.90, P<0.001) and sugar content per flower (r=0.93, P<0.001) of the two years were strongly correlated. In contrast, sugar concentration of 2011 did not correlate significantly with that of 2013 (r=-0.13, P=0.65).

Deeper flowers produced more voluminous nectar per flower in both 2011 (r=0.78, P=0.001) and 2013 (r=0.72, P=0.004). Sugar concentration was not related to nectar tube depth, neither in 2011 (r=-0.29, P=0.32) nor in 2013 (r=0.16, P=0.57). Consequently, the sugar content of the nectar did increase with increasing nectar tube depth in 2011 (r=0.77, P=0.001; Fig. 2a) and 2013 (r=0.77, P=0.001; Fig. 2b). Correlations between nectar tube depth and both nectar volume and sugar content per flower were also significant when based on phylogenetically independent contrasts (Table 2).

Nectar volume and sugar content per flower increased allometrically with nectar tube depth. There was a striking similarity between the scaling factors of 2011 and 2013 (Fig. 3). These scaling factors were approximately two and were significantly different from one (Table 3), which indicates that nectar production does not increase linear with nectar tube depth. Rather, deeper flowers produced increasingly more voluminous and sugar rich nectar. Analyses of covariance showed that the scaling relationships did not differ between the two years (Table 3), as indicated by the nonsignificant factor year and the nonsignificant interaction term between year and nectar tube depth.

In both years, plant species with deeper flowers offered more sugar per head. The scaling factor of this relationship is approximately three (Fig. 3). Flowers with deeper nectar tubes also produced more sugar per individual and more sugar per patch in both 2011 (r=0.52, P=0.06 for sugar per individual and r=0.61, P=0.02 for sugar per square meter) and 2013 (r=0.55, P=0.04 for sugar per individual and r=0.57, P=0.03 for sugar per square meter).

Table 1. Nectar tube depth, nectar tube width, number of open flowers per head, number of open heads per individual and number of heads per m^2 of the Asteraceae species studied. The table gives mean ± s.e.m. and sample size (n).

Subfamily	Plant species	Nectar tube depth (mm)	Nectar tube width (mm)	Number open flowers/head	Number open heads/individual	Number heads/m^2
Carduoideae						
Cardueae	*Cirsium sp.1*	6.31 ± 0.16 (36)	1.71 ± 0.03 (36)	106.25 ± 9.27 (8)	3.47 ± 1.34 (6)	7.40 ± 2.12 (10)
	Cirsium sp.2	4.33 ± 0.07 (39)	1.38 ± 0.02 (18)	96.13 ± 14.93 (8)	4.83 ± 0.61 (23)	9.65 *
Asteroideae						
Senecioneae	*Senecio bigelovii*	4.76 ± 0.06 (24)	1.54 ± 0.02 (24)	16.14 ± 1.71 (21)	7.84 ± 1.27 (25)	18.30 ± 2.90 (10)
	Packera werneriifolia	3.29 ± 0.06 (36)	0.90 ± 0.04 (36)	9.31 ± 0.94 (13)	12.23 ± 1.75 (20)	36.30 ± 5.12 (10)
	Senecio integerrimus	4.19 ± 0.08 (36)	1.23 ± 0.04 (36)	8.53 ± 0.65 (43)	4.78 ± 0.44 (20)	20.70 ± 1.89 (10)
Astereae	*Heterotheca villosa*	3.56 ± 0.03 (22)	0.62 ± 0.01 (22)	7.27 ± 0.65 (33)	36.96 ± 6.15 (8)	55.69 ± 14.64 (10)
	Pyrrocoma crocea	5.23 ± 0.05 (37)	0.80 ± 0.03 (23)	35.53 ± 2.19 (15)	12.23 ± 1.75 (25)	10.91 ± 1.07 (10)
	Solidago multiradiata	1.76 ± 0.08 (20)	0.68 ± 0.03 (20)	9.42 ± 0.85 (31)	18.21 ± 5.67 (12)	47.60 ± 19.81 (10)
Heliantheae	*Arnica parryi*	4.64 ± 0.05 (40)	1.46 ± 0.04 (26)	10.63 ± 0.73 (35)	2.39 ± 0.22 (28)	6.78 *
	Helianthella quinquenervis	4.13 ± 0.05 (32)	1.49 ± 0.04 (20)	34.84 ± 2.77 (38)	7.35 ± 1.84 (20)	9.10 ± 1.58 (10)
	Heliomeris multiflora	2.33 ± 0.03 (22)	0.74 ± 0.02 (22)	22.81 ± 1.52 (41)	16.07 ± 1.97 (24)	28.10 ± 2.77 (10)
	Rudbeckia occidentalis	4.22 ± 0.06 (43)	1.51 ± 0.02 (42)	108.80 ± 8.01 (22)	4.37 ± 0.48 (25)	4.15 ± 0.59 (10)
	Wyethia amplexicaulis	6.83 ± 0.07 (40)	1.72 ± 0.04 (20)	25.13 ± 1.49 (39)	5.16 ± 0.60 (37)	13.60 ± 1.19 (10)
Helenieae	*Hymenoxys hoopesii*	3.20 ± 0.04 (38)	1.11 ± 0.02 (20)	58.74 ± 3.77 (31)	7.08 ± 0.93 (50)	21.50 ± 3.48 (10)

* Flower head density was measured based on one plot of ten square meters instead of ten plots of one square meter.

Table 2. Pairwise correlation of nectar tube depth and nectar production rate (NPR) based on conventional and phylogenetic (independent contrast) analyses. For conventional analyses, nectar volume and sugar content were $\log_{10}$ transformed.

	2011		**2013**	
	Conventional	PIC	Conventional	PIC
NTD – VOL	r = 0.78	ρ = 0.82	r = 0.72	ρ = 0.70
	P = 0.001	P = 0.001	P = 0.004	P = 0.010
NTD – SUG	r = 0.77	ρ = 0.74	r = 0.77	ρ = 0.82
	P = 0.001	P = 0.006	P = 0.001	P < 0.001

NTD = nectar tube depth (mm), VOL = nectar volume (µL day^{-1} per flower), SUG = sugar content (mg day^{-1} per flower). All p-values are for two-tailed tests.

Table 3. Analyses of covariance (method backward selection of variables) with nectar production rate (volume and sugar content) as function of nectar tube depth and year. Nectar tube depth, nectar volume and sugar content were $\log_{10}$-transformed prior to analysis. The interaction between nectar tube depth and year and year itself were excluded from both models because they were not significant. The explained variance of the presented model for nectar volume is 52% (R^2=0.52, F=24.58, DF=26, p<0.001). The explained variance of the presented model for sugar content is 55% (R^2=0.55, F=31.26, DF=26, p<0.001).

	Nectar volume			**Sugar content**		
	Coefficient	95% CI	t-value	Coefficient	95% CI	t-value
Intercept	-2.26***	-2.83 – -1.69	-5.59	-2.42***	-2.92 – -1.92	-9.93
Nectar tube depth	2.24**	1.31 – 3.17	3.63	2.20***	1.39 – 3.01	5.59
Year 2013	n.s.			n.s.		
Nectar tube depth: year	n.s.			n.s.		

** 0.01<P<0.001, *** P<0.001

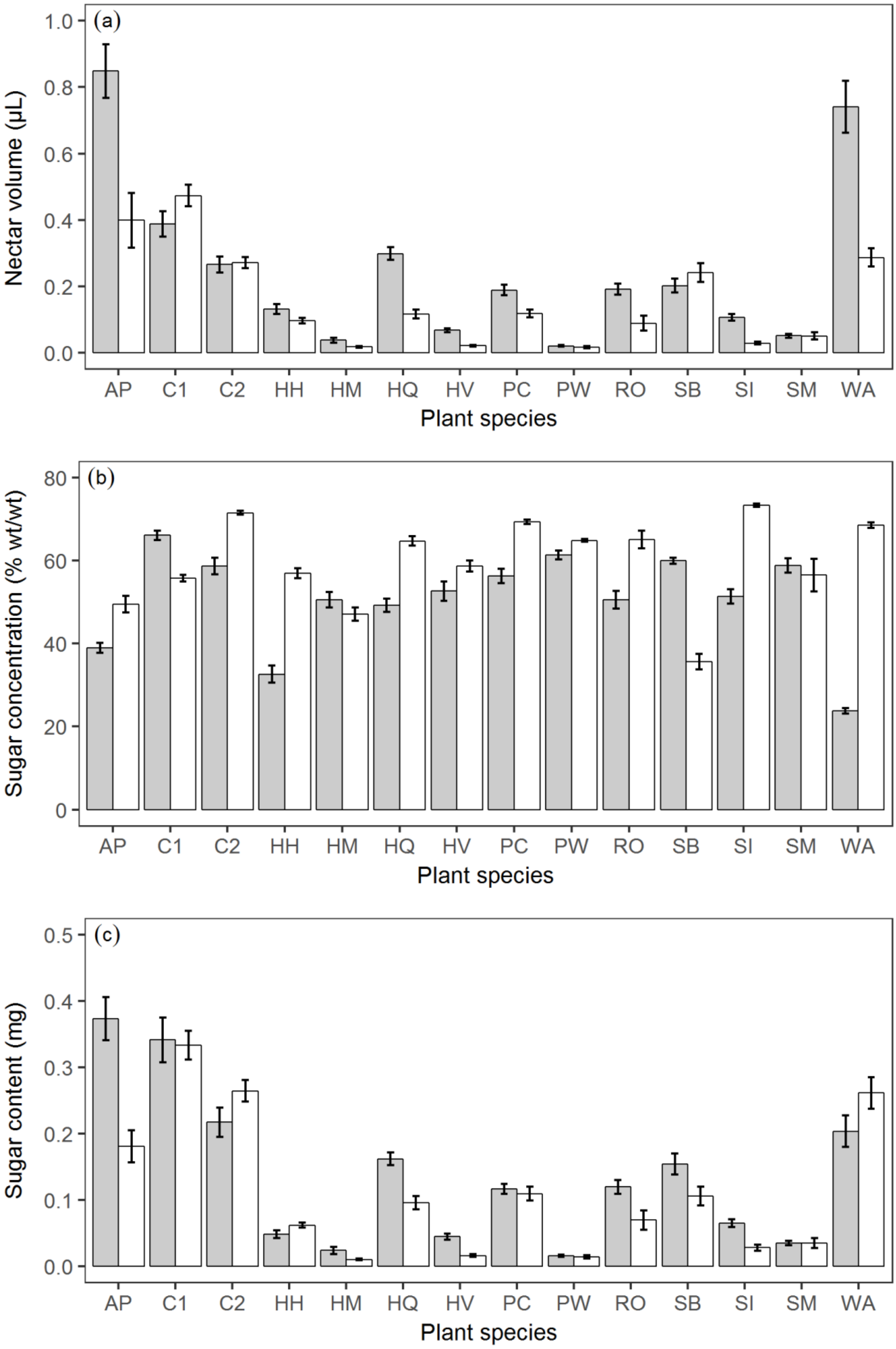

Fig. 2. Average (a) nectar volume, (b) sugar concentration, and (c) sugar content of the nectar after 24-hours bagging ± s.e. (vertical lines). Gray bars represent the average nectar production in 2011 and white bars represent the nectar production in 2013. AP: *A. parryi*, C1: *Cirsium sp.1*, C2: *Cirsium sp.2*, HH: *H. hoopesii*, HM: *H. multiflora*, HQ: *H. quinquenervis*, HV: *H. villosa*, PC: *P. crocea*, PW: *P. werneriifolia*, RO: *R. occidentalis*, SB: *S. bigelovii*, SI: *S. integerrimus*, SM: *S. multiradiata*, and WA: *W. amplexicaulis*.

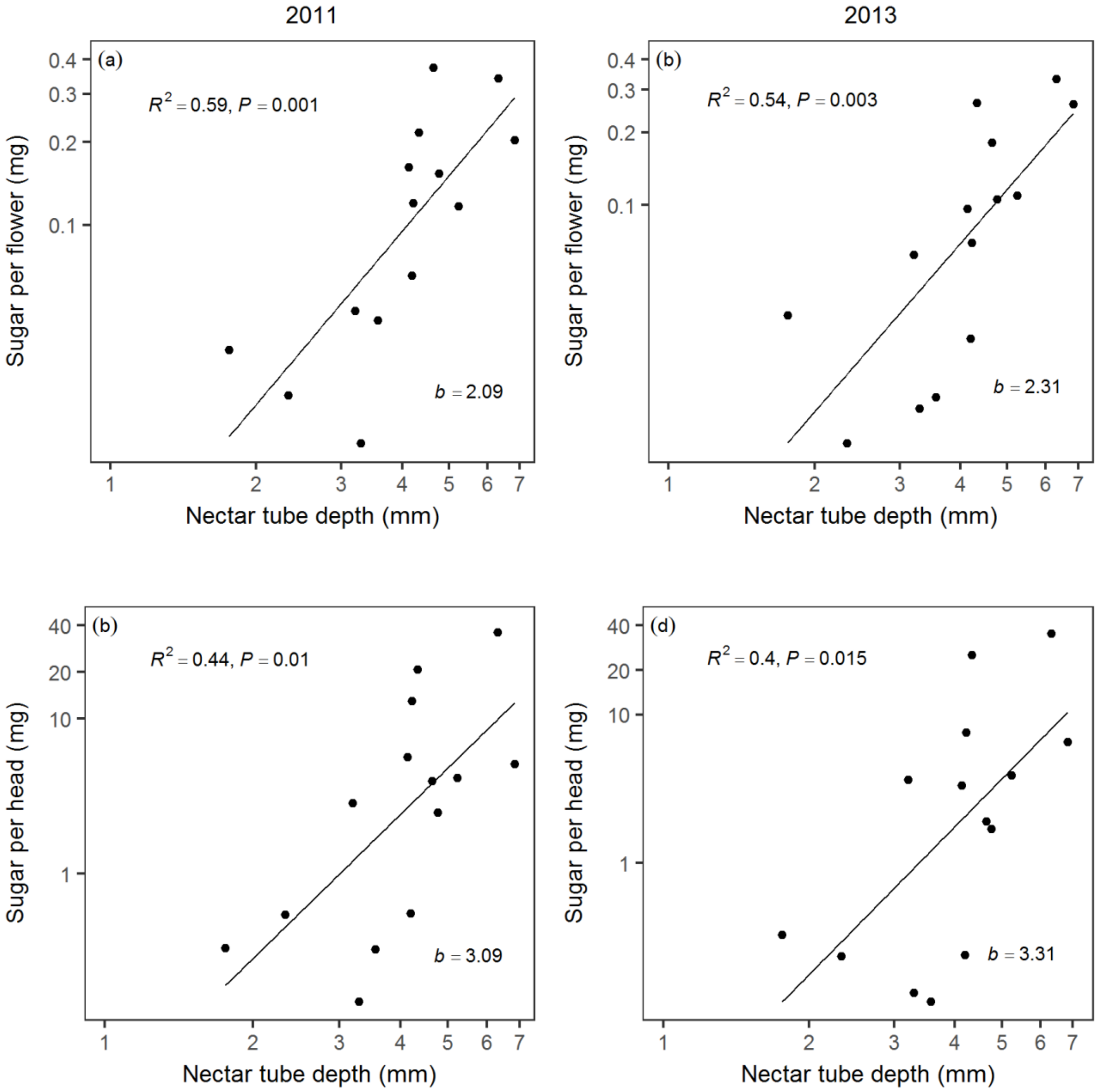

Fig. 3. Average sugar content after 24-hour bagging per single flower in 2011(a) and 2013 (b) and per flower head in 2011 (c) and 2013 (d) in relation to the nectar tube depth. All variables were $\log_{10}$ transformed prior to analyses. Each data point corresponds to a single plant species (n=14).

Nectar tube depth, nectar production and number of open flowers

Across species, there was a large variation in the number of open flowers per head, which ranged between 7 and 96 flowers. There was also a large variation in the number of heads per individual, which ranged from 2 to 37, and per square meter, which ranged from 2 to 64 (Table 1). Plant species with deeper flowers tended to have more flowers per head (r=0.36, P=0.20; Fig. 4). In contrast, plants with deeper flowers had less heads per individual (r=-0.64, P=0.01; Fig. 4) and less heads per square meter (r =-0.60, P=0.02; Fig. 4). Species with more open flowers per head produced more sugar per flower in 2011 (r=0.46, P=0.1) and in 2013 (r=0.58, P=0.03; Fig. 4). In contrast, species with more heads per individual produced less sugar per head in 2011 (r=-0.73, P=0.003) and in 2013 (r=-0.71, P=0.004; Fig. 4). Also species with more heads per square meter produced less sugar per head in 2011 (r=-0.87, P<0.001) and in 2013 (r=-0.81, P<0.001; Fig. 4).

Nectar standing crop and nectar production rate

Nectar standing crop was on average only 35% (nectar volume) and 28% (sugar content) of the 24-hour nectar production rate. Sugar content- and volume of the nectar standing crop were positively correlated with sugar content (ρ =0.79, P=0.03; Fig. 5a) and nectar volume (ρ =0.71, P=0.06; Fig. 5b) after 24-hours bagging. In contrast, sugar concentration measured after 24-hours bagging was not significantly correlated with the sugar concentration of the nectar standing crop (ρ=-0.18, P=0.67; Fig. 5c).

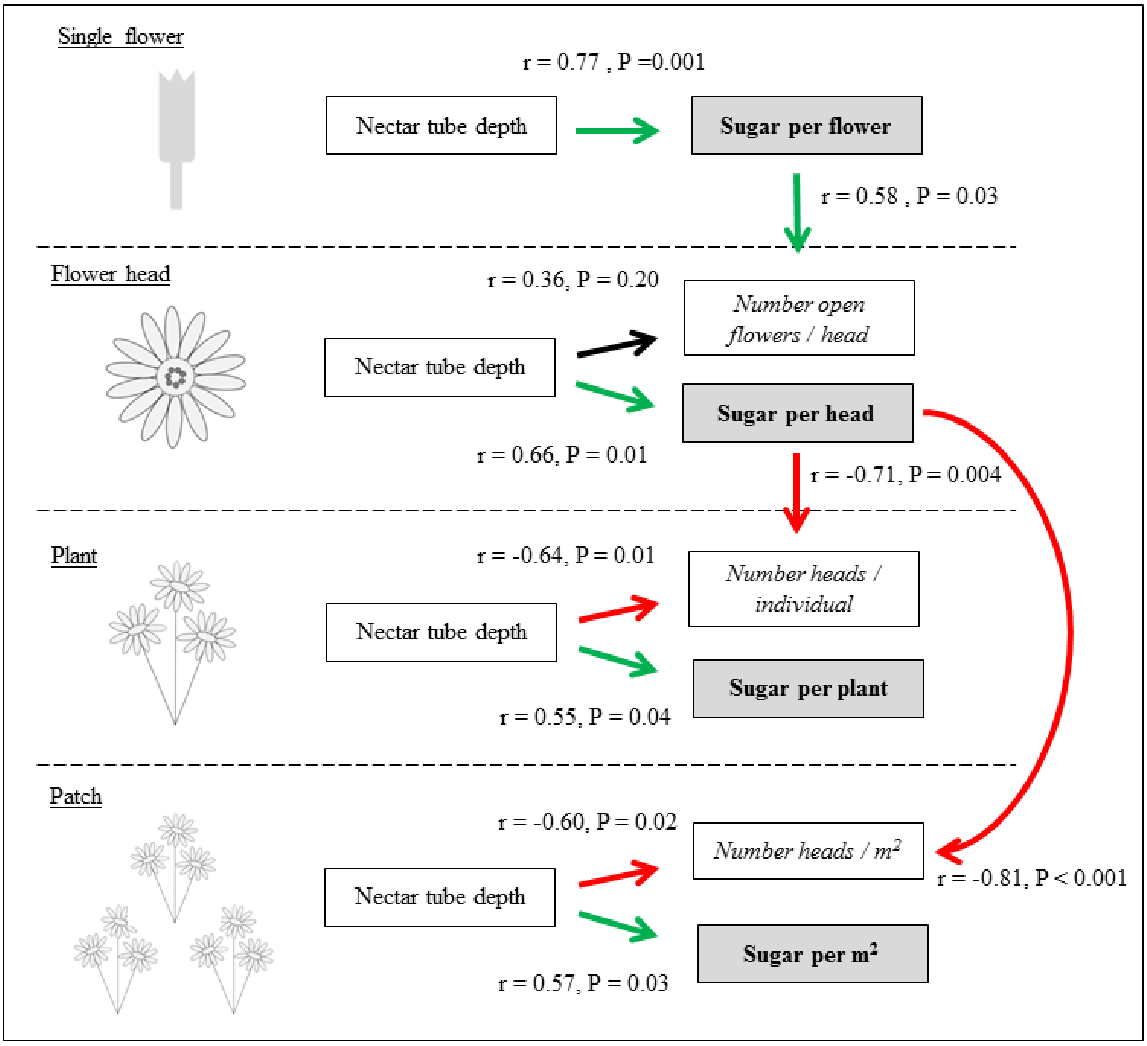

Fig. 4. Correlations among plant traits and sugar production in 2013. All variables, except nectar tube depth, are $\log_{10}$ transformed prior to analyses (n=14).

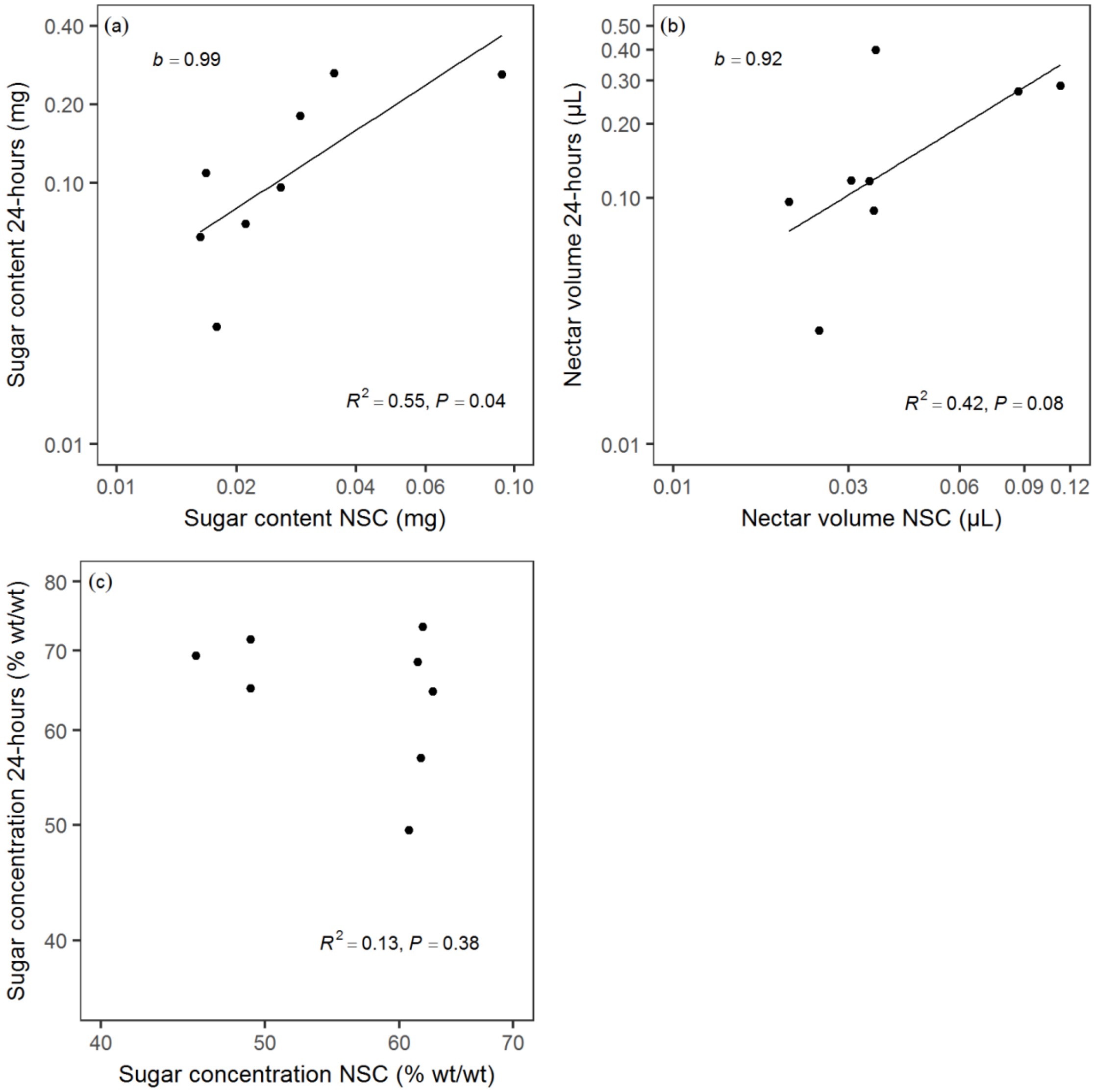

Fig. 5. Relationship between nectar standing crop and nectar production after 24-hours bagging. (a) Sugar content per flower, (b) nectar volume per flower and (c) sugar concentration per flower. All variables were $\log_{10}$ transformed prior to analyses. Each data point corresponds to a single plant species (n=8).

Discussion

Although previous studies have shown that deeper flowers produce more nectar (Galetto & Bernardello 2004; Kaczorowski, Gardener & Holtsford 2005; Petanidou 2007; Martins & Johnson 2013; Johnson *et al.* 2017), to our knowledge, this is the first study to show that also inflorescences of species with deeper flowers produce more nectar and that species with deeper flowers offer more nectar per individual and per patch. In comparison to other species, the Asteracease produced minute amounts of nectar which never filled the nectar tube for more than 11%. In this study, we payed specific attention to the scaling relationships, which have received little attention thus far. The amount of sugar they produced increased allometrically with the depth of their nectar tube, with a scaling factor of approximately two. This implies that nectar production does not increase proportionally with nectar tube depth. Rather flowers with deeper nectar tubes produce increasingly more nectar. These results suggests that even for generalized plant species, nectar production might be an adaptation to the energetic requirements of their flower-visitors. Flowers with deeper nectar tubes are expected to have a stronger incentive to produce more nectar as they have to compete stronger for their flower-visitors than flowers with shallow tubes (Ornelas *et al.* 2007; Martins & Johnson 2013). This is because deeper flowers can only be visited by flower-visitors with a longer proboscis which can potentially visit a wide variety of plant species (Borrell 2005). At the same time, shallow flowers might produce relatively low amounts of nectar to avoid attracting larger flower-visitors since they might be less effective flower-visitors (Willmer & Finlayson 2014). We are aware that nectar production of flowers might also depend on the volume of nectariferous tissue (Fahn, 1949; Dafni *et al.,* 1988; Petanidou 2000). However, analyses showed that nectar tube depth was better correlated with nectar sugar content compared to the width of the nectar tube, which is an indication of the size of the nectary (Petanidou, Goethals & Smets 2000). Consequently, the nectar production of plants might explain the high degree of morphological matching that occurs between nectar tube depth and the average proboscis length of their flower-visitors in plant-flower-visitor communities (Stang *et al*., 2009).

Flowers with deeper nectar tubes also tended to have more open flowers per head and consequently, not only single flowers but also flower heads of plant species with deeper flowers were more rewarding. Specifically, the sugar content per flower head increased with increasing nectar tube depth with a scaling factor of approximately three. We expect that the scaling factor of nectar tube depth with flower head is larger than that of single flowers to compensate for the lower flower head density of deeper flowers.

Harder and Cruzan (1990) have hypothesized that there is a trade-off between nectar tube depth, daily inflorescence size and nectar production, either because plants have limited resources or because coexisting plant species (which are potentially pollinated by the same flower-visitors) will become equally attractive to their flower-visitors over time. This is because without this equality one species would draw flower-visitors away from other species, which would result in reduced pollen movement for the latter species. Previous studies have indeed found a negative relationship between nectar tube depth and daily inflorescence size among species (Harder & Cruzan 1990; Worley *et al.* 2000; Sargent *et al.*

2007; Kettle *et al.* 2011), however, the relationship between nectar production and daily inflorescence size has not elaborately been studied (Harder & Cruzan 1990). We did not find a negative relationship between the number of open flowers per head, and either nectar tube depth and nectar production per flower. However, we did find a negative relationship between the number of flower heads and both nectar tube depth and nectar production per flower head. Moreover, plants with deeper flowers and more rewarding flowers heads also had less heads per square meter. Nevertheless, despite these negative relationships between sugar production per flower head and both number of flower heads per individual and patch, species with deeper flowers offered more nectar per individual and patch. We therefore emphasize that a negative correlation among these plant traits does not necessarily leads to equal attractiveness among plants.

The interspecific variation in nectar production has been interpreted in terms of adaptations of plants to their flower-visitors (Kaczorowski, Gardener & Holtsford 2005; Ornelas *et al.* 2007; Martins & Johnson 2013; Johnson *et al.* 2017). For flower-visitors to be able to exert selection pressure on a plant’s nectar production, nectar production rate needs to be (i) at least in part, genetically determined and (ii) positively correlated with nectar standing crop, since this is what flower-visitors encounter in the field. Although studies have proven that there is a genetic basis for nectar production rate (Leiss, Vrieling & Klinkhamer 2004; Mitchell 2004; Kaczorowski, Juenger & Holtsford 2008), previous studies have found only a weak or no correlation between nectar production rate and nectar standing crop (Zimmerman 1988). We have found not only a strong correlation between nectar production rate and nectar standing crop but our results also show that the interspecific scaling relationship between nectar tube depth and sugar content per flower was remarkably similar in both years. This indicates that the relative differences in nectar production rate, and thus the relative profitability of different plant species for flower-visitors, are constant across years. Overall, these results suggest that selection on nectar production among the Asteraceae by flower-visitors might be possible.

Overall, our results shed more light on the variation and co-variation of plant traits among plant species. We have found that flowers with deeper nectar tubes not only produce more nectar per flower but also offer more nectar at larger spatial scales and that these relationships are remarkably similar across years. Although these relationships are based on nectar production rate, nectar production rate is strongly correlated with nectar standing crop. Overall, these results provide indications that nectar production of plant species, even among generalized plants, might be under selection pressure by their flower-visitors.

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Handling time and flower choice of bees, butterflies and flies foraging on shallow flowers

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Abstract

The time it takes flower-visitors to handle a flower and extract nectar from it affects their visitation rate and foraging efficiency, and thus potentially flower-visitor flower choice and the structure of plant-flower-visitor networks. Flower-visitors with a long proboscis are assumed to handle shallow flowers less efficiently, as a long proboscis might be of hindrance when foraging on these flowers. Consequently, flower-visitors with a long proboscis may avoid shallow flowers. Here we examined whether handling time and flower choice of bumblebees, solitary bees, butterflies and flies, foraging on shallow flowers of the Asteraceae, is related to their proboscis length and both the nectar tube depth and nectar volume of the flowers they visit. We found that bees, butterflies and flies had intrinsically different handling times. Bees handled flowers faster than flies, while butterflies had longer handling times. Handling time actually decreased with increasing proboscis length, but only when flower-visitors visited flowers of which the nectar tube depth matched the length of their proboscis. Moreover, among bumblebees and solitary bees, handling time increased with increasing nectar tube depth and nectar volume. Among flies, handling time increased with increasing nectar tube depth only, while among butterflies, handling time increased with increasing nectar volume only. Further, bumblebee species with a longer proboscis tended to visit fewer plant species. Among solitary bees, butterflies and flies, the number of plant species they visited was not related to their proboscis length. Finally, bumblebees, solitary bees and flies with a longer proboscis visited on average flowers with deeper nectar tubes. Among butterflies, we did not find such a relationship. Overall our results show that a long proboscis is not of hindrance when foraging on shallow flowers of the Asteraceae. Nevertheless, bees and flies with a longer proboscis seem to prefer flowers with deeper nectar tubes. Therefore, handling efficiency cannot explain flower-visitor flower choice and thus neither plant-flower-visitor network structure.

Keywords: Asteraceae, bumblebees, butterflies, flies, proboscis length, nectar tube depth, nectar volume, solitary bees

Introduction

The time flower-visitors need to handle a flower and extract nectar from it (handling time) influences their visitation rate (Carothers 1982; Harder 1983; Herrera 1989) and flower-visitor foraging efficiency (Heinrich 1975; May 1988). Consequently, handling time may affect flower-visitor foraging behaviour and flower choice and thus plant-flower-visitor interaction patterns. Therefore, insight in how plant- and flower-visitor traits influence flower handling time may be important for understanding plant-flower-visitor network structure.

Floral traits, such as nectar tube depth, nectar volume and sugar concentration, are important determinants of handling time. Generally, it takes flower-visitors longer to handle flowers with deeper nectar tubes. This is either due to the increasing depth itself (Harder 1983; Harder 1986) or due to the underlying relationship between nectar tube depth and nectar reward (Harder 1983). Especially when the nectar tube is longer than the flower-visitor's proboscis, the efficiency of nectar extraction decreases rapidly (Hainsworth 1973; Inouye 1980; Harder 1983; Montgomerie 1984; Harder 1986; Temeles & Roberts 1993). Moreover, it takes flower-visitors longer to handle flowers that offer more voluminous or more concentrated nectar (Wolf, Stiles & Hainsworth 1972; Whitham 1977; Montgomerie 1984; Harder 1986; Hainsworth, Precup & Hamill 1991).

Handling time is also determined by flower-visitor proboscis length. In general, flower-visitors with a long proboscis handle flowers faster (Inouye 1980; Herrera 1989; Temeles & Roberts 1993; Graham & Jones 1996; Balfour, Garbuzov & Ratnieks 2013). However, when foraging on shallow flowers, it has been suggested that a long proboscis is of hindrance. Therefore, flower-visitors with a long proboscis may endure longer handling times than those with short proboscises when foraging on shallow flowers (Inouye 1980; Plowright & Plowright 1997; Kunte 2007; Karolyi *et al.* 2013; Bauder *et al.* 2015). Nevertheless, exact measurements of handling time rates, combined with quantitative morphological data of a variety of plant species with shallow-tubed flowers, to substantiate this suggestions are scarce.

Flower-visitor proboscis length does not only determine flower handling efficiency but also the ability of flower-visitors to extract nectar from flowers and consequently their flower choice and plant-flower-visitor interaction patterns. Potentially, flower-visitors with a longer proboscis can visit a wider diversity of plant species (Borrell 2005). However, longer-tongued flower-visitors often visit flowers with deeper nectar tubes (Barrow & Pickard 1984; Stang *et al.* 2009) and some bumblebee species with a long proboscis are less generalized (Goulson & Darvill 2004). These patterns consequently lead to size-matching between the proboscis length of insects and the nectar tube depth of the flowers they visit (Stang *et al.* 2009). A reduced handling efficiency when flower-visitors forage on flowers of which the nectar tube depth does not match the length of their proboscis might be another important underlying mechanism that determines this pattern of size-matching.

The main aim of this study is to investigate whether handling time is related to the proboscis length of flower-visitors and both nectar tube depth and nectar volume of the flowers they visit and how this affects flower-visitor flower choice, when flower-visitors forage of shallow flowers. To this end we analysed the handling time and flower choice of a wide diversity of insects, including bumblebees, solitary bee, butterflies and flies foraging on plant species of the Asteraceae. Although there have been numerous studies on the effects of plant and flower-visitor traits on flower choice and foraging efficiency of flower-visitors, literature is dominated by studies on bumblebees, in comparison to other flower-visitors such as solitary bees (Borrell 2005), butterflies (Tudor *et al.* 2004; Kunte 2007; Stefanescu & Traveset 2009; Bauder *et al.* 2015) and flies (Gilbert 1981; Karolyi *et al.* 2013). Nevertheless, for generalized plant species, these flower-visitor taxa can constitute the main flower-visitor fauna (Arroyo, Primack & Armesto 1982; Kearns 1992; Weiss 2001; Johnson 2010). We asked the following questions: (1) Do bees, butterflies and flies differ in handling time and is their handling time related to their proboscis length and both nectar tube depth and nectar volume of the flowers they visit? And (2) do bees, butterflies and flies with a longer proboscis visit more plant species and/or plant species with flowers with deeper nectar tubes?

Materials and methods

Study system and handling time

Data were collected in the Colorado Rocky Mountains, USA, in the vicinity of the Rocky Mountain Biological Laboratory in June-August 2011. We included fifteen co-flowering Asteraceae species belonging to the subfamilies Carduoideae and Asteroideae: two species of *Cirsium*, *Arnica parryi*, *Erigeron speciosus*, *Helianthella quinquenervis*, *Heliomeris multiflora*, *Heterotheca villosa*, *Hymenoxys hoopesii*, *Packera werneriifolia*, *Pyrrocoma crocea*, *Rudbeckia occidentalis*, *Senecio bigelovii*, *S. integerrimus, Solidago multiradiata* and *Wyethia amplexicaulis*.

Flower-visitor visits were observed in five different meadows around the Rocky Mountain Biological Laboratory, which were approximately 200 meter apart. These meadows were at approximate 3,000 m elevation, with slopes between 0° and 34°, exposed mostly to the south. Flower-visitors could move freely between these meadows.

Flower visits of natural foraging bumblebees, solitary bees, butterflies and flies were recorded with a digital video camera. Only nectar-foraging insects were included in the analysie. Video recordings were analysed frame by frame (50 frames/s) with the software Adobe Premiere Pro CC 2014. We measured the handling time, as the time that an insect has its proboscis in the flower. Therefore, our measurement of handling time includes the time an insect needs to enter a flower with its proboscis and extract the nectar. In total, we recorded the handling time of 309 individuals representing 40 (morpho)species (186 bumblebees belonging to 8 species, 32 flies belonging to 11 species, 49 butterflies belonging to 13 species, 42 solitary bees belonging to 8 species). Insects were identified based on these video recordings, up to species or morphospecies level.

Nectar volume

We measured the nectar volume of disk flowers which were bagged for 24-hours. Ray flowers are often sterile and do not produce nectar (Mani & Saravanan 1999). Flowers were bagged between 8 and 9 AM and collected 24-hours later. We used bags made of fine gauze that do not change the temperature within the bag and thus will not influence the nectar solute concentration (Corbet 2003). Nectar volume was measured in the lab under a dissecting microscope, mostly within an hour after collection. On average, 43 disk flowers were sampled for every plant species. Nectar was extracted with microcapillary tubes, ranging in size between 0.2, 0.25, 0.5 and 1.0 µL. Capillary size depended on the quantity of the nectar produced by the flowers and the nectar tube width. With the microcapillary tubes we probed the base of the nectar tube until no more nectar could be removed.

Nectar tube depth

The corolla tube of disk flowers of most Asteraceae consists of two parts: a (very) narrow lower part and an upper wider bell-shaped part. The wider part roughly begins where the stamens insert and ends at the beginning of the corolla lobes, at the base of the deepest cleft in the corolla. Nectar is produced at the base of the narrow part of the corolla tube and migrates up this part into the wider upper part of the this tube. As the lower part of the corolla tube of these species is almost filled by the style, it only contains traces of nectar and it is unlikely that flower-visitors can access this part of the corolla tube (Graenicher 1909). Therefore, for each plant species, we measured the depth of the upper wider part of the corolla tube, hereafter referred to as nectar tube. We measured the nectar tube depth for at least twenty freshly picked flowers, to the nearest 0.01 mm, under a dissecting microscope.

Proboscis length

Proboscis length measurements of solitary bees, butterflies and flies were based on specimens from a reference collection. These specimens in this reference collection were collected in 2009 and 2010, at the same study site as where our study was conducted. The proboscis lengths of these specimens were measured according to the methods described by (Stang, Klinkhamer & van der Meijden 2006). Data on the average proboscis lengths for bumblebees were obtained from (Macior 1974) (for queens and workers) and from (Inouye 1976) (for males).

Statistical analyses

Statistical analyses were performed using R version 3.3.2 (R Development Core Team 2014). Prior to all analyses, we tested whether the variables were normally distributed, using Shapiro-Wilk test. To achieve normality, handling time was $\log_{10}$ transformed prior to all analyses. We tested whether the handling time of the flower-visitor taxa differed with analysis of covariance (ANCOVA), including flower-visitor taxon (bees, butterflies and flies), proboscis length, nectar tube depth and nectar volume as independent variables. Ordinary least square (OLS) linear regression analyses were used to analyse the relationships between handling time and proboscis length. We did this for each flower-visitor taxon (bumblebees, solitary bees, butterflies and flies) separately. For these analyses, we divided the plants into different classes, based on the nectar tube depth (1-3 mm, 3-5 mm and 5-7 mm). OLS linear regression analyses were also used to investigate the relationships between handling time and both nectar tube depth and nectar volume. We did this for each flower-visitor taxon (bumblebees, solitary bees, butterflies and flies) separately as well. For these analyses, we divided the flower-visitors in different classes, based on their proboscis length (1-3 mm, 3-5 mm, 5-7 mm, 7-9 mm and >9 mm). We did not perform multiple regression analyses because nectar tube depth and nectar volume where highly correlated. To analyse the relationship between the proboscis length of bumblebees, solitary bees, butterflies and flies and the average nectar tube depth of flowers they visited, also OLS linear regression analyses were used. Further, to test whether there was a correlation between proboscis length of bumblebees, solitary bees, butterflies and flies and the number of plant species they visited correlation analyses, either Pearson or Spearman, were used, depending on whether the number of plant species visited was normally distributed.

Results

Handling time

The average handling time of bees ranged from 0.33s to 1.13 s. For flies, the average handling time range from 0.73s to 1.28s and for butterflies the average handling time ranged from 0.7s to 3.41s. Bees had a significantly shorter handling time than flies while butterflies had a significantly longer handling time than flies (Table 1).

Interestingly, proboscis length only affected handling time when the length of the proboscis closely matched the depth of the nectar tube. Among solitary bees, handling time decreased with increasing proboscis length, but only when species visited flowers of which the tube depth closely matched the length of their proboscis (Fig. 1). Among the other flower-visitor taxa, handling time was not related to proboscis length. These other flower-visitor taxa did not visit flowers of which the nectar tube depth closely matched the length of their proboscis.

The handling time of solitary bees and bumblebees increased with both increasing nectar tube depth (Fig. 2) and nectar volume (Fig. 3). However, among solitary bees, this relationship was not significant for species with a short proboscis (<5 mm). When foraging on these flowers, the length of their proboscis closely matched the nectar tube depth. Among flies, the handling time increased with increasing nectar tube depth only (Figs 2,3), while among butterflies, the handling time increased with increasing nectar volume only (Figs 2,3).

Table 1. ANCOVA analyses (method backward selection of variables) with handling time as the dependent variable and insect group (flies, bees and butterflies) as a fixed factor and nectar tube depth, proboscis length and sugar concentration as independent variables. Handling time, nectar tube depth, proboscis length and sugar concentration were ln transformed. The explained variance of the presented model is 53.4% ($R^2 = 0.534$, $F = 19.13$, $df = 100$, $P < 0.001$)

	Estimate	**s.e.**	**t**	**P**
Intercept (Flies)	-7.244	2.171	-3.337	0.001
Bees	-0.492	0.176	-2.797	0.006
Butterflies	0.966	0.213	4.541	<0.001
Nectar tube depth	6.343	1.605	3.951	<0.001
Proboscis length	-0.491	0.174	-2.822	0.006
Sugar concentration	1.904	0.583	3.265	0.002
Nectar tube depth : sugar concentration	-1.526	0.424	-3.602	<0.001

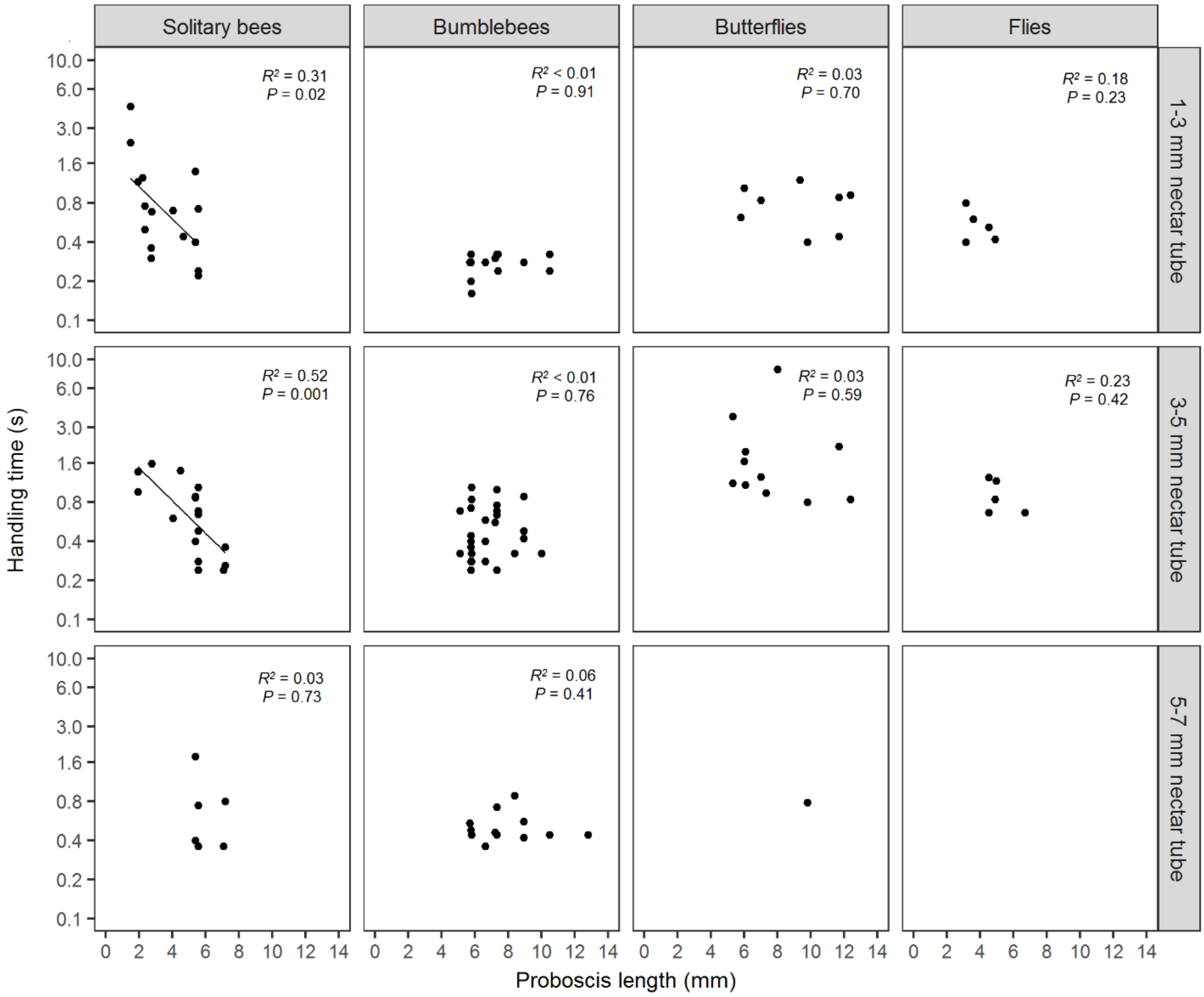

Fig. 1. Handling time in relation to the proboscis length, for solitary bees, bumblebees, butterflies and flies. Each data point corresponds to a single insect species visiting a single plant species and is shown as the median handling time. Observation are divided into different groups, according to the depth of the nectar tube of the plant species (1-3 mm, 4-5 mm and 5-7 mm).

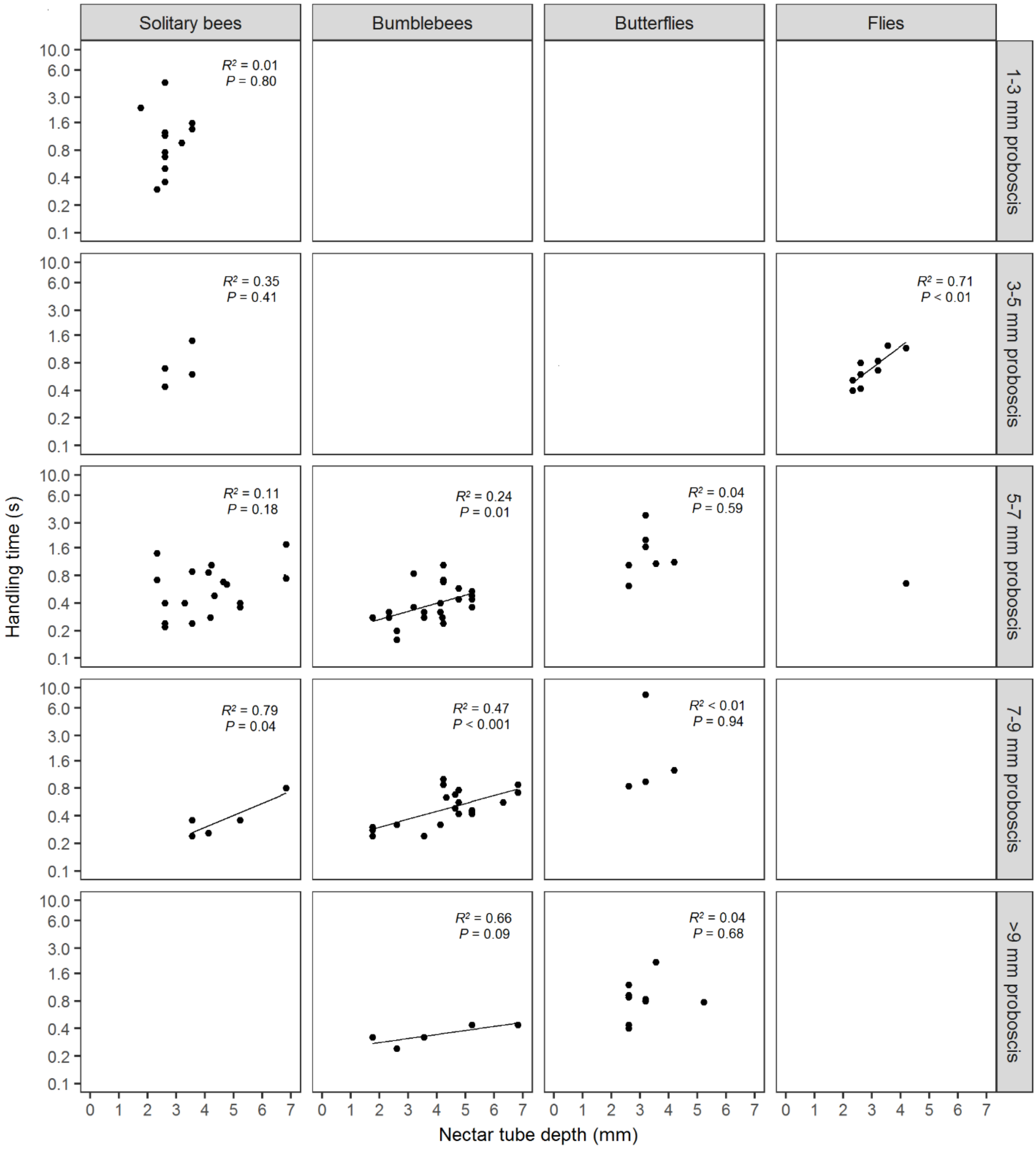

Fig. 2. Handling time in relation to nectar tube depth, for solitary bees, bumblebees, butterflies and flies. Each data point corresponds to a single insect species visiting a single plant species and is shown as the median handling time. Observations are divided into different groups, according to the proboscis length of the insects (1-3 mm, 3-5mm, 5-7mm and >9mm).

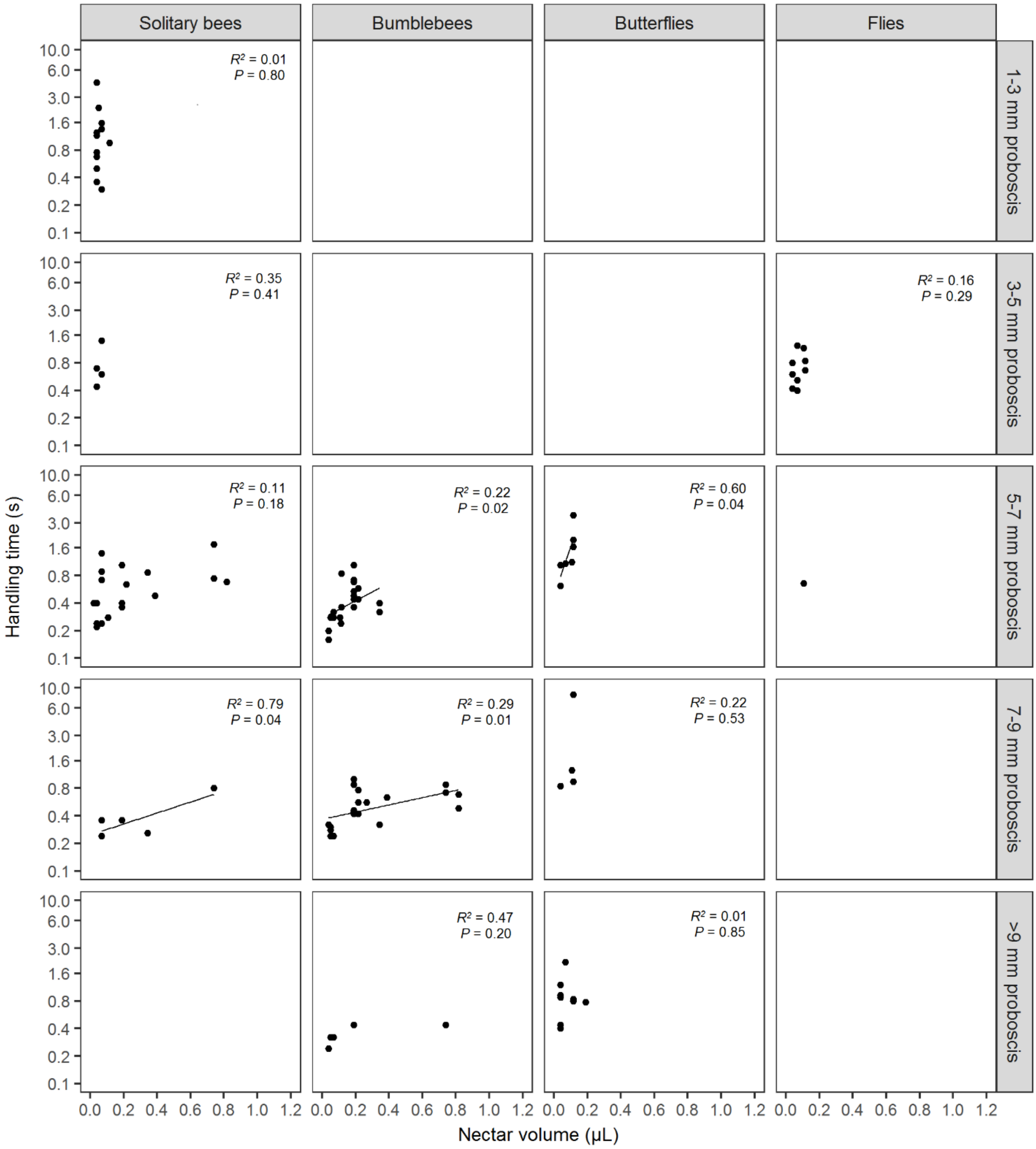

Fig. 3. Handling time in relation to the nectar production (nectar volume) of the plant species, for solitary bees, bumblebees, butterflies and flies. Each data point corresponds to a single insect species visiting a single plant species and is shown as the median handling time. Observations are divided into different groups, according to the proboscis length of the insect species (1-3 mm, 3-5mm, 5-7mm and >9mm).

Flower choice

The number of plant species that insects visited ranged between 1 and 11 plants. Bumblebees had the most diverse nectar diet, as these species visited on average six different plant species. Solitary bees, flies and butterflies were more selective, as these species visited on average only 2 or 3 different plant species. The number of plant species which solitary bees, butterflies and flies visited was not related to the length of their proboscis (Fig.4a,c,d). Bumblebee species with a longer proboscis tended to visit fewer plant species, although this was only marginally significant (Fig. 4b).

Solitary bee- and fly species with a longer proboscis visited on average flowers with deeper nectar tubes. The proboscis of these species was shorter than the nectar tube depth of the flowers of most plant species (Fig. 5a, d). Surprisingly, also bumblebee species with a longer proboscis visited on average plant species with flowers with deeper nectar tubes, although most bumblebee species had a proboscis long enough to visit all plant species (Fig. 5b). In contrast, butterfly species with a longer proboscis did not visit on average plant species with flowers with deeper nectar tubes (Fig. 5c). Most butterfly species too had a proboscis long enough to visit all plant species.

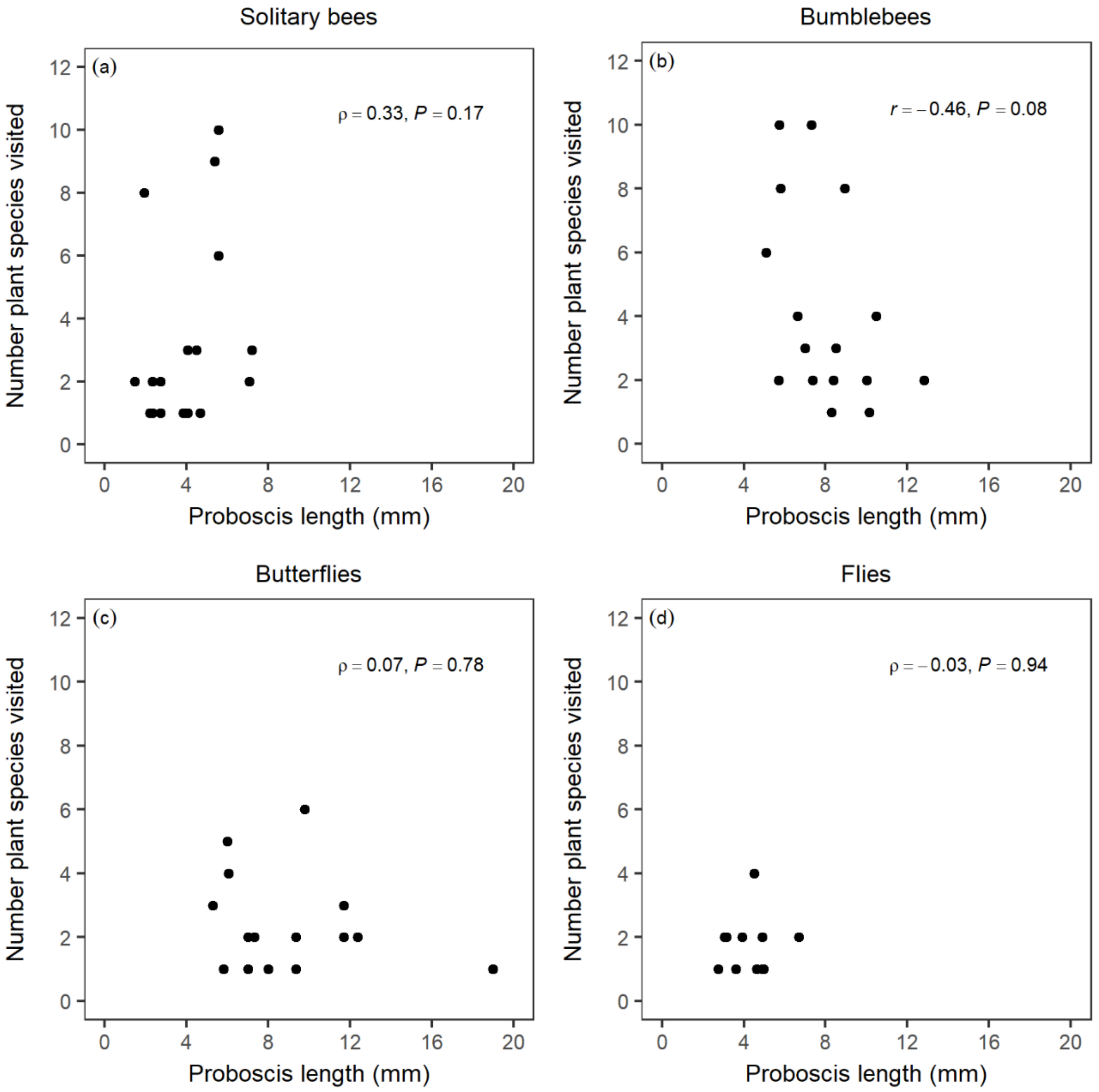

Fig. 4. The number of plant species that (a) solitary bees, (b) bumblebees, (c) butterflies and (d) flies visited in relation to their proboscis length. Each dot represent a flower-visitor species.

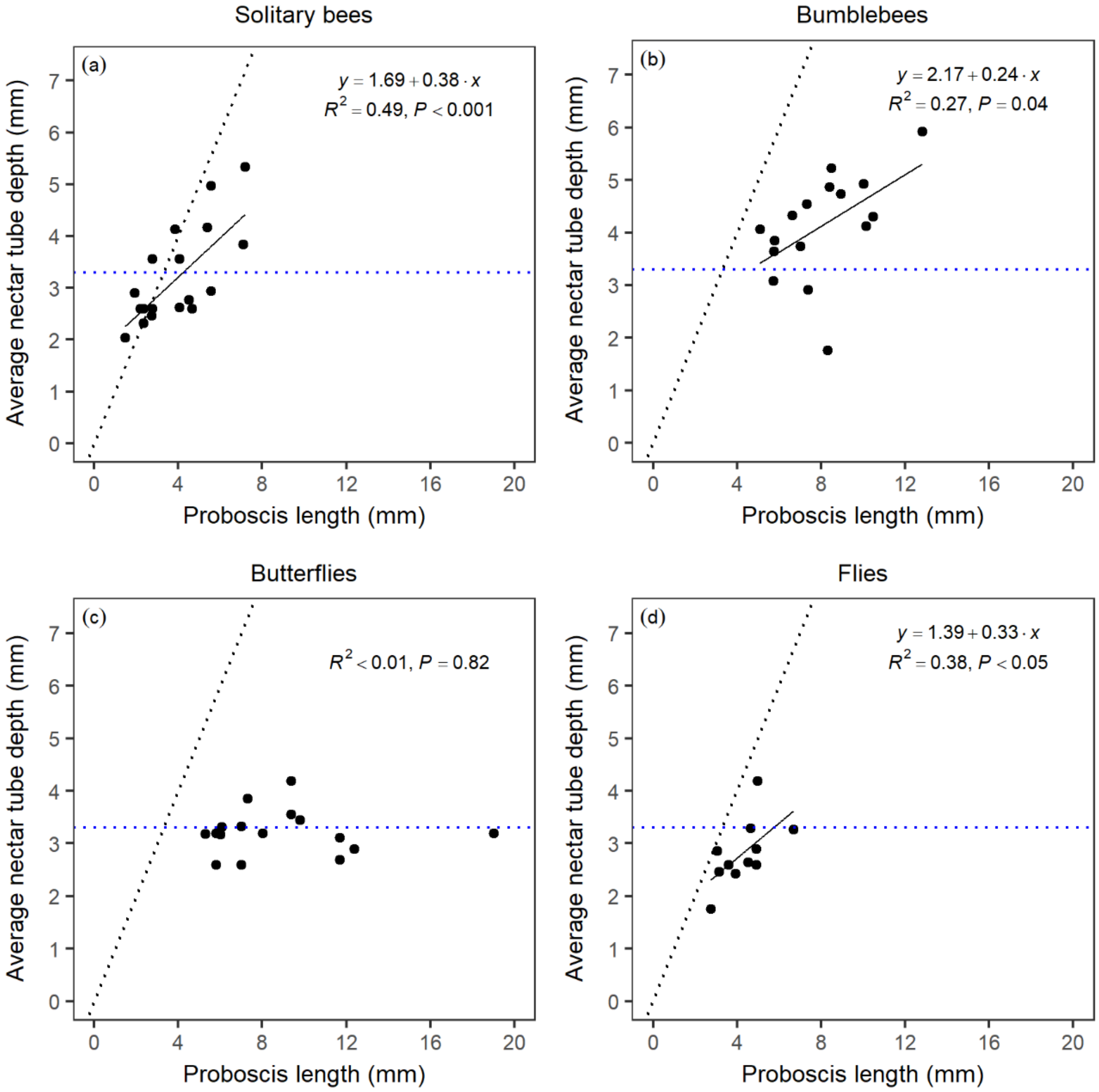

Fig. 5. Average nectar tube depth of the flowers that (a) solitary bees, (b) bumblebees, (c) butterflies and (d) flies visited in relation to their proboscis length. Each dot represent a flower-visitor species. The dotted black line (x=y) represents the expected size threshold and the dotted blue line presents the expected average nectar tube depth, based on random encounters.

Discussion

Although it has been argued that a long proboscis might be of hindrance when foraging on shallow flowers (Inouye 1980; Plowright & Plowright 1997; Stout 2000; Peat, Tucker & Goulson 2005), our results show that insects with a longer proboscis do not experience longer handling times when foraging on relatively shallow flowers of the Asteraceae, compared to insects with a short proboscis. When insect visited flowers of which the nectar tube depth closely matched the length of their proboscis, their handling time even decreased with increasing proboscis length. These were solitary bees. However, when insects visited flowers which were (much) more shallow than their proboscis, their handling time was unaffected by their proboscis length. These results might be explained by the flower morphology of the Asteraceae. The Asteraceae have relatively simple, open flowers which are aggregated in disk shaped inflorescences. This aggregation might provide a large alignment place, in contrast to single flowers, which might make it easier for insects with a long proboscis to unfold their proboscis when visiting these flowers. Moreover, when foraging on flowers of the Asteraceae, insects do not need to open or crawl into the flowers. We expect a long proboscis to be more of hindrance when insects have to open and crawl into complex flowers.

Although previous studies have examined the handling time of bumblebees (Inouye 1980; Herrera 1989), butterflies (Herrera 1989; Kunte 2007; Martins & Johnson 2013; Bauder *et al.* 2015) and flies (Gilbert 1981), this study provides the unique opportunity to compare handling time and flower choice of these flower-visitor taxa foraging on a diversity of flowers (but see Herrera 1989 for comparison of hymenopterans and lepidopterans). Our results show that bees, butterflies and flies have intrinsic different handling times. Bees handled flowers faster than flies, while butterflies had longer handling times, independently of any insect or floral traits. Moreover, whether nectar tube depth and nectar volume affected handling time differed among these flower-visitor taxa. For flies, handling time was determined by nectar tube depth only, while handling time of butterflies was determined by nectar volume only. In contrast, handling time of bees (both bumblebees and solitary bees) was related to both nectar tube depth and nectar volume. Interestingly, nectar tube depth did not affect the handling time among solitary bee species with a relatively short proboscis (<5 mm). These species visited flowers of which the nectar tube depth closely matched their proboscis length. Apparently, when insects forage on plant species of which the nectar tube closely matches the length of their proboscis, and thus can barely reach the nectar, proboscis length might be a more important determinant of handling time than floral traits. Also experimental studies have shown that when an insect’s proboscis is shorter than the nectar tube of flowers it forages on, insects forage less efficiently, in comparison to when they forage on flowers where there proboscis length exceeds the nectar tube depth (Harder 1983). In contrast, when foraging on relatively shallow flowers, and thus when nectar is easily accessible, floral traits, such as nectar tube depth and volume, are more important.

Both the intrinsic differences in handling time of bees, flies and butterflies, and the different effects of floral traits on handling time among these flower-visitor taxa may be due to different nectar extraction technique and/or body mass. Bees drink nectar by lapping with their glossae (Harder 1982), while butterflies and flies actively suck up the nectar (Kingsolver

& Daniel 1979; Gilbert 1981). Some flies even spit fluid on to the nectary, most likely to produce a medium in which the nectar sugar can dissolve (Gilbert 1981). Especially when confronted with the nectar environment of Asteraceae flowers (minute amounts and concentrated nectar), lapping proboscides are able to extract nectar at a much faster rate than sucking ones of similar length (Herrera 1989). Further, butterflies are expected to have a more narrow proboscis compared to flies which makes sucking up nectar more difficult, hence the different handling times among butterflies and flies. Moreover, body mass has a positive effect on the lapping or suction rate (Harder 1986). In general (bumble)bees have a greater body mass compared to butterflies and flies and consequently, these differences in body mass may also explain why bees handle flowers faster.

Most insect species visited only a small proportion of the plant species they could potentially visit. Bumblebees were more generalized, compared to the other flower-visitor taxa, as they visited on average eight of the fifteen plant species, while solitary bees, butterflies and flies visited on average only two or three plant species. This might not necessarily imply that solitary bees, butterflies and flies are more selective. These results might also be explained by the differences in abundances of the species (Goulson & Darvill 2004), as bumblebee species were far more abundant, compared to the other flower-visitor taxa (S.G.T. Klumpers, unpublished data). Moreover, insect species with a longer proboscis did not visit more plant species, which is in accordance with previous studies on butterflies (Tudor *et al.* 2004; Stefanescu & Traveset 2009). Bumblebee species with a longer proboscis even tended to visit fewer plant species. The higher energetic requirements of species with a longer proboscis (Heinrich 1983) might make them more selective (Goulson & Darvill 2004). As these species have higher energetic requirements, they should restrict their diet to more rewarding flowers. Also, partitioning of floral resources among insect species to avoid interspecific resource competition may make insects more selective in general (Pyke 1982; Stout, Allen & Goulson 1998; Brosi & Briggs 2013).

Solitary bee-, bumblebee- and fly species with a longer proboscis visited on average flowers with deeper nectar tubes, which is in accordance with previous studies (Brian 1957; Morse 1978; Gilbert 1981; Harder 1985; Shmida & Dukas 1990; Plowright & Plowright 1997; Borrell 2005; Stang *et al.* 2009; Johnson 2010). Among butterflies, we did not find this relationship. In contrast to these previous studies, the Asteraceae have relatively shallow flowers. Consequently, most insect species, especially bumblebees and butterflies, had a proboscis long enough to potentially visit all Asteraceae species. Therefore, the positive relationship between proboscis length and nectar tube depth cannot fully be explained by possible size-constraints that the nectar tube depth poses on the proboscis length of their flower-visitors. Neither can flower-visitor flower choice and this pattern of size-matching be explained by their handling efficiency. Bee- and fly species with a longer proboscis visited flowers with deeper nectar tubes although their handling time actually increased with increasing nectar tube depth. Thus, apparently bee- and fly species prefer to visit those flowers on which they experienced longer handling time, rather than those flowers which they handle the quickest. Patterns of size-matching between the proboscis length of flower-visitors and the nectar tube depth of the flowers they visit, which occurs in plant-flower-visitor communities (Stang *et al.* 2009), might rather be explained by other aspects of foraging efficiency, such as nectar production.

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Appendix 1

Table S1. Proboscis length and handling time of the flower-visitors and nectar tube depth and nectar volume of the plants. The table gives mean ± s.e.m. and sample size (n). Plant species are named according to the accepted names in The Plant List.

Insect species	Proboscis length (mm)	Plant species	Handling time (s)	Nectar tube depth (mm)	Nectar volume (µL)
Bumblebees					
Bombus appositus worker	10.48 ± 0.13 (50)	*Erigeron speciosus*	0.27 ± 0.05 (8)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Pyrrocoma crocea	0.45 ± 0.02 (56)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Solidago multiradiata	0.52 ± 0.10 (20)	1.76 ± 0.08 (20)	0.05 ± 0.006 (68)
Bombus appositus queen	12.81 ± 0.05 (50)	*Wyethia amplexicaulis*	0.50 ± 0.05 (28)	6.83 ± 0.07 (40)	0.74 ± 0.060 (75)
Bombus bifarius male	6.63 ± 0.18 (7)	*Helianthella quinquenervis*	0.41 ± 0.02 (92)	4.13 ± 0.05 (32)	0.34 ± 0.016 (189)
		Heterotheca villosa	0.32 ± 0.02 (54)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Pyrrocoma crocea	0.36 ± 0.02 (23)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Senecio bigelovii	0.60 ± 0.04 (64)	4.76 ± 0.06 (24)	0.22 ± 0.021 (39)
		Heliomeris multiflora	0.27 ± 0.01 (22)	2.33 ± 0.03 (22)	0.07 ± 0.021 (12)
Bombus bifarius worker	5.75 ± 0.05 (50)	*Erigeron speciosus*	0.24 ± 0.01 (260)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Helianthella quinquenervis	0.41 ± 0.02 (163)	4.13 ± 0.05 (32)	0.34 ± 0.016 (189)
		Heterotheca villosa	0.28 ± 0.02 (39)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Hymenoxys hoopesii	0.42 ± 0.02 (196)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
		Pyrrocoma crocea	0.55 ± 0.03 (153)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Rudbeckia occidentalis	0.79 ± 0.08 (29)	4.22 ± 0.06 (43)	0.19 ± 0.016 (106)
		Senecio bigelovii	0.44 ± 0.03 (25)	4.76 ± 0.06 (24)	0.22 ± 0.021 (39)
		Senecio integerrimus	0.85 ± 0.58 (19)	4.19 ± 0.08 (36)	0.11 ± 0.010 (34)
		Solidago multiradiata	0.78 ± 0.26 (95)	1.76 ± 0.08 (20)	0.05 ± 0.006 (68)
		Heliomeris multiflora	0.63 ± 0.33 (28)	2.33 ± 0.03 (22)	0.07 ± 0.021 (12)
Bombus bifarius queen	8.38 ± 0.06 (50)	*Helianthella quinquenervis*	0.30 ± 0.02 (13)	4.13 ± 0.05 (32)	0.34 ± 0.016 (189)
		Wyethia amplexicaulis	1.67 ± 0.47 (9)	6.83 ± 0.07 (40)	0.74 ± 0.060 (75)

Insect species	**Proboscis length (mm)**	**Plant species**	**Handling time (s)**	**Nectar tube depth (mm)**	**Nectar volume (µL)**
Bumblebees					
Bombus californicus worker	10.01 ± 0.13 (32)	*Heterotheca villosa*	0.38 ± 0.04 (11)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
Bombus flavifrons male	8.95 ± 0.18 (6)	*Arnica parryii*	0.60 ± 0.23 (3)	4.64 ± 0.05 (40)	0.82 ± 0.079 (54)
		Cirsium sp.1	0.58 ± 0.02 (82)	6.31 ± 0.16 (36)	0.27 ± 0.024 (44)
		Pyrrocoma crocea	0.49 ± 0.04 (12)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Rudbeckia occidentalis	0.99 ± 0.06 (53)	4.22 ± 0.06 (43)	0.19 ± 0.016 (106)
		Senecio bigelovii	0.45 ± 0.02 (74)	4.76 ± 0.06 (24)	0.22 ± 0.021 (39)
		Solidago multiradiata	0.29 ± 0.02 (16)	1.76 ± 0.08 (20)	0.05 ± 0.006 (68)
Bombus flavifrons worker	7.31 ± 0.11 (50)	*Arnica parryii*	0.99 ± 0.11 (43)	4.64 ± 0.05 (40)	0.82 ± 0.079 (54)
		Cirsium sp.2	0.68 ± 0.04 (74)	4.33 ± 0.07 (39)	0.39 ± 0.039 (40)
		Erigeron speciosus	0.33 ± 0.03 (19)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Heterotheca villosa	0.25 ± 0.01 (28)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Pyrrocoma crocea	0.45 ± 0.04 (14)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Rudbeckia occidentalis	0.98 ± 0.06 (42)	4.22 ± 0.06 (43)	0.19 ± 0.016 (106)
		Senecio bigelovii	0.90 ± 0.10 (105)	4.76 ± 0.06 (24)	0.22 ± 0.021 (39)
		Wyethia amplexicaulis	1.16 ± 0.34 (18)	6.83 ± 0.07 (40)	0.74 ± 0.060 (75)
Bombus mixtus male	7.21 ± 0.06 (8)	*Pyrrocoma crocea*	0.51 ± 0.03 (58)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Senecio bigelovii	0.50 ± 0.05 (6)	4.76 ± 0.06 (24)	0.22 ± 0.021 (39)
		Solidago multiradiata	0.32 ± 0.03 (12)	1.76 ± 0.08 (20)	0.05 ± 0.006 (68)
Bombus mixtus worker	5.09 ± 0.05 (50)	*Heterotheca villosa*	0.31 ± 0.01 (4)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Rudbeckia occidentalis	0.70 ± 0.04 (4)	4.22 ± 0.06 (43)	0.19 ± 0.016 (106)
Bombus occidentalis worker	5.71 ± 0.04 (50)	*Pyrrocoma crocea*	0.52 ± 0.05 (11)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Solidago multiradiata	0.27 ± 0.02 (25)	1.76 ± 0.08 (20)	0.05 ± 0.006 (68)
Bombus sylvicola male	7.38 ± 0.12 (3)	*Erigeron speciosus*	0.31 ± 0.01 (7)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Solidago multiradiata	0.26 ± 0.01 (28)	1.76 ± 0.08 (20)	0.05 ± 0.006 (68)

Insect species	Proboscis length (mm)	Plant species	Handling time (s)	Nectar tube depth (mm)	Nectar volume (µL)
Solitary bees					
Bombus sylvicola worker	5.79 ± 0.08 (50)	*Erigeron speciosus*	0.16 ± 0.01 (13)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Helianthella quinquenervis	0.38 ± 0.03 (75)	4.13 ± 0.05 (32)	0.34 ± 0.016 (189)
		Hymenoxys hoopesii	0.95 ± 0.11 (16)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
		Pyrrocoma crocea	0.46 ± 0.03 (17)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Rudbeckia occidentalis	1.05 ± 0.13 (6)	4.22 ± 0.06 (43)	0.19 ± 0.016 (106)
		Senecio integerrimus	0.35 ± 0.04 (42)	4.19 ± 0.08 (36)	0.11 ± 0.010 (34)
Psithyrus	-	*Helianthella quinquenervis*	0.38 ± 0.02 (22)	4.13 ± 0.05 (32)	0.34 ± 0.016 (189)
		Pyrrocoma crocea	0.39 ± 0.03 (35)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Wyethia amplexicaulis	1.08 ± 0.12 (58)	6.83 ± 0.07 (40)	0.74 ± 0.060 (75)
Ammophilia sp.1	2.75 (1)	*Erigeron speciosus*	0.72 ± 0.07 (35)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Ammophilia sp.2	2.75 (1)	*Heterotheca villosa*	1.67 ± 0.35 (6)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
Anthidium sp.1	4.67 (1)	*Erigeron speciosus*	0.96 ± 0.20 (19)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Bee sp.1	2.73 (1)	*Erigeron speciosus*	0.40 ± 0.07 (13)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Heliomeris multiflora	0.29 ± 0.02 (4)	2.33 ± 0.03 (22)	0.07 ± 0.021 (12)
Bee sp.2	2.34 ± 0.10 (18)	*Erigeron speciosus*	0.76 ± 0.06 (15)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Bee sp.3	2.34 ± 0.10 (18)	*Erigeron speciosus*	0.66 ± 0.10 (16)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Bee sp.4	1.92 ± 0.08 (46)	*Erigeron speciosus*	1.47 ± 0.11 (57)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Heterotheca villosa	1.97 ± 0.29 (25)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Hymenoxys hoopesii	1.11 ± 0.39 (5)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
Bembex rostrata gorytes	7.08 ± 0.63 (3)	*Heterotheca villosa*	0.37 ± 0.04 (48)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Pyrrocoma crocea	0.49 ± 0.15 (3)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
Coelioxys	4.06 ± 0.22 (2)	*Heterotheca villosa*	0.69 ± 0.11 (11)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)

Insect species	**Proboscis length (mm)**	**Plant species**	**Handling time (s)**	**Nectar tube depth (mm)**	**Nectar volume (µL)**
Solitary bees					
Hylaeus	1.48 ± 0.06 (33)	*Erigeron speciosus*	5.26 ± 0.98 (8)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Solidago multiradiata	2.10 ± 0.20 (7)	1.76 ± 0.08 (20)	0.05 ± 0.006 (68)
Megachile sp.1	5.57 ± 0.22 (13)	*Arnica parryii*	0.81 ± 0.14 (5)	4.64 ± 0.05 (40)	0.82 ± 0.079 (54)
		Cirsium sp.2	0.49 ± 0.03 (29)	4.33 ± 0.07 (39)	0.39 ± 0.039 (40)
		Erigeron speciosus	0.21 ± 0.02 (6)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Heterotheca villosa	0.27 ± 0.03 (32)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Pyrrocoma crocea	0.39 ± 0.02 (110)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Rudbeckia occidentalis	0.97 ± 0.12 (5)	4.22 ± 0.06 (43)	0.19 ± 0.016 (106)
		Senecio bigelovii	0.68 ± 0.11 (3)	4.76 ± 0.06 (24)	0.22 ± 0.021 (39)
		Wyethia amplexicaulis	0.87 ± 0.13 (20)	6.83 ± 0.07 (40)	0.74 ± 0.060 (75)
Megachile sp.2	5.57 ± 0.22 (13)	*Erigeron speciosus*	0.26 ± 0.01 (90)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Senecio integerrimus	0.28 ± 0.02 (11)	4.19 ± 0.08 (36)	0.11 ± 0.010 (34)
		Heliomeris multiflora	0.70 ± 0.07 (8)	2.33 ± 0.03 (22)	0.07 ± 0.021 (12)
Megachilidae sp.1	4.50 ± 0.17 (3)	*Heterotheca villosa*	1.30 ± 0.09 (13)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
Nomada	2.20 (1)	*Erigeron speciosus*	1.34 ± 0.13 (27)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Osmia sp.1	4.05 ± 0.10 (5)	*Erigeron speciosus*	0.59 ± 0.06 (14)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Osmia sp.2	5.39 ± 0.39 (9)	*Erigeron speciosus*	0.41 ± 0.05 (13)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Helianthella quinquenervis	0.73 ± 0.13 (8)	4.13 ± 0.05 (32)	0.34 ± 0.016 (189)
		Heterotheca villosa	1.11 ± 0.16 (9)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Packera werneriifolia	0.49 ± 0.05 (83)	3.29 ± 0.06 (36)	0.02 ± 0.003 (23)
		Pyrrocoma crocea	0.51 ± 0.13 (7)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)
		Heliomeris multiflora	1.52 ± 0.30 (8)	2.33 ± 0.03 (22)	0.07 ± 0.021 (12)
		Wyethia amplexicaulis	2.62 ± 0.76 (12)	6.83 ± 0.07 (40)	0.74 ± 0.060 (75)

Insect species	Proboscis length (mm)	Plant species	Handling time (s)	Nectar tube depth (mm)	Nectar volume (µL)
Solitary bees					
Osmia sp.3	7.19 ± 0.56 (6)	*Helianthella quinquenervis*	0.28 ± 0.03 (4)	4.13 ± 0.05 (32)	0.34 ± 0.016 (189)
		Heterotheca villosa	0.45 ± 0.12 (5)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Wyethia amplexicaulis	0.80 ± 0.08 (8)	6.83 ± 0.07 (40)	0.74 ± 0.060 (75)
Butterflies					
Cercyonis oetus	7.00 (1)	*Erigeron speciosus*	1.10 ± 0.37 (9)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Chlosyne nycteis	8.01 ± 0.45 (12)	*Hymenoxys hoopesii*	8.69 ± 1.05 (6)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
Coenonympha tullia	5.30 ± 0.10 (20)	*Hymenoxys hoopesii*	4.67 ± 1.08 (23)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
		Senecio integerrimus	1.18 ± 0.18 (11)	4.19 ± 0.08 (36)	0.11 ± 0.010 (34)
Erebia epipsodea	7.31 ± 0.14 (13)	*Hymenoxys hoopesii*	3.13 ± 0.83 (26)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
Lepidoptera sp.1	11.69 ± 0.69 (2)	*Erigeron speciosus*	0.52 ± 0.13 (9)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Lepidoptera sp.5	9.37 ± 0.82 (5)	*Erigeron speciosus*	1.11 ± 0.45 (3)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Lycaena helloides	5.80 ± 0.14 (7)	*Erigeron speciosus*	1.36 ± 0.54 (20)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Lycaeana nivalis	6.07 ± 0.13 (7)	*Heterotheca villosa*	1.39 ± 0.33 (3)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Hymenoxys hoopesii	3.37 ± 0.94 (23)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
Oarisma edwardsii	11.69 ± 0.69 (2)	*Erigeron speciosus*	1.07 ± 0.13 (16)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Heterotheca villosa	2.44 ± 0.65 (6)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
Plebejus icarioides	6.00 ± 0.00 (2)	*Erigeron speciosus*	1.11 ± 0.10 (15)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Hymenoxys hoopesii	3.31 ± 0.88 (17)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
Poladryas arachne	7.00 (1)	*Senecio integerrimus*	2.15 ± 0.72 (8)	4.19 ± 0.08 (36)	0.11 ± 0.010 (34)
Polites draco	12.38 ± 1.14 (4)	*Erigeron speciosus*	1.50 ± 0.35 (11)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Hymenoxys hoopesii	1.01 ± 0.35 (6)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
Speyeria	9.79 ± 0.23 (12)	*Erigeron speciosus*	0.52 ± 0.05 (57)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Hymenoxys hoopesii	1.34 ± 0.16 (46)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
		Pyrrocoma crocea	1.24 ± 0.15 (86)	5.23 ± 0.05 (37)	0.19 ± 0.016 (63)

Insect species	Proboscis length (mm)	Plant species	Handling time (s)	Nectar tube depth (mm)	Nectar volume (μL)
Flies					
Bombylius systoechus	6.69 ± 0.34 (7)	*Senecio integerrimus*	0.94 ± 0.15 (32)	4.19 ± 0.08 (36)	0.11 ± 0.010 (34)
Eristalis stipator	4.51 (1)	*Heterotheca villosa*	1.28 ± 0.20 (15)	3.56 ± 0.03 (22)	0.07 ± 0.006 (33)
		Hymenoxys hoopesii	0.69 ± 0.07 (4)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)
		Heliomeris multiflora	0.60 ± 0.06 (26)	2.33 ± 0.03 (22)	0.07 ± 0.021 (12)
Hemipenthes morio	3.60 (1)	*Erigeron speciosus*	0.67 ± 0.07 (3)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
Sarcophagidae sp.1	3.15 ± 0.31 (7)	*Erigeron speciosus*	0.97 ± 0.14 (13)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Heliomeris multiflora	0.34 ± 0.04 (5)	2.33 ± 0.03 (22)	0.07 ± 0.021 (12)
Sericomyia flagrans	4.97 (1)	*Senecio integerrimus*	1.49 ± 0.11 (100)	4.19 ± 0.08 (36)	0.11 ± 0.010 (34)
Tachinidae sp.1	4.91 ± 0.03 (3)	*Erigeron speciosus*	0.41 ± 0.03 (8)	2.60 ± 0.04 (20)	0.06 ± 0.004 (22)
		Hymenoxys hoopesii	1.29 ± 0.19 (21)	3.20 ± 0.04 (38)	0.11 ± 0.012 (68)

Why do bumblebees prefer deeper flowers?
An optimal foraging approach

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Abstract

The proboscis of flower-visitors plays an important role in their flower choice. Often, flower-visitors preferentially visit the deepest flowers on which they can forage, which are the ones of which the nectar tube depth matches the length of their proboscis. This flower preference might be an adaptation for efficient foraging, as optimal foraging theory assumes that animals forage in such a way that maximizes their fitness. In this study we investigated (1) how foraging efficiency of natural foraging bumblebees of *Bombus bifarius* is related to the nectar tube depth of the Asteraceae flowers they forage on, and (2) whether bumblebees visit those species of flowers which produce more nectar or on which they have a higher foraging efficiency more frequently. As an indication of bumblebee foraging efficiency we measured their sugar intake rate per flower, per flower head and per patch. This is the amount of sugar ingested per time unit while extracting nectar from a flower, while foraging on a flower head or while foraging in a patch respectively. In addition we calculated their net energetic gain when foraging within a patch, accounting not only for the amount of sugar bumblebees take in per time unit, but also for the energetic content of the nectar and the energy bumblebees spend while foraging. Bumblebees extracted more nectar per second when foraging on flowers with deeper nectar tubes. Also, the amount of sugar extracted while foraging on a flower head or at a patch increasing nectar tube depth. In contrast, the energy bumblebees spend was not related to the nectar tube depth of the flowers. Consequently, their (rate of) net energy gain increased with increasing nectar tube depth. Further, bumblebees visited the flowers on which they experienced a higher net (rate of) energy gain more frequently. Net energy gain and rate of net energy gain explained respectively 72% and 60% of the variation in visitation rate. Visitation rate was also positively related to the sugar content of the flowers, however, sugar content explained (only) 55% of the variation in visitation rate. Visitation rate was not related to flower head density nor nectar tube depth. Overall, our results show that bumblebees forage more efficiently on flowers with deeper nectar tubes and that they do not visit them at random, but visit those flowers which offer more sugar and provide a higher energy gain more frequently. These results explain why bumblebees prefer visiting flowers with deeper nectar tubes and, because the deepest flowers on which they can forage are the ones that match the length of their proboscis, size-matching in plant-flower-visitor communities.

Key words: Asteraceae, *Bombus bifarius*, flower head density, nectar tube depth, rate of net energy gain, sugar intake rate, visitation rate

Introduction

Morphological traits of animals play an important role in the selection of their diet. For example, among flower-visitors, the length of their proboscis is known to determine the flowers that they visit for nectar. In general, flower-visitors can only visit those flowers of which the nectar tube depth does not exceed the length of their proboscis (Corbet 2000; Stang, Klinkhamer & van der Meijden 2007). Among the flowers which they can potentially visit, they visit flowers with deeper nectar tubes more often, which leads to size-matching between the length of their proboscis and the nectar tube of the flowers they visit (Stang *et al.* 2009). Patterns of size-matching has often been explained by these size-constraints in combination with species abundance (Stang *et al.* 2009) or resource competition (Rodriguez-Girones & Santamaria 2006). However, foraging efficiency, which is determined by both nectar reward of flowers and the energy spent while foraging, might be an important underlying factor influencing flower-visitor flower choice.

Optimal foraging theory assumes that animals forage in a way that maximizes their fitness. Therefore, they should select their diets and structure their foraging activities to maximize their net energy gain per time unit (Schoener 1971; Pyke, Pulliam & Charnov 1977; Pyke 1980; Pyke 1984; Stephens & Krebs 1986; Stephens, Brown & Ydenberg 2007). Consequently, optimal foraging theory predicts that flower-visitors should restrict their foraging efforts to those flowers offering the greatest rates of net energy gain. The net energy gain depends on the nectar reward of flowers and its energetic value, the number of flowers that flower-visitors visit per time unit and the energy they spend while foraging. The number of flowers that flower-visitors visit per time unit depends on the time it takes to (i) handle flowers and ingest the nectar (handling time) and (ii) to fly from flower-to-flower (interfloral flight time). The energetic expenditure depends on whether flower-visitors can walk or need to fly from flower to flower and the time they need for this, which depends on the flower clustering and density. In fact, flight time is the major component of foraging costs, as walking can require about 100 times less energy per second than flying (Heinrich & Raven 1972).

In some flowers nectar is easy accessible while in other flowers it is hidden in a long nectar tube. Both the amount of nectar that flowers produce and flower handling efficiency is related to the length of this tube. In general, deeper flowers produce more nectar, both in volume and sugar content (Galetto & Bernardello 2004; Kaczorowski, Gardener & Holtsford 2005; Petanidou 2007; Martins & Johnson 2013) and it takes flower-visitors more time to handle these flowers and extract the nectar (Inouye 1980). Yet, it is unknown how the nectar extraction rate and the overall (rate of) net energetic gain of natural foraging bumblebees differs among plant species and whether these aspects are related to nectar tube depth of the flowers they visit. The effect of floral traits, such as nectar tube depth, on nectar extraction rate, have mostly been studied under experimental laboratorial conditions (Hainsworth & Wolf 1976; Harder 1983; Montgomerie 1984; Harder 1986). Comparative studies which have examined the foraging energetics of flower-visitors naturally foraging on a diversity of plant species are rare (but see May 1985 for butterflies).

The present study was designed to get more insight in the foraging efficiency of natural foraging bumblebees and the role this plays in their selection of flowers. Specifically, we asked whether natural foraging bumblebees can forage more efficiently on flowers with deeper nectar tubes and consequently, whether they visit those flowers which provided the largest foraging efficiency more frequently. If so, this offers a possible explanation for bumblebee preference for flowers with deeper nectar tubes, which match their proboscis length (Stang *et al.* 2009). As an indication of bumblebee foraging efficiency we measured their sugar intake rate per flower, flower head and patch. Sugar intake rate is the amount of sugar ingested per time unit while extracting nectar of a single flower, while visiting a flower head or while foraging in a patch respectively. In addition we calculated the net energetic gain of bumblebees while foraging within a patch, accounting not only for the amount of sugar bumblebees take in per time unit, but also for the energetic content of the nectar and the energy bumblebees spend while foraging (energy expenditure). We asked the following questions: (1) is the sugar intake rate of bumblebees, while extracting nectar from a single flower and while foraging on a flower head or in a patch, related to the depth of the flowers they forage on? (2) Is the energetic expenditure of bumblebees related to the depth of the flowers they forage on? (3) Is the (rate of) net energy gain of bumblebees related to the depth of the flowers they forage on? (4) Is the (rate of) net energy gain of bumblebees related to other aspects which determine bumblebee foraging efficiency, including flower head density, sugar content per flower, number of open flowers per head, flight time between heads and the time it takes to visit a flower head? And (5) do bumblebees visit those flowers that have deeper nectar tubes, are more abundant, produce more nectar or provide a larger (rate of) net energy gain more frequently?

Materials and methods

Study site and plant species

Data were collected in the vicinity of the Rocky Mountain Biological Laboratory, Gunnison County, Colorado, USA. In total, seven Asteraceae species were sampled, which belong to the subfamilies Carduoideae and Asteroideae (Table 1). The Asteraceae have tiny flowers (flowers) which are aggregated in flower heads (capitula). Due do their relatively shallow flowers, bumblebees have a proboscis which is longer than the depth of these flowers and therefore, potentially, can visit all plant species.

Nectar tube depth

As nectar tube depth, we measured the length of the upper, wider part of the corolla tube, which roughly begins where the stamens insert and ends at the beginning of the corolla lobes, at the base of the deepest cleft in the corolla. The lower part of the corolla tube of the studied species is almost filled by the style (Graenicher 1909), therefore it is unlikely that flower-visitors can access this part of the corolla tube. We measured the depth of the nectar tube for at least twenty freshly picked flowers, to the nearest 0.01 mm, under a dissecting microscope.

Nectar production

We measured nectar production of disk flowers that were bagged for 24-hours, as an estimate for the nectar production rate (NPR). Flowers were bagged between 8 and 9 AM and collected 24-hours later. We used bags made of fine gauze that do not change the temperature within the bag and thus will not influence the nectar solute concentration (Corbet 2003). For each species, we only measured the nectar production rate of disk flowers as ray flowers are often sterile and do not produce nectar (Mani & Saravanan 1999). We did this for 14-100 flowers per plant species.

Nectar was extracted with microcapillary tubes of 0.25, 0.5 and 1.0 μL. The capillary size depended on the quantity of the nectar produced by the flowers and the nectar tube width. With the microcapillary tubes we probed the base of the nectar tube until no more nectar could be removed and then used digital callipers to measure the distance the nectar had migrated up the microcapillary tubes. Nectar volume was determined by converting the distance measured to volumes (μL). Nectar sugar concentration (expressed in % w/w in a solution) was measured with a hand-held refractometer. When nectar samples were too small for sugar concentration measurements, nectar samples of flowers from the same head were pooled to obtain a single concentration measurement. Sugar content (mg) was quantified as the product of the nectar sugar concentration, which was first converted from wt/wt to wt/vol, multiplied with the nectar volume, as suggested by Bolten *et al.* (1979).

Sugar intake rate per single flower

We calculated the sugar intake rate per flower based on the nectar production of bagged flowers and the handling time of bumblebees when foraging on bagged flowers. As nectar production rate is less variable than nectar standing crop, measuring the nectar production rate and the handling time of insects on bagged flowers gives a more reliable measure of the sugar intake rate per single flower.

Handling time was recorded with a digital video camera. We analysed these video recordings frame by frame (50 frames/s) with the software Adobe Premiere Pro CC 2014. For each flower head, only the first visit was analyzed. For each plant species, we analyzed 50-201 probes of *B. bifarius*. All handling time measurements were conducted between 8 and 10 am. Sugar intake rate per flower was calculated by dividing the mean sugar content (mg) by the handling time (s). We assume that flower-visitors completely empty the flowers.

Sugar intake rate per flower head and patch

Sugar intake rate per flower head and patch was calculated based on bumblebees foraging on unbagged flowers and the nectar standing crop of these flowers. For these calculations, nectar standing crop (NSC) was estimated, based on the regression analyses between NSC and NPR (Chapter 3 of this thesis). In contrast to NPR, NSC gives a more reliable indication of the foraging energetics of natural foraging bumblebees because NSC is what bumblebees actually encounter while foraging. We calculated the sugar intake rate per flower head and per patch using the following equations:

$$SIR_{fh} = \frac{nS}{t_h} \tag{1}$$

and

$$SIR_{patch} = \frac{nS}{t_h + t_f} \tag{2}$$

where SIR_{fh}= sugar intake rate per flower head (mg s^{-1}), SIR_{patch}= sugar intake rate per patch (mg s^{-1}), n=mean number of flowers bumblebees probed per flower head, S=average quantity of sugar per flower (mg), t_h=average time spent handling flower heads (s) and t_f=average time spent flying from flower head to flower head (s).

The time bumblebees spend on a flower head and the time it takes bumblebees to fly from flower head to flower head (inter-flower head flight time) within a patch, was measured using a stopwatch.

Energy expenditure

We calculated the energy bumblebees spend while handling a flower head and during inter-flower head flight time using the following equations:

$$H_E = wt_hk_h \tag{3}$$

and

$$F_E = wt_fk_f \tag{4}$$

where H_E=energy spend while handling a flower head, F_E=energy spend during inter-flower head flight time, w=bumblebee mass, t_h=average time spend handling a flower head (s), t_f=average time spend flying from flower head to flower head (s), k_f=bumblebee mass-specific rates of energy expenditure during inter-floral flight ($Jg^{-1}s^{-1}$) and k_h = bumblebee mass-specific rates of energy expenditure while handling a flower head ($Jg^{-1}s^{-1}$). We derived k_f from published values by Heinrich (1975) and k_h from published values by Pyke (1980). Following Pyke (1980), we assumed that all bumblebees had the same body mass of 200 mg and maintained their body temperature at a constant level of 37 °C.

Net energy gain

We calculated the net energy gain (J) using the following equation, modified from Dedej and Delaplane (2005) and Harder and Cruzan (1990):

$$N_E = \frac{I_E - E_E}{t_f + t_h} \tag{5}$$

where N_E= net energy gain, I_E= intake energy, and E_E= energy expenditure (energy spend during both inter-flower head flight and flower head handling activity). Intake energy (J) was calculated as:

$$I_E = nSe \tag{6}$$

where n=mean number of flowers per flower head that bumblebees probe, S=average quantity of sugar per flower (mg), and e=energy content (J) of 1 mg of sucrose (15.48 J) (Heinrich 1972a; Heinrich 1972b). And energy expenditure (J) was calculated as:

$$E_E = F_E + H_E \tag{7}$$

where F_E=energy spend by the bumblebees during inter-flower head flight time and H_E=energy spend while handling a flower. F_E and H_E were calculated following the equations above.

Visitation rate

In plots of two-by-two meter, we observed the number of visits per flower head by *B. bifarius*. Plant were observed during peak flowering time, at sites where the plant species were flowering abundant. Each plant species was observed during twelve intervals of 15 minutes.

Data analyses

Statistical analyses were performed, using R 3.3.2 (R Development Core Team 2014). Prior to all analyses, we tested whether the variables were normally distributed, using Shapiro-Wilk test. To increase normality, all variables were $\log_{10}$, prior to most analyses. We used single ordinary least square (OLS) linear regression analyses to examine whether the sugar intake rate per single flower, flower head and patch and the energetic expenditure of bumblebees was related to the nectar tube depth of the flowers they forage on. OLS regression analyses were also used to test whether the (rate of) net energy gain of bumblebees was related to the nectar tube depth, flower head density, sugar content per flower, number of open flowers per head and/or both the time it takes to fly between flower heads and to visit a flower head. Finally, to test whether flower head visitation rate was related to nectar tube depth, flower head density, nectar production and/or (rate of) net energy gain, we used OLS linear regression analyses.

Results

Sugar intake rate

The sugar intake rate per single flower and while foraging on a flower head varied fifteen- and twelvefold, while the sugar intake rate while foraging in a patch varied fivefold (Fig. 1). Flowers with deeper nectar tubes, which were bagged for 24-hours, produced more sugar rich nectar (r=0.79, P=0.03). It did not take bumblebees longer to handle flowers with deeper nectar tubes (r=0.52, P=0.23). Consequently, sugar intake rate on a single flower (mg sucrose ingested per second) increased with increasing nectar tube depth (r=0.86, P=0.01; Fig. 1a).

The number of flowers that bumblebees probed when visiting a flower head was not related to the depth of the flowers of those flower heads (r=0.26, P=0.57). Consequently, bumblebees did not spend more time on flower heads of flowers with deeper nectar tubes (r=0.36, P=0.42). As a result of these relationships, sugar intake rate when visiting a flower head increased with increasing nectar tube depth (r=0.83, P=0.02; Fig. 1b).

The time it took bumblebees to fly from flower head-to-flower head was not related to the nectar tube depth (r=0.61, P=0.15). Consequently, also the sugar intake rate while foraging in a patch increased with increasing nectar tube depth (r=0.79, P=0.03; Fig. 1c).

Energetic expenditure and net energetic gain

When foraging, bumblebees spend between 2.08 to 15.62 seconds visiting a flower head, and between 0.72 to 2.47 seconds flying from flower head-to-flower head, depending on the plant species they visited (Table 1). Thus, they spend most of their time (between 72% to 87%) visiting a flower head when foraging in a patch. As bumblebees spend more energy during flight, the energetic expenditure while visiting a flower head and during flight were comparable (Fig. 2). Neither the amount of energy spent while visiting a flower head (Fig. 2a) nor when flying from flower head-to-flower head (Fig. 2b) was related to nectar tube depth of the flowers.

The rate of net energy gain was strongly positively correlated with nectar tube depth (Fig. 3a), sugar content per flower (Fig. 3c) and both time spent flying between flower heads (Fig. 3e) and visiting a flower head (Fig. 3f). In contrast, the rate of net energy gain decreased with increasing flower head density (Fig. 3b), most likely because flower head density is strongly negative correlated with sugar content (r= -0.73, P=0.003), and was unrelated to the number of open flowers per flower head (Fig. 3d).

Table 1. Sugar production, number of flowers probed, handling time and interflower head flight time of *B. bifarius* while foraging on the different plant species studied. The table gives mean ± s.e.m. and sample size (n).

Plant species	**Sugar production bagged flowers (mg)**	**Number of flowers probed of a flower head**	**Handling time of a single flower (s)**	**Handling time of a flower head (s)**	**Inter flower head flight time (s)**
Cirsium sp.	0.26 ± 0.026 (14)	12.0 ± 1.5 (91)	1.85 ± 0.10 (88)	15.62 ± 1.51 (91)	1.94 ± 0.15 (91)
Helianthella quinquenervis	0.10 ± 0.010 (100)	14.2 ± 1.0 (131)	1.37 ± 0.11 (71)	8.07 ± 0.48 (201)	2.06 ± 0.47 (188)
Heliomeris multiflora	0.01 ± 0.001 (33)	7.6 ± 0.6 (86)	0.95 ± 0.05 (166)	4.80 ± 0.34 (108)	1.06 ± 0.07 (110)
Heterotheca villosa	0.02 ± 0.002 (34)	4.5 ± 0.3 (104)	0.63 ± 0.10 (50)	2.08 ± 0.10 (167)	0.72 ± 0.04 (173)
Hymenoxys hoopesii	0.06 ± 0.005 (77)	9.7 ± 0.6 (129)	1.11 ± 0.04 (201)	8.44 ± 0.55 (198)	1.21 ± 0.07 (215)
Pyrrocoma crocea	0.11 ± 0.011 (24)	8.5 ± 0.5 (139)	1.42 ± 0.09 (93)	8.12 ± 0.53 (214)	1.52 ± 0.08 (224)
Senecio bigelovii	0.11 ± 0.014 (45)	8.5 ± 0.6 (92)	1.20 ± 0.08 (88)	6.23 ± 0.54 (183)	2.47 ± 0.17 (181)

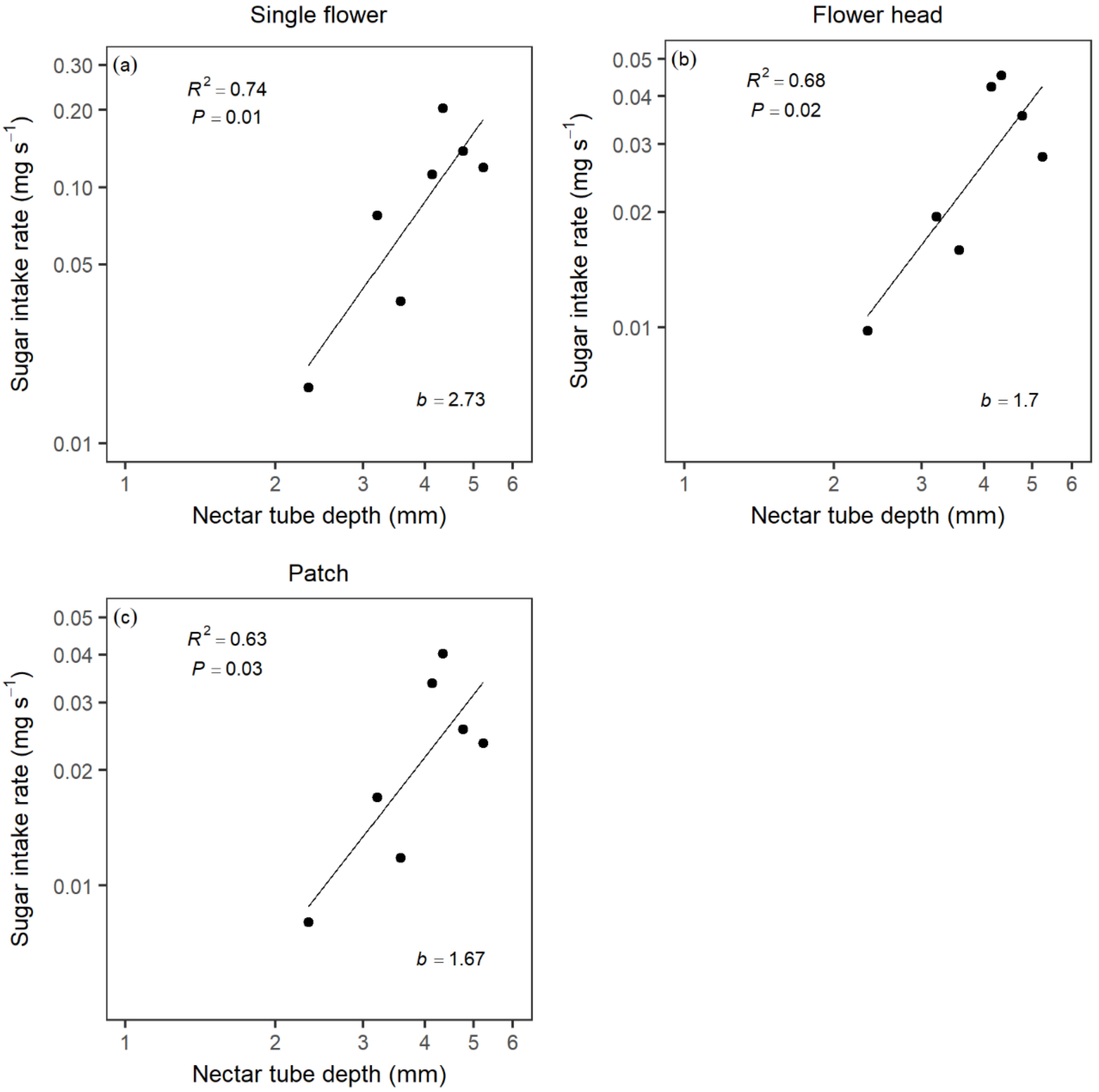

Fig. 1. Sugar intake rate while (a) extracting nectar from a single flower, (b) visiting a flower head and (c) foraging in a patch of *B. bifarius* individuals in relation to the nectar tube depth of the flowers. The sugar intake rate while extracting nectar from a single flower was based on nectar production rate measurements. Both the sugar intake rate while visiting a flower head and foraging in a patch were based on nectar standing crop measurements. Each dot represents a plant species (n=7).

Visitation rate

How often flower heads were visited by *B. bifarius* was not related to their nectar tube depth (Fig. 4a) nor flower head density (Fig. 4b). Flower heads of plant species which produced more nectar per flower (Fig. 4c) and which offered a higher net energy gain (Fig 4d) and rate of net energy gain (Fig. 4e) were more frequently visited by *B. bifarius*. Net energy gain explained 72% of the variation in visitation rate while rate of net energy gain and sugar per flower explained 60% and 55% of this variation respectively.

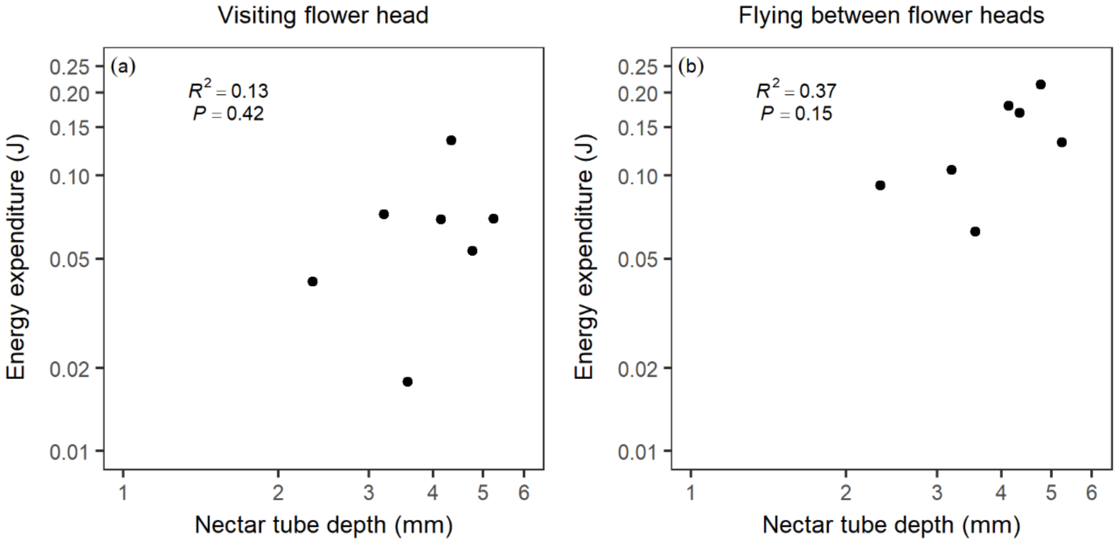

Fig. 2. Energy expenditure of *B. bifarius* individuals when (a) visiting a flower head and (b) when flying from flower head-to-flower head, in relation to the nectar tube depth of the flowers. Energy expenditure calculations were based on nectar standing crop measurements. Each dot represents a plant species (n=7).

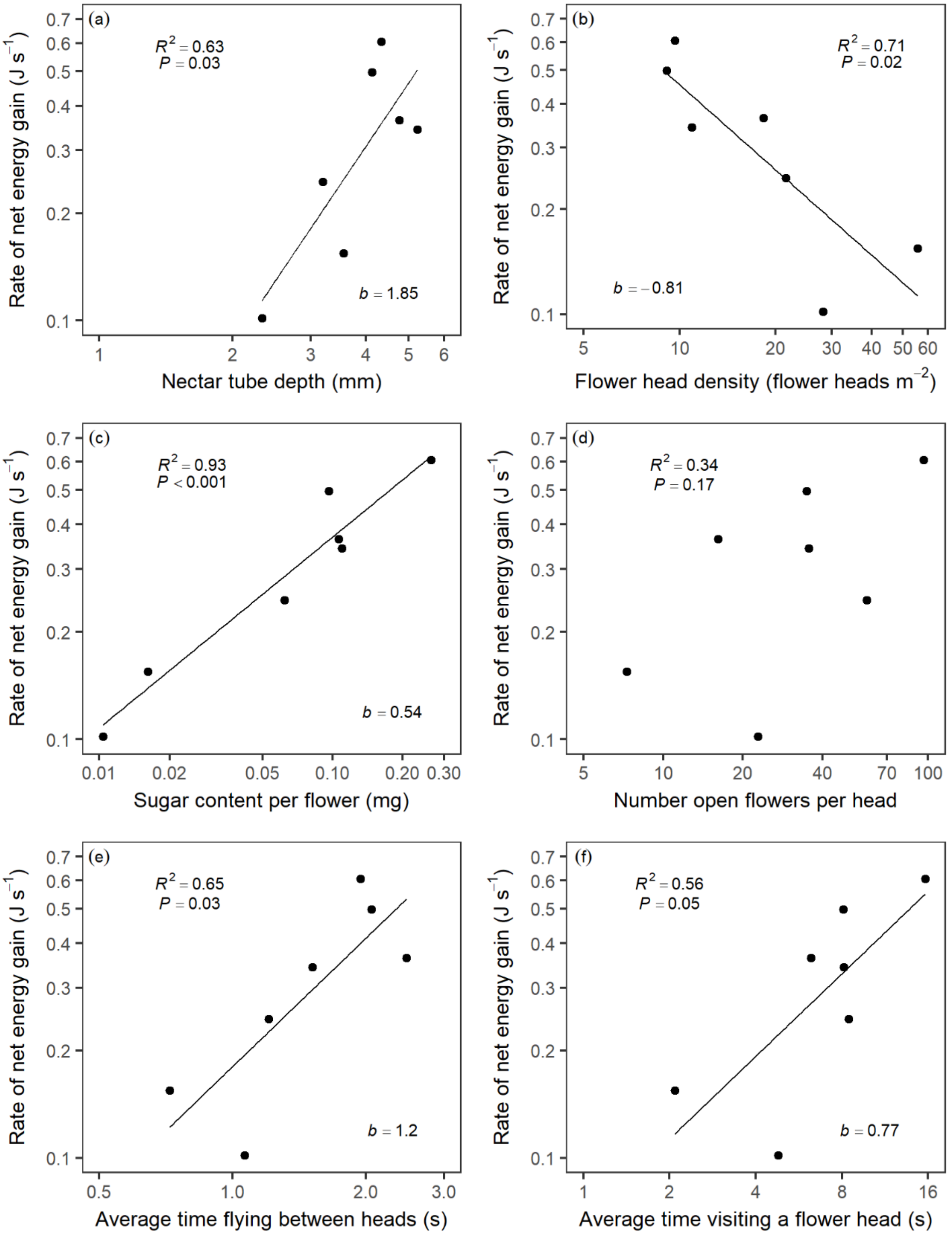

Fig. 3. Rate of net energy gain of *B. bifarius* individuals when foraging in a patch, in relation to (a) the nectar tube depth of the flowers, (b) flower head density, (c) sugar content per flower (NPR), (d) number of open flowers per flower head, and both (e) time spend flying between heads, and (f) time spend visiting a flower head. Rate of net energy gain was based on nectar standing crop measurements. Each dot represents a plant species (n=7).

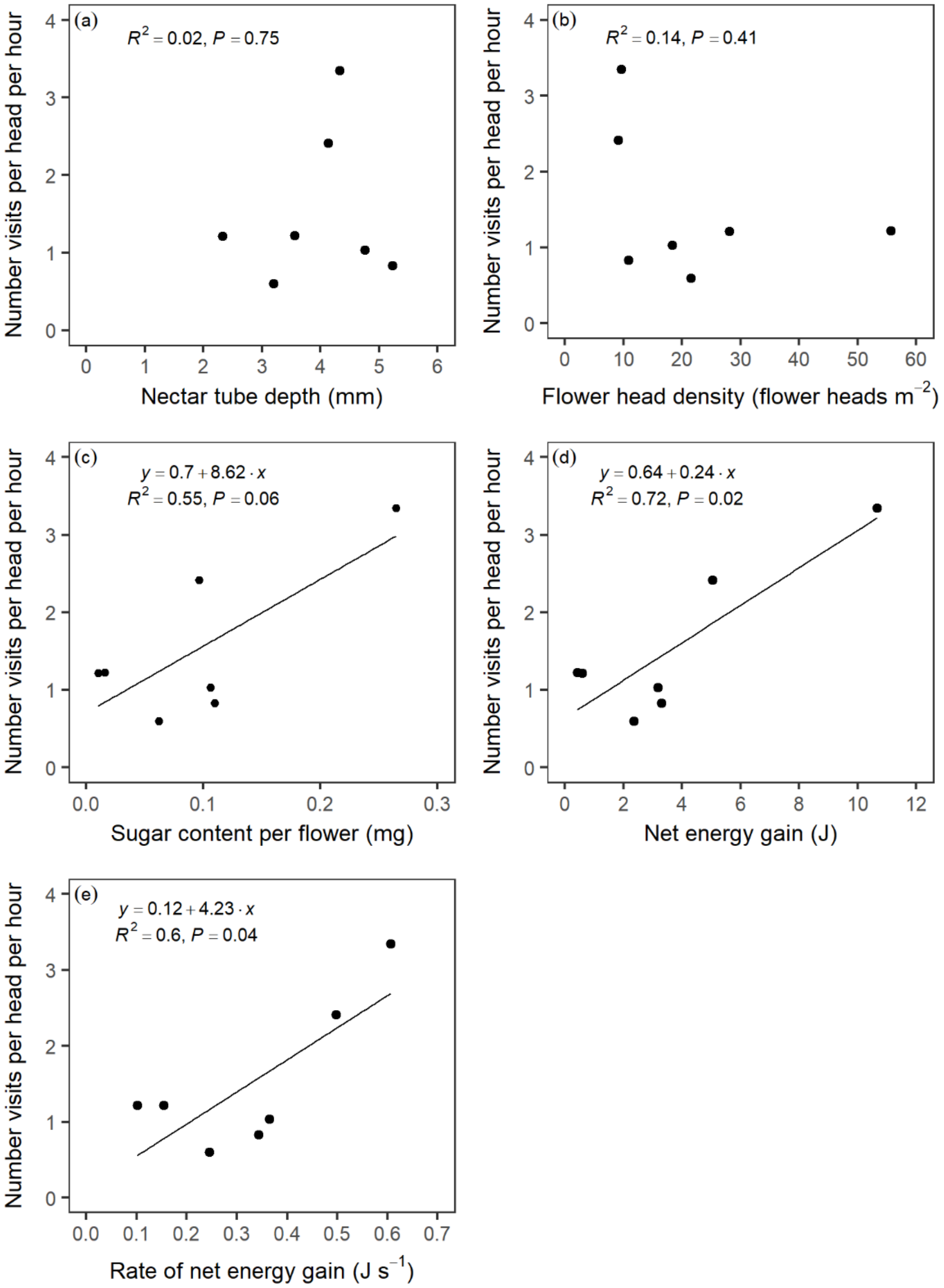

Fig. 4. Visitation rate of *B. bifarius* individuals, in relation to (a) nectar tube depth, (b) flower head density, (c) sugar content per flower (NPR) and both (d) net energy gain and (e) rate of net energy gain that *B. bifarius* individuals experience while foraging in a patch. Both net energy gain and rate of net energy gain were based on nectar standing crop measurements. Each dot represents a plant species (n=7).

Discussion

This study shows that the foraging efficiency of bumblebees is related to the nectar tube depth of the flowers on which they forage. Not only did the sugar intake rate when extracting nectar from a flower increase with increasing nectar tube depth, but also while visiting a flower head and foraging in a patch. Sugar intake rate while visiting a flower head and foraging in a patch varied less among plant species, compared to the sugar intake rate when extracting nectar from a single flower. This is most likely due to differences is number of open flowers per flower head and flower head density, which affect the time bumblebees spend visiting a flower head and flying between heads. Apparently, by clustering their flowers, plants species with flowers that produce less nectar become more profitable for bumblebees.

While foraging, bumblebees spend about 90% of their energy during flight (Heinrich & Raven 1972). Therefore, minimizing flight time, and thus choosing flowers which are more abundant, would be expected to be an effective means to increase the rate of net energy gain. Surprisingly, our result show that the rate of net energy gain actually decreased when bumblebees foraged on plant species of which the flower heads were more abundant. Consequently the rate of net energy gain increased when bumblebees foraged on plant species on which they experienced longer flight times. This can be explained by the fact that flower heads which were less abundant actually produced more nectar. Therefore, bumblebees experience longer flight times on more rewarding flowers. Apparently, flowers of less abundant plant species offer much more energy than the extra energy bumblebees spend due to increased flight times. Furthermore, although bumblebees spend most energy in flight, when they are in a patch, they spend on average 80% of their time on a flower head and only 20% flying from flower head-to-flower head. Consequently, when foraging within a patch, their energetic expenditure while visiting a flower head and during flight was comparable. However, we only observed bumblebee foraging behavior and -energetics within a meadow. Flower abundance and the distance of meadows from the nest might still be important cues for bumblebees when choosing meadows.

Although the energy expenditure of bumblebees tended to increase with increasing nectar tube depth, bumblebees which foraged on flowers with deeper nectar tubes experienced also a higher rate of net energy gain. Rate of net energy gain was also positively correlated with sugar content of bagged flowers. Therefore, both nectar tube depth and sugar content are potential cues for flower selection. This is in accordance with the results of May (1988), who found that nectar volume and nectar tube depth were strongly correlated with the foraging profit that two butterfly species experienced when foraging on a diversity of plant species.

Many network studies assume that flower-visitors distribute themselves randomly among flowers, proportional to flower abundance (Bascompte *et al.* 2003; Jordano, Bascompte & Olesen 2003; Vazquez 2005). If flower-visitors indeed forage randomly, than the number of visits per flower head should not differ among plant species. In contrast, we found that plant species of which the flowers produced more nectar and on which bumblebees experienced a higher (rate of) net energy gain were more frequently visited by bumblebees.

Net energy gain and rate of net energy gain explained 72% and 60% of the variation in visitation rate respectively and sugar content of bagged flowers explained 55% of this variation. In contrast, we did not find a relationship between visitation rate and flower head density. This indicates that bumblebees do not choose flowers based on their abundance, but on the nectar reward or the (rate of) net energy gain they provide, as assumed by theoretical models on optimal foraging (Pyke 1984). Surprisingly only few previous studies have empirically tested this optimal foraging approach and only for flower-visitors foraging behaviour when foraging on a single plant species (Whitham 1977; Pyke 1981; Pyke & Waser 1981; but see Schaffer & Schaffer 1979).

If bumblebees indeed select flowers in order to optimize their rate of net energy gain, based on floral cues such as nectar tube depth and the amount of nectar that flowers produce, the nectar production rate of flowers might be the results of this selective pressure by flower-visitors. If plant reproduction is pollen limited, than selection would favour an increase in nectar sugar content in order to attract bumblebees. Then why do not all plant species produce more nectar in order to ensure many bumblebee visits? First of all, shallow flowers might not have a strong incentive to produce more nectar, compared to deeper flowers. In contrast to shallow flowers, deeper flowers can only be visited by flower-visitors with a long proboscis (Corbet 2000; Stang *et al.* 2009), which can (potentially) visit a wide diversity of flowers (Borrell 2005). As bumblebee handling time, and thus their foraging costs, increases with increasing nectar tube depth (Inouye 1980; Harder 1983; Harder 1986), in order to attract flower-visitors, flowers with a deeper nectar tube might need to produce more nectar than shallow tubed flowers. Further, plants should not produce too much nectar, either to ensure outcrossing or to reduce geitonogamy (Klinkhamer & de Jong 1993). Finally, in our study, we did not include other flower-visitors than *B. bifarius*. Nevertheless, most of the studied plant species are visited by a variety of other insect species, mostly other bumblebees and solitary bees, butterflies and flies (Chapter 2 of this thesis). Therefore, a higher visitation rate by *B. bifarius* does not necessarily imply a higher visitation rate in general.

Overall our study shows that bumblebees experience a higher foraging efficiency when foraging on flowers with deeper nectar tubes and that flowers of plant species on which bumblebees experience a higher net energy gain are visited more frequently by these bumblebees. This indicates that bumblebees do no forage randomly, but optimally. These results may explain why bumblebees preferentially visit flowers with deeper nectar tubes and, because these flowers are the ones of which the nectar tube depth matches the length of their proboscis, why size-matching between flower- and flower-visitor morphology occurs in plant-flower-visitor communities.

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How plants can cope with unpredictable environments: pollinator species number – species predictability trade-off

6

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Manuscript in preparation

Abstract

Plants differ widely in the number of flower-visitor species visiting them and this generalization degree has important ecological implications. Specialized plants are thought to be at risk of being poorly pollinated when the abundance of their flower-visitor species fluctuate strongly from year-to-year. In contrast, plants with a generalized pollination system might have a higher visitation rate and are thought to be more resilient to the loss of flower-visitor species. However, to ensure pollination, specialized plants might specialize on those flower-visitor species which are less prone to short-term temporal fluctuations. In this study, we tested the hypothesis that plants can either be generalized on flower-visitors which are prone to temporal fluctuations or specialized on flower-visitors which are less prone to temporal fluctuations, which will result in equal visitation rates. In other words plants face a pollinator species number (SN) – species predictability (SP) trade-off. We tested this hypothesis among subalpine Asteraceae and their flower-visitors. Our results provide support for the SN-SP trade-off hypothesis. Many flower-visitor species were highly unpredictable across years, as they were only present in one of the three years, most of which were butterflies. However, specialized plants attracted those few flower-visitor species which were present in all three years and therefore less prone to temporal fluctuations. Species which were less prone to temporal fluctuations were on average more generalized and were mostly bees. Additionally, among bees, species with a longer proboscis where less prone to temporal fluctuations, compared to species with a short proboscis. Furthermore, visitation rate was not related to plant generalization degree. Overall, our results show that plants might cope with the yearly fluctuation of flower-visitors and ensure flower-visitor visitation, either by attracting a large number of flower-visitor species, which are specialized and more prone to temporal fluctuations, or by specializing on flower-visitors which are more generalized and less prone to temporal fluctuations.

Keywords: Asteraceae, *Bombus bifarius*, generalization degree, nectar tube depth, temporal fluctuation, visitation rate

Introduction

Plants differ widely in the number of flower-visitor species or functional groups visiting them (Bascompte *et al.* 2003; Fenster *et al.* 2004; Ollerton *et al.* 2007; Lazaro, Lundgren & Totland 2009). While some species are highly specialized (engaged in one or a few interactions), others are extremely generalized (engaged in many interactions) and this generalization degree has important evolutionary (Waser *et al.* 1996; Aigner 2001; Fenster *et al.* 2004) and ecological implications (Aizen, Ashworth & Galetto 2002; Bascompte *et al.* 2003; Memmott, Waser & Price 2004). Specialized plant species, which depend on only a few flower-visitor species, are more likely to experience strong directional selection on floral traits (Johnson & Steiner 1997; Alexandersson & Johnson 2002; Harder & Johnson 2009; Pauw, Stofberg & Waterman 2009) and consequently are better adapted to their flower-visitors (Herrera 1988). At the same time, it has been hypothesized that specialized plants are at risk of being poorly pollinated, especially when their flower-visitor species fluctuate from year-to-year (Waser *et al.* 1996). In contrast, plants with relatively generalized pollination systems might have a higher visitation rate and are thought to be more resilient to the loss of some flower-visitor species.

Especially when there is a high temporal year-to-year turnover in flower-visitor species, which causes uncertainty as to whether a specific species will be consistently present across years, generalization is thought to be a more successful strategy (Waser *et al.* 1996; Memmott, Waser & Price 2004). However, the reliability of pollination services not only depends on the number of potential flower-visitor species, but also on the temporal dynamics of these species. In the last ten years, there has been an increased interest in temporal variation of plant flower-visitor communities (Alarcon, Waser & Ollerton 2008; Olesen *et al.* 2008; Petanidou *et al.* 2008; Burkle & Irwin 2009; Dupont *et al.* 2009; Lazaro, Nielsen & Totland 2010; Olesen, Stefanescu & Traveset 2011; CaraDonna *et al.* 2017). These studies have shown that, although most flower-visitor species presence and their interactions fluctuate from year-to-year, certain flower-visitor species and plant-flower-visitor interactions are temporally stable. Therefore, to ensure pollination, specialized plants might specialize on those flower-visitor species which are less prone to short-term temporal fluctuations.

To ensure pollination in these unpredictable environments, where the abundance of most flower-visitor species highly fluctuates from year-to-year, plants should either attract numerous flower-visitor species or attract those few flower-visitor species which are less prone to temporal fluctuation. In fact, plants might even face a trade-off between attracting numerous flower-visitors, which are rare and prone to temporal fluctuations, or attracting only a few flower-visitor species which are less prone to temporal fluctuations. This pollinator species number (SN) – species predictability (SP) trade-off hypothesis is based on three assumptions. First of all, the number of flower-visitor species by which a plant is visited, is related to the nectar tube depth of its flowers. In general, because flowers with a deep nectar tube can only be visited by flower-visitors with a long proboscis (Stang, Klinkhamer & van der Meijden 2007), plant species with shallow flowers are more generalized (Herrera 1996; Agosta & Janzen 2005; Stang, Klinkhamer & van der Meijden

2006). Secondly, plants species with flowers with deeper nectar tubes produce more nectar (Galetto & Bernardello 2004; Kaczorowski, Gardener & Holtsford 2005; Ornelas *et al.* 2007; Petanidou 2007; Martins & Johnson 2013; Johnson *et al.* 2017). And third, flower-visitors which are less prone to temporal fluctuations are those species which are large, generalized, abundant and/or social and consequently may have higher energetic requirements. From these three assumptions it follows that plant species with flowers with deeper nectar tubes will be specialized. However, these plants will produce enough nectar to attract those flower-visitor species which are less prone to temporal fluctuations. For plant species to be generalized, they need to have shallow flowers. However, these plant species might not produce enough nectar to fulfill the energetic requirements of flower-visitors which are less prone to temporal fluctuations. Therefore, generalized plant species either may not attract flower-visitor species which are less prone to temporal fluctuations, or if they do attract these flower-visitors, they will not visit them frequently. Consequently, generalists and specialists will have comparable visitation rates.

Several studies have shown that long-tongued and specialized flower-visitor species are more prone to extinction (Biesmeijer *et al.* 2006; Burkle, Marlin & Knight 2013). However, how the year-to-year fluctuation of flower-visitor species is related to species characteristics is yet unclear. Studies that have investigated short-term yearly interaction turn-over among plants and their flower-visitors have not documented how this temporal variation is related to species traits, such as species generalization degree (but see Olesen, Stefanescu & Traveset 2011) for a butterfly-plant network) or morphology. Therefore, we still know remarkably little about the actual temporal fluctuation of flower-visitors in relation to their traits and the traits of the plants they visit.

In this paper, we tested the pollinator species number – species predictability trade-off (SN-SP hypothesis) for subalpine Asteraceae. Therefore, we investigated the fluctuation of flower-visitor species abundances across three years and how this is related to both flower-visitor- and plant species traits. Specifically we asked the following questions: (1) Is the fluctuation of flower-visitor species abundance related to their generalization degree and proboscis length? (2) Does nectar tube depth determine plant generalization degree and how does this relationship fluctuate across years? (3) Do specialist plant species remain more specialized across years? (4) Are specialized plants visited by species that are temporarily more stable? And (5) is flower visitation rate and its fluctuation among years related to plant species generalization degree and its fluctuation among years?

Materials and methods

Study system and flower-visitor observations

We studied the flower-visitors of 11 co-flowering subalpine Asteraceae species occurring in the Colorado Rocky Mountains, USA: *Arnica parryi*, *Cirsium sp.1*, *Cirsium sp.2, Helianthella quinquenervis*, *Heliomeris multiflora*, *Heterotheca villosa*, *Hymenoxys hoopesii*, *Pyrrocoma crocea*, *Senecio bigelovii*, *Solidago multiradiata* and *Wyethia amplexicaulis*.

Flower-visitor species were observed combining both random transect walks and plot-observations. For observations, we used a focal plant species approach were we observed plant-flower-visitor interactions in meadows in which the plant species were flowering abundantly. We did this to minimize the effect of plant abundance on flower-visitors. In total, plant-flower-visitor interactions were observed in five different meadows, which were approximately 200 meter apart. Flower-visitors could move freely between these meadows. Plot observations were performed by observing a 2x2 meter plot and transect walks were performed by randomly walking within the meadow. Each year, plant species were observed for 4-6 hours in total. For all plant species, equal observation periods were used, as differences in observation effort can alter the number of observed flower-visitors independent of size constraints and flower abundance (Ollerton & Cranmer 2002). Observations took place between 08:00-16:00h under favorable weather conditions and the observation periods were randomly distributed across days. Every year, we sampled during a 4-6 weeks period in July and August. We included all flower-visitors that were foraging for nectar.

In order to avoid changing flower-visitor densities, distributions and behaviour, most flower-visitor species were not caught but photographed or filmed for later identification. For identification, a reference collection with voucher specimens was created and used. These specimens were collected in 2009 and 2010, in the same site as where our study was conducted. This reference collection is kept by M. Stang. Flower-visitor specimens were identified to species- or morphospecies level.

Flower-visitor proboscis length

Proboscis length measurements of solitary bees, butterflies and flies were based on specimens from the reference collection mentioned above. Proboscis length of these specimens was measured as described in Stang, Klinkhamer and van der Meijden (2006). For bumblebees, data on the average proboscis lengths were obtained from Macior (1974) (for queens and workers) and Inouye (1976) (for males).

Plant- and flower-visitor generalization degree

We estimated the generalization degree of flower-visitors as the average number of plants they visited within a year. The generalization degree of plants was estimated as both the number of insect species that visited them each year and the total number of flower-visitors of all three years (total generalization degree).

Yearly fluctuation of flower-visitors

We classified flower-visitors according to the fluctuation in their availability in the community among years, namely species which we observed in one, two or three years. This measure gives an indication of how species abundances fluctuate across years. Species which were not observed on the plants in a particular year, were not necessarily absent at all in that year. However, it does indicate that their abundances were very low. For each plant species, we calculated the proportion of flower-visitor species that were observed in one, two and all three years.

Nectar tube depth

As nectar tube depth, we measured the length of the upper, wider part of the corolla tube, which roughly begins where the stamens insert and ends at the beginning of the corolla lobes, at the base of the deepest cleft in the corolla. It is unlikely that flower-visitors can access the lower part of the corolla tube, since this part of the corolla tube of is almost filled by the style (Graenicher 1909). We measured the depth of the nectar tube for at least twenty freshly picked flowers, to the nearest 0.01 mm, under a dissecting microscope.

Data analyses

Statistical analyses were performed, using R 3.3.2 (R Development Core Team 2014). We used a Shapiro-Wilk test to test if the variables were normally distributed. To achieve normality, flower visitation rate was $\log_{10}$ transformed prior to all analyses. We used analysis of variance (ANOVA) and Tukey HSD post hoc tests, to analyse whether the temporal stability of flower-visitors was related to their average generalization degree (average number of plants they visited). To analyse whether the temporal stability of flower-visitors was related to their proboscis length, we used Fisher's exact tests. For these analyses, flower-visitor species were divided into three groups: species with a short proboscis (< 4 mm), medium proboscis (4-8 mm) and long proboscis (>8 mm). We analysed whether temporal stability of butterfly species was related to their proboscis length separately since the abundance and distribution of butterflies is highly related to their larval diet preferences and habitat specialization, rather than to the abundance of flowering plants (Eskildsen *et al.* 2015; Aguirre-Gutierrez *et al.* 2016). To test whether nectar tube depth was related to plant specialization we used single ordinary least square (OLS) linear regression analyses. To test whether plant generalization degree of the different years were related, we used Pearson correlation analyses. Furthermore, OLS regression analyses were used to test whether the proportion of flower-visitor species which were observed in all three years was related to plant generalization degree. We also used OLS linear regression analyses to test whether flower head visitation rate and its fluctuation among years was related to plant species generalization degree and its fluctuation among years.

Results

Yearly fluctuation of flower-visitor species

During the three years, we observed 83 flower-visitor species in total, most of which were butterflies (37%) (Table 1). More than half of the species (46 species) were observed in only one of these three years, most of which were butterflies (24). Only 18 species were observed in all three years, most of which were bumble bees (7) and solitary bees (6) (Fig. 1). Flower-visitor species that were observed in all three years were on average more generalized than species that were present in only one or two years (Fig. 2). Furthermore, a higher proportion of bees and flies with a medium or long proboscis were observed in three years, compared to bee- and fly species with a short proboscis. We did not find such a relationship among butterflies (Fig. 3).

Table 1. Number of flower-visitor species.

Insect orders	**2011**	**2013**	**2014**	**Total**
Hymenoptera	24	16	25	34
Bumblebees	9	7	9	9
Solitary bees	13	8	12	18
Wasps	2	1	4	7
Lepidoptera	14	15	10	31
Diptera	8	5	9	14
Others	4	2	2	4
Total	49	37	47	83

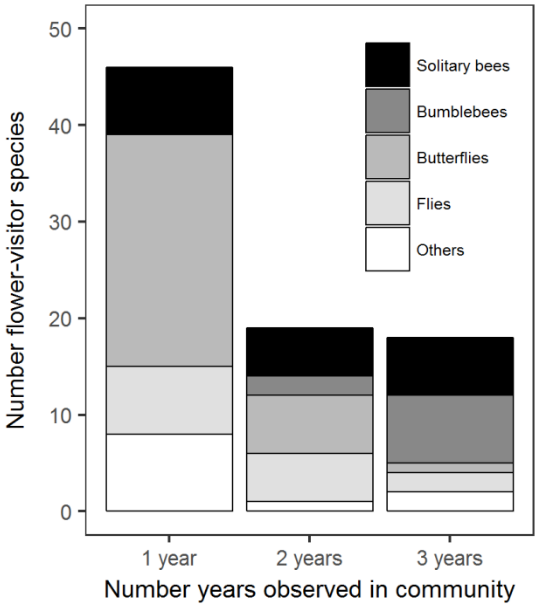

Fig. 1. Number of flower-visitors species that were present in respectively one, two or three years.

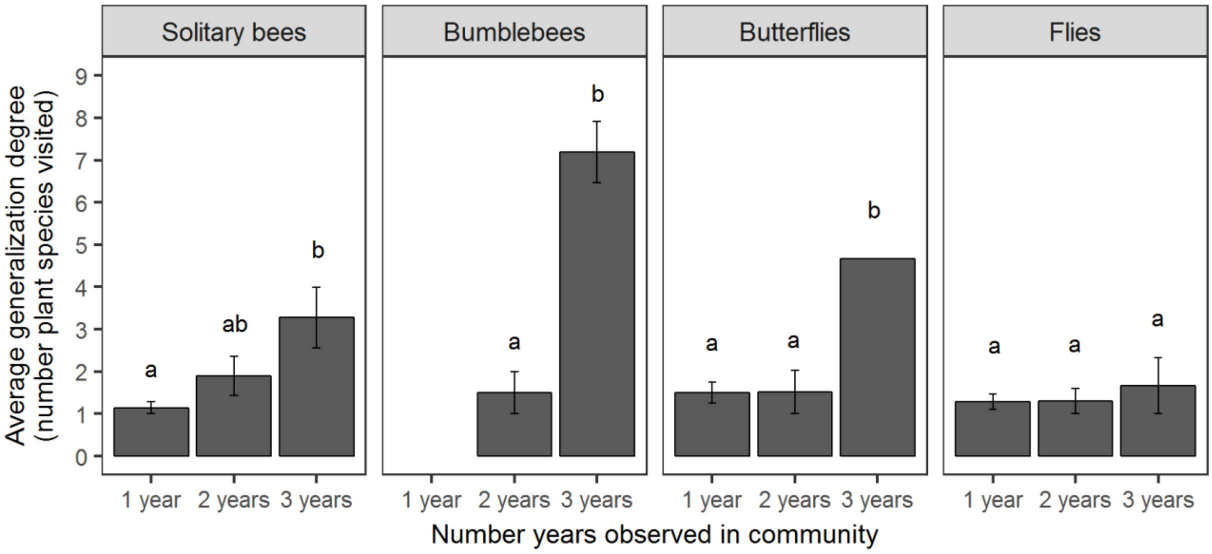

Fig. 2. Average generalization degree of flower-visitor species (number of plants that they visited) in relation to the number of years they were observed in the community. Bars indicate SE of the mean. The different letters above the bars refer to statistically significant differences ($p<0.05$; Tukey HSD post hoc tests) between groups.

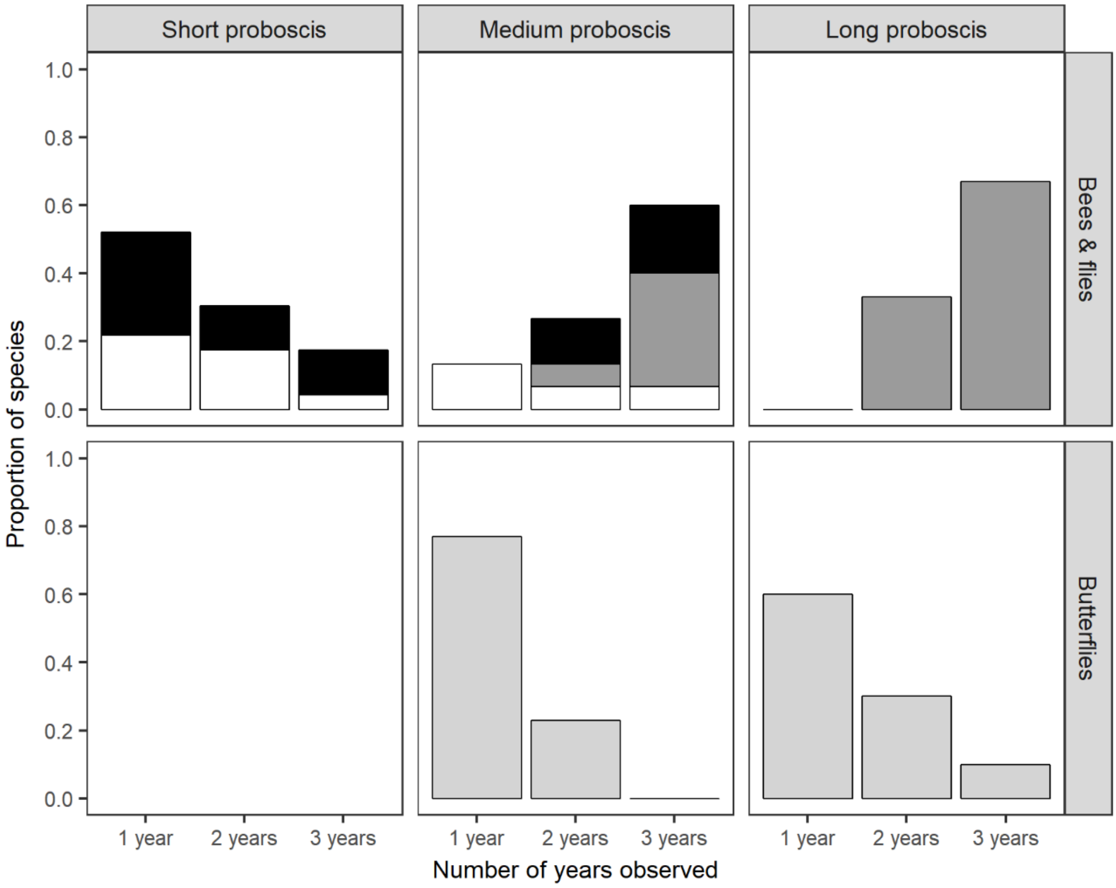

Fig. 3 Proportion of flower-visitor species observed in the community in one, two or three years in relation their proboscis length. Black bars are solitary bees, dark grey bars are bumblebees, light grey bars are butterflies and white bars are flies. Proportion of species observed in one, two or three years was significantly different among short-, medium- and long-tongued bees and flies (P=0.02), but not among butterflies (P=0.48).

Nectar tube depth and plant generalization degree

Among plant species, the total number of flower-visitor species that we observed in all three years ranged from 5 to 38 species and this number was negatively correlated to nectar tube depth (r=-0.52, P=0.04; Fig 4a). However, surprisingly, this negative relationship between nectar tube depth and the number of flower-visitor species was not significant in all three years (Fig. 4b-d).

Plant generalization degree and yearly fluctuation of their flower-visitors

For all plant species, the number of flower-visitor species varied across years. However, plant generalization degrees were positively correlated between different years (r=0.81, P<0.003 for 2011-2013; r=0.77, P=0.005 for 2011-2014; and r=0.54, P=0.09 for 2013-2014). This indicates that although the number of flower-visitor species of all plant species varied among years, specialized plants remained relatively specialized. Furthermore, a higher proportion of flower-visitors of specialized plant species were present in all three years (Fig. 5), which indicates that the flower-visitor species of specialized plants were less prone to temporal fluctuations.

Visitation rate

In both 2011 and 2013, flower visitation rate was not related to plant generalization degree (Figs. 6a, b). Further, the yearly fluctuation in visitation rate was not related to the fluctuation in plant generalization degree (Fig. 6c).

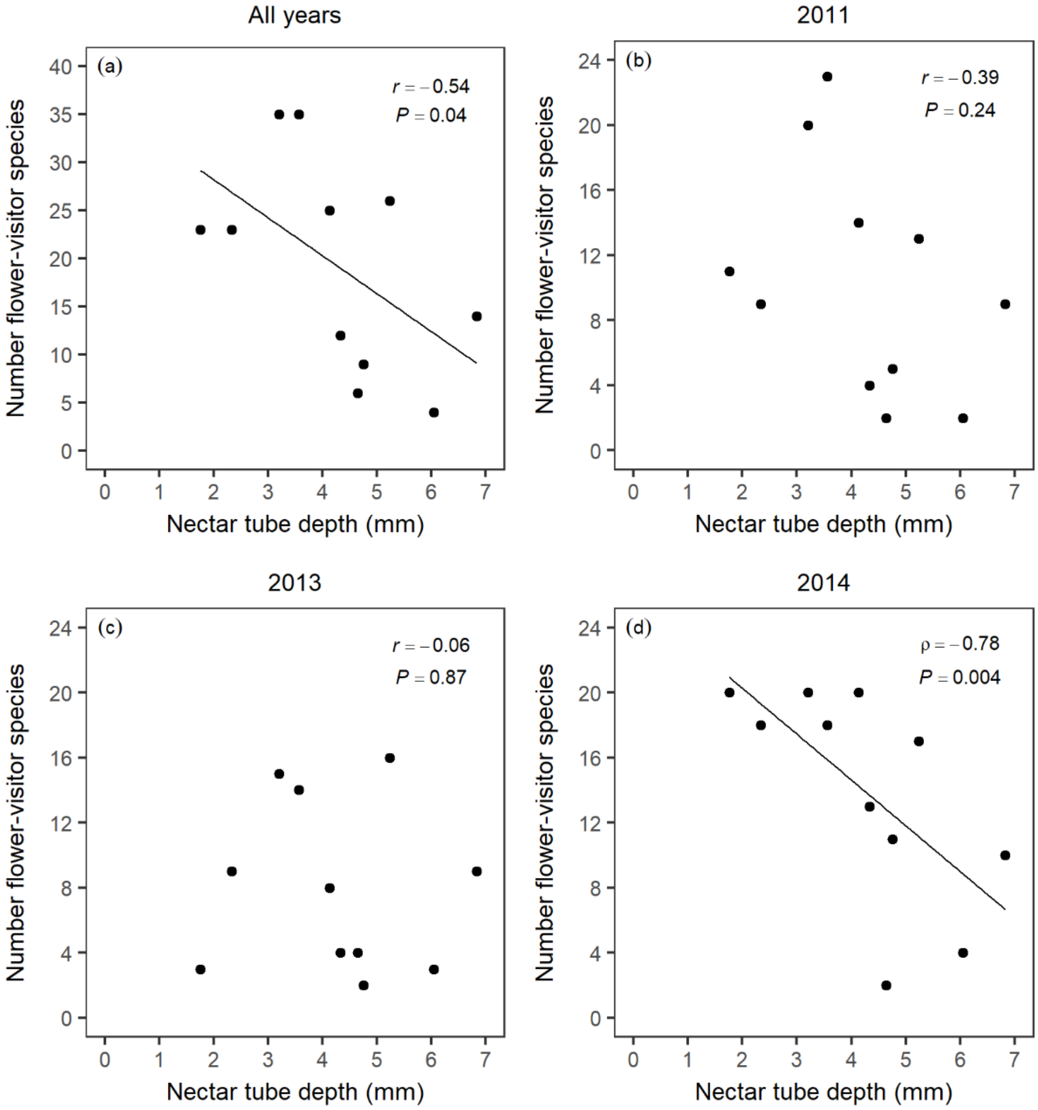

Fig. 4. Relationship between the depth of flowers and (a) their total number of flower-visitor species in all three years and their number of flower-visitor species (b) in 2011, (c) in 2013, and (d) in 2014. Each data point corresponds to a single plant species (n=11).

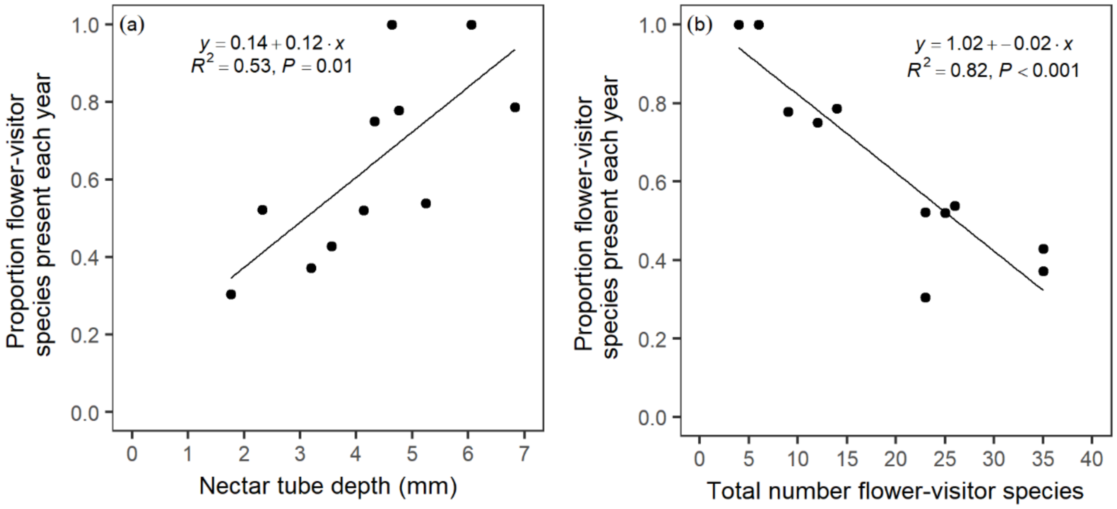

Fig. 5. Relationship between plant generalization degree (total number of flower-visitor species) and the proportion of these species that are present in the community each year. Each data point corresponds to a single plant species (n=11)

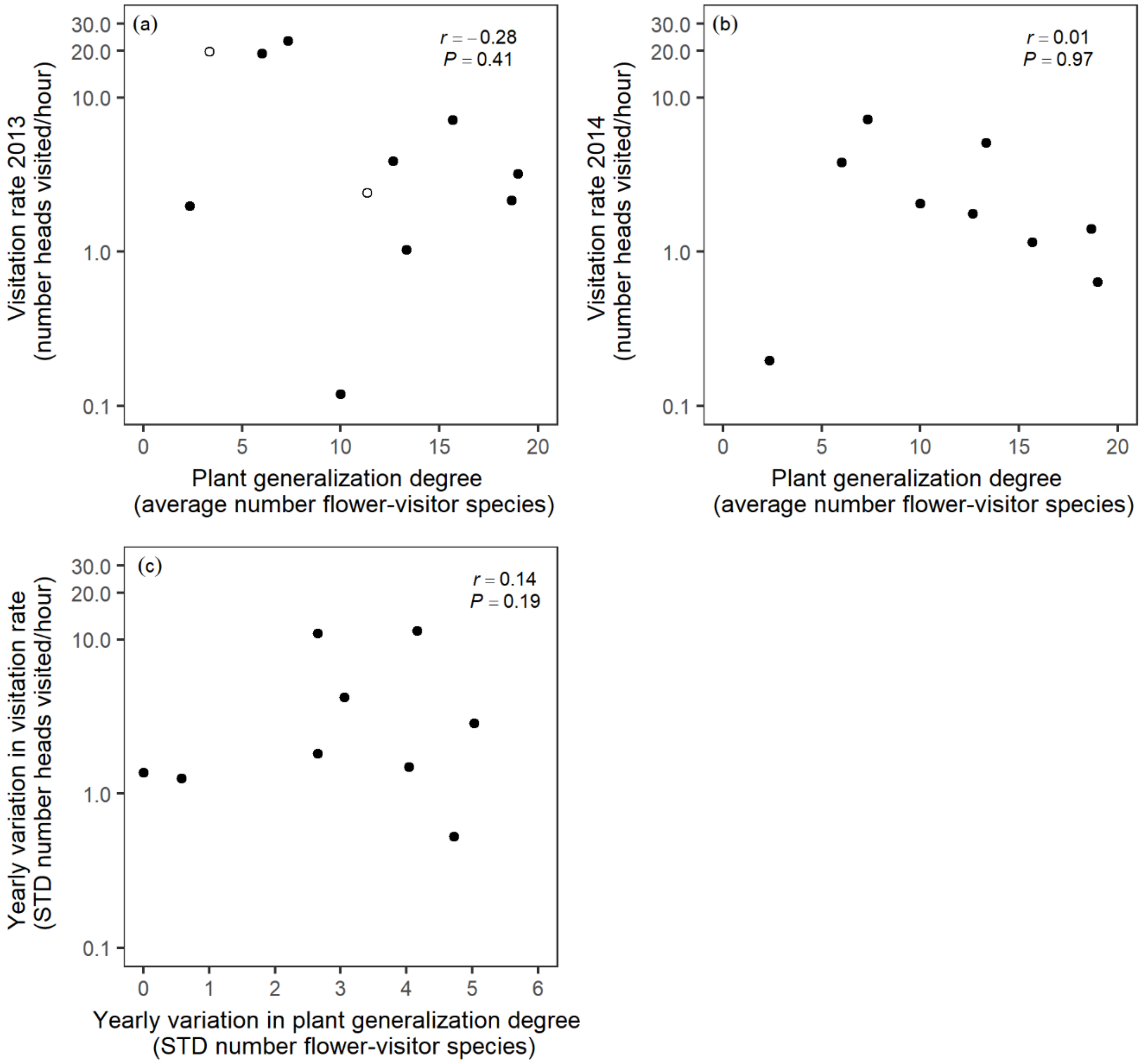

Fig. 6. Visitation rate in (a) 2013 and (b) 2014 in relation to plant generalization degree and (c) yearly variation in visitation rate in relation in relation to yearly variation in plant generalization degree. Each data point corresponds to a single plant species and is shown as the number of heads visited per hour. Filled circles are the plant species of which we measured the visitation rate in both years and open circles are the plant species of which we measured the visitation rate only in 2013.

Discussion

Our results show that specialized plants might be less vulnerable, in regard to pollination, than expected based on their generalization degree and we found support for the SN-SP trade-off hypothesis. We showed that although most flower-visitor species abundances fluctuated across years, as they were only observed in one of the three years, specialized plant species attracted those flower-visitors which were less prone to temporal fluctuations. Their flower-visitors were observed in the community in all three years. Although generalized plants were also visited by flower-visitor species which were less prone to temporal fluctuations, these flower-visitors visited them less frequently. Consequently, flower visitation rate was unrelated to plant generalization degree. In one year, flower visitation rate even tended to decrease with increasing visitation rate.

The year-to-year fluctuation of flower-visitor species abundance was related to their generalization degree and morphology. Flower-visitors which were less prone to temporal fluctuation where those species which were on average more generalized. As in our study, Olesen, Stefanescu and Traveset (2011) found that for butterflies, most specialists were sporadic, only occurring in one or two years, while the temporally stable species varied in their generalization degree although they all had more than one interaction partner. Moreover, our results indicate that among bees and flies, species with a longer proboscis tended to be less prone to temporal fluctuation. Interestingly, we did not find such a relationship among butterflies. This is likely because their abundance and distribution is strongly related to their larval diet preferences and habitat specialization (Eskildsen *et al.* 2015; Aguirre-Gutierrez *et al.* 2016). The abundance of bee- and fly species with a short proboscis might fluctuate stronger among years, compared to bee- and fly species with a long proboscis, because species with a short proboscis occur more locally. As species with a short proboscis are smaller, they fly shorter distances and thus have a smaller foraging range. Higher year-to-year fluctuation in species abundance does not necessarily imply that these species are also less prone to extinction. Studies by Biesmeijer *et al.* (2006) and Burkle, Marlin and Knight (2013) both show that a higher proportion of flower-visitor species with a longer proboscis went extinct in the past few decades. However, it does indicate that, for plants, in the short term, these species are less reliable as flower-visitors.

The specialized nature of pollination systems have been debated, as several studies have stressed that most pollination systems are more generalized and dynamic when considering temporal and spatial variation in plant-flower-visitor interactions (Ollerton 1996; Waser *et al.* 1996). Indeed, a high year-to-year interaction turnover is a general feature of plant-flower-visitor communities (Alarcon, Waser & Ollerton 2008; Olesen *et al.* 2008; Petanidou *et al.* 2008; Burkle & Irwin 2009; Dupont *et al.* 2009; Lazaro, Nielsen & Totland 2010; Olesen, Stefanescu & Traveset 2011; CaraDonna *et al.* 2017). In accordance, our results show that most plant species experienced temporal variation in their flower-visitor spectra. However, plant generalization degree was highly correlated among years, which indicates that specialized plants nevertheless remain relatively specialized. Further, we have shown that, due to species interaction turnover, the negative association between floral nectar tube depth and plant species generalization degree was not found in each year. This might explain why some studies did not find a correlation between nectar tube depth and number of flower-visitor species (Torres & Galetto 2002). Overall, these results stress the importance of considering temporal variation in plant-flower-visitor interactions.

Although our results show that flower visitation rate is not related to plant generalization degree, both plant generalization degree and plant-flower-visitor interaction turnover might still affect seed set of plants and thus their fitness. Different flower-visitors, especially different flower-visitor taxa or -species, which differ in size, might differ in pollination efficiency (Willmer & Finlayson 2014). For example, bees forage efficiently and are more constant, visiting numerous flowers of the same plant species. In contrast, butterflies may visit less flowers and might be less constant (Pohl, Van Wyk & Campbell 2011), but travel over longer distances which makes them important for genetic outcrossing. Therefore, we encourage further research on yearly interaction turnover to include measures of pollination efficiency, to get more insight on how plant generalization degree and interaction turnover affect seed set.

Overall, our results provide important insights in the year-to-year temporal dynamics of pollination networks. They show that in uncertain environments, in which most flower-visitor species abundance fluctuate highly from year-to-year, plants ensure flower-visitor visitation, either by attracting a large number of flower-visitor species, which are more specialized and more prone to temporal fluctuations, or by specializing on those flower-visitor species which are more generalized and less prone to temporal fluctuations.

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General discussion

7

In recent years, plant-flower-visitor community studies have shown general interaction patterns such as asymmetry (generalist flower-visitors interact with specialist plants and vice versa) and nestedness (a core group of generalists interacts with each other and a few specialists mostly interact with generalists) (e.g. Bascompte *et al.* 2003; Vazquez & Aizen 2004; Bascompte, Jordano & Olesen 2006). These interaction patterns are often explained by random foraging behaviour of flower-visitors (Vazquez 2005; Vazquez *et al.* 2007), sometimes in combination with the existence of forbidden interactions (Stang, Klinkhamer & van der Meijden 2006; Rezende, Jordano & Bascompte 2007; Stang, Klinkhamer & van der Meijden 2007; Stang *et al.* 2009; Olesen *et al.* 2011). Forbidden interactions imply that plants are not visited by certain flower-visitor, due to a mismatch in either phenology or morphology. Surprisingly, although nectar reward and foraging efficiency is one of the most likely aspects to determine flower-visitor foraging behaviour, we lack knowledge on how these aspects affect the type and number of interaction partners and consequently plant-flower-visitor network structure (but see Junker *et al.* 2013; Carvalheiro *et al.* 2014; Tinoco *et al.* 2017). The main aim of this study was to investigate whether flower-visitor foraging efficiency can explain species generalization degree and plant-flower-visitor interaction patterns, such as asymmetry, nestedness and size-matching between the nectar tube depth of flowers and the average proboscis length of their flower-visitors. In addition, I used my results to investigate the ways plants can mitigate the risk that in a particular year there are insufficient numbers of flower-visitors to ensure pollination. I specifically hypothesized that plants can either be generalized on flower-visitors which are prone to temporal fluctuations or specialized on flower-visitors which are less prone to temporal fluctuations, which will result in equal visitation rates. In other words plants face a pollinator species number (SN) – species predictability (SP) trade-off.

I have analyzed the interactions of subalpine Asteraceae species, co-occuring in the Colorado Rocky Mountains, and their flower-visitors. The Asteraceae are visited by a wide diversity of flower-visitor taxa, including bumblebees, solitary bees, butterflies and flies and thus are considered to be generalists (Graenicher 1909; Lane 1996; Proctor, Yeo & Lack 1996; Torres & Galetto 2002). Therefore, they provide the unique opportunity to investigate how different flower-visitor taxa respond to variation in plant traits and how interactions are structured among generalized plant species. More important, they are an ideal study system to investigate the effect of foraging efficiency on plant-flower-visitor interaction patterns, because they have relatively shallow flowers, which do not pose strong morphological constraints on their flower-visitors. A large proportion of their potential flower-visitors, mostly bumblebees and butterflies, have a proboscis long enough to visit all plant species.

In the first experimental chapter, **Chapter 2**, I analyzed the interaction structure among the Asteraceae and their flower-visitors. I investigated (1) whether interactions among generalized plants and their flower-visitors were more generalized and connected and less asymmetric and nested, (2) whether plant generalization degree (both the number of flower-visitor species and Shannon diversity index) and the number of flower-visitor visits were related to local flower head density, nectar tube depth and display area and (3) whether plant species with flowers with deeper nectar tubes are visited by flower-visitor species which have a longer proboscis and how this degree of size-matching differs among flower-visitor taxa, including bumblebees, solitary bees, butterflies and flies. In general, among generalized plant species, which have simple and easy accessible flowers, it is assumed that interactions are more generalized and connected and therefore less structured and predictable. Surprisingly, I found that the level of interaction asymmetry and nestedness was comparable to complete pollination networks (Traveset *et al.* 2016). These results might be explained by the (unexpected) presence of specialized flowers. However, I did find that the interactions among the Asteraceae and their flower-visitors were more generalized and connected, compared to other pollination networks (Traveset *et al.* 2016). This indicates that flower-visitors had a higher niche overlap and thus that species of the Asteracae share a greater proportion of their flower-visitor species which implies that there is a high redundancy among these interactions. This indicates that plant phylogeny affects flower-visitor visitation patterns (e.g. Rezende, Jordano & Bascompte 2007).

Further, plant generalization degree (both the number of flower-visitor species and their Shannon diversity index) increased with increasing local flower head abundance and display area and decreased with increasing nectar tube depth. However, the number of visit increased with increasing display area but was unrelated to nectar tube depth or flower head abundance. In contrast to what has often been assumed (Vazquez & Aizen 2004; Vazquez 2005), these results suggest that plant-flower-visitor interactions cannot simply be explained by random encounters among flower-visitors and the flowers they visit. This is because random foraging by flower-visitors implies that more abundant plant species should not only interact with more flower-visitor species, but also receive more visits. Rather, in this community, it is likely that plant-flower-visitor interactions are influenced by proboscis length frequency distribution of flower-visitors and the negative correlation between flower density and nectar tube depth. More abundant plant species had flowers with shallow nectar tubes and therefore could be visited by a wider diversity of insect species. Moreover, because flower-visitor species with a short proboscis also visited the plants less frequently and therefore apparently were also less abundant, the number of visits was unrelated to flower head density.

Furthermore, in accordance with previous studies (Stang *et al.* 2009), flowers with deeper nectar tubes were, on average, visited by bumblebee-, solitary bee- and fly species with a longer proboscis. Among butterflies, I did not find these relationships. This is likely because butterflies might select flowers on different floral cues, such as nectar concentration (Watt, Hoch & Mills 1974; Pyke & Waser 1981) or floral display area. In contrast to previous studies (Stang *et al.* 2009), in this study system, most bumblebees species had a proboscis which was long enough to visit all plant species. Therefore, this positive relationship between nectar tube depth and bumblebee proboscis length indicates that bumblebee species with a long proboscis avoided shallow flowers, while species with a short proboscis avoided deep flowers. These results indicate that size-matching between the nectar tube depth of flowers and the average proboscis length of their flower-visitor species even occurs in the absence of size constraints.

In **Chapter** 3 I investigated whether flowers with deeper nectar tubes produced more nectar, as a possible explanation for the size-matching pattern. Nectar is one of the major floral rewards for flower-visitors. Therefore, flower-visitors are expected to prefer more rewarding flowers and to visit them more frequently (Leiss & Klinkhamer 2005). I addressed the following questions: (1) Do flowers with deeper nectar tubes produce more nectar per single flower and offer more nectar per flower head, individual plant and patch? (2) Are nectar tube depth and nectar production related to the number of open flowers per head and the number of flower heads per individual or patch? Since nectar standing crop is what flower-visitors actually encounter while foraging I also tested (3) whether nectar standing crop is related to nectar production rate. In accordance with previous studies (Petanidou, Goethals & Smets 2000; Galetto & Bernardello 2004; Kaczorowski, Gardener & Holtsford 2005; Ornelas *et al.* 2007; Martins & Johnson 2013; Johnson *et al.* 2017), I found that nectar production of single flowers increased with increasing nectar tube depth. However, this relationship has not yet been studied at the level of flower heads (or inflorescence), individual plant or patch. Nevertheless, the attractiveness of a plant is likely to be determined by the total reward it offers, which is also determined by the number of open flowers and clustering of these flowers. I found that, indeed flowers with deeper nectar tubes also produce more nectar per flower head, individual plant and patch. Plant species with deeper flowers will have a stronger incentive to produce more nectar, because they need to attract flower-visitor with a long proboscis, which can potentially visit a wide diversity of flowers (Ornelas *et al.* 2007; Martins & Johnson 2013; Johnson *et al.* 2017). Further, I found that the specific scaling relationship between nectar tube depth and nectar production were remarkably similar among two years and that nectar standing crop was significantly correlated with nectar production rate. These results, together with the fact that the Asteraceae produced minute amounts of nectar, which never filled the nectar tube for more than 11%, strongly indicate that nectar may act as an underlying factor promoting flower-visitor selection for deeper flowers.

In **Chapter 4** and **Chapter 5**, I investigated whether the time it takes flower-visitor to handle a flower and extract nectar from it (handling time) and flower-visitor foraging efficiency might be alternative explanations for why size-matching between the nectar tube depth of flowers and the average proboscis length of their flower-visitors occurs. Because a long proboscis might be of hindrance when foraging on shallow flowers, longer-tongued insects are assumed to handle these flowers less efficiently (Inouye 1980; Plowright & Plowright 1997; Kunte 2007; Karolyi *et al.* 2013; Bauder *et al.* 2015). Consequently, flower-visitors with a long proboscis may visit shallow flowers less frequently or avoid them. In **Chapter 4**, I analyzed how handling time was related to insect proboscis length and the nectar tube depth and nectar volume of the flowers they visit and how this affected their flower choice. I asked the following questions: (1) Do bumblebees, solitary bees, butterflies and flies differ in handling time and is their handling time related to their proboscis length and both nectar tube depth and nectar volume of the flowers they visit? And (2) do insects with a longer proboscis visit more plant species and plant species with flowers with deeper nectar tubes? Surprisingly, in contrast to what has been suggested, a longer proboscis did not appear to be of a hindrance when foraging on shallow flowers of the Asteraceae. Handling time was not related to flower-visitor proboscis length when flower-visitors foraged on relatively shallow flowers. If insects visited flowers of which the nectar tube depth matched the length of their proboscis, handling time even decreased with increasing proboscis length.

Floral traits did determine handling time of flower-visitors, but this differed among flower-visitor taxa. Among bees, handling time increased with both nectar tube depth and nectar volume, while among flies handling time only increased with increasing nectar tube depth. Among butterflies handling time only increased with increasing nectar volume. Although previous studies have examined the handling time of bumblebees (Inouye 1980; Herrera 1989), butterflies (Herrera 1989; Kunte 2007; Martins & Johnson 2013; Bauder *et al.* 2015) and flies (Gilbert 1981), this study provides the unique opportunity to compare handling time and flower choice of these flower-visitor taxa foraging on a diversity of flowers (but see Herrera 1989 for comparison of hymenoptera and lepidoptera). Different flower-visitor taxa had intrinsic different handling times. Bees handled flowers faster than flies while butterflies had longer handling times. Both the intrinsic differences in handling time of bees, flies and butterflies, and the different effects of floral traits on handling time among these flower-visitor taxa may be due to different nectar extraction technique and/or body mass. Bees drink nectar by lapping with their glossae (Harder 1982), while butterflies and flies both suck up the nectar actively (Kingsolver & Daniel 1979; Gilbert 1981). Some flies even spit fluid on to the nectary, most likely to produce a medium in which the nectar sugar can dissolve (Gilbert 1981). Especially when confronted with the nectar environment of Asteraceae flowers (minute amounts and concentrated nectar), lapping proboscides are able to extract nectar at a much faster rate than sucking ones of similar length (Herrera 1989). Further, butterflies are expected to have a more narrow proboscis compared to flies which makes sucking up nectar more difficult, hence the different handling times among butterflies and flies. Differences in body mass among flower-visitor taxa might also have affected nectar extraction rate. In general, bees have a greater body mass, compared to butterflies and flies and insects with a greater body mass can drink nectar at a higher rate (Harder 1986).

Although flower-visitors with a longer proboscis can potentially forage on a wider diversity of flowers (Borrell 2005), the number of plant species that solitary bees, butterflies and flies visited was not related to their proboscis length. Bumblebee species with a longer proboscis even tended to visit fewer plant species and thus were more specialized. Because bumblebees with a longer proboscis are larger and thus have higher energetic requirements (Heinrich 1983), they might be more selective in their food choice (Goulson & Darvill 2004), only selecting high rewarding flowers. Also, partitioning of floral resources among species to avoid interspecific resource competition might have made flower-visitors more selective (Pyke 1982; Stout, Allen & Goulson 1998).

In Chapter 2 of this thesis, I analyzed whether flowers with deeper nectar tubes were, on average, visited by bumblebee, solitary bee and fly species with a longer proboscis (plants' point of view). In accordance, I found that bumblebee, solitary bee and fly species with a longer proboscis visited, on average, flowers with deeper nectar tubes (insects' point of view). Again, I did not find this relationship among butterflies. These results are in accordance with previous studies (Brian 1957; Morse 1978; Gilbert 1981; Harder 1985; Shmida & Dukas 1990; Plowright & Plowright 1997; Borrell 2005; Stang *et al.* 2009; Johnson 2010). However, handling time cannot explain these patterns of size-matching, as it took flower-visitors longer to handle flowers with deeper nectar tubes.

In **Chapter 5**, I investigated how foraging efficiency of natural foraging bumblebees of the species *Bombus bifarius* was related to the nectar tube depth of the Asteraceae flowers they foraged on, and whether *B. bifarius* individuals visited flowers of plant species on which they experience a higher foraging efficiency more frequently. Foraging efficiency might be more important for flower-visitor flower choice than handling time, because foraging efficiency accounts not only for the time it takes to handle a flower and extract nectar, but also for the amount of nectar, the number of flowers they visit, flower abundance and clustering and the time it takes to fly from flower-to-flower. I asked the following questions: (1) is the sugar intake rate of bumblebees, while extracting nectar from a single flower and while foraging on a flower head or in a patch, related to the depth of the flowers they forage on? (2) Is the energetic expenditure of bumblebees related to the depth of the flowers they forage on? (3) Is the (rate of) net energy gain of bumblebees related to the depth of the flowers they forage on? (4) Is the (rate of) net energy gain of bumblebees related to other aspects which determine bumblebee foraging efficiency, including flower head density, sugar content per flower, number of open flowers per head, flight time between heads and the time it takes to visit a flower head? And (5) do bumblebees visit those flowers that have deeper nectar tubes, are more abundant, produce more nectar or provide a larger (rate of) net energy gain more frequently? My results show that bumblebee sugar intake rate when extracting nectar from a single flower, while visiting a flower head and while foraging in a patch increased with increasing nectar tube depth. The energy bumblebees spent was not related to the nectar tube depth of the flowers. Consequently, their (rate of) net energy gain increased with increasing sugar production and nectar tube depth, which is also shown by May (1988) for butterflies. Moreover, bumblebees indeed visited the flowers on which they experienced a higher net energy gain more frequently. Net energy gain and rate of net energy gain explained 72% and 60% of the variation in visitation rate, respectively. Visitation rate was also positively related with the sugar content of 24-hour bagged flowers and explained 55% of the variation in visitation rate. In contrast, visitation rate was not related to nectar tube depth and flower head density. These results indicate that bumblebees do not forage randomly. Rather, they choose flowers based on their nectar reward or the (rate of) net energy gain they provide, as assumed by many theoretical models (Pyke 1984), and thus that bumblebees forage optimally. Surprisingly only few previous studies have empirically tested this optimal foraging approach and only for flower-visitors foraging behaviour when foraging on a single plant species (Whitham 1977; Pyke 1981; Pyke & Waser 1981; but see Schaffer & Schaffer 1979). These results may explain why bumblebees preferentially visit flowers with deeper nectar tubes and, because these flowers are the ones of which the nectar tube depth matches the length of their proboscis, why size-matching between flower- and flower-visitor morphology occurs in plant-flower-visitor communities.

In the last chapter, **Chapter 6**, I tested the hypothesis that, to ensure pollination in unpredictable environments in which most flower-visitor species abundance fluctuate yearly, plants can either be generalized on those flower-visitor species which are prone to temporal fluctuations or specialized on the few flower-visitor species which are less prone to temporal fluctuations, which will result in equal visitation rates. In other words plants face a pollinator species number (SN) – species predictability (SP) trade-off. This hypothesis is based on three assumptions. First of all, the number of flower-visitor species by which a plant is visited, is related to the nectar tube depth of its flowers. In general, plant species with shallow flowers are more generalized (Chapter 2 of this thesis) because flowers with a deep nectar tube can only be visited by flower-visitors with a long proboscis. Secondly, plants species with flowers with deeper nectar tubes produce more nectar (Chapter 3 of this thesis). And third, flower-visitors which are less prone to temporal fluctuations are those species which are large, generalized, abundant and/or are social and consequently may have higher energetic requirements. Thus, for plants to be generalized, their flowers need to be relatively shallow. However, these shallow flowers may not produce enough nectar to fulfill the energetic requirements of flower-visitors which are less prone to temporal fluctuations. Therefore, generalized plant species either may not attract flower-visitor species which are less prone to temporal fluctuations, or if they do attract these flower-visitors, these flower-visitors will not visit them frequently. In turn, plant species which have deep-tubed flowers are able to produce enough nectar to attract flower-visitors which are less prone to temporal fluctuations. However due to their deep nectar tubes, these flowers cannot attract a wide diversity of flower-visitor species and thus are specialized. To test this hypothesis I asked the following questions: (1) Is the fluctuation of flower-visitor species abundance related to their generalization degree and proboscis length? (2) Does nectar tube depth determine plant generalization degree and how does this relationship fluctuate across years? (3) Do specialist plant species remain more specialized across years? (4) Are specialized plants visited by species that are temporarily more stable? And (5) is flower visitation rate and its fluctuation among years related to plant species generalization degree and its fluctuation among years? My results indicate that indeed a pollinator species number (SN) – species predictability (SP) trade-off may exist. The majority of flower-visitor species was only observed in one of the three years, and thus experienced high fluctuation in abundance across years. However, specialized plant species attract those flower-visitors which were less prone to temporal fluctuations. Their flower-visitors were observed in the community in all three years. Although generalized plants were also visited by flower-visitor species which were less prone to temporal fluctuations, these flower-visitors visited them less frequently. Consequently, flower visitation rate was unrelated to plant generalization degree. In one year, flower visitation rate even tended to decrease with increasing visitation rate.

Flower-visitors which were less prone to temporal fluctuation where those species which were on average more generalized. As in our study, Olesen, Stefanescu and Traveset (2011) found that for butterflies, most specialists were sporadic, only occurring in one or two years, while the temporally stable species varied in their generalization degree although they all had more than one interaction partner. Moreover, our results indicate that among bees and flies, species with a longer proboscis tended to be less prone to temporal fluctuation. We did not find such a relationship among butterflies. The abundance of bee- and fly species with a short proboscis might fluctuate stronger among years, compared to species with a long proboscis because species with a short proboscis occur more locally. As species with a short proboscis are smaller, they fly shorter distances and thus have a smaller foraging range, compared to species with a long proboscis. Higher year-to-year fluctuation in species abundance does not necessarily imply that these species are also less prone to extinction.

Studies by Biesmeijer *et al.* (2006); Burkle, Marlin and Knight (2013) and Burkle, Marlin and Knight (2013) both show that a higher proportion of flower-visitor species with a longer proboscis went extinct in the past few decades. However, it does indicate that, for plants, in the short term, these species are less reliable as flower-visitors. Overall, our results show that in unpredictable environments, where most flower-visitor species abundances fluctuate from year-to-year, being generalized on those species which are more prone to temporal variations, or being specialized on those species which are less prone to temporal variations are two strategies with which plants might cope with the yearly fluctuation of flower-visitors and ensure flower-visitor visitation. An important next step is to investigate how these different strategies affect (conspecific) pollen transfer and with that reproductive success and plant fitness.

Overall, my results provide a better understanding of how plant- and flower-visitor morphological traits affect plant-flower-visitor interaction patterns. My results indicate that flower abundance is less important to determine plant-flower-visitor interactions than floral traits. In contrast to what has been assumed by many network studies (Vazquez & Aizen 2004; Vazquez 2005; Vazquez *et al.* 2007), flower-visitors do not distribute themselves randomly among flowers. Rather, plant-flower-visitor interactions are structured by species traits, their frequency distributions and correlations among them. First of all, although, more abundant plant species are visited by a wide array of flower-visitor species, the number of visits was unrelated to flower head density. Plant species with more dense flower heads had shallow tubed flowers and there were relatively few flower-visitor species with a short proboscis. These two aspects are likely to affect the positive relationship between flower head density and plant generalization degree, rather than random foraging behaviour. Secondly, if flower-visitors forage randomly, flower-visitors would distribute themselves among plant species proportional to plant species abundance. Consequently, all plant species would experience comparable visitation rates. In contrast, I found that flower heads of plant species on which bumblebees foraged more efficiently (a higher (rate of) net energetic gain), where more frequently visited by bumblebees.

Furthermore, this study emphasizes the importance of nectar production and flower-visitor foraging efficiency for plant-flower-visitor interaction patterns, such as size-matching (between the nectar tube depth of flowers and the average proboscis length of their flower-visitors), and even the temporal stability of plant-flower-visitor interactions. Larger- and longer-tongued flower-visitors have higher energetic requirements and therefore might prefer plant species with a deeper nectar tube, because these plant species produce more nectar and because flower-visitors forage more efficiently on these flowers. Specialized plants might be less vulnerable to yearly fluctuations than previously thought because they are visited by longer-tongued and generalized flower-visitors which are less prone to local temporal fluctuations. Therefore these interactions are more predictable. Deeper- and specialized flowers are likely to attract these longer-tongued and generalized flower-visitors, because they produce more nectar or because flower-visitors forage more efficiently on these flowers. Therefore, to predict the response of plants to yearly fluctuations in plant-flower-visitor communities, is it important to, not only, have insight in species generalization degree, but also an in-depth understanding of the mechanisms behind plant-flower-visitor interactions.

Therefore, the importance of species traits, especially nectar production and flower-visitor foraging efficiency as mechanisms behind plant-flower-visitor interaction patterns should receive more attention in plant-flower-visitor network studies.

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Summary

Plants differ widely in the number of species by which they are visited and in turn flower-visitors differ widely in the number of plants they visit. This generalization degree is an important aspect in pollination ecology, since it influences species coexistence, community stability and ecosystem services. Generalization promotes species- and network stability because if a species is lost, links in the network will be maintained by other species with similar interaction partners, which decreases the chance for subsequent secondary extinctions. Because the majority of plants highly depend on flower-visitors, an in-depth understanding of the mechanisms behind plant-flower-visitor interactions is important to predict the dependency of species on each other and potential responses of species and functions to global changes, such as the introduction of invasive species and climate change.

In recent years, plant-flower-visitor community studies have shown general interaction patterns such as asymmetry (generalist flower-visitors interact with specialist plants and vice versa) and nestedness (a core group of generalists interacts with each other and a few specialists mostly interact with generalists). Moreover, within these communities, most species abundances and interactions fluctuate through time. Still, surprisingly little is known about the mechanisms driving plant-flower-visitor interaction patterns and their fluctuations, i.e. why certain interactions between plants and flower-visitors are realized while others are not and why certain species abundance and interactions fluctuate among year, while others do not.

Plant-flower-visitor interaction patterns are often explained by random foraging behaviour of flower-visitors, sometimes in combination with the existence of ‘forbidden interactions’. Random foraging behaviour implies that flower-visitors distribute themselves among plants according to plant species abundance. Consequently, more abundant plant species will overall receive more visits from a wider diversity of flower-visitor species. This will result in equal visitation rates per single flower among plant species. Forbidden interactions imply that certain plant species are not visited by certain flower-visitor species, due to a mismatch in either phenology or morphology. For example, flowers with long corollas exclude flower-visitors with short proboscises since these animals are not able to reach the nectar produced by the flowers. Consequently, plant species with a deeper nectar tube are, on average, visited by flower-visitors with a longer proboscis, which is referred to as size-matching. However, the importance of other morphological traits, such as display area (the surface area of the flowers), in determining the generalization degree of plants are not often investigates. Surprisingly, although nectar reward and foraging efficiency are two of the most likely aspects to determine flower-visitor foraging behaviour, we also lack knowledge on how these aspects affect the type- and number of interaction partners and consequently plant-flower-visitor network structures, such as size-matching.

The main aim of this study was to investigate whether, besides flower abundance and flower- and flower-visitor morphology (floral nectar tube depth, floral display area and flower-visitor proboscis length), nectar production and flower-visitor foraging efficiency can

explain species generalization degree and plant-flower-visitor interaction patterns, such as asymmetry, nestedness and size-matching between the nectar tube depth of flowers and the average proboscis length of their flower-visitors. To this end, I investigated the generalization degree of 16 subalpine Asteraceae and their flower-visitors and their interaction patterns, including nestedness, assymmetry and size-matching. The Asteraceae have flowers with relative shallow nectar tubes and are visited by a wide diversity of insects, including solitary bees, bumblebees, butterflies and flies. The nectar tube depth does not pose strong morphological constraints because most flower-visitors have a proboscis which is longer than the nectar tube. This makes the Asteraceae an ideal study system to investigate the importance of nectar production of plants and foraging efficiency of flower-visitors for plant-flower-visitor interaction patterns. Specifically, I investigated whether size-matching occurs among the flowers of these plants and their flower-visitors, and if so, whether this pattern might be explained by the nectar production of plants, the time it takes flower-visitors to handle the flowers, and/or the foraging efficiency of flower-visitors. This because plants that either produce more nectar or have flowers that flower-visitors can handle faster or forage more efficiently on, will be more attractive to flower-visitors.

In addition, I used my results to investigate how the fluctuation (among three years) of flower-visitor species abundance and their interactions with plants is related to both plant- and flower-visitor generalization degree and morphology. Specifically, I investigated whether plants face a pollinator species number (SN) – species predictability (SP) trade-off. I expect plants to have two strategies to mitigate the risk that in a particular year there are insufficient numbers of flower-visitors to ensure pollination. To ensure pollination, plants can attract a wide diversity of flower-visitors and thus be generalized. To be generalized, plants will need to have flowers with a shallow nectar tube and therefore generalized plants may not be able to produce enough nectar to attract flower-visitor species which are large and have a long proboscis. This because these species have higher energetic requirements and thus need more nectar. However, larger flower-visitors species which have a long proboscis are expected to be those species which are more generalized and therefore less prone to temporal fluctuations. Plant species which have flowers with a deep nectar tube are specialized but can also produce more nectar and thus are expected to attract those flower-visitor species which are larger, have a longer proboscis and are more generalized and therefore less prone to temporal fluctuation. Therefore, plants can either be generalized on flower-visitors which are prone to temporal fluctuations or specialized on those flower-visitors which are less prone to temporal fluctuations, which will result in equal visitation rates.

My results show that plant generalization degree is related to flower abundance and morphological traits, including nectar tube depth and display area. Plant species which were more abundant and had flowers with a shorter nectar tube and a larger display area were visited by more flower-visitor species. However, surprisingly, the number of individuals by which plants were visited was only related to the display area. These results indicate that flower abundance by itself does not attract a more individuals and thus more flower-visitor species and thus that flower-visitor species do not forage randomly. The fact that plants with more abundant flowers were visited by a larger diversity of flower-visitor species might be the result of a strong negative correlation between flower abundance and nectar tube depth.

Furthermore, size-matching between the nectar tube depth of the flowers and the proboscis length of the flower-visitors occurred, even in the absence of strong morphological constraints. Flies, solitary bees and bumblebees with a longer proboscis visited on average flowers with a deeper nectar tube, even though bumblebees had a proboscis which was long enough to visit all plant species studied. Among butterflies, I did not find this relationship. Thus, flies, solitary bees and bumblebees had a preference for flowers with a deeper nectar tube. This preference cannot be explained by a shorter handling time when foraging on flowers of which the nectar tube matches the proboscis length of flower-visitors. This because handling time was not related to flower-visitor proboscis length. Therefore, in contrast to what has been assumed in the literature, my results show that a longer proboscis is not of hindrance when long tongued flower-visitors forage on relatively shallow flowers. The preference for plant species with a deeper nectar tube can rather be explained by the fact that flowers with a deeper nectar tube produce more nectar and that flower-visitors forage more efficiently on deeper flowers. Plant species with flowers with a deeper nectar tube produced more nectar per single flower, per flower head, per individual plant and per patch. Foraging efficiency (both the amount of nectar/sugar that bumblebees extracted from a flower per second and the overall energy they gained while foraging) increased with increasing nectar tube depth. Plant species on which bumblebees foraged more efficiently were more frequently visited by bumblebees.

Finally, my results indicate that the positive association between nectar tube depth and both nectar reward and foraging efficiency of flower-visitors may indeed pose a trade-off between the number- and type of flower-visitors which plants (can) attract and the temporal stability or predictability of these visitors. Plant species which were more specialized, were visited by those flower-visitors that were less prone to local temporal fluctuations. Their flower-visitors visited them each year (during three years). These were bees and flies (no butterflies) which were more generalized and tended to have a longer proboscis.

Overall, my results provide a better understanding of how flower- and flower-visitor morphology, nectar production and flower-visitor foraging efficiency affect plant-flower-visitor interaction patterns. In contrast to what has been assumed by many network studies, my results indicate that flower abundance is less important to determine plant-flower-visitor interactions than floral traits. Flower-visitors do not forage randomly as they do not distribute themselves among flowers in proportion to flower abundance. Rather, plant-flower-visitor interactions are structured by species traits, their frequency distributions and correlations among them. This study is one of the first to show the importance of nectar production and flower-visitor foraging efficiency for plant-flower-visitor interaction patterns, such as size-matching (between the nectar tube depth of flowers and the average proboscis length of their flower-visitors), and even the temporal stability of plant-flower-visitor interactions. Specialized plants might be less vulnerable to yearly fluctuations than previously thought because they are visited by generalized and longer-tongued flower-visitors which are less prone to local temporal fluctuations. Therefore these interactions are more predictable. Generalized and larger, longer-tongued flower-visitors have higher energetic requirements and therefore plants which have flowers with a deep nectar tube and are specialized are likely to attract these visitors, because these plants produce more nectar or because flower-visitors forage more efficiently on their flowers. Thus, to predict the response of plants to yearly fluctuations in plant-flower-visitor communities, is it important to, not only, have insight in species generalization degree, but also an in-depth understanding of the mechanisms behind plant-flower-visitor interactions. Therefore, the importance of species traits, especially nectar production and flower-visitor foraging efficiency as mechanisms behind plant-flower-visitor interaction patterns should receive more attention in plant-flower-visitor network studies.

Samenvatting

Planten verschillen sterk in het aantal bestuiverssoorten waardoor ze worden bezocht, en bestuivers zelf verschillen sterk in het aantal plantensoorten dat ze bezoeken. Deze mate van generalisatie kan variëren van één tot meer dan honderd interactiepartners. Hoe generalistisch planten en bestuivers zijn is een belangrijk aspect in de bestuivingsbiologie omdat dit het voorbestaan van soorten en de stabiliteit van de gemeenschap en '*ecosystem services*' beïnvloed. Wanneer soorten generalistisch zijn is namelijk de kans dat zij uitsterven wanneer één van hun interactiepartners verdwijnt vele malen kleiner dan wanneer zij afhankelijk zijn van slechts één of enkele interactiepartners. Omdat de meeste planten voor hun reproductie afhankelijk zijn van bestuivers is het belangrijk om inzicht te krijgen in de mechanismen achter plant-bestuiver interacties, om daarmee te kunnen voorspellen hoe afhankelijk planten en bestuivers van elkaar zijn en wat er gebeurd met soorten wanneer de soortensamenstelling binnen plant-bestuiver gemeenschappen verandert door bijvoorbeeld de introductie van nieuwe, invasieve soorten of klimaatsverandering.

Studies naar plant-bestuiver gemeenschappen laten zien dat plant-bestuiver interacties vaak asymmetrisch en genest zijn. Asymmetrisch wil zeggen dat generalistische planten vooral door specialistische bestuivers worden bezocht en dat specialistische planten vooral door generalistische bestuivers worden bezocht. Genest betekent dat er in een gemeenschap een kern groep is van generalistische bestuivers en -planten die elkaar bezoeken en dat er slechts enkele specialisten zijn die vooral generalisten bezoeken. Hoewel deze patronen robuust zijn binnen deze gemeenschappen, fluctueert de abundantie van de soorten en de onderlinge interacties door de tijd. Verrassenderwijs zijn de mechanismen die tot de variatie in het aantal interactiepartners en de resulterende interactiepatronen leiden en hun fluctuaties door de tijd nog steeds weinig begrepen.

Er wordt vaak vanuit gegaan dat de interactiepatronen tussen planten en bestuivers (wie bezoekt wie) verklaard kunnen worden door random foerageer gedrag van de bestuivers. Planten die algemener voorkomen worden door een groter aantal bestuivers bezocht, puur gebaseerd op kans. Daarnaast zijn er bepaalde interacties die simpelweg niet kunnen voorkomen, zogenaamde 'verboden interacties', omdat bijvoorbeeld de fenologie of morfologie van bepaalde soorten niet overlapt. Bijvoorbeeld, bestuivers met een korte tong kunnen bloemen met een diepe nectarbuis niet bezoeken omdat hun tong niet lang genoeg is om bij de nectar te kunnen. Hierdoor worden bloemen met een diepere nectarbuis bezocht door bestuivers met een langere tong, wat '*size-matching*' wordt genoemd. Het belang van andere morfologische kenmerken, zoals de grootte van de bloemdisplay, voor plant-bestuiver interactiepatronen is nog maar weinig onderzocht. Daarnaast, ondanks dat de nectar productie van planten en de foerageer efficiëntie van bestuivers belangrijke aspecten zijn die het foerageergedrag van bestuivers beïnvloeden, is er verrassenderwijs ook nog erg weinig bekend over of en hoe deze aspecten het aantal- en de type bestuivers van planten beïnvloeden en daarmee de interactiepatronen in plant-bestuivers gemeenschappen.

In dit proefschrift heb ik onderzocht of en hoe de bloemdichtheid, bloemmorfologie (diepte van de nectarbuis en grote van de bloemdisplay), nectar productie van planten en foerageer efficiëntie van bestuivers de interactiepatronen tussen planten en bestuivers structuren. Ik heb hiertoe de mate van generalisatie van 16 subalpine Asteraceae en hun bestuivers onderzocht en hun interactiepatronen, zoals asymmetrie en ‘*size-matching’* tussen de diepte van de nectarbuis van de bloemen en de tonglengte van de bestuivers. De Asteraceae hebben relatief ondiepe bloemen en worden daardoor door een grote verscheidenheid aan bestuivers bezocht, zoals solitaire bijen, hommels, vlinders en vliegen. De meeste bestuivers hebben een tong die lang genoeg is om bij de nectar te kunnen waardoor deze planten een ideaal studiesysteem zijn om het belang van nectar productie en foerageer efficiëntie voor plant-bestuiver interactiepatronen te onderzoeken. Specifiek heb ik onderzocht of ‘*size-matching*’ ook voorkomt wanneer er geen sterke morfologische beperkingen zijn en zo ja, of dit verklaard kan worden door de hoeveelheid nectar die bloemen produceren, de tijd die bestuivers nodig hebben om de nectar uit de bloemen te halen en/of de foerageer efficiëntie van bestuivers. Immers, bloemen die meer nectar produceren of waarbij bestuivers minder tijd nodig hebben om de nectar eruit te halen of efficiënter kunnen foerageren zullen aantrekkelijker zijn voor bestuivers.

Ook heb ik onderzocht of de jaarlijkse fluctuatie (gedurende drie jaar) van de abundantie van bestuivers en hun interacties met planten is gerelateerd aan de mate van generalisatie- en de morfologie van zowel de bestuivers als de planten. Specifiek heb ik onderzocht of er voor planten mogelijk een *‘trade-off’* bestaat tussen het aantal soorten bestuivers waardoor ze worden bezocht en de voorspelbaarheid van de bestuivers. Planten kunnen mogelijk twee strategieën hebben om het risico dat ze in een bepaald jaar niet voldoende worden bestoven te verkleinen. Allereerst kunnen planten dit risico verkleinen door meer bestuivers aan te trekken en dus generalistisch te zijn. Om een grote verscheidenheid aan bestuivers aan te trekken moeten deze planten bloemen met een ondiepe nectarbuis hebben waardoor deze planten mogelijk minder nectar produceren. Dit omdat er minder nectar in een ondiepe nectarbuis past. Hierdoor zijn generalistische planten mogelijk minder aantrekkelijk voor grote, langtongige bestuivers, omdat deze bestuivers meer energie, en dus nectar, nodig hebben. Langtongige bestuivers zijn echter naar verwachting juist de soorten die generalistisch zijn en daardoor mogelijk minder gevoelig voor jaarlijkse lokale fluctuaties in abundantie en verstoringen. Specialistische plantensoorten hebben bloemen met een diepe nectarbuis waardoor ze naar verwachting meer nectar kunnen produceren en daardoor wel de generalistische bestuivers aantrekken die mogelijk minder gevoelig zijn voor jaarlijkse lokale fluctuaties in abundantie en verstoringen. Dit zou moeten resulteren in gelijke bezoekfrequenties tussen plantensoorten, ongeacht hun mate van generalisatie.

Mijn resultaten laten zien dat de mate van generalisatie van planten sterk afhangt van de abundantie- en de morfologische kenmerken van de bloemen, zoals de grootte van de nectarbuis en de display. Planten met bloemen die in hogere dichtheden voorkwamen en planten waarvan de bloemen een kortere nectarbuis en een grotere display hadden werden door een grotere diversiteit aan bestuivers bezocht. Verrassenderwijs was het aantal individuen waardoor planten werden bezocht enkel gerelateerd aan de grootte van de bloemdisplay. Dit duidt erop dat bloemdichtheid op zichzelf niet een groter aantal individuen en daarmee een groter aantal soorten aantrekt. Bestuivers foerageren dus niet random. Het feit dat het aantal bestuiverssoorten toenam met een hogere bloemdichtheid kan mogelijk ook verklaard worden door het feit dat bloemdichtheid sterk, negatief was gerelateerd aan de diepte van de nectarbuis.

Daarnaast laten mijn resultaten zien dat, ondanks dat de bloemen van de Asteraceae ondiepe nectarbuizen hebben er sprake is van '*size-matching*'. Vliegen, solitaire bijen en hommels met een langere tong bezochten gemiddeld diepere bloemen, ondanks dat alle hommelsoorten een tong hadden die lang genoeg was om alle plantensoorten te bezoeken. Vlinders vertoonden dit patroon niet. Vliegen, solitaire bijen en hommels met een lange tong hadden dus een voorkeur voor diepere bloemen. Deze voorkeur kan niet worden verklaard door het feit dat een lange tong mogelijk hinderlijk is wanneer insecten foerageren op relatief ondiepe bloemen, zoals in de literatuur wordt aangenomen. Mijn resultaten laten namelijk zien dat de snelheid waarmee bijen (zowel solitaire bijen als hommels) nectar uit de bloemen haalden niet gerelateerd was aan de tonglengte van de bijen of '*size-mathing*'. Het is wel aannemelijk dat langtongige bijen diepere bloemen prefereren omdat deze meer nectar bieden of omdat ze op deze bloemen efficiënter foerageren. Planten met diepere bloemen produceerden namelijk meer nectar, zowel per bloem als per inflorescentie, per individu en per patch. Ook was de foerageer efficiëntie van hommels (zowel de hoeveel suiker dat hommels per seconde uit de bloemen haalden als de totale energie die ze overhouden) groter wanneer hommels foerageerden op bloemen met een diepere nectarbuis. Daarnaast werden de plantensoorten waarop hommels efficiënter foerageerden vaker (per uur) door hommels bezocht.

Ten slotte laten mijn resultaten zien dat de positieve relatie tussen de diepte van de nectarbuis en zowel de hoeveelheid nectar als de foerageer efficiëntie van bestuivers inderdaad mogelijk zorgt voor een '*trade-off*' tussen het type- en de diversiteit van de bestuivers die planten aan kunnen trekken en de stabiliteit en voorspelbaarheid van de bestuivers. Planten die meer gespecialiseerd waren, werden namelijk bezocht door bestuivers die lokaal minder gevoelig zijn voor jaarlijkse fluctuaties, daar deze bestuivers de desbetreffende planten elk jaar (gedurende drie jaar) bezochten. Dit waren bijen en vliegen (geen vlinders) die generalistischer waren en een lange tong hadden.

Samenvattend, in dit proefschrift heb ik kunnen laten zien dat kenmerken van zowel planten als bestuivers, hun frequentie verdeling, en de onderlinge correlatie tussen deze kenmerken de interactiepatronen in plant-bestuiver gemeenschappen bepalen. In tegenstelling tot wat vaak wordt aangenomen in de literatuur zijn er sterke aanwijzingen dat bloemdichtheid een minder belangrijke rol speelt en dat bestuivers dus niet random foerageren. Mijn studie is één van de eerste studies die laat zien dat naast morfologie, nectar productie van de planten en foerageer efficiëntie van de bestuivers, belangrijke aspecten zijn die plant-bestuiver interactiepatronen, zoals '*size-matching'*, bepalen en zelfs mogelijk de stabiliteit van deze interacties. Hoewel planten met bloemen met een diepere nectarbuis specialistischer zijn, worden ze bezocht door grote, langtongige bestuivers die generalistisch zijn en die lokaal minder fluctueren door de jaren en daardoor meer voorspelbaar zijn. Dit mogelijk omdat deze bestuivers, vanwege hun lichaamsgrote, meer energie nodig hebben en daarom planten met bloemen met een diepe nectarbuis bezoeken omdat deze meer nectar produceren en omdat ze op deze bloemen efficiënter foerageren. Hiermee laten mijn resultaten zien dat gespecialiseerde soorten minder gevoelig zijn voor jaarlijkse fluctuaties in plant-bestuiver gemeenschappen en het verdwijnen van bestuiverssoorten dan vaak wordt gedacht. Om te kunnen voorspellen welke plantensoorten gevoelig zijn voor jaarlijkse fluctuaties in plant-bestuiver gemeenschappen en het verdwijnen van bestuiverssoorten is het dus belangrijk om niet alleen inzicht te hebben in de mate van generalisatie maar ook in de onderliggende mechanismen die plant-bestuiver interacties en interactiepatronen verklaren. Niet alleen morfologische kenmerken maar ook nectar productie en de foerageer efficiëntie van bestuivers spelen hierbij een belangrijke rol en dienen dan ook meer aandacht te krijgen in plant-bestuiver netwerk studies.

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Saskia

Curriculum vitae

Saskia Gerardine Theodora Klumpers was born on March 20th 1986, in Hellevoetsluis, the Netherlands. She studied both Public Administration and Biology at Leiden University. During her master degree in Evolution, Biodiversity and Conservation Biology, she studied half a year at Aarhus University in Denmark. After obtaining her master degree in 2012, she started an educational degree which she quit to pursue a PhD degree at Leiden University. She performed most of the work for her PhD at the Rocky Mountain Biological Laboratory in Colorado, USA. During her PhD, she also worked as a policy advisor for the municipality of Hellevoetsluis, from 2013 until 2015, and from 2015 until now she is working as a law enforcer (Wet natuurbescherming) for the Dutch Ministry of Economic Affairs.